UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA

TESIS DOCTORAL

Nanosystems for ophthalmic drug delivery in the treatment of chronic and inflammatory pathologies

Nanosistemas de administración oftálmica para el tratamiento de patologías crónicas e inflamatorias

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

José Javier López Cano

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SCHOOL OF PHARMACY

DOCTORAL THESIS

Nanosystems for ophthalmic drug delivery in the treatment of chronic and inflammatory pathologies

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY PRESENTED BY

José Javier López Cano

DIRECTORS

Dr. Irene Teresa Molina Martínez

Dr. Rocío Herrero Vanrell

DRA. ROCÍO HERRERO VANRELL **Y** DRA. IRENE TERESA MOLINA MARTÍNEZ **CATEDRÁTICAS DE UNIVERSIDAD DEL DEPARTAMENTO DE FARMACIA GALÉNICA Y TECNOLOGÍA ALIMENTARIA DE LA FACULTAD DE FARMACIA DE LA UNIVERSIDAD COMPLUTENSE DE MADRID**

CERTIFICAN:

Que la presente memoria titulada:

"Nanosistemas de administración oftálmica para el tratamiento de patologías crónicas e inflamatorias"

"Nanosystems for ophthalmic drug delivery in the treatment of chronic and inflammatory pathologies"

ha sido elaborada bajo su dirección por el Graduado en Farmacia D. José Javier López Cano en el Departamento de Farmacia Galénica y Tecnología Alimentaria y, hallándose concluida, autorizan su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste, expiden y firman la presente certificación en Madrid a 25 de abril de 2022.

Fdo. Rocío Herrero Vanrell **Fdo. Irene Teresa Molina Martínez**

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"Si algo es lo suficientemente importante, hazlo, incluso si las probabilidades están en tu contra"

Elon Musk
CEO of SpaceX and Tesla Motors

ÍNDICE

ABREVIATURAS

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ADN: Ácido desoxirribonucleico

AFM: Microscopia de fuerza atómica (de siglas en inglés "AFM", "Atomic Force Microscopy")

AH: Ácido hialurónico

AINEs: Antiinflamatorios no esteroideos

AMPc: Adenosín monofosfato cíclico

ARNm: Ácido ribonucleico mensajero

BHR: Barrera hemato retiniana

CMC: Carboximetilcelulosa

CNTF: Factor neurotrófico ciliar (de las siglas en inglés "CNTF", "Ciliary Neurotrophic Factor")

COX: Ciclooxigenasa

CPA: Células presentadora de antígeno

CsA: Ciclosporina A

DED: Enfermedad de ojo seco (de las siglas en inglés "DED", "Dry Eye Disease")

DMAE: Degeneración macular asociada a la edad

DMSO: Dimetilsulfóxido

ELISA: Ensayo por inmunoabsorción ligado a enzimas (de las siglas en ingles "ELISA", "Enzyme-linked Immunosorbent Assay").

EOR: Especies de oxígeno reactivas

EOS: Enfermedad de ojo seco

EPR: Epitelio pigmentario de la retina

FDA: Agencia Americana de alimentos y fármacos (de las siglas en inglés "FDA", "Food and Drug Administration

GM-CSF: factor de estimulación de colonias de granulocitos y monocitos (de las siglas en inglés "GM-CSF", "Granulocyte-macrophage- Colony Stimulating Factor

GUV: Vesículas unilamelares gigantes (del inglés "GUV", "Giant Unilamellar Vesicle")

ABREVIATURAS

HIF-1: Factor 1 inducible por hipoxia (de las siglas en inglés "HIF-1", "Hypoxia Inducible Factor)

HP-guar: Hidroxipropilguar

HPC: Hidroxipropilcelulosa

HPMC: Hidroxipropilmetilcelulosa

HyG: Hidrogel (de las siglas en inglés "HyG", "Hydrogel")

IAC: Inhibidores de la anhidrasa carbónica

ICAM-1: Molécula de adhesión intercelular (de las siglas en inglés "ICAM-1", "Intercellular Adhesion Molecule 1")

IFNγ: Interferón gamma

IgA: Inmunoglobulina A

IGF: Factor de crecimiento similar a la insulina IGF-1 (de las siglas en ingles "IGF-1", "Insulin-like Growth factor 1")

IgG: Inmunoglobulina G

IgM: Inmunoglobulina M

ILs: Interleucinas

JNK: quinasas c-Jun N-terminal (de las siglas en inglés "JNK", "c-Jun N-terminal Kinases"

LFA-1: Antígeno 1 asociado a la función leucocitaria (de las siglas en ingles "LFA-1", "Leukocyte Function-Associated Antigen-12")

LUV: Vesículas unilamelares grandes (del inglés "LUV", "Large Unilamellar Vesicle")

MAPK: Cascadas de señalización celular como las proteínas quinasas activadas a mitógenos (de las siglas en inglés "MAPK", "Mitogen Activated Protein Kinases")

MLV: Vesículas multilamelares grandes (del inglés "MLV", "Multi Lamellar Vesicle")

MMP: Metaloproteinasas de matriz (de las siglas en inglés "MMP", "Matrix Metalloproteinases")

MPs: Micropartículas

ABREVIATURAS

NFAT5: Factor nuclear 5 de células T activadas (de las siglas en inglés "NFAT5", "Nuclear factor of Activated T-cells 5"

NLC: Portadores lipídicos nanoestructurados (de las siglas en inglés "NLC", "Nanostructured Lipid Carriers")

NLS: Nanopartículas lipídicas sólidas

NMDA: N-metil-D-aspartato

NPs: Nanopartículas

OMS: Organización mundial de la salud

PAA: Ácido poliacrílico (de las siglas en inglés "PAA", "Polyacrilic Acid")

PEG: Polietilenglicol

PGA: Ácido poli glicólico (de las siglas en inglés "PGA", "Poly Glycolic Acid).

PGE2: Análogos de prostaglandinas agonistas del receptor E2

PGF2: análogos de prostaglandinas agonistas del receptor F2-α

PIO: Presión intraocular

PLA: Ácido poli láctico (de las siglas en inglés "PLA), "Poly Lactic Acid)

PLGA: Ácido poliláctico co-glicólico (de las siglas en inglés "PLGA", "Poly Lactic-co-Glycolic Acid")

PVA: Alcohol polivinílico o polivinil alcohol

PVP: Polivinilpirrolidona

RCS: Tamaño celular relativo (de las siglas en inglés "RCS", "Relative Cell Size")

RD: Retinopatía diabética

RP: Retinosis pigmentaria

SUV: Vesículas unilamelares pequeñas (del inglés "SUV", "Small Unilamellar Vesicle

TFA: Ácido trifluoroacético (de las siglas "TFA", "Trifluoroacetic Acid)

TFF-1: Factor trébol 1 (de las siglas en ingles "TFF-1", "Trefoil Factor-1")

TFOS: "Sociedad de la película lagrimal y la superficie ocular" (de sus siglas inglés "Tear Film and Ocular surface Society)

ABREVIATURAS

TMC: Trimetilquitosano (de las siglas en inglés "TMC", "Trimethyl chitosan)

TNFα: Factor de necrosis tumoral alfa (de las siglas en inglés "TNFα", "Tumoral Necrosis Factor α")

TRPM8: Canales catiónicos de los receptores de potencial transitorio de la subfamilia M, miembro 8 (de las siglas en inglés "TRPM8", "Transient Receptor Potential Cation Channel, Subfamily M, Member 8")

VEGF: Factor de crecimiento endotelial vascular (de las siglas en inglés "VEGF", "Vascular Endothelial Growth Factor").

VIP: Péptido vasoactivo intestinal (de las siglas en inglés "VIP", "Vasoactive Intestinal Peptide").

ZO-1: Zónula ocluddens

RESUMEN/SUMMARY

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RESUMEN/SUMMARY

RESUMEN

NANOSISTEMAS DE ADMINISTRACIÓN OFTÁLMICA PARA EL TRATAMIENTO DE PATOLOGÍAS CRÓNICAS E INFLAMATORIAS

Las enfermedades oculares crónicas, que cursan con inflamación, afectan tanto al segmento anterior como al posterior y, en muchas ocasionescarecen de tratamientos que sean efectivos y que al mismo tiempo resulten bien tolerados. Este tipo de patologías se pueden dividir en dos categorías bien diferenciadas: aquellas inducidas por eventos inflamatorios que acaban generando un círculo vicioso, como la uveítis o la conjuntivitis, y las generadas por transformaciones o modificaciones fisiopatológicas que evolucionan hacia procesos inflamatorios y degenerativos. Esta última categoría sería el caso de patologías prevalentes como la enfermedad de ojo seco (EOS) y el glaucoma. En la EOS se producen, entre otros procesos, evaporación de la lágrima y/o alteraciones en el perfil lipídico o acuoso de la película precorneal. En el glaucoma se produce un daño en la capa de fibras nerviosas de la retina que se asocia, en la mayoría de los casos, a un aumento de la presión intraocular (PIO). En ambos casos se genera gradualmente una inflamación crónica, dando lugar a procesos degenerativos tanto del segmento anterior (fibrosis, abrasión y ulceraciones corneales) como posterior (neuroinflamación, excitotoxicidad y otros procesos neurodegenerativos de la retina). De hecho, cada vez hay más evidencia de que las enfermedades neurodegenerativas se asocian a eventos inflamatorios.

Actualmente, en la EOS, existen terapias enfocadas al tratamiento sintomático o paliativo de la patología como las lágrimas artificiales que se prescriben, en la mayoría de los casos, para disminuir los síntomas asociados a la enfermedad. Los expertos recomiendan, a demanda del paciente, la administración de suplementos acuosos y lipídicos, agentes viscosizantes y mucoadhesivos, y más recientemente, sistemas que combinen compuestos lípidicos y acuosos en la misma formulación. En determinados casos se acude también al empleo de antiinflamatorios, inmunosupresores o los llamados sustitutos biológicos de las lágrimas empleando, en muchos casos, plataformas diseñadas inicialmente como lágrimas artificiales. De hecho, en los últimos años se ha acrecentado el interés en la introducción de activos con efecto osmoprotector y/o antiinflamatorio con el fin de mejorar los preparados.

RESUMEN/SUMMARY

En lo que respecta al glaucoma, se trata de un conjunto de enfermedades que desencadenan la muerte de las células ganglionares y de otras células de la retina, siendo el principal factor de riesgo conocido, y sobre el que se puede actuar, el aumento de la presión intraocular. Si la elevación de la PIO no se controla y se cronifica se desencadenan una serie de eventos inflamatorios y de muerte celular que culminan en procesos neurodegenerativos de la retina. La mayoría de los tratamientos farmacológicos se centran en reducir la presión intraocular y su utilización, de forma crónica, se asocia a efectos adversos considerables. De hecho, se sabe que el uso prolongado de los tratamientos tópicos hipotensores puede provocar eventos inflamatorios en la superficie ocular que desencadenan a su vez la aparición de la EOS o el empeoramiento de los síntomas para quienes lo padecen, lo que provoca una falta de adherencia al tratamiento y en consecuencia la perdida de efectividad. Además, hay que tener en cuenta que el glaucoma no sólo aparece en pacientes que cursan con valores elevados de la PIO, sino que hay sujetos que desarrollan la enfermedad con valores normales de la PIO en lo que se conoce como glaucoma normotensional. Por esta razón resulta crucial establecer un tratamiento neuroprotector de la enfermedad con independencia de los valores de la presión intraocular que presente el paciente.

En esta tesis doctoral, se han desarrollado distintos nanosistemas de administración tópica e intraocular para el tratamiento de procesos crónicos e inflamatorios que afecten tanto al segmento anterior como al segmento posterior. En primer lugar, se realizó una revisión centrándose, fundamentalmente, en la importancia de la utilización de nanosistemas que aporten en el mismo preparado componentes de naturaleza acuosa y oleosa para el tratamiento de la EOS y de otras alteraciones de la superficie ocular (**capítulo I**). En esta revisión se pudo constatar además la importancia de la inclusión de activos en este tipo de preparados. También se profundizó en las distintas estrategias para aumentar el tiempo de retención de las formulaciones sobre la superficie ocular, así como posibles métodos de esterilización, que suponen un punto crítico a tener en cuenta ya que se podrían modificar la eficacia o la tolerancia de los preparados desarrollados. Se prestó especial interés a la fosfatidilcolina de soja y a los ácidos grasos insaturados ya que aportan poder antioxidante, disminuyen posibles reacciones inmunogénicas, poseen excelentes propiedades surfactantes y son altamente compatibles con la película precorneal. Otro punto a destacar en este tipo de nanosistemas es la esterilización mediante filtración y los

"métodos en frío" considerados de elección como métodos de esterilización final de los preparados.

Ante la importancia de incluir activos con propiedades antiinflamatorias y osmoprotectoras, antes de comenzar con el desarrollo de nuevos nanosistemas basados en componentes lipídicos y acuosos, se estableció un método de cribado para identificar posibles activos que tuvieran dichas propiedades (**capítulo II**). Para ello, se puso a punto un método para evaluar la influencia de diferentes ambientes hiperosmolares en células epiteliales corneales. En dicho modelo, se estableció una concentración de 470 mOsm/L como ambiente hiperosmolar óptimo capaz de inducir muerte celular, apoptosis y modificación del volumen celular de las células epiteliales corneales permitiendo detectar e identificar, de una manera óptima, candidatos potenciales con efectos protectores de la superficie ocular. Se ensayaron sustancias potencialmente osmoprotectoras (betaína, L-carnitina y taurina) así como polímeros empleados en administración tópica ocular como es el caso de ácido hialurónico (AH) e hidroxipropilmetilcelulosa (HPMC) a diferentes concentraciones. De esta forma se confirmaron las propiedades de las sustancias contempladas y se investigaron más a fondo sus posibles propiedades protectoras en el modelo desarrollado. Aunque todas ellas mostraron actividad osmoprotectora, la betaína exhibió los mejores resultados de osmoprotección a concentraciones elevadas, resultando el AH el polímero más efectivo para prevenir la muerte celular inducida por un ambiente hipertónico. Entre los osmoprotectores empleados la betaína y la taurina a concentraciones medias-altas demostraron también una alta actividad antiapoptótica, así como el AH y HPMC una reducción de los eventos apoptóticos en los estudios de citometría de flujo. Los compuestos osmoprotectores estudiados demostraron la capacidad de regular el volumen celular en condiciones similares a las basales y los polímeros fueron capaces de aumentar dicho valor por encima del 100%. De forma paralela, se seleccionó una línea celular de macrófagos para estudiar la actividad antiinflamatoria de las sustancias deseadas mediante la determinación de la inhibición del factor de necrosis tumoral alfa (TNFα). En este sentido, los osmoprotectores y los polímeros mostraron valores de inhibición del factor de necrosis tumoral superiores al 20 %; A concentraciones elevadas la L-carnitina y la taurina mostraron valores en torno al 60%. También el AH a concentraciones bajas presentó valores altos de inhibición de TNFα).

RESUMEN/SUMMARY

El desarrollo de una microemulsión como vehículo novedoso para la administración de fármacos por vía tópica ocular y el tratamiento de enfermedades de la superficie ocular se muestra en el **capítulo III**. Las principales ventajas de este tipo de sistemas de administración tópica ocular estriban en el aporte de componentes acuosos y lipídicos a la película precorneal, y en la posibilidad de introducir activos de distinta naturaleza en la formulación. Esto unido su estabilidad y facilidad de escalado les convierten en preparaciones muy interesantes en la terapia de la EOS. En esta tesis doctoral se desarrolló inicialmente una microemulsión *base* por autoemulsificación con tamaños de gotícula cercanos a 20 nm. Las microemulsiones desarrolladas se caracterizaron desde el punto de vista fisicoquímico y se estudió el efecto de la adición de AH y dextrano a estos preparados. En estas formulaciones únicamente el AH dio lugar a una modificación en los tamaños (30 nm), generando también un aumento de la viscosidad. Las microemulsiones desarrolladas, presentaron propiedades adecuadas para administración tópica oftálmica con valores adecuados de la tolerancia *in vitro* en células epiteliales de córnea y conjuntiva.

Posteriormente se realizó un ensayo de estabilidad a temperatura ambiente y en condiciones de refrigeración para comprobar la existencia de factores que pudieran alterar las propiedades fisicoquímicas, así como la tolerancia celular de los preparados durante un período de 9 meses. Las microemulsiones desarrolladas fueron estables en condiciones de refrigeración durante 9 meses y en particular aquellas que contenían polímeros. Al mismo tiempo, a partir de la microemulsión modelo se prepararon dos tipos diferentes de microemulsiones osmoprotectoras (A y B) conteniendo betaina y leucina la primera y ácido oleanólico y clusterina la segunda, añadiéndose AH a la microemulsión A (A-HA) y dextrano a la microemulsión B (B-DXT). Las características fisicoquímicas de las mismas no cambiaron significativamente en comparación con las formulaciones inicialmente desarrolladas y todos ellas mostraron una buena tolerancia in vitro (> 80%) en células epiteliales de córnea y conjuntiva. Estas formulaciones también demostraron una elevada capacidad osmoprotectora evitando la muerte celular ante un ambiente de estrés hiperosmolar en células epiteliales de la córnea. Para confirmar la seguridad de las mismas, se realizaron ensayos de tolerancia *in vivo* en conejos demostrándose que todas las formulaciones finales osmoprotectoras resultaron bien toleradas.

RESUMEN/SUMMARY

Partiendo de los nanosistemas inicialmente desarrollados, se elaboraron microemulsiones osmoprotectoras cargadas con latanoprost como nuevo tratamiento para el glaucoma, con propiedades protectoras de la superficie ocular (formulaciones híbridas) (**capítulo IV**). La adición de latanoprost no afectó significativamente a la caracterización fisicoquímica de las misma y los valores de tolerancia *in vitro* mostraron que el latanoprost resultó bien tolerado en las microemulsiones cargadas en comparación con la formulación comercializada (utilizada como referencia) que presentó una tolerancia muy baja. También cabe mencionar, que estas formulaciones hipotensoras exhibieron una alta osmoprotección en el modelo hiperosmolar previamente desarrollado al inicio de esta tesis doctoral.

Con el fin de establecer una relación entre la eficacia y la capacidad de las microemulsiones para internalizar, se realizaron estudios preliminares de permeación celular. Estos estudios demostraron la alta capacidad de las microemulsiones *base* cargadas con el agente fluorescente cumarina para internalizarse en las células epiteliales de la córnea tras tiempos cortos de exposición (5 minutos) y retener el agente fluorescente durante más de 10 días.

La microscopía electrónica mostró la capacidad de las microemulsiones desarrolladas para interaccionar con las membranas celulares, así como distribuirse y acumularse dentro de las diferentes estructuras de células epiteliales de córnea. Se contempló la posibilidad de que estos resultados estuvieran relacionados con la actividad hipotensora evaluada en conejos donde todas las microemulsiones desarrolladas con latanoprost exhibieron una notable superioridad sobre la formulación de referencia. Sorprendentemente, las microemulsiones *base* sin latanoprost exhibieron cierto efecto hipotensor, y aquellas que incluían polímeros mostraron un efecto hipotensor similar al de la formulación de referencia.

En el último capítulo de la tesis doctoral, se desarrolló un hidrogel termosensible de administración intravítrea con el objetivo de conseguir liberaciones sostenidas de agentes neuroprotectores (**capítulo V**). Una vez iniciado el proceso de neurodegeneración es urgente administrar agentes neuroprotectores capaces de frenar o al menos retrasar los procesos neurodegenerativos. Para esto, se deben mantener las concentraciones de agentes neuroprotectores en el lugar de acción durante largos periodos de tiempo. Dentro de los sistemas capaces de liberar sustancias activas de forma controlada se encuentran los

hidrogeles termosensibles cuyas principales ventajas estriban en su consistencia ya que pueden ser fácilmente administrados mediante inyección intraocular, gelificando en el lugar de administración. Con el objeto de obtener este tipo de sistemas se sintetizaron y caracterizaron dos polímeros basados en ácido poliláctico-co-glicólico y polietilenglicol con diferentes proporciones de ácido láctico y glicólico, conocidos como copolímeros tribloque, que originan hidrogeles biodegradables termosensibles a temperaturas fisiológicas. El hidrogel que contenía mayor concentración de ácido láctico, conocido como Hidrogel 1, presentó mejores propiedades de estabilidad y comportamiento de gelificación para administración intravítrea que el Hidrogel 2. Los hidrogeles desarrollados se disolvieron en un tampón de carbonatos para adaptar el pH a las condiciones fisiológicas. Se seleccionaron el ketorolaco y la dexametasona como principales agentes antiinflamatorios. También se incluyeron, como adyuvantes y sustancias potencialmente neuroprotectoras, la idebenona y la vitamina E pegilada (TPGS). Las propiedades reológicas de las formulaciones hidrogeles finales exhibieron características adecuadas para su inyección intravítrea. Los estudios de tolerancia *in vitro* en células epiteliales pigmentarias de la retina (ARPE) mostraron que el hidrogel 1 presentó una mayor tolerancia que el hidrogel 2. Estos resultados, junto con las propiedades fisicoquímicas anteriormente mencionadas, demostraron que el hidrogel 1 poseía propiedades más adecuadas para la administración intravítrea que las presentadas por el hidrogel 2. Las formulaciones neuroprotectoras finales mostraron una buena tolerancia *in vitro*, así como una elevada capacidad protectora de las células epiteliales pigmentarias de la retina frente a eventos de muerte celular inducidos por estrés oxidativo. En particular, con la formulación cargada con dexametasona e idebenona se obtuvieron los mejores resultados neuroprotectores frente al estrés oxidativo. Además, todas las formulaciones fueron capaces de inhibir la producción del factor de necrosis tumoral alfa, aunque los hidrogeles cargados con ketorolaco presentaron una mayor respuesta antiinflamatoria que los hidrogeles cargados con dexametasona.

Finalmente, los perfiles de liberación *in vitro* obtenidos con los hidrogeles desarrollados se ajustaron al modelo de Korsmeyer-Peppas. De acuerdo con los valores calculados para el coeficiente exponencial “n”, la velocidad de cesión de la mayoría de los hidrogeles, excepto el que contenía únicamente dexametasona, se correspondía con la situación de “transporte anómalo” que corresponde a mecanismos mixtos de difusión y erosión. En el

caso del hidrogel de dexametasona el mecanismo de cesión corresponde a difusión de Fick. En todos los sistemas desarrollados se obtuvieron cesiones de dexametasona o ketorolaco durante al menos 50 días. Al comparar la similitud entre los perfiles de liberación utilizando el factor f_2, se encontraron diferencias entre los hidrogeles cargados con dexametasona que contenían idebenona o TPGS frente a los hidrogeles cargados solo con dexametasona. Sin embargo, entre los hidrogeles que contenían ketorolaco no se encontraron diferencias.

RESUMEN/SUMMARY

RESUMEN/SUMMARY

SUMMARY

NANOSYSTEMS FOR OPHTHALMIC DRUG DELIVERY IN THE TREATMENT OF CHRONIC AND INFLAMMATORY PATHOLOGIES

Chronic ocular diseases that involve inflammatory conditions affects both the anterior and posterior segments of the eye, which in many cases lack of effective and well tolerated treatments at the same time. These types of pathologies can be subdivided into two well-differentiated groups: those induced by inflammatory processes that end up developing a vicious cycle, such as uveitis or conjunctivitis, and those generated by pathophysiological transformations, or modifications that evolve towards inflammatory and degenerative processes. This last instance would be the case of prevalent pathologies such as dry eye disease (DED) and glaucoma. In DED, changes on the ocular surface occur such as tear evaporation, change in the lipid or aqueous profile of the precorneal tear film among others. Meanwhile, in glaucoma a damage in the nervous fibers of the retina is produced associated in most cases to an IOP increment. In both cases a chronic inflammation is induced giving rise rise to chronic and degenerative processes of both the anterior segment (fibrosis, abrasion, corneal ulcerations, and vision loss) and the posterior segment of the eye (neuroinflammation, excitotoxicity and retinal neurodegeneration). In fact, there is growing evidence about the relation between neurodegenerative diaseases and inflammatory events.

Currently, regarding DED, there are therapies focused on symptomatic or palliative treatment such as artificial tears indicated in most cases to reduce associated symptoms. The latest recommendations aim at the administration of aqueous and lipid supplementation to increase the stability of the precorneal tear film, viscosity enhancers and more recently, systems that include both hydrophilic and lipophilic agents. Anti-inflammatory agents, immunosuppressants or so-called biological tear substitute are employed in some specific cases and frequently included in originally developed artificial tears platforms. Over the last few years, the importance of including active ingredients with both anti-inflammatory and osmoprotective effects has become an interesting strategy.

Besides and with regards to glaucoma, it is a group of diseases that triggers retinal ganglion cells (RGC) death mechanisms, progressing to other areas of the retina, being

intraocular pressure (IOP), the main risk factor known that can be modified. If IOP is not properly controlled and it becomes chronic, usually triggers a series of inflammatory and cell death mechanisms that culminates in neurodegenerative processes of the retina. Therefore, many treatments or combination of these, aiming at lowering intraocular pressure have considerable adverse effects. Moreover, their prolonged use can trigger inflammatory processes on the ocular surface such as DED usually ending in poor patient compliance and loss of effectiveness.

However, it should be taken into account that glaucoma is not only given in patients with high IOP values, but also in those that develop the disease with normal IOP values, which is commonly known as normotensive glaucoma. For this reason, it is crucial to establish a neuroprotective therapy independently of the IOP values.

In this doctoral thesis, with the purpose of developing new tools as well as nanosystems of ocular administration for the treatment of chronic and inflammatory processes, both anterior and posterior segment diseases have been addressed. Firstly, a review focused on the importance of nanosystems with lipid and aqueous components for DED and other ocular surface alterations was carried out (**chapter I**). In this review, the inclusion of actives in this kind of systems was addressed. Besides, it was delved into new strategies to increase the retention time on the ocular surface, as well as the sterilization methods, that entails a critical point to take into account, due to the possible altearation of the efficacy or tolerability of the formulations developed. In addition, special interest has been given to soy phosphatidylcholine and unsaturated fatty acids as they provide the formulations with great antioxidative power, reduce possible immunogenic reactions, have excellent surfactant properties and are highly compatible with the precorneal tear film. Another point to highlight regarding this type of systems, is sterilizing filtration and the "cold methods" which are considered as ideal after preparation, in order to reduce the risk of microbiological contamination.

Given the importance of including antiinflammatory and osmoprotective agents and before, getting immerse into the development of new lipid-based nano systems, a screening method to identify potential substances wich such properties (**chapter II**). For this purpose, a new method evaluatin the influence of different hyperosmolar environments in corneal epithelial cells was optimized. In this method, 470 mOsm/L was established as the

optimal hyperosmolar environment capable of inducing cell death, apoptosis and cell shrinkage that allowing optimal detection and identification of potential candidates with protective effects on the ocular surface. Potentially osmoprotective substances (betaine, L-carnitine and taurine), as well as polymers used for topical ocular administration, such as hyaluronic acid (HA) and hydroxypropylmethylcellulose (HPMC) at different concentrations were used. In this way, it was intended to confirm the properties already studied of the substances contemplated and to further investigate their possible protective properties in the developed model. Although all the substances showed promising results, betaine exhibited the best osmoprotection results at high concentrations and HA was the most effective polymer to prevent cell death induced by a hypertonic environment. Among the different osmoprotectants used, betaine and taurine at medium-high concentrations showed high antiapoptotic activity, while HA and HPMC also showed a reduction in apoptotic events. In addition, the osmoprotective substances studied, demonstrated under hypertonic stress, the ability to regulate cell volume in a similar way that under basal conditions, while polymers overcompensated, increasing volumes by over 100%. In addition, a macrophage cell line was selected to study the anti-inflammatory activity of the desired substances by inhibiting tumor necrosis factor-alpha assessment. In this sense, the osmoprotectants and polymers showed tumor necrosis factor inhibition values greater than 20%, although L-carnitine, taurine at high concentrations and HA at its lowest concentration showed values around 60%.

Once an in-depth investigation of topical ophthalmic lipid nanosystems had been carried out, the main composition and characteristics to be included in lipid nanosystems were studied, and a hyperosmolar stress model had been established to detect osmoprotective and anti-inflammatory substances, a microemulsion was developed as a novel vehicle for topical ophthalmic administration for ocular surface diseases **(Chapter III)**. The main advantages of this type systems aim to provide the precorneal tear film with lipids and aqueous components, as well as the possibility to incorporate different active susbtances. These, together with their easy scalability and stability entail very interesting strategies in the treatment of DED. In this doctoral thesis, a base microemulsion was first developed by self-emulsification with droplet sizes close to 20 nm. Likewise, the microemulsions developed were physiochemically characterized and the addition of HA and dextran studied, where only HA presented a change in size (30 nm) producing an

increase in viscosity. The developed microemulsions presented suitable properties for topical ophthalmic administration showing good *in vitro* tolerance values in corneal and conjunctival epithelial cells. Subsequently, a stability test was carried out at room temperature and under refrigeration conditions to verify the existence of factors that could alter the physicochemical properties, as well as cell tolerance for a period of 9 months. The developed microemulsions were stable under refrigeration conditions for 9 months, and in particular, those containing polymers. At the same time, two different types of osmoprotective microemulsions (A and B) were prepared from the model microemulsion, adding HA to microemulsion A (A-HA) and dextran to microemulsion B (B-DXT). Their physicochemical characteristics did not significantly change compared to the initially developed formulations. Besides, all of them showed good in vitro tolerance (>80%) in corneal and conjunctival epithelial cells. These formulations also demonstrated a high osmoprotective activity, thus preventing cell death in corneal epithelial cells under a hyperosmolar environment. *In vivo* tolerance studies were carried out in rabbits in order to confirm their safety, showing that all the final osmoprotective formulations were well tolerated.

After confirming the development of microemulsions and starting from the previously developed microemulsions, osmoprotective microemulsions loaded with latanoprost were developed (hybrid formulations) as a new treatment for glaucoma with protective properties for the ocular surface (**chapter IV**). The addition of latanoprost did not significantly affect their physicochemical properties. Furthermore the *in vitro* tolerance studies showed that latanoprost was very well tolerated when included in the developed microemulsions, in comparison to the marketed formulation (used as reference) that presented very poor cell tolerability. It is also worth mentioning, that the developed hypotensive formulations exhibited high osmoprotection in the hyperosmolar model previously developed at the beginning of this doctoral thesis. Furthermore, in order to establish a relationship between the efficacy and the ability of the microemulsions to internalize, cell permeation studies were performed. These studies demonstrated the high capacity of base microemulsions loaded with fluorescent coumarin to internalize corneal epithelial cells after 5 minutes of exposure as well as retaining the fluorescent agent for more than 10 days.

In addition, electronic microscopy showed the ability of the developed microemulsions to interact with cell membranes and internalize. These studies also assessed their distribution and accumulation within the different cellular structures of corneal epithelial cells. The possibility that these results were related to the hypotensive activity evaluated in rabbits was contemplated, where all the microemulsions developed with latanoprost exhibited a notable superiority over the reference formulation. Surprisingly, *base* microemulsions without latanoprost exhibited some hypotensive effect, and those that included polymers showed a hypotensive effect similar to that of the reference formulation.

Finally, a thermoresponsible hydrogel for intravitreal administration was developed with the aim of achieving sustained release of neuroprotective agents **(chapter V)**. Once the neurodegenerative processes have been established, it is urgent to administer effective treatments that stop or delay these processes. To achieve this, two polymers based on polylactic-co-glycolic acid and polyethylene glycol with different proportions of lactic and glycolic acid, known as triblock copolymers, were synthesized and characterized. Hydrogels were developed with the purpose of creating a thermosensitive biodegradable hydrogel at physiological temperatures capable of maintaining effective concentrations at the target site of action. The hydrogel containing a higher concentration of lactic acid, known as Hydrogel 1, presented better stability properties and gelation behaviour for intravitreal administration than Hydrogel 2. The developed hydrogels were dissolved in a carbonate buffer to adjust the pH to physiological conditions. Ketorolac and dexamethasone were selected as the main anti-inflammatory agents with neuroprotective properties, idebenone and pegylated vitamin E (TPGS). The rheological properties of the final hydrogel formulations exhibited suitable characteristics for intravitreal injection. *In vitro* tolerance studies in retinal pigmented epithelial cells showed that hydrogel 1 was much better tolerated than hydrogel 2. These results, together with the aforementioned physicochemical properties, demonstrated that hydrogel 1 exhibited better properties for intravitreal administration than hydrogel 2. The final neuroprotective formulations showed good *in vitro* tolerance, as well as high protective activity against oxidative-induced cell death in retinal pigmented epithelial cells. In particular, with those formulations loaded with dexamethasone and idebenone, the best neuroprotective results against oxidative stress were obtained. In addition, all formulations were able to inhibit the production of

tumour necrosis factor alpha, although the hydrogels loaded with ketorolac presented a greater anti-inflammatory response than those loaded with dexamethasone.

Finally, the *in vitro* release profiles obtained with the developed hydrogels were adjusted to the Korsmeyer-Peppas pharmacokinetic model. According to the values calculated for the exponential coefficient "n", the release rate for most hydrogels, except for the one that contained single dexamethasone, fitted to the situation of "anomalous transport", which implies mixed diffusion and erosion mechanisms. In the case of the dexamethasone hydrogel, the release mechanism corresponds to Fick diffusion. In all the systems developed, releases of dexamethasone or ketorolac were obtained for at least 50 days. Comparing the similarity between release profiles using the f_2 factor, differences were found between dexamethasone-loaded hydrogels containing idebenone or TPGS versus hydrogels loaded with dexamethasone alone. However, no differences were found between hydrogels containing ketorolac with or without neuroprotective agents.

INTRODUCCIÓN

INTRODUCCIÓN

INTRODUCCIÓN

1. Estructura anatómica y fisiológica del globo ocular

El globo ocular, es un órgano par con forma esferoidal, de 2,5 cm de diámetro, con un peso aproximado de 7 gramos y un volumen de 6,5 mL. Se encuentra localizado en una estructura ósea piramidal llamada orbita, rodeado y separado del resto de elementos de la cavidad orbital por una estructura fibrosa denominada cápsula de Tenon, que se extiende desde el extremo corneal hasta el nervio óptico. Esta cápsula se encuentra unida a la capa más externa de la esclerótica, denominada lamina epiescleral y ligada al mismo tiempo a los músculos extrínsecos oculares.

El globo ocular se encuentra dividido en dos segmentos conocidos como segmento anterior y segmento posterior, delimitados por el cristalino. El **segmento anterior** posee a su vez la cámara anterior donde se encuentra la córnea, el iris, el canal de Schlemm y parte del cuerpo ciliar. La cámara posterior del segmento anterior se encuentra en contacto con la zona posterior del iris, cuerpo ciliar y al mismo tiempo con el cristalino (1). El cristalino se encuentra suspendido desde el cuerpo ciliar por unos ligamentos denominados zónulas de Zinn, formados por una glicoproteína polimerizada, la fibrilina (2). Las cámaras anterior y posterior del segmento anterior se encuentran bañadas por el humor acuoso. El **humor acuoso** supone 0,25 mL, es producido por los cuerpos ciliares y fluye entra la superficie posterior del iris y la superficie anterior del cristalino entrando a través de la pupila en la cámara anterior. Está compuesto en un 99,9% de agua y el 0.1% restante por proteínas y aminoácidos. En la fracción acuosa se encuentran disueltos diversos azucares, iones y productos como glucosa, urea, ascorbato, ácido láctico, inositol, sodio, potasio y bicarbonato. Sus principales funciones son las de mantener un adecuado flujo de oxígeno en las diferentes estructuras corneales del iris y desechar restos del metabolismo celular. Los movimientos oculares y de convección hacen que el humor acuoso proveniente del interior se mezcle con aquel mas templado de la cámara anterior proporcionando un flujo adecuado a través del endotelio corneal. El humor acuoso cumple todas aquellas funciones parecidas a las que cumpliría la circulación sanguínea, pero permitiendo el paso de la energía luminosa a través de las diferentes estructuras oculares.

INTRODUCCIÓN

La circulación del humor acuoso se encuentra relacionada en el mantenimiento de la presión intraocular. Las estructuras implicadas en la circulación del humor acuoso son las siguientes: la malla trabecular, el canal de Schlemm y los canales colectores. Estas estructuras pueden ser apreciadas en la figura 3.

- La malla trabecular es una estructura en forma de "tamiz" a través de la cual él se elimina el humor acuoso. A su vez, está conformada por tres niveles desde el interior hacia el exterior: malla uveal, corneoescleral y yuxtacanalicular.
- El canal de Schlemm es un conducto ovalado revestido por células endoteliales, irregulares fusiformes con vacuolas gigantes presentando un gran contenido acuoso. La parte exterior del canal esta recubierta por células planas y lisas conteniendo las aperturas de los canales colectores.
- Los canales colectores denominados también vasos acuosos intraesclerales abandonan el canal de Schlemm para terminar en las venas epiesclerales. Estos vasos intraesclerales a su vez se pueden dividir en "venas acuosas" a través de las ramas o derivaciones acuoso-venosas o tener un trayecto más sinuoso a través del plexo intraescleral el cual forma los canales colectores más pequeños.

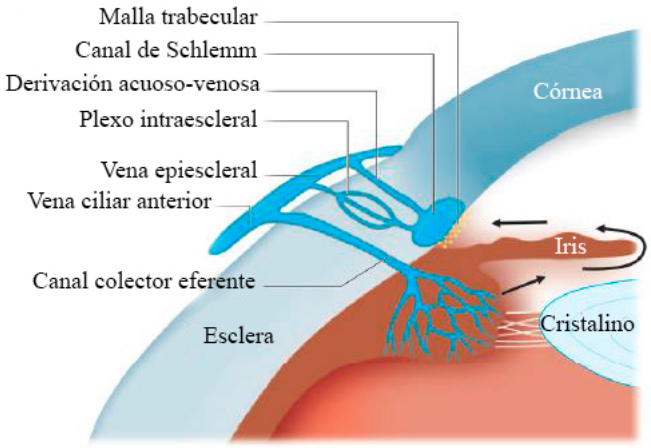

Figura 3. Sistema circulatorio y de drenaje del humor acuoso. Imagen adaptada de "Khurana A. Comprehensive Ophthalmology. 6th ed. Jaypee Brothers Medical Publishers (P) Ltd. Jaypee brothers; 2015. 623 p".

INTRODUCCIÓN

El **segmento posterior** comprende la retina, la coroides y el disco óptico. La estructura anatómica se puede observar en la figura 1.

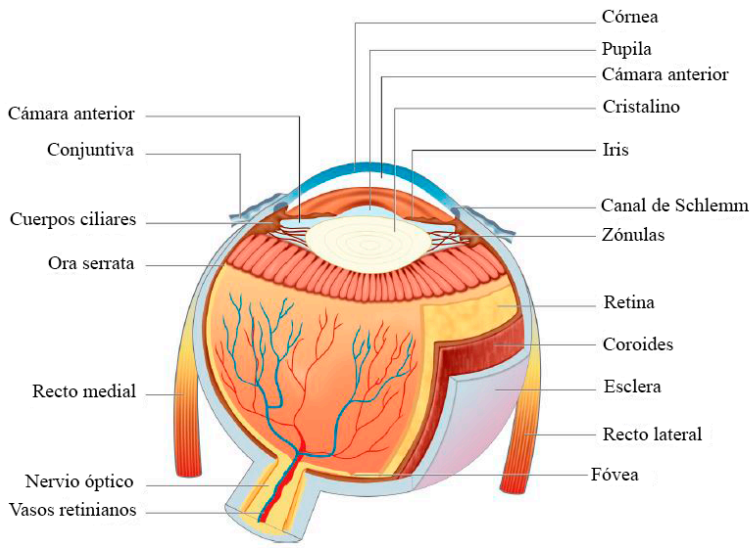

Figura 1. Anatomía y estructura del globo ocular. Imagen adaptada de "Khurana A. Comprehensive Ophthalmology. 6th ed. Jaypee Brothers Medical Publishers (P) Ltd. Jaypee brothers; 2015. 623 p".

El **humor vítreo** rellena el espacio comprendido entre la superficie posterior del cristalino y la externa de la retina. Se trata de una sustancia inerte, transparente y altamente viscosa la cual comprende dos tercios del volumen del globo ocular, constituyendo unos 4 mL aproximadamente. Entre el 98-99,7% de su contenido es acuoso. Se trata de un material con una consistencia de gel sin contenido celular salvo por la presencia de algunas células ovaladas e irregulares conocidas como hialocitos encargadas de la síntesis de diversos componentes de la matriz extracelular del humor vítreo. La resistencia, viscosidad y flexibilidad del vítreo se debe en gran medida a la presencia de proteínas fibrilares como el colágeno con más de veintisiete tipos distintos y otras macromoléculas como el ácido hialurónico o la fibrilina que ayudan a la formación redes matriciales. La presencia de glicosaminoglicanos como el ácido hialurónico o el condroitín sulfato favorece la absorción de agua y iones lo que incrementa la expansión de la matriz permitiendo un adecuado espaciamiento entre todos los elementos que la componen tal y como se puede ver en la figura 4 (3). Entre sus principales funciones cuenta con la de estabilizar mecánicamente la estructura del globo ocular y hacer oxigenar el cristalino y retina (1).

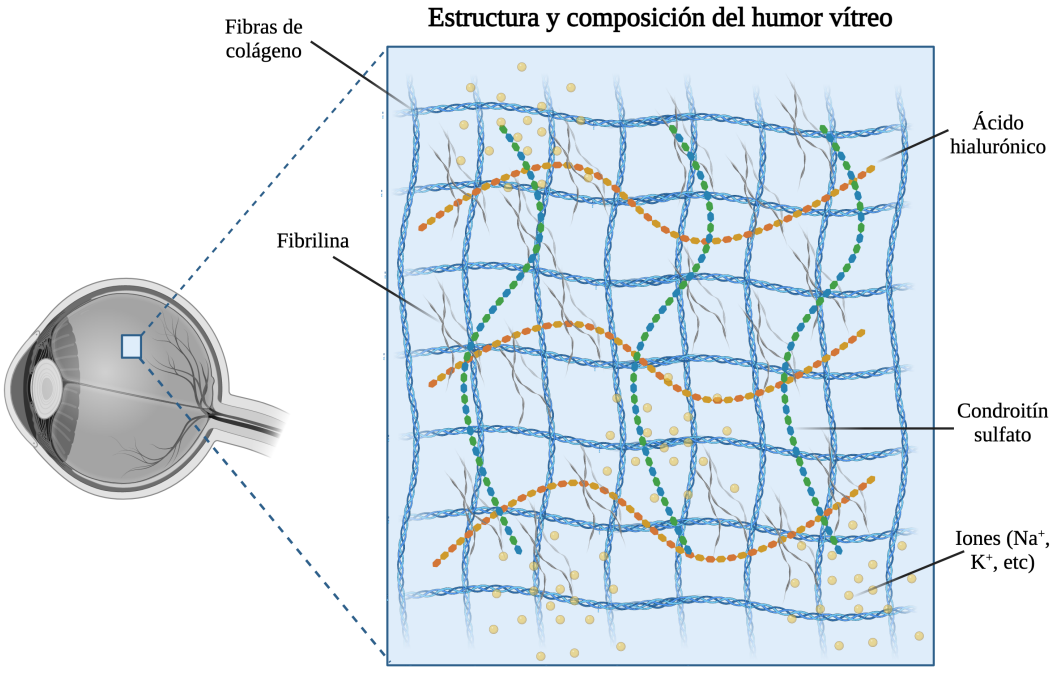

Figura 4. Estructura y composición resumida del humor vítreo. Imagen creada con BioRender.com.

Además, el globo se encuentra formado por tres capas: la mas externa llamada **fibrosa**, una capa intermedia denominada **vascular** y la mas interna conocida como capa o túnica **nerviosa** (1). Las diferentes capas del globo ocular se pueden ver en la figura 2.

La **capa fibrosa** posee una pared densa y compacta y engloba una parte opaca llamada esclerótica. En la zona anterior se sitúa la córnea. La unión entre la cornea y la esclerótica se denomina limbo corneal con la conjuntiva firmemente adherida al mismo.

- La esclerótica o esclera supone la base rígida o el "andamiaje" que mantiene la forma característica del globo proporcionando protección física a las diferentes estructuras oculares internas. Se encuentra principalmente conformada por colágeno, elastina y proteoglicanos los cuales interaccionan entre si para conferirle una estructura semitendinosa.

  Así mismo, posee diferentes puntos de anclaje donde se produce la inserción de los músculos extraoculares confiriendo una alta resistencia a las cargas dinámicas producidas por los movimientos oculares. Así mismo, la gran opacidad de la misma fuera del eje establecido evita la transducción externa de señales (4). Aunque los

requerimientos metabólicos de la esclerótica son bajos, la nutrición de la misma proviene principalmente de las redes vasculares epiesclerales y coroideas (5).

- La córnea de apariencia horizontalmente ovalada mide de 11 a 12 mm en el eje horizontal y de 9 a 11 mm en el vertical. Esta compuesta por estructuras tanto celulares como acelulares. Se encuentra formada por 6 capas: epitelio corneal, membrana de Bowman, estroma, capa de Dua, membrana de Descemet y endotelio.

  En cuanto a la nutrición de la misma, la córnea se trata de un tejido avascular donde la principal fuente de nutrientes de la misma proviene del humor acuoso. El suministro sanguíneo proviene de pequeños vasos en el borde corneal asi como ramas terminales de las arterias oftálmicas y faciales (6).

La **capa vascular** también conocida como úvea, tracto uveal o túnica vascular es aquella encargada de nutrir las diferentes estructuras oculares y consta a su vez del iris, cuerpo ciliar y coroides.

- El iris es una estructura de unos 12 mm de diámetro cuya función principal se basa en la regulación de la cantidad lumínica que penetra al interior de las estructuras oculares. Se encuentra formado casi en su totalidad por fibras musculares lisas y tejido conectivo altamente vascularizado con melanocitos, gránulos de melanina y cromatóforos. Posee dos capa de células epiteliales fuertemente pigmentadas, un musculo dilatador, un esfínter y un borde celular rugoso específico de cada individuo (7).

- El cuerpo ciliar de manera sencilla se podría definir como la continuación de la retina y la coroides conteniendo pequeños capilares y fenestraciones en su interior. Se encuentra constituido por dos partes bien diferenciadas conocidas como la parte posterior o *"pars plana*" y la parte anterior o *"pars plicata"* (8).

  La *"pars plana"* se encuentra en contacto con la zona anterior del vítreo mientras que la *"pars plicata"* da lugar a una serie de rugosidades o crestas conocidas como procesos ciliares principales responsables de la producción del humor acuoso, secreción de proteínas y factores de crecimiento (9). Las dos funciones principales

del cuerpo ciliar son intervenir en el proceso de acomodación del cristalino mediante la musculatura lisa presente en su estroma y la renovación del humor acuoso (8).

- La coroides, con un espesor medio de 0,15 mm constituye un tejido altamente vascularizado y pigmentado localizándose entre la esclerótica y la retina. A su vez, se encuentra conformada por tres capas: capa de Haller, capa de Sattler y lamina capilar o coriocapilar.

  El flujo sanguíneo ingresa en las diferentes capas de la coroides a través de las arterias ciliares posteriores donde convergen en venas vorticosas que al mismo tiempo desencadenan en el ecuador del globo ocular. Gracias a este circuito circulatorio la coroides se encarga de la nutrición y oxigenación de la retina, principalmente de la retina externa (10).

La tercera **capa** llamada túnica **nerviosa,** es la encargada de la función visual y en ella se encuentra la retina, disco óptico y estructuras adyacentes (1). Desde un punto de vista del desarrollo, la retina y el nervio óptico se consideran una prolongación del sistema nervioso central. La retina se encuentra a su vez diferenciada en epitelio pigmentario de la retina (EPR) o retina externa y retina sensorial o interna.

- El EPR se compone de una monocapa de células epiteliales pigmentarias cuboidales cuya principal función es la de mantener la integridad funcional y estructural de la retina. Además, juega un papel determinante en la protección frente a la fotoxidación mediante la absorción del exceso de energía lumínica. También participa en la producción de cerca del 50% de toda la rodopsina que se transforma a 11-cis-retinal contribuyendo al proceso de la visión.

  Así mismo, el EPR forma una parte importante de la barrera hematoretinina (BHR) externa, permitiendo el paso selectivo de moléculas desde la coroides hacia la capa mas interna de la retina conteniendo los fotoreceptores. La expresión de diferentes factores tales como moléculas de adhesión o citocinas juegan un papel importante en la regulación de respuestas inmunomediadas. Su alta actividad metabólica y la presencia de pliegues con gran superficie apical también permite que el EPR regule la composición iónica del área subretiniana para mantener la excitabilidad de los fotoreceptores (11).

INTRODUCCIÓN

- La retina sensorial o neurosensorial se compone de tres tipos de neuronas principales: los fotoreceptores (conos y bastones), las células bipolares y las ganglionares. Otros tipos de células neuronales tales como las células horizontales, amacrinas y de Müller, presentes en capas más internas de la retina cumplen funciones de soporte y nutrición. De mismo modo, la zona donde se separa la parte sensorial de la *"pars plana"* se denomina *"ora serrata"*, donde se reduce a una monocapa de células que continuarán en el EPR (1).

Respecto al proceso de transmisión de la señal, los fotones procedentes de la energía lumínica son absorbidos por los diferentes fotorreceptores, (conos y bastones). Los conos presentan un mayor umbral de excitabilidad y son los encargados de la visión a color. Se encuentran conectados con las células bipolares en la capa plexiforme externa. Por el contrario, los bastones están asociados a las imágenes en blanco y negro, siendo sensibles al contraste y luminosidad. La densidad de ambos tipos celulares varía en función del área de la retina. Por ejemplo, los bastones están ausentes en la zona de mayor agudeza visual conocida como fóvea, incrementándose su número en zonas periféricas (12).

Las señales procedentes de los fotorreceptores son transmitidas a las células bipolares que realizan sinapsis, a su vez, con las células horizontales. Las células horizontales son un tipo de interneurona con función moduladora que utilizan el ácido gamma-aminobutírico (GABA) como señal de retroalimentación negativa. Así mismo, cada célula bipolar realiza sinapsis con múltiples dendritas de las células ganglionares y células amacrinas. Estas últimas también poseen funciones moduladoras estando especialmente vinculadas con las células ganglionares.

Finalmente, la retina cuenta con dos tipos de células con funciones metabólicas y de protección (las células de Müller y los astrocitos). Mientras que las células de Müller se encargan de la eliminación de productos de desecho, síntesis de ácido retinoico o proveen de protección ante un exceso de neurotransmisores, los astrocitos se encuentran en su gran totalidad en el nervio óptico. Estos últimos actúan protegiendo a las neuronas adyacentes de especies reactivas de oxígeno, regulando la excitabilidad de los neurotransmisores.

INTRODUCCIÓN

Tanto las células de Müller como los astrocitos expresan factor de crecimiento endotelial vascular (de sus siglas en ingles *"VEGF, Vascular Endotelial Growth Factor*) el cual está implicado en la creación y modulación de vasos sanguíneos para un adecuado mantenimiento de la función vascular de la retina (11).

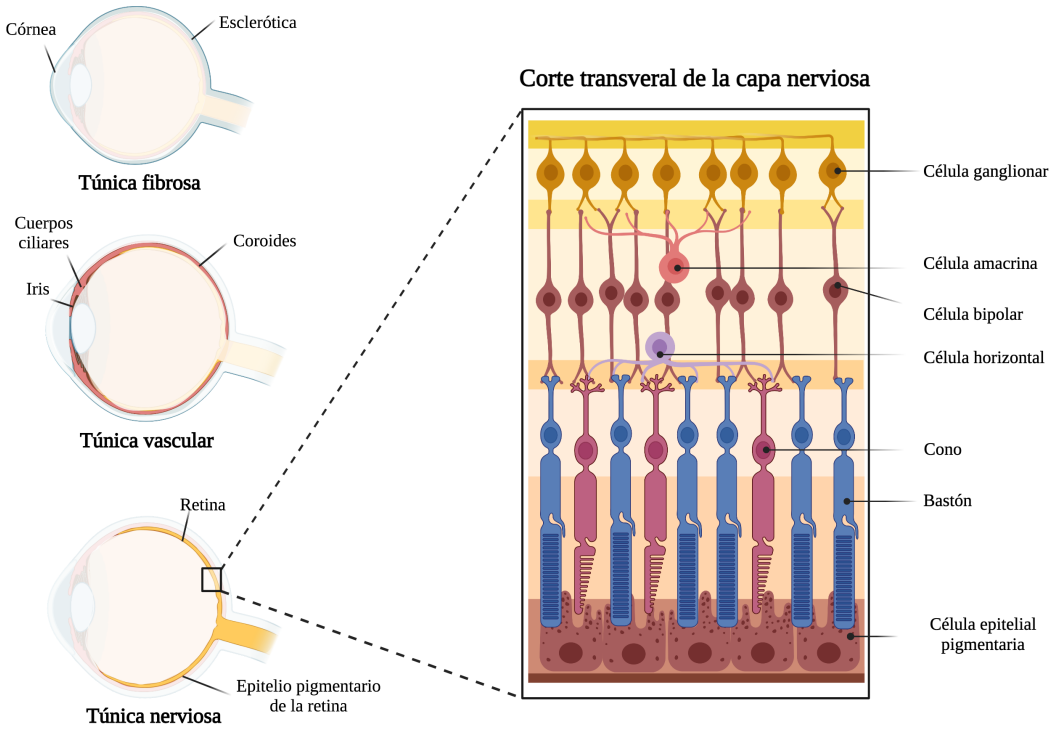

Figura 2. Clasificación de capas que conforman el globo ocular y descripción breve de la disposición celular en la capa nerviosa. Imagen creada con BioRender.com.

2. Superficie ocular: organización y distribución

El término superficie ocular se puede utilizar para describir todas aquellas estructuras que comprenden la córnea, la conjuntiva, las glándulas lacrimales y los párpados funcionando todas ellas como una única unidad tal y como se muestra en la figura 5. Todas ellas tienen como principal función mantener la integridad de superficie ocular ya sea protegiéndola mecánicamente de agentes externos o bien regulando la composición de la película precorneal (6).

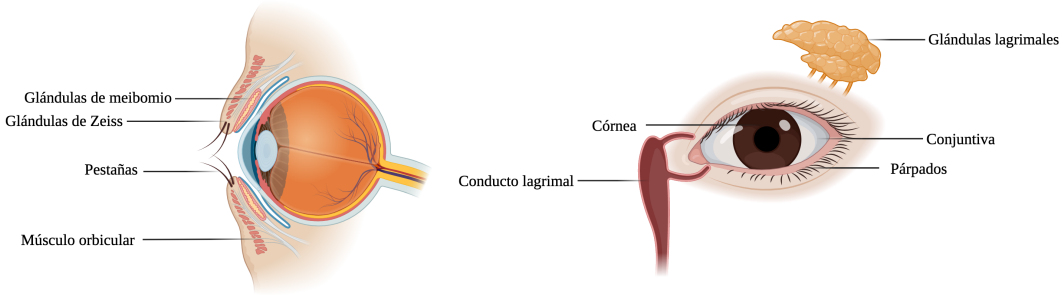

Figura 5. Estructuras que conforman la superficie ocular y tejidos anejos. Imagen creada con BioRender.com.

La **película precorneal** también llamada película lagrimal o *film* precorneal se extiende sobre la córnea, la conjuntiva y la esclerótica, descrito en la figura 6. Se compone de una fracción lipídica y una fracción acuomucinosa (13) con una osmolaridad aproximada entre 296-302 mOsm/L.

- La fracción lipídica de la película precorneal es la capa más externa y su composición está directamente relacionada con la reducción de la tensión superficial, favoreciendo la extensibilidad de la lágrima sobre la superficie y evitando también la evaporación del agua de la película precorneal (14). Las glándulas de meibomio son las principales responsables de la producción de lípidos (15). La composición de la fracción lipídica que se ha denominado lipidoma cuenta con una gran cantidad de lípidos anfifílicos, así como de lípidos apolares que aportan diferentes funciones. La fosfatidilcolina, fosfatidiletanolamina, esfingolípidos y lisofosfatidilcolina en menor proporción constituyen los principales fosfolípidos anfifílicos de la película precorneal (16,17).

Los lípidos con características anfifílicas actúan como nexo de unión entre la capa lipídica y acuosa orientando las cabezas polares hacia la fracción acuosa y las apolares hacia la fracción lipídica. Los compuestos apolares están principalmente formados por ésteres de cera y de colesterol, así como de triglicéridos.

También se encuentra cuenta en su composición con ciertas proteínas como es el caso de la lipocalina incrementan la estabilidad de la película lipídica (18). Por ejemplo, la lipocalina lagrimal es la única proteína lagrimal con la capacidad de interaccionar con diferentes lípidos como el colesterol, los ácidos grasos y los fosfolípidos permitiendo su solubilización, transporte y contribuyendo a la disminución de la tensión superficial (19,20).

- La fracción acuosa denominada acuomucinosa se encuentra secretada por la glándula lagrimal y tejidos accesorios. Entre los principales componentes de esta, se encuentran iones como el sodio, el potasio o el cloro, azucares como la glucosa, urea, albúmina y ciertos tipos de inmunoglobulinas. Dentro de sus funciones se encuentra el suministro de nutrientes y oxígeno a la córnea. También está involucrada en la eliminación de cuerpos extraños y de toxinas que puedan afectar a la superficie ocular. Además, es importante destacar la presencia de proteínas con características antimicrobianas, como es el caso de la lisozima o la lactoferrina que contribuyen también a la estabilidad de la película precorneal (21). Aunque en menor proporción la capa acuosa cuenta con la presencia de inmunoglobulinas, como la inmunoglobulina G, la inmunoglobulina M y la inmunoglobulina A siendo esta última la más abundante con un papel clave en la protección frente a agentes microbiológicos externos (22).

La presencia de mucinas en la capa acuosa es de gran relevancia siendo una de las razones por las que recibe el nombre de fracción acuomucinosa. Las mucinas son un grupo de glucoproteínas de alto peso molecular y proporcionan propiedades lubricantes, viscoelasticas e hidratantes a la superficie ocular. También proporcionan la capacidad de disminuir la viscosidad aparente de la lágrima ante las fuertes fuerzas de cizalla que se dan durante el parpadeo debido a su comportamiento no Newtoniano (23). Las mucinas se producen en las células caliciformes, el epitelio superficial estratificado de la córnea y las glándulas epiteliales (24) y se clasifican en

mucinas solubles, formadoras de geles y en mucinas asociadas a membranas. Las mucinas solubles poseen carga negativa y la habilidad de disminuir la tensión superficial contribuyendo a facilitar la extensibilidad de la película lagrimal (22,25). Las mucinas con capacidad para gelificar e incrementar su viscosidad son de alto peso molecular dentro de las que destacan la MUC5AC (26). La MUC5AC, producida por las células caliciformes de la conjuntiva, es considerada como una de las más representativas ya que contribuye a la hidratación y lubricación de la superficie ocular. En cuanto a las mucinas asociadas a membranas, destacan las MUC1, MUC4 y MUC16, que gracias a sus dominios hidrofóbicos se encuentran ancladas a las células epiteliales de la córnea y conjuntiva formando un sustrato para la interacción con todas aquellas mucinas secretadas con cargas opuestas lo que favorece el deslizamiento de los parpados, evitando interacciones con el epitelio y facilitando su lubricación.

La eliminación y renovación de la fracción acuosa de la película precorneal es debida al drenaje causado por el parpadeo, la evaporación y la presencia de los canales de acuaporina en el epitelio corneal (27,28).

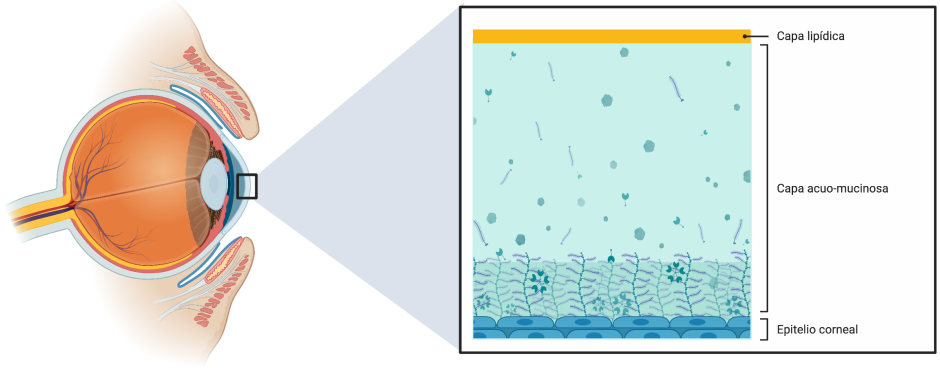

Figura 6. Estructuras que conforman la superficie ocular y tejidos anejos. Imagen creada con BioRender.com.

La córnea, es la estructura principal de la superficie ocular y se encarga de realizar funciones básicas como permitir la visión gracias a sus propiedades refractivas, y prevenir contra agresiones o daños externos (29). La córnea se divide en seis capas: epitelio corneal, membrana de Bowman, estroma corneal, capa de Dua, membrana de Descemet y endotelio (1), presentes en la figura 7.

INTRODUCCIÓN

- El epitelio corneal se encuentra diferenciado entre 5 y 7 capas. Una de las principales funciones del epitelio es la de actuar como barrera física ante la entrada de agentes externos. Es por ello que, particularmente en las zonas más cercanas a la superficie, las células epiteliales muestran una gran expresión de zónula occludens (ZO-1), componente principal de los complejos que forman las uniones estrechas. Estos complejos están formados por dominios transmembrana, ZO-1 y filamentos de actina sellando el epitelio y evitando el paso de componentes dañinos para la superficie (30). La capa más externa de la córnea contiene las células escamosas, que se encuentran en constante renovación. Dichas células se desprenden por la zona central de la córnea y se renuevan en la zona limbal donde se encuentran las células madre corneales. A continuación, se encuentran las células aladas, denominadas así por su forma característica seguidas de las células basales, encargadas de reconstruir la córnea ante posibles lesiones debido a su alta capacidad para dividirse (31,32). Las células basales o de la membrana basal del epitelio corneal se ubican entre las capas más superficiales del epitelio corneal y el estroma junto con la membrana de Bowman. Esta capa tiene un papel especialmente importante en la regulación de la secreción de citocinas y factores de crecimiento. Es por ello que está implicada en procesos de proliferación y diferenciación celular así como en la migración y adhesión del epitelio corneal al estroma (31,33). A continuación, se encuentra la capa de Bowman de características acelulares formada íntegramente por colágeno. La principal función de esta estructura es la de proteger el plexo nervioso subepitelial.

- El estroma representa una de las estructuras más amplias de la córnea suponiendo casi el 90% de su espesor (34). Su componente principal es el colágeno siendo el más abundante el de tipo I, IV y XII. También destaca su alta composición en proteoglicanos principalmente el lumicano y queratocano. Entre la matriz descrita se halla un tipo de células con apariencia pseudo-fibroblástica denominadas queratocitos, los cuales ocupan alrededor del 4% de la matriz estromal y se encargan de la fabricación de la mayor parte de los componentes del estroma. Así mismo, la interacción entre los diferentes tipos de colágeno y los proteoglicanos permiten la característica distribución del estroma haciendo posible la correcta refracción de la luz a través del colágeno y evitan así su dispersión. Los queratocitos, tienen la

habilidad para responder a determinados estímulos proporcionados por las células epiteliales y desencadenar procesos apoptóticos o pro-reparadores.

- En cuanto a la membrana de Descemet, cuya principal función es mantener la hidratación corneal y resistir las fuerzas generadas por la presión intraocular (35), está compuesta por una capa anterior formada principalmente por colágeno de tipo IV y VIII y al mismo tiempo en contacto con una capa posterior de células endoteliales (34).

- El endotelio es la capa más profunda de la córnea. Se encuentra formada por una monocapa de células hexagonales que, debido a su alto contenido en canales de acuaporina (36), es capaz de mantener el estroma hidratado y por tanto contribuir a la transparencia corneal.

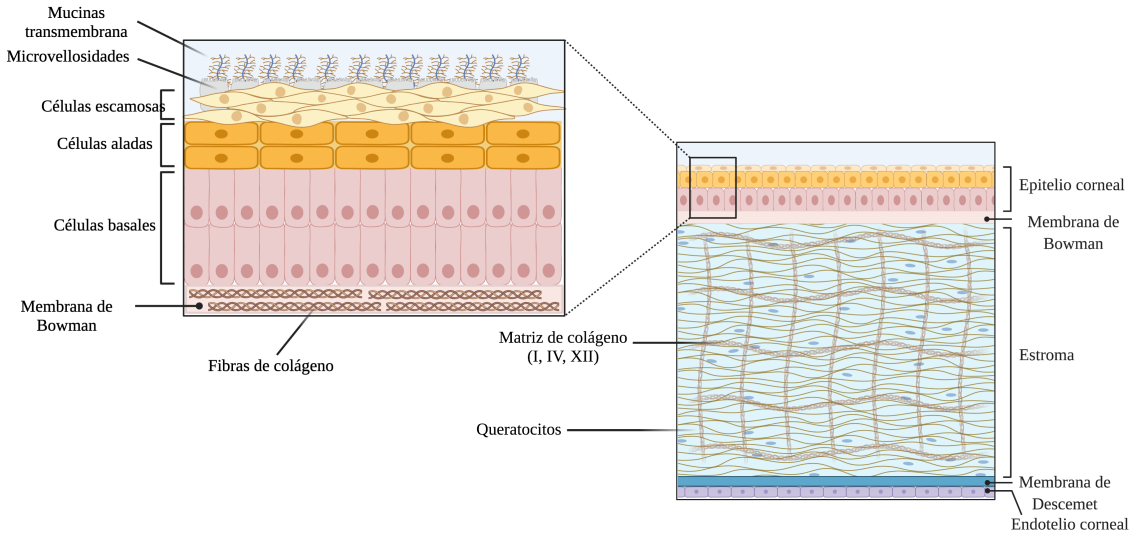

Figura 7. Organización y estructura de las diferentes capas que conforman la córnea. Imagen creada con BioRender.com.

La conjuntiva es una capa mucosa transparente que abarca desde la región anterior del globo ocular hasta la parpebral posterior. A diferencia de la córnea, la conjuntiva es un tejido muy vascularizado. Se encuentra dividida en diferentes zonas en función de su localización: palpebral o tarsal, conjuntiva del fórnix o fondo del saco y conjuntiva bulbar (figura 8).

INTRODUCCIÓN

La conjuntiva palpebral recubre la región posterior de los parpados, la conjuntiva del fórnix se encuentra en una zona intermedia plegada sobre si misma entre el globo y los parpados mientras que la conjuntiva bulbar es aquella que se encuentra cubriendo la región anterior del globo ocular alrededor de la córnea sin llegar a recubrirla (37).

- El epitelio conjuntival está conformado por entre tres y cinco capas de células epiteliales no queratinizadas que pueden ser superficiales, intermedias o basales (12). Al igual que en la córnea, la conjuntiva también posee células madre limbales pero aunque no se conoce muy bien su localización, se cree que se encuentran uniformemente distribuidas por toda la conjuntiva (37).

  El epitelio conjuntival varía estructuralmente dependiendo de donde se localice, encontrándose así un epitelio estratificado no queratinizado cerca de los parpados mientras que en el globo ocular puede observarse un epitelio columnar. También existen diversos tipos celulares que residen en el epitelio conjuntival cumpliendo diferentes funciones.

  - Las *células caliciformes* componen casi el 10% del epitelio conjuntival (1) y son las responsables principales de la secreción de mucinas solubles contribuyendo a la estabilidad de la película precorneal.

  - También destaca la presencia de *melanocitos conjuntivales* cuya cantidad depende de su localización.

  - Las células dendríticas intraepiteliales o también conocidas como *células de Langerhans*, son un tipo de célula inmunitaria cuya principal función es la fagocitosis de elementos externos y presentación antigénica en nodos linfáticos locales o en el tejido linfoide asociado a la conjuntiva. Allí interaccionan con linfocitos T o linfocitos B para generar respuestas inmunes primarias o humorales respectivamente.

  - Por último, los *linfocitos intraepiteliales* predominantemente linfocitos T CD3+, se encuentran cercanos al subepitelio conjuntival y están involucrados en la secreción activa de interleucinas (ILs), así como en procesos inflamatorios.

- La mayor parte del tejido linfoide presente en la conjuntiva se encuentra en la capa adenoidea. Esta posee una gran cantidad de centros linfoides y actúa como la principal barrera inmunológica no física de la superficie ocular donde existe una gran comunicación con el tejido epitelial y las células intraepiteliales mencionadas anteriormente.

- La capa fibrosa conjuntival está compuesta por una red elástica de colágeno y posee un mayor grosor que la capa adenoidea excepto en la región tarsal. Se encuentra compuesta por vasos sanguíneos e inervaciones nerviosas de la conjuntiva. Tal y como se describe inicialmente, se produce una unión entre esta capa de la conjuntiva y la cápsula de Tenon subyacente en la región bulbar conjuntival (12).

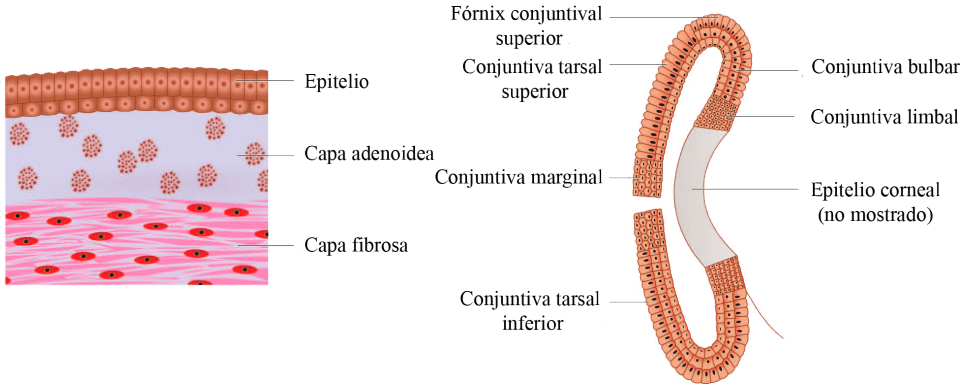

Figura 8. Distribución y estructura de las diferentes capas de la conjuntiva. Imagen adaptada de "Khurana A. Comprehensive Ophthalmology. 6th ed. Jaypee Brothers Medical Publishers (P) Ltd. Jaypee brothers; 2015.

3. Patologías oftálmicas crónicas con etiología inflamatoria

3.1. Fisiopatología de la superficie ocular: enfermedad de ojo seco

3.1.1. Epidemiología, definición y homeostasis en el ojo seco

La enfermedad de ojo seco (EOS) es una de las patologías oculares más prevalentes a nivel mundial que, de acuerdo con los últimos informes de la "Sociedad Internacional de la Película lagrimal y la Superficie ocular" (de sus siglas inglés "TFOS", "Tear Film and Ocular Surface Society") y basándose en un metaanálisis global, afecta entre un 5 y un 50% de la población mundial. Respecto a la prevalencia por sexo, se ha podido confirmar que la prevalencia de la EOS es mayor en mujeres que en hombres y tiende a incrementarse con la edad. También se ha podido observar que existe un papel importante de los diversos factores socioeconómicos y socioculturales como el tiempo de exposición a pantallas de ordenador, tiempo transcurrido en el trabajo, así como la exposición ambiental a partículas en suspensión tales como humo, contaminación, polen, etc. (38).

En cuanto a su definición, esta ha ido modificándose a lo largo del tiempo ya que se han ido identificando diferentes factores y mecanismos por los que se produce dicha enfermedad. Actualmente, la más aceptada es la acordada en el año 2017 por el subcomité de la TFOS en la segunda conferencia internacional de la EOS que establece lo siguiente: "*El ojo seco es una enfermedad multifactorial de la superficie ocular caracterizada por una pérdida en la homeostasis de la película lagrimal, acompañada de síntomas oculares, en la cual la inestabilidad e hiperosmolaridad, el daño y la inflamación de la superficie ocular así como las anomalías neurosensoriales desempeñan papeles etiológicos*" (39).

Por tanto, la EOS contempla numerosos factores que afectan a las estructuras que componen la superficie ocular y supone una pérdida de la homeostasis de la lágrima, produciéndose una alteración en el equilibrio de todos aquellos componentes que contribuyen a mantener las condiciones óptimas y fisiológicas de la superficie ocular que permiten la visión. En la enfermedad de ojo seco se encuentra presente el daño celular, un ambiente hipertónico, secreción de mediadores inflamatorios, alteraciones sensitivas y la propia inestabilidad de la lágrima como causas asociadas al desarrollo y aparición de la EOS.

Respecto a la clasificación de la EOS, existen dos grandes subtipos: el ojo seco evaporativo y ojo seco acuo-deficiente. La existencia de un tipo u otro no son absolutos y que puede producirse una transición donde puedan existir elementos mixtos entre los dos subtipos. Por esta razón, aunque exista una clasificación definida, merece la pena mencionar que se recomienda el uso de algoritmos diagnósticos establecidos por el subcomité de la TFOS tal y como se muestra en la figura 8.

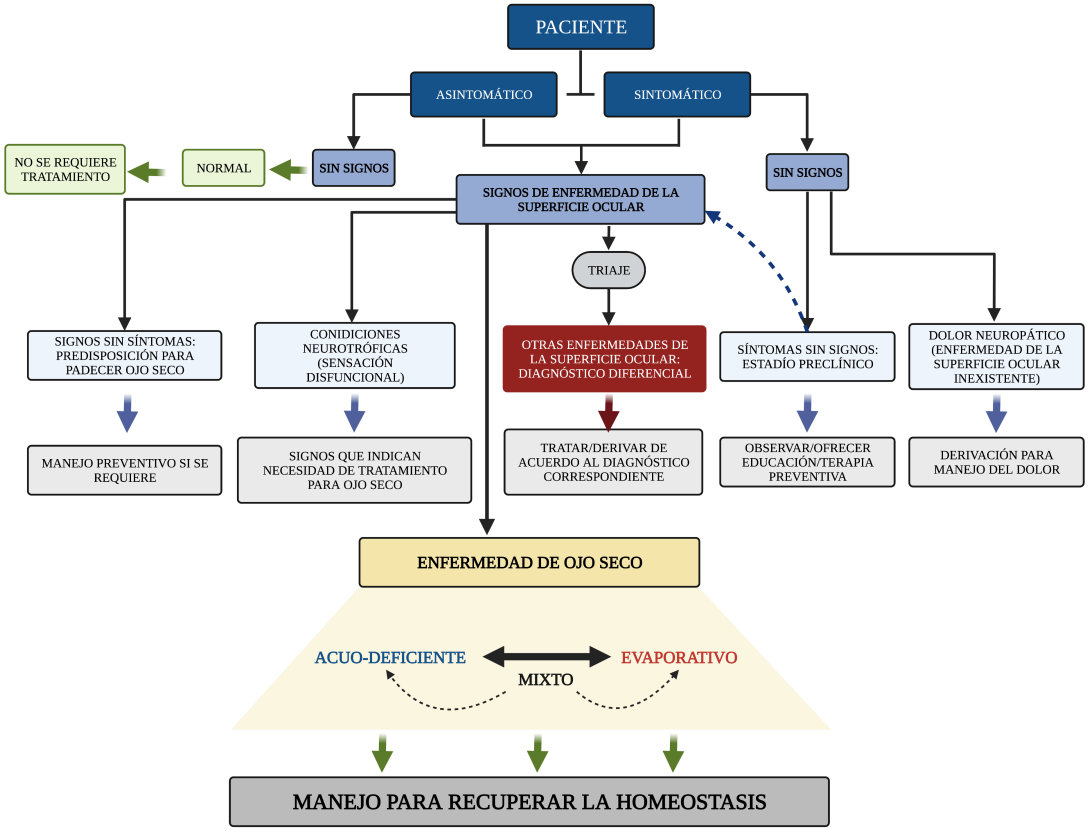

Figura 8. Adaptación del algoritmo ilustrativo para la clasificación de la enfermedad de ojo seco asi como su manejo de acuerdo al informe TFOS DEWS II del 2017.

3.1.2. Mecanismos fisiopatológicos

Respecto a los mecanismos que desencadenan la EOS, una de las características principales radica en un incremento en la osmolaridad de la lágrima, lo que da lugar a un entorno hiperosmolar o hipertónico. Normalmente los valores de hiperosmolaridad lagrimal en pacientes con EOS oscilan entre 316-380 mOsm/L aunque en zonas específicas de la superficie corneal pueden oscilar hasta los 800-900 mOsm/L (40) o entre los 440-600 mOsm/L dependiendo de diferentes modelos predictivos (41). La hiperosmolaridad es la

responsable del desencadenamiento de una serie de procesos inflamatorios y de estrés celular en cascada que favorecen la aparición de la enfermedad y de los síntomas asociados tal y como se puede ver en la figura 9.

Con respecto a los procesos de retroalimentación generados en la EOS, durante los últimos años se ha estado profundizando en su conocimiento, la presencia y papel del sistema inmune juega un papel decisivo en su funcionamiento y control, así como en la aparición de enfermedades, entre las que se encuentra la EOS (42). Una pieza clave es la célula presentadora de antígeno (CPA). Las CPAs están constituidas por células dendríticas, células de Langerhans y macrófagos. Estas se encargan de fagocitar, digerir y presentar los diferentes antígenos así como activar mediante la secreción de ILs a las células T conjuntivales, las que a su vez pueden ser de origen intraepitelial, hallarse en los centros linfoides del subepitelio conjuntival o en los nódulos linfáticos cercanos (43).

En la EOS existe un elevado número de CPAs, que, ante un daño epitelial producido por un ambiente hiperosmolar (44), se polarizan hacia fenotipos proinflamatorios. A su vez las CPAs activadas incrementan la producción de ARNm para diferentes citocinas pro-inflamatorias como el TNFα, el interferón gamma (IFNγ), IL-23 e IL-6. Son estas dos últimas las que están directamente involucradas en la proliferación y diferenciación de los linfocitos T CD4+ hacia linfocitos especializados Th17. Una vez activados, los Th17 promueven la producción del factor de estimulación de colonias de granulocitos y monocitos (de las siglas en inglés “GM-CSF”, “Granulocyte-Macrophage - Colony Stimulating Factor), el cual favorece la migración e incremento de CPAs inmaduras de más fácil activación hacia el tejido corneal, iniciando de nuevo la respuesta inflamatoria y generando lo que se conoce como círculo vicioso inflamatorio en la EOS (43).

Una de las principales citocinas pro-inflamatorias involucrada en la patogénesis de esta enfermedad es el factor de necrosis tumoral alfa (de las siglas en inglés “TNFα”, “Tumoral Necrosis Factor α”). Entre la gran cantidad de acciones pro-inflamatorias que desempeña el TNFα se encuentra su capacidad de activación de las cascadas de señalización celular como las proteínas quinasas activadas a mitógenos (de las siglas en inglés “MAPK”, “Mitogen Activated Protein Kinases”) en respuesta a un ambiente hiperosmolar. Así mismo, esos procesos desencadenan una serie de eventos apoptóticos conduciendo a la muerte celular. También se ve incrementada la producción de metaloproteinasas de la

matriz extracelular (de las siglas en inglés “MMP”, “Matrix Metalloproteinases”), como la MMP-9 y la MMP-7 que se encuentran involucradas en la digestión de ciertos componentes celulares entre los que se encuentra la ZO-1, afectando la integridad del epitelio. Debido a que la MMP-9 también realiza funciones de regulación sobre la descamación de las capas más superficiales del epitelio corneal, una sobreexpresión de la misma en este tipo de ambientes produce un desprendimiento excesivo del epitelio corneal, generando irregularidades en el mismo, conocidas como erosiones punteadas epiteliales y alterando las funciones de la barrera.

Estos eventos desencadenan retroalimentación en el círculo vicioso inflamatorio de la EOS que a su vez activa otras vías celulares relacionadas también con las MAPK como las quinasas c-Jun N-terminal (de las siglas en inglés “JNK”, “c-Jun N-terminal Kinases”), las quinasas reguladas por señales extracelulares (de las siglas en inglés “ERK”, “extracellular-signal-regulated kinase”), y la MAPK p38. Todas ellas contribuyen al mismo tiempo a la expresión de multitud de citocinas proinflamatorias y eventos de muerte celular.

Así mismo y en combinación con lo anteriormente expuesto, aunque los mecanismos innatos celulares ante un ambiente hiperosmolar tienden a incrementar el volumen celular, acumular sales y electrolitos, así como promover la supervivencia celular acaban sucumbiendo ante una presión osmótica elevada. De esta forma se produce a una disminución del volumen intracelular acompañado de una serie de eventos de estrés oxidativo que conllevan a la fragmentación del ácido desoxirribonucleico (ADN) y detención del ciclo celular. Asociado a esta cascada de procesos inflamatorios y de daño se genera un mal plegamiento de ciertas proteínas con funciones vitales, una disminución de procesos metabólicos y anomalías en el transporte intracelular de proteínas y elementos claves para el correcto funcionamiento celular (40). La pérdida de células caliciformes, el daño epitelial, así como la inflamación genera la disminución en la secreción de componentes fundamentales para la película lipídica y la capa acuomucinosa contribuyendo al incremento del ambiente hiperosmolar y por tanto de la cascada inflamatoria y de eventos apoptóticos (ver figura 9). Además, merece la pena mencionar que la expresión y activación de todas las vías mencionadas ha sido directamente relacionada con la aparición de signos y síntomas de la EOS según la evidencia clínica (45).

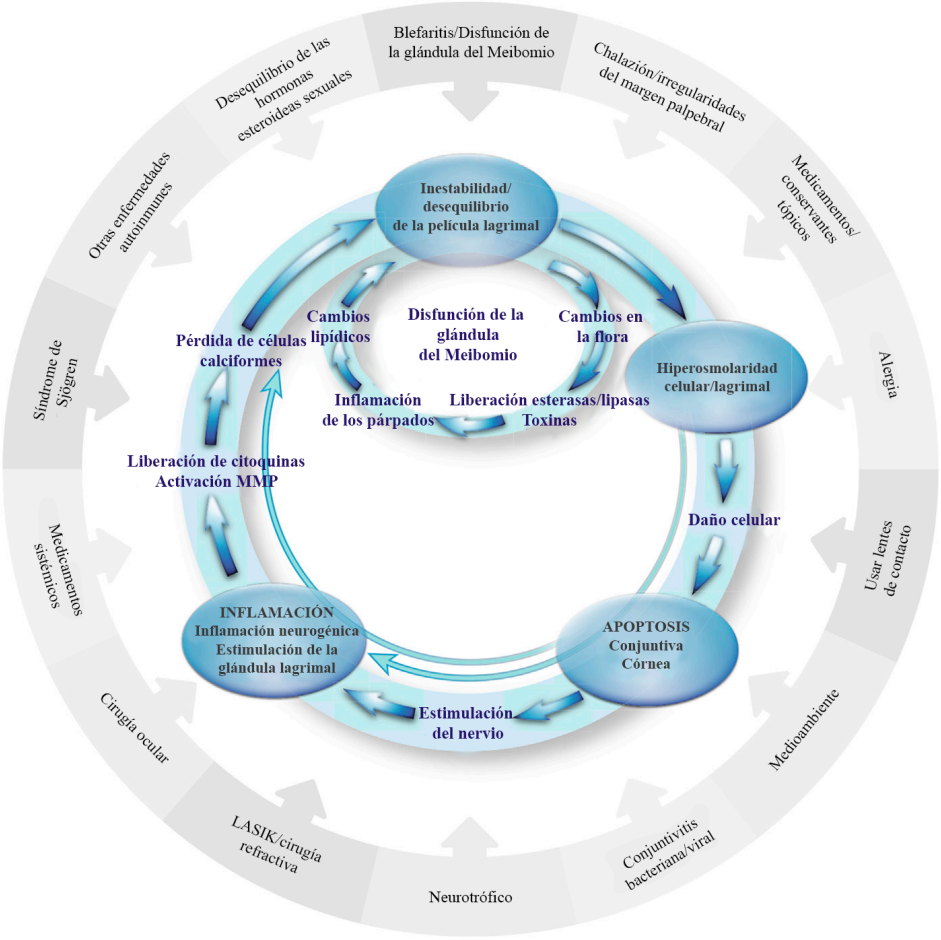

Figura 9. Circulo vicioso inflamatorio generado en la EOS produciendo inestabilidad de la película lagrimal y daño en la superficie. Imagen adaptada de "Baudouin C, Aragona P, Messmer EM, Tomlinson A, Calonge M, Boboridis KG, et al. Role of hyperosmolarity in the pathogenesis and management of dry eye disease: Proceedings of the ocean group meeting. Ocul Surf. 2013;11(4):246–58".

De este modo, aunque el ojo seco evaporativo y el acuo-deficiente tienen etiologías supuestamente diferentes tienden a compartir mecanismos fisiopatológicos similares donde el ambiente hiperosmolar acompañado de una cascada de eventos inflamatorios es común a ambos.

Como indica su nombre, el ojo seco evaporativo se produce cuando existe un exceso en la evaporación y las glándulas lagrimales funcionan correctamente. Este subtipo de ojo seco es el más común y normalmente se producen casos mixtos con el acuo-deficiente. Se caracteriza principalmente porque hay una disminución en la calidad de los lípidos que componen la película precorneal o una disminución en su secreción por parte de las glándulas de meibomio (40) acompañado en la mayoría de los casos por disminución en la frecuencia del parpadeo. También puede estar causado por eventos extrínsecos tales como

el uso de lentes de contacto, deficiencia de vitamina A, modificaciones en la superficie ocular, fármacos o conservantes presentes en formulaciones tópicas oculares.

El ojo seco acuo-deficiente se produce por una disminución en la secreción de lágrima y se puede dividir principalmente en dos tipos: asociado a síndrome de Sjögren y ojo seco no asociado a síndrome de Sjögren. Aquellos asociados al síndrome de Sjögren se caracterizan por una destrucción de diversas glándulas secretoras entre las que se encuentran las glándulas lagrimales y salivales, como consecuencia de procesos autoinmunes. Se divide en síndrome de Sjögren primario si afecta exclusivamente a las glándulas lacrimales y salivales o síndrome de Sjögren secundario si posee una afectación sistémica.

Con respecto al ojo seco acuo-deficiente no asociado al síndrome de Sjögren, cabe mencionar que se trata de un tipo de ojo seco producido por una disfunción lagrimal donde se ha descartado que esté relacionado con el síndrome de Sjögren y tienden a relacionarse con edad avanzada. Todo ello contribuye a un incremento de la osmolaridad lo que conlleva al inicio de la secreción de ILs y daño celular desencadenando los mecanismos mencionados anteriormente.

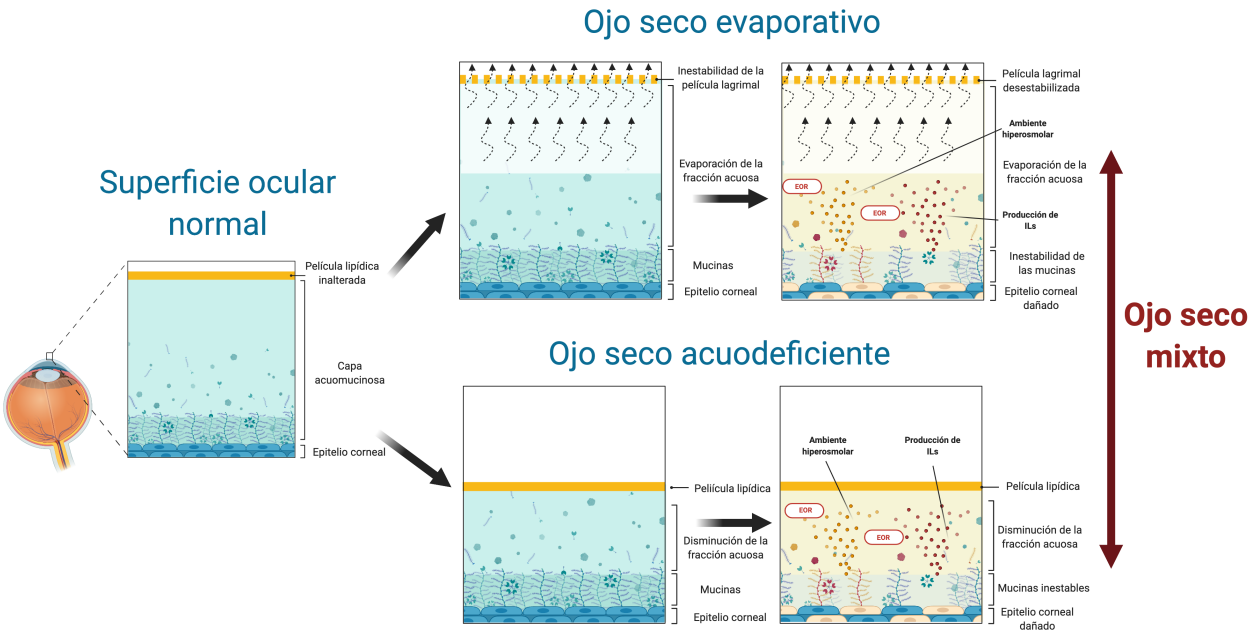

Figura 10. Resumen esquemático sobre la fisiopatología involucrada en la enfermedad de ojo seco causada por una inestabilidad de la película lipídica (evaporativo), una disminución en la producción de lágrima (acuo-deficiente) o una combinación de ambos (mixto).

3.1.3. Tratamientos para la enfermedad de ojo seco

Existen diversos tratamientos para la enfermedad de ojo seco dependiendo de la presencia o no de signos y síntomas, el tipo de ojo seco diagnosticado y las características a tratar.

Así mismo, de acuerdo al último informe internacional emitido por el comité de expertos del TFOS DEWS II 2017 (46), y por orden de prioridad según la severidad, las estrategias para el manejo y el tratamiento de la EOS se pueden clasificar en: tratamientos para **la insuficiencia lagrimal,** tratamientos destinados a **corregir anomalías en los párpados, terapia antiinflamatoria y** tratamientos **quirúrgicos**.

Entre los distintos tratamientos enfocados a corregir una **insuficiencia en la producción de lágrima** se encuentran el reemplazo, conservación y estimulación lagrimal.

Las terapias de reemplazo lagrimal son las más empleadas actualmente tanto por parte de la clínica como por parte de la propia población como medida preventiva debido mayormente a la gran cantidad de productos comercializados, muchos de ellos sin necesidad de prescripción. Además, se recomiendan en cualquier estadio de la enfermedad por su capacidad de producir un alivio en los síntomas asociados a la enfermedad.

A continuación, se detallan diferentes subcategorías de los agentes de reemplazo lagrimal:

- Sustitutos artificiales de la lágrima

  Los agentes sustitutos artificiales de las lágrimas conocidos también como lágrimas artificiales suponen una gran variedad de productos, que van dirigidos a varias capas de la película precorneal. Respecto a su seguridad, han sido catalogados como seguros, aunque en una revisión muy detallada se han descrito ciertos efectos adversos, la mayoría de ellos relacionados con la alta viscosidad de algunas formulaciones las cuales dificultan la visión o problemas asociados a la presencia de conservantes (47).

  En función de la naturaleza de sus componentes los sustitutos lagrimales pueden ser acuosos u oleosos.

- Acuosos

Los sustitutos de base acuosa tienen como objetivo lubricar la superficie ocular, incorporarse a la fracción acuo-mucinosa de la película precorneal y en muchos casos incrementar el tiempo de residencia. Entre ellos se encuentran los viscosizantes, osmóticos, osmoprotectores, antioxidantes, así como otros agentes sin actividad conocida.

- *Viscosizantes*

Poseen capacidad para incrementar el espesor de la película precorneal, proteger contra la desecación, incrementar la retención acuosa, proteger la superficie ocular as como regular la densidad de células caliciformes (48). Un problema asociado a la alta viscosidad de este tipo de agentes es la generación de visión borrosa o residuos no deseados en la periferia ocular.

Entre los agentes viscosizantes más utilizados se incluyen la hidroxipropilmetilcelulosa (HPMC), carboximetilcelulosa (CMC), hidroxipropilcelulosa (HPC), el ácido hialurónico (AH), hidroxipropilguar (HP-guar), dextrano, alcohol polivinílico (PVA), ácido poliacrílico (PAA), polivinilpirrolidona (PVP) y polietilenglicol (PEG). Algunos de ellos tienen propiedades mucoadhesivas como la CMC, la HPMC y el AH, que resultan muy interesantes por su interacción con las mucinas oculares (46).

El HPMC también conocido como hipromelosa presente en formulaciones comercializadas como el Optrex® y el CMC conocido como carmelosa presente en Viscofresh®, son agentes viscosizantes derivados de la celulosa, que tienen una amplia variedad de usos en la industria farmacéutica y cosmética, siendo ampliamente utilizados en lágrimas artificiales para el tratamiento de la EOS. Hay derivados celulósicos como la HPC que se emplean en la fabricación de insertos oculares como el LACRISERT® que protege contra la deshidratación y lubrica la superficie ocular con una liberación sostenida de polímero.

El AH pertenece al grupo de los mucopolisacaridos, cuya viscosidad se encuentra condicionada por su peso molecular. Su comportamiento no newtoniano se caracteriza por la disminución de la viscosidad cuando se

produce incremento de las fuerzas de cizalla que se dan durante el parpadeo y un aumento de la misma cuando el ojo permanece abierto (46). El HA es uno de los componentes más empleados en la elaboración de sustitutos lagrimales demostrando no solo aumentar el tiempo de retención en la superficie ocular de la formulación si no también contribuir a la reepitelización y regeneración del epitelio corneal (49) (50). Merece la pena mencionar que, recientemente, se han atribuido propiedades antiinflamatorias a los AH de altos pesos moleculares (particularmente aquellos cercanos a 1500 KDa)mientras que los de bajo peso molecular podrían ejercer un efecto contrario (51) (52).

La composición de las lágrimas artificial se encuentra en constante evolución. Una de las estrategias empleadas para conseguir preparados con una mayor eficacia ha sido la combinación de distintos biopolímeros. Un ejemplo es la combinación de AH (0,1%) con CMC (0,5%) que ha demostrado su capacidad protectora para la superficie ocular en el preparado comercializado Optive Fusion™ (46). También el AH (0,2%) se encuentra en combinación con HPMC (0,2%) en lágrimas artificiales comercializadas como es el caso de Optrex®. Otras tecnologías incluyen la gelificación "in situ" como la HP-guar en combinación con el ácido bórico como la presente en Systane® ULTRA en la que determinados estudios atribuyen una capacidad de retención de agua y actividad para reducir marcadores inflamatorios de la superficie ocular en la EOS y después de cirugía de cataratas (53). Además se ha estudiado la efectividad en modelos *in vitro* de desecación y la alta capacidad incrementar el tiempo de retención del HP-guar en combinación con AH en Systane® ULTRA HYDRATION (46).

El dextrano es un polisacárido de alto peso molecular utilizado en este tipo de preparaciones particularmente aquellos con pesos moleculares cercanos a 70KDa como agente lubricante por su capacidad para absorber agua (54) por lo que ha sido comúnmente utilizado como expansor plasmático. Tanto el PVA como el PAA constituyen polímeros formadores de geles "suaves" con una alta capacidad para retener agua. Se han empleado en preparados de lágrimas artificiales desde hace más de una treintena y continúan empleándose en la actualidad. Ambos mejoran los síntomas asociados a la EOS aumentando el

tiempo de ruptura de la película lagrimal (de las siglas en ingles "TBUT", "Tears break-up time). Aunque presentan eficacias similares el PAA podría mejorar los síntomas asociados a la EOS aunque algún estudio atribuye una baja tolerancia en células de córnea relacionada con este polímero (55)(56).

El PVP es utilizado por incrementar la retención acuosa (57) como en Vidisan®. El PEG suele ser incorporado en combinación con otros agentes exhibiendo al mismo tiempo propiedades demulcentes, es decir capacidad para proteger las mucosas de forma similar a las mucinas o secreciones fisiológicas (58). Estos últimos como el PVA, PAA o PEG se encuentran presentes por ejemplo en una gran variedad de colirios de la marca Systane®.

- *Osmóticos*

Otro aspecto importante en el desarrollo de las lágrimas artificiales ha sido el empleo de formulaciones ligeramente hipotónicas tratando así de contrarrestar la hiperosmolaridad generada en la EOS. De hecho la combinación de soluciones hipotónicas con agentes viscosizantes y reepitelizantes como por ejemplo el AH puede ser altamente beneficiosa en pacientes con EOS (59).

- *Osmoprotectores*

Se trata de compuestos osmóticamente activos con capacidad para modificar la captación de agua celular y proteger las células del epitelio corneal ante un estrés hiperosmolar (60). El uso de osmoprotectores en lágrimas artificiales comercializadas es relativamente novedoso con potenciales beneficios para el tratamiento ambientes hiperosmolares como es el caso de la EOS. Algunos ejemplos de osmoprotectores comercializados en colirios indicados para la EOS son el hylodual®, con ectoina la cual se ha visto muy efectiva en EOS con componente alérgico (61), Optava® advance con L-carnitina y eritritol ambos estudiados como potentes osmoprotectores (62) e Isomar® Occhi plus también con L-carnitina y con betaína, siendo esta última menos conocida con capacidad para regular altamente los mecanismos apoptóticos producidos durante estrés hiperosmolar (63).

- *Antioxidantes*

Un claro ejemplo que ha supuesto una estrategia efectiva en la reducción de la inflamación generada por estrés oxidativo en EOS es el uso de acetilcisteína tópica (64) como la que contiene el preparado comercializado con el nombre de Ilube®. El uso de la forma lipídica de la Vitamina A (ácido retinoico), palmitato de retinilo ha demostrado también una gran actividad antioxidante en pacientes con EOS bajo el nombre de VIVA Drops® como terapia coadyuvante (65). Otros compuestos como la quercetina o epigalocatequina poseen propiedades antioxidantes que resultan beneficiosas para la EOS. Otros agentes como el SKQ1, con actividad especifica mitocondrial, se encuentra comercializado con el nombre de Visomitin ® la (66). También la Selenoproteina P es una proteína plasmática que ha demostrado actividad extracelular especifica (67) capaz de suprimir los marcadores de estrés oxidativo en pacientes afectados de la EOS. Esta, es capaz de transportar específicamente selenio al interior celular, esencial en el correcto funcionamiento ante el estrés oxidativo de la enzima glutatión peroxidasa (68).

Las lágrimas artificiales incluyen "*otros componentes"* acuosos como los tampones empleados en la regulación del pH. El tampón fosfato ha sido empleado en muchas lágrimas artificiales, aunque su uso está disminuyendo debido a la aparición de úlceras corneales asociadas al empleo de preparados conteniendo dicho agente (69). Actualmente se emplean otros tampones como el tampón citrato, tampón boratos, este último también contribuyendo a la sustitución de agentes conservantes gracias a su capacidad antimicrobiana.

También se emplean soluciones electrolíticas que simulan el perfil salino de la lágrima como es el caso de TheraTear® con iones calcio, sodio, potasio, cloro y magnesio en concentraciones fisiológicas (70).

- Lipídicos

La suplementación con componentes lipídicos está ganando cada vez más interés y juega un papel muy importante en la integridad de la película precorneal. En la mayoría de los casos de EOS se acaba generando una inestabilidad en la

fracción lipídica de la película precorneal, muchos de ellos con una disfunción en las glándulas de meibomio, lo que contribuye a empeorar esta situación. Actualmente, una de las mejores estrategias para aportar componentes lipídicos a la superficie ocular como fosfolípidos o aceites minerales es mediante la elaboración de sistemas farmacéuticos que incluyan elementos lipídicos y acuosos como son los liposomas y emulsiones, que se describirán en el apartado de nuevas formulaciones en el tratamiento del ojo seco. Por ejemplo, la formulación comercializada bajo el nombre de Aquoral Lipo, se trata de una lágrima artificial lubricante a base de liposomas y AH. Hay en el mercado otros preparados basados en liposomas que no se podrían considerar como lágrimas artificiales ya que se administran fuera del ojo. Es el caso de Optrex™ ActiMist y Opticalm LYPOMIST® son espráis liposomales que se aplican sobre los párpados e incorporan los componentes lipídicos de los liposomas tanto a la película precorneal como a la cara interna palpebral donde se sitúan las glándulas de meibomio.

- Sustitutos biológicos

Considerados en algunos casos como los tratamientos tópicos actualmente más efectivos para el tratamiento de los síntomas asociados a la EOS con menos efectos adversos, los sustitutos biológicos se basan en compuestos bioactivos presentes en el ser humano con la intención de restaurar las condiciones fisiológicas de la superficie ocular, como diversos factores de crecimiento (epidérmico, nervioso, plaqueta, etc.) (71). Los sustitutos biológicos más empleado en el tratamiento de patologías de la superficie ocular, y en especial para las complicaciones de la EOS, son: el suero autólogo, el suero alogénico, el suero de cordón umbilical, las preparaciones y base de plaquetas.

- Suero autólogo

El suero autólogo consiste en la separación de la fracción sérica sanguínea, después de la coagulación. A continuación, se purifica seguido de su posterior dilución con cloruro sódico isotónico antes de la administración al paciente (72). La ventaja de este tipo de preparados es que posee características bioquímicas similares a las de las lágrimas, tales como factores de crecimiento, vitaminas o

nutrientes. Su empleo ha demostrado una capacidad superior para mejorar los parámetros de pacientes con EOS y en diferentes patologías de la superficie ocular (73). Sin embargo, existen algunos problemas aun por solventar como estandarización en los protocolos de fabricación, conservación, presencia de factores inflamatorios asociados a enfermedades que pueda padecer el paciente en ese momento así como la concentración adecuada a la que se debe emplear (46).

- Suero alogénico

Se trata de la fracción sérica sanguínea igual que el suero autólogo, pero proviene de otros individuos. De manera similar al suero autólogo, la presencia de factores biosimilares a los fisiológicos le convierte en una estrategia atractiva evitando algunos de los problemas asociados a la extracción o la presencia de factores inflamatorios ya que el suero proviene de un banco de sangre. Sin embargo, aunque ha demostrado beneficios a la hora de incrementar el número de células caliciformes y mejorar los síntomas asociados a la EOS (74), existe una preocupación creciente sobre la aparición de reacciones inmunológicas cruzadas.

- Suero de cordón umbilical

El suero procedente de cordón umbilical presenta las ventajas del suero alogénico, pero a diferencia del mismo, se puede utilizar para varios pacientes a partir de una única donación. Además, posee una mayor cantidad de factores del crecimiento que los presentes en las propias lagrimas demostrando una importante mejora de los valores presentes en citologías de impresión en pacientes con la EOS particularmente en aquellos con resistencia al tratamiento convencional (75). Una comparación también mostró en pacientes la superioridad del suero de cordón umbilical frente al suero alogénico (76).

- Preparaciones de plaquetas

Las plaquetas contienen una mayor cantidad de factores de crecimiento que el suero o plasma incrementando la proliferación celular de las células epiteliales de córnea expuestas a plaquetas procedentes de plasma rico en plaquetas (77,78). Las preparaciones a base de plaquetas para el tratamiento de patologías de la

superficie ocular y en concreto de la EOS suponen a menudo una preparación compleja cuyos beneficios sobre el uso de suero autólogo o alogénico aún no se han confirmado. Sin embargo, existen algunos preparados comercializados como el ENDORET® PRGF® obtenido a partir de una pequeña muestra sanguínea, demostrando una gran capacidad de regeneración de heridas (79).

- Otros agentes de reemplazo

Otros tratamientos propuestos que pueden mejorar la sintomatología y complicaciones de la EOS suponen el uso de mucolíticos por vía oral como el ambroxol o bromhexina los cuales han demostrado los síntomas asociados a la EOS en pacientes con síndrome de Sjögren (80). Tal y como se ha descrito anteriormente el uso de acetilcisteína por sus propiedades antioxidantes y también por su capacidad mucolítica mejoran los síntomas oculares (64).

En los últimos años ha adquirido un interés especial el empleo de ARN de interferencia en el tratamiento del ojo seco. En la superficie ocular existen receptores de potencial transitorio vanilloide tipo 1 (TRPV1) los cuales tienen la capacidad para activarse ante un estrés hiperosmolar, desencadenando eventos proinflamatorios y pro apoptóticos (81). El preparado Tivanisirán contiene un ARN de interferencia dirigido a los receptores TRPV1, produciendo una mejora significativa de los síntomas en pacientes con EOS (82). La inhibición selectiva de este receptor puede suponer una estrategia terapéutica para el desarrollo de nuevos tratamientos potenciales. (62).

Además, las estrategias de conservación lagrimal como su nombre indica, tienen como principal objetivo evitar una disminución en la producción, así como una perdida lagrimal. Entre la mayoría de los enfoques propuestos y aplicados se encuentran procedimientos quirúrgicos como la oclusión puntal evitando el drenaje de las lágrimas (83), aunque se desconoce los posibles problemas asociados ante un incremento de la concentración de interleucinas inflamatorias en la superficie. También se ha propuesto el uso de humificadores o cámaras de humedad a modo de gafas como estrategias para incrementar la humedad y por tanto disminuir la evaporación lagrimal (84).

INTRODUCCIÓN

En última instancia de tratamientos enfocados a la insuficiencia lagrimal se encuentran aquellos basados en la estimulación lagrimal, donde se administran diferentes agentes capaces de conseguir una hiperestimulación de la secreción endógena tanto de los componentes acuosos y mucinosos como de los lipídicos que componen la lágrima.

- Secretagogos tópicos

  Este tipo de tratamiento tiene como objetivo estimular la producción endógena de mucinas y lágrimas. El diquafosol comercializado bajo el nombre de Diquas®, es un ejemplo de secretagogo acuoso, que activa los receptores purinérgicos P2Y2, y la producción de agua y mucinas por parte de las células conjuntivales y caliciformes. El diquafosol ha demostrado producir una disminución en los marcadores inflamatorios de la superficie así como una mejora en el *tear break-up time* (TBUT) en pacientes con EOS (85). Dentro de los estimulantes de la producción de mucinas se incluye la rebamipida, actualmente aprobada en Japón para el tratamiento de la EOS, agente capaz de estimular la producción de glicoproteínas por parte de las células epiteliales de córnea, aumentando así los niveles de MUC1, MUC4 y MUC16 mediante la regulación del factor de crecimiento epidérmico (86,87). También la galectina-3 es cuyo mecanismo de acción se centra en el aumento de la expresión de la mucina formadora de geles MUC5AC, y ha demostrado reducir el daño corneal en modelos de ojo seco (88).

  Otro tipo de agente estimulante de la secreción acuosa es la lacritina, una glicoproteína con actividad mitogénica, pro-secretora y estimulante de la supervivencia. La lacritina posee un gran potencial terapéutico para el tratamiento de la EOS concretamente en aquellos pacientes afectados por síndrome de Sjögren (89).

  Otro secretagogo interesante, es el micofenolato de mofetilo, inhibidor linfocítico, que a bajas concentraciones puede aumentar la proliferación de células caliciformes conjuntivales así como la expresión de ácido ribonucleico mensajero (ARNm) para MUC5AC (90).

  El factor trébol 1 (de las siglas en ingles “TFF-1”, “Trefoil factor-1”), pertenece a una subfamilia de factores del tipo lectinas, productos de degradación de las mucinas, cuyas concentraciones se encuentran aumentadas en la EOS y cuya

inhibición se postula como una posible diana terapéutica (91). Finalmente, cabe destacar la eupatilina, compuesto tipo flavona con una potente actividad para inducir la secreción de mucinas tanto *in vitro* como *in vivo* (92).

- Estimulación de la secreción lipídica

Existen pocos compuestos que sean capaces de estimular de forma directa la secreción de componentes lipídicos de la película precorneal. Uno de ellos es el factor de crecimiento similar a la insulina IGF-1 (de las siglas en ingles "IGF-1", "Insulin-like Growth factor 1") agente capaz de estimular la secreción lipídica por parte de las células presentes en las glándulas de meibomio (93). También los andrógenos como la testosterona administrados por vía tópica ocular han demostrado resultados prometedores incrementando la secreción lipídica de las glándulas de meibomio en pacientes con EOS en ensayos clínicos de fase II reduciendo los síntomas asociados (94) (95).

- Secretagogos orales

El grupo de secretagogos orales son particularmente utilizados en pacientes con síndrome de Sjögren. La pilocarpina y la cevimelina, son dos claros ejemplos de secretagogos orales que son capaces de mejorar los síntomas asociados a la EOS, pero sin incrementar significativamente la producción de lágrima (96). Se emplean fundamentalmente para prevenir la xerostomía en este tipo de pacientes (97).

- Neuroestimulación nasal

La neuroestimulación nasal consiste en promover la estimulación por vías físicas o químicas del arco reflejo nasolacrimal que acaba en la innervación parasimpática de glándulas lacrimales y por tanto estimula la producción de lágrimas (98).

Existe un dispositivo llamado TrueTear™ que se compone de un extremo estimulador con base de hidrogel que suministra pequeñas descargas eléctricas al nervio etmoidal anterior, estimulando las glándulas lacrimales. Se ha comprobado su efecto para incrementar la producción de lágrimas en pacientes afectados de la EOS (99).

- Otros métodos de estimulación

  Entre los diversos métodos restantes encaminados a la estimulación de las glándulas lacrimales como respiración abdominal o administración de cafeína, la activación de los canales catiónicos de los receptores de potencial transitorio de la subfamilia M, miembro 8 (de las siglas en inglés "TRPM8", "Transient Receptor Potential Cation Channel, Subfamily M, Member 8") parece que podrían ser de utilidad en el tratamiento de la EOS. De hecho, se cree que el mentol, es capaz de activar los receptores TRPM8 en el extremo terminal de los termorreceptores del frío (100).

Los **tratamientos destinados a corregir anomalías en los párpados** suponen una estrategia para incrementar el funcionamiento de las glándulas de meibomio, es por ello que están especialmente indicados en pacientes con anomalías importantes en dichas glándulas.

Algunos de estos tratamientos se basan en la higiene de párpados y pestañas para evitar obstrucciones en las glándulas, lubricantes con sustancias lipídicas como los mencionados anteriormente o antimicrobianos cuando dichas anomalías están ocasionadas por infecciones.

Otros métodos físicos comprenden el uso de apósitos de calor en los párpados para incrementar la fluidez de los lípidos, pulsos de luz, y debridamiento físico cuando se genera una obstrucción glandular por depósitos de material queratinizado (46).

La **terapia antiinflamatoria** es de gran interés ya que es capaz de de regular los mecanismos por los cuales se inicia el circulo vicioso generado por un incremento en la osmolaridad lagrimal en pacientes con la EOS (101).

- Glucocorticoides tópicos

  De acuerdo a la amplia experiencia en el manejo de la EOS, durante el paso de los años, ha sido ampliamente demostrado que el uso de glucocorticoides tópicos resulta altamente eficaz y es capaz de romper o atenuar los mecanismos inflamatorios anteriormente mencionados. El papel del sistema inmune en patologías inflamatorias de la superficie ocular y particularmente en la EOS es reconocido por la comunidad

científica por lo que explicaría la alta efectividad de los tratamientos a base de esteroides y otros inmunomoduladores (102).

Un claro ejemplo es el uso de metilprednisolona (1%) tópica agente capaz de inhibir la expresión de interleucinas y MMPs (103). La metilprednisolona ha demostrado también gran efectividad en reducir los niveles de citocinas proinflamatorias en pacientes con EOS (104). Por otro lado, la fluorometolona (0,1%) produce incluso un incremento de la producción lagrimal demostrando resultados altamente efectivos en combinación con ciclosporina A, de la cual se hablará posteriormente (105).

Recientemente se está estudiando la efectividad de una combinación triple de metilprednisolona (1%), un antagonista del receptor de interleucina 1 (5%) y ciclosporina A (0,05%) (106). La combinación de agonistas selectivos de los receptores de glucocorticoides con glucocorticoides parece ser una terapia efectiva en el tratamiento de la inflamación asociada a la EOS sin mostrar una elevación en los niveles de PIO (107), aunque el uso de dexametasona tópica se relaciona con un aumento de la PIO y aparición de cataratas.

El etabonato de loteprednol, se trata de uno de los glucocorticoides tópicos más estudiados para el tratamiento de patologías de la superficie ocular incluido la EOS y se asocia con una menor cantidad de efectos adversos incluida la posible generación de cataratas (108). Su uso exclusivo para el tratamiento a corto plazo de síntomas moderados de la EOS no fue aprobado por la agencia Americana de alimentos y fármacos (de las siglas en inglés “FDA”, “Food and Drug Administration) hasta el año 2020 a una concentración de 0,25% bajo el nombre de Eysuvis® (109).

Con respecto a otros glucocorticoides alternativos se encuentra comercializada una emulsión tópica ocular al 0,05% de difluprednato, el cual ha demostrado una alta capacidad antiinflamatoria y efectividad para ciertas patologías oculares con mínimos efectos adversos (110) pero que se encuentra bajo estudio para su empleo como tratamiento para la EOS.

- Inmunomoduladores no esteroideos

Tal y como se describe anteriormente en la EOS existen componentes inflamatorios e inmunológicos por lo que el uso de inmunomoduladores ha supuesto una línea de tratamiento efectiva.

Probablemente, los agentes inmunomodulares más conocidos para este tipo de patologías son la ciclosporina A y el tacrolimus. La ciclosporina A (CsA) supone uno de los tratamientos más efectivos para la EOS, siendo comúnmente utilizada en casos severos y moderados desde su aprobación por la FDA en el año 2003. La CsA se ha prescrito como fórmula magistral en un gran número de casos ya que la primera formulación comercializada Restasis® no fue aprobada para su uso en Europa. Actualmente existe una formulación comercializada en España por los laboratorios Santen con el nombre de Ikervis®. La CsA presenta una elevada unión a ciclofilinas, proteínas responsables de la activación de los linfocitos T mediante la IL-2. El complejo formado por CsA-ciclofilina se une inhibiendo la acción de las calcineurinas, enzimas dependientes de calcio encargadas de activar los linfocitos T y estimular la producción de interleucinas proinflamatorias. Por esta razón, la CsA ha demostrado producir una reducción del marcador inflamatorio HLA-DR en células de conjuntiva y córnea de pacientes con EOS. También posee la capacidad para reducir la apoptosis en células de conjuntiva de modelos de EOS y disminuir la excesiva proliferación conjuntival que normalmente conlleva una metaplasia asociada. Como consecuencia de ello, el tratamiento tópico con CsA produce una elevación del número de células caliciformes y por tanto un incremento en la producción de mucinas, mejorando la estabilidad de la película precorneal y disminuyendo considerablemente los síntomas asociados a la EOS (111). Cabe la pena destacar que la efectividad entre la CA (0,05%) y el tacrolimus (0,03%) es similar para aliviar los síntomas moderados asociados a la EOS en pacientes con síndrome de Sjögren, probablemente debido a su mecanismo de acción similar (112).

Respecto al uso de antiinflamatorios no esteroideos (AINEs) para el tratamiento de la EOS, el diclofenaco y el ketorolaco han resultado útiles para aliviar los síntomas asociados a la inflamación generada en la superficie ocular. El ketorolaco reduce los síntomas asociados a la EOS, demostrando alta eficacia también en

combinación con la CsA (113). Sin embargo, el uso de diclofenaco está siendo estudiado debido a la gran capacidad antiinflamatoria que posee frente a estrés hiperosmolar. Este mecanismo se debe a que es probable que se encuentre involucrado en la regulación del factor nuclear 5 de células T activadas (de las siglas en inglés "NFAT5", "Nuclear Factor of Activated T-cells 5"), el cual participa en la osmoprotección celular frente a estrés hiperosmolar. Puede que este mecanismo sea exclusivo del ketorolaco y por ello suponga una excepción dentro de los AINEs ya que otros no han demostrado estas mismas características (114).

Finalmente, como inmunomodulares de interés cabe destacar la lubricina, así como los neuropéptidos sustancia P y péptido vasoactivo intestinal (de las siglas en inglés "VIP", "Vasoactive Intestinal Peptide"). La lubricina es un proteoglicano con un dominio común similar al de las mucinas, con altas propiedades lubrificantes responsable de reducir fricciones entre tejidos, la superficie ocular concretamente entre la córnea y la conjuntiva. La lubricina ha demostrado resultados muy prometedores en ensayos clínicos a concentraciones de 150 μg/mL para reducir los signos y síntomas en pacientes con EOS (115). Respecto a la sustancia P o VIP así como otros "neuromediadores", su elevación en pacientes con EOS temprana y disminución ante la enfermedad crónica han sugerido una importante función protectora y su uso tanto como marcadores de la enfermedad (116) como futuros tratamientos (117).

- Antagonistas del antígeno 1 asociado a la función linfocitaria (LFA-1)

  También conocido como Lifitegrast, se trata de un antagonista que bloquea la unión del antígeno 1 asociado a la función linfocitaria (LFA-1) y la molécula de adhesión intercelular (de las siglas en inglés "ICAM-1", "Intercellular Adhesion Molecule 1") a los receptores de las células T, para de esta forma evitar su activación (118). Lifitegrast ha demostrado una mejoría significativa de los síntomas de pacientes con EOS en ensayos clínicos (119).

- Modulación inflamatoria con antibióticos tópicos y sistémicos

  El uso de terapia antibiótica como tetraciclina, doxiciclina o macrólidos para el tratamiento de la EOS, se ha empleado con el objetivo de regular la flora microbiana

presente en los párpados. Se cree que la microflora juega un papel importante en la degradación de los lípidos producidos por las glándulas de meibomio y que por tanto una disminución de su degradación contribuiría a la secreción de una fracción lipídica más estable en ciertos pacientes. Por ejemplo, la azitromicina y dexametasona en combinación han conseguido una importante mejoría de casos con EOS en ensayos clínicos (120). Sin embargo, el riesgo de aparición de resistencias bacterianas, efectos adversos y el bajo número de datos de eficacia clínica genera cierta reticencia ante su posible aprobación (46).

Los tratamientos quirúrgicos

Los tratamientos quirúrgicos se encaminan principalmente hacia la modificación física de ciertos componentes estructurales que puedan estimular la producción de lágrima, o de lípidos por parte de las glándulas de meibomio. También se contempla el transplante de glándulas salivares o microvasculares submandibulares que mejoran los síntomas notablemente (46).

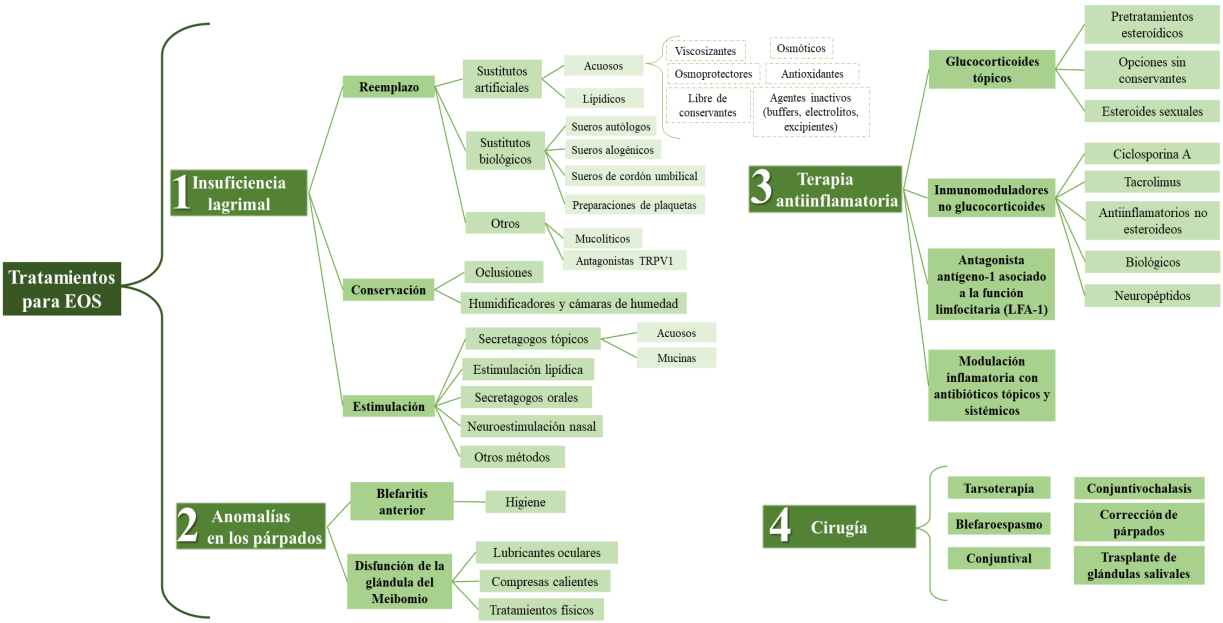

Figura 11. Clasificación de los tratamientos disponibles y aquellos en evaluación para la enfermedad de ojo seco por orden de prioridad de menor a mayor gravedad, adaptado del informe TFOS DEWS II 2017 sobre terapias para la EOS "Jones L, Downie LE, Korb D, Benítez-del-Castillo JM, Dana R, Deng SX, et al. TFOS DEWS II Management and Therapy Report. Ocul Surf 2017; 15:575–628. doi:10.1016/j.jtos.2017.05.006".

3.1.4. Nuevos sistemas de administración tópica ocular para patologías del segmento anterior

Se ha investigado mucho durante los últimos años para conseguir desarrollar nuevos sistemas efectivos de administración tópica para el tratamiento de patologías de la superficie ocular entre las que se encuentra la EOS. Así mismo, aquellos con uso potencial para el tratamiento de la EOS se pueden clasificar en: sistemas farmacéuticos con componentes **lipídicos, sistemas poliméricos y otros sistemas**.

Los sistemas con componentes **lipídicos** a son de particular interés, ya que los componentes que los conforman pueden contribuir a incrementar la estabilidad de la fracción lipídica de la película precorneal. Además, muchos de ellos contienen un compartimento acuoso y otro lipídico por lo que se pueden adoptar diferentes estrategias en función de que zonas de la película precorneal o del segmento anterior se quieran alcanzar. Dentro de éstos se encuentran los liposomas, niosomas, nanopartículas lipídicas, emulsiones y exosomas.

- Liposomas

  Los liposomas probablemente son unos de los sistemas más atractivos para el tratamiento de la EOS y patologías de la superficie ocular, ya que al estar constituido por compartimentos acuosos y oleosos permiten administrar en un único preparado sustancias de naturaleza hidrófila y lipófila. Los liposomas se pueden definir como vesículas compuestas una fase lipídica que, en proporciones específicas, permitan la formación de bicapas con un núcleo acuoso en su interior (121).Los fosfolípidos se emplean en muchos casos para la elaboración de liposomas.

  La incorporación de fosfolípidos con un alto contenido en ácidos grasos insaturados como el caso de la fosfatidilcolina de soja en la elaboración de liposomas para el tratamiento de la enfermedad de ojo seco , ha supuesto una estrategia atractiva ya que se consigue dotar de mayor estabilidad a la película lipídica corneal aportando a la vez capacidad antioxidante (122). Además, la inclusión en los liposomas de otros lípidos que se encuentran presentes en la película lagrimal ayuda a estabilizar la capa lipídica de la película precorneal (123). El colesterol es uno de los componentes de la película lipídica y además de contribuir a una mejora de la

capa lipídica de la película precorneal, desde el punto de vista tecnológico se puede incorporar a los liposomas con el fin de dotar a a la bicapa de estabilidad y rigidez tal y como se puede observar en la figura 12. Además, La incorporación de agentes antioxidantes como la vitamina E ha sido adoptado como estrategia en algunas ocasiones para evitar una excesiva oxidación de ácidos grasos insaturados (123). En los últimos años están apareciendo estudios en los que se desarrollan liposomas a partir de fosfolípidos sintéticos cuya estabilidad es superior a la de los fosfolípidos naturales (124).

En cuanto a su clasificación, existen cuatro tipos fundamentales de liposomas en función de su tamaño. Los SUVs o vesículas unilamelares pequeñas (del inglés "SUV", "Small Unilamellar Vesicle"), los LUVs o vesículas unilamelares grandes (del inglés "LUV", "Large Unilamellar Vesicle"), los MLVs o vesículas multilamelares grandes (del inglés "MLV", "Multi Lamellar Vesicle") y los GUVs o vesículas unilamelares gigantes (del inglés "GUV", "Giant Unilamellar Vesicle").

Los SUVs poseen un único compartimento con una sola bicapa lipídica con tamaños comprendidos entre 20 y 100 nm. Los LUVs también tienen una única bicapa lipídica, pero presentan un mayor tamaño (entre 100 y 1000 nm). Por el contrario, los MLVs no solo poseen un tamaño mayor, entre 1 y 50 μm, sino que además poseen múltiples bicapas lipídicas formando una estructura a capas de tipo cebolla. Sin embargo, también existe un tipo de liposomas, los GUVs con tamaños de entre 1 y 50 μm, pero a diferencia de los MLVs, los GUVs poseen un único compartimento y por tanto una única bicapa lipídica (121).

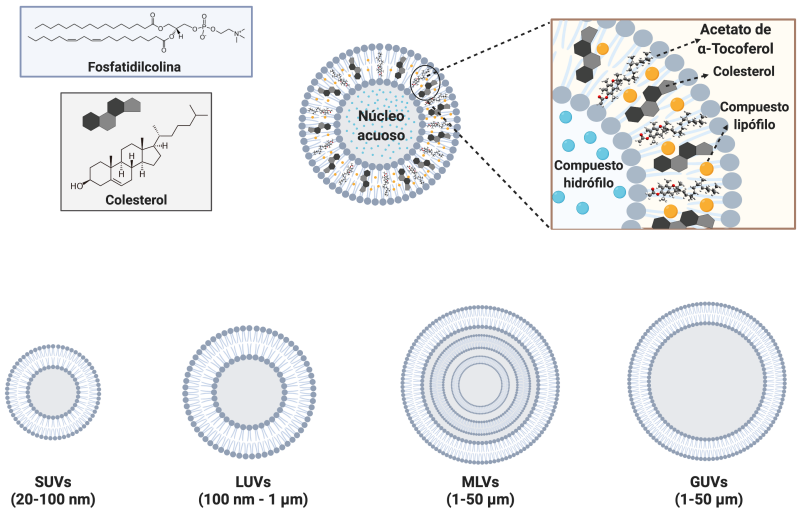

Figura 12. Estructura y clasificación de los liposomas. Imagen adaptada de "López-cano JJ, González-cela-Casamayor MA, Herrero-Vanrell R, Molina-Martínez IT, Javier J, González-cela-Casamayor MA, et al. Liposomes as vehicles for topical ophthalmic drug delivery and ocular surface protection. Expert Opin Drug Deliv 2021;18:819–47. doi:10.1080/17425247.2021.1872542.".

En cuanto a los métodos de fabricación, se encuentran el método de Bangham o rehidratación de la película lipídica, evaporación de fase reversa, deshidratación-rehidratación, congelación-descongelación, sonicación, inyección de éter o etanol, fusión liposomal inducida por calcio y por sistemas de microfluídica. Probablemente el método de Bangham, es el más conocido, aunque presenta el inconveniente de tener que someter la muestra normalmente a procesos de extrusión o de reducción de tamaños ya que tiende a formar MLVs con una elevada polidispersión (123). El método de evaporación de fase reversa es similar al de Bangham, aunque en este se crean dos fases invertidas que finalmente darán lugar a la formación de nuevo de MLVs (125). La rehidratación-deshidratación se utiliza para producir tamaños de vesícula específicos, ya que al tener lugar una rehidratación lenta y en condiciones controladas se evita la polidispersión (126). La congelación-descongelación se ha usado tradicionalmente para incrementar la eficacia de encapsulación de ciertos compuestos hidrosolubles en el núcleo acuoso del liposoma descubriéndose que además se reduce el tamaño de los MLVs (127). El método de sonicación realmente se utiliza para reducir el tamaño de MLVs a SUVs (128). El método de inyección etanólica o de éter se emplea a menudo para generar un escalado industrial de SUVs

mediante una adición lenta de una mezcla de lípidos en etanol o éter a una solución acuosa (129). El método de fusión inducida por calcio se basa en la interacción de iones calcio con las membranas lipídicas para fusionarlas entre sí y generar GUVs (130). Finalmente, el método por microfluídica es uno de los de elección debido a la alta capacidad para controlar el tamaño de partícula así como generar un escalado a nivel industrial si se desea (131).

Cabe la pena mencionar que uno de los mayores inconvenientes con respecto a la producción de liposomas son los métodos de esterilización, ya que los sistemas formados no soportan las elevadas temperaturas del autoclavado y el calor seco. Es por eso que se recurre a la filtración esterilizante siempre y cuando los tamaños de partícula sean inferiores a 200 nm o el vapor de óxido de etileno, el cual ha demostrado ser un método eficaz de esterilización sin alterar su estructura ni comportamiento (132).

Los liposomas son herramientas terapéuticas muy útiles para restaurar la película precorneal pudiendo incorporar además sistemas de gelificación in situ para incrementar su retención (133).

- Niosomas

Los niosomas, son vesículas similares a los liposomas compuestas por una membrana lipídica normalmente estabilizada por colesterol pero sin fosfolípidos y con tensoactivos no iónicos (134). Los niosomas son químicamente más estables que los liposomas a la presencia de los tensoactivos no iónicos debido a la mayor estabilidad de estos últimos en comparación con los fosfolípidos. Además, presentan costes más bajos de producción y mayor adaptación para el escalado. Aunque por lo general presentan buena biocompatibilidad y baja citotoxicidad la concentración de tensoactivos es un factor determinante a la hora de desarrollar una formulación de este tipo (135).

Los niosomas presentan un rango de tamaño más estrecho, y es más sencillo disminuir su tamaño mediante ciclos de congelación-descongelación (136). Al igual que los liposomas también pueden existir SUVs, LUVs o MLVs (135). Con respecto al tratamiento a su uso en enfermedades de la superficie ocular, los niosomas han

demostrado ser una herramienta potencial para la administración de distintos agentes como la doxiciclina como terapia para la rosácea y la EOS de manera efectiva (137). Debido a sus características fisicoquímicas y a que también han demostrado efectividad para la administración terapia génica (138), podrían suponer un vehículo eficaz en la administración de moléculas génicas como ARNm o de interferencia en la EOS.

- Nanopartículas lipídicas sólidas

Las nanopartículas lipídicas sólidas (NLS) son sistemas matriciales formados por lípidos que se encuentran al estado sólido a temperatura ambiente y surfactantes. Los NLS forman sistemas altamente estables, con una menor descomposición lipídica que los sistemas en estado líquido, capaces de generar liberaciones sostenidas (139) e incorporar un perfil lipídico biocompatible con la película precorneal.

Una variación de las NLS son los portadores lipídicos nanoestructurados (de las siglas en inglés "NLC", "Nanostructured Lipid Carriers"). Los NLC incorporan lípidos en estado líquido, como por ejemplo aceites. De esta forma los NLC son sistemas mixtos con una parte sólida cristalizada, una parte liquida y los surfactantes (140).

Aunque se tata de sistemas poco explorados para el tratamiento de la EOS, existen NLS incorporando CsA como tratamientos innovadores para tratar la inflamación y los síntomas de la EOS (141). También se ha incorporado exitosamente el glucocorticoide dexametasona en NLC como tratamiento para la EOS, mostrando una alta reducción de las MMP y citocinas proinflamatorias (142).

- Emulsiones

Las emulsiones son mezclas de componentes acuosos y lipídicos inmiscibles entre sí que se estabilizan mediante el empleo de agentes surfactantes y/o cosolventes. A su vez pueden ser de fase externa acuosa y fase interna oleosa llamadas emulsiones O/A o de fase externa oleosa y de fase interna acuosa, llamadas emulsiones A/O. Para administración tópica ocular las más utilizadas son las de tipo O/A y existen

diferentes tipos en función de su tamaño y estabilidad termodinámica (macroemulsiones, nanoemulsiones o microemulsiones).

Las macroemulsiones o emulsiones son sistemas termodinámicamente inestables cuyo tamaño de gotícula de la fase interna tiene un tamaño superior a 1μm pudiendo oscilar hasta los 100 μm. En los preparados empleados para la superficie ocular, aportan la ventaja de suministrar grandes cantidades de componentes lipídicos, aunque experimentan un rápido aclaramiento y debido a su opalescencia y consistencia ocasionan a menudo visión borrosa o presencia de residuos en la periferia ocular. Dentro de las lágrimas artificiales en forma de emulsión y que aportan la ventaja de aumentar el espesor de la capa lipídica se encuentra Refresh Endura® y Systane® o Soothe® (143)(144). Otras emulsiones comercializadas son Retaine OCuSOFT® o NEOVIS® TOTAL para el alivio de los síntomas leves de la EOS.

Las nanoemulsiones, constituyen un tipo de emulsiones más estables que las macroemulsiones, pero termodinámicamente siguen siendo inestables con tamaños de gotícula que oscilan desde los 20 hasta los 500 nm aunque tiende a situarse en la región más elevada del rango de tamaños (145). Poseen siempre una morfología de gotícula esférica con polidispersiones que oscilan entre el 10 y el 20%. Las nanoemulsiones pueden ser tanto translucidas como opalescentes y sus métodos de fabricación comprenden aquellos tanto de baja como de alta energía, siendo estos últimos más comunes, debido a la necesidad de sobrepasar ciertas barreras energéticas para su formación (146,147).

Su tamaño de gotícula reducido y su mayor estabilidad, así como la posibilidad de incluir una alta concentración de componentes lipídicos, las convierte en sistemas atractivos para suplementar la fracción lipídica y estabilizar la película precorneal. De hecho, junto con los liposomas, son de los sistemas más estudiados para la administración de fármacos por vía tópica ocular y para el tratamiento de la EOS (148,149).

Actualmente existen algunas nanoemulsiones comercializadas para el tratamiento de la EOS Una de ellas se encuentra basada en la tecnología denominada Novasorb®, donde mediante la adición de un agente conservante llamado cloruro de cetalconio en

una baja concentración, dotan a la misma de una carga positiva incrementando las interacciones con las cargas negativas de la superficie ocular y aumentando así su tiempo de retención. La lágrima artificial basada en esta tecnología recibe el nombre de Cationorm® (150). Utilizando esta misma tecnología, se ha comercializado en España Ikervis®, como una nanoemulsión catiónica incluyendo el principio activo ciclosporina para el tratamiento de la queratitis grave en casos de xeroftalmia (148).

Systane® COMPLETE es una nanoemulsión de consistencia lechosa y opalescente para el alivio de los síntomas leves de la EOS. desarrolla la nanoemulsion comercializada que utiliza este sistema recibe el nombre de

Por otro lado, Restasis® también se trata de una nanoemulsión oftálmica (151), aniónica en este caso incorporando CsA para el tratamiento de los síntomas moderados de la EOS (107) pero que no se encuentra aprobado en la Unión Europea.

Las microemulsiones suponen sistemas termodinámicamente estables con tamaños nanométricos muy reducidos, entre 5 y 100 nm, desarrollados mediante métodos de baja energía y con dispersiones de tamaño inferiores al 10% (146)(145). Estos pueden presentar morfologías esféricas o adoptar otro tipo de forma según la curvatura óptima del surfactante, dependiendo que sistema sea más estable desde un punto de vista energético. Aunque a veces, es complicado establecer las diferencias entre microemulsiones y nanoemulsiones, quizás uno de los factores que más las diferencia sea su estabilidad. A esto hay que añadir las características fisicoquímicas de las microemulsiones que permanecen inalterables en el tiempo teóricamente a menos que se produzca una degradación química o microbiológica de alguno de sus componentes (145). Esta característica hace de ellas, sistemas idóneos para aumentar el tiempo de retención, suplementar la película lipídica con componentes acuosos y oleosos que incrementen su estabilidad (152) o incrementar la penetración de fármacos en los tejidos oculares (153). Otra gran ventaja de las microemulsiones es el bajo coste de fabricación debido al empleo de métodos de baja energía, así como su fácil escalabilidad y adecuadas características fisicoquímicas para el almacenamiento a largo plazo. Las microemulsiones se están explorando como sistemas novedosos para la elaboración de lágrimas artificiales, así como para la administración de sustancias activas poco hidrosolubles. De hecho la CsA ha sido

una de los primeros fármacos que se han encapsulado en este tipo de emulsiones para incrementar su efectividad (153).

- Exosomas

Los exosomas son nano vesículas formadas por una bicapa lipídica similares a los liposomas de producción endógena celular que pueden oscilar entre los 40-100 nm. A diferencia de los liposomas, los exosomas a parte de la propia composición lipídica y acuosa, contienen una gran cantidad de sustancias biológicas procedentes del interior celular, como proteínas, marcadores, aminoácidos o material genético (154). Aunque no sean de producción sintética, el uso reciente de exosomas como potencial tratamiento para la EOS, ha demostrado resultados muy prometedores.

El empleo de exosomas provenientes de células mesenquimales estromales para el tratamiento de EOS asociada a la enfermedad injerto contra huésped demostró resultados muy prometedores tanto en animales como ensayos clínicos con humanos (155). Recientemente también se ha mostrado la habilidad de ciertos exosomas para actuar como inmunomoduladores en enfermedades de origen inmune como el síndrome de Sjögren, incrementando considerablemente la regeneración corneal y disminuyendo los factores inflamatorios asociados a la enfermedad (156).

Los sistemas basados en **polímeros** ofrecen ventajas atractivas a la hora de suministrar agentes protectores a la superficie ocular. Principalmente su uso está justificado debido a la presencia de polímeros biodegradables que pueden conseguir cesiones sostenidas en el tiempo con un alta biocompatibilidad y permeación asi como la posibilidad de desarrollar también dispositivos biodegradables que permanezcan en superficie durante tiempos prolongados.

- Nanopartículas

Entre la mayoría de los sistemas basados en nanopartículas que están actualmente en investigación, las nanopartículas de ácido poliláctico co-glicólico (de las siglas en inglés “PLGA”, “Poly Lactic-co-Glycolic Acid”) suponen un enfoque interesante a la hora de vehiculizar fármacos altamente lipófilos como la ciclosporina A (157). El

uso de nanopartículas de PLGA es particularmente atractivo ya que se trata de un polímero aprobado por la FDA para la elaboración de dispositivos (158).

- Insertos

El uso de insertos para el tratamiento de patologías como la EOS está debido a la baja absorción y retención de los principios activos en la superficie ocular. El implante ocular denominado LACRISERT® supone uno de los primeros de este tipo, biodegradable y compuesto por HPMC ha demostrado mejorar considerablemente los síntomas leves-moderados asociados a la EOS. Se administra en el cul-de-sac y se ha comprobado que su seguridad es adecuada aunque existe la necesidad de educar a la población antes de su uso ya que la mayoría de los posibles efectos asociados a la formulación derivan de una colocación inadecuada (159).

- Microesferas

Se tratan de sistemas matriciales en los que se pueden incorporar diferentes fármacos de manera homogénea. Si el polímero empelado es biodegradable desparece del lugar de administración tras la liberación de la sustancia activa. (160). Aunque poco explorado, el uso de las microesferas de PLGA o polímeros similares ha sido estudiado por su biodegradabilidad y biocompatibilidad. De igual manera que con los insertos anteriormente mencionados y recubiertas por agentes mucoadhesivos que incrementen su adhesión al saco conjuntival, se pretenden depositar en el cul-de-sac o saco conjuntival para la liberación prolongada de fármacos como tratamiento sostenido de la EOS (161).

- Dendrímeros

Los dendrímeros son moléculas bien definidas con simetría radial de estructura ramificada esférica (162). Poseen tamaños reducidos y pueden ser utilizados como vehículos de fármacos lipófilos mediante su conjugación. La administración subconjuntival de dendrímeros conjugados con dexametasona ha demostrado la normalización de marcadores proinflamatorios asi como del TBUT en modelos in vivo de EOS (163).

- Hidrogeles

Los hidrogeles son sistemas formados por una agrupación de redes fibrosas o micelares de origen polimérico que se agrupan y entrecruzan entre sí, de manera química o física formando estructuras matriciales tridimensionales atrapando agua y componentes hidrofílicos en su interior.

Así mismo, el proceso de gelificación puede estar mediado tanto por un entrecruzamiento físico o químico entre las diferentes cadenas poliméricas como deberse a una agrupación micelar para cierto tipo de hidrogeles inteligentes (164).

Respecto a este tipo de sistemas, aquellos compuestos por AH han ganado mucho interés debido a su conocida actividad para tratar los síntomas asociados a la EOS. Por ejemplo, se han desarrollado hidrogeles de ácido hialurónico tiolado entrecruzados, demostrando en ensayos preclínicos en animales su alta efectividad para atenuar los síntomas inflamatorios y mejorar los síntomas de la enfermedad (165).

Por otro lado, el uso de AH modificado con grupos vinisulfona genera hidrogeles entrecruzados más suaves pudiendo resultar mejor tolerados y más fluidos, mejorando también los síntomas de inflamación ocular (166). Actualmente comercializadas existen las lágrimas artificiales Systane® ULTRA con goma gelano, un polisacárido vegetal capaz de gelificar in situ expuesto al calcio lagrimal (167).

- Lentes de contacto

El uso de lentes de contacto ha supuesto recientemente una plataforma para la administración de fármacos eficaces en la EOS. Según se ha especulado para las lentes de contacto blandas, existe una película lagrimal post lente (entre la lentilla y la superficie corneal) que se renueva cada 30 minutos lo que permite una liberación más sostenida de fármacos o suplementos para reponer la película precorneal (168).

La mayor parte de las lentes de contacto que se han desarrollado hasta la fecha en la búsqueda de este tipo de tratamiento alternativo están compuestas por hidrogeles de silicona incluyendo muchas de ellas ácido hialurónico y algunas HPMC. Una de las principales ventajas es que este tipo de sistemas pueden alcanzar liberaciones que

se encuentran comprendidas entre horas y días dependiendo también de su tolerancia. Algunas conteniendo PVP, han conseguido mayores tiempos de liberación (169). Además, para el tratamiento de los procesos inflamatorios que se dan en la EOS, se han incluido algunos glucocorticoides como la dexametasona consiguiendo reducciones significativas de la inflamación en la superficie ocular (170). La inmersión de las lentes de contacto en soluciones de vitamina E o soluciones coloidales de nanopartículas o emulsiones suponen estrategias recientes que podrían mejorar considerablemente el futuro de los pacientes con EOS (168).

También existen **otros sistemas** los cuales pueden suponer herramientas alternativas permitiendo combinar varias características de los mencionados anteriormente (figura 13).

- Nanomicelas

Las Nanomicelas son dispersiones coloidales transparentes, formas por micelas de tamaño nanométrico entre 10 y 100 nm, altamente homogéneas con un núcleo hidrofóbico y presencia de surfactantes no iónicos. Actualmente suponen una herramienta muy útil no solo para incrementar la solubilización de sustancias poco hidrosolubles sino también para incrementar su estabilidad y evitar su posible degradación (171).

Debido a las características de estos nanosistemas semejantes a las disoluciones y homogeneidad de los principios activos encapsulados, se han propuesto como transportadores de fármacos para patologías tanto de la superficie ocular como la EOS como del segmento posterior (172).

- Nanosuspensiones

Son sistemas sólidos en suspensión de tamaño nanométrico, con una alta capacidad para incrementar la solubilidad de sustancias altamente liposolubles y mejorar su biodisponiblidad (173). Un claro ejemplo de nano suspensión para el tratamiento de la EOS es la formulación comercializada bajo el nombre de Eysuvis®, con etabonato de loteprednol, demostrando resultados prometedores para disminuir los síntomas de inflamación en la superficie ocular (174).

El hecho de que se encuentre comercializada y que se estén desarrollando nuevos sistemas de administración oftálmica basados en nanosuspensiones abre la puerta para el desarrollo de nuevas terapias para la EOS y otras patologías de la superficie basadas en Nanosuspensiones (173).

Figura 13. Esquema y clasificación de los Nuevos sistemas de administración ocular para enfermedades del segmento anterior y de aplicación a la EOS.

3.2. Patologías neurodegenerativas del segmento posterior

3.2.1. Patologías más relevantes, impacto y mecanismos fisiopatológicos

Las patologías neurodegenerativas pueden ser descritas de manera general como un conjunto de enfermedades que se caracterizan por generar una disminución y deterioro progresivo funcional y estructural del sistema nervioso periférico o central. El nervio óptico se considera como una prolongación del sistema nervioso central, y al tratarse además la retina de células nerviosas las patologías que afectan a esta zona se consideran dentro de esta clasificación. (175). Al estar localizadas en el segmento posterior reciben la denominación de patologías neurodegenerativas del segmento posterior. Entre las

patologías más destacadas que cursan con neurodegeneración en el segmento posterior se encuentran: el glaucoma, la retinopatía diabética (RD), el edema macular diabético, la degeneración macular asociada a la edad (DMAE) y patologías genéticas como la retinosis pigmentaria.

La RD, la DMAE y el glaucoma se encuentran entre las principales causas de ceguera en el mundo siendo esta última la principal causa de ceguera irreversible según la organización mundial de la salud (OMS) (176). Las tres enfermedades comparten mecanismos fisiopatológicos similares como son el desarrollo de eventos ligados al estrés oxidativo y la estimulación de procesos inflamatorios (177).

El glaucoma, se puede definir como un grupo de enfermedades caracterizadas por la muerte progresiva de las células ganglionares y una neuropatía del nervio óptico que produce un daño irreversible en el campo visual. El aumento de la presión intraocular (PIO) es el principal factor de riesgo asociado con el comienzo y la progresión de la enfermedad, aunque hay pacientes con valores normales de la PIO que desarrollan la enfermedad. Por esta razón, la terapia del glaucoma se centra en la disminución de la PIO en aquellos pacientes que cursan con valores elevados y la neuroprotección en todos los casos. La terapia hipotensora tópica es la primera línea de tratamiento en los sujetos con PIO elevada. Si ésta disminuye de un 30 a un 50%, la progresión de la enfermedad se reduce considerablemente. El glaucoma más común es el glaucoma primario o de ángulo abierto La edad avanzada así como la presión intraocular elevada son los principales factores de riesgo para el desarrollo de glaucoma primario o de ángulo abierto (178).

La PIO elevada de forma crónica tiende a producir una compresión en las dendritas de las fibras axonales y estructuras adyacentes, especialmente de la lámina cribosa conllevando cambios morfológicos celulares irreversibles y modificando la función celular (179). A raíz de estos procesos de muerte celular y modificaciones estructurales se desencadenan una serie de cambios metabólicos y proinflamatorios incrementándose la producción de EOR (180). Además, la disminución de la presión intracraneal es considerada un factor de riesgo importante junto con el incremento de la presión intraocular. Con respecto al glaucoma normotensivo, factores como la disfunción autonómica, problemas vasculares que produzcan una hipoxia transitoria en el nervio

óptico o casos de hipotensión sistémica nocturna también están vinculados al desarrollo de este tipo de glaucoma con la consiguiente degeneración de las neuronas de la retina (181). Es por ello por lo que la terapia neuroprotectora cobra especial importancia en todos los pacientes que padecen de glaucoma.

La RD es una complicación derivada de la presencia de una hiperglucemia crónica que produce una desregulación en el metabolismo de la microvasculatura ocular principalmente en la retina y la coroides. El exceso de glucosa crónico produce una alteración en el metabolismo celular afectando principalmente a las mitocondrias y desencadenando procesos apoptóticos en las células endoteliales que componen los capilares y vasos sanguíneos de la retina. Al producirse un engrosamiento de los capilares y vasos sanguíneos se pueden originar hemorragias y sangrados (182). En función de si existe o no angiogénesis la RD se puede dividir en proliferativa y no proliferativa. Una complicación asociada a la RD es el edema macular diabético (EMD). El EMD se produce por una alteración y engrosamiento en la estructura de la retina, cuando debido a la elevada permeación vascular e inflamación se produce una acumulación de líquido excesiva en las diferentes capas de la retina que no puede drenarse correctamente (183). Inicialmente se reduce la función visual ya que la acumulación de líquidos actúa como barrera física entre la luz incidente y los fotoreceptores. Cuando esta se instaura de forma crónica, la repetición de eventos apoptóticos y la activación de la microglía conlleva la degradación de la capa de fotorreceptores. Como resultado e incluso posteriormente a su resolución, la alteración estructural así como las micro isquemias persistentes disminuyen considerablemente la capacidad visual (184).

La DMAE, esta principalmente causada por la pérdida de fotoreceptores y células del epitelio pigmentario de la retina. Los mecanismos fisiopatológicos de la DMAE se encuentran principalmente asociados al estrés derivado por la fotooxidación lumínica y al estrés oxidativo debido al envejecimiento Los procesos de daño inducido por fotoxidación lumínica están asociados a longitudes de onda corta como es el caso de la radiación ultravioleta y la luz azul. Cuando la luz altamente energética incide en los cromóforos principalmente melanina y lipofuscina, presentes en los fotoreceptores y las células del EPR, se produce un cambio químico molecular produciendo la formación de “tripletes” muy reactivos con moléculas adyacentes desencadenando un daño progresivo (185). En función del estado en el que se encuentre el daño, y en función de su progresión

(neovascularización, atrofia y daño persistente), la DMAE puede clasificarse en temprana, intermedia o avanzada.

El progreso de la DMAE a etapas avanzadas puede darse con o sin neovascularización, que es lo que comúnmente se describe como DMAE húmeda o seca, respectivamente (186). La aparición del estrés oxidativo en la DMAE se debe principalmente a la acumulación de elementos celulares degradados y que experimentan una oxidación celular, normalmente conocidos como drusas. La oxidación de los mismos genera al mismo tiempo cambios metabólicos e incremento en la producción de interleucinas (187).

Tanto en la RD, la DMAE y el glaucoma se producen una serie de modificaciones metabólicas que conllevan la producción excesiva de radicales libres incluidas las EOR, estimulando la producción de una cascada inflamatoria así como distintos mecanismos de muerte celular (188). Como consecuencia se produce una disminución del crecimiento axonal e impulso sináptico, produciéndose muerte neuronal. Paralelamente, la producción excesiva de EOR incrementa la expresión de diferentes vías inflamatorias como la NF-κB, estimulando principalmente la producción de TNFα, IL-6, IL-8, factor ICAM-1 y factor de crecimiento endotelial vascular (de las siglas en inglés "VEGF", "Vascular Endothelial Growth Factor"). En la RD, la evolución hacia RD proliferativa ocurre principalmente cuando se produce un exceso de factor 1 inducible por hipoxia (de las siglas en inglés "HIF-1", "Hypoxia Inducible Factor)(177).

También es importante resaltar que, en las diferentes patologías descritas anteriormente, se produce una activación excesiva de las células de la glía, astrocitos y células de Müller incrementándose sustancialmente la cantidad de glutamato y N-metil-D-aspartato (NMDA). Una elevación de glutamato y NMDA genera una hiper excitación lo que provoca una entrada masiva de iones calcio al interior celular (figura 14). La entrada de calcio provoca la activación desmesurada de endonucleasas, proteasas y fosfolipasas produciendo daños sustanciales en el citoesqueleto asi como diferentes estructuras celulares. Como respuesta, se produce una gran cantidad de radicales libres lo que favorece, junto con el calcio intracelular, la apoptosis de los diferentes tipos neuronales (189).

Todos estos mecanismos generan un daño axonal primario desencadenando la muerte celular de neuronas adyacentes y promoviendo la sobre activación de la microglía cerrando asi el círculo vicioso (177).

Finalmente, la gran cantidad de EOR junto con los procesos anteriormente mencionados (figura 15), disminuyen la producción de diferentes factores neurotróficos esenciales para el correcto funcionamiento de las células neuronales de la retina así como las células ganglionares o bipolares conllevando hacia una neurodegeneración irreversible (177). Con respecto a la retinosis pigmentaria, se trata de un grupo heterogéneo de enfermedades de origen genético que cursan con una degeneración progresiva de los fotoreceptores y del EPR. Una de las causas fundamentales son las mutaciones recesivas producidas en el gen RPE65, el cual codifica para la enzima isomerasa que produce el 11-cis-retinal esencial al mismo tiempo para la supervivencia de los fotoreceptores. Además, también se han relacionado una gran variedad de genes tanto dominantes como recesivos, involucrados en alteraciones mitocondriales desencadenando alteraciones metabólicas, procesos inflamatorios y apoptosis celular(190). Del mismo modo que con las mencionadas anteriormente existe un aspecto común, la producción de EOR, generando una serie de eventos de estrés oxidativo empeorando la evolución de la enfermedad y acelerando la muerte de los fotoreceptores y el EPR (191).

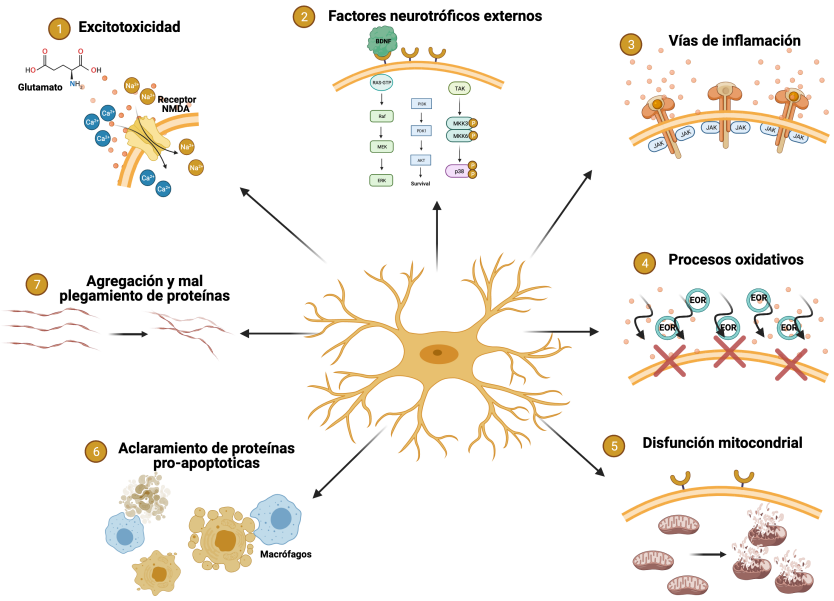

Figura 14. Mecanismos generales de los procesos degenerativos de la retina que surgen a partir de las células gliales.

3.2.2. Tratamientos para el tratamiento de patologías neurodegenerativas del segmento posterior

Tal y como se ha mencionado anteriormente las patologías neurodegenerativas que afectan al segmento posterior son multifactoriales y por lo tanto, no existe un único tratamiento o estrategia para abordarlas.

En el caso del glaucoma, en aquellos pacientes que cursan con valores elevados de la PIO se acude como terapia de primera elección a los agentes hipotensores que se administran por vía tópica. Dentro de los agentes hipotensores se encuentran los siguientes: bloqueantes β-adrenérgicos, agonistas adrenérgicos α-2, análogos de prostaglandinas, inhibidores de la anhidrasa carbónica, inhibidores de la rho-quinasa y agonistas de los receptores de adenosina.

- Bloqueantes β-adrenérgicos

  Los fármacos betabloqueantes ejercen su acción sobre el epitelio de los procesos ciliares, responsables de la estimulación de la producción de humor acuoso, produciendo un bloqueo de la inervación simpática de los mismos. Su paso a circulación sistémica puede conllevar algunos efectos adversos como broncoespasmos, bloqueo cardiaco o bradicardia. A su vez, existen bloqueantes β-adrenérgicos selectivos y no selectivos. Algunos ejemplos clásicos de no selectivos, comúnmente indicados para el tratamiento del glaucoma son el maleato de timolol con el que se consiguen reducciones importantes de la PIO (30%) o el carteolol que también protegen contra estrés oxidativo inducido por foto exposición (192). En cuanto a los selectivos, el betaxolol es un bloqueante β1 selectivo que reduce la PIO en torno a un 22%, presentando menos reacciones adversas. También merece la pena destacar el bamosiran, un ARN de interferencia que bloquea la expresión de los receptores β2-adrenérgicos (193).

- Agonistas adrenérgicos α-2

  Los agonistas adrenérgicos α-2 actúan mediante la activación de los receptores α-2 que disminuyen los niveles de adenosín monofosfato cíclico (AMPc) en el epitelio del cuerpo ciliar, generando un descenso en la producción de humor acuoso. Se cree

también que aumentan el drenaje del humor acuoso a través de la vía uveoescleral, pero esto último mecanismo es aún incierto (194). Un ejemplo bien conocido de agonista α-2 adrenérgico es el tartrato de brimonidina, comercializado bajo el nombre del Alphagan®, siendo administrados como segunda línea de tratamiento.

- Análogos de prostaglandinas

Las prostaglandinas suponen una de las primeras líneas de tratamiento para la reducción efectiva de la PIO en pacientes con glaucoma de ángulo abierto. A su vez, los más comunes son los análogos de prostaglandinas agonistas del receptor F2-α (PGF2). Su mecanismo principal de acción se basa en el remodelado de la matriz extracelular de los músculos ciliares así como en su relajación, incrementando el drenaje del humor acuoso a través de la malla trabecular y uveoescleral, consiguiendo así una reducción media de la PIO de alrededor de un 30% (195). Latanoprost, travoprost, tafluprost y bimatoprost son los principales análogos de prostaglandinas actualmente disponibles bajo los nombres de Monoprost®, Travatan®, Tapros® y Lumigan®. Merece la pena destacar, que existe comercializado un implante biodegradable de bimatoprost (Durysta®) aprobado por la FDA. Dicho dispositivo se administran en cámara anterior consiguiendo un efecto hipotensor sostenido durante varios meses sin la necesidad de llevar a a cabo instilaciones repetidas.

Por otro lado, también existen otro tipo de análogos de prostaglandinas más novedosos, los cuales actúan sobre los receptores E2 (PGE2) incrementando el drenaje del humor acuoso por la vía uveoescleral. El isopropilo de omidenepag como de aganepag son dos ejemplos de PGE2, que aumentan el drenaje de humor acuoso tanto por la vía uveoescleral como a través de la malla trabecular. Su efectividad es similar a la del latanoprost y bimatoprost, pero con un mejor perfil de seguridad (193).

Finalmente existen un tipo de prostaglandinas mixtas donantes de óxido nítrico. Están compuestas por una fracción análoga de PGF2 y otra donante de óxido. Mediante la liberación de óxido nítrico se consigue un efecto relajante en la malla trabecular y el canal de schelem, facilitando la salida del humor acuoso y la

activación de los receptores de prostaglandina incrementando el drenaje. Algunos ejemplos son el latanoprosteno bunod y el NCX-470 (196).

- Inhibidores de la anhidrasa carbónica

Los IAC, disminuyen la producción de humor acuoso mediante la inhibición de la enzima anhidrasa carbónica, evitando por tanto la formación de bicarbonato. Actualmente los más usados son los IAC de segunda generación como la dorzolamida (Trusopt®) o brinzolamida (Azopt®), con muchos menos efectos adversos asociados que los de primera generación.

- Inhibidores de la rho-quinasa

La unión de este tipo de agentes a las proteínas las rho-quinasas produce un cambio conformacional en las mismas. La activación de las rho-quinasas regulan los mecanismos de permeabilidad de membranas, contractilidad asi como adhesión celular y reorganización de la matriz extracelular teniendo un papel importante en el drenaje del humor acuoso en la malla trabecular. Su bloqueo por tanto produce un aumento tanto en la salida del humor acuoso como en el drenaje a través de la malla trabecular (197). El ripasudil es un inhibidor de la rho-quinasa que modifica la malla trabecular incrementando la permeabilidad a través del canal de Schlemm, mientras que el nertasudil, reduce la presión en las venas epiesclerales e incrementa el drenaje través de la malla trabecular (193).

- Agonistas de los receptores de adenosina

La adenosina es un componente modulador local que está implicado en diferentes funciones metabólicas, entre las que se encuentra la neuroprotección mediante la activación de la xantina oxidasa y bloqueo de los radicales superóxidos. También está implicada en la regulación de las vías de producción y drenaje del humor acuoso. Por tanto, su activación estimula los mecanismos de control sobre las rutas de drenaje y formación del mismo (198). El trabodenoson, es un agonista del receptor A1 de adenosina, que produce un aumento del drenaje del humor acuoso disminuyendo sustancialmente la PIO.

Es importante destacar que muchas de las terapias tópicas hipotensoras, como el maleato de timolol, latanoprost, o brimonidina poseen efectos adversos significativos en la superficie ocular (199). En muchos casos, la administración crónica (una vez al día) y la combinación de distintos agentes hipotensores favorece la aparición de efectos indeseados en la superficie ocular como el desarrollo de procesos inflamatorios e inmunomediados conllevando la aparición de la EOS o exacerbando la sintomatología en aquellos pacientes que ya la padecían (200). Además, el uso de conservantes en muchos de ellos, como el cloruro de benzalconio, ampliamente conocido por su toxicidad sobre el epitelio corneal y conjuntival, desencadena una serie de eventos, que contribuyen a la la desestabilización de la película precorneal (201) o la perdida de células caliciformes, que acaban con la perdida de adherencia por parte del paciente e incremento de la PIO, lo que facilita el progreso hacia eventos neurodegenerativos de la retina. Por tanto, urge la necesidad de desarrollar por tanto nuevas terapias que promuevan la protección de la superficie ocular e incrementen la efectividad hipotensora incrementando la adherencia y evitando la aparición de procesos degenerativos de la retina.

Como ya se señaló anteriormente, tanto la RD, como la DMAE, el glaucoma y la retinosis pigmentaria cursan con neurodegeneración. Dada la etiología multifactorial de estas enfermedades un tratamiento neuroprotector efectivo debería combinar distintas sustancias activas capaces de prevenir la muerte neuronal mediante la regulación e inhibición de las cascadas patológicas involucradas que tienen como efecto resultante una disfunción y muerte celular (202).

Aunque se está investigando la utilidad de los tratamientos orales y tópicos para el tratamiento de las enfermedades neurodegenerativas del segmento posterior, el acceso de las sustancias activas al lugar de acción se encuentra dificultada debido a la existencia de las barreras hematoacuosa y hematoretiniana en la administración oral y la baja biodisponibilidad de los principios activos administrados cuando se emplea la vía tópica. (203).

La **terapia oral** está, en la mayoría de los casos, destinada al tratamiento de aquellas causas sistémicas que puedan desencadenar un empeoramiento de ciertas enfermedades neurodegenerativas de la retina. Por ejemplo, en algunos casos de retinosis pigmentaria que desarrollan edema macular cistoide, se ha administrado por vía oral inhibidores de la

anhidrasa carbónica (IAC) como la acetazolamida (Diamox^{TM}). Estos son capaces de disminuir la permeabilidad vascular e incrementar el transporte de sustancias a través de la barrera hematoretiniana (BHR) (204). Aunque menos común, también se emplean en algunas ocasiones los IAC en el manejo del glaucoma, ya que se ha visto que mejoran el flujo retrobulbar , por sus propiedades vasodilatadoras además de su efecto hipotensor (205).

También se han empleado análogos del ácido fíbrico como el fenofibrato, suplementos con pigmentos xantofílicos o antioxidantes como el tocoferol, oxido de zinc o ácidos insaturados, resveratrol o coenzima Q10 que también han demostrado mejorar considerablemente los parámetros de agudeza visual en pacientes con RP (206). De hecho la coenzima Q10 y la citicolina se postulan como tratamientos potenciales en la degeneración retiniana en el glaucoma (205). También se han empleado inhibidores de la enzima convertidora de angiotensina (IECA) como el Enalapril y los bloqueantes del receptor de angiotensina II (ARAII), losartán y candesartán en el tratamiento de la RD y el edema macular diabético (206).

Existen ciertas terapias por **vía tópica** que parecen contribuir a la mejora de la progresión de algunas enfermedades de la retina como la RD o la RP. Por ejemplo, el uso de los AINEs ketorolaco, nepafenaco administrados por esta vía se ha visto que mejoran el engrosamiento de la mácula y los parámetros de agudeza visual en pacientes con RD (206). Los antagonistas de los receptores de bradiquinina β1, los inhibidores de la tubulina (OC-10X) o los promotores de la cardiolipina mitocondrial (MTP-131) son algunos ejemplos de tratamientos tópicos que se encuentran actualmente en ensayos clínicos o preclínicos con resultados alentadores con respecto a la RD (206).

La administración intraocular es la más empleada en la práctica clínica ya que se realiza el depósito directo en el lugar de acción o en una zona cercana al mismo. Los principales tratamientos por vía intraocular comprenden la terapia anti angiogénica, antiinflamatoria, génica y terapia celular.

- Anti angiogénicos

  Como su nombre indica, este tipo de tratamientos están indicados con el objeto de inhibir la angiogénesis que se produce en algunas enfermedades neurodegenerativas

como la DMAE o RD, que genera a su vez un aumento un de la permeabilidad vascular y alteraciones en el metabolismo que conllevan a la muerte celular (207). Este efecto, se consigue bloqueando el VEGF mediante la administración intravítrea de agentes anti-VEGF. Por ejemplo, el aflibercept (Eylea®) y ranibizumab (Lucentis®) son de uso común tanto en la DMAE como en la RD, produciendo una reducción significativa en la formación de nuevos vasos sanguíneos. Pegaptanib, conbercept y brolacizumab son agentes anti-VEGF indicados para la DMAE o neovascularización en el glaucoma. Otro agente conocido es el bevacizumab, utilizado en el tratamiento de la RD (208).

- Antiinflamatorios

El uso de antiinflamatorios es ampliamente utilizado en el tratamiento de las patologías degenerativas de la retina, ya que en estas enfermedades se genera un círculo vicioso que acelera el proceso lesivo de la retina. La administración intravítrea de glucocorticoides como la dexametasona, triamcinolona o fluocinola es eficaz y ha demostrado disminuir la progresión de enfermedades como la RD o la DMAE (206). También el uso de AINEs intravítreos como el diclofenaco suponen una estrategia útil en el manejo de estas enfermedades (206). Sin embargo, estos fármacos poseen una semivida muy corta, y se tiene que acudir a la administración de inyecciones repetidas, con la consiguiente incomodad del paciente asi como los riesgos asociados a la inyección (hemorragias, cataratogénesis, desprendimiento de retina etc.). Además, el riesgo de aparición de efectos secundarios aumenta con el número de inyecciones. Es por ello que en las últimas décadas se han desarrollado implantes intravítreos (biodegradables y no biodegradables) capaces de liberar de forma sostenida agentes antiinflamatorios y evitar así las administraciones sucesivas. Dentro de los de implantes no biodegradables de agentes de antiinflamatorios se encuentran Retisert®, Iluvien® y el recientemente aprobado Yutiq®. Todos ellos cargados con acetónido de fluoncinola, se encuentran indicados para evitar la progresión del edema macular diabético (209). La desventaja de estos implantes es que permanecen en el interior del ojo durante el resto de vida del paciente. Hasta el momento, existe un único implante intraocular biodegradable de agentes antiinflamatorios comercializado con el nombre de Ozurdex®. Se trata de un implante biodegradable de dexametasona, gracias al cual se consigue una cesión

sostenida del agente activo. Dicho implante ha, demostrado su seguridad con una tasa de resolución del 78% de casos de edema macular diabético, con menos de 3 administraciones durante más de dos años (210).

- Terapia génica

El uso de oligonucleótidos anti sentido con el objeto de evitar la transcripción de los exones asociados a determinados genes relacionados con la RP ha potenciado la investigación en terapia génica para el tratamiento de esta enfermedad (211). También se ha investigado el uso de vectores adenovirales destinados a corregir diversas mutaciones en el gen RPE65 durante los últimos años (212). Probablemente el mayor avance con respecto a la terapia génica sea el desarrollo de el vector voretigene neparpovec comercializado bajo el nombre de Luxturna®, medicamento huérfano, indicado para el tratamiento de la amaurosis congénita de Leber y la RP bialélica. Se trata de un vector viral adeno-asociado conteniendo dos copias sanas del gen RPE65 que se administra gracias a una inyección subretiniana en la zona central de la fóvea, de. Tras una única inyección , se consigue la resolución en una gran parte, recuperando la visión completamente(213).

- Terapia celular

El NT-501 Neurotech es un implante intravítreo que se encuentran en fase de ensayos clínicos y contiene células en su interior células genéticamente modificadas productoras del factor neurotrófico ciliar (de las siglas en inglés “CNTF”, “Ciliary Neurotrophic Factor”). EL CNTF es capaz de retardar la degeneración de los fotoreceptores, incrementando su supervivencia y mejorando sus funciones en la RP (214). Además, para el tratamiento de la DMAE se encuentra en ensayos clínicos un implante de subretiniano, de células madre embrionarias derivadas de EPR (215).

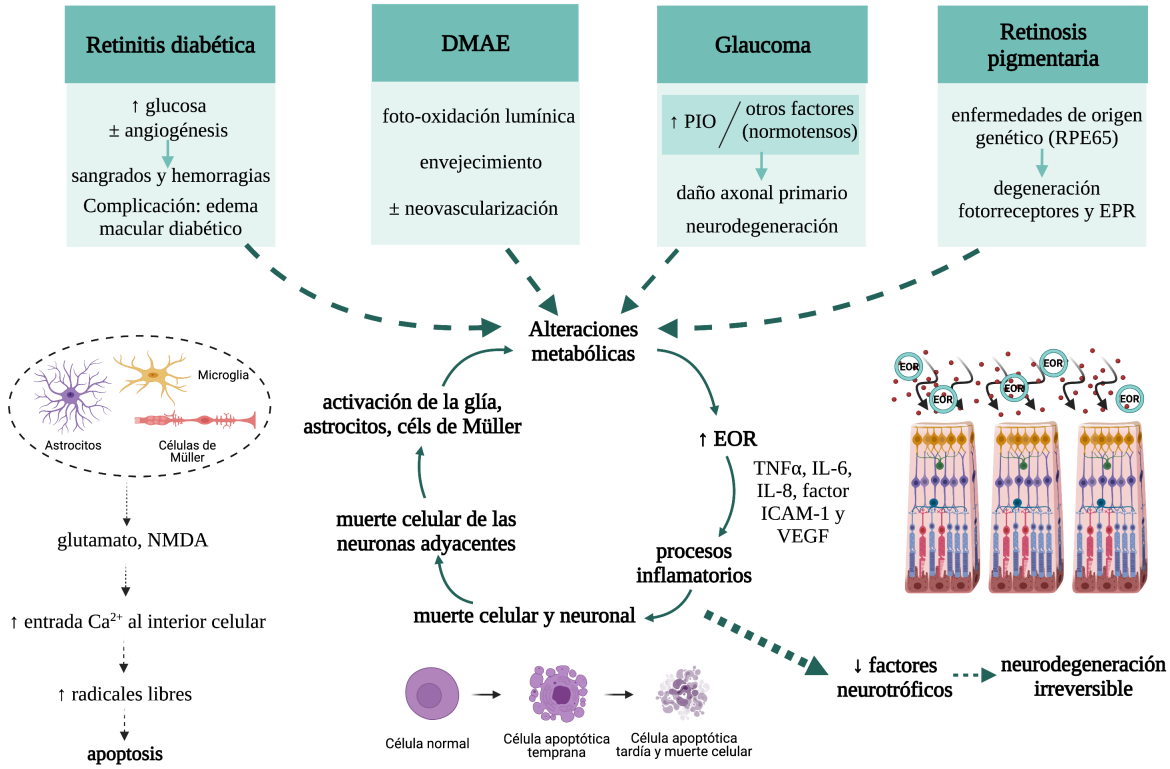

Figura 15. Mecanismos fisiopatológicos comunes sobre la inflamación y el estrés oxidativo de las principales patologías neurodegenerativas de la retina.

3.2.3. Nuevos sistemas de administración de fármacos para patologías neurodegenerativas de la retina

En la línea de lo anteriormente expuesto, existen numerosos estudios relacionados con el desarrollo de nuevos sistemas de liberación de fármacos para el tratamiento de patologías degenerativas de la retina. de En cuanto a la administración tópica, el diseño de nanosistemas con capacidad para acceder al segmento posterior y ejercer una acción neuroprotectora están cobrando un gran interés. El desarrollo de colirios tópicos de nanomicelas o nanopartículas de agentes antiinflamatorios como la dexametasona asi como liposomas con sustancias neuroprotectoras han demostrado ser los sistemas más prometedores para la administración tópica de sustancias neuroprotectoras (216). Hasta el momento actual, la vía de administración intravítrea ha demostrado ser la más eficaz en controlar el progreso de este tipo de enfermedades. Es por ello que muchos de los nuevos sistemas de liberación controlada de fármacos tienen como objetivo incrementar la eficacia de las inyecciones intravítreas asi como disminuir la frecuencia de administración. Dentro

de este tipo de sistemas se encuentran utilidad para la administración de fármacos las micropartículas (principalmente las microesferas) y nanopartículas poliméricas, hidrogeles, dendrímeros, liposomas, y niosomas, asi como exosomas.

- Microesferas y nanopartículas poliméricas

  Tal y como se han descrito anteriormente, las microesferas son sistemas matriciales con capacidad de liberar de manera sostenida en el tiempo, agentes terapéuticos. Si se fabrican con materiales biodegradables tienen la ventaja de desaparecer de la zona de administración una vez ejercido el efecto. Dentro de estos, los polímeros derivados de los ácidos láctico y glicólico son los más empleados ya que se encuentran aprobados para su empleo en dispositivos intraoculares ya comercializados. Hay numerosos trabajos que demuestran el potencial de las microesferas biodegradables en el tratamiento de patologías neurodegenerativas del segmento posterior y es una de las líneas del grupo de nuestro grupo de investigación (217–221). La posibilidad de encapsular productos biotecnológicos como los factores neurotróficos derivados de la glía y el cerebro, abre la puerta a la cesión sostenida durante varios meses de agentes que estimulen la protección y eviten la perdida de fotoreceptores asi como de las diferentes neuronas involucradas (222). También permiten la encapsulación liberación de otros fármacos como los glucocorticoides, agentes anti apoptóticos y antioxidantes, entre otros, todos ellos de utilidad como neuroprotectores.

  Las nanopartículas, como las de PLGA están siendo también estudiadas en este ámbito debido a su capacidad internalización mediante endocitosis. Por ejemplo la encapsulación del factor derivado del epitelio pigmentario (de las siglas en inglés, “PEDF”, “Pigment Epithelium-Derived Factor”), dio lugar a una disminución de los niveles de glutamato y por tanto mejoro la supervivencia de las células de la retina asi como una mejora en la permeabilidad vascular, en un modelo animal de hipoxia retiniana (223). A pesar de sus ventajas, el empleo de este tipo de sistemas para la cesión controlada de sustancias activas durante largos periodos de tiempo como es el caso de las patologías neurodegenerativas del segmento posterior se encuentra limitado por su elevada superficie específica.

- Hidrogeles

Los hidrogeles son sistemas formadores de matrices acuosas y por tanto resultan muy atractivos desde el punto de vista de la liberación de sustancias neuroprotectoras en el segmento posterior. Además, si son biodegradables tienen la ventaja de desaparecer del lugar de administración. Sin embargo, uno de los principales inconvenientes asociados a los mismos es la dificultad en su inyectabilidad, debido fundamentalmente a sus elevadas viscosidades. Es por ello que, durante los últimos años, se han estudiado distintos hidrogeles capaces de gelificar *in situ*, y evitar así los problemas asociados a la inyectabilidad. Los hidrogeles basados en ácido hialurónico modificado y entrecruzado con diferentes compuestos como grupos vinilsulfona o dextrano tiolado suponen sistemas altamente biocompatibles ya que el ácido hialurónico es uno de los componentes del humor vitreo. No obstante, la dificultad de controlar la velocidad de gelificación produciendo altas viscosidades antes de poder inyectarse, asi como factores como el pH o la formación de productos de degradación pudiendo alterar su tolerancia y degradación están todavía por resolver (209).

Hay otros sistemas de gelificación *in situ*, como los formados a partir del quitosano que han demostrado ser altamente biocompatibles, pero debido a que el este no es soluble en agua a pH fisiológico, se está investigando más a en profundidad su tolerancia y degradación. Por ejemplo, el uso de quitosano modificado con carboximetilcelulosa ha mostrado una mayor solubilidad y mejor tolerancia por administración periocular (224) (209).

Los polímeros solubles en disolventes acuosos y capaces de formar hidrogeles ante determinadas circunstancias suponen un gran avance en este tipo de terapias. Un tipo de hidrogeles “inteligentes” son los sensibles a la luz o fotosensibles, capaces de entrecruzarse instantáneamente ante un estímulo de luz ultravioleta y un fotoiniciador. De hecho, este tipo de sistemas han mostrado la capacidad para ceder productos biotecnológicos antiangiogénicos encapsulados en microesferas como el anti-VEGF bevacizumab durante 90 días (225). Una de las limitaciones en el empleo de estos preparados es la necesidad de un estímulo no fisiológico para su formación. Por esta razón los hidrogeles termosensibles han despertado un gran interés en la

comunidad científica para este tipo de terapias, ya que permanecen líquidos y transparentes en condiciones normales de almacenamiento, y tienen la capacidad de formar geles de forma espontánea una vez inyectados al alcanzar la temperatura corporal. De esta forma se pueden incorporar fácilmente sustancias hidrosolubles en la solución acuosa y tras la administración intravítrea se formará un matriz en estado de gel, liberando lentamente los principios activos. Los más conocidos son aquellos formados a base de poli N-isopropilacrilamida o PNIPAM, poloxaminas o los llamados tribloque como el PLGA-PEG-PLGA, formado por unidades repetidas de polietilenglicol y ácido poli láctico co-glicólico (209). De hecho, no solo se ha demostrado el uso seguro de formulaciones de hidrogeles termosensibles de PLGA-PEG-PLGA en modelos preclínicos si no también su habilidad neuroprotectora de forma sostenida tras la inclusión del anti-VEGF comercializado Avastin® (226).

- Dendrímeros

El uso de dendrímeros se ha mostrado particularmente útil también en las terapias neuroprotectoras en fase de investigación para el tratamiento de patologías neurodegenerativas del segmento posterior. La administración intravítrea de dendrímeros conjugados con acetónido de fluocinola y modificados específicamente para unirse al tejido inflamado han conseguido lograr una atenuación considerable de los mecanismos de neuroinflamación. Además la modificación selectiva, evita su penetración en los tejidos de individuos sanos (227). También pueden ser específicamente diseñados para actuar sobre la microglía y evitar si su activación (228).

- Liposomas y niosomas

Los liposomas administrados por vía intravenosa se emplean ya en la práctica clínica para el tratamiento de patologías neurodegenerativas del segmento posterior. Un ejemplo son los preparados liposomales de doxorrubicina, Vysudine® para el tratamiento de la DMAE húmeda, o de nistatina Nyotran® empleados junto con la terapia fotodinámica para evitar el crecimiento de nuevos vasos sanguíneos (229). Los liposomas administrados por vía intravítrea se encuentran en fase de investigación y hay trabajos en los que se funcionalizan los fosfolípidos con ligandos específicos de unión a determinadas estructuras de la retina (230).

Los niosomas a su vez, similares en estructura a los liposomas, pero con algunas ventajas comentadas anteriormente, suponen una herramienta muy útil y en desarrollo principalmente como sistema de entrega genético. La modificación de su superficie para acomplejar material genético, les permite su internalización en las células retinianas y estimular así la expresión del vector que transportan (231). De las misma forma, niosomas cargados con un vector plasmídico codificando una proteína verde fluorescente han mostrado una gran eficacia de transfección del vector en animales tanto por administración intravítrea como subretiniana (232).

- Exosomas

Los exosomas suponen una ventaja como terapia para las enfermedades de la retina, ya que permiten un abordaje multifactorial al contener diferentes proteínas, factores neurotróficos moléculas de ADN y ARN. Además, en la DMAE se ha relacionado una disminución en el número de exosomas producidos por las células de la retina con un aumento del daño celular. En cuanto a la administración intravítrea de exosomas, se ha visto que los procedentes de células madre mesenquimales poseen propiedades protectoras ante eventos isquémicos en modelos preclínicos de isquemia y neovascularización (233). Además, tanto en modelos de RD como de retinosis pigmentaria, la administración intravítrea de exosomas provenientes de células del epitelio pigmentario de la retina, ARPE-19, han mostrado proteger a los fotorreceptores asi como mostrar una disminución considerable en los niveles de interleucinas sustentando el interés de este tipo de tratamientos (234).

OBJETIVOS Y PLANTEAMIENTO

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La administración de fármacos por vía tópica para el tratamiento del glaucoma conlleva a menudo una serie de inconvenientes tales como una biodisponibilidad limitada en el lugar de absorción, dificultad para atravesar las distintas barreras oculares o la aparición de efectos adversos tales como ojo seco. Esto se debe en muchos casos a la limitada solubilidad de principios activos liposolubles en medio acuoso o la presencia de conservantes los cuales desencadenan una serie de eventos perjudiciales para la superficie ocular, desestabilizando la película precorneal y facilitando la falta de adherencia al tratamiento. En cuanto a los eventos neurodegenerativos que afectan directamente a la retina y el segmento posterior, su tratamiento se encuentra limitado por las barreras efectivas que existen en el ojo y se tiene que recurrir a la terapia intravítrea lo que a menudo origina complicaciones asociadas a la frecuencia de administración y a la incomodidad por parte del paciente.

Dentro de las líneas de investigación más recientes en este campo, se encuentra el desarrollo de nuevos nanosistemas capaces de aumentar la estabilidad de la película precorneal, incrementar la absorción y el tiempo de residencia de los fármacos (hidrosolubles y liposolubles) administrados sobre la superficie ocular tanto para el tratamiento de patologías del segmento anterior como posterior. Del mismo modo, en el ámbito de la administración intraocular, una de las líneas novedosas se centra en el desarrollo de sistemas de gelificación *in situ* que, en general, permiten modular la cesión de fármacos y, en particular, la de agentes neuroprotectores que se utilizan en la terapia avanzada para el tratamiento de patologías neurodegenerativas como el glaucoma.

El objetivo general de esta tesis doctoral es el desarrollo de nuevos nanosistemas para el tratamiento de patologías crónicas e inflamatorias oculares de afectación tanto del segmento anterior como posterior. Se pretende obtener formulaciones innovadoras basadas en nanosistemas capaces de incorporar sustancias de naturaleza acuosa y oleosa, así como incrementar la absorción y tiempo de residencia de diferentes agentes antiglaucomatosos, mejorando su eficacia y protegiendo al mismo tiempo la superficie ocular. Así mismo, se desea conseguir nuevos nanosistemas basados en hidrogeles intravítreos inteligentes con capacidad de liberar diferentes agentes neuroprotectores.

OBJETIVOS Y PLANTEAMIENTO

Para llevar a cabo el objetivo general anteriormente mencionado se han planteado los siguientes objetivos específicos:

Primer objetivo. Diseñar y optimizar modelos de estrés hiperosmolar e inflamación en células epiteliales humanas de córnea y en macrófagos, respectivamente, para evaluar la idoneidad de posibles agentes osmoprotectores y de naturaleza polimérica como posibles tratamientos para la enfermedad de ojo seco.

Para el cumplimiento de este objetivo, se realizaron las siguientes actividades:

- Evaluación de los procesos de muerte celular mediante el empleo de diferentes técnicas en líneas celulares humanas de córnea bajo la exposición crónica a diferentes concentraciones hiperosmolares.
- Estudio del volumen celular ante la exposición prolongada a diferentes ambientes hiperosmolares.
- Selección de un ambiente hiperosmolar óptimo para el estudio de la capacidad osmoprotectora de diversos agentes en células epiteliales corneales.
- Estudio de la capacidad para evitar los procesos de muerte celular producidos por un ambiente hiperosmolar de diferentes agentes osmoprotectores, así como sustancias poliméricas tales como el ácido hialurónico o la hidroximetilpropilcelulosa.
- Estudio de la actividad de los diferentes agentes osmoprotectores y poliméricos para revertir o regular la modificación del volumen celular ocasionado por un escenario de estrés hiperosmolar.
- Análisis de la capacidad antiinflamatoria para inhibir la producción de factor de necrosis tumoral alfa de compuestos osmoprotectores y poliméricos en macrófagos.

<u>Segundo objetivo.</u> Desarrollar una microemulsión de administración tópica oftálmica como plataforma para la administración de agentes osmoprotectores de la superficie ocular.

Para el cumplimiento de este objetivo se realizaron las siguientes actividades:

- Desarrollo de una microemulsión por autoemulsificación a temperatura ambiente con componentes lipídicos compatibles con la película precorneal como vehículo para incrementar la absorción y tiempo de residencia de sustancias tanto liposolubles como hidrosolubles.
- Estudio de la estabilidad de la microemulsión modelo y de la misma con la adición de sustancias poliméricas como ácido hialurónico y/o dextrano.
- Desarrollo de una microemulsión con agentes osmoprotectores seleccionados anteriormente (betaína, leucina, clusterina, y ácido oleanólico) y polímeros como ácido hialurónico o dextrano.
- Caracterización fisicoquímica de la microemulsión y las formulaciones osmoprotectoras desarrolladas para evaluar su idoneidad para la administración tópica ocular.
- Evaluación de la tolerancia en células epiteliales de córnea y conjuntiva tanto de la microemulsión modelo desarrollada como de aquellas incorporando sustancias osmoprotectoras.
- Determinación de la capacidad osmoprotectora de las microemulsiones incluyendo agentes osmoprotectores y polímeros en un modelo de estrés hiperosmolar previamente desarrollado.
- Estudio de la tolerancia *in vivo* en conejos de las formulaciones finales escogidas por su buena tolerancia *in vitro* y su actividad osmoprotectora.

<u>Tercer objetivo.</u> Puesta a punto de microemulsiones de administración tópica ocular con actividad osmoprotectora cargadas con latanoprost como formulaciones híbridas para el tratamiento del glaucoma.

Para el cumplimiento de este objetivo se establecieron las siguientes actividades:

- Elaboración de una microemulsión osmoprotectora cargada con latanoprost como agente hipotensor.
- Incorporación de agentes osmoprotectores a la microemulsión hipotensora desarrollada con o sin ácido hialurónico o dextrano como polímeros mucoadhesivos.
- Análisis y caracterización fisicoquímica de las microemulsiones osmoprotectoras con latanoprost.
- Determinación de la eficacia de encapsulación de latanoprost en las microemulsiones desarrolladas.
- Evaluación de la tolerancia *in vitro* en líneas humanas de cornea y conjuntiva a diferentes tiempos de exposición.
- Análisis de la capacidad osmoprotectora de las formulaciones desarrolladas cargadas con el agente hipotensor en un modelo previamente desarrollado de ambiente hiperosmolar crónico en células epiteliales de cornea humana.
- Evaluación de la internalización celular de las microemulsiones cargadas con el agente fluorescente lipófilo cumarina.
- Valoración de las interacciones a nivel microscópico entre las microemulsiones desarrolladas y las diferentes estructuras celulares.
- Estudios *in vivo* en conejos para evaluar la actividad hipotensora de las microemulsiones osmoprotectoras desarrolladas incorporando latanoprost.

<u>Cuarto objetivo.</u> Síntesis y evaluación de hidrogeles termosensibles a base de PLGA-PEG-PLGA como nuevas plataformas inyectables para terapias combinadas neuroprotectoras en el tratamiento de enfermedades degenerativas de la retina.

Para el cumplimiento de este objetivo se realizaron las siguientes actividades:

- Síntesis y caracterización de polímeros a base de PLGA-PEG-PLGA para la elaboración de hidrogeles sensibles a la temperatura.
- Incorporación de agentes activos neuroprotectores y antiinflamatorios en los hidrogeles seleccionados.
- Ensayos de liberación de los diferentes hidrogeles desarrollados incluyendo agentes neuroprotectores.
- Estudios de tolerancia en células de epitelio pigmentario de la retina de los hidrogeles desarrollados y de las formulaciones finales incorporando activos terapéuticos.
- Evaluación de la capacidad de las formulaciones desarrolladas para evitar procesos de muerte celular en un modelo optimizado de estrés oxidativo.
- Evaluación de la actividad antiinflamatoria de las formulaciones finales neuroprotectoras desarrolladas sobre el factor de necrosis tumoral alfa.

OBJETIVOS Y PLANTEAMIENTO

CAPITULO I. LIPOSOMES AS VEHICLES FOR TOPICAL OPHTHALMIC DRUG DELIVERY AND OCULAR SURFACE PROTECTION

CAPÍTULO I

CAPÍTULO I

Liposomes as vehicles for topical ophthalmic drug delivery and ocular surface protection

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CAPÍTULO I

CAPÍTULO I

ABSTRACT

Introduction: The development of ophthalmic formulations able to deliver hydrophilic and hydrophobic drugs to the inner structures of the eye and restore the preocular tear film has been a leading topic of discussion over the last few years. In this sense, liposomes represent a suitable strategy to achieve these objectives in ocular drug delivery.

Areas covered: Knowledge of the different physiological and anatomical structures of the eye, and specially the ocular surface are critical to better understanding and comprehending the characteristics required for the development of topical ophthalmic liposomal formulations. In this review, several features of liposomes are discussed such as the essential materials used for their fabrication, basic structure and preparation methods, from already established to novel techniques, allowing the control and design of special characteristics. Besides, physicochemical properties, purification processes and important strategies to overcome delivery or encapsulation challenges are also presented.

Expert opinion: Regarding ocular drug delivery of liposomes, there are some features that can be re-designed. Specific biocompatible and biodegradable materials presenting therapeutic properties, such as lipidic compounds or polymers significantly change the way of tackling ophthalmic diseases. Besides, liposomes entail an effective, safe and versatile strategy for the treatment of diseases in the clinical practice.

Keywords: Ocular topical liposomes dry eye, ocular surface, ophthalmology, ocular drug delivery.

CAPÍTULO I

Article highlights box:

- The precorneal tear film preserves ocular surface integrity, cornea and conjunctiva.
- Corneal low permeability entails a challenge to deliver active substances that target both the anterior and posterior segment of the eye.
- Liposomes are biocompatible and biodegradable lipid-made spherical vesicles that resemble cell membranes able to permeate and deliver both hydrophilic and hydrophobic drugs.
- The use of liposomes with similar components to those present in the precorneal tear film entails a novel strategy in the treatment of dry eye disease.
- Methods based on ethanol injection and microfluidics resulted the best options for liposome scaling-up due to their feasibility, robustness and optimization potential.
- Technological strategies such as the incorporation of bioadhesive biocompatible polymers or positively charged phospholipids help to increase mucoadhesion, retention time and permeation of liposomes in the cornea.
- One of the major issues that limits the use of liposomal formulations is the sterilization. A combination of sterilizing filtration and cold methods seems to be the most suitable alternative to industrial fabrication of liposomes.
- A simultaneous administration of topical ophthalmic liposomal formulations with supplements, such as vitamins or fatty acids, represent an important strategy for the recovery of the tear film lipid layer in ocular surface pathologies such as the dry eye syndrome.
- Development of technological strategies that increase the stability of liposomal dispersion is required.
- The attachment of highly specific biomolecules to the liposomal surface and using intrinsic therapeutic materials might entail the next generation of nano-liposome formulations.

CAPÍTULO I

1. Introduction.

The development of drug delivery systems for the treatment of ocular diseases is a great challenge mainly owing to the numerous mechanisms of eye protection against exogenous substances that act as effective barriers hindering the entry of drugs [1].

Anatomically, the eye can be divided into anterior and posterior segments. The anterior segment is formed by the first third of the eyeball, made up of structures such as the cornea, conjunctiva, iris, ciliary body, the lens and aqueous humor. The most important pathologies related to the anterior segment include dry eye disease, cataracts, conjunctivitis and keratitis. On the other hand, the posterior segment encompasses the retina, optic nerve, choroid, and vitreous humor. It can be affected by diseases that cause a significant damage in vision, including irreversible blindness, such as glaucoma, age-related macular degeneration and diabetic retinopathy, among others [2]. The treatment of the majority of ocular pathologies, requires that the drug overcomes anatomical barriers such as the cornea, conjunctiva, sclera and retina, and the blood-aqueous and blood-retinal barriers. If the formulation is administered topically physiological barriers such as eye drainage, blinking, dilution in tears and blood and lymphatic flow also limit the access of ophthalmic drugs to the intraocular target tissues.

The routes of ocular drug administration differ according to the desired site of action. The most frequent treatments of the anterior segment diseases consist in the topical administration of eye drops on the ocular surface, which has important advantages over the systemic route: Less toxicity, quicker onset of action and less dose required. Furthermore, the topical route is less invasive than other routes of ocular administration. However, the main problem is often the low bioavailability of the topical administration if the drug has to reach intraocular targets: It is estimated that only 5% of the administered drug reaches the aqueous humor [3][4][5]. For the treatment of posterior segment diseases, the challenge is much greater. Drugs administered by the topical route do not achieve the target site as easily due to the ocular barriers, so intravitreal and periocular administrations such as intravitreal, subconjunctival and retrobulbar injections are preferred.

As a drawback, these routes must be used repeatedly to maintain therapeutic drug levels, which entails numerous adverse effects [5][6][7][8]. The main limitations for the topical ocular administration are the tear drainage and dilution of the eye drops, the low

residence time of the formulation, the poor corneal and conjunctival absorption of the drug and the drug loss in systemic circulation. To improve topical ocular bioavailability, several resources are used to increase penetration and minimize drug loss (i.e., use of polymers that increase viscosity and mucoadhesion). Another alternative involves the use of drug delivery systems to enhance ocular delivery, such as nanosystems or microsystems capable of raising the bioavailability of the active substance and providing a controlled and sustained drug release [3][9][10].

Micro- and nanotechnologies are widely used for the development of controlled drug delivery systems because they are able to protect the drug from external factors and increase its bioavailability. Some of these systems include microparticles, nanoparticles, nanoemulsions, microemulsions and liposomes.

Microparticles (MPs) are drug delivery systems with sizes between 1 and 1000 μm. For ophthalmic use, the biodegradable polymers polylactic acid (PLA), polyglycolic acid (PGA) and poly (lactic-co-glycolic acid) (PLGA) are the most commonly employed. Microparticles can be classified into microspheres and microcapsules depending on whether the active ingredient is dispersed in the polymeric matrix (microspheres) or is surrounded by the polymeric membrane (microcapsules) [11]. Microparticles are under investigation for the intraocular administration of drugs whose objective is the treatment of diseases affecting the posterior segment of the eye [5]. They have the advantage that can be injected as a suspension in a physiological vehicle, thus allowing the sustained release of the drug and therefore the reduction of the number of administrations, which supposes a reduction of the risks associated to repeated interventions. Furthermore, bioadhesive polymers such as hydroxypropyl methylcellulose (HMPC) or hyaluronic acid (HA) can be used to increase the viscosity of the vehicle and enhance injectability [11]. Nanoparticles (NPs) are smaller in size (1-1000 nm) and can be also classified into nanospheres and nano capsules depending on their structure.

The small size of nanoparticles makes them available to be easily taken up by cells and being used to treat retinal pathologies. When used for topical administration, their small sizes also reduce eye discomfort and improves their contact and retention time with the ocular surface, onto the ocular surface [11]. However, they have a more limited sustained release capacity when comparing to microparticles. Microemulsions (ME) and

nanoemulsions are capable of incorporating hydrophilic and lipophilic drugs. They are made up of an aqueous phase, an oily phase and surfactants combined in different proportions allowing the system to be stabilized. That is the reason why ME are normally considered thermodynamically stable systems. Thanks to the small droplet sizes (<150 nm) and due to its low viscosity and surface tension these pharmaceutical systems spread easily over the ocular surface, making it a good alternative for topical administration. In addition, positively charged components can be added to increase the retention time in the cornea after topical administration [5][11].

Liposomes are lipidic spherical vesicles biocompatible and biodegradable formed by lipid bilayers with a size range between 10 nm and 10 μm. Its lipid bilayer structure surrounding an aqueous core allows the incorporation of both hydrophilic and lipophilic active substances. In this way, the hydrophilic drugs can be entrapped inside the liposomes or dissolved in the vehicle in which the vesicles are dispersed, while the lipophilic ones are incorporated in the lipid bilayers. Several factors are important in determining the effectiveness of liposomal formulations, such as the properties of the encapsulated active substance, the size of the liposomes and their charge. The use of liposomes for the treatment of ocular diseases has been widely studied due to their good tolerance and their capacity to increase both hydrophilic and lipophilic drugs penetration when applied topically. This is due to their ability to interact with eye tissues such as the cornea. Liposomes present various alternatives for drug release, being able to increase the retention time of drugs on the ocular surface, as well as providing a sustained release after their administration. For example, lipid nanosystems and liposomes in combination with siRNA (lipoplexes) silencing specific genes has been employed for treating some degenerative diseases of the posterior segment of the aye such as diabetic retinopathy through injection [12].

Furthermore, the topical administration of liposomal formulations does not require the use of invasive methods [11][13][14]. Besides, apart from being used as drug delivery systems, liposomes have been also developed to be used as artificial tears, demonstrating their ability to restore the lipid layer of the tear film, improving the symptoms of pathologies such as dry eye disease (DED) [15].

The following sections will review the improvements provided by the use of liposomes in topical ocular administrations, including their composition, technological requirements and methods of preparation and encapsulation of drugs with different properties. In addition, the efficacy of the several developed technological strategies to enhance topical bioavailability and its extended use to different types of active substances will be discussed. In this review, the role of liposomes in tear film recovery and the future prospects in this area will also be emphasized.

2. The ocular surface

2.1. Precorneal tear film

The precorneal tear film is constituted by a thin layer that broadens all over the ocular surface, including cornea, conjunctiva and sclera. This structure plays a decisive function in nurturing and protecting the eye surface. The tear film is composed of an aqueous mucinous gel covering by a lipid layer [16].

The aqueous layer is secreted by the lacrimal gland and accessory lacrimal tissues. Its composition includes salts, glucose, urea, albumin and immune proteins that help to protect the ocular surface [17][18]. The function of the aqueous layer is extremely important providing the cornea with nutrients and oxygen as well as eliminating foreign bodies and toxins from the ocular surface. Furthermore, there are several proteins that play an important antimicrobial role, such as lysozyme that contributes to the stability of the tear film, or lactoferrin [19].

Other proteins present in the precorneal tear film are antibodies, such as Immunoglobulin A (IgA) immunoglobulin G (IgG) and immunoglobulin M (IgM) [18]. IgA is the main immunoglobulin present in the preocular tear film (10–80 mg/dL) playing an important role in immunity protecting against viruses, bacteria and parasites [18]. Besides, lipocalin is worth to be mentioned due to its binding properties to lipids in tears [17].

Mucins present in the preocular tear film are divided in membrane associated mucins and secreted mucins. Secreted mucins are divided into gel-forming and soluble mucins. The presence of soluble mucins has been shown to play an important role in tear film extensibility due to its ability to reduce surface tension [18][20]. Gel-forming mucins,

which can reach molecular weights of 40 MDa, provide the necessary rheological properties that allow to adjust the viscosity of the preocular tear film when blinking [21]. One of the most important gel-forming mucins is MUC5AC, which is produced by conjunctival caliciform cells and grant the hydration of the ocular surface. The membrane associated mucins including MUC1, MUC4, and MUC16 are anchored to the plasma membrane of corneal and conjunctival epithelial cells via their hydrophobic terminal transmembrane domain. In addition, they form a glicocalix which main function appears to be the anchoring of a layer made out of secreted mucins. Secreted mucins are hydrophilic and negatively charged, which favors a repulsion that allows the secreted ones to slide over the epithelial mucins. In addition, this facilitates the sliding of the eyelid without adhesion to the epithelium. All these creates a lubricating layer on the ocular surface [17][22][23].

High molecular weight mucins along with proteins confer the tear film a non-Newtonian viscoelastic behavior, regulating tear viscosity when blinking and thus protecting the surface [24]. They are synthesized by the corneal and conjunctival epithelium as well as conjunctival goblet cells. The presence of terminal residues such as sialic acid gives to these glycoproteins a high negative charge, which favors the movement of mucins on the surface and helps to repel pathogens from the epithelium. Further, the high content of sialic acid could favor the blocking of the adhesion of pathogenic bacteria by binding to the adhesins to which these pathogens bind [18][25].

The production and volume management of tears is controlled by the lacrimal gland and accessory lacrimal tissues as previously mentioned and also by regulating the water flow through the cornea. Fluid removal occurs through drainage caused by the eye blink and evaporation. Thus, when the ocular surface is exposed to adverse environmental conditions evaporation occurs with a consequent increment in the tear tonicity. These events will create a flow of water through the corneal epithelium due to the channels of aquaporin, recovering the initial tone [17][26].

The lipid layer is the outermost layer of the preocular tear film and has been widely associated to the reduction of the surface tension favoring the spread of the tear film over the entire surface and the protection against tear evaporation [22][27]. Its production occurs in the Meibomian glands [24][28] and include a complex variety of lipids. The tear lipidome contains amphiphilic and nonpolar lipids which have different function. The

group of amphiphilic lipids is composed of phospholipids including phosphatidylcholine, lysophosphatidylcholine and phosphatidylethanolamine and others such as sphingolipids [28][29][30]. Amphiphilic lipids appear to form a sublayer capable of interacting with polar and nonpolar tear compounds. The polar heads are oriented towards the aqueous layer and the apolar ones interact with the non-polar lipids. In this way, the amphiphilic sublayer allows the formation of a stable non-polar lipid sublayer on the surface and its spreading [30][31]. Non-polar lipid sublayer comprehends mainly wax esters, cholesteryl esters, and triglycerides. This sublayer is directly in contact with the air and when it is in proper amounts prevents the evaporation of aqueous layer. If the lipid layer is destabilized, aqueous evaporation increases, causing pathologies such as dry eye disease (DED) [31][32].

The stability of the lipid film is also related to the presence of proteins. An example of this is lipocalin, which as previously mentioned plays an important role related to the lipid layer. Tear lipocalin (TLc) is able to bind lipids such as cholesterol, fatty acids and phospholipids enabling its solubilization and transport. Therefore, lipocalin acts as a scavenger removing lipids from the corneal surface and transporting them to the lipid layer stabilizing the tear film by reducing the surface tension [27][30][33][34]. Lipocalin also binds with lipids and reinforces tears viscosity [24][35].

2.2. Cornea and conjunctiva

The cornea, a transparent tissue with refractive properties [36], is the central structure of the ocular surface. It performs essential functions such as allowing vision, protecting against damage and preventing infections [37]. The cornea is divided in five layers: Epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium [38][39].

The stratified, scaly and not keratinized corneal epithelium is composed by 5 to 7 layers. The deepest layer is composed of basal cells with mitotic properties, followed by wing cells and finally superficial cells [40]. One of its characteristics is that it is constantly renewed [41]. It is responsible for protecting the cornea and can rebuilt itself after injury by sliding epithelial cells to cover the region followed by a mitotic process [42][43]. In addition, epithelial corneal cells express aquaporin-5 channels in charge of transporting water through the epithelium [44]. The epithelial basement membrane (BM), with a high content of type IV collagen and laminin produced by the basal cells [41][43], is located

between the corneal epithelium and the stroma and regulates the levels of cytokines and growth factors in both of them. In addition is in charge of the adhesion of epithelial cells to the stroma and is involved in the migration, proliferation, and differentiation of epithelial cells [42] [45].

Bowman's layer is an acellular structure formed by collagen which has no regenerative capacity. It represents the superficial layer of the stroma, enclosed by the basement membrane and the anterior stroma. This layer appears to form as a result of the interaction between the corneal epithelial cells and the stromal keratocytes [39][46]. It provides protection to subepithelial nerve plexus [47].

The stroma represents 90% of the corneal thickness [39]. It is a highly innervated layer [47] composed of collagen (the major component disposed in regular lamellae), keratocytes and proteoglycans such as lumican and keratocan. The characteristic distribution allows light to pass through the collagen and prevents its dispersion [39][48]. This process is possible because proteoglycans interact with collagen (type I, IV and XII), allowing collagen fibrils to maintain their position [49]. Keratocytes, arranged among the lamellae, are in charge of synthesizing the components of the stroma.

Moreover, they can respond to signals from corneal epithelial cells, going into apoptosis or activate into reparative phenotypes in the presence of damage [50]. Descemet's membrane is composed of an anterior layer formed by collagen and a posterior layer secreted by the endothelium, which thickens over time [39]. The presence of type IV and VIII collagen is characteristic in this membrane, forming a hexagonal grid. Its function is to maintain corneal hydration and protect the endothelium. Furthermore, it seems to have resistance capacity against intraocular pressure [51][52][53].

The deepest corneal layer is the endothelium, a single layer of hexagonal cells [39]. Although endothelial cells have proliferative capacity, it is too slow to replace cell loss, so the number of these cells decreases with age [54][55]. Its main function is to regulate the hydration of the stroma, allowing the transparency of the cornea to be maintained. The underlying mechanisms by which it is regulated involves the presence ionic pumps [56]. Also, the aquaporin-1 channels present in the endothelium have been suggested as responsible for regulating the transport of water through the endothelium and as a key to preventing corneal edema [44].

CAPÍTULO I

With regard to conjunctiva, it is a thin transparent mucous layer. Unlike the cornea, the conjunctiva is highly vascularized. It covers the sclera, which is made up of collagen fibrils and proteoglycans as well as the stroma, and the inner part of the eyelids. According to its location, it is divided into two areas: bulbar and palpebral conjunctiva. Bulbar conjunctiva covers the anterior part of the eye and surrounds the cornea. Palpebral conjunctiva is in charge of covering the back of the eyelids [40][57].

Conjunctival structure is formed by the epithelium with 3–5 cell layers resting on the basal membrane and the lamina propria. The lamina propria is composed of connective tissue and is highly vascularized. The conjunctival epithelium is separated from the corneal epithelium by the limbal epithelium and contains two main types of cells: stratified squamous cells and goblet cells. Both types of cells appear to be regulated by growth factors, and while the stratified squamous cells secrete water and electrolytes, the goblet cells, as mentioned above, are responsible for the secretion of mucins present in the tear film.

This function is essential to maintain a correct lubrication of the eye, and its reduction may be responsible for pathologies such as dry eye disease [58][59][60][61]. Moreover, it has been shown the presence of Langerhans cells involved in the immune response, capable of migrating to the cornea when inflammation of the conjunctiva occurs [62]. Another important feature of the conjunctiva is the presence of the conjunctival associated lymphatic system (CALT) attached to the immune protection of the ocular surface. It is composed of lymphoid follicles as accumulations of B lymphocytes and follicular dendritic cells, specialized vessels, intraepithelial lymphocytes and a lymphoid layer located in the lamina propria which contain lymphocytes, mainly T cells, and plasma cells which mostly produce IgA [63][64][65].

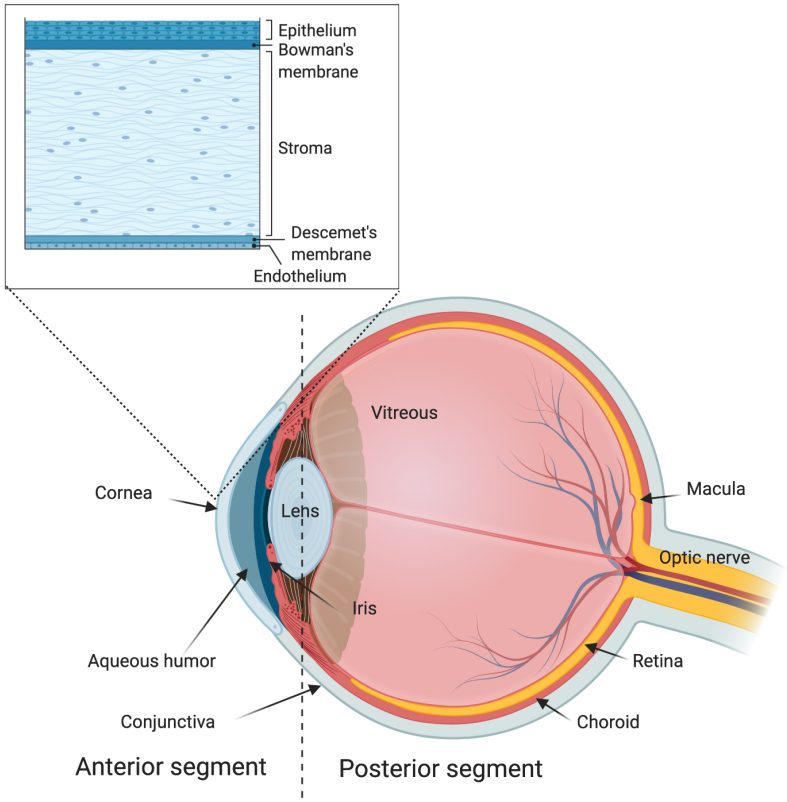

Figure 1. Eye structure. the structures of the anterior segment of the eye such as cornea with their main layers are represented. Besides, posterior segment showing the retina and adjacent structures are also illustrated.

2.3.Drug delivery across the ocular surface

As previously mentioned, topical administration of drugs, whose site of action is usually the anterior segment of the eye, including the ocular surface presents low drug bioavailability. The passage of drug through the cornea allows it to reach internal tissues such as the iris, the ciliary body and the lens. Otherwise, conjunctival penetration allows the drug to enter tissues such as the sclera, the choroid, and even the retina.

For the ocular topical administration of drugs, the physiological role of the tear film must be taken first into account. The tear film has a volume of 7 µl and a restoration time of 2 to 3 minutes. The maximum volume of eye drops that the eye can contain is 30 µl, which means that a limited volume of the ophthalmic formulation can be deposited in the eye. In addition, most of the eye drops are eliminated rapidly from the human eye surface due to blinking and tear turnover: 16% of the tear will be replenished in one minute. This

means that less than 5% of the drug reaches the intraocular tissues due to the short time retention, which supposes a great loss of drug. [66][67][68]. The mucin layer attached to the corneal surface presents hydrophilic properties, and also present a negative charge due to its composition. Thus, the use of positive charged delivery systems implies an increase in the residence time on the surface, and therefore in its permeability [69].

Regarding the passage through the cornea, its low permeability and small surface area also becomes a challenge. Drugs can pass through the cornea via the transcellular or paracellular routes. The former involves dealing with the different layers of the tissue that act as a barrier. The corneal epithelium is a lipophilic layer, which supposes a resistance to the penetration of hydrophilic molecules. The corneal stroma composed of collagen fibrils has hydrophilic properties, making it difficult for lipophilic molecules to pass through. Endothelium, the barrier among the stroma and aqueous humor, as well as epithelium, is a lipophilic layer. With regards to the paracellular route, in the epithelium the superficial cells have a small junction space that hinders the paracellular penetration of the drug.

Nevertheless, in the endothelium the leaky junctions between the cells are easier for macromolecules to traverse between stroma and aqueous humor, being less limiting than the epithelium. Accordingly, the main barriers for hydrophilic and lipophilic substances are the epithelium and stroma respectively [66][70].

The ability of drugs to cross the cornea is conditioned by the size and the distribution coefficient of the active substance. The higher the diffusion coefficient, the greater the importance of the transcellular pathway. For values of distribution coefficient between 0,01-10, the pass through the lipophilic epithelium and endothelium becomes more viable. When the value is higher than 10, almost all the passage occurs through the transcellular route and the stroma becomes the limiting barrier.

This is the reason why when the distribution coefficient is too large the permeability stops increasing. However, in the case of solutes with a low distribution coefficient, that is, substances with a hydrophilic nature, the main impediment is the epithelium and the main passage through the cornea is the paracellular route. In this sense, the passage of hydrophilic substances depends on their size or molecular weight, being this process easier for small solutes with a molecular weight less than 500 Da, and especially difficult for macromolecules [71][72][73][74]. After penetration through the cornea, the drug will

reach the intraocular tissues. First, the drug reaches the aqueous humor, from where it will pass to the intraocular tissues of the anterior segment. By this way, the drug will have to go through the anterior segment to reach the posterior segment [75].

Absorption through the conjunctiva is less productive due to the presence of blood and lymphatic vessels that cause a loss of the drug through the systemic circulation. Blood and lymphatic clearance are important dynamic barriers for the administration of drugs through the eye. It has been observed that the clearing produced by the blood and lymphatic vessels is related to the size of the drugs, being easier the elimination of small molecules [66]. When the drug enters the palpebral conjunctiva, a systemic absorption occurs.

However, when it is absorbed through the bulbar conjunctiva which covers the sclera, the majority is lost in the systemic circulation but a small part of it passes to the intraocular tissues, being postulated as a possible via for the topical posterior segment treatment [70][76][77]. The sclera, with a similar composition to the corneal stroma, owns hydrophilic properties. In addition, because the negatively charged proteoglycans, the passage of positively charged molecules thought this layer is hindered by their binding to them [78][79].

Apart from passive diffusion, the presence of efflux and influx membrane transporters in the corneal and conjunctiva cells also plays an important role in drug delivery. The efflux transporters are responsible for decreasing bioavailability expelling the molecules out of the cells. Examples of efflux transporters are P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP) and Multidrug resistance protein (MRP). P-gp is a transporter of lipophilic molecules, which reduce the absorption of lipophilic drugs.

Otherwise, MRP transporter effluxes organic anions and conjugated substances and BCRP transporter is also related to drug resistance [66][68]. On the other hand, the role of influx transporters is related to the transport through the membrane of nutrients and xenobiotics, so they are capable of transporting drugs with targeted modifications [70]. There are many types of influx transporters identified in ocular tissues, such as vitamins, glucose, nucleoside and monocarboxylate transporters. Among those, peptide and amino acid transporters are widely applied in ocular drug delivery. Transporter knowledge enables the development of targeted prodrugs capable of being recognized by carriers as substrates increasing ocular absorption [70][80][81][82].

CAPÍTULO I

3. Development and technological aspects of liposomes

3.1. Components and structure

As previously mentioned, liposomes are defined as spherical vesicles composed of lipid bilayer membranes dispersed in an aqueous solution or buffer [83]. The composition of such membranes can be tailored depending on the different physicochemical properties or characteristics that are required for the system. Normally, one type of phospholipid or a combination is chosen to engineer the liposome basic structure. All these constitutes the basic scaffold for adding the rest of the components including excipients, drugs or other substances.

Regarding ocular topical administration, soy phosphatidylcholine [84] and other phospholipids such as dioleoylphosphatidylglycerol (DOPG) have been employed due to their low immunoreactivity and benefits to corneal regeneration [85]. Besides, it is worth mentioning that soybean phosphatidylcholine is one of the most commonly used and interesting phospholipids, since contains phosphatidylcholine, the most common phospholipid present in cell membranes and incorporates a remarkably wide and rich profile of fatty acids, such as palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3). Some of them are unsaturated, that means that might provide an antioxidant effect for the ocular surface and the formulation itself [86].

Another essential component that stabilizes liposomal membranes and provides bilayer rigidity is cholesterol [87]. In fact, cholesterol was previously described as an stabilizer of intermolecular forces between phospholipids improving stability and avoiding dispersion in liposomes [88]. According to the total amount or number of bilayers present as well as their size distribution, liposomes can be classified in multilamellar vesicles (MLVs) and unilamellar vesicles (ULVs). ULVs are also subdivided into small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) [83]. MLVs are commonly obtained in the first steps of liposome fabrication, being reduced up to LUVs or SUVs by mechanical procedures. Despite that, MLVs are composed of different superposed bilayers with diameters between 1-50 μm.

On the contrary, SUVs and LUVs only contain a single lipid bilayer but differing in the vesicle size. Furthermore, while SUVs tend to have 20-100 nm sizes, LUVs are in the

range of 100 nm - 1 µm of diameter. Besides, a fourth type of liposomes has been proposed, giants unilamellar vesicles (GUVs). GUVs like MLVs, approaches to 1-50 µm but unlike them they are composed of a single lipid bilayer (Figure 2). Therefore, it could be said that GUVs share properties according to size of the different types of the above-mentioned vesicles, particularly MLVs and LUVs [89].

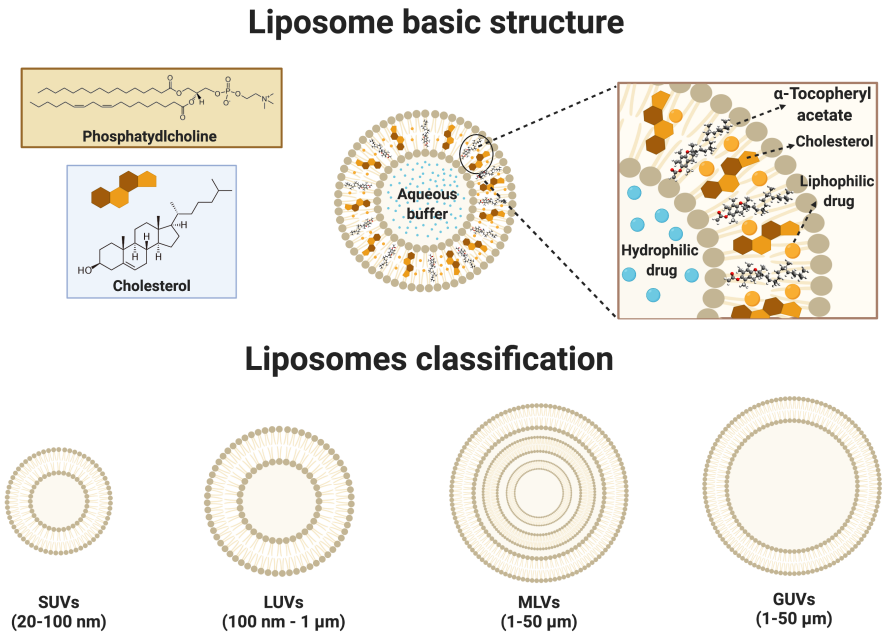

Figure 2. Basic structure of liposomes and classification according to the number of layers: small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), multilamellar vesicles (MLVs) and giant unilamellar vesicles (GUVs).

3.2. Methods for liposome preparation

Several manufacturing procedures can be used for liposome preparation. Some of them have been widely used for decades, and others that have recently become of great interest. Before getting into any method, it is important to note that in any selected method the phase transition temperature (Tc) of the phospholipids is critical in order to successfully prepare the liposomal dispersion. Working conditions below Tc and in particular while re-hydration and extrusion, could hamper the process and avoid the lipid mixture to go from gel state into the preferred 'fluid' or crystalline state [90]. For example, Tc of DOPG is -18 ºC and soy phosphatidylcholine -20ºC to -30ºC, but DMPC (dimyristoyl phosphatidylcholine) or DPPC (dipalmitoyl phosphatidylcholine) have 23ºC and 41ºC Tc

respectively [91]. In this section, different methods are described as well as their applications, optimization, advantages and disadvantages.

3.2.1. Lipid film rehydration

Perhaps, one of the most famous methods for liposome preparation is the film rehydration method, first described by Bangham et al. where a lipid mixture is dissolved in an organic solvent to be later evaporated under vacuum ('Bangosomes'). Afterwards, the dry lipid film is rehydrated in a buffer solution forming the typical and well-known multilayer structures called liposomes [92].

Cholesterol is commonly added to provide rigidity to the membranes [84]. Normally, film rehydration methods tend to yield MLVs and that is why extrusion, freeze thawing and sonication methods are needed to homogenize sizes and stabilize the dispersion [93].

On one hand, the main benefits of using lipid film rehydration are the simplicity of the process and its capability of being used with different types of lipid mixtures. On the other hand, the main difficulties associated are poor encapsulation ratios of drugs associated to its chemical properties, low vesicle size homogeneity and the need of other techniques to tackle the issue and problems in industrial up-scaling [94].

3.2.2. Reverse-phase evaporation method

The reverse-phase evaporation technique first intends to form a two-phase system composed of inverted micelles in an aqueous phase or a water in oil (W/O) emulsion, and an organic phase such as chloroform, ethanol, methanol or a combination of those. Sometimes can be hard to distinguish this method from the lipid film rehydration since the first steps are usually the same. In the reverse-phase evaporation method, when the lipid film is formed in the rotary evaporator, an organic solvent and a buffer are added. Then the organic solvent is again removed by the rotary evaporator. Finally, the liposomal sample can undergo other processes discussed in the rest of the section, such as sonication, extrusion or freeze-thawing to obtain the desired liposomal dispersion [95]. Currently there are improved versions of this technique that have been further optimized through supercritical fluid technology. If a supercritical fluid is use, it dissolves the lipid film and while the aqueous buffer is added the solvent is completely removed. Supercritical CO2 is

one of the best supercritical fluids that could be chosen for this method due to its environmentally friendly properties [96].

3.2.3. Dehydration-rehydration method

This method aims to develop new liposomes by fusion of already made liposomes. It uses dehydration and controlled rehydration in order to obtain MLVs and SUVs. With this technique large molecules such as DNA could be entrapped achieving high loading ratios. It was also described that encapsulation of small molecules is unstable [97]. Furthermore, liposomes are normally centrifuged, freeze-dried and slowly undergo a very controlled rehydration. The loss of lipids and materials during the different cycles can alter the osmotic conditions of the dispersion, thus changing concentrations and activity of entrapped compounds [98].

3.2.4. Freeze-thaw method

Freeze-thawing is a widely known technique that is generally is utilized in MLVs in order to increase their encapsulation efficiency or drug loading. This process occurs because in every freeze-thawing step MLVs are destroyed and reassembled again, thus decreasing the number of layers in every step. Normally the liposomal dispersions are immersed in a cooling bath or a freezer with a temperature range from -20 to -70ºC overnight. Finally, they can be introduced in a water bath at the desired optimized temperature or at room temperature [99].

3.2.5. Sonication

Ultrasounds have also been used to considerably homogenize and reduce the size of MLVs to form SUVs [100]. Normally the lipid mixture is achieved by means of an ultrasound bath or a sonication probe in order to achieve higher homogeneity ratios as well as smaller vesicles. The high pressures created by the ultrasounds violently breaks the vesicles that are spontaneously reassembled into small ones, forming SUVs. Furthermore, there is the inconvenience that, some metallic traces from the sonicator probe can stay in the sample, being difficult to be completely removed [101].

3.2.6. Ether and ethanol injection

Ether injection has been previously used to achieve single and homogeneous SUVs suspensions ranging from 100-300 nm [102]. Ether and ethanol injection consist of firstly prepare a lipid solution in ether, diethyl ether or ethanol and then slowly add it into an aqueous solution, normally containing a buffer that will finally form the liposomal dispersion [103] [104]. Ethanol injection together with microfluidics and micro emulsification are the chosen methods for scaling-up [105][106].

3.2.7. Calcium-induced liposome fusion method

This preparation method aims to obtain LUVs or even GUVs liposomes. The procedure is based on the fact that when SUVs interact with calcium, 'cochleate cylinders' structures are created by fusion of vesicles. Then, a planar sheet like figure is rolled in order to create circular structures [107]. Subsequently, ethylenediaminetetraacetic acid (EDTA) is added in order to create LUVs liposomal dispersions. As the main disadvantage, it is important to remark that this procedure can be only achieved with phospholipids that enclose an acidic nature.

3.2.8. Microfluidics

Microfluidics is a novel technology that as the name states, aims to manipulate fluids, such as lipid mixtures and aqueous solutions at micro or nano scale. This technique allows to monitor every parameter and therefore being able to control and adjust size distribution, polidispersity index and multi or mono-layered structures [108].

In comparison with other above-mentioned methods, microfluidics in general can be considered a novel technique that has provided significant advantages over other conventional methods. Thus, that allows to control sizes in a much more precise way and certain parameters such as flow rate as well as the ratios between injections of lipid mixtures and aqueous buffers. All these controlled features lead to obtain superior quality formulations and enhance drug loadings.

3.3. Physicochemical properties of liposomes, liposomal formulations and purification methods

As previously mentioned, it is worth noting that liposomes are thermodynamically unstable systems. Therefore, physicochemical characterization is a critical step in order to ensure that drug loading, stability and biocompatibility of the developed formulation. All

these physicochemical properties can be tailored in order to make them acceptable for ocular surface drug delivery.

3.3.1. Physicochemical properties

3.3.1.1. Size distribution and zeta potential measurements

Normally, size depends on the type of liposomes that have been developed (GUVs, MLVs, LUVs or SUVs) according to the different procedures shown before. The ideal method for measuring size distribution is DLS (Dynamic Light Scattering), although cryo-TEM (cryo-Electron Transmission Microscopy) can be also used. The size and number of layers will affect drug loading as well as entrapment efficiency, depending on the nature of the loaded drug [109]. As mentioned in previous sections, extrusion, sonication or freeze thawing are some of the most effective methods to reduce size and increase homogeneity in size distribution [99].

Zeta potential determine the overall charge of the particles; therefore, it is going to play an important function when topical liposomes are in contact with the epithelial barrier and interact with cell membranes. Cells membranes are negatively charged, so in order to improve the pass of liposomes through membranes some cationic lipids or surfactants can be used. However, special care should be taken when adding cationic substances since they have been described as potentially toxic for the ocular surface [110].

According to some studies SUVs present the highest permeation ratio through the corneal epithelial barrier while MLVs the lowest [14]. Besides, some studies with the lipophile fluorophore cumarin-6 have demonstrated that liposomes around 190-200 nm pass through every corneal epithelial layer and are able to reach the stroma [111]. Regarding topical ophthalmic administration, liposomes close to 200 nm are normally desired to deliver drugs to the ocular surface [112]. Besides sizes of liposomes carrying hypotensive drugs are between 100 - 200 nm [113]. Furthermore, liposomes with sizes between 100 and 200 nm have been studied for avoiding the mononuclear phagocytic system uptake [114].

3.3.1.2. Morphology

Changes in liposome morphology can result in alteration of layers, liposome types (GUVs, MLVs, LUVs or SUVs) or even the drug loading efficiency. [115].
Optical microscopy is a good option when micrometric liposomes are developed. Fluorescence can be a proper tool to evaluate the presence of labelled proteins internalization in the inner aqueous compartment, particularly in GUVs [116]. However, a wide variety of them (SUVs and LUVs) are in the range of nanometers. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are not suitable techniques since freeze-dried samples and negative staining normally produce significant changes in structure and morphology of the vesicles. Besides, placing the sample in TEM grids dehydrate the sample and the high vacuum that experiment before taking the images break in many cases the liposomes and hamper the visualization of the structures [117]. Therefore, the most ideal technique and widely used for studying the morphology of liposomes is cryo-TEM which allows observation of the inner architecture and structure of liposomes [118].

When using Environmental scanning electron microscopy (ESEM) vesicles remains rehydrated during image acquisition. However, a main disadvantage entails the lack of information that can be achieved since only external structure can be analyzed [119]. Last but not least, atomic force microscopy (AFM) is presented as an interesting and useful method to study liposomes. AFM provides with information about the surface of liposomes and nanoparticles. In some studies AFM has been used to study in detail the attachment of certain antibodies, pegylated phospholipids or even polymers to the surface of the liposomes [120]. It entails a very useful method to find out whether specific bioadhesive polymers or potential therapeutic substances are fixed to the surface of the liposome [121].

3.3.1.3. Viscosity

Generally, liposomal dispersions for ocular topical administration present low viscosity values that are close to those of the natural tears (1 to 8.3 mPa·s), since higher viscosity values might cause blurry vision and discomfort [35]. In some cases, the use of viscosity enhancers in liposomal ophthalmic formulations increased their retention on the ocular surface for longer periods of time. For this purpose, also bioadhesive polymers can be included in the formulations. These compounds can also interact with the mucins on the

preocular tear film increasing, by a complementary mechanism, the contact time of formulations on the ocular surface [122].

3.3.1.4. Surface Tension

Ophthalmic formulations with surface tension values similar to those of the natural tears (43.6 +/- 2,7mN/m) show a proper spreadability when blinking [123]. Surface tension values must be close to the one of the precorneal tear film to ensure proper spreadability. Caution must be taken with low surface tension values because the inner structure of the preocular tear film and the epithelium can be damaged [84]. One of the most important features that should be taken into account is that drugs and auxiliar substances may change surface tension properties of the liposomal formulation resulting in incompatibilities with the ocular surface [124].

It is well known that some components present in liposomal formulations, such as cholesterol and phospholipids (i.e., soy phosphatidylcholine) have relatively low surface tension values which result to be highly compatible with rather adhesive surfaces such as the ocular surface [125].

3.3.1.5. Osmolarity

Isotonicity of formulations is a rather important feature to adjust, but particularly in topical ocular liposomal dispersions. Tear osmolarity is minimum at night when the lids are closed and show its higher value during the day. Besides, alteration in osmolarity can make cellular tight junctions weaker and decrease the number of mucus secreting globet cells [126]. Generally, in healthy individuals the average osmolarity of tears and ocular surface is 300 mOsm/L [127]. In fact, tear osmolarity values higher than 308 mOsm/L are indicators of instability of the preocular tear film and are related to initial dry eye disease and over 316 mOsm/L moderate or severe dry eye disease [128]. That is the reason why some liposomal formulations developed for ocular surface diseases like keratoconjunctivitis sicca present hypotonic osmolar values between 200-290 mOsm/L in order to tackle hypertonic environment [129].

3.3.1.6. pH

PH of the ophthalmic formulations must be compatible with natural tears (pH 6.6 - 7.6) [130]. According to some of the established requirements for administering topical ophthalmic formulations the acceptable range of pH for topical ophthalmic drug delivery systems is between pH 6 – 9 [111].

The acidic pHs on the ocular surface may result in discomfort, inflammation and reduced wound healing capacity. Besides, a decrease in cell viability has been associated to pHs below 6 or above 8, so this parameter is an important feature to control when optimizing a novel liposomal formulation for topical ocular drug delivery [131].

Table 1. Physicochemical characteristics of a desired ophthalmic formulation

Physicochemical parameters	Value
PC/ cholesterol ratio	8/1
Size	100-200 nm
PDI	<0.2
Viscosity	1 - 8.3 mPa·s
Surface Tension	43.6 ± 2.7mN/m
Osmolarity	200-290 mOsm/L
pH	6-9
Zeta potential	0-10 mV

3.3.2. Purification methods

A common inconvenient when developing liposomal formulations with hydrophilic or partially water-soluble drugs, is that a portion of the drug is free in the aqueous buffer or inside the aqueous core, and concentrations are balanced depending on the gradient. Normally, partially water-soluble drugs try to keep balance between the outer and the inner core of the liposome. However, when it comes to full or almost full water-soluble drugs it is important to purify the liposomal dispersion and discard the excess of the drug that has not been encapsulated [132]. That could be the case of potentially toxic drugs dissolved in the aqueous media. For this reason, purifying methods are of vital importance, because they can help also to remove lipidic debris that are not forming the liposomes and could generate toxic degradation products. The common purifying methods are dialysis, gel filtration column chromatography, ion exchange chromatography, centrifugation,

ultrafiltration, protamine aggregation, liposome extruder purification and microfluidic [133].

3.3.3. Freeze drying of liposomes

Freeze drying has been widely used in the industry and research facilities to increase the stability, long storage capability of formulations such as nanoparticles as well as decreasing the risk of potential contaminations [134]. Regarding liposomal formulations, freeze-drying has been used for some authors to prepare lipidic materials in order to create liposomal transfection agents [135]. However, the freezing process and undergoing vacuum make liposomal dispersions unstable thus disrupting the vesicles leading to drug leakage as the potential disadvantage. However, recent research points out that when appropriate amounts of cryo-protectants such as trehalose are added to liposomal dispersions these problems could be avoided. Besides, coating liposomes with smart polymers could resolve the stability and leakage issues [106], therefore allowing researchers and industries to storage them as a powder.

Another study has visualized liposomes through confocal and transmission electron microscopy techniques in order to demonstrate that liposomes that underwent freeze drying exhibited similar sizes and polidispersity indexes than those that were not freeze dried [117].

3.3.4. Sterilization

It seems that sterilization of liposomal dispersions is still unclear. The methods that can be used to sterilizing liposomal formulations are particularly challenging since, due to their nature, many of the lipid substances that create the system are rather unstable at high temperatures or susceptible to denaturation. Filtration through a sterilizing membrane is one of the best options because no heat is produced and as far as the liposomes size is below 200 nm, they can pass through 0.2 μm sterilizing filters. However, some difficulties that are related to viscosity and surface tension of the formulation can lead to a quick blockage of the filtration membrane, and an increment in permeation of bacteria and pathogens may occur. Therefore, it is important to choose the most appropriate membrane depending on the conditions for the liposomal sample [136]. For that reason, although these conventional methods might be enough, a final sterilizing process is still required by some manufacturers.

The most common used procedures for the industry involve the use of irradiation (γ or UV), which links to a direct damage of the DNA through the formation of free radicals that make DNA strand unstable. Furthermore, lipid peroxidation is the main problem that occurs when γ-irradiation or UV-irradiation hit phospholipids and cholesterol present in the liposomal dispersions, thus creating O_2^- and •OH radicals respectively [137].

Another well-known sterilization method is steam sterilization with the use of an autoclave (121ºC or 134ºC for 15-20 minutes). Although lipid peroxidation is avoided in this process due to the lack of oxygen and generation of free radicals, hydrolysis of the lipidic materials might occur. These could lead to an alteration in drug loading efficiency as well as a variation in size distribution [137]. However, when selecting an ideal aqueous buffer these issues could be minimized [138].

Regarding the use of dry heat sterilization, it has been described as an unsuitable method for liposomal formulations because of the constant heating ratios leads to the evaporation of the aqueous phase and the alteration of every property of the mixture [137].

Finally, sterilization through ethylene oxide is discussed as an alternative 'cold' method commonly used for thermosensitive preparations. According to previous published works this method does not alter neither vesicle size nor structure and liposomes are reconstituted upon lyophilization without any apparent changes [139]. However, one of the main drawbacks of this technique when used for industry manufacturing is the potential risk that encompass the presence of ethylene oxide vapors residues such as mutagenicity, flammability or being a potential carcinogen [140]. Therefore, according to the above-mentioned methods, sterilizing filtration can be considered as the best option combined with cold methods if scalability or an industry approach is desired.

4. Liposomes as drug delivery systems in anterior and posterior segment diseases

Liposomes have been extensively studied for topical ocular administration due to their properties of biodegradability and biocompatibility and their ability to act as drug carriers. Furthermore, as previously mentioned, both hydrophilic and lipophilic drugs can be entrapped. Liposomes facilitate drug penetration in intraocular tissues by coming into intimate contact with the corneal and conjunctival surface, which resulted in special

relevance for high molecular weight drugs, poorly soluble drugs or those with low distribution coefficients [141] [142].

Liposomes are able to interact with cells (Fig.3) and release the entrapped active substance, facilitating the entry of drugs by various mechanisms. Among them, specific or unspecific adsorption on the surface of cells, fusion with the membrane, lipid exchange by the transfer-protein-mediated exchange or endocytosis are the most employed. When endocytosis of liposomes occurs, the endosome can break and release the content in the cell cytoplasm or reaching lysosomes where they are degraded (Figure 3) [14] [143] [144] [145].

Many authors have studied the advantages of liposomal formulations for ophthalmic application of active substances, reducing their potential toxicity and increasing their penetration and bioavailability compared to the free drug.

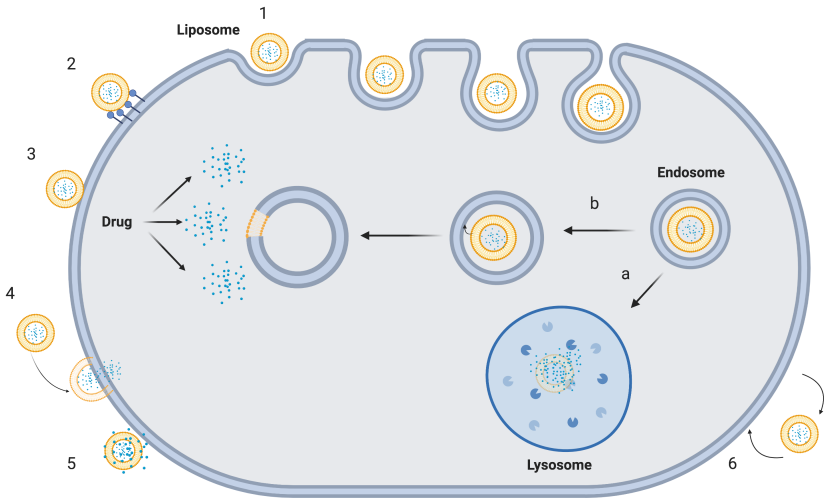

Figure 3. Liposome and cell interaction mechanisms. Liposomes can penetrate cells via endocytosis (1) and be transported to the lysosome (a) or be released into the cytoplasm after destabilizing the endosome. Liposomes can also be adsorbed specifically (2) or nonspecifically (3) by cells. Liposomes can penetrate into the cell after the fusion of the liposome membrane with the cell membrane, favoring the entrance of the drug in the cytosol (4). Liposomes, when adsorbed, can be destabilized and release the drug, which would enter the cell by micropinocytosis (5). Finally, liposomes can enter the cell by exchanging its lipid components with lipids from the membrane (6)

CAPÍTULO I

4.1. Antimicrobial agents

Liposomes are extremely versatile systems able to entrap a wide variety of substances such as hydrophilic, hydrophobic or biotechnological products like antibodies, genetic material or proteins. It is worth mentioning that entrapping substances of different nature into liposomes could entail an interesting strategy to increase the stability of potential therapeutic drugs that in solution could suffer hydrolytic or proteolytic processes as well as enzymatic degradation [113].

Although encapsulation is the common term to explain drug internalization or uptake in liposomes, entrapment efficiency is the most suitable one, since it may refer to adhered drug to the surface, entrapment in the bilayers or inclusion in the aqueous core of the liposomes [146]. For instance, these features have been used to entrap antibiotics, reduce their toxicity and increase their effectivity. Water soluble and moderate soluble antibiotics vancomycin, teicoplanin and rifampin were successfully encapsulated in liposomes, achieving high encapsulation efficiencies for teicoplanin and rifampin, 82.7% and 84.1% respectively through the reverse phase evaporation method [147]. For example, tobramycin is one of the most well-known and used topical antibiotics that has been successfully entrapped in liposomes. In fact, a volume of 0,4 mL of a 'mega' liposomal dispersion (10-100 μm) containing entrapped tobramycin (35 mg/mL) administered in a single dose to rabbits demonstrated higher or comparable efficacies compared to rabbits that received repeated instillations every hour [112] [148].

Liposomes have been studied as transporters in ocular infections due to their properties, being a vehicle that requires less dosage, increases its effectiveness, avoid systemic exposure and decreases antibiotic resistance [143][149]. Ciprofloxacin is a fluoroquinolone effective against gram-positive and gram-negative bacteria. Besides, 1 mg/mL of ciprofloxacin formulated in liposomes allows the *in vitro* controlled release of the drug for 24 hours in contrast to the drug solution at same concentration, which showed a 92,62% of the released drug in only 2-hour [150], This ability was previously reported in other studies [151]. In another *in vivo* study, after topical application of 50 μl of different formulations in rabbits, at least 3-folds greater bioavailability was obtained for liposomal formulations with doses ranging 107.63-114.52 μg ciprofloxacin compared to the dose contained in commercial eye drops (150 μg ciprofloxacin). Besides, higher concentrations of

ciprofloxacin were found in the aqueous humor. In this sense, the liposomal formulation which reached the highest concentration in the aqueous humor obtained 3.87 μg/ml, compared to 2.68 μg/ml obtained with the commercial aqueous formulation [152]. Azithromycin liposomes also showed an increase in corneal permeability, increasing the permeability coefficient from 4.43 ± 0.27 cm/s in solution to 8.92 ± 0.56 cm/s for the liposomal formulation.

On the other hand, in a dry eye rat model, after topical instillation for 7 days, 3 times a day of 20 μL of different eye drops, an improvement in symptoms were observed when using azithromycin liposomes, compared to the drug in solution (10 mg/mL of azithromycin in both of them), with a significantly greater improvement in tear break up time and fluorescein staining score ($P < 0.01$) [153].

4.2. Antiviral therapy

Topical treatment of viral eye infections like herpes simplex virus (HSV) or secondary herpes simplex keratitis has also been improved with the use of liposomes [154] [155]. A study in rabbits compared ganciclovir liposomes (1 mg/mL) with a ganciclovir solution (1 mg/mL) after the topical instillation of 50μL. The results shown a greater corneal permeability, resulting in an apparent coefficient of permeability 3.9 times higher than the drug solution, and an increase in absorption, obtaining an area under the curve of the concentration in aqueous humor 1.7 times higher [156]. Also, distamycin A liposomes used for acyclovir-resistant HSVs were reported to have the same antiviral capacity as distamycin in solution and to be less cytotoxic on rabbit corneal epithelial cells (the 48-hour viability of the liposomal formulation resulted 80% versus 60% of the drug solution).

Moreover, the amount of drug detected in the corneal tissues 30 minutes after 50 μL instillation of eye drops (0.05 mg of distamycin) in rabbits was greater for the liposomal formulation than for the solution, being 2,028 ± 0.063 ng / mg and 1,579 ± 0.087 ng / mg respectively [157].

4.3. Antifungal agents

Regarding to fungal keratitis, several authors have carried out studies with liposomal antifungal formulations [158][159]. A study in 40 rabbits with Candida albicans showed that the administration of 50 μl of topical liposomes loaded with fluconazole (2 mg/mL)

significantly improved the healing compared to the same concentration of fluconazole solution, obtaining a whole healing at 3 weeks in 86.4% of cases, compared to 50% obtained in rabbits to which the fluconazole solution had been administered.

The drops were administered during the first 3 days with a frequency of 4 times a day, and subsequently with a frequency of 3 times a day [160]. On the other hand, a clinical study was carried out with 11 patients with keratitis caused by Candida albicans. Patients were administered a 2 mg/mL fluconazole liposomal formulation 3 times a day. The mean diameter of the ulcers presented by the patients decreased from 5.5 mm to 1.3 mm after one month of treatment, obtained an amelioration in the rate of recovery and a decrease in the frequency of administration for fluconazole liposomes. [161]. It is worth mentioning that a liposomal collyrium of Amphotericin B 0.5% is developed and used for fungal infections of the ocular surface in hospitals [162].

4.4. Hypotensive agents as glaucoma treatment

Numerous studies have been conducted with drugs capable of reducing intraocular pressure carried with liposomes for topical administration. Brinzolamide, a carbonic anhydrase inhibitor, was characterized and tested *in vitro* and *in vivo* compared to a drug suspension with ten times more concentration (0.1% and 1% respectively). The transcorneal permeability study with the Franz diffusion chamber showed an increase in permeability compared to a commercial brinzolamide suspension, resulting 6 times higher for liposomes (2.58±0.04 in liposomes versus 0.35±0.01 in suspension). Furthermore, *in vivo* studies in rabbits showed that one topical instillation of 50 µL of the liposomal formulation was more effective in reducing long-term intraocular pressure, so that for liposomes the sustained effect in the reduction of intraocular pressure lasted 12 hours, while the suspension was no longer effective 30 minutes later [163].LUVs have reported to be an interesting way of achieving a sustained release of lipophilic drugs into the eye. A good example of that, is a liposomal formulation of LUVs made out of egg phosphatidylcholine at a concentration of 18 mM (109 ± 18 nm average size) that was able to release latanoprost through a single subconjunctival injection for up to 90 days in rabbits. The animals were instilled with 1.5 µg of latanoprost per drop daily. The liposomal formulation presented high ratios of drug loading (94% ± 5%) and was able to lower the IOP in rabbits in more than 2.8 folds with residual effect of 50 days [113].

In addition, the incorporation of viscous polymers such as hypromellose (HPMC) 0.3% or hyaluronic acid (HA) 1.2% [11] has been previously reported for increasing drug uptake and efficacy of topical hypotensive liposomal formulations. Apart from increasing the time the formulation was in contact with the eye, therefore increasing permeation, these two polymers were hypothesized to contribute to the ocular surface protection [84].

4.5. Anti-inflammatory agents

Triamcinolone acetonide, usually employed in intravitreal injections for vitreoretinal diseases, was administered in a topical liposomal formulation in rabbits: 50 µL every 2 hours, 6 times a day for 14 days. The formulation (2 mg/mL) was able to reach the vitreous and the retina. Drug concentrations at 12 hours were 252.10 ± 90.00 ng / g in the retina, and 32.6±10.27 ng / g in the vitreous humor [164]. In a recent pilot study, 2 mg/mL triamcinolone acetonide liposomal formulation was tested in 12 patients suffering from refractory pseudophakic cystoid macular edema. In this study, a drop of the topical liposomal formulation was applied every 2 hours for 90 days, providing an adequate tolerability and therapeutic activity. Results showed an improvement of 20.08 ± 10.35 letters in the best corrected visual acuity (BVCA) and a reduction of 206.75 ± 135.72 µm in the central foveal thickness (CFT) at 20 weeks after the beginning of the study [165]. A liposome-based formulation also had the capacity of encapsulate the corticoid medroxyprogesterone acetate (MPA) (0,2 mg/mL) to treat inflammatory eye processes. The anti-inflammatory effect was tested in vitro after a 60-minute exposure, showing a further improvement of the effect in Human corneal epithelial cells than reference non-liposomal formulation (Medrivas®). The cytokine production after TNFα stimulation was determined by an enzyme-linked immunosorbent assay (ELISA). While the MPA solution only showed a reduction in the IL-6 cytokine, the liposomal formulation reduced both IL-6 and IL-8 production. The uptake of the liposomes by the cells was also evaluated in rabbits using coumarin-6 (C6) liposomes. After 5 minutes, the corneal epithelium showed fluorescence and after a 60-minute exposure, also the corneal stroma. [111].

Similar liposomal formulation was employed to encapsulate the thrombospondin-1-derived peptide KRFK. The liposomal formulation was tested in an in vitro model of corneal and conjunctival epithelium, obtaining an apparent enhancement in corneal permeability [166].

The use of antiangiogenic and anti-inflammatory-loaded liposomes entails a potential effective therapy for the treatment of diseases that affect the posterior segment of the eye. However, precipitation and crystallization are one the main drawbacks when co-encapsulating these compounds. Some of the strategies to solve these difficulties involves the use of different cholesterol/lecithin ratios or the adjustment of the pH, that help increase the active drug loading. With respect to this issue, Lai S et al studied the difficulty of encapsulating drugs of different polarities in liposomal formulations since two drugs that differ too much between their polarities could result in precipitation or decrease in encapsulation [167].

4.6. Antitumoral substances

Other strategies to increase drug entrapment or encapsulation include the attachment of PEG-like polymers to the surface of the liposomes that may increase interactions between the drug and polymers. In reference to this, liposomes have been used as useful and specific tools to encapsulate immunoreactive substances such as anti-tumoral products targeting HER2/ neu and CD20 receptors [168].

4.7. Gene delivery

Furthermore, over the last few years cationic liposomes containing DOTAP or DOTMA [169] loaded with genetic material have gained much attention since they constitute a useful approach to tackle genetic diseases, cancer, or even deliver vaccines [170]. For example, an interesting strategy to increase the DNA plasmid loading capacity of cationic liposomes is to combine it in appropriate ratios that allows DNA condensation and to include it together with cyclodextrins [114], as well as adding a spacer, based on amino acids, introduced between the polar head and the hydrocarbon tail.

This strategy aims to increment the cellular uptake of liposomes containing a plasmid and to decrease lysosomal degradation. According to this study, liposomes containing a tailored lysine group as a spacer achieved much higher encapsulation ratios (22%) and transfection efficiencies than others. Furthermore, the combination of cationic liposomes and these poly-lysine spacer present encapsulation efficiencies similar to transfections reagents but showing a decrease in toxicity [135].

With respect to the use of liposomes that contain genetic material for topical ophthalmic administration, surface engineered liposomes have been developed in order to carry siRNA molecules to the retinal pigmented epithelium or regulate VEGF expression in age-related macular degeneration (AMD). This strategy could entail a less invasive and more effective strategy as an alternative for intravitreal injections [171]. SiRNA liposomes tackling heat shock protein 47 (HSP47) combined with vitamin A are presented as an attractive strategy for dry eye disease [172]. It is important to remark that liposomes are very interesting and attractive systems that allow to encapsulate and deliver topical ophthalmic substances that administered systemically or via intravitreal injections could entail risks or generate side effects [173].

4.8. Immunosuppressants

Immunosuppressive drugs such as cyclosporine, tacrolimus or everolimus have been successfully employed for treating mild to severe symptoms of dry eye disease or avoiding graft versus host disease after allogeneic transplants [174][175][176]. For example, a study published by Y. Dai demonstrated that liposomes carrying bile salts such as sodium deoxycholate, sodium taurocholate and sodium glycocholate as an alternative to cholesterol, and together with tacrolimus, experimented higher transcorneal permeation ratios (29.50 ± 5.78, 36.24 ± 3.51 and 29.73 ± 4.03 cm/sec respectively) than conventional liposomes loaded with the single drug (8.00 ± 2.05 cm/sec) [177].

Another comparative study between cyclosporine liposomes and the commercial emulsion Restasis® demonstrated enhanced corneal permeation and uptake by immunosuppressive liposomes. It seems that stabilization of the tear film by liposomes may seem an interesting and effective strategy to increase drug permeation through corneal layers [178]. Besides, latanoprost has been also included in liposomal systems for subconjunctival administration to treat ocular hypertension in glaucoma. Liposomes containing latanoprost 0.005% demonstrated enhaced drug permeation in clinical trials (NCT02466399) [179][164].

Table 2. Liposomes for topical ocular drug delivery.

Phospholipids used and other lipid components	Lipidic components ratio (weight)	Polymer	Preparation method	Vesicle size	Active substance (concentration)	Encapsulation efficiency	Applications	Reference
DOPE: DOTAP: Ch: CD	7.7:7.3:0.001:5.2	CD	Lipid film rehydration	186 nm	DNA	22%	Transfection	[114]
DPPC: Ch	3:1	-	Reverse- phase evaporation	100-200 nm	Vancomycin Hydrochloride	39.4%	Bacterial infections	[147]
				100 nm	Teicoplanin	82.7%		
				100 nm	Rifampin	84.1%		
DMPG: triolein: Ch	0.26:0.29:1	-	Lipid film rehydration	10-100 µm	Tobramycin (35 mg/mL)	60%	Keratitis caused by Pseudo- monas aeruginosa	[112] [148]
Egg PC: Ch: DCP	-	-	Reverse- phase evaporation	6.67 nm	Ciprofloxacin (1 mg/mL)	71.4%	Bacterial infections	[150]
Soy PC: Ch: DODAB	7:1:2	-	Reverse- phase evaporation	387 nm	Ciprofloxacin (2 - 3 mg/mL)	39.6%	Bacterial infections	[152]
DPPC: Ch: DODAB	7:1:2			388 nm		37.1%		
DMPC	-			410 nm		36.7%		
CHEMS: Egg PC DSPE-PEG 2000: MCT: Vit E	1.85:10:0.5:2:0.05	-	Lipid film rehydration	60 nm	Azithromycin (10.0 mg/mL)	95.6%	Bacterial infections	[153]
Soy PC: Ch: SDC	12:1.7:1	-	Reverse- phase evaporation	210 nm	Ganciclovir (1.0 mg/mL)	51.2%	Viral eye infections	[156]
Soy PC: Ch	4:1	-	Reverse- phase evaporation	358.4 nm	Distamycin A (1.38 mg/mL)	34.5%	Acyclovir-resistant herpes simplex virus (HSVs)	[157]
-		-	Reverse- phase evaporation	-	Fluconazole (2 mg⁄mL)	-	Candida albicans infection	[160]
-		-	Reverse-phase evaporation technique	-	Fluconazole (2 mg⁄mL)	-	Candida albicans infection	[161]

Table 2. (Continued)

Phospholipids used and other lipid components	Lipidic components ratio (weight)	Polymer	Preparation method	Vesicle size	Active substance (concentration)	Encapsulation efficiency	Applications	Reference
Soy PC: Ch	4:1	-	Lipid film rehydration	84.3 nm	Brinzolamide (1 mg/mL)	98.3%	Glaucoma	[163]
Egg PC	-	-	Lipid film rehydration	109 nm	Latanoprost	94%	Glaucoma	[113]
PEG -12 glyceryl dimyristate: Kolliphor HS 15	1:2	-	Self-forming (Qsomes)	187.8 nm	Triamcinolone Acetonide (2 mg/mL)	21– 35%	Retinal disorders	[164]
PEG-12 glyceryl dimyristate: Kolliphor HS 15	1:2	-	Self-forming (Qsomes)	187.8 nm	Triamcinolone Acetonide (2 mg/mL)	-	Cystoid Macular Edema After Cataract Surgery	[165]
Soy PC: Ch: Vit E	13:3:0.2	-	Lipid film rehydration	185.5 nm	Medroxyprogesterone acetate (0.2 mg/mL)	94%	Inflammatory processes associated with ocular surface diseases	[111]
Soy PC: Ch: Vit E	13:3:0.2	-	Lipid film rehydration	230.4 nm	Thrombospondin-1-derived peptide (0.25 mg/mL)	>80%	Ocular surface inflammation	[166]
Soy PC: SDC	3:1	-	Lipid film rehydration	100 nm	Tacrolimus	94.1%	Graft versus host disease	[177]
Egg PC: Ch:SA / DCP	1.6:1:0.15	-	Lipid film rehydration	-	Acyclovir (1.24 mg/mL)	-	Viral eye infections	[155]
Egg PC: Ch:SA	7:4:1	-	Lipid film hydration	9.3 µm	Acetazolamide (10 mg/mL)	34 %	Glaucoma	[180]
Egg PC: Ch	7:4			7.0 µm		23.6%		
DPPC*: Ch:SA	7:2:1	-	Lipid film hydration	5.3 µm	Prednisolone acetate (1.25 mg/mL)	90.3%	Uveitis	[181]
Soy PC: DOTAP	2:1	-	Lipid film rehydration	96.5 nm	Voriconazole (2.5 mg/mL)	85.1 %	Fungal keratitis	[158]
Egg PC: DOTAP	10:1	-	Lipid film rehydration	84.6 nm	Astaxanthin (200 µM)	101.9%	DED	[182]

Table 2. (Continued)

Phospholipids used and other lipid components	Lipidic components ratio (weight)	Polymer	Preparation method	Vesicle size	Active substance (concentration)	Encapsulation efficiency	Applications	Reference
Egg PC: Ch: DCP	2:1:0.5	CH 1%	Reverse- phase evaporation	8.0 µm	Ciprofloxacin -	60.3%	Bacterial conjunctivitis	[183]
Egg PC: Solutol HS-15	7.5:1	CH 0.1%	Ethanol injection method	144.5 nm	Flurbiprofen -	92.5%	-	[184]
Soy PC: Ch	5:1	CH 0.5%	Lipid film rehydration	150.7 nm	Timolol maleate -	75.9%	Glaucoma	[185]
Soy PC: Ch	8:1	CH 0.5%	Lipid film rehydration	135.5 nm	Triamcinolone acetonide (1.5 mg/mL)	90.7%	Macular edema	[186]
Soy PC: Ch: DCP	8:1:2	CH 0.5%	Lipid film rehydration	100.3 nm	Triamcinolone acetonide (0.16 mg/mL)	98%	Macular edema (ME)	[187]
Soy PC: Ch	5:1	CH 0.3 %	Lipid film rehydration	176 nm	Triamcinolone acetonide (2.5 mg/mL)	74%	Choroidal neovascularization	[188]
Soy PC: Ch: PS	3:1:0.1	LCH 0.5%	Ethanol injection	84.0 nm	Diclofenac (1 mg/mL)	99.9%	Postoperative ocular inflammation and pain	[189]
Soy PC: Ch: PS	4:1:0.1	LCH 0.25%	Ethanol injection	89.6 nm	Cyclosporin A (1 mg/mL)	-	Immune and Inflammatory ocular diseases	[190]
Soy PC: Ch: Vit E	4.88:1:X	TMC 0.5%	Ethanol injection	91.9 nm 307.4 nm	Coenzyme Q10 -	95.9%	Cataract	[191]
Egg PC: Ch	4.33:1	TMC 0.5%	Reverse-phase evaporation	158.3 nm	Cyanidin-3-glycoside -	53.7%	Oxidative stress	[192]
PC: Ch:SA	15:3:2	HA -	Lipid film rehydration	134.6 nm	Doxorubicin (0.8 mg/mL)	84.9%	Proliferative vitreoretinopathy (PVR)	[193]
Soy PC: Ch	4:1	HA 0.7%	Lipid film rehydration	218.5 nm	Fluconazole (9 mg/mL)	42.8%	Ocular fungal infections	[194]
Soy PC: Ch	8:3	DGG 0.4%	Reverse -phase evaporation	136.0 nm	Timolol maleate (2.5 mg/mL)	47%	Glaucoma	[195]

Table 2. (Continued)

Phospholipids used and other lipid components	Lipidic components ratio (weight)	Polymer	Preparation method	Vesicle size	Active substance (concentration)	Encapsulation efficiency	Applications	Reference
Soy PC: Ch: Vit E	8:1:0.08	SH 0.2%	Lipid film rehydration	162.8 nm	5-MCA-NAT (27.5 µg/mL)	-	Glaucoma	[196]
		CMC 0.5%		188.5 nm				
Soy PC: Ch: Vit E	8:1:0.08	HPMC 0.3%	Lipid film rehydration	169.7 nm	Acetazolamide (0.7 mg/mL)	64.9%	Glaucoma	[84]
DPPC	-	PMA 0.1% m/m	Lipid film rehydration	-	Ciprofloxacin (0.1% m/m)	13.7%	Eye infections	[151]
		PVA 0.14% m/m				13%		
Soy PC: Ch:SA	5:3:1	Carbopol 940 1.5%	Reverse-phase evaporation	-	Ciprofloxacin (3 mg/mL)	82%	Eye infections	[197]
Soy PC: Ch	5:3	Carbomer 934 0.17%	Lipid film rehydration	138.2 nm	Ciprofloxacin (3 mg/mL)	68.5%	Eye infections	[198]
Soy PC: Ch	7:3	Pluronic F-127 20% w/w	Reverse-phase evaporation	1.35 µm	Latanoprost (50 µg/mL)	98.4%	Glaucoma	[199]
PC: Ch:SA	2:1:X	Pluronic F-127 -	Lipid film rehydration	2.90 µm	Ketorolac (10 mg/mL)	63%	Postoperative inflammation and pain	[200]
Soy PC: Ch:SA	30:10:1	SF 1%	Ethanol injection	286.5 nm	Ibuprofen -	62.8%	-	[201]

PC: Phosphatidylcholine; PS: Phosphatidylserine; DMPC: Dimyristoyl-sn-glycero-3-phosphocholine; DPPC: Dipalmitoylphosphatidylcholine; DPPC*: 1,2 dipalmitoyl-sn-glycerol-3-phosphocholine ; MCT: Medium-chain triglyceride; Ch: Cholesterol; CHEMS: Cholesteryl hemisuccinate; SA: Stearylamine; DODAB: Dioctadecyldimethylammonium bromide; DOTAP: dioleoyl-3-trimethyleammonium propane chloride; DCP: Dicetyl phosphate; SDC: Sodium deoxycholate; PEG: Polyethylene glycol; CH: Chitosan; LCH: low molecular weight chitosan; TMC: Trimethyl chitosan; HA: Hyaluronic acid; HS: Sodium hyaluronate; CMC: Carboxymethyl cellulose; HPMC: Hydroxypropyl methyl cellulose; DGG: Deacetylated gellan-gum; PMA: Polymethacrlic acid; PVA: poly (vinyl alcohol); SF: Silk fibroin; Vit E: Vitamin E; 5-MCA-NAT: Methoxycarbonylamino-N-acetyltryptamine; CD: Carboxymethyl-β-cyclodextrin; X: Information not provided by authors.

5. Strategies to increase ocular retention time of topical liposomal formulations

Different technological approaches have been developed to increase the retention time of liposomal ophthalmic formulations applied topically. To this, the use of positively charged liposomes or the addition of polymers with different properties to liposomal formulations have been assayed.

Positively charged liposomes have shown a more prolonged interaction with the negatively charged ocular surface allowing the formation of a layer that completely covers the eye surface. Electrostatic interactions between positively charged liposomes and the negative charges of the mucin layer increase the retention time of the ophthalmic formulation [112]. On this subject, stearylamine is a lipid that included in liposomes confers a positive charge on the lipid bilayer and has been studied to encapsulate different drugs. In a study conducted with acyclovir-loaded liposomes, stearylamine and dicetylphosphate were used to confer positive and negative charge to the vesicles respectively. Researchers reported an increase in the absorption of the drug into the cornea of rabbits when using positively charged liposomes. The charge of the liposomes influenced the amount of the drug in the cornea. Corneal concentration 2.5 hours after topical administration of 50 μL (1.24 mg/mL) resulted 1093.3 ± 279.7 ng/g and 571.7 ± 105.3 ng/g after the use of positively and negatively charged liposomes respectively, and 253.3±72.0 ng/g after the administration of the solution. In addition, the positively charged liposomes seemed to bind more intensely to the corneal surface [155]. The use of liposomes with different charges to encapsulate acetazolamide, has also been studied. In this case, liposomes were formulated with phosphatidylcholine and cholesterol in different molar ratios and stearylamine or dicetylphosphate as charge inducing agents. The effect of both formulations was compared *in vivo*.

Results showed that positively charged liposomes provided a more effective decrease in intraocular pressure in rabbits: The best results were obtained using multilamellar liposomes prepared with phosphatidylcholine: cholesterol: stearylamine in a molar ratio of 7: 4: 1, so that 3 hours after topical administration of positively charged liposomes (50 μL) with a 1% concentration of acetazolamide provided an IOP reduction of 7.8 mmHg versus 5.5 mmHg when neutral liposomes were used. [180]. A different investigation with 1.25

mg/mL prednisolone acetate positively charged liposomes composed of 1,2 dipalmitoyl-sn-glycerol-3-phosphocholine, cholesterol and stearylamine yielded similar results, observing a 2-times slower release rate than the solution (1.25 mg/mL) and an increase in the concentration of the drug in the aqueous humour of rabbits about 27-40% with respect to the drug solution after a topical administration of 50 μL. Furthermore, the AUC of positively charged liposomes was higher than that of neutral liposomes [181].

Another lipid component used to confer a positive charge on liposomes is dioleoyl-3-trimethyleammonium propane chloride (DOTAP). A study with a voriconazole liposomal formulation with DOTAP concluded that liposomes were capable of adhering to mucins *in vitro,* so that when adding a suspension of mucins with 500 nm size to the liposomal formulation, the size of liposomes increased from 96.5 ± 2.2 nm to 2441.3 ± 164.5 nm, showing the formation of aggregates, which was not observed with vesicles without positive charge. In addition, tolerance measured using the HET-CAM test (Hen´s Egg *Test* corioallantoic membrane) showed a weak irritation. In an *ex-vivo* permeation test performed with porcine corneas, the DOTAP-liposomal formulation with 2.5 mg/mL of voriconazole also managed to reach a voriconazole concentration of 45.31 ± 2.02 and 62.14 ± 7.84 μg / cm^2 after exposures times of 30 and 60 minutes respectively. [158].

In a recent study, researchers developed positively charged liposomes using DOTAP to encapsulate the antioxidant astaxanthin. The formulation was tested in a dry eye disease rat model. They observed a higher corneal affinity when using positively charged liposomes in comparison with a neutral liposomal formulation. The antioxidant positive liposomal formulation also appeared to be more effective in suppressing the up-regulated expression of age-related markers presented in the DED rat model [182].

Different polymers have been used together with liposomes to increase the retention time and mucoadhesion of ophthalmic formulations. One of the most studied polymers is chitosan, a biodegradable cationic heteropolysaccharide with great biocompatibility and low toxicity capable of interacting with the negatively charged corneal surface, increasing the retention time and drug penetration. Ciprofloxacin HCl loaded-liposomes (composed of phospholipid, cholesterol, and dicetylphosphate as negatively charged agent) were coated with 1% medium-molecular-weight chitosan. In this study a reduction in encapsulation efficiency was observed for the chitosan coated liposomes, being 60.280% ± 0.642

compared to 71.400% ± 0.247 in the non-coated liposomes. The apparent permeability coefficient was higher than that of the non-coated liposomes and the free drug in *ex vivo* permeability studies (8,632 ± 0.354, 4.412 ± 0.113 and 5,188 ± 0.228 respectively). Moreover, the chitosan-coated liposomes were able to inhibit the growth of *Pseudomonas aeruginosa* 24 hours after a single dose administration of 50 µL in rabbits with induced bacterial conjunctivitis [183]. In a different study, chitosan coating of flurbiprofen deformable liposomes improved transcorneal permeation *in vitro*, with an apparent permeability coefficient 1.29 folds higher than the uncoated ones and 4.59-fold higher than the 0.03% flurbiprofen solution. The study of the residence time *in vivo* also showed a significant improvement, being more than 2 times greater than the results of deformable liposomes without chitosan [184]. Chitosan coated timolol maleate liposomes (50 µL) resulted also more effective in reducing intraocular pressure than eye drops. In addition, the chitosan coated liposomes showed the ability to bind to mucins in a mucoadhesive study, showing an increase in the size of the liposomes from 151.2 ± 20.3 nm to 1013 ± 81.2 nm due to the formation of aggregates when adding mucins and an increase in the apparent diffusion coefficient in 3 times with respect to eye drops [185].

Recently, several studies have been conducted with triamcinolone acetonide encapsulated in chitosan-coated liposomes. *In vitro* and *in vivo* studies of chitosan 0.5% coated liposomes containing 1.5 mg/mL triamcinolone acetonide (TA) were performed. The authors reported a more sustained release profile for the coated liposomes than the ones observed for a suspension or non-coated liposomes of the drug.

Moreover, the results of a histological study after the administration of the formulation in C57BL/6 mice showed an absence of corneal and conjunctive cell toxicity. In addition, they assessed the ability of chitosan-coated liposomes to reach the anterior and posterior segment eye tissues after topical application using Coumarin-6 (C6) as a fluorescent marker. C6 was carried in both liposomes with and without chitosan and in solution. The results suggested a higher up-take in corneal epithelial cells (HCEC) and retinal pigment epithelial cells (ARPE-19) for chitosan-coated liposomes, showing greater fluorescence. 5 µl of these formulations were also administered topically to C57BL/6 mice. The results showed higher fluorescence intensity in the anterior and posterior segment for C6 encapsulated in liposomes coated with chitosan [186]. After these results, the same researchers studied the application of TA in chitosomal liposomes for the treatment of

macular edema (ME) in a rat model with induced retinal edema. The results after the administration of 20 µL with a TA concentration of 160 mg/L showed a remission of the edema after 10 days, similar to that produced by intravitreal injection of a suspension of triamcinolone acetate, being suggested as an alternative to intraocular injections, reducing the resulting complications [187]. Mehanna et al. also studied a triamcinolone acetate chitosan coated liposomal formulation in a rat model with induced Choroidal neovascularization (CNV), obtaining as a result a sufficient level of drug in the vitreous humour after topical administration of 2.5 mg/mL (0.5 mL) TA chitosan liposomes 3 times per day for 15 days [188].

One of the problems with chitosan is its low solubility in water. However, its aqueous solubility can be improved by using chitosan derivatives, such as low molecular weight chitosan or trimethyl chitosan. Low molecular weight chitosan coated liposomes loaded with diclofenac showed greater stability at 25ºC for 30 days than the conventional vesicles and the drug in solution. Furthermore, coated liposomes showed an extended release of the drug (23.8% at 6 hours compared to 38.9% with conventional liposomes) and an increase in the apparent permeability coefficient (1.174 ± 0.080 versus 0.789 ± 0.069 in the non-coated liposomes). *In vivo* studies in rabbits showed a significant increase in the retention time compared to non-coated liposomes and the free drug (0.1% diclofenac in all cases). Also, *in vivo* tolerance studies showed no irritation in either the short or long term after the instillation of 150 µL (3 times each 10 minutes and 5 times per day for 7 days respectively) [189]. The same authors prepared liposomes carried cyclosporine A (CsA) coated with low molecular weight chitosan. The formulation showed no cytotoxicity in conjunctival epithelial cells (cell viability greater than 90% after 2 h of exposure). *In vivo* studies were conducted in rabbits, administering 100 µL (1 mg/mL CsA) of the formulation topically and subsequently measuring the concentration of drug in the eye tissues. This study showed an increase in concentration compared to conventional liposomes at 2, 6, 12 and 24h in the sclera, conjunctiva and cornea [190]

Coenzyme Q10, an antioxidant used to reduce cataract formation, was loaded in N-trimethyl chitosan (TMC) coated liposomes. Rabbit assays reported decreased liposome drainage and therefore a longer retention time on the corneal surface for TMC-coated liposomes compared to ^{99m}Tc-DTPA solution (1.5-fold higher). The effectiveness of Coenzyme Q10 loaded in TMC-coated liposomes was evaluated in rats with induced

selenite cataracts being the opacity of the lens at 8 days 52% after the administration of the formulation (5µl/20 g) three times per day for 8 days, compared to 95% of the untreated group [191]. Similar results were found for the antioxidant cyanidin-3-glycoside (C3G), with a residence time in the cornea 3.3 times greater than the drug solution and 1.7 times higher than the conventional liposomes. The authors also reported a decrease in lens peroxidation with TMC-coated liposomes compared to non-coated liposomes in the rat animal model [192].

The low viscosity of liposomal formulation in an aqueous vehicle does not allow the retention time on the corneal surface to be sufficiently high, so the use of polymers with viscosizing and gelling properties allows the formation of a viscous layer covering the entire corneal surface protecting the drug from tear drainage. Also, mucoadhesive polymers improve the retention time thanks to the interaction with mucins of the preocular tear film.

Polysaccharide-derived polymers have been extensively employed in the preparation of ophthalmic formulations. Hyaluronic acid (HA) is an anionic polymer present in the extracellular matrix of animal tissues, being one of the main components of these in our body. HA is biocompatible and biodegradable with low toxicity. HA is able to retain water forming a hydrogel with mucoadhesive properties. Liposomes loaded with Doxorubicin and coated with HA showed a longer *in vitro* release, which continues at 24 hours. In addition, fluorescence cellular uptake studies in corneal epithelial cells also showed that liposomes in HA solution reached the cell nucleus the most compared to liposomes in an aqueous solution and the free drug. The authors also carried out *in vivo* studies in rabbits, with topical instillation of different formulations (50 µL with 0,8 mg/mL Doxorubicin). In addition, samples of tear fluid or samples of aqueous humour were collected to measure the retention time and the pharmacokinetic profile respectively. The results showed an increase in the retention time of the formulation with respect to the formulation without HA or the free drug: The mean retention time was 527.11 ± 604.89 min compared to 211.45 ± 52.04 and 152.73 ± 3.72 for the non-modified liposomes and free drug respectively. Furthermore, the liposomal formulation with hyaluronic acid also had the highest bioavailability, being 1.7 times greater than the free drug [193]. Another study combined the use of HA to form a hydrogel and liposomes, integrating HA into and out of fluconazole-loaded liposomal vesicles. This formulation was compared with a

conventional liposomal formulation and a fluconazole suspension in studies *ex vivo* and *in vivo* (rabbits). *Ex vivo* corneal permeation studies showed promising results in relation to increased corneal permeation in liposomes with 0.7% HA. The cumulative concentration of fluconazole in corneal tissues resulted much higher compared to conventional liposomes and drug suspension (0.9% fluconazole), being 1.86 and 2.6 folds higher respectively after 6 hours. Moreover, *in vivo* studies in which drug concentrations in aqueous humour were measured over time after a topical administration of 50 µL of the formulations showed a more sustained permeation profile and a higher value of area under the curve (AUC) after 24 hours in the case of the liposomal formulation with 0.7% HA and 0.9% fluconazole compared to fluconazole suspension (0.9% fluconazole). The AUC measured by the linear trapezoidal method was 530.62 ± 44.94 and 204.34 ± 7.46 respectively [194].

Gellan gum is another biocompatible and biodegradable polymer widely used in the pharmaceutical industry. In a study with liposomes loaded with timolol maleate, the derivative deacetylated gellan gum (DGG) was employed. DGG is an anionic polymer that forms a gel in the presence of a positive charge. In this study, liposomes were incorporated into the DGG to form an ion-sensitive gel *in situ* and the formulation was compared to commercial drops of timolol maleate. The researchers reported a 1.93-fold increase in the apparent partition coefficient when compared to conventional eye drops. Furthermore, *in vitro* release studies comparing formulations with DGG, and conventional liposomes showed a longer release profile due to DGG. *In vivo* studies in rabbits after 50 µL of topical administration showed an absence of eye irritation and a longer corneal retention time of the DGG liposomal formulation relative to eye drops, timolol maleate liposomes, and gel formulations view by fluorescence imaging. Furthermore, intraocular pressure measurements in rabbits after the topical administration of 50 µL eye drops or DGG liposomes (0.25% timolol maleate) showed greater long-term efficacy for the liposomal gel, obtaining a minimum IOP of 11.96 ± 0.74 mm Hg (1 hour after instillation) and an effect duration of 300 minutes for the liposomal formulation with DGG, and a minimum of 13.61 ± 0.95 mm (2 hours after instillation) and an effect duration of 180 minutes for eye drops [195].

Cellulose-derivative polymers such as carboxymethyl cellulose (CMC) or hydroxypropyl methyl cellulose (HMPC) have also been extensively studied in ocular topical administration. A study carried out with 5-methoxycarbonylamino-N-

acetyltryptamine (5-MCA-NAT) a hypotensive melatonin analogue (100 µM 5-MCA-NAT) formulated in solution, in liposomes and in liposomes combined with different polymers showed a more sustained *in vitro* release profile with the use of liposomes, being even slower if used together with polymers. The formulation with a slower release profile was the liposomal formulation dispersed in 0.5% CMC. The reduction of intraocular pressure in normotensive rabbits *in vivo* also after the instillation of 25 µL (0.7 µg 5-MCA-NAT) eye drops showed greater effectiveness in the case of liposomal formulations combined with polymers. The liposomal formulations that provided the significantly greatest reduction were the ones prepared with 0.2% sodium hyaluronate (SH) and in 0.5% CMC, with intraocular pressure reduction values of 39.13 ± 2.21% and 36.72 ± 2.77%, respectively. Furthermore, these formulations did not cause discomfort or eye irritation in 24-hour *in vivo* tolerance studies [196]. Tolerance and efficacy studies of acetazolamide formulations were carried out by comparing a liposomal formulation loaded with acetazolamide versus solution of the drug (0.7 mg/mL) and the vehicle, and subsequently the liposomal formulation of acetazolamide (0.7 mg/mL) with and without 0.3% HPMC. The reduction in intraocular pressure after topical administration of 25 µL of the formulations was measured every hour for 8 hours. The results first showed a significant decrease in the IOP values when acetazolamide was formulated in liposomes. The maximum intraocular pressure reduction was 16.6% in the case of liposomes compared to 10.1% in the acetazolamide solution. Furthermore, the 8-hour AUC was more than 2 times higher. Secondly, the addition of 0.3 % HPMC to the liposomal formulation showed a significant improvement in the reduction of intraocular pressure, increasing the maximum reduction in intraocular pressure by 1.4 times and providing an 8-hours AUC 1.5-fold higher compared to liposomal formulation without HPMC. Furthermore, none of the liposomal formulations showed signs of ocular toxicity in rabbits after being administered topically every 30 minutes for 6 hours [84].

Researchers used different concentrations of poly (vinyl alcohol) (PVA) and polymethacrylic acid (PMA) derivatives to increase the viscosity and enhance release profile of ciprofloxacin. The results showed that the release resulted extended when increasing the polymers concentration. Furthermore, when the polymers were used in combination with a liposomal formulation of ciprofloxacin the release was more prolonged. The obtained values of half-time of release were 4600 minutes when combining

the encapsulation of ciprofloxacin (0.1% (m/m)) in α-l-dipalmithoyl-phosphatidylcholine (DPPC) liposomes and the use of 0.1% (m/m) PMA, compared to 85 minutes in the case of not using liposomes. In the case of PVA, the half-time of release increased from 72 minutes to 644 minutes when combining 0.14% (m/m) PVA with the liposomal formulation [151].

Another example is carbopol, a polymer of acrylic acid with a large number of carboxyl groups in its structure that allow it to form a gel in the presence of water. Carbopol 940 was used by researchers to prepare a liposomal hydrogel with ciprofloxacin. The results of the study showed that the use of carbopol allowed a more delayed and prolonged release of ciprofloxacin compared to the liposomal suspension and the drug solution (0.3% ciprofloxacin). In addition, the use of the liposomal hydrogel showed an increase in the permeation in a study performed with albino rabbit corneas, being 5-folds higher than for the aqueous solution of ciprofloxacin. In addition, liposomal hydrogel showed a percentage permeated of 30.6% in contrast with the 20.4% of the liposomal suspension after 6 hours [197]. Mostafa Feghhi et al carried out a recent study with Carbomer 934 for coating 0.3% ciprofloxacin liposomes. They reported an enhancement in permeability and bioavailability, which resulted 4 times higher than commercial formulation, and antimicrobial effect in an *in vivo* study in rabbits. However, the results showed no improvement over those obtained with non-coated liposomes [198].

Poloxamers are co-polymers formed by a polypropylene central chain and two polyethylene side chains. In this way, the central chain has hydrophobic properties, and the lateral ones have hydrophilic properties. An example is Pluronic F-127, a temperature sensitive polymer capable of changing from a liquid to a gel state when in contact with body temperature. The use of Pluronic F-127 as a vehicle in liposomal formulations has been studied by several authors as a method to increase the residence time of the drug and to provide a sustained release. This co-polymer has been employed as a vehicle for a latanoprost liposomal formulation, observing a longer release of the drug when compared with conventional liposomes or the use of other polymers such as HPMC, so that after 24 hours approximately 30% of the drug had been released, compared to the 40% in other liposomes. This is probably due to the hydrophobic interactions of the central block with latanoprost, which allows a slower diffusion through the gel. Moreover, the use of latanoprost-loaded liposomes vehiculized with Pluronic F-127 showed more reduction of

intraocular pressure in normotensive rabbits compared to commercialized eye drops (both 50 μg/mL latanoprost). In this sense, while the intraocular pressure returned to baseline values 24 hours after topical administration of 50 μL eye drops, in the case of latanoprost liposomal gels the values did not return to baseline until 72 hours [199]. This thermosensitive Pluronic F-127 gel was also used by other authors to disperse ketorolac liposomes. The 24-hour *in vitro* release study showed that the liposomal gel formulation provided a more sustained release than the liposomal formulation without Pluronic F-127, proving to be able to maintain the release for around 24 hours. [200].

Protein polymers have also been used to coat liposomes. This is the case of silk fibroin (SF), a non-toxic natural mucoadhesive polymer that can be degraded by proteolysis suitable for drug delivery. SF is capable of binding to proteoglycans and glycoproteins of the mucous layer. Yixuan Dong et al. performed a study comparing SF-coated liposomes with conventional ibuprofen-loaded liposomes and a drug solution, showing more sustained release profile and a greater corneal permeability *in vitro*. The apparent permeability coefficient in the case of SF liposomes was 1.23 ± 0.24 cm/s compared to 1.16 ± 0.23 for normal liposomes. Also, cell viability was always above 85% after the addition of SF in a concentration range of 0.25-2% to human corneal epithelial cells for 2-8 hours. Furthermore, the toxicity and adhesion capacity of SF-coated liposomes was also tested on human corneal epithelial cells, resulting in an absence of toxicity and rapid cell adhesion and strong cellular up-take by observing the fluorescence [201].

Biomaterials with binding properties to glycan residues on the corneal surface have been also used to increase the retention time of drugs on the cornea as it is the case of succinyl- Concavalin A. Changyou Zhan et al. studied functionalized liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG) and cholesterol loaded with 1 mg/mL of tetrodotoxin (TTX) and 100 μg/mL of dexmedetomidine (DMED) and functionalized with 25 μg/mL succinyl-Concanavalin A to increase the duration of the anaesthetic effect of TTX and DMED by ocular topical application. The size of the resulting liposomes was 508 nm, and the encapsulation efficiency was 43% for TTX and 62% for DMED. Cellular toxicity was tested in corneal limbal epithelial cells and corneal keratocytes by 24-hour exposure, showing an absence of toxicity (approximately 100% viability). The sConA-functionalized liposomal formulation was compared to a non-functionalized formulation and the drugs in

solution. The study in rats showed a significant increase in duration of anaesthesia when 30 μL of sConA-Lip/TD was administered, being between 2 and 3.9 times greater than in the case of non-coated liposomes. Furthermore, corneal persistence tests performed with fluorescent dye rhodamine 6G reported a greater persistence in the cornea when liposomes were conjugated with sConA [202].

6. Recent applications of liposomes for tear film restoration/recovery

6.1. Ocular surface protection.

As mentioned previously, the tear film performs important functions in the hydration, homeostasis and protection of the ocular surface. Many ocular pathologies, such as DED, involve an alteration of the tear film and increase of tear evaporation. Dry eye disease is a pathology whose prevalence varies between 5% and 50% depending on the criteria, increasing with age and being more common in women than in men. This disease has a great impact on visual function and quality of life [203]. The integrity of the lipid layer plays an important role in preventing evaporation of the tear film. Furthermore, the thickness of the lipid layer has been related to the evaporation of the tear [203] [204]. For this reason, the inclusion of lipids in artificial tears intended to restore the lipid layer has attracted a lot of attention. In addition, bioadhesive polymers and components with osmoprotective or anti-inflammatory properties can also be included in the formulations to improve effectiveness against DED [205] [15].

Tear film dysfunction has also been linked to environmental factors or as a consequence of medication or systemic diseases. When there is an alteration in the tear film, damage to the ocular surface and symptoms of discomfort can occur. Furthermore, tear film dysfunction has been associated with an increase in hyperosmolarity due to a loss of the aqueous component of the tear [206] [207].

These circumstances have made it necessary to develop treatments based on artificial tears to restore the protection of the ocular surface. Artificial tears have been widely used to lubricate the ocular surface. Furthermore, as mentioned previously, bioadhesive compounds are widely used in eye drops because of their interaction with the negatively charged ocular surface. Polymers such as CMC, HPMC, HA and carbomers, have

commonly been included in its composition, which increases the retention time on the ocular surface [206].

Lipid based eye drops have been shown to be well tolerated and to decrease the symptoms of DED. Among these we can find ointments, which do not have aqueous components, emulsions and liposomes [15]. The internal phase of emulsions is made up of oils that form small drops due to the presence of surfactants.

Depending on the components of the emulsions, they can be anionic or cationic emulsions. Cationic emulsions, composed of positively charged nanodroplets due to the presence of substances like stearylamine or DOTAP, are able to interact with the negatively charged ocular surface, resulting in a longer residence time and spreading of artificial tears in the eye and therefore a greater improvement in symptoms [15] [208].

In the market there are several lipid-based artificial tears, such as Neovis® (Horus Pharma, Saint-Laurent du Var, France) which contains hyaluronic acid, lipoic acid and phospholipids, Systane® Balance (Alcon, Fort Worth, Texas) which is composed of an emulsion and propylene glycol or Systane® Complete (Alcon, Fort Worth, Texas) formed by nanoparticles based on lipids and propylene glycol. Both Systane® Balance and Systane® Complete contain the phospholipid dimyristoylphosphatidylglycerol. Systane® Balance was administered in an investigator-masked controlled clinical trial in 49 dry eye patients with meibomian gland dysfunction. Patients were randomly administered Systane® Balance (n = 25) or saline (n = 24) as a control group, 4 times daily topically. After 4 weeks of treatment, patients treated with Systane® Balance experienced an increase in lipid film stability, with a non-invasive tear film break-up (NITBUT) of 2.83 ± 0.74 seconds compared to 0.66 ± 0.55 in the control group ($p < 0.001$). In addition, in the treated patients there was an increase in the expression of the meibomian glands and the density of globet cells (NCT01718028) [209]. Systane® Complete has also been tested in a clinical trial in patients with symptomatic dry eye for the use of contact lenses. The investigator-masked clinical trial was conducted in 46 patients, of whom 22 received Systane® Complete and 24 were untreated. After two weeks of treatment, the treated patients showed better results on the Contact Lens Dry Eye Questionanire-8 (CLDEQ-8), showing an improvement in symptoms, the results were 12.86 ± 6.40 compared to 17.92 ± 5.30 in the untreated group [210].

Cationorm® (Santen) is a hypotonic cationic nanoemulsion (150-300 nm) free of preservatives used for dry eye treatment. Cationorm® is based on Novasorb®, a groundbreaking technology employing high pressure homogenization. That aims to use cationic nanoemulsions in specifically designed buffers able to bind the cornea and conjunctiva in order to tackle different ocular surface diseases [211]. Besides, there are electrostatic interactions between the positive charged nano system and the ocular surface epithelium negatively charged (cornea and conjunctiva [212]. Ikervis® (Cyclosporine 1mg/mL) also based on the previously described Novasorb® technology is a nanoemulsion system entrapping cyclosporine as an effective immunomodulator and anti-inflammatory drug able to reduce symptoms and control some level of inflammation caused by keratoconjunctivitis sicca or dry eye disease [213]. It constitutes a novel technology that increase the drug permeation and efficacy due to the enhanced permeation of nanoemulsions through corneal epithelium [214].

Liposomes results of great interest in the treatment of DED. Soy phosphatidylcholine is widely used in the manufacture of liposomes. The main advantage of using phosphatidylcholine is that it is the main component present in the lipid layer of the tear film. In these liposomes, other lipophilic components such as vitamin A and vitamin E can also be added to the lipid bilayer preventing the oxidation of unsaturated lipids due to their antioxidant's properties [15]. A liposomal spray formulation for the treatment of dry eye is currently marketed under the name of Tears Again®. This liposomal spray is made up of phospholipids (phosphatidylcholine) and vitamins A and E and is intended to be applied to the surface of the eyelid with the eye closed. A comparative study between Tears Again and a saline spray was reported. The controlled, double-blind, prospective and randomized study in design was carried out in 22 subjects with dry eye. The liposomal spray was applied once to the treated eye, while a saline spray was administered to the other eye (control). The results showed a significant increase in the thickness of the lipid layer ($p < 0.005$) at 30, 60 and 90 minutes after the application of the liposomal spray. Furthermore, there was an increase in tear film stability in the treated eyes ($p < 0.001$). Moreover, 70% patients reported greater comfort after 30 minutes of applying the liposomal spray [215].

The use of liposomes for the formulation of topical eye drops for the treatment of pathologies such as DED has been developed. There is a currently commercialized liposome-based artificial tears called Aquoral Lipo® (ESTEVE, Farmigea, Pisa, Italy)

(EX3652-19-01). Aquoral Lipo is made up of liposomes, cross-linked hyaluronic acid and crocin. It is designed to be instilled in the eye topically for the treatment of dry eye [216]. Another commercialized formulation is Lacrisek® Ofta (BIOOS Italia, Italy), a product based on liposomes with vitamins A and E, intended for topical instillation for the treatment of dry eye. This topical formulation was tested on evaporative dry eye patients in a single instillation. Results showed that 60 minutes after instillation, improvements in tear film evaporation and tear break-up time (TBUT) continued, unlike Artelac Rebalance®, an aqueous formulation with polyethylene glycol and hyaluronic acid, whose protection only lasted 10 minutes [15] [217].

An interesting formulation with lipid components similar to the one present in the preocular tear film has been developed by Vicario-de-la-Torre M et al. to treat the DED. These authors designed a formulation based on phosphatidylcholine, cholesterol and vitamin E in an 8:1:0.8 ratio prepared by the lipid film hydration method. The formulations dispersed in water and 0.9% NaCl were characterized. In addition, a liposomal formulation dispersed in 0.9% NaCl diluted in proportions 1/2 with 0.2% sodium hyaluronate to increase the corneal surface adhesion was also studied. The *in vitro* tolerance of the formulations with 0.9% NaCl and 0.2% sodium hyaluronate were evaluated at 15 minutes, 1 hour and 4 hours in immortalized human corneal-limbal epithelial cells (HCLE) and normal human conjunctive cells (IOBA-NHC). The results always showed a cell viability greater than 90% in HCLE and IOBA-NHC cell lines. Furthermore, *in vivo* tolerance studies in New Zealand rabbits after topical administration of 30 µL of the liposomal formulation with 0.9% NaCl (20 mg/mL PC) and 0.2% sodium hyaluronate (10 mg/mL PC) every 30 minutes and a total duration of 6 hours showed an absence of symptoms of discomfort and disturbances [218]. The same group dispersed the liposomes in a solution with trehalose, which protects cells from desiccation, and a borate buffer solution as a dispersion vehicle. The liposomes had 186.3 nm size. This liposomal formulation diluting with sodium hyaluronate (10 mg/mL PC and 0.2 % SH) gave *in vitro* cytotoxicity results greater than 80% in HCLE and IOBA-NHC cell lines and showing good tolerance *in vivo* after the topical administration of 30 µL each 30 minutes for 6 hours [129]. Also, the liposomal formulation composed of phosphatidylcholine and cholesterol was enriched using vitamin E and vitamin A to form the liposomes. Furthermore, in order to achieve in situ gelling artificial tears, gellan gum and hydroxypropyl methylcellulose were used, with

a final concentration of 0.25% and 0.12% respectively. Other compounds such as levocarnitine, with osmoprotective activity, have been included in the formulation to attenuate the hyperosmolarity produced in DED. The resulting liposomes prepared by the lipid film hydration method had a size of 200.1 ± 4.4 nm. Cell viability in human carcinoma epithelial cells (HeLa) and J774 macrophages was greater than 90% after 2 hours of exposure. Furthermore, *in vivo* studies in rabbits showed good tolerance after administration of 30 μL of the formulation (0.5% PC) every 30 minutes for 6 hours [219].

6.2. Liposomal formulations as supplementation in dry eye treatment

In addition to phospholipids and other lipids such as cholesterol to replace the lipid layer of the tear film, other components can be added to the lipid bilayer in order to provide additional supplementation. An example of this is the use of vitamin E and vitamin A, mentioned in the previous section. Vitamin E can be incorporated into the liposomal lipid bilayer enabling the stabilization and preventing degradation of phospholipid chains. Vitamin E avoids the oxidation of unsaturated phospholipids, such as phosphatidylcholine, thus increasing the stability of liposomes. Furthermore, its antioxidant properties, making it capable of protecting cells from damage [218]. Regarding vitamin A, in addition to its antioxidant properties, a study in an animal model of dry eye in mice showed that vitamin A had the ability to reduce apoptosis of corneal epithelial cells. Moreover, it also showed an increase in the volume of the tear film, as well as its stability [220]. Vitamin A is capable of regulating the differentiation and proliferation of corneal epithelial cells, and its supplementation is important to maintain adequate vision. For this reason, the use of liposomes has been studied to increase retention in the cornea, and therefore the bioavailability of vitamin A [221].

Another interesting possibility for supplementation of the dry eye disease could be the use of fatty acids omega 3 and omega 6. These fatty acids have been shown to be effective in reducing symptoms as an oral supplement due to its anti-inflammatory properties. However, recently they have also been shown to be effective topically in eye drops [222]. A study with eye drops containing hyaluronic acid and omega 3 essential fatty acids showed a decrease in corneal irregularities, in addition to reducing oxidative stress and inflammation in a mouse model of dry eye disease, compared to eye drops containing only hyaluronic acid [223]. Another study used various types of fatty acids formulated in

emulsion. The authors tested alpha-linolenic acid omega-3 (ALA) and linoleic acid omega 6 (LA). These fatty acids were tested alone and in combination, compared to the vehicle in a mouse animal model. The formulations were applied topically every 48 hours, up to a total of 3 doses. In the case of treatment with alpha-linolenic acid, the results showed a decrease in damage to the corneal epithelium. Furthermore, the use of ALA showed a decrease in proinflammatory cytokines TNF-alpha and IL-1. These results may mean a decrease in inflammation produced in dry eye pathology [224]. The introduction of these fatty acids in liposomal formulations could be of great interest.

Other compounds, such as squalene, that can be used as supplements in artificial tears. Squalene, which has been found in the tear, has numerous properties, including antioxidant, anti-inflammatory and hydrating capacity. Squalene appears to be placed on the thinnest regions of the lipid layer of the tear film, thus allowing the entire surface to be covered by this film, increasing protection [225].

7. Limitations and future prospects.

Liposomes represent a great advance in topical ocular administration, with the advantage of being well tolerated by the eye thanks to their biodegradable and biocompatible properties. Phosphatidylcholines, which are the main phospholipids that constitute the lipidic bilayer of liposomes, are present in the tear film, providing an adequate tolerability and allowing to improve the stability of the tear film. Furthermore, their capacity to act as drug carriers, allows to reduce the dose, and therefore the toxicity of the administered drugs [15].

Moreover, it is possible to manufacture them by simple methods. However, they also have limitations and there is much to improve in the field. As mentioned in this review, liposomes are capable of increasing the bioavailability of drugs, one of the main problems of ophthalmic topical administration. Despite this, there is still much to improve and research in this regard. Currently, to solve this problem, there are numerous resources to increase mucoadhesion and retention time of topical ophthalmic formulations and subsequently, ocular drug bioavailability. These strategies, mentioned in depth in this review, include the use of charged components or polymers with biodegradable and biocompatible properties, capable of increasing drug penetration.

One of the biggest limitations of liposomes is their stability. On the one hand, the unsaturated lipids present in the lipid bilayer are easily oxidizable and can also undergo hydrolysis processes, which makes liposomes less chemically stable. However, this problem can be mitigated by including antioxidant compounds, such as vitamin E, in the formulation. On the other hand, liposomes can also become physically unstable. For example, they can undergo aggregation, forming larger particles that will be more difficult to absorb and that produce a greater tendency to be phagocytized. Liposome aggregation can be prevented by the development of charged liposomes and phagocytosis can be prevented by using polymers that coat the liposomes, such as polyethylene glycol. Another stability problem related to liposomes is the loss of the encapsulated drug, or the leaked of lipid components of the bilayer, being released into the aqueous phase. To avoid the loss of encapsulated drug and favor the stability of the liposomes, it is possible to resort to the incorporation of appropriate amounts of cholesterol in the structure [112].

Regarding sterilization, there is still a need to develop robust methods that do not alter the composition of the formulation, since as previously mentioned the nature of the sample may be affected by the selected sterilization method. The big limitation for sterilizing liposomal dispersion would be testing their posterior efficacy and safety, ensuring that no toxic or degradation have occurred. Perhaps, studying and developing alternative 'cold methods' such as ethylene oxide could solve the problem out and provide with an established method to sterilize every single liposomal formulation on the market without any risks associated [137].

As above mentioned, lyophilization of liposomal formulations is still controversial since some have described that presents stability problems, but others ensure to have developed optimized protocols that allow to sort these issues and achieve long periods of storage therefore avoiding alteration of the formulation or physicochemical changes [117]. Although this still needs to be further investigated, great advances are being developed and perhaps in the future the industry could create freeze drying protocols to storage liposomes for long times.

Regarding stability of liposomes, there are some important issues that are perhaps the limiting step when reaching a clinical translational approach. The shelf-life of liposome dispersions using phospholipids are related to oxidative and hydrolytic degradation

pathways. Oxidation of phospholipids molecules takes place via a free radical chain mechanism in the absence of specific oxidants. Peroxidation of phospholipids in liposomes can be minimized thanks to the use of hydroperoxides purified raw materials, less unsaturated fatty acyl chain-containing phospholipids and antioxidants. Also, storage at low temperature with protection from light and oxygen and working under inert gas atmosphere reduces the oxidation of the phospholipids. Hydrolysis kinetic of phospholipids depends on pH, temperature, buffer concentration and ionic strength. For long-term stability, storage of liposomes in an aqueous dispersion at low temperatures (4-6ºC) and pH adjustment to values of maximum stability of liposomes is recommended [226].

Scalability is another delicate issue that is gaining importance since liposomal formulations are entering the market. Developing large amounts of liposomes in a fast, easy, and not very expensive way is strongly associated to the type of liposomes and products that are going to be encapsulated. Nonetheless, novel techniques such as microfluidics chips and micro emulsification and optimization of other better-known methods, such as ethanol injection, are being investigated since they constitute a potential source of liposome making for industries [90].

Moreover, it bears mentioning the exosomes, innovative systems considered an evolution of liposomal formulations, have gained much interest over the last few years. Exosomes are sphere-like extracellular vesicles that are produced in endosomes of all eukaryotic cells. They constitute an effective and fast mechanism of communication between cells and their environment with different specialized functions depending on the cell type. A clear example are exosomes present in dry eye patients, which can modify the activity of matrix metalloproteinases and therefore play an important role in remodeling the extracellular matrix [227].

Exosomes from mesenchymal stem cells have shown anti-inflammatory activity, regenerative properties and being able to regulate the immune response in the eye. Although in many ocular therapies involving exosomes, these systems have been administered through intravitreal injections [228], a very recent study has shown that exosomes isolated from corneal mesenchymal stromal cells can be useful for wound-

healing purposes [229]. Besides, a clinical trial is being conducted using exosomes from umbilical mesenchymal stem for relieving dry eye associated symptoms (NCT04213248).

Finally, some interesting liposomal formulations have been developed as a novel approach to treat DED [111]. These formulations contain natural phospholipids and lipidic components similar to those present in the tear film. In addition, they aim to restore the precorneal tear film by not only treating the dry eye symptoms but also restoring normality in the ocular surface and suppressing the inflammation cascade given in DED. A good example of these type of formulations is one containing soy phosphatidylcholine (20 mg/mL), cholesterol (2,5 mg/mL) and vitamin E (0,2 mg/mL). Besides, the formulation is made hypotonic regarding tears by containing trehalose and a borated-buffer solution [129]. These technological approaches aim, not only to restore the preocular tear film but also to tackle the hypertonic environment commonly given in DED and supply the ocular surface with osmoprotective properties.

8. Conclusion

Although very accessible, the ocular surface has been for many years and still is a rather complex and delicate structure to deliver drugs and formulations. Different mechanisms and physiological structures work together making permeation and delivery a very difficult task. Fortunately, liposomes are tremendously useful systems that were developed with the purpose of entering cells and tissues when other common substances could not. Due to their similarity with cell membranes, liposomes entrapping a wide variety of therapeutic products are an effective strategy to surpass the physiological barriers present in the ocular surface such as tear clearance, tight junctions of the corneal epithelium and even corneal stroma and endothelium. Despite their suitability and usefulness, there is still a need to study scalability and market adaption so everyone can benefit from their countless applications.

9. Expert opinion

Drug delivery in ophthalmology constitutes a particular challenge since many physiological and anatomical barriers work perfectly together in order to avoid alterations in tears balance, tear film stability, pH and osmolar changes. Besides, the ocular surface is

widely prepared to fight and prevent invasion and permeation of bacteria and other pathogens.

Over the last few years ocular drug delivery formulations have experimented a dramatic growth and improvement. This is due to the increment in novel and interesting techniques that allow to design, and tailor new formulations targeting specific structures of the eye and increase drug effectivity. Among many of the systems employed with this purpose, liposomes are well studied lipid-based carriers, very similar to cell membranes and cell structures. They are in many cases nano-scaled and can be specifically design and tailored to interact with specific structures of the tissues which allow them to effectively deliver the drug. Furthermore, one of the many reasons why liposomes are ideal nanocarriers is the wide variety of designs that can be made depending on the target place and function that is desired. Moreover, they have gained much interest by its suitability of entrapping both hydrophilic and hydrophobic drugs, increasing their stability and lowering drug associated toxicity. Despite their long background, liposomes continue being the alternative for many researchers to create novel and innovative formulations, particularly in ocular drug delivery. High hydrophobic drugs, almost no soluble in aqueous media, have been entrapped in liposomes and delivered successfully to the ocular surface. Also, liposomes have been assayed to treat posterior segment diseases such as glaucoma or AMD.

Some of the last advances in liposome technology highlights the recent use of bioactive molecules such as annexin V associated to liposomes to enhance bevacizumab topical delivery in AMD. Normally annexin V has been widely used for staining techniques in apoptosis detection, but researchers have discovered that helps liposomes to go through cell membranes by a trans-cytosis mechanism [230]. Furthermore, including agents and substances in liposomes that tackle oxidative strategies for ocular surface disease is becoming a different and innovative approach like including carotenoids in liposomes, regulating gene expression and tear volume balance [182].

Despite the great progress in this area, there is still much to improve and optimize but important advances are being made. Perhaps, immunogenicity can be tuned and modified in order to avoid a disproportionate reaction of the tissue. That is the reason why, soy phospholipids are eligible and are being optimized although they show a profile rather complex to characterize since their extraction may cause some changes in the fatty acid

profile [86]. For instance, DOPG has very recently been discovered to promote tissue regeneration of the corneal epithelium, so this means that specific liposomal systems that inherently possess therapeutic properties can be designed [85]. SiRNA gene therapy is evolving in the field of liposomes and especially in topical administration for diseases previously mentioned such as DED or AMD. Apart from the containing siRNA molecules recent advances point out the importance of combining liposomes with some polymers such as HA and specific target molecules like CD44 that could enhance adhesion and cell permeation [173].

All these strategies demonstrate that liposomal field is constantly evolving and taking advantage of all the new discoveries and growing technology, highly specified systems with great tissue affinities can be created so low toxicities, doses and reduced administrations may be possible. Moreover, a combination between liposomal formulation and exosome technology could be used to specifically re-design these systems and fight these diseases from another different perspective. Ocular disease needs from these breakthroughs to discover and develop new treatments and strategies that focus not only on pathways and therapeutic substances but also in substances and materials that already possess beneficial characteristics such as antioxidative, anti-inflammatory or wound-healing properties.

Furthermore, the previously mentioned therapies containing natural components that resembles the preocular tear film has gained much interest and creates a new area of research that could be further investigated to develop new potential therapies that allows to treat more effectively ocular surface pathologies. To our view, this novel approach opens a new possibility to treat DED and ocular surface pathologies. It aims to be the next generation of liposomal formulations not necessarily containing active drugs in order to treat pathologies of the ocular surface presenting tear film instability, alteration of the physiological properties of ocular surface and ocular inflammation. Liposomal formulations with components resembling the preocular tear film could also be used as vehicles for active drugs in long term treatments avoiding the associate side effects related to chronic therapy.

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CAPÍTULO II. COMBINED HYPEROSMOLARITY AND INFLAMMATORY CONDITIONS IN STRESSED HUMAN CORNEAL EPITHELIAL CELLS AND MACROPHAGES TO EVALUATE OSMOPROTECTIVE AGENTS AS POTENTIAL DED TREATMENTS.

CAPÍTULO II

CAPÍTULO II

Combined hyperosmolarity and inflammatory conditions in stressed human corneal epithelial cells and macrophages to evaluate osmoprotective agents as potential DED treatments

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CAPÍTULO II

CAPÍTULO II

ABSTRACT

PURPOSE. To develop an easy-to-perform combined model in human corneal epithelial cells (HCECs) and Balb/c mice macrophages J774.A1 (MP) for preliminary screening of potential ophthalmic therapeutic substances.

METHODS. HCECs were exposed to different osmolarities (350 to 500 mOsm/L) and MTT assay was employed for cell survival and flow cytometry to assess apoptosis-necrosis and relative cell size (RCS) distribution. Effectiveness of Betaine, L-Carnitine, Taurine at different concentrations (ranging from 20 mM to 200 mM). Also, mucoadhesive polymers such as Hyaluronic acid (HA) and Hydroxypropylmethylcellulose (HPMC) (0.4 and 0.8 %) were evaluated. Cells were pre-incubated with the compounds (8h) and then exposed to hyperosmotic stress (470 mOsm/L) for 16h. Moreover, anti-inflammatory activity was performed in LPS-stimulated MP.

RESULTS. Exposure to hyperosmotic solutions between 450-500 mOsm/L promoted the highest cell death after 16h exposures ($p<0.0001$) with a drop in viability to 34.96%±11.77 for 470 mOsm/L. Pre-incubation with Betaine at 150 mM and 200 mM provided the highest cell survival against hyperosmolarity (66.01%±3.65 and 65.90%±0.78 respectively) while HA 0.4% was the most effective polymer in preventing cell death (42.2%±3.60). Flow cytometry showed that Betaine and Taurine at concentrations between 150-200 mM and 20-80 mM respectively presented the highest anti-apoptotic activity. Also, HA and HPMC polymers reduced apoptotic-induced cell death. All osmoprotectants modified RCS, and polymers increased their value over 100%. L-Carnitine 50 mM, Taurine 40 mM and HA 0.4% presented the highest TNF-α inhibition activity (60%) albeit all of them showed anti-inflammatory inhibition percentages higher than 20%.

CONCLUSIONS. HCECs hyperosmolar model combined with inflammatory conditions in macrophages allows the screening of osmoprotectants by simulating chronic hyperosmolarity (16h) and inflammation (24h).

Keywords: Hyperosmolarity, osmoprotectants, DED, inflammation, screening, osmoprotection model.

CAPÍTULO II

CAPÍTULO II

1. Introduction

Dry Eye Disease (DED) is a multifactorial pathology affecting the ocular surface and tears that in all cases leads to tear film instability (Aggarwal and Galor, 2018). TFOS DEWS Epidemiology report analyzed series of large-scale international epidemiological studies and concluded that the prevalence of DED was from 5 to 30% in individuals that were over their 50s. In addition, it has been established that DED is boosted with age and females are more affected (Stapleton et al., 2017). Severe clinical signs such as tear film instability and hyperosmolar conditions are given among others, constituting a potential damage to the ocular surface. In fact, one of the most important and well-established causes of DED relapses in tear evaporation, that subsequently leads to a raise in osmolarity of the tear film (Baudouin et al., 2013)(Messmer, 2015). Sjögren syndrome (SS), Sjögrens' syndrome-associated keratoconjunctivitis sicca (SS-KCS) or LASIK-induced neurotrophic epitheliopathy (LNE) constitute also some examples of diseases linked to hyperosmolar stress (Pflugfelder et al., 2018). Hyperosmolarity of the surface epithelial cells, results in an imbalance of water and electrolytes between intracellular and extracellular compartments reducing cell volume. A dramatic shrinkage of cells may lead to cell survival alteration, cell membrane and cytoskeletal integrity changes as well as denaturation of cytosolic proteins (Khandekar et al., 2013)·(Jeng, 2013).

An increase in hyperosmolarity of tears can lead to ocular surface impairment by producing a wide amount of proinflammatory markers such as tumor necrosis factor and some matrix metalloproteinases such as MMP-9 or MMP-7 (Stevenson et al., 2012). This environment also produces a decrease in conjunctival globet cells. As the osmolarity of the ocular surface increases the inflammation events persist developing in a vicious cycle (Blalock et al., 2008).

An increase in osmolarity (450, 500 and 550 mOsm/L) of tears has been associated to a subacute inflammation of the ocular surface by triggering some essential cytokines such as tumor necrosis factor alpha (TNF-α) (Luo et al., 2007). TNF-α has been widely studied as the classical proinflammatory factor produced by some important signaling pathways such as MAPK cascades in response to hyperosmolar stress (Luo et al., 2004).

According to TFOS DEWS management and therapy report, tear insufficiency, lid abnormalities and inflammation are some key treatments for DED management (Jones et

al., 2017). Treatment of tear insufficiency and supplementation aims to replace tears by artificial tear substitutes, tear production stimulation and tear conservation approaches (Albietz et al., 2002). Hydroxypropylmethylcellulose (HPMC) and Hyaluronic acid (HA) are well known examples of viscosity-enhancing agents able to increase tear film density, thickness, and preserve the surface from damage against desiccation stress, alleviating symptoms (Jones et al., 2017), (Andrés-Guerrero et al., 2011). For decades, osmoprotection has been demonstrated to play a decisive role in protecting the ocular surface damage produced as consequence of tear evaporation (Hazarbassanov et al., 2018). According to this, some osmoprotective substances have been studied in the search of useful therapies to manage DED (Corrales et al., 2008; Hua et al., 2015a). Osmoprotectants are osmotically active biocompatible compounds able to modify cellular water uptake. They are usually small, neutral and hydrophilic substances that do not interfere with cellular function (Mateo Orobia et al., 2018). Each osmoprotectant works in a different way with a wide variety of kinetics and cell internalization (Kempf and Bremer, 1998). Cell uptake of osmoprotective substances can be given when a hypertonic environment occurs or if there is a potential cell damage caused by hyperosmotic stress (Sharma et al., 2014). Besides some of them can be internalized in the cells via specific mediated transporters even before hyperosmotic environment (Slama et al., 2015). The amount of substance and period of time that cells can retain them play a critical and important role in their effectiveness (Baudouin et al., 2013). Some clear examples are Betaine (tri methylglycine) and L-Carnitine. They have been well studied for their role as cell membrane stabilizers. Both agents act by avoiding protein misfolding and conformational changes (Corrales et al., 2008). In the ocular surface, BGT-1 (betaine-GABA transporter) is expressed in human corneal epithelial cells and allows betaine to be internalized and accumulated in order to protect cells against osmotic stress (Garrett et al., 2013; Lehre et al., 2011). Moreover, Betaine has been studied to stimulate the intake of osmoprotectants and inhibit p38 pathway resulting in apoptosis inhibition (Garrett et al., 2013). L-Carnitine has also been hypothesized to be crucial during oxidative process by blocking reactive oxygen species (ROS) production in the ocular surface (Hua et al., 2015a) and inhibit some inflammation pathways such as TRPV1 (transient receptor potential cation channel) (Hua et al., 2015b) (Hua et al., 2015a). Although it is still unknown how some osmoprotectants inhibit TRPV1 pathway, the efficacy of this protective agents in avoiding its activation has been established (Khajavia et al., 2014). Besides, L-Carnitine has been thought to get into cells

through some non-specific transporters, regulating apoptosis, inflammation and osmolytes inside corneal and conjunctival cells (Khandekar et al., 2013) (figure 1). In addition, taurine has been studied as an antioxidant and osmoprotective substance capable of preventing damage in the ocular surface, entering cells through different surface taurine transporters (Bucolo et al., 2017; Shioda et al., 2002). Taurine has been hypothesized to upregulate the storage of osmolytes inside the cell therefore regulating volume loss and cell survival (Schaffer et al., 2000). Among all this substances, L-carnitine is available in the market and HPMC and HA are well known in different commercial formulations but at low concentrations (Jones et al., 2017; Monaco et al., 2011).

Until now, few studies have evaluated osmoprotection in the ocular surface focusing only on specific markers or pathways that play critical roles in DED (Bucolo et al., 2017; Khandekar et al., 2013; Mateo Orobia et al., 2018). The present work aims to recreate some of the DED chronic hyperosmolar conditions in a simple and reproducible *in-vitro* cellular model that allows the study of cell survival and apoptosis. As inflammation is also present in DED, macrophages were used to evaluate the potential anti-inflammatory activity of the osmoprotectants and polymers. The present works aims to develop a useful tool set to screen osmoprotective substances that can be potentially used in DED treatment and also as ocular surface protectants in chronic treatments such as the case of hypotensive topical formulations in glaucoma. To this, an immortalized human corneal epithelial cell line (hTERT-HCECs) was used to carry out osmoprotection studies. hTERT-HCECs expresses typical epithelial cell markers, such as ZO1 (zonula occludens) and KRT3 (keratin 3), present in healthy corneal tissues. HTERT-HCECs was developed without tumorigenic transformation (Kasetti et al., 2016). Apoptosis was also determined by flow cytometry. For the anti-inflammatory studies, Balb/c mice macrophages J774.A1 were stimulated to express TNF-α (De Stefano et al., 2010). We demonstrated that the combination of these cellular models entails a robust mechanism to identify and screen potential therapeutic substances or formulations for ocular surface diseases that undergo with hyperosmolar stress and inflammation.

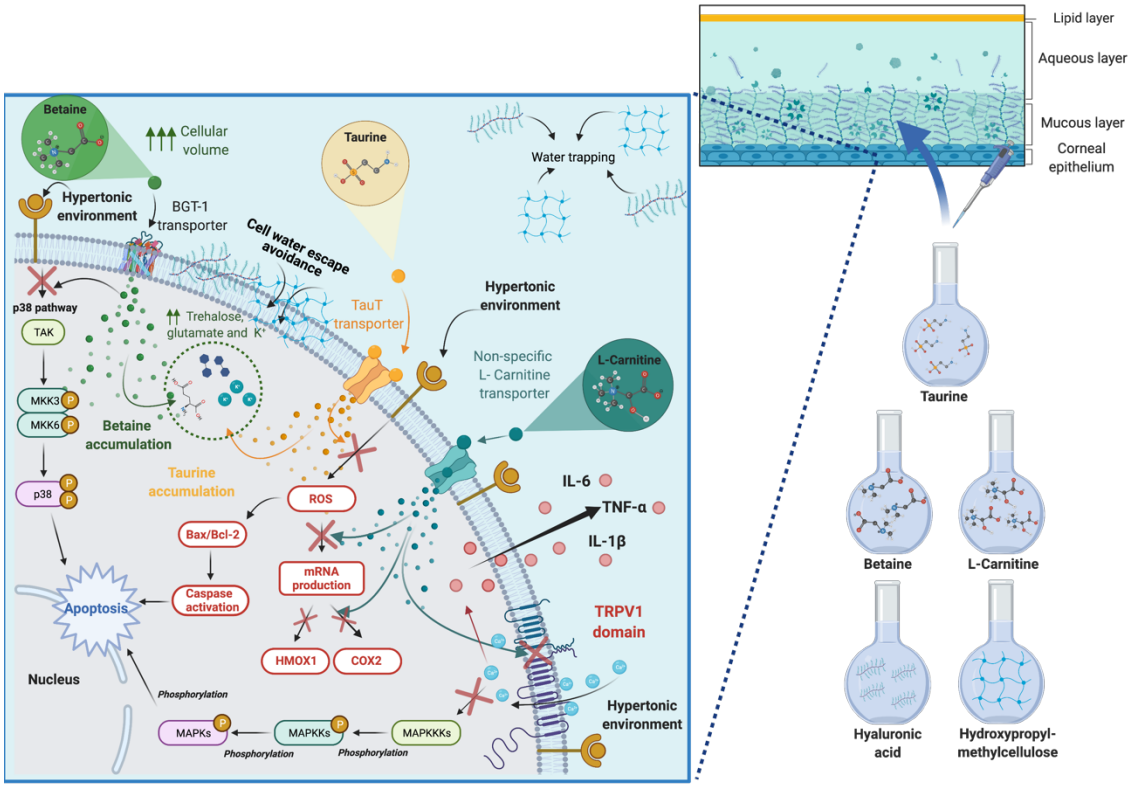

Figure 1. Proposed mechanism of action of the different potential osmoprotective substances studied according to the bibliography (Corrales et al., 2008; Garrett et al., 2013; Hua et al., 2015b, 2015a; Lehre et al., 2011).

2. Material and methods

2.1. Reagents

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Trypan-Blue, Dulbecco's phosphate-buffered saline (DPBS), Dimethyl sulfoxide (DMSO), sodium chloride (NaCl) solution 5M, Betaine ≥98%, Taurine ≥98% were purchased from Sigma Aldrich (Madrid, Spain). L-Carnitine was provided by Fagron Ibérica SAU (Barcelona, Spain). Trypsin-EDTA 0.05%, defined trypsin inhibitor, DRAQ5 (647⁄681) and YO-PRO™-1 Iodide (491/509) were supplied by Life Technologies (Madrid, Spain). 7-AAD Viability Staining Solution and cell staining buffer from Bio Legend were obtained from Palex Medical (Madrid, Spain). Hydroxypropyl methylcellulose (HPMC) was supplied by Dismadel, S.L (Madrid, Spain) and MMW (400.000-800.000 Da) hyaluronic acid (HA) was provided by Abarán Materias Primas (Madrid, Spain).

2.2. Cell cultures

Immortalized Human corneal cells (hTERT-HCECs; Evercyte GmbH, Vienna, Austria) were cultured at 37ºC under 5% CO_2 in a humid saturated atmosphere. The cells were seeded in EpiLife® media (Life Technologies, Madrid, Spain) supplemented with EDGS® 1X (Life Technologies, Madrid, Spain) and penicillin-streptomycin 1% (Sigma-Aldrich, Madrid, Spain). The media was changed every 48 hours and cells were subcultured at 80-85 % confluence, by rinsing with DPBS (Dulbecco's phosphate-buffered saline, Sigma-Aldrich, Madrid, Spain) and detaching with trypsin-EDTA 0.05% (Sigma-Aldrich, Madrid, Spain). Cell passages for *in vitro* studies were in the range of 5-10 (7,5-15 population doubling level).

Murine macrophages J774A.1-TIB67™ (American Type Culture Collection, Manassas, Virginia, USA) were cultured at 37ºC under 5% CO2 as mentioned above. The cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) (Sigma-Aldrich, Madrid, Spain) supplemented with penicillin-streptomycin 1% (Sigma-Aldrich, Madrid, Spain) and FBS 10% (Fetal Bovine Serum) (Cultek, Madrid, Spain). The media was changed every 48 hours and cells were subcultured at 70% confluence. Passages between 4-7 (6-10.5 population doubling level) were used for the anti-inflammation studies.

2.3. Preparation of osmoprotectants and polymers

Aqueous isotonic solutions of the osmoprotective substances Betaine, L-Carnitine and Taurine at different concentrations were prepared: Betaine (50 mM, 100 mM, 150 mM and 200 mM); L-Carnitine (50 mM, 100 mM and 150 mM) and Taurine (20 mM, 40 mM and 80 mM). Isotonicity of solutions was adjusted with NaCl. HA and HPMC at two different concentrations (0.4 and 0.8%) were dissolved in NaCl 0.9%. Final sterilization was performed by filtration using 0.22 μm filters.

2.4. *In vitro* toxicity assessment

The cytotoxicity of osmoprotectant substances and polymers at the selected concentrations was evaluated via MTT assay described previously (Vicario-de-la-Torre et al., 2018). To this, HTERT-HCECs were cultured in 96 well plates (20000 cells/well) and incubated overnight. Following incubation, the cells were exposed to the afore-mentioned substances (Betaine, L-Carnitine and Taurine, HA and HPMC) for 8 hours. After that, the

supernatant was removed. Then, cells were exposed to an MTT (0.33 mg/mL) mixture in cell culture media and incubated for 4 hours. Afterwards, the supernatants were discarded and 100 µL of DMSO were added to each. Finally, the plates were shaken gently for 5 minutes well for the complete solubilization of formazan crystals in the dark and immediately measured at 550 nm in the spectrophotometer. Benzalkonium chloride at 0.005% was employed as positive control for cell toxicity, since is commonly used as a preservative in artificial tears causing cell dead.

2.5. Hyperosmolar stress simulation in human corneal cells

2.5.1. Cell viability determination under hyperosmolarity

Sodium chloride was employed to reproduce a hyperosmolar environment similar to the one found in DED. Firstly, HCECs cells were seeded in 96 well plates (20000 cells/well) and incubated overnight. Then, the supernatants were removed, and the wells were filled with cell culture media (100 µL) and NaCl 0.9% (300 mOsm/L) (100 µL) for 8 hours. After that, the supernatants were discarded and hyperosmolar environment conditions were stablished in each well by using NaCl (350, 400, 450, 460, 470, 480, 490 and 500 mOsm/L). This step was carried out to study the response of HCECs under different hyperosmolar stress to select the most appropriate one. Each hyperosmolar solution was made by mixing NaCl and cell culture media (1:1) (eq.1). Finally, the supernatants were removed, and cell viability was measured by MTT as previously described in section 2.4. The following equation was employed to prepare the different hyperosmolar solutions (Eq.1):

$$\textbf{\textit{Stock hyperosmolarity}}\ (\boldsymbol{mOsm/L}) = \frac{\boldsymbol{DO}\ (\boldsymbol{mOsm/L}) \times \boldsymbol{WV1}\ (\mu\text{L}) - \text{OM}\ (300\ \text{mOsm/L}) \times \text{WV2}\ (\mu\text{L})}{\boldsymbol{HV}\ (\mu\text{L})} \qquad \text{(Eq.1)}$$

Stock hyperosmolarity (expressed in mOsm/L) refers to the osmolality concentration needed to be added to the well in order to obtain the final desired osmolality. The desired osmolality of the mixture (DO) is expressed in mOsm/L, WV1 is the total volume of DO in the well (200 µL), OM states for the osmolality of the media (300 mOsm/L), WV2 is the volume of OM added to the well (100 µL) and HV expresses the volume of the stock hyperosmolar solution needed (100 µL). Once the model was optimized, in vitro testing of the substances and polymers was evaluated. The hypertonic solution 470 mOsm/L was selected as it provides the optimal cell survival to evaluate osmoprotective protection.

Cells were incubated with betaine (50 mM, 100 mM, 150 mM and 200 mM), L-carnitine (50 mM, 100 mM and 150 mM), taurine (20 mM, 40 mM and 80 mM), HA (0.4% and 0.8%), HPMC (0.4% and 0.8%) for 8 hours. After that, all the supernatants containing the substances or polymers were discarded, and hyperosmotic stress conditions (470 mOsm/L) were created in all sample wells according to eq.1, including the positive control. Then, the cells were incubated for 16 hours. Finally, cell viability was determined by the MTT method, as described in section 2.4. Each experiment was made by triplicate (n=3; 7 wells per compound replicated in 3 separate plates).

2.5.2. Apoptosis and necrosis by flow cytometry

Different dye-mediated parameters such as necrosis and apoptosis were evaluated in hTERT-HCECs in response to different hyperosmolar concentrations (350, 400, 450, 460, 470, 480, 490 and 500 mOsm/L, as described in section 1.5.1). Briefly, 1 x 10^6 cells/well were seeded in petri dishes and incubated for 24 hours until 80% of confluence was reached. Then, the supernatants were removed and 5 mL of cell culture media and NaCl 0.9% mixture was added as described in 2.5.1 section. Cells were incubated for 8 hours. After that, the supernatants were discarded, and different hyperosmolar environments were simulated as previously described for 16 hours in order to optimize the model. After the incubation time, all the supernatants were withdrawn, and each dish was rinsed with DPBS twice. HTERT-HCECs were detached after 3 minutes treatment with trypsin-EDTA 0.05%. Cells were centrifuged at 850 x g for 10 minutes and re-suspended in 600 µL of cell staining buffer. Finally, 5 µL of 7-AAD viability staining solution and 0.5 µL of YO-PRO™-1 Iodide were added to study the apoptosis-necrosis mechanisms according to the manufacturer instructions. The cell suspension was incubated for 20 minutes in the dark and taken to the flow cytometer FC 500 (2-laser, 5-color analysis) with FC 500 CXP software (Beckman Coulter, Madrid, Spain) for data acquisition. The different instruments were calibrated weekly by the flow cytometry core personnel. Before use, the system and fluidics were allowed to warm up and stabilize for at least 20 minutes. All samples were measured at a medium flow rate of <10000 events/s during a time frame of 300s. Briefly, 7-AAD and YO-PRO™-1 Iodide signals were evaluated using the blue/red laser at 535/617 excitation/emission and the blue/green laser at 491/509 excitation/emission respectively.

As cited previously, 470 mOsm/L was_selected to perform viability, apoptosis and necrosis studies. Polymers and therapeutic substances at different concentrations (5 mL final volume) were incubated for 8 hours as shown previously (section 2.5.1). Then, all the supernatants were discarded. After that, hyperosmolar environment (470 mOsm/L) was achieved in all sample dishes. Samples were prepared as afore mentioned and taken to the flow cytometry core facility. Cells exposed to 470 mOsm/L without previous treatment were established as the positive control. Each experiment was made by triplicate (n=3).

For apoptosis detection under the fluorescence microscope, cells were seeded in 24 well plates following the same procedure with hypertonic stress when 80% of confluence was reached as previously shown. Cells were stained with YO-PRO1 for 10 minutes under the dark and examined under the fluorescence microscope with the blue laser (535/617).

2.5.3. Cell size analysis in response to osmotic stress

Cell size of hTERT-HCECs after 16 hours exposure to the different hyperosmolarity environments (350, 400, 450, 460, 470, 480, 490 and 500 mOsm/L) were analyzed. A comparison was performed between each concentration and the basal values under isotonic conditions (300 mOsm/L). All sample tubes were analyzed as previously measured (section 2.5.2) at a medium flow rate of <10000 events/s during a time frame of 300s. After selecting the optimal hyperosmolar condition for the model (470 mOsm/L) the fluctuation of cell size was analyzed.

Cell size regulation of the different osmoprotective substances (osmoprotectants and polymers) was tested by their pre-incubation with the cells (8h) prior to the addition of hypertonic solution. Then, the experiments were carried out exactly as described above in section 2.5.2.

2.6. Determination of TNF-α in an LPS-induced inflammation model

A macrophage J774A.1-TIB67™ cell line was used as inflammation model. Briefly, 20000 cells were seeded in 24 well plates and incubated overnight at 37ºC 5% CO_2. After that, all the supernatants were discarded and LPS (12.5 ng/mL) was added in each well. Afterwards polymer or osmoprotective compounds were also included. Negative and positive controls were made by adding isotonic NaCl 0.9% or LPS (12.5 ng/mL) in cell media. After incubation at 37ºC 5% CO_2 (24 h), all the supernatants were collected,

centrifuged at 850 xg 10 min and analyzed. TNF-α was determined through the Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Life Technologies, Madrid, Spain). The plates were measured at 450 nm and 570 nm. Optical densities at 450 nm were subtracted to 570 nm to eliminate background signal according to the manufacturer's instructions. Since TNF-α levels (pg/mL) could vary between assays in samples and controls, the results were normalized in terms of inhibitory effect (described in eq. 2).

$$\textbf{\textit{Inhibition}}\ (\%) = \frac{\textit{Sample TNF alpha} - \textit{Negative control TNFalpha}}{\textit{Positive control TNFalpha}\ (\textit{LPS}\ 12.5\ ng/mL) - \textit{Negative control TNFalpha}} \qquad \text{(Eq.2)}$$

As above-described (eq. 2), sample TNF-α levels (pg/mL) were subtracted to those of the negative control (basal levels) since basal levels do not represent an inflammation response. Furthermore, the normalized sample TNF-α values were divided by the normalized positive control TNF-α levels (calculated the same as normalized sample TNF-α levels) and expressed in TNF- α inhibition percentage.

2.7. Statistical Analyses

Each experiment was carried out by triplicate (n=3) and the data are shown as the mean ± SD. Ordinary one-way ANOVA combined with Dunnett's multiple comparisons test was used to determine if specific groups achieved some levels of significance (*; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$ or ****; $p \leq 0.0001$) by using GraphPad software Inc. Prism Version 7, US. Beckman Coulter Kaluza Analysis Software, US, was employed to visualize and analyze flow cytometry data.

3. Results

3.1. *In vitro* toxicity assessment

HTERT-HCECs were exposed for 8 hours with solutions of Betaine (50 mM, 100 mM, 150 mM and 200 mM), L-Carnitine (50 mM, 100 mM and 150 mM) and Taurine (20 mM, 40 mM and 80 mM) as potential osmoprotective substances and mucoadhesive polymers such as HA and HPMC at concentrations of 0.4 and 0.8% (Figure 2).

According to previous studies 80% of cell survival was selected as the minimum tolerance for ocular formulations (Ayaki et al., 2010), (Gómez-Ballesteros et al., 2019). All

Betaine and L-Carnitine solutions presented viability values higher than 89.4% and 93.36% respectively. Taurine resulted in viability values close to 80% with excellent tolerance at 20 mM. HA and HPMC viability values were higher than 80% with the highest ones observed for concentrations of 0.4% in both polymers (94.46 ± 4.90% and 95.31 ± 10.73% for HA and HPMC respectively). Regarding multiple comparison among betaine concentrations, no statistically significant differences ($p>0.05$) were observed. However, L-Carnitine 100 mM resulted significantly higher in comparison with 150 mM ($p = 0.0022$). Taurine demonstrated high statistically significant results when compared 20 mM against 40 mM and 80 mM ($p<0.0001$). Regarding polymers, HA 0.4% showed significantly higher viability values in comparison with HA 0.8% ($p = 0.0002$). HA 0.4% also showed significant differences when compared to HPMC 0.8% ($p = 0.0342$). Besides, HA 0.8% was highly significant in comparison with HPMC 0.4% ($p<0.0001$). Finally, HPMC 0.4% showed significant results when compared to HPMC 0.8% ($p = 0.0108$).

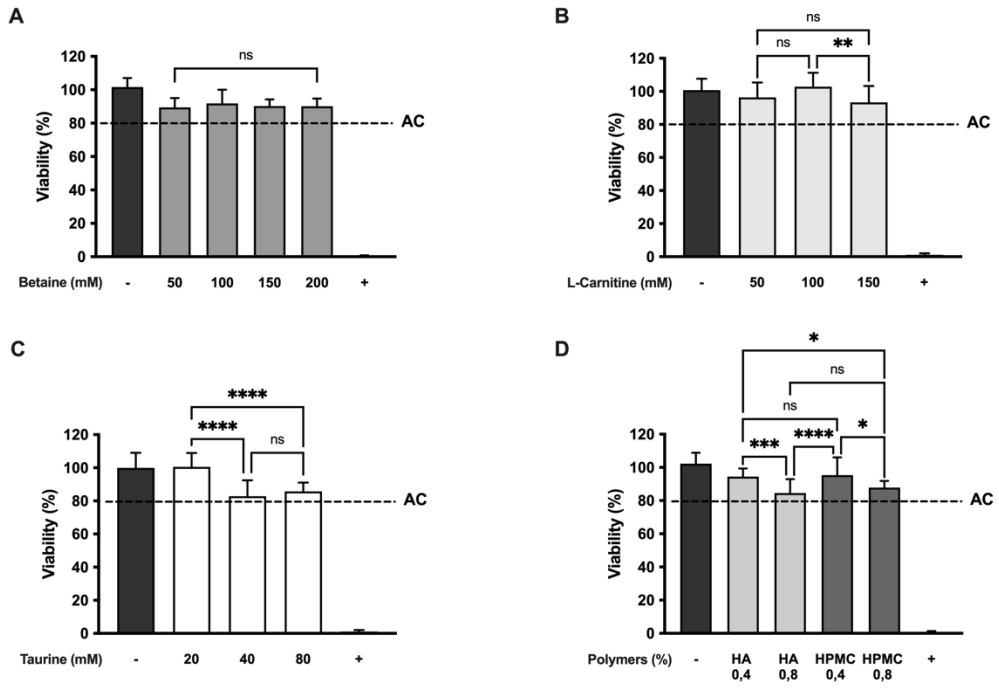

Figure 2. *In vitro* toxicity evaluation at 8 hours of the substances and polymers are illustrated. The negative control (-) represent untreated cells while the positive one (+) are BAK 0.005% treated cells as above mentioned. Broken line AC represents the acceptance criteria for ocular in vitro cell viability.

CAPÍTULO II

3.2. Hyperosmolar stress simulation in human corneal cells

3.2.1. Cell viability by MTT

Viability of cells exposed under different hyperosmolar NaCl concentrations is shown in figure 3. HTERT-HCECs cells were treated with different hyperosmolar concentrations of NaCl for 16 hours to simulate chronic hypertonic stress present in DED. All groups were compared with the negative control (isotonic conditions). Viability values showed a slight decrease in cell viability every 50 mOsm/L addition without significant results ($p = 0.9932$ for 350 mOsm/L and $p = 0.5020$ for 400 mOsm/L) compared with isotonic conditions (300 mOsm/L). Cell survival did not significantly change until 450 mOsm/L (66.66% ± 13.54), which was found to be the threshold concentration at which cell survival linearly decreases with increments of 10 mOsm/L in osmolarity up to 500 mOsm/L (12.77% ± 5.57). All values between 450-500 mOsm/L were significantly lower than 300 mOsm/L baseline ($p < 0.0001$).

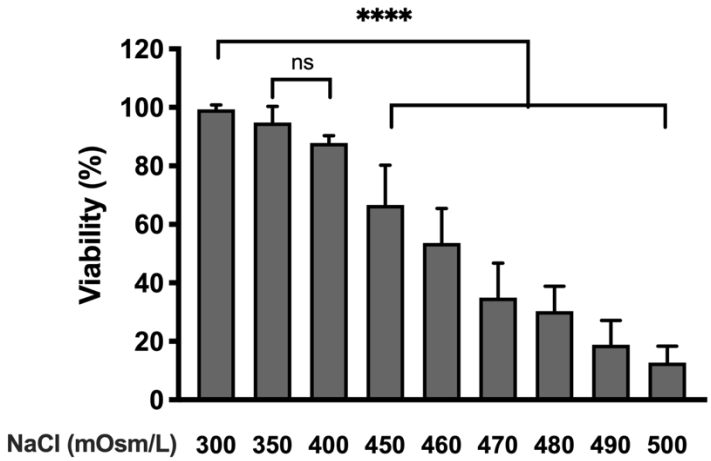

Figure 3. Decrease of hTERT-HCECs cell survival (%) in different hyperosmolar conditions. 450 mOsm/L shows a threshold concentration where osmolar sensitivity of the cells increase. Hyperosmolar concentrations from 450 to 500 mOsm/L showed statistically significant drop in viability (%) compared to 300 mOsm/L (****; $p < 0.0001$).

Cells exposed to 470 mOsm/L exhibited a mean viability of 34.96% ± 11.77 and was chosen as the most appropriate control concentration to screen osmoprotection. This hyperosmolar concentration showed a significant decrease in cell viability and avoided extreme values that could lead to confusion when assessing protection by substances with osmoprotective properties.

Figure 4 shows the osmoprotection observed for Betaine, L-Carnitine, Taurine, HA and HPMC. Betaine significantly increased viability at all the studied concentrations ($p = 0.0003$ for 50 mM and $p<0.0001$ for 100, 150 and 200 mM) in comparison with the positive control (470 mOsm/L). Betaine concentrations of 150 mM and 200 mM displayed similar percentages (66.01% ± 3.65 and 65.90% ± 0.78 respectively) of viability. L-carnitine, at all concentrations was able to increase cell viability with concentrations of 100 mM and 150 mM exhibited higher cell viability values (46.2% ± 0.41 and 49.90% ± 6.37, respectively) with p values of 0.0038 and 0.0010, respectively. Cell viability values with taurine exposure was similar, albeit the same pattern as previously reported with L-carnitine was shown and high concentrations (40 mM and 80 mM) demonstrated greater statistically significant values (***; $p = 0.0005$ for 40 and $p = 0.0002$ for 80 mM) than the smallest concentration (20 mM) (*; $p = 0.0039$). The conditions provided by HPMC 0.8% were not able to provide an improvement in comparison with the positive control ($p = 0.1070$). While the lowest concentrations (0.4%) of HA and HPMC showed an increase in cell viability (42.2% ± 3.60 and 45.50% ± 9.71 respectively) (*; $p = 0.0498$ for HA 0.4% and $p = 0.0149$ for HPMC 0.4%). HA 0.8% solution increase cell viability values to 60.24% ± 6.29 (***; $p = 0.001$).

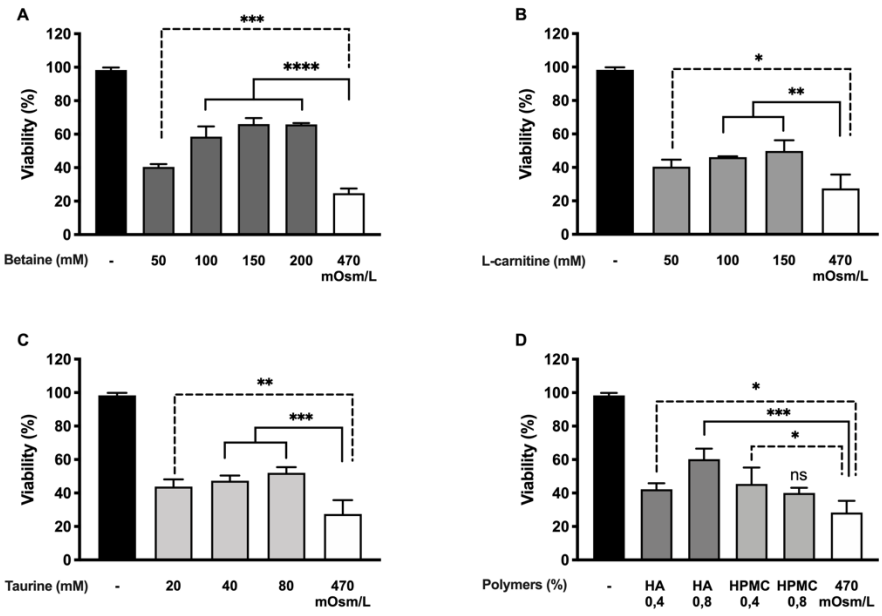

Figure 4. Osmoprotection of Betaine (A), L-Carnitine (B), Taurine (C) and polymers (HA and HPMC) (D) in response to 470 mOsm/L in hTERT-HCECs cells.

3.2.2. Apoptosis and necrosis by flow cytometry

Flow cytometry analysis was employed to determine different cell death mechanisms (early, late apoptosis and necrosis) involved in the cellular response under prolonged hyperosmotic stress as well as evaluating the protective properties of the different osmoprotective active substances and polymers. Briefly, side scatter (SSC) was plotted against forward scatter (FSC) to gate cells in order to identify the population of single cells (singlets). Moreover, SSC was plotted against YO-PRO1 to identify the total apoptotic population and 7AAD was represented against YO-PRO1 to identify the different stages involved in cell death (viable cells, early apoptotic, late apoptosis and necrotic).

Firstly, cell recovery and total apoptosis was firstly assessed after 16 hours exposure to different hyperosmolar concentrations using the isotonic (NaCl 0.9%) solution as control (100%) (figure 5). Regarding cell recovery, 350 mOsm/L was the only concentration considered as non-significant in comparison with isotonic media ($p = 0.1038$). From 400 mOsm/L to 500 mOsm/L a significant decrease was observed with every 10 mOsm/L increase in osmolarity ($p < 0.0001$). With regards to apoptosis, some levels of significance were shown at 460 mOsm/L ($p = 0.0122$) and high levels of significance were exhibited from 470 to 500 mOsm/L ($p < 0.0001$).

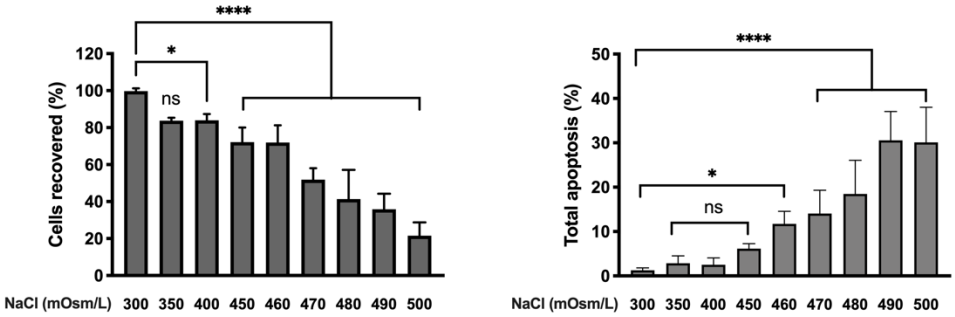

Figure 5. Total hTERT-HCECs cells recovered following exposure to different hypertonicities (left) and percentage of total apoptosis, measured by flow cytometry analysis.

Briefly, representative flow plots of cells exposed to 300 mOsm/L or 470 mOsm/L is illustrated in figure 6 (A and B respectively). Total apoptosis (YO-PRO1/SSC) for the negative control (A) was 1.41% ± 0.52 and 17.99% ± 5.27 for the selected hypertonic concentration (B).

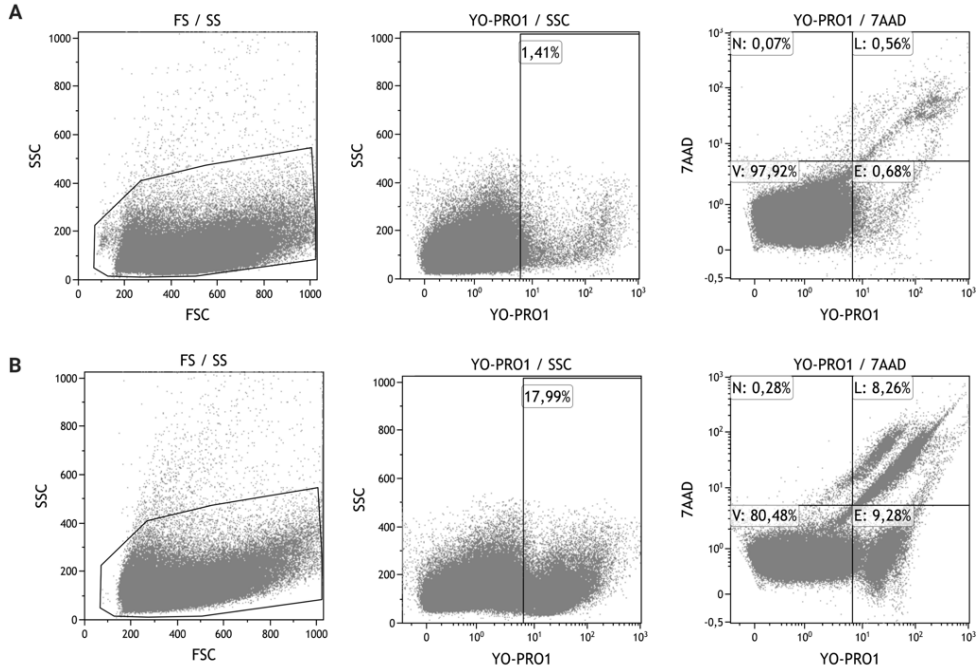

Figure 6. Representative examples of viable fraction of cells, early apoptosis, late apoptosis and necrosis of cells exposed to 300 mOsm/L (A) and cells exposed to hyperosmolar 470 mOsm/L (B). V: viable; E: early apoptosis; L: late apoptosis and N: necrosis. YO-PRO1/SSC states for the total apoptosis values.

As shown in figure 7, cells exposed to 470 mOsm/L for 16 hours after pre-incubation with different concentrations of Betaine (8h) showed a significant drop in apoptosis when compared to pre-incubation with the control solution (NaCl 0.9%). Moreover, betaine appeared to be concentration-dependent with its lowest apoptosis value at 200 mM with a dramatic decrease in early (2.82% ± 1.02) and late apoptosis (1.33% ± 0.03).

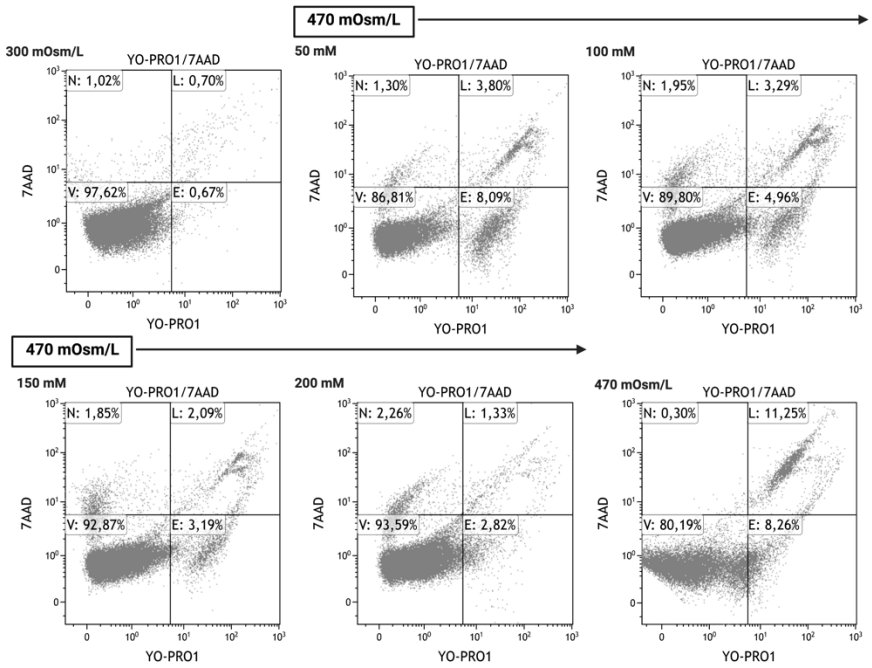

Figure 7. Cell survival of negative control (300 mOsm/L), untreated cells under osmotic stress (470 mOsm/L) and pre-treated cells with different concentrations of betaine (50-200 mM) later exposed to osmotic stress. V: viable; E: early apoptosis; L: late apoptosis and N: necrosis.

In the case of cells preincubated with taurine, early apoptosis was between 2 and 4%, close to the negative control or basal values (supplementary figure 1). Taurine showed the ability to revert the entrance of cells into early stages of apoptosis when compared to the positive control (470 mOsm/L). Taurine at 40 and 80 mM exhibited similar values to those present at basal conditions (300 mOsm/L).

L-Carnitine at all concentrations (50,100,150 mM) decreased apoptosis, but L-Carnitine at 150 mM showed the highest response by preventing cells from early apoptosis in comparison with the positive control (470 mOsm/L) by more than 4-fold. Besides, at 50 mM and 100 mM early apoptosis was reduced almost 2-fold in comparison with the positive control (supplementary figure 2).

Hyaluronic acid (HA) and hydroxypropyl methylcellulose (HPMC) prevented the cells from entering apoptosis, but HA showed higher ability than HPMC to protect against late apoptosis stages. Cells pre-incubated with HA exhibited early apoptosis values between 2,5 and 3% while those exposed to HPMC showed values ranging from 3,6 to 4,4%. Regarding late apoptosis events, HA showed expressions between 1,5 and 3,3% of apoptosis while HPMC showed values between 4 and 5%. In contrast to other substances,

both polymers at different concentrations decrease the ability of 470 mOsm/L to induce late apoptosis stages (supplementary figure 3).

Furthermore, total apoptosis of cells pre-incubated with the osmoprotective substances and polymers was assessed after hypertonic stress exposure by flow cytometry (figure 8). Betaine showed a significant decrease in apoptosis ($p < 0.0001$) at all concentrations (50, 100, 150 and 200 mM). Furthermore, betaine exhibited a concentration dependent activity with its maximum at 200 mM. Betaine 50 mM and 100 mM demonstrated similar anti-apoptotic activity (9.47% ± 3.53 and 8.67% ± 1.35). Betaine at 150 mM appeared to be a threshold concentration where its anti-apoptotic activity is increased (4.29% ± 1.34). Finally, Betaine at 200 mM showed the highest anti-apoptotic activity although similar to 150 M (3.31% ± 1.05).

All concentrations of L-Carnitine were also very significant in comparison with the positive control (470 mOsm/L) ($p < 0.0001$). Each one, showed similar protective activity (11.88% ± 0.17 for 50 mM, 11.07% ± 0.35 for 100 mM and 9.98% ± 0.26 for 150 mM). Furthermore, taurine demonstrated higher activity at all concentrations ($p < 0.0001$) although very similar between them. Taurine at 20 and 40 mM were almost identical (4.96% ± 0.43 and 4.35% ± 0.54 respectively). Taurine at 80 mM exhibited higher anti-apoptotic activity (3.65% ± 0.58) than 20 and 40 mM. Finally, both polymers HA and HPMC at 0.4% and 0.8% for each one exhibited similar activity regarding apoptosis inhibition ($p < 0.0001$). On the one hand, HA at 0.4% diminished apoptosis up to 9.78% ± 0.86 and HA 0.8% to 8.64% ± 0.92 in comparison with the positive control (24.50% ± 6.26). On the other hand, cells pre-incubated with HPMC 0.4% and 0.8% exhibited similar apoptosis values (10.05% ± 5.05 and 8.72% ± 3.22 respectively).

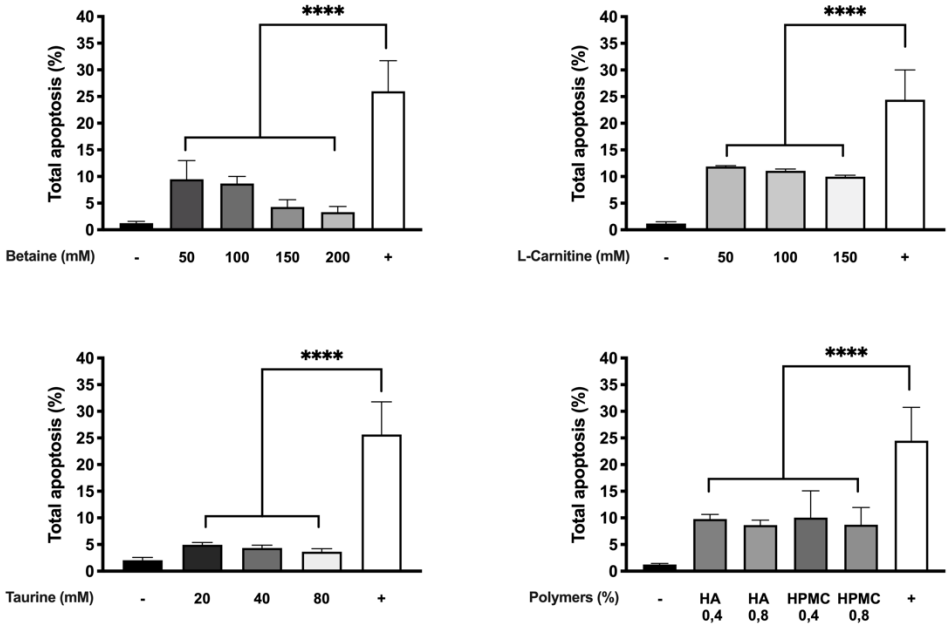

Figure 8. Total apoptosis of cells pre incubated with the different substances and polymers studied (Betaine, L-Carnitine, taurine, HA and HPMC) at different concentrations in comparison with the positive control (+; 470 mOsm/L). Negative control (-) shows cells pre-incubated with NaCl 0,9% (isotonic).

Visualization of apoptosis under different conditions as previously mentioned (hyperosmotic stress and controls) was performed under the fluorescence microscopy (figure 9) confirming the antiapoptotic effect observed by flow cytometry for of different osmoprotective substances and polymers.

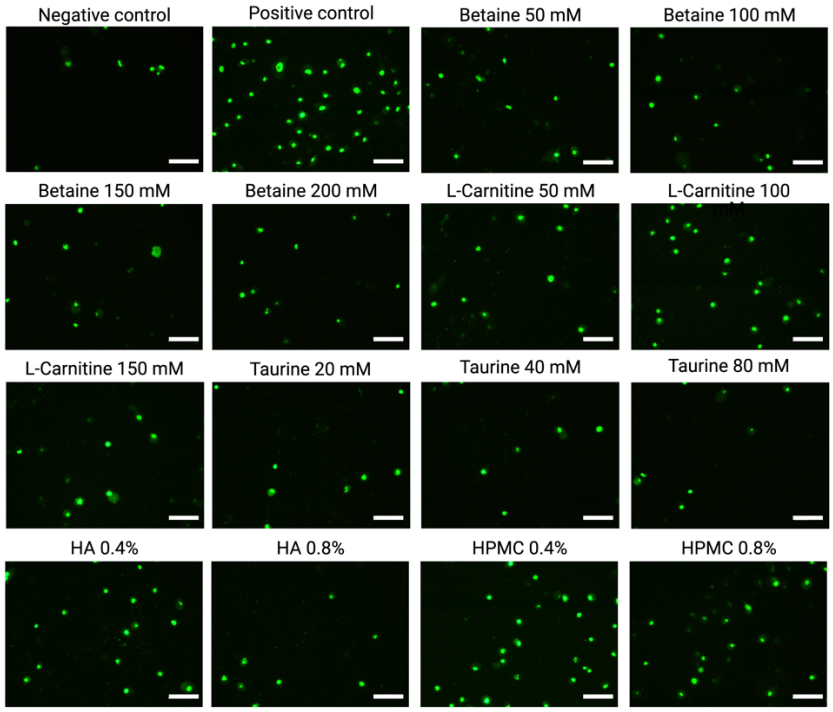

Figure 9. Apoptosis images of different cell populations pre incubated with osmoprotectants or polymers (Betaine, L-Carnitine, taurine, HA and HPMC), negative control (300 mOsm/L) and positive control (470 mOsm/L). Cells stained with YO-PRO1 and visualized under the fluorescence microscope (scale bar of 50 µm at 20x amplification).

3.2.3. Analysis of cell size in response to different hyperosmolarity

Taking into consideration that the forward scatter (FSC) is comparable to the size and volume of cells, FSC was evaluated to determine whether osmolarities modified cell volume in response to the studied osmolarities previously mentioned. Cell count was plotted against FSC using the values of cells exposed to 300 mOsm/L as control of a healthy population. The average FSC signal at each concentration was determined and the relative cell size (RCS) in percentage (figure 10) was calculated in comparison with the control population (300 mOsm/L). Cell sizes at 350 mOsm/L were practically identical to isotonic conditions (100.4% ± 2.51) with no significant differences compared to isotonic conditions ($p = 0.999$). Moreover, cells exposed at 400 and 450 mOsm/L presented a slightly increase in size (108.5% ± 2.38 and 108.6% ± 2.25 respectively) compared to isotonic control showing significant differences ($p = 0.0092$ and $p = 0.0088$ respectively). Moreover, 460 mOsm/L started to decrease the RCS in a significant manner ($p = 0.0143$).

Conversely, a dramatic decrease in cell sizes were observed from 470 mOsm/L to 500 mOsm/L as expected from previous results ($p<0.0001$). RCS at selected hyperosmolar concentration for the model (470 mOsm/L) were 82.15% ± 2.93 of control. The changes in flow cytometry histograms of RCS in response to different hyperosmolar concentrations can be seen in supplementary figure 4.

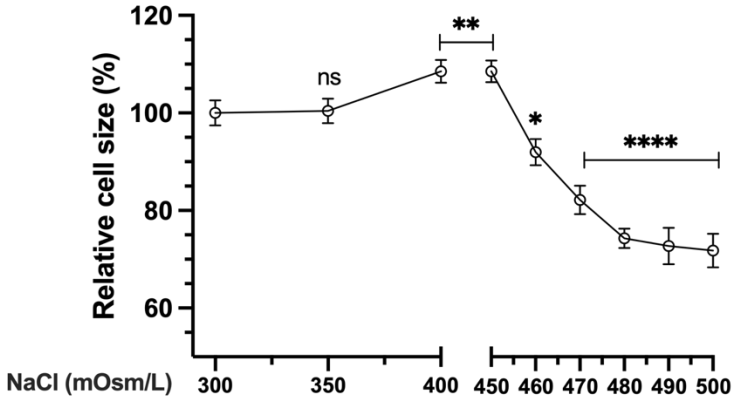

Figure 10. Representation of normalized FSC (%) in comparison with unstressed cell population (300 mOsm/L) showing the progression of cell size modifications under different hyperosmolar concentrations.

Furthermore, cells pre-incubated with Betaine at all concentrations (50, 100, 150 and 200 mM) showed a highly significant normalization ($p < 0.0001$) in cell sizes in comparison with the positive control (470 mOsm/L) presenting similar values (ranging from 99.41% to 97.08%) (histogram can be seen in supplementary figure 5). However, those exposed to L-carnitine demonstrated significant changes in cell sizes at 50 mM ($p< 0.0001$) and 100 mM ($p = 0.0005$) (supplementary figure 6). On the contrary, cell sizes from those exposed to 150 mM showed no significant changes in comparison with the positive control ($p = 0.8640$). Besides, Taurine exhibited a concentration dependent capacity to counteract cell shrinkage with 40 mM and 80 mM being the most effective concentrations ($p < 0.0001$) by increasing cell volume (106.2% ± 3.12 and 106.6% ± 1.34 respectively) (supplementary figure 7). Taurine at 20 mM increased RCS up to 90.52% ± 1.66 being statistically significant in contrast to untreated cells under hyperosmotic stress ($p = 0.0453$). Finally, among all polymers hyaluronic acid at 0.4% increased significantly ($p = 0.0007$) the cell volume (101% ± 3.97). The highest increase was observed for HA 0.8 % and both concentrations of HPMC ($p < 0.0001$). HA 0.8% counteracted the shrinking action by increasing considerably cell volume (113.8% ± 4.11). HPMC increased the RCS

up to 106.7% ± 3.84 whereas HPMC 0.8% achieved 110.2% ± 4.73 (supplementary figure 8). All the changes in cell sizes can be seen in figure 11.

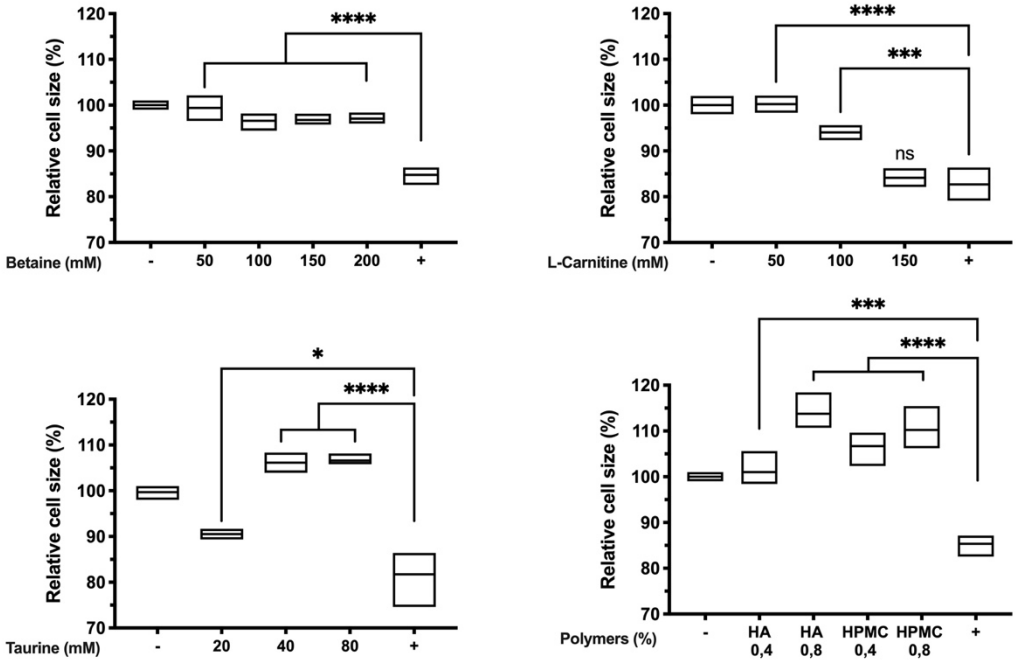

Figure 11. Activity of different osmoprotective substances and polymers on preventing cell size modification under hyperosmolar concentration (470 mOsm/L).

3.3. Determination of inflammatory markers

Macrophages were incubated with the potential osmoprotective substances or polymers were all exposed to 12.5 ng/mL of LPS for 24 hours to mimic inflammatory conditions. The ability of the substances and polymers to reduce the production of TNF-α was expressed as the inhibitory effect in percentage (%) of LPS-induced inflammation (shown in figure 12).

Betaine produced a significant decrease in TNF-α at the different concentrations studied (50 mM, 100 mM and 150 mM). All of them had similar inhibitory activities (25.59% ± 1.82, 21.29% ± 2.86 and 25.81% ± 2.63 respectively), showing no statistical difference between them ($p = 0.2611$ for 50 mM vs 100 mM, $p = 0.9994$ for 50 vs 150 mM and $p = 0.2287$ for 100 vs 150 mM). Moreover, 200 mM constituted the highest inhibitory concentration (39.15% ± 2.10) (***; $p = 0.0008$, $p = 0.0003$, $p = 0009$ for 50 vs 200 mM, 100 vs 200 mM and 150 vs 200 mM respectively). Regarding L-carnitine, it also presented

a high anti-inflammatory capacity. Furthermore, the lower the concentration showed greater anti-inflammatory activity, with 50 mM (56.39% ± 6.12) being the most effective one, followed by 100 mM (46.69% ± 9.58) and 150 mM (32.37% ± 3.83) respectively. L-Carnitine at 150 mM demonstrated significance when compared with 50 mM ($p = 0.0300$) but no difference was observed when compared with 100 mM ($p = 0.1375$). Taurine showed great anti-inflammatory values at all concentrations. Nevertheless, both 20 mM and 40 mM had the highest inhibitory values (55.71% ± 3.43 and 63.85% ± 2.26 respectively) presenting statistically significance in comparison with 80 mM ($p = 0.0046$ for 20 mM and $p = 0.0006$ for 40 mM) in comparison with the highest concentration (80 mM). Conversely, taurine at 20 and 40 mM were similar and presented no differences between them in terms of significance ($p = 0.0907$).

Significant inhibition values were obtained with regards to the above-mentioned polymers. Although polymers at all concentrations exhibited values of inhibition superior to 40%, HA 0.4% had the highest one (62.81% ± 5.96) with high statistically significance in in comparison with both concentrations of HPMC $p = 0.0155$ for HA 0.4% vs HPMC 0.4% and $p = 0.0026$ for HA 0.4% vs HPMC 0.8%).

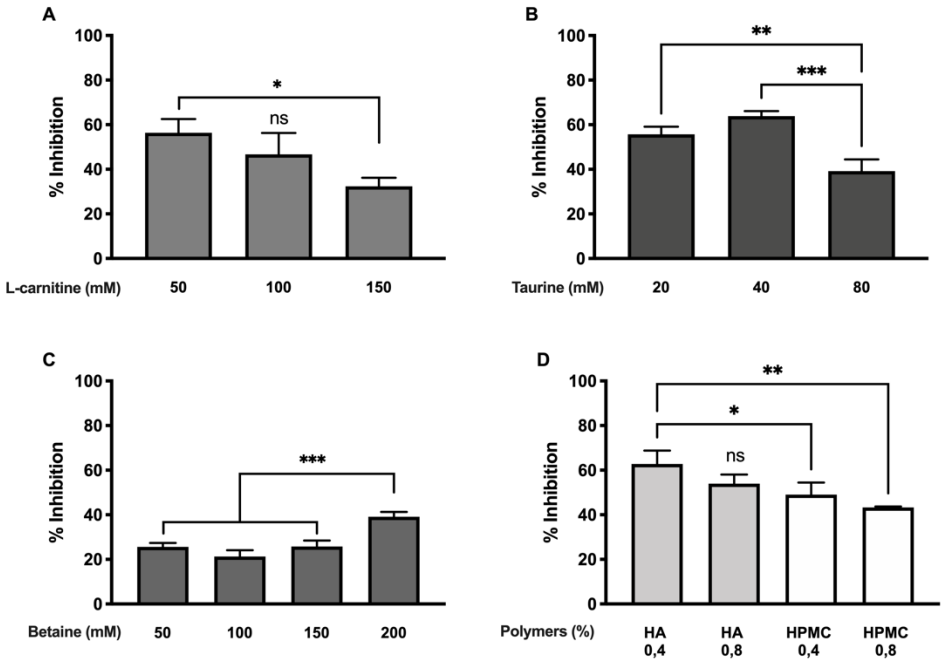

Figure 12. Inhibition of TNF-α production of L-Carnitine (A), Taurine (B), Betaine (C) and HA and HPMC polymers (D) in response to LPS 12,5 ng/mL in macrophage J774A.1-TIB67™ cell line.

4. Discussion

Hyperosmolarity is one of the most important key elements in DED, responsible for triggering an inflammation cascade, ROS production and apoptosis of corneal and conjunctival cells (Wang et al., 2019). According to some authors, increased osmolarity leads cells to volume modification and shrinking (Garrett et al., 2013). Besides, an increase in solute concentrations cause dysfunction in DNA repair, DNA rupture and consequently cell apoptosis (Baudouin et al., 2013). These highlight the importance of searching for fast and effective screening methods to find and develop potential therapies. With regards to DED screening models and techniques, an ex vivo irritation test to test efficacy of DED therapies has been developed (Spöler et al., 2010). However, the main problem associated to these methods are their costs, due to equipment such as OCT (optical coherence tomography) or a device to maintain the globes with the appropriate conditions of moist, heat and tools to induce lacrimation with the main purpose of mimicking a flow of dry air to resemble DED conditions. Besides, the requirement of animal corneas may hamper the process of rapidly screening potentially useful substances. Another interesting strategy is the development of 3D co-cultures in air-lift conditions mimicking the ocular surface, avoiding the use of animals and increasing reproducibility. *Lu et a*l. developed a complex 3D *in vitro* model based on the use of lacrimal spheroids in combination with conjunctival epithelial cells as a way to reproduce the DED pathophysiology associated to inflammatory events (Lu et al., 2017). Besides, *Barabino et* al. developed an air-lift culture with human corneal epithelial cells as a strategy to study the restoration capacity of a new modulator in the ocular surface under changes produced by a mixed sorbitol and desiccation induced hyperosmolarity model (Barabino et al., 2017). In another study, *Puleo* and collaborators developed a corneal micro-culture using collagen-based gelling materials through microfluidic techniques (Puleo et al., 2009). They claimed to have developed an extracellular matrix (ECM) -like tissue for growing corneal cells and study ocular surface processes. Despite the few complex and groundbreaking models that have been studied (Barabino et al., 2020), there is still a need to quickly and easily study the basic features that allow researchers to rapidly screen specific osmoprotective systems or substance so afterwards more complex models that gather specific information can be applied.

According to the results shown in the present study, we have developed a successful combination of cell models to evaluate osmoprotective agents based on tolerability

screening, cell survival under osmotic stress by MTT, study of cell death mechanisms through flow cytometry analysis under chronic hypertonic stress (16 hours) in human corneal cells as well as anti-inflammatory efficacy (24 hours) in macrophages. The use of both cellular models allows to test and screen the potential anti-inflammatory and osmoprotective activity of different substances and provides useful combined evaluated information to check for effective therapeutic approaches. Combining cell survival by tetrazolium salt reduction and flow cytometry analysis (YO-PRO1/7AAD) provide an overview of cell survival and investigation of apoptosis and necrosis protective mechanisms of the tested compounds. Furthermore, cell volume analysis provides extra information about hypertonic stress counteraction mechanisms. Hypertonic environment causes damaged cells to blow up when detached for flow cytometry analysis, thus combining these data provide us with more comprehensive evaluation (Criollo et al., 2007). Finally, adding TNF-α inhibition in macrophages gives additional value to survival studies and complete the study with more information about each substance.

These combined tools could provide an extremely useful strategy as a first “in vitro” step in ocular surface pathologies that progress with hyperosmolarity and trigger a series of inflammatory and cell death events. Among all the parameters that proves useful to check for a system suitability for topical ophthalmic administration are cell tolerability of individual substances, an in-vitro assay that reproduce some environmental parameters of the disease which allows to rapidly check for cell death and protection, an easy but more specific detailed view of cell death insights that provide information about protective mechanisms, morphology and finally determination of anti-inflammatory activity of testing compounds. We selected some compounds that are currently under study by their proposed activity as ocular surface cell protection in the case of Betaine, L-Carnitine and taurine.

Betaine, a known osmolyte by its capacity to protect plant cells against osmotic and temperature stress, has been previously studied for its ability to inhibit the production of interleukins as well as downregulating the mRNA production of certain chemokines (Hua et al., 2015b). In the present work betaine was well tolerated in corneal epithelial cells after 8h exposure and was also able to decrease the levels of TNF-α in the macrophage inflammation assay at all the concentrations studied (50, 100, 150 and 200 mM). Betaine at 200 mM was demonstrated to have the highest anti-inflammatory efficacy (close to 40%) compared to lower concentrations. Previous studies have shown that betaine was able to

protect human epithelial corneal cells from cell death and regulate their volume under hyperosmotic stress (Garrett et al., 2013). Accordingly, the first hyperosmolar model developed in this work, shows that preincubation of corneal cells with betaine is able to provide protection and increase cell survival. Particularly, betaine exhibited a high osmoprotective efficacy in a concentration dependent manner up to 150 mM. From 150 mM to 200 mM, seemed to have similar protective values (around 60%). When apoptosis was assessed in the flow cytometry studies, total apoptosis reduction was more prominent at 150 and 200 mM, but highly significant from the lowest concentration (50 mM). An important finding was that betaine at 200 mM was the most effective in reducing early apoptosis and normalizing late apoptosis at almost basal levels. With regards to cell size, betaine regulated cell size close to normal levels, although 50 mM apparently was closer to the basal levels. Moreover, the rest of the concentrations were also able to revert cell size close to 100 % (97-99%).

L-Carnitine has been described by *Hua* et al. in co-culture with a hyperosmolar concentration (NaCl 450 mOsm/L) for its ability to decrease the production of specific oxidative markers such as 8-hydroxy-2' -deoxyguanosine (8-OHdG), 4-Hydroxy-2-nonenal (HNE) or Aconitase-2 protein (Hua et al., 2015a). These authors also demonstrated that L-carnitine was able to reduce the production of specific mRNA for pro-inflammatory mediators such as IL-6, IL-1β and TNF-α respectively. Our results for L-Carnitine are in agreement within the range of well tolerated concentrations (50, 100 and 150 mM) L-Carnitine was able to considerably reduce TNF-α production, particularly at 50 mM. We hypothesize that this could be explained due to transporter saturation or chronic toxicity at long periods of exposure. Besides, L-Carnitine has been also described to regulate cell shrinking produced by hypertonic stress (Khajavia et al., 2014). In this study, L-Carnitine was able to protect cells from death in a concentration dependent manner in the MTT based assay exhibiting its high efficacy at 150 mM. However, apoptosis was mostly decreased at the lowest concentration but very similar in all the three concentrations. Conversely, albeit it was able to regulate cell size at 50 and 100 mM, no differences in cell sizes were observed at 150 mM in comparison with the positive control. These results suggest that one of the main mechanisms of action of L-Carnitine is through inhibition of inflammatory processes and regulation of intracellular pathways involved in apoptosis.

Regarding taurine, it is widely known to be present inside cells in a natural way and has been hypothesized to play an important role in the transport of ions through membrane channel and transporters (Schaffer et al., 2000). Bucolo et. al showed the ability of taurine to decrease ROS production under H_2O_2 stress in corneal epithelial cells, decreasing the level of specific metalloproteinases (MMP-9) as well as normalizing the tear breakup time (TBUT) in a DED rabbit model induced with atropine 1% (Bucolo et al., 2017). We demonstrate that the concentrations tested in this work (20, 40 and 80 mM) when incubated with cells prior to hypertonic exposure are able to prevent cell death. In the cytometry studies we also observed that it is a powerful inhibitor of apoptosis, particularly at early apoptosis stages. The anti-inflammatory properties increased at 20 and 40 mM in a similar pattern that with L-carnitine but presenting higher activity. Cell sizes, conversely, to betaine and L-carnitine appear to be upregulated when exposed to 40 and 80 mM. We propose that taurine, apart from inhibiting cell death mechanisms through apoptotic pathways is also preventing cell death by increasing cell volume. Apparently, as previously depicted in the illustration (figure 1), taurine would penetrate through specific TauT transporter triggering specific pathways.

Moreover, we also studied the features in the model of some polymers that already available in marketed tears such as HPMC or HA. It is also worth mentioning that HPMC concentrations raging from 0.2% to 0.8% have been employed as mucoadhesive polymers for novel topical antiglaucomatous formulations (Esteban-Pérez et al., 2020) and as lubricants by improving DED symptoms (Jones et al., 2017). Although both concentrations (0.4% and 0.8%) resulted in high viability values, HPMC 0.4% demonstrated to have some protecting effects under hypertonic stress. Although HPMC 0.8% demonstrated higher significance in reducing inflammation, we could argue that HPMC 0.4% would be more suitable than HPMC 0.8% as the cellulose derivative at high concentrations exhibits high viscosities, thus hampering cells survival and nutrients intake without improving osmolar conditions. In fact, HPMC 0.4% seems to have sufficient viscosity to retain enough water to avoid part of the harmful effects caused by hyperosmotic environment.

We have demonstrated that preincubation with HA protected cells by increasing cell survival under osmotic stress. HA also showed an important ability to decrease apoptosis and also increased cell volume, particularly at 0.8%. Besides, we prove that HA at 0.4% and 0.8% is not only well tolerated but also possesses anti-inflammatory properties at both

concentrations. HA has been also described as a mucoadhesive polymer showing high viscosity depending on the concentration. These fact makes hyaluronic to adhere to the cell surface and prolong the protective efficacy. The use of HA in dry eye treatment has been widely described. *Orobia* et al. exposed the ability of high molecular weight HA to enhance wound healing (Jones et al., 2017), epithelial cell migration, as well as hydrating properties (Mateo Orobia et al., 2018). Also, in a study of patients with glaucoma treated with beta blockers and having tear film instability, sodium hyaluronate was used to increase tear film stability and improve patient discomfort (Monaco et al., 2011).

These findings support the use of both HPMC and HA as co-adjuvant polymers in different therapies to avoid cell surface damage. HPMC acted by upregulating cell volume much more than HA, however HA was able to revert cell death mechanisms in a more effective manner.

Furthermore, this is one of the first studies that show the ability of these specific substances to prevent cells from hyperosmotic damage before being subjected to hypertonic stress. One of the key factors in the management of certain diseases, particularly in DED is the ability of certain substances and therapies to prevent inflammatory processes and cell death mechanisms in response to cell stresses. Many of the present studies, focus on isolated parameters and evaluate the protective capacity of certain substances while osmotic stress administered. However, we demonstrate that exposure to these substances in early stages are able to protect cells from osmotic damage without the need of the substances to be present in the media. According to some authors and as stated in figure 1, we hypothesize that the compounds studied in this article excepting polymers, are able to accumulate inside cells and trigger specific inner mechanisms that provide protection against osmotic stress overtime even when the substance is no longer present in the media. According to the results obtained in the present work, the use of osmoprotective active substances and bioadhesive polymers as the ones evaluated in the present study emerge as a useful strategy to prevent DED or treat ocular surface pathologies that develop with hyperosmolarity, ocular inflammation and oxidative stress.

5. Conclusions

Hyperosmolarity can trigger a cascade of inflammatory events as well as cell death mechanisms leading to patient discomfort and ocular surface damage, causing in some circumstances visual impairment. In the present study, we developed a hyperosmolar model based on different techniques that allows to identify well tolerated osmoprotective substances easily and quickly. Besides, we also demonstrated the ability of the osmoprotective substances Betaine, L-Carnitine, Taurine, and the polymers HA and HPMC to protect corneal epithelial cells from cell death under chronic hyperosmolarity or inflammatory stress (after LPS induced conditions in macrophages) but also to prevent them from these events when the substances have already disappeared from the environment. The use of this substances alone or in combination could help in the development of new potential therapies for the treatment of ocular surface pathologies. The present tools developed in this study might also serve as a starting point to continue refining and studying different osmoprotective and anti-inflammatory characteristics of substances and promising formulations. Finally, this could entail a new approach to rapidly screen potential osmoprotective activity before more complex in vitro or in vivo studies are designed.

Conflicts of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

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CAPÍTULO II

Appendix A. Supplementary data

Appendix A include supplementary images 1, 2 and 3 of the different flow charts showing the same as illustrated in figure 7 but with the rest of osmoprotective compounds. The charts represent 7AAD vs YO-PRO1.

Appendix B. Supplementary data

Appendix B include supplementary images of flow cytometry showing the modification sizes under the exposition of cells preincubated with the protective substances to the hypertonic selected concentration (470 mOsm/L). The charts represent Count vs FSC (Forward size scatter). Supplementary figure 4 shows the size modification under different hyperosmolar concentrations of NaCl. Supplementary 5, 6, 7 and 8 represents the size changes in the cells that were preincubated with betaine, L-Carnitine, Taurine and polymers respectively and exposed to hypertonic stress.

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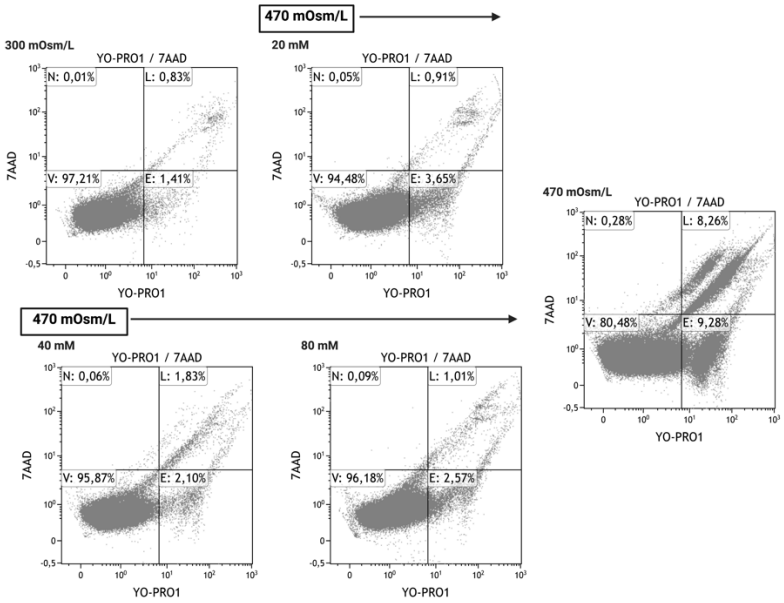
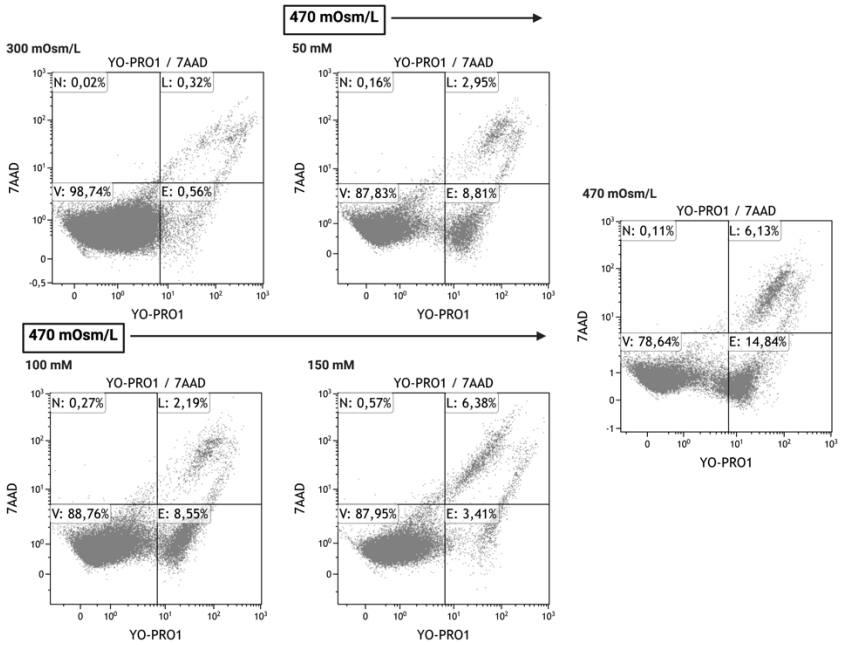
CAPÍTULO II

Supplementary material

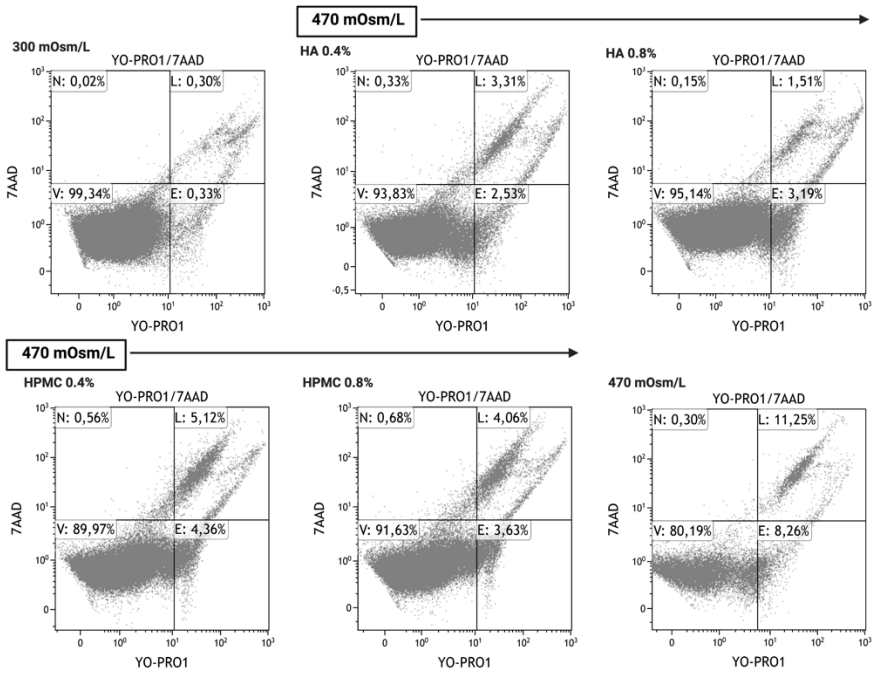
Appendix A



Supplementary figure 1. Cell survival of cells exposed to isotonic medium (300 mOsm/L) and hypertonic conditions without (470 mOsm/L) and with taurine pre-incubation (20, 40 and 80 mM). V: viable; E: early apoptosis; L: late apoptosis and N: necrosis.



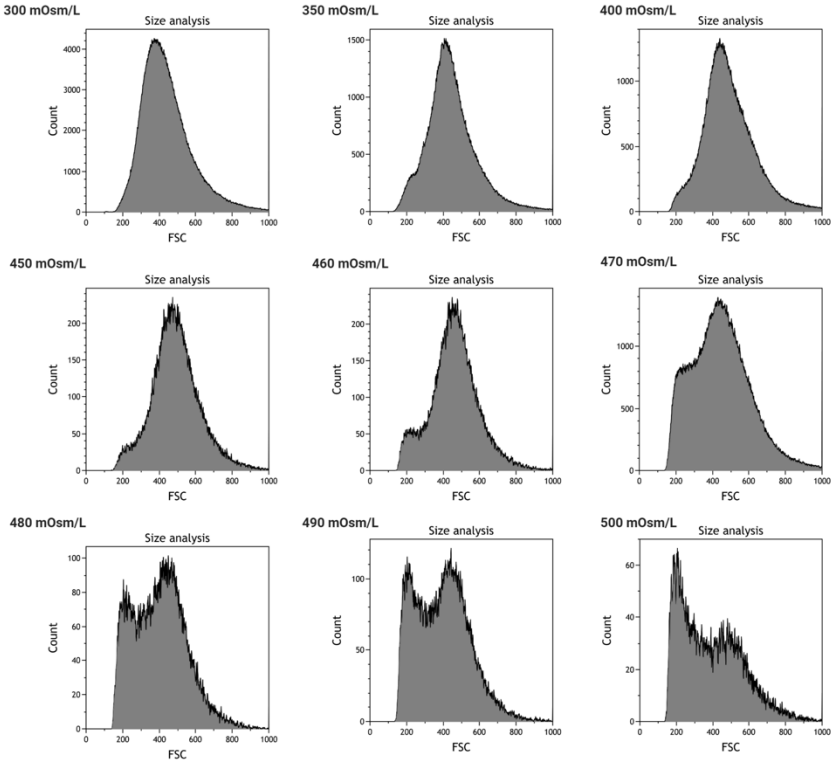
Supplementary figure 2. Cell death protection of cells pre-incubated with different concentrations of L-carnitine in comparison with the positive (470 mOsm/L) and negative control (300 mOsm/L). V: viable; E: early apoptosis; L: late apoptosis and N: necrosis.



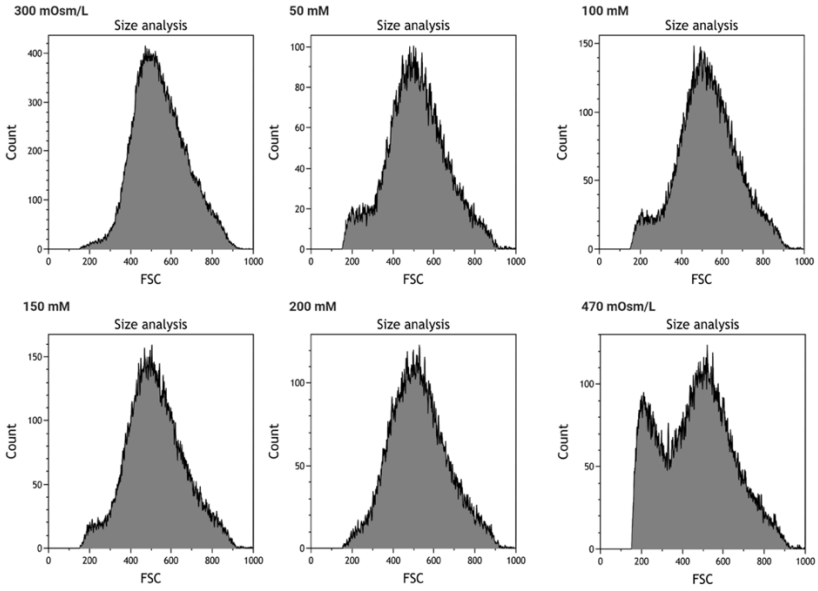
Supplementary figure 3. Cell survival of cells preincubated with the different polymers and exposed to hyperosmotic conditions (470 mOsm/L) as previously mentioned. V: viable; E: early apoptosis; L: late apoptosis and necrosis.

CAPÍTULO II

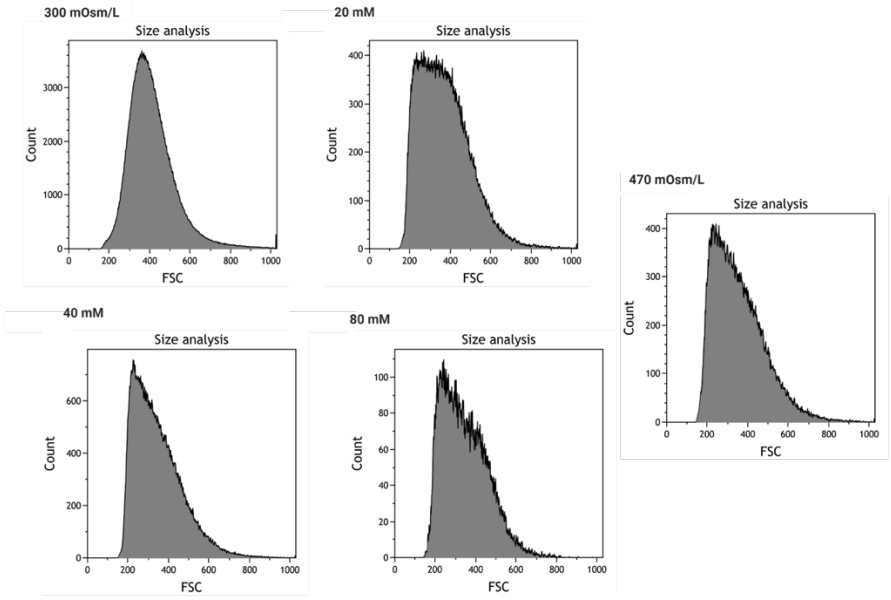
Appendix B



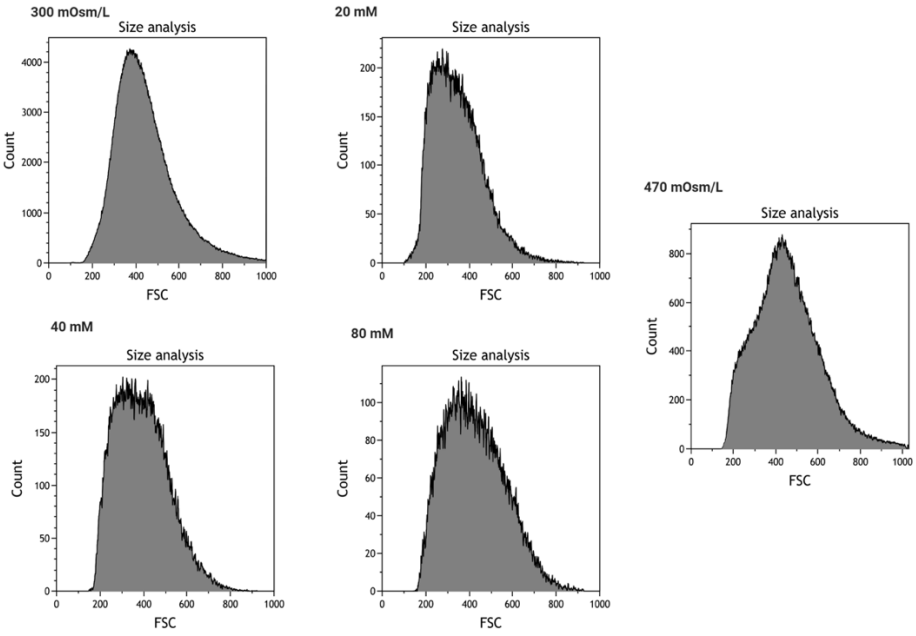
Supplementary figure 4. Cell size modification in response to different hyperosmolar concentrations (300-500 mOsm/L).



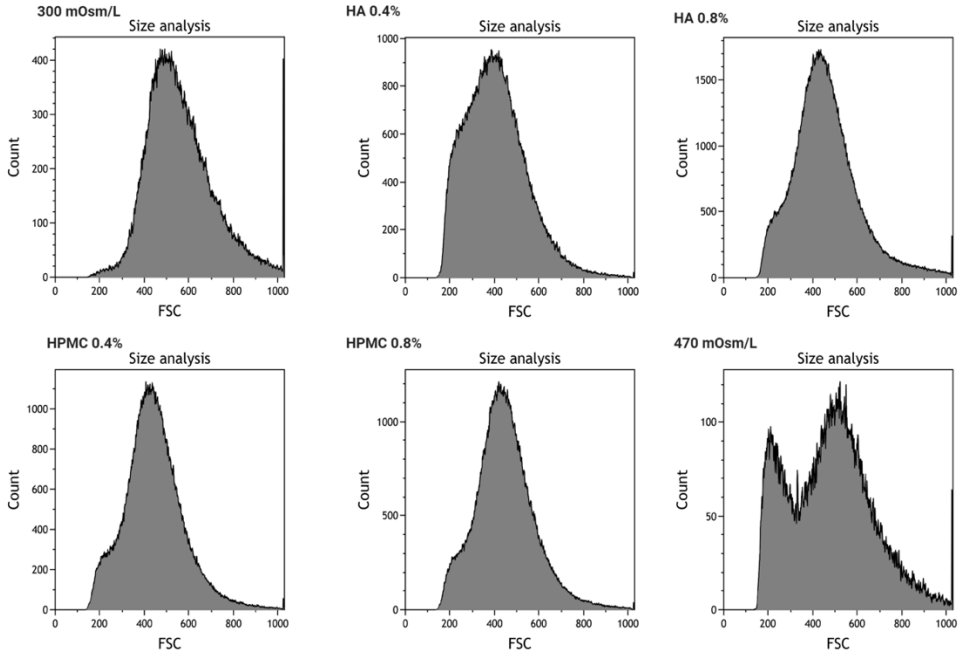
Supplementary figure 5. Activity of different concentrations of betaine to prevent cell size modification when exposed to 470 mOsm/L of NaCl.



Supplementary figure 6. Modification of cell sizes of cells pre-incubated with different concentrations of L-Carnitine and exposed to hypertonic stress (470 mOsm/L of NaCl).



Supplementary figure 7. Modification of cell sizes of cells pre-incubated with different concentrations of taurine and exposed to hypertonic stress (470 mOsm/L of NaCl).



Supplementary figure 8. Modification of cell sizes of cells pre-incubated with different concentrations of HA and HPMC at different concentrations and exposed to hypertonic stress (470 mOsm/L of NaCl).

CAPITULO III. DEVELOPMENT OF AN OSMOPROTECTIVE MICROEMULSION AS A THERAPEUTIC PLATFORM FOR OCULAR SURFACE PROTECTION.

CAPITULO III

CAPITULO III

Development of an osmoprotective microemulsion as a therapeutic platform for ocular surface protection

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CAPITULO III

CAPITULO III

ABSTRACT

Osmoprotective ophthalmic microemulsions (O/A) were prepared by self-emulsification by combining betaine/leucine, clusterin/oleanolic acid and hyaluronic acid, or dextran. Microemulsions contained an inner oily phase (1.2%), an external aqueous phase (96.3%), co-solvents (1%) and surfactants (1.5%). Physicochemical characterization, *in vivo* and *in vitro* tolerance were performed. Osmoprotective *in vitro* activity of the formulations was assayed in a hyperosmolar model in human corneal cells. Average sizes of the inner phase were between 16–26 nm for microemulsions including dextran, and the addition of hyaluronic acid increased its size range (25–39 nm). Addition of osmoprotectants did not change the size of the nanodrops. The formulations were isotonic 280-290 mOsm/L), with neuter pH (≈7) and Z potential (-10 to 0 mV), low surface tensions (≈35-40 $mN \cdot m^{-1}$), and low viscosities (≈1 mPa·s) except for microemulsions with hyaluronic acid (≈4-5 mPa·s). All formulations exhibited sphere-shaped morphology on SEM and cryo-TEM, with good cell tolerance (≈100%) and stable at 8ºC for 9 months. Osmoprotective formulations were well tolerated *in vitro* and *in vivo* protecting cells from hypertonic stress. Therefore, we developed stable microemulsions, compatible with the ocular surface that could entail a novel tool for the treatment of ophthalmic diseases.

Keywords: ophthalmic microemulsions, osmoprotection, stability, in vivo tolerance, hyperosmolar model, ocular drug delivery

CAPITULO III

CAPITULO III

1. Introduction

Homeostasis of the ocular surface is conditioned by physiological and environmental factors. Among the pathologies associated with ocular surface impairment are dry eye disease (DED), blepharitis, meibomian gland dysfunction, limbal stem cell deficiency [1], Vernal keratoconjunctivitis related to allergic diseases [2] as well as corneal ulcers or wounds associated with secondary diseases such as diabetes or Sjögren syndrome [3]. The most critical factors affecting the ocular surface are related to air (temperature, relative humidity, quality and contamination) and illumination [4]. Some microorganisms (ocular microbiota or external) could be associated with an increment in suffering from ocular surface diseases such as blepharitis, keratitis or trachoma [5]. Besides, the use of screens (mobile/computer) or other visual displays has been linked to a decrease in the blinking rate specially among young users. This together with smoking habits leads to tear film instability, corneal damage and DED symptoms [6]. In particular, DED is one of the most common ocular surface diseases having a major negative impact in vision and quality of life [6]. DED is well known in most cases for increasing the osmolarity of tears thus producing a hyperosmotic stress leading to a vicious inflammatory cycle [7]. These events also lead to an augment in cell death mechanisms such as apoptosis resulting in an overall decrease of cell survival [8]. Developing novel pharmaceutical systems that contain osmoprotective substances tackling DED could also be useful to treat other ocular surface diseases that also associated with cell damage processes.

The main approaches that have been discussed for the treatment management of dry eyes includes biological (autologous serum, umbilical cord serum and recombinant human nerve growth factor), viscosity enhancing agents (hyaluronic acid, hydroxypropylmethylcellulose or carboxymethylcellulose), aqueous secretagogues (diquafosol), mucin secretagogues (ophthalmic rebamipide), oral secretagogues (pilocarpine and cevimeline), lipid stimulation agents (insulin like growth factor), ocular lubricants (human recombinant lubricin) as well as topical glucocorticoids (loteprednol etabonate or methylprednisolone) and immunomodulators (tacrolimus or cyclosporine A) [9].

CAPITULO III

According to TFOS DEWS II management and therapy report, a 4-step algorithm has been established to identify and treat DED. Firstly, educational, and hygienic measures should be taken followed in some cases by lubricants administration. When these measures are insufficient, the next step includes tear conservation, reducing preservative-induced damage and antibiotic, corticosteroids or secretagogues administration. Furthermore, autologous serum, oral secretagogues or contact lenses are considered in step 3 when further damaged has been established. Finally, when severe and advanced DED has occurred, a surgical approach (punctual occlusion or tarsorrhaphy), amniotic grafts and topical corticosteroids are needed [10]. Artificial tears are the first line of treatment for DED. There are currently different eye drops present in the market based on viscosity enhancers such as dextran, hydroxypropyl methylcellulose (HPMC), carboxy methylcellulose (CMC), hyaluronic acid (HA) or hydroxypropyl guar (HP-guar), among others, that relieving the symptoms of DED [10]

Despite the broad number of artificial tears in the market, there is still a need for the developing of more effective treatments with less administrations. Since several years ago, the inclusion of osmoprotective and anti-inflammatory actives in artificial tears has gained a lot of attention as potential therapies for the treatment of DED and ocular surface pathologies that progress to cell death events [8]. The design of novel formulations is linked to the development of pharmaceutical systems friendly with the ocular surface able to improve the quality of the precorneal tear film in DED patients. As the precorneal tear film contains lipids and aqueous components, formulations including these two phases, results in a great advance. In this sense, microemulsions could give a high effective response to the previous mentioned requirements.

Microemulsions are defined as clear or hardly turbid systems with two immiscible phases (aqueous and an oil phase) including surfactants, cosurfactants and cosolvents. These pharmaceutical systems were firstly created as transparent continuous water in oil micelle dispersions showing Tyndall effect by Hoar, T.P. et al [11]. Recent studies have widened the knowledge about microemulsions, nanoemulsions and macroemulsions [12]. Nowadays, we know that microemulsions are transparent, thermodynamically stable nano systems with small droplet sizes (10-100 nm) and low surface tensions [12] [13] able to include poor water soluble or non-soluble drugs in the oil phase and increase the penetration of water-soluble drugs (present in the aqueous phase) [12]. Their unique

characteristics (transparency, low surface tension, small size, and high cell membrane permeability) make them ideal systems to tackle different diseases that affect both anterior and posterior segments of the eye being able to deliver poor soluble molecules unable to be included in other aqueous systems [14].

In this study, we have developed a novel microemulsion system as a potential therapy for the treatment of DED and diseases that causes tear film instability. Osmoprotective substances and polymers with potential protective properties of the ocular surface, were also included.

2. Material and Methods

2.1. Materials

Lipoid soy phosphatidylcholine (PC) (Phospholipon 90G®) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Kolliphor EL®, propylene glycol, ethyl oleate, squalene (≥ 98%) and soybean oil (dietary source of long-chain triglycerides and other lipids) were acquired from Merck life sciences (Madrid, Spain). Trehalose dihydrate from fisher scientific, oleanolic acid (97%) and betaine (98%) were purchased from Acros Organics™, Fisher Scientific (Madrid, Spain). Leucine was acquired from Merck life science (Madrid, Spain). Human recombinant clusterin was purchased from Cloud Clone, Biogen científica (Madrid, Spain). Hyaluronic acid (HA), medium molecular weight (400-800 KDa) ophthalmic degree was purchased from Abarán materias primas (Madrid, Spain). Dextran (DXT) 60-80 KDa was acquired from Fisher Scientific (Madrid, Spain). MTT was also purchased from Merck life science (Madrid, Spain).

2.2. Development of the microemulsion systems

2.2.1. Microemulsions design and elaboration

An O/W microemulsion from now on named as base microemulsion (BM) was initially prepared according to the self-emulsification method. This BM was posteriorly employed to include polymeric substances and/or osmoprotective agents.

BM was composed of an inner oily phase (1.2%), an external aqueous phase (96.3%), co-solvents (1%) and surfactants (1.5%). The oily phase was comprised of 0.8 % ethyl oleate, 0.2% soybean oil and 0.2% squalene. As the main co-solvent, 1% propylene glycol was used. Surfactants were 1% Kolliphor EL® and 0.5% soy phosphatidylcholine (PC). This microemulsion has been patented by our group [15]. Briefly, oily phase, surfactants and cosolvents were mixed in a 10 mL glass vial under constant stirring (400 rpm) at room temperature (25ºC) until a continuous clear translucid oily mixture was achieved. Di-hydrated trehalose (4.975%), used for achieving theorical isotonicity (≈300 mOsm/L). was dissolved in ultra-pure water, thus forming the aqueous phase. After that, the previous mentioned aqueous phase was poured over the oily solution in one single step. The mixture was maintained at room temperature under agitation (1000 rpm) for 10 minutes until a clear transparent solution was formed. After that, microemulsions were kept overnight at 2-8ºC for stabilization. Hyaluronic acid (0.2%) and dextran (0.1%) was included in the aqueous phase.

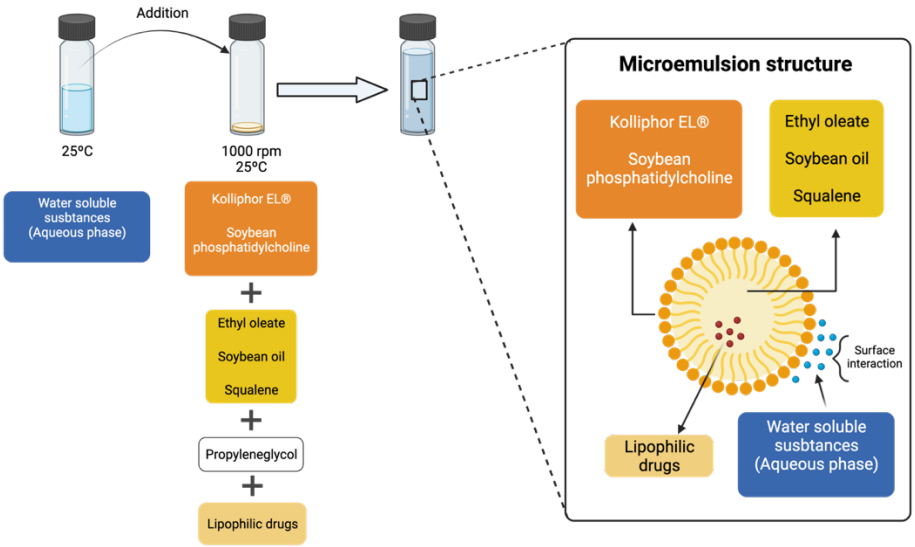

Figure 1. Elaboration scheme of the developed microemulsion system as a platform for drug delivery

2.2.2. Osmoprotective microemulsions as artificial tears for ocular surface diseases

Based on the above-mentioned BM, four different ophthalmic formulations of microemulsions were developed by combining different lipophilic, hydrophilic, or polymeric substances with potential protective properties of the ocular surface, especially against hypertonic environment. To this, a combination of betaine/leucine, or oleanolic acid (OA)/clusterin (CLU) were formulated in BM. Also, HA was included in the betaine/leucine and DXT in the oleanolic acid/clusterin formulations. The osmoprotective emulsions (with and without polymer addition), were named as follows: A (Betaine/Leucine), A-HA (A containing HA 0.2%), B (Oleanolic acid/Clusterin) and B-DXT (B containing dextran 0.1%). The composition of each formulation based on BM is depicted in table 1.

Table 1. Microemulsion formulations combining different substances including osmoprotectants based on BM.

	Osmoprotectant 1	**Osmoprotectant 2**	**Polymer**
BM	/	/	/
BM-HA	/	/	Hyaluronic acid (0.2%)
BM-DXT	/	/	Dextran (0.1%)
A	Betaine (0.4%)	Leucine (0.9%)	/
A-HA	Betaine (0.4%)	Leucine (0.9%)	Hyaluronic acid (0.2%)
B	Oleanolic acid (0.01%)	Clusterin 50 µg/mL	/
B-DXT	Oleanolic acid (0.01%)	Clusterin 50 µg/mL	Dextran (0.1%)

2.3. Physicochemical characterization of microemulsions

2.3.1. Size and zeta potential analysis

Size of the inner phase was analyzed by DLS (dynamic light scattering) (Microtrac® S3500 Series Particle Size Analyzer, Montgomeryville, PA, USA) at room temperature by diluting the sample in ultrapure water (1:2). Furthermore, zeta potential of different microemulsion was evaluated by Autosizer 4700 (Malvern, UK).

2.3.2. Morphology evaluation through electronic microscopy

Morphology and appearance of formulations were confirmed by transmission electron cryo-microscopy (cryo-EM; 200 kV FEI TALOS Arctica) and scanning electron microscopy (SEM; JEOL JSM 7600F). For cryo-EM analysis, microemulsions (3 μL) were applied to one side of quant foil Lacey Carbon film Cu/Rh lacey carbon grids, blotted, and plunged into liquid ethane in a FEI Vitrobot Mark IV. Samples were analyzed in a Talos Arctica with a X field emission gun operating at 200 kV. To acquire the images, EPU Software (ThermoFisher Scientific ®) installed on a Falcon III was used. They were recorder under low-dose conditions at a nominal magnification of 73000 (1.4 Å/pixel sampling rate respectively). The images were processed with ImageJ (Fiji) analysis software 2.1.0/1.53c (National Institute of Mental Health, Bethesda, Md USA).

To perform scanning electron microscopy (SEM) visualization, microemulsions were fixed in Whatman® Nuclepore™ polycarbonate membranes (25 mm diameter, 0.1μm pore size) with green malachite fixation method as previously described [16] [17] with some modification. All the different staining steps were carried out in 6 well plates. Firstly, microemulsions were placed in different wells. After that polycarbonate membranes were immersed in each sample (≈500 μL) and incubated for 10 min at room temperature. Briefly, the excess of moisture was removed with filter papers in each step. Furthermore, samples were immersed in 500 μL of a glutaraldehyde (3%) - malachite (1%) mixture in phosphate-buffered saline (PBS) for 1 hour at 4ºC. After that, the membranes were placed in a solution of osmium tetroxide (1%) for 1 hour at room temperature. Following that, 7 serial dehydration steps of 15 min each with ethanol were performed (30%, 50%, 70%, 90% and three times 100%) and the polycarbonate membranes were let in a freeze-dryer overnight. Finally, fixated samples were attached to a conductive carbon adhesive tape in

SEM disks, then coated with 8 nm of chromium oxide and visualized by SEM. The magnification employed was 100000 at 5 kV.

2.3.3. pH

The pH of the different formulations was measured with a pH-meter (model 230, Mettler, Barcelona, Spain) supplied with a microelectrode (InLab, Mettler, Madrid, Spain).

2.3.4. Surface Tension

A K-11 (Kruss) tensiometer was employed to analyze the surface tension by Wilhelmy plate method of the developed microemulsions. MilliQ water was employed for calibration (72.0±1 mN/m) previously to samples measurement. Before analysis, each microemulsion was pre warmed at 33ºC and equilibrated for 3 min.

2.3.5. Rheological studies

A parallel plate system (60 mm diameter and 0.6 mm gap) attached to a Discovery HR1 hybrid Rheometer – TA instruments (New Castle, DE, USA) was employed to measure viscosity of the samples. Viscosity was measured by increasing shear rates from 0 to 1000 s^{-1} in 30 steps. The study was carried out at room temperature.

2.3.6. Osmolarity

A vapor pressure osmometer (Knauer, model k-7000) was employed with the same working temperature as the ocular surface (33-36°C) [18] [19]. For a correct measurement of the samples, the osmometer was calibrated with 400 mOsm/L NaCl standards before each determination and equilibrated overnight.

2.3.7. Long-term physicochemical stability of base microemulsion and combined with polymers

Single BM and BM in combination with HA 0.2% (BM-HA) or DXT 0.1% (BM-DXT) were placed in 10 mL amber sealed glass vials under set 25 ºC (room temperature) and at 2-8 ºC. Firstly, fresh microemulsions were characterized to determine the physicochemical properties characteristics at 0 time. Then, samples were evaluated at different time sets (3, 6 and 9 months of storage) in terms of droplet size of the microemulsion, zeta potential,

pH, surface tension, viscosity and osmolarity to evaluate long-term physical stability of the model nano systems.

2.4.*In vitro* studies in cell cultures

2.4.1. Cell cultures

Two different human cell lines were employed to assess *in vitro* tolerance and effectiveness of the developed formulations. Immortalized human corneal epithelial cells (HCECs; Evercyte GmbH, Vienna, Austria) were maintained at 37 ºC under 5% CO_2 in a humid atmosphere (95%) and media was changed every 48-72 hours. HCECs were kept in EpiLife® cell culture media (Life Technologies, Madrid, Spain) with EDGS® 1X (Life Technologies, Madrid, Spain) and penicillin-streptomycin 1% (Life Technologies, Madrid, Spain) as supplementation. Besides, immortalized human conjunctival epithelial cells (HConEpiCs, Innoprot, Bizkaia, Spain) were maintained at the same conditions of temperature, humidity, and CO_2 with a change of media every 48 hours. HConEpiCs were cultured by using the IM-Ocular Epithelial Cell Medium Kit (Innoprot, Bizkaia, Spain). For the correct attachment and maintenance of the cells, the flasks were coated with collagen I by employing the collagen I, coating kit (1 mg/ml) (Innoprot, Bizkaia, Spain).

2.4.2. Viability in human corneal and conjunctival epithelial cells

Cell viability of BM and the osmoprotective formulations (with and without polymer addition) was performed in HCECs and HConEpiCs. For HCECs and HConEpiC cell viability assays 20.000 cells/well and 30.000 cells/well were seeded respectively in 96 well plates and incubated overnight. Briefly, the cells were exposed to the formulations at different exposure times (1- and 4-hours) that mimic short and chronic ocular administration respectively. After that, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) 1X twice to mimic tear clearance of the developed microemulsions. Briefly, cells were incubated for 4 hours with MTT (0.33 mg/mL) solution made in cell culture media. Following, supernatants were removed and DMSO (100 µL) was added to each well. Finally, to ensure the solubilization of formazan crystals, the plates were taken to the spectrophotometer, shaken for 5 min, and measured at 550 nm. Benzalkonium chloride (BAK) has been commonly employed by our group at 0.005% [20] as positive control for cell toxicity.

2.4.3. Osmoprotection studies in an *in vitro* model of hyperosmolarity environment in human corneal epithelial cells

In a first step, the preventive osmoprotective activity of BM, BM formulated with the single osmoprotective substances (BM/Betaine, BM/Leucine, BM/CLU, and BM/OA) and final formulations of microemulsions with and without the addition of polymers (A, A-HA, B and B-DXT) was assessed in HCECs. To this, cell survival was assayed in a model of hypertonic stress conditions previously developed and published by our group [8]. Briefly, HCECs were cultured in 96 well plates at density of 20.000 cells/well following overnight incubation. Then, supernatants were removed, and cells were exposed to the developed microemulsions for 3 hours. Wells without treatment were filled with cell culture media and NaCl 0.9%. After the incubation time, all supernatants were discarded, and wells were washed with DPBS 1X twice to mimic tear clearance. Then, 470 mOsm/L final concentration was set in all wells except for the negative controls (300 mOsm/L made with NaCl 0.9%). Following that, cells were incubated at 37ºC under 5% CO_2 for 16 hours (overnight). Then, supernatants were discarded, and cell viability was measured following the same procedure as previously described (section 2.4.2).

2.5.*In vivo* tolerance studies

Tolerability of A, A-HA, B and B-DXT was assessed by applying repeated instillations of 25 µL each 30 minutes on the right eye (n = 6) of six male New Zealand albino rabbits (Granja San Bernardo, Navarra, Spain) for 6 hours following 24 hours evaluation. The contralateral eye (left) was established as the control eye following the same procedure as with the right eye but performing a single instillation of sterile saline solution of NaCl 0.9%. Final osmoprotective microemulsions (A, A-HA, B and B-DXT) were chosen for tolerance studies. Macroscopic evaluation was performed before instillation as well as at the preset times 3, 6 and 24 hours after instillation. Ocular signs were classified according to the descriptions stated in the guidelines for testing of chemicals of the Organization of Cooperation and Development in 2020, specifically for the identification of potential eye hazards in short time exposure [21]. The scoring system employed is based on a modification of the protocols described by Enriquez et al [22] where different parameters are evaluated such as any reactions that may cause animal discomfort, alterations in cornea

and conjunctiva or any signs of discharge and swelling that could be associated to inflammation (table 2).

All the employed procedures were in accordance with the European Communities Council Directive (2010/63/UE of the European Parliament and the Council of 2010 September 22 on the protection of animals used for scientific purposes) and with the Statement for the Use of Animals in Ophthalmic and Vision Research present in the Association for Research in Vision and Ophthalmology (ARVO) guidelines. Procedures were also approved by Universidad Complutense de Madrid and the autonomous Community of Madrid (PROEX 091.5/21).

Table 2. Scoring system employed to evaluate macroscopically the *in vivo* tolerance of the microemulsions developed.

Grade	Discomfort	Cornea	Conjunctiva	Discharge	Lids
0	No reaction	No alteration	No alteration	No discharge	No swelling
1	Blinking	Mild opacity	Mild hyperemia/mild oedema	Mild discharge without moistened hair	Mild swelling
2	Enhanced blinking/intense tearing/vocalizations	Intense opacity	Intense hyperaemia/intense oedema/haemorrhage	Intense discharge with moistened hair lids	Obvious swelling

2.6. Statistical analysis

Each microemulsion composition was prepared in different batches (n=3) and each batch were analyzed by triplicate. For cell culture experiments, each sample was analyzed in 7 different wells (technical replicates) and that was repeated in 3 different days (biological replicate), to ensure reproducibility of the experiments. Cryo-TEM and SEM images were analyzed with imageJ2 2.3.0 (Fiji) software and sizes of the nano-droplets were measured from 6 different images of each formulation. GraphPad software Inc. Prism Version 9 was employed to perform the statistics. Two-way ANOVA in combination with Šidák correction was employed to perform multiple comparison of multiple variables of the physicochemical stability grouped charts. Furthermore, ordinary one-way ANOVA with Dunnett's multiple comparisons was performed to determine the level of significance

of cell viability and osmoprotection studies. APA P value style was used to describe the level of significance (ns; *; $p \leq 0.05$, **; $p \leq 0.01$ or ***; $p \leq 0.001$).

3. Results

3.1. Characterization of artificial tear model microemulsions and osmoprotective formulations

Base microemulsion as a model nanosystem to develop osmoprotective artificial tears (BM) as well as BM in combination with the two polymers (BM-HA and BM-DXT respectively) were physiochemically characterized and their suitability for topical ocular drug delivery evaluated (table 3). The size of microemulsion nanodroplets for BM resulted in 21.78±4.88 nm and it was not affected by the addition of DXT (0.1%). By the contrary, the inclusion of HA (0.2%) generated an increase of nanodroplets to 32.07±7.11 nm. Zeta potential values were slightly different with the addition of polymers while pH values were neutral in all cases. Surface tension values were between 35 and 36 $mN \cdot m^{-1}$ for the three formulations, resulting lower than the reference sample (water value: 72.0 ± 1 mN/m). As expected, the viscosity of formulations increased more than 3.5-fold when including HA 0.2%. Osmolarity did not considerably changed with the addition of polymers and was in the physiological range value of tears (≈300 mOsm/L) [23].

Table 3. Physicochemical characterization of model microemulsion as platform for ocular drug delivery.

	Size (nm)	PDI	Zeta potential (mV)	pH	Surface tension ($mN \cdot m^{-1}$)	Viscosity (mPa·s)	Osmolarity (mOsm/L)
BM	21.78±4.88	0.05	-8.28±0.16	7.27±0.03	35.87±0.51	1.17±0.06	271.07±1.43
BM-HA	32.07±7.11	0.05	-0.07±0.04	7.07±0.04	35.69±0.51	4.36±0.08	285.37±5.00
BM-DXT	21.88±5.57	0.04	-5.92±0.14	7.43±0.10	35.69±0.44	1.25±0.11	278.73±2.46

Size distribution resulted unimodal in all cases with low PDI values (0.04-0.05). For BM and BM-DXT the percentage of nanodroplets higher than 36 nm were 3.3% and 2.07% respectively. Besides, the percentage of particles between 36 and 72 nm for BM-HA was 30.94% (figure 2).

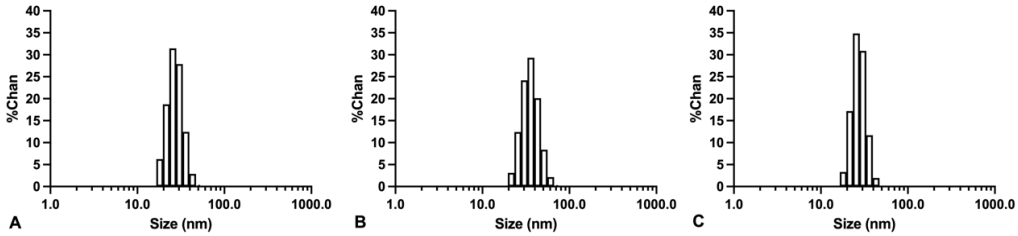

Figure 2. Size distribution of developed microemulsions BM (A), BM-HA (B) and BM-DXT (C) respectively.

The different artificial tear formulations in microemulsions were developed by the addition of osmoprotective actives as previously described. As expected, A-HA formulation exhibited higher sizes (32.07±7.11 nm) than BM and BM-DXT formulations. Moreover, B-DXT showed slightly higher size values (26.45±3.35 nm) than BM. Z potential values resulted similar to the one obtained for base microemulsion with slight modifications depending on the addition of the osmoprotective agents and polymers. pH and surface tension values were neutral and close to 35 $mN \cdot m^{-1}$ in all cases. The addition of HA exhibited a 4-fold increase in viscosity while the addition of dextran did not promote a remarkable change in viscosity. Osmolarity values were almost the same as previous findings for model base microemulsions. Besides, size distributions were not represented since are the same as BM, BM-HA and BM-DXT respectively. Physicochemical characterization of osmoprotective developed microemulsions can be seen in table 4.

Table 4. Physicochemical characterization of osmoprotective microemulsions for the treatment of ocular surface diseases.

	Size (nm)	**PDI**	**Zeta potential (mV)**	**pH**	**Surface tension ($mN \cdot m^{-1}$)**	**Viscosity (mPa·s)**	**Osmolarity (mOsm/L)**
A	21.40±2.22	0.01	-3.10±0.19	7.36±0.03	38.07±0.40	1.12±0.04	281.17±6.75
A-HA	31.96±2.23	0.01	-0.91±0.20	7.00±0.09	35.97±0.55	4.53±0.13	285.18±5.57
B	21.89±2.60	0.01	-8.79±0.15	7.06±0.13	36.32±0.13	1.19±0.05	286.82±2.43
B-DXT	26.45±3.35	0.01	-9.75±0.20	7.35±0.02	37.44±0.22	1.27±0.06	291.67±5.45

Cryo-EM was performed in order to assess morphological characteristics (inner and outer structure) of the developed microemulsions as well as to confirm sizes and homogeneity (figure 3). All microemulsions showed spherical shapes with conserved sizes. BM images exhibited an aggregated behaviour highly homogeneous and according to the images, an average size of 22.48±3.53 nm. Besides, BM-HA were less aggregated, more dispersed and increased in size (31.23±3.18 nm). Moreover, the behaviour of BM-DXT microemulsion was similar to BM-HA in distribution but with small sizes similar to BM (21.13±2.86 nm).

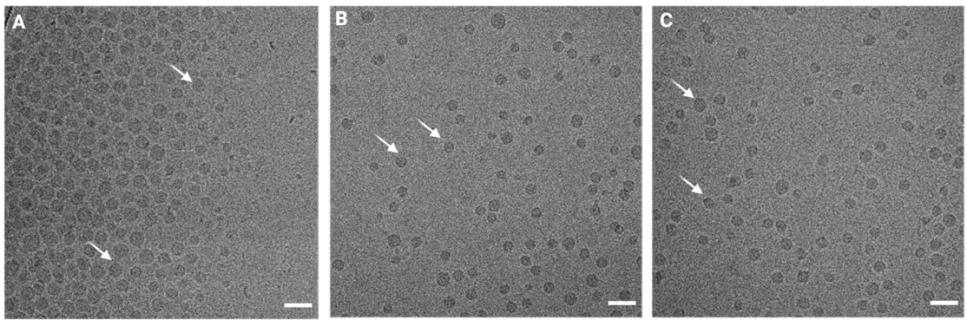

Figure 3. Cryo-EM images showing BM (A), BM-HA (B) and BM-DXT (C) microemulsion images. Examples of nano-sized drops are also illustrated (white arrows). Scale bar size set at 50 nm.

SEM images showed also sphere-shaped morphology of fixed microemulsions according to the previously described protocol (BM, BM-HA and BM-DXT) in 50 nm polycarbonate filter membranes. BM and BM-DXT SEM images exhibited particle sizes of 23.27±6.99 nm and 24.90±5.26 nm respectively. Besides, as previously shown, SEM illustrates BM-HA sizes higher (36.04±8.33 nm) than microemulsions without hyaluronic acid. It was also shown that these particles tended to fuse with an increased adhesive behaviour as it can be seen in figure 4.

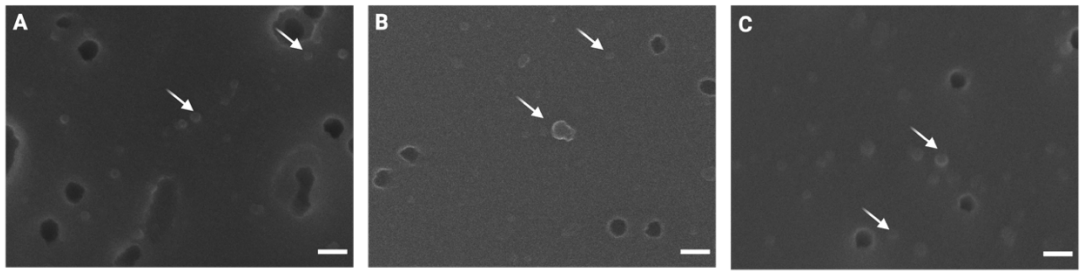

Figure 4. SEM images illustrating 3D morphological structure of BM (A), BM-HA (B) and BM-DXT (C) microemulsions. Scale bar size set at 100 nm.

The morphological structure of the osmoprotective formulations was also assessed (figure 5). As previously described, sphere-like shape was identified for all formulations. However, formulation A presented more irregular structures than the rest of formulations. As expected, the calculated size for formulation A (21.40±2.65 nm) was smaller than formulation A-HA (30.12±3.07 nm). Moreover, formulation A-HA showed more adhesive behaviour than formulation A tending to form aggregates of particles but still presenting conserved distributions. Formulation B and B-DXT were equally distributed with similar sizes (23.91±3.59 nm and 27.11±3.32 nm respectively). Furthermore, small debris-like structures were identified in formulation B, associated to aggregation of the protein included (CLU) but not present in B-DXT images.

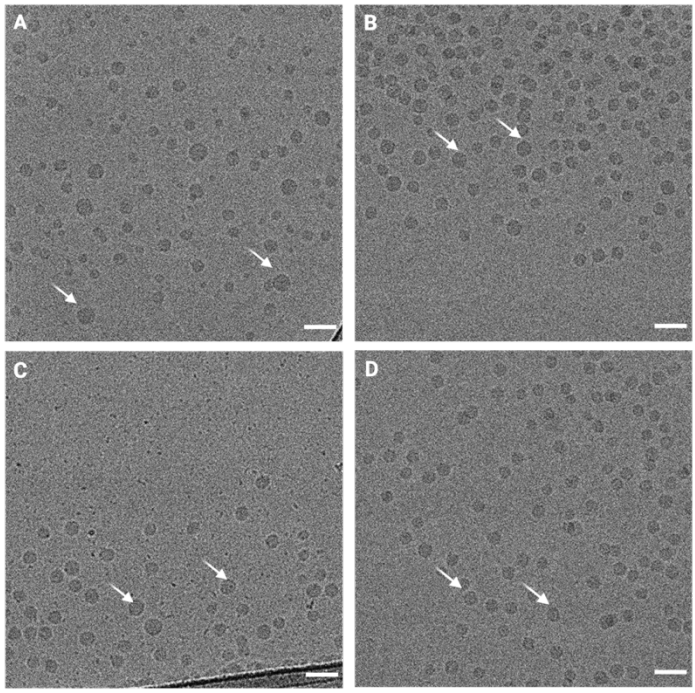

Figure 5. Cryo-EM images of final osmoprotective formulations A (A), A-HA (B), B (C) and B-DXT (D). Scale bar size set at 50 nm.

Regarding SEM performed for the osmoprotective formulations, all of them showed an equal distribution in 50 nm polycarbonate membranes. As previously found, formulation A (23.31±5.48 nm) images showed and increment in size when HA was added (39.15±6.57 nm). The same way as shown in cryo-EM experiments, B and B-DXT sizes did not considerably change (25.53±5.06 nm and 36.05±11.50 nm respectively) as can be seen in figure 6.

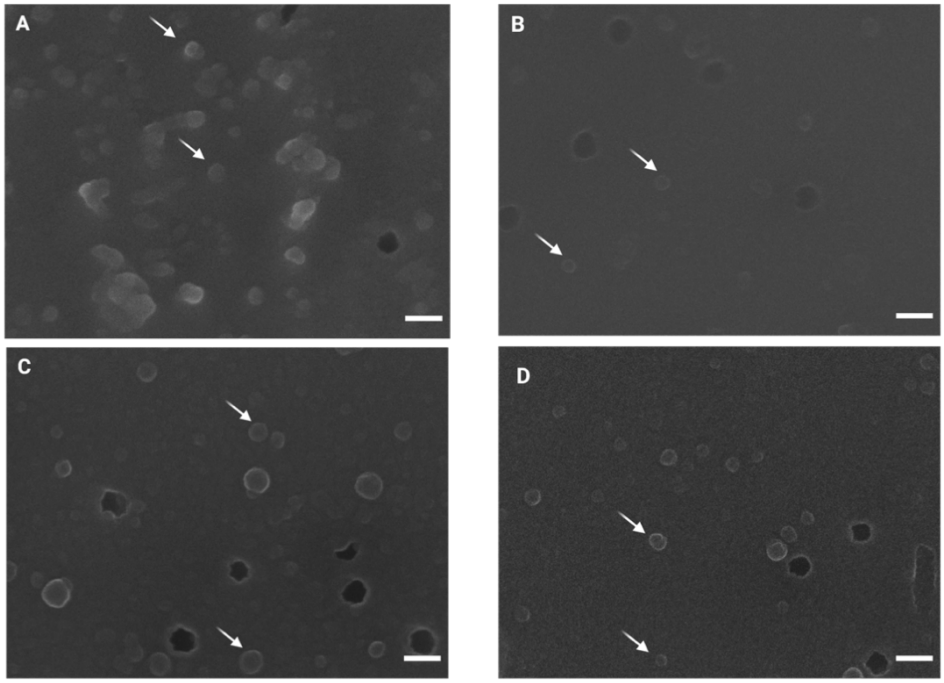

Figure 6. Osmoprotective microemulsions (A, A-HA, B and B-DXT) visualized at SEM represented in A, B, C and D images respectively. Scale bar size set at 100 nm.

3.2. Cell viability studies

In order to evaluate suitability of the developed formulations, the toxicity of BM, BM-HA and BM-DXT as well as osmoprotective formulations (A, A-HA, B and B-DXT) was assessed in HCECs and HConEpiCs after exposures of 1 hour and 4 hours. BM resulted well tolerated in cornea and conjunctiva at all exposure times with survival values close to 100%. The addition of HA and dextran did not significantly affect *in vitro* tolerability in cornea at 1 hour and conjunctiva at 1 hour and 4 hours ($p>0.05$). Furthermore, BM-HA tolerability in corneal epithelial cells was significantly higher at 4 hours in comparison with BM ($p < 0.01$) and BM-DXT ($p < 0.01$) respectively. However, cell viability was higher than the acceptance criteria (>80%) previously established for the development of topical ophthalmic formulations [20][8].

Besides, toxicity of the developed osmoprotective formulations in corneal and conjunctival epithelial cells was also assessed. No significative differences were found at all exposure times among formulations ($p > 0.05$). As with BM with and without polymers, viability of osmoprotective formulations followed the acceptance criteria for ocular surface application (figure 7).

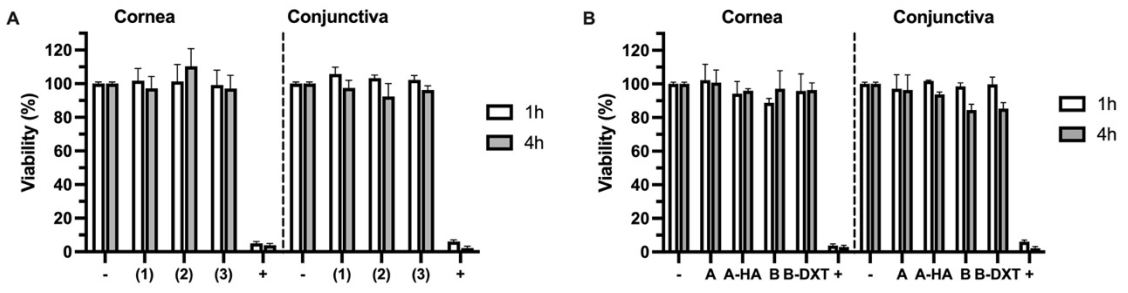

Figure 7. Cell viability of BM (1), BM-HA (2) and BM-DXT (3) (graph A) and osmoprotective formulations (graph B) in HCECs and HConEpiCs under different incubation times (1h and 4h).

3.3. Osmoprotective studies in human corneal epithelial cells

A hyperosmolar model previously developed by our group [8] was used to assess the osmoprotective activity of the developed microemulsions (figure 8). Firstly, BM and single osmoprotective susbtances added to BM were pre-exposed to cells before creating any hyperosmolar conditions (470 mOsm/L). Base microemulsion already exhibited a degree of osmoprotection (42.19%±5.02) ($p < 0.01$) in comparison with the positive control (16.52%±1.92 in untreated cells exposed to 470 mOsm/L). Betaine at 0.4% exhibited the highest protective activity (49.87%±11.98) ($p < 0.001$) and BM with OA at 0.01% produced also a significant increase in cellular viability (43.07%±8.16) ($p < 0.01$). Besides, CLU and Leucin provided similar osmoprotective effect (34.58%±2.33 and 38.26%±6.48) ($p < 0.05$ and $p < 0.01$ respectively).

According to previous results, osmoprotective substances were combined in the ophthalmic formulations trying to assess synergic protective effect under hyperosmolar stress. A-HA including betaine/leucine following by A resulted in the most increased viability values (61.19%±9.00 and 59.75%±11.47 respectively) in comparison with the positive control (22.70%±5.46) ($p < 0.001$). B (oleanolic acid/clusterin) was also very highly protective (43.07%±2.49) as well as B-DXT (41.54%±0.56) both showing significant results ($p < 0.01$ and $p < 0.05$ respectively).

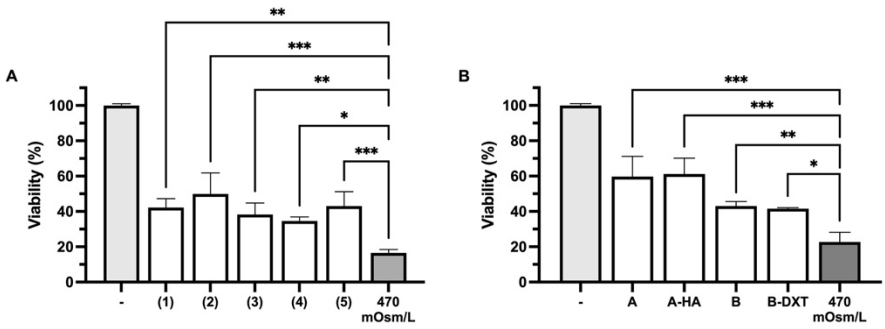

Figure 8. Osmoprotective efficacy of BM (1), the single agents included in the base microemulsion, BM/Betaine (2), BM/Leucine (3), BM/CLU (4) and BM/OA (5) (graph A) and combined therapy (graph B) in the developed microemulsion model after preincubation following exposure to hyperosmolar environment.

3.4.*In vivo* tolerance studies of osmoprotective formulations

In vivo tolerance results of the osmoprotective formulations in New Zealand albino rabbits exhibited no signs of discomfort of ocular surface damaged. According to the scoring system employed for this work (table 2), rabbits exhibited grade 0 in all categories except for some isolated cases that showed grade 1 for secretion or discharge 6 hours after exposure with grade 0 at 24 hours (see appendixes B) (figure 9).

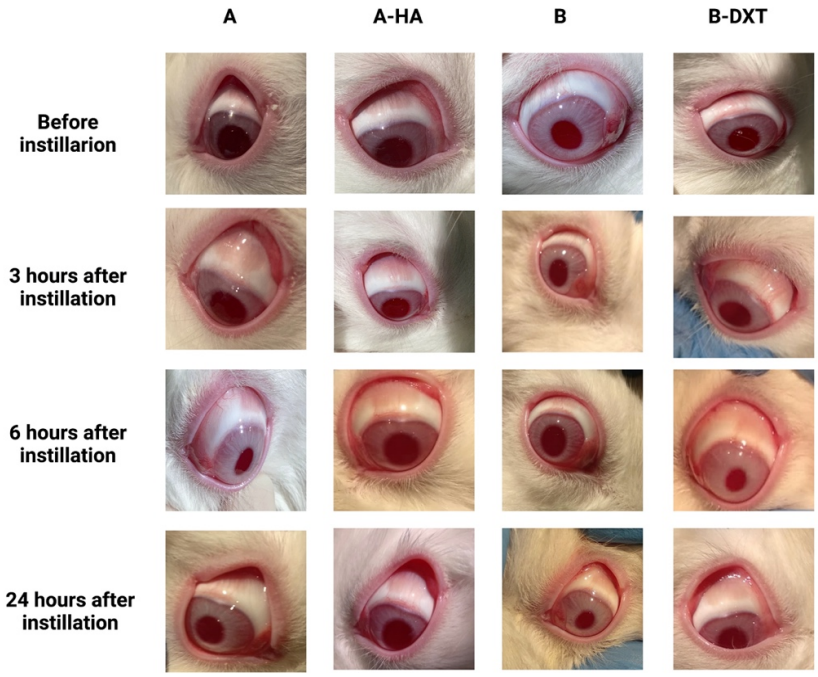

Figure 9. *In vivo* tolerance images of osmoprotective formulations in New Zealand albino rabbits after different exposure times.

3.5. Long-term storage conditions of base microemulsions and in combination with polymers

Physicochemical characterization of BM, BM-HA and BM-DXT was performed to assess their stability from a physicochemical point of view at different conditions (8ºC and 25ºC) during a certain period (1, 3, 6 and 9 months). As can be observed in figures 10 and 11, size distribution remained unimodal and did not considerably change over 9 months, independently of the storage conditions.

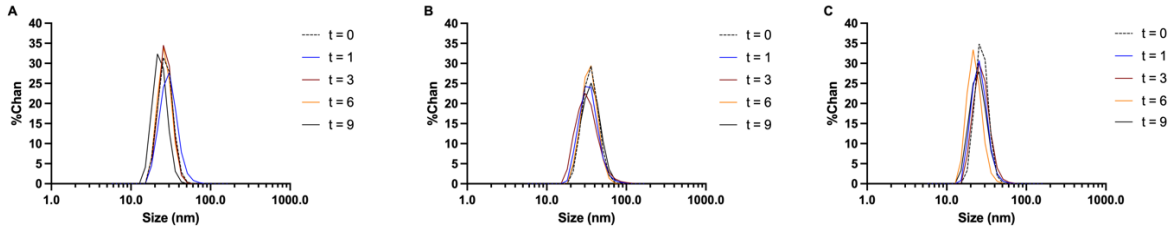

Figure 10. Size distribution of BM (A), BM-HA (B) and BM-DXT (C) microemulsions during a period of 9 months at 8ºC.

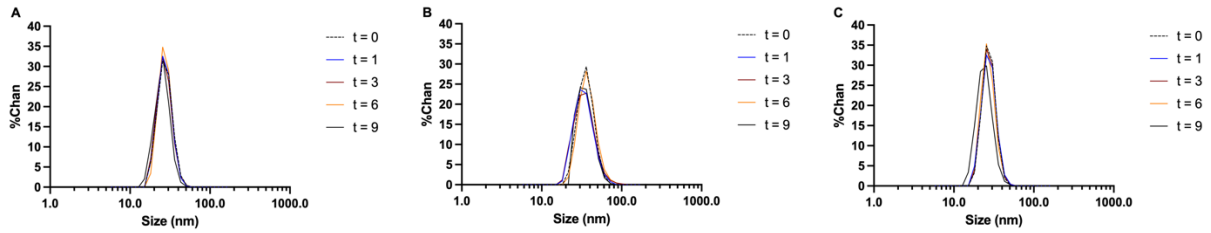

Figure 11. Size distribution stability of BM (A), BM-HA (B) and BM-DXT (C) at different time periods at 25ºC.

Besides, as can be seen in appendix A, average sizes of BM increased from 21.78±4.88 nm to 25.34±4.52 and 25.38±4.69 nm at 8ºC and 25ºC respectively. However, sizes were stabilized with little variations during the following months. BM-HA sizes experienced some variations over the period evaluated but always oscillating from 32 to 35 nm at 8ºC and 32 to 40 nm at 25ºC. BM-DXT was similar to BM with limited variation, from 21.88±5.79 nm at the beginning of the study to 25.12±5.51 nm (8ºC) and 25.28±5.78 nm (25ºC) both at 1 month, remaining almost without changes for the rest of the study. In all cases, PDI values were between 0.03 and 0.05.

Regarding Z potential, although it can be considered neutral (pH≈7), MB zeta potential increases over 9 months from -8.28±0.30 mV to -6.16±0.42 mV at 8ºC and from 8.28±0.30 mV to -3.00±0.52 mV. Besides, BM-HA exhibited similar results at both temperatures. BM-HA Z potential decreases to a maximum of -11.36±1.76 mV at 3 months 8ºC and to -11.02±0.11 mV at 6 months 25ºC. Furthermore, BM-HA Z potential increases again up to -6.25±0.41 mV and –7.54±0.44 mV at 8ºC and 25ºC respectively. Regarding MB-DXT, Z potential did not suffer considerable changes over time. BM-DXT Z potential resulted in -5.92±0.43 mV at 0 months and was -5.39±0.49 mV and -5.07±0.20 mV at 8ºC and 25ºC respectively.

In addition, at 8ºC, BM pH decreased from 7.27±0.12 to 6.45±0.01 for 9 months but diminished up to 5.78±0.01 at 25ºC. Besides, BM-HA exhibited a change in pH from 7.07±0.05 to 6.14±0.03 and 5.73±0.01 at 8ºC and 25ºC respectively. Moreover, BM-DXT recently prepared has a pH of 7.43±0.26 and ranged up to 6.57±0.02 at 8ºC and 5.81±0.04 at 25ºC. Surface tension did almost not change for 9 months being in all cases between 35 and 40 $mN \cdot m^{-1}$. Moreover, viscosity values did not experience dramatic changes over time, preserving almost the same results as freshly prepared. Finally, osmolarity was evaluated and only BM and BM-HA at 25ºC slightly increased. All the stability results regarding physicochemical characterization are shown in appendix A.

Viability studies of BM, BM-HA and BM-DXT in HCECs also performed for 9 months to assess the suitability of the formulations at different conditions (8ºC and 25ºC) over 9 months (figure 12). As can be seen in figure 12, all the formulations at 1 hour and 4 hours exposure resulted well tolerated (>80%) after storage at 8ºC for 6 months. Moreover, when polymers HA 0.2% and Dextran 0.1% were added, viability at 9 months was improved up to acceptable criteria for topical ophthalmic administration (>80%). Conversely, formulations at 25ºC, presented acceptable results of tolerability only after 1 month of storage, dramatically decreasing at 3 months exposure.

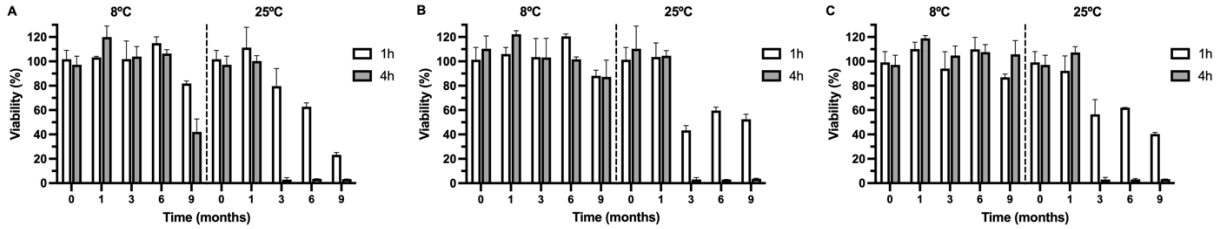

Figure 12. Toxicity assessment in HCECs regarding stability of BM (A), BM-HA (B) and BM-DXT (C) microemulsions during a period of 9 months.

4. Discussion

Microemulsions, commonly known as swollen micelles are described as fully transparent and thermodynamically stable colloidal systems presenting small sizes (<100 nm) [24]. The physicochemical differences between microemulsions and nanoemulsions have been discussed over the last few years being the main one attributed to their thermodynamic stability [24]. The different microemulsions developed in the present work are fully transparent aqueous systems with low tyndall effect and high physicochemical stability thus achieving some of the main requisites for these systems. In fact, we have proven to develop a successful microemulsion system by self-assembly through a low energy method and low surfactant concentrations at room temperature (≈21-25ºC) presenting small sizes (≈20 nm). The novel microemulsion contains lipid-based substances (soy phosphatidylcholine, ethyl oleate, squalene and soybean oil) intended to restore and stabilize the precorneal lipid film. According to different authors, lipid-containing eye drops have been developed to better resemble the aqueous and lipid components present in the preocular tear film [25]. Among the components of the lipid layer of the precorneal tear film are sphingolipids, phospholipids such as phosphatidylethanolamine or phosphatidylcholine, wax-based lipid substances (ceramides) as well as monoglycosylceramides (cerebrosides) [26].

The inclusion of phosphatidylcholine in formulations for the treatment of DED has been already employed by different authors. Heiko Pult et al. included soy phosphatidylcholine in a liposomal spray for the stabilization of the ocular tear film based on the fact that phosphatidylcholine is one of the most abundant phospholipids present in the precorneal tear film [27]. The microemulsion developed in the present work contained soy phosphatidylcholine due to its well-known benefits for the treatment of ocular surface

diseases and in particular DED. Besides, under a technological point of view soy phosphatidylcholine exhibit excellent surfactant properties enhancing the system stabilization [20]. The inclusion of ethyl oleate was upheld in the ability of ethyl esters to confer high stability and in particular thermal stability by avoiding the reaction of carboxylic groups of the fatty acid with other susbtances, since they can easily form hydrogen bounds [28]. Furthermore, this ethyl ester of oleic acid is commonly employed in intramuscular oil-based and micellar drug delivery systems [29] being some microemulsions containing ethyl oleate described as safe and well tolerated by the literature [12]. Squalene was added as a groundbreaking component in the microemulsion as it has previously demonstrated antioxidant and hydrating properties with potential application for ocular surface diseases [26]. Some authors have already stated the ability of squalene to stabilize lipid emulsions and its capability to increase tolerability [30]. Finally, the inclusion of soybean oil has promoted a general increment in viability of the developed formulations and could provide the ocular surface with potential benefits. Finally, soybean oil has been previously included in the marketed formulation Emustil® as an enhancer of precorneal lipid film stability, increasing tear volume as well as counteracting hyperosmolarity present in DED [25].

Microemulsions for ocular application have been already described in the literature. Haße et al. developed a microemulsion by self-assembly containing pilocarpine hydrochloride for topical ocular administration with high viscosity values, nano-droplet sizes between the range of 30 and 55 nm and hyperosmolar values (1000-2500 mOsm/L) [31]. Other authors claim to have developed microemulsions presenting neutral pH (≈7) with droplet sizes oscillating between 220 nm and 480 nm [32]. The novel microemulsion developed by our group resulted in a small nano-droplet size (21.78±2.21 nm), slightly hypotonic (271.07±1.43 mOsm/L), with neutral pH (≈7), low surface tension (35.13±0.40 mN/m^{-1}), neutral zeta potential (-8.28±0.16 mV) and viscosities very similar to aqueous solutions (1.16±0.06 mPa·s). According to these results the present prepared microemulsions showed ideal physicochemical properties for ophthalmic applications with special relevance in the development of artificial tears. For these reasons, we decided to firstly include different polymers such as hyaluronic acid and dextran as mucoadhesive and viscosity enhancers since they have been previously described for their ability to increase the ocular retention time and possess therapeutic properties for ocular surface diseases [8]

[33]. The addition of HA (0.2%) promoted a slightly increase in droplet size from 21.78±2.21 nm to 32.07±7.11 nm. These results are consistent with those obtained by other authors in which nanoparticles coated with hyaluronic acid increased in sizes in comparison with uncoated ones [34]. As expected, HA increases viscosity about 4-fold more than the base microemulsion which could be beneficial to increase retention time of drugs or protective susbtances on the ocular surface. Regarding dextran, some authors have studied its addition to topical ophthalmic formulations for the treatment of ocular surface diseases in patients affected by glaucoma [35]. It has been reported that coating iron nanoparticles with dextran did not promote dramatic changes in size [36]. In our case, the sizes of BM developed with dextran 0.1% (BM-DXT) did not increase or change and remained the same as BM. Viscosity of formulations containing dextran was also similar to BM and aqueous solutions. Dextran has been reported to establish binding to different cell types, proinflammatory cytokines as well as other cell markers. Besides, it has been also studied for its ability to induce corneal wound healing [37] so it could have potential benefits for corneal drug delivery and ocular surface protection. It is also worth mentioning that BM as well as those containing polymers possessed properties in terms of osmolarity, surface tension and pH compatible with the ocular surface and the precorneal tear film [25]. The developed base microemulsions (with and without polymer addition) were also well tolerated (>80%) both in conjunctival epithelial and corneal epithelial cells simulating short (1 hour) and chronic exposure (4 hour), so they were employed for the preparation of osmoprotective formulations.

Since several years ago, the advantages of including osmoprotective agents to artificial tears has been discussed. Attending to that, two different types of osmoprotective microemulsions (A and B) were also developed. According to the literature and previous studies [38] designed by our group [8], different osmoprotective susbtances were chosen for the development of osmoprotective microemulsions (betaine, leucine, OA and CLU). Betaine has been widely studied for its ability to protect cells exposed under hypertonic and desiccation stress [39] as well as regulating cell volume and diminishing apoptosis [8][40]. Furthermore, leucine possesses anti-inflammatory properties over the ocular surface, and it is one of the major components of collagen I, and one of the most abundant proteins of the corneal stroma [41]. OA is an important protective lipidic substance since it has been studied for its anti-inflammatory properties and its role in ocular allergic diseases.

OA has demonstrated to improve cell viability by stabilizing cell membranes as well as inhibiting nitric oxide synthase and matrix metalloproteinases [42]. Finally, the last substance to be included was the secreted form of the protein clusterin. CLU is a molecular chaperone that has previously demonstrated protective efficacy of corneal cells [43] against desiccation stress. CLU also preserved the integrity of the ocular surface barrier and inhibited apoptosis in a mice model of desiccating stress [44]. According to previous results [8] obtained by our group we decided to combine betaine/leucine and oleanolic acid/clusterin in the microemulsions to provide additive effects (Formulations A and B respectively). Finally, due to the good results obtained with the addition of HA and DXT to BM, HA was incorporated to A (A-HA) and DXT was added to B (B-DXT).

Regarding physicochemical properties, the sizes of nano-droplet did not change with the inclusion of different osmoprotective combinations (betaine/leucine or oleanolic acid/clusterin). As expected, hyaluronic acid increased droplet sizes as previously shown with BM-HA. Moreover, Z potential of BM-HA and A-HA were higher than those without HA. According to some authors, medium (400-800 KDa) or high molecular weight (> 800 KDa) HA has been related to higher z potential of nanoparticles [45]. However, although aminoacid-based formulations (A and A-HA) Z potentials were slightly higher than B and B-DXT, all formulations could be considered as neutral. All formulations presented neutral pH and surface tension values compatible with the ones observed in the precorneal tear film (40-46 $mN \cdot m^{-1}$). All the formulations developed in the present work resulted in surface tension values between 35 and 40 $mN \cdot m^{-1}$. Artificial tear drops tension values near to 44 $mN \cdot m^{-1}$ have demonstrated to enhance spreadability onto the ocular surface [46]. Viscosity values in the developed microemulsions were comparable to aqueous solutions (≈1 mPa·s) except for BM-HA and A-HA that presented a considerable increase due to HA addition. Besides, the formulations were slightly hypotonic (280-290 mOsm/L) which could be helpful to counteract the hyperosmolarity given in desiccating stress conditions as reported elsewhere [47]. All the microemulsions were visualized by cryo-TEM and SEM. The images confirmed the presence of clear and uniform sphere-like vesicles with compatible sizes to those obtained by DLS analysis.

Viability of osmoprotective formulations was studied in HCECs and HConEpiCs showed that they were well tolerated after a short (1 hour) and chronic (4 hours) exposure simulation. The obtained results agreed with previous findings. Betaine tolerability was previously evaluated by our group [8] and established as non-toxic and well tolerated osmoprotective agent. Other authors stated that free amino acids such as leucine were well tolerated by topical ophthalmic administration [41] and CLU has been regarded as safe for ocular administration in animal studies [43]. OA was also safely employed in corneal epithelial cells [42].

The ability of formulations to counteract cell death was assessed in a hyperosmolar model in HCECs previously developed by our group [8]. To this, HCECs were exposed to microemulsions during 3 hours before the hyperosmolar conditions were stablished. After that they were removed, a chronic hypertonic (470 mOsm/L) environment was established for 16 hours. In comparison to other studies that expose cells to high hypertonic stress for short time periods [48] and treat cells with protective therapies while stressed, the present study assesses the preventive efficacy of the formulations when they are not in contact with cells. To assess the possible synergic effect of the osmoprotective substances their osmoprotective activity was studied for the isolated substances added to BM. As it can be seen in figure 8, that the combination of betaine and Leucine increased the cellular viability up to 60% in comparison with the positive control (values lower than 20% in untreated cells). The presence of HA did not significantly affect its effectivity. Conversely, clusterin and oleanolic acid resulted significantly effective in comparison with the positive control although did not experience any apparent synergistic effects.

Regarding *in vivo* tolerability of microemulsions, some authors have previously demonstrated microemulsion suitability for ocular administration in animal experiments [49]. In the present work, the osmoprotective formulations were tested in New Zealand albino rabbits to check their acute *in vivo* tolerance. After 6 hours treatment (12 instillations) and 24 hours post exposure, the formulations resulted well tolerated and suitable for topical ophthalmic administration supporting the *in vitro* tolerance data.

Very few studies about microemulsions and their stability have been performed. However, there are some authors that have evaluated size of the inner phase stability over a long period of time at 8ºC. Furthermore, in the best of our knowledge this is the first study

in which a complete stability assessment (microemulsion inner phase size, surface tension, pH, viscosity, osmolarity and cell viability) of the model BM and BM in combination with two different polymers suitable for ocular administration (HA and dextran) has been performed over 9 months at two different temperatures (8ºC and 25ºC). As can be seen in previous studies with microemulsions [50], the particle size and distribution for our formulations did not change over the period of study with low PDIs. In all cases, Z potential did not seem to be highly influenced by temperature or time and all formulations presented neutral Z potential (-10 to +10 mV) overtime. Besides, those containing hyaluronic acid experimented a neutralization of charges reaching zero net charge (0 mV). Our results are in agreement with previous study as the case of Soha et.al who coated liposomes with chitosan and medium weight (400 KDa) hyaluronic acid demonstrating that the coating increased z potential from -38 mV to >30 mV indicating that polysaccharides-based polymers could stabilize the charges of different lipids present in an interface or bilayer [51]. In the case of BM-DXT, although the use of dextrans have been associated in some cases to a decrease of z potential, Gardner et.al demonstrated that dextran 70 KDa was able to counteract charges and reach 0 zeta potential in some cases [52]. This seems consistent with our results since we can see that using low concentrations of dextran 70KDa (0.1%) the polymer tends to diminish the negative charge. As can also be seen in appendix A, the rest of physicochemical parameters did not experiment variations regarding those at the beginning of the study, matching all the time the main desired characteristics for topical ophthalmic artificial tears.

Finally, *in vitro* tolerance studies of the storage microemulsions showed that at 25ºC they become toxic between 1 month and 3 months, probably because of some level of component degradation. We speculate that some of the unsaturated fatty acids present such as those in soybean oil or soy phosphatidylcholine could undergo lipid peroxidation at 25ºC and result toxic. Furthermore, at 8ºC the developed microemulsions were much more stable and well tolerated over the period of study. BM exhibited some level of toxicity at 9 months of study that was counteracted in those containing polymers (HA and dextran) supporting the advantage of including these polymers in the formulations. However, further stability studies could be performed.

In summary, according to the obtained results the developed microemulsion can be considered a stable and useful tool for the development of future ocular therapies.

5. Conclusions

We have successfully proven to develop a self-emulsified microemulsion system suitable for topical ophthalmic administration with biocompatible characteristics with the ocular surface and the precorneal tear film that could successfully carry lipophilic active susbtances. Osmoprotective substances such as betaine and leucine, the triterpenic acid derivative of oleanolic acid (OA) and the human recombinant clusterin (CLU) protein in combination with polymers (dextran and hyaluronic acid) have been included in the developed microemulsion. The osmoprotective formulations resulted in good tolerance and showed *in vitro* osmoprotective activity in a cellular model of hypertonic environment. In summary, all the above-mentioned characterization, *in vitro* tolerance, efficacy, and stability studies together with the *in vivo* studies in rabbits support the use of the present microemulsion as a novel system for both the treatment of ocular surface pathologies and drug delivery of potential therapeutic substances for ocular diseases.

Acknowledgements

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Appendices

Appendix A shows physicochemical characterization in the stability assessment at the temperatures (8ºC and 25ºC) and storage time (0,1,3,6 and 9 months). Appendix B states the tables with the results of the in vivo tolerance evaluation for each formulation and different rabbits.

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CAPITULO III

Supplementary material

Appendix A

Appendix A. Physicochemical characterization of BM, BM/HA, BM/DXT at 8ºC and 25ºC during different time sets (0, 1, 3, 6 and 9 months).

		8ºC						25ºC					
Microemulsion	**Time (months)**	**Size (nm)**	**PDI**	**Z potential (mV)**	**pH**	**Surface tension ($mN \cdot m^{-1}$)**	**Viscosity (mPa·s)**	**Osmolarity (mOsm/L)**	**Size (nm)**	**PDI**	**Z potential (mV)**	**pH**	**Surface tension ($mN \cdot m^{-1}$)**	**Viscosity (mPa·s)**	**Osmolarity (mOsm/L)**
BM	**0**	21.78±4.88	0.05	-8.28±0.30	7.27±0.12	35.87±1.67	1.17±0.06	271.07±1.43	21.78±4.88	0.05	-8.28±0.30	7.27±0.12	35.87±1.67	1.17±0.06	271.07±1.43
	1	25.34±4.52	0.03	-7.15±0.51	7.48±0.18	32.72±0.68	1.14±0.10	278.97±5.92	25.38±4.69	0.03	-6.14±0.08	6.60±0.09	34.50±0.46	1.17±0.05	273.10±3.95
	3	25.22±4.36	0.03	-6.02±0.88	7.06±0.06	35.17±0.50	1.24±0.01	279.47±9.06	24.90±4.47	0.03	-3.99±0.46	5.89±0.11	36.60±0.96	1.16±0.02	275.13±8.43
	6	25.40±5.01	0.04	-5.94±0.17	6.71±0.19	37.67±0.91	1.21±0.01	285.22±1.40	26.19±4.67	0.03	-3.12±0.16	5.53±0.01	38.37±0.33	1.24±0.01	284.22±1.27
	9	23.41±4.74	0.04	-6.16±0.42	6.45±0.01	39.87±0.47	1.27±0.03	282.67±0.86	23.26±4.81	0.04	-3.00±0.52	5.78±0.01	39.02±0.67	1.22±0.02	293.67±1.54
BM/HA	**0**	32.07±7.11	0.05	-0.07±0.08	7.07±0.05	35.90±0.08	4.36±0.08	285.37±5.00	32.07±7.11	0.05	-0.07±0.08	7.07±0.05	35.69±0.08	4.36±0.08	285.37±5.00
	1	34.41±6.49	0.04	-5.72±0.90	7.12±0.08	32.91±0.71	4.11±0.23	283.43±12.36	39.96±6.64	0.03	-5.17±0.07	6.24±0.02	32.27±1.18	4.14±0.05	286.60±3.37
	3	35.08±6.47	0.03	-11.36±1.76	7.03±0.11	34.53±0.46	4.50±0.09	270.36±11.86	38.72±6.61	0.03	-10.27±0.12	5.63±0.20	34.56±0.48	4.63±0.24	267.05±7.00
	6	35.70±8.45	0.06	-8.09±0.22	6.40±0.09	35.47±1.30	4.77±0.04	275.89±2.74	40.95±7.49	0.03	-11.02±0.11	5.79±0.01	38.63±0.42	4.65±0.06	263.89±1.86
	9	34.32±8.26	0.06	-6.25±0.41	6.14±0.03	34.80±0.47	4.78±0.01	279.33±0.89	34.93±10.35	0.09	-7.54±0.44	5.73±0.01	42.07±0.47	4.77±0.04	291.44±1.22
BM/DXT	**0**	21.88±5.79	0.06	-5.92±0.43	7.43±0.26	35.81±0.40	1.25±0.11	278.73±2.46	21.88±5.79	0.07	-5.92±0.43	7.43±0.26	35.81±0.40	1.25±0.11	278.73±2.46
	1	25.12±5.51	0.05	-5.26±0.86	7.53±0.05	35.22±0.89	1.23±0.04	288.60±6.22	25.28±5.78	0.05	-5.16±0.73	6.43±0.10	35.40±0.82	1.12±0.03	286.97±16.02
	3	26.24±5.44	0.05	-4.60±1.47	7.39±0.08	35.03±0.73	1.31±0.02	278.47±2.06	24.80±5.54	0.05	-4.40±1.19	5.88±0.06	35.51±1.81	1.22±0.06	279.50±12.30
	6	25.08±4.77	0.04	-5.24±0.19	7.04±0.23	33.48±1.37	1.25±0.02	281.22±4.25	26.30±4.61	0.03	-4.62±0.06	5.86±0.01	37.80±0.43	1.23±0.01	270.56±1.27
	9	24.25±5.50	0.05	-5.39±0.49	6.57±0.02	38.30±0.40	1.26±0.01	271.33±1.18	24.25±5.50	0.05	-5.07±0.20	5.81±0.04	39.53±0.43	1.26±0.01	278.67±2.73

CAPITULO III

Appendix B

Appendix B1. *In vivo* tolerance of osmoprotective formulation A before instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	2	2	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0

Appendix B2. *In vivo* tolerance of osmoprotective formulation A 3 hours after instillation.

Animal	Eye	Formulation	3 hours after instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	2	2	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0

Appendix B3. *In vivo* tolerance of osmoprotective formulation A 6 hours after instillation.

Animal	Eye	Formulation	6 hours after instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	1	1	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	1	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	2	1	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0

Appendix B4. *In vivo* tolerance of osmoprotective formulation A 24 hours after instillation.

Animal	Eye	Formulation	24 hours after instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	1	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	A	0	0	0	0	0

Appendix B5. *In vivo* tolerance of osmoprotective formulation A-HA before instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	1	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0

Appendix B6. *In vivo* tolerance of osmoprotective formulation A-HA 3 hours after instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	1	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	1	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0

Appendix B7. *In vivo* tolerance of osmoprotective formulation A-HA 6 hours after instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	1	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0

Appendix B8. *In vivo* tolerance of osmoprotective formulation A-HA 24 hours after instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	1	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	1	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	1	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	A-HA	0	0	0	0	0

Appendix B9. *In vivo* tolerance of osmoprotective formulation B before instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	1	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0

Appendix B10. *In vivo* tolerance of osmoprotective formulation B 3 hours after instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	1	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0

Appendix B11. *In vivo* tolerance of osmoprotective formulation B 6 hours after instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	1	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	1	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	1	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0

Appendix B12. *In vivo* tolerance of osmoprotective formulation B 24 hours after instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	1	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	B	0	0	0	0	0

Appendix B13. *In vivo* tolerance of osmoprotective formulation B-DXT before instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0

Appendix B14. *In vivo* tolerance of osmoprotective formulation B-DXT 3 hours after instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	1	1	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	1	1	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0

Appendix B15. *In vivo* tolerance of osmoprotective formulation B-DXT 6 hours after instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	1	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	1	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	1	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0

Appendix B16. *In vivo* tolerance of osmoprotective formulation B-DXT 24 hours after instillation.

Animal	Eye	Formulation	Before instillation				
			Discomfort	Cornea	Conjunctiva	Discharge	Lids
1	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
2	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
3	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
4	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
5	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0
6	Right	NaCl 0.9%	0	0	0	0	0
	Left	B-DXT	0	0	0	0	0

CAPITULO IV. NEW TRENDS TOWARDS GLAUCOMA TREATMENT: TOPICAL OSMOPROTECTIVE MICROEMULSIONS LOADED WITH LATANOPROST

CAPITULO IV

CAPITULO IV

New trends towards glaucoma treatment: topical osmoprotective microemulsions loaded with latanoprost

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CAPITULO IV

CAPITULO IV

ABSTRACT

Glaucoma management relapse into hypotensive formulations, but adverse effects on the ocular surface eventually leads to poor adherence and patient compliance. Chronic use of hypotensive drugs is frequently related to ocular surface side effects. Thus, new drug delivery systems with long-term efficacy and protective properties for the ocular surface are needed. This work aimed to develop an osmoprotective self-emulsifying latanoprost-loaded microemulsion formulations as a new potential glaucoma treatment. The developed nanosystems include osmoprotective substances as well as mucoadhesive polymers. Microemulsions were physiochemically characterized and latanoprost encapsulation efficacy determined. In vitro studies were carried out in cell cultures (in-vitro tolerance and osmoprotective efficacy) as well as cell internalization and cell-microemulsion interactions and distribution. In vivo hypotensive efficacy was conducted in rabbits to determine intraocular pressure reduction and relative bioavailability. Hypotensive microemulsions met the desired physicochemical characteristics for ocular surface administration. Nanodroplet sizes were within 20-30 nm with low polydispersity. In vitro tolerance showed 80-100% viability at 1h and 4h exposure in corneal and conjunctival cells. Besides, microemulsions prevented cell death in a hyperosmolar model in corneal human cell line with 2-3-fold more survival values than the untreated cells. Internalization studies shown cell fluorescence for 11 days after a short exposure to coumarin-loaded microemulsions (5 minutes). Also, fast internalization with nanodroplet accumulation in different cell compartments were observed using electronic microscopy. Surprisingly, non-loaded microemulsions displaced intrinsic hypotensive activity. One single dose of latanoprost-loaded microemulsions reduced the intraocular pressure for several days (4-6 days without polymer addition and 9-13 days with polymers). Relative bioavailability was between 4.5 and 19 times more than the reference one for all formulations. These findings suggest the use of these new microemulsions with a superior effect as a potential novel strategy for glaucoma treatment as well as protecting the ocular surface.

Keywords: osmoprotection, microemulsion, latanoprost, osmoprotective microemulsion, glaucoma, cell internalization.

CAPITULO IV

CAPITULO IV

1. Introduction

Among all the different causes of vision loss in the world, there are some chronic ophthalmic diseases that are inherently more difficult to treat than others such as it is the case of age-related macular degeneration, diabetic retinopathy, glaucoma and genetic diseases related with retinal degeneration. That is one of the main reasons why the world health organization (WHO) created the "Vision 2020: The Right to Sight" program in order to achieve a reduction of preventable vision loss diseases by 2020 [1]. Glaucoma is considered a group of eye diseases that causes irreversible blindness in the world being the intraocular pressure the main risk factor. When prolonged periods of chronic hypertension occurs, glaucoma patients eventually experiment optic nerve degeneration and ultimately, vision loss [2]. One of the first line therapeutic strategies to tackle glaucoma progression is the topical administration of hypotensive agents. There are different pharmacological groups of topical hypotensive therapies for the management of glaucoma, such as beta-blockers, carbonic anhydrase inhibitors, α-adrenergic agonists, prostamides or prostaglandin analogs. In many cases, a combination of hypotensive drugs is needed to successfully control intraocular pressure (IOP). Among all different therapies, topical prostaglandins have been studied for being highly more effective than other antiglaucomatous agents in lowering IOP in certain periods of treatment (> 3 months) [3]. Particularly, latanoprost is considered one of the most effective hypotensive drugs achieving good effectivity for 24-hour periods [4].

Despite their advantages in lowering IOP, many glaucoma drugs are related to local and systemic side effects such as high risk of low heart rate, hyperaemia (beta-blockers), altered taste, blurred vision (alpha agonists and anhydrase inhibitors) and eye lid changes or darkening as well as irritation (prostaglandins) [5]. Furthermore, patients exposed to chronic treatments with topical hypotensive drugs might present signs and symptoms of dry eye disease. These correlation between glaucoma treatment and dry eye are frequently due to a destabilization of the precorneal tear film being the main responsible the components of the ophthalmic formulations (frequently preservatives) or even the hypotensive substances. To all these, it must be added the low bioavailability of topical formulations due to their fast elimination from the ocular surface (less than 5% of the administered drug, is able to permeate the different barriers and reach intraocular tissues) [6]. Therefore, enhancing permeation and increasing residence time of hypotensive drugs

is a critical factor to bear in mind when developing future therapies for glaucoma treatment.

In summary, there are several critical factors that need to be considered when selecting the appropriate treatment for glaucoma such as patient compliance, effectiveness or absence of adverse effects [7]. It is evident that the development of novel ophthalmic formulations based on drug delivery systems able to avoid successive applications and at the same time protecting the ocular surface is a challenge in the treatment of glaucoma.

Drug delivery systems overcoming corneal barriers for the treatment of glaucoma and able to enhance ocular drug delivery of hypotensive agents are under study. Among them, pharmaceutical nanosystems are of great interest. Polymeric nanoparticle suspensions made of different biomaterials have been studied for enhancing corneal drug delivery. In the group of nanoparticulate systems, biodegradable polymers (PLGA, PLA), natural polymers (chitosan, gelatin, sodium alginate) or proteins (albumins) have successfully demonstrated their ability to surpass ocular barriers [8]. Liposomes have been widely a studied as drug delivery systems for ocular surface applications since their intrinsic properties such as low surface tension, lipidic nature and bilayer membranes make them suitable for restoring ocular surface tear film while increasing drug permeation through corneal structures [9]. Besides, the possibility to modify their structure and provide them with specific charges offer attractive possibilities [10]. Alike liposomes, niosomes are lipidic structures with double bilayers but made up of non-ionic surfactants. Their advantages over liposomes are their chemical stability, less immunogenicity and better fluidity. Niosomes have demonstrated both high efficacy and encapsulation efficiency [8] and in particular encapsulating hypotensive drugs [11]. Dendrimers are substances chemically synthesized forming branch-like structures. They can be sized and functionalized to interact with certain cellular domains [12]. Moreover, they can be combined with mucoadhesive polymers and provided with high solubilization ratios, however they might cause blurry vision or discomfort [8]. Cyclodextrins are able to create inclusion complexes entrapping hypotensive drugs presenting different properties. These oligosaccharides can act as depot systems in some cases, increase topical bioavailability and also improve tolerability [13]. Microemulsions and nanoemulsions have gained a lot of interest over the last few years as topical therapies for the treatment of ocular pathologies, mainly dry eye disease, since they combine both aqueous and lipid-base components [14].

Besides, as both pharmaceutical systems include surfactants the permeation and delivery of lipophilic substances could be facilitated [15]. The term microemulsion is used to described thermodynamically stable systems with very small sizes (often less than 100 nm) that can be prepared with low energy methods with spheric or non-spheric structures depending on the surfactant optimum curvature. Nanoemulsions comprises usually higher sizes than microemulsions (100-500 nm) although less than 100 nm could also be present. They are often developed by high energy methods such as high homogenization and resulted always sphere-shaped [16]. Ikervis® is an example of a marketed cationic cyclosporin nanoemulsion for the treatment of DED [17]. This novel therapy has been developed through the Novasorb technology, based on the preservative-free nanoemulsion system (Cationorm®) initially developed as an artificial tear formulation [18].

Among the most relevant characteristics of microemulsions is the stability of the nanodroplet sizes that remain almost unaltered over time unless chemical or microbiological degradation of their components occurs. These mentioned properties make them ideal to achieve high retention of lipophilic drugs. Furthermore, they required low energy for the fabrication procedure facilitating their scalability [19]. Attending to their composition, microemulsions requires several components such as an oily phase, surfactant, cosurfactant, an aqueous phase and also may include co-solvents. Since several years ago, microemulsions are acquiring a lot of attention in the treatment of dry eye disease because they provide lipid and aqueous substances that help preocular tear film restoration [20]. These micellar systems own low surface tension, enhancing their extensibility on the ocular surface after instillation [21]. Furthermore, microemulsions have also been studied for their ability to increase drug bioavailability and retention time more than other pharmaceutical systems [21].

Therefore, their compatibility with the ocular surface and precorneal tear film, together with their high retention time and permeability make microemulsions suitable systems for the incorporation of hypotensive drugs as anti-glaucoma treatment. Beside hypotensive drugs, some specific actives such as amino acids, sugars, proteins, re-epithelizing substances or polymers can be included in topical ophthalmic microemulsions to provide hypotensive activity with and additional antioxidant, anti-inflammatory or osmoprotective properties able to protect the ocular surface [22]. Recently, the addition of actives with

different protective properties have gain special interest to help the homeostasis of the damaged ocular surface [23].

Among the substances with potential protective activity on the ocular surface are amino acids as betaine, L-carnitine, leucin, proline and glycine. The first two ones are able to regulate cellular volume, decrease inflammation, increase anti-oxidation or decrease cell death linked to hypertonic stress on the ocular surface caused by tear film destabilization [24]. Leucin with anti-inflammatory properties on the ocular surface along with proline or glycine, all responsible of collagen fibrils formation [22]. Clusterin (CLU) is a promising agent described for the treatment of DED and ocular surface diseases promoting the sealing of disrupted corneal barrier. CLU possess antiapoptotic activity with the capability to counteract barrier disruption events produced by inflammation and desiccating stress both present in DED [25]. Another enticing substance is oleanolic acid (OA), a lipidic pentacyclic triterpene able to inhibit matrix metalloproteinase (MMP) activity promoting cell survival of corneal cells. OA has also been reported for inhibiting nitric oxide synthase (NOS) activity, thus decreasing inflammation and oxidative stress [26]. Squalene is a potent antioxidant with potential protective properties for the ocular surface, such as anti-inflammatory or ROS scavenger [27]. Also, mucoadhesive polymers such as hyaluronic acid (HA) or hydroxypropylmethylcellulose (HPMC) have been widely employed for the treatment of mild to moderate ocular surface diseases. On the one hand, HA have previously shown anti-inflammatory properties [28] in the ocular surface on human trials [29] and anti-apoptotic activity in *in vitro* cell culture models of hyperosmolarity [30]. On the other hand, HPMC is commonly employed in artificial tears increasing retention time and protective properties on the ocular surface (anti-inflammatory, antiapoptotic and cell volume regulator) [31] [30].

In this study we aimed to develop a long-acting topical ophthalmic microemulsions containing the prostaglandin Latanoprost and osmoprotective and mucoadhesive substances with ocular surface protecting activity and high residence time. This aims to create a new long-lasting generation of hypotensive glaucoma therapies with increased activity promoting surface protection and eliminating drawbacks associated to glaucoma therapies.

2. Methods and materials

2.1. Materials

Propylene glycol, squalene (≥ 98%), soybean oil (unsaturated triglycerides), Kolliphor EL® and ethyl oleate, and Leucine were purchased from Merck (Madrid, Spain). Lipoid soy phosphatidylcholine (PC) (Phospholipon 90G®) was acquired from Lipoid GmbH (Ludwigshafen, Germany). Betaine (98%), Trehalose dihydrate, Dextran clinical grade 60-80 KDa and oleanolic acid (OA) (97%) were purchased from Fisher Scientific (Madrid, Spain). Human recombinant clusterin (CLU) was purchased from Biogen científica (Madrid, Spain). Latanoprost (Lt) was purchased from MedChem Express (Sollentuna, Sweden). MTT, glutaraldehyde solution 50 wt % and osmium tetroxide 99.8%, paraformaldehyde DAC 95-100.5% (PFA) were also purchased from Merck (Madrid, Spain). Hyaluronic acid (HA), MMW (400-800 KDa) was purchased from Abarán materias primas (Madrid, Spain).

2.2. Hypotensive microemulsions development

A o/w microemulsion was developed by the self-emulsification method. The microemulsion is composed of 0.8% ethyl oleate, 0.2% squalene and 0.2% of soybean oil as the oily phase. Besides, 1% propylene glycol as well as 0.5% soy phosphatidylcholine (PC) and Kolliphor EL® were included as cosolvent and surfactants respectively. In this sense in order to create the hypotensive microemulsion, Latanoprost was dispersed in ethyl oleate in a concentration of 50 µg/µL. Then 10 µL of ethyl oleate (0.085%) containing latanoprost (500 µg) were taken and added to the rest of the oily phase (0.715% ethyl oleate, 0.2% squalene, 0.2% of soybean oil and 1% of Kolliphor EL®). The rest of the components were added as described above. The oily phase, cosolvents and surfactants were mixed and dissolved (400 rpm and 25ºC) in a 10 mL glass vial until a transparent oily solution was formed. Furthermore, the formulation was made isotonic by adjusting the amount of included di-hydrated trehalose and osmoprotective susbtances. After that, the aqueous phase was added in a single step over the oil phase under agitation (1000 rpm). Finally, microemulsions were kept overnight at 2-8ºC for maturation. The osmoprotective hypotensive microemulsions were made as follows: betaine (0.4%) and leucin (0.9%) were combined for the development of formulation A-Lt and adding HA (0.2%) to create

formulation A-HA-Lt. Moreover, the combination of OA (0.01%) and CLU (50 μg/mL) was made in order to create B-Lt formulation, adding dextran (0.1%) for formulation B-DXT-Lt. All the osmoprotective susbtances were included in the aqueous phase except for the OA that was included in the oil phase.

2.3. Physicochemical characterization of microemulsions

2.3.1. Particle size determination

Microtrac® S3500 Series Particle Size Analyzer (Montgomeryville, PA, USA) was employed for DLS (dynamic light scattering) analysis. The samples were diluted in ultrapure water (1:2) following the manufacturer's procedure.

2.3.2. SEM and Cryo-TEM experiments

Cryo-EM; 200 kV FEI TALOS Arctica was employed to evaluate the morphology of the developed nanosystems. Besides, SEM; JEOL JSM 7600F was used to assess the surface and 3D structure of the microemulsion´s nanodroplets. Cryo-EM was performed by placing 3 μL of microemulsions onto Lacey Carbon film Cu/Rh lacey carbon grids, blotted, using a FEI Vitrobot Mark IV to plunge them into liquid ethane. Furthermore, a Talos Arctica was employed to analyze the microemulsions by using a X field emission gun operating at 200 kV. EPU Software (ThermoFisher Scientific ®) on a Falcon III was used to acquire the images. They were recorder under low-dose conditions at a nominal magnification of 73000 (1.4 Å/pixel sampling rate respectively). Finally, ImageJ (Fiji) analysis software 2.1.0/1.53c (National Institute of Mental Health, Bethesda, Md USA) was used to analysis the images. Microemulsions were fixed in Whatman® Nuclepore™ polycarbonate membranes (25 mm diameter, 0.1μm pore size) by staining and fixation with green malachite according to previous studies [32] with some modifications based on our developed nanosystems. After that, the polycarbonate membranes were submerged for 10 min in each sample. Furthermore, samples were stained for 1 hour at 4ºC in a malachite (1%) - glutaraldehyde (3%) solution prepared in phosphate-buffered saline (PBS). Finally, polycarbonate membranes containing the samples were posteriorly fixated for 1 hour with osmium tetroxide (1%) at room temperature. Following that, serial dehydration with ethanol (30%, 50%, 70%, 90% and three times 100%) was performed and samples were freeze dried overnight.

For SEM visualization, fixated samples in the above-mentioned membranes were placed in a conductive carbon adhesive tape and adhered to SEM disks. SEM disks were coated with chromium oxide (8 nm) (Leica EM ACE600) and visualized in a JEOL JSM 7600F. The magnification employed was 50000 at 15 kV.

2.3.3. Zeta potential

Autosizer 4700, Malvern was employed for zeta potential analysis of the different nanodroplets of the microemulsions. The analysis was carried out at room temperature by using folded capillary zeta cells.

2.3.4. pH

The developed hypotensive microemulsions were measured with a pH-meter (model 230, Mettler, Barcelona, Spain) equipped with a microelectrode (InLab, Mettler, Madrid, Spain). The pH-meter was properly calibrated with different pH solutions (pH 9 and pH 4 respectively) weekly.

2.3.5. Surface Tension

A force tensiometer (K-11, Kruss) equipped with Wilhelmy plate was used to determine the mean average surface tension of the microemulsions developed in the present article. As a calibration step, MilliQ water was analyzed (72.0 ± 1.5 mN/m) before sample measurements. The tensiometer was preset at 33ºC and warmed for 3 min before microemulsion analysis.

2.3.6. Rheological studies

A Discovery HR1 hybrid Rheometer – TA instruments (New Castle, DE, USA) equipped with a 60 mm diameter parallel plate system adjusted to 0.6 mm gap. The conditions employed for the analysis included shear rates from 0 to 1000 s^{-1} in 30 steps at room temperature.

2.3.7. Osmolarity

The osmolarity of formulations was measured by a freezing point depression osmometer Fiske micro-osmometer, model 210 (Tecil, Madrid, Spain) calibrated with 50, 290 and 850 mOsm/L calibration standards.

2.4. HPLC determination

An isocratic method was employed for the quantification of Latanoprost by HPLC. The analysis was carried out using RP-HPLC in an Acquity Arc Bio® (Waters, Madrid, Spain) UHPLC equipped with a photodiode array detector (2998 PDA Detector), a bioSample Manager FTN-R and a bioQuaternary Solvent manager-R. For the separation, an Ascentis® C18 (25 cm x 4.6 mm; 5 µm) column was employed. The mobile phase was comprised of acetonitrile and water acidified with TFA 0.1% (60:40) HPLC grade based on a pre-existing method [33]. A flow rate of 1 mL/min and a wavelength of 210 nm were used. The column temperature was set at 30 ºC and the sample injection volume was 10 µL. Different standard concentrations were prepared to elaborate the calibration curve. The range of the concentrations used was 200, 100, 50, 25, 10 and 5 µg/mL prepared in ethanol absolute from a stock solution of 1 mg/mL in ethanol. Limit of detection (Y-intercept divided by slope) and quantification (3.3 times the limit of detection) were also calculated to determine the maximum range of quantification when no sample is detected.

2.5. Encapsulation efficiency

For the quantification of the total amount of latanoprost present in the formulations, the microemulsions (A-Lt, A-HA-Lt, B-Lt, and B-DXT-Lt) were freeze dried overnight, incubated in ethanol absolute 10 minutes, spin, passed through 0.22 µm nylon filter and injected to the chromatographic system. Total yield was calculated by dividing concentration of latanoprost present in each sample by the theoretical concentration (50 µg/mL). To determine the residual latanoprost present in the aqueous phase and calculate the entrapped Latanoprost, an ultrafiltration method was used. To this, 0.5 mL of each sample was introduced in Amicon® Ultra - 0.5 mL tubes comprised of Ultracel® - 50 KDa centrifugal filters. Afterwards, samples were centrifuged at 14.000 rpm for 15 min in a Mikro 220R centrifuge (Hettich, Tuttlingen, Germany).

Briefly, the filtered aqueous phases were freeze dried overnight, redissolved in 1 mL of ethanol absolute (solubilization of residual latanoprost) and spined for 3 minutes. Finally, samples were spined and filtered through 0.22 μm nylon filters to remove any remanent trehalose or water-soluble substances (insoluble in ethanol) and injected into the chromatographic system (10 μL). To calculate the encapsulation efficiency (EE), total amount of detected latanoprost was subtracted to the drug present in the aqueous filtrate and divided by the total amount times % (Equation 1).

$$\boldsymbol{E}\ (\%) = \frac{\boldsymbol{Total\ amount\ of\ latanoprost - latanoprost\ in\ aqueous\ filtrate}}{\boldsymbol{Total\ amount\ of\ latanoprost}}\ \boldsymbol{x\ 100} \qquad (1)$$

2.6. Cell culture studies

2.6.1. Human conjunctival and corneal epithelial cells

Human immortalized conjunctival epithelial cells (HConEpiCs, Innoprot, Bizkaia, Spain) and human immortalized corneal epithelial cells (HCECs; Evercyte GmbH, Vienna, Austria) were used for the different experiments carried out. Besides HCECs were employed for osmoprotective efficacy determination. The cells were kept under controlled conditions (37ºC, 5% CO_2 and 95% humidity) and cell culture media was changed every 48 hours. HConEpiCs was cultured in IM-Ocular Epithelial Cell Medium (Innoprot, Bizkaia, Spain) and the cells culture in collagen I coated flasks at 5 μg/cm^2 (Innoprot, Bizkaia, Spain). Furthermore, HCECs were maintained in EpiLife® cell culture media (Life Technologies, Madrid, Spain) supplemented with penicillin-streptomycin 1% and EDGS® 1X (Life Technologies, Madrid, Spain).

2.6.2. Viability in human corneal and conjunctival epithelial cells

HConEpiCs and HCECs were used for the *in vitro* tolerance studies. Cells were exposed to osmoprotective microemulsions including Latanoprost for 1 h and 4h. HConEpiCs and HCECs were seeded in 96 well plates at 30.000 and 20.000 cells/well respectively and incubated overnight. Briefly, supernatants were discarded, and cells exposed to the different selected times. Following that, Dulbecco's phosphate-buffered saline (DPBS) was added to the wells twice to wash any remaining formulation. Furthermore, MTT and cell culture media were mixed (1:6) to obtain a final working concentration of 0.33 mg/mL. MTT working solution was added to the wells and incubated for 4 hours. Finally,

supernatants were discarded and 100 μL of DMSO added to each well to dissolve formazan crystals. The plates were shaken for 5 min and measured in the spectrophotometer (550 nm). In all toxicity assays benzalkonium chloride (0.005%) was used as positive control [31].

2.6.3. Osmoprotection studies in an *in vitro* hyperosmolar model of human corneal epithelial cells

The different hypotensive formulations (A-Lt, A-HA-Lt, B-Lt and B-DXT-Lt) with osmoprotective properties were evaluated in a hyperosmolar stress model developed and optimized previously by our group [30]. For the osmoprotection assessment, 20.000 cells/well were seeded in 96 well plates and incubated overnight. Following that, supernatants were removed and the microemulsions and isotonic sodium chloride for the controls (used for positive and negative control) were added to each well following a 2-hour incubation. After that, supernatants were again removed, and the wells washed twice with DPBS. Then, a hyperosmolar environment was created by the addition of a hypertonic mixture of NaCl and cell medium (470 mOsm/L) in all wells (except for the negative control) and incubated for 16 hours at 37ºC and 5% CO_2. The positive controls were the cells initially in contact to an isotonic sodium chloride (NaCl 0.9%) and then exposed to the hyperosmolar environment. Finally, all the supernatants were discarded, and cell viability measured by MTT addition as previously described.

2.6.4. Internalization studies

2.6.4.1. Fluorescence microscopy

In order to assess the ability of microemulsion internalization, three non-loaded formulations named as base microemulsion (BM), BM with hyaluronic acid (BM-HA) and BM with dextran (BM-DXT) containing coumarin (2 μg/mL) as model fluorescence lipophilic substance were incubated with HCECs and washed twice with DPBS. A coumarin suspension at the same concentration was used to confirm minimum cell fluorescence. The cells were exposed to the different treatments for 5 min to simulate physiological retention time of topical ophthalmic administered treatments (1-10 min) [34,35]. Briefly, $5x10^5$ cells/well were seeded in 6-well plates and incubated overnight. The supernatants were discarded and 100 μL of each formulation, sodium chloride

(negative control) or coumarin suspension (fluorescence control) were added to previously added 900 μL of cell culture medium for 5 min. After that, the cells were washed twice with DPBS, and each well was renewed with fresh media. Finally, HCECs were visualized under a Nikon Eclipse TS100 Inverted phase Microscope equipped with an epi-illumination module using FITC and TRITC filters (Izasa Scientific, Madrid, Spain). Cells were examined under the blue laser (535/ 617) at 20X magnification and pictures were taken for different time periods (0, 2, 4, 7, 9 and 11 days) until fluorescence was no longer present. Fiji (Image J) was employed to analyze fluorescence intensity from the images. Corrected total cell fluorescence (CTCF) was assessed to minimize background effect (Equation 2). Integrated fluorescence density is subtracted to the product of the cell fluorescence mean area and the background fluorescence.

$$\text{CTCF} = \boldsymbol{Integrated\ density} - (\boldsymbol{mean\ area\ of\ cells\ x\ mean\ background\ fluorescence}) \quad (2)$$

2.6.4.2. Flow cytometry

Flow cytometry was also performed to quantify the level of cell fluorescence during the different exposure times until basal values were obtained. Similarly, to the microscopy experiments, $5x10^5$ cells/well were seeded in 6-well plates, incubated, and exposed for 5 min to microemulsions. After each incubation time, cells were detached by adding trypsin-EDTA 0.05% for 3 min. Besides, cells were centrifuged at 850 xg for 10 min, re suspended in DPBS and taken to the flow cytometry core facility. A FC 500 (2-laser, 5-color analysis) flow cytometer equipped with FC 500 CXP software (Beckman Coulter, Madrid, Spain) was employed. Before data analysis and gathering, the system was allowed to warm up for at least 15 min. The flow rate was established at a medium flow rate and each sample recorded data from 100000 events. In order to detect the coumarin signal, the blue/red laser (535/617) was used. Moreover, after SSC/FSC gating, and histogram showing count vs coumarin signal was employed. Besides, fluorescence signal of the negative control was adjusted to 10^0 in the X-axis.

2.6.5. Cell-Microemulsion interaction studies by electronic microscopy

Interactions between the Latanoprost microemulsions (A-Lt, A-HA-Lt, B-Lt and B-DXT-Lt) and HCECs were studied by SEM and TEM. Different parameters were observed and studied such as cell morphology, adhesion of microemulsions to cell structures, permeation capacity as well as distribution across the surface and inside cellular structures.

For SEM visualization, HCECs were seeded on 13 mm Nunc™ Thermanox™ Coverslips (Thermo Fisher, Madrid, Spain) inside 6 well plates at $5x10^5$ cells/well and incubated for 24 hours. Then, supernatants were discarded, and cells were exposed to 100 µL of different microemulsions or the negative control (NaCl 0.9%) + 900 µL of cell culture during 5 min to simulate retention on the ocular surface as previously reported.

A staining buffer (SB) was previously prepared by mixing 2.26% sodium dihydrogen phosphate (NaH_2PO_4) and 2.52% sodium hydroxide (NaOH) solutions in specific proportions (83:17 ratio respectively) adjusting the final pH to 7.2-7.4 with NaOH. A fixation mixture (FM) (4% paraformaldehyde, 3% glutaraldehyde and 1% green malachite) was made in the SB to ensure both fixation of microemulsions and HCECs. Afterwards, cells were washed twice with DPBS and firstly incubated for 4 hours in FM. Then, coverslips were stained in osmium tetroxide (1%) for 1 hour at room temperature, incubated overnight in SB and serial-dehydrated with ethanol as above-mentioned and critical point dried (Leica EM CPD 300). Finally, coverslips were mounted in SEM disks and coated with chromium oxide (8 nm) (Leica EM ACE600) as previously mentioned. The magnification employed was x2000, x15000 and x30000 by using a JEOL JSM 7600F.

JEOL JEM 1400 was used for TEM visualization and the procedure was carried out similarly. HCECs were cultured in 6-well plates ($1x10^6$/well) and grown for 24 hours. Afterwards, supernatants were removed, and cells were exposed to microemulsions as above mentioned for 5 min. Briefly, cells were trypsinized for 3 min and centrifuged (850xg for 10 min) in 2 mL centrifuges tubes. Pellets were re suspended in FM and incubated at 4ºC for 6 hours. Then, cells were washed twice with SB, centrifuged, and overnight incubated in SB at 4ºC. For the post-fixation procedure, the cells were pelleted and incubated in a 1% osmium tetroxide (OsO_4) and 0.8% potassium ferrocyanide ($C_6FeK_4N_6$) mixture in H_2O for 1 hour at 25ºC. Afterwards, cells were washed twice with

ultrapure (milliQ) water. Besides, serial-dehydration as previously was performed in ethanol. After dehydration, the samples were embedded in spurr resin (SR) mixed with acetone at different volume ratios for different times (1: 3 for 1 hour, 1:1 for 1 hour and 3:1 for 2 hours SR to acetone respectively) and with pure spurr resin overnight at 25ºC. Polymerization of resin was carried out by introducing samples into a 65-70ºC oven for 48 hours with tube caps opened. Finally, samples were processed in an ultra-microtome, stained with uranyl acetate, and visualized at 8000x and zoomed 30000x and 150000x.

2.7.*In vivo* hypotensive effect

Normotensive New Zealand albino rabbits were purchased from Granja San Bernardo (Tarazona, Navarra, Spain) and had an average weigh of 2.5-3.5 kg. Animals were kept ad libitum under controlled conditions of humidity and temperature (50% and 22ºC respectively) with 12/12 h light/dark cycles. Animals were handled following the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic Vision Research, the European regulatory system for the use of animals in research as well as the European Communities Council Directive (86/609/EEC) and Spanish Regulation of Experimental Studies with Animals (RD 53/2013, February 1; Ref PROEX 114.4/21, July 16, 2020).

Hypotensive efficacy studies of the developed latanoprost microemulsions (A-Lt, A-HA-Lt, B-Lt, B-DXT-Lt), and marketed formulation with a 50µg/mL Latanoprost (Monoprost®) was assessed in 5 rabbits (n = 10 eyes). Non loaded microemulsions (BM, BM-HA, BM-DXT) were also studied. Briefly, 25 µL of each hypotensive formulation were instilled in both eyes of each rabbit. To determine basal IOPs (100%), two different measurements were made before instillation (30 minutes before and just prior to administration). After instillation, IOPs were recorded every hour for 11 hours during the first day, 3 times a day the next day and twice daily until the end of the study using an Icare® Tonovet tonometer (Tiolat, Helsinki, Finland). A minimum wash period of 10 days was set before consecutive treatments. PBS was instilled and stablished as the base line.

IOP pressure reduction (ΔIOP) was established as a parameter to evaluate the activity of the different treatments applied. Maximum percentage of ΔIOP (ΔIOPmax), onset time (t_{onset}), effective time ($t_{effective}$) as well as area under the ΔIOP (%)-time curve from 0 to the time at which ends the study (t') ($AUC_{0-t'}$) were determined for each sample evaluated and

calculated by the lineal trapezoidal rule. Besides, bioavailability was assessed with 90% interval confidence according to the guidelines on investigation of bioequivalence [36].

2.8. Statistical analysis

Microemulsions were made and analyzed in triplicate (n = 3) and the data for *in vitro* experiments is shown as the mean ± SD. ImageJ2 2.3.0 (Fiji) was employed for cryo-TEM and SEM analysis. Fluorescence images were obtained and treated with ProgRes® CapturePro Satellite DLL 2.10.0 software. Finally, flow cytometry results were studied by Beckman Coulter Kaluza Analysis Software, US. Hypotensive curves described in the *in vivo* experiments are described as mean ± SEM and AUC graphs as mean ± SD. GraphPad software Inc. Prism Version 9, US, was used to perform Two-Way ANOVA for column comparison combined with Tukey test correction in cell viability studies. Furthermore, one-way ANOVA analysis in combination with Dunnett's test was employed to analyze the level of significance of cell viability between formulation at specific times, osmoprotective efficacy and AUC of IOP by using APA style (*; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$ or ns; $p > 0.05$). Besides xy regression analysis was employed to obtain Y intercept and calculate detection and quantification limits. Furthermore, SPSS Statistics was used to analyze the influence of different parameters (eye, rabbit, and formulations) as well as the mean square error of the residuals in order to calculate bioavailability by means of a nested ANOVA.

3. Results

3.1. Physicochemical characterization

Hypotensive microemulsions with osmoprotective properties were characterized and compared to the physicochemical properties of human tears (Table 1). Sizes of nanodroplets for A-Lt and B-Lt were similar (21.37±2.34 nm and 20.81±2.56 nm respectively) with low polidispersity index (PDI). Microemulsion containing hyaluronic acid 0.2% (A-HA-Lt) increased its size over 10 nm up to 30.51±3.20 nm. However, the addition of DXT (B-DXT-Lt) did not produce a change in the size (23.44±5.34 nm) in comparison with A-Lt or B-Lt. Z potential was neuter in all cases (-10 to + 10 mV), although A-HA-Lt presented values close to 0 mV and B or B-DXT-Lt were slightly more negative than formulations including betaine and leucine with an without AH (-8.72±0.48 mV and -6.76±0.30 mV respectively). Regarding pH, all were neuter and suitable with the pH of the ocular surface (pH ≈ 7) except for the one with HA that decreased to 6.58±0.01. Surface tension values of the microemulsions were between 34-36 $mN \cdot m^{-1}$, lower to those of the ocular surface (40-46 $mN \cdot m^{-1}$) [37] ensuring proper extensibility on the ocular surface. Regarding viscosity, A-Lt, B-Lt and B-DXT-Lt presented similar values to aqueous solutions and human tears at high shear rates (0.97 mPa·s). As expected, A-HA-Lt viscosity resulted higher (> 4-fold) than its analog without HA (A-Lt). Finally, osmolarity of the developed microemulsions loaded with latanoprost presented values close to the osmolarity of natural tears [38].

Table 1. Characterization of hypotensive latanoprost microemulsions in terms of size, PDI, zeta potential, pH, surface tension, viscosity and osmolarity.

	Nanodroplet Size (nm)	PDI	Zeta potential (mV)	pH	Surface tension ($mN \cdot m^{-1}$)	Viscosity (mPa·s)	Osmolarity (mOsm/L)
A-Lt	21.37±2.34	0.01	-2.57±0.24	7.04±0.02	34.70±0.44	1.13±0.09	281.49±3.69
A-HA-Lt	30.51±3.20	0.01	-0.03±0.07	6.58±0.01	36.77±0.55	4.58±0.08	283.43±5.21
B-Lt	20.81±2.56	0.01	-8.72±0.48	7.43±0.01	35.93±0.29	1.22±0.07	278.83±1.87
B-DXT-Lt	23.44±5.34	0.05	-6.76±0.30	7.10±0.01	36.87±0.56	1.30±0.04	288.80±2.15

All Lt loaded microemulsions exhibited a unimodal distribution with narrow histograms, typical of microemulsion systems. Microemulsion A-Lt exhibited less than 4 % with sizes higher than 36 nm. Conversely, A-HA-Lt presented a frequency of 46% between 36 and 72 nm. B-Lt and B-DXT-Lt showed a 3.5% and 21.5% of sizes above 36 nm respectively.

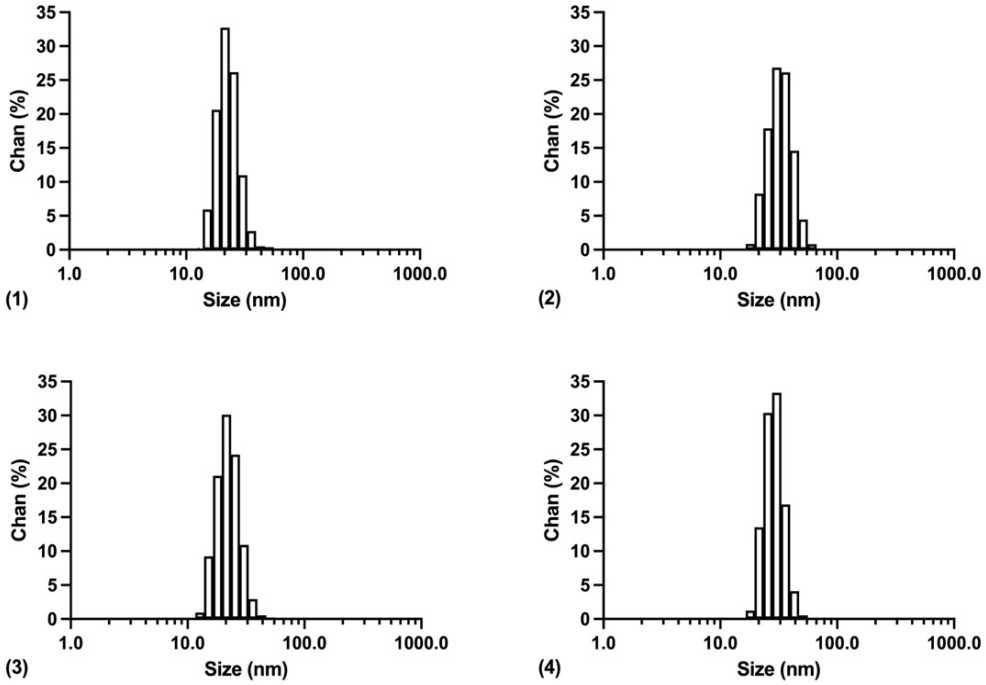

Figure 1. Size distribution of the hypotensive microemulsions developed with osmoprotective properties (1), (2), (3) and (4) for A-Lt, A-HA-Lt, B-Lt and B-DXT-Lt respectively.

Hypotensive formulations were visualized under Cryo-EM microscopy in order to assess and study morphological characteristics as well as inner structure (figure 2). Novel microemulsions presented uniform sizes, however A-HA-Lt microemulsions showed more homogeneous structures than formulation A-Lt. Calculated sizes of cryo-EM sizes were also useful to confirm DLS data. Furthermore, A-Lt microemulsion exhibited 19.72±6.30 nm sizes while A-HA-Lt demonstrated vesicles sizes of 30.07±4.96 nm. B-Lt showed 20.03±4.15 nm sizes similar to A-Lt. Meanwhile the addition of dextran (B-DXT-Lt) did not change vesicle sizes 21.27±3.11nm.

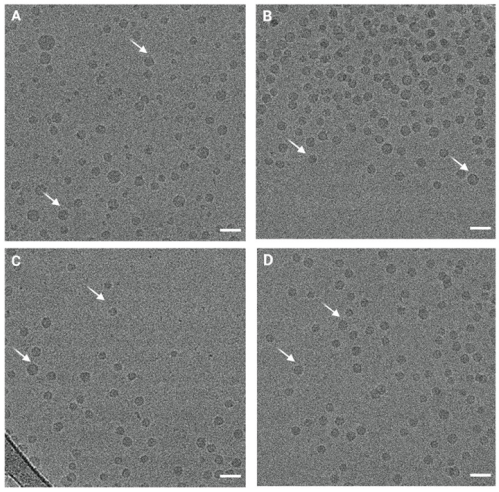

Figure 2. Cryo-TEM microscopy displaying hypotensive microemulsions A-Lt (A), A-HA-Lt (B), B-Lt (C) and B-DXT-Lt (D). Some nano-drops are illustrated (arrows) and the scale bar is set at 50 nm.

SEM evaluation of osmoprotective microemulsion containing Latanoprost showed that all microemulsions including Latanoprost (A-Lt, A-HA-Lt, B-Lt and B-DXT-Lt) were homogeneous and similar in size. As previously described, formulation A nanodroplets sizes analyzed from SEM images showed 23.64±7.39 nm while formulation A-HA-Lt increased sizes up to 31.32±3.53 nm. Moreover, according to the images (figure 3) formulation B-Lt and B-HA-Lt were very similar in distribution, morphology and measured sized (23.09±2.84 and 24.12±3.03respectively). Besides images of the microemulsions containing aminoacids (A-Lt and A-HA-Lt) were prone to exhibit charging phenomenon presenting some anomalous contrast.

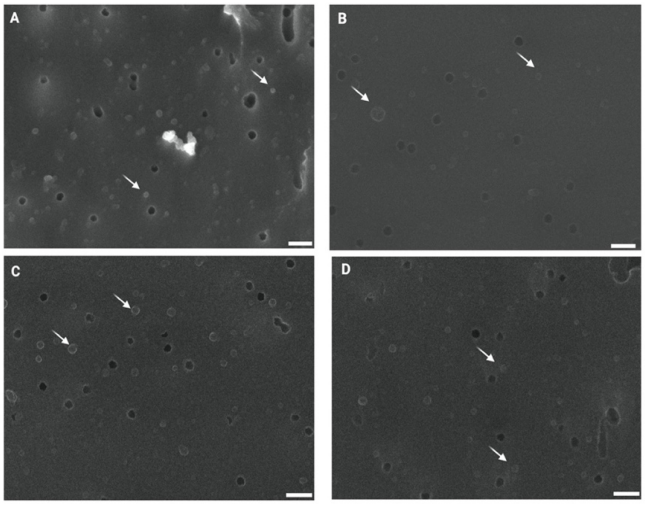

Figure 3. SEM images showing some groups of microemulsions developed (A-Lt, A-HA-Lt, B-Lt and B-DXT-Lt illustrated in A, B, C and D respectively with a scale bar of 200 nm.

3.2. Encapsulation efficiency

Latanoprost EE was quantified in the developed osmoprotective microemulsions (A-Lt, A-HA-Lt, B-Lt and B-DXT-Lt) and resulted ≥ 98%. This value was calculated taking into account the limit of detection and quantification of the calibration curve that were set at 0.93 μg/mL and 2.81 μg/mL respectively. Since no peak was detected in the filtrate, any possible concentration would be lower than the detection limit which entails 1.9% over the total latanoprost. Therefore, when latanoprost is not detected ≥ 98% of encapsulation efficiency can be ensured. (table 2).

Table 2. Latanoprost yield and EE quantification in osmoprotective hypotensive microemulsions. *ND: Non detected presenting a concentration lower than the limit of quantification.

Formulation	Total concentration (μg/mL)	Yield (%)	Ultrafiltrate concentration (μg/mL)	EE (%)
A-Lt	45.78±0.65	91.55±1.31	ND	≥ 98%
A-HA-Lt	45.18±0.75	90.36±1.50	ND	
B-Lt	46.44±1.04	92.89±2.08	ND	
B-DXT-Lt	47.65±0.73	95.31±1.46	ND	

3.3. *In vitro* cell tolerability

Cell tolerance of hypotensive microemulsions (A-Lt, A-HA-Lt, B-Lt, B-DXT-Lt) were evaluated in HCECs and HConEpiCs for different time periods (1h and 4h). All hypotensive microemulsions presented cell viabilities higher than 80% in both cell lines tested [30]. A-Lt and A-HA-Lt formulations presented the highest tolerance values at 1 hour exposure (102.81±11.36% and 113.40±13.86% respectively). At 4 hours exposure, those containing polymers (A-HA-Lt and B-DXT-Lt) exhibited greater values (101.69±17.31% and 92.92±11.58) than formulations without polymer addition (86.14±9.49% for A-Lt and 86.96±0.56) for B-Lt respectively) although no significant differences were found ($p < 0.05$). Regarding *in vitro* tolerance in HConEpiCs, all formulations had similar values (≈ 94-100%) at 1 hour exposure while longer exposure (4h) showed higher viability values for A-Lt and A-HA-Lt (96.48±5.61% and 96.01±1.89%) than for B-Lt and B-DXT-Lt respectively (83.81±2.14% and 86.52±1.97%)

following all the acceptance criteria (viability values >80%). Conversely, marketed formulation (Monoprost®) showed poor viability values at 1h in cornea and conjunctiva (51.82±8.65% and 50.59±8.21%) respectively and even less at 4 hours of exposure (38.42±4.13% and 24.09±3.64%respectively).

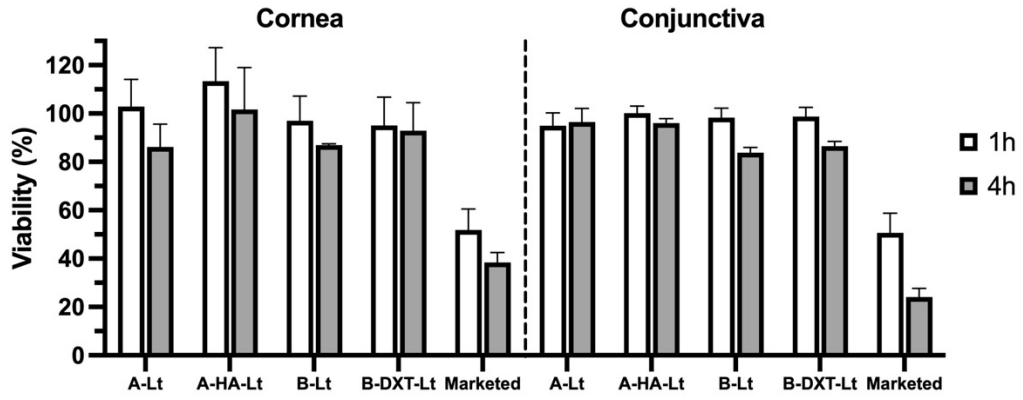

Figure 4. *In vitro* tolerance of osmoprotective hypotensive microemulsions in HCECs and HConEpiCs at different exposure times (1h and 4h) in comparison with the marketed one (Monoprost®).

1.1. Osmoprotective efficacy in cellular model of hyperosmolarity

Osmoprotective efficacy of the hypotensive microemulsions was assayed in a hyperosmolar stress model in HCECs. All formulations exhibited significant osmoprotective activity after 2 hours exposure ($p < 0.001$). Cells in contact to hyperosmolar environment (470 mOsm/L) without previous osmoprotective exposure showed viability values of 19.75±4.91% (positive control) while the viability values are increased to 44-60% (2-3-fold more) when cells were pretreated with the osmoprotective microemulsions. A-Lt showed higher osmoprotective efficacy than A-HA-Lt (58.85±1.41% and 51.24±1.63% respectively) without statistical differences. B-Lt and B-DXT-Lt osmoprotective values were also similar without significant differences (47.10±4.91% and 43.94±3.37% respectively). Furthermore, no differences were found when comparing the different formulations (A-Lt and B-Lt).

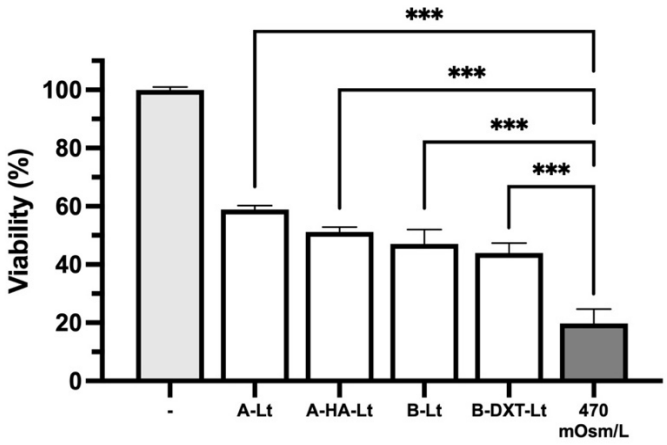

Figure 5. Osmoprotective preventive efficacy of developed microemulsions with latanoprost under a hyperosmolar environment.

3.4. Permeation studies

3.4.1. Microscopy

Non loaded microemulsion and in combination with polymers (HA and dextran) containing coumarin were incubated with HCECs for 5 minutes as previously mentioned and their mean fluorescence was monitored and determined for 11 days. All formulations showed a remarkable peak of intensity at day 0 with a decrease in time up to 11 days when almost all fluorescence has disappeared. BM-HA images (figure 6) showed a fluorescent intensity higher than BM and BM-DXT respectively at day 0 and 2. Besides, cells when exposed to BM-HA showed fluorescence longer than BM and BM-DXT. BM and BM-DXT showed similar fluorescence although BM-DXT seems have slightly more fluorescence than BM. BM images almost showed a completely disappearance in fluorescence at day 9.

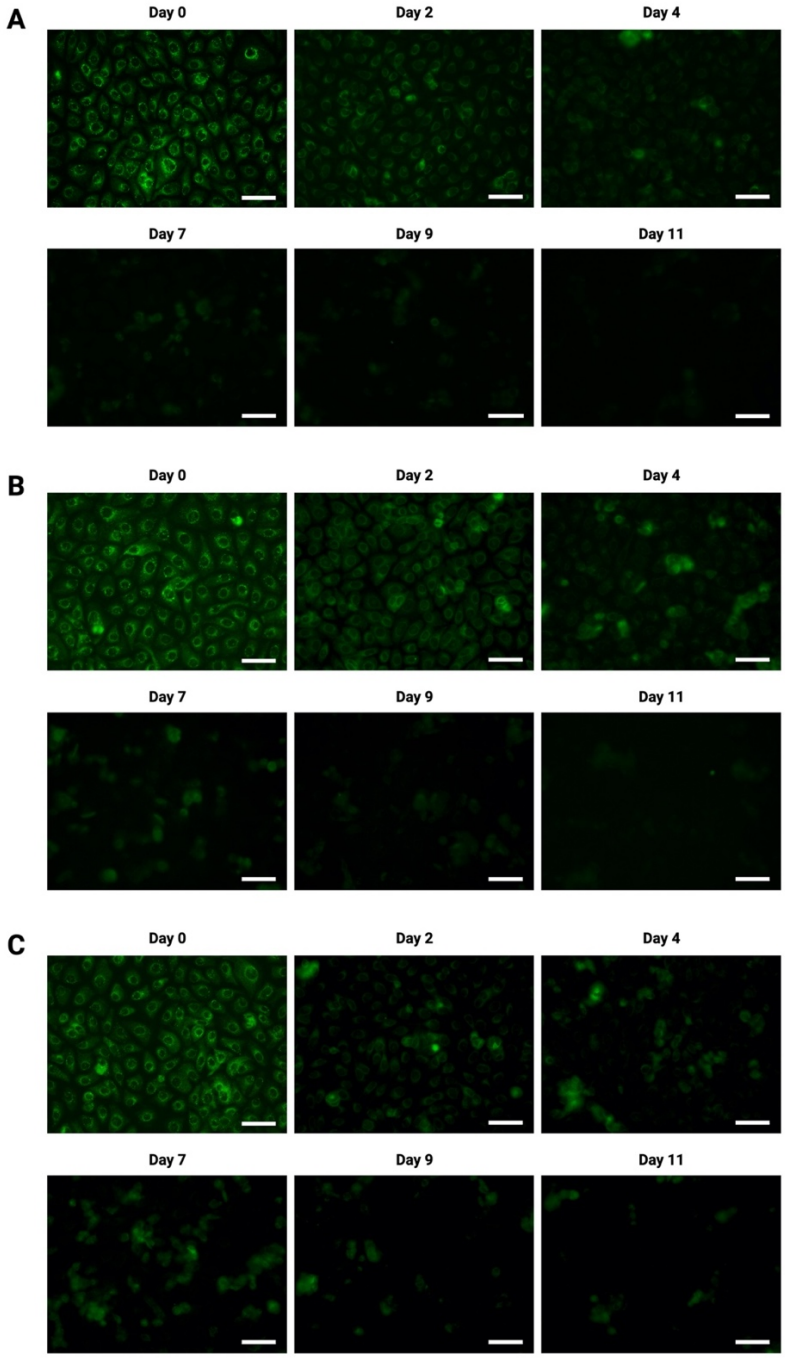

Figure 6. Fluorescence images showing internalization of BM (A), BM-HA (B) and BM-DXT (C) in HCECs for 11 days after 5 min exposure.

Regarding calculated CTCF BM-HA demonstrated remarkable fluorescence peak at day 0 higher (333265.31±32391.85) than BM and BM-DXT respectively (figure 9) ($p < 0.01$). Besides mean fluorescence of BM-HA was significantly higher (51931.83±7531.89) than BM and BM-BM-DXT (7417.51±2640.59 and 26702.10±12403.22 respectively) up to 9

days reaching similar results at the end of the study (day 11). Conversely BM and BM-DXT presented similar values of CTCF in all images with slightly increases at 2, 6 and 9 days without significant differences.

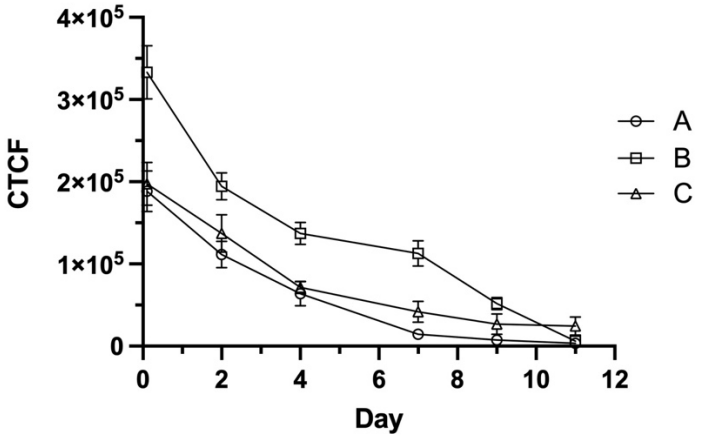

Figure 7. Corrected total cell fluorescence (CTCF) of BM (A), BM-HA (B) and BM-DXT (C) respectively for 11 days.

3.4.2. Flow cytometry

Flow cytometry experiments showed the evolution of fluorescence within 11 days after exposition to blank microemulsions (BM, BM-HA, BM-DXT). Similarly, to the results obtained with CTCF from the images, BM-HA and BM-DXT exhibited higher mean fluorescence at 0, 2, 4 and 7 days than BM as can be seen in figure 8. Besides, maximum peak of fluorescence at day 2 for BM-HA (515.28±12.12) and BM-DXT (356.50±10.85) were higher than the ones obtained for BM (172.94±13.96). Besides, BM-HA and BM-DXT showed maximum peaks at 4 days (220.53±8.47 and 256.25±7.03 respectively) and 7 days (252.09±4.56 and 226.28±8.19 respectively) superior to BM (67.41±4.8 for 4 days and 43.02±3.17 for 7 days). Finally, fluorescence at day 11 was superior in those containing polymers although all of them were almost negative in FL1 signal (10^0).

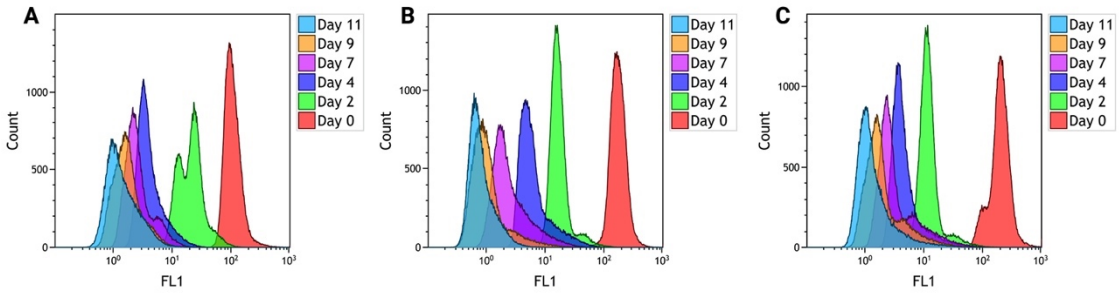

Figure 8. Flow cytometry histograms showing cell count vs fluorescence intensity (FL1) in logarithmic scale at different shown periods of BM (A), BM-HA (B) and BM-DXT (C).

3.5. Cell-Microemulsion interactions

3.5.1. Scanning electron microscopy

Morphology of HCECs exposed to microemulsions was assessed in the SEM as previously described (Figure 9). Cells without any treatment exposed to NaCl 0.9% (control) exhibited normal morphology with few common membrane pores of 100.58±35.24 nm. An increase of pore numbers of different sizes in the cell membranes were observed after exposure to Latanoprost microemulsions (A-Lt, A-HA-Lt, B-Lt, B-DXT-Lt). Those exposed to A-Lt presented an average pore size of 74.40±16.43 nm with regular shapes while A-HA-Lt presented bigger pore sizes with irregular distribution (139.27±13.07 nm). B-Lt showed irregular pattern of membrane pores in some regions with an average size of 104.61±29.01 nm whereas B-DXT-Lt exhibited smaller pore sizes (56.21±3.06 nm) with a conserved distribution.

It is also worth mentioning that in all images microemulsión droplets could be appreciated entering corneal epithelial cells by opening small nanometric pores through the cell membrane which were not present in untreated cells. It was also observed that those containing Polymers (A-HA-Lt and B-DXT-Lt) seemed to be more aggregated in some specific membrane regions than those without mucoadhesive polymers. Moreover, nano droplets were particularly abundant and localized on cell protrusions and pseudopodia in cell surroundings.

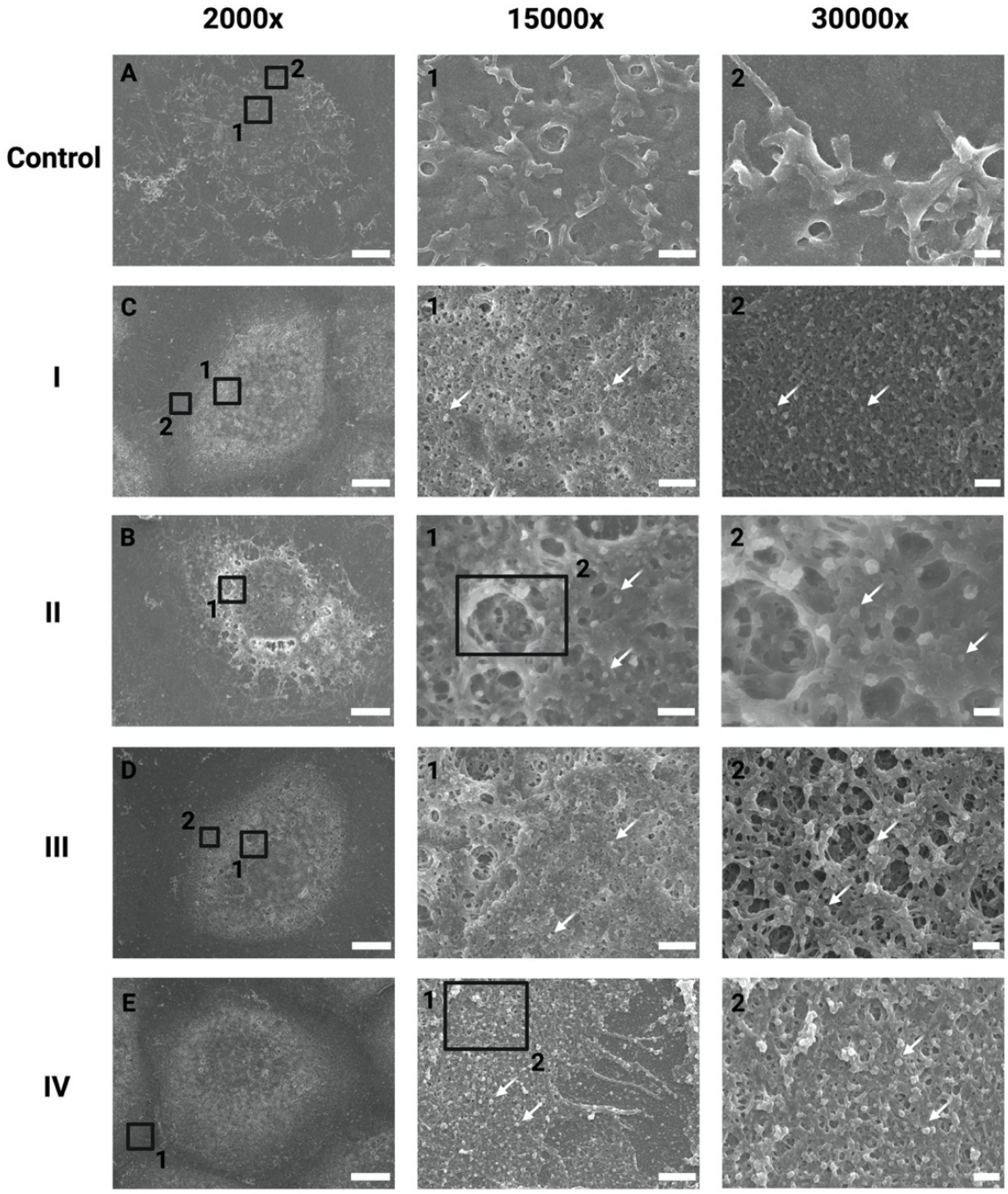

Figure 9. SEM images of HCECs exposed to no treatment (control) and cells exposed for 5 minutes to the developed formulations A-Lt, A-HA-Lt, B-Lt and B-DXT-Lt (I, II, III and IV respectively). Different augments were employed to assess cell microemulsion interactions (x2000: 8μm, x15000: 1μm and x30000: 200nm scale bars).

3.5.2. Transmission electron microscopy

TEM was employed to analyze the interaction between microemulsions and the inner structures of HCECs as well as their cellular distribution in the ultrathin cellular sections (figure 10). Control cells exposed to NaCl 0.9% (untreated) presented a normal appearance showing empty vacuoles and clear fields of the cytoplasm. Regarding microemulsions containing latanoprost, clear lipid deposits associated to microemulsions internalization were appreciated in the darkest tone (black arrows). Most of them were located in vacuoles creating reservoir-like structures.

Formulations without polymers (A-Lt and B-Lt) showed a higher number of oily deposits than those containing polymers (A-HA-Lt and B-DXT-Lt). In addition, individual microemulsion droplets can be seen moving across the cytoplasm and particularly concentrated in the surroundings of vacuoles and inner cellular membranes. These, are particularly intense dark-colored and well defined in the case of A-AH-Lt. Moreover, these nano-droplets (20-30 nm) tend to pass across inner cellular membranes such as the nuclear one. A-Lt and B-Lt can be identified inside nucleus and B-Lt specifically passing through the cellular membrane.

Generally, microemulsions were also localized in mitochondria although in a lesser extent and particularly concentrated close to the marginal side of cellular membrane.

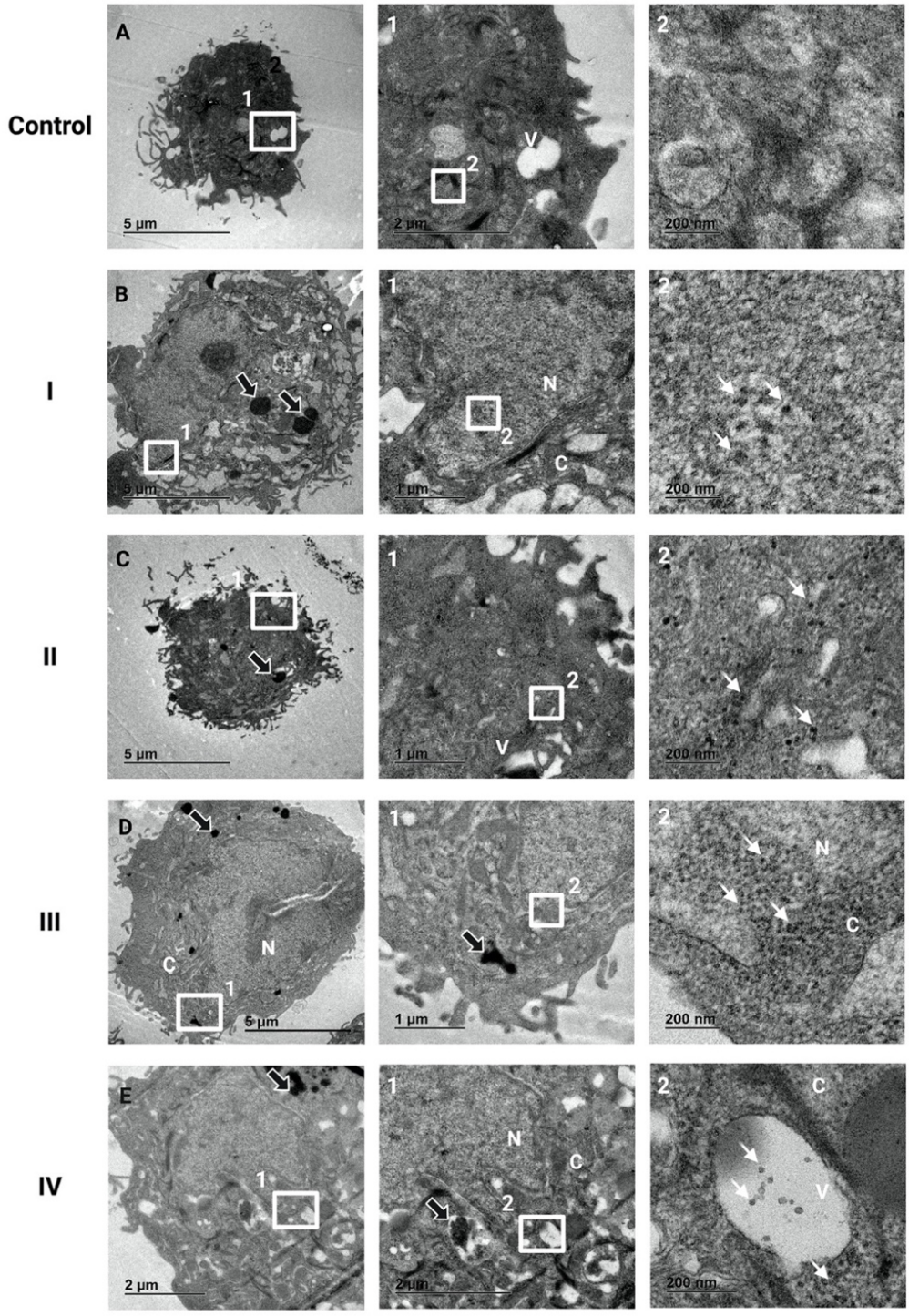

Figure 10. TEM images of HCECs unexposed cells (control) and cells exposed for 5 minutes to the developed formulations A-Lt, AHA-Lt, B-Lt, and BDXT-Lt (I, II, III and IV respectively). Different augments were employed to assess cell microemulsion interactions in different cell structures (C: cytoplasm, V: vacuole and N: nucleus). All formulations were visualized at 8000x and zoomed 30000x and 150000 (1 and 2 respectively) except for IV that was zoomed at 150000x (1 and 2). Black arrows represent lipid deposits while white arrow single microemulsions.

3.6. Hypotensive efficacy

Hypotensive effect of developed microemulsions with latanoprost (A-Lt, A-HA-Lt, B-Lt, B-DXT-Lt) and marketed formulation (Monoprost®) was measured. Non loaded microemulsions were also tested (BM, BM-HA and BM-DXT). PBS (control) was instilled and IOPs monitored to ensure that no hypotensive effect was given. Blank microemulsions showed hypotensive effect for 24h that was increased for the formulations including polymers (BM-HA and BM-DXT) being similar to the effect observed in the marketed formulation (figure 11). While marketed formulation exhibited a ΔIOPmax of 19.70±9.49%, BM-HA and BM-DXT showed values of 18.15±6.20% and 14.53±7.86% respectively. Conversely, BM showed lower ΔIOPmax values (8.27±4.99%) than marketed one although showing some level of hypotensive activity between 3h and 10h ($p < 0.05$). Furthermore BM-HA demonstrated hypotensive activity between 3h and 36h while marketed one (between 1h and 30h).

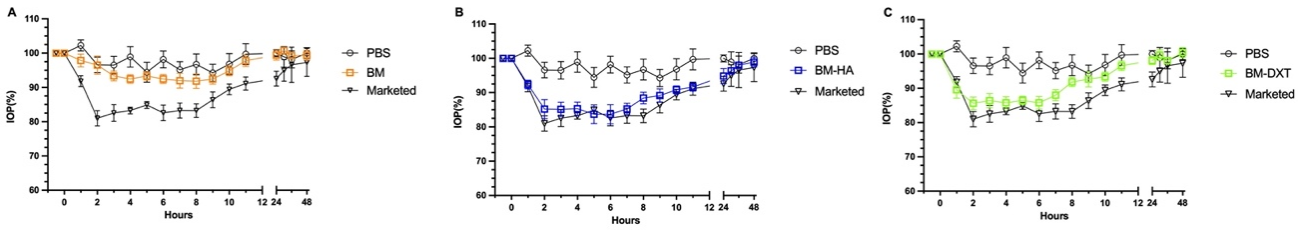

Figure 11. Intrinsic hypotensive effect of model developed microemulsions BM (A), BM-HA (B) and BM-DXT without latanoprost in comparison with the marketed formulation.

Regarding latanoprost microemulsions, hypotensive activity in rabbits was substantially higher in comparison with the marketed one (figure 12). While hypotensive activity for marketed formulation lasted for 24h-30h, A-Lt activity lasted for 82h (3.42 days). Besides, the addition of hyaluronic acid (A-HA-Lt) prolonged its hypotensive activity up to 310h (12.92 days). Moreover B-Lt lasted for 130h (5.42 days) while the addition of dextran lasted for 202h (8.42 days). ΔIOPmax were higher in comparison with the marketed one. One the one hand A-Lt and A-HA-Lt showed ΔIOPmax of 24.24±7.09% and 34.16±5.67% respectively. On the other hand, B-Lt exhibited a ΔIOPmax of 22.55±3.96% while B-DXT-Lt showed an ΔIOPmax of 25.40±4.25%. ΔIOPmax for A-HA-Lt resulted significantly higher in comparison with marketed formulation ($p < 0.05$). Although the rest of formulations were not significantly different in terms of ΔIOPmax in comparison with

the marketed formulations the hypotensive activity, area under the curve and relative biodisponibility was much higher (table 3) and statistically significant.

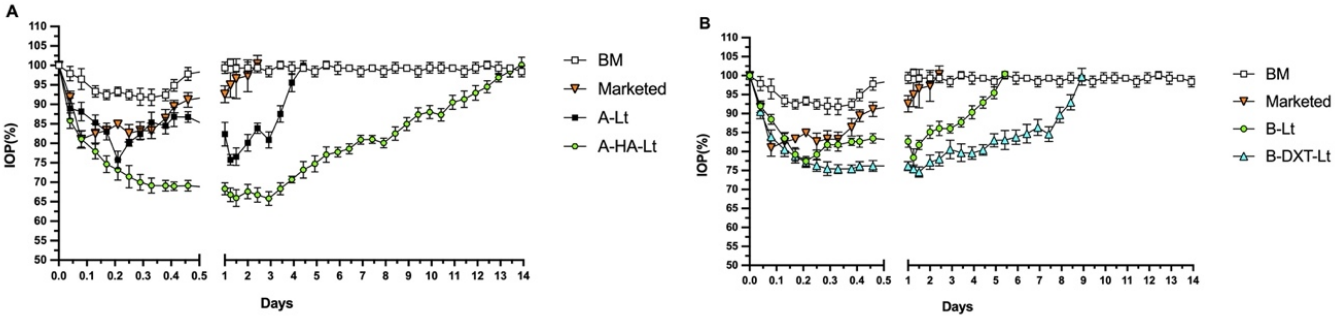

Figure 12. Comparison of hypotensive developed formulations containing latanoprost A-Lt, A-HA-Lt (A), B-Lt, B-DXT-Lt (B) with BM and marketed formulation.

Regarding calculated $AUC_{(0\text{-}t')}$, all the developed formulations with and without latanoprost were compared with the marketed formulation (Monoprost®) (figure 13). Firstly, for those developed microemulsions without latanoprost, BM-HA was similar to the marketed formulation in terms of hypotensive effect ($p > 0.05$). $AUC_{(0\text{-}t')}$ of microemulsions without polymers (A-Lt and B-Lt) were much superior (65.73±12.48 and 73.58±19.36 respectively) than marketed formulation (13.52±4.83) ($p < 0.001$). Generally, all microemulsions containing latanoprost were very significant in comparison with the marketed one ($p < 0.001$). Particularly the addition of HA increased the $AUC_{(0\text{-}t')}$ from 65.73±12.48 (A-Lt) up to 271.50±38.15 (A-HA-Lt) ($p < 0.001$). It is also worth noting that dextran increased $AUC_{(0\text{-}t')}$ from 73.58 ± 19.36 (A-Lt) to 157.40 ± 37.84 (A-DXT-Lt) ($p < 0.01$) as can be seen in table 3.

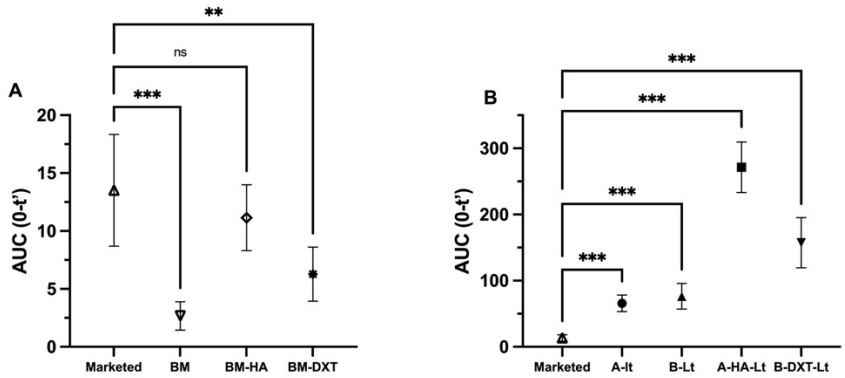

Figure 13. AUC until effectiveness of the formulations (AUC 0-t') without latanoprost (A) and those containing latanoprost in comparison with the marketed formulation (B).

Finally, relative bioavailability (RB) calculated from AUC (0-t') data, demonstrated that the microemulsions of the present study showed superior results in comparison with the marketed formulation employed as reference. A-Lt exhibited an average RB value of 4.52 times higher than the reference while the addition of the mucoadhesive polymer, HA made the difference 18.55 times higher than the control formulation. With regards to formulation B-Lt, RB value showed 5.11 times more than marketed one, increasing to 10.50 times more when dextran was added.

Table 3. Microemulsions developed showing ΔIOPmax, $AUC_{(0\text{-}t')}$ and relative bioavailability (RB) representing confidence intervals.

Formulation	ΔIOP_{max} (%)	$AUC_{(0\text{-}t')}$ (%.h)	RB*
Marketed	19.70 ± 9.49	13.52 ± 4.83	1
A-Lt	24.24 ± 7.09	65.73 ± 12.48	4.52 (4.01 – 5.32)
A-HA-Lt	34.16 ± 5.67	271.50 ± 38.15	18.55 (17.42 – 19.96)
B-Lt	22.55 ± 3.96	73.58 ± 19.36	5.11 (4.55 – 5.97)
B-DXT-Lt	25.40 ± 4.25	157.40 ± 37.84	10.50 (9.62 – 11.67)

* Relative bioavailability is expressed in times superior to reference formulation with 90% significance (p = 0.01).

4. Discussion

The design of novel systems and therapeutical strategies that increase residence time of drugs (both lipophilic and hydrophilic) as well as enhance their permeation through different ocular tissues is a technological challenge [39]. Besides, the tolerance of the eye to formulations is a critical point to be considered. In the case of topical formulations, a relevant number of existing hypotensive formulation for glaucoma treatment include different excipients, mainly preservatives, that affect the ocular surface integrity or produces adverse effects such as ocular surface discomfort, dryness, or hyperemia hampering patient adherence [4]. Also, the active substance could be the responsible to different side effects. Furthermore, a combination of different anti-glaucoma drugs as well as repeated administrations is needed in many cases to achieve a suitable IOP control [40]. All those issues (preservatives, repeated doses, and combination of drugs) bring to

evidence the need of novel therapies to overcome the side effects in glaucoma therapy. Nowadays, therapies are expected to be not only well tolerated but also include protective substances able to preserve the ocular surface integrity against harmful potential effects. For that reason, in the present study we have try to develop topical ophthalmic pharmaceutical nanosystems (microemulsions) including the hypotensive drug (latanoprost) accompanied by osmoprotective substances in combination with mucoadhesive polymers with the objective to extend the ocular contact time protecting, at the same time, the ocular surface. O/A microemulsions containing antiglaucoma drugs have been previously developed by some authors. This is the case of brimonidine tartrate in a microemulsion (90-100 nm nanodroplet size) with high viscosity values [41]. Other authors prepared bimatoprost microemulsions made out of isopropyl myristate and tween 20 (25-30 nm droplet size and neuter z potential) to be included in contact lenses [42].

Self-emulsified ophthalmic microemulsions have been previously developed by our group with osmoprotective properties. They have been able to protect corneal cells from hyperosmolar stress for the treatment of ocular surface diseases that develop with hypertonic stress such as DED. In the present work, latanoprost has been loaded in a osmoprotective microemulsion with nanodroplet sizes around 20 nm which were increased to around 30 nm with the inclusion of hyaluronic acid. All formulations presented neuter pH values (6.5-7.5) and almost isotonic values (278-290 mOsm/L). The low surface tensions obtained in the different formulations guarantee a good extension onto the ocular surface and the addition of mucoadhesive polymers enhance their residence time [43].

In accordance with previous studies, Cryo-TEM and SEM visualization showed that all formulations had very conserved sphere-like morphology [20]. In all formulations, the inclusion of the hypotensive agent and osmoprotective substances did not affect the morphology. Some authors have visualized microemulsions at ordinary TEM showing nanodroplets of 90-100 nm using negative staining [44]. However, for lipid-based nanosystems and in particular nano- and microemulsions this can be problematic since environmental modifications and conditioning of the sample can affect the systems stability. For this reason, one of the most appropriate techniques for their visualization is cryo-TEM [45,46]. We showed that cryo-TEM did not modify sizes since a modification of the environment is not artificially changed, being nanodroplet sizes similar to those analyzed with DLS. Besides, we used a modified protocol of staining for SEM morphology

visualization [32] by combining green malachite, commonly known for its ability to fixate lipids and particularly with glutaraldehyde [47].

We also assessed the ability of the developed microemulsions to entrap the hypotensive prostaglandin latanoprost. All the microemulsions showed yields higher than 90%. We hypothesized that the 10% prostaglandin lost could be due to the small amount (10 μL) added from the ethyl oleate latanoprost solution during the microemulsion preparation. The results are similar to the ones obtained by other authors encapsulating travoprost in a nanoemulsion confirming that the lipophilic substances could be successfully included in these pharmaceutical systems [48].

The osmoprotective microemulsions loaded with latanoprost showed good *in vitro* tolerance in both, corneal and conjunctival epithelial cell cultures with 1h and 4h exposures in the conditions previously established by our group to simulate chronic administration of topical ophthalmic formulations in cell cultures [31]. According to other authors, the poor tolerability obtained in our conditions (close to 50% after 1h and 20-30% after 4h in both corneal and conjunctival cells) for the marketed formulation Monoprost® can be attributed to the high percentage of the hydrogenated castor oil (5%) [49] in combination with the long-term toxicity of latanoprost [50]. In the present work, we have included a combination of osmoprotective substances (leucine/betaine or clusterin/oleanolic acid) in two different types of formulations with and without mucoadhesive polymer addition (hyaluronic acid for A-HA-Lt or dextran for B-DXT-Lt) with the idea to protect the ocular surface in chronic treatments. To test our hypothesis, we study the cell survival of the different formulations in a hypertonic stress cellular model previously published by our group [30]. In this model, the cellular viability decreased to values lower than 20% after exposure to a hyperosmolar solution (470 mOsm/L). Then, to test the protective ability of the osmoprotective microemulsions the cells were previously exposed to the different formulations and then the hyperosmotic stress conditions stablished. In all microemulsions, an increase in cell survival resulted in 25-40% compared with the untreated control under the hypertonic conditions. In fact, the osmoprotective formulations containing latanoprost showed 2-3-fold more cell survival than the positive control (without treatment). Therefore, we hypothesized that microemulsions not only exhibit the ability to increase cell tolerance and decrease toxicity associated to latanoprost but also avoid cell death induced by chronic hypertonic environment with only 2 hours

pre-exposure thanks to the substances included in the formulations. In previous studies by our group, betaine and hyaluronic acid have demonstrated to be protective to cell shrinkage, inflammation and apoptosis induced events by hypertonic environment [30].

In order to confirm the ability of internalization of the microemulsions in corneal cells blank microemulsions (BM, BM-HA and BM-DXT) were loaded with coumarin. HCECs exposed to coumarin-loaded microemulsions at 2 μg/mL for a short time (5 min) demonstrated a high internalization activity. The assessed fluorescence showed that HA containing microemulsions had higher fluorescence retention than BM and BM-DXT overtime and presented residual fluorescence up to 12 days. Interestingly, the time of exposure resulted lower than the one employed by other authors using coumarin-loaded liposomes (20 μg/mL) to assess internalization efficacy of an anti-inflammatory liposomal formulation after 60 min exposure at the fluorescence microscope [51]. The results obtained by fluorescence were confirmed by flow cytometry experiments. The polymers included in the formulations (HA and dextran) seemed to increase retention time. These findings indicate that these polymers entail interesting biomaterials to promote penetration of encapsulated active drugs in microemulsions.

The modified SEM protocol in combination with PFA previously described for lipid visualization [32] was employed to assess the interaction of the osmoprotective microemulsions loaded with latanoprost with the outer part of cell membranes. PFA has been used in SEM studies with macrophages and gold nanoparticles [52]. According to SEM images, an increase of pore numbers in cell membranes were observed after the exposure to hypotensive microemulsions (figure 9). According to other authors this reversible pore formation could be due to the surfactant concentration used to stabilize the nanodroplets. [53]. Nevertheless, it is important to take into account that the small size of nanodroplets probably allows penetration in the cells through natural occurring pores and avoid endosomal formation which increase internalization rate and efficacy. It is important to remark that there are previous studies in which cell distribution has been studied by TEM [54]. However, they were performed without lipid specific fixation. To our knowledge, this is the first study to show cell-microemulsion interaction and in particular in human corneal epithelial cells. In the present work, *in situ* fixation of lipid nanodroplets (20-30 nm) at the moment of internalization allow to visualize the inner structures of the cell. As previously shown, vacuoles and cytoplasm of untreated cells are clear with

structures associated with cellular physiological conditions. Regarding those exposed to osmoprotective microemulsions containing latanoprost, intense lipid fixation inside vacuoles is present which can be explained by microemulsions accumulation allowing their release overtime. Moreover, fixed individual nanodroplets are visualized along the different cellular structures (vacuoles, nucleus, cytoplasm, endoplasmic reticulum, mitochondria, and cell membrane) although they are particularly concentrated on the surrounding of vacuoles.

All these findings support the high hypotensive effect assessed *in vivo*. Surprisingly, blank microemulsions exhibited hypotensive activity and even the one with HA presented the same pattern than the marketed formulation ($p > 0.05$). We contemplate the possibility of an intrinsic hypotensive effect of the developed formulations due the high amount of lipidic compounds with unsaturated fatty acids present on the formulation (ethyl oleate, soybean oil, soy phosphatidylcholine and squalene). In fact, Bellenger-Germain et al. showed already the antihypertensive activity of rich fatty acid in hypertensive rats [55]. Also, Nowacki et al. established a direct relation between unsaturated fatty acids (specially lecithin derived) and the regulation of high-conductance Ca^{2+} activated potassium channels (BK) of vascular smooth muscles [58]. Furthermore, other studies assess the importance the presence of smooth muscles in the trabecular meshwork [59] and their regulation in aqueous blood flow pathways [60]. In addition, Cuppoletti et al. showed the ability of the prostaglandin analogue unoprostone to activate BK channels present in the Schlemm's canal involved in volume regulation which contributes to lowering IOP and increasing aqueous humour outflow [61]. We speculate that due to the high and fast internalization rate of the developed microemulsions, they are able to reach the trabecular meshwork and according to the above-mentioned mechanisms in the literature, activate BK channels thus lowering the IOP. Therefore, this could be the first study to describe a hypotensive effect produced by unsaturated fatty acids in the eye. No effects other than osmoprotection and possible IOP decrease were described for the rest of the raw materials of the formulation. In any case, further studies are necessary to explain this intrinsic effect. Microemulsions loaded with Latanoprost (A-Lt and B-Lt) resulted effective after one single instillation up to 4 and 6 days respectively with slightly higher values of ΔIOPmax (%). The hypotensive effect observed resulted longer than the one described by other authors. Xu et al. described a reduction for 72h and 96h in New Zealand rabbits with a bimatoprost-loaded

microemulsion in contact lenses with 50μg/mL and 75μg/mL latanoprost concentrations respectively [42]. Moreover, according to the $AUC_{(0\text{-}t')}$, A-Lt and B-Lt were significantly higher in comparison with the commercial formulation. Besides, calculated relative bioavailability was 4.52 (4.01 – 5.32) and 5.11 (4.55 – 5.97) times more for A-Lt and B-Lt respectively than the marketed formulation.

After the addition of one single drop (25 μL) in each eye with A-HA-Lt, hypotensive effect lasted for almost 13-14 days, exhibiting a high increase in the hypotensive activity (34.16±5.67% ΔIOPmax). A-HA-Lt showed superior values of RB (18.55 times more; CI90%: 17.42 – 19.96) than the marketed one. Dextran did also show long hypotensive values (lasting for 8-9 days) and exhibiting RB of 10.50 (9.62 – 11.67) times higher in comparison with the reference. This in the line of previous studies that developed prostaglandin nanoemulsions with multiple dose administration for the treatment of glaucoma [62].

It is important to highlight that currently there is not any hypotensive formulation present in the market that combine ocular surface protection and long-lasting action as the one presented in the present work. In this sense, all these novel findings suggest that the combination of the studied osmoprotective agents in this novel microemulsion technology loaded with latanoprost could entail a new and ground-breaking tool in the treatment of glaucoma. It has been demonstrated that the addition of mucoadhesive polymers enhance the benefits of the formulation. All these factors (tolerability, suitable physicochemical properties, osmoprotection, internalization capacity and long-lasting hypotensive effects) would increase patient adherence, improving effectiveness and quality of life by avoiding excessive repeatedly dose administrations as well as drug combinations eventually leading to ocular adverse effects (DED, hyperaemia, discomfort, and poor adherence). The developed microemulsion could be employed for future ocular therapies increasing the residence time and permeation of therapeutic agents (both hydrophilic and lipophilic) for long periods of time to the desired tissues. However, a model of ocular hypertension as well as pharmacokinetic studies would be interesting to confirm this promising results.

5. Conclusions

The present work studied the development of a new self-emulsifying microemulsion system displaying high cell permeability and distribution, good tolerance, and suitable physicochemical properties for topical ophthalmic administration. The addition of combined osmoprotective substances in a prostaglandin-loaded microemulsion entails a novel strategy for the treatment of glaucoma and management of ocular surface diseases associated to chronic hypotensive therapies. Besides, developed microemulsions with mucoadhesive polymers provide a remarkable long-term activity after one single instillation. All developed microemulsions seem to outperform previously developed formulations for glaucoma treatment. Nevertheless, further efficacy and kinetic studies are needed to confirm the benefits of this novel therapeutic tool.

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7. Conflict of Interest

The authors declare no conflict of interest in any subjects treated in the present work.

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CAPITULO V. THERMO-RESPONSIVE PLGA-PEG-PLGA HYDROGELS AS NOVEL INJECTABLE PLATFORMS FOR NEUROPROTECTIVE COMBINED THERAPIES IN THE TREATMENT OF RETINAL DEGENERATIVE DISEASES.

CAPITULO V

CAPITULO V

Thermo-responsive PLGA-PEG-PLGA hydrogels as novel injectable platforms for neuroprotective combined therapies in the treatment of retinal degenerative diseases

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CAPITULO V

CAPITULO V

ABSTRACT

The present study aims to develop a thermoresponsive-injectable hydrogel (HyG) based on PLGA-PEG-PLGA to deliver neuroprotective agents to the retina overtime. Two PLGA-PEG PLGA copolymers with different PEG:LA:GA ratios (1:1.54:23.1 and 1:2.25:22.5) for HyG-1 and HyG-2 development respectively were synthetized and characterized by different techniques (GPC, NMR, DLS, CMC, gelation and rheological behaviour). According to the physicochemical characterization, HyG-1 was selected for further studies and loaded with anti-inflammatory drugs: dexamethasone (0.2%), ketorolac (0.5%) alone or in combination with the antioxidants idebenone (1µM) and D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS) (0.002%). *In vitro* drug release and cytotoxicity studies were performed for the active substances and hydrogels (loaded and drug-free). A cellular model based on oxidative stress was optimized for anti-inflammatory and antioxidant screening of the formulations by using retinal-pigmented epithelial cell line hTERT (RPE-1). The copolymer 1, used to prepare thermoresponsive HyG-1, showed low polydispersity (PDI=1.22) and a strong gel behaviour at 25% (w/v) in an isotonic buffer solution close to the vitreous temperature (31-34°C). Sustained release of dexamethasone and ketorolac was achieved between 47 and 62 days, depending on the composition. HyG-1 resulted well tolerated (84.5±3.2%) in retinal cells, with values near 100% when the anti-inflammatory and antioxidant agents were included. The combination of idebenone and dexamethasone, promoted high oxidative protection in the cells exposed to H_2O_2, with viability values of 86.2±14.7%. Ketorolac and dexamethasone-based formulations ameliorated the production of TNF-α showing significant results ($p \leq 0.0001$). The hydrogels developed in the present study entail a novel biodegradable tool to treat neurodegenerative processes of the retina overtime.

Keywords: PLGA-PEG-PLGA, thermoresponsive hydrogel, micelles, neurodegenerative diseases, intravitreal drug delivery, oxidative stress, inflammation, ketorolac and dexamethasone

CAPITULO V

CAPITULO V

1. Introduction

Retinal diseases comprise one of the global leading causes of visual loss in the world. The proportion of the total visual impairment and blindness caused from age related macular degeneration (AMD), glaucoma and diabetic retinopathy (DR) involve neurodegenerative events and are currently greater than from infective causes [1]. AMD is the leading cause of irreversible vision loss in the world [2]. Although the pathophysiology is still on research, several studies have focused on the retinal pigmented epithelium (RPE)/photoreceptor/Bruch's membrane complex [3][4]. RPE is of vital importance in maintaining visual function of the photoreceptors present in the retina by supporting the metabolism and energetic requirements of photosensitive cells [3]. AMD can be subclassified into early, intermediate and late AMD. The main differences between them are the damage and progression in the fundus structures including neovascularization, atrophy and retinal degeneration [5]. If the disease progress to late AMD, neovascularization can be present of absent receiving named as wet or exudative AMD and dry or atrophic AMD. While wet AMD rapidly results in untreatable blindness, dry AMD is considered more like a chronic disease, that slowly evolves into irreversible vision loss [6] [7]. Oxidative stress has also been related to AMD and its progression, being hypothesized that undesired metabolic debris that have not been properly eliminated get oxidized promoting inflammation and cellular damage [8].

Glaucoma is another debilitating disease related to neurodegeneration. Among all the types of glaucoma, primary open angle (POAG) and closed angle (PACG) glaucoma are the most common [9]. Intraocular pressure (IOP) is the main risk factor of glaucoma, and it is linked to the compaction of axonal dendrites and surrounding structures, leading to morphological and irreversible changes in the lamina cribosa [10]. These alterations together with the interruption of flow and nutrients to retinal ganglion cells cause cell death, apoptosis and loose of function. These alterations trigger an excessive production of reactive oxygen species (ROS), interacting with cell structures and up-regulating cell death [11]. There are others degenerative ocular diseases that inevitably cause visual disfunction, like diabetic retinopathy (DR) and retinitis pigmentosa (RP). DR is widely recognized as one of the main complications of diabetes mellitus and is classified into non-proliferative, as the early stage, and proliferative, as the advanced stage of DR. In diabetes mellitus, the

extended time of hyperglycemia promotes retinal blood vessels dilation and micro-vascularization which has been associated to pericytes apoptosis and inflammation mechanisms [12] [13]. Besides, massive ROS are produced, worsening inflammation and progression of DR [12]. Retinitis pigmentosa (RP) is also a major ocular debilitating disease that cause the loss of photoreceptors. RP pathophysiology has been widely studied and it seems that there is not a single specific mechanism that may triggers the disease. Despite its complexity, RP has been linked to genetic mutations from dominant, recessive to alterations in mitochondrial genetic information. As some of the diseases mentioned above, in RP, patients experiment RPE degeneration progressing to cell death. Some of the genes affected in RP directly cause a dysregulation in cell apoptosis, inflammation mechanisms and cytoprotection to light-induced damage [14]. Furthermore, oxidative stress and ROS production are one of the worsening effects of RP by triggering inflammation and apoptosis cascades of endothelial, RPE cells and ultimately photoreceptors, leading to vision loss [15].

There are some therapies that have been developed in order to slow or revert blindness progression in these neurodegenerative diseases of the retina. Corticosteroids and particularly dexamethasone have been investigated, especially in combined therapies for its potent anti-inflammatory effects as well as their ability to stabilize the blood-retinal barrier and reduce exudative processes [16]. Furthermore, some non-steroidal anti-inflammatory drugs (NSAID), such as ketorolac or bromfenac have been also used alone [17] or in combination with anti-VEGF therapies, in order to reduce retinal thickness caused by macular edema, counteract the inflammation processes given in degenerative processes and avoid the inflammation caused by the intravitreal injection itself [18].

D-alpha-tocopherol polyethylene glycol succinate (TPGS) has been included in various drug delivery systems to enhance bioavailability and drug efficacy [19]. This vitamin-derivative possess the antioxidant properties of the vitamin E and can be solubilized in an aqueous media [20]. Q10 coenzyme has also been studied for its capacity to protect retinal ganglion cells against damage and oxidative stress [21]. Recently, the partially soluble analogue of Q10 coenzyme known as idebenone has gained much interest as a neuroprotective therapy for Alzheimer, Parkinson and retinal degeneration. Idebenone has been described to possess a strong antioxidant activity that is currently being deeply

studied and could entail an interesting option in a combined therapy for neuroprotection in retinal degenerative diseases [22].

The intravitreal administration is used to deliver drug therapies to the posterior segment of the eye [23]. However, difficulties related to the drug such as short residence time and high clearance, as well as the inflammation associated to frequently repeated injections and risk of visual impairment, are commonly some of the drawbacks of this kind of therapy. To overcome these limitations, investigations are directed towards the design of intraocular drug delivery systems that are able to resolve some of the above-mentioned difficulties. This is the case of PLGA-PEG-PLGA triblock copolymers which constitutes a family of amphiphilic water-soluble polymers made out of poly- (DL-lactic acid co- glycolic acid) (PLGA) and polyethylene glycol (PEG) monomer units [24]. In aqueous solution, PLGA-PEG-PLGA copolymers are able to create micelle-like structures with a high hydrophobic core (PLGA) and a surrounding corona-like structure made of PEG tails [25]. Although, the features can be specifically designed, normally at room temperature, the micelles are well separated and distributed conferring the solution a sol state. However, at higher temperatures, micelles increase their size and start to aggregate entering the gel state thus creating the thermo-responsive hydrogel response. It has been suggested that at higher temperatures a micelle destruction occurs due to PEG chains dehydration as well as polymer precipitation [26]. PLGA-PEG-PLGA copolymers could be a useful delivery platform to increase the residence time of active substances in the posterior segment of the eye, allowing the design of therapies avoiding frequent administrations [27]. In the present study we assess the potential of a PLGA-PEG-PLGA triblock based hydrogel in a buffer isotonic solution for intravitreal injection. The hydrogel aims to deliver dexamethasone phosphate and ketorolac tris salt in combination with idebenone or TPGS to the posterior segment of the eye, as a novel neuroprotective therapy for retinal degenerative diseases.

2. Materials and Methods

2.1. Materials

Polyethylene glycol (PEG 1500), stannous 2-ethylhexanoate, dexamethasone phosphate, idebenone and D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS) and ketorolac tris salt were purchased from Sigma-Aldrich (Leinster, Ireland). DL-Lactide and glycolide were purchased from Corbion® (Gorinchem, The Netherlands). The dialysis membranes

(Spectra-Por® Float-A-Lyzer® G2, 5 mL, MWCO 3.5-5 KDa) were from Sigma-Aldrich (Madrid, Spain). DMEM/F12, Hank's Balanced Salt Solution, H_2O_2 50% and MTT were purchased from Sigma-Aldrich (Leinster, Ireland). FBS, L-glutamine and sodium bicarbonate were purchased from Invitrogen (Dublin, Ireland). TNF and IL1b kit for inflammation studies were purchased from Invitrogen (Dublin, Ireland).

2.2. Synthesis of the PLGA-PEG-PLGA triblock copolymers

Two different PLGA-PEG-PLGA [lactic acid (LA): glycolic acid (GA): polyethylene glycol (PEG)] triblock copolymers were synthesized according to the ring opening polymerization (ROP) method described previously by Yu L *et al* [12] and Zentner *et al* [13], with some modifications. In order to select the method with the most desirable behaviour for intravitreal injection, two different ratios of PEG:LA:GA were selected (1:1.54:23.1 and 1:2.25:22.5), establishing PEG 1500 as the initiator.

For the preparation of Copolymer 1, 15 g of PEG 1500 (0.01 mol) were dried in a two-necked flask under vacuum and stirred at 100°C overnight. Under the protection of argon, 1.8 g of GA (0.015 mol) and 33.3 g of LA (0.231 mol) (GA:LA 1:15) were added to the mixture and heated with stirring at 130°C for 1h under reduced pressure. After all the monomers melted, 0.070 g of stannous 2 ethyl hexanoate (0.2% w/w of the monomers) were added, and the reaction mixture was further heated at 150°C for 8 h under argon atmosphere. Unreacted monomers were removed under vacuum for 60 min. Crude copolymer was dissolved in ice-cold water (5-8°C). After complete dissolution, the copolymer solution was precipitated at 80°C and water-soluble low molecular weight copolymer and unreacted monomers were eliminated by decanting the supernatant. Once the supernatant was decanted, crude copolymer was redissolved in ice-cold water. To obtain the final purified copolymer, the same process of heating, precipitation and decantation was repeated three times. Residual water was removed by freeze-drying, and the copolymer was stored at -30°C. Copolymer 2 (GA:LA 1:10) was synthesized following the same procedure described above.

The chemical structure of the PLGA-PEG-PLGA triblock was determined by ^{1}H-NMR. Spectra were recorded at 400 MHz on a Bruker Ascend spectrometer at 25°C in deuterated chloroform ($CDCl_3$). The weight average molecular weight was determined by gel permeation chromatography (GPC) using an Agilent 1260 series with a refractive index

detector, a viscometer detector, a dual-angle light-scattering detector (LS 15° and LS 90°) and two series of Polargel-M (7.5 × 300 mm^2) columns. The analysis was performed at 35°C using dimethyl formamide as the eluent with a flow rate of 1 mL/min.

2.3. Preparation of the combined therapy formulations

Ophthalmic formulations based on HyG-1 and HyG-2 developed with copolymer 1 and 2, respectively, were prepared by dissolving the corresponding copolymer (25% w/v) in an optimized bicarbonate buffer composed of $NaHCO_3$ 0.095M, Na_2CO_3 0.005M (buffer pH = 8.81). After addition of the corresponding active compounds, trehalose was also included at 3.25% or 2.33% in the preparation of dexamethasone or ketorolac formulations, in order to adjust the final pH (≈ 7-7.4) and isotonicity (≈300 mOsm/L). Furthermore, idebenone and TPGS were also combined with dexamethasone or ketorolac as described in table 1.

Table 1. Composition of formulations containing the combined therapy

Formulation	Polymer concentration	GA:LA ratio	Dexamethasone	Ketorolac	Idebenone	TPGS
A	25%	1:15	0.2%	-	-	-
B			0.2%	-	1μM	-
C			0.2%	-	-	0.002%
D			-	0.5%	-	-
E			-	0.5%	1μM	-
F			-	0.5%	-	0.002%

2.4. Characterization of polymers and final formulations

To study the thermo-responsive behaviour of the hydrogel in the different aqueous media, different polymer solutions at given concentrations of 15%, 20% and 25% (w/v) were prepared in several vehicles such as distilled water, PBS, Hanks, NaCl 0.9% and the above-mentioned isotonic bicarbonate buffer including trehalose. The sol (flow) – gel (nonflow) transition of the copolymers in the different aqueous vehicles was determined

using the inverting test method in a 10 mL vial by increasing the temperature 1°C every minute from a starting point of 25°C to 40°C.

The viscosity of PLGA-PEG-PLGA hydrogels (HyG-1 and HyG-2) prepared in water and the other isotonic solutions were studied using a Discovery HR-hybrid Rheometer (New Castle, USA) with a parallel plate system (8- or 20-mm diameter). To this, shear rates increasing from 0 to 1000 s^{-1} in 20 steps were used. Viscosity was measured when the steady state was reached. The determination was performed within the temperature range from 25°C to 45°C at a heating rate of 0.6°C/min at 10 rad/s. The rheology (G' module vs time) of final formulations (Table 1) were studied under the same conditions.

The critical micelle concentration (CMC) in the bicarbonate buffer/trehalose was measured with a tensiometer (K-11, Kruss) by using the Wilhelmy plate method. Before sampling analysis, the tensiometer was calibrated with MilliQ water (72.0 ± 1 mN/m) and the single bicarbonate buffer was measured (67.3 ± 0.5 mN/m). In order to obtain the desired curve, thirteen different concentrations of the hydrogels were used ranging from 0.010 mg/mL to 5 mg/mL. The equilibration time was set to 3 minutes and every concentration was measured by triplicate. In addition, the average size and size distribution of the micelles were determined using a laser scattering spectrophotometer (Autosizer 4700, Malvern), with a vertically polarized incident beam at 532 nm supplied by an argon ion laser. The scattering angle of measurements was set at 90°C.

2.5.*In vitro* release studies

In order to evaluate the drug release profile of HyG-1 based formulations, *in vitro* studies were conducted as depicted in figure 1. Firstly, 5 mL of each formulation described in section 2.3 were introduced in a pre-activated dialysis membrane (Spectra-Por® Float-A-Lyzer® G2, 5 mL, MWCO 3.5-5 KDa) and put in 50 mL closed falcon tubes filled with 40 mL of PBS at 37°C. The tubes were introduced in a water bath at the same temperature. The release media was extracted and replaced with pre-warm (37°C) fresh media at preset times maintaining *sink* conditions. During the first day, intervals of 1h, 4h, 8h 12h and 24h were used. In the next 10 days, the media was replaced every 24 h, then twice a week until the end of study. Each formulation was developed and assessed in triplicate.

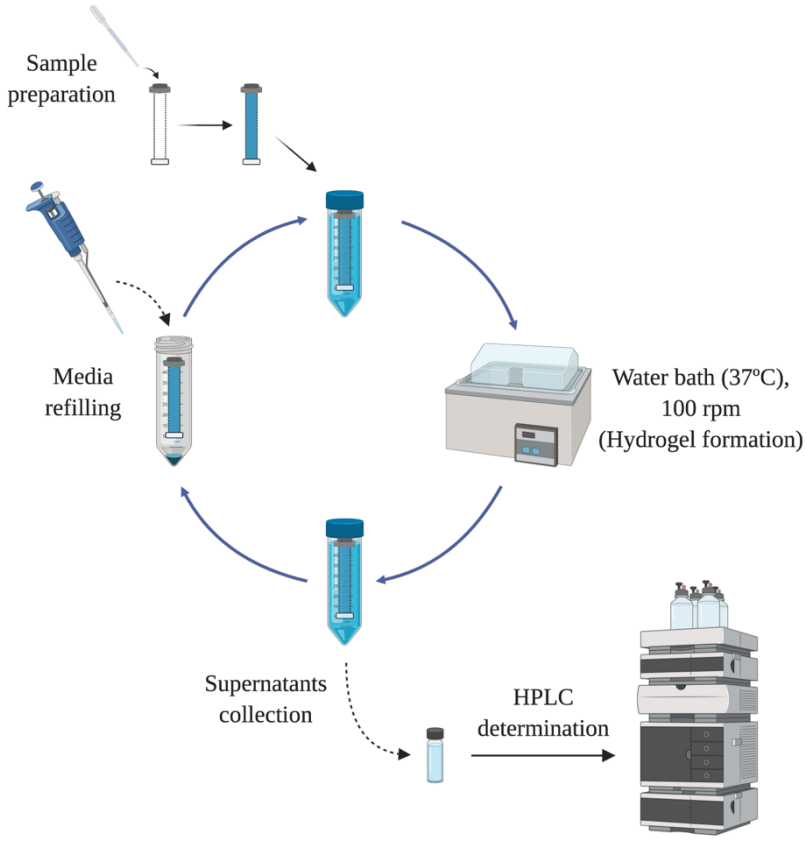

Figure 1. Design of the *in vitro* release experiments of dexamethasone and ketorolac.

Drug release of dexamethasone phosphate and ketorolac tris salt was quantified by HPLC as previously described by H. AlAani *et al* with some modifications [28]. The study was performed with a Waters Alliance 2695 separation module equipped with Waters photodiode array 2996 (Barcelona, Spain). Dexamethasone was determined at a wavelength of 240.5 nm and ketorolac at 317 nm. For both determinations, the mobile phase was composed of 0.05 M potassium dihydrogen phosphate in water (adjusted to pH 4 with formic acid) and acetonitrile (70:30) at a flow rate of 1 mL/min. The column used for the chromatographic separation was a Hypersil silica column (250 × 4 mm 5 µm particle size) and Empower3 software (Waters, Barcelona, Spain) was employed for data acquisition and processing.

The release kinetics of dexamethasone and ketorolac from the respective hydrogel formulations were analyzed according to Korsmeyer-Peppas model (Eq. 1). This semi-empirical model offers information about the mechanism involved in drug release. Although it was initially proposed for describing release behavior from solid monolithic systems such as tablets, it has been widely used later to successfully describe the drug

release mechanism from other drug delivery systems more sophisticated such as microparticles [29], liposomes [30] or semisolid systems such as hydrogels [31] [32].

$$\frac{M_t}{M_\infty} = K \cdot t^n \qquad \text{(Eq. 1)}$$

Where t is the release time, M_t is the amount of drug delivered at time t, M_∞ is the total amount of drug delivered, K is a kinetic constant, and n the diffusional exponent that indicates the drug release mechanism. M_t / M_∞ values lower than 0.6 were used for fitting, and *sink* conditions were maintained all over the dissolution assay. According to authors, the value of n equals 0.5 describes a Fickian diffusion of the drug through the polymeric matrix (Case I), values between 0.5–1.0 describes an "anomalous (non-Fickian) transport", in which not only diffusion but other mechanisms (i.e., a mixed diffusion and chain relaxation mechanisms) are involved in the control of the drug release. When n takes a value of 1.0 means a Case II transport, describing a predominant influence of polymer relaxation on the guest molecules movement within the matrix [33]. These values are valid for slab geometry. In fact, they have been modified, for example in the case of cylinders or spherical systems [34]. However, for hydrogels evaluation the initial values are commonly assumed [31] [32] and they will be followed also in the present study.

The similarity factor (f_2) (Eq. 2) was chosen to compare the dissolution profiles. It is a logarithmic transformation of the sum-squared error of differences between the test and reference products over all time points.

$$f_2 = 50 \cdot \log\left[\frac{1}{\sqrt{1+\frac{\sum_{t=1}^{t=n}(R_t - T_t)^2}{n}}} \cdot 100\right] \qquad \text{(Eq.2)}$$

Where n is the number of experimental points in the *in vitro* dissolution assay, R_t and T_t are the mean percentages of dissolved drug from the reference and test formulations, respectively, at each time point t. No more than one sampling time point after 85% dissolution was considered. When values of f_2 are between 50 and 100, it can be ensured that the difference between the two release profiles under study is lower than 10% [35].

2.6. Cell cultures

A retinal-pigmented epithelial cell line hTERT (RPE-1) kindly donated by the UCD Conway Institute was used to evaluate cytotoxicity of the formulations, screen anti-inflammatory properties and develop an oxidative stress model. Cells were cultured at 37 °C (5% CO_2) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with FBS 10%, L-Glutamine 1% and 3.5% of sodium bicarbonate 7.5%. RPE-1 cells were split at 80% of confluence, 1–2 times per week and the number of passages at the moment of the experiments was 10.

2.7. Cell viability

In vitro cytotoxicity was assessed using mitochondrial-dependent reduction of the tetrazolium salt, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Cytotoxicity studies were performed with different solutions of ketorolac, dexamethasone, HyG-1 and HyG-2, as well as the final developed formulations. Briefly, 50 μL of the tested materials were added to each well (96 well plates) and incubated at 37°C 5% CO_2 for 1 hour to ensure proper gelation. Then, in order to evaluate toxicity by contact, cells were seeded at a final number of 7.000 cells/well, on top of each polymer/formulation. As positive control, a solution of DMSO 15% was used. Cells were grown for 24 hours and the supernatant was removed cautiously in order to avoid the aspiration of the polymers in gel state. Finally, the wells were filled with a mixture of MTT 5 mg/L and DMEM/F12 with FBS 1%, L-Glutamine 1% and 3.5% of sodium bicarbonate 7.5% in a 1:6 ratio (final concentration of MTT = 0.83 mg/mL). After the plates were incubated overnight, DMSO was added to each well in order to solubilize the formazan crystals. The plate was measured at 550 nm in the spectrophotometer.

2.8. Protective activity to oxidation in cell cultures

The protective oxidation activity of final formulations prepared with HyG-1 was performed via MTT assay in cells exposed to H_2O_2. In order to optimize the H_2O_2 concentration to be used as oxidative stress positive control, different solutions were prepared from a H_2O_2 50% concentrated standard (200, 150, 100, 50, and 25 μM).

To evaluate the protective antioxidant activity of the hydrogel-based formulations, each well was filled with 50 μL of the formulations and incubated at 37°C (5% CO_2) for 1 hour to ensure proper gelation. Then, cells were seeded (7000 cells/well) and incubated overnight. Briefly, the supernatants were discarded, and each well was filled with a mixture of H_2O_2 in NaCl 0.9% (100 μL) and supplemented DMEM/F12 (100 μL) (1:1 ratio) to achieve a final concentration of 150 μM H_2O_2. After that, the cells were incubated for 24 hours. The supernatants were removed and the MTT was added following an overnight incubation. Finally, DMSO was added, and the plates were measured at 550 nm as previously described.

2.9. *In vitro* anti-inflammatory activity in response to LPS stimulation

Anti-inflammatory activity was evaluated in RPE-1 cells by human ELISA kit anti TNF alpha. Insert transwells (0.4 μm pore size) were filled with 300 μL of each hydrogel formulation in a 24 well plate and $2{,}5x10^5$ cells were seeded on the bottom of each well. Plates were incubated for 24 hours, and the media was then removed. Then 5 μg/mL of LPS in supplemented DMEM/D12 with FBS 1% were added to each well and the cells were incubated for another 24 hours. Supernatants were centrifuged at 850 xg and measured by ELISA anti TNF alpha as stated in the protocol of the commercial kit.

2.10. Statistics

The experiments were performed in triplicate (n=3). Data are represented as mean ± standard deviation (SD). One-way ANOVA with Dunnett's test was used to determine significance ($p \leq 0.05$, significant; $p \leq 0.01$, very significant or $p \leq 0.001$, highly significant) using GraphPad software Inc. Prism Version 9, US.

3. Results

3.1. Synthesis and characterization of crude copolymers

The molecular weight of the PLGA-PEG-PLGA copolymer was selected based on the needs of the ophthalmic application, in order to achieve a proper and quick gelation of the polymer solution at intravitreal temperature (31-34°C) [26]. The main structure of the PLGA-PEG-PLGA triblock copolymers 1 and 2 was confirmed by hydrogen nuclear magnetic resonance (^{1}H-NMR) (Figure 2). ^{1}H-NMR results showed that experimental

ratios of copolymer 1 were closer to the theoretical and more homogeneous than those of copolymer 2. Moreover, chemical shifts from PLGA-PEG-PLGA were identified showing the typical structure of triblock copolymers. Briefly, CH groups of lactic acid (LA) were found at 5.20 ppm (a), CH_2 of glycolic acid (GA) at 4.75 ppm (b), CH_2 pertaining both signals to PEG at 4.30 ppm (c) and 3.65 ppm (d) and CH_3 of LA at 1.55 ppm (e). Experimental and theoretical ratios of both copolymers are calculated in table 2 according to other authors [36].

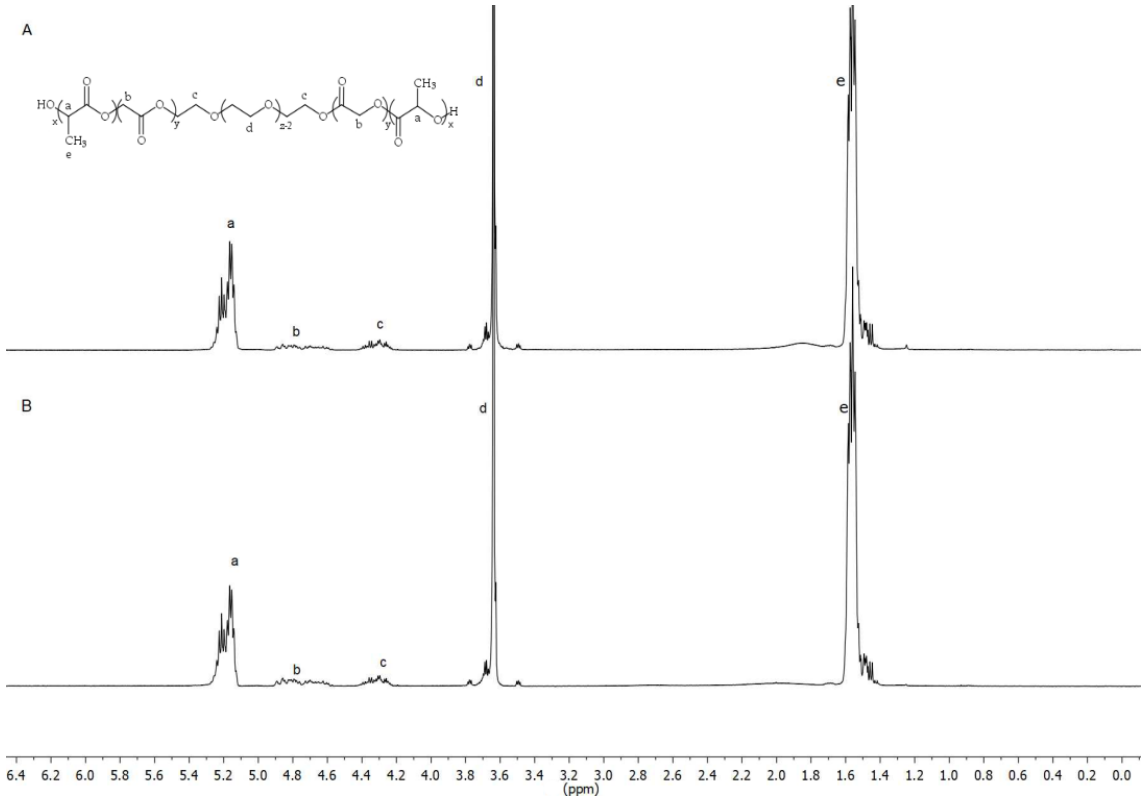

Figure 2. NMR spectrum of copolymer 1 and 2 (A and B) showing their chemical structure.

The GPC results for copolymer 1 and 2 showed that the polydispersity index (PDI) values were between 1.22 and 1.36 respectively, resulting in unimodal molecular weight distributions. Number average molecular weight, weight average molecular weight and PDI are shown in table 2. Furthermore, Figure 3 shows the molecular weight distribution for both copolymers (1 and 2). As it can be appreciated, the distribution of areas appears unimodal.

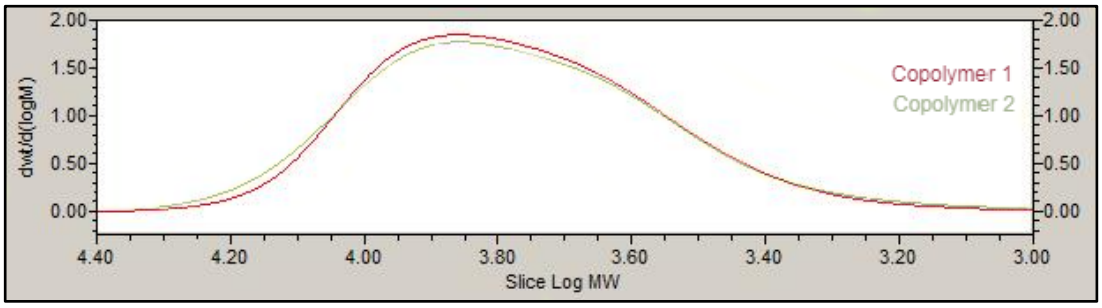

Figure 3. GPC $d_{wt}/d_{(logM)}$ vs log MW showing the molecular weight distribution for copolymers 1 and 2.

Table 2. GPC and NMR copolymers characterization

Polymers synthesized	Theoretical ratio (PEG:GA:LA)	Theoretical ratio (GA:LA)	NMR experimental ratio (PEG:GA:LA)	NMR experimental ratio (GA:LA)	Mn[1]	Mn[2]	Mw[2]	PDI[3]
Copolymer 1	1:1.54:23.10	1:15	1:1.51:20.19	1:14	4585.25	5377.70	6581.35	1.22
Copolymer 2	1:2.25:22.50	1:10	1:1.83:17.17	1:9.80	4235.87	4994.73	6792.03	1.36

[1] Molecular weight (Mw) calculated by ^{1}H-NMR. [2] Number average Mw and weight average Mw obtained by GPC. [3] Polydispersity (Mw/Mn) calculated by GPC

3.2. Physicochemical characterization

3.2.1. Sol-gel transition temperature

The transition temperature from sol to gel state is shown in the phase diagram of Figure 4. Copolymer 2 showed a sol gel transition at earlier temperatures (from 27 °C to 29 °C) than copolymer 1 (from 31 °C to 33 °C). Besides, regarding the precipitation temperature, copolymer 1 exhibited a higher stability at temperatures between 33-39 °C with a precipitation window starting at 39 °C. On the contrary, copolymer 2 displayed a precipitation window between 34-39 °C. In both cases, the transition temperature was found to be dependent on the concentration and reversible. When cooled, the copolymers exhibited hysteresis by reversing from gel to sol slower than from sol to gel. Both copolymers showed a sol state fully transparent, and a gel state transparent at the beginning. While the temperature was increased, hydrogels slowly became opaque until a

precipitate state was shown. At this point an irreversible transition to sol and a phase separation was experimented. Copolymer 1 demonstrated a wider range of transparence at gel state and higher stability than copolymer 2 at physiological temperatures of the posterior segment of the eye (32-37 °C).

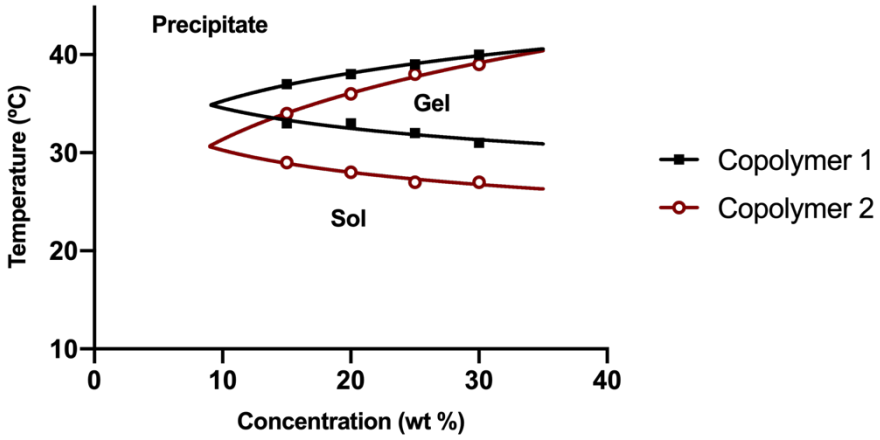

Figure 4. Phase diagram of copolymer 1 (HyG-1) and 2 (HyG-2) in the final bicarbonate buffer.

3.2.2. Rheometry and viscosity analysis

Rheological behaviour of copolymer 1 and 2 at different concentrations in water, bicarbonate buffer as well as the final loaded formulations were studied. Both copolymers 1 and 2, exhibited sol behaviour with low viscosities at room temperature (25 °C) demonstrating their suitability for injectable applications (Figure 5). However, copolymer 2 showed lower viscosity intensity than copolymer 1, lower gelation temperatures as well as more instability at 37 °C. On the contrary, copolymer 1 exhibited stronger gelation, more stability at physiological temperatures and wider gelation spectrum than copolymer 2. Besides, copolymer 1 in the injectable bicarbonate buffer appeared more stable with a more sustained gelation overtime.

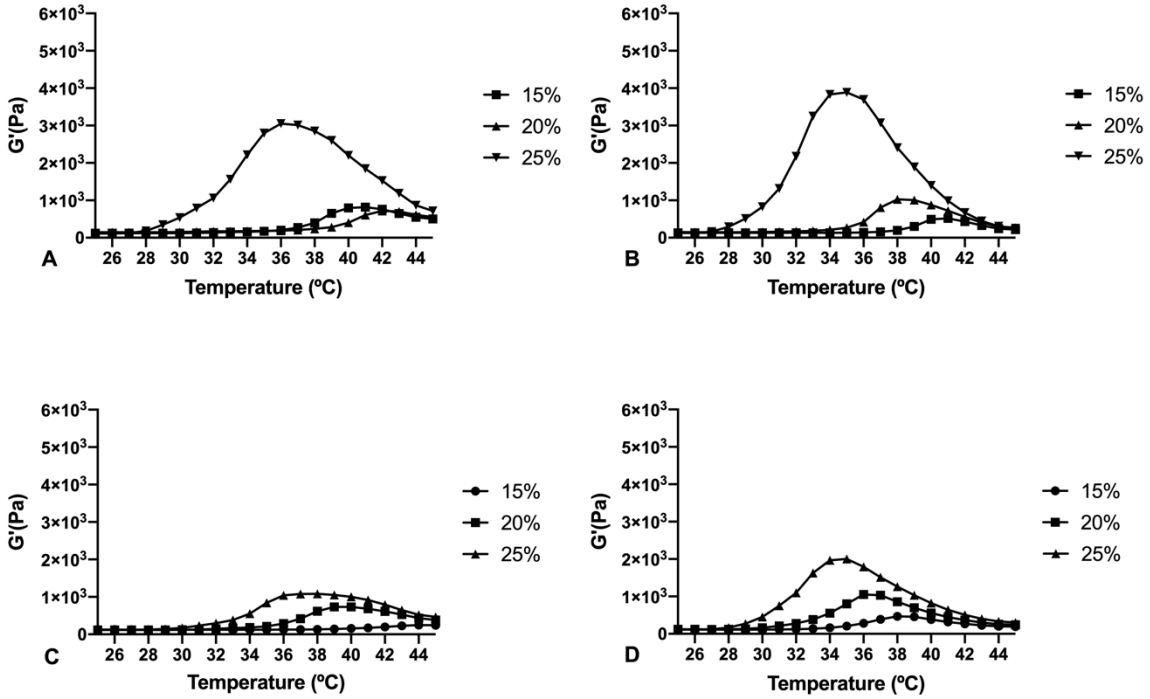

Figure 5. Rheological behaviour values (G' modulus) of PLGA-PEG-PLGA HyG-1 at different concentrations dispersed in water (A) and the bicarbonate buffer (B) and HyG-2 hydrogels also dispersed in water (C) and the bicarbonate buffer (D).

Final formulations with HyG-1 were prepared by dissolving copolymer 1 in the selected bicarbonate buffer followed by and addition of the active substances. Their rheological behavior is shown in figure 6. Both formulations (with dexamethasone phosphate or ketorolac tris salt) showed sol state properties at room temperature, starting the sol-gel transition around 30°C for both groups, and reaching the highest point at 34°C. Dexamethasone phosphate-based formulations exhibited a wider range of variation between temperatures, being the one that combined DX and idebenone 1μM the formulation with the strongest gelling behavior. For both set of groups the gel started to tear apart when it reached 39-40°C, decreasing dramatically the gel state.

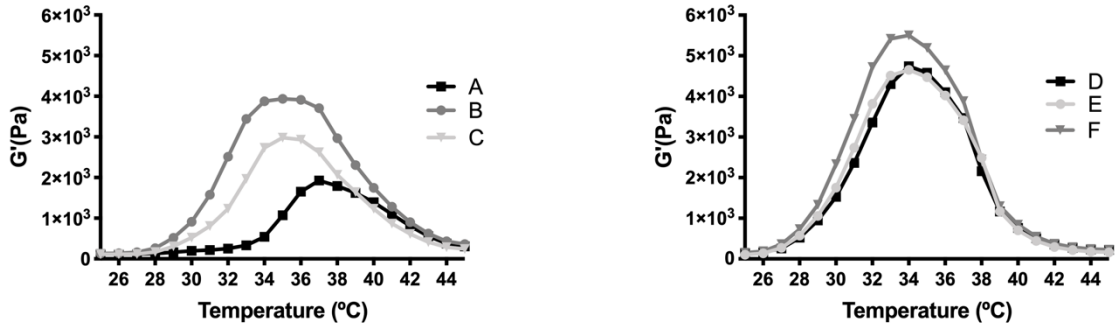

Figure 6. Rheology of Dexamethasone (left) and Ketorolac (right) formulations with selected HyG-1 in bicarbonate buffer at 25% of PLGA-PEG-PLGA. DX 0.2% (A), DX 0.2% + Idebenone 1μM (B), DX 0.2% + TPGS 0.02% (C), KT 0.5% (D), KT 0.5% + Idebenone 1 μM (E) and KT 0.5% + TPGS 0.02% (F).

3.2.3. Micelles size

The size of the micelles of HyG-1 in the selected buffer and HyG-1 developed formulations was measured by dynamic light scattering (DLS) with a Malvern laser spectrometer (Figure 6). The average size for selected copolymer 1 in the bicarbonate buffer solution at 25% (w/v) without drugs was 24.12 ± 0.17 nm. All the formulations with dexamethasone phosphate and ketorolac tris salt are shown in figure 7. Among the different formulations developed, micelles from formulation containing DX 0.2% + TPGS 0.02% were found to have the smallest size (23.03 ± 0.23 nm) while formulations with KT 0.5% presented the highest values (34.80 ± 0.81 nm). All of them presented similar PDI values with unimodal distributions (Figure 7).

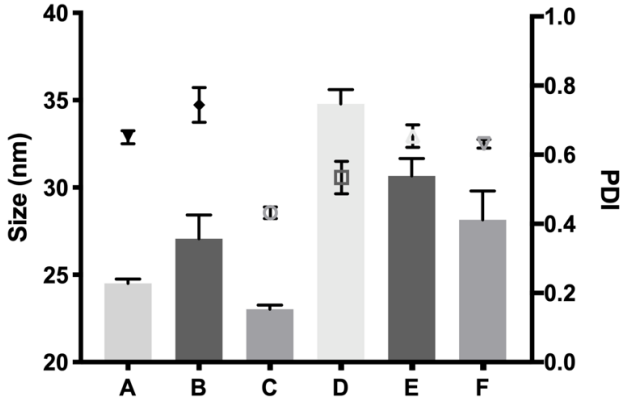

Figure 7. Size of the micelles in nanometers in comparison with the single icons indicating the polydispersity index of sizes (PDI) of DX 0.2% (A), DX 0.2% + Idebenone 1 μM (B), DX 0.2% + TPGS 0.02% (C), KT 0.5% (D), KT 0.5% + Idebenone 1μM (E) and KT 0.5% + TPGS 0.02% (F), respectively.

3.2.4. Critical micelle concentration (CMC)

CMC measurements were used to confirm the critical concentration at which the polymer formed stable micelles, which then will assemble into a group of micelles to create the thermoresponsive gel. The CMC for the selected copolymer 1 was 0.13 mg/mL at 25 °C. This value was calculated by extrapolation of the two lines that create the surface tension/concentration curve, when crossing each other (figure 8). Also, critical micelle concentration (CMC) is a useful way to confirm the concentration at which PLGA-PEG-PLGA chains start to aggregate and create micelles. Our selected copolymer 1 with a CMC of 0.13 mg/mL is in the range described in a previous published study (0.1-0.3 mg/mL) [26].

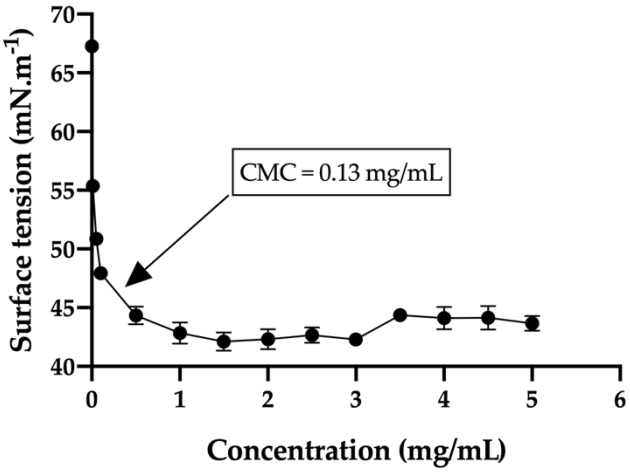

Figure 8. Surface tension diagram of the selected copolymer 1 showing the critical micelle concentration (CMC).

3.3.*In vitro* release studies

The cumulative *in vitro* release profile of dexamethasone or ketorolac alone and in combination with TPGS 0.02% or idebenone 1 µM from hydrogels is presented in Figures 10 and 11. Regarding dexamethasone formulations, the three of them showed a similar initial 24 h burst with values of 8.60 ± 0.11%, 8.94 ± 0.53% and 10.02 ± 0.86% for DX 0.2% (A), DX 0.2% + idebenone 1 µM (B) and DX 0.2% + TPGS 0.02% (C) respectively. A similar biphasic release profile was observed subsequently in the three cases. In the first phase, a sustained slow release was observed until day 51, with a release of 54.09 ± 0.87% for formulation A, of 73.25 ± 0.87% for formulation B and 73.30 ± 0.88% for formulation

C. After that, an increment in release rate from day 51 to the end of the assay at day 62 was also reported for the three hydrogel formulations. According to Korsmeyer-Peppas model (Table 3), while the hydrogel formulation that contained only dexamethasone showed a predominant diffusion mechanism, with n values slightly lower than 0.5 [37] [31], whether both TPGS or Idebenone were included in the formulation, an anomalous transport (n values between 0.5 and 1) was observed. This indicated that not only diffusion but also polymer chains related-events, for example hydrogel erosion [32] have to be considered in these systems. The high values observed for the coefficient of determination of the fitting obtained for the three profiles confirm the applicability of the release model to describe the mechanism behavior.

The similarity factor calculated with formulation A as reference (only loaded with dexamethasone), demonstrated that the inclusion of both TPGS or Idebenone significantly modified the drug release profile, considering them not similar (f_2 values of 44 for Formulation C and of 41 for formulation D)

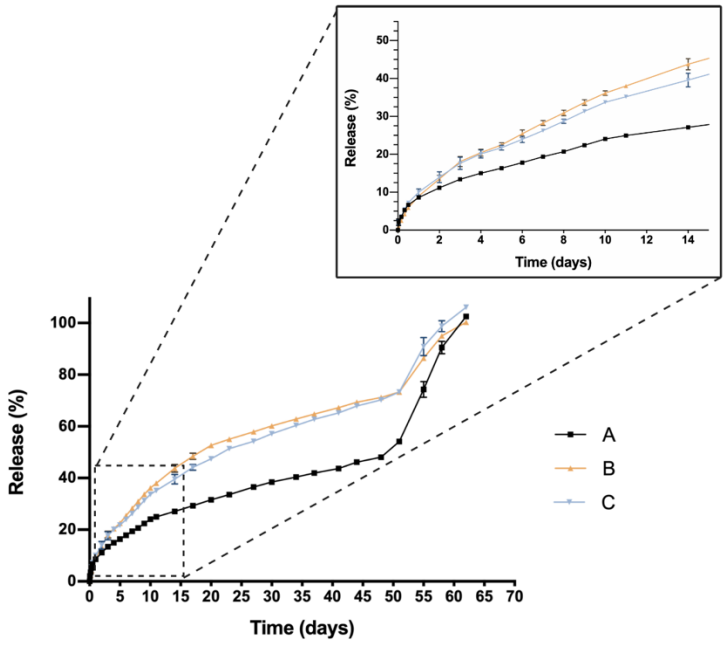

Figure 9. In vitro drug release profile of dexamethasone-based formulations with TPGS or Idebenone respectively. DX 0.2% (A), DX 0.2% + Idebenone 1µM (B), DX 0.2% + TPGS 0.02% (C). Data are represented as mean ± standard deviation (SD) from 3 different batches (n=3).

Table 3. Kinetic parameter and release mechanism proposed

Hydrogels	R^2	"n" value	Mechanism
A	0.9981	0.45	Fickian diffusion
B	0.9969	0.62	Anomalous transport
C	0.9988	0.52	Anomalous transport
D	0.9891	0.63	Anomalous transport
E	0.9970	0.63	Anomalous transport
F	0.9966	0.57	Anomalous transport

Ketorolac-based formulations exhibited higher initial 24 hours burst in comparison with dexamethasone-loaded hydrogels. Values of 16.89 ± 1.86%, 21.87 ± 0.70% and 15.40 ± 0.06% were observed for KT 0.5% (D), KT 0.5% + Idebenone 1 μM (E) and KT 0.5% + TPGS 0.02% (F) respectively (Figure 10). After that, a single release phase was observed for the three hydrogel formulations until the complete release of ketorolac, that occurred at day 47 for formulation E and at day 53 for formulations D and F. The Korsmeyer-Peppas model resulted suitable to describe the release mechanism involved (Table 3). According to the exponent n value, in the three cases an anomalous transport of the drug was observed, meaning that the release mechanism involved might be a mixture of drug diffusion and other polymer chains related events.

Calculations of f_2 considering formulation D as reference (only loaded with ketorolac), demonstrated that formulations E and F were similar to the reference, with values of 52 for the formulation including Idebenone and 90 for the formulation including TPGS.

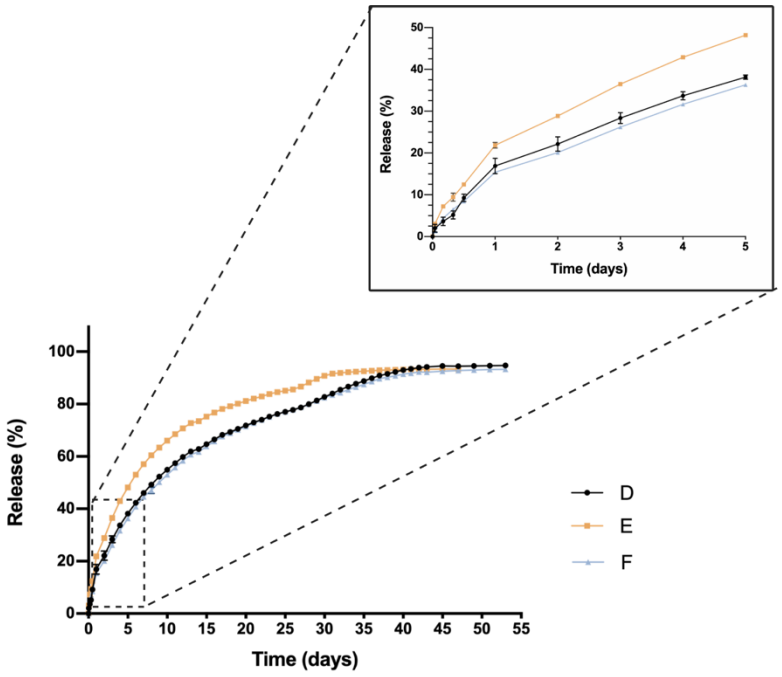

Figure 10. *In vitro* drug release profile of ketorolac-based formulations with TPGS or Idebenone respectively; KT 0.5% (D), KT 0.5% + Idebenone 1 µM (E) and KT 0.5% + TPGS 0.02% (F). Data are represented as mean ± standard deviation (SD) from 3 different batches (n=3).

3.4. Toxicity assessment of PLGA-PEG-PLGA copolymers and formulations

Cellular toxicity in RPE-1 cells of single active ingredients (dexamethasone phosphate and ketorolac tris salt) to be incorporated in the hydrogel were assessed in order to determine which concentration would be the most suitable in terms of tolerability (Figure 11).

Dexamethasone phosphate demonstrated high cell viability values at all concentrations evaluated, with values higher than 80% in all cases. Conversely, ketorolac tris salt showed a correlation between concentration and toxic effects, obtaining acceptable tolerance values (higher than 80%) only for concentrations lower than 0.5%.

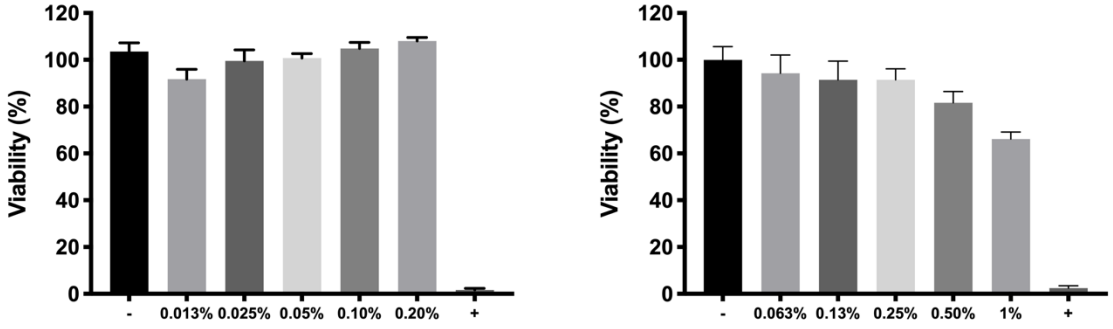

Figure 11. Cell viability of dexamethasone phosphate (left) and ketorolac tris salt (right) at different concentrations in RPE-1 cells. Data are expressed as mean ± standard deviations. Cell viability (%) is calculated in relation to the negative control (100% of viability).

The toxicity of copolymer 1 and 2 was evaluated in order to select the most appropriate one with the best above-mentioned characteristics. Copolymer 1 resulted in higher tolerance values than copolymer 2 at all concentrations studied, particularly at the concentration of more interest (25%) (Figure 12).

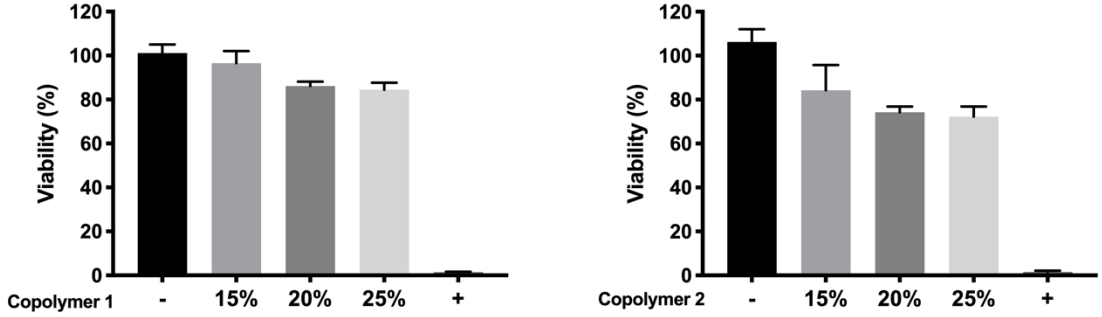

Figure 12. Viability of copolymers 1 and 2 respectively in bicarbonate buffer without drugs.

After determining cell viability of the active ingredients and polymers separately, the tolerance of the final formulations was also tested and shown in figure 13. At 24h, cell viabilities for dexamethasone-based formulations (A, B and C), were 133.10 ± 13%, 135.4 ± 8.44% and 129.4 ± 10.75%, respectively. For ketorolac-based formulations (KT 0.5%, KT 0.5% + Idebenone 1µM and KT 0.5% + TPGS 0.02%) cell viabilities were 102.7 ± 6.49%, 107.0 ± 11.21% and 103.4 ± 15.71%, respectively.

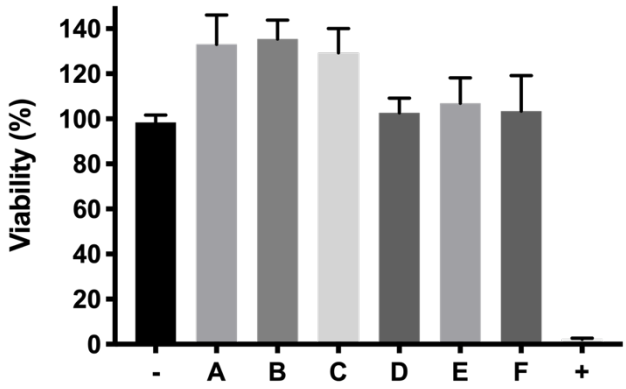

Figure 13. Viability of final formulations (%) prepared with copolymer 1. DX 0.2% (A), DX 0.2% + Idebenone 1 µM (B), DX 0.2% + TPGS 0.02% (C), KT 0.5% (D), KT 0.5% + Idebenone 1 µM (E) and KT 0.5% + TPGS 0.02% (F) respectively.

3.5. Evaluation of protective properties in an oxidative stress model

An oxidative stress model based on the MTT viability assay was developed in order to assess the protection of the formulations developed under an oxidative environment, simulating the conditions given in retinal degenerative processes. Different concentrations of H_2O_2 (200, 150, 100, 50 and 25 µM) were recreated as explained section 2.8. The different values of cell survival under H_2O_2 exposure are shown in figure 14. According to the results, 150 µM H_2O_2 was selected to further evaluate the protection of final formulations against oxidative environments (25.91 ± 5.48 % cell survival).

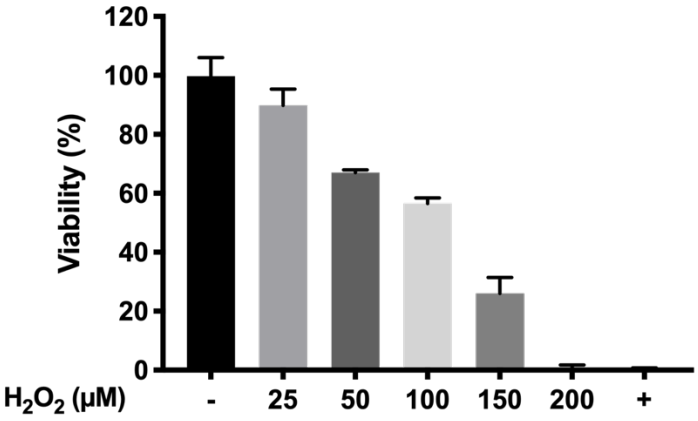

Figure 14. Optimization of cell viability in the oxidative stress model at different H_2O_2 concentrations.

As mentioned previously, cells were exposed to the protective final formulations and to the oxidative stress conditions selected from a previous optimization process (150 µM) (figure 14). According to the results, all the formulations exhibited high levels of protection to cell death caused by H_2O_2 showing high statistically significant results ($p \leq 0.0001$) in comparison with the positive control. Among all, the combination of DX 0.2% + Idebenone 1 µM and KT 0.5% alone were able to protect cells from oxidation, showing the highest viability values (86.24 ± 14.68% and 84.34 ± 8.65%, respectively) (Figure 15).

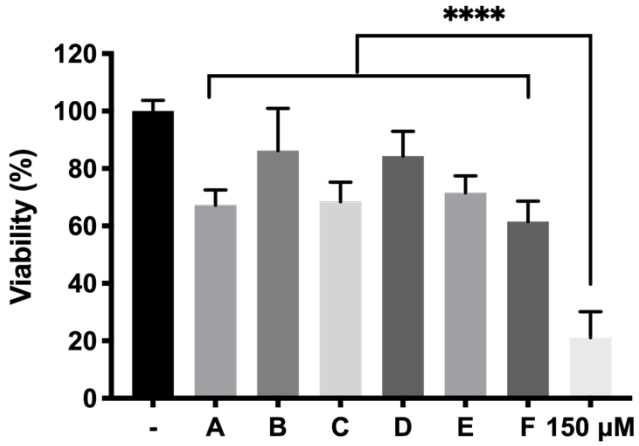

Figure 15. *In vitro* protection of the final formulations DX 0.2% (A), DX 0.2% + Idebenone 1 µM (B), DX 0.2% + TPGS 0.02% (C), KT 0.5% (D), KT 0.5% + Idebenone 1 µM (E) and KT 0.5% + TPGS 0.02% (F) respectively, in response to 150 µM of (positive control, +) oxidative stress in RPE-1 cells. High statistically significant values ($p \leq 0.0001$; ****).

3.6.*In vitro* anti-inflammatory activity in response to LPS stimulation

The effect of LPS in RPE-1 activates the stimulation of TNFα production. By exposing the cells to LPS and the formulations in the transwells for 24 hours, a clear inhibitory effect of TNFα can be seen (Figure 16). As a reference, the basal TNFα production was 4.79 ± 2.41 pg/mL and the positive control (cells exposed to 5 µg/mL of LPS without anti-inflammatory agents) showed a TNFα concentration of 34.69 ± 3.07 pg/mL. Ketorolac-based formulations demonstrated to have the highest anti-inflammatory effect with TNFα values of 12.79 ± 2.24 pg/mL, 12.83 ± 4.64 pg/mL and 15.53 ± 5.57 pg/mL for KT 0.5%, KT 0.5% + Idebenone 1µM and KT 0.5% + TPGS 0.02% each, with very high statistically significance ($p \leq 0.0001$) (Figure 16). Dexamethasone and their combinations were also able to decrease the TNFα values with lesser extent than KT with values between 21.64

and 24.86 pg/mL, being statistically significant in comparison with the positive control for inflammation ($p<0.01$).

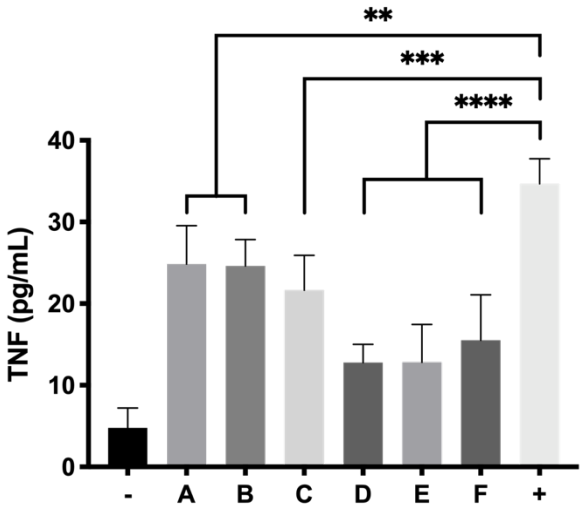

Figure 16. TNFα activity of RPE-1 cells exposed to final formulations DX 0.2% (A), DX 0.2% + Idebenone 1μM (B), DX 0.2% + TPGS 0.02% (C), KT 0.5% (D), KT 0.5% + Idebenone 1 μM (E) and KT 0.5% + TPGS 0.02% (F) respectively, in response to LPS induced inflammation. $P \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

4. Discussion

Thermoresponsive hydrogels have been suggested as a novel tailored tool to successful administer injectable therapies [26] [38]. In this work, two PLGA-PEG-PLGA based copolymers have been developed and characterized as suitable platforms for injectable formulations that deliver neuroprotective agents with anti-inflammatory and antioxidant activity to the posterior segment of the eye. In previous studies, some authors have studied the potential of PLGA-PEG-PLGA hydrogels at 10% w/v with glycolic to lactide ratios (1:3) and PEG 1000 to deliver NSAIDs, such as naltrexone, in a sustained manner, therefore avoiding repeated daily administrations and achieving more effective drug concentrations [24]. In ocular applications, Yuan Gao *et al*. studied the development of a dexamethasone acetate (0.1%) loaded PLGA-PEG-PLGA hydrogel at 20% w/v as an alternative of effective dexamethasone eye drops and also suggested the possibility of using PLGA-PEG-PLGA to develop therapies for the posterior segment of the eye [36]. Also, thermoresponsive nanocomposite-based hydrogel made out of cefuroxime nanoemulsions and solid lipid nanoparticles dispersed in Pluronic®F127 have been developed for the treatment of endophthalmitis [39]. To our knowledge, there have not

been previous studies that propose and develop a complete optimized and fully characterized thermoresponsive formulation biocompatible and biodegradable to include a combination of various neuroprotective agents to be administered in the posterior segment of the eye.

The basic structure of PLGA-PEG-PLGA copolymer 1 and 2 developed in this work presented the same groups, chemical shifts and were similar to other PLGA-PEG-PLGA polymers studied by other authors according to ^{1}H-NMR spectrum [36][24][26]. Besides, GPC peaks and PDI for both copolymers 1 and 2 (1.22 and 1.36) showed low polydispersity, uniformity and a unimodal distribution. One important aspect in the development of preparations for intravitreal administration is the resultant pH, to avoid any harmful effects due to pH deviation from physiological values [40]. Some studies have previously described the use of different aqueous vehicles like acetate buffer [41], water [42] or sodium chloride (NaCl) to prepare PLGA-PEG-PLGA based hydrogel formulations. However, in the present work low pH values (around 2-3) were rendered when using these aqueous vehicles in the PLGA-PEG-PLGA hydrogels development. To avoid this, both copolymers were dissolved in a bicarbonate buffer that counteracted the acidic nature of the polymer and equilibrated isotonicity with trehalose, so the formulations could be well tolerated and effective.

Copolymer 1 exhibited a crystalline sol behaviour at room temperatures (20-27°C) which makes it ideal for injections. When reaching 32°C, the dissolved copolymer 1 in a concentration of 25% (w/v) creates a transparent hydrogel, which is maintained for at least two to three weeks, depending on the substances entrapped. In addition, the gel modifies its structure by absorbing water and evolving into a high viscous semi-gel state. Furthermore, at this concentration the precipitation temperature starts at 39°C that being considered as a stable system for intravitreal delivery. On the contrary, lower concentrations (15% and 20%w/v) of hydrogel solutions, started to gel at higher temperatures (Figure 4). Furthermore, in these conditions the gel was not strong enough and lasted only for few days, and precipitation underwent close to physiological temperatures (36-37°C). Regarding copolymer 2, 15%, and 20% (w/v) solutions also developed weaker gels although fully transparent. However, all the tested concentrations of HyG-2 (15%, 20%, and 25%) presented sol-gel transition temperatures very close to room

temperature, showing some viscous behaviour with normal room temperature variation. These features made difficult to work with it and to check its suitability for injection.

The rheological behaviour of copolymer 1 based hydrogels confirmed the strength mentioned previously. They also show a maximum gel point around 34-36°C just in the range of physiological temperatures of vitreous humour. At higher temperatures the gel starts to tear down, which confirms the precipitation process. The optimized bicarbonate buffer increases the gel strength and more sustained hydrogels over time as well. These findings and others that will be further discussed, envisaged that copolymer 1 at 25% (w/v) presented more suitable characteristics to develop an optimal formulation for intravitreal injections. Another important feature is the size distribution of the micelles that creates the hydrogel, since their aggregation and therefore gelation could differ from each other. Some studies have shown that DLS is the common technique to study size distribution, and that PLGA-PEG-PLGA micelle sizes around 21-31 nm, such as those obtained in the present work, are within the common scope, although aggregation could occur and therefore increase the size [26]. In the present study, final formulations prepared with copolymer 1 showed different micelles sizes depending on the substances included. Dexamethasone-HyG-1 based formulations (A, B, C) showed smaller micelle sizes than ketorolac-based formulations (D, E, F). According to the sizes presented, we hypothesize that dexamethasone interacts more closely with PLGA chains that ketorolac since dexamethasone phosphate present almost tenth times less of water solubility (1.52 mg/mL) than ketorolac tris salt (15 mg/mL) [43] [44]. Additionally, formulations C and F containing TPGS presented smaller sizes distributions than the rest of their group. These findings could suggest that the presence of TPGS at 0.02% could interact in some way with micelles stabilizing them, thanks to its amphiphilic nature, promoting lower sizes and PDI values [45]. Polydispersity index values resulted relatively high in all cases. This behavior has also been observed by other authors and explained due to the high molecular weights and some level of branching that are sometimes observed in triblock polymers [45]. Additionally, some micelles aggregation due to the relatively high viscosity of the sample cannot be discarded.

One critical characteristic that is worth mentioning is the *in vitro* release profile. Sustained releases that ensure certain levels of drug overtime entails a great advance in order to reduce the number injections needed, increase effectiveness and patient's quality of life. In this sense, all HyG-1 based formulations proposed in this work showed sustained release of the loaded active compounds. The initial bursts during the first 24 h were well controlled in both dexamethasone and ketorolac-based hydrogels although ketorolac presented slightly higher burst than dexamethasone-based ones, probably due to its higher hydrophilicity. These initial bursts were followed by a sustained release, showing the utility of HyG-1 based formulations as sustained drug delivery systems. In the case of ketorolac-based hydrogels, the systems were able to release the drug for 47-53 days depending on the combinations, being this time extended for dexamethasone hydrogels until 62 days. A sustained *in vitro* release of drugs was also observed for a thermoresponsive hydrogel based on PEG-PCL (40% at 14 days) [46]. Comparison of dexamethasone-based hydrogels drug release profiles demonstrated that the inclusion of both, TPGS or idebenone, increased dramatically the release of dexamethasone, considering them "not similar" according to f_2 values. We hypothesize that, according to partition coefficient, idebenone (sparingly soluble in aqueous buffers with a logP of 4.3) [47] interacts with HyG-1 PLGA-PEG-PLGA micelles and shifts dexamethasone phosphate (logP value of 1.9) from interacting with micelles, therefore increasing the amount of free drug that is released [43]. TPGS presents higher hydrophilicity than idebenone and dexamethasone respectively with a solubility in water of 20% w/v [48]. It could also affect the capacity of dexamethasone to interact with HyG-1 micelles and increased its release. It has been previously reported that TPGS is an absorption enhancer and possess emulsification properties. Mustafa S *et al*. developed PLGA nanoparticles loaded with kanamycin and emulsified with TPGS. They experimented faster release profiles with TPGS based nanoparticles stating the ability of TPGS to interact with PLGA chains [49]. We cogitate whether TPGS emulsification properties could have enhanced dexamethasone release. This modulation in the release could be due to the ability of PEG chains present in TPGS to interact with several PEG chains of PLGA-PEG-PLGA micelles and shift dexamethasone to the aqueous phase, therefore increasing its release. Conversely, dexamethasone formulations without any additional substances seemed to decrease its release ratio until late stages, where they seemed to undergo the rupture of micelles and hydrogel, thus releasing the rest of the drug (Figure 9). The addition of both TPGS or

idebenone in dexamethasone based-hydrogels also seemed to influence the mechanisms involved in the drug release until delivering 60% of total dose, shifting from a simple diffusion of the drug through the hydrogel network in the case of dexamethasone alone to a mixture of that phenomena with others related with micelles movement/rearrangement or hydrogel erosion [32]. Regarding ketorolac *in vitro* release, there are no studies that combine ketorolac with idebenone in a thermoresponsive hydrogel, so few is known about their interactions. However, and according to what has been previously claimed for dexamethasone, for us it is plausible that as well as it occurs with dexamethasone, idebenone is shifting the active ingredient to the aqueous phase and increasing its release. Nonetheless, taking into account that ketorolac tris salt is much more soluble than dexamethasone phosphate, this would not be affected by the addition of TPGS. Furthermore, TPGS is not included in high concentrations (0.02%) and ketorolac tris salt release rate is elevated. These could be the reason why single ketorolac and its combination with TPGS would not be as affected as in the case of dexamethasone. It is also worth mentioning that between the three *in vitro* profiles of ketorolac-based formulations, differences in the release profile are more subtle than for dexamethasone. In fact, this difference is lower than 10%, according to f_2 values, and in the three cases a similar release mechanism based on diffusion plus polymeric chains rearrangement/erosion can be described, according to Korsmeyer-Peppas model. As mentioned earlier, in part that would be due to the solubility of both active ingredients, being ketorolac tris salt almost 8-fold more soluble in water than dexamethasone phosphate [50][43].

For cell *in vitro* tolerability studies, we took as an initial step in the preparation of the copolymer 1 based formulations the screening of safe concentrations of dexamethasone phosphate (0.2%) and ketorolac tris salt (0.5%). Moreover, the toxicity of HyG-1 and HyG-2 prepared at different concentrations (15, 20, and 25% w/v) was also tested after 24 h contact in order to ensure that the hydrogels were well tolerated in retinal cells. For testing the toxicity of hydrogels, a new protocol was developed, since the gels were so strong that did not allow the MTT to penetrate into the cells. Therefore, proper gelation onto the plates needed to be established before seeding the cells on top of the gels. This way entails a novel way of testing toxicity by direct contact of these biomaterials since, until now, these types of hydrogels are generally tested by indirect toxicity by exposing cells to degradation products of the hydrogels [51]. According to the so performed toxicity

assay, HyG-1 is much better tolerated than HyG-2, being the concentration of 15% the only one showing viability values superior to 80%, a limit commonly accepted as well tolerated according to the criteria for ocular drug delivery testing in cell cultures [52]. These toxicity studies, together with the physicochemical data (low sol-gel-precipitation transition temperature, unstable rheological behaviour and higher polydispersity) observed for HyG-2 allowed to discard it for further steps of the experimental work. On the contrary, HyG-1 gather the criteria of well tolerated, creating stable and transparent gels at the physiological temperatures of the vitreous, exhibiting high precipitation temperatures avoiding its degradation as well as good injectability at room temperatures. Among the three concentrations of copolymer 1, 25% (w/v) was the one selected by presenting the best characteristics in terms of sol-gel properties, long-term *in vitro* drug release and good tolerability. HyG-1 formulations at 25%, including dexamethasone or ketorolac and their combinations were also tested in terms of cell tolerance. Among them, dexamethasone-based formulations exhibited exceptional good tolerance in RPE-1 (with values even higher than 100%) after 24 h exposure. According to some studies, these findings could be linked to the ability of dexamethasone to stimulate cell proliferation together with the low toxicity of drug combination and polymers [53][54] [55]. In addition, ketorolac-based formulations also showed viability values close to 100%.

It is well known that oxidative stress plays a very important role in the development of neurodegenerative diseases of the retina [56]. Recently, Masuda *et al*. highlights the close relation between oxidative stress and neurodegenerative retinal diseases. They gather some studies where inflammation is studied as a triggering factor of ROS starting an oxidative stress and inflammatory cascade. Among the cytokines studied, TNF-α entails a pivotal point of the inflammation cascade [57]. Besides, many neurodegenerative processes of the retina such as AMD or diabetic retinopathy start or progress with lack of retinal perfusion leading in many cases to ischemia [58]. Some studies have already established a solid relation between ischemic processes and ROS generation due to neovascularization process, hyperglycemic states leading to lipid peroxidation or finally hypoxia [58]. The massive production of ROS and inflammation eventually leads to cell apoptosis, loss of functionality of retinal pigmented epithelium, photoreceptor loss and irreversible blindness. In this study, we have used a method to evaluate the protection of different formulations by generating cell death in RPE-1 cells, produced by the oxidative stress of

H_2O_2. Since H_2O_2 is one of the main radicals produced under oxidative stress in many retinal degenerative processes [59], we selected it to screen potential neuroprotective agents. As mentioned previously, different concentrations of H_2O_2 were tested in order to select one that allowed us to evaluate protective effects. Among them, 150 μM was chosen as the oxidative stress stimulus that showed cell viability values of 25.91 ± 5.48 %. This model allowed evaluating whether a developed formulation can preserve cell death provoked by oxidative stress. According to the studies performed, all the developed formulations showed statistically significant results ($p < 0.0001$) resulting in high protective formulations. Among them, formulation B (DX 0.2% + Idebenone 1μM) and formulation D (KT 0.5%) showed the highest survival ratios (86.24 ± 14.68 % and 84.34 ± 8.65 % respectively), more than 4-fold higher than the cells directly exposed to H_2O_2 without protection used as positive control. With regards to these results, it has been reported that dexamethasone is able to suppress some important signaling pathways such as p38 MAPK and NF-κB, both involved in retinal inflammation. Besides, they also reported that dexamethasone protected against ROS and increased survivability of RPE cells and photoreceptors [60]. In its turn, idebenone is an analogue of Q10 coenzyme that has been demonstrated to act as a very effective electron carrier, therefore trapping ROS generated by the excess of H_2O_2, and avoiding their interaction with cell membranes [61]. In the case of Ketorolac, its antioxidant effect could be also attributed to its capacity to inhibit cyclooxygenase (COX). In fact, some authors have previously reported that COX triggers ROS production that feeds the inflammatory cycle. Besides this, the activity of ketorolac to protect cells from ROS has been well studied [62]. Besides, TPGS in combination with dexamethasone (formulation B), also demonstrates to preserve cell death provoked by oxidative stress (68.48 ± 6.81 %). TPGS appears as very attractive hydrophilic alternative to vitamin E since can be fully dissolved in aqueous buffers. Moreover, TPGS has been described to protect retinas from ischemia-reperfusion injuries as well as against free radicals produced by lipid peroxidation [63]. On the other hand, ketorolac is a very potent NSAID that has been proposed to decrease inflammation in retinal degenerative diseases such as AMD [64] and appears to enhance its activity in combination with idebenone (71.54 ± 5.89% cell survival). It is worth noting that, although some studies have point out that dexamethasone might induce oxidative stress [65], our findings showing potential protective properties are supported by some authors that encourage the use of intravitreal dexamethasone for retinal degenerative diseases [66].

Regarding inflammation, there is no doubt that all the formulations decreased the levels of TNFα and particularly those containing ketorolac, supporting its potent anti-inflammatory properties. These findings support the use of ketorolac in combination with a hydrogel formulation as a potential effective therapy for retinal diseases. As future recommendations, *in vivo* studies in animal models of retinal degeneration are crucial to confirm the security and efficacy of this novel therapy.

5. Conclusions

Ultimately, our work introduces six different fully developed biodegradable thermoresponsive injectable hydrogel formulations with preliminary evaluation of their *in vitro* tolerance and efficacy (anti-inflammatory and antioxidant activity) in retinal pigmented epithelial cells. These novel platforms for neuroprotective combined therapy resulted well tolerated in retinal cells and also show robust, long-lasting and controlled *in vitro* release profiles overtime. These formulations could entail a new generation of sustained neuroprotective treatments that could improve the efficacy of current treatments and the quality of life of their patients.

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Conflicts of Interest: The authors declare no conflict of interest.

CAPITULO V

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DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

El objetivo general de la presente tesis doctoral es el desarrollo de nanosistemas para el tratamiento de patologías crónicas e inflamatorias tanto del segmento anterior como del segmento posterior del ojo. Por tanto, se estableció como hipótesis, que "los nanosistemas desarrollados en la presente memoria, tanto lipídicos para el tratamiento del glaucoma y protección de la superficie ocular, como los hidrogeles hidrofílicos inyectables termosensibles de liberación sostenida pueden ser empleados para el tratamiento eficaz de patologías oculares crónicas e inflamatorias".

La revisión exhaustiva realizada en el **capítulo I "Liposomes as vehicles for topical ophthalmic drug delivery and ocular surface protection"** supone una parte esencial para diseñar y elaborar nuevos nanosistemas lipídicos con precursores de la película precorneal. Para ello, se estableció como punto de partida la revisión de distintos nanosistemas lipídicos de administración tópica oculares capaces de vehiculizar fármacos y activos protectores de la superficie.

Tal y como se detalla anteriormente en el capítulo I, gracias al empleo de micro y nanosistemas como las micropartículas, nanopartículas, nanoemulsiones, liposomas o microemulsiones se puede conseguir incrementar la absorción y el tiempo de residencia de diversos agentes terapéuticos. Sin embargo, el uso de nanosistemas lipídicos ha sido de gran interés para la administración de sustancias terapéuticas en la superficie ocular para el tratamiento de diversas patologías (235). Una de las principales ventajas que justifica su uso es la vehiculización de principios activos y compuestos poco solubles en medio acuoso que presenten una baja biodisponibilidad en la superficie ocular. La presencia de barreras físicas como el epitelio corneal capaz de controlar el paso activo de moléculas, principalmente hidrofílicas, así como el estroma con transporte selectivo de moléculas acuosas, dificultan el paso de sustancias activas al interior del ojo (236). La inclusión de estas sustancias en sistemas nanoparticulares lipídicos puede suponer una estrategia conveniente para vehicular sustancias activas tales como son los agentes antiglaucomatosos hasta el lugar de acción deseado.Asímismo, la utilización de compuestos lipídicos presentes de forma natural en la película precorneal para la elaboración de dichos nanosistemas, puede suponer una estrategia efectiva para incrementar la estabilidad de la lágrima y disminuir los síntomas asociados a la enfermedad de ojo seco (25). Concretamente, la incorporación de fosfatidilcolina de soja ha sido ampliamente estudiada por su alta composición en ácidos grasos insaturados, que

además de presentar un alto poder antioxidante (238), presentan una tolerancia superior a la obtenida con otros compuestos lipídicos (237) (238).

Respecto al uso de polímeros mucoadhesivos, el empleo del ácido hialurónico en formulaciones de administración ocular, incluido el tratamiento de la enfermedad de ojo seco (60), se encuentra ampliamente extendido debido a sus innumerables propiedades, además de su capacidad para incrementar el tiempo de retención en la superficie ocular (199). También se menciona la capacidad de este polímero para mejorar la internalización de ciertos nanosistemas en tejidos corneales (239), lo que supone un valor añadido a la hora de incluirlo en las formulaciones a desarrollar en el presente trabajo. Un punto clave que se trata en esta revisión ha sido la necesidad de dotar a las preparaciones tópicas oftálmicas de una adecuada tensión superficial. Tal y como se ha estudiado ampliamente por diversos autores, con una tensión superficial reducida se consigue una lágrima de mayor estabilidad y se facilita su extensibilidad sobre la superficie ocular (201). Esto se puede llevar a cabo mediante la adición de surfactantes, además de la fosfatidilcolina de soja mencionada anteriormente, para facilitar una adecuada extensibilidad de los nanosistemas desarrollados e incrementar la estabilidad de la lágrima (240). Otro punto de especial importancia a la hora de desarrollar formulaciones oftálmicas es su isotonicidad con la lágrima (310 mOsm/L) siendo de interés, en ciertas ocasiones, una hipotonicidad entre 215 y 300 mOsm/L (241) lo cual contrarresta las situaciones de estrés hiperosmolar que aparecen en la enfermedad de ojo seco. También es importante en el diseño de formulaciones tópicas oculares que el pH que se encuentre próximo a valores fisiológicos, 6,6 y 7,80 (242), así como que la viscosidad no dificulte la visibilidad ni altere la estabilidad película precorneal.

Uno de los puntos críticos y esenciales para la adecuada conservación y administración de este tipo de formulaciones y que también se trata en el capítulo I, es el método de esterilización y el almacenamiento. Debido a la degradación de la mayor parte de componentes lipídicos presentes en este tipo de formulaciones, los métodos por calor húmedo y seco no se consideran adecuados para la esterilización de dichos sistemas. La filtración esterilizante por filtros con tamaño de poro de 0,2 μm junto con los llamados métodos fríos de esterilización como el óxido de etileno se consideran las alternativas más adecuadas para asegurar la ausencia de patógenos o elementos que puedan desencadenar una respuesta inflamatoria y facilitar el posible escalado a nivel industrial (243).

DISCUSIÓN GENERAL

Para la selección de los posibles activos que se pueden incluir en los nanosistemas mencionados anteriormente para el tratamiento de patologías de la superficie ocular que van asociadas a un ambiente hiperosmolar se llevó a cabo el trabajo expuesto en el **capítulo II, "Combined hyperosmolarity and inflammatory conditions in stressed human corneal epithelial cells and macrophages to evaluate osmoprotective agents as potential DED treatments".** Se ha conseguido desarrollar un método sencillo y accesible para evaluar la capacidad de diferentes sustancias o tratamientos que eviten procesos de muerte celular asociados a entornos hiperosmolares, modulando el volumen celular y evadiendo procesos inflamatorios asociados. Diversos autores han estudiado las consecuencias de un ambiente hiperosmolar en las células de la superficie ocular, principalmente de la córnea, tratando de establecer una relación entre la hiperosmolaridad y, los síntomas y progresión de la enfermedad de ojo seco (40). En este sentido, Garret et al. han investigado la modificación del volumen celular y eventos de muerte celular (apoptosis) asociados a un ambiente hiperosmolar en células epiteliales de córnea (244). También la creación de co-cultivos 3D mediante el uso de esferoides lagrimales junto con células de epitelio conjuntival ha servido para intentar reproducir de la mejor forma eventos de daño celular e inflamatorios complejos asociados a la enfermedad de ojo seco (245). Por otro lado, existen modelos complejos desarrollados con técnicas *ex vivo* que incluyen la preservación de globos oculares en ambientes de calor y humedad óptimas, y otros métodos, también complejos para su evaluación, como es el caso de la tomografía de coherencia óptica (de las siglas en ingles "OCT", "Optical Coherence Tomography") (246). Sin embargo, el uso de tejidos provenientes de animales de experimentación y de técnicas complicadas y/o costosas dificulta los procesos de selección y cribado rápidos para elegir posibles compuestos o potenciales terapias.

Con el modelo desarrollado en esta tesis doctoral se ha podido evaluar de una forma rápida y eficaz, el efecto de diferentes ambientes hiperosmolares sobre células de córnea, así como los procesos apoptóticos y las modificaciones en el volumen celular que se derivan en estas situaciones. Como primer objetivo de este trabajo se estableció la selección de un ambiente hiperosmolar óptimo que permitiera estudiar los mecanismos por los que diferentes sustancias osmoprotectoras o poliméricas ejercen una acción protectora. El comité de expertos de la sociedad sobre "película lagrimal y superficie ocular" (de las siglas en inglés "TFOS", "Tear Film and Ocular Surface Society") relacionó un estrés

hiperosmolar prolongado con apoptosis de las células de la superficie ocular así como de posibles sustancias denominadas osmoprotectoras capaces de revertir dichos procesos de muerte celular en modelos experimentales (46).

El ensayo MTT por reducción de la sal de tetrazolio mostró una disminución leve de la viabilidad celular en aquellas células expuestas a concentraciones ligeramente hipertónicas como es el caso de 350 y 400 mOsm/L lo cual no supuso una disminución significativa en comparación con el control isotónico (300 mOsm/L). Por el contrario, 450 mOsm/L fue la concentración "gatillo" donde la viabilidad celular experimentó un descenso brusco. De hecho, algunos autores han estudiado el estrecho vínculo entre ambientes hiperosmolares e inestabilidad de la película precorneal en la enfermedad de ojo seco y han establecido que fluctuaciones por encima de 450 mOsm/L están asociadas a la sensación de ardor y escozor que experimentan los pacientes con esta enfermedad (247). A partir de esta osmolaridad, incrementos leves de 10 mOsm/L produjeron un descenso dramático en la viabilidad hasta una estabilización entre 490 y 500 mOsm/L. Estos hallazgos concuerdan con los obtenidos por Luo et al. demostrando que la exposición de células epiteliales de córnea a osmolaridades de 500 mOsm/L durante 24 horas provocó una intensa fragmentación nuclear con una elevada expresión de la caspasa 3 (248).

Mediante la técnica de citometría de flujo y bajo las mismas condiciones hiperosmolares comentadas se estudió el número de células recuperadas en un tiempo limitado, el nivel de apoptosis y su volumen celular. Con esta técnica se obtuvieron recuperaciones celulares similares a los ensayos de MTT, pero con resultados que, aunque mostraban cierta significación a 400 mOsm/L, seguían exhibiendo un descenso elevado a partir de 450 mOsm/L con una elevación significativa en la apoptosis total a partir de 460 mOsm/L. Algunos autores investigando el papel de las quinasas de Janus (JNK) y la vía de las MAPK bajo estrés hiperosmolar obtuvieron resultados de apoptosis similares en células epiteliales corneales expuestas a 450 mOsm/L durante 24 horas (249). Respecto al volumen celular, Baudouin et al. demostraron que, en procesos de apoptosis temprana, el tamaño celular tiende a disminuir incluso cuando no han sido sometidas a ambientes hiperosmolares. Sin embargo, el mismo autor también destacó el hecho de que en las primeras etapas de exposición a un ambiente hiperosmolar o con concentraciones no demasiado elevadas, existía un mecanismo compensatorio con incremento del volumen celular (40).

DISCUSIÓN GENERAL

Los resultados obtenidos en estos ensayos permiten establecer que la exposición a ambientes hiperosmolares moderados produce un aumento de tamaño leve, aunque significativo, y a partir de osmolaridades de 460 mOsm/L, el volumen celular disminuye radicalmente coincidiendo con un incremento de los procesos apoptóticos. De acuerdo con estos resultados de viabilidad celular, apoptosis y modulación del volumen celular bajo un ambiente hipertónico se decidió escoger la concentración de 470 mOsm/L como osmolaridad óptima para realizar el cribado de sustancias osmoprotectoras y continuar con el segundo objetivo del presente capítulo.

El segundo y principal objetivo del capítulo II fue seleccionar sustancias ya conocidas y otra menos estudiadas en el tratamiento de enfermedades de la superficie ocular para confirmar y/o profundizar en su capacidad para prevenir y tratar eventos de estrés celular asociados a ambientes hiperosmolares, así como determinar las concentraciones mejores toleradas. Dichas sustancias comprenden la L-carnitina, hidroxipropilmetilcelulosa y el ácido hialurónico de peso molecular medio (400-800 KDa) ampliamente estudiadas y utilizadas en lágrimas artificiales comercializadas y otras en fase de investigación por sus potenciales usos para enfermedades de la superficie ocular como es el caso de la betaína y la taurina. Del mismo modo se estableció la capacidad de dichas sustancias para inhibir la producción del factor de necrosis tumoral alfa en macrófagos como células modelo de inflamación, ya que la presencia de esta citocina ha sido ampliamente relacionada con la continuidad de eventos inflamatorios en cascada en diversas enfermedades de la superficie ocular, incluyendo la enfermedad de ojo seco (250). En primer lugar, todos los compuestos osmoprotectores empleados (betaína, L-carnitina y taurina) y sustancias poliméricas (ácido hialurónico e hidroxipropilmetilcelulosa) resultaron bien tolerados a las concentraciones empleadas. De acuerdo con autores expertos en la materia, y según las guías establecidas por la Organización para la Cooperación y el Desarrollo Económico para el ensayo de la tolerancia de sustancias a nivel ocular, se estableció como criterio de aceptación el 80% como tasa mínima de supervivencia celular para considerar un compuesto o una formulación con tolerancia óptima (251) (252). Entre todas las sustancias con potencial osmoprotector empleadas, la betaína mostró los valores más elevados de viabilidad celular y a su vez una disminución muy pronunciada de la apoptosis concretamente los preparados de mayor concentración. Además, mostró también capacidad para regular el volumen celular, obteniéndose volúmenes celulares similares al de las células que no habían sido

expuestas a estrés hipertónico. Cabe mencionar que el papel osmoprotector de la betaína fue estudiado por Garret et al. mediante su adición e incubación en un entorno hipertónico mostrando buenos resultados para regular los volúmenes celulares y disminuir la apoptosis (244). Tanto la L-carnitina como la taurina fueron altamente protectoras. En particular la taurina fue altamente eficaz para evitar procesos apoptóticos y con gran capacidad para regular el volumen celular. Así mismo, la L-carnitina a concentraciones elevadas mostró una disminución de su eficacia, donde es probable que exista una cierta toxicidad crónica de acuerdo a los estudios de tolerancia realizados.

Los polímeros ensayados demostraron capacidad anti apoptótica, tratándose de una de las propiedades menos estudiadas. Tanto el ácido hialurónico como la hidroxipropilmetilcelulosa incrementaron la viabilidad celular, produciendo una sobrecompensación en el volumen celular, probablemente, por su capacidad de retención de agua, lo que esta consonancia con hallazgos previos (253).

Finalmente y tal y como se menciona previamente, la capacidad para inhibir la producción de factor de necrosis tumoral alfa es una característica atractiva para el tratamiento de enfermedades de la superficie ocular que cursan con ambiente hiperosmolar, ya que esta interleucina se encuentra asociada a entornos hipertónicos y contribuye a la continuidad de la cascada inflamatoria (40). En este sentido, la taurina y la L-carnitina consiguieron la capacidad máxima para inhibir la producción de esta interleucina a concentraciones intermedias. Los polímeros demostraron una gran capacidad antiinflamatoria sobre todo el ácido hialurónico. Precisamente, estudios recientes han hecho especial hincapié en la actividad antiinflamatoria del ácido hialurónico de alto peso molecular en comparación con el de bajo peso molecular (51). Aunque menos estudiado respecto a la capacidad antiinflamatoria, la hidroxipropilmetilcelulosa demostró importantes resultados en cuanto a su capacidad para inhibir la producción del factor de necrosis tumoral, lo cual explica su extensa relación con la mejoría de los síntomas y parámetros clínicos asociados al incremento de la osmolaridad de la lagrima como ocurre en la enfermedad de ojo seco (254). Algunos estudios han atribuido una cierta capacidad antiinflamatoria a los polímeros descritos como formadores de geles entre los que se encuentra el ácido hialurónico por su supuesta capacidad para absorber agua y diluir los diferentes factores proinflamatorios (255).

DISCUSIÓN GENERAL

Cabe destacar que las herramientas desarrolladas en este capítulo suponen una importante herramienta para el rápido cribado de compuestos, sistemas farmacéuticos o potenciales terapias para el tratamiento de patologías inflamatorias y que cursen con hiperosmolaridad de la superficie ocular, sin el uso inmediato de animales de experimentación o modelos costosos y complejos. Concretamente, las sustancias y polímeros utilizadas en estos ensayos demostraron una alta capacidad para proteger *in vitro* a las células epiteliales corneales frente a entornos hiperosmolares y para inhibir la producción de citocinas inflamatorias. Estas sustancias tanto de forma individual como en combinación pueden suponer una estrategia efectiva a tener en cuenta a la hora de diseñar y desarrollar futuras formulaciones para el tratamiento de patologías de la superficie ocular.

Siguiendo con los objetivos de esta memoria experimental, la siguiente etapa es el desarrollo de una microemulsión de administración ocular. Para ello, tal y como se describe en el **capítulo III, "Development of an osmoprotective microemulsion as a therapeutic platform for ocular surface protection"** se procedió a la preparación de una microemulsión por autoemulsificación con características de lágrima artificial incluyendo activos osmoprotectores. Esta microemulsión puede ser al mismo tiempo empleada para la vehiculización de fármacos de administración tópica ocular. La preparación, características y utilización de dicha microemulsión ha sido objeto de la presentación de una solicitud de una patente española (ver Anexo I).

Las microemulsiones han sido estudias en diversas áreas de aplicación tópica, sobre todo en el ámbito de la cosmética (256), debido a su gran capacidad para actuar como portadores de moléculas activas poco solubles. Aunque menos exploradas, son diversos los estudios que sientan la base para su uso en el campo de administración de fármacos por vía ocular (257). Se ha propuesto su utilización en enfermedades de la superficie ocular y en concreto han sido aprobadas por la FDA como tratamiento para aliviar los síntomas producidos por la enfermedad de ojo seco (258).

Para desarrollar la microemulsión *base* oftálmica se emplearon el oleato de etilo, aceite de soja y escualeno como fase oleosa de la misma. El oleato de etilo fue escogido debido a que se trata de un éster de ácido graso de bajo peso molecular, que puede incrementar la estabilidad del sistema, al contrario que ocurre con ácidos grasos de alto peso molecular o

triglicéridos (259). También algunos autores han estudiado la capacidad de los ácidos grasos insaturados como el ácido oleico para ejercer una acción estabilizadora de la película precorneal incrementando la compresibilidad y la elasticidad de la película sin alterar la baja tensión superficial de la lágrima (260). Por otro lado el empleo del aceite de soja, está sustentado por su alto contenido en triglicéridos insaturados favoreciendo la estabilidad de la película lagrimal (261). En la formulación comercializada a base de aceite de soja Emustil® (262), el aceite de soja, demostró buenos resultados para mejorar los valores de tiempo de ruptura lacrimal ("TBUT", "Tear break-up time"). El último componente escogido para formar parte de la fase oleosa fue el escualeno. El escualeno ha sido estudiado por sus supuestos efectos beneficiosos cardiovasculares y también por poseer diversas propiedades farmacológicas descritas por algunos autores como son su actividad antioxidante, emoliente, antiinflamatoria y antibacteriana (263). Se ha identificado su presencia a bajas concentraciones en lágrimas de pacientes y, se ha comprobado que su aplicación externa era segura y contribuye a la estabilidad de la película lagrimal (264). También supone un componente de vital importancia desde el punto de vista tecnológico, ya que hay estudios donde la adición de escualeno a sistemas basados en nanoemulsiones o microemulsiones produjo una mejora en la estabilidad física de los mismos (265).

Como surfactantes-cosurfactantes para conferir estabilidad al sistema se utilizaron la fosfatidilcolina de soja y el aceite de ricino poli etoxilado comúnmente conocido como Cremophor EL® o Kolliphor EL®. La fosfatidilcolina de soja ha sido muy utilizada en formulaciones liposomales para el tratamiento de la enfermedad de ojo seco o para la administración de fármacos por vía tópica ocular (266). Debido a que la fosfatidilcolina es uno de los lípidos más predominantes en la superficie ocular (267), su adición supone una estrategia atractiva en el desarrollo de nanosistemas lipídicos de administración tópica ocular. El aceite de ricino polietoxilado se ha convertido en una alternativa a la hora de emulsificar sustancias poco hidrosolubles, así como de conferir estabilidad a los sistemas micelares. Su empleo en el desarrollo de nanoemulsiones y microemulsiones también se ha asociado a una disminución de los tamaños de partícula y a una toxicidad más reducida que la asociada a tensoactivos aniónicos convencionales (268). También se empleó el propilenglicol como cosolvente de sustancias lipídicas y/o posible cosurfactante del resto de compuestos mencionados. El propilenglicol ha sido empleado previamente por su buena

tolerancia en el desarrollo de micro y nanoemulsiones para administración tópica ocular de antiinflamatorios (269).

La microemulsión planteada se desarrolló utilizando la habilidad de autoemulsificación (método de baja energía), con bajas concentraciones de surfactantes, en ausencia de disolventes orgánicos y a temperatura ambiente. Esto supone una ventaja frente a otras microemulsiones desarrolladas mediante agitación elevada o elevadas temperaturas (269) ya que se evita la posible degradación térmica de los compuestos a incluir y se mejora la escalabilidad de la misma al prescindir de métodos muy energéticos. Además, presentó transparencia, tamaños de partícula reducidos, alrededor de 20 nm, isotonicidad, potencial Z neutro, viscosidad semejante a soluciones acuosas, con valores fisiológicos de pH y baja tensión superficial lo que la hace con la película lagrimal y a su vez idónea para facilitar su extensibilidad en la superficie ocular. También se decidió comprobar la influencia de la adición de dos polímeros ampliamente conocidos en el campo de la administración tópica ocular: el ácido hialurónico de peso molecular intermedio y el dextrano. La adición de dextrano no modificó de forma significativa ninguna de las características de la microemulsión *base* inicialmente desarrollada. La baja concentración utilizada de dextrano estaría relacionada con que su viscosidad sea similar a la microemulsión *base*. También se pudo confirmar la estructura esférica de las nanogotículas mediante la técnica de cryo-microscopia electrónica de transmisión considerada como la más adecuada para examinar nanosistemas coloidales y lipídicos (270). La correcta visualización de la estructura tridimensional de estos sistemas en el microscopio electrónico de barrido se pudo realizar mediante la modificación de un método de fijación de lípidos empleando verde malaquita en combinación con otros agentes fijadores convencionales (271); en estas preparaciones se pudo apreciar la curvatura de las microemulsiones. La leve neutralización del potencial Z podría estar relacionado con los hallazgos de algunos autores que demostraron la capacidad del dextrano para contrarrestar ligeramente las cargas (272). En el caso de la adición de ácido hialurónico se incrementó considerablemente la viscosidad y levemente los tamaños; este último hecho es probable que sea originado por la tendencia de algunos polímeros hidrofílicos a recubrir gotículas. Este fenómeno ha sido descrito por Wang et al. al desarrollar nanopartículas de quitosano que incrementaron su tamaño al incluir ácido hialurónico (273).

DISCUSIÓN GENERAL

Es necesario indicar, que son pocos los trabajos en los que se han realizado estudios de estabilidad de microemulsiones a largo plazo. En uno de los ellos en particular, se indicó una la leve variación del tamaño de partícula en microemulsiones que fueron almacenadas a 4 ^{o}C durante 2 años (274). En el capítulo III de esta tesis se realiza un estudio de estabilidad completo a 8 ^{o}C y a 25 ^{o}C durante nueve meses donde se evalúa la evolución de las distintas propiedades fisicoquímicas, así como la tolerancia *in vitro* en células epiteliales de córnea. Las microemulsiones empleadas para este estudio de estabilidad comprendieron la microemulsión *base*, una microemulsión con ácido hialurónico (*base* + ácido hialurónico) y una microemulsión con dextrano (*base* + dextrano). En este estudio, se evaluaron los diferentes parámetros anteriormente mencionados de las microemulsiones recién preparadas y al mes, tres meses, seis meses y 9 meses tras su preparación. Los resultados indicaron que las características fisicoquímicas, incluyendo la modificación de tamaños, no experimentaron cambios significativos a lo largo del tiempo, cumpliendo con aquellas características deseadas para la administración ocular (242) (275). Con respecto al valor del potencial Z, se encontraron modificaciones a medida que aumentaba el tiempo de almacenamiento; concretamente aquellas que contenían ácido hialurónico mostraron una neutralización progresiva de la carga con el paso del tiempo, siendo este efecto más pronunciado a temperatura ambiente. En relación a estos resultados, hay estudios que confirman la modificación de la carga de superficie en partículas recubiertas con ácido hialurónico (276).

Se completo el estudio de estabilidad con los ensayos de tolerancia *in vitro* en una línea celular de epitelio corneal mostrando valores adecuados de tolerancia en aquellas microemulsiones conservadas a 8 ^{o}C durante 6 meses de almacenamiento. En las que contenían polímero esta buena tolerancia se amplió hasta el final del ensayo (9 meses). Estos resultados apoyan el uso de estos polímeros también como agentes estabilizadores de la microemulsión *base*, pudiendo mejorar su tolerancia. Bussio et al. demostraron la alta estabilidad coloidal de nanocápsulas de ácido hialurónico atribuyendo dicha propiedad a este polímero. Así mismo, también se ha demostrado un incremento de tolerancia en pacientes de soluciones salinas con ácido hialurónico, especulando el posible efecto antiinflamatorio y/o de bloqueo de cualquier tipo de hiperreactividad externa (277). El dextrano también ha sido estudiado por su habilidad para mejorar la estabilidad de nanosistemas y de encapsulación proteica (278) lo que podría explicar el aumento en la

estabilidad y tolerancia de las microemulsiones desarrolladas. Por el contrario, las microemulsiones conservadas a 25 ^{o}C mostraron ya toxicidad, a partir de los 3 meses de almacenamiento.

De forma paralela al estudio de estabilidad, se decidió añadir sustancias y/o polímeros con capacidad osmoprotectora a la microemulsión *base* inicial; algunos de los compuestos fueron seleccionados a partir del estudio desarrollado en el capítulo II. Se desarrollaron dos tipos de microemulsiones osmoprotectoras denominadas A y B. La microemulsión A contenía la combinación de betaína como agente osmoprotector (279) capaz de regular la apoptosis y mecanismos de regulación del volumen celular (244) y leucina. La incorporación de leucina se debe a la alta cantidad de este aminoácido en el estroma corneal (280) y su empleo como suplemento junto con otros aminoácidos en la enfermedad de ojo seco (281). La microemulsión B incluyo la combinación del activo liposoluble ácido oleanólico y la proteína clusterina. El ácido oleanólico es un triterpeno pentacíclico con similitud estructural al ácido ursólico propuesto para el tratamiento de patologías de la superficie ocular por su poder antiinflamatorio y en particular en aquellos procesos conjuntivales alérgicos (282). El ácido oleanólico a concentraciones similares a las empleadas en la formulación había mostrado previamente ser bien tolerado en el epitelio corneal (283). La clusterina es una proteína que posee estructura heterodimérica unida por puentes disulfuro con una fuerte actividad de chaperona altamente involucrada en el aclaramiento de restos celulares, apoptóticos y procesos antioxidativos (284). Fue incluida en la formulación B debido a su potencial uso como agente protector de la superficie ocular; estudio previos en un modelo animal de ojo seco demostraron la capacidad de sellar la superficie ocular y prevenir el estrés por desecación (285). Mas recientemente se ha propuesto esta proteína como un agente bioterapéutico para el tratamiento de patologías de las superficie ocular (286).

A partir de las microemulsiones osmoprotectoras, A y B, y con el fin de aumentar su tiempo de permanencia sobre la superficie ocular se incluyó en ambos preparados un polímero bioadhesivo: a la microemulsión A se le añadió ácido hialurónico (formulación A-HA) y a la formulación B, se decidió añadir dextrano (B-DXT). Sus caracterizaciones fisicoquímicas demostraron que ambas tenían propiedades adecuadas para la administración tópica ocular (242) (287–289); los de datos obtenidos fueron similares a los comentados para las microemulsiones *base*. En la formulación que contiene ácido

hialurónico (A-HA) se vuelve a observar un incremento de la viscosidad, una leve neutralización del potencial zeta (290) y un aumento del tamaño de partícula. La visualización por técnicas de microscopía electrónica volvió a confirmar la presencia de nanogotículas.

En cuanto a los estudios *in vitro*, tal y como se muestra en los resultados del capítulo, tanto la adición individual de cada uno de los componentes como la combinación de estos junto con la adición de los polímeros fueron muy bien tolerados en células de córnea y de conjuntiva de acuerdo a los criterios establecidos (viabilidad > 80%) (251). Algunos autores en estudios de administración oftálmica de ciertos aminoácidos obtuvieron resultados de seguridad similares a los de esta memoria (281). Además la clusterina también ha sido empleada de manera segura (285), así como el ácido oleanólico (283). Para continuar con los ensayos de tolerancia, una vez comprobada su seguridad *in vitro* se procedió a evaluar la seguridad de las formulaciones osmoprotectoras finales *in vivo* en conejos neozelandeses albinos. La buena tolerancia de las microemulsiones osmoprotectoras desarrolladas, tal y como se observa en este capítulo, apoya el uso de estas como potencial terapia para el tratamiento de patologías de la superficie ocular, aunque se necesitarían estudios de eficacia en modelos *in vivo* de hiperosmolaridad o daño de la superficie ocular.

Partiendo del desarrollo de las microemulsiones anterior diseñadas para administración tópica ocular y teniendo en cuenta los datos obtenidos de tolerancia *in vitro* e *in vivo*, de estabilidad, y de eficacia osmoprotectora, se decidió incluir en las mismas el latanoprost como agente hipotensor. Los resultados obtenidos se recogen en el **capítulo IV, "New trends towards glaucoma treatment: topical osmoprotective microemulsions loaded with latanoprost".** Se trata de desarrollar una formulación híbrida para el tratamiento del glaucoma y al mismo tiempo con actividad protectora de la superficie ocular. En el tratamiento del glaucoma resulta crítico el mantenimiento de una tensión ocular adecuada durante todo el tratamiento (291) ya que su falta de control determina la evolución de los pacientes hacia eventos neurodegenerativos de la retina.

Tal y como se encuentra descrito en la bibliografía, en los cuadros de glaucoma, existe una amplia relación entre el tratamiento hipotensor crónico y la presencia a largo plazo de efectos adversos sobre la superficie ocular. Se producen la perdida de células caliciformes

y situaciones de inflamación que contribuyen a un daño permanente de la córnea y de la conjuntiva (292). Así mismo, la falta de efectividad a largo plazo de los tratamientos hipotensores antiglaucomatosos hace que los clínicos se vean obligados a prescribir en muchas ocasiones combinaciones de los mismos (293) lo cual incrementa el riesgo de efectos adversos en la superficie (294). También merece la pena mencionar que la superficie tiene un tiempo de lavado inferior a 5 minutos con una reposición de la película precorneal entre 2 y 3 minutos por lo que se produce una rápida perdida del principio activo en más de un 95% disminuyendo así su eficacia. Según se ha discutido, en general, solo el 5% restante de la dosis administrada es capaz de alcanzar tejidos intraoculares (295). En este trabajo se combinan agentes osmoprotectores (betaína y leucina o clusterina y ácido oleanólico) y sustancias poliméricas (ácido hialurónico o dextrano), todas con potenciales propiedades para proteger la superficie ocular, con un agente hipotensor. La selección del latanoprost como agente hipotensor de la formulación, se basó en el hecho de la alta efectividad de esta prostaglandina (296).

Las características fisicoquímicas de las microemulsiones hipotensoras con latanoprost y agentes osmoprotectores fueron similares a las desarrolladas previamente sin agente hipotensor y por tanto óptimas para administración tópica ocular. La visualización por microscopia electrónica confirmó que no se produjo ningún tipo de modificación aparente en cuanto a morfología y/o tamaño de las nanogotículas. De forma similar a estudios con nanoemulsiones (297) conteniendo prostaglandinas, la encapsulación fue muy elevada con valores cercanos al 100%.

Según algunos autores, la tolerancia del latanoprost en colirios sin conservantes ha mejorado considerablemente a la vez que se mantiene su eficacia hipotensora (298). A pesar de ello, tal y como se muestra en este trabajo y de acuerdo con estudios publicados, el latanoprost presenta cierta toxicidad en córnea y conjuntiva (252)(252). Es por ello por lo que podemos ver una elevada disminución de la toxicidad cuando el latanoprost se halla incluido en las formulaciones osmoprotectoras frente a la convencional comercializada, obteniéndose valores de viabilidad celular superiores al 80% tanto en córnea como en conjuntiva. El efecto osmoprotector evaluado en el modelo de hiperosmolaridad previamente desarrollado siguió demostrando una gran capacidad osmoprotectora frente al estrés hiperosmolar y, es probable, que cierta disminución del efecto protector que se manifiesta esté asociada al latanoprost.

DISCUSIÓN GENERAL

Intentando establecer la razón de la elevada eficacia hipotensora del principio activo cuando éste se vehiculiza en las microemulsiones, se decidió estudiar la capacidad de internalización de estos sistemas en cultivos celulares. Debido a que la cumarina 6 es altamente liposoluble y existen estudios previos de su encapsulación en liposomas (299) se decidió utilizar este marcador (2 μg/mL) para preparar microemulsiones fluorescentes que permitiesen monitorizar su internalización en células epiteliales de córnea y conjuntiva. La larga permanencia de las microemulsiones con baja concentración de agente fluorescente junto con el corto periodo de exposición (5 minutos) sugiere una alta internalización celular. Concretamente el empleo de ácido hialurónico mostró una elevada fluorescencia indicando un incremento de la permanencia de las nanogotículas, lo que ha sido indicado ya por otros autores (300). La citometría de flujo también confirmó la alta internalización de estos sistemas y en particular produciendo un incremento sostenido de la señal en aquellas microemulsiones que incorporaban ácido hialurónico o dextrano hasta 11 días de estudio. La técnica de citometría ha sido anteriormente empleada y validada como método robusto para evaluar la internalización celular de nanosistemas, en particular de las nanoemulsiones y emulsiones (301,302).

En la misma dirección de los estudios de internalización, y para profundizar en los mecanismos de penetración y distribución de los sistemas desarrollados en cultivos celulares de córnea, se pretendió estudiar las interacciones de las microemulsiones finales osmoprotectoras conteniendo latanoprost con las membranas celulares. También se pretendió evaluar su distribución y almacenamiento en las diferentes estructuras celulares. Son algunos autores los que han estudiado la interacción de nanosistemas como nanopartículas de oro en microscopia electrónica de barrido (303) y de nanoemulsiones sin tinciones específicas para lípidos en microscopia electrónica de transmisión (304). De acuerdo con una revisión previa y desde nuestro conocimiento, este es el primer estudio que muestra en líneas celulares, en particular de córnea, la interacción con membranas y la distribución en el interior celular de microemulsiones. La fijación *in situ* de las nanogotículas lipídicas permitió poder detallar como dichos sistemas poseían la habilidad para abrir pequeños poros en la pared celular y por tanto internalizarse rápidamente. Estos hallazgos confirman las especulaciones de ciertos autores que atribuían, a ciertas microemulsiones, la capacidad para abrir pequeños poros en las membranas de manera reversible probablemente debido a los surfactantes (305). También se hizo patente la

distribución de las microemulsiones en las diferentes estructuras celulares, desde mitocondrias pasando por retículo endoplásmico hasta el núcleo, aunque particularmente su localización se centraba en las vacuolas. En este trabajo se postula la posibilidad de que las microemulsiones se almacenen en las vacuolas y de esta manera actúen como pequeños reservorios consiguiendo una liberación sostenida del principio activo durante largos periodos de tiempo.

Esta internalización explicaría el efecto hipotensor observado, tan a largo plazo, en las formulaciones de las microemulsiones. Con una sola instilación de 25 µL se consiguió un efecto hipotensor significativo de entre 4 y 6 días para aquellas formulaciones que no contenían agentes mucoadhesivos, de 8-9 días para aquellas con dextrano y de 13-14 días para las que incluían ácido hialurónico. En relación con estos hallazgos, hay un estudio que demostró mediante la inclusión de microemulsiones en lentes de contacto una reducción de la presión intraocular prolongada durante 3-4 días (306).

Es interesante resaltar, que las microemulsiones *base* sin incluir agentes hipotensores mostraron también un cierto efecto hipotensor, llegando este a ser similar a la formulación comercializada particularmente en el caso de las microemulsiones *base* incluyendo polímeros mucoadhesivos. Por tanto, el efecto hipotensor intrínseco de las formulaciones desarrolladas podría asociarse a sus componentes, como son los ácidos grasos insaturados (oleato de etilo, aceite de soja, fosfatidilcolina de soja y escualeno). Algunos autores han apuntado la actividad hipotensora de estas sustancias en modelos animales de hipertensión (307).

También, es importante destacar, que estudios anteriores han establecido una relación directa entre los ácidos grasos insaturados, sobre todo los extraídos de la lecitina de soja y la regulación de los canales BK de potasio activados por Ca^{2+} de alta conductividad presentes en la musculatura lisa (308). Además, otros trabajos han evaluado la función de esta musculatura lisa en la malla trabecular para regular el drenaje del humor acuoso (308,309). En la misma línea, Cuppoletti et al. demostraron que el análogo de prostaglandina, unoprostona, poseía una alta capacidad hipotensora debido a la activación de los canales de alta conductividad BK mencionados anteriormente, cuya presencia en el canal de Schlemm ha sido confirmada. Su activación mostró una función reguladora del volumen acuoso en el segmento anterior, incrementando el drenaje del humor acuoso y

consiguiendo una reducción considerable de la PIO (310). Por ello, es muy probable que debido a la alta tasa de internalización de las microemulsiones desarrolladas, estas atraviesen la malla trabecular y debido a la presencia de ácidos grasos insaturados en su composición activen los canales BK reduciendo la PIO.

Las biodisponibilidades relativas entre formulaciones calculadas a partir de las áreas bajo las curvas del efecto hipotensor-tiempo (311) utilizando la formulación comercial como referencia, tomaron valores que oscilan desde 4-5 veces superiores para las microemulsiones sin polímeros hasta de 10 y 18 veces superiores en aquellos incluyendo dextrano y ácido hialurónico respectivamente. Estos resultados mostraron una marcada superioridad de los nanosistemas desarrollados frente al colirio comercializado.

Estos resultados supondrían una elevada eficacia de las formulaciones desarrolladas en esta memoria para vehiculizar sustancias hipotensoras al mismo tiempo que presentan propiedades protectoras de la superficie ocular.

Desafortunadamente en muchas ocasiones el tratamiento crónico de la terapia antiglaucomatosa fracasa y se desarrollan eventos de estrés y muerte celular que desencadenan los procesos degenerativos de la retina y del nervio óptico. Por ello, es necesario la investigación de nuevos fármacos y el desarrollo de nuevos sistemas de liberación sostenida que sean fáciles de administrar, biodegradables y biocompatibles. El último capítulo de esta memoria se ha dedicado a la síntesis de un nuevo polímero sensible a la temperatura, útil para la preparación de formulaciones acuosas capaces de gelificar *in situ* en condiciones fisiológicas mediante mecanismos de agregación micelar. Para su obtención se parte de ácido poli(láctico-co-glicólico) y de polietilenglicol. El trabajo recogido en el **capítulo V, "Thermo-Responsive PLGA-PEG-PLGA Hydrogels as Novel Injectable Platforms for Neuroprotective Combined Therapies in the Treatment of Retinal Degenerative Diseases"**, trata de solventar los problemas asociados a la liberación sostenida de fármacos por vía intravítrea que en muchos casos es breve, ineficaz y conlleva ciertos problemas asociados a la administración.

Normalmente uno de los inconvenientes más comunes asociados a la administración intravítrea es la alta frecuencia de administraciones para mantener concentraciones efectivas en el lugar de acción, lo que puede dar lugar a complicaciones asociadas a la inyección tales como inflamación, hemorragia o incluso desprendimiento de retina (312).

La falta de concentraciones efectivas también es una de las causas por las que la progresión de la neurodegeneración continua (313). Del mismo modo, la administración repetida puede causar finalmente disconformidad al paciente y una falta de adherencia al tratamiento. Otra dificultad a tener en cuenta a la hora de administrar posibles hidrogeles o sistemas de cesión sostenida es la inyectabilidad del preparado. Respecto a ese tema, se describen inconvenientes dependiendo del sistema utilizado. Así, existen algunos hidrogeles que gelifican espontáneamente en menos de 1 minuto tras el mezclado de dos fases, otros con alta viscosidad difíciles de administrar y aquellos que pueden agregarse y llegar a colmatar agujas de pequeños calibres (209).

En ese sentido, la síntesis de polímeros que permiten la creación de soluciones acuosas con capacidad de gelificación espontánea a temperaturas fisiológicas facilita la administración de agentes enfocados al tratamiento a largo plazo de eventos degenerativos de la retina. Así mismo, los dos polímeros desarrollados con proporciones distintas de ácido láctico, ácido glicólico y polietilenglicol se caracterizaron fisicoquímicamente para evaluar si eran adecuados para la administración intravítrea. Los polímeros desarrollados en este trabajo fueron producidos por uno de los métodos de síntesis considerados “verdes” ya que no emplea ningún tipo de solvente, llamado método de apertura del anillo (314). Mediante esta técnica se desarrolló un tipo de polímero perteneciente a los llamados copolímeros tribloque formado por unidades repetidas de ácido poli(láctico-co-glicólico) junto con polietilenglicol conocido como iniciador de la reacción. Este tipo de polímeros son particularmente atractivos debido a su biodegradabilidad en medios acosos y a su aprobación por la FDA (315). El polímero dio lugar a un sistema translucido en agua, completamente fluido y con fácil inyectabilidad capaz de formar geles a temperaturas fisiológicas. La caracterización se realizó mediante técnicas de resonancia magnética nuclear y GPC, para determinar los pesos moleculares y la estructura de los polímeros desarrollados. Los estudios mediante la prueba del “frasco invertido” mostraron que el copolímero 2 con menor proporción de ácido glicólico y láctico tendía a gelificar y precipitar antes; lo cual podría suponer ciertos inconvenientes, ya que la ventana de precipitación se situaba a temperaturas cercanas a las fisiológicas del humor vítreo. Algunos autores han relacionado un incremento en la proporción de ácido glicólico con la velocidad de degradación de este tipo de polímeros (315). Debido a la alta acidez que este tipo de sistemas pueden proporcionar a las soluciones acuosas, se optimizó el preparado

con tampón carbonato de pH 8,8. Algunos estudios han demostrado que la adición de tampones carbonato a ciertos compuestos orgánicos puede modificar la viscosidad del sistema (316), y que la concentración crítica micelar también puede disminuir en presencia de tampones reguladores del pH (317). Debido a este efecto, es probable que los valores de viscosidad del módulo G' fueran más elevados para aquellos polímeros disueltos en el tampón carbonato al ser mayor la cantidad de micelas y aumentar la agregación.

En algunos estudios se indica que los fármacos y sustancias neuroprotectoras incluidas en los hidrogeles también podrían tener un efecto en el tamaño de las micelas y por ello modificar el comportamiento de los hidrogeles (318). En las formulaciones finales desarrolladas en esta memoria con el copolímero 1 y que contenían activos, se obtuvieron valores de viscosidad más elevados que aquellos sin fármacos. El perfil de liberación de los principales compuestos a estudiar (dexametasona y ketorolaco) también se vio influenciado en cierto modo por la presencia de otros activos siendo de 62 días para el fosfato de dexametasona y de 53 días para el ketorolaco trometamina. Tal y como se muestra en ciertos trabajos, y debido a su coeficiente de partición (319) es probable que la idebenona desplace parte de la dexametasona por interacción con las micelas incrementando su presencia en la solución acuosa y por ende incrementando su perfil de liberación (320). Las cinéticas de liberación para todos ellos se ajustaron al modelo de Korsmeyer-Peppas. Según la ecuación de dicho modelo, todos los hidrogeles cargados con ketorolaco mostraron un transporte anómalo el cual puede explicar la difusión de fármaco a través del hidrogel asícomo liberación explicada por mecanismos de erosión de la matriz, mostrando un comportamiento similar en los hidrogeles de dexametasona salvo el de dexametasona sin sustancias coadyuvantes. En este último solo se cumple difusión de la sustancia activa a través de la matriz. Además, estos resultados se corroboran con el factor de similitud f_2 el cual muestra una similitud entre los hidrogeles de dexametasona con idebenona o TPGS, pero diferencias con el de dexametasona sin coadyuvante. Así mismo, el factor f_2 para los hidrogeles de ketorolaco mostró que los tres eran similares desde el punto de vista de la liberación. El incremento de la liberación de la dexametasona en el hidrogel que contenía vitamina E pegilada puede deberse a las propiedades emulsificantes de esta vitamina, incrementando por tanto la cantidad de dexametasona disuelta en el medio acuoso. También se ha descrito la habilidad de la idebenona para interaccionar directamente con las cadenas de ácido poli(láctico-co-glicólico) (321) y por tanto

modificar los perfiles de liberación de sustancias afines a ellas con hidrosolubilidad limitada. Al igual que con la dexametasona se especuló la posibilidad de que tanto la idebenona como la vitamina E pegilada estuvieran involucradas en la liberación de ketorolaco, pero a diferencia de la dexametasona, el ketorolaco trometamina es mucho más soluble en soluciones acuosas (322) lo que podría explicar que la liberación del mismo estuviese mucho menos influenciada por la adición externa de sustancias activas.

La toxicidad celular *in vitro* se utilizó para seleccionar que tipo de hidrogeles eran mejor tolerados, determinar la citocompatibilidad de las sustancias y seleccionar las concentraciones mejor toleradas. Al contrario que con la mayoría de estudios que emplean hidrogeles, se adaptó el ensayo de reducción de la sal de tetrazolio para evaluar la toxicidad directa por contacto entre los hidrogeles y las células de epitelio pigmentario de la retina (323). Los hidrogeles con mayor proporción de ácido glicólico (copolímero 2) presentaron una mayor toxicidad celular tras 24 horas de exposición por lo que se descartaron; además estos hidrogeles tenían una tendencia a la precipitación a temperaturas fisiológicas similares a las del vítreo. Respecto a las formulaciones finales desarrolladas, aquellas con dexametasona presentaron una mayor viabilidad en consonancia con los datos de viabilidad individuales de diferentes concentraciones de dexametasona. Esto es debido según algunos estudios, a la cierta capacidad de la dexametasona para inducir proliferación de células de la retina (324).

Teniendo en cuenta que gran parte de los mecanismos de muerte celular producidos en eventos degenerativos de la retina son debidos en gran medida al estrés oxidativo (325), se decidió desarrollar un modelo sencillo y reproducible de estrés oxidativo inducido por peróxido de hidrogeno durante 24 horas. Todas las formulaciones neuroprotectoras desarrolladas mostraron entre un 60 y un 80% de actividad protectora frente al control de daño sin tratamiento y en concreto, el polímero que contenía dexametasona e idebenona mostró los mayores valores de protección. Estos hallazgos coinciden con los trabajos en los que se reporta la actividad neuroprotectora de la dexametasona (326) y la neuroprotección mediada por antioxidación de la idebenona (327).

El efecto protector del ketorolaco también puede ser explicado por los estudios que demuestran su capacidad para reducir el estrés celular asociado a especies reactivas de oxígeno (328). Aunque la dexametasona tiene un carácter fuertemente antinflamatorio, el

ketorolaco ha demostrado una capacidad superior para inhibir la producción de factor de necrosis tumoral alfa en células de epitelio pigmentario de la retina. Según estos resultados, la utilización de ketorolaco podría ser de especial interés como agente neuroprotector. Además serviría también para atenuar la inflamación asociada a las inyecciones intravítreas en la administración de este tipo de tratamientos (329).

CONCLUSIONES/CONCLUSIONS

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CONCLUSIONES

1. El modelo de estrés *in vitro* desarrollado en esta tesis simulando un ambiente de estrés hiperosmolar en combinación con la evaluación de la capacidad antiinflamatoria de diferentes compuestos a estudiar, supone una herramienta sencilla y efectiva para el cribado de posibles sustancias osmoprotectoras capaces de evitar procesos de muerte celular asociadas a estrés hiperosmolar, asi como para inhibir la producción de factor de necrosis tumoral alfa.

2. Los polímeros y activos seleccionados con el modelo de estrés *in vitro* suponen una estrategia atractiva para su inclusión en formulaciones osmoprotectoras al demostrar elevada capacidad para evitar procesos de muerte celular en ambientes hiperosmolares, evitar la apoptosis, regular el volumen celular en ambientes de estrés hipertónico y contrarrestar procesos inflamatorios asociados a la enfermedad del ojo seco.

3. Se ha desarrollado una microemulsión base que presenta características adecuadas para la administración oftálmica. Esta microemulsión sirve de base para la preparación de lágrimas artificiales y como vehículo de principios activos altamente lipófilos. Dicho nanosistema presentó una estabilidad adecuada desde el punto de vista fisicoquímico en condiciones de refrigeración durante al menos 9 meses. La estabilidad además se mantuvo y/o mejoró con la adición de ácido hialurónico y dextrano.

4. Las microemulsiones desarrolladas exhibieron una alta internalización celular junto con una amplia distribución en las membranas y en los diferentes orgánulos celulares, particularmente en las vacuolas. Estas características permiten su consideración potencial como sistemas transportadores en terapia génica.

5. Las formulaciones osmoprotectoras desarrolladas como lagrimas artificiales de nueva generación presentan características óptimas para la administración tópica ocular, demostrando una tolerancia *in vitro* e *in vivo* adecuadas, así como la capacidad para incrementar la supervivencia celular en ambientes hipertónicos.

6. Las formulaciones osmoprotectoras que incluyeron latanoprost como agente antiglaucomatoso, formulaciones híbridas, demostraron características fisicoquímicas adecuadas para la administración por vía tópica ocular, con una elevada encapsulación del agente hipotensor, buena tolerancia *in vitro,* así como propiedades osmoprotectoras

en células epiteliales de córnea presentándose como sistemas de interés para evitar el daño crónico asociado a la administración crónica de estos agentes hipotensores.

7. Las formulaciones hibridas (microemulsiones osmoprotectoras con latanoprost) mostraron una actividad hipotensora en animales de experimentación, de intensidad y duración muy superior a la formulación comercializada (establecida como referencia) siendo, todavía, más pronunciada con la adición de agentes poliméricos. Asi mismo, aquellas microemulsiones base sin incluir agente hipotensor demostraron también cierta actividad hipotensora incrementándose con la adición de agentes mucoadhesivos.

8. Los polímeros desarrollados a base de polietilenglicol y ácido poli(láctico–co–glicólico) sintetizados en la presente tesis producen hidrogeles translucidos, con gelificación (temperatura-dependiente) en las condiciones fisiológicas del segmento posterior y características óptimas para su inyectabilidad intraocular

9. Los hidrogeles termosensibles desarrollados han demostrado buena tolerancia en células epiteliales pigmentarias de la retina, habilidad para proteger frente a eventos de muerte celular asociados a estrés oxidativo y capacidad antinflamatoria. Además, los hidrogeles seleccionados han mostrado una liberación sostenida de principios activos antiinflamatorios y neuroprotectores durante al menos 50 días.

CONCLUSIONES/CONCLUSIONS

CONCLUSIONS

1. The *in vitro* model developed in these manuscripts, simulating a hyperosmolar environment in combination with the evaluation of the anti-inflammatory capacity of different potential compounds, entails a simple and effective tool for the screening of osmoprotective substances capable of avoiding cell death processes associated with hyperosmolar stress as well as to inhibit the production of tumor necrosis factor alpha.

2. The substances studied in the hyperosmolar model are an attractive strategy for their inclusion in osmoprotective formulations since they have demonstrated the ability to avoid cell death induced by hyperosmolar environments, avoid apoptosis, up- or down-regulate cell volume under hypertonic stress and counteract inflammatory processes.

3. It has been developed a base microemulsion with suitable properties for ophthalmic administration. This microemulsions entails a starting point for the development of artificial tears as a vehicle for the inclusion of lipophilic susbtances. This microemulsions exhibited good stability from the physicochemical point of view under refrigeration for at least 9 months. The stability remained/improve with the addition of hyaluronic acid and dextran.

4. The osmoprotective formulations developed as artificial tears show optimal characteristics for topical ophthalmic administration, exhibiting suitable *in vitro* and *in vivo* tolerance, as well as the ability to increase cell survival in hypertonic environments.

5. The osmoprotective formulations including hypotensive agent latanoprost, hybrid formulations, demonstrated suitable properties for topical ophthalmic administration, high encapsulation rates of hypotensive agent, well good *in vitro* tolerance as well as presenting osmoprotective properties in corneal epithelial cells.

6. The hybrid formulations (osmoprotective microemulsions with latanoprost) showed hypotensive activity in experimental animals, of much greater intensity and duration than the marketed formulation (established as a reference), being even more pronounced with the addition of polymeric agents. Likewise, those *base* microemulsions without including hypotensive agent also showed some hypotensive activity, increasing with the addition of mucoadhesive agents.

CONCLUSIONES/CONCLUSIONS

7. The developed hybrid microemulsions exhibited a high cellular internalization together with a wide distribution across the membranes as well as the different cellular organelles, particularly in the vacuoles.

8. The polymers developed based on polyethylene glycol and poly (lactic-co-glycolic) acid synthesized in this thesis produce translucent hydrogels, with gelation (temperature-dependent) under the physiological conditions of the posterior segment as well as optimal characteristics for injectability.

9. The thermoresponsive hydrogels developed have shown good tolerability in retinal pigmented epithelial cells, ability to protect against cell death associated with oxidative stress and anti-inflammatory capacity. In addition, the selected hydrogels have shown a sustained release of anti-inflammatory and neuroprotective active ingredients for at least 50 days.

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ANEXOS

ANEXOS

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

Número de solicitud:	P202131205	
Fecha de recepción:	23 diciembre 2021, 13:45 (CET)	
Oficina receptora:	OEPM Madrid	
Su referencia:	271/21	
Solicitante:	Universidad Complutense de Madrid	
Número de solicitantes:	1	
País:	ES	
Título:	Microemulsión oftálmica, procedimiento de obtención y uso dado a la misma	
Documentos enviados:	Descripcion.pdf (14 p.) Reivindicaciones.pdf (2 p.) Resumen.pdf (1 p.) Dibujos.pdf (7 p.) OLF-ARCHIVE.zip FEERCPT-1.pdf (1 p.) FEERCPT-2.pdf (1 p.)	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	C=ES,O=HERRERO & ASOCIADOS SL,2.5.4.97=#0C0F56415445532D423238383635323336,CN=02892782A GUSTAVO ADOLFO GONZALEZ (R: B28865236),SN=GONZALEZ PECES,givenName=GUSTAVO ADOLFO,serialNumber=IDCES-02892782A,description=Ref:AEAT/AEAT0307/PUESTO 1/37016/22102021115111	
Fecha y hora de recepción:	23 diciembre 2021, 13:46 (CET)	
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DESCRIPCIÓN

Microemulsión oftálmica, procedimiento de obtención y uso dado a la misma

La presente invención se refiere a microemulsiones oftálmicas compatibles con la película precorneal que se pueden utilizar como lágrimas artificiales y como vehículo de fármacos para su administración por vía tópica ocular. De la misma manera se refiere al procedimiento de obtención de la microemulsión.

Antecedentes de la invención

La superficie ocular está compuesta por varias capas entre las cuales se encuentran el epitelio corneal, epitelio conjuntival, glándulas de meibomio y glándulas lacrimales.

Así mismo, recubriendo las mismas se distribuye una fina película conocida como película precorneal o lagrimal, con componentes acuosos, mucinosos y lipídicos cuya principal función es lubricar y proteger la superficie ocular. Esto contribuye a evitar la evaporación de la fracción acuosa y a preservar la osmolaridad normal de la lágrima (≈300 mOsm/L). Cuando se produce una evaporación excesiva del componente acuoso o un defecto en la secreción del componente lipídico encargado de evitar la evaporación de la fracción acuosa, se produce un incremento en la osmolaridad de la lágrima (>330 mOsm/L), que desencadena una serie de procesos inflamatorios asociados a alteraciones en la superficie ocular y pérdida de células del epitelio conjuntival y corneal, manifestándose la enfermedad de ojo seco (EOS). Estos procesos desencadenan una cascada de sucesivos eventos inflamatorios y muerte celular que cronifican esta enfermedad.

Se han desarrollado diferentes sistemas de administración tópica ocular de fármacos con el objetivo de disminuir la sintomatología de ojo seco y contrarrestar los diversos eventos de muerte celular e inflamación en la superficie ocular. Se hayan en investigación y desarrollo formulaciones liposomales para el tratamiento de ojo seco debido a su capacidad para proveer a la película precorneal de la película lipídica, así como para encapsular sustancias activas tanto hidrófilas como hidrófobas.

Algunos autores han señalado el potencial sobre la utilización de nanoemulsiones para la inclusión de principios activos hidrófilos y lipófilos en el tratamiento de diversas patologías oculares. Las nanoemulsiones son dispersiones coloidales tanto acuosas como oleosas en

las que la fase dispersa forma gotículas nanométricas comprendidas entre 100 y 400 nm en la fase dispersante. Estas pueden ser del tipo W/O u O/W en función de cuál sea la fase dispersante o mayoritaria (aceite o agua respectivamente). A su vez se caracterizan por necesitar un aporte energético para su elaboración siendo por tanto termodinámicamente inestables. Debido a esto, muchas nanoemulsiones son producidas mediante homogenización a alta presión, ya que se necesita superar la energía de barrera para formar un sistema estable. Por lo tanto, es de gran interés el desarrollo de emulsiones más sencillas de obtener.

En la misma línea, se ha descrito ampliamente una asociación entre el uso de agentes antiglaucomatosos de forma crónica y la aparición, el empeoramiento o el desarrollo de la enfermedad de ojo seco. En muchos casos, las formulaciones antiglaucomatosas contienen como conservante cloruro de benzalconio, el cual tiene toxicidad sobre el epitelio corneal, el conjuntival y la superficie ocular en general. El cloruro de benzalconio produce un incremento en la expresión de marcadores inflamatorios en los epitelios corneal y conjuntival, y disminuye la densidad de células caliciformes. Estos eventos conllevan una inflamación crónica, así como una desestabilización de la película prelagrimal con la consiguiente aparición de un ambiente hiperosmolar. En muchos casos la enfermedad de ojo seco producida por la inestabilidad de la película prelagrimal puede exacerbarse debido a la administración de los agentes antiglaucomatosos en régimen de dosis múltiple. Por lo tanto, es de interés el desarrollo de un producto antiglaucomatoso que no implique todos estos problemas.

En la patente EP1985298 se describe una emulsión ocular con prostaglandina.

En la presente invención se describe una microemulsión ocular con capacidad de utilizarse como lagrima artificial y como vehículo de medicamentos oculares.

Descripción de la invención

En la presente invención se ha desarrollado una microemulsión oftalmológica que tienen la capacidad de solubilizar fármacos hidrofóbicos que son difíciles de vehiculizar en medio acuoso y/o para aumentar la baja biodisponibilidad de aquellos que tienen una permeabilidad disminuida.

Las microemulsiones se caracterizan por tener tamaños de gotículas nanométricos,

normalmente comprendidos entre 10 nm y 100 nm. La reducción del diámetro de gotícula, implica un incremento de su superficie activa, viéndose incrementada la funcionalidad de la sustancia medicinal y el efecto terapéutico. Además, no necesitan aporte energético para su elaboración siendo esta espontánea y termodinámicamente estables, con características análogas a una disolución ideal. Debido a esto, su perfil de estabilidad puede durar años, ya que su perfil energético es muy distinto a las macro- y nano- emulsiones. Al igual que las anteriores, pueden existir de varios tipos en función de la naturaleza de la fase dispersa y dispersante (w/o u o/w). Contrariamente a lo que se cree, el tamaño de gotícula de las nanoemulsiones está comprendido entre las macroemulsiones y las microemulsiones (aproximadamente 100-500 nm).

Así mismo, la naturaleza de las microemulsiones es particularmente atractiva para la vía de administración ocular debido a que forman soluciones transparentes con baja tensión superficial y tamaños de gotícula muy pequeños, lo que promueve un aumento en la internalización de las sustancias activas. Las microemulsiones oftálmicas de la invención son compatibles con la película precorneal.

Por tanto, se presentan las microemulsiones como sistemas termodinámicamente estables con una capacidad superior de internalización con potencial uso para transportar sustancias activas al interior celular, concretamente cuando se trata de barreras cuya función principal es la de impedir el paso de sustancias tales como las presentes en la superficie ocular.

Las microemulsiones incluyen una fase acuosa y otra oleosa, e incorporan agentes surfactantes y cosurfactantes para la formación de la microemulsión.

Por lo tanto, el primer aspecto de la invención se refiere a una microemulsión oftálmica que comprende:
una fase acuosa y
una fase oleosa que comprende: un aceite vegetal, un éster de ácido graso farmacéuticamente aceptable, un compuesto glicol, escualeno, agentes surfactantes y agentes cosurfactantes.

El término microemulsión se refiere a una emulsión con un tamaño de gotícula comprendido entre 10 nm a 100 nm.

El término “compuesto glicol” se refiere a un compuesto con dos o más sustituyentes

hidroxilo.

El término “aceite vegetal” se refiere a una mezcla de triglicéridos de origen vegetal o similar. Ejemplos de aceites vegetales son aceite de argán, aceite de maíz, aceite de palma, aceite de coco, aceite de oliva, aceite de cacahuete, aceite de colza, aceite de girasol, aceite de sésamo, aceite de soja.

El término “éster de ácido graso farmacéuticamente aceptable” se refiere a un éster de alquilo C_{1-6} de un ácido graso C_{10-20}, que es adecuado para su uso en contacto con los tejidos de seres humanos y animales sin toxicidad, irritación, respuesta alérgica.

El término “agentes cosurfactantes” se refiere a compuestos que junto a los surfactantes estabilizan la microemulsión.

El procedimiento objeto de la invención que aquí se describe se refiere a la preparación de microemulsiones en medio acuoso compatibles con la película precorneal.

Un segundo aspecto de la invención se refiere a un procedimiento de obtención de la microemulsión de la invención que comprende las etapas de:
a) mezclar los compuestos que comprende la fase oleosa;
b) añadir la fase acuosa;
c) filtrar
d) estabilizar.

Los últimos aspectos de la invención se refieren a los usos que son la utilización de la microemulsión de la invención como lagrimas artificiales y como vehículo de principios activos con baja biodisponibilidad y/o poco hidrosolubles para facilitar su administración por vía tópica ocular.

Por lo tanto, otro aspecto se refiere a la microemulsión para su uso como lágrimas artificiales. Igualmente, la invención se refiere al uso de la microemulsión definida arriba para su uso como vehículo de fármacos para su administración por vía tópica ocular y para su uso en el tratamiento de la hipertensión ocular.

Finalmente, también se refirió al uso de la microemulsión para su uso en el tratamiento del glaucoma.

Breve descripción de los dibujos

La Figura 1 muestra la morfología de la microemulsión-base (MB) visualizada mediante cryo-TEM a 73.000 aumentos (a) y SEM a 200.000 aumentos (b). La escala situada en la parte inferior derecha comprende un tamaño de 50 nm (a) y 200 nm (b). Las microemulsiones comprendidas en el rango anteriormente descrito se muestran con una flecha.

La Figura 2 muestra una distribución de tamaños de la microemulsión-base (MB) representados en escala semi-logarítmica expresados como % Chan (porcentaje de volumen en el canal) frente al tamaño expresado en nanómetros.

La Figura 3 muestra un reograma obtenido para el estudio de viscosidad de la microemulsión-base (MB). Se representa esfuerzo de cizalla (Pa) y viscosidad (η) frente a velocidad de cizalla (1/s).

La Figura 4 muestra una viabilidad celular (%) con el vehículo base incubado a 37ºC durante 1 y 4 horas en líneas celulares epiteliales de córnea y conjuntiva. Se utilizan dos controles, uno positivo (+; cloruro de benzalconio 0,005%) y otro negativo (-; solución acuosa de NaCl 0,9%).

La Figura 5 muestra resultados del ensayo de captación celular de la formulación microemulsión-base (MB) por células epiteliales de cornea humana (HCE). Se muestran células HCE expuestas a una suspensión de cumarina 10 µg/mL durante 15 minutos (a), MB cargada con cumarina 10 µg/mL durante 15 minutos (b), suspensión de cumarina 10 µg/mL durante 30 minutos (c) y a MB cargada con cumarina 10 µg/mL durante 30 minutos (d). La barra de escala representa 50 µm.

La Figura 6 muestra una morfología de la microemulsión-base con ácido hialurónico (MB-HA) visualizada mediante cryo-TEM a 73.000 aumentos (a) y SEM a 200.000 aumentos (b). La escala situada en la parte inferior derecha comprende un tamaño de 50 nm (a) y 200 nm (b). Las microemulsiones comprendidas en el rango anteriormente descrito se muestran con una flecha.

La Figura 7 muestra una distribución de tamaños de la microemulsión-base con ácido hialurónico (MB-HA) representados en escala semi-logarítmica expresados como % Chan

(porcentaje de volumen en el canal) frente al tamaño expresado en nanómetros.

La Figura 8 muestra un reograma obtenido para el estudio de viscosidad de las microemulsión-base con ácido hialurónico (MB-HA). Se representa esfuerzo de cizalla (Pa) y viscosidad (η) frente a velocidad de cizalla (1/s).

La Figura 9 muestra la viabilidad celular (%) de la microemulsión con hialuronato sódico (MB-HA) incubada a 37ºC durante 1 y 4 horas en líneas celulares epiteliales de córnea y conjuntiva. Se utilizan dos controles, uno positivo (+; cloruro de benzalconio 0,005%) y otro negativo (-; solución acuosa de NaCl 0,9%).

La Figura 10 muestra la viabilidad celular (%) de microemulsión-base con sustancias osmoprotectoras y reepitelizantes incubadas a 37ºC durante 1 y 4 horas en líneas celulares epiteliales de córnea. Se utilizan dos controles, uno positivo (+; cloruro de benzalconio 0,005%) y otro negativo (-; solución acuosa de NaCl 0,9%).

La Figura 11 muestra presiones intraoculares (expresadas en porcentaje de la presión basal) obtenidas con la formulación MB-HA (ΔPIO>10 %) en comparación con las encontradas con la microemulsión-base que se utiliza como control (ΔPIO<10 %).

La Figura 12 muestra una morfología de la microemulsión con hialuronato sódico y latanoprost (MB-HA-Latanoprost) visualizada mediante cryo-TEM a 73000 aumentos (a) y SEM a 100000 aumentos (b). La escala situada en la parte inferior derecha comprende un tamaño de 50 nm (a) y 200 nm (b). Las microemulsiones comprendidas en el rango anteriormente descrito se muestran con una flecha.

La Figura 13 muestra una distribución de tamaños de la microemulsión con ácido hialurónico y latanoprost (MB-HA-Latanoprost) representados en escala semi-logarítmica expresados como % Chan (porcentaje de volumen en el canal) frente al tamaño expresado en nanómetros.

La Figura 14 muestra un reograma obtenido para el estudio de viscosidad de las microemulsión con ácido hialurónico y latanoprost (MB-HA-Latanoprost). Se representa esfuerzo de cizalla (Pa) y viscosidad η (Pa·s) frente a velocidad de cizalla (1/s).

La Figura 15 muestra la viabilidad celular (%) de microemulsión con hialuronato sódico y

latanoprost (MB-HA-Latanoprost) incubadas a 37ºC durante 1 y 4 horas en líneas celulares epiteliales de córnea y conjuntiva. Se utilizan dos controles, uno positivo (+; cloruro de benzalconio 0,005%) y otro negativo (-; solución acuosa de NaCl 0,9%).

La Figura 16 muestra presiones intraoculares expresadas en porcentaje de la presión basal obtenidas con la formulación MB-HA-Latanoprost y el Monoprost comercial que se usa como referencia en el ensayo. La microemulsión-base (MB) se emplea como formulación control.

Descripción de una realización preferida

Como se ha dicho el primer aspecto de la invención se refiere a una microemulsión oftálmica que comprende:

una fase acuosa y

una fase oleosa que comprende: un aceite vegetal, un éster de ácido graso farmacéuticamente aceptable, un compuesto glicol, escualeno, un agente surfactante y un agente cosurfactante.

De manera preferente el aceite vegetal está en una concentración en masa respecto a la masa total de la microemulsión en un rango comprendido entre 0,001% y el 2%, el éster de ácido graso farmacéuticamente aceptable en una concentración en masa respecto a la masa total de la microemulsión en un rango comprendido entre el 0,05% y el 4% y el compuesto glicol está en una concentración en masa respecto a la masa total de la microemulsión en un rango comprendido entre el 0,5% y el 4%.

De manera particular en la fase oleosa de la invención el aceite vegetal es aceite de soja, el éster de ácido graso farmacéuticamente aceptable es oleato de etilo y el compuesto glicol es propilenglicol que favorece la disolución de los componentes hidrofóbicos.

De manera preferente el surfactante de la microemulsión está en una concentración en masa respecto a la masa total de la microemulsión en un rango comprendido entre 0,1% y el 2% y el cosurfactante está en una concentración en masa respecto a la masa total de la microemulsión en un rango comprendido entre el 0,5% y el 3%.

De manera particular como surfactantes de la microemulsión es fosfatidilcolina de soja y el cosurfactante es ricino polioxílico 35 (Kolliphor EL®). En concreto la fosfatidilcolina, tras su administración, va a disminuir la tensión superficial de la película lagrimal lo que favorece su rápida extensibilidad e interacción con las mucinas de la superficie ocular.

En general a las microemulsiones se les pueden añadir diferentes compuestos activos que, sin ser fármacos, tienen propiedades osmoprotectoras, reepitelizantes, antiinflamatorias y/o antioxidantes y protegen a la superficie ocular de posibles efectos adversos que se pueden producir con el uso continuado de la preparación durante largos periodos de tiempo. Estos compuestos según su naturaleza se incluirán en la fase acuosa o en la fase oleosa.

De manera preferente la fase oleosa comprende activos liposolubles seleccionados entre: ácido oleanólico, ubiquinol y/o vitaminas liposolubles.

De manera preferente el escualeno se encuentra en una concentración en masa respecto a la masa total de la microemulsión en un rango comprendido entre el 0,1% y el 2%.

De manera preferente la microemulsión comprende una sustancia medicinal antiglaucomatosa, más preferentemente prostaglandinas, de manera particular la fase oleosa comprende latanoprost, número CAS 130209-82-4. El latanoprost pertenece al grupo de medicamentos conocidos como análogos de las prostaglandinas. Se utiliza para tratar enfermedades como el glaucoma de ángulo abierto y la hipertensión ocular en adultos. Ambas enfermedades están relacionadas con un aumento de la presión dentro del ojo, lo que puede llegar a afectar a la visión. El latanoprost actúa aumentando la salida natural del humor acuoso desde el interior del ojo al torrente sanguíneo.

De manera preferente la fase acuosa comprende compuestos mucoadhesivos y/o mucomiméticos. De manera preferente el compuesto mucoadhesivo y/o mucomimético de la microemulsión está en una concentración en masa respecto a la masa total de la microemulsión en una proporción inferior al 0,8%. Más preferentemente los compuestos mucoadhesivos y/o mucomiméticos se seleccionan entre: ácido hialurónico y sus sales, dextrano, derivados de la celulosa, condroitín sulfato, quitosano, alginatos, y goma gelano.

De manera particular el compuesto es ácido hialurónico o las sales del mismo, presentando características similares a la de la película precorneal. Esta microemulsión se puede utilizar como lagrimas artificiales.

En la fase acuosa se incorpora el agente isotonizante (trehalosa, cloruro sódico, glucosa) para conseguir una osmolaridad ajustada para uso clínico y el agente adecuado para tamponar el preparado hasta pH adecuado. Los preparados podrán ser isotónicos o hipotónicos.

Por lo tanto, preferentemente la fase acuosa comprende un agente isotonizante y más preferentemente el agente isotonizante se selecciona entre: trehalosa, cloruro sódico, glucosa, fructosa, manitol, sorbitol, ribitol, eritritol y dextrosa.

De manera preferente la fase acuosa comprende activos hidrosolubles seleccionados entre: vitaminas hidrosolubles, betaína, clusterina y L-carnitina, taurina, glicina, ribitol, ectoina y aminoácidos. De manera particular betaína, leucina.

De manera preferente la fase acuosa tiene una osmolaridad comprendida en un rango entre 150 mOsm/kg y 330 mOsm/kg.

De manera preferente la microemulsión comprende: agentes capaces de disminuir la presión intraocular, agentes neuroprotectores, material genético como el ADN y el ARN, proteínas y ácidos nucleicos, inmunógenos como escualeno y polisorbato 80.

Otro aspecto de la invención se refiere a un procedimiento de obtención de la microemulsión de la invención que comprende las etapas de:
a) mezclar los compuestos que comprende la fase oleosa;
b) añadir la fase acuosa;
c) filtrar
d) estabilizar.

De manera preferente la etapa a) se realiza a una temperatura comprendida entre 20ºC y 30ºC y con protección de la luz, en la etapa b) se añade la fase acuosa a la fase oleosa en agitación, en la etapa c) el filtrado se realiza con un filtro que tiene un tamaño de poro entre 0,20 µm y 0,25 µm y donde la etapa d) de estabilización se realiza a una temperatura entre 2ºC y 25ºC.

EJEMPLOS

Los siguientes ejemplos tienen únicamente carácter ilustrativo de esta invención, y no deben ser interpretados en sentido limitativo de la misma.

Ejemplo 1

1º Preparación de las fases

FASE OLEOSA (para 10 g de microemulsión). Se utilizaron oleato de etilo 0,8 % (m:m), aceite de soja 0,2 % (m:m), Kolliphor EL® 1 % (m:m), propilenglicol 1 % (m:m), fosfatidilcolina de soja 0,5 % (m:m) y escualeno 0,2 % (m:m).

Se mezclaron los diferentes componentes y dejó en agitación a 400 rpm, y a temperatura ambiente, durante un mínimo de 30 min hasta conseguir trasparencia.

La osmolaridad de la fase oleosa, sin activos liposolubles, es próxima a 170 mOsm/L.

FASE ACUOSA (para 10 g de microemulsión). Se utilizaron ácido hialurónico 0,2 % (m:m), trehalosa cs. para osmolaridad deseada (250-300 mOsm/kg), agua csp. 10 g de microemulsión (primera fase acuosa).

Se preparó una segunda fase acuosa igual a la anterior sin ácido hialurónico. La microemulsión preparada con la fase acuosa sin ácido hialurónico se denomina a partir de ahora MB, microemulsión base.

Y una tercera fase acuosa con ácido hialurónico con la adición de activos osmoprotectores y reepitelizantes a esta formulación que favorece su utilización como vehículo de fármacos hipotensores oculares como tratamientos prolongados y que posibilitan la manifestación de alteraciones de la superficie ocular.

2º Elaboración de la microemulsión

La elaboración de la microemulsión se realizó a temperatura ambiente y con protección de la luz. Se añadió la totalidad de cualquiera de las fases acuosas, en un solo paso, a la fase oleosa que se encontraba en agitación a 1.000 rpm. Se mantuvo la agitación a 1.000 rpm durante 10 minutos. Se filtró, utilizando un filtro hidrófilo de baja retención, con un tamaño de poro de 0,22 µm. Se dejó estabilizar en nevera a 2-8 º C durante 24 horas.

3º Características de la microemulsión obtenidas en el punto 1º y 2º referenciada como MB (microemulsión base sin ácido hialurónico)

Esta caracterización se limita a la microemulsión según lo descrito en este ejemplo.

La morfología de las gotículas fue estudiada utilizando las técnicas de cryo-TEM y SEM. Para ello se utilizaron los equipos 200 kV FEI TALOS Arctica y JEOL JSM 7600F, respectivamente. Las fotografías obtenidas se muestran en la Figura 1 apartados a) y b).

El preparado MB presenta valores de pH de 7,27 ± 0,12.

El diámetro de las partículas emulsionadas de la microemulsión se determinó utilizando el método de dispersión de luz dinámica (DLS) (también llamado de espectroscopia de correlación fotónica). El valor del diámetro medio volumen de las partículas obtenidas en distintos lotes de la microemulsión oscilan entre 21 y 23 nm con desviaciones estándar (SD) entre 4,5 y 5,3 nm. El porcentaje de partículas superiores 50 nm fue, en todos los casos, inferior al 1 % (Figura 2).

Los valores de potencial zeta para los distintos lotes fluctuaron entre -7 y -9 mV (las medidas se realizaron a temperatura ambiente con un Autosizer 4700, Malvern).

Las medidas de tensión superficial se realizaron con un tensiómetro de la marca comercial Kruss (K-11) utilizando el método de la placa y se obtuvieron valores entre 34 y 38 mN/m.

En la evaluación reológica del vehículo MB a velocidades de cizalla entre 0 y 1.000 s^{-1} y temperatura ambiente, se observa que aparece un comportamiento newtoniano con valores de viscosidad dinámica entre 1,1 y 1,3 mPa·s (Figura 3). Para su determinación se utilizó el equipo Discovery HR-Hybrid Rheometer DHR-1 con un sistema de placas paralelas de 60 mm.

En los ensayos de citotoxicidad *in vitro* se utilizaron líneas celulares epiteliales de córnea humana (hTERT-HCEC) y de conjuntiva humana (HConEpiC). Para el estudio se utilizó la técnica de reducción, a nivel mitocondrial, de la sal bromuro de 3(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) a un producto coloreado (formazán) (Mossman T. J Immun Methods 1983, 65:55-63). Como control negativo se utilizó cloruro sódico al 0,9 % y como control positivo cloruro de benzalconio al 0,005 %. Las soluciones se incubaron a 37 ºC durante 1 y 4 horas. Los resultados obtenidos demostraron una tolerancia superior al 90 % a los tiempos ensayados por lo que se considera que es óptima (Figura 4).

En un ensayo inicial de captación celular de las gotículas lipídicas por células epiteliales de córnea humana (hTERT-HCEC) utilizando la microemulsión marcada con un colorante fluorescente, la cumarina-6 sustancia muy lipófila, se manifestó captación a los 15 y 30 min de exposición según se muestra en la Figura 5.

4º Características de la microemulsión según el punto 1º y el 2º con hialuronato sódico al 0,2

% como polímero mucoadhesivo (se denomina MB-HNa).

La caracterización de esta formulación (MB-HNa) se llevó a cabo utilizando los mismos equipos que los indicados en apartado 3º.

La morfología de las gotículas fue estudiada utilizando las técnicas de cryo-TEM y SEM Las fotografías obtenidas se muestran en las Figuras 6 apartados a) y b).

El preparado MB-HNa presenta valores de pH comprendidos entre 6,79 ± 0,05.

El diámetro medio volumen de las partículas obtenidas en distintos lotes de la microemulsión con hialuronato sódico oscilan entre 29 y 37 nm con desviaciones estándar (SD) entre 6,9 y 7,4 nm. El porcentaje de partículas superiores a 50 nm fue inferior al 11 % (Figura 7).

El potencial zeta fluctuó entre -0,1 y 0,2 mV (las medidas se realizaron a temperatura ambiente).

Las medidas de tensión superficial sobre el vehículo de la invención con hialuronato sódico se encontraron valores entre 35 y 38 mN/m.

En la evaluación reológica del vehículo a temperatura ambiente de la invención con hialuronato sódico a velocidades de cizalla entre 0 y 1.000 s^{-1} se manifiesta un comportamiento newtoniano con valores de viscosidad dinámica entre 4,3 y 4,5 mPa·s (Figura 8).

En los ensayos de citotoxicidad *in vitro* se utilizaron líneas celulares epiteliales de córnea humana (hTERT-HCEC) y de conjuntiva humana (HConEpiC). Para el estudio se utilizó la técnica de reducción, a nivel mitocondrial, de la sal bromuro de 3(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) a un producto coloreado (formazán) (Mossman T. J Immun Methods 1983, 65:55-63). Como control negativo se utilizó cloruro sódico al 0,9 % y como control positivo cloruro de benzalconio al 0,005%. Las soluciones se incubaron a 37 ºC durante 1 y 4 horas. Los resultados obtenidos demostraron una tolerancia superior al 90 % a todos los tiempos ensayados (Figura 9).

Esta composición farmacéutica (MB-HNa) con características de película preocular tiene la capacidad de disminuir la tensión ocular en el conejo. Así se puede observar en los resultados obtenidos en esta especie animal tras la administración de 25 µL de la

formulación (Figura 11). Se observa una disminución de la presión intraocular entre valores del 15 al 17 % a partir de las 2 horas de la instilación que se mantiene durante 6 horas.

5º Características de la microemulsión según la invención con agentes opcionales

A este vehículo (MB-HNa) basado en la microemulsión objeto de la invención con hialuronato sódico se le puede añadir componentes opcionales osmoprotectores y reepitelizantes como la betaína, la leucina y la l-carnitina. No existe comercializada ninguna lágrima artificial que sea una microemulsión que incluya estos componentes.

En los estudios realizados con la línea celular de córnea humana (hTERT-HCEC), se incluyeron formulaciones con sustancias opcionales osmoprotectoras y reepitelizantes como la betaína y la leucina, manteniéndose la buena tolerancia en las formulaciones que incluyen los activos indicados (Figura 10). Esta composición derivada de la microemulsión objeto de la invención debido a las características indicadas en este apartado puede ser utilizada como sustituto lagrimal. Este hecho junto con la adición de activos osmoprotectores y reepitelizantes a esta formulación favorece su utilización como vehículo de fármacos hipotensores oculares que suponen tratamientos prolongados y posibilitan la manifestación de alteraciones de la superficie ocular.

6º Características de la microemulsión según la invención con agentes opcionales y una sustancia medicinal antiglaucomatosa

El último ensayo se realizó con una microemulsión que comprende la composición base descrita en el punto primero de este ejemplo, hialuronato sódico y latanoprost.

El colirio comercializado en forma de disolución en envases multidosis lleva cloruro de benzalconio, producto altamente citotóxico en las células de conjuntiva y cornea. El producto comercializado en envases unidosis (Monoprost) incorpora diferentes tensoactivos ajenos al cloruro de benzalconio. Este último colirio se ha utilizado como referencia en los ensayos de comparación de actividad hipotensora.

El preparado farmacéutico propósito de esta invención lleva la misma dosificación que los preparados que están comercializados (50 µg/mL).

La caracterización de esta formulación (MB-HNa-Latanoprost) se llevó a cabo utilizando los mismos equipos que los indicados en apartado 3º.

La morfología de las gotículas fue estudiada utilizando la técnica de cryo-TEM y SEM. Las fotografías obtenidas se muestran en las Figuras 12 apartados a) y b).

El preparado MB-HNa-latanoprost presenta un valor de pH de 6,58 ± 0,02.

El diámetro medio volumen de las partículas obtenidas en distintos lotes de la microemulsión con hialuronato sódico oscilan entre 29 y 32 nm con desviaciones estándar (SD) entre 6,9 y 7,1 nm. El porcentaje de partículas superiores 50 nm fue inferior al 6 % (Figura 13).

El potencial zeta fluctuó entre -0,1 y 0,0 mV y los valores de la tensión superficial oscilaron entre 36 y 38 mN/m.

En la evaluación reológica de la microemulsión con hialuronato sódico y latanoprost realizada a velocidades de cizalla entre 0 y 1.000 s^{-1}, se manifiesta un comportamiento newtoniano con valores de viscosidad dinámica entre 4,5 y 4,7 mPa·s (Figura 14).

En los ensayos de citotoxicidad *in vitro* se utilizaron líneas celulares epiteliales de córnea humana (hTERT-HCEC) y de conjuntiva humana (HConEpiC). Para el estudio se utilizó la técnica de reducción, a nivel mitocondrial, de la sal bromuro de 3(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) a un producto coloreado (formazán) (Mossman T. J Immun Methods 1983, 65:55-63). Como control negativo se utilizó cloruro sódico al 0,9 % y como control positivo cloruro de benzalconio al 0,005%. Las soluciones se incubaron a 37ºC durante 1 y 4 horas. Los resultados obtenidos demostraron una tolerancia superior al 90 % a todos los tiempos ensayados (Figura 15).

Esta composición farmacéutica (MB-HNa-latanoprost) con actividad hipotensora fue comparada con la formulación comercializada de latanoprost en envases unidosis (Monoprost) que se utilizó como referencia. Como control se empleó la microemulsión base (MB). Los resultados se indican en la Figura 16. A las 2 h después de su administración se alcanza con ambas formulaciones una disminución en la presión intraocular (ΔPIO) superior al 15 %. En el caso de la formulación comercial se alcanzó un efecto máximo hipotensor del 20% a las 2h que se conservó aproximadamente durante 9 h (ΔPIO ~15 %) y un efecto residual (inferior al 10 %) que se mantuvo hasta los 2 días. Con la composición farmacéutica objeto de la patente, además se puede observar un ΔPIO entre el 35-38 % a partir de las 24 h que se mantiene durante 3 días. A partir del cuarto día el efecto comienza a disminuir lentamente, manteniéndose la eficacia hipotensora (ΔPIO>10%) hasta los 12 días.

REIVINDICACIONES

1. Una microemulsión oftálmica que comprende:
una fase acuosa y
una fase oleosa que comprende: un aceite vegetal, un éster de ácido graso farmacéuticamente aceptable, un compuesto glicol, escualeno, agentes surfactantes y cosurfactantes.

2. La microemulsión de la reivindicación 1 caracterizada porque el aceite vegetal es aceite de soja, el éster de ácido graso farmacéuticamente aceptable es oleato de etilo y el compuesto glicol es propilenglicol.

3.La microemulsión según alguna de las reivindicaciones 1 a 2 caracterizada porque el agente surfactante es fosfatidilcolina de soja y el cosurfactante es aceite de ricino polioxílico 35.

4. La microemulsión según alguna de las reivindicaciones 1 a 3 caracterizada porque la fase acuosa comprende compuestos mucoadhesivos y/o mucomiméticos.

5. La microemulsión según la reivindicación 4 caracterizada porque los compuestos mucoadhesivos y/o mucomiméticos se seleccionan entre: ácido hialurónico y sus sales, dextrano, derivados de la celulosa, condroitín sulfato, quitosano, dextrano, alginatos y goma gelano.

6. La microemulsión según la reivindicación 5 caracterizada porque el compuesto es ácido hialurónico o las sales del mismo.

7. La microemulsión según alguna de las reivindicaciones 1 a 6 caracterizada porque la fase oleosa comprende activos liposolubles seleccionados entre: ácido oleanólico, ubiquinol y/o vitaminas liposolubles.

8. La microemulsión según alguna de las reivindicaciones 1 a 7 caracterizada porque la fase oleosa comprende latanoprost.

9. La microemulsión según alguna de las reivindicaciones 1 a 7 caracterizado porque la fase acuosa comprende un agente isonizante.

10. La microemulsión según la reivindicación 8 caracterizada porque el agente isotonizante se selecciona entre: trehalosa, cloruro sódico, glucosa, fructosa, manitol, sorbitol, ribitol, eritritol y dextrosa.

11. La microemulsión según alguna de las reivindicaciones 1 a 10 caracterizada porque la fase acuosa comprende activos hidrosolubles seleccionados entre: vitaminas hidrosolubles, betaína, clusterina, L-carnitina, taurina, glicina, ribitol, ectoina y aminoácidos.

12.La microemulsión según alguna de las reivindicaciones 1 a 11 caracterizada porque la fase acuosa tiene una osmolaridad comprendida en un rango entre 150 mOsm/kg y 330 mOsm/kg.

13.Un procedimiento de obtención de la microemulsión según las reivindicaciones 1 a 12 que comprende las etapas de:
a) mezclar los compuestos que comprende la fase oleosa;
b) añadir la fase acuosa;
c) filtrar
d) estabilizar.

14. El procedimiento según la reivindicación 13 caracterizado porque:
la etapa a) se realiza a una temperatura comprendida entre 20ºC y 30ºC y con protección de la luz, en la etapa b) se añade la fase acuosa en un paso a la fase oleosa en agitación, en la etapa c) el filtrado se realiza con un filtro que tiene un tamaño de poro entre 0,20 µm y 0,25 µm y donde la etapa d) de estabilización se realiza a una temperatura entre 2ºC y 25ºC.

15. La microemulsión según las reivindicaciones 1 a 12 para su uso como lágrimas artificiales.

16. La microemulsión según las reivindicaciones 1 a 12 para su uso como vehículo de fármacos para su administración por vía tópica ocular.

17.La microemulsión según alguna de las reivindicaciones 1 a 12 para su uso en el tratamiento de la hipertensión ocular.

18.La microemulsión según la reivindicación 8 para su uso en el tratamiento del glaucoma.