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TOLERANCE OF HIGH AND LOW AMOUNTS OF PLGA MICROSPHERES LOADED WITH MINERALOCORTICOID RECEPTOR ANTAGONIST IN RETINAL TARGET SITE

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Abstract

Mineralocorticoid receptor (MR) contributes to retinal/choroidal homeostasis. Excess MR activation has been shown to be involved in pathogenesis of central serous chorioretinopathy (CSCR). Systemic administration of MR antagonist (MRA) reduces subretinal fluid and choroidal vasodilation, and improves the visual acuity in CSCR patients. To achieve long term beneficial effects in the eye while avoiding systemic side-effects, we propose the use of biodegradable spironolactone-loaded poly-lactic-co-glycolic acid (PLGA) microspheres (MSs). In this work we have evaluated the ocular tolerance of MSs containing spironolactone in rats' eyes. As previous step, we have also studied the tolerance of the commercial solution of canrenoate salt, active metabolite of spironolactone. PLGA MSs allowed *in vitro* sustained release of spironolactone for 30 days. Rat eyes injected with high intravitreal concentration of PLGA MSs (10 mg/mL) unloaded and loaded with spironolactone maintained intact retinal lamination at 1 month. However enhanced glial fibrillary acidic protein immunostaining and activated microglia/macrophages witness retinal stress were observed. ERG also showed impaired photoreceptor function. Intravitreal PLGA MSs concentration of 2 mg/mL unloaded and loaded with spironolactone resulted well tolerated. We observed reduced microglial/macrophage activation in rat retina compared to high concentration of MSs with normal retinal function according to ERG. Spironolactone released from low concentration of MSs was active in the rat retina. Low concentration of spironolactone-loaded PLGA MSs could be a safe therapeutic choice for chorioretinal disorders in which illicit MR activation could be pathogenic.

Keywords: Mineralocorticoid receptor, spironolactone, microspheres intraocular tolerance, Poly lactic-co-glycolic acid (PLGA), *in vivo* electroretinography, *in vivo* optical coherence tomography, immunohistochemistry

Introduction

Mineralocorticoid receptor (MR), the ancestor corticoid receptor is expressed in the distal nephron where it regulates sodium and fluid reabsorption, modulating blood volume and arterial pressure. More recently, MR was evidenced in heart, blood vessels, brain, adipose tissue, skin and macrophages, recognized as non-classical mineralocorticoid targets [1, 2]. Illicit activation of MR has been demonstrated to induce inflammation, oxidative stress, fibrosis, hypertrophic remodelling and endothelial dysfunction and increasing evidences suggest the MR antagonism has therapeutic value for endothelial dysfunction, atherosclerosis, hypertension, heart failure and chronic kidney disease [3-5].

Spironolactone has been the first steroidal competitive MR antagonist drug (MRA) approved for the treatment of hypertension half a century ago. It is a potent but low-specific MRA, interacting at high dose with androgen receptors, and causing subsequent dose-dependent hormonal side-effects [6]. Eplerenone, less potent but more specific MRA, designed to reduce the hormonal effects of spironolactone, was approved by the Food and Drug Administration in 2002 for the prevention of cardiac complications after myocardial infarction and ventricular dysfunction [7, 8]. Both MRA can potentially induce hyperkalemia, particularly with impaired renal function.

The eye is also a mineralocorticoid sensitive organ, expressing MR and pre-receptor enzyme 11β -hydroxysteroid dehydrogenase type 2 in the iris/ciliary body, neuroretina, retinal pigment epithelium and endothelial cells [9-13]. MR activation contributes to retinal fluid homeostasis through regulation of specific ion and water channels in retinal macroglial cells (Kir4.1 and AQP4) [9, 14]. An over-activation of MR by excess glucocorticoid or endogenous activation has been hypothesized in the pathogenesis of central serous chorioretinopathy (CSCR) [11]. Systemic administration of MRA have effect on subretinal fluid and choroidal vasodilation, and improves the visual acuity in CSCR patients [11, 15-18]. Since our first studies, more than 15 clinical studies have confirmed that MRA reduces subretinal fluid associated with CSCR. However, to achieve and to further maintain therapeutic effect,

long-term use of MRA may be required[18], increasing the risk of side-effects. Spironolactone, 40-fold more potent MRA than eplerenone, is associated on the long-term with hormonal side effects such as gynecomastia, erectile dysfunction and menstrual irregularities [6], being particularly bad tolerated in the CSCR population consisting mostly of middle-aged men. Although the ocular bioavailability of spironolactone and eplerenone after systemic administration has not been specifically studied, it is known that spironolactone induces the expression of the P-glycoprotein, a drug efflux protein that regulates the blood-brain barrier, and is one of its ligands [19], which may limit its ocular bioavailability.

In order to avoid systemic undesirable effects and ensure optimized MR blockade, intraocular local MRA delivery is desired and sustained release drug delivery system is mandatory to avoid repeated intraocular injections. For preclinical development in animal models and for potential clinical development, we have chosen to prepare biodegradable microspheres (MSs) using poly-lactic-co-glycolic acid (PLGA) to provide a controlled delivery of the drug in a long fashion. Particle size can be reduced to allow injection of a suspension through a regular 30G needle and provide the sustained release of active products[20-22]. PLGA, approved by FDA and the European Medical Agency for intraocular use[23, 24], is progressively hydrolysed in its monomers (lactic acid and glycolic acid), and subsequently converted into CO₂ and water via Krebs's cycle. Biodegradation in PLGA microspheres is described to occur in the polymeric matrix through a homogeneous hydrolytic chain cleavage mechanism leading to a sustained release of the active compounds included in the polymeric matrix [23, 24].

Intraocular tolerance of controlled drug delivery systems is critical as they are proposed for long term treatments. As the device is formed by a biomaterial and the active substance, it is also important to evaluate the *in vivo* response of the unloaded system. In the present work, spironolactone-loaded MSs releasing the drug *in vitro* for at least 30 days, were screened for anatomical and functional tolerance in normal rat eyes. *In vivo* and *ex vivo* tolerance analyses were performed 4 weeks after intravitreal injection of low and high amounts of microspheres in rats (0.1 mg and 0.5 mg, final

concentration in the rats vitreous of 2 and 10 mg/mL respectively). As the development of a novel controlled release also requires tolerance studies of the active molecule it self in the target tissue, we also evaluated the intraocular tolerance of an intravenous commercial solution of canrenoate salt (Soludactone), one of the active metabolites of spironolactone. To our knowledge the intraocular tolerance of spironolactone and its metabolites has not been previously studied.

Material and Methods

Animals

All experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and approved by local ethical committees (Ce5/2012/113 and Approval VD2928). Adult male Wistar rats (8 weeks old) from Janvier breeding Center (Le Genest-Saint-Isle, France) or Charles-River (L'Arbresle, France) were used. Animals were kept in pathogen-free conditions with food, water and litter and housed in a 12-hour light/12-hour dark cycle. Anesthesia was induced by intramuscular ketamine (40 mg/kg) and xylazine (4 mg/kg). Animals were sacrificed by carbon dioxide inhalation.

Intravitreal injection of soludactone

Intravitreal injections (IVT) were performed using microfine (300 µL) syringes with 30G needles under topical anesthesia (tetracaine 1%, Aldrich, Lyon, France). Five µL of the Soludactone® solution (Pfizer, Paris, France) at 37 µg/µL (final concentration in the vitreous: 10 µM) was injected in the rat eyes. Control rats received 5 µL of 0.9% NaCl. Soludactone® contains trometamol and water as excipients. Trometamol containing preparations have already been injected in the vitreous of animals and humans without any detectable toxicity [25, 26]. We thus chose Soludactone® because it is a medical grade, commercialized product that could potentially be used for intraocular use in further clinical studies.

***In vivo* and histological analyses for rats injected with soludactone**

~~These experiments were performed in collaboration with department of ophthalmology of the University of Lausanne.~~ One week after intravitreal Soludactone injection, rat retinal morphology was examined *in vivo* using a spectral domain optical coherence tomography (SD-OCT) system adapted for rat eyes (Bioptigen, Leica Microsystems, Buffalo Grove, IL, USA), and a retinal camera specially designed for mouse/rat retinal imaging (Micron III, Phoenix Research Labs, USA).

Visual function of rat eyes was also evaluated 1 week after IVT using electro-retinography (ERG). ERGs were performed using Espion E2 system from Diagnosys LLC (Lowell, MA, USA). Animals were dark adapted overnight. Scotopic ERGs were performed in the dark with light intensities of flashes ranging from 0.0001 to 30 cd.s/m². For photopic recordings, animals were light adapted for 5 min and then the response to light intensities of flashes ranging from 0.1 to 30 cd.s/m² were recorded. ERG records were analyzed using 2-way ANOVA and multiple t tests. Statistical significance was determined using the Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD.

Rats were then sacrificed, each eye was dipped in Bouin's solution for 24 hours, embedded in paraffin, cut in 4.5 µm thick sections, and stained with haematoxylin - eosin. Sections through the optic nerve and through the injection site were analyzed under a microscope (Zeiss, Oberkochen, Germany) associated with a digital camera.

***In vitro* stability of spironolactone in the presence of human vitreous**

An isotonic formulation of spironolactone was prepared using 0.9% NaCl at a concentration of 6.2 µM (2.6 µg/mL). To determine whether spironolactone is stable when in contact with vitreous, 100 µL of spironolactone-containing formulation were added and mixed with 500 µL human vitreous and kept in room temperature. Fifty µL of vitreous sample were harvested at each time point at 0, 5, 10, 30 and 60 minutes, then diluted with 25 µL acetonitrile containing ISTD, filtrated and analysed using Waters AcquityUPLC system (Saint-Quentin-en-Yvelines, France). Experiments were performed in

triplicate. Kinetics of spironolactone concentration over time was established with reference to time 0 considered as 100%.

***In vivo* kinetics of spironolactone in rat eye**

Five μL of spironolactone formulation at concentration of 5 μM in 0.9% NaCl (2.07 $\mu\text{g/mL}$) were injected in rat eyes. At 15, 30 and 60 minutes after IVT, intravitreal liquid was harvested. Twenty μL of each sample were diluted with 10 μL of acetonitrile containing ISTD and analysed using Water Acquity UPLC system. (n=4 eyes / time point).

To evaluate ocular bioavailability after intravenous injection, 100 μL of 5 μM spironolactone (2.07 $\mu\text{g/mL}$) was administrated in 6 rats. Eyes were enucleated at 15, 30 and 60 minutes after injection and vitreous/ aqueous humor were analysed for the presence of spironolactone or its metabolites.

Preparation of blank and spironolactone-loaded PLGA Microspheres

PLGA ratio 50:50 (Resomer®503) was acquired from BoehringerIngelheimPharma GmbH & Co. (Ingelheim, Germany). Spironolactone (Spiro) was obtained from Sigma-Aldrich (Schnelldorf, Germany) and Polyvinyl alcohol 72,000 g/mol (PVA) from Merck KGaA (Darmstadt, Germany). All organic solvents were HPLC grade and used as received.

Spironolactone-loaded PLGA microspheres (MSs) were manufactured using an oil-in-water (O/W) emulsion solvent evaporation technique. Briefly, The O-phase was prepared with spironolactone (40 mg) in 1 mL of PLGA dissolved in methylene chloride (20% w/v); Spiro:PLGA ratio of 2:10. This oily phase was emulsified with an aqueous solution composed by 5mL of PVA MilliQ® water solution (2% w/v). The emulsification procedure was performed at 5,000 rpm (1 min) (Polytron® RECO, Kinematica GmbH PT 10-35, Lucerna, Switzerland). This O/W emulsion was subsequently poured onto 100 mL of an aqueous PVA solution (0.1%) and maintained under constant stirring for 3h to allow MSs hardening. After that, the MSs were washed, filtered, freeze-dried, and kept at -20 °C indry conditions until used. Non-loaded PLGA MSs (blank microspheres) were prepared using the same

protocol without including spironolactone. Loaded and unloaded microspheres will be named as “Spiro-MSs” and “NL-MSs” hereby.

Microsphere characterization

Production yield percentage (PY%). The PY% was calculated as the percentage of MS weight divided by the total amount of PLGA and spironolactone used in the formulation process.

$$PY\% = \frac{\text{weight of microspheres}}{\text{initial amount of PLGA and spironolactone}} \times 100$$

Mean particle size and particle size distribution. The mean particle size and particle size distribution were measured by light scattering in a Microtrac® S3500 Series Particle Size Analyzer (Montgomeryville, PA, USA). Before each measurement the particles were suspended in water.

Morphological evaluation. The external morphology of MSs was evaluated by scanning electron microscopy (Jeol, JSM-6335F, Tokyo, Japan). Before each determination, particles were gold sputter-coated.

Spironolactone quantification. Spironolactone was quantified by HPLC consisting of two Waters Millipore 510 pumps, autosampler Waters 712DWSP, Waters 490E UV/Vis detector. Analyses were performed using a 25 cm x 5 mm reverse-phase C18 5-μm column (Tracer Extrasil ODS2, Teknokroma) preceded by a guard cartridge SEA 18, 100 mm x 4.0 mm (Teknokroma). The mobile phase was composed by methanol:water 67:33 v/v at a flow rate of 1.0 mL/min. The injection volume was 20 μL. UV detection of the spironolactone was carried out at 238 nm.

Encapsulation efficiency. Spironolactone loading in the microspheres was determined as follows: an amount of MSs (5 mg) was initially dissolved in methylene chloride (1 mL), PLGA was precipitated by the addition of ethanol (4 mL), mixed and centrifuged (8500g; 5 min). Then, the supernatant was recovered and filtered (0.22 μm) for drug quantification by HPLC.

In vitro release studies. Samples (n=18) were prepared by suspending spironolactone-loaded MSs (5 mg) in 1.8 mL of medium (Isotonic phosphate buffered saline pH 7.4 with the addition of

NaCl). Samples were maintained under a constant agitation speed of 100 rpm (Clifton Shaking Bath NE5, Nikel Electro Ltd, Avon, UK) at 37°C. Sampling was performed at 4, 7, 11, 14, 18, 21, 25, 28 and 31 days of incubation. At each time point MSs suspensions were centrifuged (18,000 rpm; 3 min; 20°C). Two of the samples including the solid particles were recovered, frozen and freeze-dried. The obtained powders were treated using the same method described for encapsulation efficiency to determine the amount of spironolactone still remaining in the particles. The rest of the supernatants were removed and replaced by the same volume of fresh medium, to continue the release test. The amount of released spironolactone was calculated as the difference between the initial content and the actual content at each time point.

Intravitreal injection of microspheres in rat eyes

Suspensions of 5 µL containing either 0.5 mg of PLGA MSs (high amount) with a total dose of 53 µg of spironolactone, or 0.1 mg of PLGA MSs (low amount) containing a total dose of 10.6 µg spironolactone were injected in the rat vitreous. Particles were always suspended in NaCl 0.9%. Control rat eyes were injected with non-loaded MSs (0.5 mg or 0.1 mg) suspended in 0.9% NaCl and also 0.9% NaCl alone.

***In vivo* and *ex vivo* tolerance analysis of blank and spironolactone-loaded PLGA microspheres**

Electroretinogram

Electroretinograms (ERG) were performed before and 4 weeks after IVT using VisioSystem device (Siem Biomedicale, Nimes, France). Animals were dark-adapted overnight. Scotopic ERG was performed in the dark with light intensities of flashes ranging from 0.0003 to 10 cd.s/m². For each intensity, the average response to 5 flashes at a frequency of 0.5 Hz was recorded. For photopic recordings, animals were light-adapted for 5 min with a background light of 25 cd/m², and then the response following a single light flash of 10 cd.s/m² was recorded.

Optical coherence tomography

The *in vivo* analysis of rat retina was performed at day3, one week and 4 weeks after IVT using spectral domain optical coherence tomography (SD-OCT, Spectralis™ device) adapted for small animal eyes [27]. Scans of intravitreal MSs and underlying retina were taken. Each two-dimensional B-scan recorded at 30° field-of-view consisted of 1536 A-scans with an optical resolution reaching 3.5µm.

Four weeks after IVT, animals were sacrificed, eyes were enucleated and prepared for histology, immunofluorescence and quantitative PCR analyses.

Histology

Enucleated eyes were fixed in 4% paraformaldehyde (PFA) for 2h at room temperature and dehydrated in a graded alcohol series (70%, 95% and 100%) and embedded in historesin. Five µm sections stained with toluidine blue. The morphology and pathological modifications of ocular tissues were examined under a light microscope (DM5500B, Leica, Nanterre, France).

Immunohistochemistry

After fixed in 4% PFA for 2 h, eyes were snap frozen in Tissue-Tek OCT-compound (Bayer Diagnostics, Puteaux, France). Cryostat sections (10 µm) of rat eyes were washed and permeabilized with 0.1% Triton X-100 for 30 min. Unspecific binding sites were blocked with 5% normal goat serum for 1 h. The sections were then incubated with primary antibodies for 1 h at room temperature, washed in PBS, and further incubated with secondary antibodies for 1 h. After washing, slides were stained for 5 min with 4',6-Diamidino-2-Phenyl-Indole (DAPI, 1:3000), washed again, and mounted with gel mount. Control sections were stained without primary antibodies. Images were taken using a fluorescence microscope (Olympus BX51, Rungis, France) equipped with a CCD camera (Olympus DP70). The following antibodies were used: rabbit anti-glial fibrillary acidic protein (GFAP, 1:100, Dako, Trappes, France), rabbit anti-ionized calcium binding adaptor molecule-1 (anti-IBA1; 1:400, Wako Richmond, USA) and Alexa Fluor 488-coupled goat anti-rabbit IgG (1:200, Molecular Probes, Leiden, Netherlands). To quantify activated macrophages/microglial cells, all round IBA-1 positive cells were

counted on entire ocular cross sections at the optic nerve level. Four sections per eye were quantified (n=4 rats in high and n=5 in low amount MSs groups).

TUNEL assay

For the TUNEL assay, rat eyes were snap frozen in Tissue-Tek OCT-compound. Eye cryosections were fixed with methanol/acetic acid for 20 min at room temperature, then washed in PBS, and permeabilized with 0.1% (w/v) sodium citrate/0.1% (v/v) Triton X-100 in water for 2 min at -4°C. After washing with PBS, sections were incubated for 1 h with 50 µL of terminal deoxynucleotidyltransferase (TUNEL; Roche Diagnostics, Mannheim, Germany) reaction mixture at 37 °C and counterstained with DAPI.

Reverse Transcription and Real-Time PCR

Total RNA was isolated from rat retinas using RNeasyPlusMini Kit (Qiagen, Courtaboeuf, France). First-strand cDNA was synthesized using random primers (Invitrogen, CergyPontoise, France) and superscript II reverse transcriptase (Invitrogen). Transcript levels of neutrophil gelatinase-associated lipocalin (NGAL), NADPH oxidase subunit 2 (NOX2), chemokine ligand 2 (CCL2) and intercellular adhesion molecule-1 (ICAM-1) were analyzed by real-time PCR performed in 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR® Green (Invitrogen) detection. The 18S and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were used as internal controls. The sequences of primers: NGAL, forward, 5'-TCACCCTGTACGGAAGAACC-3', reverse, 5'-GGTGGGAACAGAGAAAACGA-3'; NOX2, forward, 5'-GCCTCCATTCTCAAGTCTGTCT-3', reverse, 5'-GGAAGTTGGCATTGTTTCTTTC-3'; CCL2, forward, 5'-CCTGCTGCTACTCATTAC-3', reverse, 5'-TCTCACTTGGTTCTGGTCC-3'; ICAM, forward, 5'-AGAAGGACTGCTTGGGGAA-3'; reverse, 5'-CCTCTGGCGGTAATAGGTG-3'; 18S, forward, 5'-TGCAATTATTCCCATGAACG-3', reverse, 5'-GCTTATGACCCGCACTTACTGG-3'; HPRT1, forward, 5'-GCGAAAGTGGAAAAGCCAAGT-3', reverse, 5'-GCCACATCAACAGGACTCTGTAG-3'. Delta cycle threshold calculation was used for relative quantification of results.

Statistics

Data are provided as mean \pm SEM. Comparison between groups was performed using 1- or 2-way ANOVA followed by Bonferroni test (GraphPad Prism 5; GraphPad Software). A *P* value less than 0.05 was considered statistically significant.

Results

Soludactone did not alter the morphology nor the function of the rat retina

One week after IVT, eye fundus and OCT sections showed that soludactone solution (Figure 1A) did not induce retinal damage as compared to saline control (Figure 1B) around the optic nerve, as well as in the other quadrant of the retina. No retinal inflammation or edema was noticed. Histological analyses confirmed *in vivo* findings. There was no inflammation observed near the optic nerve or in irido-corneal angles either in soludactone-injected (Figure 2B) or in sham-injected rat eyes (Figure 2A). Retinal structure near the IVT site was preserved. Histology also highlighted the healing process at the IVT site, in the control and soludactone groups. Scotopic and photopic ERGs showed no difference in photoreceptor function between soludactone and control groups (Figure 3) suggesting that intravitreal soludactone does not affect the retinal function of rat eyes.

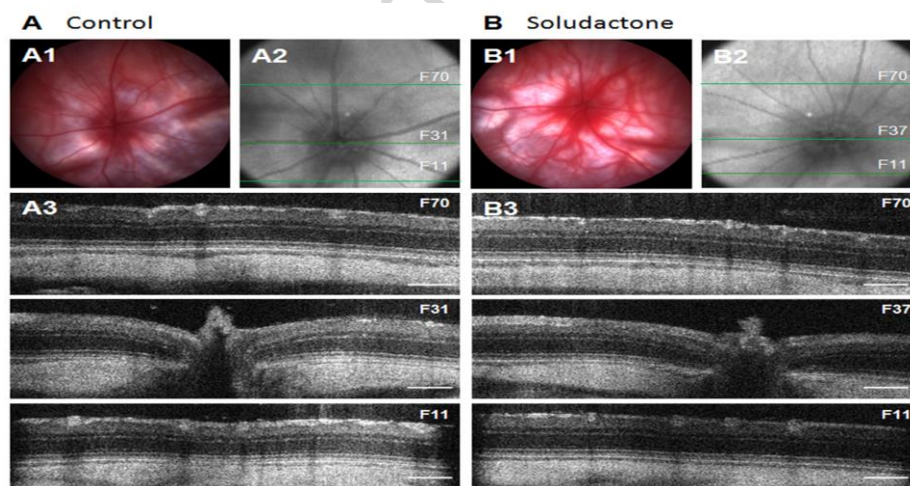


Figure 1. Fundus and optical coherence tomography (OCT) examination of the rat retinas one week after soludactone intravitreal injection (B) compared to saline control (A). (A1, B1) Eye fundi show no difference between soludactone-injected and sham-injected eyes. (A2, B2) Infrared images of the

eye fundus: each green line corresponds to the frame (F) of the OCT sections in (A3, B3). n=4 rats per group. Scale bars: 200 μ m.

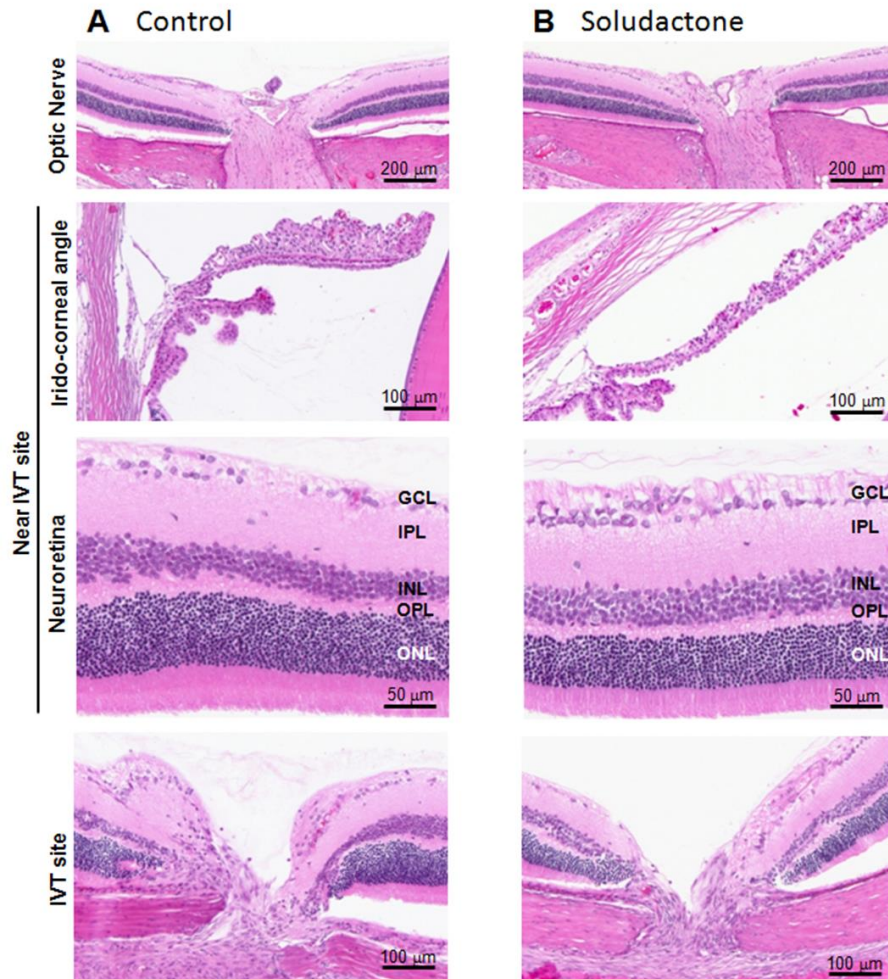


Figure 2. Histology of rat eyes one week after soludactone injection (B) compared to saline (A). No inflammatory cell is observed near the optic nerve or in the iridocorneal angle in both groups. The neuroretina maintains laminated structure in both soludactone-injected and saline-injected rat eyes. Wound healing process is observed at the IVT sites in both groups. GCL, Ganglion Cell Layer; INL, Inner Nuclear Layer; IPL, Inner Plexiform Layer; ONL, Outer Nuclear Layer; OPL, Outer Plexiform Layer. n=4 rats per group.

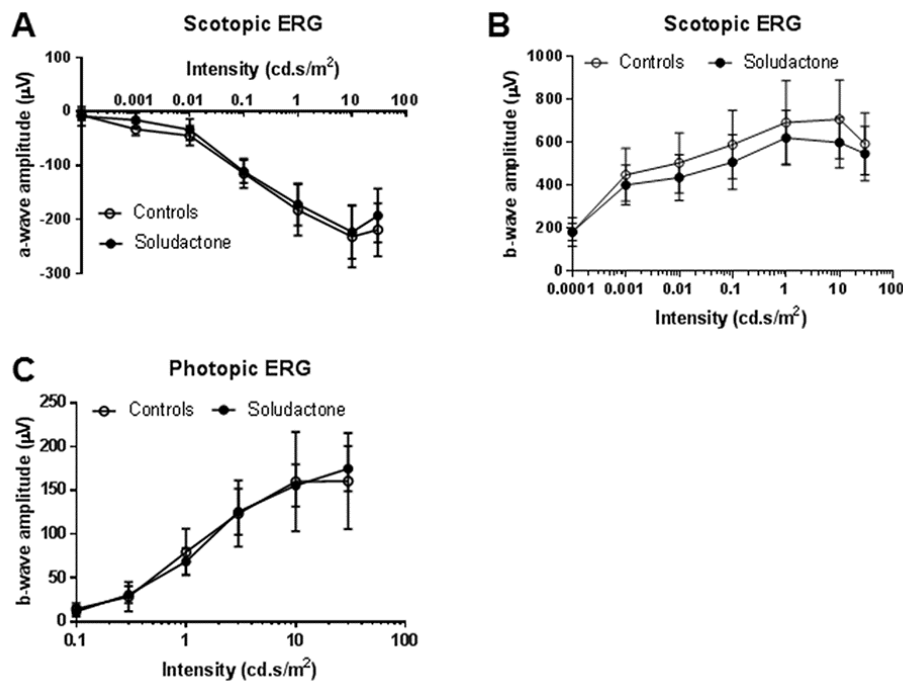


Figure 3.Electroretinograms of rat eyes one week after soludactone IVT compared to saline injection. Scotopic a-wave amplitude (A) and b-wave (B) amplitude show no significant difference between control and soludactone-injected eyes. Photopic ERG also finds no difference in b-wave amplitude between 2 groups. n=8 eyes/group.

Spironolactone has a short intravitreal half-life in the human vitreous

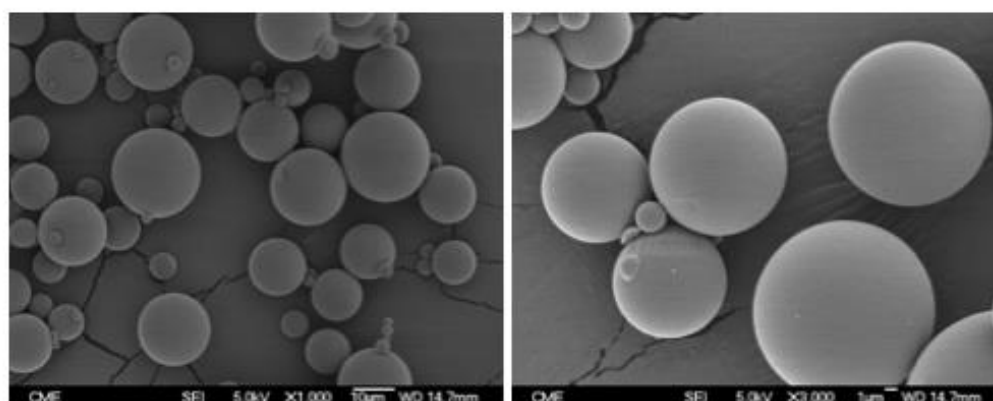
In vitro kinetics of spironolactone showed a decrease of around 30% of spironolactone after 60 minutes in contact with human vitreous. *In vivo* kinetics of spironolactone injected in the vitreous of the rat eye showed that at 15 minutes after IVT, the intravitreal concentration of spironolactone corresponded to 90% of injected dose but at 60 minutes only half of the amount of spironolactone was left in the vitreous. These findings suggest that spironolactone is relatively stable, but eliminated rapidly from the vitreous. After systemic administration, no spironolactone could be detected in ocular media at any time point indicating that spironolactone does not cross the blood-retinal barrier (results not shown).

Characterization of spironolactone-loaded PLGA microspheres

The production yield resulted 56.9%, with a mean particle size of $17.5 \pm 5.0 \mu\text{m}$. SEM pictures revealed MSs with spherical shape and absence of irregularities on their surface (Figure 4A). The amount of spironolactone in the MSs resulted $105.9 \mu\text{g}$ spironolactone/mg MSs (encapsulation efficacy of 63.5%).

The *in vitro* release study showed a sustained release of the spironolactone at a rate of $2.87 \mu\text{g}$ spironolactone/mg MSs/day (Figure 4B) during the 31-day study. According to this, high amount Spiro-MSs released approximately $1.434 \mu\text{g}$ spironolactone/day, while low amount Spiro-MSs released $0.287 \mu\text{g}$ spironolactone/day. As the microencapsulated drug remains in its solid state until it is released from microspheres, spironolactone degradation at this point is not expected.

A



B

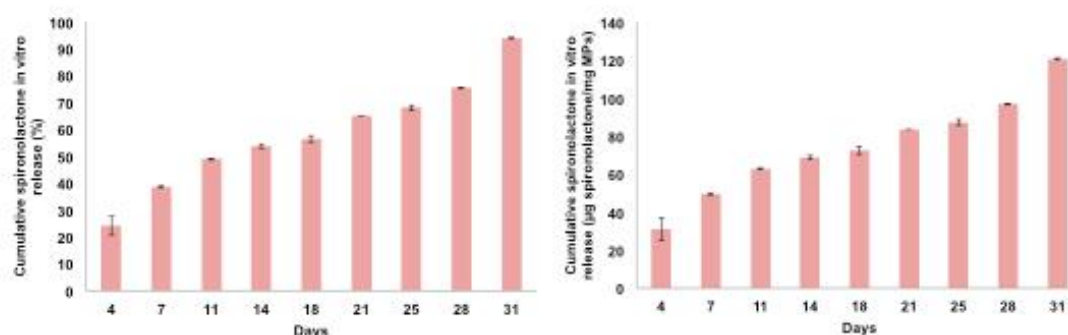


Figure 4. Scanning electron microscopy picture of spironolactone-loaded microspheres (MSs) (A) and cumulative *in vitro* release of spironolactone (μg Spiro/mg MSs and %) over 31 days from

spironolactone-loaded PLGA MSs (B). Release media: PBS (pH 7.4) isotonized with NaCl.

Spironolactone-loaded PLGA MSs did not induce change in rat retinal structure

OCT showed that at day3 after IVT, MSs tended to aggregate in the vitreous with the underlying retina maintained its normal structure in both NL-MSs and Spiro-MSs groups (Figure 5A and B). The amount of visible MSs diminished thereafter from 1 week to 4 weeks (Figure 5C-F). At 4 weeks after IVT, MSs are still present in the vitreous (Figure 5E and F). No inflammation or change in retinal lamination was observed *in vivo* at any assayed time (Figure 5C-F).

In all groups (NL-MSs and Spiro-MSs) even with high amount of microspheres (Figures 6B, 6C, 6E and 6F) histology showed that no inflammation occurs in the ciliary body or in the retina four weeks after IVT, as compared to NaCl-injected control rat eyes (Figure 6A and D). The retina maintained its lamination structure (Figure 6D-F). Morphology of eyes injected with low amount of MSs showed the same intact ocular structure (not shown).

TUNEL test showed apoptotic corneal epithelial cells as positive control (arrows in Figure 7A). In either high amount (Figure 7B and C) or low amount of MSs groups (not shown), we did not observe TUNEL-positive cells in the rat retina except for the site of the IVT.

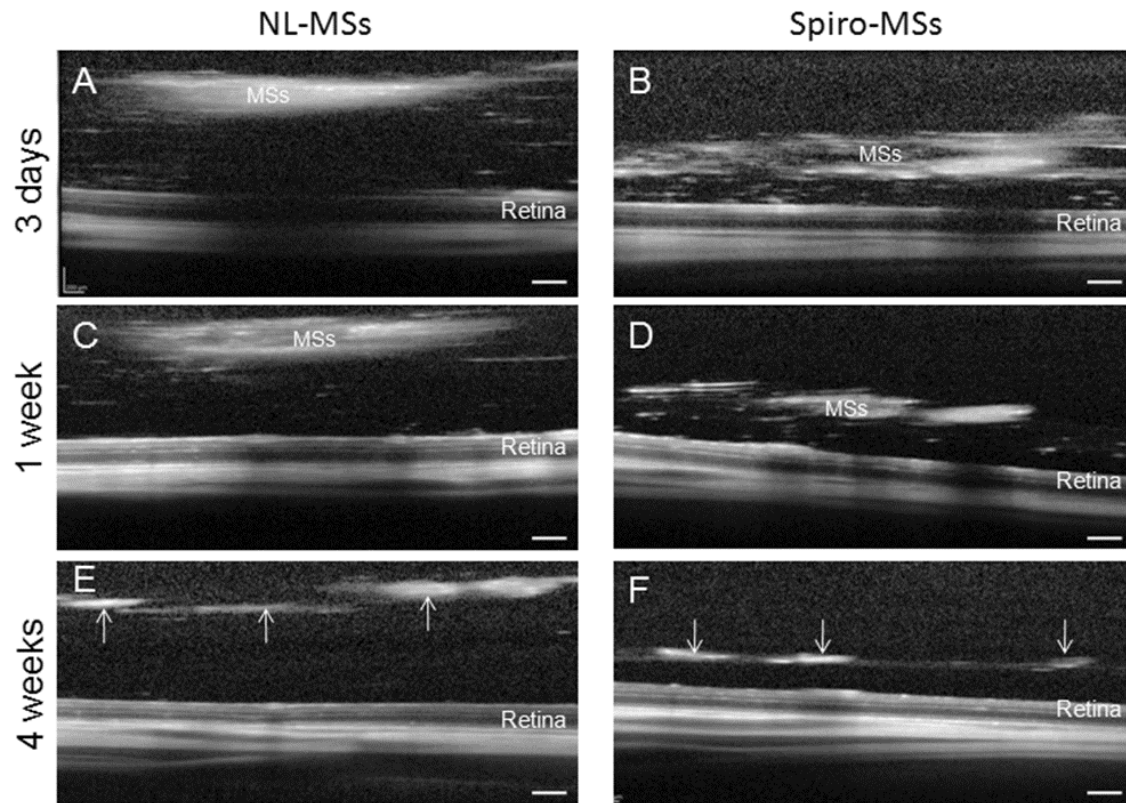


Figure 5. Optical coherence tomography (OCT) examination of the rat eyes injected with non-loaded (NL-MSs) and spironolactone-loaded PLGA microspheres (Spiro-MSs). At day3 after IVT, MSs tend to aggregate in the vitreous in both NL-MSs (A) and Spiro-MSs groups (B). MSs decrease from 1 week (C and D) to 4 weeks (E-F) and scattered at 4 weeks after IVT (E and F). The underlying retina maintains its normal structure at each time point (A-F) in both groups. Arrows indicate microspheres. Scale bars: 200 μm .

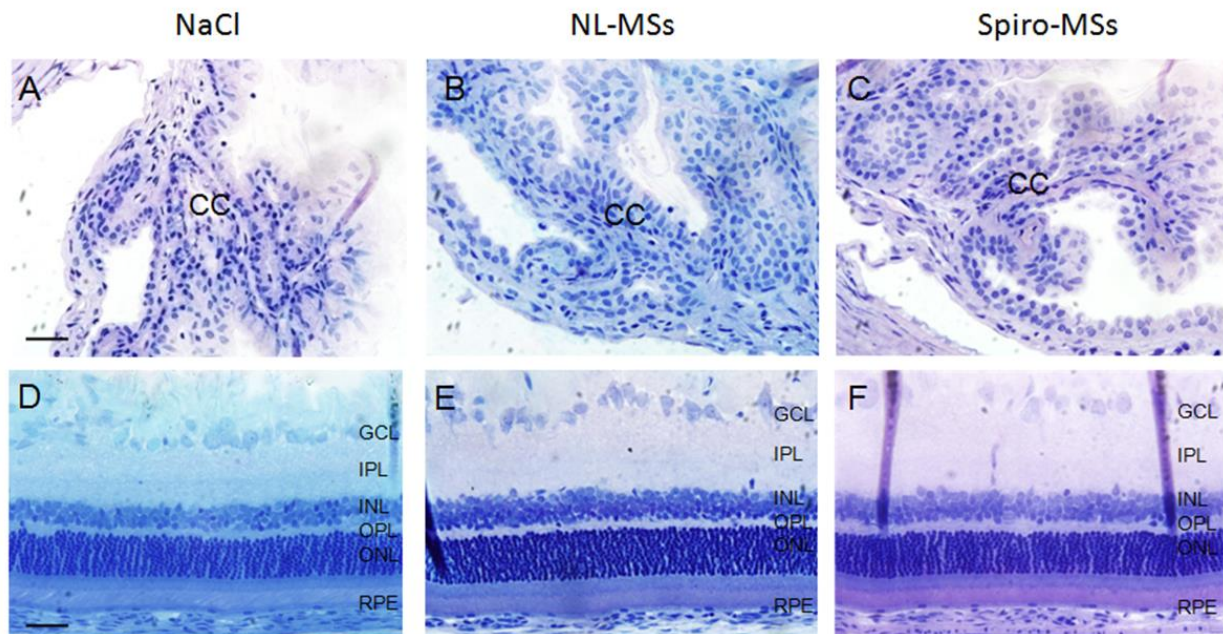


Figure 6. Histology of the rat ciliary body and retina 4 weeks after IVT of high amount of PLGA microspheres (MSs). The eyes injected with non-loaded (NL-MSs) or spironolactone-loaded PLGA microspheres (Spiro-MSs) show intact ciliary body (CC, B and C) and retinal structure (E and F) compared to saline-injected eyes (A and D). No inflammatory cell is observed. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. n=4 rats per group, scale bars: 50 μ m.

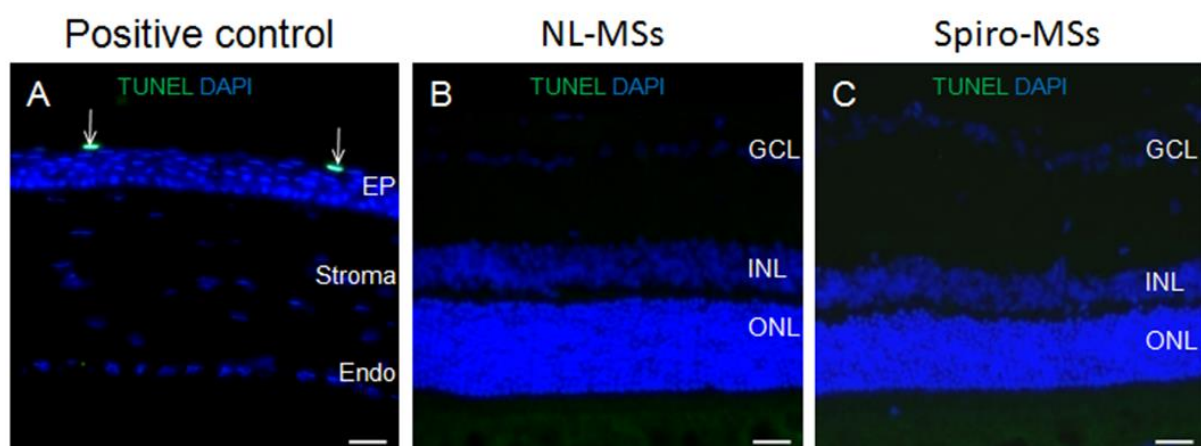


Figure 7. TUNEL assay of rat retina 4 weeks after IVT of high amount of PLGA microspheres. TUNEL positive corneal epithelial cells (green) serve as positive control (arrows in A). No TUNEL positive cell is observed in the retina of rat eyes injected with either non-loaded PLGA microspheres (NL-MSs) or

spironolactone-loaded microspheres (Spiro-MSs). EP, corneal epithelium; Endo, corneal endothelium; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. n=4 rats per group, scale bars: 50 μ m.

High amount but not low amount of PLGA microspheres may induce retinal stress

The amount of injected MSs resulted in different retinal responses. GFAP labels astrocytes and end feet of retinal Müller glial cells (RMG) in saline-injected rat eyes are shown in Figure 8A. Both high amount of NL-MSs and Spiro-MSs enhanced GFAP fluorescence in activated RMG extending up to outer limiting membrane (Figure 8B and C). Low amount of NL-MSs and Spiro-MSs induced only a moderate activation of RMG, GFAP staining was limited in the inner retina (Figure 8D and E).

Macrophages and microglial cells were immunolabeled by anti-IBA1 antibody. Activation of microglia induces modification of its shape from ramified multidirectional extensions to polarized dendrites and then to round amoeboid cells. In saline-injected rat eyes, macrophages/microglial cells maintain their inactive ramified shape (Figure 9A). In high amount NL-MSs and Spiro-MSs groups, some macrophages/microglial cells became rounded and activated (Figure 9B and C, arrows), in eyes injected with low amount NL-MSs or Spiro-MSs, only a few round amoeboid cells were observed (Figure 9E and F). Quantification of IBA1 positive cells showed that the number of activated macrophage/microglia was significantly decreased in low amounts of MSs-injected eyes. No difference was observed between NL-MSs and Spiro-MSs (Figure 9D).

These results suggest that high amount of MSs injected in the vitreous can induce glial activation, related to PLGA and not specifically to spironolactone, as no additional toxicity was observed in spironolactone-loaded MSs as compared to NL MSs.

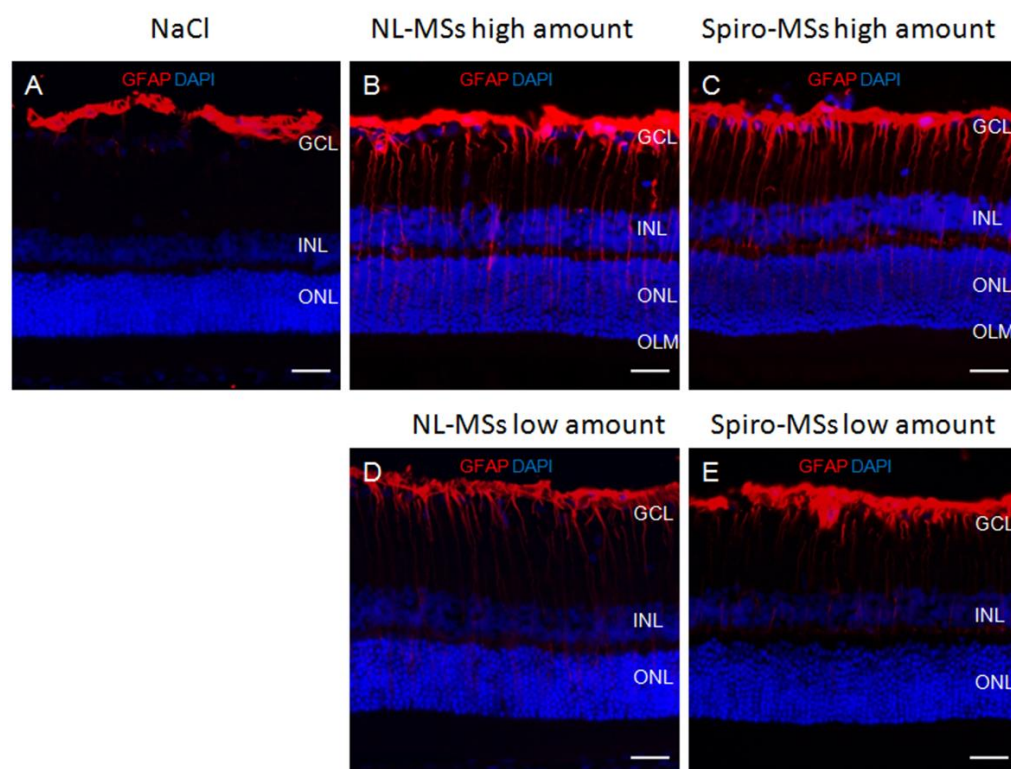


Figure 8. GFAP immunostaining in the rat retina 4 weeks after IVT of spironolactone-loaded (Spiro-MSs) and non-loaded PLGA microspheres (NL-MSs). GFAP (in red) labels astrocytes and end feet of retinal Müller glial cells (RMG) in saline-injected rat eyes (A). Both high amount NL-MSs (B) and Spiro-MSs (C) enhance GFAP fluorescence in activated RMG extending up to outer limiting membrane (OLM). GFAP staining is limited in the inner retina in the retina of rat eyes injected with low amount MSs (D and E). There is no difference between NL-MSs and Spiro-MSs. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. n=4 rats in high amount groups and 5 rats in low amount groups. Scale bars: 50 μm.

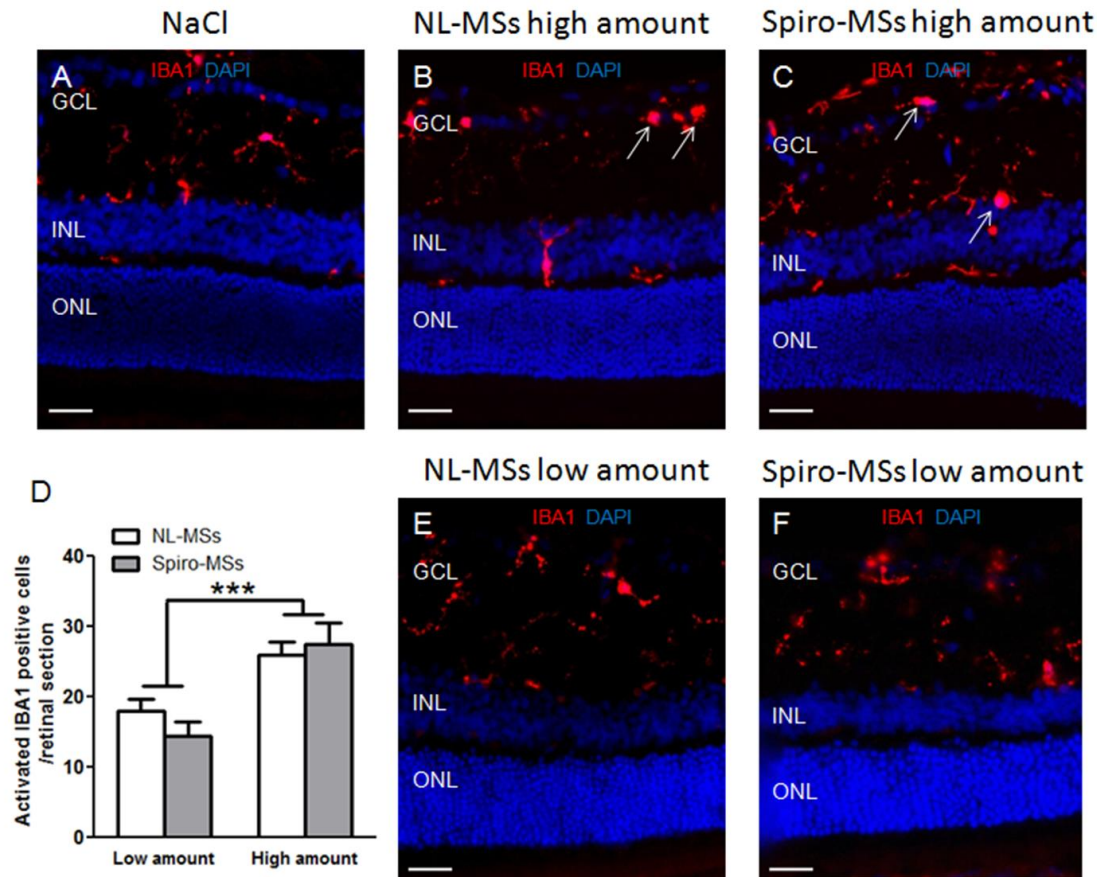


Figure 9. IBA-1 immunolabeling of macrophages and microglial cells in rat retina 4 weeks after IVT of spironolactone-loaded (Spiro-MSs) and non-loaded PLGA microspheres (NL-MSs). In saline-injected rat eyes, macrophages/microglial cells maintain their inactive ramified shape (A). In high amount NL-MSs (B) and Spiro-MSs groups (C), some macrophages/microglial cells become rounded and activated (arrows). In eyes injected with low amount NL-MSs (E) or Spiro-MSs (F), only a few round amoeboid cells are observed (E and F). Quantification of IBA1 positive cells shows a significant decrease in activated macrophage/microglia in low amount MSs-injected eyes. No difference was observed between NL-MSs and Spiro-MSs (D). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. n=4 rats in high amount groups and 5 rats in low amount groups. ***, P < 0.001.

Scale bars: 50 μ m.

High amount but not low amount of PLGA microspheres alter rat retinal function

Scotopic ERGs showed a significant reduction of a- and b-wave amplitude in eyes injected with high amount MSs (both NL- and Spiro-MSs) at intensities from 0.3 to 10 cd.s.m⁻², while low amount MSs did

not decrease the amplitude of both a and b waves, suggesting also that high dose but not low dose MSs could affect photoreceptor function (Figure 10). No significant difference was observed in photopic ERGs after IVT of high or low amount MSs (Figure 11). There was no difference between NL- and Spiro-loaded MSs on scotopic and photopic ERGs. ERG findings suggest that IVT of high amount MSs could be harmful for retinal function, while injections with low amount MSs resulted safe. No additional change in retinal function was observed for spironolactone released from MSs.

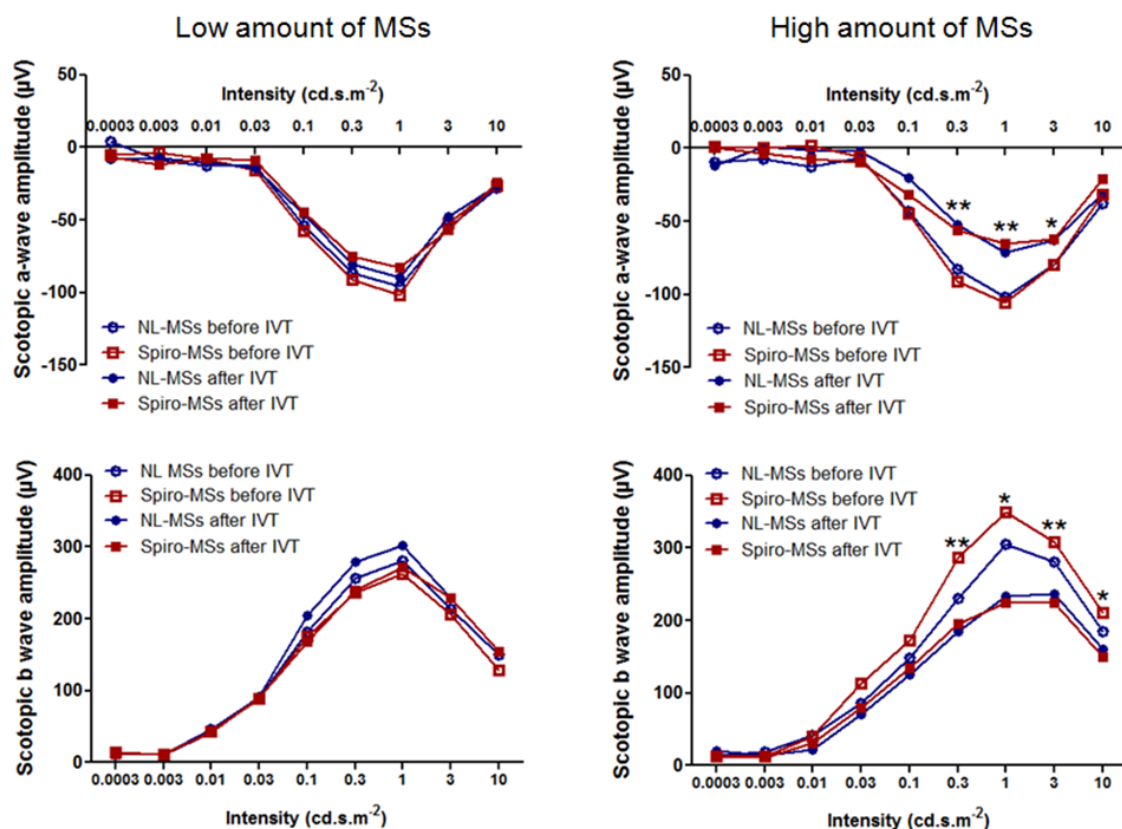


Figure 10. Scotopic electroretinograms (ERGs) of rat eyes injected with spironolactone-loaded (Spiro-MSs) and non-loaded PLGA microspheres (NL-MSs). While low amount MSs do not decrease the amplitude of both a and b waves, high amount MSs reduce significantly scotopic a-wave amplitude at intensities from 0.3 to 3 cd.s.m^{-2} as well as b-wave amplitude at intensities from 0.3 to 10 cd.s.m^{-2} . There is no difference between NL- and Spiro-loaded MSs on scotopic ERGs. $n=4-8$ rats per group. *, $P < 0.05$; **, $P < 0.01$.

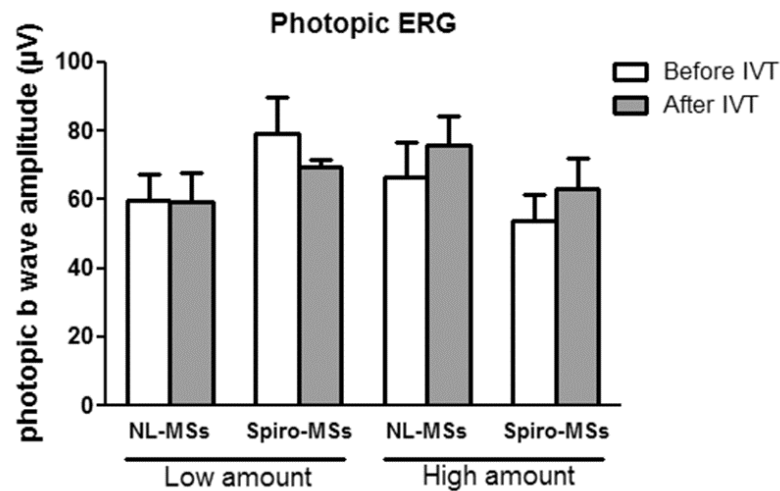


Figure 11. Photopicelectroretinograms (ERGs) of rat eyes injected with spironolactone-loaded (Spiro-MSs) and non-loaded PLGA microspheres (NL-MSs). No significant difference was observed in photopic ERGs after IVT of high or low amount MSs. n=4-8 rats per group.

Spironolactone released from intravitreal PLGA microspheres has biological effects in the rat retina

After intraocular injection of spironolactone-loaded MSs, we evaluated the effects on known biologic spironolactone regulated targets [28-30] to ensure that the released spironolactone was active in the retina.

Quantitative PCR was thus performed using rat retinas 1 month after IVT. We found that the transcription of spironolactone target genes, like NGAL, NOX2, CCL2 and ICAM-1, was down-regulated (Figure 12), suggesting that spironolactone released from PLGA MSs reached the retina and exerted biological effects in the rat retinas.

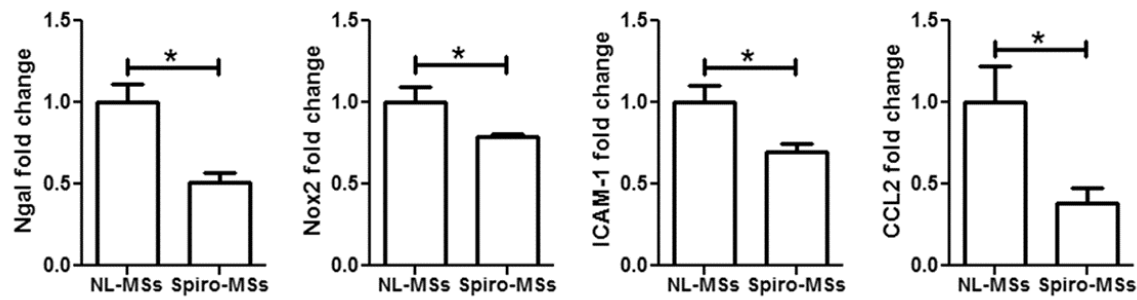


Figure 12. Quantitative PCR of the neuroretina of rat eyes injected with low amount of PLGA microspheres. Spironolactone released from microspheres (Spiro-MSs) decreases the transcripts of neutrophil gelatinase associated lipocalin (NGAL), NADPH oxidase subunit 2 (NOX2), chemokine ligand 2 (CCL2) and intercellular adhesion molecule-1 (ICAM-1) in rat neuroretina compared to non-loaded microspheres (NL-MSs). n=5 rats per group. *, $P < 0.05$.

Discussion

While the pharmacokinetic and pharmacodynamic properties of MR antagonists (MRA), and their therapeutic effects on cardiovascular disease and systemic adverse effects are well-documented, little is known about the pharmacology of MRA in the eye. We recently demonstrated that whilst MRA can exert beneficial anatomic and functional results in patients with CSCR, [15, 18, 31, 32] a long-term treatment with MRA is required in long-lasting chronic forms of the disease [18, 33]. The exact ocular bioavailability of spironolactone after oral administration was not known and a possible reduction of its ocular penetration together with a restoration of the blood-retinal barriers is suspected. In a preliminary experiment, we found that, after intravenous injection of spironolactone in rats without blood-retinal barrier breakdown, spironolactone and its metabolites were below detectable levels in the vitreous suggesting that the clinical observed effect could result partly from the effects on the choroidal vessels which are not protected by retinal barriers (reduced thickness and vasodilation) but also probably from spironolactone penetration through RPE barrier breakdown, occurring in acute episodes and in chronic forms of the diseases. We thus hypothesize that optimized efficacy and systemic tolerance of spironolactone could be achieved by the direct intraocular

administration of spironolactone. Amongst MRA, spironolactone was selected for ocular formulations because eplerenone has a 40-fold lower affinity for the MR but higher selectivity, reducing systemic hormonal effects, which cannot be feared after intraocular injection. We have first evaluated the ocular tolerance of the only commercial intravenous preparation of potassium canrenoate (Soludactone®, Pfizer), that is the active metabolite of the prodrug spironolactone for potential further clinical use. At a clinically relevant dose (10 μ M) which corresponds to minimal effective concentration blocking the mineralocorticoid activity in rat eyes [9, 14], Soludactone did not induce morphological and functional alterations in the rat retina, suggesting the safety of its formulation. However, spironolactone and canrenone have very short half-life in the vitreous *in vivo*, requiring frequent administrations to maintain therapeutic concentrations in the target site. For this reason, the use of an intraocular drug delivery systemable to release the active substance for long term would result of great interest in the clinical practice.

Biodegradable MSs for intraocular drug delivery have the advantage that they can release the drug over time with one single intravitreal injection, having the same effect as multiple injections. Among the polymers used for intraocular drug delivery, PLGA are the most employed and it is approved by FDA and European Medicines Agency (EMA) for clinical use [34]. PLGA MSs incorporating active substances have been evaluated in experimental models of uveitis [35], diabetic retinopathy [36], glaucoma [37, 38], photoreceptor degeneration [39, 40], choroidal neovascularization [41] and regenerative medicine [42]. Furthermore, Ozurdex® (Allergan), intravitreal implant composed of PLGA releasing dexamethasone, has been approved to treat macular edema and non-infectious uveitis [43].

In our study, spiro-MSs showed sustained *in vitro* release of spironolactone for 31 days due to the fact that a PLGA 50:50 (50% lactide and 50% glycolide) was used, allowing a relatively rapid degradation [22]. However, if desired, the degradation rate can be modulated by modifying the PLA:PGA ratio as well as the molecular weight of the polymer. Also the degradation rate of D- or L-

PLA, DL-PLA and PGA is slower than PLGA, making it possible to select the most adequate polymer to prepare the particles [40].

As previously described, MSs have the tendency to aggregate several days after IVT in small animals [22, 44], a phenomenon that we also observed in the present study. Among the clinical signs, inflammation can occur in early stages after IVT of PLGA MSs. These signs have been described to be similar to the observed for sutures and disappear 2-4 weeks after administration. Giordano et al explained as a non-progressive foreign body reaction [44]. The clinical follow-up performed using SD-OCT has shown a continuous degradation of microspheres from injection to the 4th week. It is therefore unlikely that additional inflammatory reaction would be observed at later time points. Previous experiments have shown that no inflammatory reaction occurred in rabbits at the end of PLA/PLGA microspheres degradation after intravitreal injection, except for activation of retinal glial Müller cells, similar to our observations [44].

In our experimental conditions, no retinal and choroidal damage were observed 1 month after administration. In human, the special concern is related to the risk of visual impairment after IVT of MSs. This clinical issue has been previously evaluated in a tolerance study, in which sustained-release triamcinolone MSs system (RETAAC) was injected in human eyes. Authors reported a tendency of the MSs to aggregate and condensate at the site of the injection leaving a free visual axis. In the same study, no inflammation was observed after MSs injection, although it should be taken into account that, the anti-inflammatory drug could attenuate the inflammatory reaction [45]. In the present study, high amount of PLGA MSs (0.5 mg) resulting in an intravitreal concentration of 10 mg/mL lead to retinal macro and microglial reactions. However, it did not alter retinal structure, neither induce apoptosis in retinal neuronal cells. A reduced scotopic ERGs was also observed, suggesting photoreceptor dysfunction. On the contrary, low concentrations of PLGA MSs (intravitreal concentration 2 mg/mL) appear well tolerated and did not alter retinal function, findings consistent with previous studies describing the safety of low concentration intravitreal PLGA MSs in rabbit eyes

[24, 44]. In larger eyes with higher vitreous cavity, reduced inflammatory reaction is expected as observed with PLGA rods. The amount of spironolactone *in vitro* released from low amount of MSs resulted sufficient to inhibit mineralocorticoid activity in the retina and choroid [9, 11].

Despite the undeniable interest of performing the *in vivo* pharmacokinetic study of the Spiro-MSs, it was technically not possible due to the small volume of intraocular fluid in rats and spironolactone concentrations below the detection limit of the assay. ~~Biological effects were instead measured at one month after Spiro-MSs administration to evaluate whether spironolactone was still present in the retina. In eyes receiving Spiro-MSs, we could identify known MR-regulated targets, reflecting effects of spironolactone in the retina. Amongst the regulated targets, oxidative stress and inflammation were reduced which could have contributed to the good tolerance of Spiro-MSs.~~ Although spironolactone PK profile observed *in vitro* cannot be simply extrapolated to the *in vivo* release of spironolactone from MS, it gives the information that the kinetic profile of spironolactone from PLGA microspheres (>1 month) is compatible with clinical use. The rat vitreous being different from the human vitreous and vitreous drug levels not being an imperfect reflect of retinal drug levels, we have chosen not to perform release kinetic in the rat (that has a very small vitreous volume, i.e. around 30µl) but rather to analyze on one hand the stability of spironolactone in human vitreous and on the other hand to test whether biological effect of spironolactone was still observed in the retina at 4 weeks. Our results have shown that indeed, known spironolactone molecular targets were still regulated in the neuroretina at 4 weeks.

This is the first study evaluating the ocular tolerance of MRA in solution and spironolactone loaded PLGA MSs. It demonstrates that intravitreal injection of soludactone alone did not affect the retinal morphology and function. PLGA MSs allows a sustained release of spironolactone. High intravitreal concentration of PLGA MSs (10 mg/mL) unloaded and loaded with spironolactone induce somehow retinal stress and photoreceptor dysfunction in rats. However, MSs concentration of 2 mg/mL (unloaded and loaded with spironolactone) in rat eyes showed excellent morphologic and functional

tolerance and could be a safe therapeutic choice for chorioretinal disorders in which illicit MR activation could be pathogenic. Optimized formulations with longer release profile could benefit to patients with severe CSCR forms requiring long-term ocular MR antagonism, whilst suppressing potential side-effects.

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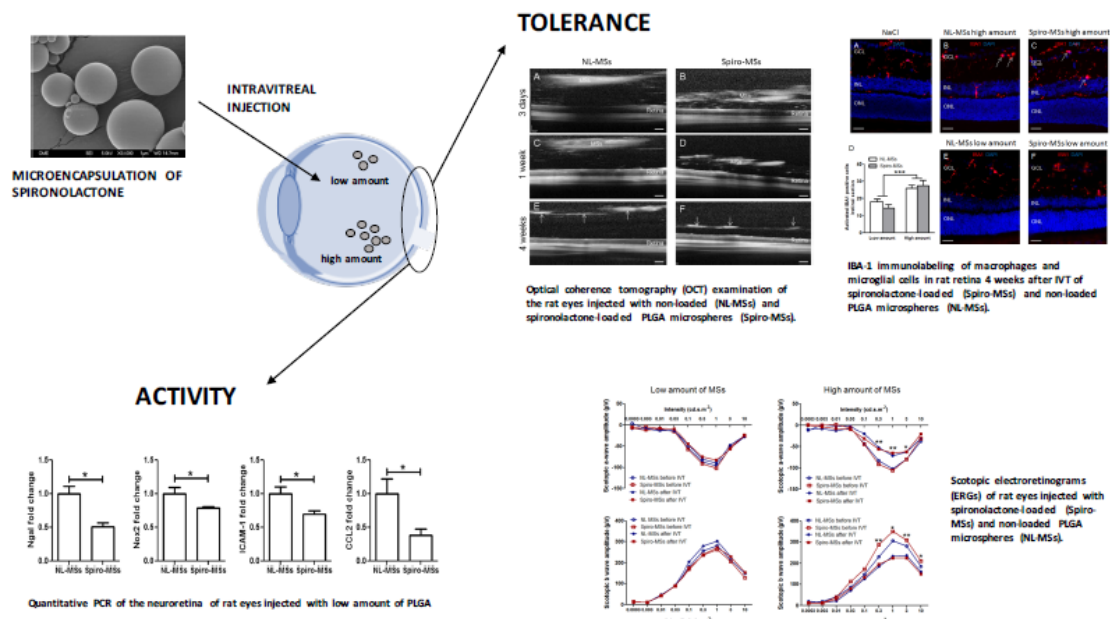
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TOLERANCE OF HIGH AND LOW AMOUNTS OF PLGA MICROSPHERES LOADED WITH MINERALOCORTICOID RECEPTOR ANTAGONIST IN RETINAL TARGET SITE

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Graphical abstract