UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Evaluación de los efectos hepáticos de la administración de Bisfenol A y Bisfenol F en un modelo experimental murino

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Beatriz Linillos Pradillo

DIRIGIDA POR

Elena María Vara Ameigeiras
Jesús Ángel Fernández-Tresguerres Fernández
Lisa Rancán

Madrid

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PROGRAMA DE DOCTORADO EN INVESTIGACIÓN BIOMÉDICA

A mis padres y a mi hermana,

A mis abuelos,

A Ismael

"Ciencia es todo aquello sobre lo cual siempre cabe discusión".

José Ortega Y Gasset

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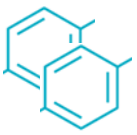


ABREVIATURAS

A

A: Adenina

ADN: Ácido desoxirribonucleico

AGPI: Ácidos grasos poliinsaturados

AIF: Factor inductor de apoptosis

ALP: Fosfatasa alcalina

ALT: Alanina aminotransferasa

Apaf-1: Factor activador de la proteasa apoptótica 1

AST: Aspartato aminotransferasa

ATP: Adenosín trifosfato

B

BP: Bisfenoles

BPA: Bisfenol A

BPAF: Bisfenol AF

BPAG: BPA-glucurónido

BPAS: BPA-sulfato

BPF: Bisfenol F

BPFG: BPF-glucurónido

BPFS: BPF-sulfato

BPS: Bisfenol S

C

C: Citosina

Ca^{2+}: Calcio

CASP: Caspasa

CAT: Catalasa

CEH: Células hepáticas estrelladas

CO: Monóxido de carbono

D

DEs: Disruptores endocrinos

DES: Dietilestilbestrol

DAMPs: Patrones moleculares asociados a daños

DPN6: Día postnatal 6

DDT: 2,2-bis-(p-clorofenil) -1,1,1-tricloroetano

E

EFSA: Autoridad Europea de Seguridad Alimentaria

eNOS: Óxido nítrico sintasa endotelial

EPA: Agencia de Protección Ambiental de los Estados Unidos

ER: Receptor de estrógenos

ERα: Receptor de estrógenos alfa

ERβ: Receptor de estrógenos beta

ERγ: Receptor de estrógenos gamma

EDCs: *Endocrine Disrupting Chemicals*

F

Fe^{2+}: Hierro

G

G: Guanina

GD: Día gestacional

GPER1: *G protein-coupled estrogen receptor 1*, Proteína G acoplada al receptor de estrógenos 1

GPR30: *G protein-coupled receptors*, receptores acoplados a proteínas G 30

GPx: Glutatión peroxidasa

GR: Glutatión reductasa

GSH: Glutatión reducido

GSSG: Glutatión oxidado

GST: Glutatión-S-transferasa

γ-GCS: Gamma-glutamilcisteína-sintetasa

H

H_2O_2: Peróxido de hidrógeno

HBPA: *High doses of BPA*, dosis alta de BPA

HBPF: *High doses of BPF*, dosis alta de BPF

HClO: Ácido hipocloroso

HO-1d: Hemo-oxigenasa inducible

HO-2d: Hemo-oxigenasa constitutiva

HOO*: Radicales hidroperoxilo

I

IDT: Ingesta diaria tolerable

IFN-γ: Interferón gamma

IL: Interleuquina

iNOS: Óxido nítrico sintasa inducible

J

JNK: Quinasas c-Jun N-terminal

K

K^+: Potasio

KC: Células de Kupffer

KDa: kilodalton

KGN: *human granulosa cells*, células de la granulosa humanas

KO: Knockout

L

LBPA: *Low doses of BPA*, dosis baja de BPA

LBPF: *Low doses of BPF*, dosis baja de BPF

LPO: Lipoperóxidos

LRR: Dominio de repetición rico en leucinas

M

MAPKs: Quinasas activadas por mitógenos

MDA*:* Malondialdehído

N

NACHT: Dominio de oligomerización de unión a nucleótidos

NADPH: Fosfato de dinucleótido de nicotinamida y adenina oxidasa

NAFLD: *Non alcoholic fatty liver disease*, Enfermedad del hígado graso no alcohólico

NF-κB: Factor nuclear kappa-potenciador de la cadena ligera de las células B activadas

NK: Linfocitos *natural killer*

NMDR: *Non-monotonic dose responses,* dosis-respuestas no monotónicas

nNOS: Óxido nítrico sintasa neuronal

NO: Óxido nítrico

NO_2: Dióxido de nitrógeno

$ONOO^-$: Peroxinitrito

O

OMS: Organización Mundial de la Salud

OH^*: Radicales hidroxilo

8-OHdG: 8-oxo-2'-desoxiguanosina

O_2^{*-}: Anión superóxido

O_3: Ozono

O_2^1: Oxígeno singlete

P

PAMPs: Patrones moleculares asociados a patógenos

PARP: Poli-ADP polimerasas

PBB: Bifenilos polibromados

PCBs: Bifenilos organoclorados

PYCARD: *Apoptosis-associated speck-like protein containing CARD*

R

RNS: *Reactive nitrogen species*, especies reactivas del nitrógeno

ROS: *Reactive oxigen species*, especies reactivas del oxígeno

S

SOD: Superóxido dismutasa

T

T: Timina

TG: Triglicéridos

TGF-β: Factor de crecimiento transformante beta

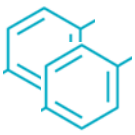
TLR: Receptores tipo Toll

TNF-α: Factor de necrosis tumoral alfa

U

UDPGT: Uridil difosfato glucuronil transferasa

UE: Unión Europea



RESUMEN

INTRODUCCIÓN

El bisfenol A (BPA) es un compuesto fenólico utilizado en la elaboración de plásticos para la protección o el envasado de alimentos. El BPA se considera un disruptor endocrino (DE) que causa daños adversos, incluyendo efectos endocrinos, reproductivos, metabólicos, cardiovasculares e incluso cáncer, por lo que se considera un riesgo para la salud pública. Debido a los riesgos asociados al BPA, su análogo, el bisfenol F (BPF) está sustituyendo al BPA en la fabricación de productos plásticos de base. Los monómeros de BPF, al igual que los del BPA, pueden liberarse en la cadena alimentaria, lo que da lugar a una exposición humana continua a dosis bajas. Dado que los bisfenoles se metabolizan principalmente en el hígado, este órgano es más vulnerable a dosis bajas de bisfenoles que otros. La gran cantidad de literatura previa sobre los efectos del BPA a diferentes dosis y en diferentes tejidos ha demostrado que puede generar un incremento del estrés oxidativo, inflamación y apoptosis, además de causar enfermedades hepáticas con un posible papel del inflamasoma NLRP3. En el caso del BPF, la literatura es más reciente y se desconoce si el BPF puede desencadenar respuestas inflamatorias a través del inflamasoma NLRP3, que desempeña un papel importante en el desarrollo de enfermedades hepáticas; además de sus efectos sobre el estrés oxidativo y la peroxidación lipídica. La mayor parte de los estudios se centran en el efecto en el organismo adulto, pero es importante estudiar la exposición durante el desarrollo prenatal. La exposición a DEs durante este período crítico podría provocar alteraciones en la ontogenia de los tejidos, aumentando el riesgo de desarrollar enfermedades en la edad adulta.

OBJETIVOS

El objetivo principal de esta tesis ha sido estudiar los posibles efectos tras la administración de una dosis baja y otra dosis alta de BPA y su análogo, BPF, en el hígado de ratas *Long Evans* gestantes y lactantes, por ser el principal órgano encargado de su metabolismo y detoxificación, así como estudiar los efectos perinatales en ambos sexos de la descendencia en el día postnatal 6 (DPN6). Para llegar a este objetivo hemos determinado los siguientes objetivos específicos:

1. Estudiar si la administración de BPA induce daño hepático en ratas lactantes y en las hembras de su descendencia afectando al equilibrio redox y generando inflamación y apoptosis.

2. Evaluar si la administración de BPF genera estrés oxidativo al afectar a las enzimas antioxidantes y el sistema del glutatión, además de inducir la peroxidación lipídica en el hígado de ratas lactantes y en ambos sexos de la descendencia.

3. Estudiar si la administración de BPF incrementa el estrés nitrosativo y si el inflamasoma NLRP3 desempeña un papel en el desarrollo de la inflamación en el hígado de ratas lactantes

y su efecto perinatal en ambos sexos de la descendencia.

MATERIAL Y MÉTODOS

Se distribuyeron aleatoriamente 36 ratas *Long Evans* según el tratamiento oral: control, grupo de dosis baja de BPA o BPF (LBPA o LBPF; 0,036 mg/kg peso corporal/día) y grupo de dosis alta de BPA (HBPF; 3,42 mg/kg peso corporal/día) o de BPF (HBPF; 3,65 mg/kg peso corporal/día). Respecto al BPA, se midieron mediante métodos colorimétricos las enzimas antioxidantes CAT, SOD, GR, GPx y GST, el sistema del glutatión GSH/GSSG y los marcadores de daño lípido-ADN MDA, LPO, NO y 8-OHdG. Los inductores del estrés oxidativo HO-1d e iNOS, la citoquina proinflamatoria IL-1β y los marcadores de apoptosis AIF, Bax, Bcl-2 y Bcl-XL se midieron mediante qRT-PCR y Western blotting en el hígado de ratas lactantes y en las crías hembras de su descendencia en el DPN6. Se evaluaron los daños hepáticos mediante los marcadores séricos AST, ALT y GGT, así como con tinción hematoxilina-eosina. Respecto al BPF, además se evaluaron los componentes del inflamasoma NLRP3 (NLRP3, PyCARD, CASP1) y las citoquinas proinflamatorias IL-1β, IL-18, IFN-γ y TNF-α mediante expresión génica y proteica en el hígado de las ratas lactantes y en las crías hembras y machos del DPN6.

RESULTADOS Y DISCUSIÓN

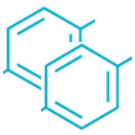
La administración de LBPA aumentó los niveles de estrés oxidativo en el hígado de ratas lactantes, disminuyendo las actividades de las enzimas antioxidantes y alterando el sistema del glutatión. Además, promovió la peroxidación lipídica y el daño oxidativo en el ADN, junto con un incremento de los niveles de NO en el plasma. A su vez, favoreció el incremento de la citoquina proinflamatoria IL-1β y la liberación de los factores proapoptóticos AIF y Bax, además de disminuir los factores antiapoptóticos Bcl-2 y Bcl-XL. En las crías hembras de la descendencia en el DPN6 también se observaron los mismos efectos: incremento del estrés oxidativo, inflamación y apoptosis con efectos más notables tras dosis bajas.

La exposición a LBPF durante la gestación y la lactancia afectó a los mecanismos de defensa del hígado (disminución de las enzimas antioxidantes y alteración del sistema del glutatión), aumentando los niveles de ROS. Este exceso de ROS afectó a las membranas celulares de los hepatocitos, aumentando los niveles de peroxidación lipídica de los mismos. Tras la exposición perinatal al BPF se observaron efectos similares en ambos sexos de la descendencia respecto a las ratas lactantes. Además, las ratas lactantes tratadas con LBPF mostraron un aumento significativo del estrés nitrosativo junto a la activación de los componentes del inflamasoma NLRP3 y se produjo la liberación de diferentes citoquinas proinflamatorias como IL-1β, IL-18, IFN-γ y TNF-α.

Los efectos fueron similares en ambos sexos de la descendencia tras la exposición perinatal, aumentando los niveles de los marcadores de estrés nitrosativo y de citoquinas proinflamatorias con un posible papel del inflamasoma NLRP3.

CONCLUSIÓN

La administración oral de BPA y BPF a dosis ambientalmente relevantes genera hepatotoxicidad induciendo alteraciones proinflamatorias, prooxidantes y proapoptóticas tanto en las ratas adultas lactantes como en su descendencia tras la exposición peri y postnatal.



ABSTRACT

INTRODUCTION

Bisphenol A (BPA) is a phenolic compound used in plastics elaboration for food protection or packaging. BPA is considered an endocrine disruptor (ED) that causes health problems in humans, including endocrine, reproductive, metabolic, cardiovascular disorders and even cancer, so that it has been considered a risk for public health. Due to the risks associated with BPA, its analogue, bisphenol F (BPF) is replacing BPA in the manufacture of basic plastic products. BPF monomers, like BPA monomers, can be released into the food chain, resulting in continuous low-dose human exposure. Since bisphenols are mainly metabolized in the liver, this organ is more vulnerable to low doses of bisphenols than others. The extensive previous literature on the effects of BPA at different doses and in different tissues has shown that it can lead to increased oxidative stress, inflammation and apoptosis, as well as causing liver disease with a possible role played by the NLRP3 inflammasome. Regarding BPF, the literature is more recent, scarce, and it is unknown whether BPF can trigger inflammatory responses through the NLRP3 inflammasome, which plays an important role in the development of liver diseases, in addition to its effects on oxidative stress and lipid peroxidation. Most studies focus on the effect on the adult organism, but it is also important to study exposure during prenatal development. Exposure to EDs during this critical period could lead to alterations in tissue ontogeny, increasing the risk of developing diseases in adulthood.

OBJECTIVES

The main objective of this PhD thesis has been to study the possible effects after the administration of low and a high doses of BPA and its analogue, BPF, in the liver of pregnant and lactating *Long Evans* rats, being this the main organ in charge of its metabolism and detoxification. Also, to study the perinatal effects in both sexes of the offspring at postnatal day 6 (PND6). To reach this objective we have determined the following specific objectives:

1. To study whether BPA administration induces liver damage in lactating rats and their female offspring by affecting redox balance and generating inflammation and apoptosis.

2. To evaluate whether BPF administration generates oxidative stress by affecting antioxidant enzymes and the glutathione system, in addition to exert lipid peroxidation in the liver of lactating rats and in both sexes of the offspring.

3. To study whether BPF administration increases nitrosative stress and if NLRP3 inflammasome may play a role in the development of inflammation in the liver of lactating rats and its perinatal effect in both sexes of the offspring.

MATERIAL AND METHODS

Thirty-six *Long Evans* rats were randomly distributed according to oral treatment: control, BPA or BPF low-dose group (LBPA or LBPF; 0.036 mg/kg body weight/day) and BPA or BPF high-dose (HBPA; 3.42 mg/kg body weight/day) or HBPF group (HBPF; 3.65 mg/kg body weight/day). Regarding BPA exposure, the levels of antioxidant enzymes CAT, SOD, GR, GPx and GST, the glutathione system GSH/GSSG, oxidative stress inducers HO-1d and iNOS, proinflammatory cytokine IL-1β and apoptosis markers AIF, Bax, Bcl-2 and Bcl-XL were measured by qRT-PCR and Western blotting in the liver of lactating rats and their PND6 female offspring. Liver damage was assessed by serum markers AST, ALT, GGT, as well as hematoxylin-eosin staining. Regarding BPF, in addition, NLRP3 inflammasome components (NLRP3, PyCARD, CASP1) and proinflammatory cytokines (IL-1β, IL-18, IFN-γ and TNF-α) were evaluated by gene and protein expression in the liver of lactating rats and in PND6 female and male offspring.

RESULTS AND DISCUSSION

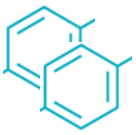
LBPA administration increased oxidative stress levels in the liver of lactating rats, decreasing the activities of antioxidant enzymes and altering the glutathione system. In addition, LBPA promoted lipid peroxidation and oxidative DNA damage, together with an increase in plasma NO levels. In turn, it favoured the increase of the proinflammatory cytokine IL-1β and release of proapoptotic factors AIF and Bax, and showed a decrease of antiapoptotic factors Bcl-2 and Bcl-XL. The same effects were observed in PND6 female offspring: oxidative stress, inflammation and apoptosis increase with more noticeable effects after the low doses.

Exposure to LBPF during gestation and lactation affected liver defence mechanisms (decreased antioxidant enzymes and altered glutathione system), increasing ROS levels. This excess of ROS affected hepatocyte cell membranes, increasing the levels of lipid peroxidation. After BPF perinatal exposure, similar effects were observed in female and male offspring with respect to lactating rats. In addition, LBPF-treated lactating rats showed a significant increase in nitrosative stress along with activation of NLRP3 inflammasome components and release of different proinflammatory cytokines such as IL-1β, IL-18, IFN-γ and TNF-α. The effects were similar in both sexes of the offspring after perinatal exposure, showing increased levels of nitrosative stress markers and proinflammatory cytokines with a possible role of the NLRP3 inflammasome.

CONCLUSION

Oral administration of BPA and BPF at environmentally relevant doses, generates hepatotoxicity inducing proinflammatory, prooxidant and proapoptotic alterations in both lactating adult rats

and their offspring after peri- and postnatal exposure.



INTRODUCCIÓN

1. DISRUPTORES ENDOCRINOS

1.1 DEFINICIÓN Y MECANISMOS DE ACCIÓN

Los plásticos son un tipo de polímeros que han cobrado relevancia en las últimas décadas al estar presentes en la mayoría de los productos de uso cotidiano. Su fabricación y uso tienen un impacto en el entorno natural, la fauna, la flora y también en la vida humana. Algunos de estos plásticos contienen sustancias químicas conocidas como disruptores endocrinos (DEs), o en inglés "*Endocrine Disrupting Chemicals* (EDCs)". Este término se propuso en 1991 tras confirmar diversas alteraciones de la función reproductiva en la vida silvestre y en los seres humanos relacionadas con el uso intensivo de plaguicidas en la agricultura (Basak et al., 2020). Un disruptor endocrino (DE) se define como una sustancia sintética que se encuentra en diversos materiales con la capacidad de interferir con el sistema endocrino produciendo efectos adversos sobre la salud de un organismo o de su progenie (Kabir et al., 2015).

Las hormonas de nuestro sistema endocrino regulan distintos procesos como el desarrollo, el crecimiento, la reproducción, el metabolismo, la inmunidad, el comportamiento, etc. Las acciones biológicas de las hormonas sintetizadas dentro de un organismo, tales como los estrógenos, progesterona, testosterona y tiroxina, son mediadas por proteínas receptoras de alta afinidad localizadas en el interior de las células diana. La interacción de una hormona con su receptor inicia una cascada de sucesos que llevan a diferentes efectos asociados con cada hormona en particular (Herrera, 2019).

Los efectos de los DEs sobre el sistema endocrino se pueden explicar por su actuación a diferentes niveles (**Figura 1**):

1. Mimetizar la acción de las hormonas confundiendo a sus receptores celulares.

2. Antagonizar la acción de las hormonas.

3. Alterar el patrón de síntesis, transporte y metabolismo hormonal.

4. Modular los niveles de los receptores hormonales correspondientes.

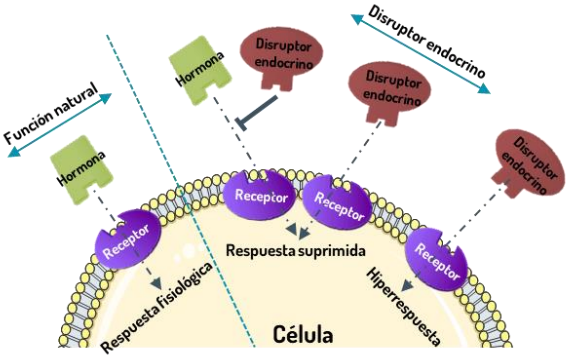


Figura 1. Mecanismos de acción de DEs. Los DEs son capaces de imitar o imitar en parte a las hormonas endógenas pudiendo unirse a receptores hormonales o incluso bloquear la unión de la hormona endógena con el receptor, alterando la respuesta fisiológica. Figura creada con Smart Servier Medical Art.

En definitiva, los DEs interfieren de alguna manera con la función endocrina de las hormonas produciendo efectos adversos en la salud humana y en la fauna silvestre. La **tabla 1** recoge las similitudes y diferencias entre las hormonas y los DEs.

	Hormonas	Disruptores endocrinos
Actúan a través de los receptores hormonales	Sí. Algunas tienen múltiples receptores Clases y subtipos de receptores específicos de tejidos Las hormonas normalmente se unen de forma similar a todos los subtipos de receptores	Sí y de otros múltiples receptores. Causarán una función anormal de los receptores Probables interacciones isoforma-específicas
Niveles en sangre	Los niveles en sangre no siempre reflejan la actividad	Los niveles en sangre no siempre reflejan la actividad
Unión a proteínas séricas	Pueden unirse con un pequeño porcentaje libre	Pueden unirse a las proteínas séricas
Bioacumulación	No hay	Posible
Relaciones no lineales de dosis-respuesta. Saturación.	Sí, activas a dosis bajas. Pueden mostrar relaciones dosis-respuesta no monotónicas. Siempre saturable con un rango dinámico variable.	Algunos actúan a dosis bajas, otros presentan rango de dosis variables. Pueden mostrar relaciones dosis-respuesta no monotónicas. Siempre saturable con un rango dinámico variable.
Efectos específicos de los tejidos y de la etapa de la vida	Sí	Sí
Efectos de desarrollo permanentes	Programas cerebro y sistema endocrino para la función adulta	Interfiere con los procesos de programación

Tabla 1. Comparación y similitudes entre las hormonas y los DEs. Figura adaptada de M. Pombo & L. Castro-Feijóo, 2005.

Por todo esto, la Agencia de Protección Ambiental de los Estados Unidos (EPA) define a un DE como un agente que interfiere con la síntesis, secreción, transporte, unión o eliminación de hormonas naturales presentes en el organismo que son responsables del mantenimiento de la homeostasis, la reproducción, el desarrollo y/o el comportamiento (M. Pombo & L. Castro-Feijóo, 2005).

A su vez, la Autoridad Europea de Seguridad Alimentaria (EFSA) respalda la definición de la Organización Mundial de la Salud (OMS) de que una sustancia debe cumplir tres criterios para ser considerada un DE: en primer lugar, la presencia de un efecto adverso, en segundo lugar, la presencia de actividad endocrina y, en tercer lugar, una relación causal entre ambos. Una característica destacable de los DEs es su capacidad de no seguir los patrones habituales de dosis-respuesta, es decir, no siempre dosis más altas pueden significar mayores efectos. Esta característica se denomina patrón dosis-respuesta "no monotónico" (NMDR, *non-monotonic dose responses)* (Vandenberg et al., 2012).

Hoy en día se conocen más de un millar de químicos con capacidad disruptora. Existen compuestos naturales que pueden comportarse como DEs: los fitoestrógenos, tales como los derivados de la soja, la alfalfa y el trébol. Sin embargo, la mayoría son compuestos industriales utilizados en la industria pesada [bifenilos organoclorados (PCBs), dioxinas], pesticidas organoclorados [clorpirifos, metoxicloro, 2,2-bis-(p-clorofenil)-1,1,1-tricloroetano (DDT) y sus metabolitos], fungicidas (vinclozolina), plásticos y plastificantes [Bisfenol A (BPA), y sus análogos [Bisfenol F (BPF), Bisfenol S (BPS) y Bisfenol AF (BPAF) y ftalatos], productos farmacéuticos [dietilestilbestrol (DES) e incluido más recientemente, el paracetamol], metales (cadmio, plomo, mercurio, uranio y un metaloide, el arsénico) (**Figura 2**) (M. Pombo & L. Castro-Feijóo, 2005).

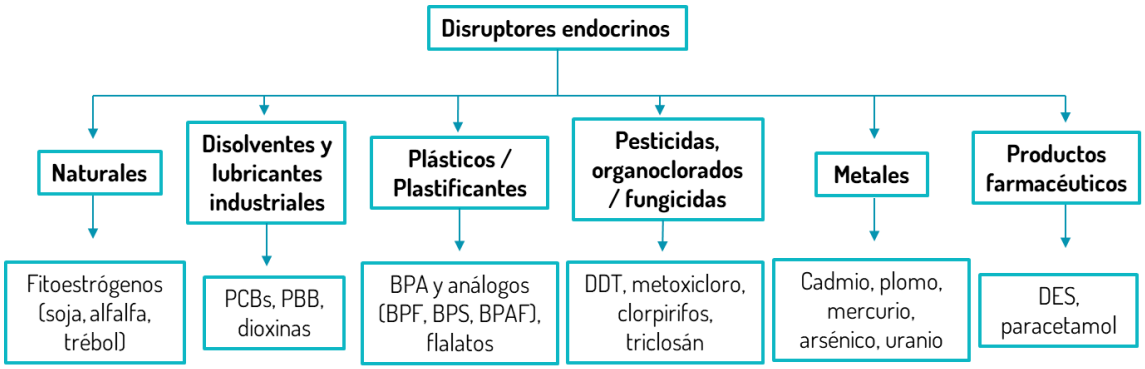


Figura 2. Clasificación y ejemplos de sustancias químicas que pueden actuar como DEs. Figura adaptada de (M. Pombo & L. Castro-Feijóo, 2005).PCBs: bifenilos organoclorados. PBB: bifenilos polibromados. BPA: Bisfenol A. BPF: Bisfenol F. BPS: Bisfenol S. BPAF: Bisfenol AF. DDT: Dicloro difenil tricloroetano. DES: Dietilestilbestrol.

1.2 BISFENOL A (BPA)

Los bisfenoles (BP) son un grupo de compuestos químicos con propiedades y estructuras similares. Se producen grandes cantidades de estos compuestos en todo el mundo y se calcula que cada año se emplean unos 2 millones de toneladas de BP en la producción de bienes fabricados con plástico. El Bisfenol A (BPA, bis (4-hidroxifenil) propano, $C_{15}H_{16}O_2$) es un compuesto xenoestrógeno sintético con una elevada prevalencia en nuestro medio ambiente formado por dos anillo fenólicos unidos en el centro por un grupo propano (Kazemi et al., 2016; Zhang et al., 2020). Se obtiene por la condensación de dos moléculas de fenol con una molécula de acetona en presencia de ácido clorhídrico. Fue sintetizado por primera vez en 1891 por el químico ruso Aleksandr Dianin, pero hasta 1930 no se conocieron sus propiedades y actividad estrogénica (Juan-García et al., 2015).

El BPA es el bisfenol más utilizado y una de las sustancias químicas de mayor volumen en todo el mundo debido a su uso en aplicaciones industriales. El BPA no es peligroso en su forma polimérica, pero es inestable en soluciones ácidas y básicas, cuando se expone a la luz ultravioleta y cambios de temperatura. Estas condiciones pueden convertir/transformar el BPA polimérico en formas monoméricas (Kazemi et al., 2016). Se utiliza principalmente en la industria alimentaria como monómero en la fabricación de plásticos de policarbonato y resinas epoxi como envases plásticos para alimentos o bebidas y en el recubrimiento de latas, protegiendo el contenido del contacto directo con la superficie metálica, pero también en determinados productos de papel (Acaroz et al., 2019; Dutta & Paul, 2019; Elswefy et al., 2016; Eweda et al., 2020; Peerapanyasut et al., 2019; Xia et al., 2014).

1.2.1 Vías de exposición

Hay tres vías principales por las que el BPA puede entrar en el organismo: oral, cutánea e inhalatoria (**Figura 3**):

- Vía oral: la vía más común es a través del consumo de alimentos y bebidas que fueron almacenadas en recipientes o botellas hechas de materiales plásticos que contienen BPA en forma de polímero. Principalmente tras la exposición a altas temperaturas, sus monómeros pueden liberarse en los alimentos o bebidas contenidos y ser ingeridos en el organismo. Las resinas epoxifenólicas a base de BPA se utilizan como revestimientos protectores para latas de alimentos y bebidas, en recipientes de plásticos, así como en el recubrimiento en cisternas de almacenamiento de agua potable residenciales (Acaroz et al., 2019; Ambreen et al., 2019; Lin et al., 2019; Xia et al., 2014).

- Vía cutánea: el BPA se aplica de manera generalizada en el papel térmico utilizado habitualmente en los recibos de las cajas registradoras. Además de estar presente en pinturas a base de resina epoxi, productos sanitarios, selladores dentales, revestimientos superficiales, tintas de impresión y retardantes de llama (Björnsdotter et al., 2017; Konieczna et al., 2015).

- Vía inhalatoria: es la vía minoritaria ya que solo los trabajadores de fábricas que producen bienes que contienen BPA están expuestos a su inhalación (Hines et al., 2017).

La mayoría de la población están expuestos por vía oral o cutánea (Acaroz et al., 2019; Ambreen et al., 2019; Eweda et al., 2020; Geens et al., 2012).

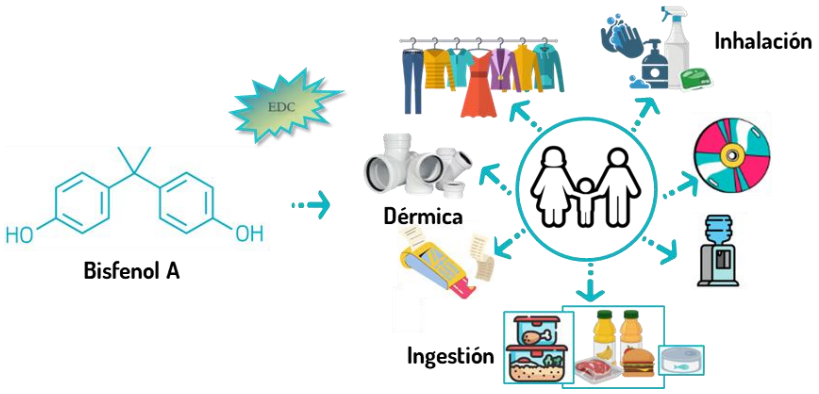


Figura 3. Vías de exposición al BPA y su estructura química. Figura creada con CANVA.

También existe contaminación ambiental del BPA (Michałowicz, 2014). Se descompone con mucha facilidad en el suelo y el aire, pero no en el agua. Estudios previos muestran que el BPA no empieza a degradarse en el agua de los ríos hasta que pasan 50 días, mientras que en el agua del mar no se encuentran signos de degradación hasta después de 150 días (Petrie et al., 2019; Wei et al., 2023).

1.2.2 Metabolismo y transmisión materno-fetal

La exposición al BPA a través de la dieta es la fuente más importante de riesgo. Tras la ingestión del BPA por el organismo, se absorbe casi por completo en el tracto gastrointestinal y llega al hígado a través de la sangre, siendo este órgano el responsable de su metabolismo en dos formas posibles: la glucuronidación y la sulfonación. La mayor cantidad de BPA se metaboliza por la enzima hepática uridil difosfato glucuronil transferasa (UDPGT) a un compuesto menos tóxico llamado bisfenol A-glucurónido (BPAG) (Inoue et al., 2005; Völkel et al., 2002); siendo ésta la principal vía de detoxificación de este xenobiótico. BPAG es su forma biológicamente inactiva, soluble en agua, no es estrogénicamente activa y se elimina de la sangre por la orina con una vida media de eliminación de 5,4-6,4 horas. Por tanto, la glucuronidación es un proceso importante de eliminación que convierte a los BP en moléculas hidrofílicas que se excretan en la orina. En menor medida, la glucuronidación puede tener lugar fuera del hígado, en el intestino y parcialmente en los riñones.

Una cantidad menor de BPA se conjuga con sulfato mediante una sulfotransferasa dando lugar a BPA-sulfato (BPAS) (Geens et al., 2012) o también puede oxidarse a un catecol seguido de una transformación posterior a una O-quinona (4,5-bisfenol-O-quinona) (Atkinson & Roy, 1995). El BPA restante que no ha sido metabolizado en el tracto gastrointestinal entra en el torrente sanguíneo, donde está presente en su forma libre y bioactiva. Su carácter lipofílico permite la distribución a diversas dianas, como el tejido adiposo, el cerebro, el tejido mamario, los órganos

reproductores, etc. (Ješeta et al., 2021).

Respecto a la entrada a través de la piel, a diferencia de los plásticos donde el BPA está presente principalmente en su forma polimerizada, el papel térmico contiene BPA en su forma libre en mg/g de papel. La entrada percutánea es rápida e incluso se ve acelerada por los geles antibacterianos que interrumpen la barrera dérmica. El BPA absorbido a través de la piel evade la metabolización primaria en el hígado y esto aumenta significativamente la concentración de formas libres y bioactivas de BPA en el torrente sanguíneo. En la población general, se transfiere aproximadamente 1 µg de BPA por un dedo tras sostener papel térmico durante 5 segundos, y en dedos húmedos o grasientos, lo aumentan a 23 µg por dedo (Biedermann et al., 2010). Se determinó que la vida media del BPA libre tras el contacto dérmico era de hasta 17,6 horas; lo que es tres veces más largo que la administración oral. Los BP que entran en el cuerpo tras el contacto dérmico se excretan más lentamente (Sasso et al., 2020).

En estudios anteriores, se ha detectado BPA en la placenta humana (Schönfelder et al., 2002), en la sangre del cordón umbilical (Wan et al., 2010), en el líquido amniótico (Ikezuki et al., 2002; Yamada et al., 2002), en el hígado fetal (Cao et al., 2012) y en la leche materna (Sun et al., 2004), así como en el suero y la orina de humanos (Liao et al., 2012) (**Figura 4**).

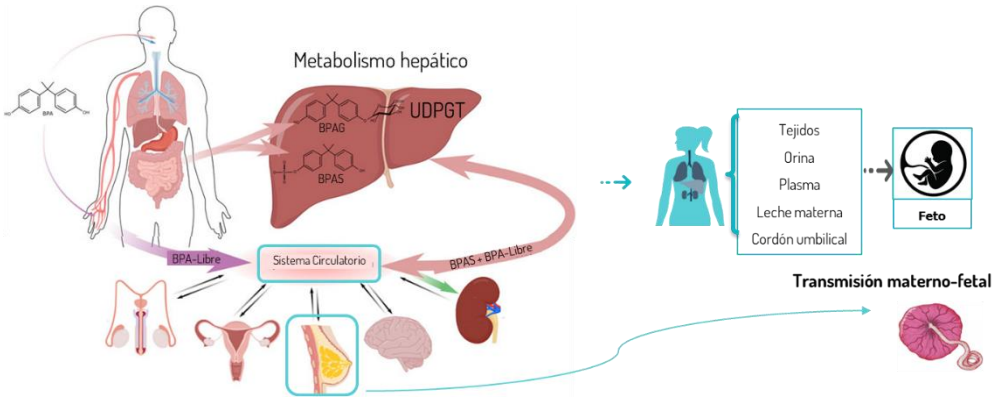


Figura 4. Metabolismo hepático del BPA y su posible transmisión materno-fetal. Figura adaptada de Ješeta et al., 2021.

Dado que el BPA se ha encontrado en los tejidos mencionados anteriormente, así como en el nacimiento (T. Zhang et al., 2013), es probable la exposición a este compuesto durante la vida prenatal. La exposición de las madres embarazadas al BPA es preocupante para el feto en desarrollo, ya que las formas libres y bioactivas del BPA son capaces de atravesar la placenta y entrar en la sangre del cordón umbilical y el líquido amniótico. Además, la enzima β-glucuronidasa es muy activa en la placenta y puede contribuir a aumentar la exposición fetal al BPA libre mediante la hidrólisis del BPA conjugado que entra en el compartimento fetal (Ginsberg & Rice, 2009; J. Wei et al., 2011). A diferencia de los humanos adultos, la enzima UDPGT fetal presenta una escasa o nula actividad enzimática para biotransformarlo en BPAG inactivo (Ikezuki et al., 2002).

La presencia de BPA libre en el hígado de ratas fetales podría ser el resultado de la transferencia directa de BPA libre a la circulación materna a través de la placenta, además de la hidrólisis de BPAG en el hígado fetal (Nishikawa et al., 2010).

El BPA también se une al receptor estrógeno gamma (ERɣ), que está altamente expresado en la placenta, facilitando la acumulación de BPA y aumentando así la exposición del feto en desarrollo a este compuesto, causando posibles efectos nocivos para la descendencia a dosis muy bajas y sostenidas.

También existe transferencia del BPA libre del torrente sanguíneo al pecho de la madre lactante. Se han detectado concentraciones altas de BPA libre en la leche materna pocas horas después de la ingesta oral. Por tanto, la transmisión materno-fetal del BPA es preocupante ya que es capaz de alterar el desarrollo de la placenta y consecuentemente promover complicaciones del embarazo y del feto (Adu-Gyamfi et al., 2022; Manzan-Martins & Paulesu, 2021).

1.2.3 Efectos endocrinos y daños de la exposición al BPA

El BPA se considera un DE con capacidad de alterar funciones y sistemas del organismo. A pesar del metabolismo eficaz del BPA en el hígado y eliminación por la orina, un porcentaje de BPA en su forma libre y activa presenta afinidad por los receptores de los estrógenos y por tanto, posee la capacidad de producir efectos estrogénicos. El BPA se considera un xenoestrógeno, pero no un imitador de estrógenos (Gould et al., 1998) debido a su capacidad de unirse a los receptores nucleares de estrógenos (ER) clásicos alfa y beta (ERα y Erβ) (Vandenberg et al., 2009); aunque en comparación con el 17β-estradiol la afinidad es unas 10.000 veces menor para el ERα y 1.000 veces más débil que la afinidad para el ERβ (Kuiper et al., 1998). También es capaz de unirse a receptores estrogénicos de membrana clásicos y no clásicos (Alonso-Magdalena et al., 2012), así como al receptor acoplado a proteína G 30 (GPR30) (Thomas & Dong, 2006), y actuar por vías no genómicas (Ropero et al., 2006) y también como activador de los receptores de hormonas tiroideas y andrógenos (Alonso-Magdalena et al., 2012; Rochester, 2013).

El BPA puede actuar como DE mostrando efectos similares a los de los estrógenos y las hormonas tiroideas. Debido a la exposición continuada, puede causar problemas de salud en los humanos, incluyendo efectos endocrinos, reproductivos y metabólicos, trastornos cardiovasculares y cáncer, por lo que se ha considerado un riesgo para la salud pública (Ambreen et al., 2019; Baralić et al., 2020; Xia et al., 2014; Y. Zhang et al., 2020). El BPA se absorbe desde el intestino delgado y llega al hígado a través de la sangre, siendo este órgano el responsable de su metabolismo transformándolo en su ácido glucurónico. Por lo tanto, existe la posibilidad de

que haya una mayor concentración y toxicidad de este compuesto en el hígado (Mahdavinia et al., 2019). También se ha observado que el BPA desempeña un papel importante en la inflamación; aumentando la expresión de citoquinas proinflamatorias como la IL-6 (interleuquina 6) y el TNF-α (factor de necrosis tumoral alfa) (Moon et al., 2012). Además, también induce un aumento del estrés oxidativo al disminuir las enzimas antioxidantes (Acaroz et al., 2019; Dutta & Paul, 2019; Elswefy et al., 2016; Eweda et al., 2020; Kazemi et al., 2016; Peerapanyasut et al., 2019) y compromete significativamente la función mitocondrial (Kovacic, 2010). El BPA también es capaz de inhibir las isoformas del citocromo P450 en el hígado de rata (Bindhumol et al., 2003; Hanioka et al., 1998; Ikezuki et al., 2002). Otros estudios experimentales *in vivo* han demostrado que la exposición al BPA también puede causar enfermedades hepáticas, como esteatosis (Jiang et al., 2014), tumores hepáticos (Weinhouse et al., 2014) y síndrome metabólico (J. Wei et al., 2011). Sin embargo, todavía no se conoce bien el efecto del BPA en la descendencia.

1.2.4 Regulación europea de la dosis

En 2006, la EFSA publicó su primera evaluación del riesgo del BPA y en 2007, crearon la legislación REACH, reglamento de la Unión Europea (UE) que se adoptó con el fin de mejorar la protección de la salud humana y el medio ambiente frente a los riesgos derivados de las sustancias y mezclas químicas, y potenciar al mismo tiempo la competitividad de la industria química de la UE.

En 2011, la UE prohibió la fabricación y venta de plásticos con BPA en los biberones, además de quedar vetada la importación a la UE por sus posibles efectos en la salud de los niños. En 2015 redujeron el nivel de ingesta diaria tolerable (IDT) de 50 mg/kg de peso corporal/día a una IDT temporal de 4 μg/kg de peso corporal/día (EFSA, 2015). El 19 de abril de 2023, la EFSA ha reconocido que la exposición actual al BPA, a través de la dieta, constituye un elevado riesgo para la salud humana. El nuevo informe considera que los actuales límites de exposición al BPA subestiman el daño que este compuesto causa a la salud humana y establece que el nuevo umbral de exposición debe ser 20.000 veces inferior al actual (IDT de 0,2 ng/kg de peso corporal/día). Para esta reevaluación han examinado una gran cantidad de publicaciones científicas, incluidos más de 800 nuevos estudios publicados desde enero de 2013 (EFSA, 2023).

En la práctica, los expertos confirman que se trata de eliminar el BPA de todos aquellos productos en contacto con comida o bebida, y se espera que la Comisión Europea adopte las medidas oportunas para ello.

1.3 BISFENOL F (BPF)

Debido al gran número de estudios que demuestran los riesgos para la salud del BPA, se ha estimulado el desarrollo y la producción de alternativas a este DE para sustituirlo en un sinfín de aplicaciones (Chen et al., 2016). El BPA está siendo sustituido por sus análogos como el Bisfenol F [BPF, bis (4-hidroxifenil) metano, $C_{13}H_{12}O_2$]. El BPF es un difenilalcano con dos anillos de fenol unidos a través de un metileno (Usman et al., 2019).

El BPF se utiliza en la fabricación de resinas epoxi y revestimientos en su forma polimérica que confieren a los materiales mayor grosor y durabilidad (Liao & Kannan, 2013). Se encuentra con frecuencia en plásticos, pinturas y barnices, selladores dentales y productos de cuidado personal y papel de oficina (Fromme et al., 2002; Liao, Liu, Guo, et al., 2012). Su presencia en la producción de envases de alimentos y latas de consumo es muy relevante (Siracusa et al., 2018).

1.3.1 Vías de exposición

Residuos de BPF pueden migrar a los alimentos y se han encontrado monómeros de BPF en alimentos enlatados y refrescos con una concentración media de 0,18 mg/dL (Cacho et al., 2012; Gallart-Ayala et al., 2011; Liao & Kannan, 2013). Además, existe contaminación ambiental con el BPF, al encontrarse en el polvo doméstico y en aguas superficiales, sedimentos y efluentes de alcantarillado (Fromme et al., 2002; Liao, Liu, Guo, et al., 2012). Por tanto, al igual que ocurría con el BPA, la exposición al BPF se produce por tres vías: oral, dérmica e inhalatoria, siendo la vía oral predominante ya que es el BP más utilizado en contacto con los alimentos (**Figura 5**).

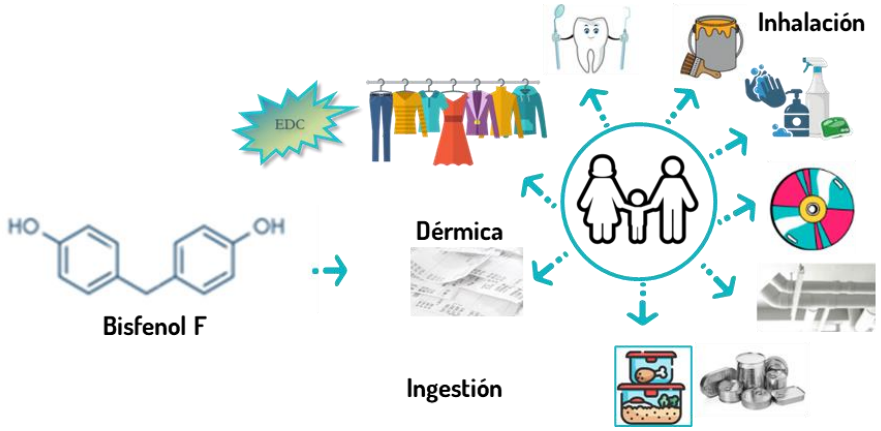


Figura 5. Vías de exposición al BPF y su estructura química. Figura creada con CANVA.

1.3.2 Metabolismo y transmisión materno-fetal

Tras la absorción oral, el BPF se metaboliza principalmente en el hígado por glucuronidación (glucurónido de BPF, BPFG) y sulfonación (sulfato de BPF, BPFS). La mayor parte del BPF se

excreta en la orina como conjugado de sulfato (BPFS).

El hecho de que el BPF no tenga sustituyentes metilo en el carbono central de la molécula lo hace ligeramente más soluble en agua que el BPA, lo que facilita su excreción urinaria. Por eso, el principal metabolito urinario en el caso del BPA es BPAG mientras que en el caso del BPF, su metabolito mayoritario excretado en orina es BPFS. EL BPFG también puede desconjugarse (hidrolizarse) más fácilmente en el intestino y posteriormente reabsorberse en grandes cantidades. En modelos experimentales de rata, es muy probable que el BPF y/o sus metabolitos experimenten ciclos enterohepáticos, lo que podría ser responsable de las cantidades relativamente altas de residuos que aún se excretan 4 días después de la administración de BPF. Entre un 7-9% permanecía en los tejidos de la rata 96 h después de la exposición al BPF. Además, en el hígado se detectaba la mayor cantidad de residuos de BPF (0.5% de la dosis), indicando ser más vulnerable al efecto de dosis más bajas de BP, ya que es el responsable del metabolismo y la detoxificación de compuestos para mantener la homeostasis en todo el organismo (Cabaton et al., 2006; Dumont et al., 2011).

Respecto a la vía de exposición a través de la piel, hay estudios previos que revelan una bioaccesibilidad dérmica humana de alrededor un 30% tras contacto con papel térmico con BPF (Hu et al., 2023).

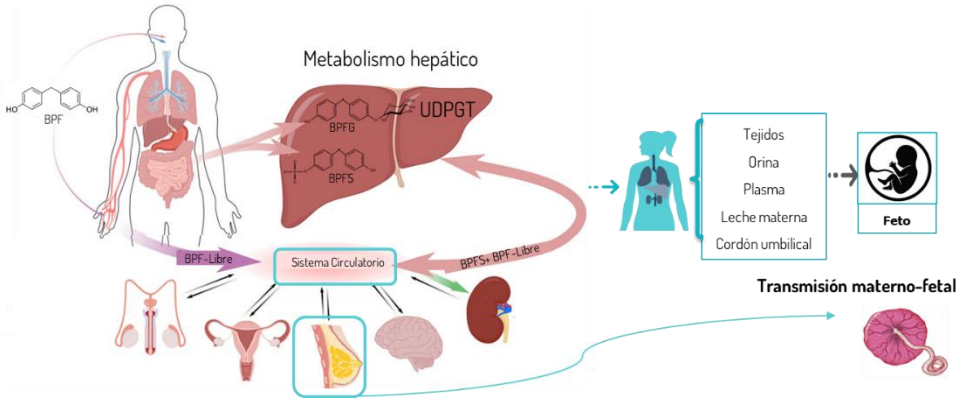


Figura 6. Metabolismo hepático del BPF y su posible transmisión materno-fetal. Figura adaptada de Ješeta et al., 2021.

El BPF se ha encontrado en muestras de orina humana en varios países europeos en concentraciones comparables a las del BPA (Frederiksen et al., 2020; H. Wang et al., 2020; Zhou et al., 2014), en la leche materna (Dualde et al., 2019) y en el suero (Liang et al., 2020). Se ha detectado en plasma humano en concentraciones tres veces superiores a las del BPA (Usman et al., 2019).

El BPF no sólo se detecta en tejidos y fluidos de adultos, sino que también puede atravesar la barrera hematoencefálica, la barrera placentaria y llegar al feto (Cabaton et al., 2006; Usman et al., 2019) (**Figura 6**).

El impacto de la exposición a los BP es crucial en los primeros años de vida, por lo que es necesario seguir investigando el efecto no sólo en los organismos adultos, sino también el efecto perinatal en la descendencia. Esto se debe a que el feto, al igual que la placenta, es vulnerable debido a la falta de las enzimas adecuadas para su detoxificación. Esto hace que la gestación y el período perinatal sean los momentos más vulnerables a la toxicidad de los DEs en la vida humana (Basak et al., 2020).

1.3.3 Efectos endocrinos y daños de la exposición al BPF

Estudios anteriores han mostrado que el BPF puede tener una toxicidad y un mecanismo de acción similares a los del BPA, debido a sus similitudes estructurales y fisicoquímicas (Li et al., 2015; Park et al., 2018; Siracusa et al., 2018). Numerosos estudios *in vivo* e *in vitro* muestran que el BPF presenta actividad estrogénica, androgénica y tiroidogénica (Higashihara et al., 2007; Rochester & Bolden, 2015). El BPF también ha mostrado otros efectos *in vitro* como citotoxicidad, disfunción celular, daños en el ADN y aberraciones cromosómicas (Audebert et al., 2011; Meng et al., 2019; Nakagawa & Tayama, 2000), además de mostrar efectos de toxicidad reproductiva, neurotoxicidad (Rochester & Bolden, 2015; Vancamp et al., 2023) y asociaciones con el desarrollo de obesidad y diabetes mellitus (Alharbi et al., 2022). Se ha demostrado que el BPF muestra acciones acumulativas, posiblemente sinérgicas, *in vivo* cuando se administra con un andrógeno (Yamasaki et al., 2003), al igual que ocurre con el BPA cuando se combina con otras hormonas o xenoestrógenos (Kang et al., 2002; Silva et al., 2002). Estudios previos han demostrado que dosis bajas de BPF elevan los niveles de estrés oxidativo, inducen la proliferación celular y ejercen actividad estrogénica mediante interacciones entre las vías ERα y GPER1 (proteína G acoplada al receptor de estrógenos 1) (Lei et al., 2018).

2. ESTRÉS OXIDATIVO

El hígado es un órgano metabólico esencial que mantiene la homeostasis en todo el organismo. Ejerce una función indispensable en la mediación de las respuestas sistémicas a las lesiones isquémicas e inflamatorias. Como se ha mencionado anteriormente, los BP son metabolizados principalmente por el hígado mediante el proceso de glucuronidación, por lo que debido a su función detoxificadora y metabólica, el hígado es más propenso a sufrir cambios que otros

órganos (Cheung et al., 2012; Ye et al., 2009).

Durante el proceso metabólico se generan las especies reactivas del oxígeno (ROS, *reactive oxigen species* por sus siglas en inglés) que son agentes citotóxicos que pueden atacar al hígado. Dentro de las ROS encontramos los radicales libres de oxígeno (oxígeno singlete (O_2^1), anión superóxido (O_2^{*-}), radicales hidroxilo (OH^*) y radicales hidroperoxilo (HOO^*) como la adicción de no radicales (peróxido de hidrógeno [H_2O_2], ácido hipocloroso [HClO]) y el ozono (O_3). También encontramos las especies reactivas de nitrógeno (RNS, *reactive nitrogen species* por sus siglas en inglés), incluidos los radicales y no radicales a base de nitrógeno, como el dióxido de nitrógeno (NO_2), radicales de óxido nítrico (NO) y el peroxinitrito ($ONOO^-$), que derivan del NO; y el superóxido a través de la óxido nítrico sintasa inducible (iNOS) y el fosfato de dinucleótido de nicotinamida y adenina (NADPH) oxidasa (Li et al., 2015) (**Figura 7**).

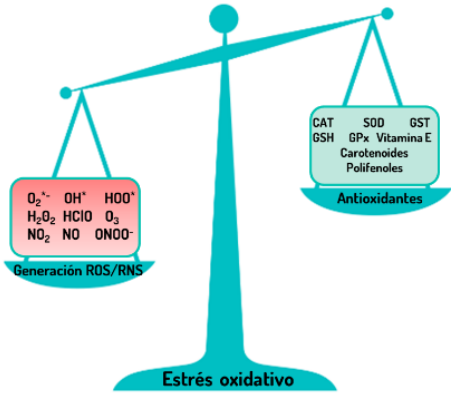


Figura 7. Estrés oxidativo. Figura adaptada de Li et al., 2015.

Estas especies reactivas no son necesariamente una amenaza para el organismo en condiciones fisiológicas ya que se requieren para importantes funciones biológicas y el cuerpo puede eliminar gran parte de ROS/RNS (McCord, 2000; Mittler, 2002). La generación de ROS es una parte natural de la vida aeróbica, responsable de la manifestación de funciones celulares: vías de transducción de señales, defensa contra microorganismos invasores y expresión génica para promover el crecimiento o la muerte (Finkel & Holbrook, 2000). Sin embargo, en la mayoría de los casos, grandes niveles de ROS inducirán muerte celular a través de mecanismos necróticos y/o apoptóticos, lo que conducirá a lesiones celulares y tisulares. Por tanto, el estrés oxidativo y/o nitrosativo es definido como un desequilibrio redox debido a la existencia de un exceso de oxidantes en el organismo o a la disfunción de los sistemas antioxidantes (Li et al., 2015).

2.1 ENZIMAS ANTIOXIDANTES Y SISTEMA DEL GLUTATIÓN

El hígado dispone de un sistema de defensa antioxidante endógeno para prevenir el daño celular causado por el estrés oxidativo como las enzimas antioxidantes y el sistema del glutatión (Li et al., 2015). La primera línea de defensa contra el ataque celular por ROS son las enzimas antioxidantes: catalasa (CAT) y superóxido dismutasa (SOD). La SOD generalmente dismuta el radical anión superóxido en peróxido de hidrógeno y oxígeno molecular. Este peróxido de hidrógeno es degradado por la CAT y permite proteger de la peroxidación a los ácidos grasos insaturados de la membrana celular (**Figura 8**).

Por otro lado, el glutatión (GSH) es una molécula antioxidante que metaboliza y desintoxica los xenobióticos que se conjugan directamente para proteger a las células del daño oxidativo. Este GSH es utilizado como cofactor por la enzima antioxidante glutatión peroxidasa (GPx) que cataliza la degradación de los hidroperóxidos en compuestos de hidroxilo. El glutatión oxidado (GSSG) producido tras la reducción de un hidroperóxido orgánico por la GPx es reciclado de nuevo a su estado reducido por la enzima glutatión reductasa (GR) y el NADPH. La relación entre GSSG/GSH es un indicador de daño oxidativo (Dutta & Paul, 2019) (**Figura 8**).

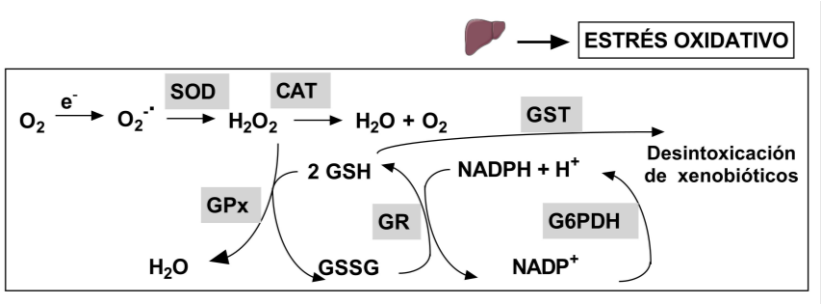


Figura 8. Enzimas antioxidantes y sistema del glutatión. Figura creada con Prism v7 (GraphPad Sofware, Inc, CA, EE. UU.).

Además, la glutatión-S-transferasa (GST) protege a la célula conjugando el GSH con sustratos electrofílicos, generando compuestos menos reactivos y más solubles, siendo una enzima detoxificadora de fase II implicada en el metabolismo de muchos xenobióticos (Sharma et al., 2004). La gamma-glutamilcisteína-sintetasa (γ-GCS) cataliza el primer paso en la síntesis de GSH. Por tanto, el sistema del glutatión proporciona la otra línea de defensa contra las ROS, ya que puede eliminar los radicales libres y reducir la formación de H_2O_2 en la célula (Boesten et al., 2014) (**Figura 8**).

2.2 PEROXIDACIÓN LIPÍDICA Y DAÑO OXIDATIVO AL ADN

Cuando las defensas antioxidantes no son capaces de neutralizar a los oxidantes, debido a sus características químicas especiales, ROS/RNS pueden iniciar la peroxidación lipídica, causar roturas de cadenas de ADN y oxidar indiscriminadamente todas las moléculas en membranas y tejidos biológicos, ocasionando lesiones. Los ácidos grasos poliinsaturados (AGPI) son una diana muy importante debido a que las ROS reaccionan con el hidrógeno de los AGPI iniciando una reacción autocatalítica en cadena. En esta reacción, en presencia de metales de transición, los hidroperóxidos de lípidos dan lugar a radicales peroxilo o radicales alcoxi, que se ciclan y degradan, dando aldehídos altamente reactivos como el malondialdehído (MDA) (Frijhoff et al., 2015; Ungurianu et al., 2019). El exceso de MDA producido como resultado del daño tisular puede combinarse con grupos aminos de proteínas lo que produce aductos de proteínas modificadas (Rossi et al., 2016), por lo que la prevención de la peroxidación lipídica es esencial en los organismos aerobios ya que los productos derivados de este proceso pueden interactuar con el ADN y son potencialmente mutagénicos. Cuando el ADN resulta dañado por radicales hidroxilo se produce la especie 8-oxo-2´-desoxiguanosina (8-OHdG). Es una forma oxidada de la guanina y se considera el mayor inductor de daño oxidativo al ADN, causando mutaciones de transversión de A:T (adenina:timina) a C:C (citosina:citosina) o G:C (guanina:citosina) a T:A (timina:adenina), debido a su apareamiento con adenina o citosina (Gassman, 2017; Rossi et al., 2016) (**Figura 9**).

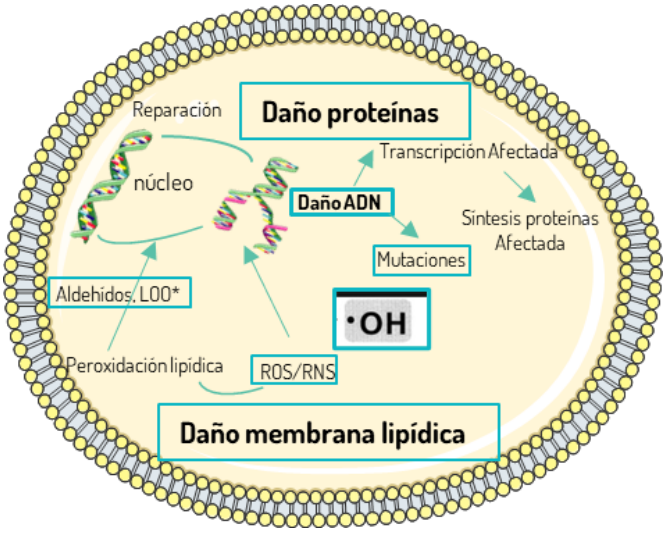


Figura 9. Lipoperoxidación lipídica y daño en el ADN. Figura adaptada de Hermes-Lima (2005) y creada con Smart Servier Medical Art.

2.3 MEDIADORES DEL ESTRÉS NITROSATIVO

Entre las RNS, el NO es una molécula de señalización que interviene en muchos procesos biológicos y funciones fisiológicas tan importantes como la vasodilatación, inhibición de la agregación plaquetaria y neurotransmisión. La función vascular depende principalmente del equilibrio entre la síntesis/degradación de NO. En condiciones fisiológicas es sintetizado por la enzima óxido nítrico sintasa endotelial (eNOS); aunque también en otras situaciones puede ser producido por la isoforma neuronal (nNOS) y en condiciones no fisiológicas bajo ciertos estímulos, como inflamación y/o incremento de ROS, por la isoforma inducible (iNOS) (Eid et al., 2015). La isoforma constitutiva eNOS se ha identificado en las células endoteliales sinusoidales y contribuye a mantener la presión portal y la distribución local de la perfusión en condiciones fisiológicas. Por otro lado, iNOS se ha encontrado en la mayoría de las células hepáticas (macrófagos, células endoteliales, células estrelladas hepáticas (CEH) y hepatocitos). La sobreproducción de NO por un aumento de la actividad de iNOS, se asocia a una mayor producción de RNS, que puede inducir daños estructurales en biomoléculas como proteínas, lípidos y ADN al igual que ocurría con las ROS (Andrew, 1999). Además, pueden provocar lesiones hepáticas por llevar a cabo la nitrosilación de los residuos de tiol de muchas enzimas celulares (Clemens, 1999) (**Figura 10**).

También destaca la hemo-oxigenasa (HO), una enzima microsomal que cataliza la degradación oxidativa del grupo hemo de las hemoproteínas a hierro (Fe^{2+}), biliverdina y monóxido de carbono (CO). Se han caracterizado dos isoformas: la hemo-oxigenasa 2 (HO-2d) constitutiva, que se expresa en condiciones fisiológicas, y la hemo-oxigenasa 1 (HO-1d) inducible, que se expresa tras diversos estímulos, como el estrés oxidativo, mediadores proinflamatorios, choque térmico y altos niveles de NO, siendo un marcador fiable de un estado proinflamatorio y prooxidante (Choi & Alam, 2012) (**Figura 10**).

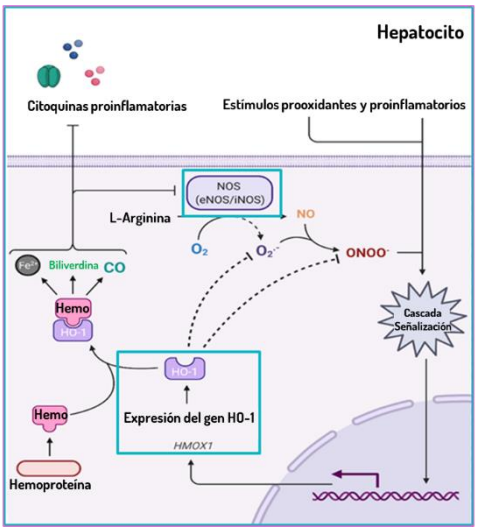


Figura 10. Mediadores del estrés nitrosativo. Figura creada con Smart Servier Medical Art y CANVA.

La función de la HO-1d es antiinflamatoria, además de poseer efectos antiapoptóticos y antiproliferativos. Se considera un sistema de protección durante el estrés celular generando productos de reacción enzimática con el fin de prevenir una mayor lesión (Hoetzel et al., 2008; Morse & Choi, 2002).

Por tanto, el estrés oxidativo ha sido considerado como un mecanismo patológico conjunto y contribuye al inicio y progresión de la lesión hepática.

3. INFLAMACIÓN

El estrés oxidativo se asocia al estrés inflamatorio (con mayor presencia de compuestos proinflamatorios frente a los antiinflamatorios), siendo la oxidación y la inflamación dos procesos relacionados, cruciales en el inicio y desarrollo de la enfermedad hepática. Por eso, el estrés oxidativo continuo puede conducir a una inflamación crónica (Li et al., 2016) y a la inversa.

La inflamación es un componente esencial de la respuesta inmunitaria y en el hígado se manifiesta como infiltración de células inflamatorias (neutrófilos, monocitos y linfocitos) principalmente para combatir la invasión de patógenos, lesión tisular u otras sustancias nocivas. Sin embargo, una vez que este proceso es excesivo, prolongado o desregulado, se producirá una inflamación patológica y una lesión tisular con acumulación de lípidos asociados con un mayor riesgo de enfermedades hepáticas graves (Asrih & Jornayvaz, 2013; H. J. Wang et al., 2012).

La inflamación hepática tiene funciones duales en el hígado: es esencial para mantener el tejido saludable y actúa como impulsor crítico de la patología hepática cuando persiste o está fuera de control. La infiltración de leucocitos es un proceso complicado que implica la participación de muchos receptores, moléculas de adhesión y quimioquinas. Además, estas células inflamatorias infiltradas también producen mediadores solubles como metabolitos de ácido araquidónico, citoquinas y quimioquinas, que activan cascadas de transducción de señales y cambian factores de transcripción, para reclutar más células inflamatorias en el tejido lesionado (Kershenobich Stalnikowitz & Weissbrod, 2003; Seki & Schwabe, 2015).

Al igual que el estrés oxidativo, la inflamación generalmente se mantiene y participa en todo el proceso de daño hepático desde la etapa inicial hasta etapas más avanzadas. La infiltración de leucocitos, la activación de las células de Kupffer (KC) y la regulación positiva de la enzima iNOS desencadenan tanto la respuesta inmunitaria innata como la adaptativa (Greuter & Shah, 2016).

A su vez, estas células producen gran cantidad de NO y citoquinas como la citoquina profibrogénica TGF-β (factor de crecimiento transformante beta) y moduladores de la inflamación como TNF-α. Numerosos mediadores inflamatorios incluyendo citoquinas proinflamatorias, quimioquinas y receptores tipo Toll (TLR) están involucrados en la regulación de la fibrogénesis hepática. Los TLR son proteínas receptoras sensibles a redox y han sido involucradas en la respuesta celular al estrés oxidativo (Greuter & Shah, 2016; Kershenobich Stalnikowitz & Weissbrod, 2003; Li et al., 2016). La respuesta inmunitaria adaptativa inducida por el estrés oxidativo, como los productos de la peroxidación lipídica (MDA), está implicada en el desarrollo de la fibrosis hepática. Al cambiar la fluidez y permeabilidad de la membrana celular, se conduce a la infiltración inflamatoria inducida por la peroxidación lipídica, resultando en mayor inflamación y necrosis del hígado, e incluso fibrosis (Albano et al., 2005). NF-κB (factor nuclear kappa-potenciador de la cadena ligera de las células B activadas) y JNK (quinasas c-Jun N-terminal) se consideran las vías de señalización más importantes que vinculan la inflamación y fibrosis. La hiperactivación de NF-κB en hepatocitos o células inflamatorias infiltradas fomentan la inflamación hepática mediante una mayor generación de citoquinas proinflamatorias como IL-1β (interleuquina-1 beta), TNF-α e IL-6 (Bubici et al., 2006).

Por tanto, el sistema inmunitario innato es la primera barrera contra los patrones moleculares asociados a patógenos (PAMPs) presentes en los patógenos microbianos como las bacterias, los virus y los parásitos. También es la primera línea de defensa frente a los patrones moleculares asociados a daños (DAMPs) derivados de las células del huésped debido al estrés celular o el ADN citosólico (Sahin & Aricioglu, 2013). En el hígado, los DAMPs más comunes provienen de los hepatocitos dañados: ATP (adenosín trisfosfato), ácido úrico, beta amiloide, fragmentos de ADN, pirofosfato de calcio, cristales de colesterol y ácidos grasos. Estos DAMPs conducen al ensamblaje del inflamasoma (Wree & Marra, 2016).

3.1 INFLAMASOMA NLRP3

Los inflamasomas son componentes clave del sistema inmunitario natural que pueden proteger en gran medida las funciones hepáticas normales frente a infecciones patógenas, enfermedades metabólicas y estrés celular (Szabo & Petrasek, 2015). Los inflamasomas suelen estar formados por tres componentes: una molécula desencadenante y sensora denominada receptor tipo NOD (NLR), una proteína adaptadora y un importante agente denominado pro-caspasa-1 (Al Mamun et al., 2020). Los inflamasomas pueden activarse directamente en las células hepáticas estrelladas (CEH), las células inmunitarias (células de KC) y los hepatocitos (Wree & Marra, 2016).

Generalmente la activación del inflamasoma requiere dos pasos. En primer lugar, el reconocimiento por las células hepáticas de las señales de peligro a través de los receptores TLR para poder ensamblar los complejos del inflamasoma. Estos TLR son estimulados por PAMPs o DAMPs, desencadenando cascadas de señalización que conducen a la maduración de proteínas quinasas activadas por mitógenos (MAPKs) y a la activación de NF-κB. Se induce la transcripción de diferentes genes en el núcleo celular, como la IL-6, TNF-α, la pro-interleuquina-1β (pro-IL-1β), la pro-interleuquina-18 (pro-IL-18), y la proteína NLRP3 que contiene el dominio *NOD-like receptor Pyrin domain containing*. La IL-6 y el TNF-α se liberan fuera de la célula tras su producción. En segundo lugar, es necesario la activación de NLRP3 y la posterior formación del complejo del inflamasoma para la secreción de las formas maduras de la IL-1 y la IL-18. En este segundo paso, el ATP puede estimular los receptores P2X7 acoplados a los canales de cationes, que provoca la salida de iones K^+ de la membrana celular, dando lugar a la oligomerización de NLRP3 y a la formación del complejo del inflamasoma. NLRP3 se autoinhibe debido a la unión del dominio de repetición rico en leucinas (LRR) con el dominio de oligomerización de unión a nucleótidos (NACHT), pero esta estimulación permite la oligomerización de NLRP3 y su unión a la pro-caspasa-1 a través de una proteína adaptadora conocida como *apoptosis-associated speck-like protein containing CARD* (PYCARD, a menudo denominada ASC). La fase de unión desarrolla la caspasa-1 (CASP1), a partir de la pro-caspasa-1. CASP1 produce formas activas de la pro-IL-1β (IL-1β) y pro-IL-18 (IL-18) que son mediadores inflamatorios (Sahin & Aricioglu, 2013).

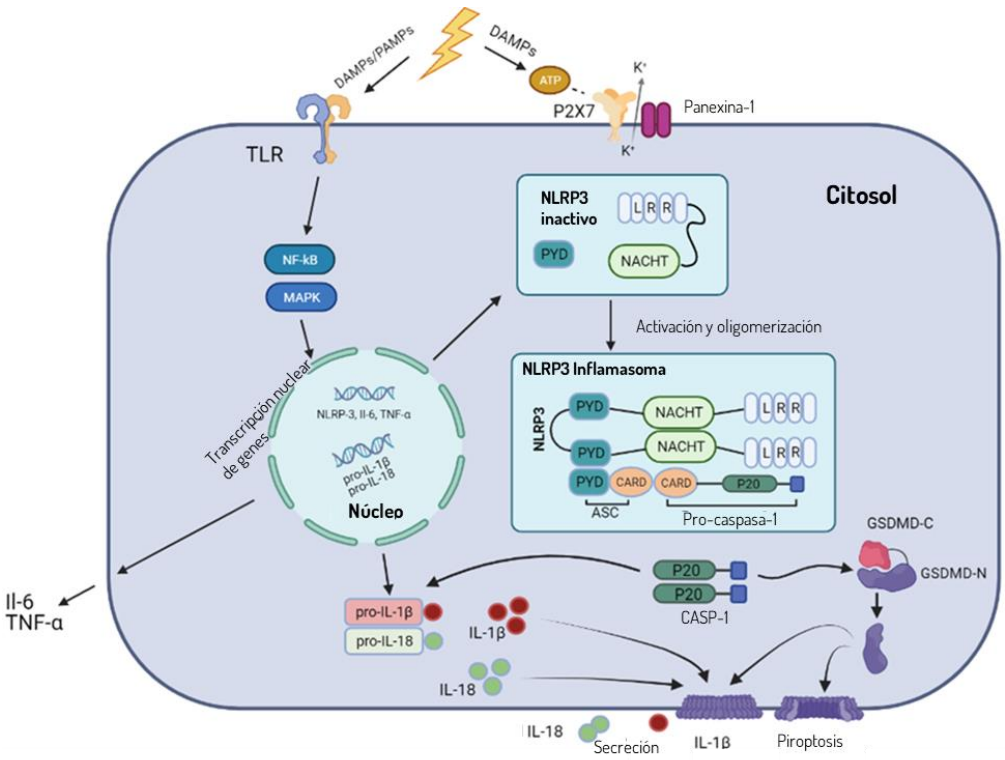


Figura 11. Vía de activación del inflamasoma NLRP3 en una célula hepática. Figura creada con BioRender.

Se ha descrito un papel importante del inflamasoma NLRP3 en las enfermedades hepáticas (Luan & Ju, 2018; Szabo & Petrasek, 2015). Como hemos visto, el inflamasoma NLRP3 es un andamiaje multiproteico que responde a DAMPs y puede mediar la activación catalítica de la caspasa-1 (CASP1), promoviendo la escisión y liberación de IL-1β e IL-18 (Guo et al., 2018). Sin embargo, una respuesta inflamatoria excesiva regulada por el inflamasoma NLRP3 desencadena la progresión de la enfermedad hepática (Al Mamun et al., 2020).

El inflamasoma NLRP3 puede activarse en respuesta a una amplia gama de estímulos, como infecciones, daños tisulares o estrés metabólico (a través de diferentes vías: ATP, mitocondrias dañadas, descomposición lisosomal, cambios en Ca^{2+}, K^{+}, y también aumentos en las concentraciones de ROS mitocondriales y no mitocondriales). Una vez activada la proteína receptora NLRP3, se une a la molécula adaptadora PyCARD que recluta y activa la pro-caspasa-1 en CASP1, capaz de promover la maduración de citoquinas proinflamatorias como la IL-1β y la IL-18. Además, CASP1 es capaz de escindir precursores proteicos que afectan al citoesqueleto celular, la glucólisis, la función mitocondrial y la inflamación (Wree et al., 2014). También induce la piroptosis, una forma inflamatoria de muerte celular programada (Sahin & Aricioglu, 2013).

La IL-1β y la IL-18, miembros de la superfamilia de citoquinas IL-1, promueven procesos asociados a la infección, la inflamación y la autoinmunidad. La IL-1β es clave en la activación de las CEH y promueve el reclutamiento de células inflamatorias, contribuyendo a la fibrosis y a la acumulación de triglicéridos en los hepatocitos y a su muerte junto con el TNF-α (Szabo & Petrasek, 2015). El TNF-α provoca inflamación hepática, proliferación y apoptosis, así como cambios en la morfología de las CEH (Yang & Seki, 2015). El TNF-α también puede promover el reclutamiento de neutrófilos y macrófagos proinflamatorios y la activación de vías fibrogénicas que conducen al desarrollo de fibrosis hepática (Wree et al., 2018).

La IL-18 induce la síntesis de IFN-γ (interferón gamma), además de activar las células NK y los linfocitos T citotóxicos, y parece estar implicada en la modulación de la microbiota intestinal (Szabo & Petrasek, 2015). El IFN-γ es un mecanismo regulador del inflamasoma NLRP3 y tiene una doble función: activa células efectoras como los linfocitos NK (linfocitos *natural killer*) y también tiende a disminuir la activación a través de la iNOS porque el NO induce la nitrosilación de la proteína NLRP3 y puede inhibir su actividad tras un tiempo prolongado (Latz et al., 2013).

Así, los mecanismos de activación de CASP1 que desencadenan los diferentes tipos de inflamasomas son complejos y diversos dando lugar a la inducción y secreción de la IL-1β, generando inflamación y la muerte celular de tipo piroptosis (Sahin & Aricioglu, 2013). Además, CASP1 es capaz de escindir precursores proteicos que afectan al citoesqueleto celular, la glucólisis, la función mitocondrial y la inflamación (Wree et al., 2014).

4. MUERTE CELULAR

La muerte celular puede ocurrir de diferentes maneras: a través de un proceso descontrolado denominado necrosis, o por el contrario, mediante un proceso muy regulado denominado apoptosis.

La necrosis se produce tras una pérdida de energía asociada a la depleción de ATP, donde la célula necrótica sufre hinchazón y disrupción de la membrana con liberación de enzimas proteolíticas, que da lugar a una inflamación de tejidos circundantes y una infiltración leucocitaria (Guicciardi et al., 2013; Nanji & Hiller-Sturmhöfel, 1997), mientras que la apoptosis ocurre en condiciones fisiológicas y es un fenómeno para el correcto mantenimiento de la homeostasis (tamaño de los tejidos, número de células, supresión de células dañinas o no funcionales, etc.) (Kumar & Jugdutt, 2003).

La apoptosis también participa en el desarrollo y embriogénesis, permitiendo la remodelación orgánica y metamorfosis, como mecanismo de defensa y en el envejecimiento. Esta muerte celular programada es dependiente de energía que se desencadena tras un daño celular no tan severo como para producir necrosis. En el proceso apoptótico hay 3 eventos importantes:

- Fase de inducción (presencia de señales de muerte como TNF-α o ligando FAS, así como un incremento de ROS o miembros de la familia de proteínas de Bcl-2 como Bax o Bad; o radiaciones ionizantes, etc.).

- Fase de ejecución: con la liberación de mediadores proapoptóticos como citocromo C o AIF (Factor inductor de apoptosis).

- Fase final de degradación: incluye una serie de eventos nucleares y citoplasmáticos, como activación de caspasas en el citosol o fragmentación de ADN en el núcleo (Seki & Schwabe, 2015).

Por tanto, dentro de los mecanismos moleculares de regulación de la apoptosis se conoce un papel activo y destacable de dos familias de proteínas: caspasas y proteínas de la familia Bcl-2.

4.1 CASPASAS Y VÍAS DE APOPTOSIS

Las caspasas son proteínas con actividad cisteína-proteasa, la cual hidroliza en residuos de aspartato específicos. Las caspasas están implicadas en eventos de apoptosis e inflamación. Se clasifican en tres clases: caspasas iniciadoras (CASP-2, 8, 9 y 10), que detectan señales de peligro, caspasas ejecutoras (CASP-3, 6 y 7), que desencadenan la muerte celular, y una tercera clase,

CASP-1, 4, 5 y 12, en humanos, involucradas en procesos inflamatorios. Estas proteasas se sintetizan como zimógenos inactivos de una sola cadena polipeptídica (proenzimas inactivas de 30 a 50 KDa), que son activadas por corte proteolítico e inician una cascada de caspasas, siendo las principales ejecutoras de la apoptosis (Jordán, 2003).

Las dos vías principales de la apoptosis dependiente de caspasas son:

- Vía extrínseca: se activa por la interacción de receptores de muerte con sus ligandos, como TNF-α/TNF-R, Fas (CD95)/Fas-ligando (CD95-L) o angiotensina AII/AII-Receptor. Esta unión da lugar a la activación de una cascada de caspasas y a la reducción de la expresión de la proteína antiapoptótica Bcl-2. Es fundamental la actividad de la CASP8 que inicia la cascada de las caspasas, activando a las efectoras (CASP-3, 6 y 7) y produciendo la fragmentación del ADN en cuerpos apoptóticos a través de la activación de la poli-ADP polimerasas (PARP) (Feldmann, 1997) (**Figura 12**).

- Vía intrínseca: se activa por estrés intracelular (ROS, RNS), radiaciones ionizantes, agentes químicos, infecciones virales, daños en el ADN, etc. Durante este proceso, miembros proapoptóticos de la familia Bcl-2 son activados y traslocados hacia la mitocondria donde inducen apertura de poros a través de la permeabilización de la membrana externa mitocondrial y liberación de proteínas solubles promotoras de la apoptosis, específicamente promotoras de la activación de CASP1, como el citocromo C o AIF (Guicciardi et al., 2013).

El citocromo C forma un complejo llamado apoptosoma junto con Apaf-1 (factor activador de la proteasa apoptótica 1) y la forma inactiva de la CASP9. Apaf-1 hidroliza ATP y estabiliza su unión al citocromo C, que reclutan a la pro-caspasa-9 e inducen su activación mediante autocatálisis. El apoptosoma activa a CASP3 que junto a las CASP6 y CASP7 fragmenta el ADN dando lugar a la apoptosis celular (Guicciardi et al., 2013).

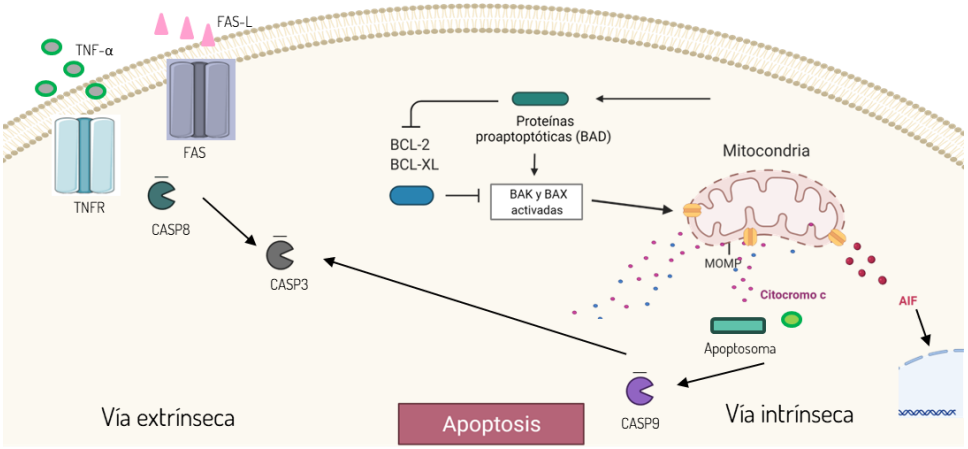


Figura 12. Vías de la apoptosis: extrínseca e intrínseca. Figura creada con BioRender.

En el caso de AIF tras una agresión apoptótica, sufre proteólisis y se transloca al núcleo, donde desencadena la condensación de la cromatina y la degradación del ADN de forma independiente de las caspasas (Sevrioukova, 2011) (**Figura 12**).

4.2 PROTEÍNAS DE LA FAMILIA DE BCL-2

La familia de proteínas de Bcl-2 es una familia de proteínas intracelulares cuya función es regular procesos de permeabilización mitocondrial, constituyendo un punto clave en el desarrollo de la apoptosis. Bcl-2 se localiza en la membrana mitocondrial externa principalmente, aunque también puede ubicarse en la membrana nuclear y retículo endoplasmático (Cazanave & Gores, 2009). Se encarga de inhibir la apoptosis estabilizando la membrana mitocondrial, así que evita la liberación del citocromo C al citosol y la formación del apoptosoma (Kale et al., 2018).

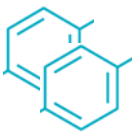
Esta familia de proteínas de Bcl-2 contienen dominios de homología con Bcl-2 (BH) y se dividen según su función: antiapoptóticas que contienen cuatro dominios de homología BH1-BH4 (Bcl-2, Bcl-XL) y otras son proapoptóticas que contienen tres dominios BH1-3 (Bax, Bak, Bcl-XS y otras), existe un tercer grupo proapoptótico con un único dominio de homología BH3 (Bad, Bid, Bik y otras) (Cazanave & Gores, 2009; Kluck et al., 1997).

Por tanto, tras diferentes estímulos, miembros proapoptóticos (Bax, Bak) se regulan al alza y miembros antiapoptóticos se regulan a la baja (Bcl-2, Bcl-XL) activando la vía intrínseca de la apoptosis.

Además, existe un tipo de muerte celular programada en respuesta a la producción de PAMPs o DAMPs, denominada piroptosis. La piroptosis es dependiente de la CASP1, ya que esta caspasa se comporta de forma similar a las caspasas iniciadoras por los complejos denominados inflamasomas (Gan et al., 2022; Zhao et al., 2022). La piroptosis tiene características tanto de la apoptosis como de la necrosis: se produce la fragmentación del ADN aunque no conduce a la ruptura de la membrana plasmática, pero sí ocurre la liberación de citocromo C y la activación de la enzima CASP3, además de desencadenar el edema de la célula, la formación de poros y la lisis celular, así como la liberación de la CASP1. La piroptosis puede tener consecuencias adversas en el organismo (Sahin & Aricioglu, 2013).

Por lo tanto, el aumento o disminución de estos procesos apoptóticos, o incluso la ausencia de estos, juegan un papel importante en el desarrollo de enfermedades (Seki & Schwabe, 2015).

La resolución de la inflamación involucra la disminución de la proliferación y maduración de células inmunitarias y la inducción de la apoptosis con inhibición de la secreción de mediadores inflamatorios (Cervantes-Villagrana et al., 2014), por lo que tanto el desbalance en el estado redox celular como la inflamación y los procesos apoptóticos son clave en el inicio, progresión y control de eventos tantos fisiológicos como patológicos.



OBJETIVOS

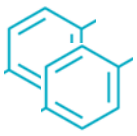
Partiendo de la base de que los bisfenoles, como el BPA, producen alteraciones moleculares y causan toxicidad en diversos órganos, se está promoviendo la búsqueda de alternativas de químicos más inocuos como el análogo BPF, donde la toxicidad de estos sustitutos químicos no se ha estudiado todavía en demasiada profundidad.

La hipótesis de trabajo es que el BPA y su análogo, el BPF, pueden tener un efecto hepatotóxico que afecta al organismo adulto y a la descendencia, y que este efecto está mediado por un incremento del estrés oxidativo y de la inflamación. El objetivo principal de esta tesis ha sido estudiar los posibles efectos hepáticos de la administración de una dosis baja y una dosis alta de bisfenol A y su análogo, bisfenol F en un modelo experimental murino, investigando los efectos de los mismos tanto en ratas adultas gestantes y lactantes, como en la descendencia en el día postnatal 6 (DPN6). Para llegar a este objetivo hemos determinado los siguientes objetivos específicos:

1. Evaluar si la administración de BPA durante la preñez es capaz de inducir daño hepático tanto en ratas lactantes como en las hembras de su descendencia en el DPN6:
   1.1. Determinar afectaciones en el equilibrio oxidante/antioxidante mediante la inducción de estrés oxidativo.
   1.2. Determinar la liberación de mediadores de estrés nitrosativo y proinflamatorios.
   1.3. Determinar la generación de apoptosis.

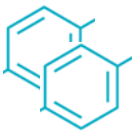
2. Evaluar si la administración de BPF genera estrés oxidativo tanto en el hígado de ratas lactantes como en ambos sexos de su descendencia en el DPN6:
   2.1. Determinar las enzimas antioxidantes y el sistema del glutatión.
   2.2. Determinar los indicadores de daño lipídico.

3. Evaluar si la administración de BPF incrementa el estrés nitrosativo y el posible papel que desempeña el inflamasoma NLRP3 tanto en el hígado de ratas lactantes como en ambos sexos de su descendencia en el DPN6:
   3.1. Determinar la generación de especies reactivas del nitrógeno.
   3.2. Determinar si se produce la activación del inflamasoma NLRP3 e incremento de marcadores inflamatorios que éste determina.



MATERIAL Y MÉTODOS

Los materiales y métodos empleados en esta tesis doctoral están detallados en los artículos presentados en el apartado de resultados.



RESULTADOS

Publicación 1: **Dosis bajas de BPA inducen lesiones hepáticas a través de estrés oxidativo, inflamación y apoptosis en ratas lactantes *Long-Evans* y su efecto perinatal en las crías hembras en el día postnatal 6 (DPN6)**

El bisfenol A (BPA) es un compuesto fenólico utilizado en la elaboración de plásticos para la protección o el envasado de alimentos. Los monómeros de BPA pueden liberarse en la cadena alimentaria, lo que da lugar a una exposición humana continua y ubicua a dosis bajas. Esta exposición durante el desarrollo prenatal es especialmente crítica y podría provocar alteraciones en la ontogenia de los tejidos, aumentando el riesgo de desarrollar enfermedades en la edad adulta. El objetivo de este estudio fue evaluar si la administración de BPA en dos dosis diferentes (0,036 mg/kg peso corporal/día y 3,42 mg/kg peso corporal/día) a ratas gestantes y lactantes podría inducir lesiones hepáticas generando estrés oxidativo, inflamación y apoptosis, y si estos efectos pudieran observarse en las crías hembras en el día postnatal 6 (DPN6). Se determinaron mediante métodos colorimétricos las enzimas antioxidantes CAT, SOD, GR, GPx y GST, el sistema del glutatión (GSH/GSSG) y los marcadores de daño lipídico y daño oxidativo al ADN MDA, LPO, NO y 8-OHdG. Los inductores del estrés oxidativo HO-1d y iNOS, la citoquina proinflamatoria IL-1β y los marcadores de apoptosis AIF, Bax, Bcl-2 y Bcl-XL se midieron mediante qRT-PCR y Western blotting en el hígado de ratas lactantes y en sus crías. Se analizaron los daños hepáticos mediante la evaluación de los marcadores séricos AST, ALT y GGT, así como con estudios histológicos (tinción hematoxilina-eosina). La dosis baja de BPA causó lesiones hepáticas en ratas lactantes y tuvo un efecto perinatal en sus crías hembras al aumentar los niveles de estrés oxidativo, desencadenar una respuesta inflamatoria y activar vías de apoptosis en el órgano responsable de la desintoxicación de este disruptor endocrino.

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Low Dose of BPA Induces Liver Injury through Oxidative Stress, Inflammation and Apoptosis in Long–Evans Lactating Rats and Its Perinatal Effect on Female PND6 Offspring

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Abstract: Bisphenol A (BPA) is a phenolic compound used in plastics elaboration for food protection or packaging. BPA-monomers can be released into the food chain, resulting in continuous and ubiquitous low-dose human exposure. This exposure during prenatal development is especially critical and could lead to alterations in ontogeny of tissues increasing the risk of developing diseases in adulthood. The aim was to evaluate whether BPA administration (0.036 mg/kg b.w./day and 3.42 mg/kg b.w./day) to pregnant rats could induce liver injury by generating oxidative stress, inflammation and apoptosis, and whether these effects may be observed in female postnatal day-6 (PND6) offspring. Antioxidant enzymes (CAT, SOD, GR, GPx and GST), glutathione system (GSH/GSSG) and lipid-DNA damage markers (MDA, LPO, NO, 8-OHdG) were measured using colorimetric methods. Inducers of oxidative stress (HO-1d, iNOS, eNOS), inflammation (IL-1β) and apoptosis (AIF, BAX, Bcl-2 and BCL-XL) were measured by qRT-PCR and Western blotting in liver of lactating dams and offspring. Hepatic serum markers and histology were performed. Low dose of BPA caused liver injury in lactating dams and had a perinatal effect in female PND6 offspring by increasing oxidative stress levels, triggering an inflammatory response and apoptosis pathways in the organ responsible for detoxification of this endocrine disruptor.

Keywords: bisphenol A; oxidative stress; inflammation; apoptosis; liver injury; perinatal offspring

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1. Introduction

Bisphenol A [BPA; 2,2-bis (4-hydroxyphenyl)] is a synthetic xenoestrogen compound with a high prevalence in our environment [1,2]. BPA is not hazardous in its polymeric form but is unstable in acidic and basic solutions and when exposed to ultraviolet light. These conditions can convert/transform polymeric BPA into monomeric forms [1]. It is used mainly in the food industry as a monomer in the manufacture of polycarbonate plastics and epoxy resins such as plastic food or beverage containers and in the coating of cans, protecting the contents from direct contact with the metal surface [3–8], but also for certain paper products. BPA residues can migrate into the food, beverages or environment due to high temperatures, causing people to inevitably be exposed to BPA in their daily lives [3,6,9,10]. The main source of human exposure is through ingestion [5,9,11], while transdermal absorption and inhalation would be possible through secondary routes of exposure [3,5,9]. BPA can act as an endocrine disruptor showing effects that are similar to those of estrogenic and thyroid hormones. Due to continuous exposure, it can cause health

problems in humans, including endocrine, reproductive and metabolic effects, cardiovascular disorders and cancer, so that it has been considered a risk for public health [2,6,9,11]. BPA is absorbed from the small intestine and reaches the liver through the blood, this organ being responsible for its metabolism into its glucuronic acid-conjugated form. Therefore, there is a very real possibility of the presence of a higher concentration and toxicity of this compound in the liver [12]. BPA has also been observed to play a major role in inflammation; as Moon et al. [13] reported, it increases the expression of pro-inflammatory cytokines such as IL-6 and TNF-α. In addition, it also induces an increase in oxidative stress by decreasing antioxidant enzymes [1,3–5,7,8] and significantly compromises mitochondrial function [14]. BPA is also able to inhibit cytochrome P450 isoforms in the rat liver [15–17]. Other in vivo experimental studies have shown that exposure to BPA can also cause liver disease, including steatosis [18], liver tumors [19] and the metabolic syndrome [20]. In previous studies, BPA has been detected in the human placenta [21], umbilical cord blood [22], amniotic fluid [17,23], fetal liver [24] and breast milk [25] as well as in human serum and urine [26]. Hence, since BPA was found in the previously mentioned tissues, as well as at birth [27], exposure to this compound during prenatal life is probable. However, the effect of BPA on the offspring is still poorly understood. The aim of this study was to evaluate whether BPA administration during pregnancy is able to induce liver damage in lactating rats by affecting the oxidant/antioxidant balance through the induction of oxidative stress, increasing inflammation and triggering apoptosis. Moreover, it was studied whether this effect can also be observed in female offspring at postnatal day 6 (PND6).

2. Results

2.1. Effects of BPA Exposure on Body Weight, Food Consumption, Number of Pregnant Dams and Mortality Rate in PND6 Offspring and Pup Weight

In this study, clinical observations were made daily and body weight of females was monitored every 3–4 days. Regarding the general appearance, the animals did not show any alteration that could be perceived visually or any unexpected behavior. During the entire experiment it was not necessary to sacrifice any animal for signs of cadence or signs of pain or aggression. Considering all rats from day 24 (before starting mating) at equal weight, an increase in body weight was observed in all females until the maximum weight was reached at the end of pregnancy. No significant differences were observed in the groups of females treated with different doses of BPA in the diet compared to the control group (Figure 1A). There were also no significant differences in food consumption between the control and BPA treatment groups, monitored during the second week of premating and the second week of pregnancy (Figure 1B). Regarding reproduction data, in the case of control females (n = 10), eight female rats were pregnant and two females were not pregnant. In dams treated with BPA, 0.036 mg/kg/b.w./day group (low-dose BPA) (n = 9), it resulted in eight pregnant females and only one non-pregnant female. Regarding dams treated with BPA, 3.42 mg/kg/b.w./day group (high-dose BPA) (n = 8), six females were pregnant and two remained non-pregnant. Therefore, pregnancy was achieved in 22 females from a total of 27 females. The highest percentage of pregnancy was observed in the BPA low dose group (88.8%) followed by the control group (80%) and the lowest percentage of pregnancy was seen in the high-dose BPA group (75%) (Figure 1C). Considering the total number of offspring, the highest number of offspring (106 pups) was obtained in the BPA low dose group followed by the control group (90 pups) and the lowest number of offspring (72 pups) was seen in the high-dose BPA group. However, the mortality rate after birth was higher in the BPA low-dose group (14.1%) compared to the control animals (13.3%) and the BPA high-dose group (8.3%), which had the lowest number of dead pups. Among the living animals, the number of female offspring was 45 in the control group, 46 in the BPA low-dose group and 31 in the high-dose BPA group. All these animals were included in the study. In addition, the offspring of both BPA treatments had

lower body weights compared to the control PND6 offspring, while no significant differences were observed between both doses of BPA (Figure 1E). No significant differences were observed between BPA and control groups in the body weight of the females during the entire experiment. In all experimental groups, a constant weight gain was observed, reaching the maximum at the end of pregnancy, as expected (Figure 1A).

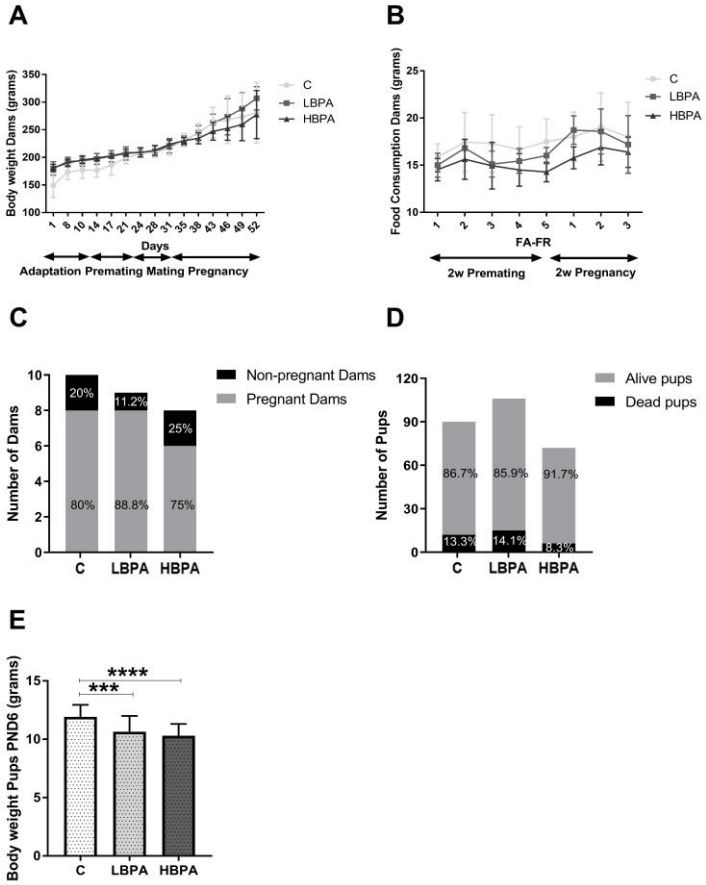

Figure 1. Body weight, food consumption and reproduction data in pregnant dams and neonatal offspring. (**A**) Body weight of dams monitored every 3–4 days; (**B**) food consumption during the second week of premating and the second week of pregnancy; (**C**) number of pregnant and non-pregnant dams; (**D**) percentage of alive and dead offspring after birth and (**E**) body weight of PND6 offspring after birth. Data are represented as mean ± SD. Statistical significance was determined by one-way ANOVA. *** $p < 0.001$, **** $p < 0.0001$.

2.2. Effects of BPA Exposure on Antioxidant Enzyme Activities and Glutathione Concentrations in Dams

Female rats exposed to low and high doses of BPA were compared in terms of antioxidant enzyme activities and glutathione concentrations; which are endogenous antioxidant defense systems to prevent cellular damage measured in the liver (Figure 2). When lactating females were treated with low-dose BPA, all antioxidant enzyme activities such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) were significantly decreased compared to the control group (Figure 2A–E, respectively). In addition, a decrease in reduced glutathione (GSH) concentration (Figure 2F) and an increase in oxidized glutathione (GSSG) concentration (Figure 2G) were observed in low BPA dose-treated dams. When lactating dams were treated with the high dose of BPA, decreased activities of antioxidant enzymes CAT, GPx and GST were observed in comparison to the control group (Figure 2A,C,E, respectively). GSSG concentration was also increased compared to the control

(Figure 2G), but no significant differences were observed in the GSH concentration (Figure 2F). The GSSG/GSH ratio, a marker of oxidative stress, was significantly increased in dams exposed to the low dose of BPA compared to the control group and significant differences were also observed between treatment groups, resulting in higher levels of oxidative stress in the low-dose BPA group (Figure 2H).

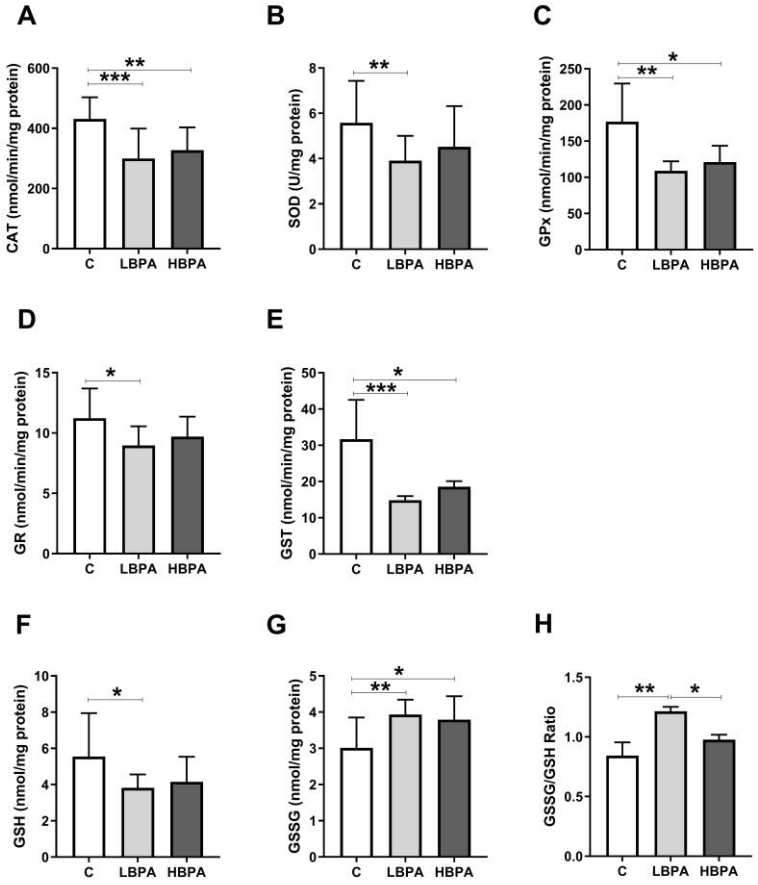

Figure 2. Antioxidant enzyme activities and glutathione concentrations in livers from lactating dams after exposure to low and high doses of BPA. (**A**) Enzymatic activity of catalase (CAT) in nmol/min/mg protein; (**B**) superoxide dismutase (SOD) in U/mg protein; (**C**) glutathione peroxidase (GPx) in nmol/min/mg protein; (**D**) glutathione reductase (GR) in nmol/min/mg protein; (**E**) glutathione S-transferase (GST) in nmol/min/mg protein. (**F**) Concentration of reduced glutathione (GSH) in nmol/mg protein; (**G**) concentration of oxidized glutathione (GSSG) in nmol/mg protein. (**H**) GSSG/GSH ratio. Data represent mean ± SD. n = 8 control dams; n = 8 LBPA dams; n = 6 HBPA dams (two replicates for each sample). Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.3. Effects of BPA Exposure on Oxidative Damage in Dams and Gene Expression Profile of GSH-Related Enzymes

Data concerning measurements of oxidative damage and the gene expression of antioxidant enzymes (GPx, GR, GST, γ-glutamylcysteine synthetase (γ-GCS)) were tested in the liver of dams (Figure 3). In the low-dose BPA dams, GPx, GR, GST and γGCS gene expressions were down-regulated versus control dams (Figure 3A–D). Lipid peroxidation is a metabolic process that causes oxidative deterioration of lipids by reactive oxygen species (ROS). This process can degrade lipids within the cell membrane leading to cell damage and eventual cell death. In lactating dams treated with the low dose of BPA, we

observed an increase in malondialdehyde (MDA) and lipid hydroperoxide (LPO), two products generated under oxidative stress situations, used to measure oxidative lipid damage (Figure 3E,F, respectively). In dams treated with the low dose of BPA, an increase in 8-oxo-2′-deoxyguanosine (8-OHdG), one of the main DNA oxidation products, used as a biomarker of oxidative DNA damage, was also observed (Figure 3I). Furthermore, an increase in adenosine triphosphate (ATP) energetic levels was observed in the low dose of BPA dams compared to the control group (Figure 3G). Nitric oxide (NO) plays a dual role in oxidative and antioxidant behavior. As an antioxidant, NO protects cells from oxidative stress. However, when produced in excess, it behaves as an important pro-oxidant factor. In this case, an increase in plasma NO metabolites of dams treated with the low dose of BPA was observed (Figure 3H). In addition, when the two BPA doses were compared, significant differences were observed in LPO concentrations, ATP levels and NO metabolites, being significantly lower than the results of the group treated with the high dose of BPA when compared to the low dose (Figure 3F,G,H, respectively).

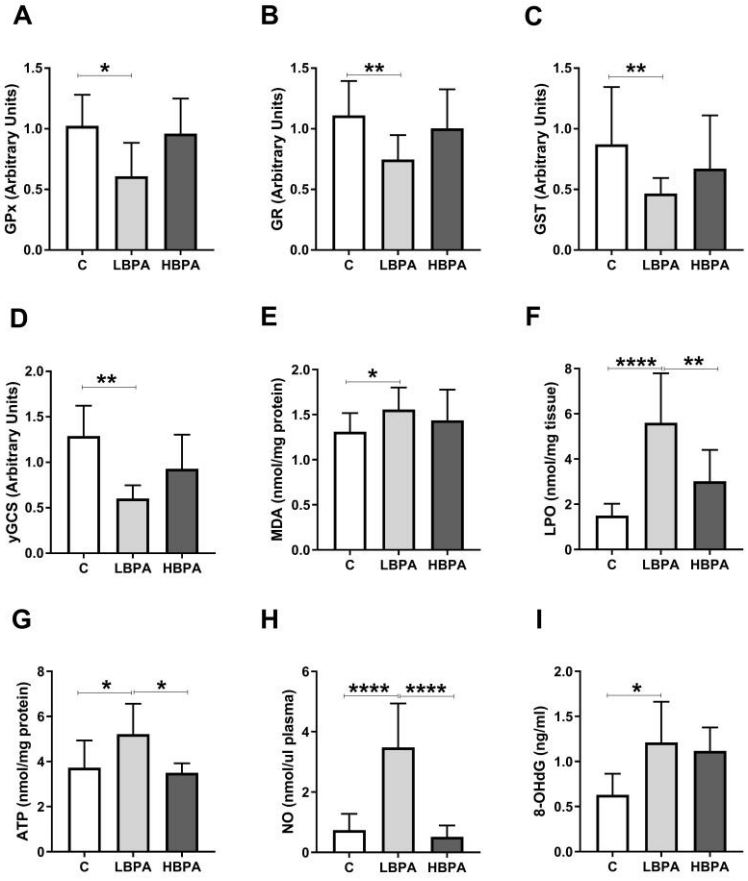

Figure 3. Gene expression profile of GSH-related enzymes and parameters of oxidative damage in livers from lactating dams after exposure to different doses of BPA. (**A**) Glutathione peroxidase (GPx), (**B**) glutathione reductase (GR), (**C**) glutathione S-transferase (GST) and (**D**) γ-glutamylcysteine synthetase (γ-GCS) relative gene expression. (**E**) Malondialdehyde (MDA) content in nmol/mg protein. (**F**) Lipid hydroperoxide (LPO) content in nmol/mg tissue. (**G**) Adenosine triphosphate (ATP) levels in nmol/mg protein. (**H**) Concentration of nitric oxide metabolites (NO) in nmol/μL plasma. (**I**) Concentration of 8-oxo-2′-deoxyguanosine (8-OHdG) in ng/mL. Data represent mean ± SD. For mRNA, n = 8 control dams; n = 8 LBPA dams; n = 6 HBPA dams (three replicates for each gene). For ELISA assay, n = 8 control dams; n = 8 LBPA mg/kg dams; n = 6 HBPA dams (two replicates for each sample). Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

2.4. Effects of BPA Exposure on Oxidative Stress-Inducing Markers in Dams

Results obtained for gene and protein expression of oxidative stress-inducing proteins: Heme oxygenase-1 (HO-1d) and NOS isoforms: Inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in the livers of dams exposed to the two doses of BPA are shown in Figure 4. In dams treated with the low dose of BPA, an up-regulation in gene and protein expressions of HO-1d and iNOS compared to the control group was observed (Figure 4A–D). In dams treated with the high dose of BPA, a significant increase in iNOS gene and protein expressions was observed versus the control dams (Figure 4C,D, respectively). However, no differences in eNOS gene and protein expressions were observed among the groups (Figure 4E,F, respectively). When the two doses of BPA were compared, the only oxidative stress-inducing protein that showed significant differences among groups was HO-1d, which was significantly higher in the low dose of BPA group compared to the high dose one (Figure 4B).

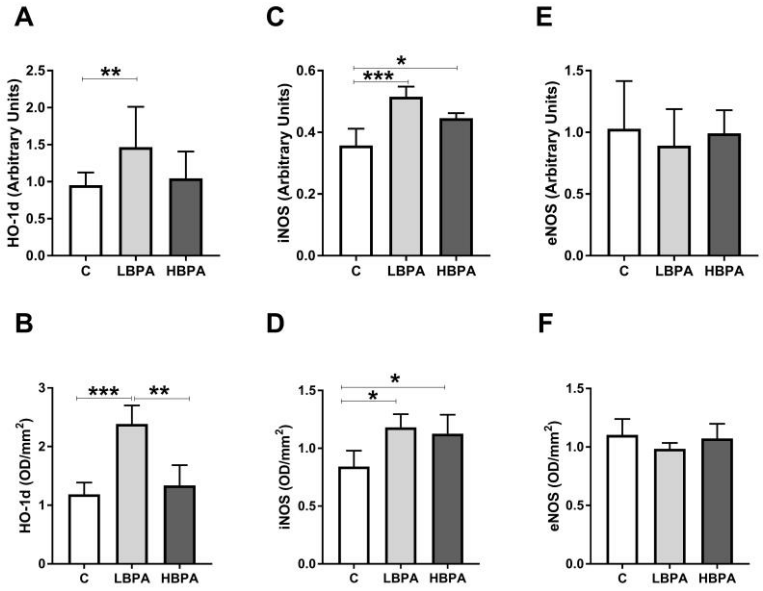

Figure 4. Oxidative stress intermediaries in livers from lactating dams after exposure to different doses of BPA. mRNA and protein expressions of HO-1d (heme oxygenase 1) (**A**,**B**); iNOS (inducible nitric oxide synthase) (**C**,**D**); and eNOS (endothelial nitric oxide synthase) (**E**,**F**). Data represent mean ± SD. For mRNA, n = 8 control dams; n = 8 LBPA dams; n = 6 HBPA dams (three replicates for each gene). For protein, n = 5 rats per experimental group. Data represent mean ± SD. Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.5. Effects of BPA Exposure in Dams on Inflammatory and Apoptosis Response in the Liver

The results of gene and protein expressions of inflammatory markers such as interleukin-1-β (IL1β) and apoptosis markers: Apoptosis-inducing factor (AIF), Bcl-2-associated X protein (BAX), B-cell lymphoma (BCL-2) and B-cell lymphoma-extra large (BCL-XL) are shown in Figure 5. The proinflammatory cytokine IL1β showed a significant increase in gene and protein expressions in dams treated with the low dose of BPA as compared with the control group (Figure 5A,B, respectively). Regarding the proapoptotic molecules, AIF gene and protein expressions were up-regulated in dams treated with the low dose of BPA (Figure 5C,D, respectively) compared with the control group. BAX gene expression was up-regulated in the low dose of BPA treated dams (Figure 5E). In dams treated with high dose of BPA, an increase in AIF gene expression was observed compared to control (Figure 5C). Considering the antiapoptotic molecules, BCL-2 and BCL-XL protein expressions were down-regulated in dams treated with the low dose of BPA (Figure 5F,G, respectively). When the two doses of BPA were compared, a significant difference was found in the protein expression of the proapoptotic molecule AIF, the low dose of

BPA group being significantly higher than the high-dose group (Figure 5D). In addition, the protein expression of BCL-XL, an antiapoptotic molecule, was significantly lower in the low dose of BPA group compared to the high dose one (Figure 5G). Representative protein blots for each tested marker are shown in Figure 5H.

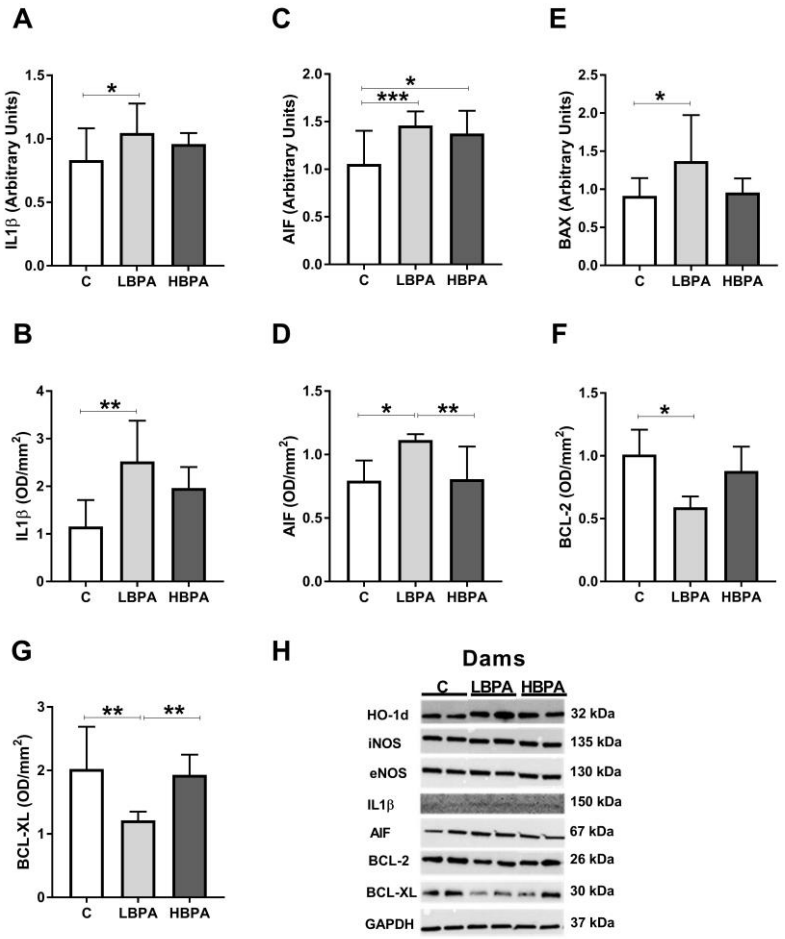

Figure 5. Inflammatory mediator and apoptosis markers in livers from lactating dams after exposure to different doses of BPA. mRNA and protein expressions of IL1β (interleukin-1-β) (**A**,**B**); mRNA and protein expression of AIF (apoptosis-inducing factor) (**C**,**D**). (**E**) mRNA expression of BAX (Bcl-2-associated X protein). (**F**,**G**) Protein expressions of BCL-2 (B-cell lymphoma) and BCL-XL (B-cell lymphoma-extra large). (**H**) Representative images of the Western blot results of the different proteins studied. Data represent mean ± SD. For mRNA, n = 8 control dams; n = 8 LBPA dams; n = 6 HBPA dams (three replicates for each gene). For protein, n = 5 rats per experimental group. Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.6. Effects of Perinatal Exposure to BPA on Antioxidant Enzyme Activities and Glutathione Concentrations in Liver of Female PND6 Offspring

Antioxidant enzyme activities and glutathione concentrations were determined in the livers of female PND6 pups to determine the effect of perinatal exposure to low and high doses of BPA (Figure 6). When PND6 offspring were perinatally exposed to low dose of BPA, all antioxidant enzyme activities (CAT, SOD, GPx, GR and GST) were significantly decreased compared to the control group (Figure 6A–E). In addition, a decrease in reduced glutathione (GSH) concentration (Figure 6F) and an increase in oxidized glutathione (GSSG) concentration (Figure 6G) were observed in the low dose of BPA offspring. These same effects were observed in lactating dams exposed to the low dose of BPA (Figure 2). When PND6 offspring were perinatally exposed to the high dose of BPA, decreased

activities of antioxidant enzymes SOD and GST were observed in comparison to the control group (Figure 6B,E, respectively). GSH concentration decreased in comparison to the control group whereas no significant changes were observed in GSSG concentration (Figure 6G). As observed in lactating dams, GSSG/GSH ratio increased in offspring exposed to low dose of BPA as compared to the control group (Figure 6H). When antioxidant enzyme activities and glutathione concentrations were compared between treated groups, a significant increase in GPx was observed in the high dose of BPA group compared to the low dose one (Figure 6C). On the contrary, an imbalance between GSSG and GSH levels was observed in the low dose of BPA group, resulting in a higher ratio as a marker of oxidative stress compared to the high dose one (Figure 6H).

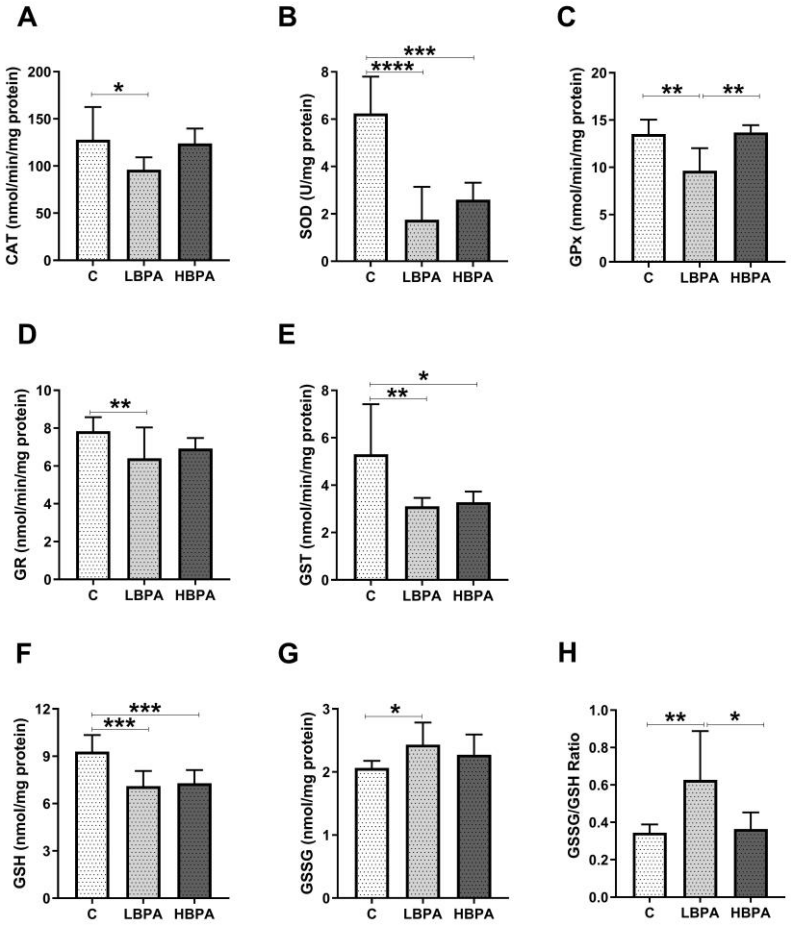

Figure 6. Effects of perinatal exposure to BPA on antioxidant enzyme activities and glutathione concentrations in livers from female PND6 offspring. (**A**) Enzymatic activity of catalase (CAT) in nmol/min/mg protein; (**B**) superoxide dismutase (SOD) in U/mg protein; (**C**) glutathione peroxidase (GPx) in nmol/min/mg protein; (**D**) glutathione reductase (GR) in nmol/min/mg protein; (**E**) glutathione S-transferase (GST) in nmol/min/mg protein. (**F**) Concentration of reduced glutathione (GSH) in nmol/mg protein; (**G**) concentration of oxidized glutathione (GSSG) in ng/mg protein. (**H**) GSSG/GSH ratio. Data represent mean ± SD. n = 12 control PND6 offspring; n = 12 LBPA PND6 offspring; n = 12 HBPA PND6 offspring (two replicates for each sample). Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

2.7. Effects of Perinatal Exposure to BPA on Oxidative Damage and Gene Expression Profile of GSH-Related Enzymes in Liver of PND6 Offspring

The transcriptional levels of antioxidant enzymes (GPx, GR, GST, γGCS) and markers of oxidative damage in the liver of female PND6 offspring are shown in Figure 7. GPx,

GR, GST and γGCS gene expressions were down-regulated in low-dose-PND6 offspring versus control offspring (Figure 7A–D). In PND6 offspring perinatally exposed to a low dose of BPA, an increase in MDA, LPO and 8-OHdG content compared to the control group was observed (Figure 7E,F,I, respectively). ATP energy levels increased in low-dose-BPA offspring compared to the control group (Figure 7G). These results suggest that perinatal exposure to low doses of BPA increased oxidative damage of lipids and DNA in offspring, as it was observed in dams exposed to low doses of BPA (Figure 3E,F,I,G). Furthermore, an increase in plasma NO metabolites of low-dose-BPA offspring was observed (Figure 7H). In offspring exposed to high doses of BPA, an increase in 8-OHdG was observed, showing oxidative DNA damage (Figure 7I). When the two BPA doses were compared, significant differences were observed in GST gene expression, which was significantly higher in the high-dose-PND6 group compared to the low dose one (Figure 7C). In addition, LPO concentrations and ATP levels showed significant differences between groups, the high-dose-PND6 group being significantly lower compared to the low dose one (Figure 7F,G, respectively). These results are similar to those observed in dams, where significant differences were also observed between treated groups the high dose of BPA group being the one that showed significantly lower levels (Figure 3F,G, respectively).

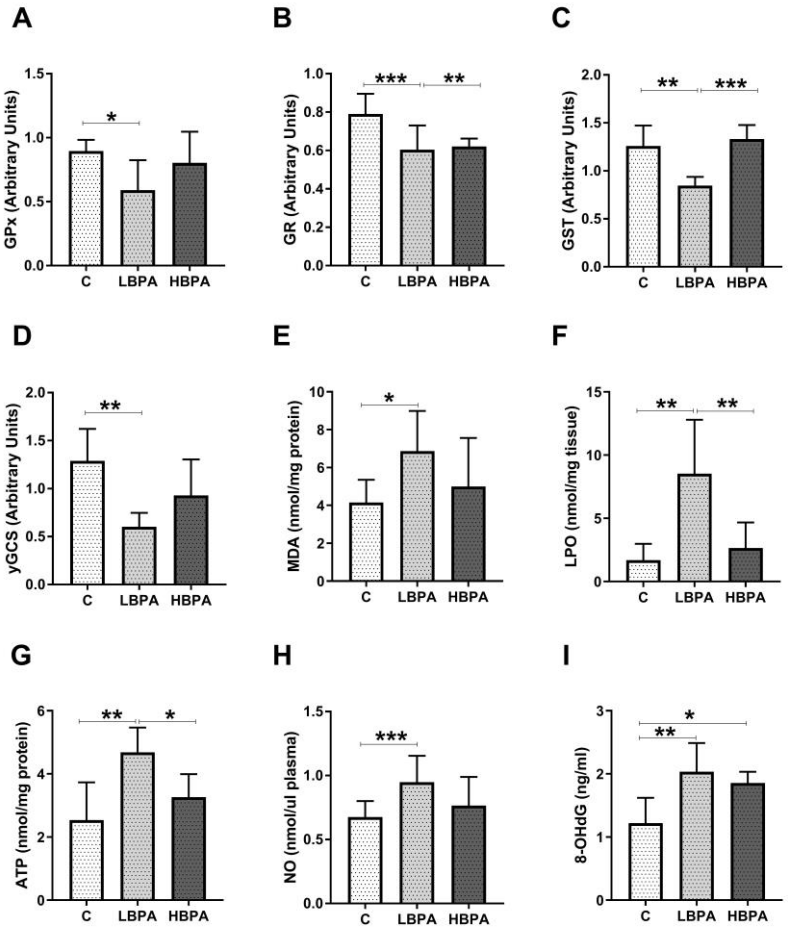

Figure 7. Effects of perinatal exposure to BPA on gene expression profile of GSH-related enzymes and oxidative damage in livers from female PND6 offspring. (**A**) Glutathione peroxidase (GPx); (**B**) glutathione reductase (GR); (**C**) glutathione S-transferase (GST); (**D**) γ-glutamylcysteine synthetase (γ-GCS) relative gene expression. (**E**) Malondialdehyde (MDA) content in nmol/mg protein. (**F**) Lipid hydroperoxide content in nmol/mg tissue. (**G**) Adenosine triphosphate (ATP) levels in nmol/mg protein. (**H**) Concentration of nitric oxide metabolites (NO) in nmol/μL plasma. (**I**) Concentration of 8-oxo-2′-deoxyguanosine (8-OHdG) in ng/mL. Data represent mean ± SD. For ELISA assay, n = 12

control PND6 offspring; n = 12 LBPA PND6 offspring; n = 12 HBPA PND6 offspring (two replicates for each sample). For mRNA, n = 12 control PND6 offspring; n = 12 LBPA PND6 offspring; n = 12 HBPA PND6 offspring (three replicates for each gene). Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.8. Effects of Perinatal Exposure to BPA on Oxidative Stress Intermediaries in Liver of PND6 Offspring

In PND6 offspring exposed to low doses of BPA, an up-regulation in gene and protein expressions of HO-1d and iNOS compared to the control group was observed (Figure 8A–D). Regarding HO-1d gene expression and iNOS protein expression, significant differences were also observed between treatment groups, where the low-dose-PND6 group showed significantly higher expressions than the high dose group (Figure 8A,D, respectively). However, no differences in eNOS gene and protein expressions were observed among groups (Figure 8E,F, respectively); these results were similar to those observed in dams exposed to low doses of BPA (Figure 4).

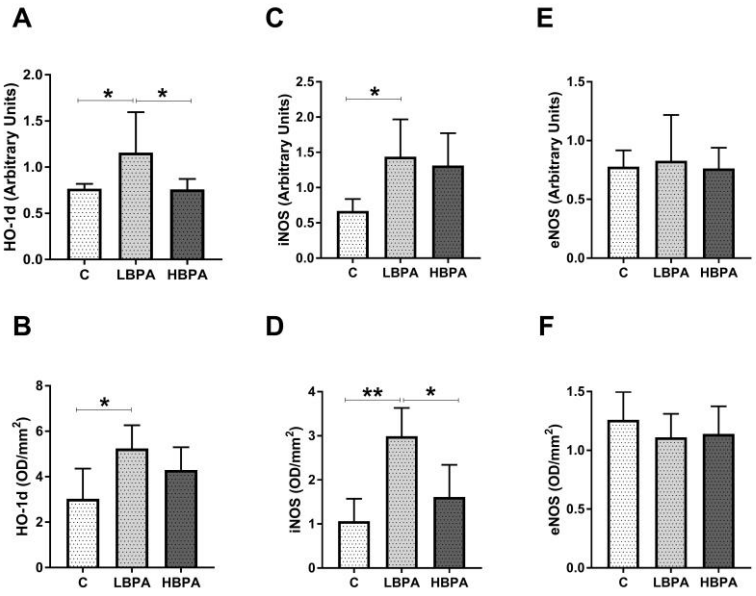

Figure 8. Effects of perinatal exposure to BPA on oxidative stress intermediaries in livers from female PND6 offspring. mRNA and protein expressions of HO-1d (heme oxygenase 1) (**A**,**B**); iNOS (inducible nitric oxide synthase) (**C**,**D**); and eNOS (endothelial nitric oxide synthase) (**E**,**F**). Data represent mean ± SD. For mRNA, n = 12 control PND6 offspring; n = 12 LBPA PND6 offspring; n = 12 HBPA PND6 offspring (three replicates for each gene). For protein, n = 5 rats per experimental group. Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$.

2.9. Effects of Perinatal Exposure to BPA on Inflammatory Mediator and Apoptosis Markers in Liver of PND6 Offspring

The results of gene and protein expressions of inflammatory markers IL1β and apoptosis markers AIF, BAX, BCL-2 and BCL-XL are shown in Figure 9. The proinflammatory cytokine IL1β showed a significant increase in gene and protein expressions in offspring treated with low doses of BPA as compared to the control group (Figure 9A,B, respectively). Regarding the proapoptotic molecules, AIF gene and protein expressions were up-regulated in offspring exposed to low doses of BPA compared to the control group (Figure 9C,D, respectively). When AIF gene and protein expressions were analyzed between treated groups, significant differences were found. In both gene and protein expressions, the low-dose-PND6 offspring showed significantly higher values than the high-dose group, whose results were not different from the control group (Figure 9C,D, respectively). BAX gene expression was up-regulated in low dose of BPA offspring (Figure 9E).

The anti-apoptotic markers BCL-2 and BCL-XL significantly reduced their protein expression in low-dose-BPA offspring versus the control group (Figure 9F,G, respectively). This imbalance between pro-apoptotic and anti-apoptotic family members shown in low-dose-BPA pups was also observed in lactating dams (Figure 5F,G, respectively). Representative protein blots for each tested marker are shown in Figure 9H.

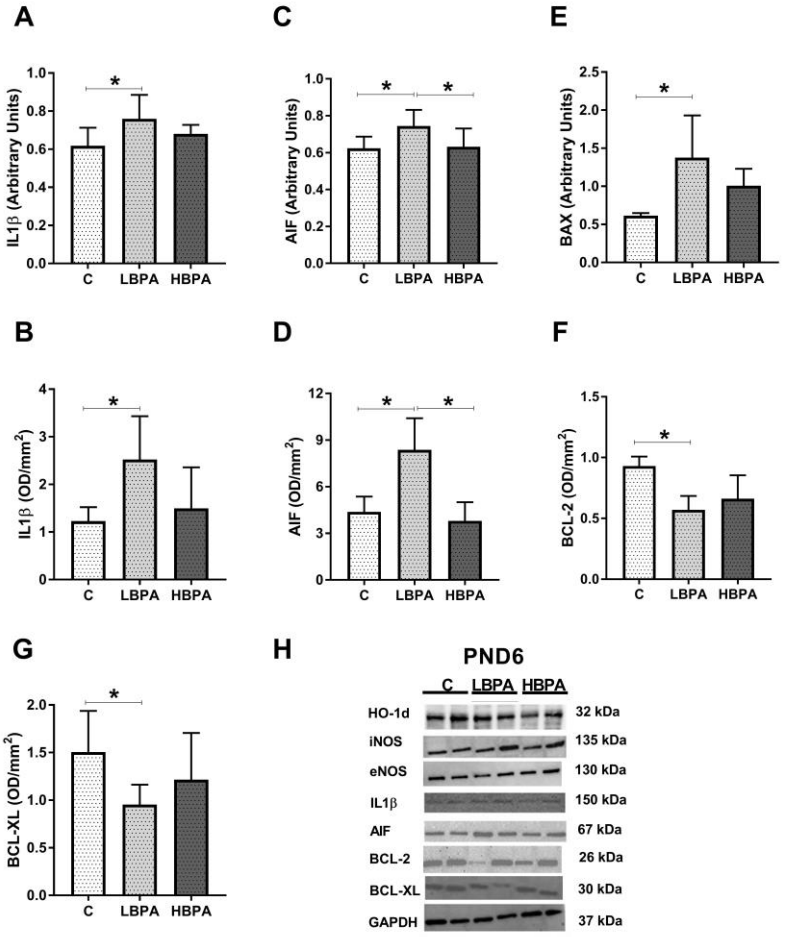

Figure 9. Effects of perinatal exposure to BPA on inflammatory mediator and apoptosis markers in livers from female PND6 offspring. (**A**,**B**) mRNA and protein expressions of IL1β (interleukin-1-β). (**C**,**D**); mRNA and protein expression of AIF (apoptosis-inducing factor). (**E**) mRNA of BAX (Bcl-2-associated X protein). (**F**,**G**) Protein expressions of BCL-2 (B-cell lymphoma) and BCL-XL (B-cell lymphoma-extra large). (**H**) Representative images of the Western blot results of the different proteins studied. Data represent mean ± SD. For mRNA, n = 12 control PND6 offspring; n = 12 LBPA PND6 offspring; n = 12 HBPA PND6 offspring (three replicates for each gene). For protein, n = 5 rats per experimental group. Statistical significance was determined by one-way ANOVA. * $p < 0.05$.

2.10. Effect of BPA on Histopathology and Hepatic Serum Markers

Hematoxylin and eosin staining was used to analyze the effect of BPA on liver injury; this is shown in Figure 10. In the livers of dams exposed to BPA, no changes were observed in cellular structure compared to control hepatocyte images (Figure 10A). However, histological staining showed that BPA administration increased nucleus aggregation and infiltration of inflammatory cells in PND6 offspring liver tissue compared to control pups. In addition, lower concentration of BPA had a noteworthy impact on liver injury compared to higher doses in PND6 offspring (Figure 10B). Results of hepatic serum marker assessment indicated that dams treated with lose-dose BPA exhibited liver injury manifested by a significant rise in the levels of aspartate aminotransferase (AST) and alanine

aminotransferase (ALP) when compared to the control (Figure 10C,D, respectively). The serum levels of gamma glutamyl transpeptidase (GGT) did not show any significant change in animals receiving BPA in comparison with the control group (Figure 10E).

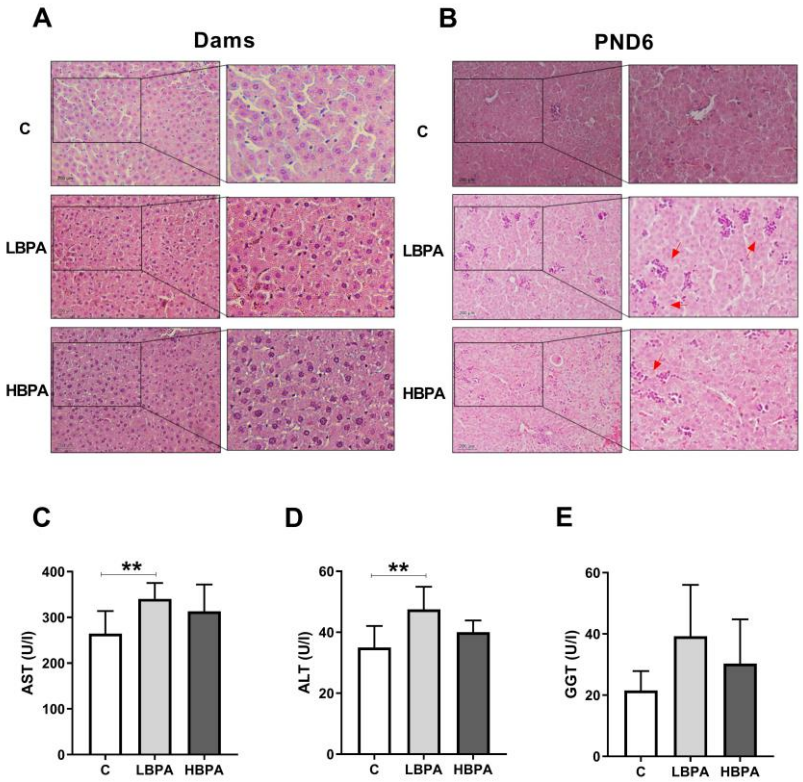

Figure 10. Histology and clinical chemistry of liver functional markers after BPA administration. Representative images of livers from dams (**A**) and female PND6 offspring (**B**) stained with H&E in control and low and high BPA treatment groups (LBPA or HBPA). Scale bars indicate 200 μm (20×) and magnified image of the characteristic tissue section (40×). Red arrows indicate the aggregation of nuclei. (**C**) Aspartate aminotransferase (AST); (**D**) alanine aminotransferase (ALP) and (**E**) gamma glutamyl transpeptidase (GGT) in U/L in serum dams. Data represent mean ± SD. n = 8 control dams; n = 8 LBPA dams; n = 6 HBPA dams. Statistical significance was determined by one-way ANOVA. ** $p < 0.01$.

3. Discussion

Bisphenol A is one of the most widely used industrial chemicals worldwide. Trasande et al. [28] reported that 90% of the general population has detectable levels of BPA. These BPA levels are 70 times higher in occupationally exposed individuals than in environmentally exposed populations [29]; therefore, BPA exposure is considered an unavoidable and concerning situation. Since BPA exposure occurs mainly through ingestion, in the present study the effects of BPA administered by the oral route at two different concentrations were evaluated: a low dose of 0.036 mg/kg/b.w./day and an almost 100-fold higher dose of 3.42 mg/kg/b.w./day, in the liver of pregnant dams and their perinatal effect in PND6 offspring.

The liver is the main organ responsible for the metabolism of BPA through conjugation by the liver enzyme uridyl diphosphate glucuronyl transferase (UDPGT) to a less toxic compound called bisphenol A-glucuronide (BPAG) [30,31]; this being the main pathway for the detoxification of this xenobiotic. A smaller amount of BPA reacts with sulfate giving rise to BPA-sulfate (BPAS) [32] or can be oxidized to a catechol followed by further transformation to an O-quinone (4,5-bisphenol-O-quinone) [33]. The catechol-O-quinone couple is capable of redox cycling with generation of ROS and oxidative stress [14]. ROS are cytotoxic agents that cause oxidative damage by attacking the cell membrane but also

DNA. The liver has an endogenous antioxidant defense system to prevent cellular damage such as antioxidant enzymes and the glutathione system. The activities of antioxidant enzymes CAT, SOD, GPx and GR were decreased in the livers of dams treated with low doses of BPA compared to the control group. There was also a decrease in the enzymatic activities of CAT and GPx in dams treated with high doses of BPA, without any significant difference with the low doses of BPA group. SOD generally dismutates the superoxide anion radical into hydrogen peroxide, which is degraded by CAT using GSH. The reduction in CAT activity may reflect the inability to remove H_2O_2 produced after BPA exposure [15,34].

GST protects the cell by conjugating glutathione (GSH) to electrophilic substrates, generating less reactive and more soluble compounds, being a detoxifying enzyme involved in the metabolism of many xenobiotics [35]. Exposure to both doses of BPA showed a significant reduction in GST activity, reflecting an inability to detoxify this compound. Exposure to low BPA dose also reflects lower gene expression levels of the γGCS that catalyzes the first step in glutathione synthesis, resulting in low cellular levels of glutathione. Glutathione provides a first line of defense against ROS as it can scavenge free radicals and reduce H_2O_2 formation in the cell. BPA produces several quinols and semiquinone intermediates that can react with glutathione producing glutathione conjugates, which, in turn, increase oxidative stress levels [7]. GPx utilizes reduced glutathione (GSH) to remove peroxides produced by oxidative stress [36]. On the other hand, GR reduces oxidized glutathione (GSSG) back to GSH using NADPH [7].

In the present study, the GSH depletion shown in the dams treated with low BPA dose along with NADPH oxidation and altered redox homeostasis seems to play an important role in the disruption of antioxidant defense, leading to elevated levels of oxidative stress in liver cells. Oxidative stress produces free radicals that can easily react with cell membrane lipids, proteins and nucleic acids, thus initiating a chain of reactions leading to the production of lipid peroxides [37] and DNA damage [38]. In our results, a significant increase in MDA levels was observed with low doses of BPA and an increase in LPO with both doses of BPA in dams, in this case being higher in the low dose of BPA group compared to the high dose one. In turn, exposure to low doses of BPA led to an increase in oxidative damage to DNA as shown by the increased values of 8-OHdG in the livers of pregnant dams.

We observed that exposure to low BPA dose induced liver damage in rats, affecting the oxidant/antioxidant balance and causing liver injury. Our results are in agreement with many others, such as Acaroz et al. [3] who demonstrated decreased SOD and CAT enzymatic activities and GSH levels in Wistar albino rats exposed to BPA at different oral doses (5, 10 and 20 mg/kg). In another study using a dose of 25 mg/kg in rats for 50 days, an increase in MDA levels and a decrease in GSH levels and SOD and CAT activities in kidney, brain and testis tissues was found [39]. Bindhumol et al. [15] also showed a reduction of antioxidant enzymes (SOD, CAT, GR, GPx) in the mitochondrial and microsome-rich fractions of the liver; while H_2O_2 and MDA levels increased in Wistar rats treated with BPA doses ranging from 0.2 to 20 μg/kg. The same occurred in the study by Hassan et al. [40] where antioxidant activities were decreased at doses of 50 mg/kg of BPA in rat livers.

To investigate the involvement of BPA in cellular oxidative stress, eNOS, iNOS and HO-1d were tested as mediators of this process. Vascular function mainly depends on the balance between synthesis/degradation of nitric oxide (NO). NO produced by eNOS is a result of a physiological response that plays an important role in mediating many processes such as vasodilation, immunity and neurotransmission. In our results, we observed no difference in eNOS gene and protein expression in both treatment groups. However, elevated plasma NO levels and higher gene and protein expression of iNOS were observed in dams treated with low BPA dose compared with the control group and high BPA dose. An increase in the synthesis of NO produced by iNOS causes vascular dysfunction and its iNOS activation may have some detrimental effects for liver function. NO is a

potent oxidant and a nitrating agent capable of attacking and modifying proteins, lipids and DNA, as well as decreasing antioxidant defenses [41].

Regarding heme oxygenase (HO), which participates in the metabolism of the heme group of hemoproteins, two isoforms have been characterized: one inducible (HO-1d) and one constitutive (HO-2d). The inducible isoform, HO-1d, is expressed under various stimuli, such as oxidative stress and cytokines such as TNFα; being a reliable marker of a proinflammatory and prooxidant state [42]. Our results showed an increase in gene and protein expression of HO-1d on low BPA dose treatment in dams. Its induction following increased oxidative stress could act as a cellular defense mechanism to prevent progression of liver fibrosis. Kazemi et al. [1] showed an increase in HO-1d gene expression with a BPA dose-dependent profile in liver cells.

High levels of oxidative stress have been linked to inflammatory processes. In this study, dams treated with low BPA dose increased gene and protein levels of the proinflammatory cytokine (IL-1β). In accordance with our results, Acaroz et al. [3] showed that BPA exposure at 25 mg/kg in male Wistar rats increased the expression of proinflammatory cytokines such as TNF-α, IL-6 and IL-1β and decreased anti-inflammatory/antifibrotic cytokine (IL-10). Elswefy et al. [8] administered 50 mg/kg of BPA to rats orally for eight weeks and reported that its administration significantly increased the serum level of IL-1β and reduced the level of IL-10. This increase in proinflammatory cytokines induced liver inflammation by transporting mononuclear and polymorphonuclear leukocytes to inflamed tissues [43]. In our study, no structural changes of hepatocytes were noticeable yet after BPA administration in the liver of the dams. However, in the histological study by Kazemi et al. [44] it was demonstrated that oral administration of BPA by gavage at low doses induced liver injury in male adult rats.

Liver tissue damage can be assessed by serum liver markers. In our study, a marked increase in AST and ALT was observed with the low BPA dose, indicating tissue damage in the liver. This is consistent with the study by Ijaz et al. [45] where a substantial increase in the levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) was also observed in BPA-treated rats. This may be because overproduction of ROS damages the structural integrity of liver cells, which is manifested by an increase in hepatic serum markers [46]. However, it is not yet apparent in histological sections because it is at an early stage of involvement after seven weeks of BPA exposure.

Previous studies found that BPA impairs hepatic mitochondrial function by releasing soluble factors into the cytosol [13,47]. This membrane permeabilization may be the initial stage of mitochondrial apoptosis [6]. One of the proapoptotic markers is AIF, which upon release into the cytosol, translocates to the nucleus where it triggers apoptotic pathways. In our study, elevated gene levels of AIF were found in the liver upon exposure to BPA. We also studied the mRNA expression of BAX, another factor that promotes apoptosis, which showed significantly elevated gene expression levels in dams treated with low BPA dose, whereas protein expression of Bcl-2 and BCL-XL, which are anti-apoptotic factors that protect the cell from various cytotoxic alterations, were found to be significantly decreased with the low dose of BPA treatment. Previous studies also showed increased proapoptotic protein caspase-3 and reduced anti-apoptotic protein BCL in the liver of male rats [6,8]. BPA weakened hepatocyte mitochondrial function and promoted cell apoptosis in the liver by up-regulating protein levels of Bax, cleaved caspase-3 and cleaved PARP1, while it down-regulated Bcl-2 in the liver using high doses of BPA [48]. Notably, cytochrome c, a key mediator of apoptosis through activation of caspases in the cytosol [49–51], was also found to be increased.

Low BPA dose treatment showed elevated ATP levels in pregnant dams compared to the control group. This maintenance of sufficient ATP levels together with the release of pro-apoptotic factors causes liver cells to enter apoptosis [52]. The mechanism of BPA-induced apoptosis probably also involves an alteration in the expression ratio of pro-apoptotic and anti-apoptotic proteins of the BCL-2-associated X family (BAX) and BCL-2 in the outer mitochondrial membrane that modulates the release of proapoptotic factors

[53,54]. Therefore, exposure of pregnant dams to low doses of BPA may exert toxic effects on liver cells through the formation of ROS, induction of inflammation and apoptosis.

At high doses of BPA these effects are not as noticeable or significant in many of the parameters studied compared to the control group. This may be because there is a higher level of vulnerability in the liver towards low doses of BPA compared to other organs due to the initial metabolism of BPA by the liver [12,13,55]. BPA is considered a xenoestrogen, but not an estrogen mimic [56] due to its ability to bind to the classical nuclear estrogen receptors (ER) ERα and ERβ [57]; although compared to 17β-estradiol the affinity is about 10,000 times lower for ERα and 1000 times weaker than the affinity for ERβ [58]. It is also able to bind to classical and non-classical membrane estrogen receptors [59], as well as to the G protein-coupled receptor 30 (GPR30) [60], and act through non-genomic pathways [61] and also as an activator of the thyroid hormone and androgen receptors [59,62]. This may explain that BPA, as an endocrine disruptor, such as some hormones, can follow non-monotonic dose–response curves (NMDR), showing more noticeable effects at low doses than at high doses [63]. The endocrine system is configured to respond to very low concentrations of hormones and a maximal biological response can be observed without a high receptor occupancy of this response. This could be due to the fact that response mechanisms become saturated before all receptors are occupied.

This is consistent with a previous study that observed a non-monotonic relationship in pregnant Wistar rats exposed to BPA (50, 250 or 1250 μg/kg) and their offspring after weaning. Only the lowest dose of 50 μg/kg of BPA produced effects such as increased body weight, elevated serum insulin and impaired glucose tolerance in adult pups. However, this study exposed rats to normal or high-fat diets, which could also play a role in the response mechanisms. Rats exposed perinatally to the higher doses showed none of the adverse effects, regardless of diet [20].

Most scientific studies have focused on the effect of high doses of BPA in adults, but the effect of low BPA dose on perinatal exposure seems to be more important to take into consideration [64]. Exposure of pregnant dams to BPA is of concern to the developing fetus since it is able to cross the placenta and enter into cord blood and amniotic fluid. This is in addition to the presence of little or no fetal enzymatic activity at all of UDPGT to biotransform it into inactive BPAG [17]. Furthermore, the enzyme β-glucuronidase is highly active in the placenta and can further contribute to increase fetal exposure to free BPA by hydrolysis of conjugated BPA entering the fetal compartment [20,65]. BPA also binds to the estrogen-related receptor gamma (ERɣ), which is highly expressed in the placenta, facilitating the accumulation of BPA and thus increasing the exposure of the developing fetus to this compound causing potential harmful effects to the offspring at very low and sustained doses.

A recent in vitro study showed that activation of the P2X7 receptor after incubation with BPA has been observed in human placental cells, leading to different pathways involved in producing preeclampsia and preterm delivery, through activation of the NLRP3 inflammasome and apoptosis [66]. Nishikawa et al. [67] showed that the presence of free BPA in the liver of fetal rats could be the result of direct transfer of free BPA into the maternal circulation via the placenta, in addition to the hydrolysis of BPAG in the fetal liver.

In the present study, we observed a decrease in the activity levels of antioxidant enzymes CAT, SOD, GPx, GR and GST in PND6 pups exposed to the low dose of BPA. SOD and GST activities were also decreased in the offspring with the high dose of BPA. GSH was reduced in the offspring exposed to the low dose of BPA, with increased levels of oxidized GSH. In addition to decreased antioxidant enzyme activity, lipid peroxidation-associated damage (increased levels of MDA and LPO) was increased in the liver of offspring exposed to low dose, along with increased DNA oxidation in offspring exposed to both doses of BPA. This is consistent with a study in pregnant mice orally exposed to a dose of 100 ng/g BPA from PND7 to PND21, showing that perinatal BPA exposure could induce oxidative damage and alter normal metabolic profiles in the liver [68].

Lin et al. [69] showed that perinatal BPA exposure causes the development of non-alcoholic fatty liver disease (NAFLD) in the offspring of pregnant Sprague-Dawley rats that had access to water containing 1 or 10 μg/mL BPA from gestational day six (GD6) to PND21. BPA exposure is associated with up-regulation of lipogenic genes, dysregulation of autophagy and activation of the inflammatory response involving PI3K/Akt/mTOR and TLR4/NF-κB pathways. This oxidant/antioxidant imbalance also became noticeable here as gene and protein expression levels of oxidative stress-inducing proteins (HO-1d and iNOS) were increased in the offspring exposed to the low dose of BPA, along with elevated plasma NO levels. Increased proinflammatory cytokine IL-1β and proapoptotic factors AIF and BAX, with the subsequent decrease in antiapoptotic factors BCL-2 and BCL-XL, led to an induction of apoptosis in liver cells in the offspring perinatally exposed to the low dose of BPA. In our study, higher aggregation of nucleus and infiltration of inflammatory cells were observed in the liver of PND6 offspring treated with low dose BPA as compared to the high dose one. Santoro et al. [70] showed that the main histological alteration of the liver was a mild to moderate microvesicular steatosis in BPA-treated rats at 10–17 PND and 45–60 PND. Mild hepatocellular hypertrophy was observed in some BPA-exposed lactating or weaned animals. Furthermore, the expression of inflammatory cytokines, Sirt1, its natural antisense long non-coding RNA (Sirt1-AS LncRNA), and histone deacetylase 1 (Hdac1) were affected in exposed animals. Another study has shown susceptibility to NAFLD in adulthood following mitochondrial dysregulation upon perinatal exposure [20]. Jiang et al. [18] showed that perinatal BPA exposure contributes to the development of hepatic steatosis in male offspring at 3, 15 and 26 weeks when postnatally treated with 40 μg/kg BPA, and that this was mediated by impaired hepatic mitochondrial function.

Therefore, exposure to low levels of endocrine disrupting chemical (EDC) BPA—these levels being easier to achieve in daily life—is of concern since it interferes with many metabolic processes and causes widespread damage to body tissues. The fact that lower levels of BPA are generally more effective than the higher doses is a very remarkable issue as previously described. Moreover, it should also be noted that the timing of BPA exposure may determine the long-term outcome, as earlier exposure points tend to exert a more severe effect [18]. Thus, fetuses and newborns are more sensitive than adults, and chemical exposure during critical developmental stages could cause irreversible long-term consequences [6,17,71]. In our study, similar effects were observed in perinatally exposed offspring as well as in their lactating dams after BPA exposure, being this a critical period influencing ontogenic development of various tissues and also increasing the risk of developing diseases later in adulthood. Further research is critical to understand the extent and effect of prenatal exposure to potentially toxic chemicals including BPA.

Our study is subject to a series of technical limitations. First, since it is part of a bigger European Union's Horizon 2020 Research and Innovation Programme project (ENDpoiNTs; grant number: 825759), at the time of extraction, livers had to be quickly divided and immediately frozen in liquid nitrogen. Therefore, the liver weight could not be measured. These data could have provided additional information regarding a possible hepatic injury. Another technical limitation regarding the analysis of liver functional enzymes is that this could only be performed with dams' sera. In the case of PND6 offspring, the collected serum volume was insufficient to perform these chemical determinations. Hence, information about hepatic functionality in PND6 offspring is missing. Furthermore, for experimental design reasons, only female pups were included. It would have been interesting to compare the effects of the BPA administration also in male pups. Finally, an important limitation of the study is that, for technical limitations, BPA level determinations either in plasma or liver biopsies are missing. Furthermore, although special attention was paid to avoid any BPA contamination throughout the entire experiment, an interference of background BPA exposure with low-dose treatment may not be completely excluded.

4. Materials and Methods

4.1. Animals

Twenty-seven female (eight weeks of age) and twelve male (ten weeks of age) Long–Evans rats (Janvier Labs, Le Genest-Saint-Isle, France) were used in the study. The animals were all housed and maintained in a well-ventilated room at 22 ± 2 °C, with automatic light cycles (12 h light/dark) and all had free access to diet and drinking water ad libitum. Rats were housed in special polypropylene cages (Sodispan Research, Coslada, Madrid, Spain) that were manufactured with the lowest chemical composition of Makrolon, a polycarbonate with bisphenol A. Water bottles were made of glass.

4.2. Treatment and Experimental Design

Animals were randomly divided into three groups consisting of: (1) Control (non-treated) group—received chow with a corresponding concentration of corn oil (n = 10 females; n = 4 males); (2) bisphenol A (0.5 mg/kg chow) low-dose group—diet intake of 0.036 mg/kg body weight/day of BPA (n = 9 females; n = 4 males); and (3) bisphenol A (50 mg/kg chow) high-dose group—diet intake of 3.42 mg/kg body weight/day of BPA (n = 8 females; n = 4 males). High dose of BPA was chosen in the range of doses (2.5 mg/kg and 50 mg/kg) that consistently induced impairment learning and memory loss in rodents when administered in the perinatal period. While low dose was 100 times lower than that.

The dose ingested by each rat was calculated based on the food intake data per animal which corresponded to 7.3% of body weight. BPA with purity >99% was purchased from Sigma Aldrich (Argovia, Switzerland) (CAS number 80-05-7; article number: 239658). It was dissolved in ethanol and then corn oil at a ratio of 10% ethanol and 90% corn oil. The chosen chow was purchased from Granovit (Argovia, Switzerland) corresponding to a diet of natural ingredients low in phytoestrogens (rather restricted concentrations so that the estrogenic effects were weak) (Granovit AG, Kaiseraugst, KLIBA NAFAG 3317.PX.L15). Rats were bred in special polypropylene cages (Sodispan Research, Coslada, Madrid, Spain) and glass drinking bottles were used to avoid the presence of substances that could also act as endocrine disruptors. A cylindrical environmental enrichment element was included.

During the entire experiment (premating, mating, pregnancy, lactation), the control group cages were kept separate from the BPA-treated groups, to avoid any chance of spreading chow containing BPA. During premating, female and male rats were treated with the diet with their corresponding dose of BPA for two weeks. Control animals received the control diet. Mating phase took place between a male and a female from the same group, after checking that the female was in the estrus phase. The following morning, a check for sperm-positive vaginal smear or sperm-plug was carried out and the process was repeated all mornings for one week. Treatment was maintained during pregnancy. After birth, the lactating dams were kept in individual cages with their offspring and dietary treatment continued until PND6. During the entire period, body weight of females was recorded every 3–4 days and clinical observations were made daily. Furthermore, food consumption (FC) was calculated by weighing the food administered (FA) on the previous day and subtracting weight of food remaining (FR) (FC: FA-FR) during the second week of premating and second week of pregnancy. With 2 rats per cage to avoid isolation stress, we divided the total quantity consumed in the cage by 2 (FC: (FA-FR)/2). In addition to counting the number of pups on the day of birth, the number of dead pups was recorded and the sex ratio and pup weight on PND6 were identified. Lactating dams were sacrificed by decapitation using a guillotine. Female offspring were sacrificed at postnatal day 6 (PND6) by decapitation using scissors. The livers were collected and immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Plasma samples were collected from the lactating dams and stored at −20 °C (Figure 11).

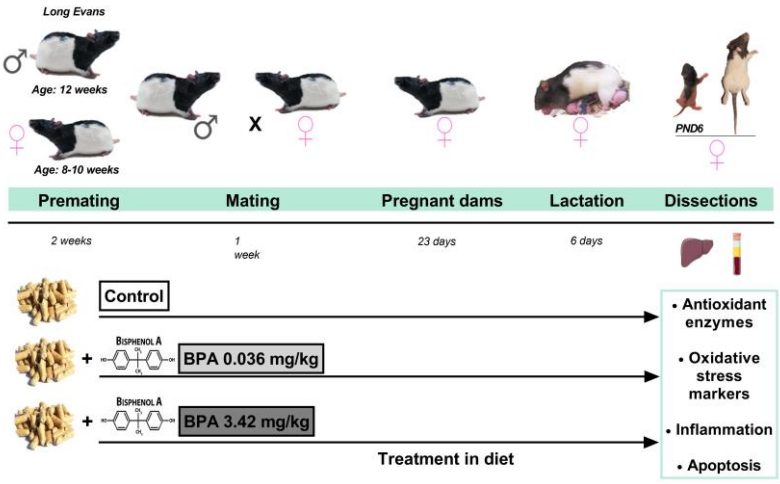

Figure 11. Experimental design. The diet of the parental generation (F0) was different according to the experimental group (control, BPA 0.036 mg/kg and BPA 3.42 mg/kg). During the entire experiment (premating, mating, pregnancy, lactation), the treatment was maintained until the time of dissection in dams and pups to postnatal day 6 (PND6). The influence of lactating dams and perinatal exposure to BPA on livers and its possible mechanism in offspring were studied. Figure created with Prism v7 (GraphPad Software Inc., La Jolla, CA, USA).

4.3. Activities of Antioxidant Enzymes and Glutathione Concentrations

4.3.1. Antioxidant Enzymes

Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) activities were measured in the liver homogenate previously lysed with the corresponding buffer and analyzed spectrophotometrically according to the manufacturer's instructions (Cayman Chemical; Ann Arbor, MI, USA). CAT activity was determined by the reaction with methanol in the presence of an optimal concentration of hydrogen peroxide (H_2O_2). The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen at 540 nm. SOD activity was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine. The standard curve generated using this enzyme provides a means to accurately quantify the activity of all three types of SOD (Cu/Zn, Mn and FeSOD). GPx activity was measured spectrophotometrically; it was coupled to the oxidation of NADPH by GR. A GR assay kit was used to measure activity of this enzyme by quantifying the rate of NADPH oxidation. GST activity was determined spectrophotometrically by measuring formation of the conjugate of reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm. Each sample was tested in triplicate. Enzyme activities were normalized according to liver protein content and were expressed as nmol/min/mg of protein except for SOD, which was expressed in U/mg of protein.

4.3.2. Glutathione Concentrations

Liver was homogenized in 50 mM phosphate buffer and 0.1 M EDTA, pH 8. Then, 10 µL of $HClO_4$ was added per mL of homogenate and supernatants were used for the quantification of both reduced (GSH) and oxidized (GSSG) glutathione by o-phthalaldehyde (OPT) at pH 12 and pH 8, respectively, resulting in the formation of a fluorescent compound. Fluorescence was measured at 350 nm excitation and 420 nm emission. Results were expressed as nmol of GSSH and GSH per milligram of protein. Moreover, the GSSG/GSH ratio was calculated for each sample.

4.4. Oxidative Stress Markers

4.4.1. Lipid Peroxidation Assay

Quantification of lipid peroxidation (LPO) was carried out in liver homogenate according to the manufacturer's instructions (Cayman Chemical; Ann Arbor, MI, USA). Lipid Hydroperoxide Assay Kit measures the hydroperoxides directly utilizing the redox reactions with ferrous ions. The amount of lipid hydroperoxide was obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample. LPO content was expressed as nmol/mg of tissue. This procedure eliminates any interference caused by hydrogen peroxide or endogenous ferric ions in the sample and provides a more sensitive and reliable assay for lipid peroxidation.

4.4.2. Thiobarbituric Acid Reactive Substances (TBARS) Assay

Lipid peroxidation was also evaluated using a commercial kit (BioVision, Mountain View, CA, USA), which measures the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) and the MDA-TBA adduct formation. Samples were resuspended in lysis buffer with the antioxidant butylated hydroxy-toluene (BHT) (0.1 mM) to prevent further formation of MDA during the preparation of the sample or during the heating step. Then, they were centrifuged at 3200× *g* for 30 min. Furthermore, 200 µL of supernatants from each sample were added to 600 µL TBA, and incubated at 95 °C for 60 min. Samples were cooled in ice for 10 min, and 300 µL of n-butanol were added (Sigma-Aldrich, Madrid, Spain) to create an organic phase in which the MDA molecules were to be placed. Samples were centrifuged and 200 µL of upper organic phase were collected and dispensed into a 96-well microplate for spectrophotometric measurement at 532 nm. Results were expressed as nmol TBARS/mg protein.

4.4.3. Adenosine Triphosphate Determination

The adenosine triphosphate (ATP) levels of liver tissue were determined using a colorimetric/fluorometric assay kit (Bio Vision, Milpitas, CA, USA) according to the manufacturer's instructions. For the assay, 50 mg of liver tissue was used and the ATP content was calculated and expressed as nmol/mg of protein.

4.4.4. Determination of Nitric Oxide Metabolites (NOx)

Levels of nitric oxide metabolites (NO_x) in plasma samples were measured by the Griess reaction as nitrite ion (NO_2^-) concentration after nitrate (NO_3) reduction to NO_2^-. Briefly, after incubation of the plasma with *Escherichia coli* NO_3 reductase and $NADPH^+$ (37 °C, 30 min), 300 µL of Griess reagent (0.5% naphthylenediamine dihydrochloride, 5% sulfonilamide, 25% phosphoric acid (H_3PO_4)) (Sigma-Aldrich, Saint Louis, MO, USA) was added. The reaction was performed at 22 °C for 20 min, and the absorbance at 546 nm was measured, using sodium nitrite ($NaNO_2$) solution as standard. Results were expressed as nmol/µL of plasma.

4.4.5. DNA Oxidative Damage Measurement

Oxidative DNA damage was assessed by means of an ELISA kit consisting of a competitive assay for the quantitative measurement of 8-hydroxyguanosine (8-OHdG) (Cell Biolabs, Inc., San Diego, CA, USA). The unknown 8-OHG samples or 8-OHdG standards were first added to an 8-OHdG/BSA conjugate preabsorbed microplate. After a brief incubation, an anti-8-OHdG monoclonal antibody was added, followed by an HRP conjugated secondary antibody. The 8-OHdG content in unknown samples was determined by comparison with a predetermined 8-OHG standard curve. Finally, results were expressed as ng/mg DNA.

4.5. Determination of Protein Concentration

The protein content of the same samples was evaluated following a bicinchoninic acid protein assay kit protocol (Sigma-Aldrich, Madrid, Spain) or by BCA Assay Pierce (Bio-Rad Laboratories, Hercules, CA, USA) using a BSA standard curve.

4.6. RNA Isolation and RT-PCR Quantification

mRNA expression of GPx, GR, GST, γ-glutamylcysteine synthetase (γGCS), heme oxygenase 1 (HO-1d), inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), interleukin-1-β (IL-1β), apoptosis-inducing factor (AIF) and Bcl-2-associated X protein (BAX) was measured using real time qRT-PCR. RNA was isolated from liver samples according to the method described by Chomczynski [72] using the TRI Reagent Kit (Molecular Research Center, Inc., Cincinnati, OH, USA) following the manufacturer's protocol. The purity of the RNA was estimated by 1% agarose gel electrophoresis, and RNA concentrations and ratio 260/280 were determined by spectrophotometry BioDrop (Fisher scientific, Waltham, MA, USA). Reverse transcription of 2 mg of RNA for cDNA synthesis was performed using the StaRT Reverse Transcription Kit (AnyGenes, Paris, France). qRT-PCR was performed using a 7500 Fast Real Time PCR System thermal cycler (Applied Biosystems, Cambridge, MA, USA) with the TB Green Ex Taq (Tli RNase H Plus) (Takara Bio Inc., Shiga, Japan) and 300 nM concentrations of specific primers (Table 1). The qPCR amplification cycles were a 95 °C 10 min cycle, followed by 45 cycles at 95 °C for 10 s and at 60 °C for 30 s and finally melting curve analysis, following the recommendations of the manufacturer (95 °C for 10 s, 65 °C for 30 s and 95 °C for 0 s). Amplification of 18S mRNA was used as a loading control for each sample. The gene expression level was analyzed in triplicate for each sample. Relative changes in mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method [73].

Table 1. Primer sequences for quantitative real-time PCR.

Target Gen	Forward (5′-3′)	Reverse (5′-3′)
GPx	CAGTTCGGACATCAGGAGAAT	AGAGCGGGTGAGCCTTCT
GR	GGGCAAAGAAGATTCCAGGTT	GGACGGCTTCATCTTCAGTGA
GST	TTGAGGCACCTGGGTCGCTCTTTAG	GGTTCTGGGACAGCAGGGTCTCAAA
γ-GCS	ATCTGGATGATGCCAACGAGTC	CCTCCATTGGTCGGAACTCTACT
HO-1d	GTCAAGCACAGGGTGACAGA	ATCACCTGCAGCTCCTCAAA
iNOS	CTTTGCCACGGACGAGAC	TCATTGTACTCTGAGGGCTGAC
eNOS	CCAGTGCCCTGCTTCATC	GCAGGGCAAGTTAGGATCAG
IL-1β	TGTGATGAAAGACGGCACAC	CTTCTTCTTTGGGTATTGTTTGG
AIF	AGTCGTTATTGTGGGGTTATCAAC	TTGGTCTTATTTAATAGTCTTGTAGGC
BAX	GTGAGCGGCTGCTTGTCT	GTCCCGAAGTAGGAGAGGA
18S	GGTGCATGGCCGTTCTTA	TCGTTCGTTATCGGAATTAAC

4.7. Protein Extraction and Western Blot Analysis

Western blotting was used to measure levels of HO-1d, iNOS, eNOS, IL-1β, AIF, Bcl-2 and B-cell lymphoma-extra large (BCL-XL). Briefly, liver samples, after homogenization with modified RIPA lysis buffer (PBS, Igepal, Sodium deoxycholate (D5670-5G), 10% SDS, PMSF, 0.5 M EDTA and 100 mM EGTA) to which protease inhibitor cocktail (#P-2714) (Sigma-Aldrich, Madrid, Spain), PMSF (#P7626, 1 mM), sodium orthovanadate (#S6506, 2 mM) and sodium pyrophosphate (#S6422, 20 mM) were added, were sonicated and boiled for 10 min at 100 °C in the ratio 1:1 with gel-loading buffer (100 mmol/L TrisHCl (pH 6.8), 4% SDS, 20% glycerol, bromophenol blue 0.1, 200 mmol/L dithiothreitol). Total protein equivalents (25 μg) for each sample were separated by SDS-PAGE by using 10% Mini-PROTEAN TGX Precast acrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and were transferred onto a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, CA, USA). The membrane was immediately placed into blocking buffer containing 5% non-fat milk in 20 mM Tris pH 7.5, 150 mM NaCl and 0.01% Tween-20. The blot was allowed to block at 37 °C for 1 h. The membrane was incubated with a rabbit polyclonal antibody (1:1000) (Table 2) for 12 h at 4 °C, followed by incubation with a goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:7000). Protein detection was performed using the Clarity Western ECL Substrate assay kit (Bio-Rad Laboratories, CA, USA) and ECL Plus (Amersham Life Science Inc.,

Buckinghamshire, UK) by chemiluminescence with the BioRad ChemiDoc MP Imaging System to determine the relative optical densities. Prestained protein markers were used for molecular weight determinations. Housekeeping gene GAPDH was used as loading control (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were quantified using BioRad Image Lab software v6.1 (Bio-Rad Laboratories, Hercules, CA, USA).

Table 2. Source of primary antibodies.

Antibody	Catalog Number	Company
HO-1d	AB1284	Chemicon International, Temecula, CA, USA
iNOS	AB16311	Chemicon International, Temecula, CA, USA
eNOS	AB16301	Chemicon International, Temecula, CA, USA
IL-1β	500-P80	PeproTech EC, Ltd., London, UK
AIF	5318	Cell Signaling Technology, Beverly, MA, USA
BCL-2	2870	Cell Signaling Technology, Beverly, MA, USA
BCL-XL	21061	Signalway antibody, Collage Park, MD, USA
GAPDH	2118	Cell Signaling Technology, Beverly, MA, USA

4.8. Histological Staining

Liver tissues were washed in 0.9% cold saline and fixed in a 10% formalin buffer solution for the histopathological assessment for 24 h. After fixation, samples were processed for embedding in paraffin. Serial sections (5 μm) were prepared using a rotary microtome Leica RM2125 RTS (Leica Biosystems, Wetzlar, Germany) for hematoxylin and eosin staining (H&E). The sections were stained with 0.1% hematoxylin (Ciba, Basel, Switzerland) for 5 min. Then slides were washed with tap water for 15 min and then a quick wash with hydrochloric alcohol (0.5% HCl in absolute ethanol) to remove excess staining on the sample (differentiation). The acid was neutralized by immersing the sections in tap water for 5 min and a final wash with distilled water. They were immersed in 0.1% eosin (Ciba, Basel, Switzerland) for 5 min. After washing with distilled water, tissue sections were dehydrated using ascending ethanol passages and finishing in xylol for 30 s. Tissue sections were cover slipped. Images were captured with Leica Microscope (Leica Biosystems, Wetzlar, Germany).

4.9. Analysis of Hepatic Serum Markers

Evaluation of aspartate aminotransferase (AST), alanine aminotransferase (ALP) and gamma glutamyl transpeptidase (GGT) levels in dams' sera was determined by a veterinary laboratory (LAV Arturo Soria, Madrid, Spain) using a KONE sequential automatic autoanalyzer (Kemia Científica, Madrid, Spain).

4.10. Statistical Analysis

Differences between obtained values (mean ± SD) were assessed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test or Bonferroni post-test to compare all pairs of means after testing for normal distribution. A confidence level of 95% ($p < 0.05$) was considered statistically significant. Statistics were calculated using Prism v7 (GraphPad Software Inc., La Jolla, CA, USA).

5. Conclusions

Bisphenol A is a molecule capable of producing estrogenic effects and its continued exposure at low doses is unavoidable. It produces adverse effects on the body, including the liver, the main organ in charge of detoxifying the organism. In this study, it was observed that exposure of female Long–Evans rats to low doses of BPA during pregnancy and lactation increased the levels of oxidative stress in the liver, decreasing antioxidant activities and the glutathione system. This loss of homeostasis generated by the excessive

accumulation of ROS and NOS caused an increase in inflammation, triggering cellular apoptosis pathways. The effect of perinatal BPA exposure on female offspring was also studied at PND6 and shows similar effects as found in the dams. Although alterations were observed at both doses of BPA, the maximum effect occurred with the low dose of BPA, resulting in an inverse dose–response relationship. We consider that this is especially important since in our everyday life we are constantly exposed to low doses of BPA, with it being present in many commonly used products.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethical Committee of the Complutense University of Madrid (Madrid, Spain) in accordance with the Guidelines for Ethical Care of Experimental Animals of the European Union (2010/63/UE). The project complies with the provisions of Government of Spain Royal Decree 53/2013 of February 1 establishing the basic rules applicable for the protection of animals used in experimentation and other scientific purposes, including teaching. This research is within a European project entitled "Novel Testing Strategies for Endocrine Disruptors in the Context of Developmental NeuroToxicity" supported by the European Union's Horizon 2020 Research and Innovation Programme (ENDpoiNTs project; grant number: 825759).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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Publicación 2: **El estrés oxidativo aumenta en el hígado de ratas lactantes tras la exposición a dosis bajas de BPF: efectos perinatales en la descendencia**

El bisfenol F (BPF) está sustituyendo al bisfenol A (BPA) en la fabricación de productos debido a sus efectos de alteración endocrina. Los monómeros de BPF también pueden liberarse al medio ambiente y entrar en la cadena alimentaria, lo que provoca la exposición humana a dosis bajas. Dado que los bisfenoles se metabolizan principalmente en el hígado, este órgano es más vulnerable a dosis bajas de bisfenoles que otros. La exposición durante el desarrollo prenatal puede aumentar el riesgo de enfermedades en la edad adulta. El objetivo de este estudio fue evaluar si la administración de BPF podía generar estrés oxidativo en el hígado de ratas lactantes, y si estos efectos podían observarse también en las crías hembras y machos del día postnatal 6 (DPN6). Las ratas *Long Evans* recibieron tratamiento oral: control, dosis baja de BPF (LBPF) 0,0365 mg/kg peso corporal/día, y dosis alta de BPF (HBPF) 3,65 mg/kg peso corporal/día. Los niveles de enzimas antioxidantes CAT, SOD, GR, GPx y GST, el sistema de glutatión (GSH, GSSG) y los marcadores de daño lipídico MDA y LPO se midieron mediante métodos colorimétricos en el hígado de tanto ratas lactantes como en las crías en el DPN6. LBPF afectó a los mecanismos de defensa del hígado (enzimas antioxidantes y sistema del glutatión), aumentando los niveles de ROS y produciendo peroxidación lipídica en las ratas lactantes. Se encontraron efectos similares en las crías hembras y machos del DPN6 como consecuencia de la exposición perinatal.

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Oxidative stress increases in liver of lactating rats after BPF-low-dose exposure: perinatal effects in the offspring

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Bisphenol F (BPF) is replacing Bisphenol A (BPA) in the manufacture of products due to endocrine-disrupting effects. BPF monomers can also be released into the environment and enter the food chain, resulting in human exposure to low doses. Since bisphenols are primarily metabolized by the liver, this organ is more vulnerable to lower doses of bisphenols than others. Exposure during prenatal development may increase the risk of diseases in adulthood. The aim was to evaluate whether BPF administration could generate oxidative stress in liver of lactating rats, and whether these effects may be also observed in female and male postnatal day 6 (PND6) offspring. Long Evans rats received oral treatment: Control, BPF-low-dose (LBPF) 0.0365 mg/kg b.w./day, and BPF-high-dose (HBPF) 3.65 mg/kg b.w./day. The levels of antioxidant enzymes (CAT, SOD, GR, GPx and GST), glutathione system (GSH, GSSG) and lipid damage markers (MDA, LPO) were measured using colorimetric methods in liver of both lactating dams and in PND6 offspring. Mean values were analyzed using Prism-7. LBPF affected liver defense mechanisms (antioxidant enzymes and glutathione system), increasing ROS levels and producing lipid peroxidation in lactating dams. Similar effects were found in female and male PND6 offspring as a consequence of perinatal exposure.

Numerous scientific studies describe the toxic effects of Bisphenol A (BPA), an endocrine disruptor widely used in industry. Its exposure is associated with adverse health effects (cardiovascular, respiratory, diabetes, renal, obesity and reproductive disorders)[1–3] and its use is being restricted.

BPA is being replaced by allegedly safer analogues such as Bisphenol F (BPF, bis (4-hydroxyphenyl) methane). BPF is used in the manufacture of epoxy resins and coatings[4] and in polymers that give materials increased thickness and durability. It is found frequently in plastic, varnish, dental sealant and personal care products and office paper[5,6]. Its presence in food packaging production and cans consumed in everyday life is very relevant[7]. In addition, there is environmental contamination with BPF, as it is found in household dust and in surface waters, sediments and sewage effluents[5,6].

Exposure to this chemical occurs via three routes: oral, dermal and inhalation. BPF has been found in human urine samples in several European countries at concentrations comparable to BPA[8–10], in breast milk[11] and in serum[12]. It has been detected in human plasma at concentrations three times higher than BPA[13]. BPF is not only detected in adult tissues and fluids, but can also cross the blood–brain barrier and the placental barrier and reach the fetus[14].

The impact of exposure to bisphenols is crucial in early life, therefore, there is a need to investigate the effect not only on adult organisms, but also the perinatal effect on offspring.

Previous studies reported that BPF may have a similar toxicity and mechanism of action to BPA, due to their structural and physicochemical similarities[7,15,16]. Li et al.[17,18] have reported that loss of the antioxidant system leads to oxidative damage in the liver. Exposure to BPA is known to impair the antioxidant defense system, increases lipid peroxidation and causes oxidative damage to the liver[19,20]. Recent studies by our research group showed that low dose of BPA caused liver injury in lactating dams and had a perinatal effect in female PND6 offspring by increasing oxidative stress levels, triggering an inflammatory response and apoptosis pathways in

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the liver, the organ responsible for detoxification of this endocrine disruptor[21]. However, the mechanisms and effects of BPF on oxidative stress in the liver remain unclear.

The aim of this study was to evaluate the effects after exposure to two doses of BPF on oxidative stress: antioxidant enzymes, glutathione system and indicators of lipid damage in the liver of lactating dams. Moreover, it was studied whether this effect can also be observed in the liver of female and male offspring at postnatal day 6 (PND6).

Results

The liver has an endogenous antioxidant defense system to prevent cell damage, consisting of antioxidant enzymes and the glutathione system.

When lactating females were treated with LBPF, all antioxidant enzyme activities (CAT, SOD, GPx, GR and GST) were significantly decreased as compared to the control group (Figs. 1a–e respectively). No significant changes were shown in antioxidant enzymes in HBPF-treated dams.

Regarding glutathione concentrations, a decrease in reduced glutathione (GSH) concentration was observed in LBPF-treated dams (Fig. 1f). GSSG concentration increased significantly in LBPF-treated dams as compared to the control group and significant differences were also observed between both treatment groups, resulting in higher levels of oxidative stress in the LBPF group (Fig. 1g). The same was observed for the GSSG/GSH ratio, as a marker of oxidative stress, indicating a more marked redox imbalance after LBPF administration (Fig. 1h).

Oxidative stress produces free radicals that can easily react with cell membrane lipids, triggering the production of lipid peroxides. Also, a significant increase in MDA and LPO levels, two products used to measure oxidative lipid damage, was observed after LBPF administration in lactating dams (Fig. 1i and j).

To study whether perinatal administration of BPF was able to generate alterations of the oxidant/antioxidant balance, we evaluated the same parameters in the liver of female and male offspring.

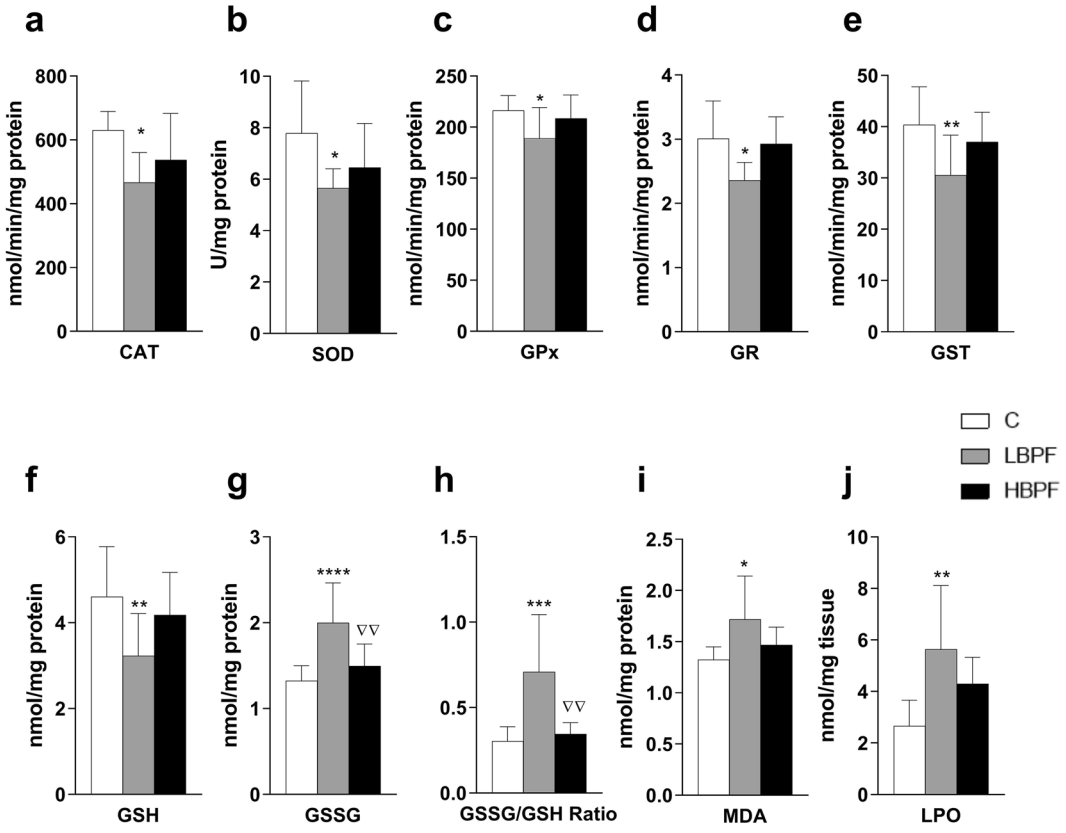

Figure 1. Effects of BPF administration on antioxidant enzymes, glutathione concentrations and oxidative stress biomarkers in liver from lactating dams. (**a**) Enzymatic activity of catalase (CAT) in nmol/min/mg protein; (**b**) Superoxide dismutase (SOD) in U/mg protein; (**c**) Glutathione peroxidase (GPx) in nmol/min/mg protein; (**d**) Glutathione reductase (GR) in nmol/min/mg protein; and (**e**) Glutathione S-transferase (GST) in nmol/min/mg protein. (**f**) Concentration of reduced glutathione (GSH) in nmol/mg protein; (**g**) Concentration of oxidized glutathione (GSSG) in nmol/mg protein. (**h**) GSSG/GSH ratio. (**i**) Malondialdehyde (MDA) content in nmol/mg protein. j) Lipid hydroperoxide (LPO) content in nmol/mg tissue. Data represent mean ± SD. n = 6 lactating control dams; n = 6 lactating LBPF dams; n = 10 lactating HBPF dams (two replicates for each sample). Statistical significance was determined by one-way ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ compared to Control group. ∇∇$P < 0.01$, LBPF vs. HBPF.

When female PND6 offspring were perinatally exposed to LBPF, all antioxidant enzyme activities (CAT, SOD, GPx, GR and GST) were significantly decreased as compared to the control group (Fig. 2a–e). Significant changes were observed between both treatment groups with respect to the activities of the antioxidant enzymes SOD, GR and GST, with the levels in LBPF-exposed female offspring significantly lower than those observed in the HBPF group (Fig. 2b, d and e respectively).

Similar effects were found in male offspring exposed perinatally to BPF, i.e., a decrease in the activity of all antioxidant enzymes (CAT, SOD, GPx, GR and GST) (Fig. 2a–e). Regarding CAT, GPx and GR levels, significant changes were observed between both treatment groups, being the levels in LBPF-exposed male offspring significantly decreased as compared to the HBPF-exposed group (Fig. 2a, c and d). There was also a decrease in SOD levels in comparison to the control group in male offspring after perinatal exposure to HBPF (Fig. 2b).

Regarding the glutathione system, a decrease in reduced glutathione (GSH) concentration (Fig. 3a) and an increase in oxidized glutathione (GSSG) concentration (Fig. 3b) were observed in LBPF-exposed female offspring. An increase in the GSSG/GSH ratio in LBPF-exposed female offspring was also observed (Fig. 3c). These three parameters showed significant differences between LBPF and HBPF treatments, with more noticeable effects in the LBPF group. There was also an increase in GSSG levels in female offspring after perinatal exposure to HBPF compared to the control group (Fig. 3b); with no significant changes in GSH levels.

When PND6 male offspring were perinatally exposed to LBPF, a decrease in GSH levels, an increase in GSSG and thus an increment in the GSSG/GSH ratio was observed compared to the control group (Fig. 3a–c). When comparing both sexes, it was noteworthy that the GSSG/GSH ratio, a marker of oxidative stress, was much higher in female offspring; therefore, female offspring showed higher levels of GSSG and thus a greater imbalance in the glutathione system (Fig. 3c). This elevation was also observed in LBPF-treated dams (Fig. 1h).

Decreased levels of antioxidant enzymes and alteration of the glutathione system increased MDA and LPO levels in both LBPF-exposed female and male offspring (Fig. 3d and e). In addition, there were significant differences in MDA levels between LBPF- and HBPF-exposed females, with higher levels after LBPF exposure (Fig. 3d).

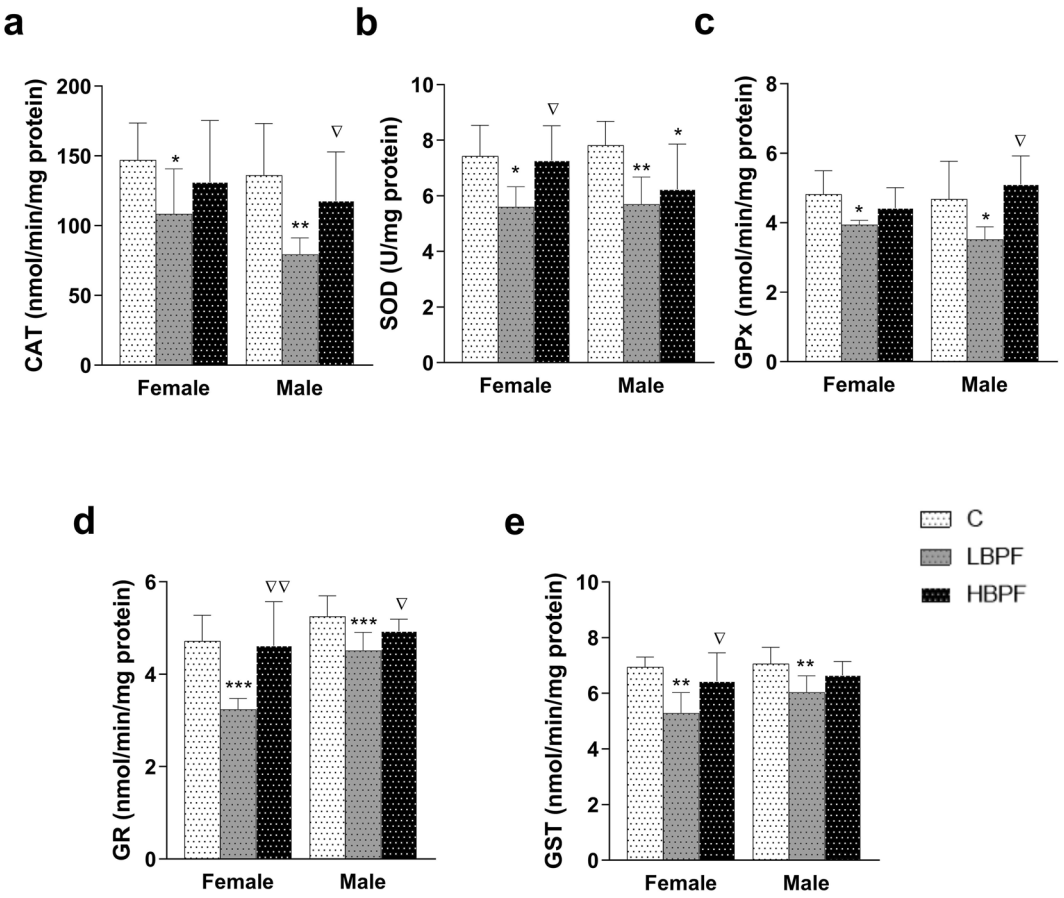

Figure 2. Effects of BPF administration on antioxidant enzymes in liver from female and male PND6 offspring. (**a**) Enzymatic activity of catalase (CAT) in nmol/min/mg protein; (**b**) Superoxide dismutase (SOD) in U/mg protein; (**c**) Glutathione peroxidase (GPx) in nmol/min/mg protein; d) Glutathione reductase (GR) in nmol/min/mg protein; and (**e**) Glutathione S-transferase (GST) in nmol/min/mg protein. Data represent mean ± SD. n = 12 female PND6 pups; n = 12 male PND6 pups for each experimental group with two replicates for each sample (control, LBPF and HBPF). Statistical significance was determined by one-way ANOVA. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to Control group. $\nabla P < 0.05$; $\nabla\nabla P < 0.01$, LBPF vs. HBPF.

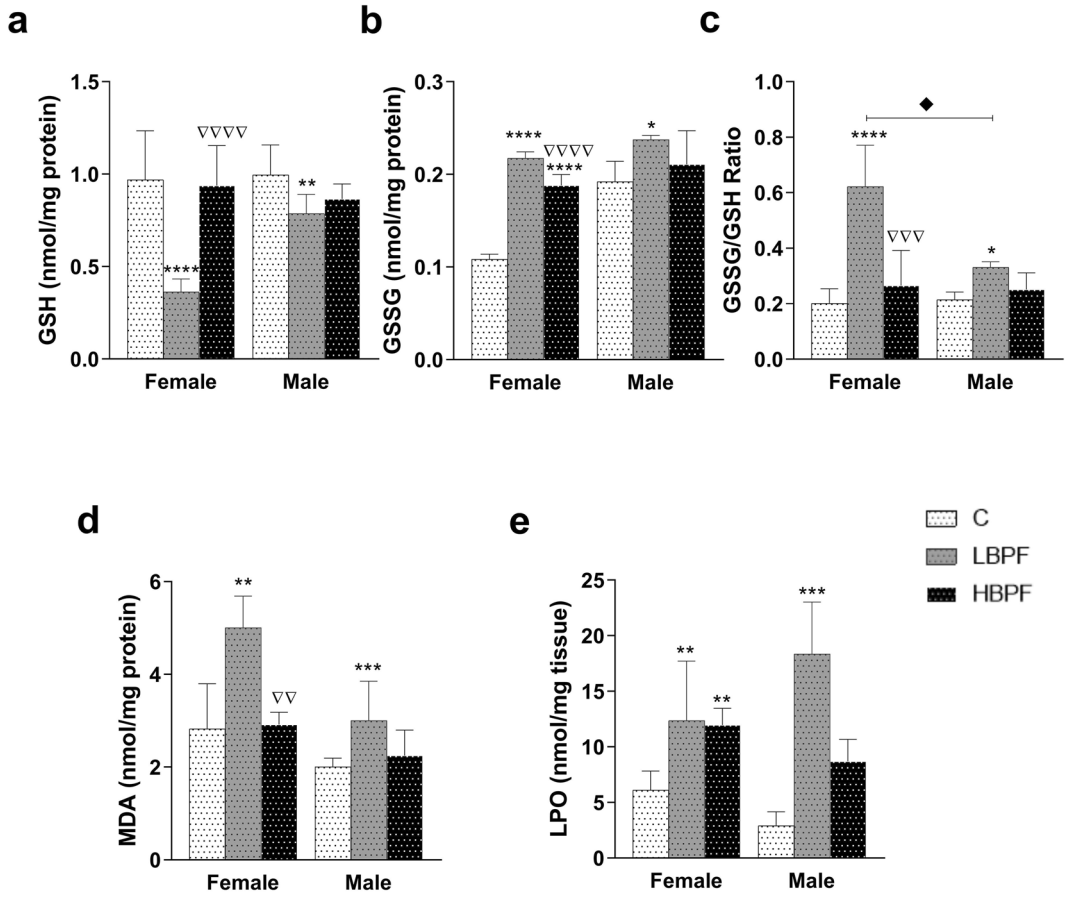

Figure 3. Effects of BPF administration on glutathione concentrations and oxidative stress biomarkers in liver from female and male PND6 offspring. (**a**) Concentration of reduced glutathione (GSH) in nmol/mg protein. b) Concentration of oxidized glutathione (GSSG) in nmol/mg protein. (**c**) GSSG/GSH ratio. (**d**) Malondialdehyde (MDA) content in nmol/mg protein. e) Lipid hydroperoxide (LPO) content in nmol/mg tissue. Data represent mean ± SD. n = 12 female PND6 pups; n = 12 male PND6 pups for each experimental group with two replicates for each sample (control, LBPF and HBPF). Statistical significance was determined by one-way ANOVA. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$ compared to Control group. $\nabla\nabla P<0.01$; $\nabla\nabla\nabla P<0.001$, $\nabla\nabla\nabla\nabla P<0.0001$, LBPF vs. HBPF. ◆ LBPF Female vs. LBPF Male.

After perinatal exposure to HBPF, there was also an increase in LPO levels in female offspring as compared to the control group (Fig. 3e).

Discussion

Due to the widespread use of BPF as an alternative to BPA, residues of BPF can migrate into food and BPF monomers have been found in canned foods and soft drinks with an average concentration of 0.18 mg/dL[4,22,23]. In a previous study with female Sprague–Dawley rats that were gavaged with a single dose of BPF (7 and 100 mg/kg/b.w.), it was shown that excretion of BPF residues occurred mainly in urine via BPF-conjugated sulfate. However, BPF residues were detectable in all tissues examined at 96 h, with the highest amounts found in the liver (0.5% of the dose). The liver is an essential organ for detoxification and metabolism, being more especially vulnerable to damage[14].

For this reason, in this study the effects of BPF administered by the oral route at two concentrations: low dose of 0.0365 mg/kg b.w./day (LBPF) and high dose of 3.65 mg/kg b.w./day (HBPF) in the liver of lactating dams were evaluated. Also, the perinatal effect in female and male PND6 offspring after BPF exposure was studied.

Oxidative stress represents the imbalance in the production and removal of reactive oxygen species (ROS) and can be considered a pathological mechanism that contributes to the initiation and development of liver injury. To respond to oxidative stress under physiological conditions, the liver has both an enzymatic antioxidant system and the glutathione system[17].

The first line of defense against cell attack by ROS are the enzymes CAT and SOD. SOD carries out the dismutation of superoxide anion radicals into hydrogen peroxide and molecular oxygen. This hydrogen peroxide is degraded by CAT to form water and oxygen allowing the unsaturated fatty acids of the cell membrane to be protected from peroxidation. In the present study, a decrease in the enzymatic activity of CAT and SOD after administration of LBPF in lactating dams was observed[24].

GSH is an antioxidant molecule that metabolizes and detoxifies xenobiotics that are directly conjugated to protect the cells from oxidative damage. Reduced GSH levels were found after exposure to LBPF-treated dams. This GSH is used as a cofactor by the antioxidant enzyme GPx which catalyzes the degradation of hydroperoxides into hydroxyl compounds. The activity of GPx is closely related to its cofactor, GSH, and after observing GSH depletion, it can lead to a decrease in the activity of this enzyme in liver cells, as observed the present investigation. Oxidized glutathione (GSSG), produced after reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. In this study, we observed a decrease in the enzymatic activity of GR and an excess of GSSG concentration, more noticeable after administration of LBPF as compared to the control and HBPF groups in lactating dams[19,25]. GST is a phase II detoxifying enzyme with a critical role in cellular protection against ROS and toxic xenobiotics; it catalyzes the conjugation of GSH in reaction to endogenous and exogenous electrophiles. The activity of GST was decreased in LBPF-treated dams, impairing the correct elimination of this xenobiotic in the liver[19,26].

Therefore, in our study, LBPF caused oxidative damage in liver cells resulting in decreased antioxidant enzyme activities and alteration of the glutathione system. This was manifested by an increased GSSG/GSH ratio, a marker of oxidative stress. Also, this ratio was also statistically significant after comparison of both doses of BPF, with higher levels implying more intense actions of LBPF found in the liver of lactating dams.

Increased ROS also acts on macromolecules such as polyunsaturated fatty acids to initiate lipid peroxidation, as well as changing cell membrane fluidity and permeability. In our results, reduced antioxidant enzyme activity contributed to increased levels of MDA and LPO, two end products of lipid peroxidation used to monitor cell membrane damage in the liver of LBPF-treated dams[27].

Our results are consistent with previous research following in vivo and in vitro BPF exposure, in liver and other organs, using different animal species. The cytotoxic effects of seven concentrations of BPF during 24 h were studied on hepatocytes isolated from the liver of rainbow trout. CAT activity was decreased with all BPF concentrations. Also, GSH content was reduced with the highest concentration of BPF, and MDA content was increased significantly at BPF concentrations between 15.63 and 250 μM[19]. In addition, BPF exposure induced NAFLD-like changes (non-alcoholic fatty liver disease), with obvious lipid droplet deposition, triglyceride (TG) and fatty acid increases in mouse livers administered with BPF (50 mg/kg/day) for 30 days via subcutaneous injection[28]. In the study by Higashira et al.[29], carried out in rats orally gavaged with different concentrations of BPF (0, 20, 100, and 500 mg/kg per day) during 28 days, BPF caused liver toxicity based on clinical biochemical parameters and liver weight, but without histopathological changes. In a recent study by Sun et al.[30], they indicated altered liver function by BPF exposure in male mice following increased hepatic ALT and increased plasma AST levels after low doses of BPF. After exposure to BPF at 100 mg/kg in the reproductive tissues of male rats, CAT and SOD activities decreased and increased ROS levels and lipid peroxidation[27]. Maćczak et al.[31] found depleted GSH levels in human erythrocytes exposed to BPF and increased levels of lipid peroxidation. In vitro study on KGN cells, showed that BPF exhibited slight toxic effects and increased the damage to biomacromolecules (MDA, 8-OHdG) after BPF exposure[32]. Furthermore, the exposure of BPF resulted in higher MDA contents in the larvae of zebrafish and finally led to apoptosis[33]. Furthermore, previous studies reported that ROS levels vary significantly depending on the cell type and hormone receptor status of the cells. Lei et al.[34] showed that low doses of BPF elevates ROS levels, induces cell proliferation, and exerts estrogenic activity by interactions between ERα and GPER1 pathways.

The alteration of homeostasis (oxidant/antioxidant imbalance and cell membrane damage) can lead to potentially severe or permanent effects, especially if exposure occurs during specially sensitive periods of life such as fetal development, infancy and puberty[35].

Our results in offspring showed a significant reduction of all antioxidant enzymes (CAT, SOD, GPx, GR and GST) in both sexes after LBPF perinatal exposure. Regarding the glutathione system, there was a much more drastic reduction of GSH content in female offspring, leading to a higher GSSG/GSH ratio compared to male offspring. Thus, female offspring showed higher levels of GSSG and thus a greater imbalance in the glutathione system.

Lipid membrane damage occurred in both sexes and levels varied depending on whether measured directly (LPO) or indirectly (MDA, TBARs). HBPF exposure effects on all these parameters were not as noticeable, except for decreased SOD activity in males.

One of the previous studies showed that perinatal exposure to BPF was associated with oxidative damage and metabolic disorders in livers of male mouse offspring. Pregnant mice (F0) were orally gavaged with BPF (100 ng/g b.w./day) from gestational day (GD) 7 to postnatal day (PND) 21. Male offspring in each treatment group were provided a normal diet for 10 weeks after weaning at day 21. Nevertheless, BPF exposure significantly reduced CAT and GSH levels, suggesting disturbances in the antioxidant defense system. Moreover, BPF exposure led to metabolic disorders in the liver due to changes observed in the levels of 8 key metabolites[20] and lipid accumulation inducing liver damage after perinatal exposure to BPF. Musachio et al.[36] found sex-specific changes in oxidative stress parameters in *Drosophila melanogaster* after BPF exposure for seven days. Female flies were more susceptible to oxidative cell damage and reduced its longevity. Male flies had higher antioxidant defenses that responded primarily to lower concentrations of BPF, minimizing oxidative cell damage. Perinatal exposure to BPF and analogues affected the body weight of certain organs in male rat offspring and the ovarian function in females. Therefore, BPF can induce reproductive toxicity at low doses[37]. There are few studies in the literature showing BPF effects on offspring, but there is an absolute need to study exposure occurring during early development. The prenatal period is a critical window[38], where exposure to exogenous compounds such as BPF can affect fetal development. The fetus is extremely vulnerable with limited capacity to metabolize and process such chemicals[39]. In this sense, hepatic UGT2B1 (UDP-glucuronosyltransferase) activity towards BPF in rats is very weak in the fetus and newborn pups[40]. Perinatal exposure and placental transfer (in rats occurs

in late gestation)[14] may also result in developmental tissue changes that contribute to adverse health outcomes in adulthood[41].

When assessing the adverse effects of bisphenols and analogues, adult organisms and the perinatal effect on offspring have to be taken into account, as they may be affected differently due to different time windows and vulnerability. In turn, when studying the effect on both sexes, even under similar exposure conditions, results in differences due to variability in metabolism, storage and excretion of xenobiotics[42]. It is also important to study different animal models; for example, studies in rodents observed a higher vulnerability to the effects of BPA and other analogues in females than in males[43,44]. Furthermore, the effect at different doses is also important. Thus, according to Vanderberg et al.[45], more intense low dose effects are common in studies of natural hormones and endocrine disruptors. When non-monotonic dose–response curves are produced, low-dose effects cannot be predicted from the effects observed at high doses. In our results, the maximum effects on antioxidant enzyme involvement, glutathione system and cell membrane damage were observed after exposure to LBPF both in lactating dams and in offspring. This could be explained by the endocrine system responding to very low concentrations of hormones, allowing a maximal biological response without high receptor occupancy of this response or, alternatively, that response mechanisms become saturated before all receptors are occupied. Although the process is very difficult to interpret, the fact is that it has been found by many other authors[46–48] showing a non-monotonic dose–response manner.

The fact that low doses of BPF produce more noticeable effects than high doses highlights the need for further research to really elucidate the effects of low and environmentally relevant doses both in adulthood and after perinatal exposure to this chemical.

Conclusions

BPF is one of the most widely used alternatives to BPA in everyday plastic products, resulting that the population is submitted to a constant exposure to low doses of this chemical. In this study, it was observed that exposure to low doses of BPF in female Long Evans rats during pregnancy and lactation increased oxidative stress levels by decreasing the activity of antioxidant enzymes and altering the glutathione system in the liver, the organ responsible for detoxification. This excess of ROS affected cell membranes of hepatocytes, increasing the levels of lipid peroxidation. Similar effects in lactating dams were found in female and male offspring after BPF perinatal exposure. The period of exposure is very important because even if it occurs during the fetal or neonatal period, the effects can influence tissue development and affect adult life. Regardless, further research is needed to elucidate the health risk of BPF exposure in adult life and in offspring.

Methods

Chemical and animals. BPF with purity > 99% was purchased from Sigma Aldrich (Switzerland) (CAS Number 620–92-8; article number: 239658). It was dissolved in ethanol and then corn oil at a ratio of 10% ethanol and 90% corn oil. The chosen chow corresponds to a diet with natural ingredients low in phytoestrogens and was purchased from Granovit (Granovit AG, Kaiseraugust, Switzerland).

Thirty-six female (eight weeks of age) and eighteen male (ten weeks of age) Long Evans rats (Janvier Labs, Le Genest-Saint-Isle, France) were housed and maintained in a well-ventilated room at 22 ± 2 °C, with automatic light cycles (12-h light/dark) and all had free access to diet and drinking water ad libitum. Rats were housed in special polypropylene cages (Sodispan Research, Coslada, Madrid) and water bottles were made of glass. A cylindrical environmental enrichment element was included.

The study was approved by the Ethical Committee of Complutense University of Madrid (Madrid, Spain) in accordance with the Guidelines for Ethical Care of Experimental Animals of the European Union (2010/63/UE). This research is within a European project entitled "Novel Testing Strategies for Endocrine Disruptors in the Context of Developmental NeuroToxicity" supported by the European Union's Horizon 2020 Research and Innovation Programme (ENDpoiNTs project; grant number: 825759). All authors complied with the ARRIVE guidelines.

Experimental design. Animals were randomly divided into three groups: (1) Control (non-treated) group, received chow with a corresponding concentration of corn oil (n = 12 females; n = 6 males) which is the solvent used for the inclusion of the two Bisphenol F dosages in the other groups; (2) Bisphenol F, low dose group (LBPF)—diet intake of 0.0365 mg/kg body weight/day of BPF (n = 12 females; n = 6 males); (3) Bisphenol F, high dose group (HBPF)—diet intake of 3.65 mg/kg body weight/day of BPF (n = 12 females; n = 6 males). The doses of BPF used were chosen according to previous studies on BPA[21,49] and the large existing literature, where the dose range of BPA (2.5 mg/kg—50 mg/kg) induced impairment learning and memory loss in rodents when BPA was administered in the perinatal period. Thus, the high dose is 3.65 mg/kg higher than 2.5 mg/kg; while the low dose was 100 times lower, to investigate whether, even with such a small dose, any effects were observed.

During premating, female and male rats were treated with the diet with their corresponding dose of BPF for two weeks. Control animals received the control diet for the same time. Mating phase took place between a male and a female from the same group, after checking by vaginal smear that the female was in the estrus phase. The following morning, a check for sperm-positive vaginal smear or sperm-plug was carried out and the process was repeated all mornings for ten days. Dietary treatment was maintained during pregnancy. Six females were pregnant in control and LBPF groups and ten females were pregnant in HBPF group. After birth, the lactating dams were kept in individual cages with their offspring and dietary treatment continued until postnatal day 6 (PND6). PND6 was chosen because the hippocampus is in full development, and they are within the critical period of sexual differentiation of the brain, related to the brain studies on which the ENDpoiNTs project is focused. During the entire experiment (Premating, Mating, Pregnancy, Lactation), the control group cages were

kept separately from the BPF-treated groups, to avoid any chance of spreading chow containing BPF that could contaminate it.

Lactating dams were sacrificed by decapitation using a guillotine. Female and male offspring were sacrificed at PND6 by decapitation using scissors. The livers were collected and immediately frozen in liquid nitrogen and stored at -80 °C until analysis (Fig. 4).

Activities of antioxidant enzymes. Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) activities were measured in the liver homogenate previously lysed with the corresponding buffer and analyzed spectrophotometrically according to the manufacturer's instructions (Cayman Chemical; Ann Arbor, MI, USA).

CAT activity was determined by the reaction with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen at 540 nm.

SOD activity was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine. The standard curve generated using this enzyme provides a means to accurately quantify the activity of all three types of SOD (Cu/Zn, Mn, and FeSOD).

GPx activity was measured spectrophotometrically; it was coupled to the oxidation of NADPH by GR. Oxidized glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP + is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A340 is directly proportional to the GPx activity in the sample.

GR assay kit was used to measure activity of this enzyme by quantifying the rate of NADPH oxidation. The oxidation of NADPH to NADP + is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A340 is directly proportional to the GR activity in the sample.

GST activity was determined spectrophotometrically by measuring formation of the conjugate of reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm.

Each sample was tested in duplicate. Enzyme activities were normalized according to liver protein content and were expressed as nmol/min/mg of protein except for SOD which was expressed in U/mg of protein.

Glutathione concentrations. Liver was homogenized in phosphate buffer 50 mM and EDTA 0.1 M, pH 8. Then, 10 µl of $HClO_4$ was added per mL of homogenate and supernatants were used for the quantification of both reduced (GSH) and oxidized (GSSG) glutathione by o-phthalaldehyde (OPT) at pH 12 and pH 8, respectively, resulting in the formation of a fluorescent compound. Fluorescence was measured at 350 nm excitation and 420 nm emission. Results were expressed as nmol of GSSH and GSH per milligram of protein. Moreover, the GSSG/GSH ratio was calculated for each sample.

Lipid peroxidation determination. Quantification of lipid peroxidation (LPO) was carried out in liver homogenate according to the manufacturer's instructions (Cayman Chemical; Ann Arbor, MI, USA). Lipid

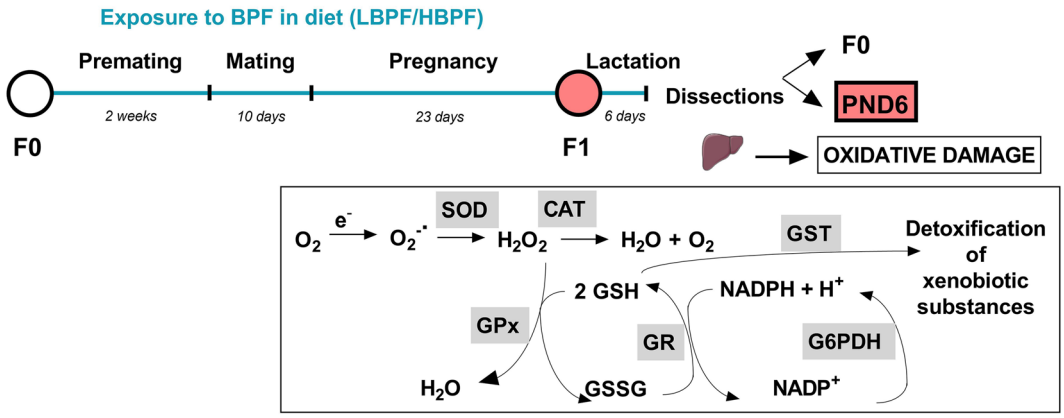

Figure 4. Experimental design. Female and male Long Evans rats (F0) were exposed to a low or high dose of BPF (LBPF or HBPF) or control diet from premating until the end of the experiment. Six days after birth and lactation of the pups, lactating dams (F0) and PND6 pups (females and males) were sacrificed to study the oxidant/antioxidant balance in the liver. Endogenous antioxidant agents include enzymes such as superoxide dismutase (SOD), which eliminates the first oxygen free radical (O_2) produced during oxygen utilization, the superoxide anion (O_2^{-}.); catalase (CAT), which is responsible for converting hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2); and the main enzymes involved in the glutathione system, glutathione peroxidase (GPx), which catalyzes the elimination of peroxides, such as hydrogen peroxide (H_2O_2), using reduced glutathione (GSH) and converting it to oxidized glutathione (GSSG), glutathione reductase (GR), which recomposes GSH so that it can be used by GPx, and also glutathione-S-transferase (GST), which catalyzes the conjugation of glutathione with xenobiotics, playing a role in the inactivation of free radicals. Figure created with Prism v7 (GraphPad Software, Inc, CA, USA).

Hydroperoxide Assay Kit measures the hydroperoxides directly utilizing the redox reactions with ferrous ions. The amount of lipid hydroperoxide was obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample. LPO content was expressed as nmol/mg of tissue. This procedure eliminates any interference caused by hydrogen peroxide or endogenous ferric ions in the sample and provides a more sensitive and reliable assay for lipid peroxidation.

Lipid peroxidation was also evaluated using the traditional Thiobarbituric acid reactive substances (TBARS) assay. The commercial kit (BioVision, Mountain View, CA, USA) measures the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) and the MDA-TBA adduct formation. Samples were resuspended in lysis buffer with the antioxidant butylated hydroxy-toluene (BHT) (0.1 mM) to prevent further formation of MDA during the preparation of the sample or during the heating step. Then, they were centrifuged at 3200 G for 30 min. 200 µL of supernatants from each sample were added to 600 µL TBA, and incubated at 95 °C for 60 min. Samples were cooled in ice for 10 min, and 300 µL of n-butanol were added (Sigma-Aldrich, Madrid, Spain) to create an organic phase in which the MDA molecules were to be placed. Samples were centrifuged and 200 µL of upper organic phase were collected and dispensed into a 96-well microplate for spectrophotometric measurement at 532 nm. Results were expressed as nmol TBARS/mg protein.

Determination of protein concentration. The protein content of the same samples was evaluated following a bicinchoninic acid protein assay kit protocol (Sigma-Aldrich, Madrid, Spain) using a BSA standard curve.

Statistical analysis. Results are expressed as the mean ± SD. Mean comparison was done by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test after testing for normal distribution. A confidence level of 95% ($p < 0.05$) was considered statistically significant. Statistics were calculated using Prism v7 (GraphPad Software, Inc, CA, USA).

Data availability

All data generated or analysed during this study are included in this published article.

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Author contributions

W.L., E.V. and J.A.F. contributed to the conception and design of the study. B.L-P., L.R., S.D.P., J.G.M and M.S. performed the experiments and the data acquisition. B.L-P., L.R. and E.V. analyzed the data. B.L-P., wrote the original draft. All authors contributed to manuscript revision, read it, and approved the submitted version.

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Competing interests

The authors declare no competing interests.

Additional information

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Publicación 3: **Activación del inflamasoma NLRP3 en el hígado de ratas lactantes *Long Evans* y sus efectos perinatales en la descendencia tras la exposición al bisfenol F**

El hígado es el órgano responsable del metabolismo y la desintoxicación del BPF, el análogo del BPA que lo está sustituyendo en los productos plásticos de base. Se desconoce si el BPF puede desencadenar respuestas inflamatorias a través del inflamasoma NLRP3, que desempeña un papel importante en el desarrollo de enfermedades hepáticas. El objetivo de este estudio fue evaluar las especies de estrés nitrosativo y la activación del inflamasoma NLRP3 en el hígado de ratas lactantes tras la exposición a BPF. Además, se estudió si este efecto también podía observarse en el hígado de las crías hembras y machos en el día postnatal 6 (DPN6). Se distribuyeron aleatoriamente 36 ratas *Long Evans* según el tratamiento oral en tres grupos: grupo control, grupo de dosis baja de BPF (LBPF; 0,0365 mg/kg peso corporal/día) y grupo de dosis alta de BPF (HBPF; 3,65 mg/kg peso corporal/día). Se midieron los niveles de proteínas inductoras de estrés nitrosativo iNOS y HO-1d, componentes del inflamasoma NLRP3 (NLRP3, PyCARD, CASP1) y las citoquinas proinflamatorias IL-1β, IL-18, IFN-γ y TNF-α mediante expresión génica y proteica en el hígado de las ratas lactantes y en las crías hembras y machos del DPN6. Las ratas lactantes tratadas con LBPF mostraron un aumento significativo de iNOS y HO-1d, la activación de componentes del NLRP3 (NLRP3, PyCARD, CASP1) y promovieron la liberación de citoquinas proinflamatorias como IL-1β, IL-18, IFN-γ y TNF-α. Se observaron efectos similares en las crías hembras y machos de la descendencia tras la exposición perinatal. La administración oral de LBPF y la exposición perinatal provocaron un aumento de los marcadores de estrés nitrosativo, liberación de citoquinas proinflamatorias y activación del inflamasoma NLRP3 en el hígado de las ratas lactantes y en las crías del DPN6.

Beatriz Linillos-Pradillo, Sergio D. Paredes, María Ortiz-Cabello, Margret Schlumpf, Walter Lichtensteiger, Elena Vara, Jesús Á. F. Tresguerres, Lisa Rancan. Activation of NLRP3 Inflammasome in Liver of Long Evans Lactating Rats and Its Perinatal Effects in the Offspring after Bisphenol F Exposure. **International Journal of Molecular Sciences, Sep 2023**, 24, 14129 doi:10.3390/ijms241814129.

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Activation of NLRP3 Inflammasome in Liver of Long Evans Lactating Rats and Its Perinatal Effects in the Offspring after Bisphenol F Exposure

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Abstract: The liver is the organ responsible for the metabolism and detoxification of BPF, the BPA analogue that is replacing it in plastic-based products. It is not known whether BPF can trigger inflammatory responses via the NLRP3 inflammasome, which plays a major role in the development of liver disease. The aim of this study was to evaluate nitrosative stress species (RNS) and NLRP3 inflammasome activation in the liver of lactating dams after BPF exposure. Moreover, it was studied whether this effect could also be observed in the liver of female and male offspring at postnatal day 6 (PND6). 36 Long Evans rats were randomly distributed according to oral treatment into three groups: Control, BPF-low dose (LBPF; 0.0365 mg/kg b.w./day) group and BPF-high dose (HBPF; 3.65 mg/kg b.w./day) group. The levels of nitrosative stress-inducing proteins (eNOS, iNOS, HO-1d), NLRP3 inflammasome components (NLRP3, PyCARD, CASP1) and proinflammatory cytokines (IL-1β, IL-18, IFN-γ and TNF-α) were measured by gene and protein expression in the liver of lactating dams and in female and male PND6 offspring. Lactating dams treated with LBPF showed a significant increase in iNOS and HO-1d, activation of NLRP3 components (NLRP3, PyCARD, CASP1) and promoted the release of proinflammatory cytokines such as IL-1β, IL-18, IFN-γ and TNF-α. Similar effects were found in female and male PND6 offspring after perinatal exposure. LBPF oral administration and perinatal exposure caused an increase of nitrosative stress markers and proinflammatory cytokines. Also, NLRP3 inflammasome activation was significantly increased in in the liver of lactating dams and PND6 offspring.

Keywords: Bisphenol F; RNS; NLRP3 inflammasome; liver; offspring

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1. Introduction

Nowadays it is well documented that bisphenol A (BPA) exposure can cause liver tissue remodeling and fibrosis due to the generation of reactive oxygen species (ROS) and an uncontrolled inflammatory cascade [1]. This liver injury can lead to diseases such as hepatic steatosis, tumors, and metabolic syndrome. An important role of the NLRP3 inflammasome has been described in liver diseases [2,3]. Inflammasomes are key components of the natural immune system that can largely protect normal liver functions against pathogenic infections, metabolic diseases, and cellular stress [4]. NLRP3 inflammasome is a multiprotein scaffold that responds to damage-associated molecular patterns (DAMPs) and can mediate the catalytic activation of caspase-1 (CASP1), promoting the cleavage and release of IL-1β and IL-18 [5]. However, excessive inflammatory response regulated by NLRP3 inflammasome triggers liver disease progression [4].

Previous studies showed that BPA promoted inflammation and fibrosis progression with a key role of the NLRP3 inflammasome in the liver of obese mice after BPA and high-fat diet administration [6]. Knockout mouse models suggested that inhibition of the NLRP3 inflammasome reduced liver inflammation, indicating that the NLRP3 inflammasome is involved in the progression of non-alcoholic fatty liver disease (NAFLD) [7,8]. Furthermore, NLRP3 upregulation and increased gene and protein expression of IL-1β, IL-18, NLRP3, and CASP1 were observed in laying hens after high doses of BPA [9].

Due to the large number of studies demonstrating the health risks of BPA, the development and production of alternatives to this endocrine-disrupting chemical (EDC), has been stimulated to replace it in a myriad of applications [10]. Some of the new alternatives to BPA are the bisphenol analogues, such as bisphenol F (BPF). BPF is a diphenylalkane with two phenol rings linked through a methylene. BPF is replacing BPA in the manufacture of plastic-based products [11]. Also, BPF is the predominant bisphenol found in foodstuffs, representing 17% of total bisphenols.

After oral absorption, BPF is mainly metabolized in the liver by BPF-glucuronide and BPF-sulfate. Most BPF is excreted in the urine as a sulfate conjugate. Nonetheless, between 7–9% remains in the rat tissues 96 h after BPF exposure [12]. The liver seems to be more vulnerable to the effect of lower doses of bisphenols as it is responsible for the metabolism and detoxification of compounds to maintain homeostasis in the whole organism. It also plays an indispensable role in mediating inflammatory responses [13]. It is particularly interesting to investigate and understand how exposure to different EDCs can affect the developmental period. This is because an unborn fetus, as well as the placenta, is vulnerable because of the lack of the proper enzymatic machinery. This makes the gestation and the perinatal period, the most vulnerable times to EDC toxicity in human life [14]. In addition, effects may manifest differently in males and females due to differences in metabolism, storage, and elimination of xenobiotics [15].

Previous studies by our research group showed that low-dose BPF increased oxidative stress by reducing antioxidant enzyme activities and altering the glutathione system in lactating rats and their offspring [16]. However, it is unknown whether BPF triggers NLRP3 inflammasome-mediated inflammatory responses in the liver.

The aim of this study was to evaluate nitrosative stress after BPF exposure, and whether reactive nitrogen species (RNS) could serve as a stimulus for NLRP3 inflammasome activation and generation of inflammation and apoptosis in the liver of lactating dams. Moreover, it was studied whether this effect could also be observed in the liver of female and male offspring at postnatal day 6 (PND6).

2. Results

In addition to reactive oxygen species (ROS), there are also reactive nitrogen species (RNS) that are produced physiologically. However, imbalances between the production and neutralization of these RNS are known as nitrosative stress.

When lactating dams were treated with LBPF, gene and protein levels of oxidative stress-inducing proteins such as iNOS and HO-1d were significantly increased compared to the control group. In addition, iNOS and HO-1d mRNA and protein levels of HO-1d were higher in the LBPF group as compared with the HBPF-treated dams. No significant changes were shown in the physiological eNOS isoform after the administration of both doses of BPF in the liver of lactating dams (Figure 1a,b). To further investigate the role of BPF on hepatic inflammation, we measured the mRNA and protein levels of NLRP3 inflammasome components. The mRNA of NLRP3, PyCARD (ASC adaptor), and CASP1 were upregulated in LBPF-treated dams when compared to the control group. Higher PyCARD mRNA levels were also shown after LBPF administration as compared to HBPF in the liver of lactating dams (Figure 1d). Higher protein expression of NLRP3, CASP1, and IL-18 were obtained after LBPF administration when compared to control dams (Figure 1e–g). CASP1 and IL-18 protein expression levels were also higher in HBPF when compared to control dams (Figure 1f,g). Regarding proinflammatory cytokines IL-1β, IL-18, IFN-γ, and TNF-α, they

were considerably upregulated in LBPF-treated dams, whereas no significant change was observed in the HBPF group as compared with the control group except for IL-18 mRNA levels. Significant differences were also observed between both treatment groups, resulting in higher gene and protein levels of IL-1β and TNF-α in the LBPF group (Figure 1h,i). Representative protein blots for each tested marker are shown in Figure 1c,j.

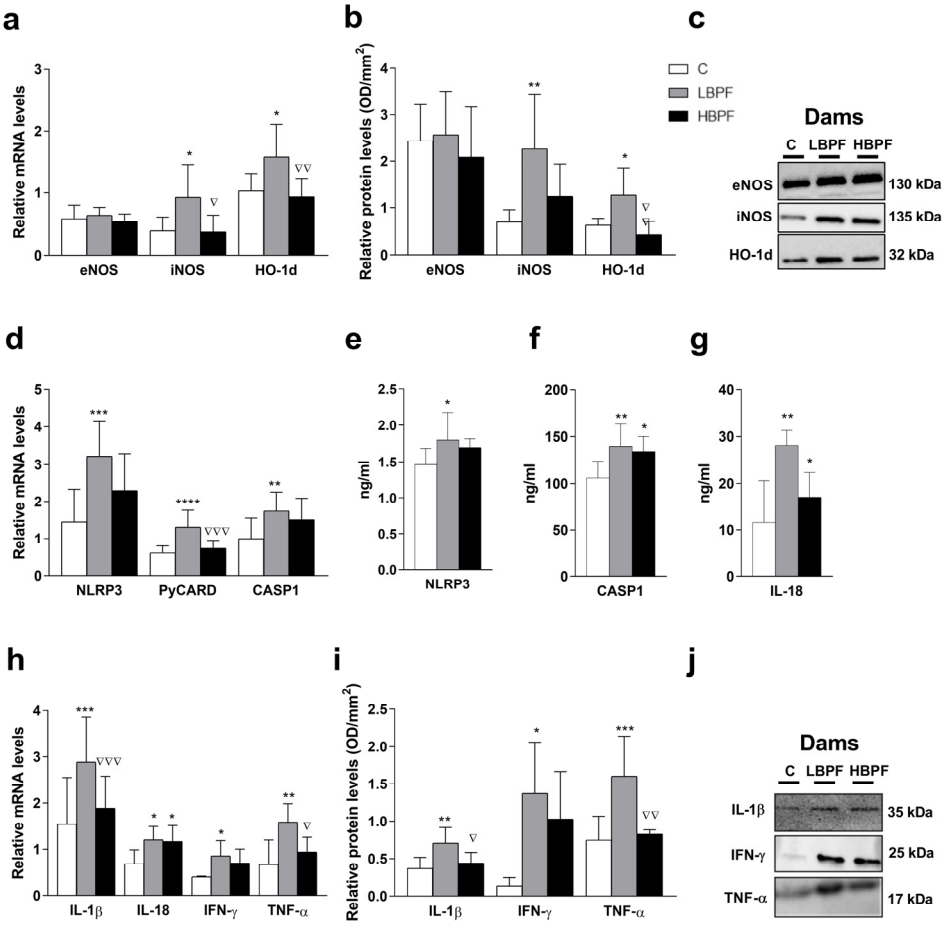

Figure 1. BPF effect on nitrosative stress markers, NLRP3 inflammasome activation and release of pro-inflammatory cytokines in the liver of lactating dams. (**a**) mRNA levels of eNOS, iNOS and HO-1d; (**b**) protein expression of eNOS, iNOS and HO-1d; (**c**) representative eNOS, iNOS and HO-1d protein blots measured by Western blotting; (**d**) mRNA levels of NLRP3 inflammasome components (NLRP3, PyCARD and CASP1); (**e**) NLRP3, (**f**) CASP1, and (**g**) IL-18 protein levels measured by ELISA; (**h**) mRNA levels of pro-inflammatory cytokines IL-1β, IL-18, IFN-γ and TNF-α; (**i**) protein expression of IL-1β, IFN-γ and TNF-α; and (**j**) representative IL-1β, IFN-γ and TNF-α protein blots evaluated by Western blotting. Data represent mean ± SD. $n = 6$ lactating control (C) dams; $n = 6$ lactating BPF low-dose (LBPF)-treated dams; $n = 10$ lactating BPF high-dose (HBPF)-treated dams. For qRT-PCR analysis, three replicates for each sample were performed. For protein, $n = 5$ rats per experimental group. Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to control group. ∇ $p < 0.05$; ∇∇ $p < 0.01$; ∇∇∇ $p < 0.001$, LBPF vs. HBPF.

Hence, LBPF increased nitrosative stress levels, which could be the stimuli to activate the NLRP3 inflammasome and to promote inflammatory responses in the liver of lactating dams.

To study whether perinatal administration of BPF generated alteration of the nitrosative balance in the liver of female and male offspring, we evaluated the same isoforms of NO and HO-1d. When female PND6 offspring was pre- and perinatally exposed to LBPF, the mRNA and protein levels of iNOS and HO-1d were increased in the LBPF group as com-

pared to the control group (Figure 2a,c). Also, higher levels of HO-1d mRNA and protein expression were observed in LBPF-exposed female offspring compared to the HBPF group (Figure 2a). Notably, eNOS isoform showed no differences between groups (Figure 2a,c).

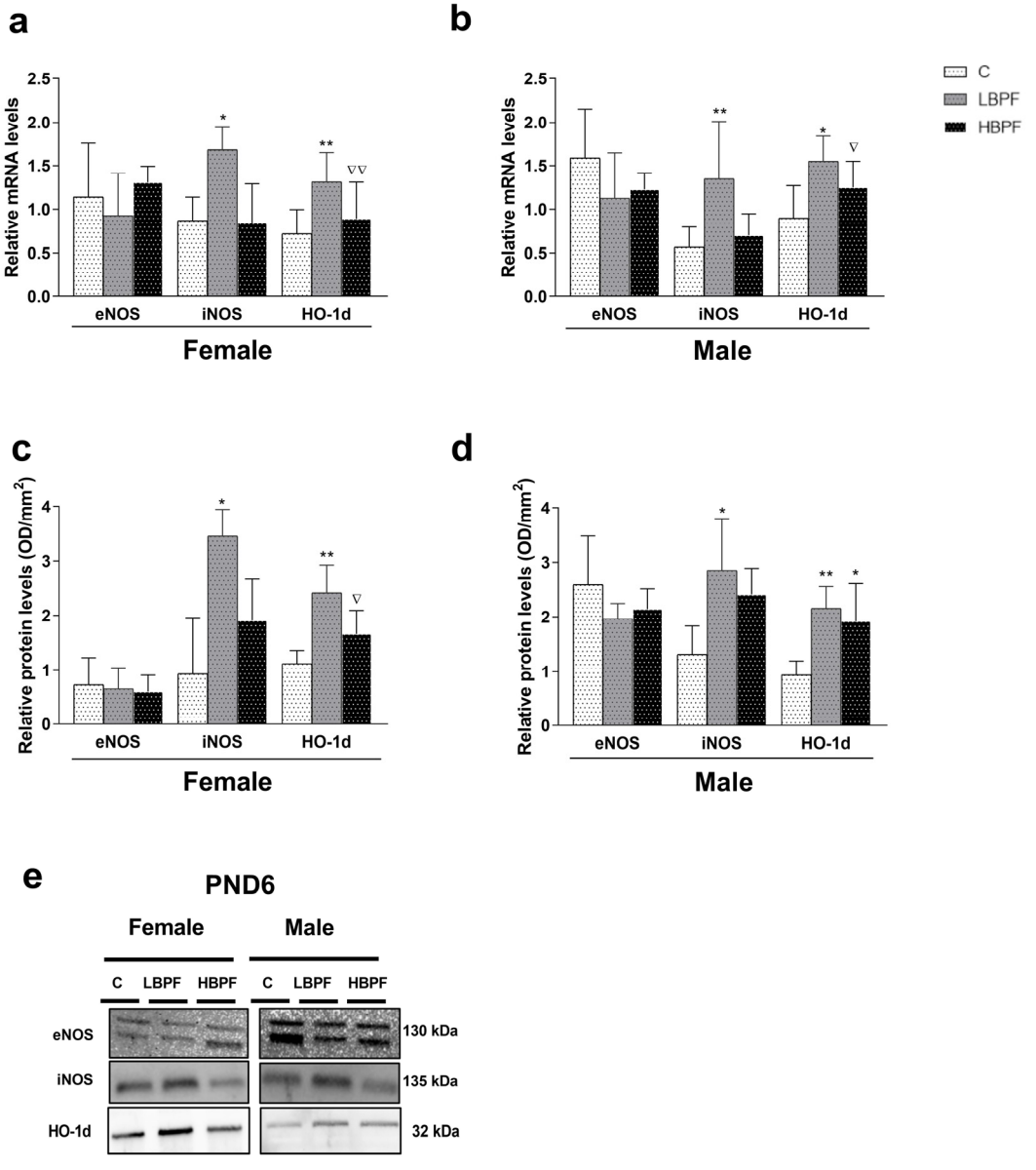

Figure 2. BPF pre- and perinatal effect on nitrosative stress markers in the liver of female and male PND6 offspring. (**a**) mRNA levels of eNOS, iNOS and HO-1d in female offspring; (**b**) mRNA levels of eNOS, iNOS and HO-1d in male offspring; (**c**) protein expression of eNOS, iNOS and HO-1d in female offspring; (**d**) protein expression of eNOS, iNOS and HO-1d in male offspring; and (**e**) representative eNOS, iNOS and HO-1d protein blots measured by Western blotting in both sexes. Data represent mean ± SD. For mRNA analysis, $n = 12$ female PND6 pups and $n = 12$ male PND6 pups for each experimental group with three replicates for each sample, control (C), low-dose BPF (LBPF) and high-dose BPF (HBPF), were evaluated, and for protein analysis, $n = 5$ female and $n = 5$ male per experimental group. Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$ compared to control group. ∇ $p < 0.05$; ∇∇ < 0.01, LBPF vs. HBPF.

In males exposed pre- and perinatally to BPF, the same results were obtained as in females. Thus, iNOS and HO-1d gene and protein levels increased in the LBPF-treated males compared to the control group, and no significant changes in eNOS isoform between groups were found (Figure 2b,d). Also, higher levels of HO-1d mRNA were observed in

LBPF-exposed male offspring as compared to HBPF (Figure 2b). HO-1d protein levels were higher in HBPF compared to the control group. Figure 2e shows eNOS, iNOS, and HO-1d representative blots analyzed in both PND6 females and males. In both sexes, there was also an enhanced expression of the inducible HO-1d and iNOS isoforms, which increased nitrosative stress levels (Figure 2).

Regarding the NLRP3 inflammasome pathway activation, an increase in NLRP3 gene expression and the following up-regulation of the adaptor ASC (PyCARD) and CASP1 mRNAs were shown after LBPF administration in female offspring (Figure 3a). In addition, increased PyCARD mRNA levels were observed in HBPF-exposed female offspring when compared to the control group (Figure 3a). Higher levels of NLRP3 and CASP1 mRNA and protein expression were observed in LBPF-exposed female offspring compared to the HBPF group (Figure 3a,c,d).

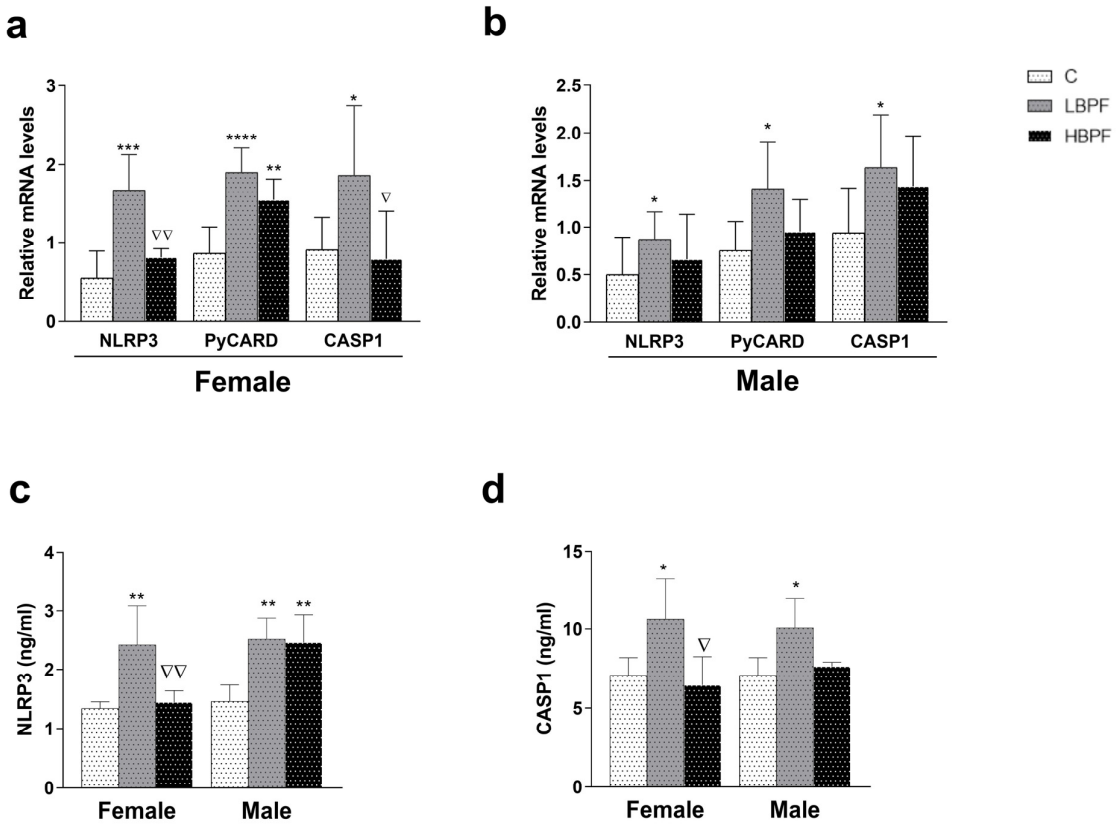

Figure 3. BPF pre- and perinatal effect on NLRP3 inflammasome induction in the liver of female and male PND6 offspring. (**a**) mRNA levels of NLRP3, PyCARD and CASP1 in female offspring; (**b**) mRNA levels of NLRP3, PyCARD and CASP1 in male offspring; (**c**) NLRP3, (**d**) CASP1 protein levels in male and female offspring measured by ELISA. Data represent mean ± SD. For mRNA analysis, n = 12 female PND6 pups and n = 12 male PND6 pups for each experimental group with three replicates for each sample, control (C), low-dose BPF (LBPF) and high-dose BPF (HBPF), were evaluated, and for protein analysis, n = 5 female and n = 5 male per experimental group. Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to control group. ∇ $p < 0.05$; ∇∇ $p < 0.01$; LBPF vs. HBPF.

When PND6 male offspring was pre- and perinatally exposed to LBPF, an increase in NLRP3, PyCARD, and CASP1 was observed as compared to the control group (Figure 3b). This was also observed with respect to the protein expression of NLRP3 and CASP1 (Figure 3c,d). NLRP3 protein expression was also upregulated in HBPF-treated offspring when compared to control male offspring (Figure 3c). Notably, NLRP3 pathway activation occurred in both sexes, allowing binding to the adaptor molecule and promoting CASP1 gene expression after pre- and perinatal exposure to LBPF (Figure 3).

When female PND6 offspring was pre- and perinatally exposed to LBPF, the mRNA and protein levels of IL-1β, IL-18, IFN-γ, and TNF-α were increased when compared to the control group (Figure 4a,c,e). Also, higher mRNA and protein levels of IL-1β and IFN-γ were observed in LBPF-exposed female offspring when compared to the HBPF group (Figure 4a,c). TNF-α mRNA levels were upregulated in HBPF-exposed female offspring compared to the control group (Figure 4a).

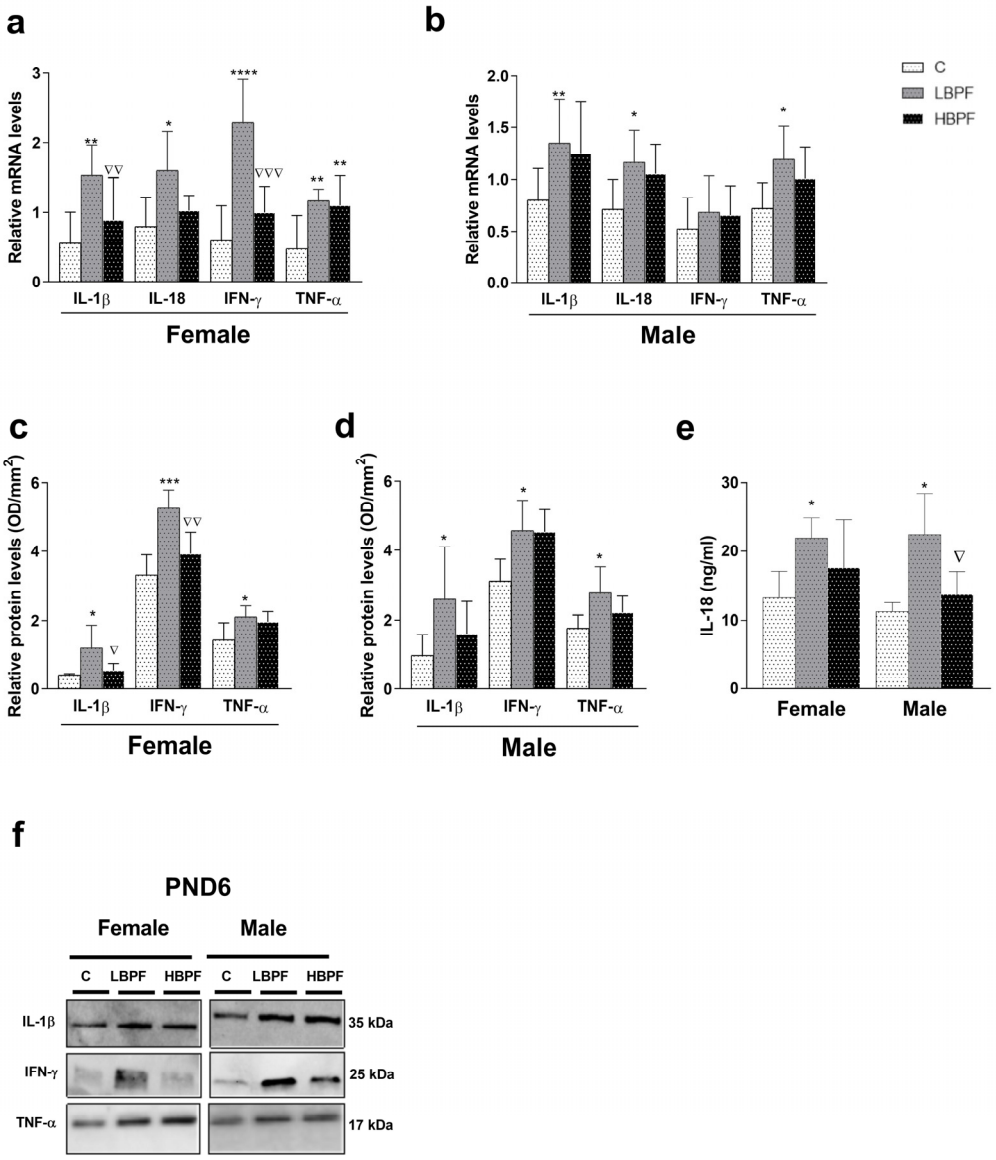

Figure 4. BPF pre- and perinatal effect on release of pro-inflammatory cytokines in the liver of female and male PND6 offspring. (**a**) mRNA levels of IL-1β, IL-18, IFN-γ and TNF-α in female offspring; (**b**) mRNA levels of IL-1β, IL-18, IFN-γ and TNF-α in male offspring; (**c**) protein expression of IL-1β, IFN-γ and TNF-α in female offspring; (**d**) protein expression of IL-1β, IFN-γ and TNF-α in male offspring; (**e**) IL-18 protein levels in male and female offspring measured by ELISA and (**f**) representative IL-1β, IFN-γ and TNF-α protein blots measured by Western blotting in both sexes. Data represent mean ± SD. For mRNA analysis, $n = 12$ female PND6 pups and $n = 12$ male PND6 pups for each experimental group with three replicates for each sample, control (C), low-dose BPF (LBPF) and high-dose BPF (HBPF), were evaluated, and for protein analysis, $n = 5$ female and $n = 5$ male per experimental group. Statistical significance was determined by one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to control group. ∇ $p < 0.05$; ∇∇ $p < 0.01$; ∇∇∇ $p < 0.001$, LBPF vs. HBPF.

In males exposed pre- and perinatally to BPF, up-regulated mRNA levels of IL-1β, IL-18 and TNF-α were observed as compared to the control group (Figure 4b). Protein levels of IFN-γ, IL-1β, and TNF-α were also higher in LBPF-exposed male offspring as compared with the control group (Figure 4d). IL-18 protein levels were also higher in LBPF-exposed animals when compared to control and HBPF-exposed male offspring (Figure 4e). Figure 4f shows representative blots of pro-inflammatory cytokines in PND6 females and males.

Regarding the histological study of the liver of lactating dams, no changes were still observed in cellular structure in the livers of BPF-treated dams compared to control hepatocyte images (Figure 5a). However, in both sexes of offspring, BPF administration induced nuclei aggregation and inflammatory cell infiltration in the liver of PND6 offspring compared to control pups with more noticeable effects at LBPF (Figure 5b,c).

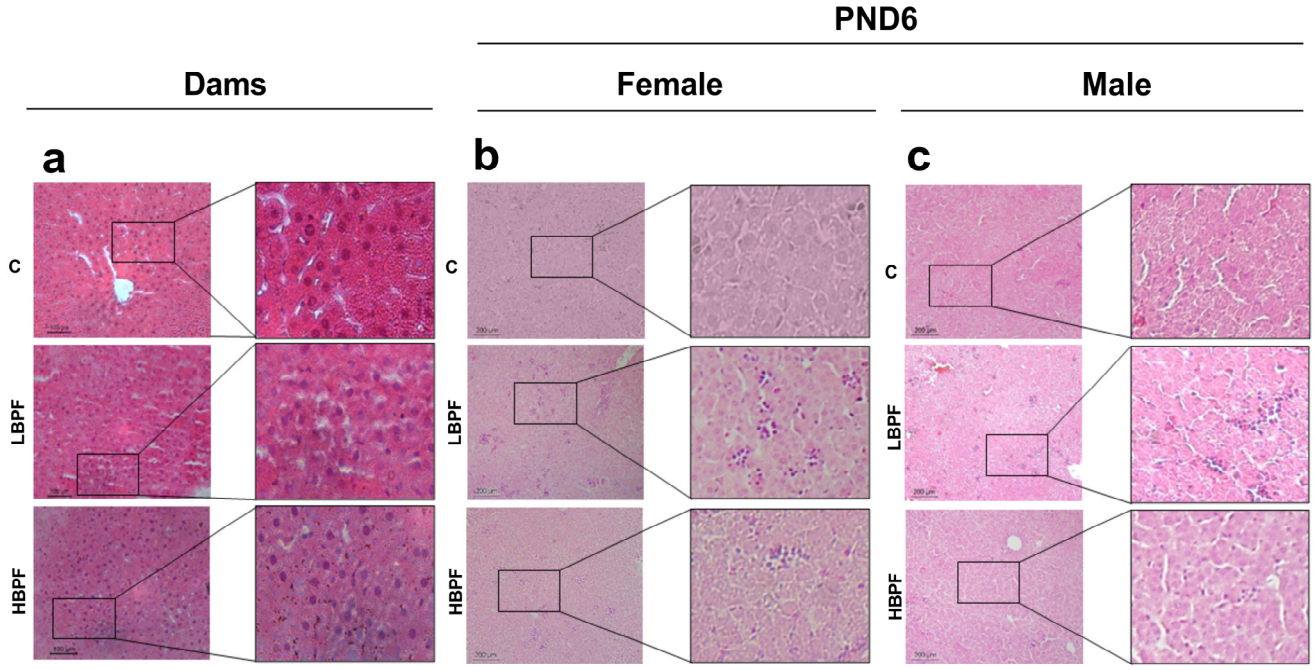

Figure 5. Histological study after BPF exposure of liver from (**a**) dams, (**b**) female and (**c**) male PND6 offspring stained with H&E. Representative images from control, LBPF and HBPF liver (10×) and magnified image of the specific tissue section (20×) indicating the aggregation of nuclei.

After BPF exposure, NLRP3 inflammasome activation and pro-inflammatory cytokines release were observed in offspring of both sexes. These same effects were observed in the liver of lactating dams with more noticeable effects after LBPF exposure.

3. Discussion

Oxidative stress and inflammation in the liver are closely correlated, as they occur simultaneously and interact with each other and are crucial in the initiation and development of liver disease [13].

In a previous study by our research group, antioxidant enzyme activities were decreased, and oxidized glutathione levels were increased after low doses of BPF in lactating Long Evans rats and their offspring, in addition to increased lipid peroxidation. Thus, LBPF increases oxidative stress [16]. However, it was unknown whether BPF could increase nitrosative stress and serve as a stimulus to trigger inflammatory responses after administration of two doses of BPF: a low dose of 0.0365 mg/kg/b.w./day (LBPF) and a high dose of 3.6 mg/kg/b.w./day (HBPF) in the liver of lactating dams and PND6 offspring after pre- and perinatal BPF exposure.

Among the reactive nitrogen species (RNS), nitric oxide (NO) is a signaling molecule involved in many biological processes: blood pressure, inhibition of platelet aggregation,

and neurotransmission; synthesized by at least three isoforms: neuronal nNOS, endothelial eNOS, and inducible iNOS. NO overproduction is associated with enhanced RNS production, which is able to induce structural damage to biomolecules, including proteins, lipids, and DNA [17].

No significant changes were found in the constitutive eNOS isoform, but increased gene and protein expression of inducible iNOS in LBPF-treated dams was observed. Excess of NO levels from increased iNOS activity can cause liver cell injury due to nitrosylation of thiol residues of many cellular enzymes, as well as a triggering effect of innate and adaptive immune responses [18]. Increased gene and protein expression of inducible HO-1d were also observed in LBPF-treated dams. HO-1d responds to transcriptional induction due to alterations in oxygen tension, inflammatory mediators, heat shock, oxidative stress, and NO levels. Therefore, HO-1d induction is elevated after nitrosative stress in order to prevent further injury [19].

Increased mitochondrial reactive oxygen species (ROS) and RNS are able to influence several physiological and pathological processes, including inflammation. Inflammation may be triggered by several different processes being the activation of the inflammasome one of the most important. The NLRP3 inflammasome can be activated in response to a wide range of stimuli such as infection, tissue damage, or metabolic stress (via different pathways: ATP, damaged mitochondria, lysosomal breakdown, changes in $Ca^{2+,}$ K^{+}, and also increases in mitochondrial and non-mitochondrial ROS concentrations). Once NLRP3 is activated, it binds to the adaptor molecule PyCARD (ASC; apoptosis-associated speck-like protein containing a CARD), which recruits and activates procaspase-1 into caspase-1 (CASP1), which is able to promote the maturation of proinflammatory cytokines such as IL-1β and IL-18. In addition, CASP1 is able to cleave protein precursors that affect the cell cytoskeleton, glycolysis, mitochondrial function, and inflammation [20]. It also induces pyroptosis, an inflammatory form of programmed cell death [21].

An increase in gene expression of the NLRP3 sensor, its adaptor molecule PyCARD, and CASP1, the three components of the NLRP3 inflammasome were observed in LBPF-treated dams. In turn, a release of proinflammatory cytokines such as IL-1β, IL-18, IFN-γ, and TNF-α occurred after exposure to LBPF, as measured by gene and protein expression in the liver of lactating dams.

IL-1β and IL-18, members of the IL-1 superfamily of cytokines, promote processes associated with infection, inflammation, and autoimmunity. IL-1β is key in the activation of hepatic stellate cells (HSC) and promotes the recruitment of inflammatory cells, contributing to fibrosis and triglyceride accumulation in hepatocytes and their death together with TNF-α [3]. TNF-α causes hepatic inflammation, proliferation, and apoptosis, as well as changes in HSC morphology [22]. TNF-α can also promote the recruitment of proinflammatory neutrophils and macrophages and the activation of fibrogenic pathways leading to the development of liver fibrosis [23].

IL-18 induces IFN-γ synthesis, in addition to activating NK cells and cytotoxic T lymphocytes, and seems to be involved in modulating the gut microbiota [3]. IFN-γ is a regulatory mechanism of the NLRP3 inflammasome and has a dual role: it activates effector cells such as NK lymphocytes and also tends to decrease activation through iNOS because NO induces nitrosylation of the NLRP3 protein and can inhibit its activity after a prolonged time [24]. The results obtained in the liver of lactating dams are consistent with a study that showed a significant increase in the levels of TNF-α and other inflammatory molecules in zebrafish after administration of BPF between 10–1000 μg/L [25].

Therefore, oral administration of LBPF to lactating dams led to an increase in liver RNS, which could stimulate the NLRP3 inflammasome and promote the release of pro-inflammatory cytokines.

There are no previous studies showing the influence of BPF on the activation or inhibition of inflammasomes, their components, or the release of products, but there is already data about the effects of BPA administration, as previously mentioned [6,9]. In a recent study by our research group, it was shown that after administration of low doses of BPA,

oxidative stress and NO levels increased, with a decrease in the endogenous antioxidant enzyme system (CAT, SOD, GST, GR, and GST) and glutathione system (GSSG/GSH ratio) in lactating dams as well as in female offspring [26]. Therefore, understanding how BPF exposure can affect the developmental period is very important, as it is the most critical and vulnerable period in human life. This exposure could cause a higher risk of developing diseases in adulthood due to their limited ability in this period of life to metabolize and process these chemicals [14,27]. Also, it is the moment in which the brain, as well as other organs, are in the phase of development.

Furthermore, human placental cells incubated with BPA and BPF are shown to activate the P2X7 receptor, promoting the NLRP3 inflammasome and increasing the activity of several caspases, showing a toxic effect. This could trigger preterm birth and pre-eclampsia in humans [28]. BPF administration also increases spontaneous abortions in pregnant dams in a dose-dependent manner [29].

Our results in PND6 offspring showed an increase in gene and protein expression of iNOS and no change in the eNOS isoform in both males and females, as well as an increase in inducible mRNA and protein HO-1d levels in both sexes. In a previous study [16], higher levels of the GSSG/GSH ratio were found in females than in males, but antioxidant enzymes were decreased in both sexes.

Regarding the components of the inflammasome, in both female and male offspring, an increase in NLRP3, PyCARD, and CASP1 was observed after pre- and perinatal exposure to LBPF, together with the consequent release of proinflammatory cytokines IL-1β, IL-18, IFN-γ and TNF-α. Therefore, one of the stimuli responsible for the activation of NLRP3 components and the release of inflammation-promoting cytokines may be the excess of RNS after exposure to this chemical.

In addition, inflammatory cell infiltration and aggregation was observed more noticeable after LBPF in both female and male offspring. However, no notable morphological changes were observed in lactating dams during exposure. Liver damage following perinatal exposure to LBPF was also observed in other studies [30,31]. This may be due to the fact that after perinatal exposure, the fetus is in the process of tissue ontogeny, being much more vulnerable to such chemical exposure, and on postnatal day 6 (PND6), structural alterations are already observed with aggregation of nuclei and infiltration of inflammatory cells in the liver. Therefore, this makes the fetus much more sensitive and vulnerable to the effect of BPF on the liver than adult dams.

Finally, the administration of LBPF had more noticeable effects than HBPF in the liver of lactating dams and their offspring. This might be due to the particular behavior of bisphenol in dose-response curves, so it may also be interesting to evaluate and analyze the effects of BPF, as well as other BPA analogues, at very low concentrations, typical of environmental exposure [32] on other organs apart from the liver. However, further research on the effect of BPF on inflammation and its mechanisms of inflammasome activation would be needed.

4. Materials and Methods

4.1. Animals and Treatments

After 10 days of acclimatization, 36 female (8 weeks of age) and 18 male (10 weeks of age) Long Evans rats (Janvier Labs, Le Genest-Saint-Isle, France) were randomly divided into three groups: control group (non-treated), low dose (0.0365 mg/kg body weight/day; LBPF) group of BPF and high dose (3.65 mg/kg body weight/day; HBPF) group of BPF. In each experimental group, there were 12 females and 6 males. Except for the control group, which received chow with a corresponding concentration of corn oil, all groups were fed their corresponding diet with BPF, and the experiment lasted 60 days. Food and water were fed ad libitum. The doses of BPF used were chosen according to previous studies on BPA [26,33] and the large existing literature, where the dose range of BPA (2.5–50 mg/kg) induced impairment learning and memory loss in rodents when BPA was administered in the perinatal period. Thus, the high dose is 3.65 mg/kg higher than 2.5 mg/kg; while the

low dose was 100 times lower, to investigate whether, even with such a small dose, any effects were observed.

All experimental procedures in this study were in accordance with the Guidelines for Ethical Care of Experimental Animals of the European Union (2010/63/UE) approved by the Ethical Committee of the Complutense University of Madrid (Madrid, Spain). This research is within a European project entitled "Novel Testing Strategies for Endocrine Disruptors in the Context of Developmental NeuroToxicity", supported by the European Union's Horizon 2020 Research and Innovation Programme (ENDpoiNTs project; grant number: 825759).

4.2. Chemicals and Experimental Design

BPF with purity > 99% was purchased from Sigma Aldrich (Buchs, Switzerland) (CAS Number 620-92-8; article number: 239658). It was dissolved in ethanol and then in corn oil at a ratio of 10% ethanol and 90% corn oil. The chosen rat chow was purchased from Granovit AG (Kaiseraugst, Switzerland) and corresponds to a diet with natural ingredients low in phytoestrogens.

Rats were housed in special polypropylene cages (Sodispan Research, Coslada, Madrid, Spain), water bottles were made of glass, and a cylindrical environmental enrichment element was included. In vivo experimental design consisted of five phases: premating (2 weeks), mating (10 days), pregnancy (23 days), lactation (6 days) and dissections. During premating, female and male rats were treated with a control diet or the corresponding dose of BPF in the diet for 2 weeks. After checking that the female was in the estrus phase, the mating phase took place between a male and a female from the same group. The following morning, a check for a sperm-positive vaginal smear or sperm-plug was carried out and the process was repeated all mornings for 10 days. Diet treatment was maintained during the whole pregnancy period. Six females were pregnant in the control and LBPF groups, and 10 females were pregnant in the HBPF group. Before the birth of the offspring, pregnant dams were separated into individual cages for lactation, and dietary treatment was maintained until postnatal day 6 (PND6). During all phases of the in vivo experiment, the cages of the control group were kept separate from the BPF-treated groups to avoid any possibility of spreading chow containing BPF.

Lactating dams were sacrificed by decapitation using a guillotine. Female and male offspring were sacrificed at PND6 by decapitation using scissors. The livers were collected and immediately frozen in liquid nitrogen and stored at −80 °C until analysis (Figure 6).

4.3. RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was isolated from liver tissues by using TRI Reagent Kit (Molecular Research Center, Inc., Cincinnati, OH, USA) and reverse transcribed into cDNA by using the StaRT Reverse Transcription Kit (AnyGenes, Paris, France). qRT-PCR was performed using a 7500 Fast Real Time PCR System thermal cycler (Applied Biosystems, MA, USA) according to the instruction of the TB Green® Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan). The related mRNA expression was normalized to 18s mRNA, and qRT-PCR data were analyzed using the comparative $2^{-\triangle\triangle Ct}$ method [34]. The following primers were used to amplify rat genes: forward (F) primer 5′-CCAGTGCCCTGCTTCATC-3′ and reverse (R) primer 5′GCAGGGCAAGTTAGGATCAG-3′ for eNOS, F primer 5′-CTTTGCCACGGACGAGAC-3′ and R primer 5′-TCATTGTACTCTGAGGGCTGAC-3′ for iNOS, F primer 5′-GTCAAGCACAGGGTGACAGA-3′ and R primer 5′-ATCACCTGCAG CTCCTCAAA-3′ for HO-1d, F primer 5′ TGAAAGCCTAGAAAGTCTGAAGAAC-3′ and R primer 5′-CGTGTTACCGTCCTTTTGC -3′ for IFN-γ, and F primer 5′- GGTGCATGGCCG TTCTTA-3′ and R primer 5′-TCGTTCGTTATCGGAATTAAC-3′ for 18S. The other rat primers (NLRP3, PYCARD, CASP1, IL-1β, IL-18, TNF-α) were custom primers and validated (AnyGenes, Paris, France).

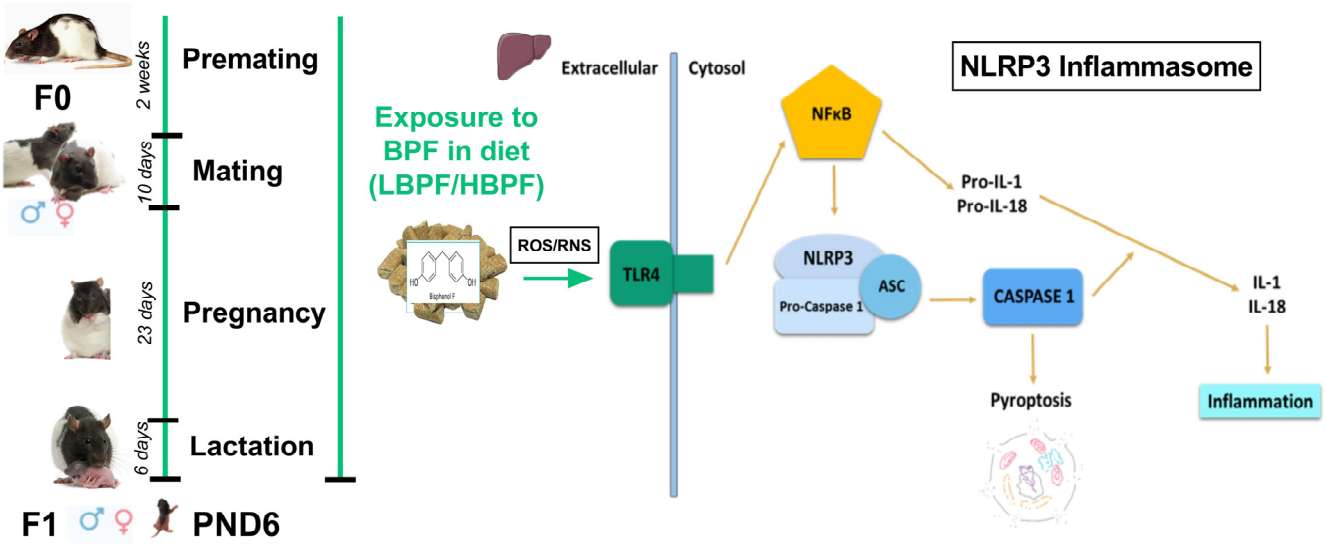

Figure 6. Experimental design. Parental generation (F0) was exposed to a diet containing a low dose (LBPF; 0.0365 mg/kg body weight/day) or a high dose (HBPF; 3.65 mg/kg body weight/day) of BPF or received a control diet (C) during the entire experiment. The levels of nitrosative stress and the NLRP3 inflammasome pathway in the liver of lactating dams and their offspring after BPF administration were studied. Activation of the NLRP3 inflammasome ultimately resulted in the release of the interleukins IL-1β, IL-18, IFN-γ and TNF-α, and could be triggered by different stimuli, including the generation of reactive oxygen species and nitrogen species (ROS/RNS). Figure created with Prism v7 (GraphPad Software Inc., San Diego, CA, USA).

4.4. Protein Preparation and Western Blot Analysis

Livers were homogenized with modified RIPA lysis buffer (PBS, Igepal, Sodium deoxycholate (D5670-5G), 10% SDS, PMSF, 0.5 M EDTA and 100 mM EGTA) to which protease inhibitor cocktail (Sigma #P-2714), PMSF (#P7626, 1 mM), sodium orthovanadate (#S6506, 2 mM) and sodium pyrophosphate (#S6422, 20 mM) were added. Samples were sonicated and boiled for 10 min at 100 °C in a ratio of 1:1 with gel-loading buffer (100 mmol/L Tris HCl [pH 6.8], 4% SDS, 20% glycerol, bromophenol blue 0.1, 200 mmol/L dithiothreitol). Total protein lysates (25 µg of dams' sample and 50 µg of female and male offspring sample) were subjected to SDS-PAGE by using 10% Mini-PROTEAN® TGX™ Precast acrylamide Gels (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, Stain Free technology was activated using the BioRad® ChemiDoc MP Imaging System (Bio-Rad Laboratories, Richmond, CA, USA) and was transferred onto a PVDF membrane using Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, Richmond, CA, USA).

The obtained membrane filter was then blocked with a blocking buffer containing 5% non-fat milk in 20 mM Tris pH 7.5, 150 mM NaCl, and 0.01% Tween-20 at 37 °C for 1 h. Rabbit polyclonal primary antibodies (dilution 1:1000) for immunoblotting were as follows: anti-eNOS (#PA1-037, Thermo Fisher Scientific, Waltham, MA USA), anti-IL-1β (#PA5-95455, Thermo Fisher Scientific, Waltham, MA, USA), anti-HO-1d (#3391, BioVision, Milpitas, CA, USA), anti-iNOS (#AB16311, Chemicon International, Temecula, CA, USA), anti-IFN-γ (#40499, Signalway Antibody, College Park, MD, USA) and anti-TNF-α (#500-P72, PeproTech EC, Ltd. London, UK) for 12 h at 4 °C, followed by incubation with a goat anti-rabbit IgG secondary antibody (dilution 1:7000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Protein detection was performed using the Clarity Western ECL Substrate assay kit (Bio-Rad Laboratories, Richmond, CA, USA) by chemiluminescence with the BioRad® ChemiDoc MP Imaging System to determine the relative optical densities. Pre-stained protein markers were used for molecular weight determinations. The intensity of the bands present in each lane was analyzed using BioRad® Image Lab software (Bio-Rad Laboratories,

Richmond, CA, USA) normalizing all measurements to the amount of total protein loaded in each well (thanks to the Stain Free technology of the Precast acrylamide Gels).

4.5. Enzyme-Linked Immunosorbent Assays

The levels of NLRP3 (#ER0800, FineTest, Wuhan Fine Biotech Co, Wuhan, Hubei, China) CASP1 and IL-18 (#MBS1600620 and #MBS8801271, Mybiosource, Vancouver, British Columbia, CA, USA) were determined in liver tissues using specific commercial enzyme-linked immunosorbent assays (ELISA) kits according to the manufacturer's instructions.

4.6. Histological Staining

Liver tissues were fixed in a 10% formalin buffer solution for 24 h and samples were processed for embedding in paraffin. Serial sections (5 µm) were prepared using a rotary microtome Leica RM2125 RTS (Leica Biosystems, Wetzlar, Germany) for hematoxylin and eosin staining (H&E). The sections were stained with 0.1% hematoxylin (Ciba, Basel, Switzerland) for 5 min. Then slides were washed with tap water for 15 min and a quick wash with hydrochloric alcohol (0.5% HCl in absolute ethanol) to remove excess staining on the sample (differentiation). The acid was neutralized by immersing the sections in tap water for 5 min and a final wash with distilled water. They were immersed in 0.1% eosin (Ciba, Basel, Switzerland) for 5 min. After washing with distilled water, tissue sections were dehydrated using ascending ethanol passages and finished in xylol for 30 s. Images were captured with a Leica Microscope (Leica Biosystems, Wetzlar, Germany).

4.7. Statistical Analysis

Results were presented as mean ± SD. Means from more than two experimental groups were compared by 1-way analysis of variance (ANOVA). To account for multiple comparisons, the Tukey-Kramer multiple comparison test after testing for normal distribution. All statistical analyses were carried out with Prism v8 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $p < 0.05$ in all the statistical analyses.

5. Conclusions

One of the BPA analogues that is replacing its use in plastic products is BPF. In this study, lactating dams treated with LBPF showed an increase in iNOS and HO-1d, activation of NLRP3 components, and promoted the release of proinflammatory cytokines. Similar effects were found in the offspring after perinatal exposure. The study found that BPF exposure caused an increase in nitrosative stress markers and proinflammatory cytokines. The activation of NLRP3 inflammasome was significantly increased in the liver of lactating dams and PND6 offspring. These findings suggest that BPF exposure can cause liver inflammation and may contribute to the development of liver disease.

Author Contributions: W.L., E.V. and J.A.F.T. contributed to the conception and design of the study. B.L.-P., L.R., S.D.P., M.O.-C. and M.S. performed the experiments and the data acquisition. B.L.-P., L.R. and E.V. analyzed the data. B.L.-P., L.R., S.D.P. and J.A.F.T. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was approved by the Ethical Committee of Complutense University of Madrid (Madrid, Spain) in accordance with the Guidelines for Ethical Care of Experimental Animals of the European Union (2010/63/UE). This research is within a European project entitled "Novel Testing Strategies for Endocrine Disruptors in the Context of Developmental NeuroToxicity" supported by the European Union's Horizon 2020 Research and

Innovation Programme (ENDpoiNTs project; grant number: 825759). All authors complied with the ARRIVE guidelines.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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DISCUSIÓN

El bisfenol A es una de las sustancias químicas industriales más utilizadas en todo el mundo. El 90% de la población general presenta niveles detectables de BPA. Trasande et al. (2013) han mostrado que los niveles de BPA en los individuos ocupacionalmente expuestos (cuando la exposición ocurre en el lugar de trabajo como el caso de trabajadores que manipulan plásticos que contienen BP) son 70 veces superiores comparados con las poblaciones ambientalmente expuestas (Hines et al., 2017); por lo tanto, la exposición al BPA se considera una situación inevitable y preocupante.

Gracias a los numerosos estudios científicos previos sobre los riesgos que conlleva el BPA, se ha llevado a cabo recientemente su regulación por la EFSA, bajando 20.000 veces la IDT. Esto ha promovido el desarrollo y la producción de alternativas a este DE para sustituirlo en un sinfín de aplicaciones. El BPF es una de las alternativas al BPA. Debido a su estructura química tiene menor viscosidad y es más resistente a los disolventes que otros BP, por ello, el BPF está sustituyendo al BPA en la fabricación de productos plásticos de base.

Dado que la exposición al BPA y al BPF se produce principalmente a través de la ingestión, en los trabajos presentados en esta tesis se evaluaron los efectos del BPA y del BPF administrado por vía oral a dos concentraciones diferentes: una dosis baja de 0,036 mg/kg/peso corporal/día de BPA o BPF (LBPA or LBPF, *low doses* of BPA or BPF) y una dosis casi 100 veces superior de 3,42 mg/kg/peso corporal/día de BPA o 3,65 mg/kg/peso corporal/día de BPF (HBPA or HBPF, *high doses* of BPA or BPF) en el hígado de ratas lactantes. Ambas dosis se consideran ambientalmente relevantes y fueron elegidas de acuerdo con el proyecto europeo denominado "*Novel testing strategies for endocrine disruptors linked to developmental neurotoxicity*" con el acrónimo: ENDpoiNTs 825759) cuyo objetivo principal es desarrollar nuevos métodos de ensayo y cribado para identificar las sustancias químicas alteradoras endocrinas que inducen neurotoxicidad en el desarrollo humano. La dosis alta de BPA se eligió en el rango de dosis (2,5 mg/kg - 50 mg/kg) que consistentemente indujo deterioro del aprendizaje y pérdida de memoria en roedores cuando se administró en el período perinatal. Por eso, la dosis alta es entre 3,42 - 3,65 mg/kg superior a 2,5 mg/kg; mientras que la dosis baja era 100 veces inferior, para investigar si, incluso con una dosis tan pequeña, se observaban efectos (0,036 mg/kg). Respecto a las dosis de BPF, se eligieron las mismas que con el BPA de acuerdo a la bibliografía previa existente y al estudio piloto del BPA (Lichtensteiger et al., 2021; Linillos-Pradillo et al., 2023), además de estudiar su efecto perinatal en la descendencia en el DPN6 centrándonos en el hígado, como órgano esencial para la desintoxicación y el metabolismo, siendo especialmente vulnerable a los daños (Cabaton et al., 2006).

La elección del DPN6 también se basa en el objetivo principal de ENDpoiNTs por tratarse del período crítico de diferenciación sexual e importante en el desarrollo del hipocampo y comportamiento.

1. ESTRÉS OXIDATIVO

En nuestro estudio, las actividades de las enzimas antioxidantes CAT, SOD, GPx y GR disminuyeron en el hígado de ratas lactantes tras la administración de LBPA. También hubo una disminución de la actividad enzimática de CAT y GPx en las ratas tratadas con HBPA, sin ninguna diferencia significativa con el grupo de LBPA. Esta reducción de la actividad de la CAT indicó una incapacidad de eliminar el H_2O_2 producido tras la exposición al BPA (Bindhumol et al., 2003; Sayed-Ahmed et al., 2010). La exposición a ambas dosis de BPA mostró una reducción significativa de la actividad de la GST, lo que refleja una incapacidad para desintoxicar este compuesto. La exposición a LBPA también muestra niveles más bajos de expresión génica de la γ-GCS que cataliza el primer paso en la síntesis de glutatión, lo que resulta en niveles celulares bajos de glutatión. Respecto a los niveles de peroxidación lipídica, observamos un aumento de los niveles de MDA con LBPA y un aumento de lipoperóxidos (LPO) con ambas dosis de BPA en las ratas lactantes, con niveles mayores en el grupo de LBPA. A su vez, la exposición a LBPA condujo a un aumento del daño oxidativo en el ADN, como lo demuestra el aumento de los valores de 8-OHdG en los hígados de las ratas lactantes.

Los resultados obtenidos concuerdan con muchos otros estudios previos, como Acaroz et al. (2019), que demostraron una disminución de las actividades enzimáticas SOD y CAT y de los niveles de GSH en ratas albinas *Wistar* expuestas a BPA en diferentes dosis orales (5, 10 y 20 mg/kg). En otro estudio en el que se utilizó una dosis de 25 mg/kg en ratas durante 50 días, se observó un aumento de los niveles de MDA y una disminución de los niveles de GSH y de las actividades de SOD y CAT en los tejidos renal, cerebral y testicular (Morgan et al., 2014). Bindhumol et al. (2003) también mostraron una reducción de las enzimas antioxidantes (SOD, CAT, GR, GPx) en las fracciones mitocondriales y ricas en microsomas del hígado; mientras que los niveles de H_2O_2 y MDA aumentaron en ratas *Wistar* tratadas con dosis de BPA de 0,2 a 20 µg/kg. Lo mismo ocurrió en el estudio de Hassan et al. (2012) donde las actividades antioxidantes disminuyeron a dosis de 50 mg/kg de BPA en hígados de ratas.

Un escenario similar encontramos tras la administración de LBPF en ratas lactantes, con la disminución de la actividad enzimática de CAT, SOD, GPx y GR junto a niveles reducidos de GSH y un exceso de concentración de GSSG, más notable tras la administración de LBPF en comparación con los grupos control y HBPF en ratas lactantes. Esta alteración en el sistema del glutatión se manifestó por el aumento de la relación GSSG/GSH como marcador de estrés oxidativo, siendo estadísticamente significativa tras la comparación de ambas dosis de BPF, con niveles más altos que implican acciones más intensas de LBPF encontradas en el hígado de las ratas lactantes. La actividad de la GST estaba disminuida en las ratas tratadas con LBPF, perjudicando la correcta eliminación de este xenobiótico en el hígado (Aykut & Kaptaner, 2021; Li, 2011). También se produjo el aumento de los niveles de MDA y LPO, dos productos finales de la peroxidación lipídica utilizados para monitorizar el daño de la membrana celular en el hígado de las ratas lactantes.

Estos resultados concuerdan con investigaciones previas realizadas tras la exposición *in vivo* e *in vitro* a BPF, en hígado y otros órganos, utilizando diferentes especies animales, ya que aún la literatura sobre el BPF en experimentos murinos sobre el efecto en hígado es limitada. La actividad de la CAT disminuyó con siete concentraciones de BPF durante 24 h en hepatocitos aislados del hígado de trucha arcoíris (*Oncorhynchus mykiss*) mostrando un efecto citotóxico. Asimismo, el contenido de GSH se redujo con la concentración más alta de BPF, y el contenido de MDA aumentó significativamente con concentraciones de BPF entre 15,63 y 250 µM (Aykut & Kaptaner, 2021). Tras la exposición oral de 100 mg/kg de BPF, en los tejidos reproductores de ratas macho, disminuyeron las actividades de CAT y SOD y aumentaron los niveles de ROS y la peroxidación lipídica (Ullah et al., 2019). Maćczak et al. (2017) encontraron niveles reducidos de GSH en eritrocitos humanos expuestos a BPF y mayores niveles de peroxidación lipídica. Un estudio *in vitro* en células KGN (*human granulosa cells*) mostró que el BPF exhibía ligeros efectos tóxicos y aumentaba el daño a las biomacromoléculas (MDA, 8-OHdG) tras la exposición al BPF (Huang et al., 2020). Además, la exposición a BPF provocó un mayor contenido de MDA en las larvas del pez cebra (*Danio rerio*) y finalmente condujo a la apoptosis (Gu et al., 2020).

En resumen, observamos que la exposición a LBPA y LBPF indujeron daños hepáticos en las ratas lactantes, afectando al equilibrio oxidante/antioxidante (disminución de las enzimas antioxidantes y alteración del sistema de glutatión) e iniciando el proceso de peroxidación lipídica.

Respecto al estrés nitrosativo tras la administración de BPA, no observamos diferencias en la expresión génica y proteica de la isoforma eNOS en ambos grupos de tratamiento. Sin embargo,

se observaron niveles elevados de NO en plasma y una mayor expresión génica y proteica de la isoforma iNOS que está asociada a inflamación y HO-1d. Lo mismo ocurrió tras la administración de LBPF, aumento de la expresión génica y proteína de las isoformas inducibles iNOS y HO-1d; sin cambios en la isoforma eNOS que es la isoforma constitutiva. Kazemi et al. (2016) mostraron un aumento de la expresión del gen HO-1d con un perfil dependiente de la dosis de BPA en células hepáticas.

El exceso de niveles de NO debido al aumento de la actividad de iNOS puede causar lesiones en las células hepáticas debido a la nitrosilación de los residuos de tiol de muchas enzimas celulares, así como un efecto desencadenante de respuestas inmunitarias innatas y adaptativas (Andrew, 1999). Además de ser un agente nitrante capaz de modificar proteínas, lípidos y ADN, así como de disminuir las defensas antioxidantes (Eid et al., 2015). La inducción de HO-1d podría elevarse tras el estrés nitrosativo y oxidativo como un mecanismo de defensa celular para prevenir una lesión mayor como la fibrosis hepática (Hoetzel et al., 2008) tras la exposición a BPA y BPF.

2. INFLAMACIÓN

El estrés oxidativo y el nitrosativo pueden influir en varios procesos fisiológicos y patológicos, incluida la inflamación, por lo que altos niveles de estrés oxidativo se han relacionado con procesos inflamatorios.

Las ratas tratadas con LBPA aumentaron la expresión génica y proteica de la citoquina proinflamatoria IL-1β. Coincidiendo con nuestros resultados, Acaroz et al. (2019) demostraron que la exposición al BPA a 25 mg/kg en ratas *Wistar* macho aumentaba la expresión de citoquinas proinflamatorias como TNF-α, IL-6 e IL-1β y disminuía la citoquina antiinflamatoria/antifibrótica (IL-10). Elswefy et al. (2016) administraron 50 mg/kg de BPA a ratas por vía oral durante ocho semanas e informaron de que su administración aumentaba de forma significativa el nivel sérico de IL-1β y reducía el nivel de IL-10. Esto estaría de acuerdo con nuestro estudio en el que tras la administración de LBPF se observó un aumento de la expresión génica y proteica de diferentes citoquinas proinflamatorias como IL-1β, IL-18, IFN-γ y TNF-α.

La inflamación puede desencadenarse por varios procesos diferentes, siendo la activación del inflamasoma uno de los más importantes. Estudios anteriores demostraron que el BPA fomentaba la inflamación y la progresión de la fibrosis con un papel clave del inflamasoma NLRP3 en el hígado de ratones obesos tras la administración de BPA y de una dieta rica en grasas (Pirozzi et al., 2020). En ratones *knockout* (KO) se observó que la inhibición del inflamasoma

NLRP3 reducía la inflamación hepática (Chen et al., 2016; Lv et al., 2019), sugiriendo que el inflamasoma NLRP3 está implicado en la progresión de la enfermedad del hígado graso no alcohólico (NAFLD). Además, se observó un aumento de la regulación de NLRP3 y de la expresión de genes y proteínas de IL-1β, IL-18, NLRP3 y CASP1 en gallinas ponedoras tras dosis elevadas de BPA (Gao et al., 2021). Sin embargo, se desconoce si el inflamasoma NLRP3 podría activarse tras la exposición a BPF desencadenando respuestas inflamatorias.

En nuestro estudio, se observó un aumento de la expresión génica del receptor NLRP3, su molécula adaptadora PyCARD y CASP1, los tres componentes del inflamasoma NLRP3 en el hígado de las ratas tratadas con LBPF con la liberación de diferentes citoquinas proinflamatorias. Los resultados obtenidos concuerdan con un estudio que mostró un aumento significativo de los niveles de TNF-α y otras moléculas inflamatorias en el pez cebra (*Danio rerio*) tras la administración de BPF entre 10-1000 µg/L (Y. Wang et al., 2021). Por lo tanto, la administración oral de LBPF a las ratas lactantes provocó un aumento de ROS y RNS, lo que podría estimular el inflamasoma NLRP3 y promover la liberación de citoquinas proinflamatorias e incluso inducir la piroptosis a través de la CASP1 (Sahin & Aricioglu, 2013).

3. MUERTE CELULAR

Una inflamación excesiva o descontrolada puede incrementar la permeabilización de la membrana pudiendo ser la etapa inicial de la apoptosis mitocondrial (Xia et al., 2014). Tras la administración de LBPA, encontramos niveles elevados en la expresión génica y proteica de AIF, que al traslocarse al núcleo puede desencadenar vías apoptóticas, además de un incremento de la expresión génica de Bax, factor promotor de la apoptosis, mientras que la expresión proteica de Bcl-2 y Bcl-XL, factores antiapoptóticos, se encontró significativamente disminuida tras la administración de LBPA.

Estudios anteriores descubrieron que el BPA deterioraba la función mitocondrial hepática al liberar factores solubles en el citosol (Moon et al., 2012; Nakagawa & Tayama, 2000) y un aumento de CASP3 junto a una reducción de Bcl-2 en el hígado de ratas macho (Elswefy et al., 2016; Xia et al., 2014). El BPA debilitó la función mitocondrial de los hepatocitos y promovió la apoptosis celular en el hígado mediante la regulación al alza de los niveles proapoptóticos de Bax, CASP3 escindida y PARP1 escindida, mientras que reguló a la baja Bcl-2 en el hígado utilizando altas dosis de BPA (Liu et al., 2022).

También se observó un aumento del citocromo C, un mediador clave de la apoptosis a través

de la activación de las caspasas en el citosol (Kroemer et al., 2007; Rönn et al., 2013; Vaux, 2011).

En nuestro estudio, la administración de LBPA mostró niveles elevados de ATP en las ratas lactantes en comparación con el grupo de control. Este mantenimiento de niveles suficientes de ATP junto con la liberación de factores proapoptóticos puede promover que las células hepáticas entren en apoptosis (Muntané et al., 2007). El mecanismo de la apoptosis inducida por BPA probablemente también implica una alteración en la relación de expresión de las proteínas proapoptóticas y antiapoptóticas de la familia X asociada a Bcl-2 (Bax) y Bcl-2 en la membrana mitocondrial externa que modula la liberación de factores proapoptóticos (Kluck et al., 1997; Xu et al., 2002).

Por lo tanto, la exposición de las ratas lactantes a LBPA puede ejercer efectos tóxicos sobre las células hepáticas a través de la formación de ROS, la inducción de la inflamación y la apoptosis. Este incremento de la inflamación tras la administración de BPA podría conducir a la apoptosis, lo que a su vez puede atraer la infiltración de células inflamatorias y originar fibrosis y mayor afectación hepática.

4. EVALUACIÓN DEL DAÑO HEPÁTICO

Aunque en nuestro estudio aún no se apreciaron cambios estructurales de los hepatocitos tras la administración de BPA en el hígado de las ratas lactantes, sí se observó un incremento de los marcadores hepáticos séricos (aumento de aspartato aminotransferasa, AST y alanina aminotransferasa, ALT) como indicador de daño tisular tras LBPA. Sin embargo, en el estudio histológico de Kazemi et al. (2017) se demostró que la administración oral de BPA por sonda a dosis bajas inducía lesiones hepáticas en ratas macho adultas. En el estudio de Elswefy et al. (2016) tras observar un aumento de las citoquinas proinflamatorias se indujo la inflamación hepática mediante el transporte de leucocitos mononucleares y polimorfonucleares a los tejidos inflamados (Wei et al., 2014). En el estudio de Ijaz et al. (2022) también se observó un aumento de los niveles de ALT, fosfatasa alcalina (ALP) y AST en ratas tratadas con BPA. Esto podría deberse a que la sobreproducción de ROS daña la integridad estructural de las células hepáticas, lo que se manifiesta por un aumento de los marcadores séricos hepáticos (Vagvala & O'Connor, 2018) al igual que lo observado en nuestro estudio.

Tampoco se observaron cambios morfológicos notables en el hígado de las ratas lactantes tras LBPF al igual que ocurría con la administración de BPA. Sin embargo en otros estudios recientes, la exposición al BPF indujo cambios similares a los de la NAFLD, con una evidente deposición de

gotas lipídicas y aumentos de triglicéridos (TG) y ácidos grasos en los hígados de ratones a los que se administró BPF (50 mg/kg/día) durante 30 días mediante inyección subcutánea (J. Wang et al., 2021). En el estudio de Higashira et al. (2007), realizado en ratas a las que se administró por vía oral diferentes concentraciones de BPF (0, 20, 100 y 500 mg/kg al día) durante 28 días, el BPF causó toxicidad hepática según los parámetros bioquímicos clínicos y el peso del hígado, pero sin cambios histopatológicos. Además se ha visto una alteración de la función hepática por exposición a BPF en ratones macho tras un aumento de los niveles hepáticos de ALT y un aumento de los niveles plasmáticos de AST tras dosis bajas de BPF (Sun et al., 2023). En nuestro estudio, el hecho de no apreciar cambios histopatológicos podría deberse a que se encuentran en una fase temprana de afectación tras siete semanas de exposición respectivamente.

5. EFECTOS PERINATALES EN LA DESCENDENCIA

La mayoría de los estudios científicos se han centrado en el efecto de dosis elevadas de BPA en adultos, pero resulta relevante tener en cuenta el efecto de dosis bajas de BPA en la exposición perinatal (Sewelam & Mokhtar, 2019). El período prenatal es una ventana crítica (Selevan et al., 2000) donde la exposición a compuestos exógenos como el BPA y el BPF puede afectar al desarrollo fetal (Selevan et al., 2000). El feto es extremadamente vulnerable, con una capacidad limitada para metabolizar y procesar tales sustancias químicas (Unüvar & Büyükgebiz, 2012) ya que la actividad de la enzima UDPGT es muy débil en el feto y posteriormente en las crías recién nacidas (Yabusaki et al., 2015). La exposición perinatal y la transferencia placentaria (en ratas se produce al final de la gestación) (Cabaton et al., 2006) también pueden dar lugar a cambios en los tejidos del desarrollo que contribuyen a resultados adversos para la salud en la edad adulta (Tucker et al., 2018).

En las hembras de la descendencia expuesta a LBPA, observamos una disminución de los niveles de actividad de las enzimas antioxidantes CAT, SOD, GPx, GR y GST. Las actividades de SOD y GST también disminuyeron en las crías hembras con HBPA. El sistema del glutatión también se vio alterado mostrando niveles reducidos de GSH y mayores niveles de GSSG, además del daño asociado a la peroxidación lipídica por incremento de los niveles de MDA y LPO tras LBPA junto a un aumento de la oxidación del ADN en la descendencia expuesta a ambas dosis de BPA. Esto concuerda con un estudio en ratones hembras preñadas expuestas oralmente a una dosis de 100 ng/g de BPA desde el DPN7 hasta el DPN21, que muestra que la exposición perinatal al BPA podría inducir daño oxidativo y alterar los perfiles metabólicos normales en el hígado (Meng et al., 2019). Lin et al. (2019) demostraron que la exposición perinatal al BPA causaba el

desarrollo de NAFLD en la descendencia de ratas *Sprague-Dawley* preñadas que tuvieron acceso a agua que contenía 1 o 10 µg/mL de BPA desde el día gestacional seis (GD6) hasta el DPN21. La exposición al BPA se asoció con la regulación al alza de los genes lipogénicos, la desregulación de la autofagia y la activación de la respuesta inflamatoria a través de las vías PI3K/Akt/mTOR y TLR4/NF-κB.

Este desequilibrio oxidante/antioxidante también fue notable en nuestro estudio, ya que los niveles de expresión génica y proteica de las proteínas inductoras de estrés oxidativo (HO-1d e iNOS) aumentaron en la descendencia expuesta a LBPA. El aumento de la citoquina proinflamatoria IL-1β y de los factores proapoptóticos AIF y Bax, con la consiguiente disminución de los factores antiapoptóticos Bcl-2 y Bcl-XL, condujo a una inducción de la apoptosis en las células hepáticas en la descendencia expuesta perinatalmente a LBPA.

Respecto a los efectos de BPF en la descendencia, existen pocos estudios en la bibliografía aún, pero es absolutamente necesario estudiar la exposición que se produce durante el desarrollo temprano en ambos sexos de la descendencia; sin embargo, por razones de diseño experimental solo fue posible estudiar el efecto perinatal al BPA en las hembras de la descendencia.

Nuestros resultados en la descendencia mostraron una reducción significativa de todas las enzimas antioxidantes (CAT, SOD, GPx, GR y GST) en ambos sexos tras la exposición perinatal a LBPF. En cuanto al sistema del glutatión, se observó una reducción mucho más drástica del contenido de GSH en las crías hembras, lo que condujo a una mayor relación GSSG/GSH en comparación con las crías machos. Así, las crías hembras mostraron niveles más altos de GSSG y, por tanto, un mayor desequilibrio en el sistema del glutatión. El daño de la membrana lipídica se produjo en ambos sexos y los efectos de la exposición a HBPF sobre todos estos parámetros no fueron tan notables, excepto por la disminución de la actividad de la SOD en los machos.

Un estudio previo demostró que la exposición perinatal a BPF se asociaba con daño oxidativo (reducción de los niveles de CAT y GSH) y trastornos metabólicos junto a la acumulación de lípidos que inducen daño hepático tras la exposición perinatal a BPF en hígados de crías de ratones macho (Meng et al., 2019). Cambios específicos por sexo se observaron en parámetros de estrés oxidativo en *Drosophila melanogaster* tras la exposición a BPF durante siete días. Las moscas macho tenían mayores defensas antioxidantes que respondían principalmente a concentraciones más bajas de BPF, minimizando el daño celular oxidativo mientras que las moscas hembra eran más susceptibles al daño celular oxidativo y su longevidad se veía reducida (Musachio et al., 2022).

Ambos sexos de la descendencia expuesta a LBPF mostraron un aumento de la expresión génica y proteica de la isoforma proinflamatoria iNOS y la proteína HO-1d y ningún cambio en la isoforma constitutiva eNOS. En cuanto a los componentes del inflamasoma, tanto en hembras como en machos se observó un aumento de NLRP3, PyCARD y CASP1 tras la exposición pre y perinatal a LBPF, junto con la consiguiente liberación de citoquinas proinflamatorias IL-1β, IL-18, IFN-γ y TNF-α, sugiriendo que uno de los estímulos responsables de la activación de los componentes NLRP3 y de la liberación de citoquinas promotoras de la inflamación puede ser el exceso de ROS y RNS tras la exposición a esta sustancia química.

Otra de las similitudes tras la exposición perinatal a BPA y BPF en las hembras de la descendencia fue la presencia de mayor agregación de núcleos e infiltración de células inflamatorias en el hígado de crías del DPN6 tratadas con LBPA o LBPF en comparación con la dosis alta respectivamente. En las crías macho también ocurrió lo mismo tras la exposición a LBPF. Esto podría deberse a que, tras la exposición perinatal, el feto se encuentra en el proceso de ontogenia tisular, siendo mucho más vulnerable a dicha exposición química, y en el DPN6 ya se observan alteraciones estructurales con agregación de núcleos e infiltración de células inflamatorias en el hígado. Por lo tanto, esto hace que el feto sea mucho más sensible y vulnerable al efecto de LBPA y LBPF que las ratas lactantes adultas.

En investigaciones recientes, se ha visto que la principal alteración histológica del hígado era una esteatosis microvesicular de leve a moderada en ratas tratadas con BPA entre los DPN 10-17 y entre los DPN 45-60 (Santoro et al., 2021). Otro estudio ha demostrado la susceptibilidad a padecer NAFLD en la edad adulta como consecuencia de la desregulación mitocondrial tras la exposición perinatal (Wei et al., 2011). Además la exposición perinatal al BPA contribuye al desarrollo de esteatosis por una función mitocondrial hepática alterada en descendientes machos a las 3, 15 y 26 semanas cuando son tratados postnatalmente con 40 µg/kg de BPA (Jiang et al., 2014). En otros estudios también se observaron daños hepáticos tras la exposición perinatal a LBPF (Maćczak et al., 2017; Sun et al., 2023). La exposición perinatal a BPF y otros análogos al BPA afectó al peso corporal de determinados órganos en las crías de rata macho y a la función ovárica en las hembras induciendo toxicidad reproductiva a dosis bajas (Kaimal et al., 2021). Además, se ha demostrado que BPA y BPF podrían desencadenar partos prematuros y preeclampsia en humanos, al activar el receptor P2X7 en células placentarias humanas y promover la activación del inflamasoma NLRP3 y apoptosis (Fouyet et al., 2021). La administración de BPF también aumenta los abortos espontáneos en madres gestantes de forma dosis-dependiente (Kaimal et al., 2021).

Por lo tanto, es muy importante comprender cómo puede afectar la exposición a dichas sustancias químicas durante el desarrollo fetal ya que es el período más crítico y vulnerable de la vida humana, además de en la infancia y la pubertad (Street & Bernasconi, 2020). Esta exposición podría causar un mayor riesgo de desarrollar enfermedades en la edad adulta debido a su limitada capacidad en este período de la vida para metabolizar y procesar estas sustancias químicas (Basak et al., 2020; Tucker et al., 2018).

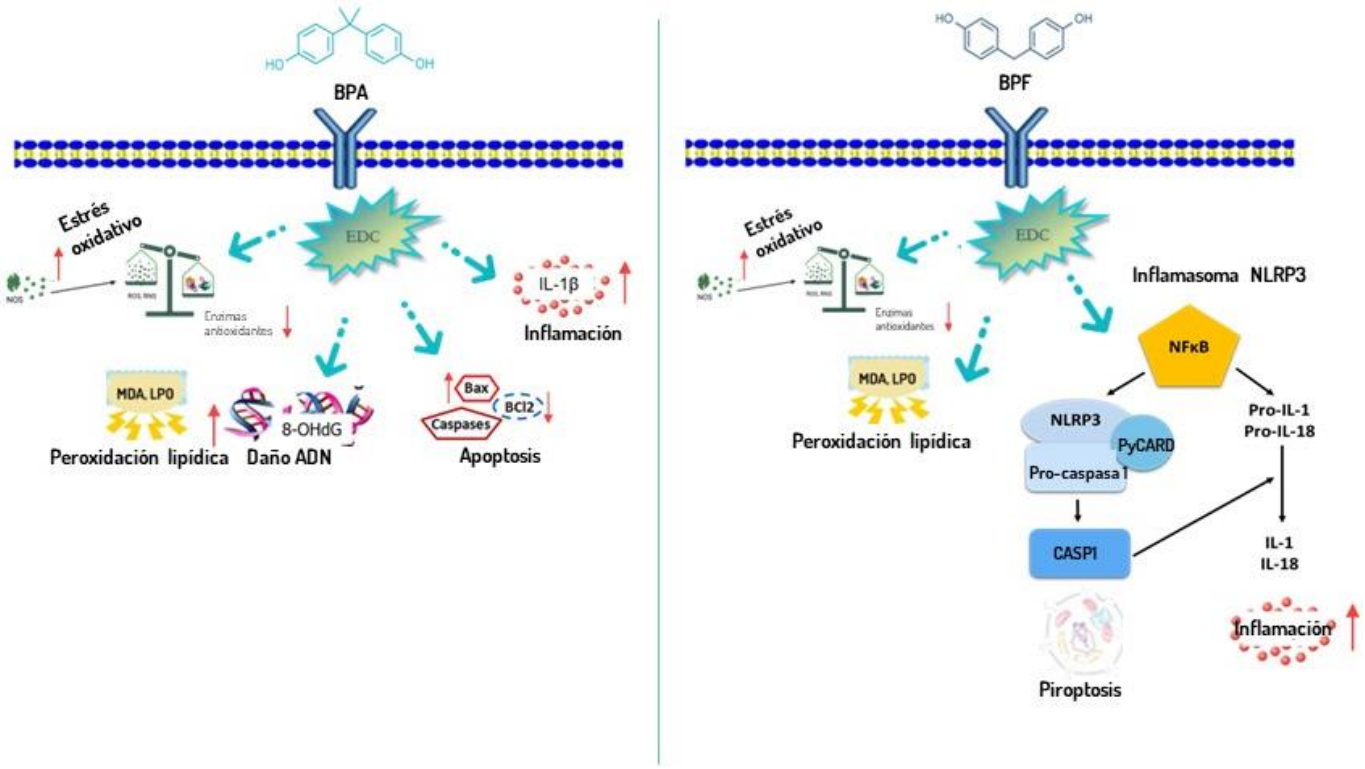


Figura 13. Resumen de los resultados obtenidos tras la exposición de BPA y BPF en las ratas lactantes y el efecto perinatal en la descendencia. Figura creada con CANVA.

6. EFECTOS A DOSIS BAJAS

Una característica notable de nuestros estudios es que a dosis altas de BPA y BPF, los efectos no son tan notables o significativos en muchos de los parámetros estudiados en comparación con el grupo control. Esto puede explicar que el BPA, como disruptor endocrino, y su análogo BPF, al igual que algunas hormonas, puedan seguir curvas NMDR, mostrando efectos más notables a dosis bajas que a dosis altas (Vandenberg et al., 2012). El sistema endocrino está configurado para responder a concentraciones muy bajas de hormonas y se puede observar una respuesta biológica máxima sin una alta ocupación del receptor de esta respuesta. Esto podría deberse al hecho de que los mecanismos de respuesta se saturan antes de que todos los receptores estén ocupados. También podría deberse a que existe un mayor grado de vulnerabilidad en el hígado frente a LBPA y LBPF en comparación con otros órganos, debido al metabolismo inicial de dichos

BP por el hígado precisamente (Mahdavinia et al., 2019; Moon et al., 2012; Pottenger et al., 2000).

Esto concuerda con la literatura donde se ha observado una relación no monotónica en ratas *Wistar* preñadas expuestas a BPA (50, 250 o 1250 µg/kg) y en sus crías después del destete (Schönfelder et al., 2002). Sólo la dosis más baja de 50 µg/kg de BPA produjo efectos como aumento del peso corporal, elevación de la insulina sérica y alteración de la tolerancia a la glucosa en las crías adultas. Sin embargo, en este estudio se expuso a las ratas tanto a dietas normales como ricas en grasas, lo que también podría desempeñar un papel en los mecanismos de respuesta. Las ratas expuestas perinatalmente a las dosis más altas no mostraron ninguno de los efectos adversos, independientemente de la dieta (Schönfelder et al., 2002). Aunque el proceso es difícil de interpretar, lo cierto es que ha sido constatado por muchos otros autores mostrando una forma dosis-respuesta no monotónica también tras la exposición a DEs (Ho et al., 2017; Xiao et al., 2021; Zhang, 2015).

7. OTROS ASPECTOS A TENER EN CUENTA CON LOS DEs

Al evaluar los efectos adversos de los BP y sustancias análogas, deben tenerse en cuenta los organismos adultos y el efecto perinatal en la descendencia, ya que pueden verse afectados de forma diferente debido a las distintas ventanas temporales y al grado de vulnerabilidad. El momento de la exposición a dichos BP puede determinar el resultado a largo plazo, ya que los puntos de exposición más tempranos tienden a ejercer un efecto más grave (Crain et al., 2008; Ikezuki et al., 2002; Jiang et al., 2014; Xia et al., 2014).

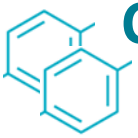
A su vez, es importante estudiar el efecto en ambos sexos, incluso en condiciones de exposición similares, pues se producen diferencias debido a la variabilidad en el metabolismo, almacenamiento y excreción de los xenobióticos (Caporossi & Papaleo, 2015). También es importante investigar en diferentes modelos animales; por ejemplo, estudios en roedores observaron una mayor vulnerabilidad a los efectos del BPA y otros análogos en hembras que en machos (Jardim et al., 2020; Rashid et al., 2009). Además, el hecho de que los niveles más bajos de BPA y BPF sean generalmente más eficaces que las dosis más altas es una cuestión muy destacable, como se ha descrito anteriormente; por tanto, estudiar el efecto a diferentes dosis también es relevante. Así, según Vanderberg et al. (2012), en los estudios sobre hormonas naturales y alteradores endocrinos son frecuentes los efectos más intensos a dosis bajas. Cuando se producen NMDR, los efectos a dosis bajas no pueden predecirse a partir de los efectos

observados a dosis altas.

Por otro lado, también es importante recalcar que los mecanismos de acción del BPA y el BPF varían, según la dosis, el tejido (tipo de célula y del estado del receptor hormonal de las células) y la etapa de exposición (Vom Saal & Vandenberg, 2021). Tienen la capacidad de imitar las acciones de los estrógenos alterando las respuestas celulares en los diferentes tejidos y mediante diferentes mecanismos de acción y a dosis muy bajas, al unirse a los ER con gran afinidad (Alonso-Magdalena et al., 2012; Lei et al., 2018), además de seguir vías no canónicas y activar receptores tiroideos y androgénicos.

Asimismo, debemos ser conscientes de que en nuestro día a día estamos expuestos no solamente a un DE sino más bien a un gran número de contaminantes. Aún no hay conocimiento suficiente de cómo estás múltiples exposiciones interaccionan dentro del cuerpo y pueden afectar conjuntamente a la salud, a lo que se une que no existe un consenso universal sobre la regulación de los DEs, debido a que, dadas sus propiedades particulares de toxicidad y la diversidad de estas entre los diferentes químicos, es muy difícil establecer un umbral seguro de exposición.

Por último, es fundamental seguir investigando para comprender el alcance y el efecto de la exposición prenatal a sustancias químicas potencialmente tóxicas a concentraciones muy bajas, típicas de la exposición ambiental (Vom Saal & Vandenberg, 2021) en otros órganos aparte del hígado, en cuanto a mecanismos de activación del inflamasoma y apoptosis.



CONCLUSIONES

1. La administración de BPA durante la preñez y la lactancia es capaz de inducir daño hepático tanto en ratas lactantes como en las hembras del DPN6 de su descendencia, con efectos más notables tras LBPA, indicando una relación dosis-respuesta no monotónica:
    1.1. LBPA indujo estrés oxidativo mediante la disminución de los niveles de las enzimas antioxidantes CAT, SOD, GR, GPx y GST y la alteración del sistema del glutatión (mayores niveles de GSSG), además de incrementar la peroxidación lipídica y el daño oxidativo en el ADN (mayores niveles de MDA, LPO y 8-OHdG).
    1.2. LBPA indujo estrés nitrosativo mediante altos niveles de NO en el plasma de las ratas lactantes y mayores niveles de expresión génica y proteica de iNOS y HO-1d junto al incremento de la citoquina proinflamatoria IL-1β en el hígado de ratas lactantes y en las hembras del DPN6 de su descendencia.
    1.3. LBPA favoreció la liberación del factor proapoptótico AIF y la activación de la vía intrínseca de la apoptosis con mayores niveles del componente proapoptótico de la familia Bcl-2 (Bax) y menores niveles de factores antiapoptóticos (Bcl-2, Bcl-XL).

2. La administración de BPF durante la preñez y la lactancia es capaz de generar estrés oxidativo hepático tanto en ratas lactantes como en ambos sexos de su descendencia en el DPN6, con efectos más notables tras LBPF, indicando una relación dosis-respuesta no monotónica:
    2.1. LBPF indujo estrés oxidativo mediante la disminución de los niveles de las enzimas antioxidantes CAT, SOD, GR, GPx y GST y alteración del sistema del glutatión tanto en el hígado de ratas lactantes como en su descendencia con mayor ratio GSSG/GSH en las crías hembras comparadas con los machos.
    2.2. LBPF incrementó la peroxidación lipídica con mayores niveles de MDA y LPO tanto en ratas lactantes como en ambos sexos de su descendencia en el DPN6.

3. La administración de BPF incrementa el estrés nitrosativo y favorece la activación del inflamasoma NLRP3 en el hígado de ratas lactantes con efectos similares tras la exposición perinatal en ambos sexos de la descendencia en el DPN6.
    3.1. LBPF incrementó el estrés nitrosativo con mayores niveles de expresión génica y proteica de iNOS y HO-1d en el hígado de ratas lactantes y en ambos sexos de su descendencia en el DPN6.

3.2. LBPF favoreció la activación de los componentes del inflamasoma NLRP3 (NLRP3, PyCARD y CASP1) promoviendo la liberación de citoquinas proinflamatorias (IL-1β, IL-18, IFN-γ y TNF-α) en el hígado de ratas lactantes como en ambos sexos de su descendencia.

Por todo ello, podemos concluir que la administración por vía oral de BPA y de BPF a dosis ambientalmente relevantes induce alteraciones proinflamatorias, prooxidantes y proapoptóticas que generan un daño hepático tanto en las ratas adultas lactantes como en su descendencia, cuando ésta se ve expuesta en la etapa perinatal y postnatal.

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ANEXOS

PUBLICACIONES OBTENIDAS DURANTE LA TESIS DOCTORAL

1. Publicaciones relacionadas con la tesis doctoral

Beatriz Linillos-Pradillo, Lisa Rancan, Sergio D. Paredes, Margret Schlumpf, Walter Lichtensteiger, Elena Vara, Jesús Á. F. Tresguerres. Low Dose of BPA Induces Liver Injury through Oxidative Stress, Inflammation and Apoptosis in Long–Evans Lactating Rats and Its Perinatal Effect on Female PND6 Offspring. **International Journal of Molecular Sciences, Feb 2023**, 24(5), 4585. doi:10.3390/ijms24054585.

Beatriz Linillos-Pradillo, Lisa Rancan, Julio García Murias, Margret Schlumpf, Walter Lichtensteiger, Jesús Á. F. Tresguerres, Elena Vara, Sergio D. Paredes. Oxidative stress increases in liver of lactating rats after BPF-low-dose exposure: perinatal effects in the offspring. **Scientific Reports, Jul 2023,** 13(1), 11229. doi:10.1038/s41598-023-38434-w

Beatriz Linillos-Pradillo, Sergio D. Paredes, María Ortiz-Cabello, Margret Schlumpf, Walter Lichtensteiger, Elena Vara, Jesús Á. F. Tresguerres, Lisa Rancan. Activation of NLRP3 Inflammasome in Liver of Long Evans Lactating Rats and Its Perinatal Effects in the Offspring after Bisphenol F Exposure. **International Journal of Molecular Sciences, Sep 2023**, 24, 14129 doi:10.3390/ijms241814129.

2. Otras publicaciones durante la tesis doctoral

Beatriz Linillos-Pradillo, Lisa Rancan, Elías Díaz Ramiro, Elena Vara, Begoña Artíñano, Javier Arias. Determination of SARS-CoV-2 RNA in different particulate matter size fractions of outdoor air samples in Madrid during the lockdown. **Environmental research**, **Apr 2021**, 195, 110863. doi:10.1016/j.envres.2021.110863.

Rafael Blanco-Domínguez, Raquel Sánchez-Díaz, Hortensia de la Fuente, Luis J Jiménez-Borreguero, Adela Matesanz-Marín, Marta Relaño, Rosa Jiménez-Alejandre, **Beatriz Linillos-Pradillo,** Katerina Tsilingiri, María L Martín-Mariscal, Laura Alonso-Herranz, Guillermo Moreno, Roberto Martín-Asenjo, Marcos M García-Guimaraes, Katelyn A Bruno, Esteban Dauden, Isidoro González-Álvaro, Luisa M Villar-Guimerans, Amaia Martínez-León, Ane M Salvador-Garicano, Sam A Michelhaugh, Nasrien E Ibrahim, James L Januzzi, Jan Kottwitz,

Sabino Iliceto, Mario Plebani, Cristina Basso, Anna Baritussio, Mara Seguso, Renzo Marcolongo, Mercedes Ricote, DeLisa Fairweather, Héctor Bueno, Leticia Fernández-Friera, Fernando Alfonso, Alida L P Caforio, Domingo A Pascual-Figal, Bettina Heidecker, Thomas F Lüscher, Saumya Das, Valentín Fuster, Borja Ibáñez, Francisco Sánchez-Madrid, Pilar Martín. A Novel Circulating MicroRNA for the Detection of Acute Myocarditis. **The New England journal of medicine, May 2021**, 384(21), 2014–2027. doi:10.1056/NEJMoa2003608.

Jesús Á. F. Tresguerres, Isabel Fernández-Tresguerres, José Viña, Lisa Rancan, Sergio D. Paredes, **Beatriz Linillos-Pradillo**, Elena Vara. Effects of GH on the Aging Process in Several Organs: Mechanisms of Action. **International journal of molecular sciences, Jul 2022**, 23(14), 7848. Review. doi:10.3390/ijms23147848.

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Lisa Rancan, **Beatriz Linillos-Pradillo**, Julia Centeno, Sergio D. Paredes, Elena Vara, Jesús A.F. Tresguerres. Protective Actions of Cannabidiol on Aging-Related Inflammation, Oxidative Stress and Apoptosis Alterations in Liver and Lung of Long Evans Rats. **Antioxidants, Sep 2023**, 12(10), 1837; doi:10.3390/antiox12101837.