Role of mitochondrial GSH on the hepatotoxicity induced by Bile Acids

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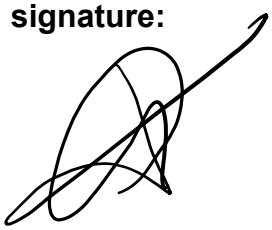

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Content

1. Abbreviations

18S	Small Subunit 18S rRNA
2OGC	2-Oxoglutarate Carrier
7D	7-Dehydroxycholesterol
ABC1	ATP-Binding Cassette Transporter 1
ACAT	Acyl-CoA Cholesterol Acyltransferase
BA	Bile Acid
BDL	Bile Duct Ligation
BSA	Bovine Serum Albumin
C4	7α-Hydroxy-4-Cholesten-3-One
CA	Cholic Acid
CDCA	Chenodeoxycholic Acid
Ct	Threshold Cycle
CYP	Cytochrome P450
CYP27A1	27-Hydroxylase
CYP7A1	Cholesterol 7α-Hydroxylase
CYP7B1	Oxysterol 7α-Hydroxylase
CYP8B1	Sterol 12α-Hydroxylase
DCA	Deoxycholic Acid
DCC	N,N' – Dicyclohexylcarbodiimide
DE	Desmosterol
DHCR24	24-Dehydrocholesterol Reductase
DHCR7	7-Dehydrocholesterol Reductase
DMSO	Dimethyl Sulfoxide
DNTB	5, 5'-Dithio-Bis-2-Nitrobenzoic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol-Bis (B-Aminoethyl-Ether)-N,N,N′,N′-Tetraacetic Acid
ER	Endoplasmic Reticulum
EtOH	Ethanol
FBS	Fetal Bovine Serum
FPP	Farnesyl Pyrophosphate
GCA	Glycocholic Acid
GRP78	Glucose-regulated protein 78
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Oxidized Glutathione
HMG-CoA	3-Hydroxy-3-Methylglutaryl-CoA
HMGR	3-Hydroxy-3-Methylglutaryl-CoA Reductase
Hmox-1	Heme Oxygenase 1
HP	(R, S)-3-Hydroxy-4-Pentenoate
HSD3B7	3β-hydroxysteroid dehydrogenase
ICAM-1	Intercellular Adhesion Molecule 1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
Insig	Insulin-Induced Gene 1
IPP	Isopentenyl Pyrophosphate
LCA	Lithocholic Acid
Ly6c	Lymphocyte Antigen 6 Complex
MBD	Mannitol – Buffer – Digitonin
mGSH	Mitochondrial Glutathione
MIM	Mitochondrial Inner Membrane
MOM	Mitochondrial Outer Membrane
mRNA	Messenger RNA
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
PBC	Primary Biliary Cholangitis
PBS	Phosphate Buffer Saline
PI	Propidium Iodide
PMH	Primary Mouse Hepatocytes
PSC	Primary Sclerosing Cholangitis
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RT	Room temperature
Scap	Sterol-Sensitive SREBP Cleavage Activating Protein
SEM	Standard Error of The Mean
Spp1	Secreted Phosphoprotein 1
SREBP2	Sterol Regulatory Element Protein 2
StARD1	Steroidogenic Acute Regulatory Protein 1
START	StAR-related lipid-transfer
STAT	Signal Transducer and Activator of Transcription
TCA	Taurocholic Acid; Trichloroacetic acid
TIC	Tumour Initiating cells
TNB	5 – thionitrobenzoic acid
TβMCA	Tauro-β-Muricholic Acid
WT	Wild Type

αMCA	α-Muricholic Acid
βMCA	β-Muricholic Acid
γ-GCS	γ-glutamylcysteine synthetase

2. Abstract

Cholestatic liver diseases can be induced by diverse reasons including genetic defects, drug toxicity, hepatobiliary malignancy, or bile duct obstruction. Acute and chronic cholestasis promotes hepatocellular injury, bile duct proliferation, fibrosis, cirrhosis, and eventually liver failure.

Diminished bile flow during cholestasis provokes the hepatic accumulation of products normally excreted into bile, such as cholesterol and bile acids (BAs). Some BAs, especially hydrophobic ones, can induce cell injury in hepatocytes. Cholesterol, a highly regulated molecule found in the lipid bilayer of cells, is the precursor of BAs mainly in the classical pathway, which is modulated by CYP7A1 and CYP8B1. Besides, cholesterol can be transported to the mitochondria by StARD1 protein, functioning as an additional source of BAs in the alternative pathway, which is controlled by CYP27A1 and CYP7B1.

Moreover, StARD1 overexpression in cholestatic liver diseases have been described. The up-regulated protein leads to the overload of cholesterol in the mitochondrial inner membrane, changing its physicochemical properties and losing its plasticity and permeability. Membrane rigidity can affect the transport of glutathione (GSH) to the mitochondrial matrix, where serves as a cofactor for several antioxidant and detoxifying enzymes.

In addition, impaired bile flow promotes the accumulation of toxic BAs in hepatocytes, causing oxidative stress, mitochondrial damage, and hepatic impairment. Given the high production of reactive oxygen species in this process and the inability to transport glutathione into mitochondria due to membrane stiffness, liver damage occurs leading to the pathophysiology of the disease.

Although it has long been believed that cholestatic liver damage was due to a direct effect of BAs toxicity, recent studies have shown that an inflammatory response might be the leading cause of injury.

This master's thesis aims to clarify the effect of BAs on primary hepatocytes and to elucidate the role of glutathione in cholestatic pathologies, such as primary biliary cholangitis and primary sclerosing cholangitis.

After exposing primary mouse hepatocytes to high concentrations of BAs *in vitro*, as cholestasis-like conditions, it has been shown that BAs do not induce cell death directly. The depletion of mitochondrial GSH sensitizes the hepatocytes to a second hit and predisposes

them to cellular damage via oxidative stress and the release of pro-inflammatory cytokines involved in the inflammatory response.

Key words: *alternative pathway, bile acids, cholestasis, cholesterol, classical pathway, glutathione, inflammation, liver, primary mouse hepatocyte.*

3. State of the art

Cholesterol is an essential structural component of mammalian cells that is obtained by *de novo* synthesis (Figure 1) or from the diet by serum lipoproteins.

This sterol is secreted in discrete domains, called lipid rafts, in close association with glycosphingolipids, determining the physical properties of the membrane, like permeability or fluidity, by membrane proteins modification (Garcia-ruiz et al., 2020).

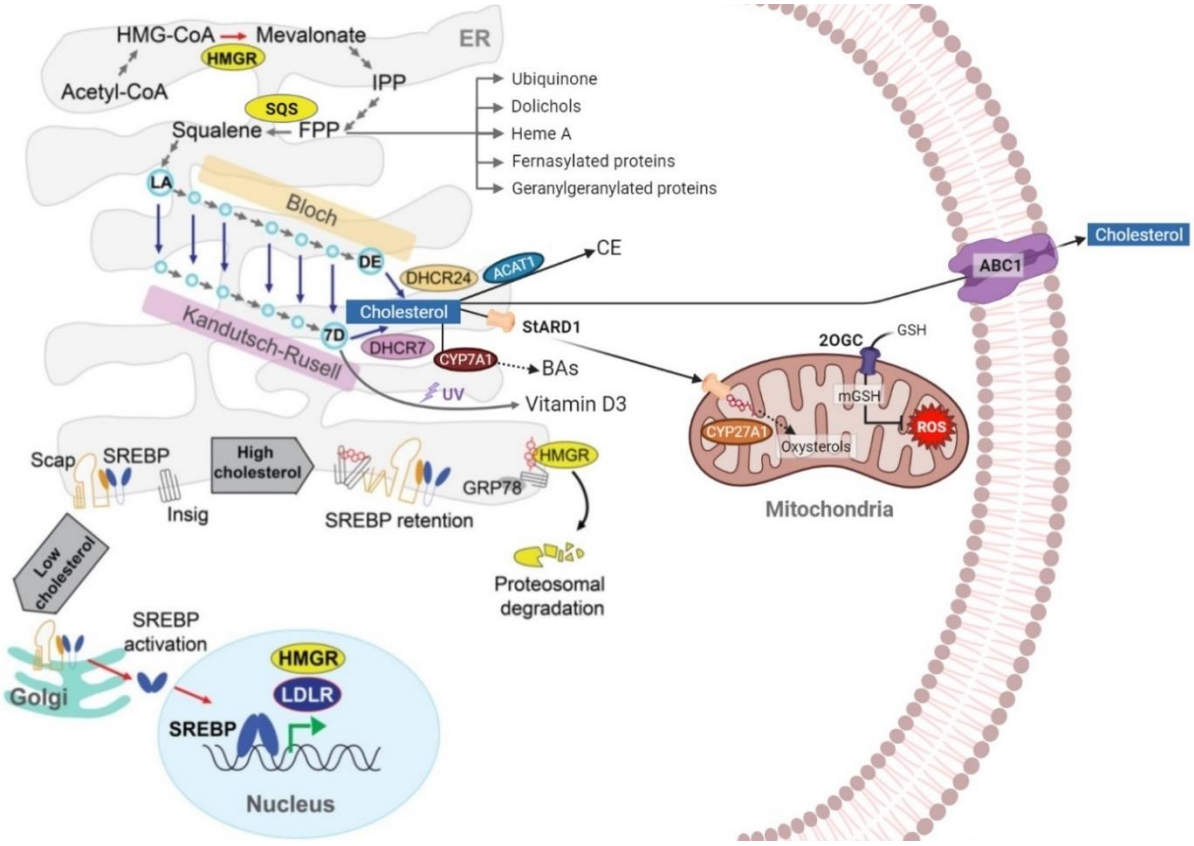

Figure 1. *De novo* cholesterol synthesis and mitochondrial cholesterol and glutathione trafficking. Cholesterol synthesis occurs via the mevalonate pathway. This process begins with acetyl-CoA, which once is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), it is irreversibly turned to mevalonate by the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). Mevalonate is catalysed to farnesyl pyrophosphate (FPP) with a previously isopentenyl pyrophosphate (IPP) production. From FPP, squalene synthase (SQS) continue the cholesterol production through the formation of squalene and subsequently lanosterol (LA), or it can give rise to ubiquinone, dolichols, heme A or isoprenoids, which modify proteins through farnesylation or geranyl generation. Desmosterol (DE) or 7-dehydroxycholesterol (7D) are formed thanks to the Bloch and Kandutsch-Russel pathways, respectively. 7D can be modified by UV light to Vitamin D3. By 24-dehydrocholesterol reductase (DHCR24) and 7-dehydrocholesterol reductase (DHCR7) action, DE and 7D give rise to cholesterol. Once synthesized, cholesterol can be esterified (CE) by acyl-CoA cholesterol acyltransferase (ACAT1), exported from cell trough cholesterol pump (e.g., ATP-binding cassette transporter 1 (ABC1)), transported to mitochondrial inner membrane by steroidogenic acute regulatory protein 1 (StARD1) for further metabolism into oxysterols or bile acids (BAs) by 27-hydroxylase (CYP27A1). Sterol-dependent feedback controls HMGR levels inducing or not its degradation through an Insulin – induced gene 1 protein/Glucose-regulated protein 78 (Insig/GRP78)-dependent mechanism and the retention of sterol regulatory element protein 2 (SREBP2), which in turn is modulated by sterol-sensitive SREBP cleavage activating protein (Scap). To regulate mitochondrial cholesterol-mediated reactive

oxygen species (ROS) generation, glutathione (GSH) is translocated into mitochondrial matrix (mGSH) trough 2-oxoglutarate carrier (2OGC). *Adapted from Arenas et al., 2017 and Garcia-Ruiz et al., 2020.*

Although cholesterol is predominantly present at the plasma membrane, where regulates a plethora of signalling pathways, once it reaches the endolysosomes, it is distributed to intracellular organelles.

Precisely, mitochondrial cholesterol transport involves lipid transfer proteins located at membrane contact sites or cytosolic diffusible lipid transfer proteins, which include the signal transducer and activator of transcription (STAT) protein family. Among their members, steroidogenic acute regulatory protein 1 (StARD1) is the principal supplier of mitochondrial cholesterol (Figure 1).

StARD1 is a highly conserved nuclear – encoded phosphoprotein synthesised in the cytosol by requirement. It is translocated to mitochondria forming part of transduceosome at mitochondrial outer membrane (MOM) or by being involved in steroidogenesis at mitochondrial matrix (Conde et al., 2021).

Its structure contains a C-terminal functional StAR-related lipid-transfer (START) domain, responsible for lipid binding, and a N-terminal leader sequence to target it to mitochondria. The resulting conformation creates a hydrophobic cavity that can bind and accommodate one molecule of cholesterol at a time and deliver it to mitochondrial inner membrane (MIM).

Despite content of cholesterol in this organelle is only 3% of the total cellular cholesterol, this small amount has a fundamental physiological role in the liver, serving as a precursor for bile acids (BAs) synthesis in hepatocytes (Figure 2).

BAs are detergent molecules composed by water, biliary salts, biliary pigments, some lipids, and bicarbonate. They emulsify lipids to facilitate digestion in the duodenum and allow the absorption of steroids and lipid-soluble vitamins (Chiang & Ferrell, 2018). Besides, BAs are capable of control gut bacteria overgrowth and protect intestinal barrier function.

Their production takes place via two routes: the classical or neutral and the alternative or acidic pathways (Figure 2).

Classical biosynthesis starts in endoplasmic reticulum (ER) and is responsible for synthetize 80% of BAs in human livers. It is regulated by cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1).

In contrast, alternative via, which depends on mitochondrial cholesterol transport by StARD1, and 27-hydroxylase (CYP27A1) and oxysterol 7α-hydroxylase (CYP7B1) action (Li & Chiang, 2014), is only a minor component of BAs production.

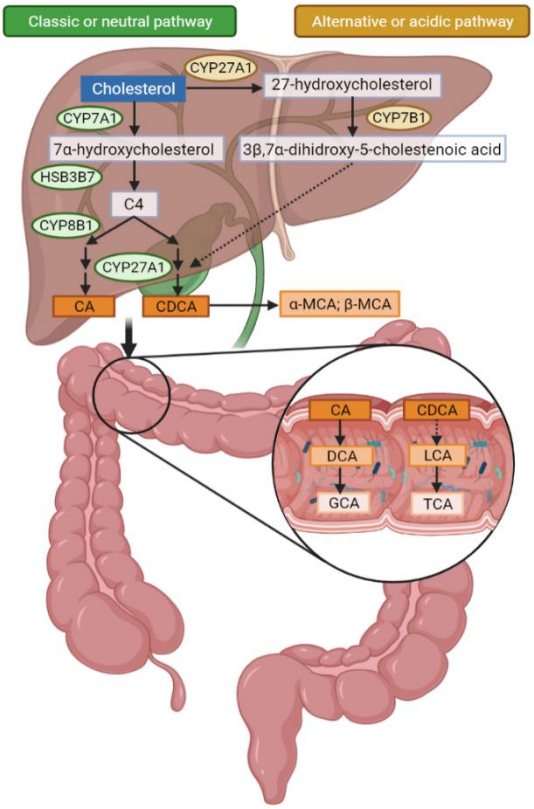

Figure 2. Bile acids synthesis pathways. Bile acids are made from cholesterol through the classic route and, in a minor rate, by alternative pathway. Classic or neutral pathway starts with 7α-hydroxycholesterol conversion from cholesterol by the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) in the endoplasmic reticulum (ER). Then, 7α-hydroxy-4-cholesten-3-one (C4) is formed thanks to 3β-hydroxysteroid dehydrogenase (HSD3B7). At this point, primary bile acids are synthesised: sterol 12α-hydroxylase (CYP8B1) lead the route to cholic acid (CA) production meanwhile chenodeoxycholic acid (CDCA) is formed from C4 without its action. Both primary bile acids are finally catalysed by 27-hydroxylase (CYP27A1). In mitochondria, alternative pathway begins with 27-hydroxycholesterol formation by CYP27A1. This metabolite is transformed to 3β,7α-dihydroxy-5-cholestenoic acid by oxysterol 7α-hydroxylase (CYP7B1), and sometimes it is converted to CDCA. In the large intestine, bacteria can form deoxycholic acid (DCA) and lithocholic acid (LCA) from CA and CDCA, respectively. Primary and secondary bile acids can be conjugated with glycine (GCA) or taurine (TCA). CDCA also acts a precursor for α – muricholic (α-MCA) and β – muricholic acids (β-MCA), specially find at high concentrations in mice. *Adapted from Nuño-lámbarri & Uribe, 2016 and Chiang & Ferrell, 2018.*

As BAs pool size is highly regulated, the alternative route is only activated if homeostasis is compromised, producing mainly hydrophobic BAs. The increase of hydrophobic components leads to a cholestatic state (Nuño-lámbarri & Uribe, 2016) in which bile flow is reduced or disrupted. The resulting cholesterol and BAs accumulation in hepatocytes promotes an inflammatory state as well as an increment of mitochondrial oxidative stress due to their high toxicity (Nuño-lámbarri et al., 2016).

To discharge toxic anionic compounds produced in this process, a tripeptide called glutathione (GSH) is synthesised in the cytosol and transported to mitochondria (Figure 1 and 3). In healthy cells and tissues, up to 98% of the total GSH pool is in the reduced form whereas in liver diseases, its oxidized state (GSSG) takes priority (Figure 3) (Marí et al., 2009). Thus, an increased GSSG-to-GSH ratio is indicative of oxidative stress (Grattagliano et al., 2014; Nuño-lámbarri et al., 2016).

In cholestatic diseases, such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), there is a slow and progressive destruction of the small bile ducts. The ensuing liver damage can lead to scarring, fibrosis and eventually cirrhosis.

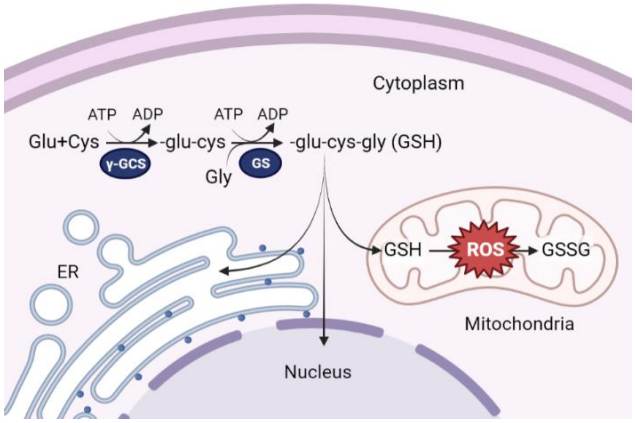

Figure 3. GSH synthesis, compartmentation, and ROS blockage. Glutathione (GSH) is a tripeptide (Glu – Cys – Gly) synthesized in cytoplasm by two ATP-dependent enzymes: γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS). Lately, GSH is distributed in the endoplasmic reticulum (ER), nucleus, and mitochondria, where executes its protective function inhibiting reactive oxygen species (ROS). *Adapted from Grattagliano et al., 2014 and Marí et al., 2009.*

Although numerous studies have proposed that liver damage in cholestasis is due to cell death caused by direct cytotoxic effect of BAs, recent publications have hypothesised that activation of the inflammatory response is the primary cause of hepatic injury (Cai & Boyer, 2021; Pastor & Collado, 2000; Zhang et al., 2013).

According to data collected, the aim of this Master's thesis is to determine *in vitro* the contribution of main BAs accumulated in cholestasis and the importance of GSH, providing a new insight into the pathophysiology of cholestatic diseases.

4. Hypothesis

Previous studies from our laboratory show that genetically modified mice (StARD1$^{\Delta Hep}$) subjected to bile duct ligation (BDL) reduce an almost 50% BAs accumulation, ameliorating liver injury but not disappearing (Conde et al., unpublished data). Therefore, we hypothesise that BAs do not exert a direct effect on cell death, but that damage occurs through the activation of signalling pathways, with mitochondrial GSH (mGSH) playing a crucial role in this process.

5. Objectives

The objectives are:

1. To analyse cell death, and inflammatory and stress gene expression in primary mouse hepatocytes (PMH) from wild type (WT) mice with Regular Chow Diet exposed to high concentrations of BAs.
2. To study the role of glutathione in these events, depleting mGSH using (R, S)-3-Hydroxy-4-Pentenoate (HP).

6. Methodology

6.1 Chemicals and Reagents

Compounds needed to elaborate HANKS solution (Annex 1, Table 1), Phosphate Buffer Saline (PBS), Trypan Blue, BAs and their vehicles for reconstitution, digitonin compartmentalization (Annex 2), GSH standards (Annex 3) and Ellman (Annex 3, Table 1) and Recycling Assay (Annex 3, Table 2) used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA).

Dulbecco's Modified Eagle Medium F-12 (DMEM:F12) and supplements (Annex 1, Table 2) were purchased from Gibco except Hepes pH 7,4 (Sigma Aldrich, St. Louis, MO, USA). Trypsin – EDTA for dissociation PMH monolayers was obtained from Invitrogen.

All primers used (Annex 6, Table 3) are purchased from Invitrogen (USA) except the housekeeping gene, which was Small Subunit 18S rRNA (18S; Metabion International AG).

6.2 Animal model

C57BL/6J male mice (8–10 weeks old) were purchased from Charles River Laboratories (USA) and were maintained in pathogen-free conditions with controlled temperature and humidity on a 12h light-dark cycle in the animal care facility at the Medical and Health Sciences School of Universitat de Barcelona. All mice were fed with a Regular Chow Diet (Teklad Global Rodent Diet, Harlan Laboratories, Madison, WI) and water *ad libitum*.

The experimental protocols used were approved and performed in accordance with the Animal Care Committee of the University of Barcelona and all procedures involving animals and their care were approved by the Ethics Committee of the University of Barcelona following national and European guidelines for the maintenance and husbandry of research animals.

At the time of sacrifice, animals were anaesthetised and exsanguinated (Annex 1); the liver was harvested and processed for subsequent analysis, removing gallbladder.

6.3 PMH isolation and culture

PMH were isolated from 8–10-week-old male WT mice by two-step collagenase perfusion (Annex 1). The viability was >90% as assessed by Trypan Blue exclusion. Hepatocytes were seeded at concentration of $3'5x10^5$ cells/ml in a 6 or 12 - well plate with the Ham's F-12/Dulbecco's modified Eagle's basal hepatocyte growth medium supplemented with 10% fetal bovine serum (FBS) (Isolation Medium). After a 3h attachment, the medium was replaced

to a serum-free basal hepatocyte growth medium (Maintenance medium) (Annex 1, Table 2). Culture plates were precoated with rat-tail collagen in order to ensure hepatocyte adhesion and once seeded, plates were incubated at 37ºC with 5% CO_2.

6.4 Treatment

PMH keep their phenotype for 24-48h after isolation (Ríos-López et al., 2020), so treatments were done during this time to avoid artifacts.

PMH were exposed to a mixture of BAs containing 200 µM β – Muricholic acid (βMCA), 1mM Tauro – β – Muricholic acid (TβMCA) and 500 µM Taurocholic acid (TCA) as these were the BAs found in concentrations >50 µM at 6h in mice subjected to BDL *in vivo* (Zhang et al., 2013). Moreover, to abolish mGSH levels (York, 1993), cells were also exposed during 5 minutes to HP and then washed with PBS. Finally, a combination of BAs and HP were added to reproduce the physiological situation occurring in cholestasis, resulting 4 experimental groups: Control, BA, BA with HP and HP.

All conditions were analysed at 6 and 12h post-treatment in order to determine time – dependent changes in the time-range with highest BAs expression (Zhang et al., 2013).

βMCA and TCA were diluted with ethanol (EtOH) while TβMCA was reconstituted with dimethyl sulfoxide (DMSO) as manufacturer's indicated.

6.5 GSH measurement

Cellular (total), cytosolic and mitochondrial GSH levels, previously fractionated by digitonin permeabilization (Annex 2) from a pool of 3 – well isolated PMH, were analysed using the enzymatic recycling method (Annex 3).
Measurement of the absorbance at 412 nm by spectrophotometer (Beckman Coulter, DU800) provided an accurate estimation of total GSH in the sample.

6.6 Protein Quantification

With the aim of correcting GSH levels by milligram of protein, protein concentration was determined using Quick Start Bradford Protein Assay (Bio-Rad, 500-0201). Assay was performed in a 96-well plate with triplicates of each sample and spectrophotometer plate reader was set at 595 nm (Annex 4).

6.7 Cell viability

Viability was determined by Höechst/Propidium iodide (PI) staining (Annex 7). Formalin 10% was used to fix cells and mounting media (Fluoromont, Sigma) was added. Digital images were taken in a fluorescence microscope (Olympus BX-41) and nucleus of alive and death cells were counted by ImageJ 1.53c (Fiji Software).

6.8 RNA extraction

We used a TRIzol-chloroform extraction (Invitrogen) to maintain the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization (Annex 5).

RNA quantification was performed at absorbance of 260-280 nm with NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 1000).

6.9 qRT-PCR

The mRNA levels of proinflammatory genes in cultured hepatocytes were determined by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) using the SensiFAST™ SYBR® No-ROX Kit (Bioline) following the manufacturer's instructions (Annex 6). Each reaction was run in triplicate to determine the threshold cycle (Ct) for each mRNA.

Pro-inflammatory and oxidative stress markers studied were interleukin 1β (IL-1β), interleukin 6 (IL-6), heme oxygenase (Hmox-1), secreted phosphoprotein 1 (Spp1), lymphocyte antigen 6 complex (Ly6c) and intercellular adhesion molecule 1 (ICAM-1). As a housekeeping gene, 18S was analysed.

6.10 Statistical Analysis

Data are presented as mean ± SEM for at least 7 different animals and each experiment was carried out in triplicates. Comparisons between more than 2 groups were performed using the One-way analysis of variance (ANOVA) with a Bonferroni's multiple comparison post-test. GSH results were analysed with Paired T-Student test. The criterion for significance was set at $p<0.05$. Statistical analyses were performed using GraphPad Prism version 9.2.1 (GraphPad Software Inc).

7. Results

To establish the impact *in vitro* of the three main BAs (βMCA, TβMCA and TCA) observed in mice subjected to BDL operation *in vivo*, PMH were exposed to a mixture of them mimicking pathological levels during 6 and 12h (Garcia-ruiz et al., 2020; Zhang et al., 2013) in the presence and absence of HP to deplete the antioxidant mGSH level in hepatocytes.

As the ability of BAs to cause cell damage or even death of hepatocytes was questioned, cell viability was analysed by Höechst/PI staining (Table 1, Figure 4). No significant differences were observed between treatments (Figure 4C, 4D), indicating that exposure to BAs or depletion of GSH levels did not induce an apoptotic or necrotic state. Vehicles were analysed and no morphological evidence of injury was observed (Annex 8, Figure 1).

Höechst/PI			
Time (h)	**Condition**		**Viability ± SEM (%)**
6	Control		96.87±0.01
	BA	βMCA and TCA	97.20±1.32
		βMCA, TβMCA and TCA	95.66±3.91
	BA+HP	βMCA, TCA and HP	92.12±1.40
		βMCA, TβMCA, TCA and HP	86.65±3.35
	HP		92.45±1.16
12	Control		99.65±0.01
	BA	βMCA and TCA	96.58±1.32
	BA+HP	βMCA, TCA and HP	92.71±1.40
	HP		98.78±1.16

Table 1. Viability analysis by Höechst/PI double fluorescence staining of live PMH. PMH were exposed to 4 conditions during 6 (n=3) or 12h (n=2): Control, BA, BA+HP and HP. BA includes 2 subgroups: PMH submitted to βMCA and TCA or PMH exposed to βMCA, TβMCA and TCA. Data are expressed as the mean ± SEM (%).

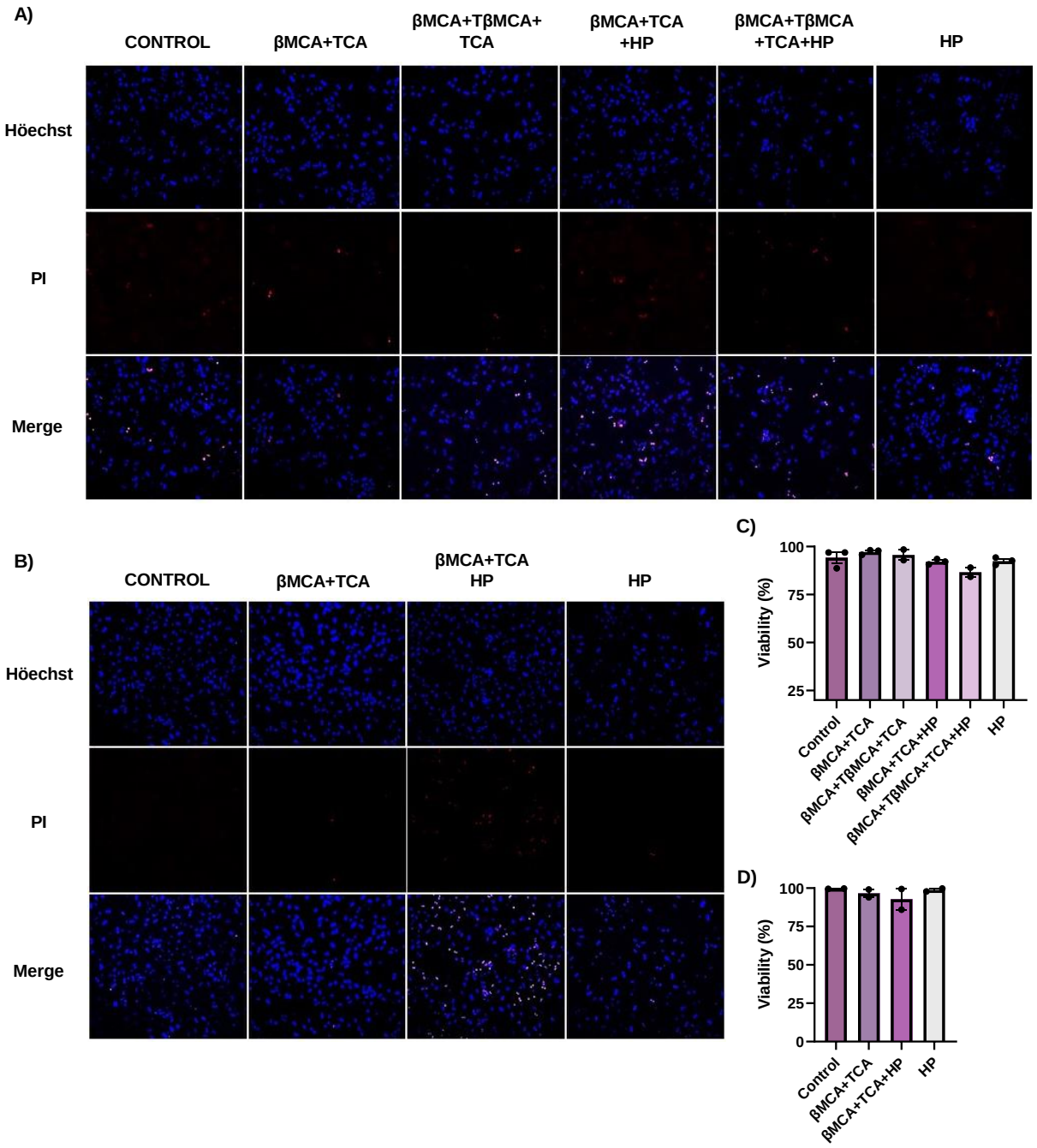

Figure 4. Viability and toxicological analysis by Höechst/PI double fluorescence staining of live PMH. Cells were treated with a mixture of bile acids (BAs), HP, or combination of both during 6 (n=3) or 12h (n=2). The blue fluorescence (top panel) indicates Höechst, and the red fluorescence (middle panel) is a consequence of PI accumulation in death cells. Höechst fluorescence imaging and cell viability quantification were performed after 6h (A, C) and 12h exposure (B, D) at 20x. Data are expressed as the mean ± SEM.

In cholestatic diseases, BAs cause oxidative stress (Cai & Boyer, 2021).To simulate this state, cells were treated with HP, which forms adducts specifically with mGSH and depletes antioxidant levels. As it can be seen in Figure 5A, 5E and 5I, HP had an immediate effect on hepatocellular (55.83 nmol/mg protein), cytosolic (23.35 nmol/mg protein) and mitochondrial

(0.89 nmol/mg protein) pool of GSH in PMH in comparison with control (234.86, 215.96 and 23.91 nmol/mg protein, respectively). Although cell exposure to BAs mixture did not exert any effect on GSH pools, the combination of BAs and HP magnified the reduction exerted by HP in total (24.31 nmol/mg protein), cytosolic (18.94 nmol/mg protein) and mitochondrial (0.62 nmol/mg protein) compartment.

Comparing controls at 0 (234.86 nmol/mg protein), 6 (107.4 nmol/mg protein) and 12h (94.24 nmol/mg protein), it was observed that cellular GSH levels decreased as the culture time increased (Figure 5D). Same effect was observed when PMH was exposed to BAs (255.57, 103.1 and 92.93 nmol/mg protein, respectively) and between compartments (Figure 5D, 5H, 5L). Moreover, PMH submitted to HP or BAs with HP, GSH levels at 6 (Figure 5B, 5F, 5J) and 12h (Figure 5C, 5G, 5K) showed a GSH recovery in comparison with basal analysis. Vehicles did not induce any change between conditions (Annex 8, Figure 2).

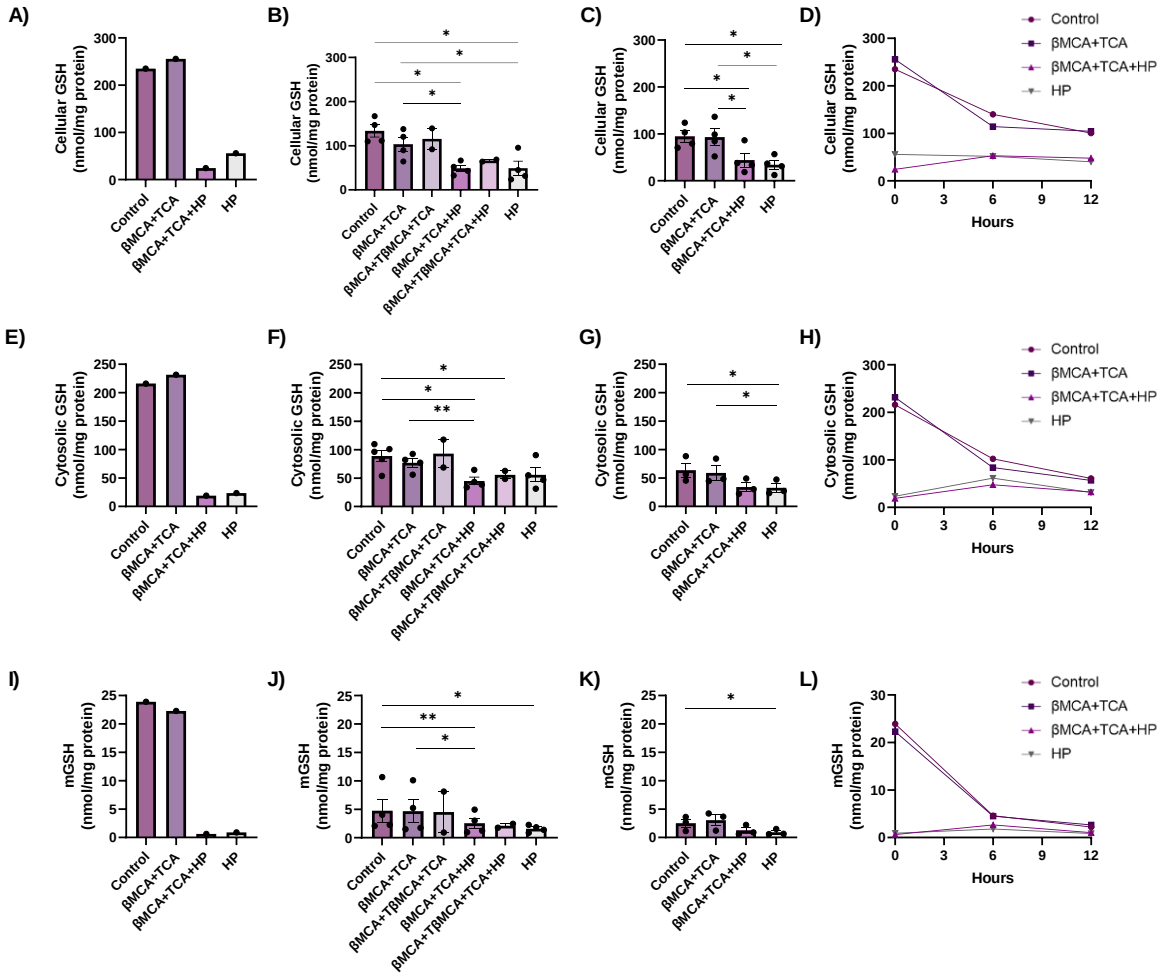

Figure 5. GSH levels. Cellular (total), cytosolic and mitochondrial GSH were measured at 0h (A, E, I; n=1), 6h (B, F, J; n=6) and 12h (C, G, K; n=4) after treatment. Temporal changes in GSH levels were plotted (D, H, L). Data are expressed as the mean ± SEM and p-values correspond to p<0.05 (*) and p<0.01 (**).

As previous studies showed that inflammatory markers were enhanced by BAs exposure in tumour initiating cells (TICs) (Conde de la Rosa et al., 2021), inflammatory and oxidative stress genes such as IL-1β, IL-6, Spp1, Ly6c, ICAM-1 and Hmox1 were analysed in our experimental design (Figure 6).

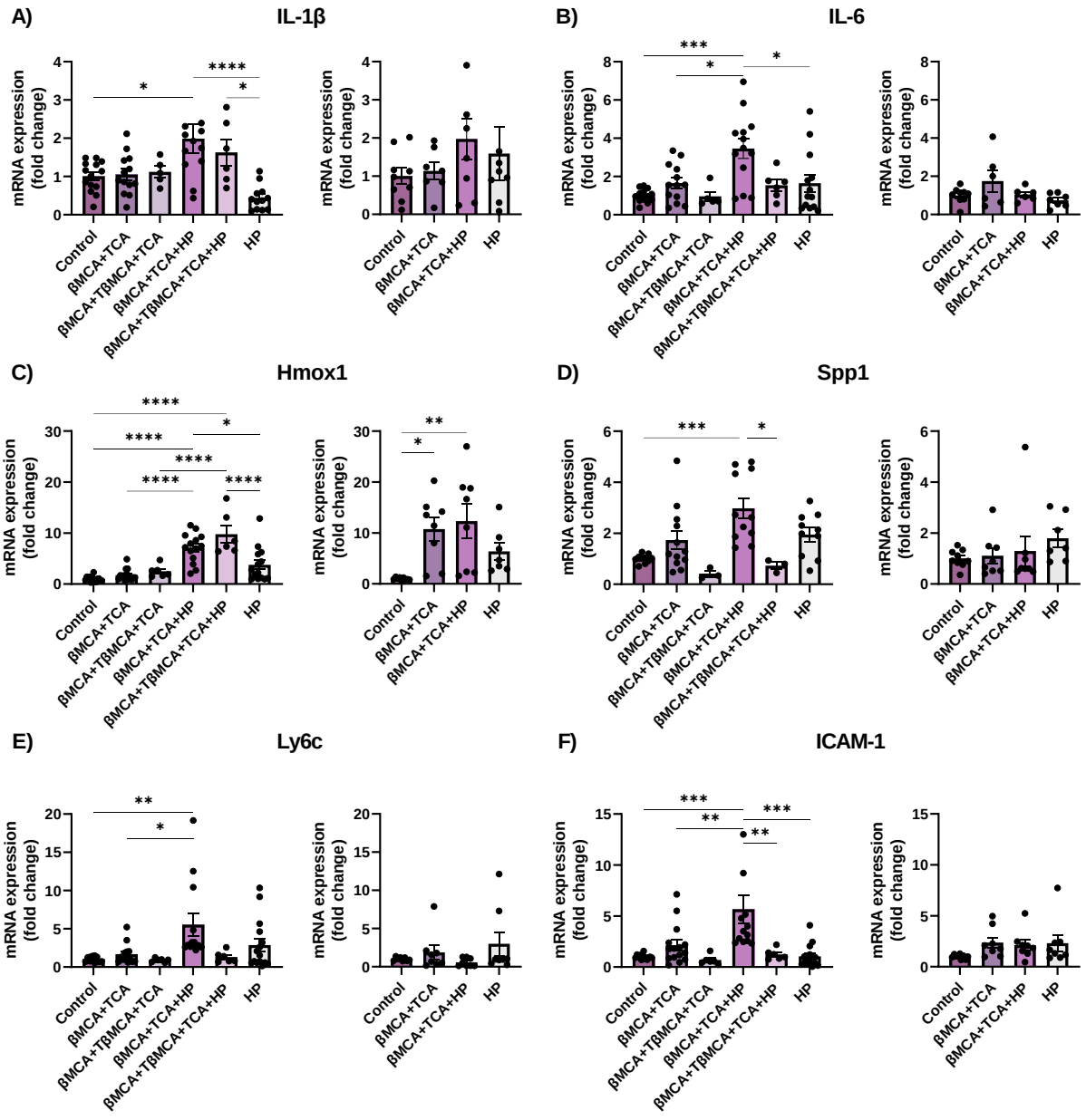

Figure 6. Analysis of the inflammatory and oxidative stress markers profile. Levels of mRNA expression of proinflammatory (A, B, D – F) and oxidative stress (C) genes were analysed by RT-PCR at 6h (right panels; n=15 from 5 PMH's isolation) and 12h (left panels; n=9 from 3 PMH's isolation). Data are expressed as the mean ± SEM.

After 6h exposure (see right panels of Figure 6), no changes were observed when PMH were cultured with BAs. In contrast, the combination of BAs and HP produced a significantly

increment of pro-inflammatory genes such as IL-1β (2 – fold) and IL-6 (3.5 – fold), Spp1 (3.5 – fold), Ly6c (5 – fold) and ICAM-1 (5 – fold), indicating the importance of depleting mGSH level to sensitize hepatocytes to the second hit of BAs exposure.
Only significantly changes were observed in inflammatory cytokines IL-1β and IL-6 (2 – fold each one) at 12h (see left panels of Figure 6A, 6B).

The oxidative stress marker Hmox1 raised it levels 5 – fold when HP was added, and this effect was duplicated with a mixture of BAs and HP at both timings (Figure 6C).

Spp1, Ly6c and ICAM-1 showed a significantly repressive effect when TβMCA was part of BAs mixture (Figure 6D, 6E, 6F). Besides, IL-6 showed similar tendency (Figure 6B). As TβMCA were diluted in DMSO, which is a potent scavenger, PMH were also exposed to vehicles and no differences were observed between experimental groups (Annex 8, Figure 3).

8. Discussion

Cholestasis is a syndrome characterised by an impairment of BAs formation or secretion that results in the accumulation of bile in the liver and circulation (Jüngst & Lammert, 2013), leading to fibrosis, cirrhosis and sometimes, liver failure.

Underlying mechanism of liver injury have not been yet elucidated in cholestatic diseases. Previous studies postulated that the accumulation of BAs in the liver, specially hydrophobic ones, exerted a cytotoxic effect on hepatocytes and consequently, cell death. However, recent publications deny this hypothesis and speculate about an inflammatory response as a main etiology of liver injury (Cai & Boyer, 2021).

In this context, our two main objectives have been to find out what effect BAs executed on liver injury and how mGSH levels contribute to the development of cholestasis. To achieve these purposes, PMH were exposed to 4 different conditions in order to analyse direct effect of BAs (BA condition), the role of mGSH (HP addition) and to mimic physiological state in cholestasis (BA and HP combination).

As described above, mixture of BAs was based on βMCA, TβMCA and TCA due to their high expression in mice subjected to BDL surgery and all conditions were analysed at 6 and 12h in accordance with findings described at Zhang et al., 2013.

Höechst/PI staining was used to determine direct cytotoxicity of BAs. As a result, exposure to BAs did not promote cell death despite HP addition, indicating that no direct effect on cell death was exert even if mGSH levels were low.

In line with previous findings linking BAs accumulation in the liver with mitochondria-mediated ROS generation and impairment in antioxidant defence (Cai & Boyer, 2021; Grattagliano et al., 2014), data obtained about cellular, cytosolic, and mGSH showed a decrement with HP addition. This fact was emphasised by the combination of HP with the BAs mixture. Also, GSH content declined earlier and at a greater extent in mitochondria than in the cytosolic compartment, pointing to selective mitochondrial damage and supporting that disruption in GSH homeostasis was an important contributor to BAs mediated hepatocellular damage.

According to other studies suggesting that most of the injury is due to an inflammatory environment and not directly by BAs, the exposure of PMH to BAs mixture did not cause cell death but upregulated pro-inflammatory markers such as Il-1β, IL-6, Hmox-1, Spp1, Ly6c and ICAM-1 when mGSH was depleted. All these cytokines, chemokines, adhesion molecules, and oxidative stress proteins, that influence immune cell levels and function in the liver, were the key factors in inflammatory pathways activation trough NF-κB activation (Cai & Boyer, 2021).

However, the dampening effect observed in some genes when TβMCA was added to the mixture is still not understood. A possible target-dependence is suspected as vehicle studies showed no alteration.

Analysing all the data together, we can conclude that the loss of antioxidant capacity due to low levels of mGSH, sensitises hepatocytes to BAs exposure. Combining the two hits, hepatocytes activate inflammatory signalling pathways that leads cell injury.

9. Conclusions

The pathophysiology of liver disease is due to a complex set of cellular alterations. Thus, in each mechanism there is a wide range of molecules involved in liver damage.

Although it was already known that mitochondrial damage and reduced antioxidant capacity occurred in cholestasis, the role of mGSH had not been studied in these diseases (Jüngst & Lammert, 2013; Zollner & Trauner, 2008).

Those studies that proposed that BAs killed hepatocytes through their direct cytotoxic/detergent effect or through induction of apoptosis, usually did not use pathological concentrations of BAs or the combination performed was not representative of cholestasis (Cai & Boyer, 2021).

This work not only disproves this hypothesis, but also demonstrates the strong involvement of mitochondrial antioxidant power in the development of hepatic disease. Moreover, it supports the current idea that the resulting inflammation is the main cause of liver damage.

In summary, the key points of this work are:

- Hydrophobic BAs accumulation does not induce hepatocyte cell death (apoptosis/necrosis).
- mGSH has an important role in hepatocellular damage sensitizing hepatocytes to a second hit, such as BAs exposure.
- Inflammatory response is dependent on antioxidant levels.
- Inflammation is a critical contributor to liver injury in cholestasis.

Importantly, this work is part of an *in vivo* study of cholestatic diseases in both Flox and StARD1$^{\Delta Hep}$ mice submitted to BDL operation, and human samples of PBC. The resulting data showed high levels of fibrosis in Flox mice with BDL surgery and PBC tissues (StARD1$^{\Delta Hep}$ mice subjected to BDL showed fibrosis in less degree) but underlying mechanism was not understood. This *in vitro* experiment has provided insight into the mechanism triggering physiopathology observed.

Even so, further investigation is needed. For instance, other cholestatic diseases can be studied, such as PSC, for seeing if there are any differences between similar pathologies.

Besides, *in vitro* study could be further improved to apport new information. Some new contributions include:

- **Animal model:** mGSH depletion could be induced by a nutritional model, such as 2% cholesterol and 0.5% cholic acid diet for two days, instead of HP addition. Besides, addition of GSHee (5mM) would test whether the recovery of antioxidant power would reverse the effects observed in this study.
- **BAs:** TβMCA exerted a repressor function on certain pro-inflammatory genes and that was not due to its vehicle (DMSO). Consequently, it would be worth studying TβMCA action.
- **GSH levels:** Although the recovery of mGSH occurred very slowly and progressively, this process could be prevented by exposing PMH to buthionine sulfoximine (BSO), which inhibits GSH synthesis through irreversibly inhibiting γ-glutamylcysteine synthetase (γ-GCS).
- **Pro-inflammatory markers:** While at 6h an increase in inflammatory genes was observed, at 12h there were no significant changes. Therefore, it would be interesting to create a dose-response curve from 0 to 6h to analyse the maximum peak of expression. Besides, if cellular markers were added in addition to the inflammation genes studied, we could find out which immune cells play a key role in this process.

10. Bibliography

1. Cai, S.-Y., & Boyer, J. L. (2021). The role of bile acids in cholestatic liver injury. *Annals of Translational Medicine*, *9*(8), 737–737. https://doi.org/10.21037/atm-20-5110
2. Chiang, J. Y. L., & Ferrell, J. M. (2018). *Invited Review Bile Acid Metabolism in Liver Pathobiology*. *18*, 71–87.
3. Conde de la Rosa, L., Garcia-Ruiz, C., Vallejo, C., Baulies, A., Nuñez, S., Monte, M. J., Marin, J. J. G., Baila-Rueda, L., Cenarro, A., Civeira, F., Fuster, J., Garcia-Valdecasas, J. C., Ferrer, J., Karin, M., Ribas, V., & Fernandez-Checa, J. C. (2021). STARD1 promotes NASH-driven HCC by sustaining the generation of bile acids through the alternative mitochondrial pathway. *Journal of Hepatology*, *74*(6), 1429–1441. https://doi.org/10.1016/j.jhep.2021.01.028
4. Conde, L., Rosa, D., Garcia-ruiz, C., Vallejo, C., Karin, M., & Ribas, V. (2021). *STARD1 promotes NASH-driven HCC by sustaining the generation of bile acids through the alternative mitochondrial pathway*. 1–13.
5. Garcia-ruiz, C., Conde, L., Rosa, D., & Ribas, V. (2020). Seminars in Cancer Biology. *Seminars in Cancer Biology*, *July*. https://doi.org/10.1016/j.semcancer.2020.07.014
6. Grattagliano, I., Calamita, G., Cocco, T., Wang, D. Q., & Portincasa, P. (2014). *Pathogenic role of oxidative and nitrosative stress in primary biliary cirrhosis*. *20*(19), 5746–5759. https://doi.org/10.3748/wjg.v20.i19.5746
7. Jüngst, C., & Lammert, F. (2013). *Cholestatic Liver Disease*. 152–154. https://doi.org/10.1159/000347210
8. Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A., & Zoric, N. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine*, *27*(2–3), 95–125. https://doi.org/10.1016/j.mam.2005.12.007
9. Li, T., & Chiang, J. Y. L. (2014). *Bile Acid Signaling in Metabolic Disease and Drug Therapy*. *October*, 948–983.
10. Marí, M., Morales, A., Colell, A., García-Ruiz, C., & Fernández-Checa, J. C. (2009). Mitochondrial glutathione, a key survival antioxidant. *Antioxidants and Redox Signaling*, *11*(11), 2685–2700. https://doi.org/10.1089/ars.2009.2695
11. Nuño-lámbarri, N., Domínguez-pérez, M., Baulies-domenech, A., Monte, M. J., Marin, J. J. G., Rosales-cruz, P., Souza, V., Miranda, R. U., Bucio, L., Montalvo-jave, E. E., Gutiérrez-ruiz, M. C., García-ruiz, C., Fernández-checa, J. C., & Gomez-quiroz, L. E. (2016). *Liver Cholesterol Overload Aggravates Obstructive Cholestasis by Inducing Oxidative Stress and Premature Death in Mice*. *2016*.

https://doi.org/10.1155/2016/9895176

12. Nuño-lámbarri, N., & Uribe, M. (2016). *Elevated cholesterol levels have a poor prognosis in a cholestasis scenario*. *June*, 1–6. https://doi.org/10.1002/jbt.21831
13. Pastor, A., & Collado, P. S. (2000). *Factors influencing the inhibition of biliary glutathione efflux induced by biliary obstruction*. *68*, 69–79.
14. Ríos-López, D. G., Aranda-López, Y., Sosa-Garrocho, M., & Macías-Silva, M. (2020). La plasticidad del hepatocito y su relevancia en la fisiología y la patología hepática. *TIP Revista Especializada En Ciencias Químico-Biológicas*, *23*, 1–19. https://doi.org/10.22201/fesz.23958723e.2020.0.225
15. York, R. N. (1993). *Selective Depletion of Mitochondrial Glut at hione Concentrations by*. 75–81.
16. Zhang, Y., Hong, J., Rockwell, C. E., Copple, B. L., Jaeschke, H., & Klaassen, C. D. (2013). *EFFECT OF BILE DUCT LIGATION ON BILE ACID COMPOSITION*. *32*(1), 58–69. https://doi.org/10.1111/j.1478-3231.2011.02662.x.EFFECT
17. Zollner, G., & Trauner, M. (2008). *Mechanisms of Cholestasis*. *12*, 1–26. https://doi.org/10.1016/j.cld.2007.11.010

ANNEXES

Annex 1. **PMH ISOLATION AND CULTURE**

Total PMH were isolated by collagenase perfusion through portal vein followed by a differential centrifugation.

After isolation, PMH were seeded on culture vessels at different densities depending on the vessel type and the purpose of the experiment. Culture plates were precoated with rat – tail collagen to ensure hepatocyte adhesion.

PROTOCOL:

- *Collagen – coated plates:*
  - Prepare 0.02 M Acetic Acid in mQ-H_2O (114µl Acetic Acid + 100ml mQ-H_2O) and filter it with a 0.2 µm filter to sterilize it.
  - Prepare a 50 µg/ml collagen solution in 0.02 M Acetic Acid (commercial Collagen I, rat tail, Gibco A10483-01). The volume of collagen solution to prepare depends on the number and type of culture vessels to be coated.
  - Cover the desired vessels with the collagen solution.
  - Incubate for 20-30 min at room temperature (RT).
  - Aspirate collagen.
  - Let dry plates in the cell culture hood for minimum 30 min.
  - Cover vessels with tin foil and keep them at 4°C until needed.
  - Prior to use, wash them once or twice with PBS to remove residual acetic acid.

- *Collagenase perfusion:*
  - Prepare buffers required for collagenase perfusion (Table 1).
  - Allow HANKS I and II buffers to reach 37°C in a water bath.
  - Clean the peristaltic pump tubes with 70% EtOH, dH_2O and then fill the circuit with warm HANKS I buffer at a speed of 6 – 8 ml/min.
  - Anesthetize the mouse with a dose of 100 mg/kg of sodium pentobarbital intraperitoneally.
  - Prepare one surgical suture about 12cm long (Suturas Aragó, Seda trenzada negra 2/0).
  - Open the mouse with a laparotomy.

HANKS 10X	
Compound	**Concentration**
NaCl	1.4 mM
KCl	53.6 mM
$MgSO_4 \cdot 7H_2O$	8.1 mM
$Na_2HPO_4 \cdot 2H_2O$	6.7 mM
KH_2PO4	8.8 mM
mQ – H_2O	-

HANKS 1X	
Compound	**Concentration**
HANKS 10X	10%
Hepes	12.6 mM
$NaHCO_3$	25 mM
mQ – H_2O	-

HANKS I	
Compound	**Concentration**
BSA	0.5%
EGTA	0.9 mM

HANKS II	
Compound	**Concentration**
Collagenase (fraction V)	160 U/ml
$CaCl_2 \cdot 2H_2O$	1 mM

Table 1. Buffers HANKS 10X, HANKS 1X, HANKS I and HANKS II composition. HANKS Buffers are used for mice perfusion. While a stock of HANKS 10X can be made and stored, HANKS 1X must be freshly prepared. After its elaboration, HANKS 1X should be saturated with 95% CO_2 and 5% O_2 for 20 min to stabilize the pH. The rest of compounds can be added to elaborate HANKS I and HANKS II and then, both buffers should be filtered trough a 0.45 µm bottle filter. EGTA is crucial in the cleaning buffer to avoid coagulation and calcium is necessary for the digestion buffer as a cofactor of the collagenase. BSA: Bovine Serum Albumin; EGTA: ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

- Place one suture under the portal vein.
- Insert the catheter (BD Insyte, 381212) into the portal vein and immobilize it with the suture around it. If the catheter does not fill with blood, fill it with warm HANKS I buffer avoiding bubble formation.
- Stop the peristaltic pump (Cole-Parmer, MasterFlex L/S, #7553-79) and connect it to the catheter. Avoid bubble formation.
- Start the pump and cut the inferior cava vein so the blood can exit the system.
- Clean the liver with HANKS I buffer. This will prepare the liver for digestion. Make pressure above the cut part of the inferior cava vein with the tweezers for 10 seconds to allow the buffer to penetrate in the liver.
- Once the liver is clean, change to HANKS II buffer to start the digestion. It will take 3 - 6 min to digest. Make pressure in the inferior cava vein with the tweezers for 10 seconds to better perfuse in the liver. The liver will turn slightly white/brown while it starts swelling. It is crucial to reach an optimal digestion point since too much or insufficient digestion will result in poor hepatocyte viability and content. The liver is

digested when it does not increase its volume when applying the pressure at the cut part of the inferior cava vein.

- Pour some HANKS II buffer in a petri dish and prepare a small Erlenmeyer with a funnel and a sterile maze to filter the non-digested parts of the liver.
- Take the liver out and disaggregate the tissue in a petri dish with HANKS II buffer using a pair of tweezers to liberate the hepatocytes from the digested liver.
- Filter hepatocytes through the maze and adjust to a final volume of 50 ml with HANKS II buffer.
- Centrifuge at 60xg for 3 min at RT.
- Discard the supernatant containing Kupffer, hepatic stellate and sinusoidal endothelial cells and resuspend the pellet containing the hepatocytes with warm isolation medium (Table 2) (twice the volume of the pellet).
- After hepatocytes resuspension, use Trypan Blue staining to determine hepatocytes viability rate and cellular concentration:
  - Dissolve 50 µl of the resuspended hepatocytes in 950 µl of 0.2% Trypan Blue (Sigma, T8154).
  - Wait a minute and start to count for alive (white) and dead (blue) cells using a Neubauer Chamber. Multiply the number of cells by the Trypan Blue dilution factor (1/20) and by the volume of the Neubauer Chamber (10^4 cells/ml). The sum of alive and dead cells will give us the total number of cells that we will use to calculate the % of viability:

$$Viability\ (\%) = \frac{alive\ cells}{total\ number\ of\ cells}\ x\ 100$$

  - Isolations with a viability rate below a 70% were discarded.

- *PMH culture:*
  - Dilute PMH in isolation medium (Table 2), which is supplemented with fetal bovine serum (FBS).
  - Seed each plate with PMH and keep plate in an incubator at 37ºC with 5% CO_2.
  - After 3h, discard isolation medium and add maintenance medium (Table 2), which is used for rest of the culture and during the treatments.

Isolation Medium	
Compound	**Concentration**
DMEM:F12	-
Penicillin/Streptomycin	100 U/ml
L-Glutamine	200 mM
Hepes pH 7,4	150 mM
FBS	10%

Maintenance Medium	
Compound	**Concentration**
DMEM:F12	-
Penicillin/Streptomycin	100 U/ml
L-Glutamine	200 mM
Hepes pH 7,4	150 mM

Table 2. Mediums used for PMH culture. Isolation Medium is supplemented with fetal bovine serum (FBS) in contrast to maintenance medium which is not. DMEM:F12: Dulbecco's Modified Eagle Medium F-12.

Annex 2. **MITOCHONDRIAL CELLULAR ISOLATION**

PMH were fractionated into cytosol and mitochondria by digitonin permeabilization.

PROTOCOL:

- Wash cultured PMH with PBS solution to remove all traces of serum that could inhibit trypsin action.
- Add Trypsin-EDTA (Invitrogen, 25200-056) to the cell culture dish and tripsinize cells normally.
- Add culture medium with FBS to stop trypsinisation and centrifuge to obtain a cell pellet.
- Resuspend the cell pellet with PBS and determine cell concentration by Trypan Blue.
- Immediately, pellet $1x10^6$ PMH should be resuspend with 600 µl of PBS (cell homogenate).
- From the cell homogenate, separate:
  - 50µl for total protein determination
  - 50µl for total GSH measurement
  - 500µl for cytosolic-mitochondrial fractioning
- Prepare 1.5 ml centrifuge tubes containing from the bottom to the top: 100 µl of 10% trichloroacetic acid (TCA) (Sigma, T6399), 500 µl of a 6:1 (v/v) mixture of silicone (Sigma, 175633) and paraffin oil (Sigma, 18512), and a 100 µl top layer of MBD (Mannitol – Buffer – Digitonin) (Table 1) at 37°C.

MBD	
Compound	**Concentration**
Mannitol	250 mM
EDTA	19.8 mM
Hepes pH 7,4	12 mM
Digitonin	0.15%
mQ – H_2O	-

Table 1. MBD Buffer (pH 7,4) used for cellular mitochondria isolation. MBD: Mannitol – Buffer – Digitonin; EDTA: Ethylenediaminetetraacetic acid.

- Rapidly, add 500 µl of cell homogenate inside the 100 µl MBD top layer and wait for 30 seconds at RT (in this short incubation, digitonin makes holes in the cell membrane

but it does not damage mitochondria).

- Subsequently, centrifuge tubes for 1 min at 13.000 rpm at RT to separate cytosolic and mitochondrial fractions.
- 3 layers appear:
    - Bottom layer: contains the mitochondrial fraction in 10% TCA.
    - Middle layer: contains the Silicon:Paraffin oil.
    - Top layer: contains the cytosolic fraction.
- To analyse cytosolic GSH levels, take a known volume of the cytosolic fraction, dilute with 10% TCA (1:1 ratio) and follow protocol for glutathione measure.
- Mitochondrial GSH levels can be read directly since the fractioning has been performed with 10% TCA in the lower phase.
- To analyse total GSH levels, the separated 50 µl aliquot of cell homogenate (first steps of the protocol) should be diluted with 10% TCA (1:1 ratio).

Annex 3. **GLUTATHIONE MEASUREMENT**

Total GSH (GSH and GSSG) levels in liver homogenate and isolated mitochondria from mice and in cellular extracts from isolated PMH or cell lines were analysed using the enzymatic recycling method.

In this assay, the sulfhydryl group of GSH reacts with 5, 5'-dithio-bis-2-nitrobenzoic acid (DNTB, Sigma, D8130) and produces a detectable yellow-coloured 5-thionitrobenzoic acid (TNB). The GSTNB (GSH linked to TNB) that is concomitantly produced, is reduced by GR to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which in turn is directly proportional to the concentration of GSH in the sample. Thus, measurement of the absorbance of TNB at 412 nm provides an accurate estimation of total GSH in the sample.

PROTOCOL:

- *Sample's preparation and harvesting:*
  - To be able to determine GSH level, all samples need to be previously diluted 1:1 with TCA 10% - PBS vortexed and spun down at 12.000 rpm for 5 min at 4ºC. This step allows cellular membranes disruption and release of GSH to the supernatant.

- *Ellman assay:*
  - Prepare a 1mM GSH (Sigma, G6529) stock in PBS.
  - Prepare 7 GSH standard solutions 0, 10, 20, 30, 40, 50 or 60 µM in a final volume of 4 ml with PBS.
  - Mix in a tube: 2 ml of Ellman buffer, 1 ml of standard and 30 µl of Ellman reagent (Table 1).
  - Mix thoroughly and wait 3 min before reading it.
  - Read in a spectrophotometer (Beckman Coulter, DU800) at an absorbance of 412 nm.

The values obtained will be multiplied by 198 (coefficient of extinction of the spectrophotometer) and will represent the real concentration of the GSH standards prepared that we would use in the recycling assay as well. We will use these data to normalize the absorbance obtained in the recycling assay for the GSH standards.

Ellman Buffer	
Compound	**Concentration**
KH_2PO_4	0.5M
mQ – H_2O	-

Ellman Reagent	
Compound	**Concentration**
DNTB	10 mM
$Na_2HPO_4 \cdot 2H_2O$	0.1 M
$NaHCO_3$	11.9 mM
mQ – H_2O	-

Table 1. Buffers for Ellman assay. Ellman Buffer (pH 8) can be stored at 4ºC meanwhile Ellman Reagent (pH 7.4) can be aliquoted and stored at -20ºC. DNTB: 5, 5'-dithio-bis-2-nitrobenzoic acid.

- *Recycling assay:*
  - Prepare the Recycling buffer (Table 2), protect it from the light and warm it at 37°C.
  - Prepare the GR at a concentration of 10 U/ml in mQ-H2O. It will be stable a few weeks at 4°C.
  - Mix in a tube: 2.5 ml of recycling buffer at 37°C, 100 µl of sample/standard and 100 µl of GR.
  - Mix by inversion.
  - Read during 40 seconds in the spectrophotometer at an absorbance of 412 nm.

GSH concentrations will be determined referring the samples to the standard curve normalized by the Ellman assay and by protein concentration.

Recycling Buffer	
Compound	**Concentration**
EDTA	1 mM
DNTB	0.1 mM
NADPH	0.15 mM
$NaPO_4$	50 mM
mQ – H_2O	-

Table 2. Buffer for Recycling Assay. Recycling Buffer should be freshly prepared, and it must be protected from light. EDTA: Ethylenediaminetetraacetic acid. DNTB: 5, 5'-dithio-bis-2-nitrobenzoic acid.

Annex 4. **PROTEIN QUANTIFICATION**

Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins that is detectable at an absorbance of 595 nm. There are certain chemical-protein or chemical-dye interactions that may interfere with Bradford assay.

PROTOCOL:

- Prepare BSA standard points to build a standard curve (e.g., 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) in PBS.
- Dilute lysates in PBS to fit in the standard curve.
- Perform the assay in a 96-well plate in triplicates.
- Add 4 µl of the diluted sample or BSA standard per well.
- Using a multichannel pipette add 200µl of Quick Start Bradford Protein Assay (Bio-Rad, 500-0201).
- Incubate at RT for at least 20 min. Samples should not be incubated longer than 1h at RT.
- Set the spectrophotometer plate reader to 595 nm. Measure the absorbance of the standards, blanks, and unknown samples.
- Samples will be referred to the BSA standard curve and they will be assigned a protein concentration.

Annex 5. **mRNA ISOLATION AND QUANTIFICATION**

To isolate messenger RNA (mRNA) we used a TRIzol-chloroform extraction (Invitrogen). TRIzol Reagent is a monophasic solution of phenol, guanidine isothiocyanate and other proprietary components, which facilitate the isolation of a variety of RNA species of large or small molecular size. It maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization. After homogenizing the sample with TRIzol, chloroform is added, and the homogenate can separate into a clear upper aqueous layer containing RNA, an interphase, and a red lower organic layer containing the DNA and proteins. Finally, RNA is precipitated from the aqueous layer with isopropanol.

RNA is very sensitive to nucleases. Thus, all material must be autoclaved, and all the solutions and organic solvents need to be molecular biology graded, free of DNases and RNase.

PROTOCOL:

- *RNA precipitation:*
  - Add 0.5 ml of 100% isopropanol to the collected aqueous phase, per each 1 ml of TRIzol used for homogenization and mix by inversion.
  - Incubate for 10 min at RT.
  - Centrifuge at 12.000xg for 10 min at 4°C.

- *RNA wash:*
  - Remove supernatant from the tube, leaving only the RNA pellet.
  - Wash the pellet with 1 ml of 75% ethanol in RNase free water at -20°C per each 1 ml of TRIzol Reagent used in the initial homogenization. From this point, the RNA can be stored in 75% ethanol at least 1 year at -20°C, or at least 1 week at 4°C.
  - Vortex the sample briefly and then centrifuge the tube at 7.500xg for 5 min at 4°C.
  - Discard the wash.
  - Air-dry the RNA pellet for 5-10 min. Do not allow the RNA to dry completely, because the pellet can lose solubility. Partially dissolved RNA samples have an A260/280 ratio <1.6.

- *RNA resuspension:*
  - Resuspend the RNA pellet in RNase-free water by passing the solution up and down several times through a pipette tip.
  - Incubate samples at least 30 min at 4°C to allow complete RNA resuspension.

- *RNA quantification:*
  - Load 2 µl of resuspended mRNA onto the NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 1000).
  - Measure the sample absorbance at 260 nm and 280 nm to calculate nucleic acid concentration (260 nm) and the purity of the sample (260/280 ratio between 1.7-2.0).

Annex 6. **qRT-PCR**

Quantitative real-time polymerase chain reaction (qRT-PCR) technique is a refinement of the original PCR, which is used to amplify nucleic acids in a cyclic process to generate many identical copies. In RT-PCR, the amount of product formed is monitored during the reaction by recording the fluorescence of dyes or probes introduced into the reaction, which is proportional to the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA molecules is registered. Assuming a certain amplification efficiency, which typically is close to a doubling of the number of molecules per amplification cycle, it is possible to calculate the number of DNA molecules of the amplified sequence that were initially present in the sample (Kubista et al., 2006).

One important step in relative quantization is the selection of an endogenous control (housekeeping gene) since normalization to the housekeeping gene allows correcting results that can be skewed by differing amounts of input nucleic acid template. Any gene expressed at the same level in all study samples can potentially be used as an endogenous control. We used 18S as endogenous control.

RT-PCR was performed using the SensiFAST™ SYBR® No-ROX Kit (Bioline, BIO-98020) following the manufacturer's instructions.

PROTOCOL:

- Dilute RNA samples with RNAse free water to reach 100 ng RNA/well.
- Prepare as many RT-PCR mixes as number of genes to check (Left Table 1). The volume of mix to prepare depends on the number of samples and replicates to be analysed.
- Load the RT-PCR plate, cover the plate with film and seal it properly.
- Spin the plate down.
- Perform the PCR run following the indicated PCR protocol in a CFX384 Real-Time PCR Detection System (Bio-Rad) (Right Table 1).
-

RT-PCR mix	
Compound	**Quantity**
SYBR Green Master Mix	5.25 µl
Oligonucleotides	0.105 µl
ng RNA/well	100 ng
Reverse transcriptase	0.105 µl
Ribosafe RNA inhibitor	0.210 µl
RNAse free water + RNA	4.83 µl
Final volume	10.5 µl

	RT-PCR Protocol		
	Time	**Temperature**	**Repetitions**
Step 1	2 min	95°C	-
Step 2	5 sec	95°C	x40 cycles
	10 sec	60°C	
	20 sec	72°C	
Step 3	1 min	95°C	-
Step 4	10 sec	10°C	x81 cycles

Table 1. RT-PCR mix and RT-PCR protocol.

Results were analysed using the relative quantification method ΔΔCt. The Cycle Threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold (e.g., exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample so, the lower the Ct level, the greater the amount of target nucleic acid in the sample. The ΔΔCT method uses arithmetic formulas to achieve the result for relative quantization. The amount of target, normalized to the housekeeping gene and relative to a calibrator (or control experimental group), is given by:

$$\text{Gene expression} = 2^{-(\Delta\Delta Ct)}$$

Where $\Delta\Delta Ct$ stands for: $\Delta\Delta Ct = \Delta Ct_{\text{experiment}} - \Delta Ct_{\text{control}}$

And ΔCt stands for: $\Delta Ct = Ct_{\text{gene "X"}} - Ct_{\text{housekeeping gene}}$

The primer sequences used to determine the expression of different genes are describe at Table 2.

Gene	Specie	Primer	
ICAM-1 (Invitrogen)	Mouse	*Forward*	GTGATGCTCAGGTATCCATCCA
		Reverse	CACAGTTCTCAAAGCACAGCG
Ly6c (Invitrogen)	Mouse	*Forward*	GCAGTGCTACGAGTGCTATGG
		Reverse	ACTGACGGGTCTTTAGTTTCCTT
18S (Metabion international)	Mouse	*Forward*	CGGCTACCACATCCAAGGA
		Reverse	CCAATTACAGGGCCTCGAAA
IL-1β (Invitrogen)	Mouse	*Forward*	GAGCTGAAAGCTCTCCACCTC
		Reverse	CTTTCCTTTGAGGCCCAAGGC
IL-6 (Invitrogen)	Mouse	*Forward*	GGCGGATCGGATGTTGTGAT
		Reverse	GGACCCAGACAATCGGTTG
Spp1(Invitrogen)	Mouse	*Forward*	TCTGCTTCTGAGATGGGTCA
		Reverse	TTGGCAGTGATTTGCTTTTG
Hmox1(Invitrogen)	Mouse	*Forward*	CCTTCAAGGCCTCAGACAAA
		Reverse	GAGCCTGAATCGAGCAGAAC

Table 2. Primers of inflammatory genes. Intercellular Adhesion Molecule 1 (ICAM-1), Lymphocyte Antigen 6 Complex (Ly6c), small subunit 18S rRNA(18S), interleukin 1β (IL-1β), interleukin 6 (IL-6), secreted phosphoprotein I (Spp1), Heme oxygenase I (Hmox1).

Annex 7. **DOBLE STAINING BY HÖECHST/PI**

The double staining apoptosis assay provides a rapid and convenient method for the compacted state of chromatin in apoptotic cells.

Propidium iodide (PI) is used as a DNA stain by intercalating between the bases with little or no sequence preference. Once the dye binds to nucleic acid, its fluorescence is enhanced 20 to 30-fold. PI is excited at 488 nm and its maximum emission wavelength is 617 nm. As a membrane impermeable dye that generally excluded from viable cells, it is commonly used as a counterstain in multicolour fluorescent techniques to distinguish between late apoptotic cells and normal cells in a population.

Höechst stains are cell-permeable nuclear counterstain that emit blue fluorescence when combined with dsDNA. These bisbenzimidazole derivatives bind into the minor groove of DNA with adenine thymine (AT) selectivity. Höechst dyes are very sensitive to DNA conformation and chromatin status in cells and are therefore used to detect the gradations of nuclear damage, such as distinguishing condensed pycnotic nuclei in apoptotic cells.

The staining pattern resulted from the simultaneous use of these dyes makes it possible to distinguish normal, apoptotic, and dead cell populations by fluorescence microscopy.

PROTOCOL:

- Add to a 12 – well plate coverslips and then, precoat vessel with collagen as is describe in Annex 1.
- After PMH culture, prepare a 10µg/ml Höechst/PI working solution in cell medium. Volume depends on the number of wells that need to be treated.
- Take the 12 – well plate with PMH cells from the incubator.
- Aspirate the medium.
- Wash with PBS (500 µl/well) several times.
- Prepare 1% Höechst stock solution in PBS.
- Prepare 1% Propidium Iodide (PI) stock solution in PBS.
- Add 250 µl of the Höechst/PI working solution per well.
- Incubate 20 minutes at 37ºC in the cell culture incubator.
- Wash twice with PBS (500µl/well).
- Add 250µl of formalin 10% per well to fix the cells.
- Wait for 15 minutes at RT.
- Wash twice with PBS (500µl/well)

- Take each coverslip and place them upside-down in a glass microscope slide with mounting media (Fluoromont).
- Store coverslips at 4ºC and dark to observe them the following day.

  Höechst = DAPI filter

  PI = TRICT filter

  Count PI-positive (dead) and Höechst- positive (dead + alive) cells

  Perform the following calculation to know the cell viability of the sample:

$$Viability\ (\%) = 100 - \left(\frac{PI\ cells\ (dead)}{Höechst\ cells\ (dead + alive)} \cdot 100\right)$$

Annex 8. **VEHICLES ANALYSIS**

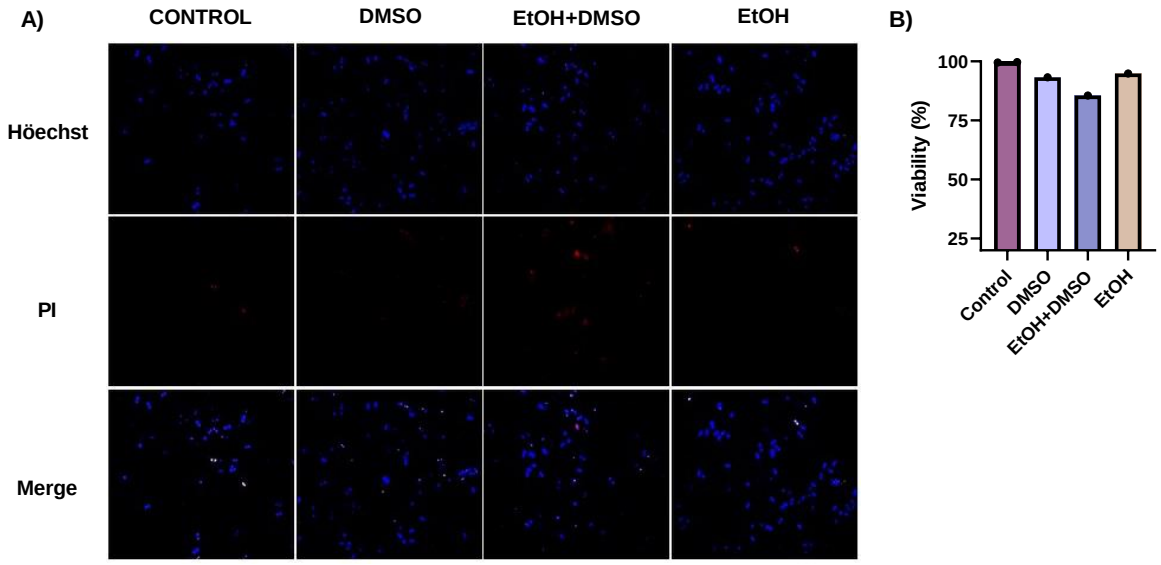

Figure 1. Viability and toxicological analysis by Höechst/PI double fluorescence staining of live PMH exposed to vehicles. PMH were analysed at 12h (n=1). Viability for DMSO was 93.29%, for DMSO+EtOH 85.53% and for EtOH 94.85%.

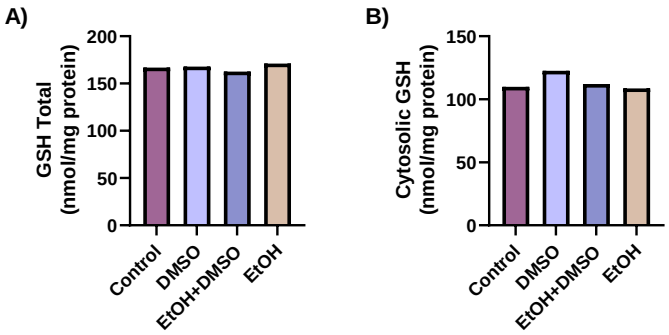

Figure 2. GSH levels. Cellular and cytosolic GSH of PMH exposed to vehicles at 12h. mGSH was not analysed because unsuccessful compartmentalization process.

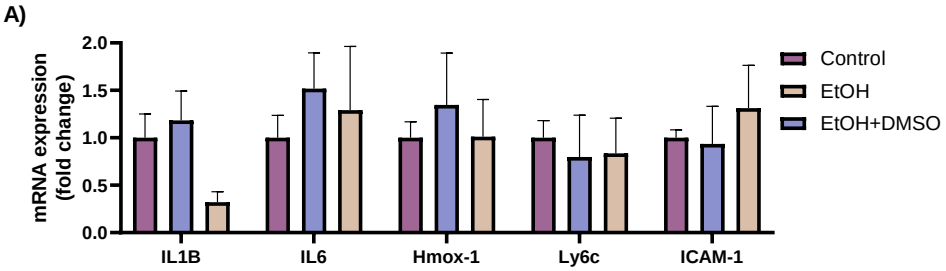

Figure 3. Analysis of the inflammatory and oxidative stress markers profile after vehicles exposition.