UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

Aquaporins involvement in hepatic ischemia reperfusion injury

Jessica Alexandra Silva Fernandes

Dissertation supervised by Professor Graça Soveral
and co-supervised by Doctor Maria Luísa Corvo

Master in Biopharmaceutical Sciences

2021

UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

Aquaporins involvement in hepatic ischemia reperfusion injury

Jessica Alexandra Silva Fernandes

Dissertation supervised by Professor Graça Soveral
and co-supervised by Doctor Maria Luísa Corvo

Master in Biopharmaceutical Sciences

2021

The studies presented in this thesis were conducted at the Laboratory of Biological Transport, Department of Biochemistry and Human Biology (DBBH), Faculdade de Farmácia, Universidade de Lisboa (FFUL), under the supervision of Professor Graça Soveral.

This thesis is part of a project regarding the study of inflammation processes in hepatic ischemia-reperfusion in collaboration with Laboratory Human Genetics and Cancer Therapeutics of UCIBIO, Department of Life Sciences, Faculdade de Ciências e Tecnologias, Universidade Nova de Lisboa (FCT-UNL), and BioNanoSciences Group from iMed.ULisboa, Faculdade de Farmácia, Universidade de Lisboa (FFUL).

Acknowledgments

Firstly, I would like to thank Professor Graça Soveral for challenging me into participate in this project, although not initially the theme I had in mind, it turn out to be an enriching experience and an interesting project. Even through all the setbacks we encountered within this year, thank you for incentivizing me to keep going and helping me develop my scientific knowledge and critic spirit. With you I learned that there are no bad results, only a chance to articulate new theories and perspectives.

Moreover, I would like to thank Doctor Maria Luísa Corvo for accepting co-orienting my thesis and helping me understand the theme which was completely new and interesting to me. Your input was essential for this project. Thank you for the teachings and the availability.

Additionally, my thanks to Doctor Inês Silva for the guidance throughout this year, for helping me understand the theme, for coaching me the better way to articulate my arguments, to teaching me the tools I needed to write the project and for all the scientific knowledge and time you've put in this project.

To all my laboratory colleagues, Inês, Catarina, Andreia, Bárbara, that through a short period of time accompanying me into long hours of desperation and some good results and finally the lunches and snack times and the long afternoons sharing experiences, arguments and laughter. Thank you for the companionship.

I would like to express my gratitude to my family, mom and dad for always persisting by me through my academic ambitions, for putting up with me in my dark days (I know there have been many) and celebrating with me my bright days and achievements, for all the love and support. I couldn't have done this without you, I hope you know how much I appreciate everything you have done for me to achieve my goals. I would also like to express my gratitude towards the rest of my family for believing in me and giving me strength for always trying to do my best and also lighting up my life with days full of laughter and good memories.

To all my close friends for insisting on maintaining my social life alive through a full schedule, for always making me laugh so much until I start crying, for accepting me completely and to get on board on my crazy ideas, for all the experiences and good moments, for all the support on the bad moments. And most importantly teaching me that when there's willingness there's no excuses, only solutions. A special thanks to my friends João (for helping me understand statistics and basic informatics, and also being my eternal buddy on our crazy dramas), Pedro (for keeping my days light with your laughter and friendship since our childhood), and finally Inês (for always being my other half on our unlucky young moments).

Also, I would like to thank my friends from the masters, Margarida for the perfect partnership and all the joyously insanity throughout the first year, Avilson, Joana, Andre, Liliana, Inês for making me feel young, for the crazy moments we experienced and the many more there is to come.

Finally, I would like to thank my work colleagues and my manager for always making it possible for me to reconcile work, thesis, and leisure time. For all the time we spend together every day, through the hard days and the days we celebrate our goals, for all the companionship and friendship created through long and stressful hours. Thank you for having faith in me, you’ve kept me going through the last year.

Abstract

Hepatic ischemia-reperfusion (IR) injury (IRI) is an unavoidable complication during liver surgery caused by the interruption of blood flow and subsequent inflammation during liver revascularization. Since this pathological condition has a high mortality rate associated, there is a growing interest in assessing methods and strategies to prevent and attenuate liver IRI.

Liver IR is characterized by an ischemia phase, where the lack of oxygen supply and ATP depletion leads to microcirculation disruption and mitochondrial damage, and a reperfusion phase distinguished by activation of the liver immune system which involves activation of macrophages and neutrophils, production of cytokines and chemokines, generation of oxidative stress, and increased expression of adhesion molecules on the hepatic endothelium. Mechanisms of inflammation resolution have been reported as some anti-inflammatory mediators are capable of inducing tissue repair and hepatocytes proliferation.

The hepatobiliary system is responsible for several metabolic processes including bile formation and secretion, detoxification and glycerol metabolism. As bile is predominantly composed of water, canalicular bile formation is an osmotic secretory process that results from the input of water in response to osmotic gradients created by active secretion of solutes. When hepatocytes are stimulated, water transport occurs significantly via transcellular pathway through the plasma membrane.

Several cells from the hepatobiliary tract have been shown to express aquaporins (AQPs), which are small transmembrane proteins known to assist on the transcellular water transport by increasing water permeability and also allow the transport of glycerol and other small solutes. Additionally, since during the liver revascularization membrane permeability is affected by inflammation processes, AQPs situated in several liver cells pose as important regulators of hemodynamic equilibrium during IR.

Considering the role that AQPs may play during liver IRI processes, the focus of this work consisted on assessing the IR effect on AQPs expression. The *in vitro* assay with HepG2 cells exposed to different IR stimulus allowed to distinguish the effect of inflammation and hypoxia stimulus on AQPs expression. Upon severe inflammation stimulus with LPS, cells induced AQPs expression in order to reestablish homeostasis and attenuate biological processes instigated by overexpression of inflammation mediators. Opposing the inflammatory response, upon severe ischemia stimulus, cells subjected to a hypoxia chamber suppress AQPs expression as a mechanism of preventing water influx into the cells and maintaining water homeostasis during the initial inflammatory process and preventing cell swelling.

Moreover, administration of liposomal drugs formulations proved effective in attenuating inflammation processes caused by the HepG2 cells exposure to toxic compounds and inflammation-related dysfunction in liver cells, as both pharmaceutical drugs

tested (prednisolone and ibuprofen) showed favorable effects in reversing LPS-induced inflammation by decreasing significantly AQP3 and cytokines expression.

The *in vivo* assay of IR effect on rat liver cells allowed to delineate a profile of AQPs expression along the different timepoints of the IR process. Cells suppressed significantly the AQPs expression after 2h of reperfusion in order to oppose the mechanisms ensued in the ischemia process and prevent water influx into the cell and cell edema within the first hours of reperfusion, then induced AQPs expression after 6h of reperfusion in order to reestablish water homeostasis and finally after 24h of reperfusion cells increased AQPs expression. As AQPs and TNF-α expression after 24h of reperfusion resembled the normoxia condition, this assay confirmed that at this timepoint the cells are undergoing processes of inflammation resolution and returning to homeostasis. Moreover, treatment with the flavonoid quercetin was inefficient in attenuating the IR-induced inflammation processes, as AQPs profiles resembled the samples without treatment. Regardless, intravenous administration of SPC liposomal quercetin presented higher AQPs expression than intraperitoneal administration of EPC liposomal quercetin.

In order to further assess the role of AQPs in liver IR mechanisms, functional assays should be carried out and, ultimately, strategies to target and regulate AQPs during liver IR should be investigated aiming to reduce the high mortality rate associated with this condition.

Key words: hepatic ischemia-reperfusion (IR), aquaporins (AQPs), ischemia stimulus, inflammation stimulus, liposomal drug formulations, cytokines expression, inflammation resolution, water homeostasis.

Resumo

A lesão de isquemia-reperfusão (IR) hepática é uma complicação inevitável durante a cirurgia hepática, causada pela interrupção do fluxo sanguíneo e subsequente inflamação durante a revascularização do fígado. Uma vez que esta condição patológica tem uma alta taxa de mortalidade associada, há um interesse crescente na investigação de métodos e estratégias para prevenir e atenuar a IR hepática.

A IR hepática é caracterizada por uma fase de isquemia, em que a falta de oxigénio e a depleção de ATP leva à interrupção da microcirculação e ao dano mitocondrial, e uma fase de reperfusão, distinguida pela ativação do sistema imunológico do fígado que envolve ativação de macrófagos e neutrófilos, produção de citocinas e quimiocinas, stress oxidativo e aumento da expressão de moléculas de adesão no endotélio hepático. Os mecanismos de resolução de inflamação no fígado são conhecidos uma vez que alguns mediadores anti-inflamatórios são capazes de induzir a cicatrização do tecido hepático e a proliferação de hepatócitos.

O sistema hepatobiliar é responsável por diversos processos metabólicos, incluindo formação e secreção biliar, desintoxicação e metabolismo do glicerol. Como a bile é predominantemente composta por água, a formação de bile canalicular é um processo de secreção osmótica que resulta da entrada de água em resposta a gradientes osmóticos criados pela secreção activa de solutos. Quando os hepatócitos são estimulados, o transporte de água ocorre significativamente pela via transcelular através da membrana plasmática.

Foi demonstrado que várias células do trato hepatobiliar expressam aquaporinas (AQPs), pequenas proteínas transmembranares conhecidas por auxiliar no transporte transmembranar de água, aumentando a permeabilidade à água e também permitindo o transporte de glicerol e outros pequenos solutos. Além disso, como durante a revascularização do fígado a permeabilidade da membrana é afectada por processos de inflamação, as AQPs situadas em várias células do fígado representam importantes reguladores do equilíbrio hemodinâmico durante a IR.

Considerando o papel que as AQPs podem desempenhar durante os processos de IR do fígado, o foco deste trabalho consistiu em avaliar o efeito da IR na expressão de AQPs. O ensaio *in vitro* com células HepG2 expostas a diferentes estímulos de IR permitiu distinguir o efeito da inflamação do estímulo de hipoxia na expressão de AQPs. Mediante estímulo de inflamação com LPS, as células induziram a expressão de AQPs a fim de restabelecer a homeostase e atenuar os processos biológicos instigados pela sobre-expressão de mediadores da inflamação. Opondo-se à resposta inflamatória, mediante estímulo de isquemia, as células submetidas a uma câmara de hipoxia suprimem a expressão de AQPs como um mecanismo de prevenção de influxo de água nas células e manutenção da homeostase da água durante o processo inflamatório inicial e prevenção de edema celular.

Além disso, a administração de formulações lipossomais de fármacos demonstrou ser eficaz na atenuação dos processos de inflamação causados pela exposição das células HepG2 a compostos tóxicos e disfunções relacionadas à inflamação nas células do fígado, já que ambos os fármacos testados (prednisolona e ibuprofeno) mostraram efeitos favoráveis na reversão da inflamação induzida por LPS, diminuindo significativamente a expressão de AQP3 e citocinas.

O ensaio *in vivo* do efeito de IR em células de fígado de rato permitiu delinear um perfil de expressão de AQPs ao longo dos diferentes tempos do processo de IR. As células suprimiram significativamente a expressão de AQPs após 2h de reperfusão, a fim de se opor aos mecanismos decorrentes do processo de isquemia e evitar o influxo de água na célula e edema celular nas primeiras horas de reperfusão, então induziram a expressão de AQPs após 6h de reperfusão a fim de restabelecer homeostase da água e, finalmente, após 24h de reperfusão as células aumentaram a expressão de AQPs. Como a expressão de AQPs e TNF-α após 24h de reperfusão se assemelhava à condição de normóxia, este ensaio confirmou que após 24h de reperfusão as células passam por processos de resolução da inflamação e retornam à homeostase. Além disso, o tratamento com o composto natural quercetina foi ineficiente em atenuar os processos de inflamação induzidos por IR, pois os perfis de AQPs assemelham-se às amostras sem tratamento. Apesar disso, a administração intravenosa de quercetina lipossomal (formada por SPC) apresentou maior expressão de AQPs do que a administração intraperitoneal de quercetina lipossomal (formada por EPC).

Para uma melhor avaliação do papel das AQPs nos mecanismos de IR do fígado, devem ser realizados ensaios funcionais e, em última análise, devem ser investigadas estratégias para direcionar e regular as AQPs durante a IR do fígado com o objetivo de reduzir a alta taxa de mortalidade associada a essa condição.

Palavras-chave: Isquemia-reperfusão (IR) hepática, aquaporinas (AQPs), estímulo de isquemia, estímulo de inflamação, formulações lipossomais de fármacos, expressão de citocinas, resolução de inflamação, homeostase de água.

List of Abbreviations

AIF1	Allograft inflammatory factor 1
AQP	Aquaporin
APC	Activated protein C
ATP	Adenosine triphosphate
BAX	Bcl2-associated X protein
Bcl2	B-cell lymphoma 2
cAMP	Cyclic adenosine monophosphate
C3a	Complement factor 3a
C5a	Complement factor 5a
$CD4^+$ T cell	T-cell helper lymphocyte
cGMP	Cyclic guanosine monophosphate
CHIP28	Channel-forming Integral Membrane Protein of 28 KDa
CXC	C-X-C motif chemokine
DAMP	Damage-associated molecular pattern
EGFR	Epidermal growth factor receptor
EPC	Egg phosphatidylcholine
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinases
ET	Endothelin
GSH	Glutathione
GTP	Guanosine triphosphate
HAT	Histone acetyltransferase
HGF	Hepatocyte growth factor
HMGB1	High-mobility group box-1 protein
HPC	Hepatic progenitor cell
HSC	Hepatic stellate cell

IFN-β	Interferon-beta
IFN-γ	Interferon-gama
IP	Intraperitoneal
IRF1	Interferon regulatory factor 1
IRF3	Interferon regulatory factor 3
IR	Ischemia-reperfusion
IRI	Ischemia-reperfusion injury
IV	Intravenous
KC	Kupffer cells
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cells
MAC	Membrane attack complement complex
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MPTP	Mitochondrial permeability transition pore
NADH/NAD$^+$	Nicotinamide adenine dinucleotide
NF-κB	Nuclear Factor κB
NK T-cell	Natural killer T-cell
NO	Nitric oxide
NOX-2	NADPH oxidase 2
NSAID	Non-steroidal anti-inflammatory drugs
PKB	Protein kinase B (also known as Akt)
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern Recognition Receptor
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase
RAGE	Receptor for Advanced Glycation End Products
RBC	Red blood cell
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
SC	Stratum Corneum
siRNA	Small-interfering RNA
SLPI	Secretory leukocyte protease inhibitor
SOD	Superoxide dismutase
SPC	Soybean phosphatidylcholine
STAT3	Signal transducer and activator of transcription 3
TIM-1	T-cell immunoglobulin and mucin domain 1
TIM-4	T-cell immunoglobulin and mucin domain 4
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TRAM	TRIF related adaptor molecule
TRAF6	TNF receptor associate factor 6
TRIF	TIR domain-containing adaptor inducing IFN-β
TXA2	Thromboxane A2
UPR	Unfolded protein response
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

List of Contents

List of Figures

List of Tables

Chapter I. **INTRODUCTION**

1. Liver ischemia-reperfusion injury

During **hepatic surgery** or liver transplantation, commonly performed in patients with terminal liver disease or hepatic tumors, cellular damage can occur, due to interruption of blood flow, in a process known as **ischemia-reperfusion (IR) injury (IRI)**. This process is characterized by the interruption of blood supply and therefore lack of oxygen and nutrients. This deprivation leads to cellular damage and can be aggravated due to inflammation during liver revascularization [1, 2]. Liver injury caused by this condition can be categorized into warm and cold ischemia-reperfusion injury. **Warm IRI** occurs *in situ* during liver transplantation or hepatic surgery, when the blood flow is interrupted, and leads to hepatocyte damage. **Cold IRI** occurs *ex vivo* when the liver is being preserved for the transplantation and mainly affects liver sinusoidal endothelial cells (LSEC), which leads to microcirculation disruption. The last type of IR is usually coupled with the first during the transplantation surgery [3, 4].

1.1 Mechanism of inflammation in liver ischemia-reperfusion

IR is constituted by two phases: an ischemia phase, where the lack of oxygen supply and ATP depletion leads to microcirculation disruption, mitochondrial damage and ultimately hepatocytes death; and a reperfusion phase characterized by activation of the liver immune system which involves activation of macrophages and neutrophils, production of cytokines and chemokines, formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and increased expression of adhesion molecules on the hepatic endothelium [4, 5].

1.1.1 Ischemia phase

Concerning the **ischemia phase** of hepatic IR, a series of molecular events originated by hypoxia leads to ATP depletion and oxidative stress and ultimately causes cell swelling and hepatocytes necrosis (**Figure 1**) [4, 6].

When the blood flow is interrupted during the liver transplantation, the lack of oxygen in liver cells causes mitochondrial dysfunction by disrupting the oxidative phosphorylation, which result in increased levels of NADH/NAD^+ and formation of ROS within the cells. These events lead to the opening of the mitochondrial permeability transition pore (MPTP) causing ATP depletion from the cells [3, 6, 7]. Decreased levels of intracellular ATP cause stimulation of glycolysis and therefore increased formation of lactate. Ischemia also induces an increase in cAMP levels that contribute to deregulation of glucose metabolism [8]. Low intracellular ATP also interferes with cells active transmembrane transport, leading to alteration on H^+, Na^+, and Ca^{2+} homeostasis and ultimately producing edema in hepatocytes and LSEC [5, 7]. Deficiency of ATP triggers endoplasmic reticulum (ER) stress by activating the unfolded protein response (UPR). In this process, a few ER transmembrane receptors are required in signaling cascades, in order to inhibit protein synthesis and also activate the transcription of genes which encode proteins involved in protein folding and degradation. This response will activate autophagy mechanisms in order to remove damage cells [6, 7].

Another source of ROS production has been pointed out as xanthine oxidase (XO), a form of xanthine oxidoreductase (XOR). Using oxygen as a terminal electron acceptor, XO generates ROS such as superoxide radical ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2). In mammalian cells, XOR exists in two interconvertible forms, the predominant form in healthy tissue xanthine dehydrogenase (XDH) and the ROS producing form XO. Upon an ischemia insult, conversion of XDH isoform into XO results from limited proteolysis and sulfhydryl oxidation [6].

Formation of ROS causes low nitric oxide (NO) levels and, along with the upregulation of endothelin (ET) and thromboxane A2 (TXA2) contributes to the contraction of the sinusoidal lumen and microcirculation dysfunction ultimately leading to a disturbance of the hemodynamic equilibrium [3, 8, 9].

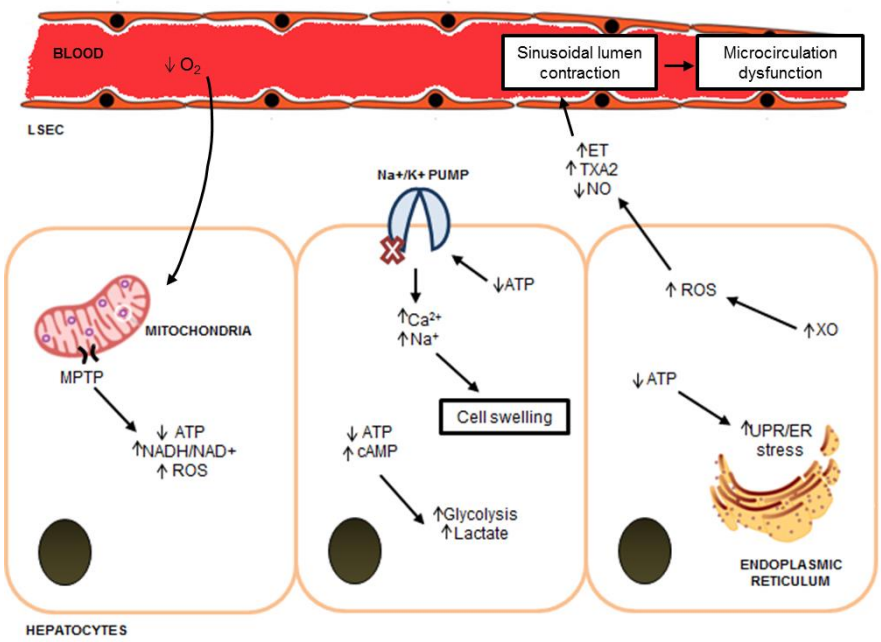

Figure 1 – Metabolic processes occurring in the ischemia phase of liver IRI. Interruption of blood flow during liver surgery leads to cellular hypoxia state with depletion of ATP levels. Decreased levels of intracellular ATP leads to glycogen consumption, mitochondrial dysfunction, increased glycolysis, production of ROS, endoplasmic reticulum stress, alteration in H^+, Na^+ and Ca^{2+} concentrations, cell swelling, sinusoidal lumen contraction and microcirculation dysfunction. Ultimately these events cause hepatocytes death mainly by necrosis.

1.1.2 Reperfusion phase

Regarding the **reperfusion phase** of IR, it can be divided into two different stages. The first stage, occurring in the initial 6h of the reperfusion, is distinguished by an impairment of microcirculation. Additionally, occurs the generation of ROS and RNS and the activation of resident liver macrophages known as Kupffer cells, leading to the initiation of the inflammatory process and causing oxidative stress. This first stage is crucial for the production and release of pro-inflammatory cytokines and chemokines, which stimulate the upregulation of adhesion molecules. These molecules are crucial mediators in the signaling pathways, assuring the recruitment of inflammatory cells such as neutrophils [4, 10]. The second stage, occurring 6 to 48h after the reperfusion onset, is associated with neutrophils recruitment and hepatocytes injury caused by the activation of NOX-2 (**Figure 2**) [4].

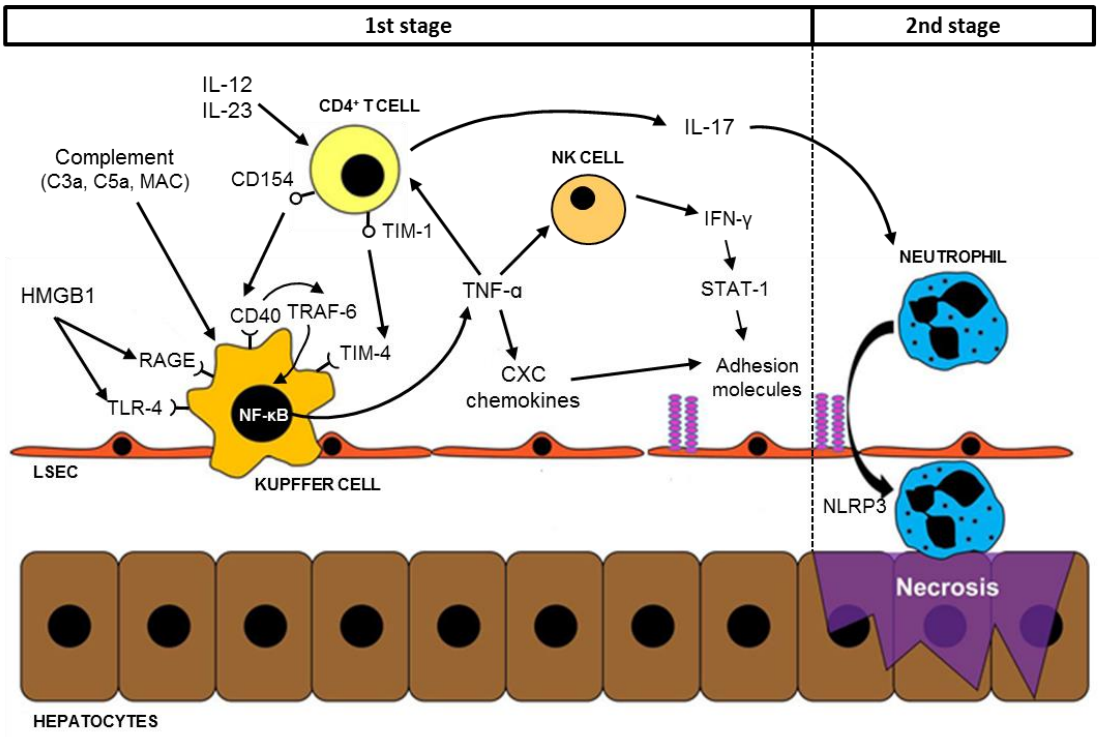

Figure 2 – Inflammatory process occurring in the reperfusion phase of liver IRI. Oxidative stress produced in the ischemia phase of IRI lead to the release of complement, HMGB1 and TNF-α, and the activation of Kupffer cells, $CD4^+$T cells and natural killer cells, resulting on the upregulation of cytokines, chemokines and adhesion molecules, which will facilitate the neutrophils recruitment into the liver parenchyma and induce oxidative stress in the hepatocytes, ultimately causing hepatocytes death mainly by necrosis. (Adapted from *Konishi et al 2017*) [11]

1st stage of reperfusion – Kupffer cells activation

Kupffer cells (KC), specialized macrophages which line the liver sinusoidal endothelial cells (LSEC), are the first immune response against pathogens by releasing pro-inflammatory cytokines and chemokines, and also have an important role on the formation of bile by breakdown of mature red blood cells (RBCs) leading to the release into the bile duct of bilirubin, a bile component [12, 13].

The first stage of reperfusion is characterized by the initial inflammatory immune response and **activation of KC**, involving complements, damage-associated molecular patterns (DAMPs), transcription factors, tumor necrosis factor alpha (TNF-α) and several other cytokines, T_H cells, natural killer (NK) cells, and chemokines such as C-X-C motif chemokines (CXC) [3, 6, 10, 14].

IR induces activation of the complement system, which is an important mediator of innate immune defense and inflammation process. In the liver, the complement system activation leads to the release of complements, a group of small soluble circulating proteins involved in tissue injury. Complement factor 3a (C3a), complement factor 5a (C5a), and membrane attack complex (MAC) inhibition have been shown to reduce KC-associated oxidative stress [6, 9, 15, 16].

High-mobility group box-1 protein (HMGB1) is an important DAMP that associate with pattern recognition receptors (PRRs) present in the KC such as toll-like receptors (TLRs) and

receptor for advanced glycation end products (RAGE). ROS formation during the ischemic phase leads to nuclear translocation of the transcription factor interferon regulatory factor 1 (IRF1), which in turn upregulates histone acetyltransferase (HAT) activity leading to HMGB1 acetylation and its extracellular release from hypoxic hepatocytes. HMGB1 has been identified as a key endogenous TLR4 ligand, and requires the recruitment of TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF related adaptor molecule (TRAM) in order to induce the translocation of interferon regulatory factor 3 (IRF3) to produce IFN-β and chemokine CXCL10, which will induce signaling cascades to upregulate pro-inflammatory cytokines. HMGB1 also binds KC receptor RAGE inducing mitogen activated protein kinases (MAPKs) which leads to production of cytokine TNF-α and chemokines CXCL10 and CXCL12 [3, 6, 10, 14, 17, 18].

The production of TNF-α, induced by oxidative stress, leads to the activation of nuclear factor κB (NF-κB) in KC, hepatocytes and also LSEC. In KC, NF-κB activation upregulates TNF-α, which in turn stimulates the activation of $CD4^+$ T lymphocytes and NK T cells. The $CD4^+$ T cells amplify the inflammatory process by continuously stimulating the KC activation through the complex CD154-CD40 and TIM-1-TIM-4 interactions. The proteins CD154 and T-cell immunoglobulin and mucin domain 1 (TIM-1) expressed in $CD4^+$ T cells surface interact with the proteins CD40 and TIM-4, expressed at the surface of KC and LSEC, respectively. The interaction CD154-CD40 stimulates CD40 recruitment of TNF receptor associate factor 6 (TRAF6) in order to activate NF-κB in KC and, ultimately, leading to the production of cytokines IL-1β, TNF-α, IL-6, chemokines CXCL2 and CXCL8, and NO [3, 6, 10, 19]. Cytokines IL-12 and IL-23 also stimulate $CD4^+$ T cells to produce IL-17, a cytokine responsible for inducing chemokines secretion and therefore facilitating neutrophils recruitment in the late phase of reperfusion [3, 6].

The upregulation of TNF-α also has an important role in the recruitment of neutrophils by upregulating adhesion molecules in the LSECs. The TNF-α induced activation of NK T cells leads to the release of IFN-γ, which in turn leads to an increased expression of adhesion molecules in the LSEC by stimulation of the transcription factor STAT1. Moreover the CXC chemokines production stimulated by TNF-α promotes upregulation of the adhesion molecules [6, 20].

2nd stage of reperfusion – Neutrophils recruitment

The final stage of reperfusion is characterized by the **recruitment of neutrophils** from the blood vessel lumen into the liver parenchyma and the respective inflammatory process which leads to damage to the hepatocytes and the LSECs. The recruitment process requires the contribution of chemotactic agents and vascular endothelial adhesion molecules such as ICAM-1, E-selectin, and VCAM-1 [6, 11, 13].

The capture of neutrophils in the blood vessels lumen is ensured by the presence of L-selectin on the neutrophil surface, which binds to its ligand expressed on the LSEC, and the presence of P-selectin on the LSEC surface, which binds to its ligand expressed on the neutrophil. The integrins expressed on neutrophils surface and the adhesion molecules ICAM-1 and VCAM-1 expressed on LSEC lead to the arrest of neutrophils and facilitate their migration to the liver parenchyma [11, 21, 22].

Additionally, NLRP3 inflammasome is also involved in neutrophils migration to the sites of hepatic necrosis through the activation of NLRP3, a PRR of the NOD-like receptor family.

Neutrophils binding to hepatocytes is ensured by interaction of integrins present on the neutrophils and ICAM-1 present on the hepatocytes [10, 11].

Once neutrophils contact with hepatocytes, there is an activation of NADPH oxidase 2 (NOX-2) in neutrophils leading to O_2 release and production of ROS, ultimately causing mitochondrial dysfunction and cell death mainly by necrosis. There are also proteases and hydrolytic enzymes released during neutrophils degranulation that are directly responsible for hepatocytes necrosis [6, 23].

1.2 Inflammation resolution and liver regeneration

Concerning the mechanisms of inflammation resolution, it has been reported that anti-inflammatory mediators are capable of inducing tissue repair and hepatocytes proliferation, such as cytokines (IL-6, IL-10 and IL-13), secretory leukocyte protease inhibitor (SLPI), chemokines, transcription factor NF-κB, and growth factors (HGF and EGFR) [11].

During the resolution of the inflammatory process, IL-6 has been shown to induce activation of signal transducer and activator of transcription 3 (STAT3), a crucial mediator for cell proliferation, while IL-13 inhibits the pro-inflammatory effects of NF-κB and protects cells from peroxide cytotoxicity [24, 25]. In addition, a small protein that inhibits enzymes with serine protease activity, known as SLPI, results in reduced expression of pro-inflammatory mediators and neutrophil recruitment [26]. Moreover, chemokines role in the resolution of the inflammatory process is a controversial matter as low concentrations of CXC induce cell proliferation, while high concentrations of CXC lead to cell cytotoxicity [27].

Although the transcription factor NF-κB is highly involved in inflammation processes, the primary function of this mediator is associated with cell proliferation and hepatocyte survival, as NF-κB activation in hepatocytes induces production of anti-inflammatory mediators such as IL-10 and HGF [28].

Simultaneously with the process of inflammation resolution, mechanisms of tissue repair take place on the liver during late IR. As the liver is an organ with a great regenerative capacity, although the stimulus for hepatic regeneration remains unknown, the hepatocytes proliferation begins shortly after IR. Hepatocytes are quiescent, highly differentiated cells, which under normal conditions have minimal proliferation capacity; however, after liver mass reduction or functional loss, hepatocytes acquire potential of replication, leaving their quiescent phase (G_0 phase) and entering phase G_1 of cell cycle. This initial phase of hepatocytes priming is mediated by several cytokines and growth factors. For instance, IL-6 and TNF-α, that are released from Kupffer cells, activate NF-κB and STAT3 in order to start transcription of target genes essential for regulation of cell cycle such as G1 cyclin and cyclin D. Moreover, hepatocytes transition from G_1 phase to S phase of DNA replication is induced by several growth factors such as HGF and EGFR, ensuring the expression of essential genes to this phase such as cyclin E, cyclin A, and DNA polymerase [11, 29]. Furthermore, the *Met* proto-oncogene signaling is essential for activation of ERK1/2 (extracellular signal-regulated kinases), needed in signaling pathways for cell cycle, and also for encoding a tyrosine kinase receptor that binds HGF to a scatter factor (SF) resulting in the complex HGF-SF. This complex induces hepatocyte proliferation in which the scatter factor functions as a motility agent and dissociates, increasing motility of the epithelial cells. Finally, once the

liver mass is restored, the replication process ceases and hepatocytes return to their quiescent phase [30].

After the loss of liver mass, there are large amounts of dead tissue originated from necrotic cell that must be removed. This process is ensured by regenerating cells, including macrophages and hepatic stellate cells (HSC). In several models of severe liver injury, where the parenchymal proliferation capacity is impaired, activation of hepatic progenitor cells (HPC) occurs and is regulated by regenerative cells [31, 32].

1.3 Strategies to prevent liver IRI

Ischemia-reperfusion injury enlaces mechanisms still not fully described. However, it is known that the response of the liver to this condition during hepatic surgery is dependent on several factors characteristic of the livers owner such as starvation, age, and hepatic steatosis [10, 33]. In order to prevent liver IR or reduce its effects has been studied pharmacology therapies, gene therapies, and surgical interventions [8].

Concerning the pharmacological therapies there are several drugs known to induce favorable effects in attenuating IRI in rat models (**Table 1**).

Table 1 – Pharmacological therapies to attenuate liver IRI according to the experimental model (warm IR and cold IR), the ischemic time, and the respective beneficial effects [8].

Drug	Ischemic time	Effect
Warm IR		
AT1R, AT2R (Ang II type I and II receptor antagonist)	60min	Low hepatic injury, MDA and nitrotirosines
Ascorbate (vitamin C)	30min	Low hepatic injury and apoptotic Kupffer cells
bpV (HOpic) (PTEN inhibitors)	60min	Low hepatic injury, apoptosis and PTEN High PKB/Akt activation
Captopril (Ang II blockers)	60min	Low hepatic injury
CORM-2 (carbon monoxide-releasing molecule-2)	60min	Low hepatic injury, neutrophils, apoptosis, NF-κB, TNF, IL-6 and ICAM expression
EGF (epidermal growth factor)	60min	Low hepatic injury
Fasudil (Rho-kinase inhibitor)	30min	Low hepatic injury, HSC activation, endothelin 1 and portal perfusion pressure High Survival
IGF-1 (insulin-like growth factor 1)	60min	Low hepatic injury
MF-1 (metron factor 1)	90min	Low hepatic injury and oxidative stress High survival and NO synthesis
n-3 PUFA (polyunsaturated fatty acid)	60min	Low hepatic injury, inflammation and oxidative stress
PD123319 (Ang II blockers)	60min	Low hepatic injury

Retinol (vitamin A)	60min	Low hepatic injury and steatosis High Regeneration
Sirolimus (immunossupressant)	60min	Low hepatic injury, apoptosis, neutrophil infiltration and MDA
Trans-resveratrol (antioxidant)	60min	Low hepatic injury and MDA High catalase, GSH and SOD
WY-14643 (PPAR-α antagonist)	60min	Low hepatic injury, inflammatory cytokines and oxidative stress
Cold IR		
A779 (Ang-(1-7) receptor antagonist)	6h	Low hepatic injury, oxidative stress and necrosis High Survival
17-β Estradiol (steroid hormone)	4h	Low hepatic injury, apoptosis, and glycogen synthase kinase-3β High survival and Akt
Fructose	26h	Hepatocyte and LSEC protection Low hepatocyte injury High ATP
GW9662 (PPARγ antagonist)	6h	Low hepatic injury
MPLA (TRL4 agonist)	6h	Low hepatic injury High survival
RBP4 (adipocytokine)	6h	Low hepatic injury and PPARγ expression
rHuEPo (glycoprotein hormone)	6h	Low hepatic injury and apoptosis High survival
rMnSOD (antioxidant)	16h	Hepatocyte and LSEC protection Low hepatic injury and inflammation Improvement in hepatic microcirculation and endothelial dysfunction in healthy and steatotic conditions
Simvastatin (HMG-CoA reductase inhibitor, vasoprotector)	16h	Hepatocyte and LSEC protection Low hepatic injury, apoptosis and inflammation Improvement in hepatic microcirculation and endothelial dysfunction
SQ22536 (adenylate cyclase inhibitor)	6h	Low hepatic injury, lactate accumulation, oxidative stress and microvascular disorders High survival
Warm/cold IR		
FK506 (immunossupressant)	60min	Low hepatic injury, edema, necrosis and AIF-1 activation
Tauroursodeoxycholate (bile acid)	60min/6h	Low hepatic injury and PPARγ expression Low ER stress

Although the mechanisms of inflammation and liver injury are more severe in warm ischemia, there is a considerable amount of adverse effects in cold ischemia that can be prevented if liver preservation is maintain under favorable conditions. For this purpose, there are some solutions that present conservative properties during several ischemic times and show favorable effects in IR rat models with cold IR (**Table 2**) [8].

Table 2 – Additives for preservation solutions to prevent cold IRI according to the ischemic time, and the respective beneficial effects [8].

Drug	Ischemic time	Effect
APC (anticoagulant serine protease)	2, 6, 20h	Low hepatic injury, apoptosis, hyaluronic acid, IL-6, TNF-α, ET-1, infiltration of neutrophils and macrophages High survival and hepatic microcirculation
Carvedilol (β-blocker and antioxidant)	24h	Low hepatic injury microcirculatory diseases, ATP depletion, oxidative stress and mitochondrial damage
EGF, IGF-1	24h	Low hepatic injury and necrosis
Oxycyte (perfluorocarbon)	8h	Low hepatic injury
Peg-Alb (polyethylene glycol-albumin)	30h	Hepatocyte and LSEC protection Low hepatic injury High portal vein inflow
rMnSOD (antioxidant)	16h	Low hepatic oxidative stress accumulation
RXP409 (MMP inhibitor)	42h	Low hepatic injury
Simvastatin (HMG-CoA reductase inhibitor, vasoprotector)	16h	Hepatocyte and LSEC protection Low hepatic injury, apoptosis and inflammation Improvement in hepatic microcirculation and endothelial dysfunction
TAT-HO-1 (heme oxygenase fusion protein)	2, 6, 12, 18h	Hepatocyte and LSEC protection Low hepatic injury and BAX High Bcl2
Tauroursodeoxycholate (bile acid)	2h	Low ER stress

Regarding gene therapies that aimed to attenuate adverse effects of IRI, some target genes and strategies have been studied in warm ischemia rat models and presented favorable effects. Amongst these strategies, there was the technique of siRNA upon adiponectin [34], the use of adipose tissue-derived mesenchymal stem cells [35], and of NF-κB decoy oligonucleotides [36]. All were administrated after 1h of ischemia and resulted in low hepatic injury, oxidative stress and apoptosis, and decreased NF-κB, IL-6, and TNF-α expression [8].

During hepatic surgery, there are a few clinical procedures that can be performed in order to attenuate and resolve the adverse effects of IRI, such as intermittent clamping, ischemic preconditioning and machine perfusion. These techniques have been studied in rat models and presented a decrease in hepatic injury and oxidative stress. However, they are not yet usually applied in the clinic, since further studies are necessary [8, 37-39].

2. Aquaporins

Aquaporins (AQPs) are small transmembrane proteins that facilitate water and solutes transport through the plasma membrane of cells triggered by osmotic or solutes gradient. AQPs play an important role in biological functions including maintenance of water homeostasis, urinary concentration in the kidney, brain function, glandular secretion, skin hydration, tissue repair and metabolism. These membrane channels also facilitate the flux of small solutes such as glycerol, ammonia, urea, carbon dioxide, peroxide, metalloids, and nitric oxide; and restrict the passage of charged molecules such as cations, protons, and ammonium, although some isoforms were reported to present permeability for anions. [40]

2.1 Water channels discovery

The existence of a water channel that facilitates water movement across biological cell membranes had been predicted for a long time [41]. While attempts to isolate the membrane protein were futile, some publications were able to provide information regarding the molecular size of the protein at around 30 KDa [42]. In 1992, while working with red blood cell membrane proteins, Peter Agre and his colleagues noticed a consistent band at their gels at around 28 KDa and persisted on investigating its importance [43]. This protein was later isolated, sequenced and antibodies showed that it was also being expressed in highly water permeable membranes such as kidney proximal tubules and the descending limb of Henle [44]. Believing that they had managed to isolate the molecular water channel, Agre conducted an experience by injecting mRNA encoding this protein into *Xenopus laevis* oocytes, which are commonly highly impermeable to water. The oocytes exhibited high osmotic water permeability, causing cells to swell and explode in hypotonic buffer. This permitted the conclusion that the mRNA they injected had allowed the oocytes to produce a new membrane protein which increased membrane permeability to water. This membrane protein was the first water channel to be identified as channel-forming integral membrane protein of 28 KDa (CHIP28), now known as AQP1[45].

2.2 Aquaporins structure

In the membrane, aquaporins are assembled as tetramers, composed of 4 linked water channels monomers (**Figure 3a**). Each AQP monomer has approximately 30 KDa, and consists of 6 transmembrane α-helices domains (M1, M2, M4, M5, M6, M8), 2 half helices (M3, M7), and 5 loops (loops a-e) that connect the helices (**Figure 3b-c**) [40].

The AQP monomer has an extracellular domain and a cytoplasmic domain that are connected by a central amphipathic pore region (**Figure 3d**). The amino and carboxyl terminals of the protein can be found in the cytoplasmic domain [40].

The hydrophilic surface of the pore is composed of polypeptide backbone α-carbonyl groups, which act as hydrogen-bond donors and acceptors, while the hydrophobic surface insures the blockage of bigger molecules [40].

Two highly conserved Asn-Pro-Ala (NPA) motifs can be found on the cytoplasmic loop b and on the extracellular loop e, forming a dipole that exclude charged species from the lumen of the pore. These NPA motifs contain asparagine polar side chains which are responsible, alongside with the α-carbonyl groups, for the coordination of the selective and

bidirectional flux of water and other solutes through the pore and also for proton exclusion (**Figure 3d**) [40, 46, 47].

On the extracellular domain, in the narrowest pore region, there is an aromatic-arginine region (ar/R constriction) known as the selectivity filter that blocks molecules larger than water and also assists on the prevention of proton conduction (**Figure 3d**) [40, 47].

The pore region constitutes a site of potential interest for the identification of small molecules that can target the AQPs [40].

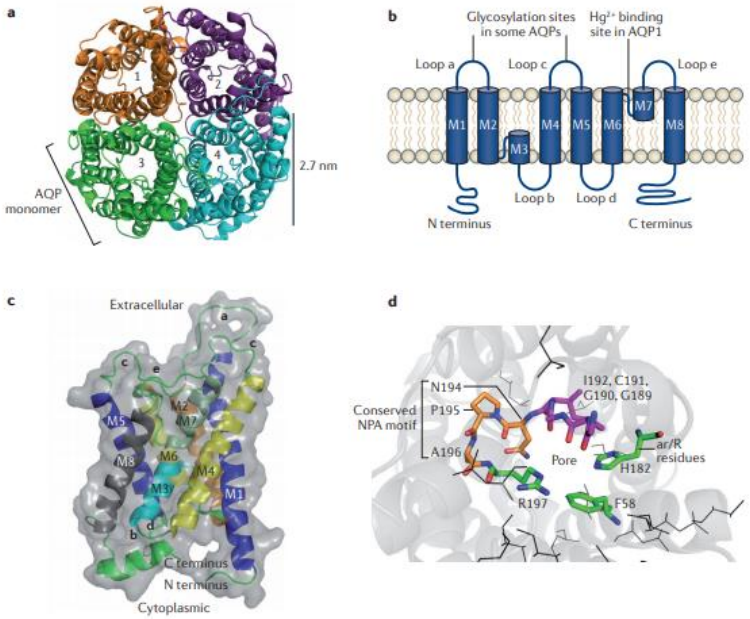

Figure 3 – Aquaporins structure. a) Top view of the extracellular domain of AQP1 homotetramer with monomers labeled 1-4, using Protein Data Bank (PDB). **b)** AQP membrane topography, which shows helical domains labeled M1-M8 and the connecting loops labeled a-e. **c)** Structure of the bovine AQP1 monomeric unit exposing the organization of the helical domains, the connecting loops, as well as the cytoplasmic and extracellular domains, with the N and C-terminal in the cytoplasmic domain. **d)** Extracellular vestibule of bovine AQP1. The ar/R constriction region is represented in green with residues Phe58, His182, and Arg197. The NPA motifs are represented in orange with residues Asn194, Pro195, and Ala196. The backbone α-carbonyl hydrogen-bond acceptors are represented in violet with residues Ile192, Cys191, Gly190, and Gly189. The hydrophobic side chains that consist on the non-polar amphipathic surface are shown in black. (Adapted from *Verkman et al 2014*) [40]

2.3 Aquaporins classification and biological functions

Aquaporins are widely expressed in epithelial and endothelial cells involved in fluid transport, such as kidney tubules, glandular epithelia, and choroid plexus, but also in tissues that have no significance in fluid transport, such as skin and adipocytes [48]. Whereas most AQPs are localized at the cell plasma membrane and intracellular membranes, some may also be found circulating in exosomes or extracellular vesicles [47].

In mammals, 17 aquaporins have been identified and are expressed widely in specific cell tissues and organs throughout the organism, although only AQP0-12 have been found in higher orders of mammals like human [49]. According to their structural and functional specificity, AQPs can be divided into three subfamilies, the classical water transporting aquaporins (AQP0-2, AQP4-6 and AQP8), the glycerol transporting aquaglyceroporins

(AQP3, AQP7, AQP9 and AQP10) and finally the unorthodox superaquaporins (AQP11 and AQP12) [49].

There are a few aquaporins capable of transporting peroxide (H_2O_2) called peroxiporins, such as AQP1, AQP3, AQP5, AQP8, AQP9 and AQP11 [47, 50-52].

2.3.1 Water transport

Classical aquaporins allow the water transport in response to osmotic gradients created by the transport of active ions and neutral solutes. In most cells and tissues, AQPs assist on the epithelial fluid transport by increasing water permeability [40]. This type of transport is transcellular, which means that the fluid flows from the cell through another cell, even by absorption on the apical membrane from the lumen to the blood, or by secretion on the basolateral membrane from the blood to the lumen [48].

Within the water homeostasis, AQPs play a major role in several processes along different tissues (**Figure 4**), such as kidney tubule water transport, epithelial fluid secretion, cell migration, brain water transport, and neural signaling [40, 48].

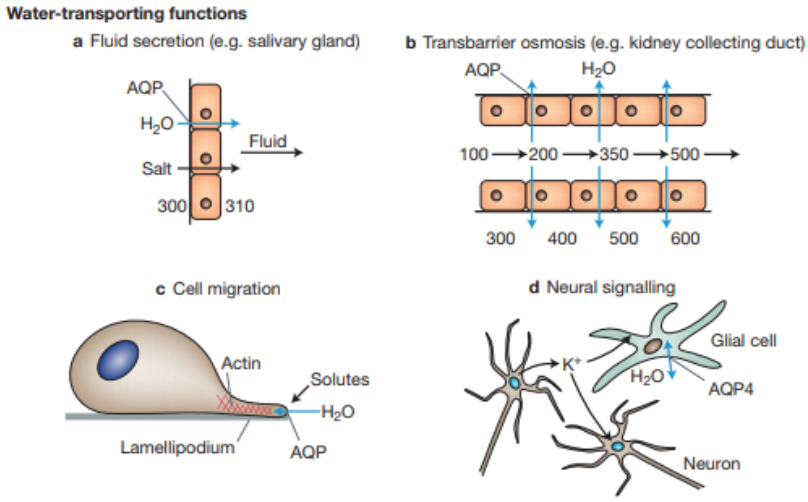

Figure 4 – Main aquaporins physiological functions associated with water transport. a) Fluid secretion is obtained through AQP-induced increased of epithelial water permeability by creation of an osmotic or solute gradient. **b)** Transbarrier osmosis occurs on the level of kidney collecting duct in order to produce concentrated urine by AQP-induced higher permeability. **c)** Cell migration induced by the formation of the protrusion called lamellipodium due to AQP-induced polarization of the leading edge of the cell. **d)** Neural signaling occurs in astrocytes mainly through AQP4 water transport allowing potassium continuing reuptake. (Adapted from *Verkman et al 2008*) [48]

Kidney tubule water transport. AQPs assist in the urine-concentrating function of the kidney by increasing the water permeability in kidney tubules and microvessels (**Figure 4b**) [40]. The increased water permeability will allow its absorption from the tubule lumen fluid, triggered by an osmotic gradient, leading to concentrated urine. If the AQPs fail to increase the permeability, on the collecting duct epithelium, a great quantity of water will remain in the lumen, leading to the excretion of a larger volume of diluted urine. As the transepithelial water transport in the collecting duct is transcellular, this deficiency of urine concentration can be explained by low water permeability of AQP2 on the apical membrane or of AQP3/AQP4 on the basolateral membrane of the epithelial cells [48, 53].

Fluid secretion. AQPs increase the transepithelial water permeability, consequence of an osmotic gradient created by the active transport of ions or solutes (**Figure 4a**). [40] This feature will assist in the production of different fluids on several tissues such as salivary gland, airway submucosal gland, lacrimal gland, choroid plexus, and sweat gland. The impairment of AQP1 and AQP5 was shown to reduce the fluid secretion although it didn't seem to affect the production of sweat. This fact allows the conclusion that the AQP influence depends on the rate of fluid transport [40, 48, 54].

Brain water transport. AQP4 is highly expressed in astrocytes throughout the brain and spinal cord, most precisely in fluid transport sites of the blood brain and blood-CSF (cerebrospinal fluid) barriers, which allows the conclusion that this AQP is crucial for the water balance in the brain. If this balance is disrupted in favor of the entrance of water into the brain, it may lead to brain swelling [40, 48, 55].

Cell migration. In cells which are going through the migration process, several AQPs are polarized to the leading edge of the cells and contribute to the formation of cell membrane protrusions, called lamellipodium, in the direction of the cell movement, facilitating their migration (**Figure 4c**) [40, 48, 56-58].

Neural signaling. In astrocytes, the water transport through AQP4 during potassium reuptake following neuroexcitation leads to contraction of the extracellular space, maintaining the force for the potassium reuptake (**Figure 4d**) [40, 48, 59, 60].

2.3.2 Glycerol transport

Aquaglyceroporins participate in several processes that require glycerol transport (**Figure 5**), such as cell proliferation, adipocyte metabolism, and epidermal water retention [40, 47, 48]. Their structure differ from classical AQPs as they have a larger pore due to the presence of an additional conserved Asp residue near the second NPA motif, allowing glycerol and other small solutes to permeate [61].

Skin hydration. The hydration of stratum corneum (SC), the most superficial skin layer, depends on several factors like the SC lipid/protein composition, external humidity, and concentration of retained water through osmolytes fraction (**Figure 5a**). AQP3, which is highly expressed in the basal membrane of keratinocytes of SC, allows the transport of glycerol which is a water-retaining osmolyte. Impairment of AQP3 will result in low hydration, skin elasticity, and wound healing [40, 48, 62, 63].

Cell proliferation. AQP3-facilitated glycerol transport is crucial on cell energy balance, as increased levels of glycerol induce production of ATP, which will in turn induce cell proliferation (**Figure 5b**) [40, 48].

Adipocyte metabolism. The high permeability to glycerol in plasma membrane of adipocytes, conferred by AQP7, prevents fat accumulation on adipocytes. AQP7 deficiency induces an increase in glycerol concentration that results in increased glycerol-3-phosphate and triacylglycerol biosynthesis through glucose metabolism (**Figure 5c**). AQP9 also has been shown to be correlated with impairment of adipocyte metabolism [40, 48, 64, 65].

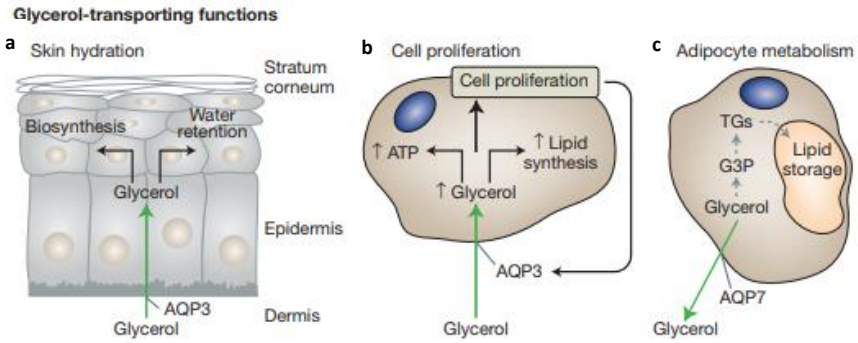

Figure 5 – Main aquaglyceroporins physiological functions. a) Skin hydration correlates with AQP3 glycerol input which will in turn retain water. **b)** Cell proliferation occurs through AQP3-facilitated glycerol transport which will increase ATP production. **c)** Adipocyte metabolism correlates with AQPs in the sense that AQP7-facilitated glycerol output prevents lipid storage. (Adapted from *Verkman et al 2008*) [48]

2.4 Aquaporins regulation

Regulation of AQPs is crucial to water homeostasis and osmoregulation of several solutes in mammalian organs involved in fluid transport [49]. Short-term regulation of AQPs is known as gating and can be achieved through intracellular signaling processes inducing protein conformational changes that alter its transport activity, intracellular location, and also its expression on the various tissues. Protein posttranslational modifications (phosphorylation, acetylation, methylation, ubiquitination, and glycosylation), subcellular distribution, degradation, and protein interactions influence the gating process of AQPs. Other environment factors such as pH, pressure, temperature, solute and osmotic gradients can also affect the regulation of AQPs [47, 66, 67].

There are different mechanisms of regulation dependent on the AQP location and function [66]. AQP1 is activated by intracellular cGMP, and its recognition is regulated by the phosphorylation of residue Y253 in the C-terminal domain of the protein [66]. The swelling of secretory vesicles in the exocrine pancreas induced by AQP1 is therefore stimulated by guanosine triphosphate (GTP) [66]. AQP2 is a vasopressin-regulated water channel, in which the increase of the hormone induces the water permeability of collecting duct cells in the kidney. Vasopressin phosphorylation of AQP2 stimulates the redistribution from the intracellular vesicles in the basal side to the apical membrane [66, 68]. AQP2 can also be regulated by exocytosis by receptor-activated adenylyl cyclase-protein kinase A phosphorylation of the residue Ser256 on the C-terminal cytoplasmic domain of the aquaporin [68, 69]. Mercury chloride is a compound that was shown to inhibit the water permeability of most AQPs, although a stimulatory effect was reported for AQP0, and AQP6 [68].

Different from the intracellular signaling regulating processes, it was shown that a membrane-tension-mediated mechanism is more efficient and faster in the closing of water channels as all the monomers cooperate, such as tested in AQP1. This mechanism is reversible [70, 71].

3. Aquaporins biological functions in the liver

The hepatobiliary system is responsible for several metabolic processes including bile formation and secretion, detoxification and glycerol metabolism [72]. Hepatocytes, the main liver cells, are highly polarized epithelial cells constituted by two plasma membrane domains; a basolateral domain in contact with the sinusoidal blood capillary, and an apical domain between each hepatocyte in which the membrane fuses to form the bile canaliculi, where the bile produced by the hepatocytes is secreted (**Figure 6**). As bile is predominantly composed of water, canalicular bile formation is an osmotic secretory process that results from the input of water in response to osmotic gradients created by active secretion of solutes. In basal conditions, water diffusion across hepatocyte plasma membrane occurs mainly via paracellular transport through tight junctions on hepatocytes apical domain. However, when hepatocytes are stimulated, water transport occurs significantly via transcellular pathway through the plasma membrane [73-76].

Several cells from the hepatobiliary tract including hepatocytes, hepatic stellate cells (HSC), cholangiocytes, intrahepatic bile duct cells, gallbladder epithelial cells, and endothelial cells have been shown to express AQPs, which are known to assist on the transcellular water transport by increasing water permeability and also allow the transport of glycerol and other small solutes (**Figure 6**) [72, 75-78]. AQPs expression has been found impaired in several pathological liver conditions such as extrahepatic cholestasis, insulin resistance syndrome, cirrhosis, fatty liver and gallstone disease, thus suggesting that AQPs can constitute biological markers for identification of liver diseases and can be targeted in order to attenuate its effects [78, 79].

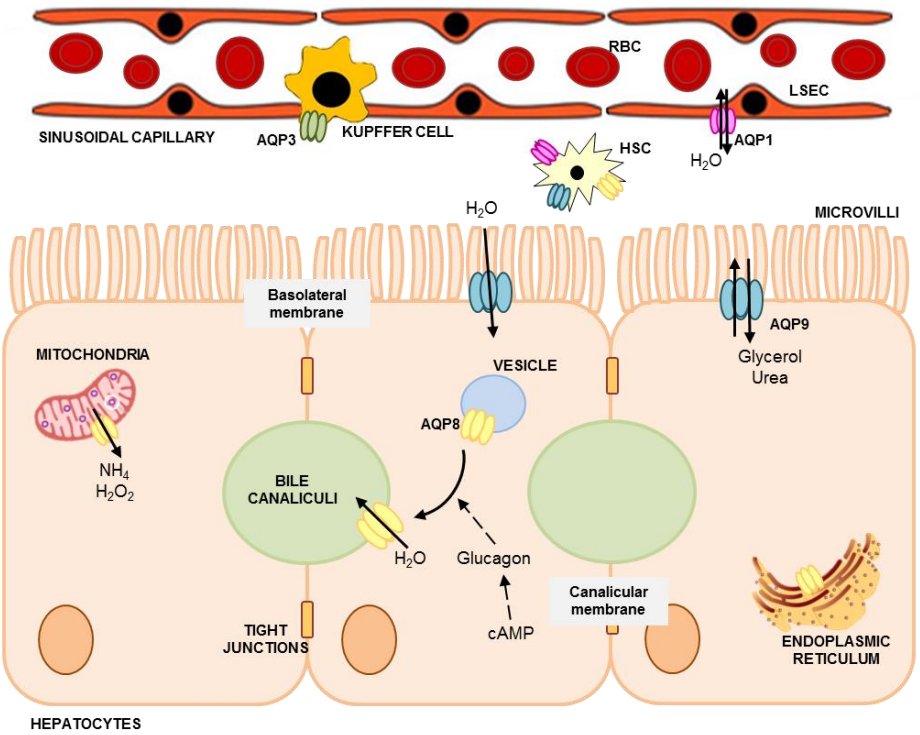

Figure 6 – AQPs location and function in hepatic cells. AQP1 (represented in pink) in LSEC and HSC mainly facilitates transcellular water transport. AQP3 (represented in green) was show expressed in Kupffer cells and considered important in inflammation processes although its function remains unclear. AQP8

(represented in yellow) is mostly expressed in intracellular vesicles, although, upon choleretic stimuli via cAMP and glucagon, is translocated to the hepatocytes canalicular plasma membrane, allowing an increase of water membrane permeability, and ultimately facilitates bile secretion to the bile canaliculi. A detoxification role is also conferred to AQP8 by allowing the efflux of mitochondrial ammonia and hydrogen peroxide during production of ROS. AQP9 (represented in blue), expressed in the basolateral membrane of hepatocytes, controls the diffusion of water, ammonia and glycerol from the sinusoidal capillary to the hepatocytes.

3.1 AQP1

AQP1, the most abundant classical aquaporin in mammals, is expressed in several cells from the hepatobiliary tract such as HSC, intrahepatic bile duct cells, gallbladder cells, and endothelial cells and small vessels [72]. Under normal conditions, its role on the liver remains unclear, although speculation points its function mainly as transcellular water transport on the various organs where this aquaporin is expressed (**Figure 6**) [76, 77]. However, when bile secretion from the hepatocytes is impaired either due to an increase or a decrease in the volume of bile secretion, under choleretic or cholestatic stimuli respectively, water can be secreted by apical AQP1 or either secreted or reabsorbed through endothelial AQP1 [72, 79].

3.2 AQP3

AQP3 is an aquaglyceroporin found expressed in Kupffer cells, resident liver macrophages which are activated during liver inflammation (**Figure 6**). As AQPs are involved in cell migration and proliferation, AQP3 has been pointed out as contributing to cell migration of KC to the inflammation site during liver diseases [77]. Moreover, in a bowel disease model, AQP3 expression has been correlated with NF-κB and cytokine TNF-α modulation, indicating that AQP3 has an important role in inflammatory processes [80]. However, more studies are needed in order to identify AQP3 specific involvement in Kupffer cells contribution on liver diseases.

3.3 AQP8

AQP8, the most abundant AQP in hepatocytes, is predominantly located in intracellular vesicles and to a lesser extent in the canalicular plasma membrane of hepatocytes. This AQP was also found expressed in HSC, gallbladder, ER, and the inner membrane of mitochondria [72-78, 81]. Besides water transport, AQP8 has been shown to allow the diffusion of ammonia and hydrogen peroxide [82]. Its main function is related with bile secretion as hepatocytes stimulation with hormone glucagon induces translocation of intracellular AQP8-containing vesicles to the canalicular plasma membrane, leading to an increase of canalicular membrane water permeability and therefore stimulating bile secretion. AQP8 canalicular membrane translocation dependent-glucagon stimulation requires cAMP activation (**Figure 6**) [74-78, 82, 83]. Moreover, as AQP8 has significantly high permeability for ammonia, it has been suggested an important role of this AQP in the hepatic mitochondrial ammonia detoxification and ureagenesis, although its mechanism remains unknown [76, 79, 82, 83]. Additionally, AQP8 facilitates H_2O_2 release from the mitochondria matrix during

generation of ROS, thus indicating a protective role against liver inflammation induced by oxidative stress (**Figure 6**) [79, 83].

3.4 AQP9

AQP9 is located at the basolateral membrane of hepatocytes and also in HSC [72-78]. Regarding transcellular water transport, this AQP facilitates water uptake from the sinusoidal capillary through the hepatocyte basolateral membrane during active bile secretion (**Figure 6**). Nevertheless, this aquaglyceroporin is mainly responsible for the hepatic metabolism of glycerol, both in fed and fasting state. AQP9 regulates liver glycerol permeability depending on the nutritional state and the circulating insulin levels. During the fasting state or in diabetes, AQP9 induces both the glycerol uptake into the hepatocyte for gluconeogenesis and the urea efflux from hepatocytes after ureagenesis (**Figure 6**) [72, 76, 78, 81, 82, 84]. Hepatic AQP9 has also been attributed a detoxification function as it induces uptake of ammonia and arsenite from the blood vessels into the periportal hepatocytes, thus facilitating their excretion and reducing their toxicity [72]. However, AQP9 permeability for these toxic solutes is also thought to lead to hepatocellular damage and cell death [82]. In fact, AQP9 has also been implicated in hepatocyte apoptosis by facilitating water efflux [81].

PROBLEM CONTEXT AND OBJECTIVES

Hepatic ischemia-reperfusion injury is an unavoidable complication in liver surgery with serious physiological consequences leading to multiple organ failure. Since this pathological condition has a high mortality rate associated, there is a growing interest in assessing methods and strategies to prevent and attenuate liver IRI [1, 2].

Due to the inflammatory process occurring in the reperfusion phase of IR where blood is restored to the liver cells, membrane permeability tends to increase, leading to cell swelling and disturbance of hemodynamic equilibrium. Since membrane permeability is affected by inflammation processes, AQPs situated in several liver cells such as Kupffer cells, hepatocytes and LSEC, pose as important regulators of hemodynamic equilibrium during IR [78, 79].

Considering the role that AQPs may play during liver IRI, the focus of this work consisted on assessing the inflammation effect on AQPs expression *in vitro* and *in vivo*. With the purpose to correlate AQPs response to IR stimulus, inflammation-related genes expression was also determined. The work plan is represented in **Table 3**.

The *in vitro* assay aimed at evaluating AQPs expression upon LPS-induced inflammation, the effect of anti-inflammatory drugs to reverse the inflammation, and the hypoxia effect in hepatocytes. The *in vivo* assay aimed at measuring AQPs expression upon inflammation induced by an ischemia-reperfusion rat model and the effect of a natural compound (quercetin) in the mitigation of the onset of the inflammatory process.

Table 3 – Project plan. The project consisted in two assays and 5 tasks.

Evaluation of AQPs inflammatory response *in vitro*
1. Assessment of the LPS inflammatory effect on AQPs expression in HepG2 cells
2. Effect of liposomal drugs formulations that reverse the LPS-induced inflammation on AQP3 expression in HepG2 cells
3. Assessment of the effect of hypoxia on AQPs expression in HepG2 cells
Evaluation of AQPs inflammatory response *in vivo*
4. Evaluation of IR effect on AQPs expression in rat liver samples
5. Assessment of the protective effect of quercetin in encapsulated form on AQPs expression in rat liver samples

Chapter II. MATERIAL AND METHODS

1. Evaluation of AQPs inflammatory response *in vitro*

1.1 Cell culture

For the evaluation of the inflammatory response *in vitro*, HepG2 human liver cancer cell line was chosen to represent liver cells and assess LPS-induced inflammation, the effect of pharmaceutical drugs to attenuate LPS-induced inflammation, and inflammation induced by a hypoxia chamber model. All samples for this *in vitro* evaluation were provided by Research Lab Human genetics and cancer therapeutics of UCIBIO, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologias, Universidade Nova de Lisboa and were prepared as following (1.2 to 1.4).

1.2 LPS-induced inflammation in HepG2 cells

HepG2 cells were plated at a concentration of $5x10^5$ cells/mL in 24-well plates and then exposed to different concentrations of lipopolysaccharide (LPS) (Sigma) for 24h: 5, 10, 100, and 200 µg/mL of LPS. After the incubation, the cells were harvested, centrifuged at 200 g for 5 min at room temperature, and total RNA was extracted for the evaluation of the inflammatory response [85].

1.3 Pharmaceutical drugs administration to reverse LPS-induced inflammation in HepG2 cells

HepG2 cells were plated at a concentration of $5x10^5$ cells/mL in 24-well plates, then incubated for 24h with 10 µg/mL of LPS and with pharmaceutical drugs design to attenuate LPS-induced inflammation such as prednisolone and ibuprofen delivered both freely or through liposomes (on the membrane or encapsulated) and its respective buffers. The added dose of prednisolone both free and in liposomes was 0.4 mM, and the dosage of ibuprofen both free and in liposomes was 0.2 mM. The buffer used for the liposomes of both drugs was citrate. As a hydrophobic compound, the free ibuprofen required solubilization with DMSO. After the incubation, the cells were harvested, centrifuged at 200 g for 5 min at room temperature, and total RNA was extracted for the evaluation of the inflammatory response [85].

1.4 Hypoxia chamber assay in HepG2 cells

HepG2 cells were incubated in a hypoxia chamber with low oxygen tension (approximate 1% O_2, 5% CO_2, and balanced with N_2) for 12h to induce hypoxic condition. Then reoxygenation was induced on a separate chamber by exposing cell cultures to hyperoxic condition (approximate 95% O_2/5% CO_2) for a few minutes in order to evacuate N_2. Finally cells were maintained in a 37ºC humidified incubator under normoxic conditions (air/5% CO_2) [86]. After 4h of reperfusion, the total RNA was extracted for the evaluation of the inflammatory response.

1.5 Reverse transcription and quantitative polymerase chain reaction (RT-qPCR) of AQPs and inflammation-related genes

Total RNA from HepG2 cells was extracted and provided for this assay and the purity and concentration of RNA were determined by measuring the absorption ratio at 260/280 nm, using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was obtained from 100 ng of total RNA and the reverse transcription was carried out with a NZY First-Strand cDNA Synthesis Kit (NZYTech) using a CFX96TM Real-Time System C1000 (BioRad).

In order to amplify the fragments of interest and quantify the RNA expression of the AQPs, real-time PCR reactions were performed using a CFX96TM Real-Time System C1000 (BioRad), with the TaqMan® Universal Master Mix II (Applied Biosystems). The specific TaqMan gene expression primers and probes for AQP1, AQP3, AQP8 and AQP9 (Applied Biosystems) are presented in **Table 4**.

For the evaluation of AQPs exposed to each LPS concentration and the evaluation of AQP3 subjected to each drug group, the relative quantification of the target genes expression was obtained by normalization with the housekeeping gene 18S rRNA, using the $2^{(-\Delta\Delta Ct)}$ or Livak method. For the assessment of AQPs subjected to the hypoxia chamber, the relative quantification of the target genes expression was obtained by normalization with the housekeeping genes HPRT1 and β-actin, using the Livak method. Finally, in order to profile the AQPs expression in the normoxia condition, the modified Livak method was used with the same 3 housekeeping genes above [87]. The Taqman references for the housekeeping genes are presented in **Table 4**.

Table 4 – Taqman gene expression primers (Applied Biosystems) used in the RT-qPCR reactions for the evaluation of the inflammation response *in vitro*.

Gene of interest	References
AQP1	Hs01028916_m1
AQP3	Hs01105469_g1
AQP8	Hs01086280_g1
AQP9	Hs00175573_m1
18S rRNA	Hs03003631_g1
HPRT1	Hs02800695_m1
β-actin	Hs99999903_m1

Moreover, the RT-qPCR reactions for the assessment of inflammation-related genes such as TNF-α, IL-6 and IL-10 in all of the *in vitro* assays were performed in Research Lab Human Genetics and Cancer Therapeutics of UCIBIO and the analyzed data were provided in order to contextualize the AQP inflammation response.

2. Evaluation of AQPs inflammatory response *in vivo*

2.1 Animals

For the evaluation of the inflammatory response in vivo, male Wistar rats older than 2.5 months and weighing 200-250g were chosen to reproduce IRI condition and assess the IRI-induced inflammation and the effect of quercetin to attenuate the IRI-induced inflammation. The animals were fed with a standard diet and water *ad libitum*. All animal experiments were carried out by the BioNanoSciences Group, iMed.ULisboa with the permission of the local animal ethical committee, and in accordance with the Declaration of Helsinki, the EUL Directive (2010/63/EUL Directive) and the Portuguese laws D.R. no. 31/92, D.R. 153 I-A 67/92, and all the following legislations for the humane care of animals in research, in BioNanoSciences Group, iMed. ULisboa, FFUL (2.2-2.3) [88].

2.2 Liver ischemia-reperfusion rat model

Random groups of 5 rats were anaesthetized with 0.2 ml of Imalgene 1000-Domitor® 1:1 (v/v), weighed, and an abdominal incision was performed. The abdomen was disinfected with an aseptic solution. The ischemia-reperfusion condition was induced through a median laparotomy exposing the liver hilum. After identification of the vascular structures, both the hepatic artery and the portal vein were clamped using a silk suture 2/0 and mosquito forceps in order to avoid vascular lesion and hemorrhage. The ischemia was considered at this moment and lasted 20 min until the silk suture was cut which led to the reestablishment of liver blood flow. To recover from the anesthesia, 0.2 ml of Antisedam® was administrated into the rats. After 2h, 6h, and 24h, animals were sacrificed, blood was collected by cardiac puncture, and the liver was extracted, washed with 0.154 mM KCl and kept in a 10% (v/v) neutral buffered Formalin solution for the liver assays [4, 88, 89].

2.3 Preparation and administration of the quercetin vesicles

For the assessment of quercetin protective effect on AQPs and inflammation-related genes expressions after hepatic IR in rats, liposomal quercetin was prepared with the main phospholipids egg phosphatidylcholine (EPC) or soybean phosphatidylcholine (SPC). A few minutes before the reperfusion process, the selected liposomal formulations were administered either intraperitoneal (EPC) or intravenous (SPC) [88].

2.4 Reverse transcription and quantitative polymerase chain reaction (RT-qPCR) of AQPs and inflammation-related genes

Total RNA from rat liver was extracted and provided for this assay and the purity and concentration of RNA were determined by measuring the absorption ratio at 260/280 nm, using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was obtained from 500 ng of total RNA and the reverse transcription was carried out with a NZY First-Strand cDNA Synthesis Kit (NZYTech) using a CFX96™ Real-Time System C1000 (BioRad).

In order to amplify the fragments of interest and quantify the RNA expression, real-time PCR reactions were carried out using a CFX96^{TM} Real-Time System C1000 (BioRad), with the TaqMan® Universal Master Mix II (Applied Biosystems). The specific TaqMan gene expression primers and probes for AQP1, AQP8 and AQP9 (Applied Biosystems) are presented in **Table 5**.

The relative quantification of the target genes expression was obtained by normalization with the reference gene 18S rRNA, using the $2^{(-\Delta\Delta Ct)}$ or Livak method for the evaluation of AQPs expression within each IR groups; and the modified Livak method in order to profile the AQPs expression in the normoxia condition [87]. The Taqman reference for the housekeeping gene is presented in **Table 5**.

Table 5 – Taqman gene expression primers (Applied Biosystems) used in the RT-qPCR reactions for the evaluation of the inflammation response *in vivo*.

Gene of interest	References
AQP1	Rn00562834_m1
AQP8	Rn00569732_m1
AQP9	Rn01530733_m1
18S rRNA	Rn03928990_g1

Furthermore the RT-qPCR reactions for the assessment of inflammation-related genes such as TNF-α, IL-6 and IL-10 in all of the *in vivo* assays were performed in Research Lab Human Genetics and Cancer Therapeutics of UCIBIO and the analyzed data were provided in order to contextualize the AQP inflammation response.

3. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) (n=3). Statistical analysis between the groups was performed by a one-way analysis of variance (ANOVA) followed by post-hoc Tukey's multiple comparisons test. When data did not follow ANOVA assumptions, an unpaired two-samples t-test was carried out. The p-value < 0.05 was considered statistically significant. Statistical analysis was carried out using GraphPad Prism 8 software (GraphPad Software, Inc.).

4. Limitations

Due to Covid-19, the work plan suffered altercations. The access to UCIBIO Lab and FFUL animal facility was not possible so a few experiments initially planned were suspended. It was also initially planned more *in vivo* assays with rat liver samples and measurement of liver cells membrane permeability through permeability tests in order to assess AQPs function. Also the assessment of inflammation-related genes expression was deferred, and data was provided in order to correlate AQP data.

Chapter III. RESULTS AND DISCUSSION

1. Assessment of the LPS inflammatory effect on AQPs expression in HepG2 cells

In order to investigate the effect of IR-related inflammatory process in AQPs expression in liver, we used LPS, an endotoxin known for inducing systemic inflammation by increasing oxidative stress and stimulating both innate immune system and non-immune cells involved in the inflammatory process [90], to activate HepG2 cells.

The gene expression of AQP3 and AQP9, two aquaglyceroporin isoforms with great importance in liver processes [72, 77], was assessed in HepG2 cells in order to determine the AQPs profile in basal conditions in liver cells (**Figure 7**).

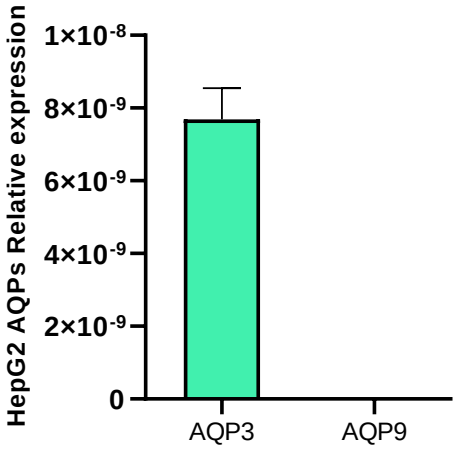

Figure 7 – AQP3 and AQP9 profile in basal conditions in HepG2 cells. Gene expression of AQP3 and AQP9 in HepG2 cells. AQP9 was not detected in HepG2 cells. Data are means ± standard deviation (SD) (n=3).

AQP3 was detected in a significant amount and, although AQP9 is reported as the predominant aquaglyceroporin in primary hepatocytes, it was not detected in this cell line (**Figure 7**). HepG2 is a hepatocellular carcinoma cell line and AQP3 is known to facilitate hepatocyte glycerol uptake, so it might substitute the role AQP9 plays in primary hepatocytes [91]. Although AQP9 was detected in several hepatocellular carcinomas, no studies have related it to carcinogenesis process [92]. The absence of AQP9 in HepG2 might be related to the tumorous nature of this cell line and the immortalization process of commercial cell lines since it is commonly verified that AQP3 is the most representative isoform in cancer cell lines [91, 93, 94].

AQP3 and AQP9 expression was then evaluated in HepG2 cells exposed to different concentrations of LPS (**Figure 8**). In parallel to the evaluation of AQPs gene expression, the alteration in gene expression of inflammation-related mediators TNF-α, IL-6 and IL-10 in LPS-treated HepG2 cells was also determined and compared to untreated cells (**Table 6**).

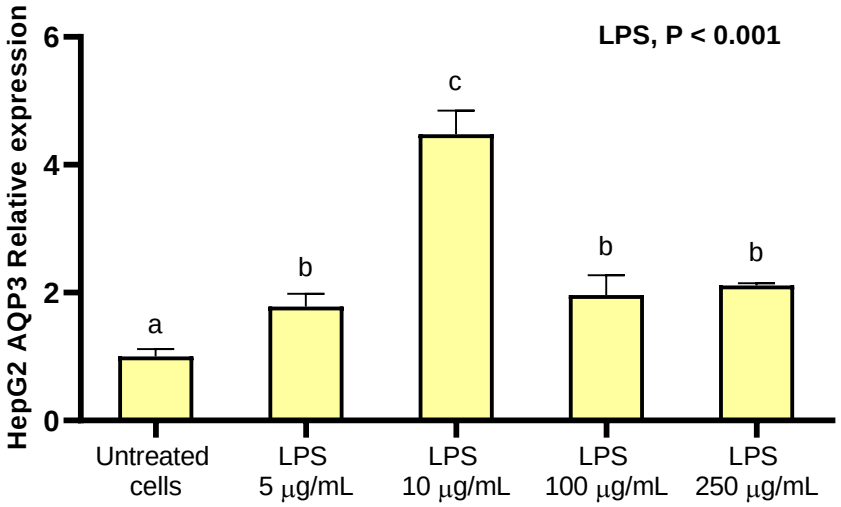

Figure 8 – AQP3 profile at different concentrations of LPS in HepG2 cells. Gene expression of AQP3 in untreated cells and cells exposed to different concentrations of LPS: 5 µg/mL of LPS, 10 µg/mL of LPS, 100 µg/mL of LPS and 250 µg/mL of LPS. Data are means ± standard deviation (SD) (n=3). Means with different letters are significantly different (Tukey's post-hoc, *P < 0.05*).

Table 6 – Relative expression of inflammation mediators at different concentrations of LPS in HepG2 cells. Gene expression of inflammation mediators (TNF-α, IL-6 and IL-10) in untreated cells and cells exposed to different concentrations of LPS: 5 µg/mL of LPS, 10 µg/mL of LPS, 100 µg/mL of LPS and 250 µg/mL of LPS.

	Relative expression		
HepG2 - LPS	**TNF-α**	**IL-6**	**IL-10**
Untreated cells	n.a.	n.a.	n.a.
LPS 5 µg/mL	+	+	+
LPS 10 µg/mL	+	+	+
LPS 100 µg/mL	+	+	+
LPS 250 µg/mL	++	++	++

Notes: n.a. non applicable as the samples are all normalized with the untreated cells as control.
+ indicates that is upregulated compared with the control.

AQP3 expression is induced when cells are exposed to LPS, while AQP9 was still not detected (**Figure 8**). The concentration of 10 µg/mL of LPS induces the maximal expression of AQP3, and although 250 µg/mL of LPS also increases AQP expression, high LPS concentrations are known to affect the extent of hepatocellular damage in several types of inflammatory liver diseases such as fatty liver disease and steatohepatitis associated with either alcohol consumption or metabolic syndrome and obesity [95, 96]. This increase of AQP3 expression can be justified by the cells necessity to reestablish homeostasis after LPS-induced inflammation [95].

Regarding the expression of inflammation-related mediators, LPS induces pro-inflammatory cytokines TNF-α and IL-6 and anti-inflammatory cytokine IL-10 expression as previously described [97, 98], where higher concentrations of LPS induce increased expression of cytokines (**Table 6**). Table 6 resumes in a qualitative way the induction of cytokines expression according to LPS concentration used. When treated with a concentration of 250 µg/mL of LPS, the expression of cytokines is significantly higher than with other concentrations which indicate a stronger cell response (**Table 6**).

LPS activates TLR4, a receptor expressed on the surface of KC, hepatocytes, HSC and LSEC. The LPS/TLR4 signalling pathway consists of activation of transcription factor NF-κB inducing cytokines expression and release from liver cells [96-98]. Pro-inflammatory cytokines TNF-α and IL-6 are secreted simultaneously with anti-inflammatory cytokine IL-10 (**Table 6**), resulting in homeostasis [98].

Since our aim is to study the involvement of aquaporins in IRI and recovery process, we decided to perform the additional experiments using 10 µg/mL of LPS, a concentration where we observe the highest induction of AQP3 expression and an established pro-inflammatory state that is observable by the increase of pro-inflammatory cytokines.

2. Effect of liposomal drugs formulations that reverse the LPS-induced inflammation on AQP3 expression in HepG2 cells

Several pharmaceutical drugs have been used to minimize the inflammatory process that features IRI. Prednisolone and ibuprofen were used to evaluate the attenuation of the pro-inflammatory effects of LPS in AQP3 expression in HepG2 cells.

There is a growing interest in using liposomal formulation as a drug-delivery system, due to their biocompatibility and facility for surface modification through the bonding of polyethylene glycol (PEG) chains, which leads to improved drug circulation and distribution, thus enhancing drug efficacy [99].

Prednisolone is a corticosteroid which has been increasingly stirring up interest in preventing inflammation in liver diseases due to its increased tissue blood flow and suppression of the activity of NF-κB, ROS and cytokine production [100]. Nevertheless, the intracellular mechanisms of this anti-inflammatory drug action on hepatic IRI remains unclear.

In this assay, prednisolone sodium sulfate was delivered to the cells in two different forms, in free form solubilized in medium or encapsulated in liposomal form with a citrate buffer, both added to the cell culture medium. The gene expression of AQP3 (**Figure 9**) and inflammation-related mediators TNF-α, IL-6 and IL-10 (**Table 7**) were determined in cells exposed to 10 µg/mL of LPS, in cells exposed to LPS plus different prednisolone deliveries, and in the respective negative controls and compared to untreated cells.

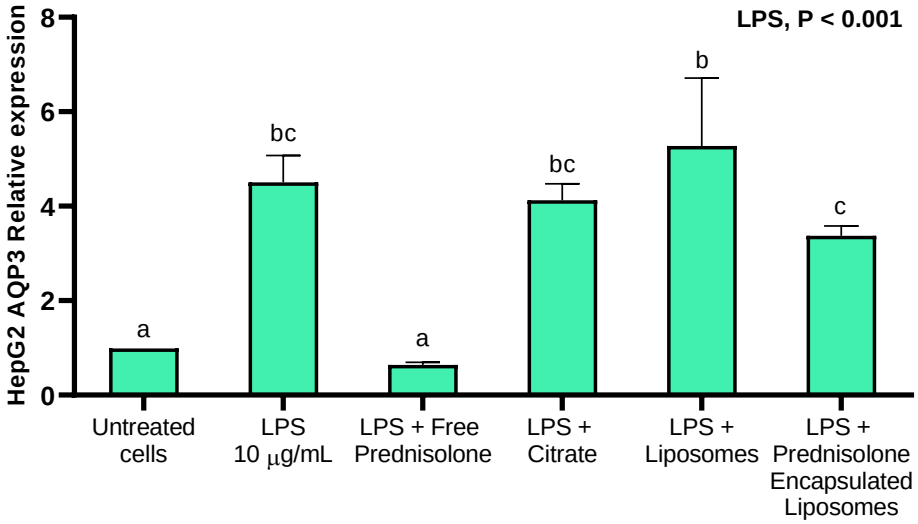

Figure 9 – AQP3 profile after administration of LPS and prednisolone to HepG2 cells. Gene expression of AQP3 in untreated cells, cells exposed to 10 µg/mL of LPS, and cells exposed to 10 µg/mL of LPS and prednisolone in the free form, the formulation form and the respective buffers citrate and empty liposomes as negative controls. Data are means ± standard deviation (SD) (n=3). Means with different letters are significantly different (Tukey's post-hoc, *P < 0.05*).

Table 7 – Relative expression of inflammation mediators after administration of LPS and prednisolone to HepG2 cells. Gene expression of inflammation mediators (TNF-α, IL-6 and IL-10) in untreated cells, cells exposed to 10 µg/mL of LPS, and cells exposed to 10 µg/mL of LPS and prednisolone in the free form, the formulation form and the respective buffers citrate and empty liposomes as negative controls.

	Relative expression		
HepG2 - LPS	**TNF-α**	**IL-6**	**IL-10**
Untreated cells	n.a.	n.a.	n.a.
LPS 10 µg/mL	+	+	+
LPS 10 µg/mL + Free Prednisolone	--	--	=
LPS 10 µg/mL + Citrate Buffer	=	=	=
LPS 10 µg/mL + Empty Liposomes	=	=	+
LPS 10 µg/mL + Prednisolone Encapsulated Liposomes	--	--	--

Notes: n.a. non applicable as the samples are all normalized with the untreated cells as control.
LPS 10 µg/mL was compared with the untreated cells.
+ indicates that is upregulated compared with the cells exposed to LPS.
- indicates that is downregulated compared with the cells exposed to LPS.
= indicates that resembles the cells exposed to LPS.

In this model, prednisolone in the free form seems to protect cells from LPS pro-inflammatory effect since AQP3 expression levels were similar to the observed in untreated cells (**Figure 9**). In addition, cells treated with prednisolone in both forms show decreased levels of cytokines compared to LPS-treated cells (**Table 7**), thus confirming this anti-inflammatory drug protective effect against LPS-induced inflammation.

Furthermore, anti-inflammatory cytokine IL-10 expression in cells treated with prednisolone in the free form was similar to the observed in cells exposed to LPS (**Table 7**), highlighting the role of this prednisolone administration on reverting mainly LPS-induced inflammatory response and ensuing mechanisms of inflammation resolution as IL-10 antagonizes pro-inflammatory cytokines [101].

The liposomal form of prednisolone seems to have no action on LPS effect over AQP3 expression. AQP3 expression in this condition was similar to LPS-treated cells and thus, much higher when compared to untreated cells (**Figure 9**). However, this formulation reduces the pro-inflammatory effect of LPS by impairing cytokines levels (**Table 7**). These data point to distinct mechanisms of regulation for cytokines and AQP3. AQP3 is involved in migration of KC and was shown to be down-regulated by TNF-α in a model of skin inflammation, thus TNF-α inhibition may prevent AQP3 downregulation [80, 102].

As the prednisolone in the liposomal form requires the use of buffers when administered in the cell culture medium, citrate buffer was also used with empty liposomes as negative controls. The negative controls used for the prednisolone in formulation, citrate buffer and empty liposomes, present a similar AQP3 (**Figure 9**) and cytokines (**Table 7**) expression levels compared to cells exposed to LPS.

Ibuprofen is one of the most prescribed non-steroidal anti-inflammatory drugs (NSAID) to treat inflammation. In liver diseases, this anti-inflammatory compound has been successfully administered in order to treat inflammation related to oxidative stress, lipid peroxidation, amongst other [103, 104]. Nevertheless, NSAID have also been associated with liver injury, although ibuprofen was reportedly the less hepatotoxic NSAID [105, 106].

In this assay, ibuprofen was administered to the cells in three different forms, delivered freely with DMSO as a solvent, incorporated in liposomal bilayer (hydrophobic form of ibuprofen), and encapsulated in liposomal inner aqueous space (hydrophilic form of ibuprofen). The assessment of AQP3 (**Figure 10**) and inflammation-related cytokines (**Table 8**) expression in cells exposed to 10 µg/mL of LPS and to LPS plus each ibuprofen formulation forms and the respective negative controls was performed and compared with untreated cells.

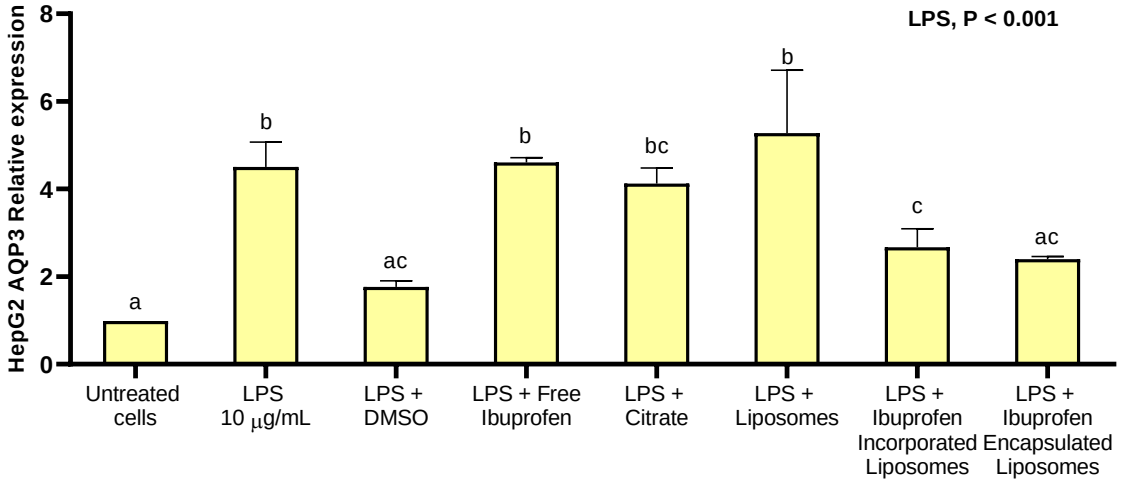

Figure 10 – AQP3 profile after administration of LPS and ibuprofen to HepG2 cells. Gene expression of AQP3 in untreated cells, cells exposed to 10 µg/mL of LPS, and cells exposed to 10 µg/mL of LPS and ibuprofen in the free form, the respective buffer DMSO, the liposomes formulations forms and the respective buffers citrate and empty liposomes as negative controls. Data are means ± standard deviation (SD) (n=3). Means with different letters are significantly different (Tukey's post-hoc, $P < 0.05$).

Table 8 – Relative expression of inflammation mediators after administration of LPS and ibuprofen to HepG2 cells. Gene expression of inflammation mediators (TNF-α, IL-6 and IL-10) in untreated cells, cells exposed to 10 µg/mL of LPS, and cells exposed to 10 µg/mL of LPS and ibuprofen in the free form, the respective buffer DMSO, the liposomes formulations forms and the respective buffers citrate and empty liposomes as negative controls.

	Relative expression		
HepG2 - LPS	**TNF-α**	**IL-6**	**IL-10**
Untreated cells	n.a.	n.a.	n.a.
LPS 10 µg/mL	+	+	+
LPS 10 µg/mL + DMSO	-	-	+
LPS 10 µg/mL + Free Ibuprofen	-	=	--
LPS 10 µg/mL + Citrate Buffer	=	=	=
LPS 10 µg/mL + Empty Liposomes	=	=	+
LPS 10 µg/mL + Ibuprofen Incorporated Liposomes	-	=	--
LPS 10 µg/mL + Ibuprofen Encapsulated Liposomes	--	+	--

Notes: **n.a.** non applicable as the samples are all normalized with the untreated cells as control.
LPS 10 µg/mL was compared with the untreated cells.
+ indicates that is upregulated compared with the cells exposed to LPS.
- indicates that is downregulated compared with the cells exposed to LPS.
= indicates that resembles the cells exposed to LPS.

In this assay, ibuprofen in both formulation forms appears to have a protective effect against LPS-induced inflammation since AQP3 expression was similar to the observed in untreated cells (**Figure 10**). On the contrary, when exposed to ibuprofen in the free form cells showed no improvement from the LPS effect over AQP3 expression (**Figure 10**). However, cells treated with ibuprofen in all delivery forms show decreased levels of cytokines TNF-α and IL-10, and similar IL-6 expression to the observed in LPS-treated cells (**Table 8**). Ibuprofen does not normalize IL-6 expression, suggesting that this cytokine may have a more predominant protective role against LPS-induced inflammation, since IL-6 induces cell proliferation through activation of STAT3 [24, 98].

Moreover, of both liposomal ibuprofen formulations, the encapsulated form induced a similar AQP3 expression to the observed in untreated cells (**Figure 10**) and a much lower cytokines expression compared with the LPS-treated cells (**Table 8**), suggesting that the hydrophilic form of ibuprofen is more effective in preventing LPS-induced inflammation [107].

Since ibuprofen is a poorly soluble compound in aqueous medium [108], the free form of this drug required the addition of DMSO, a known antioxidant, as a solvent in the cell culture medium. The AQP3 expression of cells treated with DMSO resembles to the observed in untreated cells (**Figure 10**) and also the expression of pro-inflammatory cytokines TNF-α and IL-6 is decreased (**Table 8**), confirming DMSO role in attenuating oxidative stress [109].

As the ibuprofen in the liposomal form also requires the use of buffers when administered in the cell culture medium, citrate buffer was also used with empty liposomes as negative controls. These samples present a similar AQP3 (**Figure 10**) and pro-inflammatory cytokines (**Table 8**) expression as the cells exposed to LPS.

In both drug assays, discrepancy between AQP3 and cytokines gene expression amongst the different drug delivery forms can be distinguished, perhaps due to different therapeutic dosages required to alter each gene expression. Development of drug release from the liposomal core is a critical factor in the liposomal formulation strategy. An effective therapeutic response depends on the liposome capacity to reach the target location with the drug stabilized and undergo the release of the active pharmaceutical substance to achieve the desired therapeutic concentration. The physiochemical properties of the liposomes including their membrane composition determine both the encapsulation efficiency and liposomes stability [99]. Modification of liposomes component PEG inhibits cells uptake and endosomal release which impairs the liposomal formulation delivery [110]. Hence, minimizing liposomes nonspecific binding to tissues and proteins increases the chances of them reaching liver cells with the stable pharmaceutical drug and achieve the desired therapeutic dosage [111].

3. Assessment of the effect of hypoxia on AQPs expression in HepG2 cells

HepG2 hepatocytes were cultured under a hypoxic environment to evaluate the molecular alterations occurring during ischemia-reperfusion injury *in vitro*, such as AQPs and pro-inflammatory cytokines expression.

The gene expression of AQP1, AQP3 and AQP8 was then assessed in HepG2 cells in order to determine the AQPs profile in liver cells (**Figure 11**). In liver, AQP1 facilitates transcellular water transport, AQP3 facilitates glycerol transport and finally AQP8 is involved in bile production and secretion from the hepatocytes [78, 79].

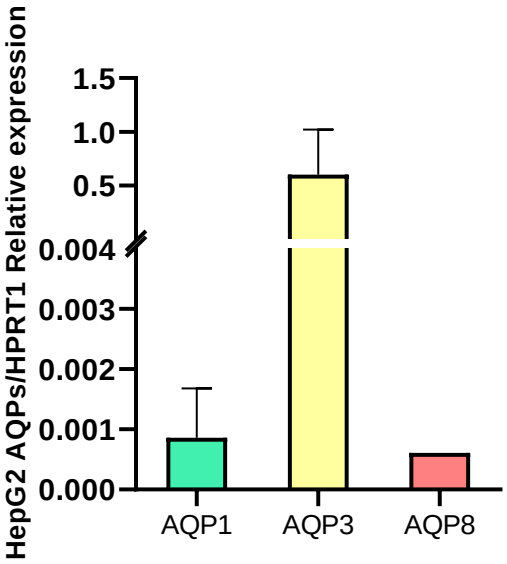

Figure 11 – AQP1, AQP3 and AQP8 profile in basal conditions in HepG2 cells. Gene expression of AQP1, AQP3 and AQP8 in HepG2 cells in normoxia condition. AQP1 and AQP8 expressions are significantly lower than AQP3 expression. Data are means ± standard deviation (SD) (n=3).

AQP3 is the main AQP isoform expressed in HepG2 cells (**Figure 11**). AQP1 and AQP8 are also detected although with lower level of expression (**Figure 11**).

The influence of hypoxia in AQPs (**Figure 12 A-C**) and cytokines TNF-α, IL-6 and IL-10 (**Table 9**) expression was evaluated in cells incubated in the hypoxia chamber and compared to cells in the normoxia condition.

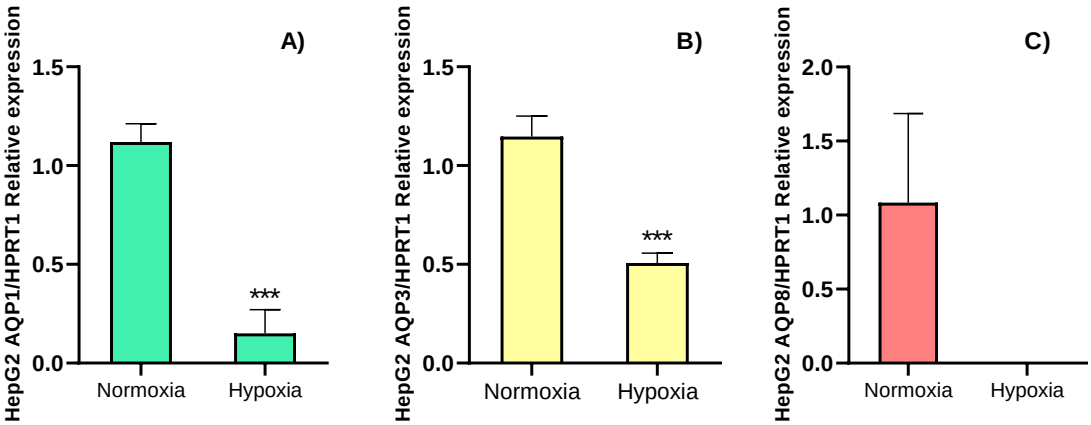

Figure 12 – AQP1, AQP3 and AQP8 profile in HepG2 cells in hypoxia chamber. Gene expression of AQP1, AQP3 and AQP8 in HepG2 cells in normoxia and hypoxia conditions with normalization with housekeeping HPRT1. **A)** Gene expression of AQP1. **B)** Gene expression of AQP3. **C)** Gene expression of AQP8. AQP8 was not detected in hypoxia condition. Data are means ± standard deviation (SD) (n=3). ***, $P < 0.001$ (unpaired t-test).

Table 9 – Relative expression of inflammation mediators in HepG2 cells in hypoxia chamber. Gene expression of inflammation mediators (TNF-α, IL-6 and IL-10) in HepG2 cells in normoxia condition and exposed to a hypoxia chamber.

	Relative expression		
HepG2 – Hypoxia chamber	**TNF-α**	**IL-6**	**IL-10**
Normoxia	n.a.	n.a.	n.a.
Hypoxia	+	+	-

Notes: n.a. non applicable as the samples are all normalized with the normoxia condition as control.
+ indicates that is upregulated compared with the control.
- indicates that is downregulated compared with the control.

In this model, the hypoxia environment seems to impair AQPs (**Figure 12**) and cytokine IL-10 (**Table 9**) expression and to induce production of cytokines TNF-α and IL-6 (**Table 9**).

Taking into account the conditions in which cells were subjected in the hypoxia chamber (12h of hypoxia followed by 4h of reperfusion), the decrease in AQPs expression suggests that, upon stimulus of ischemia, cells impair AQPs expression as a mechanism of preventing water influx into the cells in order to maintain water homeostasis within the cells during the IR process and prevent cellular edema [112, 113].

AQP8 was not detected in the hypoxia condition, which may be explained by the prolonged ischemia time in which cells were exposed. In models of cerebral ischemia, AQPs expression reaches its peak at 6h of ischemia as development of edema occurs on the first hours of ischemia, then decreases significantly after 12h and 24h of ischemia [113].

Concerning the expression of inflammation mediators, the increase of pro-inflammatory cytokines TNF-α and IL-6 expression occurs on the first hours of the reperfusion phase of liver IR upon stimulus of oxidative stress produced in the ischemia phase of IR [3, 6]. As IL-10 is on its majority an anti-inflammatory cytokine involved in the liver cells regeneration and proliferation, at 4h of reperfusion this cytokine is downregulated, as mechanisms of inflammation resolution are not yet activated [28].

When comparing these results with the *in vitro* LPS assay results, it is clear that cells react differently upon inflammation and ischemia stimulus. Liver cells overexpress AQP3 upon LPS-induced inflammation and underexpress AQPs upon a prolonged stimulus of ischemia, suggesting a different role of AQPs upon different stimulus during the IR process. Regardless, upon both inflammation and ischemia processes, cells induce expression of pro-inflammatory cytokines.

4. Evaluation of IR effect on AQPs expression in rat liver samples

In order to better understand the involvement of AQPs in hepatic IRI, we evaluated AQPs and inflammation-related cytokines expression in rat liver samples subjected to different timepoints of IR.

The gene expression of AQP1, AQP8 and AQP9 was then evaluated in rat liver cells in order to determine the AQPs profile in basal conditions in liver cells (**Figure 13**).

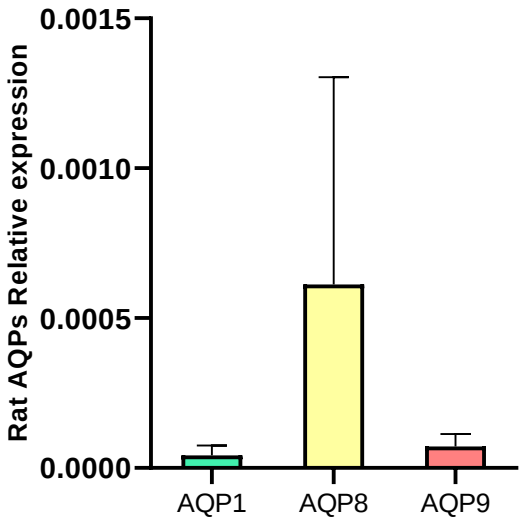

Figure 13 – AQP1, AQP8 and AQP9 profile in basal conditions in rat liver samples. Gene expression of AQP1, AQP8 and AQP9 in rat liver samples in normoxia condition. Data are means ± standard deviation (SD) (n=3).

AQP8 is the predominant AQP in liver cells as this aquaporin is involved not only on bile production and other liver functions but also in detox processes and inflammation resolution [76-80]. This analysis of AQPs profile confirms the expression of AQP8 and detects the expression of AQP1 and AQP9 (**Figure 13**), both aquaporins relevant for transcellular water transport and bile secretion in physiological conditions [72, 76].

Taking into account the AQPs profile in rat liver cells in physiological conditions, an assessment of each AQP expression in rat liver subjected to different times of reperfusion was carried out with 20 minutes of prior ischemia (**Figure 14 A-C**). Also, the expression of pro-inflammatory cytokine TNF-α in rat liver subjected to 20 minutes of ischemia and different times of reperfusion was measured and compared to the control (**Table 10**).

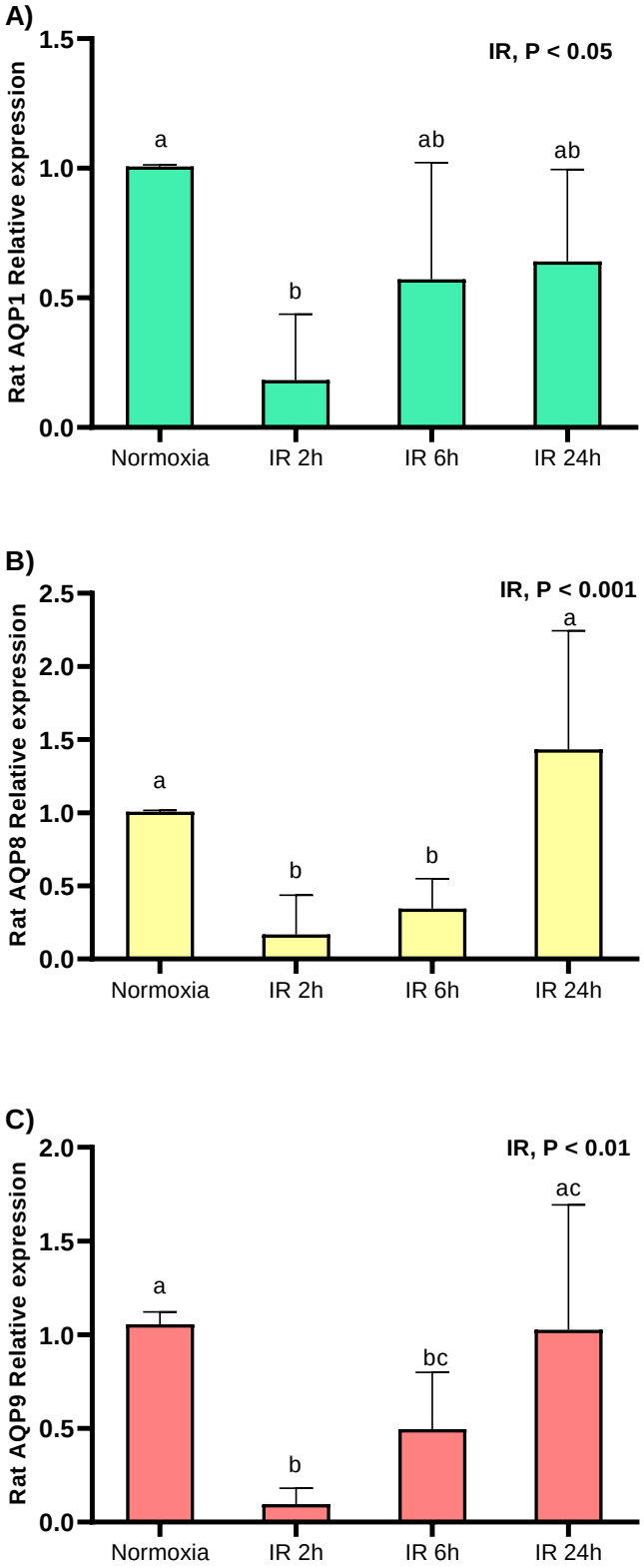

Figure 14 – AQP1, AQP8 and AQP9 profile in different conditions of IR in rat liver samples. Gene expression of AQP1, AQP8 and AQP9 in rat liver samples in normoxia and subjected to 20 minutes of ischemia and different times of reperfusion: samples subjected to 2h of reperfusion; samples exposed to 6h of reperfusion; samples subjected to 24h of reperfusion. **A)** Gene expression of AQP1. **B)** Gene expression of AQP8. **C)** Gene expression of AQP9. Data are means ± standard deviation (SD) (n=3). Means with different letters are significantly different (Tukey's post-hoc, $P < 0.05$).

Table 10 – Relative expression of inflammation mediator TNF-α in different conditions of IR in rat liver samples. Gene expression of inflammation mediator TNF-α in rat liver samples in normoxia condition and subjected to 20 minutes of ischemia and different times of reperfusion: samples exposed to 2h of reperfusion, samples subjected to 6h of reperfusion and samples exposed to 24h of reperfusion.

	Relative expression
Rat liver samples - IRI	**TNF-α**
Normoxia	n.a.
IR 2h	++
IR 6h	+++
IR 24h	=

Notes: n.a. non applicable as the samples are all normalized with the normoxia condition as control.
+ indicates that is upregulated compared with the control.
= indicates that resembles the control.

According to this assay, given an IR stimulus liver cells suppress the expression of AQPs on the first 2 hours of reperfusion, then increase their expression after 6h of reperfusion and finally the AQPs expression normalizes after 24h of reperfusion (**Figure 14**). As for the expression of cytokine TNF-α (**Table 10**), it is possible to confirm the overexpression of this inflammatory mediator during the first 6h of reperfusion since this timepoint represents the first stage of the reperfusion phase where the inflammatory process initiates due to activation of KC that induce the release of pro-inflammatory mediators [3, 6].

During the first hours of reperfusion, it is possible to verify a correlation between AQPs and TNF-α production as the expression of this cytokine increases (**Table 10**), the expression of AQPs decreases (**Figure 14**). During the ischemia phase of IR, depletion of ATP leads to alteration on H^+, Na^+, and Ca^{2+} homeostasis and ultimately produces edema in hepatocytes and LSEC [5, 7]. The first stage of IR is characterized by microcirculation impairment and production of pro-inflammatory mediators due to generation of ROS and RNS [3, 4]. This correlation can be explained by the cells necessity to suppress AQPs and prevent water influx and cell swelling while the oxidative stress process takes place and the inflammatory process initiates [114].

Meanwhile, after 6 hours of reperfusion cells overexpress AQPs (**Figure 14**) as these water channels can constitute important inflammation mediators in the immobilization of liver macrophages and therefore contribute to the inflammatory process [77, 80]. In addition, the increased AQPs expression may suggest a cellular mechanism of maintaining water homeostasis while blood flow reestablishes [89]. Also, at this IR timepoint, cells overexpress TNF-α (**Table 10**) as this cytokine induces upregulation of inflammation mediators and adhesion molecules, thus playing an important role in the recruitment of neutrophils [3, 6].

Furthermore, when cells are exposed to 24h of reperfusion the expression of TNF-α resembles the normoxia condition (**Table 10**) which in turn correlates with the results from the AQPs profiles at 24h of reperfusion (**Figure 14**). Once more this correlation indicates that after 24h of reperfusion the cells are undergoing processes of inflammation resolution and returning to homeostasis [4, 11]. AQPs represent important mediators in inflammation resolution as aquaglyceroporins participate in cell proliferation processes [40, 48]. The increased AQP8 and AQP9 expression after 24h of reperfusion (**Figure 14 B-C**) might be associated with the recovery process, in fact, these isoforms have been previously reported to be involved in mechanisms of inflammation resolution and detox processes [72, 79, 83].

5. Assessment of the protective effect of quercetin in liposomal formulation on AQPs expression in rat liver samples

Measurement of AQPs and inflammation-related cytokines expression in rat liver cells subjected to different timepoints of IR and a natural anti-oxidant compound such as quercetin was performed in order to assess the quercetin protective role on attenuating the inflammation process caused by the liver cells exposure to IR stimulus.

Quercetin, a flavonoid present in plants, has been stirring up interest as this natural compound possesses unique biological properties that include anti-carcinogenic, anti-inflammatory and antiviral functions [115]. The mechanisms in which quercetin inhibits TNF-α-related inflammation has been thoroughly described [115]. Quercetin also has a protective role against oxidative stress and inhibits adhesion molecules such as VCAM-1, preventing neutrophils recruitment and further oxidative stress processes [115].

Considering the numerous functions of quercetin that has as a protective role against liver inflammation, this flavonoid has been selected to assess IR-related inflammation and thus, evaluate its effect in AQP and pro-inflammatory cytokine TNF-α expression. Since quercetin is practically insoluble in water, the best form of administration consists in the incorporation through vesicles [116, 117]. In this assay quercetin was administered intraperitoneal (IP) or intravenous (IV), encapsulated in liposomes whose membranes were formed by egg phosphatidylcholine (EPC) or by soybean phosphatidylcholine (SPC), respectively.

The influence of quercetin in AQPs (**Figure 15**) and cytokine TNF-α (**Table 11**) expression was evaluated in cells subjected to 20 minutes of ischemia and different times of reperfusion, treated with the two different lipid composition of liposomal quercetin (EPC and SPC) and compared to cells in the normoxia condition and the samples subjected to the respective IR conditions as positive controls.

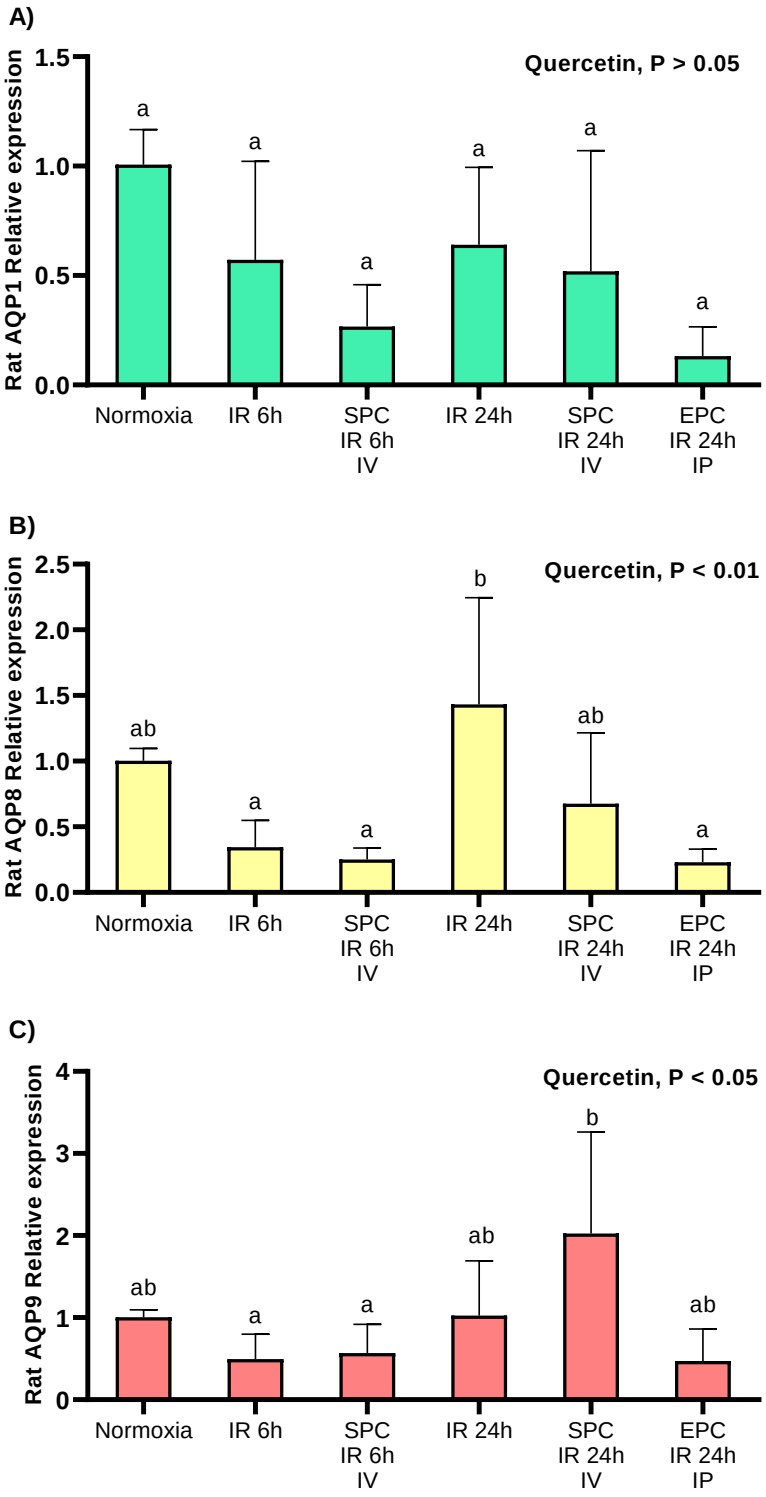

Figure 15 – AQP1, AQP8 and AQP9 profile after different treatments with quercetin in rat liver samples. Gene expression of AQP1, AQP8 and AQP9 in rat liver samples in normoxia condition, and treated with quercetin liposomes either formed by SPC (IV administration) or EPC (IP administration) and subjected to 20 minutes of ischemia and different times of reperfusion and the respective positive controls: samples with SPC liposomes subjected to 6h of reperfusion; samples with SPC liposomes exposed to 24h of reperfusion; samples with EPC liposomes subjected to 24h of reperfusion. **A)** Gene expression of AQP1. **B)** Gene expression of AQP8. **C)** Gene expression of AQP9. Data are means ± standard deviation (SD) (n=3). Means with different letters are significantly different (Tukey's post-hoc, *P < 0.05*).

Table 11 – Relative expression of inflammation mediator TNF-α after different treatments with quercetin in rat liver samples. Gene expression of inflammation mediator TNF-α in rat liver samples in normoxia condition, and treated with quercetin vesicles formed by either SPC or EPC and subjected to 20 minutes of ischemia and different times of reperfusion.

	Relative expression
Rat liver samples - IRI	**TNF-α**
Normoxia	n.a.
SPC IR 6h	+
SPC IR 24h	=
EPC IR 24h	-

Notes: n.a. non applicable as the samples are all normalized with the normoxia condition as control.
- indicates that is downregulated compared with the control.
= indicates that resembles the control.

Taking into account the two different routes of drug administration used in this assay, the intravenous administration seems to protect cells from IR-related inflammation. AQP9 expression in cells treated with SPC liposomal quercetin after 6h and 24h of reperfusion increases in comparison with the positive controls and appears to approximate the normoxia condition (**Figure 15C**). However this quercetin formulation proved inefficient on increasing AQP1 and AQP8 expression in both IR timepoints (**Figure 15 A-B**) and decreasing TNF-α production (**Table 11**).

The intraperitoneal administration seems to have no action on IR effect over AQPs expression since AQPs expression in samples treated with EPC liposomal quercetin was significantly impaired compared with the positive control (**Figure 15**). Although most substances present a fast absorption from the peritoneal cavity and the IP administration is considered more localized than the IV administration, this route subjects the molecules though metabolic processes, thus making it difficult to profile the substance pharmacokinetics [118]. For this reason, the IP administration is justifiable in order to evaluate the target engagement but not assess the properties and pharmacokinetics of a drug formulation for clinical translation [119]. Regardless, this formulation was able to inhibit TNF-α expression after 24h of reperfusion (**Table 11**), as previously described [115].

Besides transcellular water transport in the liver, AQP9 is involved in bile production, hepatic glycerol metabolism and also participates in detoxification and inflammation resolution processes [72, 78, 79]. Although for this aquaglyceroporin the quercetin treatment appears to be effective, AQP overexpression can cause osmotic imbalances and disruption of cells homeostasis that ultimately leads to cell death [49].

Altogether this data points to distinct treatment outcomes regarding the drug administration with IV administration of quercetin being more effective on treating liver IR-related inflammation associated with AQPs regulation and IP administration of quercetin successfully inhibiting TNF-α production. However analysis of other reperfusion timepoints would be advisable to verify the effectiveness of both quercetin formulations and the respective routes of administration.

Chapter IV. CONCLUSIONS AND FUTURE WORK

With the main objective of assessing and characterizing AQPs profile during inflammatory processes of liver IR, the present work demonstrated that AQPs pose as important regulators of hemodynamic equilibrium when inflammation mechanisms take place.

Upon severe inflammation stimulus, cells induced AQPs expression in order to reestablish homeostasis and attenuate biological processes instigated by overexpression of inflammation mediators.

Administration of liposomal drugs formulations proved effective in attenuating inflammation processes caused by the cells exposure to toxic compounds and inflammation-related dysfunction in liver cells, as both prednisolone and ibuprofen showed favorable effects in reversing LPS-induced inflammation by decreasing significantly AQP3 and cytokines expression. As for the drug delivery system, prednisolone in the free form manifests a significantly favorable effect in reversing LPS-induced inflammation, while ibuprofen proved more efficient in the encapsulated form. Moreover ibuprofen in formulation form seemed to increase IL-6, confirming that this cytokine is also involved in inflammation resolution mechanisms. Despite the fact that the present assay allowed to conclude the effectiveness of the studied pharmaceutical drugs in reversing LPS-induced inflammation, more studies are required to accomplish consistency in control buffers and reduce cell culture conditions associated errors.

The hypoxia chamber assay allowed the conclusion that upon severe stimulus of hypoxia cells suppress AQPs expression as a mechanism of preventing water influx into the cells and maintaining water homeostasis during the initial inflammatory process and preventing cell swelling. Additionally, this work allowed to confirm the stimulation of cytokines production upon IR. Altogether the timepoint of 12h of hypoxia tested in this assay proved unfavorable to mimic the IR model and assess the associated inflammatory processes in liver cells. For deeper understanding of the hypoxia stimulus effect on AQPs and inflammation-related mediators, different timepoints of hypoxia should be assessed in a hypoxia chamber model. Moreover, it was possible to distinguish the effect of inflammation and hypoxia stimulus on AQPs expression. The present assay demonstrated ineffective in recreating IR conditions as highlighted the ischemia stimulus over the inflammation stimulus associated with the reperfusion phase of liver IR.

The *in vivo* assay of IR effect on rat liver samples confirmed a majority of AQP8 in liver cells and allowed to delineate a profile of AQPs expression along the different timepoints of IR. In general, cells suppressed significantly the AQPs expression after 2h of reperfusion in order to oppose the mechanisms ensued in the ischemia process and prevent water influx into the cell and cell edema within the first hours of reperfusion, then induced AQPs expression after 6h of reperfusion in order to reestablish water homeostasis and finally after 24h of reperfusion cells maintained AQPs expression. As AQPs and TNF-α expression after 24h of reperfusion resembled the normoxia condition, this assay confirmed that at this timepoint the cells are undergoing processes of inflammation resolution and returning to homeostasis. AQP8 and AQP9 showed higher expression after 24h of reperfusion which

most likely is associated with the involvement of these AQPs in detoxification processes and inflammation resolution mechanisms.

Treatment with quercetin showed poor results as AQPs profiles resembled the previous assay in rat liver samples and although IV administration of SPC liposomal quercetin presented higher AQPs expression than IP administration of EPC liposomal quercetin, it was still not efficient in attenuating the IR-induced inflammation processes. Even though the aim of quercetin administration was to prevent IR-associated inflammation, overexpression of AQPs can lead to osmotic imbalances which can further dysregulate liver cells. Regarding the assessment of inflammation mediators, it would be advisable to verify the effectiveness of both quercetin formulations on several more timepoints in order to conclude of its protective role against inflammation and the appropriate route of drug administration.

Although this project encountered several drawbacks, the developed work contributed considerable advances in comprehending and characterizing hepatic IRI mechanisms. Not only allowed to evaluate the AQPs profiles during liver IR but proved that some liposomal drugs formulations may be effective in attenuating this condition during liver surgery. In order to further assess the role of AQPs in liver IRI mechanisms, functional assays should be carried out and, ultimately, strategies to target and regulate AQPs during liver IR should be investigated aiming to reduce the high mortality rate associated with this condition.

REFERENCES

[1] Serracino-Inglott, F., Habib, N.A., Mathie, R.T. (2001) Hepatic ischemia-reperfusion injury. Am J Surg 181(2), 160-166.

[2] Kupiec-Weglinski, J.W., Busuttil, R.W. (2005) Ischemia and Reperfusion Injury in Liver Transplantation. Transplant Proc 37(4), 1653-1656.

[3] Abu-Amara, M., Yang, S.Y., Tapuria, N., Fuller, B., Davidson, B., Seifalian, A. (2010) Liver Ischemia/Reperfusion Injury: Processes in Inflammatory Networks—A Review. Liver Transpl 16, 1016-1032.

[4] Marcelino, P., Marinho, H. S., Campos, M. C., Neves, A. R., Real, C., Fontes, F. S., Carvalho, A., Feio, G., Martins, M.B.F., Corvo, M. L. (2017) Therapeutic activity of superoxide dismutase-containing enzymosomes on rat liver ischaemia-reperfusion injury followed by magnetic resonance microscopy. Eur J Pharm Sci 109, 464–471.

[5] De Groot, H., Rauen, U. (2007) Ischemia-Reperfusion Injury: Processes in Pathogenetic Networks: A Review. Transplant Proc 39(2), 481–484.

[6] Marinho, H.S., Marcelino, P., Soares, H., Corvo, M.L. (2018) Gene Silencing using siRNA for Preventing Liver Ischaemia-Reperfusion Injury. Curr Pharm Des 24(23), 2692-2700.

[7] Kosieradzki, M., Rowiński, W. (2008) Ischemia/Reperfusion Injury in Kidney Transplantation: Mechanisms and Prevention. Transplant Proc 40(10), 3279–3288.

[8] Peralta, C., Jiménez-Castro, M.B., Gracia-Sancho, J. (2013) Hepatic ischemia and reperfusion injury: Effects on the liver sinusoidal milieu. J Hepatol 59(5), 1094-106.

[9] Montalvo-Jave, E. E., Escalante-Tattersfield, T., Ortega-Salgado, J. A., Piña, E., Geller, D. A. (2008) Factors in the Pathophysiology of the Liver Ischemia-Reperfusion Injury. J Surg Res 147(1), 153–159.

[10] Zhai, Y., Petrowsky, H., Hong, J. C., Busuttil, R. W., Kupiec-Weglinski, J. W. (2012) Ischaemia-reperfusion injury in liver transplantation—from bench to bedside. Nat Rev Gastroenterol Hepatol 10(2), 79–89.

[11] Konishi, T., Lentsch, A.B. (2017) Hepatic Ischemia/Reperfusion: Mechanisms of Tissue Injury, Repair, and Regeneration. Gene Expr 17(4), 277-287.

[12] Bilzer, M., Roggel, F., Gerbes, A.L. (2006) Role of Kupffer cells in host defense and liver disease. Liver Int 26, 1175–1186.

[13] Kolios, G., Valatas, V., Kouroumalis, E. (2006) Role of Kupffer cells in the pathogenesis of liver disease. World J Gastroenterol 12(46), 7413–7420.

[14] Van Golen, R. F., van Gulik, T. M., Heger, M. (2012) The sterile immune response during hepatic ischemia/reperfusion. Cytokine Growth Factor Rev 23(3), 69–84.

[15] Zhang, J., Hu, W., Xing, W., You, T., Xu, J., Qin, X., Peng, Z. (2011) The Protective Role of CD59 and Pathogenic Role of Complement in Hepatic Ischemia and Reperfusion Injury. Am J Pathol 179(6), 2876–2884.

[16] Jaeschke, H., Farhood, A., Bautista, A.P, Spolarics, Z., Spitzer, J.J. (1993) Complement activates Kupffer cells and neutrophils during reperfusion after hepatic ischemia. Am J Physiol 264(4), G801–G809.

[17] Tsung, A., Sahai, R., Tanaka, H., Nakao, A., Fink, M.P., Lotze, M.T., Yang, H., Li, J., Tracey, K.J., Geller, D.A., Billiar, T.R. (2005) The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 201(7), 1135-43.

[18] Zeng, S., Dun, H., Ippagunta, N., Rosario, R., Zhang, Q.Y., Lefkowitch, J., Yan, S.F., Schmidt, A.M., Emond, J.C. (2009) Receptor for advanced glycation end product (RAGE)-dependent modulation of early growth response-1 in hepatic ischemia/reperfusion injury. J Hepatol 50(5), 929-36.

[19] Hanschen, M., Zahler, S., Krombach, F., Khandoga, A. (2008) Reciprocal activation between CD4+ T cells and Kupffer cells during hepatic ischemia-reperfusion. Transplantation 86(5), 710-8.

[20] Jaruga, B., Hong, F., Kim, W.H., Gao, B. (2004) IFN-gamma/STAT1 acts as a proinflammatory signal in T cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: A critical role of IRF-1. Am J Physiol Gastrointest Liver Physiol 287(5), G1044-52.

[21] Farhood, A., McGuire, G.M., Manning, A.M., Miyasaka, M., Smith, C.W., Jaeschke, H. (1995) Intercellular adhesion molecule 1 (ICAM-1) expression and its role in neutrophil-induced ischemia-reperfusion injury in rat liver. J Leukoc Biol 57(3), 368-74.

[22] Peralta, C., Fernández, L., Panés, J., Prats, N., Sans, M., Piqué, J.M., Gelpí, E., Roselló-Catafau, J. (2001) Preconditioning protects against systemic disorders associated with hepatic ischemia-reperfusion through blockade of tumor necrosis factor-induced P-selectin up-regulation in the rat. Hepatology 33(1), 100-13.

[23] Jaeschke, H., Woolbright, B.L. (2012) Current strategies to minimize hepatic ischemia-reperfusion injury by targeting reactive oxygen species. Transplant Rev 26(2), 103–14.

[24] Tiberio, L., Tiberio, G.A., Bardella, L., Cervi, E., Cerea, K., Dreano, M., Garotta, G., Fra, A., Montani, N., Ferrari-Bravo, A., Callea, F., Grigolato, P., Giulini, S.M., Schiaffonati, L. (2006) Mechanisms of interleukin-6 protection against ischemia-reperfusion injury in rat liver. Cytokine 34(3-4), 131–42.

[25] Lentsch AB, Shanley TP, Sarma V, Ward PA. (1997) In vivo suppression of NF-kappa B and preservation of I kappa B alpha by interleukin-10 and interleukin-13. J Clin Invest 100(10), 2443–8.

[26] Lentsch, A.B., Yoshidome, H., Warner, R.L., Ward, P.A., Edwards, M.J. (1999) Secretory leukocyte protease inhibitor in mice regulates local and remote organ inflammatory injury induced by hepatic ischemia/reperfusion. Gastroenterology 117(4), 953–61.

[27] Kuboki, S., Shin, T., Huber, N., Eismann, T., Galloway, E., Schuster, R., Blanchard, J., Edwards, M.J., Lentsch, A.B. (2008) Hepatocyte signaling through CXC chemokine receptor-2 is detrimental to liver recovery after ischemia/reperfusion in mice. Hepatology 48(4), 1213–1223.

[28] Muriel, P. (2008) NF-κB in liver diseases: a target for drug therapy. J Appl Toxicol 29, 91–100.

[29] Fausto, N., Campbell, J.S., Riehle, K.J. (2006) Liver regeneration. Hepatology 43(2 Suppl 1), S45–53.

[30] Borowiak, M., Garratt, A.N., Wüstefeld, T., Strehle, M., Trautwein, C., Birchmeier, C. (2004) Met provides essential signals for liver regeneration. Proc Natl Acad Sci U S A. 101(29): 10608–10613.

[31] Mochizuki, A., Pace, A., Rockwell, C.E., Roth, K.J., Chow, A., O'Brien, K.M., Albee, R., Kelly, K., Towery, K., Luyendyk, J.P., Copple, B.L. (2014) Hepatic stellate cells orchestrate clearance of necrotic cells in a hypoxia-inducible factor-1alphadependent manner by modulating macrophage phenotype in mice. J Immunol 192(8), 3847–57.

[32] Katoonizadeh, A., Nevens, F., Verslype, C., Pirenne, J., Roskams, T. (2006) Liver regeneration in acute severe liver impairment: A clinicopathological correlation study. Liver Int 26(10), 1225–33.

[33] Zamboni, F., Franchello, A., David, E., Rocca, G., Ricchiuti, A., Lavezzo, B., Rizzetto, M., Salizzoni, M. (2001) Effect of macrovescicular steatosis and other donor and recipient characteristics on the outcome of liver transplantation. Clin Transplant 15(1), 53–57.

[34] Massip-Salcedo, M., Zaouali, M., Padrissa-Altés, S., Casillas-Ramírez, A., Rodés, J., Roselló-Catafau, J. (2008) Activation of peroxisome proliferator-activated receptor-alpha inhibits the injurious effects of adiponectin in rat steatotic liver undergoing ischemia-reperfusion. Hepatology 47(2), 461–472.

[35] Sun, C.K., Chang, C.L., Lin, Y.C., Kao, Y.H., Chang, L.T., Yen, C.H., Shao, P.L., Chen, C.H., Leu, S., Yip, H.K. (2012) Systemic administration of autologous adipose-derived mesenchymal

stem cells alleviates hepatic ischemia-reperfusion injury in rats. Crit Care Med 40(4), 1279–1290.

[36] Watanabe, G., Uchinami, H., Yoshioka, M., Abe, Y., Kikuchi, I., Iwasaki, W., Kume, M., Yamamoto, Y. (2012) Transfection of naked nuclear factor-jB decoy oligodeoxynucleotides into liver by rapid portal vein infusion in rats: its effect on ischemia-reperfusion injury of liver. Hum Gene Ther 23(4), 428–436.

[37] Hong F, Yang S. (2012) Ischemic preconditioning decreased leukotriene C4 formation by depressing leukotriene C4 synthase expression and activity during hepatic I/R injury in rats. J Surg Res 178(2), 1015–1021.

[38] Schiesser, M., Wittert, A., Nieuwenhuijs, V.B., Morphett, A., Padbury, R.T., Barritt, G.J. (2009) Intermittent ischemia but not ischemic preconditioning is effective in restoring bile flow after ischemia reperfusion injury in the livers of aged rats. J Surg Res 152(1), 61–68.

[39] Vairetti, M., Ferrigno, A., Carlucci, F., Tabucchi, A., Rizzo, V., Boncompagni, E., Neri, D., Gringeri, E., Freitas, I., Cillo, U. (2009) Subnormothermic machine perfusion protects steatotic livers against preservation injury: a potential for donor pool increase? Liver Transpl 15(1), 20–29.

[40] Verkman, A. S., Anderson, M. O., Papadopoulos, M. C. (2014) Aquaporins: important but elusive drug targets. Nat Rev Drug Discov 13(4), 259–277.

[41] Brown, D. (2017) The Discovery of Water Channels (Aquaporins). Ann Nutr Metab 70(1), 37-42.

[42] van Hoek, A.N., Hom, M.L., Luthjens, L.H., de Jong, M.D., Dempster, J.A., van Os, C.H. (1991) Functional unit of 30 kDa for proximal tubule water channels as revealed by radiation inactivation. J Biol Chem 266(25), 16633–16635.

[43] Denker, B.M., Smith, B.L., Kuhajda, F.P., Agre, P. (1988) Identification, purification, and partial characterization of a novel Mr 28,000 integral membrane protein from erythrocytes and renal tubules. J Biol Chem 263(30), 15634–15642.

[44] Preston, G.M., Agre, P. (1991) Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: member of an ancient channel family. Proc Natl Acad Sci U S A 88(24), 11110–11114.

[45] Preston, G.M., Carroll, T.P., Guggino, W.B., Agre, P. (1992) Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science 256(5055), 385–387.

[46] Fu, D., Lu, M. (2007) The structural basis of water permeation and proton exclusion in aquaporins (Review). Mol Membr Biol 24(5-6), 366–374.

[47] Dajani, S., Saripalli, A., Sharma-Walia, N. (2018) Water transport proteins–aquaporins (AQPs) in cancer biology. Oncotarget 9(91), 36392-36405.

[48] Verkman, A. S. (2008) Mammalian aquaporins: diverse physiological roles and potential clinical significance. Expert Rev Mol Med Vol.10, e13.

[49] Khan, S., Ricciardelli, C., Yool, A.J. (2021) Targeting Aquaporins in Novel Therapies for Male and Female Breast and Reproductive Cancers. Cells 10(2), 215.

[50] Erudaitius, D., Huang, A., Kazmi, S., Buettner, G.R., Rodgers, V.G.J. (2017) Peroxiporin Expression Is an Important Factor for Cancer Cell Susceptibility to Therapeutic H2O2: Implications for Pharmacological Ascorbate Therapy. PLoS ONE, 12(1), e0170442.

[51] Tamma, G., Valenti, G., Grossini, E., Donnini, S., Marino, A., Marinelli, R.A., Calamita, G. (2018) Aquaporin Membrane Channels in Oxidative Stress, Cell Signaling, and Aging: Recent Advances and Research Trends. Oxid med cell long 2018(2), 1-14.

[52] Bienert, G.P., Møller, A.L.B., Kristiansen, K.A., Schulz, A., Møller, I.M., Schjoerring, J. K., Jahn, T. P. (2006) Specific Aquaporins Facilitate the Diffusion of Hydrogen Peroxide across Membranes. J Biol Chem 282(2), 1183-92.

[53] Vallon, V., Verkman, A.S., Schnermann, J. (2000) Luminal hypotonicity in proximal tubules of aquaporin-1 knockout mice. Am J Physiol 278(6), F1030-F1033.

[54] Song, Y., Verkman, A.S. (2001) Aquaporin-5 dependent fluid secretion in airway submucosal glands. J Biol Chem 276(44), 41288-41292.

[55] Zhang, H., Verkman, A.S. (2008) Aquaporin-4 independent Kir4.1 Kþ channel function in brain glial cells. Mol Cell Neurosci 37(1), 3-10.

[56] Papadopoulos, M. C., Saadoun, S. (2015) Key roles of aquaporins in tumor biology. Biochim Biophys Acta 1848(10), 2576-83.

[57] Verkman, A. S., Hara-Chikuma, M., Papadopoulos, M. C. (2008) Aquaporins—new players in cancer biology. J Mol Med 86(5), 523-9.

[58] Saadoun, S., Papadopoulos, M.C., Watanabe, H., Yan, D., Manley, G.T., Verkman, A.S. (2005) Involvement of aquaporin-4 in astroglial cell migration and glial scar formation. J Cell Sci 118(24), 5691-5698.

[59] Binder, D.K., Yao, X., Zador, Z., Sick, T.J., Verkman, A.S., Manley, G.T. (2006) Increased seizure duration and slowed potassium kinetics in mice lacking aquaporin-4 water channels. Glia 53(6), 631-636.

[60] Amiry-Moghaddam, M., Williamson, A., Palomba, M., Eid, T., de Lanerolle, N.C., Nagelhus, E.A., Adams, M.E., Froehner, S.C., Agre, P., Ottersen, O.P. (2003) Delayed K+ clearance associated with aquaporin-4 mislocalization: phenotypic defects in brains of alpha-syntrophin-null mice. Proc Natl Acad Sci U S A 100(23), 13615-13620.

[61] Hub, J.S., de Groot, B.L. (2008) Mechanism of selectivity in aquaporins and aquaglyceroporins. Proc Natl Acad Sci U S A, 105(4), 1198–1203.

[62] Hara, M., Verkman, A.S. (2003) Glycerol replacement corrects defective skin hydration, elasticity, and barrier function in aquaporin-3-deficient mice. Proc Natl Acad Sci U S A 100(12), 7360-7365.

[63] Ma, T., Hara, M., Sougrat, R., Verbavatz, J.M., Verkman, A.S. (2002) Impaired stratum corneum hydration in mice lacking epidermal water channel aquaporin-3. J Biol Chem 277(19), 17147-17153.

[64] Rojek, A.M., Skowronski, M.T., Füchtbauer, E.-M., Füchtbauer, A.C., Fenton, R.A., Agre, P., Frøkiaer, J., Nielsen, S. (2007) Defective glycerol metabolism in aquaporin 9 (AQP9) knockout mice. Proc Natl Acad Sci U S A 104(9), 3609-3614

[65] Hara-Chikuma, M., Sohara, E., Rai, T., Ikawa M., Okabe, M., Sasaki, S., Uchida, S., Verkman, A.S. (2005) Progressive adipocyte hypertrophy in aquaporin-7 deficient mice: adipocyte glycerol permeability as a novel regulator of fat accumulation. J Biol Chem 280(16), 15493-15496.

[66] De Ieso, M. L., Yool, A. J. (2018) Mechanisms of Aquaporin-Facilitated Cancer Invasion and Metastasis. Front Chem 6:135.

[67] Törnroth-Horsefield, S., Hedfalk, K., Fischer, G., Lindkvist-Petersson, K., Neutze, R. (2010) Structural insights into eukaryotic aquaporin regulation. FEBS Lett 584(12), 2580–2588.

[68] Agre, P., King, L.S., Yasui, M., Guggino, W.B., Ottersen, O.P., Fujiyoshi, Y., Engel, A., Nielsen, S. (2002) Aquaporin water channels – from atomic structure to clinical medicine. J Physiol 542(1), 3-16.

[69] Fushimi, K., Sasaki, S., Marumo, F. (1997) Phosphorylation of serine 256 is required for cAMP-dependent regulatory exocytosis of the aquaporin-2 water channel. J Biol Chem 272(23), 14800–14804. [70] Ozu, M., Dorr, R. A., Gutiérrez, F., Teresa Politi, M., Toriano, R. (2013) Human AQP1 Is a Constitutively Open Channel that Closes by a Membrane-Tension-Mediated Mechanism. Biophys J 104(1), 85–95.

[71] Soveral, G., Madeira, A., Loureiro-Dias, M. C., Moura, T. F. (2008) Membrane tension regulates water transport in yeast. Biochim Biophys Acta 1778(11), 2573-9.

[72] Laforenza, U., Bottino, C., Gastaldi, G. (2016) Mammalian aquaglyceroporin function in metabolism. Biochim Biophys Acta 1858(1), 1-11.

[73] Lehmann, G.L., Larocca, M.C., Soria, L.R., Marinelli, R.A. (2008) Aquaporins: Their role in cholestatic liver disease. World J Gastroenterol 14(46), 7059-7067.

[74] Marinelli, R.A., Tietz, P.S., Caride, A.J., Huang, B.Q., LaRusso, N.F. (2003) Water Transporting Properties of Hepatocyte Basolateral and Canalicular Plasma Membrane Domains. J Biol Chem 278(44), 43157-62.

[75] Jessner, W., Zsembery, A., Graf, J. (2008) Transcellular water transport in hepatobiliary secretion and role of aquaporins in liver. Wien Med Wochenschr, 158(19-20), 565–569.

[76] Masyuk, A.I., LaRusso, N.F. (2006) Aquaporins in the Hepatobiliary System. Hepatology, 43(2 Suppl 1), S75-81.

[77] Gregoire, F., Lucidi, V., Zerrad-Saadi, A., Virreira, M., Bolaky, N., Delforge, V., Lemmers, A., Donckier, V., Devière, J., Demetter, P., Perret, J., Delporte, C. (2015) Analysis of aquaporin expression in liver with a focus on hepatocytes. Histochem Cell Biol 144(4), 347-63.

[78] Marinelli, R.A., Gradilone, S.A., Carreras, F.I., Calamita, G., Lehmann, G.L. (2004) Liver Aquaporins: Significance in Canalicular and Ductal Bile Formation. Ann Hepatol 3(4), 130-6.

[79] Portincasa, P., Palasciano, G., Svelto, M., Calamita, G. (2008) Aquaporins in the Hepatobiliary Tract. Which, Where and What They Do in Health and Disease. Eur J Clin Invest 38(1), 1-10.

[80] Meli, R., Pirozzi, C., Pelagalli, A. (2018) New Perspectives on the Potential Role of Aquaporins (AQPs) in the Physiology of Inflammation. Front Physiol 9:101.

[81] Calamita, G., Ferri, D., Gena, P., Liquori, G.E., Cavalier, A., Thomas, D., Svelto, M. (2005) The Inner Mitochondrial Membrane Has aquaporin-8 Water Channels and Is Highly Permeable to Water. J Biol Chem 280(17), 17149-53.

[82] Marinelli, R.A., Lehmann, G.L., Soria, L.R., Marchissio, M.J. (2011) Hepatocyte aquaporins in bile formation and cholestasis. Front Biosci 16, 2642-52.

[83] Portincasa, P., Calamita, G. (2012) Water channel proteins in bile formation and flow in health and disease: When immiscible becomes miscible. Mol Aspects Med 33(5-6), 651-64.

[84] Carbrey, J.M., Gorelick-Feldman, D.A., Kozono, D., Praetorius, J., Nielsen, S., Agre, P. (2003) Aquaglyceroporin AQP9: Solute Permeation and Metabolic Control of Expression in Liver. Proc Natl Acad Sci U S A, 100(5), 2945-50.

[85] Fernandes, A.R., Mendonça-Martins, I., Santos, M.F.A., Raposo, L.R., Mendes, R., Marques, J., Romão, C.C., Romão, M.J., Santos-Silva, T., Baptista, P.V. (2020) Improving the Anti-inflammatory Response via Gold Nanoparticle Vectorization of CO-Releasing Molecules. ACS Biomater Sci Eng 6(2), 1090–1101.

[86] Samarasinghe, D.A., Farrell, G.C. (1996) The central role of sinusoidal endothelial cells in hepatic hypoxia-reoxygenation injury in the rat. Hepatology, 24(5), 1230–1237.

[87] Livak, K.J., Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods 25(4), 402-408.

[88] Martins, M.B.A.F., Corvo, M.L., Marcelino, P., Marinho, H.S., Feio, G., Carvalho, A. (2014) New long circulating magnetoliposomes as contrast agents for detection of ischemia–reperfusion injuries by MRI. Nanomedicine 10(1), 207-214.

[89] Karimi, S., Khatami, S.R., Azarpira, N., Galehdari, H., Pakbaz, S. (2018) Investigate of AQP gene expression in the liver of mice after ischemia–reperfusion. Mol Biol Rep 45, 1769–1774.

[90] Hamesch, K., Borkham-Kamphorst, E., Strnad, P., Weiskirchen, R. (2015) Lipopolysaccharide-induced inflammatory liver injury in mice. Lab Anim 49(1), 37-46.

[91] Tozawa, H., Maekawa, T., Kimura, H., Fujii, T. (2015) A novel effect of parylene-based surface coating on HepG2 cell function. Mater Sci Eng C Mater Biol Appl 46, 190–194.

[92] Lindskog, C., Asplund, A., Catrina, A., Nielsen, S., Rützler, M (2016) A Systematic Characterization of Aquaporin-9 Expression in Human Normal and Pathological Tissues. J Histochem Cytochem 64(5), 287–300.

[93] Ramaiahgari, S. C., den Braver, M. W., Herpers, B., Terpstra, V., Commandeur, J. N. M., van de Water, B., Price, L. S. (2014) A 3D in vitro model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. Arch Toxicol 88, 1083–1095.

[94] Guo, L., Dial, S., Shi, L., Branham, W., Liu, J., Fang, J.L., Green, B., Deng, H., Kaput, J., Ning, B. (2011) Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. Drug Metab Dispos. 39(3), 528-38.

[95] Ganz, M., Csak, T., Nath, B., Szabo, G. (2011) Lipopolysaccharide induces and activates the Nalp3 inflammasome in the liver. World J Gastroenterol. 17(43), 4772-8.

[96] Szabo, G., Bala, S. (2010) Alcoholic liver disease and the gut-liver axis. World J Gastroenterol. 16(11), 1321-9.

[97] Zhai, Y., Qiao, B., Shen, X.D., Gao, F., Busuttil, R.W., Cheng, G., Platt, J.L., Volk, H.D., Kupiec-Weglinski, J.W. (2008) Evidence for the pivotal role of endogenous toll-like receptor 4 ligands in liver ischemia and reperfusion injury. Transplantation. 85(7), 1016-22.

[98] Kawaratani, H., Tsujimoto, T., Douhara, A., Takaya, H., Moriya, K., Namisaki, T., Noguchi, R., Yoshiji, H., Fujimoto, M., Fukui, H. (2013) The effect of inflammatory cytokines in alcoholic liver disease. Mediators Inflamm. 2013, 495156.

[99] Solomon, D., Gupta, N., Mulla, N.S., Shukla, S., Guerrero, Y.A., Gupta, V. (2017) Role of In Vitro Release Methods in Liposomal Formulation Development: Challenges and Regulatory Perspective. AAPS J. 19(6), 1669-1681.

[100] Wang, M., Shen, F., Shi, L.H., Xi, T., Li, X.F., Chen, X., Wu, M.C. (2008) Protective effect of prednisolone on ischemia-induced liver injury in rats. World J Gastroenterol 14(27), 4332–4337.

[101] Hill, D.B., D'Souza, N.B., Lee, E.Y., Burikhanov, R., Deaciuc, I.V., de Villiers, W.J. (2002) A role for interleukin-10 in alcohol-induced liver sensitization to bacterial lipopolysaccharide. Alcohol Clin Exp Res. 26(1), 74-82.

[102] Horie, I., Maeda, M., Yokoyama, S., Hisatsune, A., Katsuki, H., Miyata, T., Isohama, Y. (2009) Tumor necrosis factor-alpha decreases aquaporin-3 expression in DJM-1 keratinocytes. Biochem Biophys Res Commun. 387(3), 564-8.

[103] Konukoglu, D., Taşci, I., Cetinkale, O. (1998) Effects of cyclosporin A and ibuprofen on liver ischemia-reperfusion injury in the rat. Clin Chim Acta 275(1), 1-8.

[104] Ward, P.H., Maldonado, M., Roa, J., Manríquez, V., Vivaldi, E. (1995) Ibuprofen Protects Rat Livers From Oxygen-Derived Free Radical-Mediated Injury After Tourniquet Shock. Free Radic Res 22(6), 561-569.

[105] García Rodríguez, L.A., Williams, R., Derby, L.E., Dean, A.D., Jick, H. (1994) Acute liver injury associated with nonsteroidal anti-inflammatory drugs and the role of risk factors. Arch Intern Med 154(3), 311-316.

[106] Andrade, R.J., Lucena, M.I., García-Cortés, M., García-Ruiz, E., Fernández-Bonilla, E., Vázquez, L. (2002) Chronic hepatitis C, ibuprofen, and liver damage. Am J Gastroenterol 97(7), 1854-1855.

[107] Narayanan, D., M.G., G., H., L., Koyakutty, M., Nair, S., Menon, D. (2013) Poly-(ethylene glycol) modified gelatin nanoparticles for sustained delivery of the anti-inflammatory drug Ibuprofen-Sodium: an in vitro and in vivo analysis. Nanomedicine. 9(6), 818-28.

[108] Irvine, J., Afrose, A., Islam, N. (2018) Formulation and delivery strategies of ibuprofen: challenges and opportunities. Drug Dev Ind Pharm 44(2), 173-183.

[109] Zhou, Z., Wang, L., Song, Z., Lambert, J.C., McClain, C.J., Kang, Y.J. (2003) A critical involvement of oxidative stress in acute alcohol-induced hepatic TNF-alpha production. Am J Pathol. 163(3), 1137-46.

[110] Hatakeyama, H., Akita, H., Harashima, H. (2013) The polyethyleneglycol dilemma: advantage and disadvantage of PEGylation of liposomes for systemic genes and nucleic acids delivery to tumors. Biol Pharm Bull. 36(6), 892-9.

[111] Jayaraman, M., Ansell, S.M., Mui, B.L., Tam, Y.K., Chen, J., Du, X., Butler, D., Eltepu, L., Matsuda, S., Narayanannair, J.K., Rajeev, K.G., Hafez, I.M., Akinc, A., Maier, M.A., Tracy, M.A., Cullis, P.R., Madden, T.D., Manoharan, M., Hope, M.J. (2012) Maximizing the potency

of siRNA lipid nanoparticles for hepatic gene silencing in vivo. Angew Chem Int Ed Engl. 51(34), 8529-33.

[112] Papadopoulos, M.C., Krishna, S., Verkman, A.S. (2002) Aquaporin water channels and brain edema. Mt Sinai J Med. 69(4), 242-8.

[113] Yang, M., Gao, F., Liu, H., Yu, W.H., Sun, S.Q. (2009) Temporal changes in expression of aquaporin-3, -4, -5 and -8 in rat brains after permanent focal cerebral ischemia. Brain Res. 1290, 121-32.

[114] Ikaga, R., Namekata, I., Kotiadis, V.N., Ogawa, H., Duchen, M.R., Tanaka, H., Iida-Tanaka, N. (2015) Knockdown of aquaporin-8 induces mitochondrial dysfunction in 3T3-L1 cells. Biochem Biophys Rep. 4, 187-195.

[115] Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M.T., Wang, S., Liu, H., Yin, Y. (2016) Quercetin, Inflammation and Immunity. Nutrients 8(3), 167.

[116] Liang, C.H., Ho, W.Y., Yeh, L.H., Cheng, Y.S., Chou, T.H. (2013) Effects of 1-hexadecyl-3-methylimidazolium ionic liquids on the physicochemical characteristics and cytotoxicity of phosphatidylcholine vesicles. Colloids Surf A Physicochem Eng Asp 436, 1083-1091.

[117] Li, H., Zhao, X., Ma, Y., Zhai, G., Li, L., Lou, H. (2009) Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. J Control Release. 133(3), 238-44.

[118] Baowen, Q., Yulin, Z., Xin, W., Wenjing, X., Hao, Z., Zhizhi, C., Xingmei, D., Xia, Z., Yuquan, W., Lijuan, C. (2010) A further investigation concerning correlation between anti-fibrotic effect of liposomal quercetin and inflammatory cytokines in pulmonary fibrosis. Eur J Pharmacol. 642(1-3), 134-9.

[119] Al Shoyaib, A., Archie, S.R., Karamyan, V.T. (2019) Intraperitoneal Route of Drug Administration: Should it Be Used in Experimental Animal Studies? Pharm Res. 37(1), 12.