UNIVERSITAT DE BARCELONA

Polyethylene glycol conditioning: An effective strategy to protect against liver ischemia reperfusion injury

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TESIS DOCTORAL

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To my family, for the patience

To all my professors, for the knowledge

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INDEX

ABBREVIATIONS

AMPK: adenosine monophosphate-activated protein kinase

Akt: protein kinase B

ATF-6: activating transcription factor 6

ATP: adenosine triphosphate

CHOP: C/EBP homologous protein

CytC: cytochrome c

DCD: Donation after cardiac death

eIF2α: eukaryotic translation initiation factor 2α subunit

ER: endoplasmic reticulum

eNOS: endothelial nitric oxide synthase

ERK ½: Extracellular signal regulated kinases

FoxO: Forkhead box-containing protein O

HSPs: Heat shock proteins

HES: hydroxyethyl starch

HIFs: hypoxia-inducible factors

ICAM-1: intracellular adhesion molecule

iNOS: inducible nitric oxide synthase

IRE1α: inositol requiring enzyme 1

IGL-1: Institute Georges Lopez-1

INF-γ: interferon-γ

IL-1: interleukin-1

IL-6: interleukin-6

IL-10: interleukin-10

IRI: ischemia-reperfusion injury

GRP78: glucose regulated protein 78

LT: Liver transplantation

MAPK: mitogen activated protein kinases

mTOR: (mammalian Target of rapamycin)

mPTP: mitochondria permeability transition pore

NADPH: nicotinamide adenine dionucleotide phosphate

MnSOD: Mn-superoxide dismutase

NO: nitric oxide

NF-kB: nuclear factor kappa B

PERK: RNA-activated protein kinase (PKR)-like ER kinase

ppar-α:peroxisome proliferator-activated receptor-α

PGC1α: peroxisome proliferator-activated receptor-γ coactivator

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

PC: ischemic preconditioning

p70S6k: protein S6 kinase

ROLT: reduced orthotopic liver transplantation

ROS: reactive oxygen species

RAS: renin-angiotensin system

SEC: Sinusoidal endothelial cells

SLT: split liver transplantation

SIRT: sirtuin

TNF-α: tumour necrosis factor

UCP2: uncoupling protein 2

UPR: unfolded protein response

UW: University of Wisconsin

XBP-1: X box-binding protein 1

I. INTRODUCTION

1 Introduction

Ischemia is defined by the arrest of blood flow in the organ cutting thus oxygen and metabolite supply indispensable for its survival and function. Reperfusion occurs when blood flow is restored and it is associated with tissue injuries. Ischemia reperfusion injury (IRI) remains one of the major problems in liver surgery and transplantation; it determines the viability of hepatic tissue after resection and the recovery of the grafted organ.

Therapeutic strategies against IRI have been developed during the last 60 years and great advances into the mechanisms responsible of injuries have been achieved. However, efficient therapy against IRI is still lacking and few clinical studies in phase III have proven their effectiveness. This could be due, in part, to the complexity of the mechanisms responsible of IRI and to the specific drugs activity and their potential adverse effects.

Polyethylene glycols (PEG) are water soluble and nontoxic polymers. PEG is non immunogenic, shows little toxicity and is eliminated from the body intact by either the kidneys (for PEGs < 30 kDa) or in the faeces (for PEGs > 20 kDa) [1]. Also, PEG has been associated with the majority of events occurring during IRI. Indeed, it has been shown that PEG reduces reactive oxygen species, prevent cell death, maintain mitochondrial integrity and reduce inflammation and endoplasmic reticulum stress [2-6]. From this perspective, it is reasonable to expect that PEG administration may be an effective therapeutic strategy against IRI.

The aim of this thesis is to investigate the beneficial effects of PEG 35 in different models of IRI that mimic clinical situation of liver surgery. In the first study, we investigated the impact of the intravenous administration of PEG 35 before liver warm IRI. In the second one, we investigated whether PEG 35 administrated intravenously could protect against cold IRI in steatotic rat livers. Finally, we developed a new washout solution containing PEG 35 to prevent reperfusion injury after prolonged cold preservation.

2 The Liver

The liver is located in the right upper quadrant, between the fifth intercostal space in the midclavicular line and the right costal margin. It weighs approximately 1800 grams in men and 1400 grams in women. The surfaces of the liver are smooth and convex in the superior, anterior and right lateral regions. It is separated incompletely into lobes, covered on their external surfaces by a thin connective tissue capsule. The main hepatic function is the uptake of substrates from the intestine in order to be stored, metabolized and distributed to the peripheral circulation for being used by other tissues. Furthermore, it is the main detoxifying organ of the body, which removes wastes and xenobiotics by metabolic conversion and biliary excretion.

2.1 Organization of the liver

The liver is structurally and functionally complex and has been considered second only to brain in its complexity. Awareness of the complexities and heterogeneity of the liver will add greater understanding of disorder that lead to toxicity, cancer, and other diseases.

2.1.1 Liver anatomy and vasculature

Anatomically human liver is divided into right and left lobes by the falciform ligament, which connects the liver to the anterior abdominal wall and the diaphragm. The right lobe is further subdivided into two smaller lobes, the caudate and the quadrate lobes. The left part of the liver can also be divided into medial and lateral sections by the tissue named as ligamentum teres. Furthermore, the right lobe is firmly attached to the gall bladder, a pear-shaped pocket that stores and evacuates bile. The liver can also be divided into eight segments, where each one has its own vascular and biliary supply [7] (Figure 1A).

The liver contains blood equivalent to approximately 25% of the cardiac output [8]. The portal vein and the hepatic artery are the two main vascular systems that supply blood to the liver. The portal vein supplies about 70% of the blood flow and 40% of the oxygen while the hepatic artery supplies 30% of the flow and 60% of the oxygen [8]. The portal blood drains from the mesenteric, gastric, splenic, and pancreatic veins and travels to the liver where it branches into the right and left sides of the liver.

The conducting portal vessels deliver blood to the parenchymal vessels called preterminal and terminal portal venules, respectively. Blood from the terminal portal venules enters the sinusoids [9]. The hepatic artery generally accompanies the portal veins in the portal triads and its smaller branches feed the sinusoids at varying levels and biliary tracts (which most often subsequently drains into sinusoids; a so-called portal-portal flow). The sinusoidal blood flow is carefully regulated and collects into terminal hepatic venules (also called central veins) prior to emptying into larger hepatic veins and eventually to the vena cava [10].

The portal triad is defined by the portal vein, bile duct, and hepatic artery, however the portal area contains on average about 6 profiles (range is 2–35) with an average of 1–2 arteries, 1 portal vein, 1–2 bile ducts, lymphatics, nerves in a connective tissue matrix comprised mainly by type 1 collagen [10].

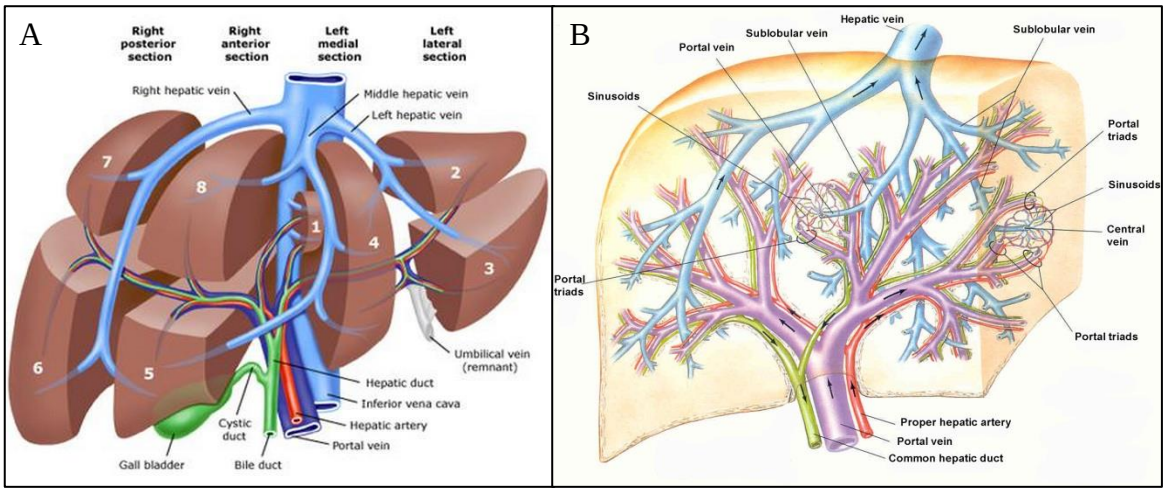

Figure 1: Liver anatomy (A) and vasculature (B)

2.1.2 Hepatic Functional units

The lobule, proposed by Matsumoto in 1979, has been gaining acceptance as the functional unit of the liver over other conceptual views because it's based on vessel architecture [10]. Hepatic lobule is a small division of the liver defined at the histological scale. The classic hepatic lobule is a polygonal structure where the hepatic venule forms its central axis and in its periphery boundaries are regularly distributed the

portal triads, containing a bile duct and a terminal branch of the hepatic artery and portal vein (Figure 2).

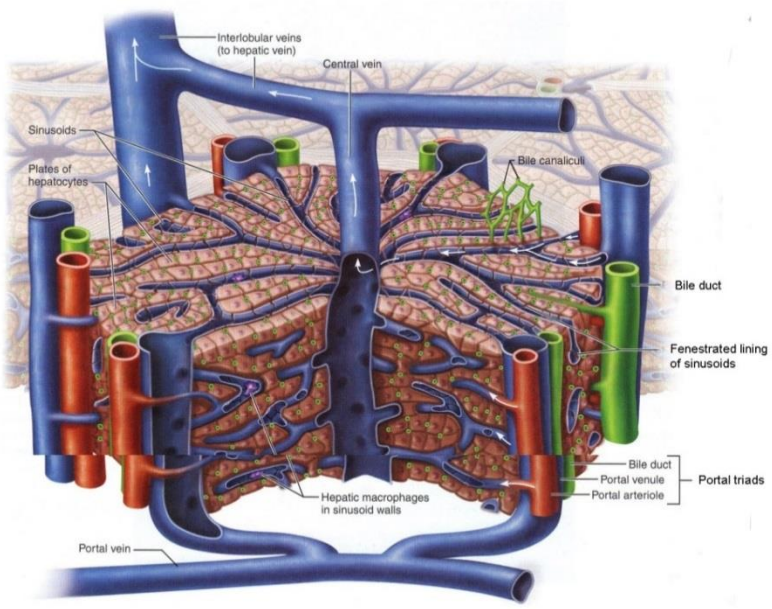

Figure 2: Schematic representation of the lobule

2.2 Liver cells

Hepatocytes are the most numerous and comprise 60% of the total cells and 80% of the volume of liver. Sinusoidal endothelial cells (SECs), Kupffer cells, hepatic stellate cells (HSC) and biliary epithelium make up a significant number (3–20% each) of the remaining biologically important cells [7, 10] (Figure 3).

2.2.1 Hepatocytes

Hepatocytes are arranged in plates or laminae of cords of 1 cell thick (called muralium) that branch and anastomose in a continuous labyrinth with limiting plates being at the capsule and portal regions. The 6 or more surfaces of the hepatocyte either are adjacent parenchymal cells, border bile canaliculi, or are exposed to the peri-sinusoidal space (this surface being covered by microvilli) [10]. Being the workhorses of the liver, hepatocytes contain the machinery necessary to carry out the thousands of vital functions. Normally, about 15% of the cell volume is composed of smooth and rough

endoplasmic reticulum and there are about 30 lysosomes and 500 peroxisomes (microbodies) per cell. The mitochondria number about 1,000 per hepatocyte and there are numerous free ribosomes, Golgi complex, cytoskeleton elements (such as microfilaments, intermediate filaments, and microtubules), and varying levels of cytoplasmic lipid and glycogen. One of the main hepatocyte functions is the production of bile, which averages about 15 ml/kg/day in humans. With age, the number of hepatocytes decreases and hypertrophy, polyploidy, lysosomes, and smooth endoplasmic reticulum increases. The mitochondria and microbodies remain unchanged with age and the microsomal drug-metabolizing capabilities decrease [10-12].

2.2.2 Hepatic sinusoid cells

The walls of hepatic sinusoid are lined by three different cell types: sinusoidal endothelial cells (SEC), Kupffer cells (KC), and hepatic stellate cells (HSC, formerly known as fat-storing cells, Ito cells, lipocytes, perisinusoidal cells, or vitamin A-rich cells). Additionally, intrahepatic lymphocytes, including pit cells, i.e., liver-specific natural killer cells, are often present in the sinusoidal lumen [13].

i) Sinusoidal endothelial cells

SECs are a layer of cells between the hepatocytes and the blood flowing in sinusoids. SEC contains numerous fenestrae (pores) which are clustered together in groups known as “sieve plates” and allow the exchange between the blood and the surrounding tissue. The endothelial fenestrae are dynamic structures whose diameters are affected by luminal blood pressure, vasoactive substances, drugs and toxins.

SECs represent an important blood clearance system, as all transport between the lumen and the hepatocytes has to pass through this filter. Furthermore, it permits rapid access to substances in the blood. SECs play an important role in immunity and inflammation, as secrete pro-inflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6), interferons and eicosanoids. They facilitate also adhesion of leucocytes and lymphocytes by secreting chemokines and expressing molecules, such as intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Thus, along with the Kupffer cells, the endothelium participates in host defense mechanisms.

Furthermore, they contribute to the formation of new blood vessels and regulate sinusoidal blood flow by releasing vasoconstrictor and vasodilator factors.

ii) Kupffer cells

Kupffer cells represent 15% of the liver cells (30% of sinusoidal cells) and are derived from circulating monocytes. They can proliferate locally, are phagocytic, are the major producers of cytokines as mediators of inflammation and provide "cross-talk" with other cells. Kupffer cells remove through endocytosis toxicants and bacteria from the circulation, as well as toxic and infective substances of intestine origin. Kupffer cells also produce both beneficial and toxic substances that contribute to host defense and liver injury respectively [10].

iii) Stellate cells

Stellate cells are located in the space of Disse, between hepatocytes and sinusoidal endothelial cells. In this way, they are able to interact with the surrounding cell types. Stellate cells contain fat droplets and constitute the most important storage site of retinoids, including vitamin A. In healthy liver, they are quiescent. However, when activated, they synthesize collagen and thus contribute to the development of cirrhosis [14].

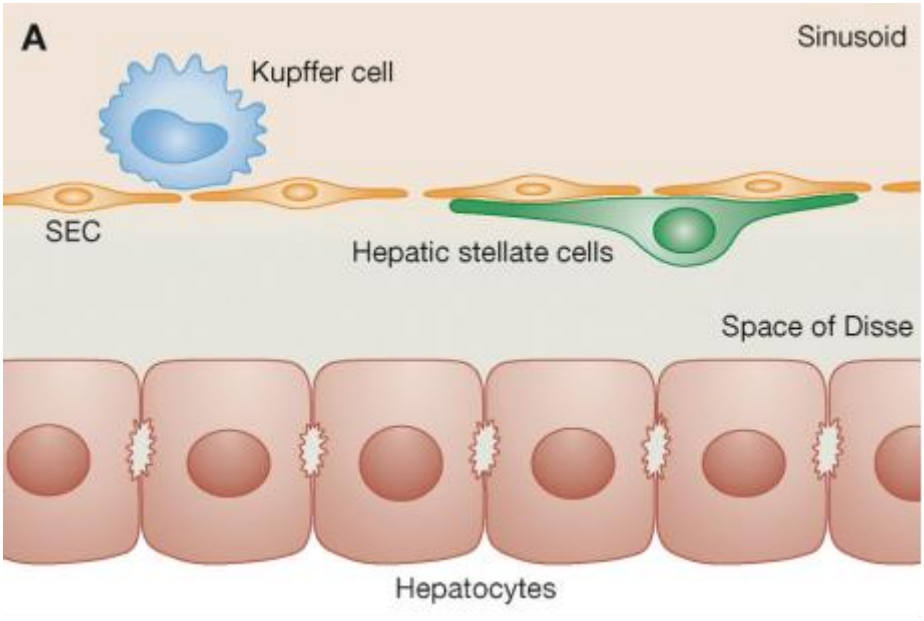

Figure 3 Liver cells organization [15]

3 Hepatic ischemia reperfusion injury: insight into the mechanism

3.1 Ischemic injury

Two major types of ischemic injury that are attributable to IRI can be distinguished: The 'warm' IRI which is developed *in situ* during liver surgery or during various forms of shock or trauma, and might lead to liver or even multiorgan failure. The 'cold' IRI, which occurs during *ex vivo* preservation in storage solution during liver transplantation surgery [16]. Hepatic cells response to the two kind of ischemia is different, hepatocytes are very susceptible to warm ischemia; however, SEC damage occurs predominantly during cold ischemia [17].

The mechanisms involved in both type of ischemia are multifactorial and share the most common signalization pathway. Understanding these mechanisms is of great interest because it permits the development of new effective strategies against IRI.

3.1.1 ATP depletion

The liver, a well-differentiated tissue, requires large amounts of oxygen to support its various specialized functions. These functions are maintained by energy derived from aerobic metabolism. As cells become anoxic, oxidative phosphorylation ceases and adenosine triphosphate (ATP) reserves are consumed rapidly. Lack of ATP leads to impairment of all energy-dependent functions, thus, ATP depletion could be considered as the initial common pathway of cell injury, responsible for cellular edema, cytoskeleton disorganization, proteasome activation and cell death [18-21]. ATP can be saved by cooling the organ, which reduces the tissue's metabolic demands for nutrients and oxygen (cold ischemia) [22-24]. However, intracellular homeostatic functions are decelerated equally which induces cellular edema.

3.1.2 Cellular edema

Disturbance of the electrolyte balance is a major consequence of slowing the metabolism by hypothermia. Normally, the cells are bathed in an interstitial fluid high in sodium (Na^{+1}) and calcium (Ca^{2+}) and low in potassium (K^{+}), compared with the

intracellular electrolyte concentrations (44). The intracellular concentrations of Na^+ and K^+ are maintained by an energy dependent cation transport system in the cell membrane [25]. This enzyme system uses ATP to make Na^+ impermeable outside the cell, counteracting the colloidal osmotic pressure derived from the intracellular proteins and other impermeable anions, and causes K^+ to accumulate in the cell. Hypothermic preservation suppresses the activity of this Na^+ pump and decreases the membrane potential of the plasma membrane. Consequently, chloride ($C1^-$) enters the cell down a concentration gradient with water and cause cell edema [26-28].

3.1.3 Acidosis

During hepatic ischemia, ATP-dependent cellular metabolic activities are gradually stopped, intracellular ATP is rapidly depleted and the redox process of the hepatocytes is blocked and as a consequence the metabolic pattern is shifted from aerobic to anaerobic. The enhanced anaerobic glycolysis leads to the accumulation of acidic metabolites, such as lactic acid and ketone bodies. This is accompanied by hypofunction of mitochondrial oxidative phosphorylation, resulting in the decrease of pH values between tissues and cells, known as metabolic acidosis [29]. This metabolic naturally occurring acidosis actually protects against the onset of necrotic cell death in hepatocytes. Conversely, restoration of a normal pH during reperfusion of ischemic cells enhance ATP-dependent enzyme such as proteases and phospholipases and accelerates cell killing, a phenomenon called the « pH paradox » [30].

3.1.4 Proteasome activation

Ubiquitin proteasome system (UPS) is the principal non-lysosomal proteolytic pathway responsible for the degradation of misfolded, aged and damaged proteins [31, 32]. In addition, UPS is an energy dependent system that modulates many regulatory proteins involved in a wide range of cellular processes which include inflammatory process, cell cycle, metabolism, growth, and differentiation [32, 33]. Majetschak *et al*. provided evidences that the 26S proteasome is under direct control of the cellular energy status and that a subset of 26S proteasomes is a cell-destructive protease, which is activated as the tissue ATP level declines [18]. Thus, UPS inhibition is protective against IRI in different organs [34-39].

3.2 Reperfusion injury

Efforts to restore blood flow in hypoxic tissue can paradoxically result in more destructive than beneficial effects, depending on the length of the ischemic period. Previous research indicates that the main damaging effects in IRI involve reactions following restoration of blood flow to the tissue rather than the ischemia itself [40].

3.2.1 Oxidative stress

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are one of the earliest and most important components of tissue injury after reperfusion of ischemic organs. The major ROS include the superoxide radical, hydroxyl, and hydrogen peroxide. The prime sources of ROS production in ischemic livers include cytosolic xanthine oxidase (XO), Kupffer cells, and adherent PMN [41, 42]. ROS-induced injury targets proteins, enzymes, nucleic acids, cytoskeleton, cell membranes, and lipid peroxides, resulting in decreased mitochondrial function, and lipid peroxidation [43]. The endothelial cells damage caused by ROS leads to the loss of microvascular integrity and decreased blood flow. Endogenous antioxidant compounds, such as superoxide dismutase, catalase, glutathione, and beta-carotene, may limit the effects of ROS but these systems can become overwhelmed by large quantities of ROS [44].

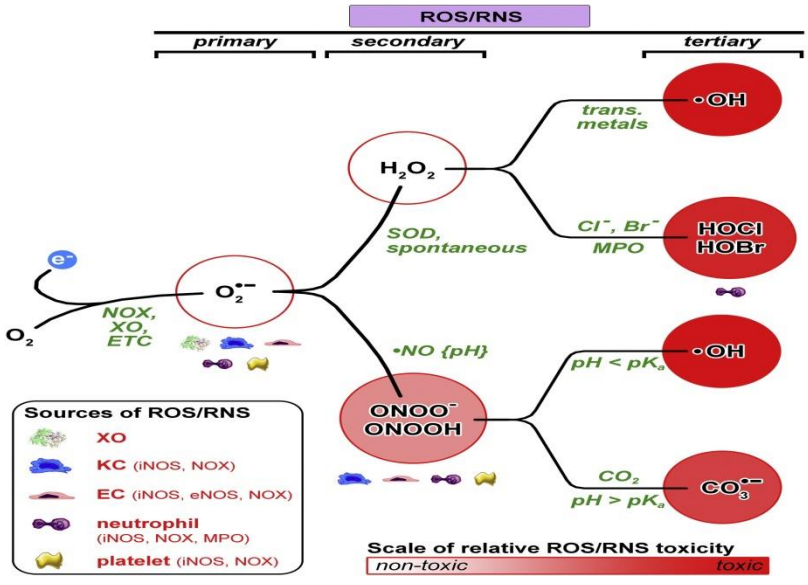

Figure 4 The formation of ROS and RNS during IRI (modified from [40]) (XO, xabthine oxidase; SOD, superoxide dismutase; ETC, electron transport chain; NOX, NADPH oxidase; MPO, myeloperoxidase; KC, kupffer cells)

3.2.2 Inflammation

The activation of inflammatory cells is a key event in the development of liver injury during ischemia and reperfusion. Kupffer cells are activated during reperfusion which generate ROS, RNS and pro-inflammatory cytokines, such as Tumor necrosis Factor α (TNFα), interferon-γ (INF-γ), interleukin-12 (IL-12) and Interleukin-1 (IL-1). These chemokines promote the expression of adhesion molecules, such as the intercellular adhesion molecule (ICAM), potentiating thus the activation, recruitment, and adhesion of neutrophils to the EC. Adhered neutrophils trigger cell death by releasing various proteases (elastases, proteinases, and collagenases), which degrade components of the extracellular matrix, attack cells, and inactivate various proteins such as immunoglobulins and proteins of complement. Furthermore, neutrophils generate ROS, like hydrogen peroxide, through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. In addition, the produced cytokines, ROS and the increased translocation of P-selectin (endothelial adhesion molecule) to the surface of endothelial cells and platelets promote the adherence of leukocytes to the microvascular endothelium. The inflammatory cascade induces significant organ infiltration and injury [45-47].

3.3 Cellular consequences of IRI

3.3.1 Mitochondrial dysfunction

The mitochondria are the location where oxidative phosphorylation mainly takes place, and the mitochondria participate in multiple pathophysiological processes of IRI. A large number of ROS and RNS are generated in the mitochondria during the state of ischemia. Hypoxia impair the process of oxidative phosphorylation in cells and obstructs the production of ATP, causing disorders of the cytoplasmic ions such as Ca^{2+}, Na^{+}, and H^{+} in the mitochondria, and leads to the opening of high-conductance pores in the mitochondrial inner membrane. These pores conduct both positively and negatively charged solutes of up to 1,500 Da. Pore opening induces mitochondrial permeability transition (MPT) responsible for membrane depolarization, swelling, and uncoupling of oxidative phosphorylation [29, 48]. MPT was implicated in lethal cell injury associated with anoxia, reperfusion, and oxidative stress to heart and liver cells [40, 48]. Treatments that ameliorate mitochondria recovery have been associated with less graft damage and better recovery [48-51].

3.3.2 Apoptosis and necrosis

Hepatocytes and SECs are the two main cell types that are injured in IRI. Hepatocytes are more sensitive to warm ischemic injury, while SECs are more sensitive to cold ischemia. Physiologically, exclusive injury of one cell type is not found and there is evidence that both cell types have been injured directly in both cold and warm IRI. There has been debate about what the primary mode of cell death is in liver IRI: apoptosis or necrosis. Apoptosis is an energy dependent process, so in theory when there is greater depletion of ATP, necrosis should dominate. Also, necrosis takes longer to become apparent, normally more than 3h. This is challenging to show experimentally *in vivo*, as tissue ATP before and after reperfusion would need to be measured as well as the change in metabolic state of the cell. Many of the same initiators and pathways are involved for both types of cell death, so there is much overlap, referred as necroptosis [19, 52].

3.3.3 Autophagy

Autophagy is a tightly regulated pathway implicated in many physiological and pathological processes. Autophagy is essential for normal development and embryogenesis, as contributes to the clearance of apoptotic cells. Autophagic degradation of cellular constituents can efficiently recycle essential nutrients so that basic biological processes can be sustained [53]. The process of autophagy can be divided into four basic steps: induction, formation of autophagosome, autophagosome fusion with the lysosome, and degradation, where more than 30 autophagy-related proteins participate. The first step, the induction of autophagy, requires the beclin-1–class III PI3K (phosphoinositide 3-kinase) complex. After induction, the isolation membrane is elongated in order to sequester the cytosolic components and form the double membrane autophagosome. This step is primarily mediated by LC3II. LC3, the full length precursor protein, is converted to LC3-I which then is conjugated with phosphatidylethanolamine and thus is converted into LC3-II. LC3-II is inserted into the autophagosomal membrane, a process that play an essential role in the expansion of the autophagosomes [54]. Next, the outer membrane of autophagosomes fuse with lysosomes to generate the autophagolysosome and finally the contents of the autophagolysosome are degradated onto the lysosome [55].

The role of autophagy during warm and/or cold liver IRI remains discordant. Depending on the context, induction or impairment of autophagy during warm and/or cold liver IRI can be protective or detrimental for liver cells. Stimulation of impaired autophagy following warm and/or cold IRI may promote hepatocyte survival by degradation of intracellular contents to maintain ATP production and removal of damaged organelles and protein aggregates [56, 57]. Excessive and long-term upregulation of autophagy, as it occurs during severe ischemic insult of the liver, may lead to destruction of essential proteins and organelles resulting in hepatocellular apoptosis and necrosis [57].

3.3.4 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is a membrane system that forms a series of flattened sacs within the cytoplasm of eukaryotic cells and serves multiple functions, being important particularly in the synthesis, folding, modification, and transport of proteins.

It is well known that ischemia reperfusion leads to altered Ca^{2+} homeostasis [58]. Knowing that ER is the site where Ca^{2+} is stored and released, perturbation of Ca^{2+} homeostasis induces ER stress. Consequently, newly synthesized unfolded proteins accumulate in the organelle. To cope with accumulated unfolded proteins, mammalian cells trigger a specific adaptive response called the unfolded protein response (UPR). The UPR has three branches: inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor (ATF6). These proteins are normally held in inactive states in ER membranes by binding to intra-ER chaperones, particularly the 78-kD glucose-regulated/binding immunoglobulin protein (GRP78). In response to stimuli that divert ER chaperones to misfolded proteins, IRE1, PERK and ATF6 initiate signal transduction processes in order to promote the expression of genes required to fold newly synthesized proteins and to degrade the unfolded proteins [59]. However, when injury is excessive, the same ER stress signal transduction pathways induce cell death [60].

Accumulating evidence has suggested that perturbations at the ER are involved in the promotion of cell death during IRI [61]. In line with this, activation of ER stress markers has been observed in tissue biopsies from human allograft after reperfusion [62]. Prevention of ER stress by rapamicine, tauroursodeoxycholic acid, N-

acetylcyteine, trimetazidine or ischemic preconditioning (IP) has been shown to be effective in reducing IRI [63-67]. Also, the use of IGL-1 preservation solution resulted in less injury through ER stress inhibition when compared to UW solution in rat orthotopic liver transplantation [68].

3.3.5 Cytoskeleton disorganization

The cytoskeleton is the inner structural elements of a cell, composed of microtubules, microfilaments, and larger filaments that spread out through the cytoplasm. The cytoskeleton regulates cell motility, the intracellular distribution of organelles, and trans-endothelial permeability. Since cellular microfilaments and microtubules are in an energy-requiring dynamic state of continuous formation and disassembly, ATP deficiency during ischemia is responsible, in part, of cytoskeleton disturbance.

During cold ischemia, rounding and cytosolic retraction of SEC occurs and cells detach from their underlying attachments as a consequence of disruption of the cytoskeleton. Also, it has been reported that chemical hypoxia caused marked cytoskeletal disruption characterized by degradation of actin microfilaments, disappearance of focal adhesions, retraction of the cytoplasm, and the appearance of gaps between the cells [69]. These structural alterations are reported to cause organ dysfunction observed at the moment of reperfusion [70, 71].

The subcellular distribution of filamentous actin (F-actin), being an important component of the cytoskeleton, as well as the balance between F-actin and monomeric G-actin seem to largely determine the functional outcome [72]. In liver cells, F-actin forms microfilaments involved in intracellular transport processes, exo- and endocytosis, maintenance of cell shape, and canalicular motility responsible for bile flow [72]. They are located particularly around the bile canaliculi exhibiting regulatory functions on bile secretion, but also in the apical membrane region of hepatocytes ensuring stability and mobility [73]. In hepatocytes, a decrease in F-actin content determines inhibition of store-operated calcium channels (SOCs), disruption of the organization of the endoplasmic reticulum, and functional disturbances of tight junctions [74]. It has been reported that after warm ischemia, F-actin is reduced in rabbit livers resulting in the loss of cell-integrity and cytoplasmic transport in the liver causing damage to organelles and changes in cell morphology [75]. Little work has been done to

investigate protective strategies against cytoskeleton disruption. Killer *et al.* have shown that atrial natriuretic peptide (ANP) preserved F-actin content through a mechanism dependent in p38 MAPK activation [76] during liver IRI.

3.3.6 Glycocalix disruption

It is now well recognized that the luminal surface of the ECs that line our vasculature is coated with a glycocalyx (GCX) of membrane-bound macromolecules comprised of sulfated proteoglycans, hyaluronan, glycoproteins, and plasma proteins that adhere to this surface matrix [77]. The glycocalyx play a central role in modulating the oncotic forces that regulate the exchange of water in microvessels, in transducing fluid shear stress into the intracellular cytoskeleton of endothelial cells, in the initiation of intracellular signaling, and in the inflammatory response cascade [78, 79]. During liver IRI, ROS/RNS-mediated degradation of the GCX, induces vasoconstriction, facilitates leukocyte adherence, and directly activates innate immune cells. Preliminary experiments revealed that hepatic sinusoids contain a functional GCX that is damaged during murine hepatic ischemia reperfusion and major liver surgery in patients [40, 80]. However, the underlying mechanisms remains poorly defined.

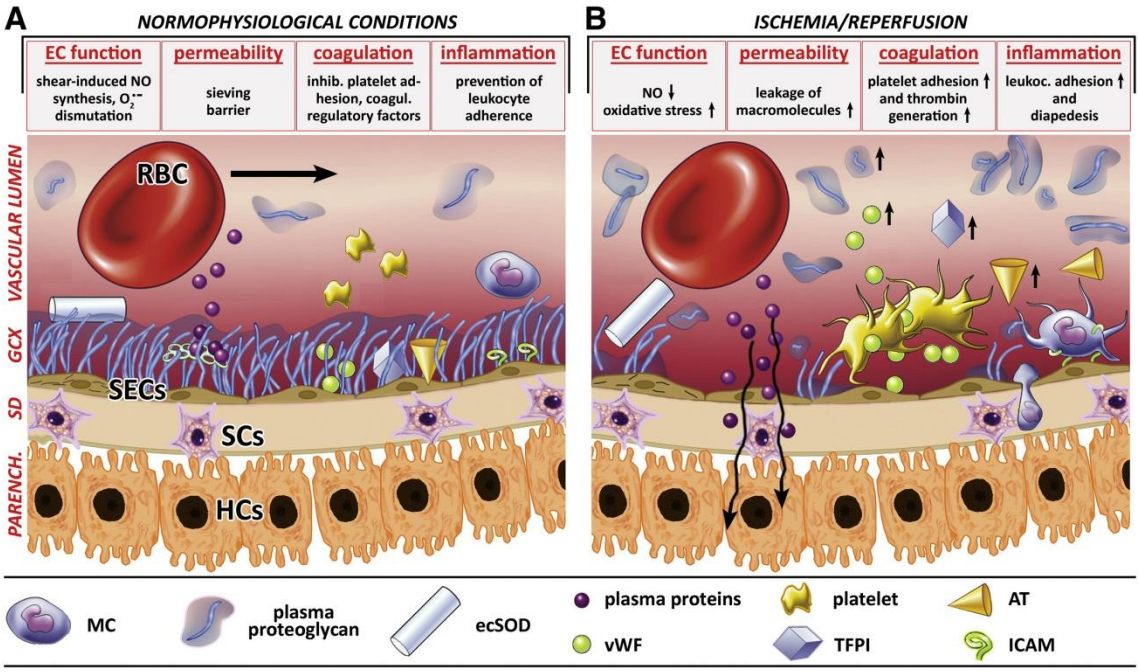

Figure 5 The glycocalyx under normophysiological (A) and ischemia/reperfusion-induced pathophysiological circumstances (B) [40] (SECs, sinusoidal endothelial cells; ecSOD, extracellular superoxide dismutase; SD, space of Disse; TFPI, tissue factor

pathway inhibitor; AT, antithrombin; vWF, von Willebrand factor; ICAM-1, intracellular adhesion molecule 1; RBC, red blood cell; MC, monocyte; SCs, stellate cells; HC, hepatocyte

3.4 Cellular mediators involved in IRI

3.4.1 Adenosin monophosphate activated protein kinase

AMPK is a serine–threonine kinase that functions as a fuel gauge and maintains energy homeostasis during cellular stress. AMPK is a heterotrimeric complex of α, β, and γ subunits. AMP and ADP binding to the γ subunit allosterically activates AMPK and facilitates the phosphorylation of the activating Thr172 site in the α subunit by upstream kinases including liver kinase B 1 (LKB1) and calcium–calmodulin-dependent kinase kinase 2 (CaMKK2), and possibly transforming growth factor-β-activated protein kinase-1 (TAK1) [81]. When ATP consumption exceeds production, there is an ensuing increase in cellular ADP content. Conversion of two ADPs to AMP (and ATP) by adenylate kinase also increases the cytosolic concentration of AMP. The increase in both AMP and ADP activates AMPK by binding to the regulatory nucleotide-binding domains of the AMPK gamma subunit. Desactivation of AMPK is mediated in part by protein phosphatase 2A and 2C, which dephosphorylate the Thr172 site. AMPK is also negatively regulated by acetylation and oxidation of cysteine residues in the AMPK α subunits [81].

AMPK activation during ischemia activates downstream targets in order to decrease cellular process that consume energy and induce cellular signaling pathway in order to provide necessary metabolite to the cell. In liver, AMPK activation has been shown to protect ischemic liver through endoplasmic reticulum stress (ERS) inhibition [65], autophagy induction [82], hypoxic inducible factor 1 alpha (HIF-1α) stabilization [83] and mitochondria protection. Indeed, melatonin has been shown to improve mitochondrial function after cold ischemia [84] through AMPK activation [85].

3.4.2 Hypoxic Inducible Factor 1

HIF-1 is a heterodimer formed by α and β subunits which are constitutively expressed. The β subunit is independent of O_2, whereas the protein stability of the α subunit depends on the cellular levels of O_2. Under normoxic conditions, the α subunit is

degraded by a complex process involving the prolylhydroxylases, the Von Hippel Landau protein and the 26 S proteasomes [86]. In ischemic conditions, hypoxia HIF-1α regulates the adaptive response of the organ to the changes in oxygenation [87]. Indeed, HIF-1α confers protection against IRI by activating others genes such as heme oxygenase-1 (HO-1) and downstream proteins including Erythropoetin (Epo), Vascular endothelia growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) [83, 88].

3.4.3 Nitric oxide

Nitric oxide (NO) is a gaseous molecule with an unpaired electron in its valence shell. This structural configuration results in NO being highly reactive, with a consequent half-life in the order of 5–10 s. Numerous clinical and non-clinical benefits of NO are described in various organ systems. The effects of NO on liver ischemia reperfusion may prove to be harmful, beneficial or a combination of both. The determining factors are the length of the ischemic insult and the enzymatic source of NO. In fact, NO generated by the eNOS isoform protects against liver I/R injury, whereas inducible NOS (iNOS)-derived NO may have either a protective or a deleterious effect during the early phase of IRI, depending on the length of ischemia, length of reperfusion and experimental model. The protective effects of NO are presented in figure 6.

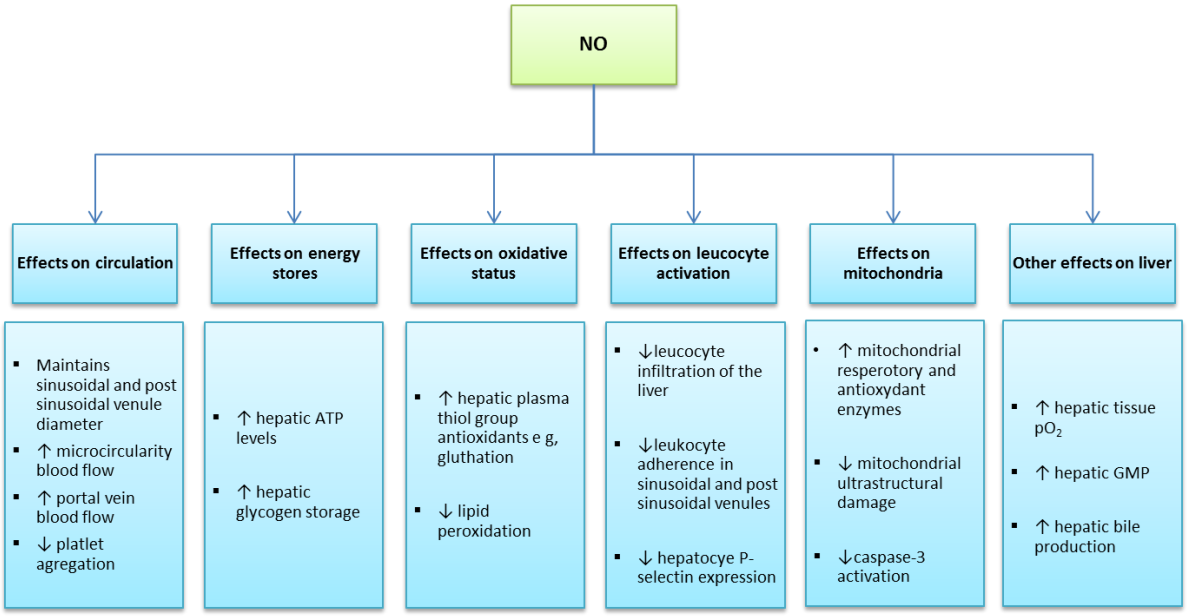

Figure 6: Mechanism of protection of nitric oxide in liver IRI. ATP, adenosine triphosphate; NO, nitric oxide; pO_2, partial oxygen pressure (modified from [89])

3.4.4 Mitogen Activated Protein Kinases

Mitogen activated protein kinases (MAPKs) play an important role in intracellular signal transduction in response to extracellular stimuli and dual phosphorylation of their threonine and tyrosine residues is necessary for their activation. Once activated, these kinases are translocated to the nucleus, where they phosphorylate and activate different transcription factors and thus the transcription of various genes. MAPKs are classified as: (1) Extracellular signal regulated kinases, ERK ½, (2) JunNH2-terminal kinases 1/2 (JNK 1/2) and (3) p38 MAPK. ERK 1/2 is usually activated by mitogenic and proliferative stimuli, like growth hormone receptors, whereas JNKs and p38s are stimulated by various cellular stresses like ROS, heat shock, inflammatory cytokines, and ischemia and for this reason are also referred as stress-activated protein kinases [90] [91]. p38 kinase regulates cell proliferation and differentiation and can modulate either pro-proliferative or pro-apoptotic signals [92]. Besides this, p38 activation has been mainly associated with the production and activation of inflammatory mediators [93].

The hepato-protective effects of preconditioning (PC) have been associated with activation of JNK-1 and p-p38 and subsequent entry of hepatocytes into the cell cycle, thus favoring hepatocyte survival against IRI [94]. However, more recent studies in steatotic livers revealed that PC, through PPAR-α activation, reduced p38 and JNK

expression. This was also associated with reduced adiponectin levels and oxidative stress [95]. Furthermore, the pro-apoptotic ERK has been shown to be activated during cardiac PC [96].

3.4.5 Metalloproteinases

Metalloproteinase 9 (MMP-9) is a zinc dependent secreted gelatinase which catalysis degradation of type IV collagen and gelatin. It has been shown that liver IRI is reduced by up to 80% in MMP-9-/- knockouts. In normal animals, increased expression of MMP-9 on macrophages and neutrophils during reperfusion was associated with pro-inflammatory cascades which increases neutrophil transmigration in liver sinusoids and increases TNF-α and interferon γ secretion and $CD4^+$ T cell activation [97, 98]. The mechanisms by which MMP-9 expression is increased in liver during ischemia reperfusion were not investigated in these knockout studies, but a possible pathway involves induction of MMP-9 by ROS and tumor necrosis factor α (TNF-α) [98].

3.4.6 Heme oxygenase-1

Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme, followed by production of CO, biliverdin, and free iron. The HO system is believed to confer cytoprotection by inhibiting inflammation, oxidation, and apoptosis, and maintaining microcirculation. HO-1, an inducible form of HO, serves a vital metabolic function as the rate-limiting steps in the heme degradation pathway, and affords protection in models of hepatic IRI [99-101]. The cytoprotection often seen in the transplanted organ following local HO-1 overexpression may include several factors, such as: a) antioxidant function, b) maintenance of microcirculation, c) anti-apoptotic function, and d) anti-inflammatory function [99, 100].

3.4.7 Heat shock proteins

Heat-shock proteins (HSPs), or stress proteins, are ubiquitous highly conserved proteins produced by cells in response to exposure to stressful conditions. HSPs constitute a large family of proteins that are often classified based on their molecular weight: hsp10, hsp40, hsp60, hsp70, hsp90, etc. They play crucial roles in folding/unfolding of proteins, assembly of multiprotein complexes, transport/sorting of proteins into correct subcellular compartments, cell-cycle control and signaling, and protection of cells

against stress/apoptosis. In addition, extracellular HSPs can stimulate antigen-presenting cells of the immune system, such as macrophages and dendritic cells [102]. HSPs are induced during exposure to a wide variety of stresses, including ischemia-reperfusion in order to protect cells from damage. They have been associated with anti-apoptotic effect, decreases pro-inflammatory mediators, such as NF-kB and enhances the anti-oxidant capacity of the cell [101, 103, 104]. Various studies have evidenced that the induction of HSP72 and HO-1 expression during IP contributed to the acquisition of improved hepatic function and increased tolerance against IRI [105, 106].

3.4.8 Sirtuins

Sirtuins are highly conserved class III histone deacetylases with homology to the yeast silent information regulator 2 (Sir2). To date, seven sirtuins have been described in mammals (SIRT1 through SIRT7). Sirtuin 1 (SIRT1) is a histone deacetylase that either activates or suppresses the transcription activities of various non-histone proteins, through its NAD^+-dependent activity. SIRT1 has been associated with the pathophysiology of IRI in several organs [107]. In fact, SIRT1 is involved in a wide variety of cellular processes, including oxidative stress, DNA damage, apoptosis, cellular stress and autophagy [108-113]. It has been reported that SIRT1 deacetylates p53 thus reducing its transcriptional activity and its ability to induce apoptosis [114]. Also, Forkhead box-containing protein O 1 (FoxO1) is a target for SIRT1 and its deacetylation has been involved in detoxification of ROS and promotion of autophagy [115]. Furthermore, we have recently shown that SIRT1 activation contributes, in part, to the protective effects of liver IP against IRI [116].

4 Clinical situations associated with liver ischemia reperfusion injury

IRI is an inevitable problem in many clinical situation of liver surgery such as liver transplantation, trauma and liver resection.

4.1 Liver Transplantation

Liver transplantation (LT) is the only lifesaving therapy for the end-stage liver diseases. In LT, IRI is principally caused by blood flow disturbances which starts with the brain death and is due to severe hemodynamic disturbances in the cadaveric donors. The

clamping of hepatic artery causes a short, but severe liver ischemia during the harvesting operation. In addition, the cold ischemia during allograft liver storage may also cause a further ischemic damage. The allograft liver transplantation from living related donors is also subjected to warm ischemia, but in such condition disturbances related to brain death are not present and cold ischemia is also shorter. Indeed, IRI is less frequent and less severe in transplantation from living donors. The final and biologically more severe stage of the injury occurs during the reperfusion as a consequence of the blood flow restoration. IRI is the major cause of early allograft dysfunction (EAD), associated with worst graft and recipient survival rates. Depending on the criteria used, the reported incidence of EAD has varied in different series and ranged from 2% to 23%. Among the 23.2% of recipients with EAD, 18.8% died [117, 118].

Dr Thomas Starlz is acknowledged as the first person to successfully performed deceased LT in the 1960s. In the 1970s, developments in surgical technique helped to advance the field, but rejection of the organ was a limiting factor in survival. A vital discovery in the advancement of the field was the development of cyclosporine in 1976 by Sir Roy Calne. Cyclosporine allowed longer graft and patient survival. Since, surgical techniques have been refined, medical therapies are more sophisticated, and awareness for transplantation is more widespread.

Liver transplant activity started in Spain in 1984. Drs. Margarit & Jaurrieta carried out the first liver transplant at Bellvitge Hospital (Barcelona). Since 2003, more than 1000 liver transplants are performed annually (1,108 in 2008). As a consequence of this, the liver transplant rate in 2007 was 24.6 per million population (pmp), one of the largest ever reached in the world. According to the World Transplant Registry – developed by the Spanish Transplant Organization in collaboration with the World Health Organization – around 20,000 liver transplants are performed annually all over the world [119]. In the period between 1984 and 2013, 85.4 % of patients who underwent transplant were residents in Catalonia, 13.6% (569) were resident in the rest of Spain and 0.7% (31) were from abroad. This information was not available for 12 patients [120].

4.1.1 Indications

LT is indicated in the cases of fulminant hepatic failure, liver-based metabolic defect or, more commonly, cirrhosis with complications such as hepatic encephalopathy, ascites, hepatocellular carcinoma, hepatorenal syndrome, or bleeding caused by portal hypertension (Table 1). While the complications of cirrhosis can often be managed relatively effectively, they indicate a change in the natural history of the disease that should lead to consideration of liver transplantation [121]. Referral for transplantation should also be considered for the rare diseases state in which the liver is not failing but causing other systemic disease (e.g., amyloidosis or oxaluria). In Catalonia, the most frequent indication is cirrhosis (48.9%), followed by hepatocellular carcinoma (30.0%), although there are differences according to recipient age. In pediatric patients, the most frequent indications are congenital biliary diseases [120].

The benefit of transplantation must be weighed against the considerable potential morbidity and mortality of undergoing a major surgical procedure. Therefore, transplantation is generally reserved to those who are felt to have poor quality of life and high mortality without LT. Patients should be projected to have a longer life with transplant than in its absence, referred to as *transplant survival benefit*.

Table 1. General Indications for Liver Transplantation [121]

Fulminant hepatic failure
Complications of cirrhosis
Ascites
Encephalopathy
Synthetic dysfunction
Liver cancer
Refractory variceal hemorrhage
Chronic gastrointestinal blood loss due to portal hypertensive gastropathy
Systemic complications of chronic liver disease
Hepatopulmonary syndrome
Portopulmonary hypertension
Liver-based metabolic conditions causing systemic disease[a]
Primary oxaluria
Familial amyloidosis
α_1-antitrypsin deficiency
Wilson's disease
Urea cycle enzyme deficiencies
Glycogen storage disease
Tyrosemia

4.1.2 Complications of a liver transplant

Currently, the main limitation of liver transplantation is the poor number of donors. Although Spain has the greater number of graft donor in the world, the number of patients in waiting list exceeds the number of liver donor. Despite all the efforts made to increase organ donation in recent years, the rate of organ donation remains relatively stagnant comparing to the increased patients in waiting list [119].

To counter the lack of available organs for transplantation, various strategies have been developed to optimize the use of available organs. These strategies include the re-evaluation of the variables of acceptance of an organ to be transplanted such as marginal liver grafts and grafts with rare metabolic disorders (domino transplant), and the development of various transplantation techniques alternatives to conventional such as split technique and living donor liver transplantation.

4.1.3 Marginal liver grafts

Limited pool of donor organs for liver transplantation has led to the acceptance of marginal livers such as steatotic ones, despite their higher risk of EAD and non-function. In fact, steatotic liver grafts are associated with an EAD rate of 60% compared with less than 5% for nonsteatotic grafts [122-124]. This is due to their poor tolerance to IRI [117], evidenced by the exacerbated oxidative stress, mitochondrial damage, and microcirculatory alterations [58, 123]. For these reasons, there is an urgent need for the development of strategies in order to minimize the detrimental effects of IRI in case of steatotic livers or to eliminate the fat content.

4.2 Trauma

The liver is the most commonly injured organ in blunt abdominal trauma and the second most commonly injured organ in penetrating abdominal trauma. Most hepatic injuries are relatively minor and heal spontaneously with nonoperative management which consists of observation, and possibly arteriography and embolization. Operative intervention to manage the liver injury is needed in about 14 % of patients including those with hemodynamic instability or those who fail nonoperative management [125]. As far as surgical approaches are concerned, IRI contributed significantly to mortality

and morbidity. The modification of existing clamping techniques and the ischemic preconditioning are the most promising techniques till recently to prevent liver IRI [126]. Hence, clinicians should be familiar with the concept of hepatic IRI and respond appropriately and timely [127].

4.3 Hepatic resection

Liver resection requires occlusion of the blood supply to the liver in order to reduce bleeding from the cut liver surface. This temporary interruption of blood causes significant IRI.

5 Therapeutic strategies against liver ischemia reperfusion injury

Protective strategies against liver IRI include surgical interventions known as ischemic conditioning, the use of pharmacologic agents, the application of organ preservation techniques and the use of wash out solutions.

5.1 Ischemic conditioning

Ischemic conditioning is a form of endogenous protection induced by transient subcritical ischemia in order to diminish IRI in various organs including liver, heart and brain [128-130]. In this regard, we can distinguish three types of ischemic conditioning: 1) ischemic preconditioning (IPC), ischemic postconditioning (IPost) and remote ischemic conditioning (RIC).

5.1.1 Ischemic preconditioning

IPC is based on the application of short periods of ischemia (5-10 minutes), separated by short reperfusions (10-15 minutes) prior to a sustained episode of ischemia. In this way, hepatocytes are prepared to respond favorably against the sequential prolonged ischemic insult. IPC process involves multiple extracellular signals, and intracellular second messengers [131-133], such as diminution of ROS production, apoptosis and inflammation. IPC has been applied successfully in patients with steatotic livers undergoing major resection [126].

5.1.2 Ischemic postconditioning

IPost was developed to overcome the clinical difficulties related to the application of IC before ischemia. It involves the same surgical procedure of brief episode of ischemia but it is applied at the beginning of the reperfusion phase. IPost has been evaluated in transplantation studies of the liver, heart, brain, and kidney [134] and ameliorated organ function. Several extracellular factors produced endogenously are known to play an essential role in IPost (adenosine, bradykinin, opioid peptides, and ROS [129, 130, 134].

5.1.3 Remote ischemic conditioning

There are now results showing that, in animals, transient ischemia of a wide range of tissues induces a systemic multi-organ protection against subsequent extended IRI in a remote organ. This was first observed in the coronary arteries of the heart and has also been noted in remote organs such as the kidneys, small intestine and liver [134, 135].

5.2 Pharmacologic conditioning

Pharmacologic research for the protection against IRI is based on its pathophysiological mechanisms. In this sense, all the parameters implicated in ROS-mediated damage, inflammation, cellular death or cytoskeleton disruption represent potential targets of pharmacologic PC.

Although the advance in defining the molecular mechanisms behind the IRI and the various therapeutic strategies proposed, few clinical studies at phase III achieved to provide encouraging results that ameliorate the clinical outcome.

5.3 Graft preservation

The main goal in organ preservation is to maintain function of the organ and tissue during storage so that the graft will be viable at reperfusion (See appendix: Emerging concepts in liver graft preservation).

5.3.1 Static cold storage (SCS)

To date, the predominant organ preservation method used by most centers is SCS. The principles of SCS are based on the diminution of metabolism by hypothermia. The appropriate preservation solution is infused into the organ (the cooling phase) and then stored statically [136].

Cooling

SCS is the most widely used method for preserving organs for transplantation. Cooling is necessary to reduce cellular metabolism and the oxygen requirements in order to prevent tissue injury [137].

In order to obtain viable organs after long-term preservation, various methods have been proposed, ranging from organ freezing and vitrification [138, 139] to "*supercooling" (*subzero non-freezing at 0 ºC to -5 ºC) [140-143]. In general, long-term survival rates after transplantation are disappointing.

However, in a recent study by Berendsen *et al*, the combination of "*supercooling*" (cold preservation at -6 ºC) with other parameters achieved effective preservation of liver grafts for 4 days [23]. This promising new technique comprises three steps: first, "supercooling" of the organ at -6°C to reduce the cellular metabolism; second, subnormothermic machine perfusion at 21°C (see the dynamic preservation section below), which reinitiates the metabolism and replenishes ATP levels, and third, the use of two preservatives, 3-O-methyl-d-glucose (3-OMG) and polyethylene-glycol 35. Each of these conditions is necessary to achieve successful liver transplantation [22]. With this in mind, supercooling techniques may be a potentially useful tool for suboptimal livers which are currently discarded for transplantation purposes, and may have great impact on global organ sharing.

Preservation solutions

Although cold is a fundamental requirement for tissue preservation, it has harmful repercussions due to the induction of cell swelling [144] and cytoskeletal alteration [145]. This was, in part, the reason for the development of commercial organ preservation solutions able to prevent many of the cellular alterations associated with hypothermia and to mitigate the harmful effects of cooling.

EuroCollins (EC) solution was developed in the 1970s as a high potassium-sodium solution (intracellular composition) which does not contain oncotic agents but does contain glucose. Given that glucose is impermeable to renal cells, this preservation solution was suitable for kidney preservation when relatively short times were needed or DCD organs were used. However, the permeability of the liver and pancreatic cells to glucose leads to the loss of the osmotic effect, and also causes the subsequent anaerobic metabolization of glucose, inducing intracellular acidosis and thus limiting cell preservation. This is why glucose was later substituted by other larger sugar molecules such as lactobionate and raffinose in University of Wisconsin (UW) solution, which remains in the extracellular space and preserves its beneficial effect. The use of the UW preservation solution improved organ preservation time from 6 to 16 hours [26].

The efficacy of UW solution is based on the prevention of edema by impermeants (raffinose, lactobionate), and the addition of an ATP precursor (adenosine) and anti-oxidant components (allopurinol, reduced glutathione). Drawbacks include the presence of hydroxyethyl starch (HES) as oncotic support, which has been associated with high blood viscosity and consequent tissue saturation with the preservation solution. As a result, washout of blood from the graft and blood flow during reperfusion may be reduced [146, 147]. In addition, the high K+ concentration is associated with cellular depolarization and activation of voltage-dependent channels [148]. The problems caused by HES and K+ led to the development of other preservation solutions without oncotic agents such as Celsior and HTK (Custodiol) and others with polyethylene glycol (PEG) as oncotic agent, such as Institute George Lopez solution (IGL-1) and Tissue and Organ Conservation Solution (SCOT).

Celsior was developed initially in the 1990s as a cardiac preservation solution with a low potassium and high sodium composition. Due to its extracellular composition, Celsior was also adopted for the preservation of abdominal organs as an alternative to UW. Other solutions without oncotic agents such as histidine-tryptophan-ketoglutarate solution (HTK) were also developed. HTK presents low viscosity and for this reason provides more rapid cooling and better washout of blood elements during organ procurement than UW. Celsior and HTK solutions have been extensively used for liver transplantation [149-151]. However, some limitations for HTK use have recently been reported. Stewart et al reported that HTK is associated with reduced graft survival in

case of additional risk factors such as DCD, cold ischemia time over 8 hours, and donors over 70 years when compared to UW solution [152].

In IGL-1 preservation solution, HES was substituted by a PEG with a molecular weight of 35 KDa (PEG 35), and the high K^+/ low Na^+ ratio was reversed. Both experimental [68, 153] and clinical [154-156] studies of liver and kidney transplantation have shown the beneficial effects of IGL-1 against apoptosis, endoplasmic reticulum stress, microcirculation dysfunction and immune response. Moreover, in previous studies of cold preservation and *ex vivo* perfusion, we have reported that IGL-1 contributes to a more efficient preservation of both non-steatotic and steatotic rat liver grafts compared to UW [83, 157, 158], The beneficial effects of IGL-1 include prevention of hepatic damage, oxidative stress and mitochondrial injury, and are mediated through nitric oxide (NO) production. So, IGL-1 is the first solution reported to be advantageous in SCS of suboptimal livers. Also, SCOT preservation solution which contain PEG 20 and low K^+/high Na^+ concentrations was reported to show a higher renal protection against the immune response, mainly due to the "immunocamouflage" process provided by PEG20 [159].

5.3.2 Dynamic preservation: Machine perfusion technique

For standard liver grafts, SCS with different preservation solutions remains highly successful. However, with the increasing need for organs in recent years, the use of novel techniques for optimizing suboptimal graft preservation is arousing interest.

Machine perfusion (MP) consists of creating a controlled recirculating flow of preservation solution through the organ using a pump. This continuous perfusion permits better penetration of the preservation solution, a thorough washout of blood and equilibration of the interstitium with the perfusate medium, delivery of oxygen and nutrients (if the perfusate is oxygenated), and removal of toxic metabolites (when the perfusate is renewed or filtered). In addition, it allows real-time monitoring of the functional and biochemical performance of the graft and the provision of metabolic support during preservation [160].

Unlike the kidney, the MP protocol for the liver is determined mainly by the temperature of preservation: hypothermic (HMP) at 4ºC, normothermic (NMP) at 37ºC

and subnormothermic (SNMP) at 20ºC-25ºC. Also, several flows and pressures (pulsatile or not), single or dual perfusion (hepatic artery and portal vein), oxygenation or non-oxygenation, and different MP solution compositions have been tested in various liver graft experimental models [161].

Hypothermic Machine Perfusion (HMP)

HMP is a dynamic cold preservation method at 4ºC which ensures homogeneous and continuous supply of metabolic substrates to the graft during the *ex vivo* period [162]. This procedure is designed to overcome or reverse the injuries due to the non-controlled warm ischemic period or the hypothermia itself. During HMP, aerobic metabolism decreases but does not stop completely and the provision of metabolic substrates allows the reduction of the cellular insults seen during reperfusion.

HMP offers several advantages over SCS. Guarrera *et al*. were the first to compare HMP to SCS in human liver transplantation, and showed that HMP improves graft function and attenuates classical biochemical markers of liver preservation injury [163]. Given the fact that ROS accumulation during ischemia can lead to significant hepatocyte toxicity, HMP has been shown to protect the rodent liver from ROS by a reduction in glutathione depletion and superoxide anion release when compared with SCS [164]. And in the case of suboptimal livers, Bessems *et al*. showed that HMP improved both hepatocellular and endothelial function while reducing damage in a diet-induced rat fatty liver model [165].

In contrast to the kidney, in which successful HMP does not necessarily depend upon oxygenation, oxygenated HMP (HOPE) has been developed as a means of improving the quality of liver preservation in normal or ECD livers [166]. Oxygenated preservation enables grafts to restore tissue homeostasis and to maintain the functional integrity of hepatocytes during ischemia. In a recent study, Schlegel *et al.* also described a protective effect on the rodent biliary system using HMP in DCD grafts that underwent transplantation [167]. As expected, perfusion with the HOPE system decreased the parameters of hepatocellular injury and lowered immunogenic upregulation.

Subnormothermic machine perfusion (SNMP)

Recently it has been suggested that the use of SNMP systems may be suitable for *ex vivo* preservation and recovery of human liver for transplantation. SNMP is an intermediate status for graft conservation, using sub-thermic conditions (20–25 °C), taking advantage of the lower metabolic demand in sub-physiological temperature conditions, while still maintaining sufficient metabolism for viability testing and improvement of graft function. SNMP has already proven advantageous in reducing markers of biliary injury during preservation and in restoring normal biliary physiology [168]. A recent study by Bruinsma *et al.* is the first demonstration of the capacity of SNMP to sustain human livers. This group showed that SNMP effectively supports the human liver *ex vivo* with minimal injury, and normalizes physiological disturbances post-ischemia [169].

Normothermic Machine Perfusion (NMP)

The principle of normothermic perfusion is the maintenance of normal cellular metabolism in a physiological environment throughout the preservation period by maintaining normal temperature (37ºC) and providing oxygen and essential substrates [170]. This ensures large-scale metabolic activity and the maintenance of energy reserves such as ATP content. NMP has the advantage of allowing viability assessment prior to transplantation. As the liver metabolism is maintained during preservation, markers including bile production and liver enzymes can be measured.

NMP is an emerging technology whose potential in liver preservation has been described in several animal studies, which have shown its superiority over SCS in the preservation of liver grafts [170-172]. Interestingly, porcine and murine models of DCD livers are significantly improved by NMP compared to organs preserved by SCS [173, 174].

Recently, Kavikumar *et al.* reported the first clinical trial of transplanted livers with NMP [175]. Their study included 10 transplanted patients with relatively low risk donors and recipients, and showed that NMP is safe and feasible in human applications. This study opens up new avenues for research into liver graft preservation with NMP.

Recently, NMP has emerged as a novel tool for decreasing steatosis in a process named “defatting”. In a preliminary study using porcine livers, *ex vivo* normothermic perfusion

for 48 h led to a 50% reduction in lipid droplet size in perivenous hepatocytes, reaching the size found in control lean livers [176]. Moreover, NMP of steatotic livers from Zucker Ob rats using a "defatting cocktail" decreased the intracellular lipid content by 50% over 3 h of perfusion [177]. Decreasing steatosis prior to transplantation by short term NMP would allow the transplantation of severely steatotic livers and thus alleviate the donor liver shortage.

5.4 Graft wash out

After cold storage, preserved liver grafts need to be washed out before reperfusion to remove preservation solution and metabolic waste in order to ameliorate transplant revascularization and viability after transplantation. The washout step seems to be neglected in experimental research and washout solutions were not investigated as well as preservation solution. In clinical practice, surgeons usually use Ringer lactate solution (RLS) or albumin based solution (5% human albumin). Although RLS has a simple composition and was not designed to reduce reperfusion injury, it has been shown to be effective in liver transplantation [178]. Adam et al uses albumin rinse solution at Paul Brusse Hospital; in a prospective randomized study, they have shown that serum albumin flush solution preserved better liver grafts than RLS [179].

Since 1991, Gao *et al.* developed Carolina rinse solution which has been designed to prevent reperfusion injury in liver transplantation. Carolina rinse solution contains extracellular inorganic ions similar to RLS, adenosine, a calcium channel blocker, antioxidant and radical scavengers (allopurinol, gluthatione and desferrioxamine). Carolina rinse solution also contains fructose and mildly acidotic pH to reduce hypoxic cell death. Adenosine and mildly acidotic pH were identified as key components. It has been shown that the simple use of Carolina rinse solution enhance survival significantly after rat liver transplantation when compared to RLS. Carolina rinse diminished postoperative sinusoidal endothelial cell damage and reduced Kupffer cells activation significantly. Also, Carolina rinse solution diminished graft swelling, improved hepatic microcirculation and reduced postoperative elevation in serum enzyme 2 to 3 folds [180-182].

6 Polyethylene glycol in ischemia reperfusion injury

6.1 Structure

PEG are water-soluble polymer formed by a process of linking repeating units of ethylene glycol to form polymers with linear or branched shapes of different molecular weight (Figure 4) [183]. The FDA has approved the use of PEG as a vehicle or a base in foods, cosmetic and pharmaceuticals, including injectable, topical, rectal and nasal formulations [184]. PEG is non immunogenic, shows little toxicity and is eliminated from the body intact by either the kidneys (for PEGs < 30 kDa) or in the faeces (for PEGs > 20 kDa) [1]. Because of the high flexibility, hydrophilicity, and large number of water molecules integrated into its chains, PEG presents a hydrodynamic volume greater than would be expected from its molecular weight and has high protein-rejecting properties [184, 185]. PEG has an apparent molecular weight 5–10 times higher than corresponding soluble protein of similar molecular mass, as shown by gel permeation chromatography [184].

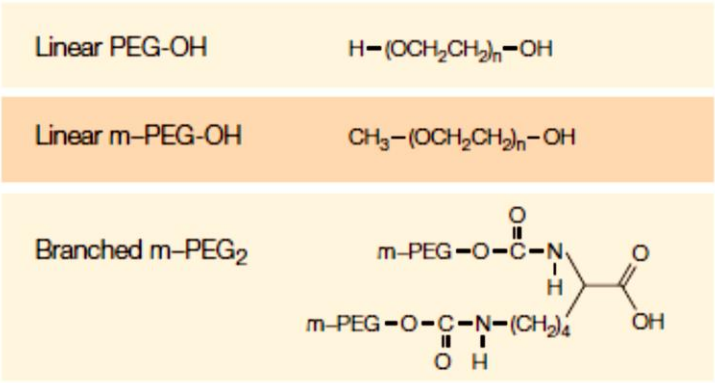

Figure 7 Structural formulae of polyethylene glycol [184]
(m-PEG, monomethoxy-PEG)

6.2 The effects of PEG

In general, PEGs prevent the generation of reactive oxygen species (ROS) [186, 187], enhance cell survival pathways in hypoxia/reoxygenation conditions [6], protected injured mitochondria [2] and repair endothelial cell damage during post-ischemic reperfusion [6, 188]. PEG exerts its cytoprotective role through the restoration of membrane integrity [6, 145, 186, 189] or by entering the cell through the disrupted

membranes and interacting with cellular organelles [3]. In hypothermic hepatocyte preservation, PEG8 (8kDa) prevented cell swelling through a mechanism that was independent of its osmotic properties [144]. Also, PEG used as cryoprotectant in supercooling technique was necessary to achieve successful liver transplantation [24]. Moreover, PEG suppressed hypothermic-induced cell swelling in hepatocyte preservation [187] and protected primary hepatocyte during supercooling preservation [190]. As a colloid, PEG changes the way proteins interact with each other and with water in the glycocalyx and it has been reported that PEG-lipids mimic the inhibiting effect of the cell glycocalyx on adhesion [191].

6.2.1 PEG as additive to preservation solution

PEG has been added to preservation solutions as a colloid agent to prevent cell swelling. However, it seems that PEG benefits are mediated by others mechanisms in addition to its oncotic properties. IGL-1 solution, which contain PEG 35 at 1g/L has been shown to be superior to UW solution in many experimental studies and was proposed as an effective alternative in steatotic liver preservation [158]. The beneficial effects of IGL-1 include prevention of hepatic damage, ERS, oxidative stress, mitochondrial injury, microcirculation dysfunction and immune response [192]. Knowing that the main difference between IGL-1 and UW preservation solution is the substitution of the colloid HES by PEG 35, we can speculate that all these protective effects are the result of PEG 35 supplementation.

Moreover, a PEG of smaller size (PEG20), is the basic component SCOT preservation solution which have been shown to be effective in kidney preservation and provides a higher renal protection against the immune response, mainly through "immunocamouflage" process provided by PEG20 [159]. Moreover, PEG20 at 15 g/L in SCOT solution has been found to reduce alloantigen recognition after liver reperfusion in comparison to UW solution [193].

Even so, the use of PEG 35 as oncotic agent has been shown to be more effective than PEG20 for liver graft preservation [194]. However, in kidney model of transplantation in pigs, it has been shown that PEG of 20 kDa was more effective than PEG 35 in ameliorating glomerular function and preventing histological injuries [195]. The authors conclude that PEG 35 act by a concentration dependent manner and high concentration

of PEG 35 of 15 or 30 g/L induces cellular damage [195]. This could be explained by the high viscosity and the lower clearance of PEG 35 in these storage solutions.

6.2.2 PEG as additive to machine perfusion liquid

PEG has been also used in MP solutions. Bessems y al have shown that substitution of HES in Polysol perfusion solution by PEG resulted in equal or better function and less damage in rat liver after 24h of HMP . Moreover, Polysol-PEG solution was more efficient than UW-Gluconate perfusion solution [196]. More recently, it has been shown that PEG 35 addition to SNMP at 5g/L in supercooling technique was necessary to achieve successful transplantation after 6 days preservation [190]. On the other hand, in a model of kidney auto transplantation in pigs after 60 min of warm ischemia and 24h of HMP, it has been shown that the use of SCOT solution which contain PEG20 at 15g/L result in more damage when compared to the two reference perfusion solutions for kidney: UW-M and KPS [195].

6.2.3 PEG as preconditioning agent

PEG is not absorbed by gastrointestinal tract, so its use was limited to its addition to preservation or perfusion solutions. Recently, it has been shown that intravenous delivery of 10% PEG 15–20 prior to reperfusion following 60 minutes of ischemia by left anterior descending artery (LAD) occlusion in rats, resulted in significant recovery of left ventricular ejection fraction, markedly less myocardial apoptosis and inhibition of ventricular fibrosis and adverse remodeling. PEG treatment led to the upregulation of myocardial prosurvival signaling pathways such as Akt, ERK1/2, GSK-3β, and eNOS [197]. PEG use as a method of pharmacological preconditioning may be a very promising strategy in clinical situation of IRI.

II. OBJECTIVES

Hepatic IRI is an inevitable clinical problem for liver surgery. PEGs are water soluble nontoxic polymers that have been extensively used in biomedical applications and proved their effectiveness in preventing tissue injuries.

In this sense, the main objective of this thesis was to study the potential contribution of PEG to liver graft protection against IRI.

For that purpose, we established the following specific objectives:

1- To investigate whether the intravenous administration of a high molecular weight PEG of 35 kDa (PEG 35) could be an effective strategy for assessing rat liver preconditioning against warm IRI.

2- To evaluate the potential protective effects of intravenous administration of PEG 35 in steatotic livers subjected to cold ischemia reperfusion.

3- To determine whether a new rinse solution containing PEG 35 could prevent reperfusion injury in liver grafts.

III. INFORME DEL DIRECTOR DE TESIS

El doctor Joan Roselló-Catafau, com a director de la tesis doctoral presentada pel Sr. Mohamed Bejaoui, fe constar que el doctorant ha participat activament en la preparació dels articles presentats en aquesta tesi. El Sr Bejaoui va dur a terme el treball experimental, ha establert els models d'experimentació animal, ha fet el disseny dels experiments en aquests estudis i ha contribuït a l'anàlisi crítica de les dades i resultats. També ha participat en la redacció dels articles:

Titol de l'article: Polyethylene glycol preconditioning: An effective strategy to prevent liver ischemia reperfusion injury`
Autores: Mohamed Bejaoui, Eirini Pantazi, Maria Calvo, Emma Folch-Puy, Anna Serafín, Gianfranco Pasut, Arnau Panisello, René Adam and Joan-Roselló-Catafau
Revista: Enviat a Oxidative Medicine and Cellular Longevity
Factor d'Impacte: 3.516

Titol de l'article: Protective effect of intravenous high-molecular-weight polyethylene glycol on fatty liver preservation
Autores: Mohamed Bejaoui, Eirini Pantazi, Emma Folch-Puy, Arnau Panisello, María Calvo, Gianfranco Pasut, Antoni Rimola, Miquel Navasa, René Adam and Joan Roselló-Catafau
Revista: Biomed Research International, 2015, Article ID 794287, 10 pages
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IV. PUBLICATIONS

POLYETHYLENE GLYCOL PRECONDITIONING: AN EFFECTIVE STRATEGY TO PREVENT LIVER ISCHEMIA REPERFUSION INJURY

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Abstract

Hepatic ischemia reperfusion injury (IRI) is an inevitable clinical problem for liver surgery. Polyethylene glycols (PEGs) are water soluble nontoxic polymers that have proven their effectiveness in various *in-vivo* and *in-vitro* models of tissue injury. The present study aims to investigate whether the intravenous administration of a high molecular weight PEG of 35 kDa (PEG35) could be an effective strategy for rat liver preconditioning against IRI. PEG35 was intravenously administered at 2 and 10 mg/kg to male Sprague Dawley rats. Then, rats were subjected to one hour of partial ischemia (70%) followed by two hours of reperfusion. The results demonstrated that PEG35 injected intravenously at 10 mg/kg protected efficiently rat liver against the deleterious effects of IRI. This was evidenced by the significant decrease in transaminases levels and the better preservation of mitochondrial membrane polarisation. Also, PEG35 preserved hepatocyte morphology as reflected by an increased F-/G-actin ratio and confocal microscopy findings. In addition, PEG35 protective mechanisms were correlated with the activation of the pro-survival kinase Akt and the cytoprotective factor AMPK and the inhibition of apoptosis. Thus, PEG may become a suitable agent to attempt pharmacological preconditioning against hepatic IRI.

Introduction

Ischemia reperfusion injury (IRI) is inherent to surgical procedures such as liver resection and liver transplantation. The deleterious effects caused by IRI are the main cause of graft primary non-function and dysfunction [1]. Many strategies have been developed to protect against IRI such as ischemic preconditioning (IPC) and the use of different drugs. However, these strategies didn't prove their effectiveness in clinical setting and efficient treatments are still lacking.

Polyethylene glycols (PEGs) are water soluble non-toxic polymers with different molecular weights and properties that have been extensively used in numerous applications (cosmetic, foods, pharmacy and biomedicine) [2]. Also, PEGs have been found to exert beneficial effects in various *in-vivo* and *in-vitro* models of tissue injury [3-8]. Recently, it has been demonstrated that intravenous administration of high molecular weight PEG of 20 and 35 kDa protected rat heart against reperfusion injury and steatotic livers against cold IRI, respectively [9, 10]. The protective effects were associated with decreased vascular permeability, decreased oxidative stress and inhibition of cell death [8, 11].

The aim of the present study is to examine the potential benefits of prophylactic intravenous administration of PEG35 in order to prevent warm IRI in rat liver, as well as to investigate the underlying mechanisms.

Meterials and Methods

Animals

Male Sprague-Dawley rats (250-300 g) were purchased from Charles River (France) and housed in a temperature and humidity controlled room under a constant 12-hour light/dark cycle. Animals had free access to water ad libitum and rat chow (standard laboratory pelleted formula A04, Panlab, Barcelona, Spain). This study was performed in accordance with European Union directive 2010/63/EU for animal experiments and approved by the Ethics Committees for Animal Experimentation of the University of Barcelona (No. 696/14).

Surgical Procedure

All the procedure was performed under isofluorane inhalation (induction dose of 5 % and maintenance dose of 1.5-2 %). Also, analgesia was applied before surgery by subcutaneous injection of buprenorphine at the dose of 0.05 mg/kg. After laparotomy, ischemia was induced by occlusion of the hepatic artery and portal vein of the left and median lobes using an atraumatic micro vascular clip (70% ischemia). After one hour of ischemia, liver reperfusion was established by removal of the clamp and the abdomen was sutured. Then, rats were kept in clean cages with free access to water and standard rodent chow. After 2h of reperfusion, animals were sacrificed by cervical dislocation under isoflurane anaesthesia for blood and tissue collection. Sham operated rats underwent the same procedure without vascular clamping.

Drug Treatment:

PEG 35 was kindly provided by Institute Georges Lopez (IGL). PEG 35 was dissolved in phosphate buffer saline (PBS) and administrated 10 min before liver ischemia by

intravenous bolus via the penile vein at the concentration of 2 mg/kg or 10 mg/kg using PEG 35 solution of 1g/L and 5g/L, respectively. For intravital microscopy study, PEG 35 was fused with fluorescein (PEG-FITC) as previously described by Mero at al [12].

Experimental Groups:

Rats were randomly distributed into four groups as follows:

Group 1: (Control: Ctr, n=6): Midline incision was performed and hepatic pedicle was dissected. Then, 500µL of PBS was injected intravenously via the penile vein and abdomen was sutured. After 2h, rats were sacrificed for blood and sample collection.

Group 2: (IR 2h, n=6): Rats were pre-treated with 500µL of PBS intravenously and then subjected to one hour of ischemia followed by 2h of reperfusion. Then, animals were sacrificed and plasma and liver samples were collected.

Group 3: (PEG 2mg/kg, n=6): Same as group 2 but rats were pre-treated with intravenous administration of PEG35 at the dose of 2 mg per kg body weight.

Group 4: (PEG 10mg/kg, n=6): Same as group 2 but rats were pre-treated with intravenous administration of PEG35 at the dose of 10 mg per kg body.

Biochemical determinations:

Hepatic injury

Plasma levels of alanine aminotransferase (AST) and aspartate amino transferase (ALT) were measured using a commercial kit from RAL (Barcelona, Spain) according to manufacturer's protocol.

Glutamate dehydrogenase activity

GLDH activity was determined using a commercial kit (GLDH, Randox laboratories Ltd., Crumlin, UK) by quantifying the decrease in absorbance at 340 nm according to the manufacturer's protocol.

Determination of Nitrites and Nitrates

Nitric oxide levels were measured as nitrate plus nitrite (NOx) in tissue samples using a commercial colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Western blot analysis:

Liver tissue was homogenized in HEPES buffer and 50µg of protein were separated on 6-10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were then incubated overnight at 4°C using the following antibodies: anti-eNOS (BD Transduction Laboratories, Lexington, KY, USA), anti-phosphorylated Akt, anti-total and anti-phosphorylated AMPK (Cell Signaling Technology Inc., Beverly, MA, USA), and anti-β-actin (Sigma Chemical, St. Louis, MO, USA). The corresponding secondary antibody was then added for 1 hour at room temperature and membranes were developed using the enhanced chemiluminescence reagents from Avision (Advansta, Menlo Park, CA, USA). Signals were quantified by scanning densitometry using the Quantity One software for images analysis. Results were expressed as densitometric ratio between the protein of interest and the correspondent control (β-actin, total AMPK, total Akt).

Histology:

Formalin-fixed paraffin-embedded liver tissues were cut in 5µm sections and stained with hematoxylin and eosin according to standard procedures. Images were analysed by an independent investigator in blind manner.

F/G-actin ratio measurements:

To analyse the levels of F-actin and G-actin, liver samples were homogenized with PHEM buffer (60 mM Pipes, 20 mM HEPES, 10 mM EGTA, 2 mM MgCl2, 1% Triton-X100, pH 7.0) and ultra-centrifuged (48.000 rcf) at 4°C for 5min to separate both fractions. Supernatant, containing G-actin, was collected; the F-actin pellet was washed twice with cold PHEM buffer and then dissolved in 1x SDS sample buffer. Equivalent amounts of proteins were separated by 10% SDS-PAGE, and F-actin and G-actin were determined by western blot and quantified by scanning densitometry. The F/G-actin ratio was calculated.

Intravital microscopy

Rats were anesthetized with isofluorane inhalation, laparotomized and putted in a prone position over a cover slip mounted on the stage of a Leica TCS SP5 resonant scan multiphoton confocal microscope (Leica Microsystems Heidelberg GmbH) equipped with an incubation system with temperature control, a HCX IR APO L 25× water immersion objective (Numerical Aperture 0.95), resonant scanner at 8000 lines/s and a near infrared Titanium:Saphire laser (MaiTai, SpectraPhysics) for two photon excitation running at 800 nm.

The following vital dyes were injected intravenously as indicated: Hoechst 33342 trihydrochloride (12mg/Kg, Invitrogen, H3570) for DNA-Nuclei staining; Rhodamine 123 (0.11mg/Kg, Sigma, R8004) for mitochondrial membrane potential dye, Evans Blue (20 mg/Kg, Sigma, E2129-10), a bulk fluid-phase albumin marker that enhances contrast of plasma and PEG35 conjugated with fluorescein isothiocyanate (PEG-FITC). Images were acquired with resonant scan at 8000 lines/second.

Confocal fluorescence microscopy:

Liver was fixed, cryoprotected with sucrose, embedded in OCT and frozen on a cooper plate on dry ice. Ten-micrometer cryosections were cut in a cryostat and post fixed in 4% buffered paraformaldehyde for 10 min and then permeabilized with PBS containing 0.1% Triton X-100 and 1% BSA for 30 min. For actin visualization, the slides were incubated with TRITC-phalloidin (dilution 2 μM, Sigma) in PBS with 1% BSA and 0.2% Triton X-100 for 30 min. Slides were washed three times for 15 min with PBS. The last PBS wash included Hoechst 33342 (dilution 1 mM, Invitrogen). Finally, cryosections were mounted using Mowiol (Calbiochem). Confocal images were acquired with a Leica TCS SP5 laser scanning microscope. Hoechst-33342 and Phalloidin-A555 images were acquired sequentially using 405 and 561 nm laser lines. The confocal pinhole was set at 1 airy unit and when 3D reconstruction was required stacks of images every 0.3mm were acquired. The hepatocytes size (in μm2) and circularity as (4π×Area)/Perimeter2 (based on Phalloidin staining) were quantified on ImageJ. The red channel (phalloidin-A555 staining) was processed to segment hepatocytes. Hepatocytes were selected and size and circularity was measured (in 1.5 mm2 of each sample). A value of 1.0 indicated a perfect circle; as the value approached 0.0, indicated a more polyhedral shape.

Statistical analysis:

Data are expressed as means ± standard error and were compared statistically by the one-way analysis of variance, followed by the Tukey test (Graph Pad Prism software). $P < 0.05$ was considered significant.

Results

In order to evaluate the effect of PEG35 in liver IRI, we firstly determined the liver damage through transaminases levels and hepatic histology after 1 hour of ischemia followed by 2 hours of reperfusion. As shown in Figure 1A and B, IR group led to significant increases in transaminases levels compared to control group, which was prevented when rats were pretreated with intravenous injection of PEG35 at 10 mg/kg. By contrast, no significant differences were observed when PEG35 at 2 mg/kg was administered. Histological findings shown in Figure 1C were in accordance with liver injury parameters. Animals subjected to IR showed extensive areas of coagulative hepatic necrosis with disruption of hepatic cords and haemorrhage randomly distributed throughout the hepatic parenchyma. PEG at 10mg/kg reduced the extent and the number of necrotic areas.

It is well known that hepatic damage after reperfusion is associated with mitochondrial alterations. For this reason, we measured GLDH activity to assess mitochondrial injury after two hours of reperfusion. As indicated in Figure 2A, the increase in GLDH levels observed in ischemic group was prevented in PEG 10 mg/kg group. No changes were observed in PEG 10 mg/kg versus control. By contrast, pretreatment with PEG35 at 2 mg/kg was not sufficient to protect mitochondria against IRI. Also, we performed intravital multiphoton microscopy in living rats at one hour of reperfusion to evaluate mitochondrial polarization status using Rh123 (Figure 2B). In the livers of control rats, bright punctate Rh123 fluorescence was observed representing cells with polarized mitochondria. However, livers from ischemic rats showed a dimmer diffuse cytosolic fluorescence indicating mitochondrial depolarization. Importantly, when rats were pretreated with PEG35 at 10 mg/kg, mitochondrial depolarization was lessened after ischemia and totally reverted after reperfusion.

Next, we investigated the potential signalling mechanisms involved in the beneficial effects of PEG35 pretreatment. In this sense, it has been reported that protein kinase B (Akt) is a pro-survival protein that decreases apoptosis in models of IRI [13]. Also, it is well known that AMPK is a cellular metabolic sensor that switches the cell to an energy conserving status under ischemic conditions [14]. Our results show that PEG35 administration at the dose of 10 mg/kg induced a significant activation of both Akt and AMPK (Figure 3A and 3B, respectively).

Both AMPK and Akt have been shown to activate endothelial nitric oxide synthase (eNOS) [15]. However, no changes in eNOS activation were found when PEG was used (Figure 3C) although a significant increase in nitrites/nitrates levels in liver tissue were observed (Figure 3D).

Given the central role played by apoptosis in liver IRI, we evaluated the effects of PEG35 on hepatic apoptosis by measuring caspases 3 and 9. As shown in Figure 3, PEG 10mg/kg promoted a significant reduction of cleaved caspases 3 and 9. No significant differences were found regarding pretreatment with PEG35 at 2mg/kg although a tendency towards a decrease was observed when compared to IR group (Figure 4).

In addition, we studied the potential PEG effects on cytoskeleton. Confocal microscopy images of F-actin stained with phalloidin showed that, in PEG35 pre-treated livers, filamentous actin associated to the membrane micro-filamentous network and the peri-canalicular band are kept preserved (Figure 5A). Also, morphology of hepatocytes in ischemic livers was compromised after ischemia reperfusion as shown in quantification of hepatocytes size and circularity (Figures 5B and 5C). Increase in size and circularity indicates that hepatocytes have swollen and lost their shape which was less evident in case of PEG treated livers. Moreover, IRI induced an important decrease of F/G-actin ratio, which was significantly prevented when PEG 10mg/kg was administered, as it is

indicated in Figure 4D. Also, it has been reported that activation of p38 leads to cytoskeletal changes by increasing the hepatocyte F-actin content after IRI [16]. For this reason, we investigated whether the cytoskeleton preservation observed with PEG pre-treatment was correlated with changes in p38 phosphorylation status. Indeed, our results showed that PEG preconditioning increased p38 activation (Figure 5E).

Finally, we performed intravital microscopy using PEG-FITC in order to study its localization in liver tissue. We observed that PEG was still present in liver vascular bed after one hour of ischemia and one hour of reperfusion, which is showed in the additional movie file [see Additional file 1].

Discussion

IRI is an important cause of liver damage occurring during surgical procedures including hepatic resection and liver transplantation, and represents the main cause of graft dysfunction and primary non function after transplantation [17]. PEGs are water soluble non-toxic polymers that are known to play an important role in the cytoprotection against ischemic damage. Recent studies have shown that PEG exerts anti-inflammatory, anti-apoptotic, immunosuppressive and membrane stabilization effects [4, 5, 8, 18-20]. From this perspective, it is reasonable to expect that PEG administration may be an effective therapeutic strategy against IRI. In this study, we demonstrate, for the first time, that PEG35 preconditioning protects rat liver against warm IRI.

We have focused our study on PEG with molecular weight of 35kDa because it has been previously demonstrated that PEG35 was effective to prevent cold IRI in liver when it was added to organ preservation solutions [21-23]. Moreover, PEG35 has been

shown to protect renal cells against cold ischemia [24]. Also, we recently evidenced that PEG35 addition to washout solution protected cold stored livers against reperfusion injury [25]. However, PEGs with different molecular weights such as PEG8 [11] or PEG20 [26] might also be useful for conferring protection against IRI.

In order to achieve an efficient hepato-protection, the most suitable concentration of PEG35 was 10 mg/kg. This concentration was well tolerated as control rats injected with PEG35 at 10 mg/kg did not present any liver damage (data not shown).

The beneficial effects induced by PEG35 are mainly associated with the preservation of the mitochondrial status, as revealed by decreases in GLDH levels and intravital microscopy findings. Since mitochondria are sensitive targets for damage during IRI [23, 24], the lessened hepatic injury observed when PEG35 was administered at 10mg/kg coincided with increased mitochondrial preservation.

We next determined whether PEG35 beneficial effect could be related to the activation of protective cell signalling pathways. Our results showed that PEG35 at 10 mg/kg contributes to AMPK and AKT activation. These facts are in line with previous reports showing that PEG20 protects against heart ischemia through AKT activation [7, 8] and PEG35 protects rat liver against reperfusion injury, in part, through AMPK activation [9]. These observations suggest that PEG protective effects are not only related to its known role as an oncotic support but also to its pharmacological properties.

Akt and AMPK activation has been related to apoptosis inhibition in many models of IRI [15, 27, 28]. As it was expected, PEG 35 at 10 mg/kg prevented caspase 3 and caspase 9 activation. These observations are in line with results observed by Malhotra *et al.* who demonstrated that PEG15-20 protected cardiac myocytes from hypoxia and re-oxygenation induced apoptosis [8]. Moreover, we have previously reported that PEG35

addition to preservation solutions acts as an oncotic agent ameliorating organ graft preservation by reducing apoptosis in rat liver transplantation [29].

Nitric oxide (NO) is a gaseous vasodilator implicated in the regulation of hepatic microcirculation, which is impaired upon IRI [25]. In this sense, we found that PEG35 at 10 mg/kg significantly increased NOx levels and this was not correlated with eNOS activation. This observation is concomitant with a previous published report showing that PEG induced arteriolar dilatation which was not correlated to eNOS activation [26]. However, we have recently evidenced that the benefits of PEG in IRI were associated with closely e-NOS activation [8, 9]. Thus, more investigations are needed to elucidate the precise mechanisms of NO generation mediated by PEG.

Structural alterations of the cytoskeleton following ischemia reperfusion have been reported to cause disturbances of intracellular transport processes, cell motility and microcirculation leading to organ dysfunction [30-33]. In liver cells, F-actin is a relevant component of liver cytoskeleton which forms microfilaments involved in intracellular transport processes, such as exocytosis and endocytosis, maintenance of cell shape, and canalicular motility responsible for bile flow [25, 32, 34, 35]. In this context, we have explored whether PEG35 pretreatment could maintain the cytoskeleton structure and preserve the morphological characteristics of hepatocytes. Indeed, our present data confirmed that F-actin/G-actin ratio is increased as a consequence of PEG administration at 10 mg/kg. Furthermore, confocal microscopy findings confirmed that PEG contributes to the regulation of endothelial cell barrier by rearranging the actin cytoskeleton. Hepatocytes presented a more normal hexagonal morphology in livers pretreated with PEG35 compared with livers submitted to IRI. All of these observations are consistent with a recent study in lung endothelial cells evidencing that PEG15-20 preserves the architecture of the endothelial cytoskeleton [36]. Moreover, it has been

demonstrated that PEG induced membrane stabilization through sarcolemmal lipid-raft architecture preservation [8]. These published data suggested that PEG interaction with cell membrane (adhesion or intercalation) preserved the cytoskeleton. In our study we further evidenced that PEG-induced-p38 MAPK activation may also be responsible for cytoskeleton preservation. However, the precise mechanisms of how PEG could affect cytoskeleton remain to be elucidated.

Currently, the proposed strategies against IRI rely on surgical procedures such as IPC, or on the use of pharmacological agents (pharmacologic preconditioning) [37]. IPC is a well-established technique that consists on the application of brief episodes of ischemia and reperfusion which cause protection against the subsequent prolonged ischemic insult [38]. However, this manipulation is not tolerated in most operation rooms. Pharmacologic preconditioning consist on the administration of drugs that block injurious pathways directly, or trigger endogenous protective mechanisms [37]. Although most of these drugs were effective in reducing IRI in many experimental models, studies that evaluate their efficacy in the clinical settings are still lacking. Moreover, their benefits are limited to the specific drug activity and their potential adverse effects. Compared to IPC and pharmacologic preconditioning, PEG present the advantageous of being safe and multi-target drug. Indeed, PEG effects are associated with the majority of the events occurring during IRI such as oxidative stress, mitochondrial preservation, cytoskeleton protection and the induction of prosurvival and cytoprotective signaling pathways.

Conclusions:

In conclusion, the present work evidences that intravenous administration of PEG35 is a useful tool for liver preconditioning against the deleterious effects of IRI. Based on these findings, PEG35 could be useful in clinical settings.

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FIGURES LEGEND

Figure 1: Hepatic injury after ischemia-reperfusion. PEG35 at 10 mg/kg decreases AST (A) and ALT (B) levels and the number of necrotic areas as shown by histological findings (eosin/hematoxylin staining) (C). Data represent mean ± SEM. $^{*}p<0.05$ vs Ctr, $^{\#}p<0.05$ vs IR 2h, +p<0.05 vs PEG 2mg/kg

Figure 2: Mitochondrial damage after liver ischemia reperfusion. PEG35 at 10 mg/kg decreases glutamate dehydrogenase (GLDH) levels (A) and preserve mitochondrial polarization status (B) (mitochondrial membrane potential dye Rhodamine123 (green color), the nuclei dye Hoechst (blue color) and the plasma albumin dye Envans blue (red color)). Data represent mean ± SEM. $^{*}p<0.05$ vs Ctr, $^{\#}p<0.05$ vs IR 2h.

Figure 3: Effect of PEG35 on Akt, AMPK, eNOS activation and NO generation. PEG 35 preconditioning at 10 mg/kg enhances AMPK and Akt activation and increase nitrite/nitrate levels without any effect in eNOS. Western blot and densitometric analysis of phosphorylated Akt / b-actin (A) phosphorylated AMPK / total AMPK (B) eNOS / b-actin (C) and biochemical determination of Nitrite+Nitrate levels in liver tissue (D). Data represent mean ± SEM. $^{*}p<0.05$ vs Ctr, $^{\#}p<0.05$ vs IR 2h.

Figure 4: Effect of PEG35 on liver apoptosis after ischemia reperfusion. PEG35 at 10 mg/kg reduced the levels of apoptotic proteins Caspase 3 and 9. Western blot and densitometric analysis of Cleaved Caspase 3 / pro-caspase 3 (A) and Cleaved Caspase 9 / pro-caspase 9 (B). Data represent mean ± SEM. #p<0.05 vs IR 2h, +p<0.05 vs PEG 2mg/kg

Figure 5: PEG35 preconditioning contribution on cytoskeleton preservation. Images of confocal microscopy show that, in PEG 35 pretreated livers, filamentous actins (red) and hepatocytes morphology were better preserved when compared with

non-treated ones. Also, PEG 35 pretreatment at 10 mg/kg enhance significantly F/G-actin ratio and phospo-p38 protein levels. Confocal microscopy for F-actin (A), determination of hepatocyte size (B) and hepatocyte circularity (C) and western blot and densitometric analysis of F-actin/G-actin (D) and phosphorylated p38 (E). Data represent mean ± SEM. $^{*}p<0.05$ vs Ctr, $^{\#}p<0.05$ vs IR 2h, $+p<0.05$ vs PEG 2mg/kg.

Additional File 1: Rats were treated intravenously with fluorescent PEG35 (PEG-FITC) and then submitted to 1 hour of hepatic ischemia followed by 1 hour of reperfusion. The confocal microscopy findings after reperfusion confirmed the presence of PEG in the liver vascular system.

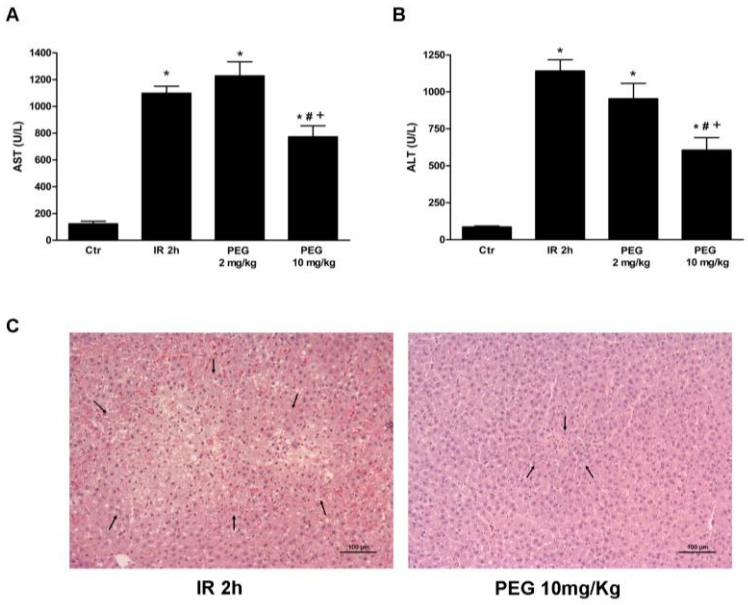

Figure 1

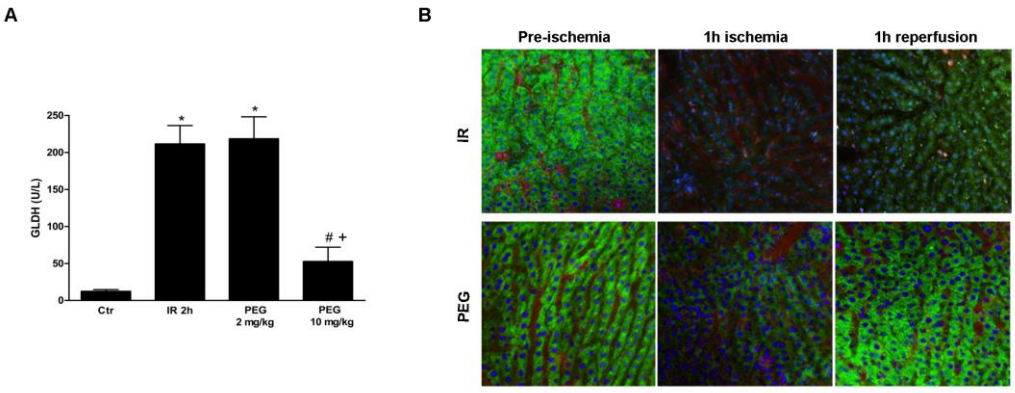

Figure 2

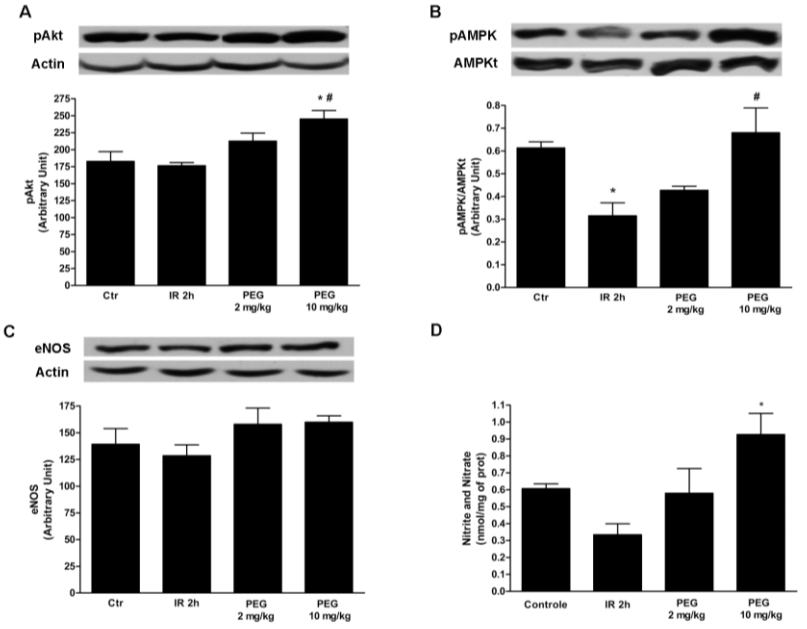

Figure 3

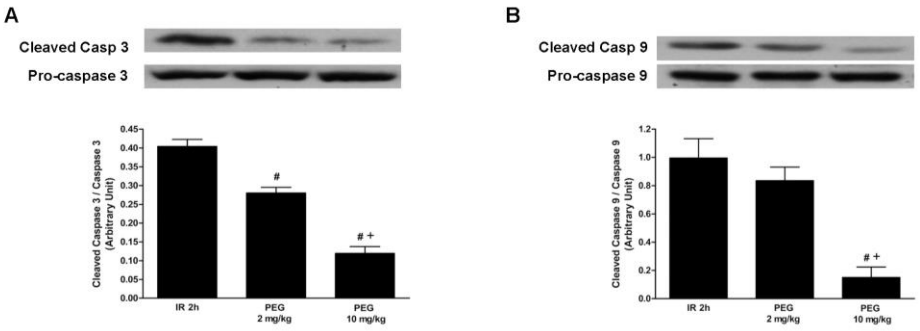

Figure 4

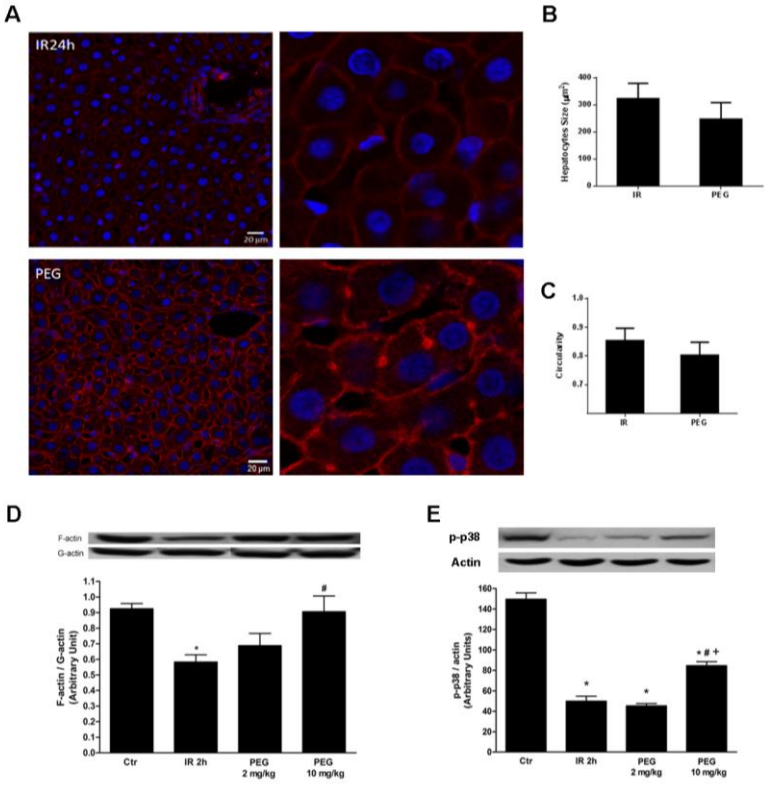
A
IR24h
20 μm
PEG
20 μm
B
Hepatocytes Size (μm²)
400
300
200
100
0
IR
PEG
C
Circularity
1.0
0.9
0.8
0.7
IR
PEG
D
F-actin
G-actin
F-actin / G-actin (Arbitrary Unit)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
#
Ctr
IR 2h
PEG 2 mg/kg
PEG 10 mg/kg
E
p-p38
Actin
p-p38 / actin (Arbitrary Units)
160
140
120
100
80
60
40
20
0
*
*
*#+
Ctr
IR 2h
PEG 2 mg/kg
PEG 10 mg/kg

Figure 5

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Research Article

Protective Effect of Intravenous High Molecular Weight Polyethylene Glycol on Fatty Liver Preservation

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Ischemia reperfusion injury (IRI) leads to significant tissue damage in liver surgery. Polyethylene glycols (PEGs) are water soluble nontoxic polymers that have proved their effectiveness against IRI. The objective of our study was to investigate the potential protective effects of intravenous administration of a high molecular weight PEG of 35 kDa (PEG 35) in steatotic livers subjected to cold ischemia reperfusion. In this study, we used isolated perfused rat liver model to assess the effects of PEG 35 intravenous administration after prolonged cold ischemia (24 h, 4°C) and after reperfusion (2 h, 37°C). Liver injury was measured by transaminases levels and mitochondrial damage was determined by confocal microscopy assessing mitochondrial polarization (after cold storage) and by measuring glutamate dehydrogenase activity (after reperfusion). Also, cell signaling pathways involved in the physiopathology of IRI were assessed by western blot technique. Our results show that intravenous administration of PEG 35 at 10mg/kg ameliorated liver injury and protected the mitochondria. Moreover, PEG 35 administration induced a significant phosphorylation of prosurvival protein kinase B (Akt) and activation of cytoprotective factors e-NOS and AMPK. In conclusion, intravenous PEG 35 efficiently protects steatotic livers exposed to cold IRI.

1. Introduction

Organ preservation is a fundamental requirement in organ transplantation; it preserves the viability of the organ during its transport from the donor to the recipient so that the graft can maintain its function after transplantation [1]. Besides advances in organ preservation, the presence of steatosis remains a limiting factor for the suitable preservation of liver grafts, as steatotic livers are particularly vulnerable to hepatic ischemia reperfusion injury (IRI) [2]. Their use is accompanied by increased risk of primary failure and lowered success of liver transplantation [3]. Currently, the increasing needs of transplantation as well as the scarce of donors pool have obliged the physicians to take advantage of suboptimal liver grafts, as steatotic ones [4]. For this reason, there is an urgent need to explore new strategies that provide a more efficient preservation of steatotic liver grafts. Minimizing the deleterious effects of hypothermia could decrease the reperfusion injury and, consequently, assure an increased rate of graft survival after transplantation.

Polyethylene glycols (PEGs) are water soluble nontoxic polymers that have been employed in many biomedical applications such as gastrointestinal disorders and drugs pegylation [5, 6]. Besides their usefulness as oncotic agents in preservation solutions [7, 8], it has been shown that PEGs molecules protect against cold injury and ischemic

damage. Indeed, PEG used as cryoprotectant in supercooling technique was necessary to achieve successful liver transplantation [9]. Moreover, PEG suppressed hypothermic-induced cell swelling in hepatocyte preservation [10] and protected primary hepatocyte during supercooling preservation [11]. Also, PEG protected cardiac myocytes from hypoxia and reoxygenation-induced cell death [12], decreased oxidative stress [13], and protected injured mitochondria [14].

With this in mind, we hypothesized whether intravenous administration of PEG in the rat prior to organ procurement could protect fatty liver graft against hypothermic and hypoxic damage occurring during preservation and the subsequent reperfusion injury. Our results demonstrated that PEG 35 prevented the deleterious effects of cold IRI when administered intravenously in obese rats.

2. Materials and Methods

2.1. Animals. Male homozygous obese Zucker rats, aged 9 to 10 weeks, were purchased from Charles River (France) and housed at 22°C with free access to water and standard chow. All experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 697/14), University of Barcelona, and were conducted according to European Union regulations for animal experiments (Directive 86/609 CEE).

2.2. Liver Procurement and Ex Vivo Perfusion. All procedures were performed under isoflurane anesthesia inhalation. After laparotomy, the common bile duct was cannulated and livers were flushed with 40 mL of chilled UW preservation solution (4°C) by the mean of catheter insertion into the aorta. After cooling, a second catheter was inserted into the portal vein to complete liver rinsing with further 10 mL of UW solution. The whole liver was then excised and preserved at 4°C for 24 h in the same solution. This procedure implicates the death of the animal under isoflurane anesthesia, and thus the application of analgesia or euthanasia was unnecessary. After 24 h of cold preservation, steatotic livers were removed from preserved solution and flushed at room temperature with 20 mL of Ringer Lactate solution to eliminate the metabolite waste accumulated during liver storage. Then, livers were perfused at 37°C via the portal vein in a closed and controlled pressure circuit. Time point 0 was considered when the portal catheter was satisfactorily connected to the circuit. During the first 15 minutes of perfusion (initial equilibration period), the flow was progressively increased in order to stabilize the portal pressure at 12mmHg (Pression Monitor BP-1; Pression Instruments, Sarasota, FL). The flow was controlled by a peristaltic pump (Minipuls 3; Gilson, France). The reperfusion liquid (150 mL for each perfusion) consisted of a cell culture medium (William's medium E; BioWhittaker, Barcelona, Spain) with a Krebs-Henseleit-like electrolyte composition enriched with 5% albumin as oncotic supply. The medium was continuously gassed with 95% O_2 and 5% CO2 gas mixture and subsequently passed through a heat exchanger (37°C) and a bubble trap prior to entering the liver. After 120 minutes of normothermic reperfusion, the effluent perfusion fluid was collected for biochemical determinations and hepatic tissues were sampled and stored at −80°C for further analysis.

2.3. Drug Treatment. PEG 35 was kindly provided by IGL-1 Company. PEG 35 was dissolved in physiological saline (5 g/L) and administrated 10 min before liver procurement by intravenous bolus through the penile vein at the concentration of 10 mg/kg.

For confocal microscopy study with PEG-FITC, PEG 35 was fused with fluorescein as previously described by Mero et al. [15].

2.4. Experimental Groups. All animals were randomly distributed into different experimental groups, as indicated below (Scheme 1).

Protocol 1: Effect of PEG 35 in Fatty Livers after Cold Storage. In order to study the effects of PEG 35 administration in cold preservation, rats were randomly divided into the following groups:

(1) Group 1: Control 1 (Ctr 1) ($n = 4$): control livers were flushed via the portal vein with Ringer's lactate solution immediately after laparotomy. Then liver samples were collected for posterior analysis.

(2) Group 2 ($n = 6$): UW: steatotic livers were preserved for 24 hours in UW solution at 4°C. Then, livers were flushed with Ringer's solution and the effluent liquid was collected for further biochemical determinations. Liver tissue was stored at −80°C.

(3) Group 3 ($n = 6$): PEG 35: livers were pretreated with PEG 35 intravenously at 10mg/kg 10 min before liver procurement and then preserved for 24 hours in UW solution as in group 2.

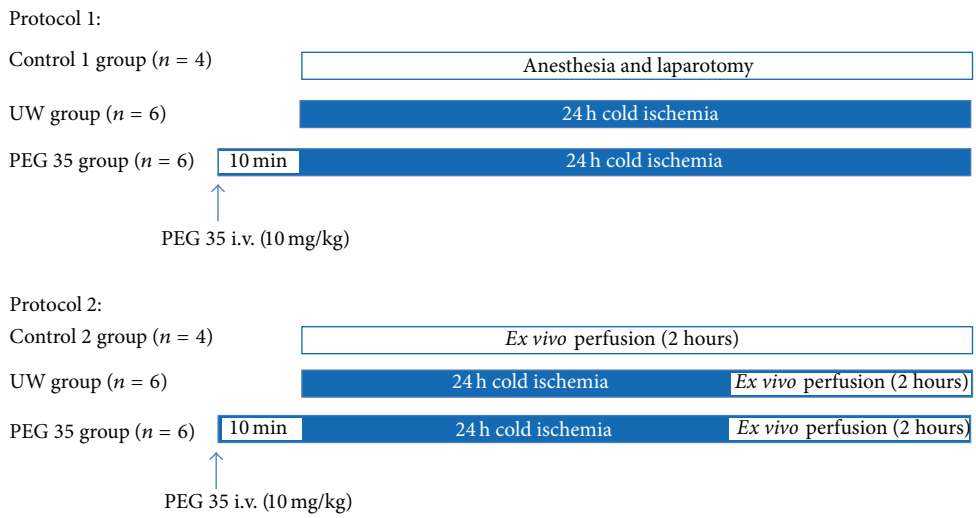
Protocol 2: Effect of PEG 35 in Fatty Livers after 24 h of Cold Storage and 2 h of Normothermic Reperfusion. To examine the effect of PEG 35 in liver injury after normothermic perfusion, fatty livers were randomized in the following groups:

(1) Control group (Ctr 2) ($n = 4$): after procurement, steatotic livers were *ex vivo* perfused for 2 h at 37°C as described above, without prior cold storage.

(2) UW group ($n = 6$): fatty livers were preserved in UW preservation solution for 24 hours at 4°C and then subjected to 2 h of normothermic reperfusion at 37°C.

(3) PEG 35 group ($n = 6$): Zucker Ob rats were pretreated with intravenous administration of PEG 35 at 10 mg/kg, 10 min before liver procurement. Then, livers were preserved for 24 h in UW solution and finally *ex vivo* perfused for 2 hours at 37°C.

2.5. Liver Injury: Transaminases Assay. Hepatic injury was assessed in terms of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels with commercial kits from RAL (Barcelona, Spain). Briefly, 100 μL of effluent



Scheme 1: Experimental designs to investigate PEG 35 effects on steatotic livers after cold ischemia at 4°C (Protocol 1) and after cold storage followed by normothermic *ex vivo* reperfusion (Protocol 2).

washout liquid or perfusate was added to 1 mL of the substrate provided by the commercial kit and then transaminases activity was measured at 340 nm with a UV spectrometer and calculated following the supplier's instructions. Results were normalized using a commercial calibrator (Biocal, RAL, Barcelona, Spain).

2.6. Mitochondrial Damage

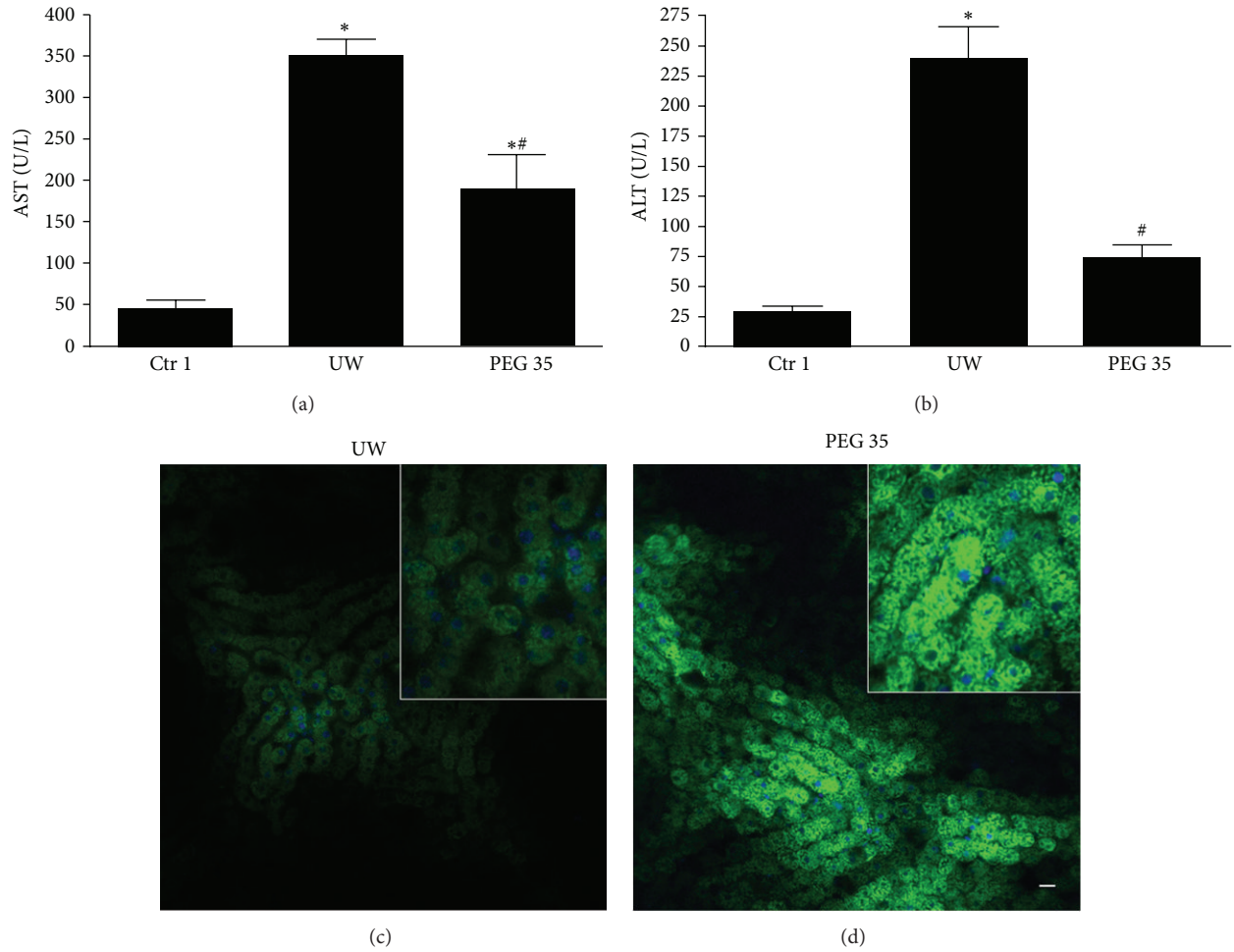
2.6.1. Glutamate Dehydrogenase Activity. Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme present predominantly in liver and contributes to the oxidative deamination of glutamate. Measurable increases in serum levels are indicative of mitochondrial damage. Serum concentrations of GLDH were determined using a commercial kit (GLDH, Randox laboratories Ltd., Crumlin, UK) by quantifying the decrease in absorbance at 340 nm according to the manufacturer's protocol.

2.6.2. Confocal Microscopy. After 24 h of hypothermic preservation, fatty livers pretreated with PEG conjugated to FITC (PEG-FITC) or saline were washed out via the portal vein with 20 mL of Ringer lactate solution containing fluorescent dyes. The fluorescent dyes were diluted in the washout liquid and injected to the preserved fatty liver at the following final concentrations: Hoechst 33342 trihydrochloride (12mg/kg body weight, Invitrogen, H3570) for DNA-nuclei staining and rhodamine 123 (0.1 1mg/kg body weight, Sigma, R8004) for mitochondrial membrane potential staining. Fatty livers were then carefully sectioned (0.5 cm^3 fragments) and the internal side of the liver was exposed on the glass coverslip mounted on the stage of a Leica TCS SP5 resonant scan multiphoton confocal microscope (Leica Microsystems Heidelberg GmbH) equipped with a HCX IR APO L 25x water immersion objective (Numerical Aperture 0.95), scanner at 400 lines/s, and a near infrared Titanium:Saphire laser (MaiTai, SpectraPhysics) for two-photon excitation running at 800 nm. Images were acquired with resonant scan at 8000 lines/second. Two-photon excitation was performed at 800 nm and emission of the different fluorescent dyes was captured at the following wavelength ranges: PEG-FITC (400–550 nm), Hoechst 33342 (400–470 nm), and rhodamine 123 (500–550 nm).

2.7. Vascular Resistance. Vascular resistance was defined as the ratio of portal venous pressure which was maintained at 12 mmHg during the reperfusion to flow rate and expressed in mmHg/min per gram of liver/mL. Perfusion flow rate was assessed continuously throughout the reperfusion period and expressed as mL/min per gram of liver.

2.8. Western Blotting Technique. Liver tissue was homogenized in HEPES buffer and proteins were separated by SDS-PAGE and transferred to PVDF membranes. Then, membranes were immunoblotted over night at 4°C using the following antibodies: anti-p-AMPKα (Thr172, #2535), anti-AMPKα (#2603), anti-p-SAPK/JNK (Thr183/Tyr185), anti-p-p38 MAP kinase (Thr180/Tyr182, #9211),and anti-p-p44/42 MAPK (Erk1/2, Thr202/Tyr204, #9101); the above antibodies were all purchased from Cell Signaling (Danvers, MA); anti-eNOS (610296) was purshased form Transduction Laboratories (Lexington KY) and anti-b-actin (A5316) was purshased from Sigma Chemical (St. Louis, MO, USA). After washing, bound antibody was detected after incubation for 1h at room temperature with the corresponding secondary antibody linked to horseradish peroxidase. Bound complexes were detected and quantified by scanning densitometry.

2.9. Statistical Analysis. Statistical analysis was performed with GraphPad Prism version 4.02 for Windows (GraphPad Software). Quantitative data are reported as mean ± SEM and statistical comparison was performed with analysis of



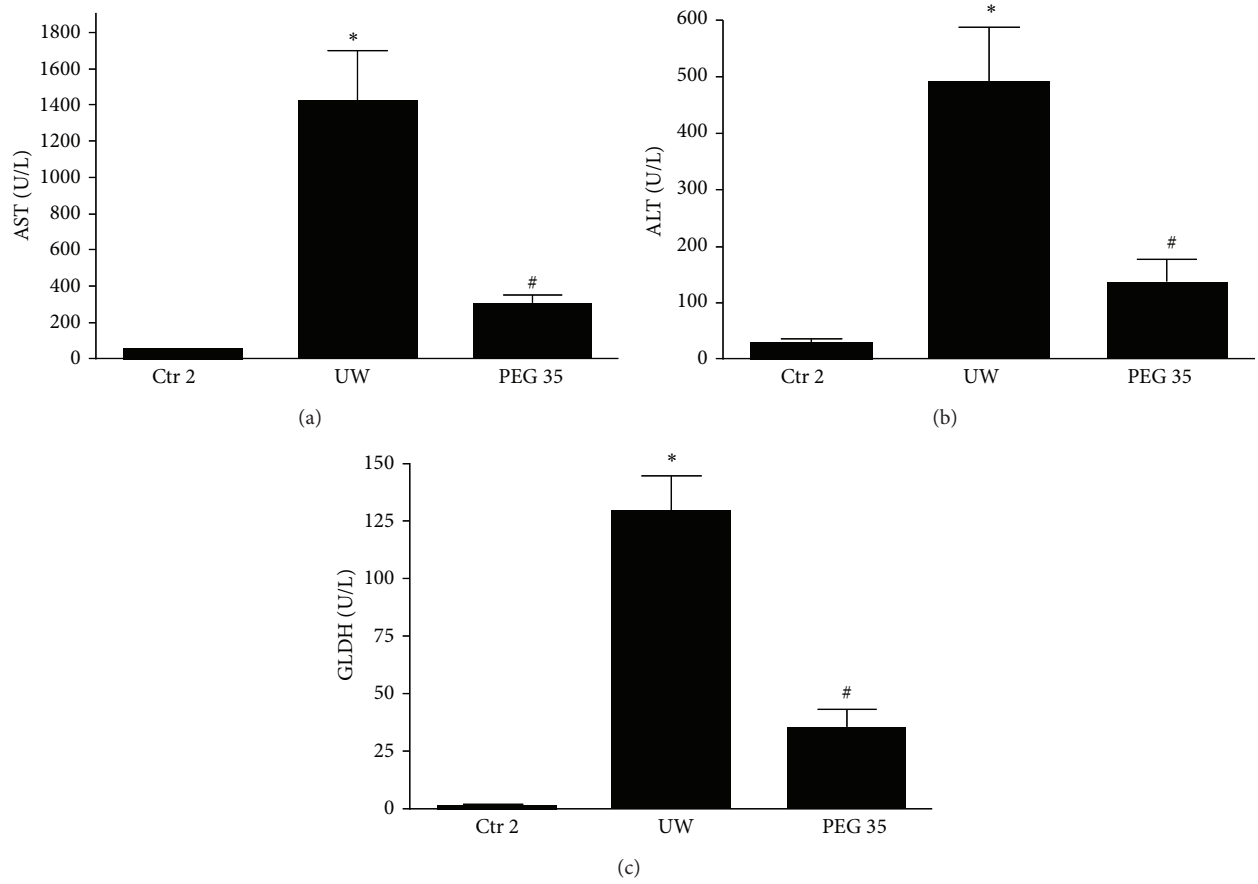
Figur e 1: Hepatic and mitochondrial injuries after cold ischemia. PEG 35 administration decreases aspartate aminotransferase (AST) (a) and alanine aminotransferase (ALT) levels (b) after 24 h of cold storage. Confocal microscopy of mitochondrial membrane potential stained with rhodamine 123 (green) after cold storage: mitochondrial depolarization occurs after preservation (c); however, in rats pretreated with PEG 35 conjugated to FITC, we observed bright punctate fluorescence showing polarized mitochondria (d). No PEG fluorescence has been detected in liver sinusoids, neither in hepatocytes nor bound to cell membrane (d). Ctr 1: anaesthesia and laparotomy; UW: livers preserved in UW preservation solution for 24 hours at 4°C; PEG 35: Zucker obese rats treated intravenously with PEG 35 at 10 mg/kg and steatotic livers were then subjected to 24 h cold ischemia. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 1; $^{\#}p < 0.05$ versus UW.

variance, followed by Tukey tests. An associated probability of $p < 0.05$ was considered to be significant.

3. Results

3.1. Effect of Intravenous PEG 35 on Cold Storage of Steatotic Rat Livers. To investigate the protective effect of intravenous PEG 35 treatment on liver preservation, we measured transaminases levels in the effluent of washout liquid after 24 h of cold storage. As shown in Figures 1(a) and 1(b), liver preservation resulted in increased AST/ALT levels versus control group and the intravenous administration of PEG 35 at 10 mg/kg decreased significantly transaminases release indicating substantially less hepatocellular damage. Also, we explored mitochondrial polarization after fatty liver preservation using rhodamine 123 vital dye. In livers pretreated with PEG 35, we observed bright punctate fluorescence standing for the cells with polarized mitochondria. By contrast, in nontreated livers, we observed a cloudy diffuse cytosolic fluorescence standing for cells with depolarized mitochondria (Figures 1(c) and 1(d)). Moreover, our result shows that when obese rats were treated with PEG-FITC, no significant PEG fluorescence was detected in liver sinusoids neither into hepatocytes or other liver cells nor bound to cell membrane (Figure 1(d)).

3.2. Effect of Intravenous PEG 35 Administration on Fatty Liver Injury after Ischemia Reperfusion. In the following, we evaluated the reperfusion injury after 2 hours of *ex vivo* perfusion at 37°C (Protocol 2). We observed a significant decrease in transaminases levels in the perfusate from rats pretreated with PEG 35 when compared to the untreated ones (Figures 2(a) and 2(b)). Moreover, the evaluation of mitochondrial damage, measured by GLDH activity, showed



Figur e 2: Hepatic and mitochondrial injuries after cold ischemia and reperfusion. PEG 35 administration decreases aspartate aminotransferase (AST) (a), alanine aminotransferase (ALT) (b), and glutamate dehydrogenase (GLDH) levels after 2 hours of *ex vivo* perfusion. Ctr 2: liver procurement and *ex vivo* perfusion; UW: livers preserved in UW preservation solution for 24 h at 4°C and then subjected to 2 h of normothermic *ex vivo* perfusion; PEG 35: rats treated intravenously with PEG 35 (10 mg/kg) and then subjected to 24 h cold ischemia followed by 2 h of normothermic *ex vivo* perfusion. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 2; $^{\#}p < 0.05$ versus UW.

significant decreases when rats were pretreated with PEG 35 (Figure 2(c)).

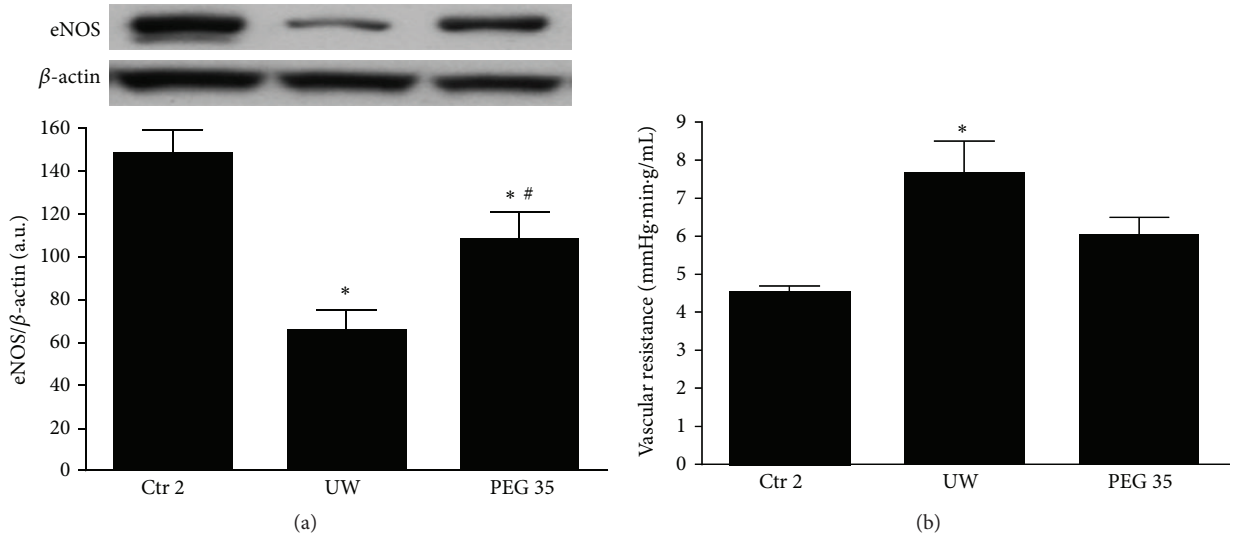
Steatotic livers present fat accumulation in the cytoplasm of the hepatocytes which causes disturbance of the sinusoidal flow during reperfusion [2, 16]. Given that, we explored vascular resistance and endothelial nitric oxide synthase (eNOS) activation after reperfusion. Figure 3(a) shows that eNOS protein expression decreased after 2 h of *ex vivo* perfusion in UW compared to control group. In contrast, pretreatment with PEG 35 clearly induced eNOS expression which was concomitant with decreased vascular resistance (Figure 3(b)).

Next, we explored whether the hepatoprotective effect of PEG 35 could be attributed to well-known cell signaling pathways associated with IRI such as adenosine monophosphate activated protein kinase (AMPK) and protein kinase B (Akt). As shown in Figure 4(a), liver preservation followed by 2 hours of *ex vivo* perfusion promoted Akt phosphorylation, which was further enhanced when rats were pretreated with PEG 35. Regarding AMPK, PEG 35 administration prior to liver procurement induced a significant activation in AMPK in comparison to non-PEG 35-treated rats (Figure 4(b)).

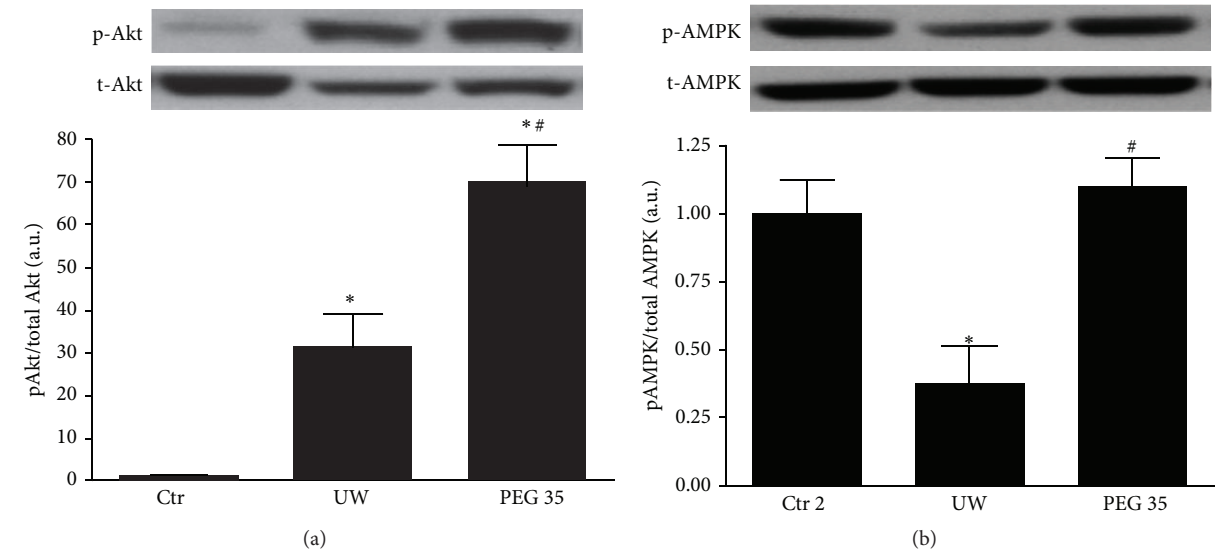
It is well known that mitogen activated protein kinase (MAPK) signaling pathway regulates inflammation and cell survival during IRI [17, 18]. We therefore assessed the possible involvement of MAPK regulation in the protective effect of PEG 35. As indicated in Figure 5, all MAP kinases (p-p38, p-JNK, and p-Erk) levels were increased at 2 h reperfusion. A significant reduction in p-p38 activation was evident after PEG 35 treatment (Figure 5(a)). On the contrary, no changes for JNK and Erk activity were found (Figures 5(b) and 5(c), resp.).

4. Discussion

The beneficial effects of PEG in tissue injury are well documented [5, 12–14, 19]. However, because PEG molecules are not absorbed in the gastrointestinal tracts, their use against ischemic damage was limited to their addition to preservation solutions as oncotic agents. The present study was thus designed to investigate if the intravenous PEG 35 administration by a unique and nontoxic dose of 10 mg/kg could protect steatotic liver grafts against the deleterious



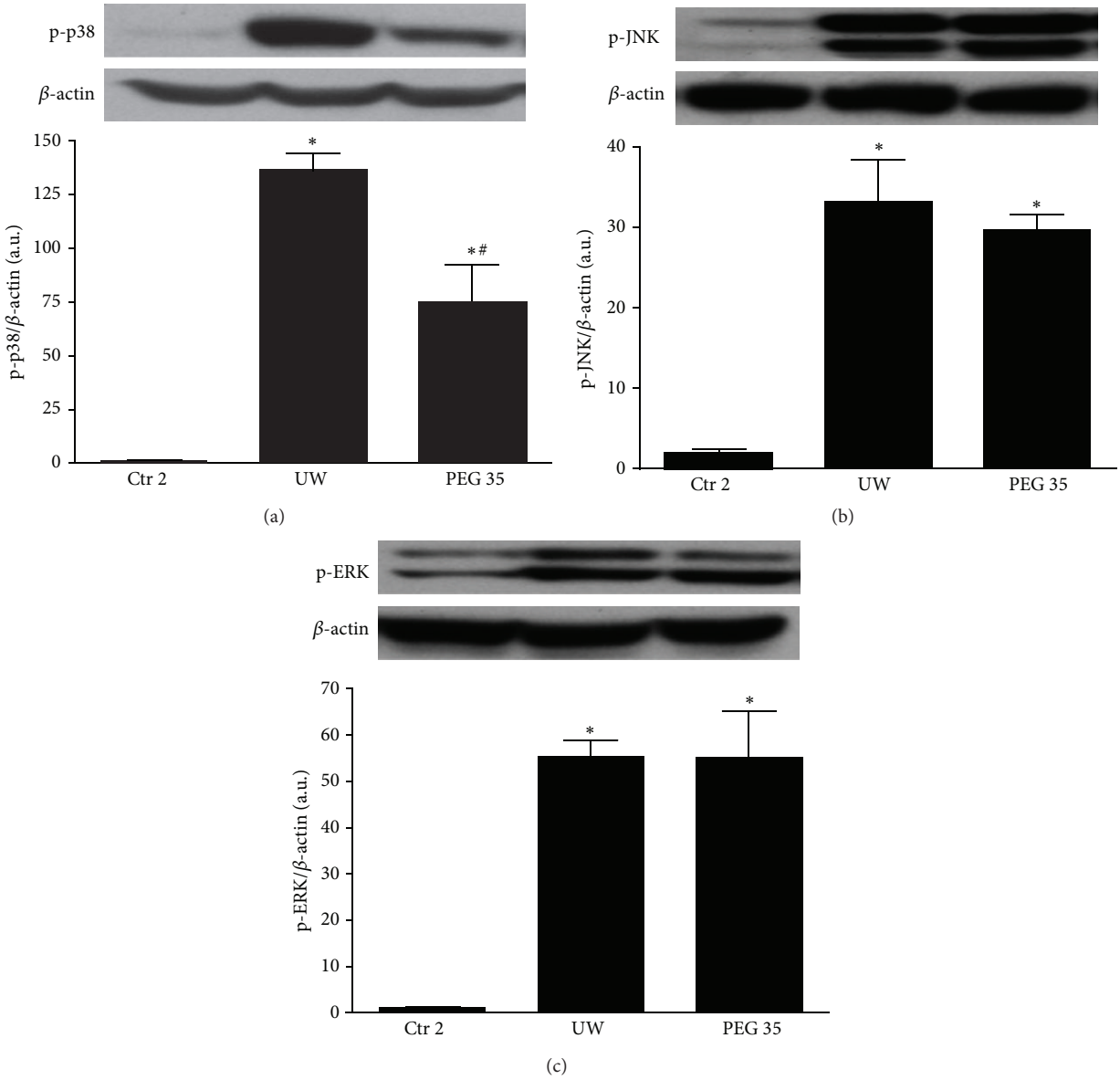
Figur e 3: Effects of PEG 35 on eNOS activation and vascular resistance. PEG 35 pretreatment activates eNOS and decreases vascular resistance: densitometric analysis of eNOS/β-actin (a) and vascular resistance (b) after 120 min of normothermic reperfusion. Ctr 2: liver procurement and *ex vivo* perfusion; UW: livers preserved in UW preservation solution for 24 h at 4°C and then subjected to 2 h of normothermic *ex vivo* perfusion; PEG 35: rats treated intravenously with PEG 35 (10 mg/kg) and then subjected to 24 h cold ischemia followed by 2 h of normothermic *ex vivo* perfusion. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 2; $^{\#}p < 0.05$ versus UW.



Figur e 4: Effects of PEG 35 on Akt and AMPK. PEG 35 administration activates Akt and AMPK: densitometric analysis of phosphorylated Akt/total Akt (a) and phosphorylated AMPK/total AMPK (b). Ctr 2: liver procurement and *ex vivo* perfusion; UW: livers preserved in UW preservation solution for 24 h at 4°C and then subjected to 2 h of normothermic *ex vivo* perfusion; PEG 35: rats treated intravenously with PEG 35 (10mg/kg) and then subjected to 24 h cold ischemia followed by 2 h of normothermic *ex vivo* perfusion. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 2; $^{\#}p < 0.05$ versus UW.

effects of cold storage and the subsequent reperfusion. Our data demonstrated that pretreatment of rats with PEG 35 lessened liver injury associated with ischemia reperfusion.

In our study, we have used the isolated perfused rat liver (IPRL) model, a widely used and appreciated method to assess cellular injury and liver function in an isolated setting. In comparison to other *in vitro* models, the IPRL-model does have considerable advantages, such as the use of the entire intact organ instead of only single cells or several layers of cells (i.e., isolated hepatocytes or the liver slice model) and an intact cellular architecture. Furthermore, the use of an acellular perfusion solution (Krebs solution) prevents alloreactivity and permits a conclusive focus on IRI effects. Regarding liver transplantation, IPRL model presents the advantages of minimizing the use of laboratory animals as well as the suppression of the immunological



Figur e 5: Effect of PEG 35 on MAPKs. PEG 35 reduces p38-MAPK activation whereas it has no effect on JNK and ERK phosphorylation. Densitometric analysis of phosphorylated p38/β-actin (a), phosphorylated JNK/β-actin (b), and phosphorylated ERK/β-actin (c). Ctr 2: liver procurement and *ex vivo* perfusion; UW: livers preserved in UW preservation solution for 24 h at 4°C and then subjected to 2 h of normothermic *ex vivo* perfusion; PEG 35: rats treated intravenously with PEG 35 (10 mg/kg) and then subjected to 24 h cold ischemia followed by 2 h of normothermic *ex vivo* perfusion. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 2; $^{\#}p < 0.05$ versus UW.

reactions and the influences of other organs occurring during transplantation. The disadvantage of the IPRL-model is the duration of reperfusion, which is limited to 90–120 minutes and the fact that it remains an *in vitro* tool that merely simulates the initial phase after liver transplantation. In this sense IPRL model could be considered as a pre-screening model before liver transplantation especially in ischemia reperfusion research [20, 21].

In contrast to the current pharmacological strategies used against IRI, PEG administration presents the advantages of being a multitarget strategy. In fact, IRI is a multifactorial disease including oxidative stress, inflammation, proteasome activation, endoplasmic reticulum stress, mitochondrial damage, and cytoskeleton alterations which lead to cell death and organ dysfunction [22–24]. PEG has been associated with the majority of these events as it has been shown that PEG reduces reactive oxygen species, prevents cell death, maintains mitochondrial integrity, and reduces inflammation and endoplasmic reticulum stress [12, 14, 19, 25, 26].

The half-life and biodistribution of the polymer and consequently its activity mainly depend on its molecular weight. Based on our experience in organ preservation, we used PEG with a molecular weight of 35 kDa. Indeed, we have previously demonstrated that PEG 35 addition to washout

solution protected the liver against reperfusion injuries [27]. Moreover, PEG 35 addition to IGL-1 preservation solution protects kidney and liver grafts against ischemic damage [7, 8, 28, 29]. PEG with a molecular weight of 20 kDa has also been used as an additive to HTK and SCOT preservation solutions and was associated with protective effects against IRI in pancreas [30], kidney [8], intestine [26], and liver grafts [31]. In addition, PEG20 has been shown to protect against cardiomyocyte apoptosis induced by hypoxia [12]. However, PEG 35 was more effective than PEG20 in protecting porcine proximal tubular epithelial cell line against cold storage at the same doses used [32].

Mitochondrial protection is essential for graft survival after transplantation [33]. Thus, we further explored mitochondrial depolarization after cold preservation and we evidenced that PEG 35 prevented fatty liver mitochondria depolarization after prolonged cold ischemia. Also, mitochondrial injury was lessened after liver reperfusion as indicated by the decrease in GLDH release. These results are in accordance with previous published data showing that PEG 2 kDa improved mitochondrial function *in vitro* and *in vivo* after acute spinal cord injury [25]. Moreover, PEG of 4 kDa inhibited mitochondrial pore transition (MPT) and cytochrome C release in rat liver mitochondria [34]. Also, PEG (1.5 and 2 kDa) was able to cross the cytoplasmic membrane and directly interact with neuronal mitochondria to preserve its structure and restore function [14]. Interestingly, PEG with higher molecular weight (4 kDa) failed to exert significant improvement in neuronal injured mitochondria indicating that PEG-mediated mitochondrial protection is dependent on the size of PEG [14]. In our study, we did not detect any PEG fluorescence after cold storage in liver sinusoids, neither in hepatocytes nor bound to cell membrane. In this sense, the mechanism by which PEG 35 decreases mitochondrial damage and exerts its protective effects needs more profound investigation.

Our results show that PEG 35 activated eNOS, the enzyme responsible for nitric oxide (NO) generation, and consequently decreased vascular resistance. This could also explain the protective mechanism of PEG toward mitochondria as it has been showed that NO protects rat hepatocytes against reperfusion injury through the inhibition of MPT [35]. Previous study from Bertuglia et al. has shown that PEG 15–20 kDa reduced vasoconstriction and the altered capillary perfusion after ischemia reperfusion [36]. However, in that case, the decreased vascular resistance of PEG were not mediated by eNOS activation [36].

In order to explore whether the beneficial effects of PEG 35 are associated with other well-known cell signaling pathways involved in IRI, we further evaluated the activation of AMPK and Akt and the regulation of MAPKs. AMPK is a metabolic fuel gauge and energy regulator activated during ischemia in order to induce an energy-saving state preventing thus the lactate accumulation and cell death [37–39]. Here, we showed that PEG 35 enhanced AMPK levels after reperfusion, which could contribute to assuring energy levels sufficient to cell survival. Another cytoprotective marker is Akt, a serine-threonine protein kinase that is linked to cell survival during reperfusion [40–42]. Data reported here revealed that PEG 35 increased Akt levels, as similarly observed with PEG 20 in cardiac myocyte submitted to IRI [12]. Regarding MAPKs signalling, we observed that PEG 35 was capable of preventing p38 activation, while no changes were found on JNK and ERK pathways. The data reported here are consistent with previously reported works showing that the inhibition of p38 prevented preservation-induced graft injury and improved the outcome of liver transplantation [43–45]. Other studies as well reported that PEG 35 decreased p38 activation while it activated JNK in cold stored porcine proximal tubular cell line [32].

The rationale of PEG 35 intravenous administration was to induce a pharmacological preconditioning against the subsequent cold storage and reperfusion injury. PEG presents the advantages of being safe and multifactorial agent and may constitute a novel strategy to increase liver graft preservation. This could be relevant in clinical situation of brain-dead donors or steatotic livers, both being risk factors in liver transplantation. Until now, PEG has been used clinically for ischemia reperfusion purpose as additive to preservation solution due to its oncotic properties. In this study, we used UW solution which contains hydroxyethyl starch as an oncotic support in order to demonstrate that the protective mechanisms of PEG are not only related to its oncotic effect, but also to other properties such as the preservation of mitochondria and the induction of protective cell signaling pathway (eNOS, Akt, and AMPK). In a previous study we have shown that PEG addition to rinse solution protected preserved liver against the subsequent reperfusion injury (PEG postconditioning). Interestingly, when liver grafts were preserved in IGL-1 solution which contains PEG 35, the rinse solution does not show any additional protective effect [46]. In this sense, PEG pre- and postconditioning would be considered as a safe and protective strategy applicable to all preservation solutions.

5. Conclusions

PEG 35 represents a potential pharmacological agent for preventing the deleterious effects of cold IRI and may constitute a novel clinical strategy to increase liver graft preservation, especially for "marginal" organs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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ORIGINAL ARTICLE

Polyethylene glycol rinse solution: An effective way to prevent ischemia-reperfusion injury

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Abstract

AIM: To test whether a new rinse solution containing polyethylene glycol 35 (PEG-35) could prevent ischemia-reperfusion injury (IRI) in liver grafts.

METHODS: Sprague-Dawley rat livers were stored in University of Wisconsin preservation solution and then washed with different rinse solutions before *ex vivo* perfusion with Ringer's lactate solution and a new rinse solution enriched with PEG-35 at either 1 or 5 g/L. We assessed the following: liver injury (transaminase levels), mitochondrial damage (glutamate dehydrogenase), liver function (bile output and vascular resistance), oxidative stress (malondialdehyde), nitric oxide, liver autophagy (Beclin-1 and LCB3) and cytoskeleton integrity (filament and globular actin fraction); as well as levels of metalloproteinases (MMP2 and MMP9), adenosine monophosphate-activated protein kinase (AMPK), heat shock protein 70 (HSP70) and heme oxygenase 1 (HO-1).

RESULTS: When we used the PEG-35 rinse solution, reduced hepatic injury and improved liver function were noted after reperfusion. The PEG-35 rinse solution prevented oxidative stress, mitochondrial damage, and liver autophagy. Further, it increased the expression of cytoprotective heat shock proteins such as HO-1, HSP70, and AMPK, and contributed to the restoration of cytoskeleton integrity after IRI.

CONCLUSION: Using the rinse solution containing PEG-35 was effective for decreasing liver graft vulnerability to IRI.

Key words: Liver washout; Liver transplantation; Rinse solution; Ischemia-reperfusion injury; Polyethylene glycol 35; Nitric oxide; Adenosine monophosphate-activated protein kinase; Heme oxygenase 1; Heat shock protein 70; Metalloproteinases

Core tip: Research into optimal rinse solutions for graft washout is limited, and their clinical application

is dependent on surgeon preference. We present a new rinse solution containing polyethylene glycol 35 (PEG-35) that is not only suitable for washing liver grafts after cold preservation, but also provided good graft protection against reperfusion injury. Using PEG-35 in the rinse solution resulted in less hepatic injury, a significant induction of cytoprotective proteins, and the preservation of cytoskeletal integrity. Thus, PEG-35 supplemented rinse solutions may contribute to liver graft protection against ischemia-reperfusion injury.

Zaouali MA, Bejaoui M, Calvo M, Folch-Puy E, Pantazi E, Pasut G, Rimola A, Abdennebi HB, Adam R, Roselló-Catafau J. Polyethylene glycol rinse solution: An effective way to prevent ischemia-reperfusion injury. *World J Gastroenterol* 2014; 20(43): 0000-0000 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i43/0000.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i43.0000

INTRODUCTION

Liver transplantation (LT) has had a profound impact on patient outcomes in end-stage liver disease, representing the most effective treatment for many patients with acute or chronic liver failure[1]. Ischemia-reperfusion injury (IRI) is an inherent risk of LT, and is often responsible for early graft failure within the first week of surgery[2-4]. IRI is initiated when the liver is recovered from the donor and placed in cold storage; it continues when the graft is re-warmed prior to LT, and persists at graft revascularization after LT[3,4]. Research has identified several strategies to reduce the impact of these multifactorial processes on graft function.

Maintaining organ viability during cold storage (*i.e.*, preservation) is an important prerequisite for a successful outcome after LT[1,5]. For this reason, the composition of organ preservation solutions is crucial; during cold storage, the solution must prevent cell swelling, impaired energy metabolism, acidosis, and the accumulation of precursors of reactive oxygen intermediates[1,5]. Currently, the University of Wisconsin (UW) solution is the one most commonly used for LT[6,7]. However, several studies have reported that its composition is limited by: (1) the high concentration of K+ ions in preserved grafts that could cause cardiac arrest in the recipient at reperfusion[8]; (2) the oncotic agent, hydroxyl-ethyl starch (HES), which confers high viscosity with incomplete distribution of the UW solution in the liver graft, particularly between the intravascular space and liver parenchyma; and (3) the hyper-aggregating effects of HES on erythrocytes, which may hamper liver graft reperfusion[9-11]. These limitations have led physicians to rinse grafts before revascularization to remove the K^+ ions, HES, and toxins that accumulate during preservation, and to ensure optimal conditions for graft revascularization and viability.

Current studies on rinse solutions for organ washout are limited, particularly for the liver. Ringer's lactate solution (RLS) was initially used, before a more effective alternative was proposed by Adam *et al*[12] who used a macromolecular albumin solution to restrict reperfusion damage. Later, the Carolina rinse (CR) solution was shown to prevent IRI[13-15], but it has a complex composition and contains HES, which induced red blood cell aggregation and incomplete washout[11]. Despite some reductions in liver injury, the optimal washout solution has yet to be established and current practice depends mostly on physician preference.

In previous studies, we have used polyethylene glycol (PEG) 35 as an oncotic agent in Institut Georges Lopez (IGL-1) preservation solutions for liver graft conservation[16-18]. PEG-35 is a non-toxic, water-soluble polymer that prevents red blood cell aggregation when compared to HES[1,11]. PEG has shown protective effects in different organs and can reduce oxidative stress through the preservation or restoration of membrane integrity[19]. Moreover, several studies have demonstrated the efficiency of PEG in kidney[20], heart[21], liver[22], pancreas[23], and small bowel[24] preservation in experimental models and clinical studies.

In the present study, we investigated the efficacy of liver washout with a new PEG-35 rinse solution after graft cold preservation in UW solution.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250 g body weight) were anesthetized under isoflurane inhalation anesthesia according to European Union regulations (Directive 86/609 EEC), and surgery was performed as previously reported[25]. Animals were randomly distributed into groups as described below.

Liver washout

After 24 h cold storage in UW solution, the liver grafts were subjected to normothermic washout with different rinsing solutions (Table 1 and Table 2) and then re-perfused for 2 h at 37 ℃. The experimental groups were as follows: Group 1, Ringer's lactate solution (RLS) (n = 8) as shown in Table 1; the liver grafts were flushed with RLS (15 min; room temperature) and then re-perfused for 2 h at 37 ℃. Group 2, Base solution (BS) (n = 8); same as Group 1, but flushed with the BS, as indicated in Table 2. Group 3, (BS + PEG1) (n = 8); same as Group 1, but flushed with BS supplemented with PEG-35 at 1 g/L (Table 2). Group 4, (BS + PEG5) (n = 8); same as Group 1, but flushed with the BS supplemented with PEG-35 at 5 g/L (Table 2).

Model of isolated perfused rat liver

After cold storage in UW solution, the liver grafts were rinsed at room temperature for 15 min using one of the rinse solutions indicated above. Then livers were connected *via* the portal vein to a recirculating perfusion

Table 1 Composition of Ringer's lactate solution

Ringer lactate solution	
Composition	**Concentration (mg/100 mL)**
NaCl	600
KCl	40
$CaCl_2.2H_2O$	27
$NaC_3H_5O_3$	312
Osmolarity (mOsm/L)	277
pH	5.0-7

system for 2 h at 37 ℃. Time zero was the point at which the portal catheter was satisfactorily connected to the circuit. As previously reported, during the first 15 min of perfusion (initial equilibration period), the flow was steadily increased until we achieved stabilization of the portal pressure at 12 mm Hg (Pressure Monitor BP-1; World Precision Instruments, Sarasota, Florida). The flow was controlled by a peristaltic pump (Minipuls 3; Gilson, France)[26,27]. The reperfusion liquid consisted of a cell culture medium (William's medium E; BioWhittaker, Barcelona, Spain) with a Krebs-Heinseleit-like electrolyte composition enriched with 5% albumin for oncotic pressure[28]. Before entering the liver, the buffer was exposed to a mixture of 95% O_2 and 5% CO_2, a heat exchanger (37 ℃), and a bubble trap[27,28]. During normothermic reperfusion lasting 120 min, the effluent was collected at 30 min intervals to measure the liver aminotransferase levels and the liver function (bile, vascular resistance). Biochemical parameters were measured 2 h after reperfusion.

Biochemical determinations

Transaminase assay: Hepatic injury was assessed by measuring transaminase levels using commercial kits from RAL (Barcelona, Spain). Briefly, 200 μL of effluent perfusate was added to the substrate provided by the commercial kit and the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined at 365 nm with an ultraviolet spectrometer and calculated according to the manufacturer's instructions[27,29].

Bile Output: Liver function was assessed by measuring bile production. Bile was collected through a cannulated bile duct and output was reported as microliter per gram of liver (μL/g liver)[26].

Vascular resistance: Liver circulation was assessed by measuring perfusion flow rate and vascular resistance. The perfusion flow rate was assessed continuously during reperfusion (expressed as mL/min/g liver). Vascular resistance was defined as the ratio of portal venous pressure to flow rate (expressed as mmHg/min/g liver/mL)[26].

Glutamate dehydrogenase activity: The mitochondrial enzyme glutamate dehydrogenase (GLDH) was used as an indirect measure of mitochondrial damage as previously reported[28].

Table 2 Composition of the different rinse solutions: BS, BS+PEG35 at 1g/L (BS+PEG1) and BS+PEG35 at 5g/L (PEG+PEG5)

Composition	BS	BS + PEG1	BS + PEG5
$CaCl_2.2H_2O$ (mmol/L)	1.3	1.3	1.3
KH_2PO_4 (mmol/L)	5	5	5
NaH_2PO_4 (mmol/L)	20	20	20
$MgSO_4.7H_2O$ (mmol/L)	5	5	5
Lactobionic acid (mmol/L)	100	100	100
Raffinose (mmol/L)	30	30	30
PEG 35 (g/L)	-	1	5
pH	7.4	7.4	7.4
Osmolarity (mOsm/L)	320	320	320

BS: Base solution; BS + PEG1: BS with PEG-35 at 1 g/L; BS + PEG5: BS with PEG-35 at 5 g/L.

Lipid peroxidation assay: Lipid peroxidation in the liver was used as an indirect measure of the oxidative injury induced by ROS. Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) with the thiobarbiturate reaction[28].

Determination of nitrites and nitrates: Nitric oxide (NO) production by the liver was determined by measuring tissue accumulation of nitrites and nitrates, as previously reported[28].

Western blot analysis

Liver tissue was homogenized as previously described[28]. Total protein was loaded in Laemmli buffer onto a SDS-polyacrylamide gel in a Mini Cell (Bio-Rad). The proteins were transferred to polyvinylidene difluoride membranes and blocked in 1 × phosphate buffered saline (PBS)/0.05% Tween 20/5% non-fat dry milk. Membranes were immunoblotted with antibodies directed against phospho-AMPK (adenosine monophosphate-activated protein kinase) (Thr172), total AMPK, Beclin-1, and LCB3 (Cell Signaling Technology Inc, Beverly, Massachusetts); anti-endothelial NO synthase (eNOS) and heat shock protein (HSP) 70 (BD Transduction Laboratories, Lexington, Kentucky); and β-Actin, actin, and heme oxygenase-1 (HO-1; Sigma Chemical, St. Louis, Missouri). The secondary antibody was added for 1 h and the membranes were developed using enhanced chemiluminescence reagents from Bio-Rad (Hercules, California) and quantified by scanning densitometry.

Zymography

Frozen tissue samples were homogenized with a lysis buffer and centrifuged as described elsewhere[30]. Supernatants were used for extraction of gelatinolytic activity with Gelatin-Sepharose 4B (Amersham Biosciences, Uppsala, Sweden). Extracted liver samples were used to perform gelatin zymography using gelatinase zymography standards. The gels were stained in 0.1% amido black in a 1:3:6 ratio of acetic acid:methanol:water for 30 min

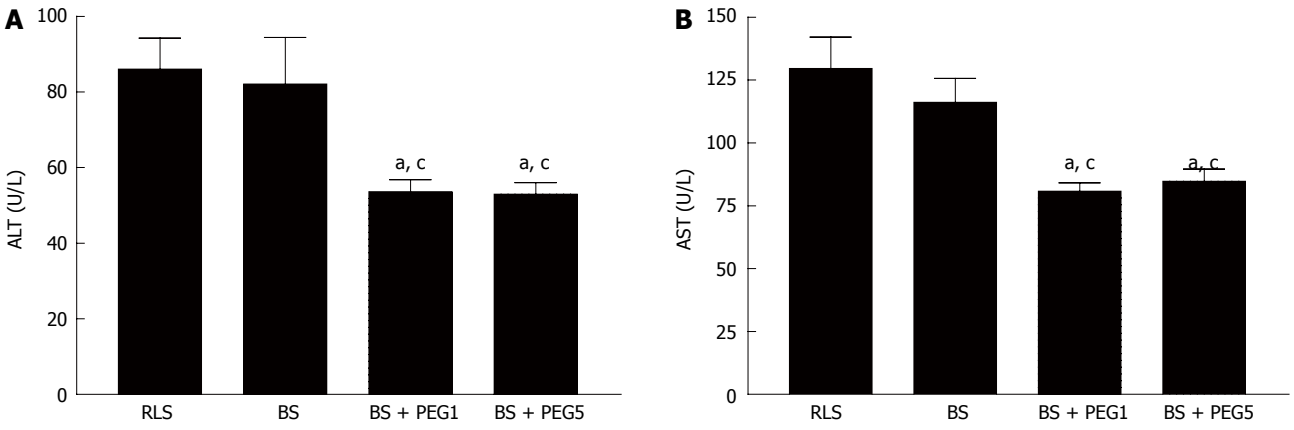

Figure 1 Alanine aminotransferase (A) and aspartate aminotransferase (B) levels in the perfusate of livers rinsed with different washout solutions (RLS, BS, BS + PEG1, and BS + PEG5) and subjected to 2 h of normothermic reperfusion. [a]*P* < 0.05 *vs* RLS; [c]*P* < 0.05 *vs* BS. Please see BS composition in Scheme 1. PEG: Polyethylene glycol; RLS: Ringer's lactate solution; BS: Base solution; BS + PEG1: BS with PEG-35 at 1 g/L; BS + PEG5: BS with PEG-35 at 5 g/L.

and destained in the solvent, followed by a final wash in distilled water. Gelatinolytic enzymes were detected as transparent bands on the gel[30].

Confocal fluorescence microscopy

The liver was fixed, cryoprotected with sucrose, embedded in optimal cutting temperature (OCT) media, and frozen on a copper plate on dry ice. Cryosections (5 μm) were cut in a cryostat and post fixed in 4% buffered paraformaldehyde for 10 min, and then permeabilized with PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA) for 30 min. For actin visualization, the slides were incubated with tetramethylrhodamine-phalloidin (TRITC-phalloidin, Sigma; dilution 2 μmol/L) in PBS with 1% BSA and 0.2% Triton X-100 for 30 min. Slides were washed 3 times for 15 min with PBS. The last PBS wash included Hoechst 33342 (Invitrogen; dilution 1 μmol/L). Finally, cryosections were mounted using Mowiol (Calbiochem).

Confocal images were acquired with a Leica TCS SP5 laser scanning microscope (Leica Microsystems, Germany) equipped with a 63x NA1.4 oil-immersion objective. Hoechst-33342 and Phalloidin-A555 images were acquired sequentially using an acousto-optical beam splitter with 405 and 561 nm laser lines and emission detection ranges of 415-450 and 570-650 nm respectively. The confocal pinhole was set at 1 Airy unit and when 3-dimensional reconstruction was required, stacks of images every 0.3 mm were acquired. The sinusoid circularity of livers (based on Phalloidin staining) was quantified on ImageJ (Wayne Rasband, National Institute of Health, United States) as $4\pi \times [(\text{Area})]/[(\text{Perimeter})2]$. Briefly, the red channel (phalloidin-A555 staining) was mean filtered (radius 1), before thresholding, conversion to a binary image, and inversion. Sinusoids were selected and circularity was measured (in 16 mm^2 of each sample). A value of 1.0 indicated a perfect circle; as the value approached 0.0, an increasingly elongated shape was more likely.

Statistical analysis

Data were expressed as mean ± standard error, and were compared statistically by variance analysis, followed by the Student-Newman-Keuls test (Graph Pad Prism software). $P < 0.05$ was considered significant.

RESULTS

Liver injury

Effluent fluid was collected to determine ALT and AST levels after 2 h of reperfusion as a predictor of organ damage after cold IRI. Figure 1 shows the AST and ALT profiles of the liver grafts subjected to washout with RLS, BS, and BS+PEG-35 solutions (Table 2). Use of the BS solution supplemented with PEG-35 prevented AST and ALT release after 2 h of reperfusion when compared to both the RLS and BS solutions. No differences were found when BS + PEG1 (1 g/L) and BS + PEG5 (5 g/L) solutions were compared.

Liver function

Liver function was assessed by measuring bile production and vascular resistance. As indicated in Figure 2A, a significant increase was observed in bile production after 2 h of reperfusion in liver grafts flushed with BS + PEG1 and BS + PEG5 rinse solutions when compared to RLS and BS alone. These results were consistent with a reduced vascular resistance after 2 h of reperfusion, as shown by the profiling studies reported in Figure 2B.

Oxidative stress and mitochondrial injury in the liver

To evaluate the effect of PEG-35 rinse solutions in oxidative stress and mitochondrial damage, we measured levels of MDA, a lipoperoxidation marker. We observed a significant MDA reduction with the PEG-35 rinse solutions when compared to BS alone (Figure 3A). This preventive effect was more marked when compared to RLS. These results were consistent with liver mitochondrial damage measured by GLDH activity in liver perfusate at the end of the 2-h reperfusion period. Livers rinsed with RLS showed the highest GLDH activity when compared to those rinsed with BS alone (Figure 3B). PEG-35 (either 1 g/L or 5 g/L) added to BS significantly reduced

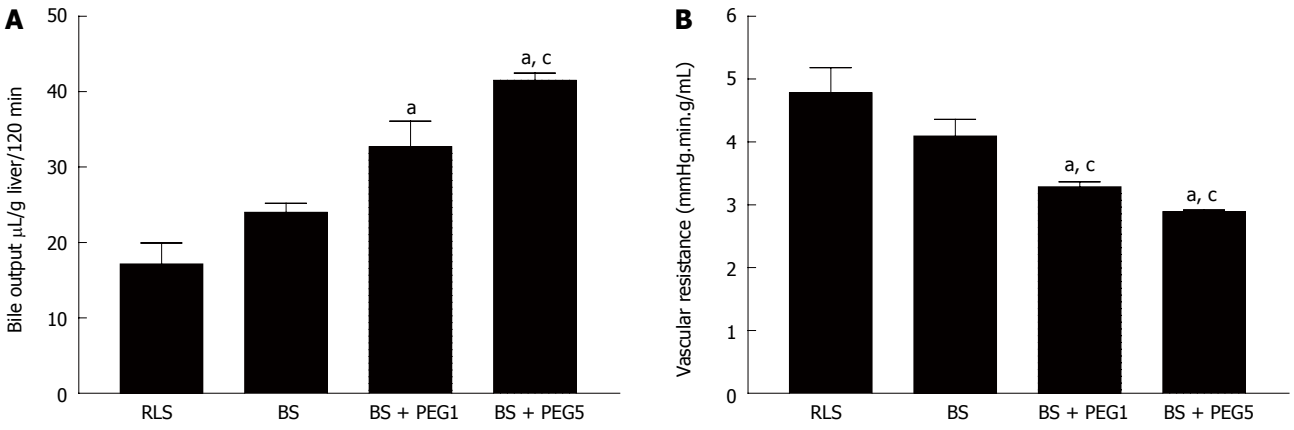

Figure 2 Bile production and vascular resistance (A), in livers rinsed with different washout solutions (RLS, BS, BS + PEG1, and BS + PEG5) and subjected to 2 h of normothermic reperfusion (B). $^{a}P < 0.05$ *vs* RLS; $^{c}P < 0.05$ *vs* BS. PEG: Polyethylene glycol; RLS: Ringer's lactate solution; BS: Base solution; BS + PEG1: BS with PEG-35 at 1 g/L; BS + PEG5: BS with PEG-35 at 5 g/L.

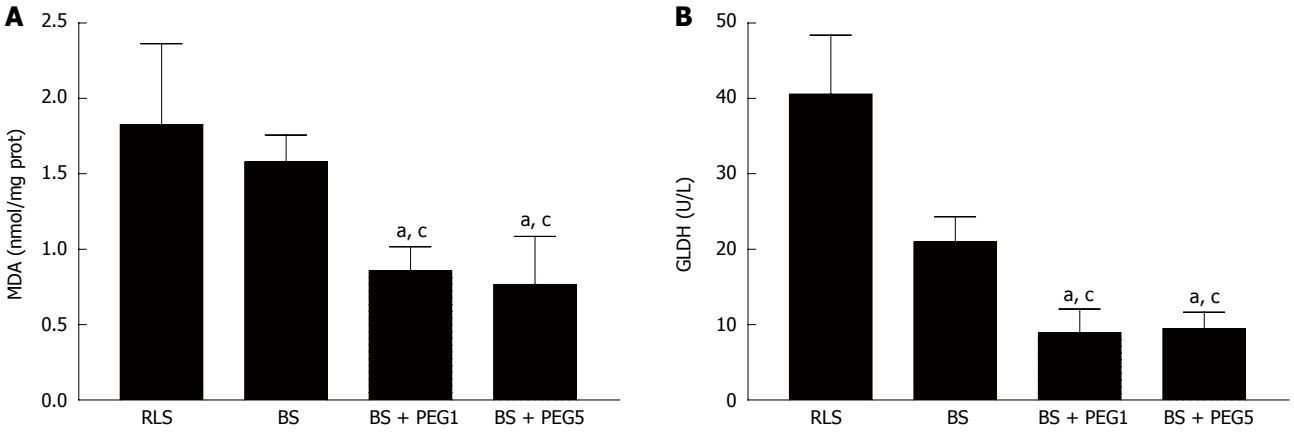

Figure 3 Hepatic malondialdehyde (A) and glutamate dehydrogenase (B) in livers rinsed with different washout solutions (RLS, BS, BS + PEG1, and BS + PEG5) and subjected to 2 h of normothermic reperfusion. $^{a}P < 0.05$ *vs* RLS; $^{c}P < 0.05$ *vs* BS. PEG: Polyethylene glycol; RLS: Ringer's lactate solution; BS: Base solution; BS + PEG1: BS with PEG-35 at 1 g/L; BS + PEG5: BS with PEG-35 at 5 g/L.

GLDH levels when compared to RLS and BS alone. No differences were observed between the PEG-35 groups.

Cell signaling pathways implicated in liver protection

We evaluated the cytoprotective cell signaling pathways involved in liver grafts by assessing the effect of PEG-35 rinse solutions on AMPK phosphorylation. We found a significant increase in phosphorylated AMPK levels in liver grafts rinsed with PEG-35 containing solutions when compared to RLS and BS (Figure 4A). This was concomitant with increases in nitrite/nitrate levels and eNOS activation (Figures 4B and C).

In addition, we examined the effect of PEG-35 rinse solutions on the induction of heme oxygenase-1 (HO-1) and HSP70 (known cytoprotective proteins involved in the reduction of liver damage after IRI). The highest HO-1 protein levels were observed in livers rinsed with PEG-35 solution when compared to RLS and BS alone. Major differences were observed between the 5 g/L and 1 g/L concentrations of PEG-35 (Figure 5A). The HSP70 protein expression pattern was similar to that described for HO-1 expression (Figure 5B).

Liver cytoskeleton alteration

Next, we evaluated the impact of PEG-35 rinse solutions on liver cytoskeleton distribution by assessing changes in filamentous actin (F-actin) and globular actin (G-actin). Our results demonstrated that the greatest G-actin fraction was observed in livers rinsed with the RLS when compared to BS and PEG-35 solutions (Figure 6). Conversely, the F-actin fraction was higher in the PEG-35 solution than in either RLS or BS alone (Figure 6). This was consistent with the metalloproteinase (MMP) activation shown in Figure 7. In fact, we observed a significant reduction in MMP9 activity in livers flushed with PEG-35 solutions when compared to those flushed only with RLS or BS. No differences were found between the PEG-35 solutions. In addition, we observed an increased MMP2 activity only when BS was used. However, no differences were found between the PEG-35 solutions and RLS (Fig-

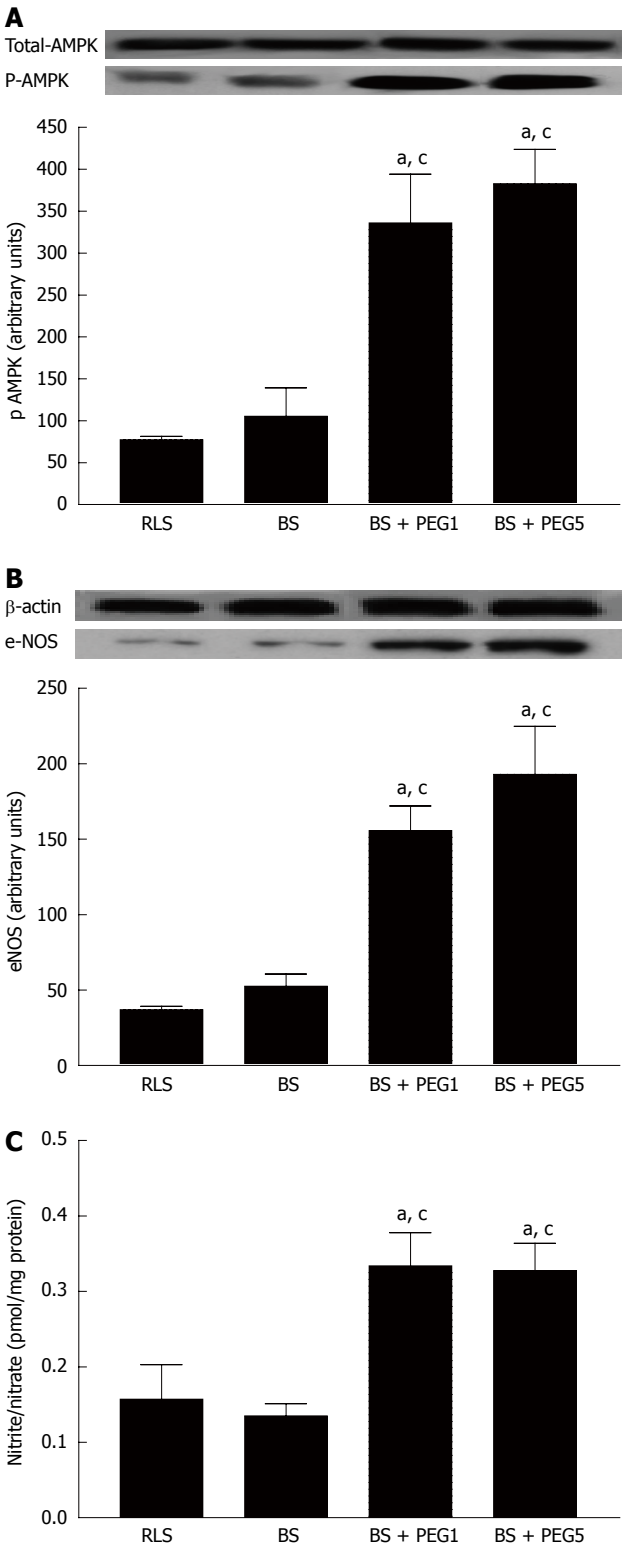

Figure 4 Phosphorylated adenosine monophosphate-activated protein kinase protein levels (A), e-NOS protein levels (B) and nitrites and nitrates (C) in livers flushed with different washout solutions (RLS, BS, BS + PEG1, and BS + PEG5) and subjected to 2 h of normothermic reperfusion. $^{a}P < 0.05$ *vs* RLS; $^{c}P < 0.05$ *vs* BS. PEG: Polyethylene glycol; RLS: Ringer's lactate solution; BS: Base solution; BS + PEG1: BS with PEG-35 at 1 g/L; BS + PEG5: BS with PEG-35 at 5 g/L.

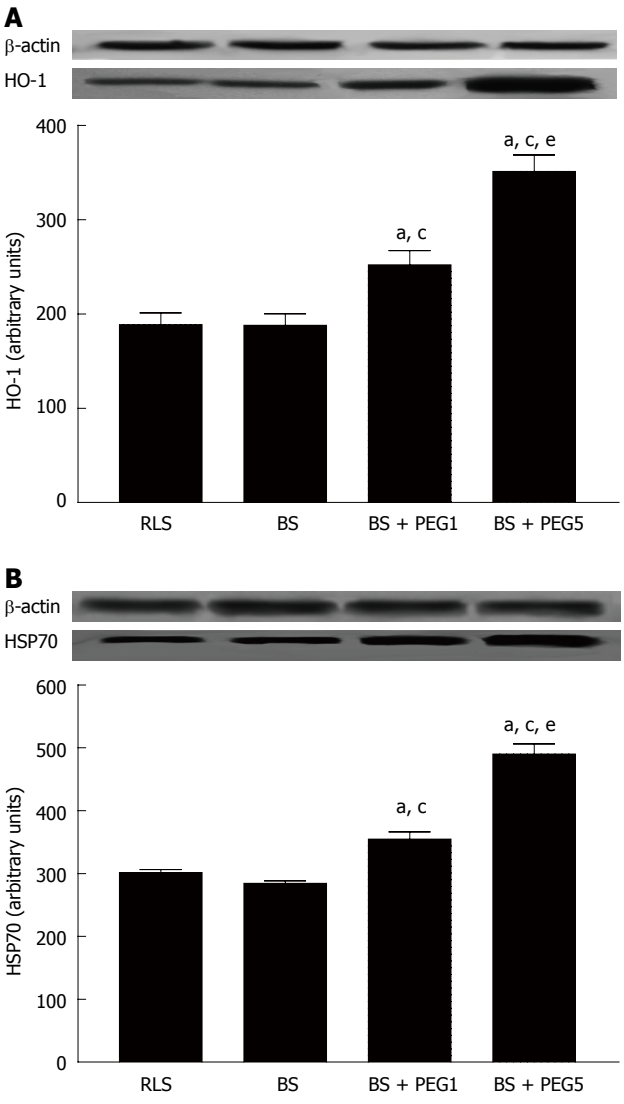

Figure 5 Heme oxygenase 1 (HO-1) (A) and HSP70 (B) protein levels in livers flushed with different washout solutions (RLS, BS, BS + PEG1, and BS + PEG5) and subjected to 2 h of normothermic reperfusion. $^{a}P < 0.05$ *vs* RLS; $^{c}P < 0.05$ *vs* BS; $^{e}P < 0.05$ *vs* BS + PEG1. PEG: Polyethylene glycol; RLS: Ringer's lactate solution; BS: Base solution; BS + PEG1: BS with PEG-35 at 1 g/L; BS + PEG5: BS with PEG-35 at 5 g/L.

ure 7B). The alterations in the actin cytoskeleton resulted in changes in cell shape and adhesiveness: we observed sinusoidal shape changes in livers rinsed with RLS as opposed to a normal hexagonal morphology in livers rinsed with the PEG-35 solutions (Figure 8).

Liver autophagy

In liver grafts subjected to 24 h of UW cold preservation and 2 h of reperfusion, we evaluated the incidence of liver graft autophagy by measuring the levels of established markers, Beclin-1 and LCB3. As evidenced in Figure 9, Beclin-1 and LCB3 levels fell significantly in livers washed with PEG-35 rinse solutions compared to those washed with either RLS or BS alone.

DISCUSSION

Several rinse solutions have been proposed for the efficient washout of liver grafts before transplantation. In

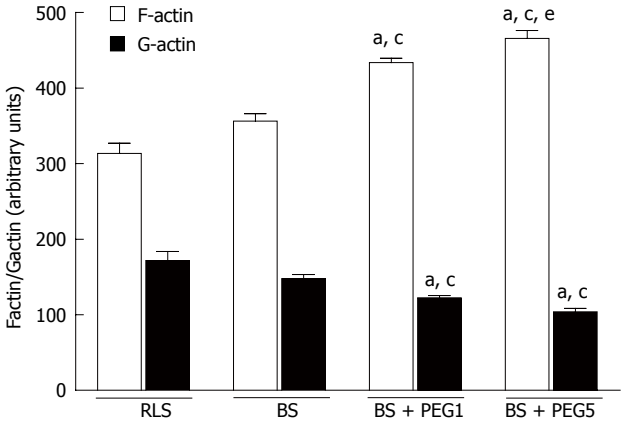

Figure 6 Effects of washout on liver cytoskeleton changes: changes in filamentous actin (F-actin) and globular actin (G-actin) ratio in livers flushed with different washout solutions (RLS, BS, BS + PEG1, and BS + PEG5) and subjected to 2 h of normothermic reperfusion. $^{a}P < 0.05$ *vs* RLS; $^{c}P < 0.05$ *vs* BS; $^{e}P < 0.05$ *vs* BS + PEG1. PEG: Polyethylene glycol; RLS: Ringer's lactate solution; BS: Base solution; BS + PEG1: BS with PEG-35 at 1 g/L; BS + PEG5: BS with PEG-35 at 5 g/L.

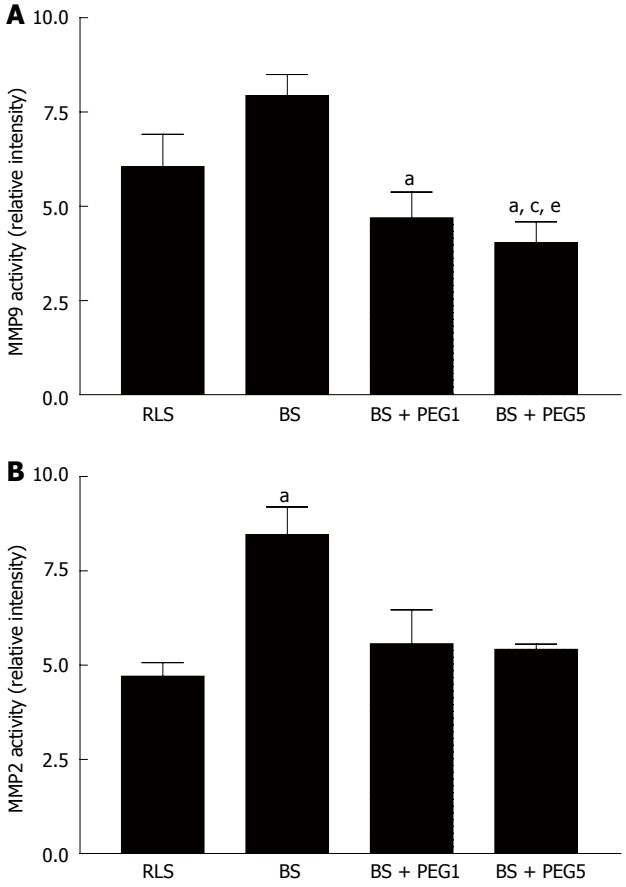

Figure 7 Effects of washout on metalloproteinase MMP2 (A) and MMP9 (B) protein activity levels in livers flushed with different washout solutions (RLS, BS, BS + PEG1, and BS + PEG5) and subjected to 2 h of normothermic reperfusion. $^{a}P < 0.05$ *vs* RLS; $^{c}P < 0.05$ *vs* BS; $^{e}P < 0.05$ *vs* BS + PEG1. PEG: Polyethylene glycol; RLS: Ringer's lactate solution; BS: Base solution; BS + PEG1: BS with PEG-35 at 1 g/L; BS + PEG5: BS with PEG-35 at 5 g/L.

this paper we demonstrate that the washout step is necessary for both the removal of remaining preservation solution, and also for protecting the liver graft against IRI. Following our experience in the use of PEG-35 for fatty liver preservation[17,28,29], we have explored its use as a rinse solution. Due to the fact that PEG-35 is the oncotic agent at low concentrations in the IGL-1 preservation solution, we compared rinse solutions supplemented with PEG-35 at 1 g/L and 5 g/L.

PEG polymers are water-soluble, biocompatible, non-toxic materials that are commercially available at different molecular weights[1,19]. PEG is approved for use in humans by the FDA, and its applications range from use as an excipient in drug formulations, cosmetics, and food preparations to use as a conjugating polymer for therapeutic protein delivery[31]. To date, the use of PEG in preservation solution has been associated with several advantages, but it has not yet been tested in rinse solutions. In this study, we demonstrated that the PEG-35 rinse solution prevented liver injury after 2 h of reperfusion when compared to the grafts flushed with either RLS or BS alone. This effective protection was concomitant with a subsequent improvement in hepatic function, reflected by bile production as a marker of biliary epithelial cell integrity after ischemia[17], and a significant reduction in vascular resistance. These results are consistent with previous reports evidencing the protective role of different PEG molecules for organ preservation[16,20,28].

A growing body of evidence indicates that mitochondrial dysfunction is a critical pathological process in liver IRI. Impaired mitochondrial function results in defective energy use and excessive reactive oxygen species generation[32]. Here we demonstrated that the addition of PEG-35 to a rinse solution prevented mitochondrial damage and oxidative stress, when compared to either RLS or BS solutions. Our results are in line with previously reported data demonstrating the effective antioxidant role of PEG, through the suppression of lipid peroxidation, after rewarming cold-stored hepatocytes[33]. This prevention was also accompanied by subsequent increases in HSP70 and HO-1 expression. Overexpression of both HSPs, well known markers of graft survival after transplantation[34], was more relevant for PEG-35 at 5 g/L when compared to 1 g/L suggesting that the higher concentration was more suitable for increasing liver graft tolerance to IRI.

In our study, the PEG-35 rinse solution increased phosphorylated AMPK levels. AMPK is an enzyme involved in cellular energy balance that regulates the downstream signaling pathways towards an energy-conserving state[35]. AMPK activation before or during organ preservation helps to limit organ injury and maintain graft quality[36,37]. We have previously reported that AMPK inducers ameliorated fatty liver graft preservation when added to preservation solutions[38]. We have now demonstrated that PEG-35 rinse solutions contribute to AMPK activation; furthermore, rinsing liver grafts with solutions containing PEG-35 resulted in activation of constitutive eNOS and subsequent NO generation[36,37]. NO is a gaseous vasodilator that protects liver endothelial

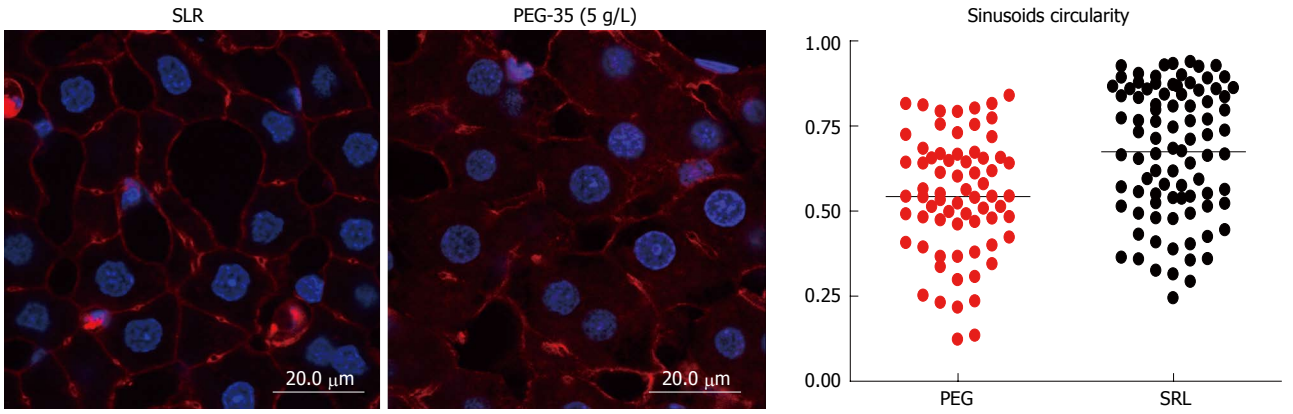

Figure 8 Confocal microscopy findings of a sinusoidal shape when livers were rinsed in a 5 g/L concentration of the polyethylene glycol 35 (PEG-35) solution. PEG: Polyethylene glycol; RLS: Ringer's lactate solution.

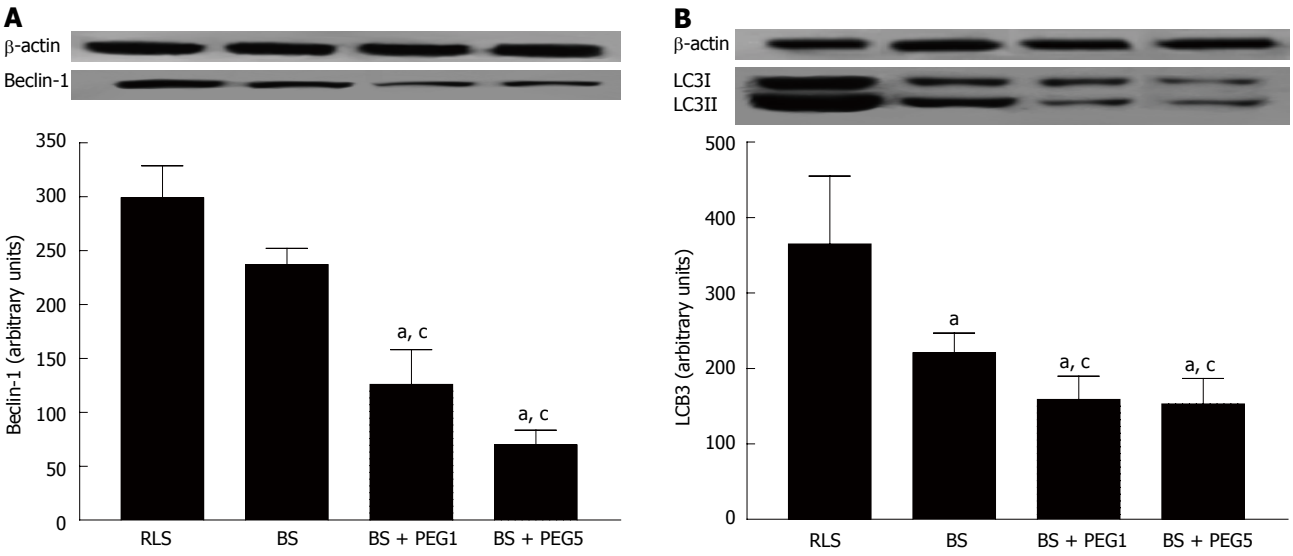

Figure 9 Effects of washout solution on liver graft autophagy: Beclin 1 (A) and LCB3 (B) protein levels in liver rinsed with different washout solutions (RLS, BS, BS + PEG1, and BS + PEG5). $^{a}P < 0.05$ *vs* RLS; $^{c}P < 0.05$ *vs* BS; $^{e}P < 0.05$ *vs* BS + PEG1. PEG: Polyethylene glycol; RLS: Ringer's lactate solution; BS: Base solution; BS + PEG1: BS with PEG-35 at 1 g/L; BS + PEG5: BS with PEG-35 at 5 g/L.

cells against IRI[39]. Its activation is consistent with the observed decreases in vascular resistance after washing out with PEG-35 rinse solutions, which are associated with NO generation and contribute to preventing microcirculation alterations after liver graft revascularization.

Changes in cytoskeletal structure and cell morphology occur in the liver following IRI that can ultimately lead to graft dysfunction[40-42]. Recently, it has been reported that PEG contributes to the regulation of endothelial cell barrier by rearranging the actin cytoskeleton[43]. Subcellular F-actin is an important component of the cytoskeleton, and the balance between F-actin and monomeric G-actin largely determines the functional outcome. It is important to note that F-actin forms microfilaments in liver cells, which are involved in intracellular processes, the maintenance of cell morphology, and bile canalicular motility necessary for bile secretion[42,44,45]. With this in mind, we explored the effects of PEG-35 rinse solutions on the liver cytoskeleton by measuring F- and G-actin distribution and ultrastructural changes by confocal microscopy. Livers subjected to washout with RLS and BS showed low F-actin content reflecting actin cytoskeletal derangement, probably associated with ischemia. In contrast, PEG-35 use reversed the loss of F-actin by increasing its polymerization, as reflected by an increased F-actin ratio. This confirms the protective effect of PEG-35 rinse solution in preventing the loss of cell integrity during IRI.

Extracellular matrix turnover, influenced by MMP9 and MMP2, occurs during tissue remodeling after IRI[46,47]. In experimental models of hepatic IRI, inhibiting MMP2 and MMP9 has also reduced tissue damage[30,48]. In this study, we demonstrated that the PEG-35 rinse solutions inhibited MMP9 and MMP2 activity, suggesting cytoskeletal stability compared to either RLS or BS. This fact is consistent with the reduction of morphological alterations on confocal microscopy. Specifically, we observed that liver graft cells rinsed with RLS or BS acquired a slightly deformed round shape, while those

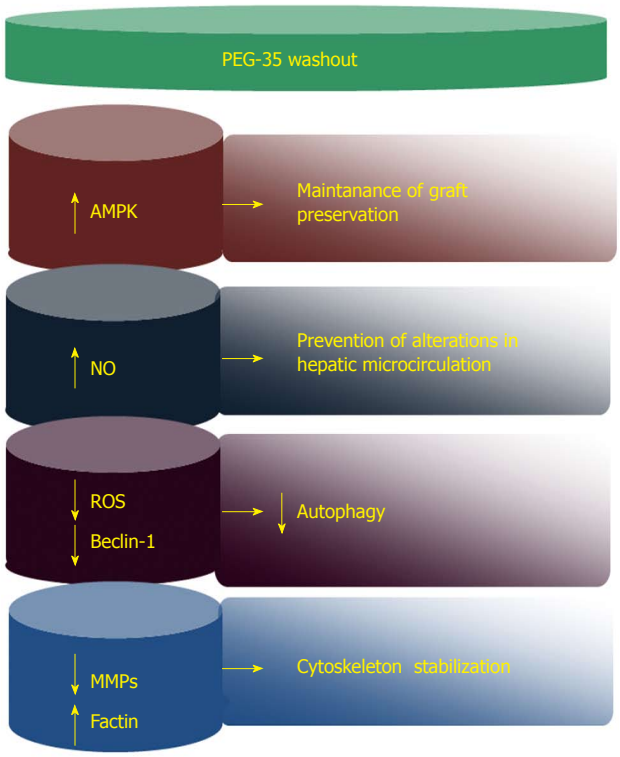

Figure 10 Benefits of Polyethylene glycol-35 washout solution. PEG: Polyethylene glycol; AMPK: Adenosine monophosphate-activated protein kinase.

washed with PEG-35 rinse solutions demonstrated normal morphology.

Our results suggest that cytoskeletal integrity is better preserved by the presence of PEG-35 in rinse solutions. This is consistent with another study where high-molecular-weight PEG physically bound to cardiac myocyte plasma membranes was observed to activate signaling pathways that protect against hypoxia-reoxygenation associated cell death[49]. It has also been reported that PEG joins various regulatory elements of the endothelial cell barrier, thus providing beneficial effects on the architecture of the endothelial cytoskeleton[43].

Autophagy is associated with the turnover of long-lived proteins, cytosolic components, or damaged organelles. It is a highly regulated process involving the formation and delivery of autophagosomes to lysosomes for degradation. Based on growing evidence linking autophagy to IRI[50-52], we evaluated its potential involvement during the liver graft washout process. Indeed, autophagy has long been recognized to occur in organs under stress conditions such as IRI[52,53], although its precise role remains unclear and controversial. Although we know that the activation of autophagy during ischemia is essential for cell survival and maintaining organ function, through AMPK dependent mechanisms, its role during reperfusion could be detrimental[52,54,55]. Autophagy in reperfusion is accompanied by a robust up-regulation of Beclin-1, which in turn is exacerbated by the generation of reactive oxygen species, leading to a massive degradation of vital molecules and autophagic cell death[55,56].

In this study, we demonstrated that liver graft washout using a rinse solution containing PEG-35 prevented liver autophagy. This is demonstrated through diminished Beclin-1 and LC3B levels, similarly to that reported for post-conditioning in a rat brain model[57]. The data reported here are consistent with studies carried out by Gotoh *et al*[58], who implicated autophagy in the initiation of graft dysfunction after rat liver transplantation. Thus, we can speculate that the prevention of mitochondrial damage and ROS production by PEG-35 could explain the decreases in Beclin-1 and LC3B after liver graft washout. The overall benefits of using PEG-35 as a rinse solution are summarized in Figure 10.

In conclusion, we have demonstrated that the use of a new rinse solution containing PEG-35 protects rat liver grafts against IRI and it could therefore be a useful clinical tool for increasing liver graft protection against reperfusion injury.

ACKNOWLEDGMENTS

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COMMENTS

Background

Ischemia-reperfusion injury (IRI) is a determinant factor of graft function during and after liver transplantation. IRI is a complex process accompanied by oxidative stress, loss of cell membrane integrity, and cell death. Liver graft washout prior to revascularization is an obligatory step to remove any remaining preservation solution; however, this practice is dependent on individual surgical practices. Liver graft washout strategies are not standardized and have not been investigated in detail. Here, we propose a new rinse solution containing polyethylene glycol (PEG) 35 as suitable for liver graft protection against IRI. In addition, the underlying mechanisms of IRI are investigated.

Research frontiers

PEG is a non-toxic, water soluble polymer that has been associated with beneficial effects after various insults, including IRI. PEG is known to decrease reactive oxygen species, to protect liver mitochondria, to protect against cell death, and to help preserve cell membrane integrity. The presence of PEG-35 (35000 k-daltons) in the novel Institut Georges Lopez (IGL) 1 preservation solution (as an oncotic agent) has been associated with the prevention of hepatic IRI. The benefits are due, in part, to adenosine monophosphate protein kinase (AMPK) and endothelial nitric oxide synthase (eNOS) activation. Thus, we added PEG-35 to a rinse solution to evaluate the potential benefits in washing-out and increased graft protection against IRI.

Innovations and breakthroughs

The authors provide evidence that washing out the liver grafts prior to reperfusion with a rinse solution that contains PEG-35 is an effective tool for providing a more effective protection against IRI. This is confirmed by decreases in hepatic injury and oxidative stress, ameliorated hepatic function, and more efficient preservation of liver endothelial integrity.

Applications

Liver graft washout with a PEG-35 rinse solution is a useful strategy for efficient graft rinse and provides superior prevention before graft revascularization in clinical practice.

Terminology

Rinse solution is used for removing the remaining preservation solution and any toxic agents produced during cold storage of the graft. The use of rinse solution favors the most suitable graft revascularization and survival after transplantation.

Peer review

In this study, the authors studied a new rinse solution containing PEG-35 for

preventing IRI in the liver graft. Using biochemical determinations, Western Blot Analysis, zymography and confocal fluorescence microscopy, they studied the function of PEG-35 in the processes of liver injury, liver function, oxidative stress, mitochondrial injury, liver cytoskeleton alteration and liver autophagy. While the role of PEG-35 in the protection against IRI is not surprising, I believe that there are merits in this study because it may give some cues for future research and clinical application in LT.

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V. DISCUSSION

IRI is an important cause of liver damage occurring during surgical procedures including hepatic resection and liver transplantation, and represents the main cause of graft dysfunction and primary non function after transplantation [117]. PEGs are water soluble non-toxic polymers that are known to play an important role in the cytoprotection against ischemic damage. Recent studies have shown that PEG exerts anti-inflammatory, anti-apoptotic, immunosuppressive and membrane stabilization effects [2-4, 6, 198, 199]. From this perspective, it is reasonable to expect that PEG administration may be an effective therapeutic strategy against liver IRI.

The aim of this thesis was to investigate the beneficial effects of PEG 35 in different models of IRI that mimic clinical situation of liver surgery. In the first study, we investigated the impact of the administration of intravenous PEG 35 before liver warm IRI. In the second one, we investigated whether intravenously administrated PEG 35 could protect against cold IRI in steatotic rat livers. Finally, we developed a new washout solution containing PEG 35 to prevent reperfusion injury after prolonged cold preservation.

We have focused our studies on PEG with molecular weight of 35kDa because it has been previously demonstrated that PEG 35 was effective to prevent cold IRI in liver when it was added to IGL-1 preservation solutions [23, 194, 200]. Moreover, PEG 35 has been shown to protect renal cells against cold ischemia [201]. However, PEGs with different molecular weights such as PEG8 [187] or PEG20 [202] might also be useful for conferring protection against IRI. However, PEG 35 was more effective than PEG20 in protecting porcine proximal tubular epithelial cell line against cold storage at the same doses used [201]. Ramified PEGs showing the some molecular weight as linear ones could have more interactions with hepatocytes membrane and thus afford an additional protection of liver cytoskeleton.

1. PEG preconditioning in warm IRI

In order to achieve an efficient hepato-protection, PEG 35 was injected to Sprague Dawley rats at 2 and 10 mg/kg before the induction of one hour of partial ischemia (70%) and two hours of normothermic reperfusion. Our results showed that PEG 35 efficiently protects rat liver against IRI and that this effect is dose dependent. Only the dose of 10 mg/kg shows a significant decrease in liver injuries.

The lessened hepatic injury observed when PEG 35 was administered intravenously coincided with increased mitochondrial preservation, as revealed by decreases in GLDH levels and intravital microscopy findings. This fact is of great importance since mitochondrial protection is essential for graft survival after transplantation [203]

We next went inside into the mechanisms of protection of PEG 35 and determined whether PEG 35 beneficial effect could be related to the activation of protective cell signalling pathways. Our results showed that PEG 35 at 10 mg/kg contributes to AMPK and AKT activation. This is in line with previous reports showing that PEG20 protects against heart ischemia through AKT activation [6, 197]. Moreover, a crosslink between AMPK and Akt activation has been demonstrated in IRI [204]. These observations suggest that PEG protective effects are not only related to its known role as an oncotic support but also to some pharmacological properties.

Nitric oxide (NO) is a gaseous vasodilator implicated in the regulation of hepatic microcirculation, which is impaired upon IRI [29, 188, 205, 206]. In this sense, we found that PEG 35 at 10 mg/kg significantly increased NOx levels and this was not correlated with eNOS activation. This observation is concomitant with a previous published report showing that PEG induced arteriolar dilatation was independent of eNOS induction [188]. The authors suggested that PEG vasodilation effect might be related to its hydrophilicity and ability to insert into the endothelial surface layer, which preserves shear stress mediated vasodilation during ischemia reperfusion [188].

Given that apoptotic cell death is involved in IRI, we then investigated the levels of the apoptotic markers caspase 3 and 9. As it was expected, ischemia reperfusion provoked an important induction of apoptosis, which was significantly prevented when PEG 35 was administered at 10 mg/kg. These observations are in line with the results observed by Malhotra *et al.* who demonstrated that PEG15-20 protected cardiac myocytes from hypoxia and re-oxygenation induced apoptosis [6]. Moreover, we have previously reported that PEG 35 addition to preservation solutions ameliorated liver graft preservation by reducing apoptosis after transplantation [68].

Structural alterations of the cytoskeleton following ischemia reperfusion have been reported to cause disturbances of intracellular transport processes, cell motility and microcirculation leading to organ dysfunction [75, 207-209]. In liver cells, F-actin is a

relevant component of liver cytoskeleton which forms microfilaments involved in intracellular transport processes, such as exocytosis and endocytosis, maintenance of cell shape, and canalicular motility responsible for bile flow [209-212]. In this context, we have explored whether PEG 35 preconditioning could maintain the cytoskeleton structure and preserve the morphological characteristics of hepatocytes. Indeed, our present data confirmed that F-actin/G-actin ratio is increased as a consequence of PEG administration at 10 mg/kg. Furthermore, confocal microscopy findings confirmed that PEG contributes to the regulation of endothelial cell barrier by rearranging the actin cytoskeleton. Hepatocytes presented a more normal hexagonal morphology in livers pretreated with PEG 35 compared with livers submitted to IRI. All of these observations are consistent with a recent study in lung endothelial cells evidencing that PEG15-20 preserves the architecture of the endothelial cytoskeleton [213]. Moreover, it has been demonstrated that PEG induced membrane stabilization through sarcolemmal lipid-raft architecture preservation [6]. These published data suggested that PEG interaction with cell membrane (adhesion or intercalation) preserved the cytoskeleton. In our study we further evidenced that PEG-induced-p38 MAPK activation may also be responsible for cytoskeleton preservation. However, the precise mechanisms of how PEG could affect cytoskeleton remain to be elucidated.

Until now, PEG was used only as additive to preservation solution in IRI researches. This study gives the first evidences that PEG could be used as a preconditioning agent to protect against liver ischemia reperfusion damage. The innovation of this study was the route of administration of PEG: intravenously. Next, we used the effective dose obtained in this study (10 mg/Kg) to investigate the effects of PEG in a model of cold ischemia in steatotic liver grafts.

2. PEG preconditioning in cold IRI

This is the first report describing the use of PEG to protect steatotic livers against cold IRI. In this study, we have shown that the protective effects of PEG 35 that were seen in livers subjected to one hour of ischemia are applicable to an *ex vivo* rat model of cold IRI. Intravenous delivery of PEG 35 at 10 mg/kg prior to 24h cold ischemia followed by 2h resulted in significant recovery of liver graft function, markedly less mitochondrial

injury as measured by GLDH release and intravital microscopy findings, and decreased vascular resistance. Similar to our previous study, PEG treatment again led to the upregulation of prosurvival signaling as measured by the phosphorylation of Akt and AMPK. Conversely, PEG regulated MAPKs by reducing p-p38 activation in contrary to the first study where we have seen that PEG induced a significant activation of p38 MAPKs. This fact may be explained by the dual role of p38-MAPKs in IRI. Indeed, it has been shown that p38 activation was protective in warm ischemia but its induction worsened cold ischemia [76, 94, 214, 215].

In our study, we have used the isolated perfused rat liver (IPRL) model, a widely used and appreciated method to assess cellular injury and liver function in an isolated setting. In comparison to other *in vitro* models, the IPRL-model does have considerable advantages, such as the use of the entire intact organ instead of only single cells or several layers of cells (i.e. isolated hepatocytes or the liver slice model) and an intact cellular architecture. Furthermore, the use of an a-cellular perfusion solution (Krebs solution), prevents allo-reactivity and permit conclusive answer in IRI. Regarding liver transplantation, IPRL model presents the advantages of minimizing the use of laboratory animals, and the suppression of the immunological reactions and the influences of other organs occurring during transplantation. On the other hand, this model also has some disadvantages such as the duration of reperfusion, which is limited to 90-120 minutes and the fact that it remains an *in vitro* tool, and merely simulates the initial phase after liver transplantation. In this sense IPRL model could be considered as a pre-screening model.

We further explored mitochondrial depolarization after cold preservation and we evidenced that PEG 35 prevented fatty liver mitochondria depolarization after prolonged cold ischemia. Also, mitochondrial injury was lessened after liver reperfusion as indicated by the decrease in GLDH release. These results are in accordance with our first study and with previous published data showing that PEG 2 kDa improved mitochondrial function *in vitro* and *in vivo* after acute spinal cord injury [3]. Moreover, PEG of 4 kDa inhibited mitochondrial pore transition (MPT) and cytochrome C release in rat liver mitochondria [216]. Also, PEG (1.5 and 2kDa) was able to across the cytoplasmic membrane and directly interact with neuronal mitochondria to preserve its structure and restore function [2]. Interestingly, PEG with higher molecular weight (4

kDa) failed to exert significant improvement in neuronal injured mitochondria indicating that PEG-mediated mitochondrial protection is dependent on the size of PEG [2]. In this sense, the mechanism by which PEG 35 decreased mitochondrial damage needs more profound investigation.

In fatty livers, the fat accumulation in the cytoplasm of the hepatocytes provokes severe disturbances in the sinusoidal flow during reperfusion [217]. PEG 35 protective mechanisms were associated with the activation of eNOS responsible of NO generation and the subsequent diminution of vascular resistance. This could also explain the protective mechanism of PEG toward mitochondria as it has been shown that NO protects rat hepatocytes against reperfusion injury through the inhibition of MPT [205]. However, in our first study we have shown that PEG 35 increased NO production and this effect was not related to eNOS activation. The difference between the two experimental models (warm ischemia vs cold ischemia) and the duration of the ischemia may explain the contrast between the different findings in our studies.

In our previous studies we evidenced that PEG could be considered as a preconditioning drug protecting against both warm and cold IRI. In the next study, we investigated the potential protective role of PEG as a post-conditioning agent by its addition to washout solution.

3. PEG postconditioning

In this study we demonstrated that PEG 35 addition to a newly developed washout solution was necessary to achieve liver graft protection against reperfusion injury. Moreover, this protection was dose dependent as the concentration of PEG 35 of 5 g/L was more efficient than 1 g/L. The decreased hepatic injury was concomitant with the subsequent improvement in hepatic function reflected by bile production and a significant reduction in vascular resistance.

Moreover, the addition of PEG 35 to a rinse solution prevented mitochondrial damage and oxidative stress, when compared to either RLS or BS solutions. These results are in line with the two first studies demonstrating the preservation of mitochondrial integrity and with previous reported data showing the antioxidant role of PEG after rewarming cold-stored hepatocytes [187]. The prevention of reperfusion damage was also

accompanied by subsequent increases in HSP70 and HO-1 expression, two well-known markers of graft survival after transplantation [218]. Also, we observed a significant increase in AMPK phosphorylation and activation of eNOS.

In warm IRI, we demonstrated a protective role of PEG by preserving cytoskeleton integrity. With this in mind, we explored the effects of PEG 35 rinse solution on the liver cytoskeleton by measuring F and G actin distribution and ultra-structural changes by confocal microscopy. Livers subjected to washout with RLS and BS showed low F-actin content reflecting actin cytoskeletal derangement, probably associated with ischemia. In contrast, PEG 35 use reversed the loss of F-actin by increasing its polymerization, as reflected by an increased F-actin ratio. This confirms the protective effect of PEG 35 rinse solution in preventing the loss of cell integrity during IRI.

Extracellular matrix turnover, influenced by MMP9 and MMP2, occurs during tissue remodelling after IRI [219, 220]. In experimental models of hepatic IRI, inhibiting MMP2 and MMP9 has also reduced tissue damage [221, 222]. In this study, we demonstrated that the PEG 35 rinse solutions inhibited MMP9 and MMP2 activity, suggesting cytoskeletal stability compared to either RLS or BS. This fact is consistent with the reduction of morphological alterations on confocal microscopy. Specifically, we observed that liver graft cells rinsed with RLS or BS acquired a slightly deformed round shape, while those washed with PEG 35 rinse solutions demonstrated normal morphology.

Based on growing evidence linking autophagy to IRI [223-225], we evaluated its potential involvement during liver graft washout process. In this study, we demonstrated that liver graft washout using a rinse solution containing PEG 35 prevented liver autophagy. This is demonstrated through diminished Beclin-1 and LC3B levels, similarly to that reported for post-conditioning in a rat brain model [226]. The data reported here are consistent with studies carried out by Gotoh et al [227], who implicated autophagy in the initiation of graft dysfunction after rat liver transplantation. Thus, we can speculate that the prevention of mitochondrial damage and ROS production by PEG 35 could explain the decreases in Beclin-1 and LC3B after liver graft washout.

4. Perspectives

In this thesis we demonstrated the protective role of PEG 35 against hepatic IRI in three different conditions. PEG 35 preconditioning was efficient to protect against both cold and warm IRI. Also, PEG addition to washout solution (PEG postconditioning) decreased significantly reperfusion injury.

PEGs are multifunctional molecules that can target several pathological processes associated with organ injury following IRI including oxidative stress, mitochondrial dysfunction, loose of membrane integrity and cell death. They also present the advantages of being safe and could be adopted as a cost-effective strategy in clinical situation of ischemic damage. Interestingly, we have shown that the effect of PEG 35 is dose dependent which raises the question about the relationship between molecular weight, concentration and effectiveness.

In this thesis, we focused our attention on PEG 35, however, PEG of 4 ,8 and 20 kDa have been also shown to protect against various type of organ damage including ischemic injuries [5, 186, 197]. Also, it could be possible that PEG 35 is more effective in liver ischemic injury and PEG 20 is more effective in heart or kidney [6, 193]. Moreover, taking into account the interaction between PEG and cell membrane, it might be of great interest to test other types of PEG such as ramified ones, which could have more interactions with hepatocytes membrane and thus afford an additional protection of liver cytoskeleton.

In our studies, we have demonstrated the effectiveness of PEG in protecting mitochondria. However, the precise mechanism of protection still needs to be elucidated. Does PEG 35 cross the membrane under ischemic condition and interacts directly with the mitochondria or does this protective effect depend on the preservation of cytoskeleton? Does PEG with lower molecular weight is more effective in reducing mitochondrial injuries? Probably, *in vitro* studies with isolated hepatocytes and isolated mitochondria could give conclusive response to these questions.

Due to their large molecular size and hydrophilic properties, PEGs generate an oncotic sink to reduce cellular edema and stabilize the cytoskeleton. It will be also very

interesting to study the effects of PEG in glycocalyx. Indeed, a growing body of evidence suggests a key role of the glycocalyx in IRI [40, 80].

Finally, although we clearly demonstrated the protective effect of PEG against cold IRI, these results need to be confirmed in a model of liver transplantation. Such model could also reveal new mechanisms as it has been suggested that PEG reduces immune response and inflammation [228].

Taking this into account, we firmly believe that the use of PEG is a promising therapeutic approach to decrease IRI in clinical practice. The results observed with steatotic livers, the safety and cost-effectiveness of this molecule encourages the future clinical investigations of PEG in the field of IRI.

VI. CONCLUSIONS

The conclusions of the present thesis are the following:

- Intravenous administration of PEG 35 at 10 mg/kg protects the liver in an experimental model of warm IRI in rats. The protective mechanisms are associated with the activation of the pro survival pathways Akt and AMPK and the inhibition of apoptosis. PEG 35 also protects the hepatocyte morphology by increasing F-/G-actin ratio and activating p-p38.

- Intravenous administration of PEG 35 at 10 mg/kg protects steatotic livers in an experimental model of cold IRI in obese rats. The protective effects of PEG 35 are mediated by the preservation of mitochondrial status, the stabilisation of the cytoskeleton and the regulation of the cytoprotective AMPK and Akt signalling pathways.

- Liver graft washout with a PEG 35-containing rinse solution increases the protection against IRI in a model of isolated perfused rat liver. Protection was due to the inhibition of metalloproteinases, the activation of cytoprotective AMPK and eNOS signalling pathways and the preservation of cytoskeleton integrity.

VII. RESUMEN

La lesión por isquemia reperfusión (I/R) es un proceso complejo que tiene lugar cuando un órgano se ve privado del aporte sanguíneo (isquemia) de forma temporal y se manifiesta de forma predominante después del posterior restablecimiento del flujo sanguíneo (reperfusión).

Existen numerosas situaciones en la práctica clínica en las que el hígado se ve sometido a una situación de isquemia y posterior reperfusión, entre ellas, la resección hepática y el trasplante hepático. En el trasplante hepático la lesión por I/R es la causa principal del fallo del injerto y del re-trasplante tras el rechazo inmunológico. Además, la lesión por I/R hepática afecta otros órganos aparte del hígado, como el pulmón, riñón y el páncreas. El procedimiento estándar para el trasplante hepático se inicia con la extracción del hígado del donante. Antes de su extracción el hígado es perfundido con la solución de preservación, a una temperatura de 4ºC. En este momento comienza la fase de isquemia fría. A continuación, el hígado es extraído del donante y colocado en una solución de preservación a baja temperatura (4ºC), con la finalidad de enlentecer al máximo el metabolismo hepático hasta su posterior implante en el receptor. Este período de isquemia fría suele durar en la práctica clínica de 6 a 8 h tras el cual, el órgano es sometido a un período de isquemia caliente, que se prolonga desde que el órgano es situado en la cavidad abdominal del receptor hasta que se restablece el flujo sanguíneo en el hígado trasplantado. Este período de isquemia caliente corresponde al tiempo empleado en realizar la anastomosis de los vasos sanguíneos hepáticos en la intervención quirúrgica. Al restablecerse el flujo sanguíneo en el órgano comienza la fase de reperfusión.

Estos períodos de isquemia fría y caliente, y posterior reperfusión que tienen lugar en el trasplante hepático provocan la activación de una serie de mecanismos que dan lugar a la denominada lesión por I/R. La fisiopatología de la lesión por I/R es muy compleja y no está totalmente definida, ya que numerosos factores y mediadores están involucrados. La separación de los eventos celulares que ocurren durante la isquemia y la reperfusión no es absoluta, ya que durante la isquemia se inducen diversos procesos que son determinantes para la inducción de la lesión durante la reperfusión.

En la fase de isquemia, la falta de oxígeno detiene la cadena respiratoria mitocondrial y se produce la depleción de los niveles de ATP. La disminución del nivel energético

conduce hacia el metabolismo anaerobio (vía glucolisis y fermentación láctica), con un rendimiento energético inferior que en condiciones aeróbicas, y a la acumulación de sus metabolitos tales como el ácido láctico. La acidosis metabólica resultante altera la cinética normal de los enzimas. La depleción de ATP provoca la alteración de todos los sistemas ATP-dependientes, entre ellos las bombas de sodio/potasio y de calcio, con la consecuente pérdida del gradiente a través de las membranas celulares. Una consecuencia de estas alteraciones es la acumulación del Ca^{2+} citosólico, el cual activa diferentes sistemas enzimáticos, tales como fosfolipasas y proteasas implicadas en la respuesta inflamatoria que posteriormente se desarrollará en la fase de reperfusión. Las proteasas tras su activación, catalizan la conversión de la xantina deshidrogenasa (XDH) a xantina oxidasa (XOD), enzima implicada en la formación de RLO (radicales libres de oxígeno) durante la reperfusión. Todos estos fenómenos inducen alteraciones en la permeabilidad e integridad de la membrana citoplasmática y de los orgánulos intracelulares, que pueden producir el edema celular, y finalmente la muerte celular.

Durante la reperfusión se produce el restablecimiento del flujo sanguíneo y con él ocurren la mayoría de las lesiones. Aunque produce efectos beneficiosos en el tejido isquémico al recuperarse los niveles de ATP, también se ponen de manifiesto los efectos de los metabolitos tóxicos formados durante la fase previa, que tienen repercusión a nivel local y sistémico. Diversos autores sugieren que en la lesión de reperfusión se puede distinguir dos fases [17]:

- Fase precoz o aguda: comprende las primeras 3 a 6 h después de la reperfusión. El principal acontecimiento en esta fase es la activación de las células de Kupffer. Esta activación se lleva a cabo por la acción previa de componentes activados del sistema del complemento, el reclutamiento y la activación de los linfocitos T $CD4^{+}$.

- Fase tardía o subaguda: se caracteriza por una infiltración masiva de neutrófilos, alcanza su máximo a las 18–24 h de la reperfusión. Estos neutrófilos activados liberan RLO y proteasas, ambos causantes del estrés oxidativo y de la lesión hepatocelular que se produce en esta fase de la lesión por reperfusión, que supera en gravedad a la de la fase precoz. El reclutamiento de neutrófilos polimorfonucleares (PMN) en el hígado tras la I/R es tan acusado que la reducción aguda de su recuento periférico se ha propuesto como marcador intraoperatorio precoz de la lesión por reperfusión del injerto hepático [229]. Además, durante la reperfusión, el daño mitocondrial, el estrés oxidativo, el

estrés del retículo endoplasmático y las citokinas juegan un papel importante en la progresión del daño, especialmente en el caso del hígado esteatósico. También, el daño hepático por I/R está acompañado de alteraciones en el citosqueleto, lo cual resulta en perturbaciones en los procesos intracelulares de trasporte y en la microcirculación. La F-actina forma los microfilamentos de actina que contribuyen al mantenimiento de la forma celular y, durante la isquemia, la F-actina se reduce y contribuye a la pérdida de la integridad celular y finalmente al daño del órgano [21].

Los polietilenglicoles (PEGs) son polímeros solubles en agua, no tóxicos, con diferentes pesos moleculares, que se utilizan como coadyuvantes en la administración de drogas, para prolongar su farmacocinética y son muy bien tolerados en patologías gastrointestinales. Algunos de ellos, con un peso molecular de 20 kDa (PEG20) y de 35 kDa (PEG 35) forman parte de la composición de soluciones de preservación de órganos (SCOT e IGL-1) utilizadas en diversos ensayos experimentales y clínicos [157, 193, 230]. Basándonos en investigaciones previas que comparan las soluciones UW vs IGL-1, cuya única diferencia reside en el factor oncótico HES (en UW) vs PEG 35(en IGL-1) y la concentración de iones Na+/K+, podemos sugerir que el efecto protector de IGL-1 es debido a la presencia de PEG 35. Además, en varios modelos experimentales de I/R *in vivo* e *in vitro* se ha reportado que varios PEGs ejercen efectos beneficiosos, mediante la reducción de las ROS y de la muerte celular [4, 159, 187, 197].

Atendiendo a lo anteriormente expuesto, la utilización de PEGs puede constituir una excelente herramienta para prevenir el daño hepático por isquemia reperfusión asociado al trasplante. El objetivo de este estudio es investigar los efectos beneficiosos del PEG 35 en diferentes modelos de lesión por I/R, que imitan una cirugía hepática. En el primer estudio, se investigó el impacto de la administración intravenosa de PEG 35 previa a la inducción de una lesión por I/R caliente. En el segundo, se investigó si el PEG 35 administrado por vía intravenosa podría proteger contra la lesión por I/R fría en hígados de rata esteatósicos. Por último, hemos desarrollado una nueva solución de lavado que contiene PEG 35 para evitar el daño por reperfusión después de la conservación en frío prolongado.

Hemos centrado nuestros estudios en el PEG con peso molecular de 35 kDa, ya que se ha demostrado previamente que el PEG 35 reduce la lesión por I/R fría en el hígado

cuando es añadido a soluciones de preservación IGL-1 [68, 156, 158]. Así también se ha demostrado que el PEG 35 puede proteger a las células renales contra la isquemia fría [154]. Por otro lado, los PEG con diferentes pesos moleculares, tales como el PEG8 [144] o PEG20 [6] también protegen contra la I/R. Sin embargo, el PEG 35 resulta más eficaz que el PEG20 en la protección de células epiteliales porcinas del túbulo proximal contra el almacenamiento en frío a las mismas dosis usadas [201]. Los PEG ramificados que presentan el mismo peso molecular que los lineales podrían tener más interacción con la membrana de los hepatocitos y así permitir una protección adicional del citoesqueleto del hígado.

1. El precondicionamiento hepático con PEG en un modelo de I/R caliente

Con el fin de lograr una eficiente hepato-protección, se inyectó PEG 35 a ratas Sprague Dawley a 2 y 10 mg/ kg antes de la inducción de una hora de isquemia parcial (70%) y dos horas de reperfusión normotérmica. Los resultados muestran que el PEG 35 protege el hígado de rata frente a la I/R y que este efecto es dependiente de la dosis. Sólo la dosis de 10 mg / kg muestra una disminución significativa en las lesiones hepáticas.

La disminución de la lesión hepática observada cuando el PEG 35 se administra por vía intravenosa coincide con un aumento de la preservación mitocondrial, que se revela por la disminución en los niveles de GLDH y por los hallazgos en la microscopía intravital. Este hecho es de gran importancia, ya que la protección mitocondrial es esencial para la supervivencia del injerto después del trasplante [48, 205]

A continuación, se determinó si el efecto beneficioso del PEG 35 podría estar relacionado con la activación de vías de señalización de protección celular. Nuestros resultados mostraron que el PEG 35 a 10 mg / kg contribuye a la activación de AMPK y Akt. Esto está en consonancia con estudios anteriores que muestran que el PEG20 protege frente a la isquemia cardiaca a través de la activación de Akt [6, 197]. Por otra parte, una asociación entre AMPK y la activación de Akt se ha demostrado en la I/R [204]. Estas observaciones sugieren que los efectos protectores del PEG no sólo están relacionados con su papel conocido como apoyo oncótico sino también con algunas propiedades farmacológicas.

El óxido nítrico (NO) es un gas vasodilatador implicado en la regulación de la microcirculación hepática que se deteriora en la I/R [29, 89]. En este sentido, encontramos que el PEG 35 a 10 mg/kg aumentó significativamente los niveles de NOx y esto no se correlacionó con la activación de la eNOS. Esta observación es concomitante con un estudio que muestra que el PEG induce vasodilatación arteriolar independiente de la inducción de la eNOS [188]. Los autores sugirieron que el efecto vasodilatador del PEG podría estar relacionado con su hidrofilicidad y su capacidad para insertarse en la capa de la superficie endotelial [188].

Dado que la muerte celular por apoptosis está implicada en I/R, investigamos entonces los niveles de marcadores de apoptosis como las caspasas 3 y 9. Como se esperaba, la I/R provocó una inducción importante de la apoptosis, lo que fue impedido significativamente cuando se administró PEG 35 a 10 mg/kg. Estas observaciones están en línea con los resultados observados por Malhotra *et al.* quienes demostraron que el PEG15-20 protege los miocitos cardíacos de la hipoxia y reoxigenación inducida por apoptosis [6]. Por otra parte, hemos informado anteriormente de que la adición de PEG 35 a soluciones de preservación mejora la preservación del injerto hepático mediante la reducción de la apoptosis después del trasplante [68].

Se han reportado alteraciones estructurales del citoesqueleto después de la isquemia reperfusión que causan perturbaciones de los procesos intracelulares de transporte, la motilidad celular y la microcirculación, que conducen a la disfunción de órganos [21, 207]. En las células del hígado, la F-actina es un componente relevante del citoesqueleto que forma microfilamentos que participan en los procesos de transporte intracelulares, tales como la exocitosis y endocitosis, el mantenimiento de la forma celular, y la motilidad canalicular responsable del flujo de la bilis [72, 231]. En este contexto, hemos explorado si el preacondicionamiento del PEG 35 podría mantener la estructura del citoesqueleto y preservar las características morfológicas de los hepatocitos. De hecho, nuestros datos actuales confirman que la relación F-actina/G-actina incrementa como consecuencia de la administración de PEG a 10 mg/kg. Por otra parte, los resultados de microscopía confocal confirman que el PEG contribuye a la regulación de la barrera de células endoteliales reordenando el citoesqueleto de actina. Los hepatocitos presentan una morfología hexagonal más normal en los hígados tratados previamente con PEG 35 en comparación con hígados sometidos a la I/R. Todas estas observaciones son

consistentes con un estudio reciente evidenciando que el PEG15-20 conserva la arquitectura del citoesqueleto endotelial las células endoteliales pulmonares [213]. Por otra parte, se ha demostrado que el PEG induce la estabilización de la membrana a través de la preservación de la arquitectura del sarcolema [6]. Estos datos publicados sugieren que la interacción del PEG con la membrana celular (adhesión o intercalación) conserva el citoesqueleto. Además, en nuestro estudio evidenciamos que la activación de MAPK p38 inducida por el PEG también puede ser responsable de la preservación del citoesqueleto. Sin embargo, los mecanismos precisos de cómo el PEG podría afectar el citoesqueleto aún no se han dilucidado.

Hasta ahora, el PEG se ha utilizado sólo como aditivo a la solución de preservación en estudios de I/R. Este estudio da las primeras evidencias que el PEG se podría utilizar como un agente de preacondicionamiento para proteger contra el daño por isquemia y reperfusión en el hígado. La innovación de este estudio fue la vía de administración de PEG: la vía intravenosa. A continuación, se utilizó la dosis efectiva de este estudio (10 mg / kg) para investigar los efectos del PEG en la isquemia fría en injertos hepáticos esteatósicos

2. El precondicionamiento con PEG en un modelo de I/R fría

Este es el primer estudio que describe el uso del PEG para proteger el hígado esteatósico contra la I/R fría. En este estudio, hemos demostrado que los efectos protectores del PEG 35 que se observaron en los hígados sometidos a una hora de isquemia son extrapolables a un modelo ex-vivo de I/R fría en rata. La administración intravenosa de PEG 35 a 10 mg/kg previa a una isquemia fría de 24horas seguida por 2 h de reperfusion, dió como resultado una recuperación significativa de la función del injerto hepático, así como una disminución marcada de la lesión mitocondrial que se midió por la liberación de GLDH, los hallazgos de microscopía intravital, y la disminución de la resistencia vascular. Similar a nuestro estudio anterior, el tratamiento con PEG llevó de nuevo a la regulación al alza de la señalización de supervivencia del infarto medido por la fosforilación de AKT y AMPK. Sin embargo, la regulación de las MAPKs a través del PEG se traduce en una reducción de la activación del p-p38 contrariamente al primer estudio en el que hemos visto que el PEG indujo una activación significativa de p38. Este hecho puede explicarse por el doble papel de la p38 MAPK en la I/R. De hecho, se ha demostrado que la activación de la p38 cumple un

papel protector en la isquemia caliente pero su inducción en la isquemia fría es dañina [90, 92, 94, 215].

En nuestro estudio, hemos utilizado el modelo de hígado de rata aislado y perfundido (HRAP), un método ampliamente utilizado y apreciado para evaluar la lesión celular y la función hepática en un entorno aislado. En comparación con otros modelos *in vitro*, el modelo HRAP tiene ventajas considerables, tales como el uso de todo el órgano intacto en lugar de sólo las células individuales o varias capas de células y una intacta arquitectura celular. Además, el uso de una solución de perfusión a-celular, (solución de Krebs) evita alo-reactividad y permite una respuesta concluyente en la I/R. En cuanto al trasplante de hígado, el modelo HRAP presenta las ventajas de reducir al mínimo el uso de animales de laboratorio, suprimir las reacciones inmunológicas y las influencias de otro órgano que se producen durante el trasplante. La desventaja del modelo HRAP es la duración de la reperfusión, que se limita a 90-120 minutos y el hecho de que sigue siendo una herramienta in-vitro, y verdaderamente simula la fase inicial después del trasplante hepático. En este sentido el modelo HRAP podría considerarse como un modelo pre-screening.

Siguiente, hemos explorado la despolarización mitocondrial después de la conservación del hígado y se evidenció que el PEG 35 impidió la despolarización mitocondrial de hígados grasos después de una isquemia fría prolongada. Además, la lesión mitocondrial disminuyó después de la reperfusión del hígado tal como indica la disminución en la liberación de GLDH. Estos resultados están de acuerdo con nuestro primer estudio y con datos anteriores publicados que muestran que el PEG 2 kDa mejora la función mitocondrial *in vitro* e *in vivo* después de la lesión de la médula espinal aguda [186]. Por otra parte, el PEG de 4 kDa inhibe transición del poro mitocondrial (MPT) y la liberación de citocromo C en las mitocondrias de hígado de rata [216]. También, los PEGs (1,5 y 2 kDa) son capaces de atravesar la membrana citoplasmática e interactuar directamente con las mitocondrias neuronales para preservar su estructura y restaurar su función. Curiosamente, el PEG con peso molecular más alto (4 kDa) no pudo ejercer una mejora significativa en las mitocondrias neuronales lesionadas, lo que indica que la protección mitocondrial mediada por el PEG es dependiente del tamaño [3]. En este sentido, el mecanismo por el cual el PEG 35 disminuyó el daño mitocondrial necesita una investigación más profunda.

En hígados grasos, la acumulación de grasa en el citoplasma de los hepatocitos provoca graves alteraciones en el flujo sinusoidal durante la reperfusión [232]. Los mecanismos de protección del PEG 35 se asociaron con la activación de la eNOS responsable de la generación de NO y la posterior disminución de la resistencia vascular. Esto también podría explicar el mecanismo de protección del PEG hacia la mitocondria ya que se ha demostrado que el NO protege los hepatocitos de rata contra la lesión por reperfusión a través de la inhibición de la MPT [205]. Sin embargo, en nuestro primer estudio observamos que el PEG 35 aumentó la producción de NO pero que este efecto no estaba relacionado con la activación de la eNOS. La diferencia entre los dos modelos experimentales (isquemia caliente vs isquemia fría) y la duración de la isquemia podría explicar el contraste entre los diferentes resultados de nuestros estudios.

En nuestro estudio anterior se evidenció que el PEG podría ser considerado como un fármaco de preacondicionamiento de la protección contra la lesión de I/R caliente y fría. En el siguiente estudio, se investigó el potencial papel protector del PEG como agente de poscondicionamiento al ser añadido a la solución de lavado.

3. El postcondicionamiento con PEG

En este trabajo se demuestra que la adición del PEG 35 a una solución de lavado de nuevo desarrollo, es necesario para lograr la protección del injerto de hígado contra el daño por reperfusión. Por otra parte, esta protección fue dosis dependiente, siendo la concentración de PEG 35 de 5 g/L más eficiente que la de 1 g/L. Esta disminución en las lesiones hepáticas es concomitante con la consiguiente mejora en la función hepática reflejada en la producción de bilis, y una reducción significativa en la resistencia vascular.

Por otra parte, la adición de PEG 35 a una solución de enjuague previno el daño mitocondrial y el estrés oxidativo, en comparación con las soluciones Ringer Lactate (SRL) y la solución básica (SB) de lavado. Estos resultados están en línea con los dos primeros estudios que demuestran la preservación de la integridad mitocondrial y con estudios previos que muestran el papel antioxidante del PEG después del recalentamiento de los hepatocitos almacenados en frío [144]. La prevención del daño por reperfusión también estuvo acompañada por aumentos posteriores en la expresión de HSP70 y HO-1, dos marcadores bien conocidos de la supervivencia del injerto

después del trasplante [99, 106]. Además, se observó un aumento significativo de la fosforilación de la AMPK y la activación de la eNOS.

En la I/R caliente, hemos demostrado que el PEG conserva la integridad del citoesqueleto. Con esta intención, hemos explorado los efectos del PEG 35 en soluciones de enjuague sobre el citoesqueleto de los hepatocitos mediante la medición de la distribución de las actinas F y G y los cambios ultra-estructurales por microscopía confocal. Los hígados sometidos al lavado con la SRL y la SB mostraron un bajo contenido de F-actina reflejando alteración del citoesqueleto, probablemente asociada a la isquemia. En contraste, el uso del PEG 35 impidió la pérdida de F-actina mediante el aumento de su polimerización, como se refleja en una relación de F-actina/g-actina aumentada. Este hecho confirma el efecto protector de la solución de enjuague con PEG 35 s en la prevención de la pérdida de integridad celular durante I/R.

El recambio de la matriz extracelular, influenciado por MMP9 y MMP2, se produce durante la remodelación de tejidos después de la I/R [220]. En modelos experimentales de I/R hepática, la inhibición de MMP2 y MMP9 también reduce el daño tisular [221, 222]. En este estudio, hemos demostrado que las soluciones de enjuague PEG 35 inhibieron la actividad de MMP2 y MMP9, lo que sugiere la estabilidad del citoesqueleto en comparación con cualquier solución SRL o SB. Este hecho es consistente con la reducción de las alteraciones morfológicas en la microscopía confocal. Específicamente, se observó que las células hepáticas del injerto que se enjuagaron con SRL o SB adquieren una forma redonda ligeramente deformada, mientras que los que se lavaron con soluciones de enjuague conteniendo PEG 35 mantuvieron la morfología normal.

La autofagia está asociada con el catabolismo de proteínas de larga vida, componentes citosólicos, u orgánulos dañados. Es un proceso altamente regulado que implica la formación y entrega de autofagosomas a los lisosomas para degradación. En base a la creciente evidencia que vincula la autofagia a la I/R [223-225], se evaluó su potencial participación durante el proceso de lavado del injerto hepático. De hecho, hace tiempo se ha reconocido que la autofagia se produce en órganos bajo condiciones de estrés tales como la I/R [225, 233], aunque su papel exacto sigue sin estar claro y es controvertido. Aunque sabemos que la activación de la autofagia durante la isquemia es esencial para

la supervivencia celular y el mantenimiento de la función del órgano, a través de un mecanismo dependiente de AMPK, su papel durante la reperfusión podría ser perjudicial [82, 225, 234]. La autofagia durante la reperfusión se acompaña de una robusta regulación de Beclin-1, que a su vez se ve agravada por la generación de especies reactivas del oxígeno, lo que lleva a una degradación masiva de moléculas vitales y la muerte celular autofágica [55, 234]. En este estudio, hemos demostrado que lavando el injerto hepático con una solución de enjuague que contiene PEG 35 impide la autofagia hígado. Esto se demuestra a través de niveles de beclin-1 y LC3B reducidas, de manera similar a la reportada para el post-acondicionado en un modelo de cerebro de rata [226]. Los datos aquí presentados son consistentes con los estudios realizados por Gotoh et al [227], que implicó la autofagia en el inicio de la disfunción del injerto después del trasplante de hígado de rata. Por lo tanto, podemos especular que la prevención del daño mitocondrial y de la producción de ROS por PEG 35 podría explicar la disminución en Beclin-1 y LC3B después de lavado injerto hepático.

Las conclusiones de la presente tesis son las siguientes:

- EL PEG 35 administrado por vía intravenosa protege eficientemente el hígado de ratas contra la I/R caliente y fría. Los mecanismos de protección están asociados con la activación de la pro supervivencia vías Akt y AMPK y la inhibición de la apoptosis. También el PEG 35 protege la morfología de los hepatocitos mediante el aumento de la F/ G-actina y la activación de p-p38.

- La administración intravenosa de PEG 35 a 10 mg / kg protege los hígados esteatósicos en un modelo experimental de I/R fría en ratas obesas. Los efectos protectores de PEG 35 están mediadas por la preservación del estado mitocondrial, la estabilización del citoesqueleto y la regulación de las vías de señalización citoprotectores AMPK y AKT.

- La adición de PEG 35 a soluciones de lavado aumenta la protección contra la lesión por I/R en un modelo de hígado de rata aislado y perfundido. La protección era debida a la inhibición de las metaloproteinasas, la activación de vías de señalización citoprotectores AMPK y eNOS y la preservación de la integridad del citoesqueleto.

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IX. APPENDIX

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REVIEW

Emerging concepts in liver graft preservation

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Abstract

The urgent need to expand the donor pool in order to attend to the growing demand for liver transplantation has obliged physicians to consider the use of suboptimal liver grafts and also to redefine the preservation strategies. This review examines the different methods of liver graft preservation, focusing on the latest advances in both static cold storage and machine perfusion (MP). The new strategies for static cold storage are mainly designed to increase the fatty liver graft preservation *via* the supplementation of commercial organ preservation solutions with additives. In this paper we stress the importance of carrying out effective graft washout after static cold preservation, and present a detailed discussion of the future perspectives for dynamic graft preservation using MP at different temperatures (hypothermia at 4 ℃, normothermia at 37 ℃ and subnormothermia at 20 ℃-25 ℃). Finally, we highlight some emerging applications of regenerative medicine in liver graft preservation. In conclusion, this review discusses the "state of the art" and future perspectives in static and dynamic liver graft preservation in order to improve graft viability.

Key words: Static cold preservation; Suboptimal liver grafts; Preservation solutions; Graft washout solutions; Machine perfusion and liver bioengineering

Core tip: This review focuses on the latest advances in liver graft preservation, in both static cold storage and dynamic preservation by machine perfusion (MP). We describe some new trends for static cold preservation based on our experience; we stress the importance of developing washout solutions and the use of MP for suboptimal liver grafts. Finally, we discuss emerging applications of regenerative medicine in liver graft preservation.

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INTRODUCTION

Liver transplantation is the definitive treatment option for end-stage liver diseases. Besides the immunological mechanisms of graft rejection, liver transplantation outcome is also limited by ischemia-reperfusion injury (IRI). IRI is a complex multifactorial process caused, principally, by the energy depletion during graft cold storage in preservation solutions (cold ischemia) and the subsequent production of oxidative stress and inflammatory events after graft revascularization in the recipient (reperfusion)[1]. IRI is associated with delayed graft function and primary graft failure, which remains one of the major clinical problems following liver transplantation.

A common strategy to reduce ischemic injury following explantation from the donor is the rapid cooling of the organs with the use of a preservation solution to minimize enzymatic activity and energy substrate depletion. In recent decades, major advances have been made in the area of liver preservation, including the development of new preservation solutions. Their emergence has helped to decrease hypoxic injury and has reduced graft vulnerability against reperfusion insult.

Currently, the high increase in demand for organs has obliged physicians to use suboptimal grafts in order to increase the organ supply for transplantation. Suboptimal or extended criteria donor (ECD) livers include organs characterized by steatosis, old donor age, prolonged cold ischemia or donation after cardiac death (DCD)[2,3]. It is well known that suboptimal livers present increased vulnerability to IRI, and are associated with graft dysfunction and long-term survival problems after surgery. For this reason, preservation methods for suboptimal livers need to be exhaustively explored in order to identify the ones that are the most suitable for graft conservation.

Machine perfusion (MP) has emerged as an alternative preservation strategy to static cold storage (SCS). MP is already routinely used for kidney transplantation, but a great deal is still to be done before it can be regularly used in clinical liver transplantation. In this review, we examine the SCS and MP techniques in detail, describing the latest advances in the development of preservation solutions for liver grafts and providing some proposals and new strategies in order to improve current graft preservation methods.

STATIC COLD STORAGE

The main goal in organ preservation is to maintain function of the organ and tissue during storage so that the graft will be viable at reperfusion. To date, the predominant organ preservation method used by most centers is SCS. The principles of SCS are based on the diminution of metabolism by hypothermia. The appropriate preservation solution is infused into the organ (the cooling phase) and then stored statically[4].

Cooling

SCS is the most widely used method for preserving organs for transplantation. Cooling is necessary to reduce cellular metabolism and the oxygen requirements in order to prevent tissue injury[5].

In order to obtain viable organs after long-term preservation, various methods have been proposed, ranging from organ freezing and vitrification[6,7] to "supercooling" (subzero non-freezing at 0 ℃ to -5 ℃)[8-11]. In general, long-term survival rates after transplantaion using these methods are disappointing.

However, in a recent study by Berendsen *et al*[12], the combination of "supercooling" (cold preservation at -6 ℃) with other parameters achieved effective preservation of liver grafts for 4 d. This promising new technique comprises three steps: first, "supercooling" of the organ at -6 ℃ to reduce the cellular metabolism; second, subnormothermic MP at 21 ℃ (see the dynamic preservation section below), which reinitiates the metabolism and replenishes ATP levels, and third, the use of two preservatives, 3-O-methyl-D-glucose (3-OMG) and polyethylene-glycol 35 (PEG35). Each of these conditions is necessary to achieve successful liver transplantation[13]. With this in mind, supercooling techniques may be a potentially useful tool for suboptimal livers which are currently discarded for transplantation purposes, and may have great impact on global organ sharing.

Preservation solutions

Although cold is a fundamental requirement for tissue preservation, it has harmful repercussions due to the induction of cell swelling[14] and cytoskeletal alteration[15]. This was in part the reason for the development of commercial organ preservation solutions able to prevent many of the cellular alterations associated with hypothermia and to mitigate the harmful effects of cooling.

Euro-Collins (EC) solution was developed in the 1970s as a high potassium-sodium solution (intracellular composition) which does not contain oncotic agents but does contain glucose. Given that glucose is impermeable to renal cells, this preservation solution was suitable for kidney preservation when relatively short times were needed or DCD organs were used. However, the permeability of the liver and pancreatic cells to glucose leads to the loss of the osmotic effect, and also causes the subsequent anaerobic metabolization of glucose, inducing intracellular acidosis and thus limiting cell preservation. This is why glucose was later substituted by other larger sugar molecules such as lactobionate and raffinose in

Table 1 Additives for improving static cold storage in University of Wisconsin and Institute Georges Lopez preservation solutions

Additive	Preservation solution	Ref.
TMZ	UW, IGL-1	[25,30]
EGF + IGF-1	UW	[45]
IGF-1	IGL-1	[44]
EGF	IGL-1	[46]
ML	IGL-1	[43]
BZ	UW, IGL-1	[51,117]
SV	UW	[64]
BZ, MG132	UW	[50]
ML + TMZ	IGL-1	[38]
CAII	IGL-1	[54]

TMZ: Trimetazidine; EGF: Endothelial growth factor; IGF: Insulin growth factor 1; ML: Melatonin; BZ: Bortezomib; SV: Simvastatin; CAII: Carbonic anhydrase Ⅱ; UW: University of Wisconsin; IGL-1: Institute Georges Lopez.

University of Wisconsin (UW) solution, which remains in the extracellular space and preserves its beneficial effect. The use of the UW preservation solution improved organ preservation time from 6 to 16 h[16].

The efficacy of UW solution is based on the prevention of edema by impermeants (raffinose, lactobionate), and the addition of an ATP precursor (adenosine) and anti-oxidant components (allopurinol, reduced glutathione). Drawbacks include the presence of hydroxyethyl starch (HES) as oncotic support, which has been associated with high blood viscosity and consequent tissue saturation with the preservation solution. As a result, washout of blood from the graft and blood flow during reperfusion may be reduced[17,18]. In addition, the high K^+ concentration is associated with cellular depolarization and activation of voltage-dependent channels[19]. The problems caused by HES and K^+ led to the development of other preservation solutions without oncotic agents such as Celsior and HTK (Custodiol) and others with PEG as oncotic agent, such as Institute Georges Lopez solution (IGL-1) and Tissue and Organ Conservation Solution (SCOT).

Celsior was developed initially in the 1990s as a cardiac preservation solution with a low potassium and high sodium composition. Due to its extracellular composition, Celsior was also adopted for the preservation of abdominal organs as an alternative to UW. Other solutions without oncotic agents such as histidine-tryptophan-ketoglutarate solution (HTK) were also developed. HTK presents low viscosity and for this reason provides more rapid cooling and better washout of blood elements during organ procurement than UW. Celsior and HTK solutions have been extensively used for liver transplantation[20-22]. However, some limitations for HTK use have recently been described. Stewart *et al*[23] reported that HTK is associated with reduced graft survival in case of additional risk factors such as DCD, cold ischemia time over 8 h, and donors over 70 years when compared to UW solution.

In IGL-1 preservation solution, HES was substituted by a PEG with a molecular weight of 35 KDa (PEG35), and the high K^+/low Na^+ ratio was reversed. Both experimental[24,25] and clinical[26-28] studies of liver and kidney transplantation have shown the beneficial effects of IGL-1 against apoptosis, endoplasmic reticulum stress, microcirculation dysfunction and immune response. Moreover, in previous studies of cold preservation and *ex vivo* perfusion, we have reported that IGL-1 contributes to a more efficient preservation of both non-steatotic and steatotic rat liver grafts compared to UW[29-31], The beneficial effects of IGL-1 include prevention of hepatic damage, oxidative stress and mitochondrial injury, and are mediated through nitric oxide (NO) production. So IGL-1 is the first solution reported to be advantageous in SCS of suboptimal livers.

Moreover, a PEG of smaller size, PEG20, is the basic component of another solution for organ preservation: the SCOT, which furthermore contains low K^+/high Na^+ concentrations. SCOT was reported to show a higher renal protection against the immune response, mainly due to the "immunocamouflage" process provided by PEG20[32]. PEG20 at 15 g/L has been found to reduce alloantigen recognition after liver reperfusion in comparison to UW solution[33]. Even so, the use of PEG35 as oncotic agent has been shown to be more effective than PEG20 for liver graft preservation[34].

Modification of static preservation solutions

The extended use of commercial preservation solutions has improved the conditions of liver graft preservation, but with the increasing use of suboptimal grafts it seems necessary to explore new alternatives in order to prolong the ischemia times and increase graft quality during cold storage. Along these lines, new additives have been proposed to improve static liver graft preservation when UW and IGL-1 solutions are used (Table 1). Although these alternatives are promising and have been successfully applied in animal models, they require further investigation before they can be implemented in clinical transplantation.

Anti-ischemic drugs: Previous work in kidney[35,36], liver[37,38] and heart[39-41] models has demonstrated the anti-oxidant action of trimetazidine (TMZ), an anti-ischemic drug. The addition of TMZ to UW solution was tested in both steatotic and non-steatotic rat livers after cold storage and *ex vivo* perfusion[25]. The enrichment of UW solution with TMZ reduced hepatic injury by diminishing microcirculatory dysfunction, oxidative stress, and mitochondrial damage. In the same experimental conditions, supplementation of IGL-1 solution with TMZ offered better liver graft preservation than IGL-1 solution alone and induced significant activation of hypoxia inducible factor-1α (HIF1α) and increased NO production[30]. The benefits of TMZ have been shown clinically in patients undergoing hepatic surgery under vascular clamping[42]. This would suggest that TMZ has

potential for use as an additive in commercial preservation solutions for clinical transplantation purposes.

Hormones: Melatonin (ML), a hormone produced by the pineal gland in a circadian manner, has been shown to be highly beneficial for enhancing resistance of both steatotic and non-steatotic livers against IRI when added to IGL-1. ML decreased hepatic injury by overexpression of endothelial NO synthase (e-NOS) and Heme Oxygenase-1, and reduced mitochondrial damage and oxidative stress[43]. These protective effects of ML in fatty liver graft preservation were further potentiated by addition of TMZ to IGL-1 + ML solution[38]. Protective mechanisms were dependent on AMPK activation. Furthermore, UW and IGL-1 solutions enriched with trophic factors like epidermal growth factor and insulin-like growth factor-1 enhanced the resistance of steatotic livers to IRI, partly due to Akt and eNOS signaling activation, and reduced cytokine release[44-46].

Proteasome inhibitors: The ubiquitin proteasome system (UPS) is an energy-dependent system that degrades misfolded proteins and regulates various cellular processes[47]. It has been established that proteasome activation is a pathophysiologically relevant mechanism of cold ischemic myocardial injury. A subset of 26S proteasomes appears to be a cell-destructive protease that is activated as ATP levels decline[48]. The addition to UW solution of epoxomicin, a proteasome inhibitor, reduced cardiac edema and preserved the ultrastructural integrity of the post-ischemic cardiomyocyte[49]. In liver, we have recently demonstrated that the addition of the reversible UPS inhibitors bortezomib (BRZ) and MG132 to UW solution improved steatotic and non-steatotic liver preservation, and that the protective effect of BRZ was superior to that of MG132[50]. Supplementation of IGL-1 solution with BRZ also showed protective effects which were partially mediated through the activation of AMPK and Akt/mTOR signaling[51].

Carbonic anhydrase Ⅱ: Carbonic anhydrase (CA) are Zn-metalloenzymes that catalyze the reversible reaction between carbon dioxide hydration and bicarbonate dehydration. Recently the function of CAs has aroused great interest, as they contribute to the transport of CO_2 and protons across the biological membranes and are involved in pH regulation, CO_2 homeostasis and biosynthetic reactions such as gluconeogenesis, lipogenesis and ureagenesis. In mammals 16 different CAs are found, with different amino acid sequences, enzymatic properties and sites of expression[52]. Since carbonic anhydrase Ⅱ (CAⅡ) also contributes to acid-base homeostasis[53], we suggest that it could be modulated in conditions of liver preservation and that its addition to the preservation solution could be an efficient strategy for reversing pH alterations provoked by cold ischemia. Indeed, in preliminary studies at our laboratory, we have observed that fatty livers preserved in IGL-1 solution supplemented with CAⅡ showed lower injury, better function and major reductions in liver apoptosis parameters[54]. So CA enrichment of preservation solutions is an up-and-coming approach for improving the preservation of suboptimal liver grafts.

Statins: Statins, or the 3-hydroxyl-3-methylglutaryl coenzyme A inhibitor family, are a group of drugs known to decrease cholesterol levels and treat dyslipidemias[55]. They also have a variety of anti-inflammatory, antioxidant and immunoregulatory effects[56,57] and they maintain the endothelial barrier by activation of eNOS and subsequent production of NO[58-60]. Due to their various effects, statins have been proposed as effective pharmacological agents against IRI in both normal and steatotic livers[61-63]. UW supplementation with simvastatin (a synthetic analog of statin) prevented the deleterious effects of cold storage in endothelial cells, due to the enhancement of vasoprotective pathways, thus improving liver viability[64]. With this in mind, the supplementation of IGL-1 with simvastatin could promote the NO generation induced by IGL-1 solution alone, and may contribute to preventing the exacerbated microcirculation complications existing in fatty liver grafts after revascularization. In addition, increased levels of NO could contribute to stabilize cytoprotective factors such as HIF-α, which are generated as an adaptive response to the hypoxic conditions that characterize cold preservation[30].

New potential additives: Some considerations

Sirtuin activators: Sirtuin1 (SIRT1) is a deacetylase that regulates the activity of various non-histone and histone proteins and as a result is involved in various cell processes such as apoptosis and oxidative stress[65-68]. SIRT1 induces AMPK activation through LKB1 deacetylation, and favors NO production by e-NOS activation[69,70]. Further, in a recent study published by our group, we mentioned that SIRT1 is involved in the beneficial effects of ischemic preconditioning, partly *via* AMPK and eNOS activation[68]. Consequently, addition of SIRT1 activators in preservation solutions may be a promising strategy for prolonging storage periods; SIRT1 activators may activate AMPK and maintain the cell energy status, and may also increase NO levels and alleviate microcirculation disturbances, especially in fatty livers. Preliminary data obtained from our laboratory showed that SIRT1 is a differential marker in steatotic and non-steatotic livers during cold preservation. Since SIRT1 activity requires high NAD^+ levels, NAD^+ activators may also contribute to better liver graft preservation by activating not only SIRT1, but also other members of sirtuin-family such as Sirtuin3 (SIRT3). SIRT3 is located in the mitochondria and affects the acetylation status of various mitochondrial proteins[71]. Enhancement of SIRT3 activity could thus achieve better mitochondrial preservation and prevent reactive oxygen

species (ROS) production during reperfusion.

Nrf2 activators: Moreover, recent studies have demonstrated the importance of Nrf2 in IRI models[72-74]. Nrf2 is activated under conditions of oxidative stress and induces the transcription of anti-oxidant enzymes in order to eliminate redox stress. Nrf2-deficient livers exhibit enhanced liver injury upon IRI[75]. Consequently, we propose the use of Nrf2 activators in preservation solutions in order to alleviate oxidative stress during reperfusion.

All in all, extensive studies in experimental models have proposed modified preservation solutions in order to extend cold storage and to maintain graft viability as far as possible. Since IRI is a multifactorial process, preservation solutions could incorporate various pharmacological agents in order to combine diffe-rent protective mechanisms and thus improve liver preservation. Nonetheless, the use of pharmacological agents may be limited by their potential toxicity and side effects or their unsuitability for suboptimal grafts, and so novel strategies of preservation should be developed.

Liver graft wash out

After cold storage, the liver grafts preserved in commercial preservation solutions need to be washed out to remove the solution before reperfusion and also to obtain the most suitable conditions for graft revascularization and viability after transplantation. Although research into rinse solutions is limited, recent data from our laboratory show that washing out the liver grafts preserved in UW for 24 h, with a rinse solution containing PEG35, is an effective tool for reducing liver graft injury after two hours of *ex vivo* perfusion[76]. PEG35 in the rinse solution was associated with decreased oxidative stress and mitochondrial damage, increased activation of AMPK, and enhanced NO generation. In addition, it contributed to restoring cytoskeleton integrity following IRI. In contrast, when livers were preserved in IGL-1 solution, these benefits were not evident, probably due to the presence of PEG35 as oncotic agent (unpublished data).

It is well known that PEG molecules are water-soluble polymers of various molecular weights which are non-immunogenic and non-toxic[77]. In general, PEGs prevent the generation of ROS[78,79], enhance cell survival pathways in hypoxia/reoxygenation conditions and repair endothelial cell damage during post-ischemic reperfusion[80,81]. PEG exerts its cytoprotective role through the restoration of membrane integrity[15,78,81,82] or by entering the cell through the disrupted membranes and interacting with cellular organelles[83]. In hypothermic hepatocyte preservation, PEG8 (8 kDa) prevented cell swelling through a mechanism that was independent of its osmotic properties[14].

DYNAMIC PRESERVATION: MACHINE PERFUSION TECHNIQUES

For standard liver grafts, SCS with different preservation solutions remains highly successful. However, with the increasing need for organs in recent years, the use of novel techniques for optimizing suboptimal graft preservation is arousing interest.

MP consists of creating a controlled recirculating flow of preservation solution through the organ using a pump. This continuous perfusion permits better penetration of the preservation solution, a thorough washout of blood and equilibration of the interstitium with the perfusate medium, delivery of oxygen and nutrients (if the perfusate is oxygenated), and removal of toxic metabolites (when the perfusate is renewed or filtered). In addition, it allows real-time monitoring of the functional and biochemical performance of the graft and the provision of metabolic support during preservation[84].

Unlike the kidney, the MP protocol for the liver is determined mainly by the temperature of preservation: hypothermic (HMP) at 4 ℃, normothermic (NMP) at 37 ℃ and subnormothermic (SNMP) at 20 ℃-25 ℃. Also, several flows and pressures (pulsatile or not), single or dual perfusion (hepatic artery and portal vein), oxygenation or non-oxygenation, and different MP solution compositions have been tested in various liver graft experimental models[85].

HMP

HMP is a dynamic cold preservation method at 4 ℃ which ensures homogeneous and continuous supply of metabolic substrates to the graft during the *ex vivo* period[86]. During HMP, aerobic metabolism decreases but does not stop completely and the provision of metabolic substrates allows the reduction of the cellular insults seen during reperfusion.

HMP offers several advantages over SCS. Guarrera *et al*[87] were the first to compare HMP to SCS in human liver transplantation, and showed that HMP improves graft function and attenuates classical biochemical markers of liver preservation injury. Given the fact that ROS accumulation during ischemia can lead to significant hepatocyte toxicity, HMP has been shown to protect the rodent liver from ROS by a reduction in glutathione depletion and superoxide anion release when compared with SCS[88]. And in the case of suboptimal livers, Bessems *et al*[89] showed that HMP improved both hepatocellular and endothelial function while reducing damage in a diet-induced rat fatty liver model.

In contrast to the kidney, in which successful HMP does not necessarily depend upon oxygenation, oxygenated HMP (HOPE) has been developed as a means of improving the quality of liver preservation in normal

or ECD livers[90]. Oxygenated preservation enables grafts to restore tissue homeostasis and to maintain the functional integrity of hepatocytes during ischemia. In a recent study, Schlegel *et al*[91] also described a protective effect on the rodent biliary system using HOPE in DCD grafts that underwent transplantation. As expected, perfusion with the HOPE system decreased the parameters of hepatocellular injury and lowered immunogenic upregulation.

Perfusates for HMP: In general, the composition of perfusate solutions used for HMP is based on a re-formulation of UW solution, in which lactobionate is replaced by gluconate. This solution, named Belzer-MP solution (Belzer-MPS), continues to be the predominant perfusion solution.

Bessems *et al*[92] described a new HMP solution, Polysol, which contains amino acids, histidine, glutamine, tryptophan, ascorbic acid and α-tocopherol. Their studies show that Polysol improved liver preservation compared to Belzer's MPS, with lower enzyme release and increased bile production. Vasosol has also been proposed as an efficient alternative for HMP[87]. Its composition is based on Belzer-MPS but it is supplemented with antioxidants (N-acetyl-cysteine), metabolic substrates (α-ketoglutarate, *L*-arginine) and vasodilators (prostaglandin E1 and nitroglycerin). Recently, the benefits of Vasosol have been improved by the addition of α-tocopherol to further enhance antioxidant properties when HMP is used[93].

SNMP

Recently it has been suggested that the use of SNMP systems may be suitable for *ex vivo* preservation and recovery of human liver for transplantation. SNMP is an intermediate status for graft conservation, using sub-thermic conditions (20 ℃-25 ℃), taking advantage of the lower metabolic demand in sub-physiological temperature conditions, while still maintaining sufficient metabolism for viability testing and improvement of graft function. SNMP has already proven advantageous in reducing markers of biliary injury during preservation and in restoring normal biliary physiology[94]. A recent study by Bruinsma *et al*[95] is the first demonstration of the capacity of SNMP to sustain human livers. This group showed that SNMP effectively supports the human liver *ex vivo* with minimal injury, and normalizes physiological post-ischemia disturbances.

NMP

The principle of normothermic perfusion is the maintenance of normal cellular metabolism in a physio-logical environment throughout the preservation period by maintaining normal temperature (37 ℃) and providing oxygen and essential substrates[96]. This ensures large-scale metabolic activity and the maintenance of energy reserves such as ATP content. NMP has the advantage of allowing viability assessment prior to transplantation. As the liver metabolism is maintained during preservation, markers including bile production and liver enzymes can be measured.

NMP is an emerging technology whose potential in liver preservation has been described in several animal studies, which have shown its superiority over SCS in the preservation of liver grafts[96-98]. Interestingly, porcine and murine models of DCD livers are significantly improved by NMP compared to organs preserved by SCS[99,100].

Recently, Ravikumar *et al*[101] reported the first clinical trial of transplanted livers with NMP. Their study included 10 transplanted patients with relatively low risk donors and recipients, and showed that NMP is safe and feasible in human applications. This study opens up new avenues for research into liver graft preservation with NMP.

Recently, NMP has emerged as a novel tool for decreasing steatosis in a process named "defatting". In a preliminary study using porcine livers, *ex vivo* normothermic perfusion for 48 h led to a 50% reduction in lipid droplet size in perivenous hepatocytes, reaching the size found in control lean livers[102]. Moreover, NMP of steatotic livers from Zucker ob rats using a "defatting cocktail" decreased the intracellular lipid content by 50% over 3 h of perfusion[103]. Decreasing steatosis prior to transplantation by short term NMP would allow the transplantation of severely steatotic livers and thus alleviate the donor liver shortage.

Perfusates for NMP: NMP requires advanced metabolic support since the organ is fully metabolically active. Therefore, typically diluted blood-based perfusates are used. More recently, a solution initially described for lung perfusion has also been applied to liver grafts[104]. Steen is a buffered extracellular solution containing dextran and albumin at an optimized colloid osmotic pressure.

For defatting purposes, the perfusate developed contains different compounds to activate nuclear receptors such as PPARs, pregnane X receptor, and constitutive androstane receptor in order to exert an insulin-mimetic effect and to stimulate intracellular cAMP. This liquid was added into Minimum Essential cell culture medium as a perfusate to stimulate the lipid metabolism of obese rat liver grafts preserved using NMP. With this cocktail, a significant decrease (50%) in steatosis was observed after 3 h of NMP[103]. A recent study showed that the supplementation of this cocktail with *L*-carnitine, together with hyperoxic exposure, abolished the sensitivity of macrosteatotic hepatocytes to hypoxia reoxygenation (H/R)[105].

BIOENGINEERING IN LIVER GRAFT PRESERVATION

In the context of liver graft preservation, bioengineered human livers represent an opportunity to test new solutions and liver preservation methods, thus potentially bypassing the requirement of precious and scarce human organs. Bioengineering allows quicker and cheaper

Table 2 Advantages and disadvantages of machine perfusion preservation

Advantages	Disadvantages
Continuous nutrients and oxygen supply	Logistically complex
Continuous monitoring of organ viability	High cost
Removal of metabolic waste products	No optimized conditions
Extended preservation time	Need for trained personnel
Better preservation of microcirculation	
Potential "rescue" of suboptimal organs	

development and transfer to the clinic[106].

Over the past few years, organ bioengineering has come of age. The seminal study by Ott *et al*[107] in 2008 on heart decellularization and recellularization paved the way for whole organ bioengineering. After this initial study of the heart, many other organs followed. In 2009, Baptista *et al*[108] described the first methods for liver, pancreas and kidney decellularization and recellularization, and their paper was followed by an exponential growth of publications by many other authors.

Currently, with several solid organs already successfully bioengineered and under further development by several groups around the world, this technology has huge potential. However, bioengineered organs are still not available to the transplant surgeon as alternative grafts. There are already several applications that can be addressed and extended with the current generation of bioengineered organs and their acellular scaffolds. Most of these applications, like drug metabolism[106], organ/tissue physiology[106,107,109,110], matrix biology[111], developmental biology[111,112], and stem cell biology[113] are perfectly complemented by these novel bioengineered human tissues which will open up exciting new experimental avenues.

In the particular context of normothermic perfusion, the enabling bioreactor and culture media technology developed in the bioengineering process of livers may constitute a new body of knowledge that can help further the development of NMP for liver preservation, due to the similarities of the conditions used[114]. Finally, the use of normothermic perfusion bioreactors in liver preservation and bioengineered human livers may also provide a better route and environment for *ex vivo* administration of mesenchymal stem cells. The use of these cells has been proposed as a novel way to attenuate IRI and to downregulate the alloimmune response (adaptive immunity) and promotes engraftment after transplantation[115]. This has been demonstrated for rat kidneys, thus raising the hope that it may also work in the liver and other solid organs[116].

CONCLUSION

Due to its low cost and simple technical and logistical requirements, SCS is still preferred to MP as the standard method of preservation in liver transplantation. SCS is probably unsuitable for suboptimal liver grafts, because they have already suffered severe tissue damage secondary to hypoxia during the initial period of warm ischemia. Additional damage to the organ due to hypothermic conditions may limit the ability to restore cellular function, because metabolic activity is decreased at low temperatures.

The growing need to use suboptimal livers and to expand donor pool is accompanied by the drive to improve current preservation techniques before transplantation. In this situation, there has been renewed interest in liver graft preservation using machine perfusion. Both HMP and NMP have been found to be beneficial in preserving normal and suboptimal livers, and their relative merits are currently being debated. More basic research and randomized controlled trials are needed. As for SNMP preservation, it remains relatively unexplored at present.

Studies on the cost-effectiveness of MP and SCS will continue over the coming years, but considerable support for MP is beginning to emerge. Table 2 summarizes its advantages and disadvantages for liver preservation. It seems clear that MP strategies will play an increasing role and that their use should be optimized, including the subsequent development of new perfusion solutions. With this in mind, the future of liver MP preservation will also depend on the composition of perfusion solutions. At present, little attention is being paid to the potential advantages of adding cytoprotective, immunomodulating, pro-regenerative components to the MP solutions.

It is well known that PEG protects cell membranes; it has already been used as a colloid in machine perfusion, just as it was previously in SCS. The development of different PEG molecules could establish new frontiers in the design of new perfusion solutions for application in MP techniques and may increase graft conservation in the future. The revitalization of steatotic livers through defatting agents represents another interesting future application, given that the worldwide incidence of severely steatotic livers is expected to rise together with the increase in obesity rates.

Finally, bioengineering is another area with great potential for graft preservation in clinical transplantation.

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