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Protective Role of Polyethylene Glycol 35 Against Hepatic Cold and Warm Ischemia

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"No one who achieves success does so without acknowledging the help of others. The wise and confident acknowledge this help with gratitude."

Alfred North Whitehead

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Abbreviations

ΔΨm: Mitochondrial membrane potential

4-HNE: 4-hydroxy-2-nonenal

ALDH2: Aldehyde dehydrogenase 2

ALT: Alanine aminotransferase

AMPK: Adenosine monophosphate-activated protein kinase

ANT: Adenine nucleotide translocase

ATP: Adenosine triphosphate

Cyt c: Cytochrome c

DAMPs: Damage-associated molecular patterns

DCD: Donation after circulatory death

DNL: *De novo* lipogenesis

Drp1: Dynamin-related peptide-1

ECD: Extended criteria donors

ETC: Electron transport chain

eNOS: Endothelial nitric oxide synthase

FAs: Fatty acids

FFA: Free fatty acids

Fis1: Fission protein-1

GGT: γ-glutamyl transferase

GSH: Glutathione

H_2O_2: Hydrogen peroxide

HCC: Hepatocellular carcinoma

HES: Hydroxyethyl starch

HMP: Hypothermic machine perfusion

HOPE: Hypothermic oxygenated perfusion

HSC: Hepatic stellate cells

HTK: Histidine-Tryptophane-Ketoglutarate

IGL: Institute Georges Lopez

IL-1β: Interleukin 1β

IL-6: Interleukin 6

IPC: Ischemic preconditioning

IR: Ischemia-reperfusion

IRI: Ischemia-reperfusion injury

KC: Kupffer cells

KEAP1: Kelch-like ECH-associated protein-1

KLF: Kruppel-like factor

LSEC: Liver sinusoidal endothelial cells

LT: Liver transplantation

MAFLD: Metabolic associated fatty liver disease

MDA: Malondialdehyde

MetS: Metabolic syndrome

Mff: Mitochondrial fission factor

Mfn: Mitofusin

miRNAs: microRNAs

MP: Machine perfusion

MPTP: Mitochondrial permeability transition pore

MRI: Magnetic resonance imaging

mtDNA: Mitochondrial DNA

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NMP: Normothermic machine perfusion

NO: Nitric oxide

NRF1: Nuclear respiratory factor 1

NRF2: Nuclear erythroid 2 p45-related factor 2

OMM: Outer mitochondrial membrane

OPA1: Optic atrophy protein-1

OXPHOS: Oxidative phosphorylation

PEG: Polyethylene glycol

PNF: Primary nonfunction

ROS: Reactive oxygen species

SCS: Static cold storage

SNMP: Subnormothermic machine perfusion

SOD: Superoxide dismutase

T2DM: Type 2 diabetes mellitus

TFAM: Mitochondrial transcription factor A

TGs: Triglycerides

TNF-α: Tumor necrosis factor-alpha

TZDs: Thiazolidinediones

UCP: Uncoupling protein

UW: University of Wisconsin

VDAC: Voltage-dependent anion channel

VLDL: Very low-density lipoprotein

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Introduction

1. Non-Alcoholic Fatty Liver Disease

Non-Alcoholic Fatty Liver Disease, most known by its acronym "NAFLD", is characterized by a relevant intracellular lipid deposition in the liver parenchyma hepatocytes without history of alcohol abuse, drugs, chronic viral infection or autoimmunity. It was first described by Ludwig and colleagues in 1980 (Ludwig et al., 1980). The severity of liver injury in 20 patients evaluated over a 10-year period, showed histologic evidence suggestive of alcoholic hepatitis on liver biopsy despite no history of alcohol abuse.

Even though considered a relatively benign condition, NAFLD can progress from simple steatosis into a more aggressive stage with lobular inflammation, hepatocyte ballooning as well as chronic hepatocyte injury in the absence of alcohol consumption, the so-called Non-Alcoholic Steatohepatitis (NASH) (Cohen et al., 2011; Giulio Marchesini et al., 2016). NASH may range from an initial stage of inflammation and hepatic cell damage up to a latter one of fibrosis, which may further progress into cirrhosis, liver failure and hepatocellular carcinoma (HCC) (Ekstedt et al., 2017; Gastaldelli et al., 2009) (Figure 1).

NAFLD is often considered a self-inflicted disease given that the person's behavior is the main driver for the illness development. The massive consumption of highly processed food, rich in saturated fat, fructose, and cholesterol as well as the lack of exercise are considered risk factors for the disease onset (Gehrke et al., 2019; Perdomo et al., 2019). NAFLD is often association with a broad spectrum of metabolic abnormalities, including obesity, type 2 Diabetes Mellitus (T2DM), dyslipidemia and hypertension, symptoms that are collectively known as "metabolic syndrome" (MetS) (Barshop et al., 2008). Recently, international experts proposed changing the name of NAFLD to a more appropriate overarching term and suggested metabolic associated fatty liver disease (MAFLD) (Eslam et al., 2020). The consensus group has proposed this acronym given the tight connection between the current knowledge of fatty liver diseases and metabolic dysfunctions.

Most NAFLD patients are asymptomatic and do not present major clinical outcomes for decade. In several cases, the diagnosis is performed accidently, while in others, physicians may not even consider the severity of the potentially advanced disease stage.

Despite all the efforts, the molecular mechanisms regulating the onset and progression of NAFLD are still elusive. The worldwide incidence and prevalence of NAFLD burden is thought to increase even more in the near future and therefore, the unravel of key pathogenic drivers, accurate biomarkers and pharmacological therapy are of upmost importance.
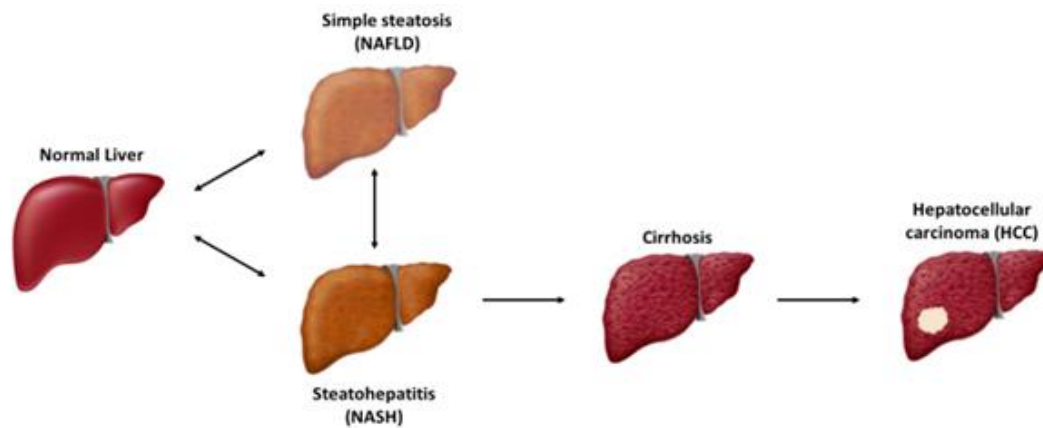
Figure 1- Schematic progression of Non-Alcoholic Fatty Liver Disease. Adapted from (Cohen et al., 2011)

1.1.   Epidemiology

NAFLD is the most common chronic liver disease in industrialized nations in the 21st century, affecting around 25% of the worldwide population (Figure 2). A meta-analysis has reported that the Middle East and South America have the highest NAFLD incidence, while Africa has the lowest one (Z. Younossi et al., 2018).

The number of people affected by NAFLD is already overwhelming, and its prevalence is likely to become even more pronounced owing the increasing percentage of elderly, obesity, unhealthy dietary habits, low physical activity and T2DM (Lazarus et al., 2020). According to the third national health and nutrition examination survey, 7.5% of normal-weight men and 6.7% of normal-weight women showed prevalence of NAFLD compared with 57% and 44% of men and women with BMI greater than 35 kg/m^2, respectively (Lazo et al., 2013). NAFLD is multifactorial and has a strong connection with obesity. However, in some cases there is the so-called "lean NAFLD", in which patients who are not obese can also present NAFLD (C. Ding et al., 2016). In accordance with obese ones, lean NAFLD patients have an altered metabolic and cardiovascular profile, leading to collective risk for comorbidities. Of note, patients with lean NAFLD display higher rates of hepatic inflammation, ballooning, fibrosis and cirrhosis in comparison to overweight patients (Denkmayr et al., 2018). Chen and colleagues have reported that in people with lean NAFLD the genetic variant rs58542926 of the transmembrane 6 superfamily member 2 (TM6SF2) had a higher prevalence when compared to obese individuals (F. Chen et al., 2020). The variant was connected to impaired very low-density lipoprotein (VLDL) production.

NAFLD has been associated with age, gender, ethnic bias and family history (Yki-Järvinen, 2014).
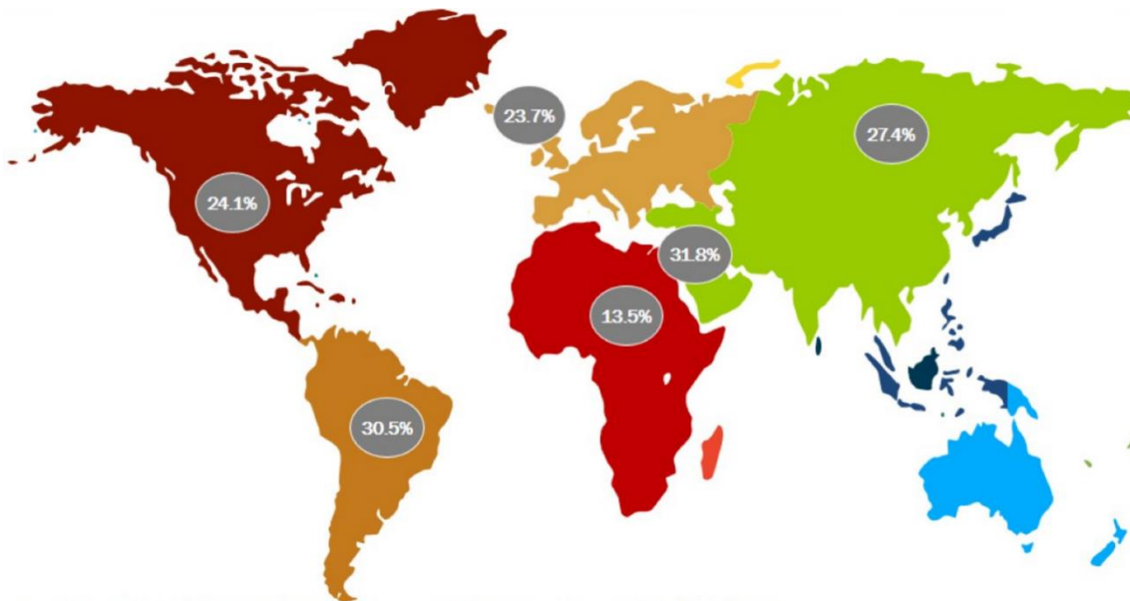
Figure 2- Worldwide prevalence of NAFLD (Z. Younossi et al., 2019).

Even though age is not a risk factor for developing NAFLD *per se*, elderly have higher probability of disease progression to fibrosis and HCC, especially in the presence of T2DM (Koehler et al., 2016). The relationship between the development of NAFLD and gender have been evaluated in the past few years. Several studies have reported that NAFLD is more prevalent in men than women, 40% and 20% respectively, with an increased prevalence in elders and those with T2DM (Byrne & Targher, 2015). However, in postmenopausal women, NAFLD occurs at a higher rate suggesting a protective effect of estrogen (Lonardo et al., 2019).

Regarding ethnicity, its role in the prevalence of NAFLD has been addressed in studies involving the multi-ethnic population of the USA (Rich et al., 2018). Hispanics hold the highest prevalence (45 to 58%), while Afro-Americans have the lowest (24 to 35%). Despite having a higher prevalence of obesity, the lower prevalence of NAFLD in the African American population underlies the influence of lifestyle factors and genetic background in the development of this pathology.

As mentioned above, the scientific community has widely accepted a close association between NAFLD and MetS. Among the general population the NAFLD prevalence is 23%, but this value goes up to 51% in case of obesity and to 58-64% in T2DM obese patients (Z. M. Younossi et al., 2016). Patients with NAFLD and T2DM have higher risk of progression to NASH, advanced fibrosis, cirrhosis and ultimately to HCC (Portillo-Sanchez et al., 2015; Z. M. Younossi et al., 2019). A cohort study observed that NAFLD patients with T2DM presented a 2.2-fold increased risk of liver-related mortality when compared to patients only with T2DM, over a 10 years' follow-up (Adams et al., 2010). Of note, subjects with T2DM are twice more likely to develop NAFLD and

an increased risk for developing NASH and fibrosis (Forlani et al., 2016). However, NAFLD continues to be overlooked by the general population and even by the global health community. Regarding NASH, the prevalence is much lower (1.5-6.5%), but also in the presence of T2DM and/or obesity, NASH incidence increases drastically. There is a correlation between NASH and obesity of 82% and a 37% correlation between NASH and T2DM (Z. M. Younossi et al., 2019). Nonetheless, NAFLD and MetS do not always coexist in the same individual, despite the tight connection between them. Several studies have suggested the influence of other processes beyond metabolic syndrome in the development of NAFLD pathogenesis, including genetic predisposition (Anstee & Day, 2013; Dongiovanni et al., 2015).

1.2. Genetic predisposition

NAFLD is an overly complex disease, and subtle genetic and environmental changes between individuals can be crucial for the disease progression. The genetic background seems to play an important role in the disease progression and severity and genetic variants of different genes encoding proteins associated with hepatic lipid metabolism have been reported for their robust correlation with the progressive nature of NAFLD (Dongiovanni et al., 2015). A genome-wide association study reported that around fifty percent of people with NAFLD carried, at least, one variant (G) allele at rs738409 in the PNPLA3 gene, which is linked to increased liver fat content, hepatic inflammation and fibrosis (Romeo et al., 2008). The correlation between steatosis and *PNPLA3* variant was described in other seven genome-wide association studies (Anstee & Day, 2013). PNPLA3 is mainly expressed in the liver in humans and its enzymatic activity is related to triglycerides (TGs) hydrolysis and Ile148Met seems to be an important player in the catalytic function given that its substitution abolishes PNPLA3´s activity (Y. Huang et al., 2011). Besides its influence in hepatic fat accumulation, PNPLA3 Ile148Met protein variants were also found to increase the susceptibility to more aggressive liver disease (Bruschi et al., 2017).

The liver is an important organ in the regulation of lipid metabolism and thus, it has several mechanisms to cope with a sudden overload of TGs accumulation. Nevertheless, in genetically predisposed subjects this protection is compromised, leading to a state of chronic inflammation and subsequent hepatocellular death and development of fibrosis.

1.3. Molecular Pathogenesis

As mentioned above, patients with NAFLD can progress to a less reversible and clinically silent stage, the NASH. A liver is considered steatotic when the fat content is >5% of the liver volume or histologically defined when 5% or more of hepatocytes contain visible intracellular fat (Kleiner et al., 2005). There are two distinct histologic patterns of hepatic steatosis which are classified

according to the size of TGs droplets: microvesicular and macrovesicular steatosis. Macrosteatosis is characterized by a single, bulky fat vacuole in the hepatocytes, which displaces the nucleus to the cell periphery. It is commonly associated with excessive alcohol intake, obesity, diabetes and hyperlipidemia (Angele et al., 2008). Whereas, in microsteatotic livers, the hepatocytes contain relatively small lipid droplets without nuclear displacement and it is commonly found in pathological conditions related to mitochondrial injury, including acute viral or drug induced liver injury, sepsis and some metabolic disorders (Álvarez-Mercado et al., 2019). NAFLD is characterized by the presence of steatosis without significant inflammation or fibrosis, while NASH is defined by ballooning necrosis in the vicinity of steatotic hepatocytes, lipid peroxidation, proinflammatory cytokine activation and most likely fibrosis, which further compromise liver function (Malik et al., 2019; Yki-Järvinen, 2014).

In 1998, Day and James described the pathogenesis of NAFLD as a two hits theory (Day & James, 1998). According to this theory, NAFLD begins with the presence of oxidizable fat within the liver (the "first hit"). This hepatic fat accumulation, in particular TGs, increases the vulnerability of the liver to subsequent injury caused by "the second hit", which includes an inflammatory response, associated with mitochondrial dysfunction, lipid peroxidation, culminating in further hepatic damage and fibrosis (Petta et al., 2016).

1.3.1. The first hit

Over nutrition and reduced physical activity leads to an imbalance between calory intake and energy expenditure, resulting in hepatic TGs accumulation (Figure 3). Dietary fatty acids (FAs) are absorbed by the intestine and subsequently stored into chylomicrons enriched in TGs before its release into circulation. The vast majority is transported to the adipose tissue, whereas the remaining part is captured by the liver (Dash et al., 2015). In physiological conditions, energy homeostasis is regulated by the adipose tissue, which works as a storage tissue for the TGs. However, excessive food intake and underexertion may lead to a dysfunction in adipose tissue metabolism. Insulin resistance in white adipose tissue is the one of the main driving forces to NAFLD due to increased hepatic free fatty acids (FFA) influx and consequent induction of lipid synthesis and gluconeogenesis, which in turn may induce peripheral insulin resistance, as demonstrated in both human and animal studies (Buzzetti et al., 2016).

The liver is the central regulator of lipid metabolism, being responsible for lipid synthesis, uptake and export to extrahepatic tissues (Nguyen et al., 2008; Ponziani et al., 2015). These pathways are tightly regulated by complex interactions between nuclear receptors, hormones and other transcription factors. Disruption in these processes may lead to defective lipid metabolism circuits, which eventually culminates with hepatocyte fat accumulation.
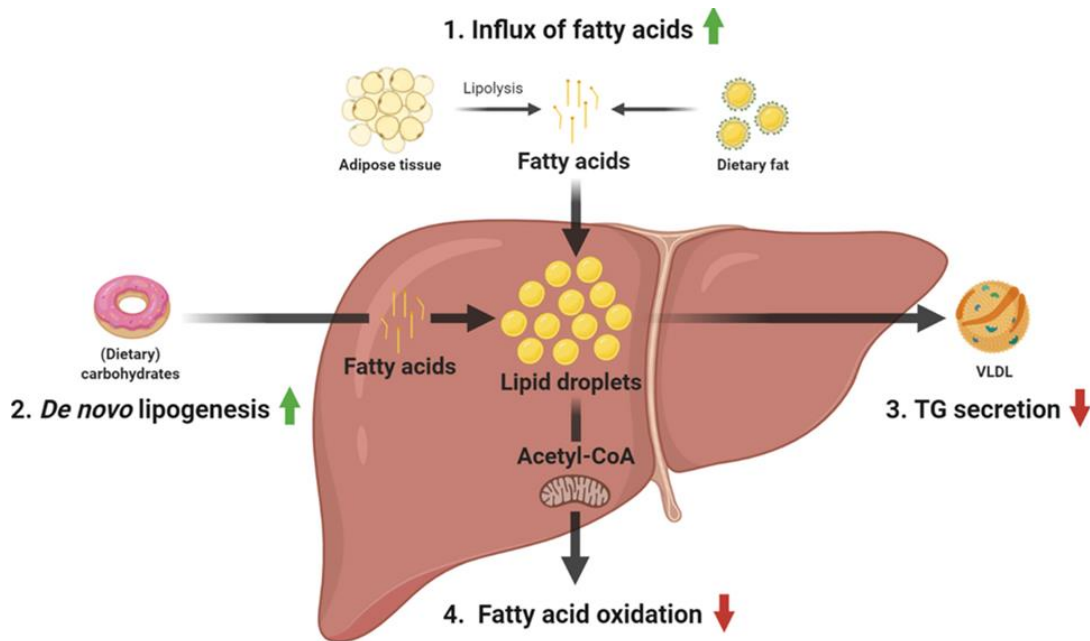
Figure 3- Main pathways involved in the accumulation of triglycerides in NAFLD. TG, triglycerides; VLDL, very-low-density lipoprotein. (Grefhorst et al., 2021)

In the postprandial state, the liver uses the surplus of glucose to store its glycogen pool (glycogenesis) and to synthesize *de novo* fatty acids (*de novo* lipogenesis, DNL), which are subsequently esterified to store TGs (Lonardo et al., 2017). Increased DNL is considered a key process in hepatic steatosis as well as progression to NASH (Lambert et al., 2014). Recently, Smith and co-workers reported that DNL contributes to 38% and 19% of hepatic TGs content in obese individuals with and without NAFLD, respectively (Smith et al., 2020).

Afterwards, the TGs resulting from the DNL are transported and stored in the adipose tissue. VLDL are the main vehicle of TGs exportation from the liver to the peripheral tissues. These lipoproteins are mainly formed by TGs, cholesteryl esters and phospholipids surrounded by Apolipoprotein B. Alterations in any of those elements may result in liver lipid metabolism perturbations (Haque & Sanyal, 2002). In NAFLD patients, the insulin insensibility leads to impair insulin capacity to suppress production of glucose and VLDL (Yki-Järvinen, 2014).

In addition to hepatic and adipose tissue insulin resistance, NAFLD is also linked to other peripheral organs including skeletal muscle. High levels of circulating FFA are related to muscle insulin resistance. In the skeletal muscle, there is a decreased GLUT4-mediated glucose disposal as a result of insulin resistance, overwhelming the liver with postprandial glucose load, which in normal conditions would be metabolized by myocytes (Meex & Watt, 2017).

Although, it remains unclear what comes first, whether steatosis or insulin resistance, it is clear a tight link among liver and whole body insulin resistance (Armandi et al., 2021).

1.3.2. The second hit

The changes observed during the first hit render the liver extremely vulnerable to secondary insults such as inflammation. Hepatic inflammation has been demonstrated to be a major aggravating factor of liver steatosis (Cha et al., 2018). Kupffer cells (KC) are the resident macrophages of the liver and when activated are known to produce a series of cytokines and chemokines including interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Wan et al., 2014). This cytokines can recruit proinflammatory cells, as well as other innate immune cells, which will promote local inflammation (Manne et al., 2018). Popko and co-workers have reported that serum concentration of both TNF-α and IL-6 were increased in obese patients with or without T2DM (Popko et al., 2010). In addition, gene expression of *Tnfa* and *Il6* was found to be upregulated in morbid obese and advanced NASH patients comparing to individuals with obesity and NAFLD (Jorge et al., 2018).

Hepatic stellate cells (HSC) are responsible for the synthesis of extracellular matrix proteins, being important mediators in the repairing processes. HSC are generally dormant, but once activated by inflammation, participate in the promotion of liver fibrosis (Mederacke et al., 2013). A recent study demonstrated that activated HSC presented almost 3000 genes overexpressed in comparison with quiescent HSC (Marcher et al., 2019). Among these genes, it is worth mention the presence of the transcriptional regulators ETS proto-oncogene 1 and Runt-related transcription factor 1, which seem to work as drivers of NASH-related HSC plasticity. Of note, increased expression of the latter has been reported in patients with NASH (Kaur et al., 2019).

An imbalance between pro-oxidative species and antioxidant defenses may augment the oxidative burden, which may culminate in macromolecules damage including lipids, DNA and proteins. Oxidative stress is an important mediator in the progression from simple steatosis to steatohepatitis. Fatty acids overload triggers the upregulation of β-oxidation as a compensatory mechanism leading to an augment in mitochondria tricarboxylic acid cycle and ATP generation, originating higher amounts of ROS. Afterwards, ROS can react with FA leading to lipid peroxidation and consequent formation of reactive aldehydes such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) (Sutti et al., 2014). Several studies have reported increased lipid peroxidation products in the liver and serum of NAFLD and NASH patients (Shearn et al., 2017; Zelber-Sagi et al., 2020). In addition, ROS can also increase the expression of different cytokines and chemokines including TNFα and IL-8, which have been related to NAFLD progression (Bahcecioglu et al., 2005).

Besides triggering lipid peroxidation and the expression of cytokines and chemokines, ROS can also induce mitochondrial DNA (mtDNA) deleterious mutations, which may affect mitochondrial integrity and ultimately, cellular homeostasis. NASH patients displayed ultrastructural

mitochondrial disturbances including loss of mitochondrial cristae, comparing to NAFLD individuals (Sanyal et al., 2001). Sookoian and colleagues have shown that the mutation rate of liver mtDNA is higher as the disease severity increases (Sookoian et al., 2016). Furthermore, dysregulation in proteins involved in mitochondrial oxidative phosphorylation, lipid metabolism and mitochondrial fatty acid oxidation have been reported in animal models of NASH (You et al., 2017). mtDNA is extremely susceptible to oxidative damage, causing its depletion. Sookoian *et al.,* demonstrated a diminished liver mtDNA/nuclear DNA ratio in obese NAFLD patients, suggesting a significant association between mtDNA depletion and NAFLD (Sookoian et al., 2010). In addition, a significantly lower mtDNA copy number was observed in obese and IR NAFLD and NASH patients, comparing to healthy individuals (Pirola et al., 2015).

1.3.3. Multiple hits

NAFLD is complex disease which involves several factors and multi-organs and thus, the initial "two-hits" theory can no longer explain its complexity. The nature of NAFLD is still unclear and highly variable and liver biopsies obtained over several years showed that the progression of NAFLD from steatosis to NASH and fibrosis is not a linear process and it is even more dynamic and complex than previously thought (Bessone et al., 2019; Negro, 2020). The current findings suggests that this nature reflects the heterogeneous and simultaneous impacts of genetics and epigenetics, microbiome, metabolism and comorbidities over NAFLD progression (Ekstedt et al., 2017; Eslam et al., 2018).

Consequently, the "two-hits" theory is now considered outdated for the simplistic way that describe such complex process and a "multiple-hits" hypothesis has been proposed alternatively (Buzzetti et al., 2016). This hypothesis is widely accepted and provides a more accurate explanation of NAFLD pathogenesis (Figure 4). In accordance with the "two-hits" theory, this new hypothesis still considers that exacerbated hepatic fat accumulation, due to increasing hepatic DNL and failure to inhibit adipose tissue lipolysis, is a consequence of insulin resistance and the responsible for the first triggers (Fang et al., 2018).

In addition, it also recognizes the importance of genetic and environmental factors as well as hepatocyte dysfunction through mitochondrial impairment, endoplasmic reticulum stress and inflammasome activation, adipose tissue dysfunction, alterations in the crosstalk between different organs and tissues, increased levels of pro-inflammatory cytokines and gut dysbiosis, among the secondary " multiple hits" (Cotter & Rinella, 2020). It is easy to understand that this "multiple hits" hypothesis highlights the importance of inflammatory cytokines in the development of NASH and its progression into fibrosis and HCC (Fang et al., 2018).
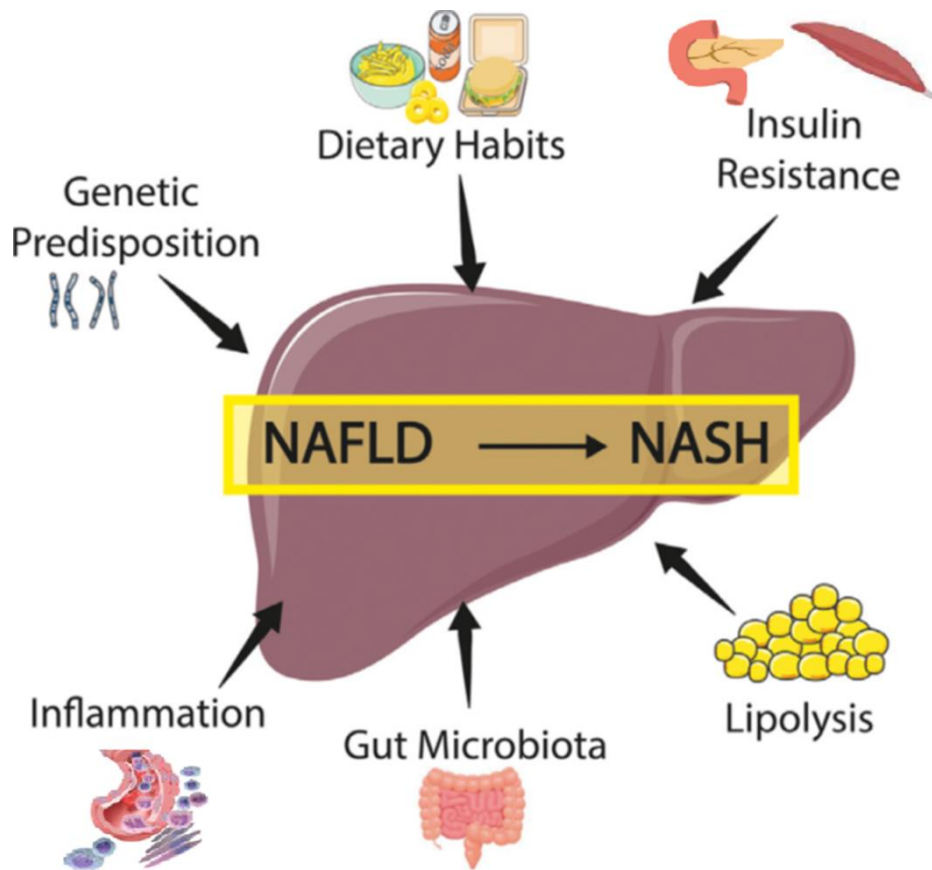
Figure 4- Multiple parallel insults and the pathogenesis of NAFLD. Adapted from (Arconzo et al., 2021)

1.4.   Diagnosis

NAFLD is a silent disease and usually, patients discover that they have fatty liver by routine analysis, which means that an early diagnosis is difficult to obtain.

NAFLD causes an abnormal increase in alanine aminotransferase (ALT) values, a hepatic enzyme considered a liver health marker, and according to recent evidence, ALT is often the first indicator of NAFLD (B & Thykadavil, 2019). Nonetheless, many NAFLD patients can still have normal ALT levels and as a result, other biomarkers have been proposed to make the diagnostic more accurate.

Serum γ-glutamyl transferase (GGT) levels, which predicts several cardiovascular diseases, are often increased in patients with steatohepatitis (Ahmed, 2007). In addition, it is also possible to establish a correlation between GGT and insulin resistance (Petta et al., 2012).

Recently, the involvement of non-coding RNAs and their use as biomarkers to the diagnosis of NAFLD have been considered due to its ability to regulate gene expression. Several studies have reported that NAFLD development and progression of NASH are intimately related to differential of expression of many hepatic microRNAs (miRNAs) (Gjorgjieva et al., 2019; C.-H. Liu et al., 2018).

Despite the huge number of miRNAs described in the literature, miR-122, miR-21 and miR-34a are the most robustly studied miRNAs in NAFLD (Calo et al., 2016; Pillai et al., 2020). Upregulation of miR-34a (Simão et al., 2019) and miR-21 (Afonso et al., 2018) as well as downregulation of miR-122 (Hsu et al., 2012) are highly associated with development of NAFLD and increased risk of fibrosis and HCC.

After the clinical suspicion of fat infiltration through the biomarkers, imaging techniques are used to confirm liver steatosis. Ultrasonography has been considered the first-line diagnostic test for the detection of liver steatosis. Ultrasounds are widely used because, besides providing little discomfort to the patient, it is a safe and cheap method. However, this technique display some clear disadvantages including poor estimation of liver steatosis when fat content is lower than 20% (Dasarathy et al., 2009). In livers presenting a degree of steatosis superior to 20%, the sensitivity increases up to 80% (Shannon et al., 2011).

Magnetic resonance imaging (MRI) is one of the leading techniques to provide accurate information regarding the intrahepatic triglyceride content across the entire liver (Dyson et al., 2014; T. H. Kim et al., 2019). This tool has a high sensitivity and specificity allowing the screening and staging of NAFLD patients without the need for ionizing radiation (X.-M. Wang et al., 2018). In addition, it also accurately classifies grades and changes in liver steatosis.

After ruling out all these other possible causes for fatty liver and given the limitations of the non-invasive methods, the final NAFLD diagnosis and staging are only confirmed through needle biopsy. NAFLD exclusion criteria includes viral hepatitis, alcoholic fatty liver disease, certain medications, some medical conditions such as Wilson and celiac disease, certain metabolic abnormalities and nutritional status (Papatheodoridi & Cholongitas, 2018).

Liver biopsy is an invasive method which allows the estimation of the steatosis degree and the presence of fibrosis or necro-inflammatory activity and ultimately, distinguish steatosis from steatohepatitis (Dyson et al., 2014). However, despite liver biopsies still remaining as the "gold standard" to evaluate steatosis, it is relatively subjective and may vary among individual observers (Angele et al., 2008). In fact, it is important to mention that needle biopsy sample only represents the 50000th part of the liver volume and since the liver is not uniformly affected in NAFLD, the biopsy sample harvested may be the cause of interpretation errors (Isabela Andronescu et al., 2018). Ratziu and colleagues reported that, in a group of 51 patients with NAFLD, two liver biopsies performed in the same day and on the same patient but in different areas of the liver revealed completely opposite levels of liver fibrosis depending on the area, in about 35% of all the patients assessed (Ratziu et al., 2005).

1.5.   NAFLD management

Given the high prevalence of NAFLD and related comorbidities, the implementation of specific treatments has become of the utmost importance. However, as of today, since the scarce understanding of the molecular mechanisms of NAFLD pathogenesis, there is no approved medications or effective treatment which can directly deal with NAFLD, despite some pharmacological approaches are undergoing clinical trials (Friedman et al., 2018). Consequently, the management for NAFLD has been weight loss and increasing insulin sensitivity via lifestyle adjustments, medications or surgical intervention (Mundi et al., 2020).

Behavior modification, physical exercise and calories reduction have beneficial effects on NAFLD progression. In some cases, lifestyle changes are enough to mitigate insulin resistance and decrease the hepatic transaminases values (Hickman et al., 2004; Katsagoni et al., 2017). In fact, Johari and colleagues have reported that obese individuals subjected to a modified alternate-day calorie restriction for 8 weeks displayed decreased levels of hepatic steatosis and fibrosis as well as diminished body mass index and ALT levels (Johari et al., 2019).

It is well known that obesity runs high among NAFLD individuals, thus weight loss is one of the suggested treatment for people suffering from NAFLD/NASH (Chalasani et al., 2012). However, weight loss should represent at least 10% of the total body weight, in order to induce a near complete resolution of NASH and improvement of liver fibrosis by at least one degree (Romero-Gómez et al., 2017).

In some severe cases and when other medical approach have failed to accomplish sustained weight loss, bariatric surgery is the treatment of choice (Musso et al., 2016). Bariatric surgery is still the most effective treatment of sustained weight loss in obese subjects. In a group of 109 morbid obese patients submitted to bariatric surgery, liver biopsy demonstrated that in 85% of the patients, NASH had disappeared one year after the surgery (Lassailly et al., 2015). Furthermore, the patients also shown decreased levels of ALT, GGT and higher insulin sensitivity. Although, as all surgical interventions, mid- and long-term complications can occur, such as intestinal obstruction, malabsorption, marginal ulcer and gallstones (I. T. Ma & Madura, 2015).

1.6.   Pharmacological treatment

To date, there are no specific pharmacological therapy for NAFLD management, despite some ongoing trials. The disease is characterized by a wide variety of causes and symptoms. Therefore, only treatments aiming to address coexisting conditions including obesity, insulin resistance and dyslipidemia have been used by their potential in reducing insulin resistance or improving liver function (Mundi et al., 2020). For instance, metformin, which is used for the management of T2DM and obesity, started to be commonly used in the treatment of NAFLD after presenting

stunning results in the amelioration of hepatic steatosis and lipogenesis (Lin et al., 2000). In NAFLD patients, metformin treatment decreased serum ALT levels, reduced liver volume by 20% and significantly improved insulin sensitivity (G Marchesini et al., 2001).

Another example are the thiazolidinediones (TZDs), which are insulin sensitizing agents. TZDs have demonstrated an enormous potential in NAFLD patients by their capacity to induce adipocyte differentiation, increase adiponectin levels and improve insulin sensitivity (Mundi et al., 2020). Rosiglitazone, a known TZD, has improved hepatic steatosis, inflammation, ballooning and fibrosis in NASH patients (Neuschwander-Tetri et al., 2003).

2. Transplantation, organ shortage and marginal donors for transplantation

Success in liver transplantation (LT) took longer to obtain than kidney transplantation and the initial attempts made in 1963 by Dr. Thomas Starlz were unsuccessful (Starzl et al., 1963). However, a few years later his work paid off and the first successful LT in humans was carried out in 1967. Since then, the surgical transplantation procedures have been improving with the implementation of new techniques, including the split technique and the reduced size orthotopic liver transplantation. The former one consists in dividing the liver in two functional parts, which are then transplanted into two different recipients, while in the latter, only one lobe of the graft with fully functionally is used (Malagó et al., 1995).

Nowadays, kidney, pancreas, heart, lung, liver, bone marrow and cornea transplantations are performed among non-identical individuals in a daily basis with a huge success increment. Different types of transplants may occur including from one part of the body to another in the same patient (autograft), between two genetically identical individuals (isograft), between two genetically non-identical individuals (allograft) or even from one specie to another (xenograft).

After transplantation, an immunosuppressive therapy is required to circumvent the recipient's immune system in order to minimize recognition and subsequent rejection (Gitto & Villa, 2016).

Despite the advances in the transplantation field, which has permitted the increase of donors' pool and the number of liver transplantations, the gap between the number of patients waiting for LT and the number of available organs has intensely increased. The worldwide raising demand for organs has led to the urgent need for the expansion of donor pool to match the growing demands. Therefore, some strategies have been designed to increase the number of organs that are transplanted by increasing the acceptance variables of an organ for transplantation and the development of various alternative and conventional transplant techniques. In this sense, the possible use of suboptimal or marginal organs as a viable approach to increase the number of available organs for transplantation has been highly encouraged. Nevertheless, this expanded criteria donors (ECD) organs are well known to be more prone to

morbidity or mortality once transplanted into the recipient. Therefore, the main challenge in the LT filed are linked to the possible use of such suboptimal or marginal organs as a viable approach to augment the number of available organs for transplantation. The discovery of new therapeutic strategies to minimize factors that overall render the organ non-functional and at the same time, increase the use of marginal livers for transplantation instead of discarding them, are extremely important due to its high prevalence among the potential donors.

Livers from elderly fit within the ECD. As the life span increases, the quality of organ donor decreases giving its advanced age, which in most cases are associated with more comorbidities. Rising evidences have shown that older donor livers are associated with primary nonfunction (PNF), defined as initial poor function requiring retransplantation or causing death within 7 days of transplantation (Feng & Lai, 2014). This increased risk of liver allograft failure has been related to, among others, relatively fewer hepatocytes and decreased regenerative capacity in older hepatic parenchyma (Schmucker, 1998). Moreover, the increased burden of medical comorbidities in older donor may further increase the susceptibility to injury.

Apart from the donor's age, the degree of steatosis has been identified as one of the main risk factors for PNF of liver graft. Excessive fat accumulation in the cytoplasm creates a state in which the cells are swollen, resulting in a partial or total obstruction of the sinusoidal space. Hence, the architectural alteration of microvessel structures reduces the blood flow, and consequently, diminish the input of oxygen and nutrients, becoming in a chronical state of hypoxia and predisposing the liver to ischemia-reperfusion injury (IRI), which is extremely relevant in LT context, as it will be discussed later.

According to the European Association for the Study of the Liver Clinical Practice Guidelines, the degree of steatosis can be divided into a) mild (≤ 30% macrosteatosis), donor organ is considered suitable for transplantation; b) moderate (30–60% macrosteatosis), may result in acceptable outcomes in selected donor-recipient risk combinations; and c) severe (> 60% macrosteatosis), are discarded because they present increased sensitivity to endotoxins, endothelial damage, decreased ATP levels, sinusoidal swelling and congestion, factors that are associated with high risk of PHF, graft failure, biliary complications, and mortality (Burra et al., 2016). Early hepatic graft dysfunction and poor recipient outcome are mainly the result of two underlying mechanisms. During the cold storage, lipid droplets have been seen to expand, changing the cellular infrastructure by displacing the surrounding organelles. Thus, the increase in the hepatocytes size tends to worsen the microcirculation in the liver (Takeda et al., 1999). In addition, KC activation, which are responsible for ROS generation, and are further activated during reperfusion, seems to play an important role in PNF. Moreover, the cell membrane

fluidity and the hepatic mitochondrial function are affected by the preservation process and IR (Fukumori et al., 1999).

Assessment of the steatosis levels of the liver are imperative, since operative mortality associated with steatosis after major liver resection exceeds 14% compared with the 2% for healthy livers, and the risks of PNF after surgery can go up to 60%, compared with only 5% for non-steatotic grafts (Canelo et al., 1999; Tashiro et al., 2014).

In the context of living donor liver transplantation, treatments for NAFLD donors intend to reduce fatty infiltration, ameliorate injury and diminish metabolic risk factors before transplantation. However, current approaches require long-standing interventions (varying from 6 to 72 weeks), which are incompatible in the case of death donors (Álvarez-Mercado et al., 2019) Therefore, further studies are needed to develop therapies and reduce the pretreatment times during emergency procedures. However, this is a point to bear in mind, since living donors could increase the number of available grafts, helping to reduce the waiting list.

The challenge in transplantation community is look for new therapeutic strategies to minimize factors that contribute to the endanger graft quality and increase the use of steatotic livers for transplantation instead of discarding them. This would lead to an increase in organ pool availability and consequently reduce the waiting list for transplantation.

2.1.   Liver preservation methods

The advances in surgical techniques and immunosuppressive drugs have established LT as a standard treatment for patients with end-stage liver disease. The transplant setting encompasses organ procurement, preservation and implantation of the graft into the recipient. It is a complex procedure associated with multifactorial reasons which may lead to graft failure, including donor factors (i.e., age, steatosis, or donation after death), organ retrieval and preservation (cold and warm ischemic times), as well as transplantation procedure itself, including surgeon expertise and possible surgical complications (Kahn & Schemmer, 2018).

The main goal in organ preservation is the maintenance of organ function during storage, so the graft works back properly at the reperfusion period. Throughout the process the liver faces events of warm ischemia, cold ischemia and rewarming ischemia which can lead to organ damage, the so-called ischemia-reperfusion injury (Teodoro et al., 2022; Weigand et al., 2012). During LT, when the organ is retrieved from the donor and stored within the preservation solution under hypothermic conditions, the liver undergoes a period of cold ischemia. Once removed from cold storage, the graft is exposed to warm ischemia before the beginning of reperfusion in the recipient. Therefore, in the context of LT, the IRI is a cumulative combination

of cold and warm ischemia followed by reperfusion. On the other hand, IRI in the context of liver surgery, trauma and shock is just due to warm ischemia reperfusion.

The use of cold preservation solutions is the main technique in organ transplantation to maintain the morphological and functional integrity of the graft. It has the purpose of reducing, as much as possible, factors that endanger graft quality, especially the ones implicated in hepatic IRI and the complications deriving from it. The composition of a preservation solution will dictate the quality and duration of graft preservation, through the prevention of energy depletion, acidosis, edema and oxidation, among others (Gores et al., 1989; N. V Jamieson et al., 1988; C Peralta et al., 2002; Robinson, 1978).

Currently, there are two main strategies for organ preservation: static and dynamic (Figure 5). Static Cold Storage (SCS) consists in organ preservation at low temperatures (0-4°C) to reduce metabolic activities that would lead to cellular damage when oxygen is removed from the donor organ (Lee & Mangino, 2009). The organ is perfused with a cold solution to wash out the blood and to enhance storage performances. Afterwards, the graft is stored statically in a container filled with the preservation solution and place into an ice-box while waiting for the transplantation.

Regarding dynamic perfusion, the retrieved organ is place into a chamber, where it is continuously perfusion, with either an oxygenated or non-oxygenated solution using a machine perfusion (MP) pump. The continuous perfusion allows a better distribution of the preservation solution throughout the graft, a washout of blood, a continuous delivery of oxygen and nutrients and toxic metabolites clearance. In addition, this technique permits also a real-time monitoring of the functional and biochemical performance of the graft as well as the administration of drugs (Taylor & Baicu, 2010). MP can be performed under different temperatures, including Hypothermic Machine Perfusion (HMP), Normothermic Machine Perfusion (NMP), Subnormothermic Machine Perfusion (SNMP) and Hypothermic Oxygenated Perfusion (HOPE) (Phillipp Dutkowski et al., 2019).

2.1.1. Static Cold preservation

The maintenance of organ viability during cold storage is of utmost importance to a successful outcome after LT. To minimize graft injury during cold storage, preservation solutions were developed many years ago. Generally speaking, preservation solutions are used to wash the organ during procurement and to preserve the graft during transportation. Hypothermia is well known to significantly reduce the body metabolism and in average, the cellular oxygen and glucose requirements decrease up to 8% for every degree of decrease in temperature (W. Wang

et al., 2020). Cold storage is a procedure that delays the depletion of ATP levels and slows down the deleterious processes, but it can also elicit damage.
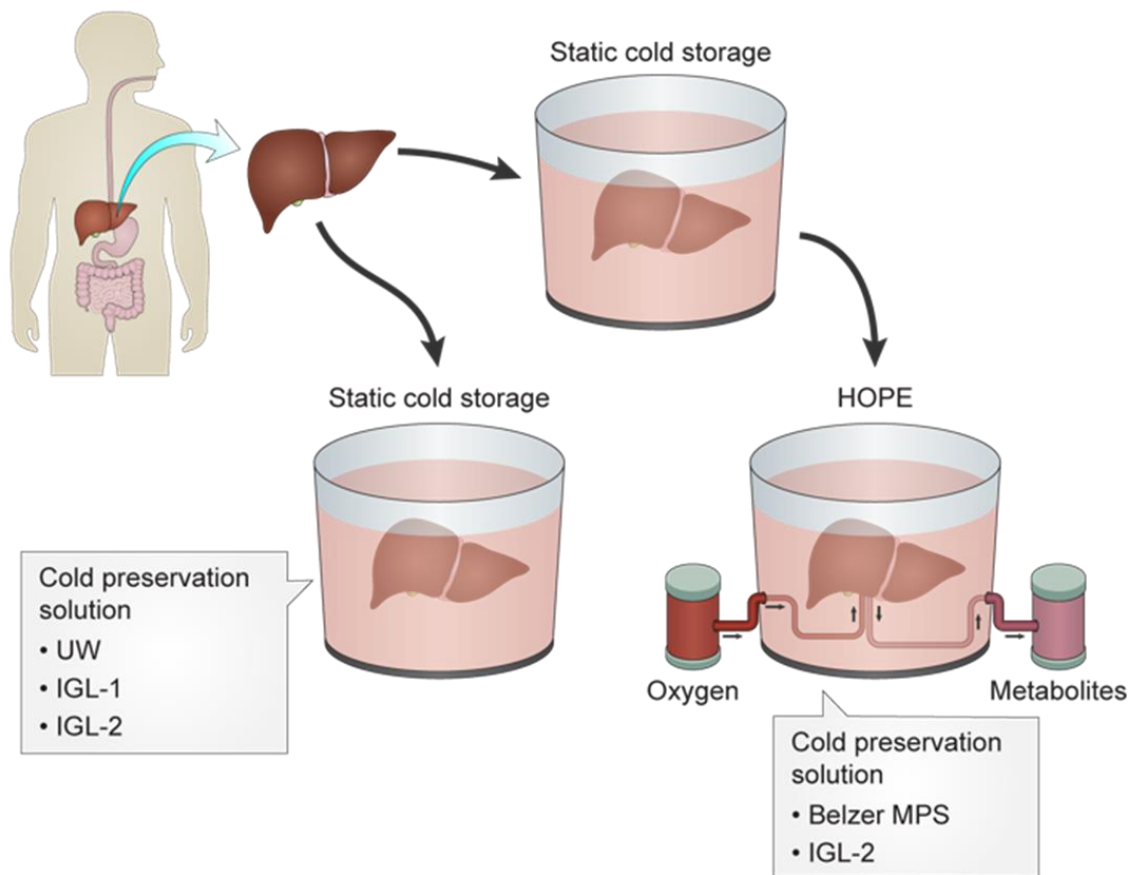
Figure 5- Strategies of organ preservation. Static cold storage and hypothermic oxygenated machine perfusion (HOPE) (Teodoro et al., 2022).

In the first time of organ transplantation, the adverse effects of cold storage were attributed to the inhibition of the Na^+/K^+-ATPase and subsequent buildup of intracellular sodium levels followed by chloride influx and cell swelling (Rauen & de Groot, 2008). Therefore, to enhance organ preservation, solutions mimicking the intracellular composition and protecting intracellular spaces during the onset of ischemia have been developed. In 1969, Collins and colleagues developed the first solution, Collins' solution (Collins et al., 1969). This solution contained a high potassium ion content avoiding cellular swelling and a high glucose concentration that acted as a cell impermeant. This solution increased SCS period to 24 hours for kidneys. In the following years, alterations in the Collins' solution were made, such as Euro-Collins solution, in which magnesium phosphate was removed due to its precipitation causes crystals, and glucose was replaced by mannitol or sucrose, which provides major protection during prolonged cold ischemia (Andrews & Bates, 1985). However, this solution kept having

poor success in pancreatic and hepatic transplantation and in the 80's Belzer developed the University of Wisconsin (UW) solution.

Prior to the discovery of the UW solution, liver graft preservation was limited to 6 hours. With the UW solution, storage improved up to 16 hours, allowing long distance procurement of the donor organ. In that way, UW solution became the gold standard for transplantation. The UW solution contains high potassium and low sodium concentrations along with the impermeant trisaccharide raffinose (to prevent cell swelling), and the impermeant hydroxyethyl starch (HES) (to prevent expansion of the extracellular space). In addition, UW solution formulation contains also lactobionate and raffinose to attenuate hypothermia-induced cell swelling and edema, phosphate to prevent acidosis allopurinol and reduced glutathione to prevent oxidative stress and adenosine and ribose to stimulate ATP synthesis (Table 1) (Belzer & Southard, 1988).

The UW solution is now used for other donor organs including kidneys, hearts, pancreas, intestine and lungs. However, several studies have concluded that in different cell types such as liver endothelial cells, hepatocytes and renal tubular cells, the intracellular sodium accumulation and cell swelling did not seem to have a crucial role (Rauen & de Groot, 2008). The high potassium present in the UW solution induces cellular depolarization and vasoconstriction which impairs the organ perfusion during washout and reperfusion (Rauen & de Groot, 2004). In addition, HES, the oncotic agent used in UW, has been associated with red blood aggregation, macrophage invasion and tubular damage (Hüter et al., 2009).

During the 90's other solutions caught some attention, Histidine-Tryptophane-Ketoglutarate (HTK) and Celsior. The main HTK components are histidine (strong buffer), tryptophan and alpha-ketoglutaric acid, low permeable amino acids, and mannitol, which provides the osmotic barrier. On contrary to the UW solution, the potassium, sodium and magnesium concentration are low and its low viscosity makes the flushing more effective and the cooling of organs more rapid and efficient (Guibert et al., 2011). Celsior solution contains the same buffer of HTK solution, lactobionate and mannitol belonging to UW solution, but with a high content of sodium. Celsior is considered a "mixture of solutions", which in the beginning was used for heart transplantation but has also shown efficacy in lung (Wittwer et al., 1999), kidney (Nunes et al., 2007) and pancreas (Hackl et al., 2010) preservation.

The replacement of HES for another oncotic agent (polyethylene glycol (PEG) 20 kDa) in the UW solution was reported to improve rabbit heart performance after 24h preservation (Wicomb et al., 1990). In addition, liver grafts stored in UW solution and then rinsed with a solution containing PEG 35 kDa (PEG35) showed reduced hepatic injury and improved liver function after reperfusion (Mohamed Amine Zaouali et al., 2014). The better outcome was associated with the prevention of oxidative stress, mitochondrial damage and liver autophagy.

Table 1- Composition of UW, IGL-1, HTK and Celsior solutions

Components	UW	IGL-1	HTK	Celsior
K^+ (mmol/L)	125	25	10	15
Na^+ (mmol/L)	27	125	15	100
Mg_2^+ (mmol/L)	5	5	4	13
SO_4^{2-} (mmol/L)	4	5	-	-
Ca^{2+} (mmol/L)	-	0.5	0.015	0.25
Cl^- (mmol/L)	-	-	50	40
Diphosphate (mmol/L)	25	25	-	-
Histidine (mmol/L)	-	-	198	30
Histidine-HCl (mmol/L)	-	-	18	-
Tryptophan (mmol/L)	-	-	2	-
Raffinose	-	30	-	-
Mannitol (mmol/L)	-	-	-	60
Lactobionic acid (mmol/L)	105	100	-	80
Mannitol (mmol/L)	-	-	30	-
Hydroxyethyl starch (g/L)	50	-	-	-
Polyethylene glycol 35 (g/L)	-	1	-	-
Glutathione (mmol/L)	3	3	-	3
Allopurinol	-	1	-	-
Adenosine (mmol/L)	5	5	-	-
Glutamic acid (mmol/L)	-	-	-	20
Ketoglutarate (mmol/L)	-	-	1	-
pH	7.4	7.4	7.4	7.4
Osmolarity (mosmol/L)	320	320	310	320

The concentration of some components may vary among manufacturers

The beneficial effects of PEGs are known for decades. Back in the decade of 1970, Daniel and Wakerley demonstrated increased cellular viability by using PEG 20 kDa during the cold preservation of renal pig cells (Daniel & Wakerley, 1976), whereas lower molecular weight PEG (6 kDa) protected myocardium from cellular edema and membrane damage during preservation (Ganote et al., 1977). Since then, several studies have demonstrated the protective role of different molecular weight PEG during cold preservation using different animal models and the satisfying results obtained led the use of PEG to a clinical level.

In France, a solution similar to UW solution was developed in the beginning of the century: the Institute Georges Lopez (IGL)-1 solution. This solution is characterized by a high sodium and low potassium concentrations. The main trait of this solution is the use of PEG35 as colloid instead of HES (M. A. Zaouali et al., 2011) and its efficiency in the abdominal organ's preservation has been reported in several publication (Codas et al., 2009; Dondéro et al., 2010; Panisello-Roselló, Alva, et al., 2018).

IGL-1 is a PEG based solution routinely used in clinical LT and it has been considered as a suitable alternative to UW solution by the European Liver Transplant Registry (Adam et al., 2015). Franco-Gou et al., 2007 demonstrated the superiority of IGL-1 compared to UW in cold rat liver

preservation after 24 hours. More recently, it has been reported that IGL-1 solution more efficiently protected steatotic livers against IRI than UW solution (Ben Mosbah et al., 2006). Liver grafts preserved in IGL-1 solution have been found to possess increased the mitochondrial enzyme, aldehyde dehydrogenase 2 (ALDH2), expression and activity, which in turn were associated with reduced mitochondrial damage and ATP breakdown prevention (Panisello-Rosselló, Alva, et al., 2018).

Despite these beneficial effects, the protective mechanisms of IGL-1 are complex. They may include cytoprotective mechanisms exerted, at least in part, by the activation of AMPK (cytoprotective factor), Nitric oxide (NO) generation (vasodilator agent), as well as the mitochondrial protection among others (Mohamed Amine Zaouali et al., 2010). Until now, IGL-1 is being successfully used in both human liver and pancreas preservation.

2.1.2. Dynamic preservation

The need for organ is continuously increasing and consequently, the use of novel techniques for optimizing suboptimal graft preservation is of utmost importance. Dynamic preservation aims to recondition and achieve extension of preservation time window in otherwise rejected organ donations (Tara et al., 2021).

Machine perfusion allows the dynamic perfusion of the organs, and it was developed decades ago. However, the logistic nuisance (portability) and possible damage in vital organ structures, including the endothelium, were pointed as drawbacks in MP usage for the transplantation community and therefore, those difficulties to implement the logistics kept their impact to a low profile. Nowadays, with the advance in innovation, their design is more portable and efficient, and consequently, a more promising therapeutic approach for graft preservation.

The explanted organ is placed into a chamber, under continuous perfusion with either an oxygenated or non-oxygenated solution using a pump (Figure 5). The continuous flow facilitates a better distribution of the preservation solution throughout the graft and the continuous supply of oxygen and nutrients while flushing cellular waste products from the liver, preventing the damage cascade buildup that occurs in static cold storage. In addition, MP is also capable of maintaining the hemodynamic stimulation on the vasculature of the graft, which plays a crucial role in vascular function under physiological conditions (Yuan et al., 2010).

MP can be performed under different temperatures including hypothermic, subnormothermic and normothermic. The preservation of steatotic liver using MP has been showing better results than SCS. Bessems and co-workers showed that followed 24 hours of hypothermic preservation, bile and urea production, ammonia clearance, oxygen consumption and ATP levels were significantly higher after MP comparing to SCS (Bessems et al., 2007). Subnormothermic MP on

rat liver (Vairetti et al., 2009) and normothermic MP in a porcine model (Jamieson et al., 2011) demonstrated a reduced preservation injury in steatotic organs, comparing to SCS.

Over the years, different variants of MP have been developed. The monitorization of the organ conditions during the entire process and the possibility to obtain a time window for a pharmacological intervention made this technique an efficient alternative to simple cold storage.

2.1.2.1. Normothermic Machine Perfusion

In this technique, a solution is perfused at body temperature (37°C) to maintain the liver at physiological temperatures outside the body while maintaining its metabolic functions. At this temperature, the demand of oxygen is extremely high and to meet the metabolic requirements of the graft, red blood cells are used in the perfusate as an oxygen carrier (Martins et al., 2020). NMP has been evaluated in several animal studies, showing better results than SCS in liver graft preservation (Fondevila et al., 2011). However, this technique required more logistics than the other different types of MP and it has a risk for bacterial contamination during the preservation period (Vekemans et al., 2008).

2.1.2.2. Subnormothermic Machine Perfusion

SNMP systems intend to play an intermediate role between NMP and HMP. The continuous perfusion of the graft with a perfusate at room temperature (21°C) enables lowering the metabolic demands while still maintaining sufficient metabolism for viability testing and improvement of graft function (Bruinsma et al., 2014). SNMP demonstrated a better outcome of steatotic rat liver when compared to SCS (Vairetti et al., 2009). Fatty liver preserved by SNMP showed reduction in hepatic injury and oxidative stress, while the ATP levels and bile production were higher.

2.1.2.3. Hypothermic Machine Perfusion

Hypothermic Machine Perfusion (HMP) is a method of dynamic cold preservation where the graft is perfused with either a oxygenated (Op den Dries et al., 2014) or non-oxygenated solution (Guarrera et al., 2010).

Oxygen and mitochondria play an important role during hepatic IRI. Hepatic perfusion in HOPE benefits in great extent of the oxygenation of the perfusate, which is responsible for maintaining the integrity and function of the mitochondrial population (Schlegel et al., 2020). HOPE embraces a dynamic perfusion at the range of 4–11°C with active oxygenation of the perfusate (Brüggenwirth et al., 2020; Czigany et al., 2020; Phillipp Dutkowski et al., 2019). The supply of

oxygen to the tissue permits the ATP synthesis through mitochondrial electron transport chain, helping the restoration of cellular homeostasis and preventing mitochondrial collapse (Hessheimer; et al., 2015). HOPE has been reported to improve the quality of liver preservation by maintaining the functional integrity of hepatocytes during ischemia via oxidative stress reduction (Kron et al., 2018).

In a comparison study using a rodent model of donation after circulatory death (DCD) liver grafts, MP strategy proved to be more protective compared to static cold storage (Schlegel et al., 2014). In addition, comparison among different machine perfusion approaches were also performed (warm vs cold perfusion). Normothermic oxygenated perfusion failed to protect from lethal injury in grafts exposed to 1h warm ischemia, with a concomitant activation of Kupffer- and endothelial cells. On the other hand, HOPE prevented the development of lethal graft injury, probably by the downregulation of electron transfer rates, which hampers the initial oxidative stress. These results suggest better outcome for the HOPE technique to rescue DCD livers. Moreover, cold oxygenation also diminishes mitochondrial ROS release, triggering less oxidative damage in mitochondria in early reperfusion (Schlegel et al., 2018). Additionally, the buildup of some metabolites including succinate, during the ischemic period have been described to lead to mitochondrial dysfunction on several tissues (Chouchani et al., 2014). Hence, the removal of such metabolites by the dynamic flow observed in HOPE might be an important way to increase the odds of a proper mitochondrial function during early normothermic reperfusion.

1h of HOPE treatment after SCS protected liver grafts from initial ROS and damage-associated molecular patterns (DAMPs) release after transplantation alongside with decrease activation of inflammatory pathways (Kron et al., 2018). This HOPE period showed also to be appropriate to recover ATP loading prior to reperfusion and reduce cell death during reperfusion (Philipp Dutkowski et al., 2006).

2.1.2.4. Preservation solutions in machine perfusion

The composition of perfusion solutions for hepatic hypothermic oxygenated perfusion are identical to those used for static cold storage (Table 2). Belzer-MPS is one of the most used solutions for HOPE perfusion and like its mother-solution, UW solution, has HES as oncotic agent in its composition. The main drawback of solutions containing HES is the high viscosity, which may lead to sinusoidal shear stress and subsequent destruction of the glycocalyx of hepatocytes (Zeng et al., 2018). Glycocalyx comprises the thin luminal sugar monolayer that protects the graft endothelia and its damage has been related to graft injury and function in clinical liver transplantation (Lopez et al., 2018; Schiefer et al., 2020).

To overcome these shortcomings, a new solution containing PEG35 instead of HES was developed, called Polysol (Maud Bessems et al., 2005). As an oncotic agent, PEG35 exerts an oncotic pressure similar to HES but with a relatively lower viscosity and consequently less shear stress in the hepatic sinusoid. A better liver function and less liver damage was observed using Polysol solution compared to Belzer-MPS (M. Bessems et al., 2005). In addition, 24 hours of MP using Polysol showed a better preservation compared to the same solution but with HES instead of PEG (Polysol-HES), highlighting the protective role of PEG. Furthermore, a lower molecular weight PEG, PEG20 when supplemented to Celsior solution offered a better protection of pig kidneys recovered after cardiac death using MP, compared to Celsior without supplementation or Belzer-MPS (Maio et al., 2007).

Schlegel et al., 2013 showed that the endothelial cleaning or repair of the glycocalyx represents an important mechanism of protection confer to the liver by HOPE. The glycocalyx is known to be cleaved through enzymatic cleavage of the proteoglycan core proteins or direct oxidative stress by ROS underlying IR, inducing endothelial permeability and edema (Van Golen et al., 2014). Lopez et al., 2018 showed compelling evidences on the benefits of IGL-1 solution on steatotic livers, highlighted by the importance of glycocalyx protection during SCS. It is known that PEG35, present in IGL-1 solution, prevents cell swelling and vascular endothelial damage through the stabilization of lipid membranes and by lowering membrane permeability. To deepen the findings made by Lopez and colleagues regarding the role played by glycocalyx during hepatic IRI and its connection to PEG35, the shear stress should be taken into account. The HOPE seems a valid strategy to investigate the shear stress inherited to IR and its effect on the glycocalyx integrity using a perfusate containing PEG35. Such therapeutic approach of glycocalyx protection could potentially enhance the organ viability of marginal grafts and diminish the severity of IRI.

This approach represents an alternative to simple cold preservation, enabling the monitorization of organ viability as well as the possibility to apply pharmacologic intervention.

Table 2- Dynamic preservation solutions compositions. Adapted from (Wei et al., 2007)

Components	Polsol	Belzer-MPS
K^+ (mmol/L)	5	25
Na^+ (mmol/L)	135	120
Mg_2^+ (mmol/L)	4	5
SO_4^{2-} (mmol/L)	-	5
Ca^{2+} (mmol/L)	2	0.5
Diphosphate (mmol/L)	21.7	25
HEPES (mmol/L)	20	10
Histidine (mmol/L)	6.3	-
Raffinose (mmol/L)	3	-
Trehalose (mmol/L)	5.3	-
Mannitol (mmol/L)	-	30
Dextrose (mmol/L)	-	10
Ribose (mmol/L)	-	5
Na^+-Gluconate (mmol/L)	75	85
K^+-Gluconate (mmol/L)	20	-
Hydroxyethyl starch (g/L)	-	50
Polyethylene glycol 35 (g/L)	20	-
Glutathione (mmol/L)	3	3
α-tocopherol (mmol/L)	5×10^{-5}	-
Ascorbic acid (mmol/L)	0.11	-
Allopurinol (mmol/L)	1.2	-
Adenosine (mmol/L)	5	-
Amino acids (mmol/L)	various[1],11	-
Vitamins (mmol/L)	various[2],0.17	-
Glucose (mmol/L)	11.1	-
Adenine (mmol/L)	5	5
Sodium pyruvate (mmol/L)	0.23	-
pH	7.4	7.4
Osmolarity (mosmol/L)	320	320

[1]alanine (1.01), arginine (1.18), asparagine (0.08), aspartic acid (0.23), cystine (0.33), cystine (0.25), glutamic acid (0.34), glutamine (0.002), glycine (0.67), isoleucine (0.38), leucine (0.57), lysine (0.48), methionine (0.30), ornithine (2.00), phenylalanine (0.30), proline (0.78), serine (0.29), threonine (0.34), tryptophan (0.88), tyrosine (0.19), and valine (0.43).

[2]ascorbic acid (0.11), biotin (0.21), Ca-pantothenate (0.004), choline chloride (0.01), inositol (0.07), ergocalciferol (3× 10-4), folic acid (0.002), menadione (4 × 10-5), nicotinamide (0.01), nicotinic acid (0.004), pyridoxal (0.005), riboflavin (0.003), thiamine (0.03), vitamin A (3 × 10-4), vitamin B12 (1 × 10-4) and vitamin E (5 × 10-5).

The concentration of some components may vary among manufacturers.

3. Ischemia Reperfusion Injury

Ischemia reperfusion injury (IRI) is a major hurdle in many clinical scenarios, including liver resection, trauma and the aforementioned, liver transplantation (Varela et al., 2011). Aside from immunological reasons, IRI is the major cause for graft dysfunction and even mortality (Deschenes, 2013).

During liver surgery, vascular occlusion is performed to avoid excessive blood loss, whereas liver transplantation involves liver graft perfusion with a preservation solution before cold storage.

The paradigm of IRI is based on two distinct but interconnected phases, the ischemic and the reperfusion phase, respectively. Ischemia is characterized by an inadequate supply of blood to

the tissues, causing a shortage of oxygen and nutrients required for normal cellular metabolism. On the other hand, reperfusion is defined as the restoration of blood flow, which reestablishes the oxygen and nutrients delivery to support cell metabolism and at the same time, removes potential damaging by-products produced during the ischemic period (Montalvo-Jave et al., 2008).

The severity of IRI is multifactorial and the extension of the injury will depend, among others, on the organ initial conditions, type of ischemia and duration of the ischemic period. The importance of the state of the liver can be easily understood in the following example. After 40 minutes of ischemia and 2 hours of reperfusion, there was a 9% decrease in the number of functional sinusoids in lean mice, while in ob/ob mice was observed almost a 50% decreased compared with sham-operated controls (Hasegawa et al., 2007). In addition, the injury might be reversible if the organ is subjected to a short ischemia period, but when longer ischemia period occurs, the injury might become irreversible.

In regard to the type of ischemia, it can be divided into warm or cold ischemia: the former takes place at body temperature (37°C), while the latter at 4°C (Baumann et al., 1989). In the context of liver resection or transplantation, warm ischemia is initiated when the blood flow is temporarily suspended by the surgeon, as in the case of living donor transplantation. While, cold ischemia occurs when the liver is removed from the donor, and it is placed in a cold preservation solution, prior liver transplantation (Zhai et al., 2013). Among all hepatic cells, liver sinusoidal endothelial cells (LSEC) and hepatocytes have been described as the more vulnerable to IR. The former is compromised in both cold and warm ischemia, while the latter is mainly affected during warm ischemia. The biomechanical stimulus is important for the normal functioning of LSEC and the lack of such stimuli during liver procurement and preservation, leads to the downregulation of the transcription factor Kruppel-like factor 2 (KLF2). In which turn, KLF regulates the transcription of protective genes like endothelial nitric oxide synthase (eNOS) or the nuclear erythroid 2 p45-related factor 2 (NRF2) (Álvarez-Mercado et al., 2019). NRF2 is considered the master regulator of redox homeostasis (Azzimato et al., 2020). Through its interaction with Kelch-like ECH-associated protein-1 (KEAP1), NRF2 is targeted for proteasomal degradation, under normal physiological conditions. On the contrary, upon stress conditions, the complex NRF2-KEAP1 dissociates, and NRF2 translocate to the nucleus, driving an antioxidant response.

The mechanisms of organ damage followed IR has been widely studied and involves the complex interactions of multiple pathways. Organ injury is triggered by the synergistically effect triggered by the disruption in the blood flow and hypothermia during the cold storage, which are likely to act synergistically during reperfusion further exacerbating the cellular damage. The

reestablishment of blood supply after a period of hypoxia contributes to reoxygenation injury, rewarming after a hypothermic period leads to rewarming injury/cold-induced apoptosis, and the reintroduction of blood into the injured organ gives rise to ROS production, release of proinflammatory cytokines and chemokines, and activation of immune cells to promote inflammation and consequent tissue damage (Rauen & de Groot, 2004).

The warm and the cold ischemia reperfusion injury share common pathological mechanisms: morphological changes, ATP depletion, local inflammatory innate immune activation and oxidative stress (Zhai et al., 2011).

There are many processes involved in hepatic IRI events. The lack of oxygen shifts the metabolism from aerobic respiration (i.e., oxidative phosphorylation (OXPHOS)) towards anaerobic respiration, or glycolysis. As a result, there is a severe reduction in ATP generation capacity and overtime, this ATP depletion may lead to cell death and destruction of the parenchymal tissue.

Other consequence of ATP depletion is the cellular edema (Reddy et al., 2004). The Na^+/K^+ ATPase, which helps to maintain the cell homeostasis, due to the ATP depletion is inhibited, leading to an imbalance in the intracellular Na^+ and Ca^{2+} concentrations and ultimately resulting in a K^+ accumulation in the cell. Accordingly, Cl^- enters to the cell through a water gradient, causing edema (Ramalho et al., 2006). The cell swelling underlying edema will originate narrowing of the sinusoidal space, which will be increased in steatotic livers triggering cell death. Moreover, the metabolic shift leads to an accumulation of lactic acid and ketone bodies, resulting in the acidification of the cellular milieu, known as metabolic acidosis. Thus, intracellular acidosis alters the physiological functioning of the cell by changing the affinity of proteins and their tertiary structures, inhibiting enzymes and disrupting the function of sarcoplasmic pumps and carriers, which accelerates the cell injury (Martin & Parton, 2006).

During the first moments of the reperfusion, the blood flow is reestablished and with it, two main consequences to the cell: elimination of the noxious elements generated during the ischemic period and the capacity to produce ATP via OXPHOS. However, the reestablish of oxygen during reperfusion may further aggravated the injury verified during the ischemic period, since ROS might be formed by the O_2 interaction with some metabolic products produced during ischemia. ROS cause also the release of inflammatory cytokines and lipid peroxidation. The synthesis of MDA and 4-HNE, which are substances with higher half-life and mobility than ROS, may migrate to different tissue amplifying oxidative stress (Esterbauer et al., 1991). The damage caused lead to a loss of microvascular integrity and a decrease of blood flow and ultimately to cell death (van Golen et al., 2012).

As mentioned above, KC are specialized macrophages in the liver with a crucial role in the defense against bacteria, viruses and other exogenous compounds. In addition, KC are important players in IRI. During the early stages of reperfusion, KC activation and consequent recruitment of neutrophils and macrophages triggers a sterile inflammation process (Weigand et al., 2012). Cytokines overexpression increase the oxidative stress damage and induce hepatocyte death. Furthermore, cytokines promote the secretion of selectins and integrins, that may occlude sinusoids, prolonging hypoxia and triggering a further activation of KC (Figure 6).

The evaluation of hepatic IRI can be performed using three different approaches*: in vivo* models, *in vitro* cell culture systems and *ex vivo* intact organ models. The main *in vivo* model was described by Yamauchi and colleagues in 1982 (Yamauchi et al., 1982), consisting in a model of partial hepatic ischemic (70%) where the hepatic artery and portal vein to the left and median liver lobes were occluded. This model prevents the mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. The *ex vivo* models use the isolated perfused liver, in which the excised organ is perfused via the portal vein using a system with buffer as perfusate, where buffer flow rates can be adjusted. Regarding the *in vitro* models, despite failing to reproduce the dynamic conditions that liver cell types are exposed *in vivo*, are a good model to decipher molecular mechanisms underlying IRI.

3.1.   NAFLD and increased susceptibility to IRI

Fatty organs are well known for the increased susceptibility to IRI, and different hypothesis have been proposed. Among others, impaired microcirculation, ATP depletion, Kupffer cell dysfunction, impaired mitochondrial function and increased adhesion of leukocytes, but the role that each of these mechanisms play in injury is not yet elucidated.

Fat deposition within the hepatocytes is associated with an increase in hepatocellular volume causing sinusoidal space narrowing and consequently, reduction on hepatic blood flow. Fatty livers have a decrease in blood flow at approximately 50% compared with non-fatty livers, which can induce chronic hypoxia (Carmen Peralta et al., 1999). Microcirculation impairment has been considered one of the major causes of IRI in steatotic livers (Álvarez-Mercado et al., 2019). The obstruction of hepatic flow, which confers important changes in liver microcirculation, may compromise the suitable graft revascularization and consequent viability after transplantation (Pantazi et al., 2015). Diverse authors propose that these alterations in hepatic sinusoidal microcirculation in fatty livers can amplify the negative effects produced by I/R and worsen the hepatic damage (Sun et al., 2001; Clemens et al., 1999).
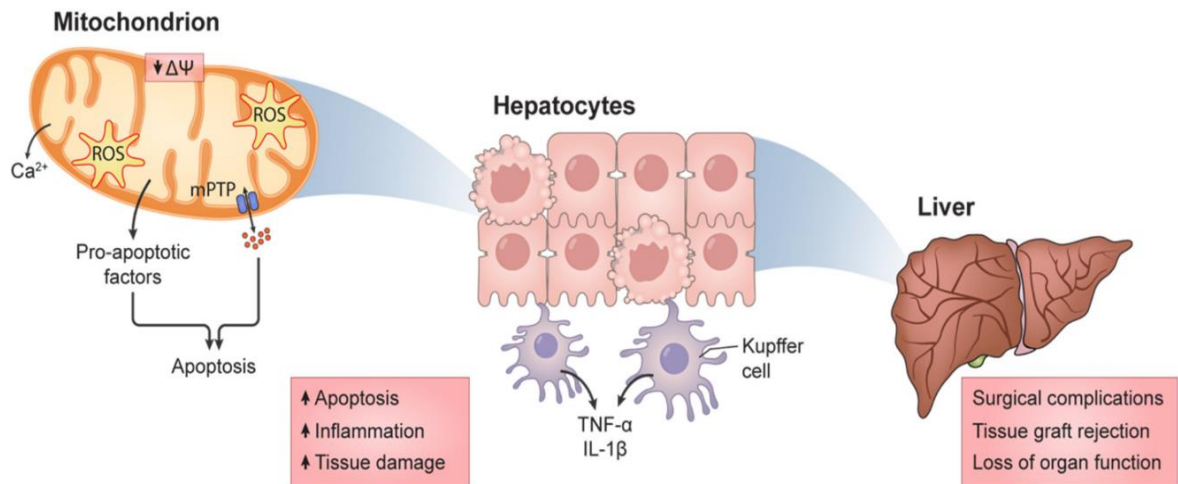
Figure 6- IRI and the escalation of the hepatic injury. Impairment of mitochondrial function triggers ROS production and the release of pro-apoptotic factors, which may lead to Kupffer cells activation and cytokines overexpression and ultimately triggers a sterile inflammation process. All these processes, if not properly controlled, may cause tissue loss and in the end, organ failure (Teodoro et al., 2022).

KC dysfunction is another reported cause for the increased susceptibility of fatty organs to IRI. Several studies have demonstrated higher presence of KC and increased phagocytic activity of these cells in fatty livers than in normal livers after IR (Wanner et al., 1996). These hepatic resident macrophages produce substances that can modulate sinusoidal blood flow and are an important source of ROS and cytokines, such as TNF α and IL-1, which increases during reperfusion. These cytokines promote the recruitment and activation of neutrophils to the endothelial cells by increasing adhesion molecules, including ICAM-1 and P-selecting, triggering an inflammatory cascade and ultimately cell injury (Iñiguez et al., 2008). Therefore, inhibition of KC activity in models of fatty livers reduces hepatic IR injury and increases the survival after transplantation (Mosher et al., 2001).

During hepatic IRI, NO reduction is related to the worsening of the hepatic damage. In this particular condition, the reduced activity of eNOS leads to lower NO levels. Conversely, restoration of NO to more physiological levels diminishes the liver ischemic injury, enhancing hepatic oxygenation and sinusoidal microcirculation (Siriussawakul et al. 2010).

The vasodilatation and antioxidant properties of NO play a key role in the protection of steatotic livers which are more susceptible to dysfunctional microcirculation. In some extent, the capacity to increase NO levels, which leads to a protective effect against liver IRI and alterations in the hepatic microcirculation, is thought to be, at least in part, the reason how IGL-1 exert its beneficial effects (Tabka et al., 2015).

Another process occurring affecting fatty livers during IR is the ATP depletion. Lean hepatocytes are able to restore rapidly the ATP levels, but steatotic livers cannot restore ATP content after reperfusion. Steatosis is associated with a decreased ability of the liver to generate ATP (Teodoro et al., 2008).The lower ATP levels in fatty livers, associated with higher levels of oxidized lipids and proteins, is the probable cause for higher necrotic cell death after IRI, comparatively to lean livers.

Taken together studies both from humans and experimental animals suggest that fatty livers are more prone to a variety of insults such as IR, being hepatic steatosis a major risk factor for liver surgery and success in the transplant of fatty donor organs.

4. Mitochondria

Mitochondria are the essential player in the metabolism of eukaryotes, producing more than 95% of cell's total energy requirement. The number of mitochondria present in each cell depends on their metabolic requirements and may range from hundreds to thousands. Unlike what was thought to a few years ago, mitochondria are dynamic organelles since they continuously change their shape and numbers through frequent fusion, fission and movement throughout the cell (McBride et al., 2006), processes that are intimately linked to the cell's energetic needs.

4.1. Mitochondrial structure

A double phospholipid membrane limits the structure of the mitochondria. The outer membrane separates the mitochondrion from the cytosol and defines the outer perimeter and the inner membrane, that is invaginated, forms the cristae and defines the matrix of the organelle.

The outer membrane has a high percentage of lipid and is rich in cholesterol. Voltage-dependent anion channel (VDAC) is the most abundant protein in the outer membrane, and it is responsible for the passage of low molecular-weight molecules between the cytoplasm and the intermembrane space. The inner membrane has various elements with a crucial role in metabolic pathways, such as components of the electron transport chain (ETC) and ATP synthase, the phosphate carrier, the adenine nucleotide translocase (ANT) and uncoupling proteins (UCP) (Protasoni & Zeviani, 2021). The large content in cardiolipin reduces the permeability of the phospholipid bilayer to protons, permitting a proton-motive force to be established across the inner membrane.

The most well-known function of mitochondria involves the production of energy through the citric acid (Krebs) cycle and OXPHOS. While Krebs cycle occurs exclusively on the mitochondrial matrix, taking in acetyl-CoA and generating reducing equivalents (nicotinamide adenine

dinucleotide, NADH and flavin adenine dinucleotide, $FADH_2$), OXPHOS takes places mainly within the inner mitochondrial matrix, where electrons provided by NADH and $FADH_2$ are transported along the respiratory chain. Alongside with the electronic transport, there is also the transport of protons from the matrix towards the intermembrane space. The intermembrane space is an important compartment on mitochondria, which acts as a reservoir of protons, establishing an electrochemical gradient (with electric and pH components) across inner membrane. This gradient holds a tremendous potential energy, which is used to generate ATP from ADP and a molecule of ionic phosphate. The ADP phosphorylation occurs in the ATP synthase, a protonic channel bound to a catalytic head (Nolfi-Donegan et al., 2020).

Moreover, besides energy production, mitochondria assume other functions such as heme synthesis, β-oxidation of free fatty acids, metabolism of certain amino acids, formation and export of Fe/S clusters, iron metabolism, and play also a crucial role in calcium homeostasis and cell death (Michel et al., 2012).

4.2.   Mitochondrial reactive oxygen species

The generation of mitochondrial ROS is a normal consequence of OXPHOS because electrons may escape along the ETC and react with O_2, generating free radicals. The term ROS is used to generally describe a variety of molecules and free radicals derived from molecular oxygen: singlet oxygen molecules (O), superoxide anions (O2•-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH) (Turrens, 2003).

Oxidative stress refers to an unbalanced cellular state in which the ROS production overwhelms the cellular mechanisms of defense, such as Superoxide Dismutase (SOD), reduced glutathione (GSH), vitamins A, E, C, and other molecules involved in the counterbalancing ROS production and inactivation (Pieczenik & Neustadt, 2007).

First thought as unwanted by-products of cellular respiration, at low levels, ROS are now considered important physiological molecules, being critical effector in proliferation, expression of antioxidant enzymes and insulin signaling. Conversely, when its balance is lost, high ROS levels result in oxidative stress which may lead to cell damage (Kasai et al., 2020).

Under normal conditions, cells are able to deal with high levels of ROS through a variety of defense mechanisms. However, when ROS production overcomes the cellular antioxidant defenses, it might lead to transient or permanent damage to biological molecules. Mitochondria are especially vulnerable to oxidative stress since many of its components might be severely impaired by ROS. For instance, thiol groups within respiratory chain complexes are readily oxidized leading to increased ROS generation thanks to electron transport mishandling (Lesnefsky et al., 2017). Furthermore, ROS generation can cause lipoperoxidation at the

mitochondrial membrane, among others in cardiolipin, which is involved in the protein folding and ETC complexes activity. Several studies have shown that cardiolipin peroxidation is associated to impairment of ETC activity. Therefore, this event can create a cycle of ROS production leading to lipid peroxidation, mitochondrial DNA (mtDNA) damage and OXPHOS impairment and ultimately causing mitochondrial dysfunction (Fontes et al., 2019). Damaged mitochondria produce gradually higher amounts of ROS, which in turn may further aggravate mitochondrial damage.

In the liver, oxidative stress accounts for one of the main reasons of hepatocyte death. The generation of high ROS levels can lead to the buildup of reactive aldehydes such as 4-HNE and MDA by lipid peroxidation. In a lipid-rich environment, as in the case of steatotic livers, giving the huge amount of lipids the effects are even more detrimental.

Lower antioxidant defenses, mainly SOD and GSH, and higher production of ROS from mitochondria and xanthine/xanthine oxidase system are features that made steatotic livers more prone to lipid peroxidation (Fernández et al., 2004). The oxidative stress enhancement may induce mtDNA damage, which further exacerbates ATP depletion and mitochondrial dysfunction.

4.3.   Mitochondrial dysfunction in NAFLD

Mitochondria are in the core of fatty acids metabolism. FFA are transported to the mitochondrial matrix where are converted to acetyl-CoA through mitochondria β-oxidation, which in turn can either enter the Krebs cycle for complete oxidation or be transformed in ketone bodies, providing energy to other tissues (Rolo et al., 2012).

NAFLD is characterized by a massive lipid deposition inside the hepatocytes due to an excessive overflow of FFAs. In order to counteract this excessive fat storage, hepatic mitochondria have to undergo bioenergetic remodeling. Several compensatory mechanisms are activated in response to the overwhelming FFAs load into the liver, including increased mitochondrial β-oxidation and proliferation and enlargement of liver peroxisomes. In fact, peroxisomes are responsible for the metabolism of long chain and branched chain fatty acids that cannot enter directly into the mitochondria (Ready & Mannaerts, 1994).

Hepatic mitochondria are recognized to be harmfully affected in the pathophysiology of NAFLD. The increase in nutrient availability causes systemic metabolic alterations that lead to an increase in hepatic mitochondrial respiration as well as alterations in the mitochondrial lipid membrane composition (Fontes et al., 2019). Impairment of hepatic ATP synthesis, reductions in the activities of complexes of the ETC and increased ROS production have been demonstrated (Cortez-Pinto et al., 1999; Longo et al., 2021). These alterations are linked to ultrastructural

abnormalities indicative of impaired OXPHOS. Hepatic mitochondria appear scarce in number, swollen and rounded with loss of cristae (Pérez-Carreras et al., 2003; Teodoro et al., 2008).

The close vicinity to the main source of ROS production renders mtDNA extremely vulnerable to oxidative damage (Mansouri et al., 2018). mtDNA fragmentation, genetic mutations and increased 8-hydroxydeoxyguanosine levels (a marker of mitochondrial oxidized DNA) have been reported as a consequence of ROS overload in NASH patients (Fujita et al., 2009). mtDNA encodes proteins required for the normal function of the ETC. As a result, mtDNA damage may impair the electron flow within the ETC, enhancing the leakage of electrons from complexes I and III and consequently, further increase ROS production and oxidative stress (Begriche et al., 2013; I. García-Ruiz et al., 2006). Of note, decreased activity of hepatic mitochondrial complexes have been observed in NASH patients (C. García-Ruiz & Fernández-Checa, 2018; Pérez-Carreras et al., 2003). Moreover, lipid peroxidation products including 4-HNE and MDA may interfere with elements of mitochondrial complexes, leading to impairment of ETC (Mansouri et al., 2018). All these processes create a vicious cycle of oxidative damage, which may affect the surrounding mitochondria. Fatty livers display a reduced antioxidant capacity, although the molecular mechanism is still subject of uncertainty. Recently, Azzimato and colleagues reported the production of miR-144 from liver macrophages, which impairs the antioxidative response in the livers of obese insulin-resistant humans and mice (Azzimato et al., 2020).

Mitochondrial antioxidant capacity is not sufficient to deal with the burst of ROS production. Mitochondrial GSH (mGSH) depletion has been observed in animal and human NASH. In fact, the liver in steatotic mice and primary hepatocytes treated with free cholesterol, demonstrated that high levels of cholesterol reduce mGSH (Ribas et al., 2014; von Montfort et al., 2012). Moreover, induction of MPTP, cyt *c* release, lipid oxidative stress and ATP depletion were also observed. Besides GSH, other antioxidant enzymes such as SOD and catalase have been found decreased in NASH patients (Liu et al., 2015).

As mentioned above, fatty livers are exposed to an overload of substrate supply that largely exceeds cellular energy demands. Similarly, to other cells types, when confronted with such scenario, hepatocytes upregulate pathways that are not efficiently coupled to ATP synthesis, leading to the increase of energy expenditure. The induction of UCPs has been reported as one of the pathways activated under this overwhelming situation.

UCPs are mitochondrial inner membrane proteins, responsible of regulate proton channel. In this way, UCP1 is an important regulating fuel metabolism, meanwhile UCP2 is responsible for attenuating ROS production through partial decoupling, regulated by ROS themselves (Azzu et al., 2010). These proteins affect the uncoupling of OXPHOS system and consequently, diminish the redox pressure on the mitochondrial ETC and ultimately, suppressing mitochondrial ROS

generation. UCP2 upregulation and a consequent mitochondrial proton leakage are proposed events underlying NAFLD development. Liver mitochondria may activate ways of substrate oxidation not coupled to energy production, to cope with an increase in energy supplies over energy requirements (Stevanović-Silva et al., 2021). In addition, hepatic UCP2 expression is well known to be stimulated by fatty acids via a PPARα-mediated pathway.

4.4.   Mitochondrial alterations related to IRI

For the maintenance of physiological activities is crucial a functional intact mitochondrion. The deleterious effects associated to hepatic IRI leads to mitochondrial damage, which in turn can produce a large amount of ROS to further attack healthy mitochondria and ultimately triggering cell death. Consequently, the removal of dysfunctional mitochondria is an important process to avoid cell death.

The mitochondrial function is compromised in IRI, resulting in an alteration of energy metabolism (Figure 7). The OXPHOS comes to a halt, leading to incomplete oxidation of FAs and accumulation of acetyl-CoA and Krebs cycle intermediates in mitochondria. The cessation of OXPHOS causes tissue ATP and creatine phosphate concentrations to decrease with a concomitant rise in ADP, AMP and Pi, which leads to a compensatory increase of glycolytic flux to maintain the ATP levels. The increased anaerobic glycolysis and ATP degradation produce H^+-maintaining mitochondrial membrane potential. The maintenance of ion gradient across the plasma membrane and between cellular compartments is highly dependent on ATP-driven reactions and thus, perturbation in the normal metabolism may swiftly disturb cellular ion homeostasis (Palmeira et al., 2019).

During ischemia, there is an elevation of intracellular H^+, Na^+ and Ca^{2+} levels, which induces osmotic stress and causes mitochondrial damage. The stimulated Na^+ influx via Na^+/H^+ exchanger, caused by intracellular H^+ accumulation, and the reduced Na^+ efflux via inhibition of Na^+/K^+-ATPase lead to increased Na^+ concentrations during ischemia (Murphy & Steenbergen, 2008). Although Na^+ overload stimulates Ca^{2+} influx through the Na^+/Ca^{2+} exchanger, during ischemia Ca^{2+} levels are kept relatively low since acidosis inhibits the Na^+/Ca^{2+} exchanger, and cytosolic Ca^{2+} is taken up by the mitochondria while the membrane potential is maintained.

In the first moment of ischemia, there is an increase in ROS production that is believed to play a crucial role in damaging the organ during ischemia and sensitizing it to reperfusion. Complexes I and III of the mitochondrial ETC, or xanthine/xanthine oxidase system are a possible source of ROS production (Palmeira et al., 2019). The combination of ATP depletion with increased intracellular Ca^{2+} levels and ROS production culminate in a gradual decline of cellular integrity as

degradative enzymes are activated and ATP-dependent repair processes are incapable of operating.
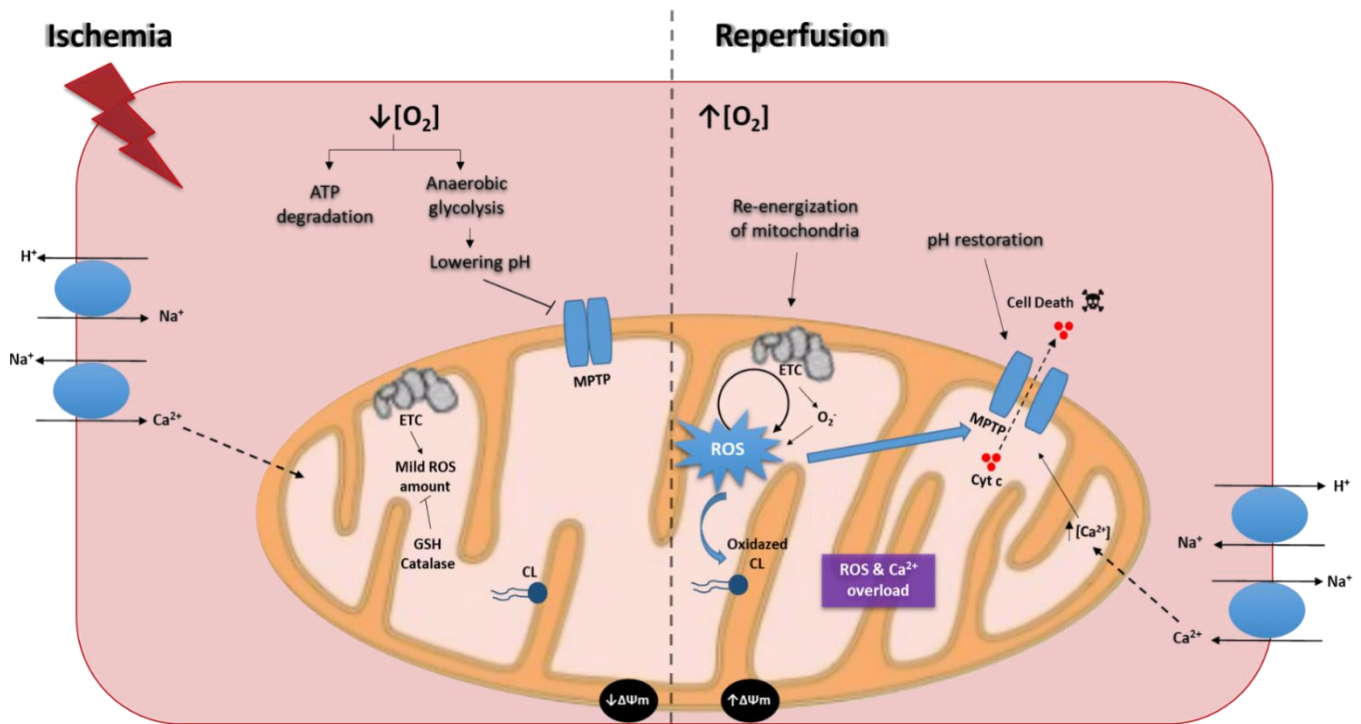
Figure 7- The mitochondrial molecular mechanisms involved in IRI. $\Delta\Psi_m$, mitochondrial membrane potential; CL, cardiolipin; Cyt c, cytochrome c; ETC, electronic transport chain; GSH, glutathione; MPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species. Adapted from Palmeira et al., 2019.

Mitochondrial integrity is crucial for the maintenance of physiological activities. Hence, if the tissue remains ischemic for a short period and the mitochondria remain sufficiently intact to produce ATP, tissue damage can be reversible and repaired. However, when a critical point is reached, the recovery is not possible. In this case, although reperfusion restores ATP production, it will actually cause further damage to the organ due to the metabolic disorders that accumulate during ischemia, causing cell death.

Reperfusion is related to increased Ca^{2+} and bursts of ROS production. Mitochondria seem to be the main responsible for the ROS generation after reintroduction of oxygen due to the faulty transfer of electrons and consequent leakage from the mitochondrial respiratory chain. As a consequence, there is the formation of the superoxide anion radical, which can be converted by superoxide dismutase to molecular oxygen and H_2O_2. The latter can be degraded by catalase, GSH or interact with iron to form the highly reactive hydroxyl radical. ROS causes lipid

peroxidation of cardiolipin of the inner mitochondrial membrane, impairing electron flow (Petrosillo et al., 2003). Moreover, the elevation of mitochondrial ROS is known to cause the release of several DAMPs, which may activate toll-like receptors and NLRP triggering an upregulation of several proinflammatory cytokines (Philipp Dutkowski & Clavien, 2018).

ROS and mitochondrial Ca^{2+} overload are potent inducers of the mitochondrial permeability transition pore (MPTP) opening, leading to mitochondrial-initiated cell death (Rauen & de Groot, 2004). The major consequences of MPTP induction are the mitochondrial depolarization, uncoupling OXPHOS and mitochondrial swelling driven by colloid osmotic forces. In addition, MPTP has also been related to the release of apoptotic factors into the cytosol, including cytochrome c (cyt c) and other apoptosis-inducing factors (Forbes et al., 2001). Nevertheless, in conditions of ATP depletion, apoptosis may vary to necrosis. Alterations in mitochondrial morphology achieved through mitochondrial dynamics might play a crucial role in cell viability and in IRI.

4.5.   Mitochondrial quality control

The maintenance of a healthy mitochondrial network is a determinant factor for cellular homeostasis and cell survival. Removal of damaged mitochondria by selective degradation and their replacement by new ones via mitochondrial biogenesis, alongside with changes in mitochondrial dynamics, form an effective quality control system to combat adverse conditions and maintain mitochondrial function.

4.5.1. Mitochondrial dynamics

Mitochondrial biogenesis as well as mitochondrial fission and fusion mediate cellular energetics and metabolic demands, which ultimately ensures mitochondrial turnover, content and number. A network of transcription factors and co-mediators are involved in the regulation of mitochondrial biogenesis, including PGC-1α and the downstream nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM) (Dusabimana et al., 2019). Reduced PGC-1α activity and decreased TFAM have been observed in NAFLD and NASH patients, suggesting diminished mitochondrial biogenesis (Piccinin et al., 2019).

The maintenance of a healthy mitochondrial network is a determinant factor for cellular homeostasis and cell survival. Mitochondria are highly dynamic organelles regulated by fission and fusion events, in response to cellular stress and consequent alterations in intracellular environment (Youle & Van Der Bliek, 2012).

In the liver of NAFLD individuals, mitochondria are normally round and swollen, with depletion of cristae structure, suggestive of mitochondrial dysfunction (Mansouri et al., 2018; Anabela P Rolo et al., 2012).

Mitochondrial fusion and fission are two essential quality control mechanisms which favor the segregation and clearance of dysfunctional mitochondria to achieve homeostasis. Mitochondrial fission is regulated my several proteins including dynamin-related peptide-1 (Drp1), fission protein-1 (Fis1) and mitochondrial fission factor (Mff) leading to mitochondrial fragmentation, which creates new mitochondria, an essential process in growing and dividing cells. Contrary to fission, mitochondrial fusion produces elongated mitochondria and the formation of mitochondrial networks which are mediated by mitofusin 1 and 2 (Mfn1, Mfn2) and optic atrophy protein-1 (OPA1) (Youle & Van Der Bliek, 2012).

Recent studies have found that MFN2 is decreased in the liver, muscle and adipose tissue of obese, and NAFLD and NASH patients (Hernández-Alvarez et al., 2019). Conversely, DRP1 is increased in the muscle of NASH patients (Simão et al., 2019), and it is also related to liver steatosis in experimental obesity and NAFLD (Cruz Hernández et al., 2020).

4.5.2. Mitophagy

Mitochondrial selective autophagy, the so-called mitophagy, together with the ubiquitin/proteasome system (UPS), has gained some attention in the regulation of mitochondrial quality control.

Mitophagy is tightly regulated by several cellular signal mechanisms including phosphatase and tensin homolog-induced putative kinase 1 (PINK1), Parkin and mitophagy receptors and adaptors (Yang et al., 2019). PINK1 and Parkin have a crucial role in the canonical ubiquitin-mediated mitophagy pathway. In healthy mitochondria, PINK1 is generally undetectable because it is degraded by presenilin associated rhomboid-like (PARL) protein and mitochondrial peptidases. On the other hand, in stressed mitochondria, activated PINK1 accumulates on the outer mitochondrial membrane (OMM), recruiting Parkin from the cytosol to mitochondria. In turn, activated Parkin polyubiquitylates a number of OMM proteins which can be recognized by LC3 adapters, triggering mitophagy(Yang et al., 2019).

Different studies have considered mitophagy as a hepatocellular mechanism due to the elimination of defective mitochondria resultant from IRI(Shin & Lee, 2017; Zheng et al., 2020). Hong and colleagues demonstrated that hepatic IRI promotes auto- and mitophagy, shown by the higher levels of PINK1/Parkin and LC3-II(Hong et al., 2016). In addition, pharmacological stimulation of mitophagy improved the hepatic IR outcome, supporting the hepatoprotective role of mitophagy (Kang et al., 2018; Shin & Lee, 2017). The mitophagy protective role was also

demonstrated in humans. Hepatic biopsies obtained after transplantation, demonstrated that aging aggravates hepatic IRI through the decreased parkin expression(Y. Li et al., 2018).

The role of mitophagy has also been investigated in other organs. Parkin knockdown augmented cardiomyocyte death due to the increased vulnerability of cardiac cells to IRI, suggesting a cardioprotective role of Parkin through mitophagy activation (C. Huang et al., 2011). Acidic postconditioning induced parkin-dependent mitophagy which protects against cerebral ischemia injury and extend the reperfusion window (Shen et al., 2017). Furthermore, ablation of PINK1 increased the susceptibility of cardiomyocytes to IRI (Siddall et al., 2013). In addition to its role in mitophagy, Parkin has also been suggested to activate the UPS for proteolysis of damaged OMM proteins subjected to IRI (Kulek et al., 2020).

However, there are still some contradictory findings whether mitophagy is a protective or detrimental in IRI. Ma and colleagues reported autophagy paradox in the protective role of the mitochondrial enzyme, ALDH2 against cardiac IRI(H. Ma et al., 2011a). The authors demonstrated that ALDH2 promoted autophagy during ischemia but inhibited it during reperfusion. The results suggested an autophagy-mediated cardioprotection in the ischemic period while being detrimental in reperfusion phase. Similarly, ALDH2 increased cell viability and protection against IRI through the suppression of PINK1/Parkin-mediated mitophagy(Ji et al., 2016). Alda-1 pretreatment also diminished ROS generation and showed a partial preservation of mitochondrial membrane potential. The observed results may indicate that ALDH2 activation induces a better maintenance of mitochondrial integrity and thereby inhibits excessive activation of mitophagy. Many studies have suggested a dual effect of mitophagy in IRI. Induction of mitophagy during the ischemia phase seem to play a protective role, while it might be detrimental in reperfusion (Anzell et al., 2018; Kubli et al., 2013; Yang et al., 2019).

Apart from Parkin, accumulating findings have reported that some mitochondrial proteins are also able to recruit autophagosomes to mitochondria through direct interaction with LC3. FUN14 domain containing 1 (FUNDC1) is an OMM, which has been seen to act as a mitophagy receptor under hypoxic conditions. Under normal conditions, FUNDC1 is involved in the control of mitochondrial fusion through its interaction with OPA1. Conversely, under hypoxia settings, there is the dissociation of OPA1 from FUNDC1, which in turn associates with DRP1 promoting fission(M. Chen et al., 2016). Moreover, in the hypoxic state, increased expression of Unc-51 like autophagy activating kinase 1 (ULK1) leads to FUNDC1 phosphorylation at Ser17, enhancing its binding with LC3 to promote mitophagy(Wu et al., 2014).

4.5.3. Mitochondrial fusion and fission

Unlike what was thought over the past decades, mitochondria are dynamic organelles, continuously dividing and elongating through frequent fusion and fission in response to cellular stress and consequent alterations in intracellular environment (Youle & Van Der Bliek, 2012).

Compelling evidences of the interplay between mitochondrial quality control and cell fate in a number of organs subject to IR have been gathered over the last years. Modifications in regulators involved in fission or fusion processes, loss of cristae integrity alongside with inefficient removal of damaged mitochondria by mitophagy have all been implicated to play a vital role in IRI (Kulek et al., 2020).

To undergo a successful mitophagy, damaged mitochondria must be set apart from the healthy mitochondrial network and fragmented to smaller size through fission. Drp1, Mfn1/2 or Opa1 may interact with various number of mitophagy receptor proteins in order to collaborate in mitochondrial dynamics and mitophagy (M. Chen et al., 2016; Murakawa et al., 2015). Drp1 knockdown promoted mitochondrial elongation and accumulation of damaged mitochondria through the suppression of mitophagy, thereby promoting cardiac dysfunction and increased susceptibility to IRI (Ikeda et al., 2015). Moreover, inhibition of Fis1 or DRP1 caused an accumulation of oxidized mitochondrial proteins and a decreased mitophagy(Twig et al., 2008). OPA1 knockdown has been reported to exacerbate the deleterious effects provoked by IR(Guan et al., 2019; Le Page et al., 2016).

Besides mitophagy, Drp1 has also been linked to apoptosis. The proapoptotic molecules Bax and Bak were suggested to stimulate the sumoylation of Drp1 and its association with mitochondrial membranes(Wasiak et al., 2007). This stimulation of mitochondrial fragmentation alongside with proapoptotic molecules induces mitochondrial permeabilization and the cyt *c* release, triggering apoptotic cell death.

OMA1 is a mitochondrial protein located at the inner membrane which plays a role in both physiological and pathological conditions, and it is tightly associated with mitochondrial bioenergetic function and respiratory stability (Nan et al., 2019). In response to stress such as IRI, OMA1 cleaves OPA-1 leading to the destruction of cristae structure, cyt c release and mitochondrial malfunction. The use of epigallocatechin gallate (EGCG) inhibited the self-cleavage of OMA1 and as a result, attenuated OPA1 cleavage and loss of cristae integrity and reduced cyt *c* release in cardiomyocytes subjected to hypoxia/reperfusion injury (Nan et al., 2019).

4.6.   Aldehyde dehydrogenase 2

Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial enzyme, mostly expressed in the liver, where it plays an important role in the ethanol metabolism (C. H. Chen et al., 2014). In addition, ALDH2 is also involved in the clearance of toxic aldehydes originated from the lipoperoxidation of mitochondrial and plasma membranes, mainly 4-HNE and MDA, under oxidative stress conditions. 4-HNE is well known to impair mitochondrial and membrane integrity as well as other cellular function including apoptosis (Panisello-Roselló, Lopez, et al., 2018). Some studies have found the inhibition of ALDH2 activity in the presence of high levels of 4-HNE (C. Chen et al., 2008; Gomes et al., 2015).

Alda-1 is a well-known ALDH2 activator and several reports have demonstrated a significant improvement against IRI in the presence of Alda-1 in various types of organs, including heart, brain, lung, kidney and intestine (C. Chen et al., 2008; J. Ding et al., 2016; Zhu et al., 2017). Recently, Li and colleagues reported the protective effect of Alda-1 against liver IRI in mice (M. Li et al., 2018). The authors suggested that the protective mechanism was related to the clearance of reactive aldehydes (decreased accumulation of 4HNE and MDA) and autophagy enhancement by AMPK activation. These results suggest that Alda-1 pretreatment could increase ALDH2 activity which in turn scavenges reactive aldehydes.

In accordance, Alda-1 pretreatment protected the liver in a rat model of hepatic IRI, resulting in decreased hepatic enzyme release, oxidative stress and inflammation, through the autophagy enhancement which might be dependent on the AKT/mTOR and AMPK signaling pathways (Liu et al., 2020). Moreover, reduction of liver mitochondrial damage and attenuation of hepatocyte apoptosis were also observed.

Besides its aforementioned role in ethanol metabolism and toxic aldehydes detoxification, ALDH2 can also interact with other enzymes promoting NO formation. Furthermore, it can also have a dual role in autophagy (H. Ma et al., 2011b). The activation of AMPK by ALDH2 during ischemia increases the cytoprotective autophagy, while during reperfusion, ALDH2 inhibits AMPK and activates Akt leading to a detrimental Akt-dependent inhibition of autophagy (Cursio et al., 2012; Panisello-Roselló, Lopez, et al., 2018; Van Erp et al., 2017). Therefore, the importance of ALDH2 rely not only in its direct interaction with several enzymes, but also for its side effect by cleansing the 4-HNE that would impair them.

5. Protective strategies

In the past few years, a sizeable body of literature suggested that different drugs could have a beneficial role in the prevention and/or damage reduction against IR. There is an urgent need

to find efficient strategies against IRI in fatty livers, given there are susceptibility to acute stressors.

Although the perfect drug does not exist at the moment, there are some synthetic and natural derivatives showing metabolic and/or antioxidative effects that can improve hepatic function following IR (Varela et al., 2010). Besides the use of pharmacological treatment, ischemic preconditioning (IPC) is one of the strategies that have been used to attenuate the harmful effect of IRI. IPC comprises a short period of ischemia followed by reperfusion (usually, 10 min ischemia plus 10 min reperfusion). Both experimental (Pantazi et al., 2014; Anabela Pinto Rolo et al., 2009) and clinical settings (Clavien et al., 2000) have demonstrated the beneficial effects of IPC in IRI in steatotic livers. NO generation by eNOS is considered, at least in part, one of the mechanisms responsible for the hepatoprotection exerted by IPC (Pantazi et al., 2014). In addition, important clinical observation is that graft protection can be achieve by the activation of signaling pathways after reperfusion. Postconditioning is based on the induction of brief periods of ischemia and reperfusion, at the immediate onset of reperfusion. Several studies have demonstrated that in different organs, including heart, brain, kidney and liver, this approach seems to be simple to apply and leading to the improvement of graft function, reduction oxygen free radicals' production and cytokines expression, culminating in effective reduction of IRI.

5.1.   Polyethylene glycol

Polyethylene glycols (PEGs) are non-toxic, neutral, water-soluble compounds containing a linear polymer of ethylene oxide with hydroxyl terminal groups (Figure 8), approved by the Food and Drug Administration (FDA) for their use in cosmetics, foods and drugs.

In 1971, Robinson reported the importance of PEG in edema prevention for the first time (Robinson, 1971). PEG cannot cross the plasma membrane and acts as a compound that exerts an oncotic pressure which limits tissue edema without breaking the transmembrane ionic balance. A few years later, Daniel and Wakerley, demonstrated increased cell viability using PEG 20 kDa during the cold preservation of renal pig cells (Daniel & Wakerley, 1976). Since then, several studies have demonstrated the protective role of different molecular weight PEG during cold preservation using different animal models. The satisfying results obtained led the use of PEG to a clinical level. The use of PEG35 in the IGL-1 solution showed to be protective for liver graft preservation prior liver transplantation. Recently, intravenous PEG pretreatment proved to be more effective in liver preservation compared to UW solution alone in a rat model of warm ischemia reperfusion (Bejaoui et al., 2015).
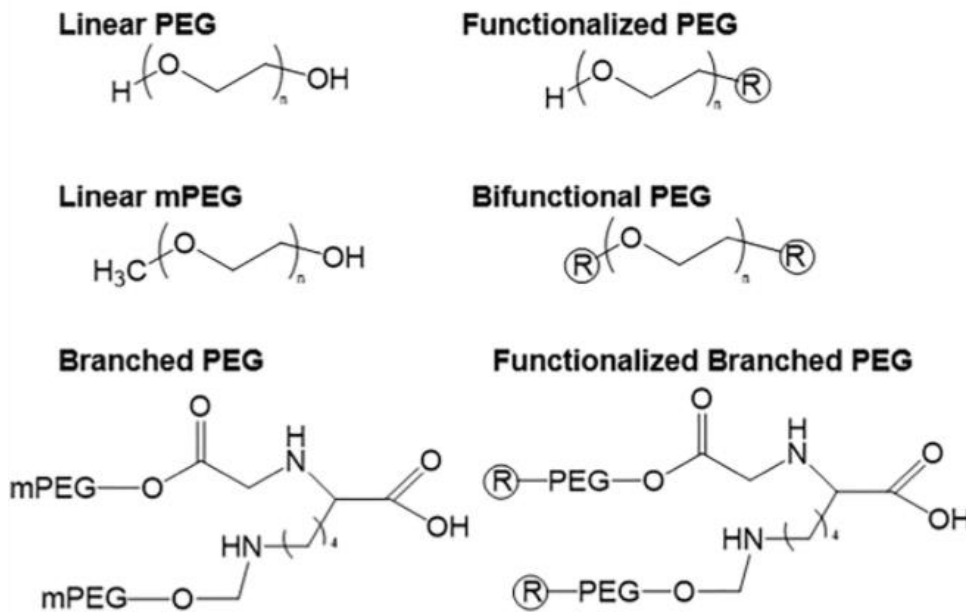
Figure 8- Chemical structures of PEG. R represents the functional group. Adapted from (Freire Haddad et al., 2021)

A sizeable body of literature have been demonstrating the beneficial effects of PEG. PEG has been reported to protect cell membrane by creating a physical barrier which hampers antigen recognition (Eugene, M. 2004). This barrier is created by the high number of water molecules which binds on PEG chain making the cell membrane less permeable to extracellular elements, a phenomenon called immunocamouflage or immunomaskage (Giraud et al., 2018). Clinicians have been taking advantage of such immunomasking effect to reduce host immune reaction by limiting cellular infiltration of the graft after organ transplantation (Bejaoui, 2020).

Both *in vitro* and *in vivo* studies have demonstrated that high molecular weight PEGs have the ability to reduce cytokine production and neutrophil activation (Ackland et al., 2010; Ferrero-Andrés, Panisello-Roselló, Serafín, et al., 2020). Although the mechanisms for such reduction are still elusive, immunocamouflage could play an important role in the decrease of leukocyte adhesion. In fact, the number of leukocytes, in a model of rat peritoneal inflammation, decreased by 43% in PEG-treated group (Nagelschmidt et al., 1998). PEG may also reduce inflammation by its ability to decrease oxidative stress through preservation of membrane integrity and thereby breaking the cycle of cellular damage and free radical generation. Malhotra and co-workers observed that PEG was able to preserve the sarcolemmal lipid-raft architecture due to membrane stabilization (Malhotra et al., 2011). PEG is capable of seal and progressively eliminate membrane disruptions. Still, some PEG molecules can pass through the membrane openings and interact with mitochondrial membrane, preventing the formation of

the so called, mitochondrial permeability transition pore (Shi, 2013). Consequently, there is a reduced mitochondrial swelling, which allows the maintenance of the mitochondrial membrane potential and inhibits the release of Cyt *c* and subsequent cell death.

PEG has shown multiple benefits in cell and organ preservation, including antioxidant capacity, preventing edema, membrane stabilization and, in the context of subzero preservation, freeze-protection (Puts et al., 2015). Malhotra and colleagues reported that PEG inhibits apoptosis in isolated rat cardiomyocytes followed IRI (Malhotra et al., 2011). The protective effect is suggested to be linked to PEG's capacity to decrease ROS production and lipoperoxidation, which in turn leads to membrane stabilization and consequently, maintenance of cell integrity and inhibition of apoptotic cell death. Furthermore, in an *in vivo* model of rat hearts subjected to 1 hour of artery occlusion followed by either 48 hours or 4 weeks reperfusion, PEG postconditioning improved myocardial protection. The mortality within the first 24 hours had significantly a better outcome in the presence of PEG, 40% vs 10% in the non-PEG group and PEG-treated rats, respectively. Beside its role in cold liver preservation, Bejaoui and colleagues have also shown PEG35 protective effect in nonfatty livers, as a preconditioning agent, against warm IRI, which may occur during liver resections (Bejaoui et al., 2016).

Objectives

Steatotic livers are considerably more prone to IRI, in which turn, it is known to impair mitochondrial function. Our working hypothesis lies on the fact that impaired mitochondrial function and reduced protection against oxidative stress are increased risk of complications of hepatic surgery in NAFLD patients. Thus, the main challenges in this field are based on the identification of new pharmacological interventions for enhancing hepatic mitochondrial function and capacity for a better clinical outcome of hepatectomy.

The main objective of this doctoral thesis is evaluating the possible protective role of PEG35 against hepatic cold and warm ischemia using different experimental models, more specifically:

1- Understand the role that PEG35 plays in the IGL solutions. In particular, we analyzed the efficacy of fatty liver cold storage using three solution, IGL-0, IGL-1 and IGL-2 containing 0 g/L, 1g/L and 5 g/L of PEG35, respectively (Study 1).

2- Explore a possible synergetic effect on the mitochondria, where PEG35 could enhance HOPE protection, by using the new IGL-2 solution (Study 2).

3- Highlight the use of PEG35-containing solutions as a key factor for hepatic and mitochondrial protection (Study 3).

4- Investigate the utility of PEG35 pharmacological intervention to decipher the mechanisms underlying PEG35 preconditioning-induced protection against IRI, using a model of hypoxia/reoxygenation injury in human hepatoma cell line (Study 4).

Directors´ Statement

Barcelona, 16th of June 2022

Dr. Joan Roselló-Catafau and Prof. Carlos Palmeira, as directors of this doctoral thesis state the following:

Mr. Rui Teixeira da Silva has actively participated in the preparation of the articles presented in this thesis, carried out the design of the experiments and the experimental work and contributed to the critical analysis of the data and results. In addition, he has also participated in the writing of the articles.

1- Polyethylene glycol 35 as a perfusate additive for mitochondrial and glycocalyx protection in hope liver preservation. Panisello-Roselló A*, Teixeira da Silva R*, Castro C, Bardallo RG, Calvo M, Folch-Puy E, Carbonell T, Palmeira C, Roselló-Catafau J, Adam R. Int J Mol Sci 2020; 21(16), 1–16.
(*) Contributed equally to this study.
    Impact Factor: 5.924. Q1, Biochemistry and molecular biology (index SJR)

2- Glycocalyx as a Useful Marker of Endothelial Injury in Liver Transplantation: The Role of Preservation Solution. Panisello-Roselló A, Castro Benitez C, Lopez A, Teixeira da Silva R, Roselló-Catafau J, Adam R. Transplantation 2020; 104(12), e356–e357.
    Impact Factor: 4.939. Q1, Transplantation (index SJR)

3- Role of PEG35, Mitochondrial ALDH2, and Glutathione in Cold Fatty Liver Graft Preservation: An IGL-2 Approach. Bardallo RG, Teixeira da Silva R, Carbonell T, Folch-Puy E, Palmeira C, Roselló-Catafau J, Pirenne J, Adam R, Panisello-Roselló A. Int J Mol Sci 2021; 22(10), 1–13.
    Impact Factor: 5.924. Q1, Biochemistry and molecular biology (index SJR)

4- PEG35 as a Preconditioning Agent against Hypoxia/Reoxygenation Injury. Teixeira da Silva R, Machado IF, Teodoro JS, Panisello-Roselló A, Roselló-Catafau J, Rolo AP, Palmeira CM. Int J Mol Sci 2022; 23(3), 1156.
    Impact Factor: 5.924. Q1, Biochemistry and molecular biology (index SJR)

5- IGL-2 as a Unique Solution for Cold Static Preservation and Machine Perfusion in Liver and Mitochondrial Protection. Da Silva RT, Bardallo RG, Folch-Puy E, Carbonell T, Palmeira CM, Fondevila C, Adam R, Roselló-Catafau J, Panisello-Roselló A. Transplantation Proceedings 2022; 54(1), 73–76.
    Impact Factor: 1.066. Q3, Transplantation (index SJR)

6- Shaping of Hepatic Ischemia/Reperfusion Events: The Crucial Role of Mitochondria. Teodoro JS*, Teixeira da Silva R*, Machado IF, Panisello-Roselló A, Roselló-Catafau J, Rolo AP, Palmeira CM. Cells 2022; 11(4), 688.
(*) Contributed equally to this study.
      Impact Factor: 6.600. Q2, Cell Biology (index SJR)

7- The Use of a Single, Novel Preservation Solution in Split Liver Transplantation and Hypothermic Oxygenated Machine Perfusion. Panisello-Roselló A, Teixeira da Silva R, Folch-Puy E, Carbonell T, Palmeira CM, Fondevila C, Roselló-Catafau J, Adam R. Transplantation 2022; 106(3), e187–e188.
      Impact Factor: 4.939. Q1, Transplantation (index SJR)

8- Liver Graft Hypothermic Static and Oxygenated Perfusion (HOPE) Strategies: A Mitochondrial Crossroads. Bardallo RG*, Teixeira da Silva R*, Carbonell T, Folch-Puy E, Palmeira C, Roselló-Catafau J, Adam R, Panisello-Roselló A. Int J Mol Sci 2022; 23(10), 5742.
(*) Contributed equally to this study.
      Impact Factor: 5.924. Q1, Biochemistry and molecular biology (index SJR)

Finally, we state that the article 3 is involved in the elaboration of another doctoral thesis. In this specific article, Rui Teixeira da Silva performed the surgical procedures and evaluated the parameters involved in the hepatic lesion (AST/ALT), mitochondrial damage (GLDH) and enzymatic function of ALDH2. In addition, he also evaluated the ATP levels and parameters linked to autophagy (Beclin 1 and LC3B). Finally, he was also involved in the writing of the manuscript.

Dr. Joan Roselló-Catafau   Prof. Carlos Palmeira

Results

Role of PEG35, Mitochondrial ALDH2, and Glutathione in Cold Fatty Liver Graft Preservation: An IGL-2 Approach

The cumulative injury resulting from the combined action of ischemia and cold preservation must be minimized to achieve full recovery of the graft's function after liver transplantation, especially in the case of fatty liver grafts. IGL-1 has emerged as a good alternative to the current gold standard solution, UW solution. The main difference in their composition is the oncotic agent present, HES for UW and PEG35 for IGL-1. Having this in mind, in this study we aimed to understand the role that PEG35 plays in the IGL-1 solution.

In addition, we also evaluated the benefits of higher PEG35 and glutathione concentrations using a new preservation solution, IGL-2.

The presence of PEG35 in IGL1/IGL2 solutions was determinant for protecting liver mitochondria and preserving the energy breakdown against ischemic insult in oxygen deprived conditions. Livers preserved in PEG35-containing solutions showed a higher ALDH2 activity and, consequently, a prevention of toxic aldehydes (4-HNE) and lipoperoxides (MDA) and oxidized proteins. Furthermore, PEG35 promoted the production of NO, a vasodilator agent, that may contribute to prevent the well-known microcirculatory disturbances occurred in fatty livers due to fat accumulation in sinusoidal space.

In the IGL-2 solution, the augmented PEG35 content and the higher antioxidant capacity prevented oxidative stress through ALDH2 upregulation as well as promoted cytoprotective autophagy.

To sum up, PEG35 seems to play an important role as an oncotic agent in the preservation of fatty liver grafts against cold ischemia insult. This protective action is partly mediated by increased ALDH2 activity, which prevents the formation of toxic aldehyde adducts and lipoperoxides. The ALDH2/4HNE balance is decisive for improving fatty graft protection against cold ischemia insult.

Article

Role of PEG35, Mitochondrial ALDH2, and Glutathione in Cold Fatty Liver Graft Preservation: An IGL-2 Approach

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Abstract: The total damage inflicted on the liver before transplantation is associated with several surgical manipulations, such as organ recovery, washout of the graft, cold conservation in organ preservation solutions (UW, Celsior, HTK, IGL-1), and rinsing of the organ before implantation. Polyethylene glycol 35 (PEG35) is the oncotic agent present in the IGL-1 solution, which is an alternative to UW and Celsior solutions in liver clinical transplantation. In a model of cold preservation in rats (4 °C; 24 h), we evaluated the effects induced by PEG35 on detoxifying enzymes and nitric oxide, comparing IGL-1 to IGL-0 (which is the same as IGL-1 without PEG). The benefits were also assessed in a new IGL-2 solution characterized by increased concentrations of PEG35 (from 1 g/L to 5 g/L) and glutathione (from 3 mmol/L to 9 mmol/L) compared to IGL-1. We demonstrated that PEG35 promoted the mitochondrial enzyme ALDH2, and in combination with glutathione, prevented the formation of toxic aldehyde adducts (measured as 4-hydroxynonenal) and oxidized proteins (AOPP). In addition, PEG35 promoted the vasodilator factor nitric oxide, which may improve the microcirculatory disturbances in steatotic grafts during preservation and revascularization. All of these results lead to a reduction in damage inflicted on the fatty liver graft during the cold storage preservation. In this communication, we report on the benefits of IGL-2 in hypothermic static preservation, which has already been proved to confer benefits in hypothermic oxygenated dynamic preservation. Hence, the data reported here reinforce the fact that IGL-2 is a suitable alternative to be used as a unique solution/perfusate when hypothermic static and preservation strategies are used, either separately or combined, easing the logistics and avoiding the mixture of different solutions/perfusates, especially when fatty liver grafts are used. Further research regarding new therapeutic and pharmacological insights is needed to explore the underlying mitochondrial mechanisms exerted by PEG35 in static and dynamic graft preservation strategies for clinical liver transplantation purposes.

Keywords: fatty liver; PEG35; IGL-1 solution; ALDH2; 4-HNE; nitric oxide

1. Introduction

Unhealthy lifestyles associated with alcohol consumption and inappropriate diet, along with other factors such as aging, are responsible for the accumulation of fat in the liver, which leads to varying degrees of undesirable hepatic steatosis [1]. Considering the urgent lack of organs for transplantation, physicians have been obliged to take advantage

of fatty livers [1,2] to increase the donor pool and thus shorten waiting lists for clinical transplantation [3]. However, it is clear that steatotic livers show higher vulnerability against cold ischemia and reperfusion injury [4], and their use increases primary failure and compromises graft outcome after transplant [4,5].

Ischemic injury in liver transplantation is associated with mechanical organ manipulation by the physician prior to transplantation. The process includes organ recovery, washing of the graft, cold storage in an organ preservation solution, and finally, rinsing of the graft. Due to the combined action of ischemia and cold preservation, the graft may undergo damage. As a result, the cumulative injury in both mandatory steps before transplantation must be minimized in order to achieve the recovery of the graft's function after liver transplantation, especially in the case of steatotic grafts [1–4].

The most frequently used preservation solutions for liver transplant are UW, HTK, Celsior and, more recently, IGL-1 [6,7]. Certain limitations regarding the use of HTK [8,9] have been pointed out by the United Network for Organ Sharing (UNOS) and the European Liver Transplant Registry (ELTR). IGL-1 emerged as a good alternative to UW solution, which is the gold standard [9]. The only differences in their composition are the oncotic agent (HES for UW and PEG35 for IGL-1) and the reversal of Na+/K+ concentrations conferring IGL-1 the property of extracellular low potassium solution [10,11]. In addition, replacing the HES present in UW for PEG35 lessens red blood cell aggregation and favors organ rinsing, preservation, and perfusion [12]. All these changes in IGL-1 have shown to provide benefits in clinical liver transplantation in terms of reducing early allograft dysfunction [13] and improving graft survival according to the European Liver Transplant Registry (ELTR) [9].

Moreover, PEG35 presence in rinse solution for graft washing out promotes several cytoprotective factors, conferring hepatoprotection [14]. This includes the generation of nitric oxide (NO) whose vasodilation properties counterbalance the microcirculation disturbances in fatty liver grafts, favoring graft preservation and revascularization [15–18]. Keeping in mind the beneficial PEG35 properties, we recently proposed the use of IGL-2 solution (containing PEG35) as a suitable perfusate for dynamic hypothermic oxygenated strategies (HOPE) with promising results [19,20]. This might improve the only perfusate for machine perfusion currently available, Belzer-MPS and generics, containing HES [21], given that the use of a unique solution, such as IGL-2, for static preservation and machine perfusion (MP) would facilitate logistics and avoid the mixture of different solutions [19–21] when both techniques are combined. This is the criterion by which we evaluated the IGL-2 benefits in static hypothermic preservation in the present study.

Aldehyde dehydrogenase-2 (ALDH2), a liver mitochondrial enzyme that was initially implicated in the liver alcohol metabolism, has been associated with the pathophysiology of ischemia–reperfusion injury in several organs, including the liver [22–24]. Several authors reported that the use of Alda-1 (an activator of ALDH2) protects against liver ischemia–reperfusion injury (IRI) in the rat [25,26], but currently, no evidence exists on the direct PEG35 effect as a regulator of mitochondrial ALDH2 in cold old ischemic preservation strategies, although its cytoprotective action was indirectly evidenced when different organ preservation solutions were used for cold storage of fatty liver grafts [23,24]. ALDH2 could play a constitutive housekeeping role essential for the development and regulation of recycling and survival processes as those occurring in cold ischemia preservation [22,27].

With this in mind, we explored the relevance of PEG35 in IGL-2 solution (Table 1) on ALDH2. In this communication, we demonstrated for the first time how the direct effects of PEG35 on mitochondrial ALDH2 contribute towards maintaining mitochondrial functionality during ischemia preservation. Mitochondrial ALDH2 could act as a gatekeeper of ROS overproduction protecting the liver graft from the damaging effects of transient aldehydes produced [22–24] besides other additives in preservation solutions such as labile glutathione [28] that play an important role against oxidative stress.

Table 1. Composition of IGL-2 and IGL-1 solutions.

Preservation Solution	IGL-1	IGL-2
Electrolytes (mmol/L)		
K^+	25	25
Na^+	125	125
Mg^{2+}	5.5	
SO_4		5.5
Zn^{2+}		0.091
Buffers (mmol/L)		
Phosphate	25	25
Histidine		30
Impermeants (mmol/L)		
Mannitol	60	60
Lactobionic acid	80	
Colloids (g/L)		
Polyethylene glycol- 35	1	5
Antioxydants (mmol/L)		
Glutathione	3	9
Metabolic precursors (mmol/L)		
Adenosine	5	5
$NaNO_2$ (nmol/L)		50
pH	7.4	7.4
Osmolarity (mosmol/L)	320	320
Viscosity (cP)	1.2	1.4

The results reported here reveal the superior antioxidant capacity of IGL-2 (due to ALDH2 combined with increased glutathione content) against the ischemic insult during graft preservation, presenting an interesting tool to be considered for improving hypothermic fatty liver preservation by using static and dynamic approaches.

2. Results

The presence of PEG35 and glutathione (group IGL-1) is determinant for preventing liver cold ischemic injury (AST/ALT) and mitochondrial damage (GLDH) during fatty liver graft preservation and the prevention of energy breakdown during cold storage, at 4 °C during 24 h. As revealed in Figure 1, a higher ATP content is shown in liver preserved in IGL-1 solution (containing PEG35 as oncotic agent) than in liver preserved in IGL-0 (the same solution as IGL-1 but without PEG35).

Since PEG35 in IGL-1 preserved liver mitochondrial status during cold ischemia insult during experimental models [23], we evaluated its effect on mitochondrial ALDH2 and compared it to IGL-0 (which is the same as IGL-1 but without PEG35 in its composition). As shown in Figure 2, PEG35 presence in IGL-1 promoted significant increases in ALDH2, contrasting with the significantly lower levels of ALDH2 found in fatty liver grafts preserved in IGL-0 (without PEG35). (Figure 2).

Considering the relevance of PEG35 concentration according to the previously known results, we expanded our study to one additional group using IGL-2 solution [19,20]. We focused mainly on parameters relevant to the mitochondria (such as ALDH2) that regulate other cytoprotective responses. As demonstrated in Table 1, the IGL-2 solution is mainly characterized by higher concentrations of PEG35 and glutathione when compared to IGL-1 (Table 1).
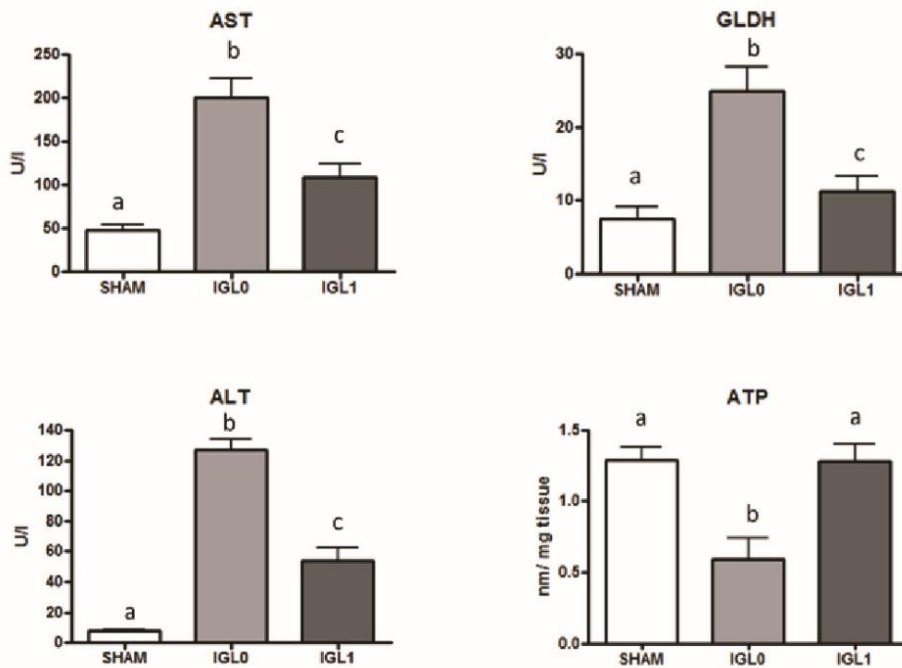
Figure 1. Transaminases (AST/ALT), mitochondrial damage (measured as GLDH), and ATP levels in steatotic livers preserved in IGL-0 (without PEG35) and IGL-1 solutions (PEG35: 1 g/L) vs. SHAM. Results are expressed as mean ± SEM ($n = 6$). Different lowercase letters indicate significant differences among treatments $p < 0.05$.
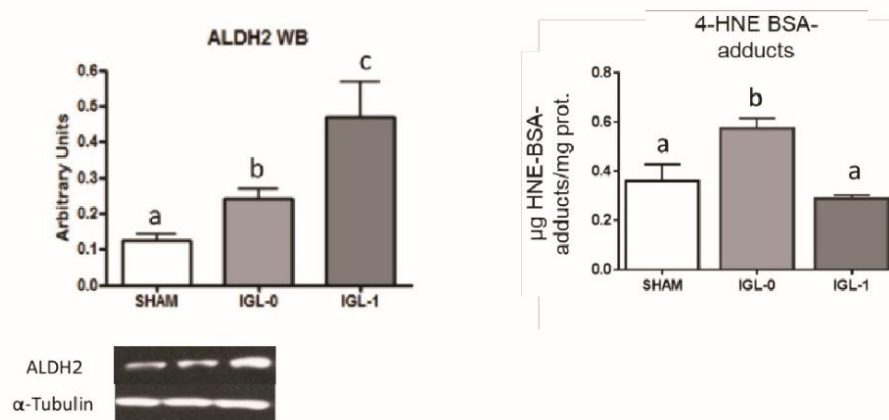
Figure 2. ALDH2 expression and 4-HNE protein-adducts (expressed as μg 4-HNE-BSA-adducts/mg protein) levels in steatotic livers preserved in IGL-0 (no PEG35) and IGL-1 solutions (PEG35: 1 g/L) vs. SHAM. Results are expressed as mean ± SEM ($n = 6$). Different lowercase letters indicate significant differences among treatments $p < 0.05$.

Firstly, we analyzed transaminases (AST/ALT) release and mitochondrial damage (GLDH), from which the total liver damage could be inferred. As shown in Figure 3, the PEG35 presence was a major factor in preventing transaminases and GLDH release with a dose-dependent PEG35 tendency. Although no significant differences between IGL-2 and IGL-1 were observed (except for AST), the presence of PEG35 seems to be a determinant factor in preventing the release of transaminases and GLDH in a dose-dependent manner.

Figure 3. AST, ALT, and GLDH levels in steatotic liver samples for SHAM, IGL-0, IGL-1, and IGL-2 groups. Results are expressed as mean ± SEM ($n = 6$). Different lowercase letters indicate significant differences among treatments $p < 0.05$.

With this in mind, we evaluated the incidence of PEG35 on the energy breakdown prevention during cold storage by measuring ATP content in preserved livers. Data reported in Figure 4 revealed higher ATP levels in PEG-containing solutions and evidenced the PEG35-dependent energy breakdown prevention during cold preservation.
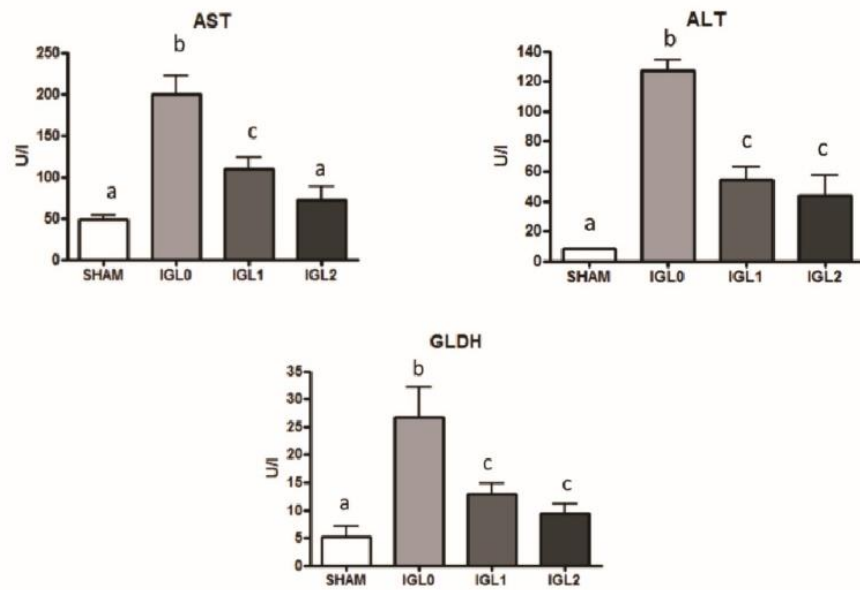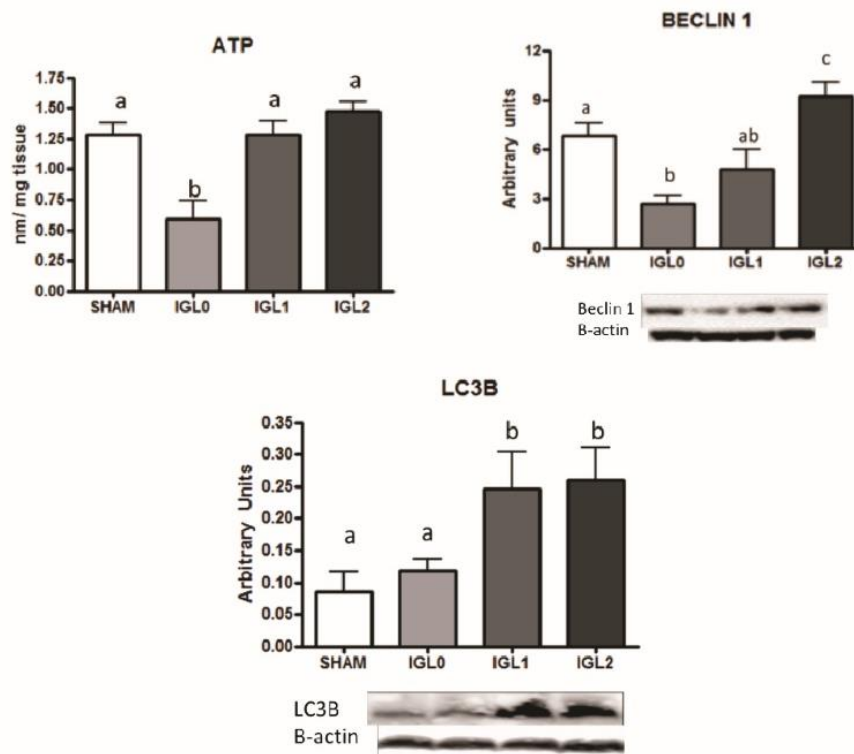

Figure 4. ATP, Beclin-1, and LC3B in steatotic liver samples for SHAM, IGL-0, IGL-1, and IGL-2 groups. Results are expressed as mean ± SEM ($n = 6$). Different lowercase letters indicate significant differences among treatments (one-way ANOVA, $p < 0.05$).

Additionally, and considering the cytoprotective autophagy as a recycling mechanism of nutrients and energy to cope with stress situations existing in cold ischemia conditions [27], we evaluated Beclin-1 and LC3B as autophagy marker [29]. Beclin-1 and LC3B levels correlated with the tissue ATP levels in preserved livers in IGL-0, IGL-1, and IGL-2. As shown in Figure 4, there was a significant upregulation of cytoprotective autophagy in the IGL-2 group compared with the others, which correlated with ATP levels in PEG35 groups, showing a positive tendance in ATP prevention for IGL solutions.

We correlated mitochondrial aldehyde dehydrogenase 2 (ALDH2) with the toxic aldehydes (4-HNE) and oxidized protein (AOPP) levels. Figure 5 shows an increase in the expression of the ALDH2 enzyme, which is concomitant with a decrease in the 4-HNE protein adducts formation, notably decreasing the levels of oxidized proteins going from IGL-0 to IGL-1 and IGL-2. When comparing IGL-0 and IGL-1, which only differ in the presence or absence of PEG, it can be seen that ALDH2 is augmented, the 4-HNE protein adducted, and AOPP decreased solely by the effect of PEG.
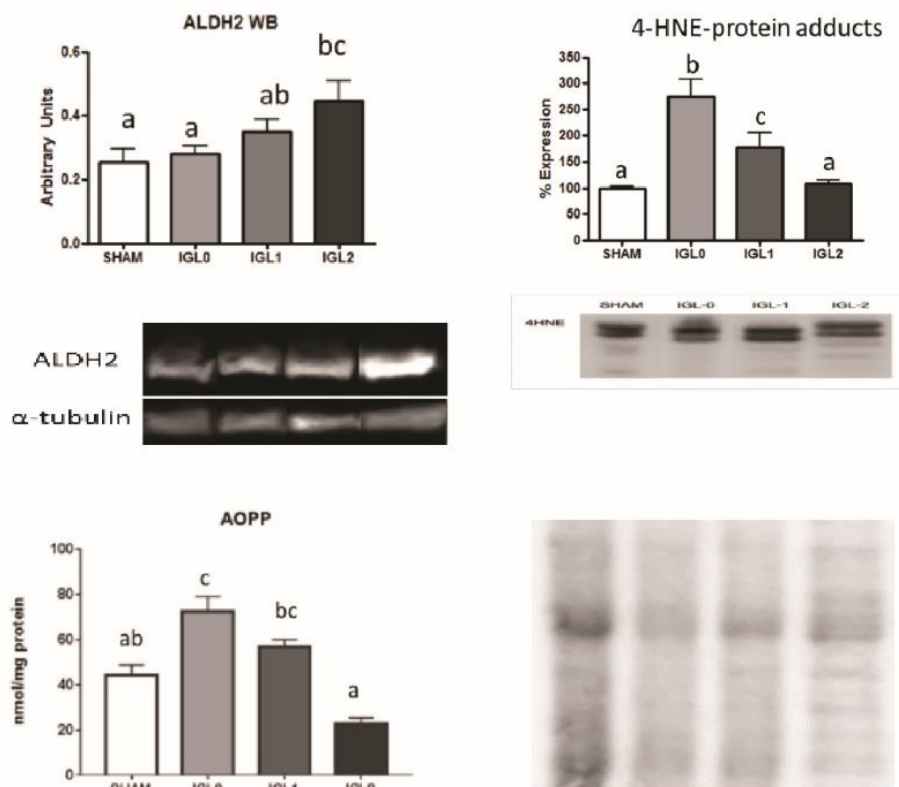
Figure 5. ALDH2 expression and levels of AOPP and 4HNE protein adducts in steatotic liver samples for SHAM, IGL-0, IGL-1, and IGL-2 groups. Results are expressed as mean $\pm$ SEM ($n = 6$). Different lowercase letters indicate significant differences among treatments (one-way ANOVA, $p < 0.05$).

Therefore, to observe the impact of glutathione on the reduction of oxidative stress (and not only by PEG35 itself, which happens when comparing IGL-0 and IGL-1), reduced glutathione was measured. There was a significant difference of reduced glutathione in IGL-2 solution, which was to be expected due to its initial increased dosage; however, high variance in the IGL-1 group might suggest that part of its glutathione (if compared to IGL-0) might be spared (therefore, not oxidized) due to other antioxidant capacities derived from PEG35 (Figure 6).

Figure 6. Reduced glutathione (GSH) in IGL-2 solution compared to IGL-1 and IGL-0 (no PEG35) in steatotic liver samples. Different lowercase letters indicate significant differences among treatments (one-way ANOVA, $p < 0.05$).

Finally, we evaluated the direct effect of PEG35 on NO production and oncotic pressure to avoid oedema. As shown in Figure 7, we evidenced respective augments in NOx products and endothelial NO synthase expression levels. Significant (endothelial NO synthase) eNOS activity and NOx levels were observed for PEG35 groups (IGL-1 and IGL-2). This fact was correlated with a significant upregulation of mitochondrial ALDH2 expression in these groups. A positive trend towards increased eNOS expression and NOx levels in IGL-2 vs. IGL-1 was observed, but statistical differences were not found.

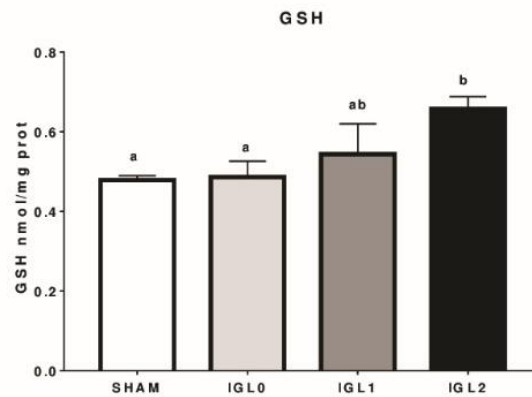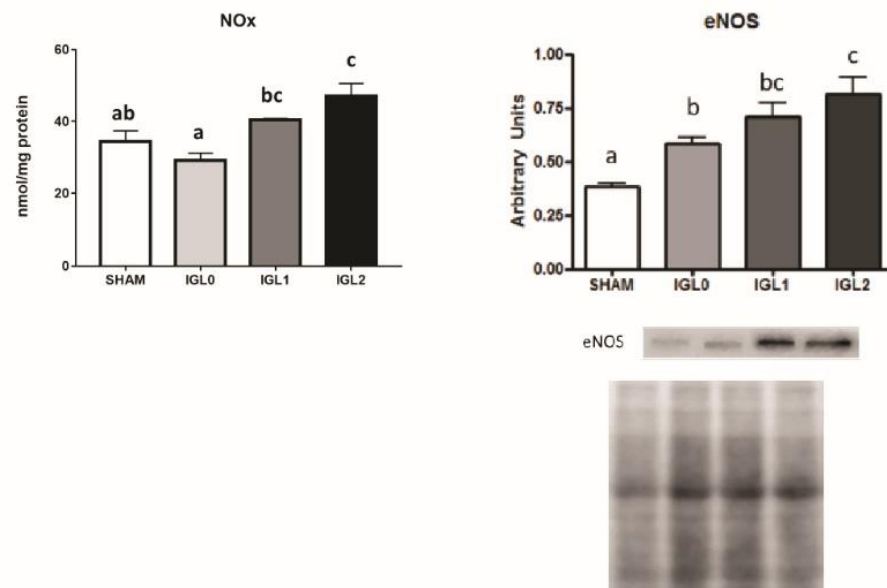**Figure 7.** NOx and eNOS in steatotic liver samples for SHAM, IGL-0, IGL-1, and IGL-2 groups. Results are expressed as mean $\pm$ SEM ($n = 6$). Different lowercase letters indicate significant differences among treatments (one-way ANOVA, $p < 0.05$).

3. Discussion

It is well known that fatty livers are more susceptible to cold ischemia during preservation than normal livers. Therefore, the aim of our research was to increase the performance of their preservation by developing a new solution (IGL-2).

PEG35 is the oncotic agent present in IGL-1 solution [11], and its protective role in IRI [30], rinse solution [14], and cold storage [23,24] was previously described. It is an established fact that any preservation solution must contain antioxidants to counteract ox-

idative stress during IR. Considering this, the IGL-2 benefits reported here for hypothermic static preservation are consistent with its suitability to be used in HOPE over the currently used perfusates (Belzer-MPS or its generics) [19,20]. Both reasons constitute a solid basis for simplifying the logistics and avoiding mixing different solutions when both static and HOPE need to be combined. This is especially interesting for rescuing fatty liver graft liver transplantation purposes [21].

PEG35 solutions (IGL-2 and IGL-1) are related to reduced AST/ALT and especially to GLDH, showing better mitochondrial protection, as previously described [14,19,20]. PEG35 improved mitochondrial machinery and ALDH2 functionality, reducing graft cold ischemic injury [23,24]. Consequently, this calls for an exploration of the underlying protective mechanisms by which PEG35 confers mitochondrial protection on fatty liver grafts preserved in IGL-1 and IGL-2 solutions whose mechanisms substantially differ from the ones induced in HOPE [31], where the transient oxygenation during hypothermic preservation is responsible for maintaining mitochondrial activity function at basal levels. This confirms the relevance of organ preservation strategies on the mitochondrial status and their subsequent benefits in liver transplantation, as recently published by Horvath et al. [32].

Recently, it has been reported that aldehyde dehydrogenase 2 ALDH2 activation is linked to protective mechanisms in several organs, such as the heart, brain, kidney, and intestine [22,33]. The cardioprotective and neuroprotective role of ALDH2 in myocardial ischemia–reperfusion has been demonstrated [34], with recent evidence showing that ALDH2 inhibition alters endothelial functions along with a deterioration of bioenergetic functions [35]. ALDH2 arises as an important gatekeeper of ROS overproduction, making the cell more tolerant to it [36]. In fact, the main function of mitochondrial ALDH2 is to protect mitochondria and cells from the damaging effect of aldehydes (by oxidizing the substrates into their corresponding non-toxic carboxylic acids), which are involved in the oxidative stress associated with IRI [22]. Zhang et al. [25] demonstrated that Alda-1, an ALDH2 activator, protects the liver against warm IRI preventing oxidative stress. In this sense, our work revealed that the oncotic agent PEG35 could be considered as an enhancer of mitochondrial ALDH2 upregulation, whose underlying protective mechanisms against cold ischemic insult have not been assessed in depth [22].

The prevention of liver injury exerted by solutions containing PEG35 contrasts with the injury observed in livers preserved in IGL-0 (without PEG35) with depleted energetic levels after 24 h of cold storage. Remarkably, the comparison of IGL-1 to IGL-0, which only differ in the presence or absence of PEG, suggests that PEG35 by itself leads to an ALDH2 upregulation. This is consistent with the IGL-2 solution, where the augmented PEG35 content further prevented oxidative stress through ALHD2 upregulation and promoted cytoprotective autophagy.

Nevertheless, the total antioxidant effects of the IGL-2 solution are mediated by the contribution of ALDH2, besides the antioxidant action of glutathione present as an additive, which should be considered as well. Thus, the higher IGL-2 antioxidant capacity compared to that of IGL-1 (due to increased PEG35 and glutathione concentrations) is also reflected by preventing AOPP and 4-HNE protein adduct formation in PEG35 solutions (IGL-1 vs. IGL-0) and, even more importantly, with the presence of increased PEG35 and glutathione content (IGL-2 vs. IGL-1). Indeed, IGl-1 shows more ALDH2 than IGL-0, and more IGL-2 than IGL-1, suggesting that ALDH2 increases with PEG35 in a concentration-dependent manner.

In addition, it must be considered that the increased expression of eNOS synthase increased the hepatoprotection mechanisms during cold preservation [15]. The beneficial effects of PEG35 on ALDH2 mitochondrial machinery are also increased by the concomitant presence of glutathione to prevent the action of toxic aldehyde adducts (4-HNE) and lipoperoxide generation [22] associated with hypothermic storage. Alternatively, PEG35, through the increased e-NOS activation and subsequent NO generation, prevents the microcirculatory disturbances that occur in fatty liver graft revascularization [37]. Furthermore, some of this NO could act as a scavenger of ROS, thus reducing the number of oxidizing

particles. IGL-2 is a suitable alternative solution for increasing cold graft preservation strategies when static cold preservation and HOPE need to be combined.

In conclusion, we demonstrated the relevance of the oncotic agent PEG35 in modulating the redox state through mitochondrial ALDH2, thus reinforcing the protection mechanisms of fatty liver graft in cold preservation in combination with glutathione. This could improve the preservation of fatty liver grafts and may help to design new static and dynamic preservation strategies using PEG-containing solutions/perfusates.

Further, in-depth research should be conducted to clarify the role of mitochondrial ALDH2 and its direct relationship with polyethylene glycols features as efficient tools for preventing IRI.

4. Materials and Methods

4.1. Animals

Homozygous (obese (ob)) Zücker male rats aged 16–18 weeks were purchased from Charles River (Charles River, Lyon, France). They were housed in a temperature-controlled environment (25 °C) with a 12 h light/dark cycle and provided water and standard chow ad libitum. The rats presented a rate of steatosis between 60% and 70%. All procedures were carried out according to the EU rules for animal experiments (EC guideline 86/609/CEE) and were approved by the University of Barcelona's Ethics Committees for Animal Experimentation (#483/16). The animals underwent general anesthesia with isoflurane inhalation.

4.2. Experimental Groups

Zücker Rats aged (16–18) weeks were divided into three groups. The abdomen was cut with a midline incision, and following bile duct cannulation, the portal vein and the splenic and gastroduodenal veins were ligated. After organ recovery, the livers were flushed with IGL-0, IGL-1, or IGL-2 (Table 1) and stored in each solution for 24 h at 4 °C. Animals were randomly distributed in different groups ($n = 6$), as follows:

Group 1 (SHAM): Obese Zücker rats underwent transverse laparotomy, and silk ligatures of right suprarenal and diaphragmatic veins and hepatic artery were performed before retrieving the liver.
Group 2 (IGL-0 solution): After organ recovery, fatty livers were flushed with 40 mL of IGL-0 preservation solution and were then stored in IGL-0 at 4 °C for 24 h.
Group 3 (IGL-1 solution): After organ recovery, fatty livers were flushed with 40 mL of IGL-1 preservation solution and were then stored in IGL-1 at 4 °C for 24 h.
Group 4 (IGL-2 solution): After organ recovery, fatty livers were flushed with 40 mL of IGL-1 preservation solution and were then stored in IGL-2 at 4 °C for 24 h.

After 24 h of cold preservation or right after surgery (in the case of Sham), liver samples were rinsed with Ringer's lactate (20 mL), and samples were taken from the flush. They were then stored at −80 °C for subsequent biochemical determinations.

4.3. Biochemical Analyses

Transaminase Assay

Liver injury was assessed by alanine aminotransferase (ALT) and aspartate aminotransferase (AST) commercial kits, purchased from RAL (Barcelona, Spain) following the manufacturer's instructions. Briefly, 100 mol of effluent washout was added to 1 mL of substrate provided by the commercial kit. Transaminase activity was measured at 340 nm using a UV spectrometer.

4.4. Glutamate Dehydrogenase (GLDH) Activity

Mitochondrial damage was measured by GLDH activity, following the manufacturer's instructions of the commercial kit purchased from RANDOX (Crumlin, United Kingdom).

4.5. Energy Metabolism (ATP Breakdown)

The determination of ATP in liver samples homogenized in a perchloric acid solution was performed using the ATP assay kit for fluorimetry (Sigma Aldrich ATP colorimetric/fluorometric assay kit, Madrid, Spain). The ATP concentration was determined by the phosphorylation of glycerol, which is a detectable product for the fluorimeter (excitation/emission 535 nm/587 nm) at 37 °C and proportional to the amount of ATP in the sample. Energy breakdown during cold storage was measured through the changes in ATP levels.

4.6. 4-Hydroxynonenal Protein Adducts Assay

4-Hydroxynonenal (4-HNE) protein adducts were measured in liver homogenate using the OxiSelect™ HNE Adduct Competitive ELISA Kit (Cell Biolabs, Inc. San Diego, CA, USA). Liver was homogenized in 10% (w/v) with a Teflon bar in a RIPA solution, (Tris 50 M pH 7.4, 1% Triton 100×, NaCl 150 mM, NaF 5 M, 0.1% sodium dodecyl sulphate, and 1% sodium deoxycholate) with antiprotease solution (aprotinin at 1.7 mg/mL, 2 μg/mL pepstatin, 2 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride, and sodium orthovanadate at 1 mM). The suspension was centrifuged at 2000 g for 5 min and the pellet discarded. Liver homogenates were added to an HNE conjugate preabsorbed ELISA plate. After a brief incubation, an anti-HNE polyclonal antibody was added, followed by an HRP conjugated secondary antibody. The quantity of HNE adduct in protein samples was determined by comparing its absorbance with that of a known HNE-BSA standard curve.

4.7. Advanced Oxidation Protein Products (AOPP)

Advanced oxidation protein products (AOPP) are biomarkers of oxidative damage to proteins, detecting dityrosine-containing and cross-linking protein products. The formation of AOPP in the liver homogenates was spectrophotometrically measured at 340 nm. Results were obtained through a standard calibration curve using 100 μL of chloramine-T solution (0–100 μmol/L). AOPP concentration was expressed in nmol/mg protein. Advanced oxidation protein products (AOPPs) in the liver were assayed by a modification of Witko-Sarsat's method [38].

4.8. Glutathione Analysis

Reduced glutathione (GSH) was measured in the liver extracts using the procedure previously described [28]. Liver samples were homogenized in cold buffer containing 5 mm phosphate–EDTA buffer (pH 8.0) and 25% HPO3. The homogenates were ultra-centrifuged at 100,000× g and 4 °C for 30 min, and the resulting supernatant, with the fluorescent probe o-phthalaldehyde, was used to determine GSH concentration. Fluorescence was determined at a wavelength emission of 420 nm and excitation at 350 nm. Results are expressed as GSH nmol/mg protein.

4.9. Nitrite/Nitrate Analysis

NO production in the liver was determined by tissue accumulation of nitrite and nitrate using a colorimetric assay kit (Cayman, Tallinn, Estonia) according to the manufacturer's instructions.

4.10. Western Blot Analysis
ALDH2, Beclin-1, and LC3B

Separated on 6–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, proteins were blotted into poly-vinylidene fluoride (PVDF) membranes (Bio-Rad, Madrid, Spain) and immunoblotted overnight at 4 °C using antibodies against ALDH2 (Abcam, Cambridge, UK. ref: ab133306), Beclin-1 (Sigma Aldrich, San Louis, Missouri, ref: SAB5700251), and LC3B (Abcam, Cambridge, UK. ref: ab48394). Detection was performed with anti-IgG-HRP (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). In all cases, the

chemiluminescence signals were quantified ChemiDoc (Bio-Rad, Madrid, Spain). Both β-actin (Abcam ref: ab8226) and α-tubulin (Abcam ref: ab7291) were used as loading controls.

4.11. 4-HNE Protein Adducts and eNOS

Liver samples were homogenized in RIPA (50 M Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 5 M NaF, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate) and centrifuged for 20 min at 10,000 g. The supernatant was denatured with the addition of Bromophenol Blue (1/2) and heating at 95 °C for 5 min. A total of 50 mg of protein per sample was loaded onto the 10% agarose gel, and wet blotting was carried out on a PVDF membrane (Bio-rad, Irvine, CA, USA). The membranes were blocked for 1 h in Odyssey® Blocking Buffer (LI-COR Biosciences GmbH, Germany) diluted in Tris Base Buffer (TBS, Tris-buffered saline) (pH = 7.4) with 0.05% Tween (TTBS). The membranes were incubated overnight with anti-4-hydroxynonenal (4-HNE) protein adducts and anti-eNOS (BD Biosciences-Europe) antibodies according to the manufacturers' recommendations.

Detection and analysis were carried out by incubation with secondary fluorescence (800 W) with the Odyssey® Fc system (LI-COR Biosciences GmbH). To quantify the expression, Image Studio 5.2.5 software (LI-COR Biosciences) was used, correcting for the total protein analyzed with the REVERTTM solution (Li-COR Biosciences) according to the manufacturer's protocol and expressing the results as a percentage with respect to the sham group.

4.12. Statistics

Data are expressed as mean ± standard error and were compared statistically by variance analysis, followed by the Student–Newman–Keuls test using GraphPad Prism version 8.1.0 for Windows (GraphPad Prism software, San Diego, CA, USA, 2018) and one-way ANOVA. A level of $p < 0.05$ was considered significant. Significant differences between groups are represented with different letters in the graphs. A group labeled with a letter has significant statistical differences compared to a group labeled with a consecutive letter with a $p < 0.05$.

Author Contributions: R.G.B., R.T.d.S., and A.P.-R. carried out surgical procedures and the experimental. E.F.-P. and J.R.-C. carried out the data analyses evaluation. E.F.-P., C.P., J.P., R.A., and J.R.-C. analyzed data and wrote the paper. T.C. and A.P.-R. designed the experiments, coordinated the study, and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Role of PEG35 and mitochondrial protection in dynamic preservation

Despite been around for a long time, machine perfusion devices were not widely used in organ preservation due to logistics problems. Nowadays, given the advances in innovation and technology, they have become more portable and efficient, and consequently, more prominent and promising strategies for graft preservation. Amongst these, HOPE has been related to better post-transplant outcomes, especially with grafts from DCD donors. HOPE is a complementary tool to improve graft preservation which combines the benefits of cold preservation conditions with the supply of oxygen to the organ in a perfusion system. This approach enables the maintenance of a basal level of mitochondrial oxidative phosphorylation, which in turn will lead to a better outcome after the unavoidable reperfusion.

Belzer Machine Perfusion Solution (Belzer MPS) and its generics, which are a variation of the original UW solution used in the SCS, are the most commonly used perfusion solution for HOPE. However, the use of Belzer MPS in liver machine perfusion has been showing some limitations due to the presence of HES, which increases the perfusate viscosity and consequently augments the shear stress. In addition, HES has also been linked to higher levels of erythrocytes hyper-aggregation, making difficult a suitable rinsing and preservation during machine perfusion. To overcome these limitations, a new IGL-2 solution/perfusate was developed as an alternative to Belzer MPS. This new PEG35-containing perfusate showed a better mitochondrial liver protection (measured as GLDH) when compared to Belzer MPS.

In this study we report some considerations about the use of PEG35 as a component of perfusate for MP strategies whose benefits are associated with the mitochondrial machinery graft preservation when dynamic hypothermic oxygenated strategies are used.

Furthermore, we also demonstrate **"newly results not published before" (Figure 3)**, suggesting that PEG35-containing perfusates in HOPE, such as IGL-2, could be a good alternative tool to Belzer MPS, for future HOPE investigations to prevent shear stress and to diminish the higher viscosity Belzer MPS vs IGL-2.

Review

Polyethylene Glycol 35 as a Perfusate Additive for Mitochondrial and Glycocalyx Protection in HOPE Liver Preservation

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Abstract: Organ transplantation is a multifactorial process in which proper graft preservation is a mandatory step for the success of the transplantation. Hypothermic preservation of abdominal organs is mostly based on the use of several commercial solutions, including UW, Celsior, HTK and IGL-1. The presence of the oncotic agents HES (in UW) and PEG35 (in IGL-1) characterize both solution compositions, while HTK and Celsior do not contain any type of oncotic agent. Polyethylene glycols (PEGs) are non-immunogenic, non-toxic and water-soluble polymers, which present a combination of properties of particular interest in the clinical context of ischemia-reperfusion injury (IRI): they limit edema and nitric oxide induction and modulate immunogenicity. Besides static cold storage (SCS), there are other strategies to preserve the organ, such as the use of machine perfusion (MP) in dynamic preservation strategies, which increase graft function and survival as compared to the conventional static hypothermic preservation. Here we report some considerations about using PEG35 as a component of perfusates for MP strategies (such as hypothermic oxygenated perfusion, HOPE) and its benefits for liver graft preservation. Improved liver preservation is closely related to mitochondria integrity, making this organelle a good target to increase graft viability, especially in marginal organs (e.g., steatotic livers). The final goal is to increase the pool of suitable organs, and thereby shorten patient waiting lists, a crucial problem in liver transplantation.

Keywords: polyethylene glycol 35 (PEG35); hydroxyethyl starch (HES); UW solution; IGL-1 solution; Belzer-MPS; HOPE; liver graft preservation

1. Introduction

For more than 100 years, a fascinating dream has been to keep organs "alive" outside the human body [1]. In the process of transplantation, the storage of organs in preservation solutions is a

mandatory step for maintaining their quality and for the success of the transplantation [2,3]. For many years, the University of Wisconsin (UW) solution was considered as the gold standard [4,5]. However, other solutions containing (or not) oncotic agent in their compositions, such as Institut Georges Lopez 1 (IGL-1) [6], Celsior [7] and histidine-tryptophan-ketoglutarate (HTK) [8], are also commonly used for static cold storage (SCS) in clinical transplantation. UW preservation solution contains the oncotic agent hydroxyethyl starch (HES) [5], in contrast to IGL-1, which contains polyethylene glycol 35 (PEG35) [6] as a main component (along with other components). Other commercially available solutions used, such as Celsior and HTK [7,8], have no oncotic agent in their formulations. All of them are considered a good alternative to UW in liver transplantation according to data from the European Liver Transplant Registry (ELTR), albeit with some limitations for HTK [9].

PEGs are polymers of ethylene oxide with hydroxyl termini. They are neutral, water-soluble, non-toxic and non-immunogenic polymers, characterized by their three-dimensional conformation and their high flexibility [10,11]. The molecular weight (MW) of the molecule depends on the length of the HO-(CH2CH2O) n-CH2-CH2-OH chain, which confers them characteristics of density and high hydrophilicity. PEGs are negligibly synthetized in vivo, and their non-toxicity makes them FDA approved, being used for many purposes (industrial, alimentary, pharmacologic, etc.) [11].

Robinson firstly reported the relevance of oedema prevention by PEG [12–14]. Further studies demonstrated that PEG cannot cross membranes, thereby exerting an oncotic pressure that limits tissue edema without breaking the transmembrane ionic balance [14]. In 1976, Daniel and Wakerley [15] used 20 kDa PEG to demonstrate increased cell viability during the preservation at 4 °C of renal pig cells. In 1977, Ganote et al. [16] demonstrated that low molecular (6 kDa) PEG avoids cellular edema in heart rat tissue during the preservation, limiting the regions damaged by hypoxia. Based on this line of studies, the use of 8 kDa PEG permitted a 24 h preservation to be obtained in isolated hepatocytes, reducing tissue edema and cell mortality by preserving actin microfilaments and the microtubules integrity [17].

Investigations by Wicomb et al. [18] resulted in the modification of the cardiac preservation solution St. Thomas by adding PEG 20 (20 kDa PEG), demonstrating an improved preservation and an interest in the use of PEGs to a clinical setting. Further studies proved that higher molecular PEGs, such as the PEG 35 used in the IGL-1 solution, is protective for liver graft preservation [19], making it an effective alternative to UW (the gold standard) used in clinical liver transplantation [9].

IGL-1 is the unique PEG based solution routinely used in clinical liver transplantation, and it contains PEG35 at 1 g/L. The use of other solutions, such as SCOT containing PEG 20 (at 15 g/L), has been limited, with non-conclusive results. In fact, in 2019, Karam et al. [20] and previously in 2017, Adam et al. [21] described a large series of transplants performed at the Paul Brousse Hospital (France) that shows a higher incidence of early allograft dysfunction in HTK- and SCOT-preserved ECD livers as compared to other solutions (e.g., UW and IGL-1) and that this negatively affects graft survival [20,21]. This could be due to the lower molecular weight of PEG 20 used in SCOT solution, which requires its concentration to be increased to 15 g/L (as compared to 1 g/L for PEG 35 in IGL-1). This in turn increases viscosity which could not only hinder an efficient graft washout after organ recovery but also promote deleterious effects on endothelial shear stress in graft preservation processes. Moreover, the presence of a glucose SCOT solution could promote deleterious effects in preserved liver graft during SCS due to the generation of acidosis in oxygen deprivation conditions [22]. Other PEG-containing solutions are IGL-1, Polysol and IGL2 [6,23,24], as shown in Table 1.

With this in mind, a PEG35 rinse solution contributes to a more efficient washout of the graft. As this avoids the hyper-aggregation action of HES against red blood cells in recovered liver graft when UW solution is used, it also confers additional protection against reperfusion [11]. Moreover, the presence of PEG35 in a rinse solution provides significantly more mitochondrial protection as compared to a rinse solution without PEG35 [19].

Table 1. PEG-based preservation solutions.

Preservation Solution	PEG (kB)	Concentration (g/L)
IGL-1	35	1
IGL-2	35	5
Polysol	35	20
SCOT	20	15

More recently, it has been demonstrated that intravenous (i.v.) PEG35 administration is also protective against liver warm ischemia reperfusion injury [25]. This protection has been reviewed in other organs, such as pancreas in an inflammation model [26]. This protective effect was demonstrated in i.v. PEG35 pre-treatment in rats [26]. Several studies reported the efficiency of PEGs in upregulating cell-survival pathways in different organs [27,28], resulting in the protection of the mitochondria, prevention of radical oxygen species (ROS) formation, cell swelling and oedema, and preservation of the cellular membrane, among others. It is thought that, under conditions of low surface pressure, PEGs stabilize the cell membranes [29]. This effect is partly associated with the ability of PEGs to reduce the lipid molecular motion, which may cause denser packing of lipids and decrease the fluidity of the membrane [29].

PEGs have been shown to lower monolayer surface pressures and to increase the area per lipid at the "collapse point", at which point glycerophospholipids are densely packaged. Conditions of hypothermia imposes lipid phase transitions in the membranes, which modify the conformation of the lipid packing. This pressure-lowering effect of PEG may be evident in membrane regions with saturated phospholipids and, to a lesser degree, on unsaturated acyl chains in membrane monolayers. Temperature (conditioning the ion strength) and pH of the environment are extremely important parameters, which have direct effects on the binding properties of PEG [29–31]. The precise mechanisms by which high molecular PEGs exert their liver cytoprotection still remain unclear, although Belzer and Southard suggested the relevance of underlying mechanisms 30 years ago [4,5]. They pointed out that "the mechanism by which PEG prevent cell swelling is not related to the osmotic or oncotic properties of the molecule but instead is apparently related to some "unknown interactions" between PEG and cells, an interaction that provides stability during hypothermic preservation of hepatocytes" [32].

It is important to note that the properties of PEGs contribute to the sustained maintenance of cold, which is a determinant parameter for graft integrity during conservation, as evidenced by several studies [33–35].

We have witnessed an increasing amount of evidence that points out to the crucial role of oxygen and mitochondria during ischemia. As a consequence, in the last decade, the hypothermic oxygenated perfusion (HOPE) has been implemented as a complementary tool to improve graft preservation [36–39]. This strategy combines the benefits of cold preservation conditions with the presence of a transient oxygen supply to the organ in a perfusion system, with the purpose to help the mitochondrial system to sustain a basic level of oxidative phosphorylation, which in turn will provide the organ with better conditions to face the unavoidable reperfusion [36–39]. In contrast to static preservation, the fluid dynamics that intrinsically occurs in any fluid-applying forces in a vascular section, as it happens in HOPE (or any kind of reperfusion), is responsible for generating the phenomenon known as shear stress [40]. Shear stress affects the surface of the section and hence the glycocalyx, the luminal thin monolayer of sugars that covers the blood vessel endothelia [41]. In this context, perfusates used in HOPE may play a determinant role in the associated mechano-transduction processes involved in shear stress [42].

The most commonly used perfusion solution for HOPE is a variation of the original SCS UW solution, the Belzer Machine Perfusion Solution (Belzer MPS) and its generics. As the same formulation is used throughout this article, we refer to them generically as Belzer MPS. This solution was designed for kidney MP and then extended to liver HOPE strategies [36–39]. However, the use of Belzer MPS–like solutions in liver MP has some limitations. First, it contains HES that contributes to increasing the

viscosity of the solution, resulting in augmented shear stress; second, the presence of glucose as an osmotic agent (which is not harmful to kidney) provokes acidosis in liver and pancreas cells [22]. Furthermore, as another side effect, HES increases the hyper-aggregation of erythrocytes as compared to PEG35 in flushing solutions, and this can create difficulties for suitable rinsing and preservation during MP [43,44]. Therefore, given the limitations of the current MP perfusates, we explored whether they can be improved based on previous evidences.

In this review we present and discuss the preliminary results on the advantages of using PEG35 perfusates for HOPE in liver graft preservation strategies. PEG35 seems to enhance mitochondrial protection. Moreover, previous evidence suggests the importance of viscosity, shear stress and signal transduction mechanisms and their effect on endothelial glycocalyx, and how PEG35 can modulate these processes during hypothermic liver machine perfusion.

2. Static Cold Storage (SCS) and PEG

SCS is based on the idea of keeping an organ in a box at a cold temperature in order to slow down its metabolism. This simple strategy has been the most widely used technique since the early beginnings of transplantation until even now, due to its simplicity and effectiveness. However, it is not ideal as does not completely halt the metabolism, as anaerobic reactions can still be found to operate. Although it is easy to handle, it has been demonstrated that it is not as effective as MP in kidney with expanded criteria donors (ECD) and donors after circulatory death (DCD) [45,46].

In previous studies, our group has seen that the presence of PEG35 in the IGL-1 solution is a key factor in mitochondria preservation and in reduction of transaminases. Figure 1 compares IGL-1, which contains PEG35 at 1 g/L), with IGL-0, which has same composition as IGL-1 but without any PEG.
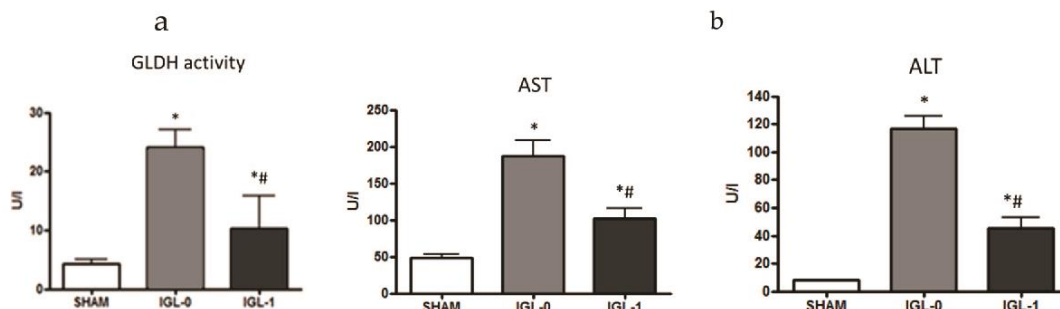

Figure 1. Differences between IGL-0 and IGL-1. (**a**) Mitochondrial damage as glutamate dehydrogenase (GLDH) activity. (**b**) Liver damage as transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Groups are divided into SHAM (no treatment), fatty livers preserved 24 h at 4 °C in IGL-0 solution (without PEG35) and commercial IGL-1 solution. Results are expressed as mean ± SEM ($n = 6$). * $p < 0.05$ significant differences as compared to SHAM; # $p < 0.05$ as compared to IGL-0.

Those results could partly explain the difference found in graft protection when IGL-1 and UW solutions were compared after 24 h SCS prior to reperfusion [6]. This was corroborated by a significant decrease in the transaminases AST/ALT release (liver injury) and in GLDH enzyme activity (mitochondrial damage). The absence of oxygen in SCS forces cells to switch to an anaerobic metabolism, which leads to an energetic breakdown, provoking cellular edema secondary to membrane imbalance, a drop in the pH and accumulation of subproducts that later on greatly contribute to ROS formation and general organ damage. Inability to generate sufficient ATP to maintain ionic homeostasis affects the integrity of the mitochondria. All these processes can be modulated by the preservation solution during cold storage in order to maintain the graft viability; for this, the presence of PEG35 is determinant, as we have previously demonstrated [47]. The presence of PEG35 in the IGL-1 solution is more effective at preventing energy metabolism failure than UW, thus favoring a better graft conservation [6].

The aforementioned energetic breakdown of the cell provokes a mitochondrial depolarization and the closing of mitochondrial permeability transition pore (MPTP), which is accompanied by increases in lactate, succinate and other ROS precursors associated in this oxygen deprivation condition. In this sense, mitochondrial protection conferred by PEG35 would be a critical point in ischemias, as recently suggested by Kulek et al. [48]

With its potential cytoprotective effects in mind, we evaluated the benefits of PEG35 in rinse solution for graft washout after cold preservation [19]. The presence of PEG35 in rinse solution after static preservation in UW confirmed that this polymer is directly responsible for mitochondrial preservation during cold storage, including as well the concomitant activation of cytoprotective factors such as AMP-activated protein kinase (AMPK) and presumably the inherent cytoskeleton rearrangement [19]. These data are in accordance with Chiang et al. [49], who reported that PEG35 could contribute to a better stabilization of the liver endothelial barrier and cytoskeleton when grafts are subjected to PEG35 rinse. These facts point to the potential benefits of using PEGs as oncotic agents in perfusates to reduce the deleterious effects inherent to dynamic preservation strategies.

Oxygen deprivation during IGL-1 cold storage activates a set of protective cell-signalling pathways, such as AMPK, as a self-response of the organ in front of the energetic breakdown [50]. PEG35 promotes AMPK activation, which acts on its downstream targets and leads the graft towards an energy–conserving strategy in ischemic conditions. This AMPK activation through PEG35 contribute to limiting the impact of IRI, and in this sense, PEG35 could be considered as a preconditioning agent [51–53].

One of the main AMPK targets is the endothelial nitric oxide synthase (eNOS), which is responsible for the generation of nitric oxide (NO), a well-known vasodilator agent that protects the liver against IRI [51–53]. Thus, any activation of AMPK and HIF, triggered either by intrinsic cytoprotective mechanisms of the cell or as a result of a PEG35 upregulation, would reduce the graft injury during preservation, as happens when IGL-1 solution is used [50,51]. Further, AMPK counterbalances the exacerbated microcirculatory alterations through NO production, which is specially narrowed in the livers presenting steatosis [54,55]. In this sense, PEG35-derived NO in IGL-1 solution would at least partly explain AMPK activation as well as the improved vascular resistance and function during reperfusion [6,56,57].
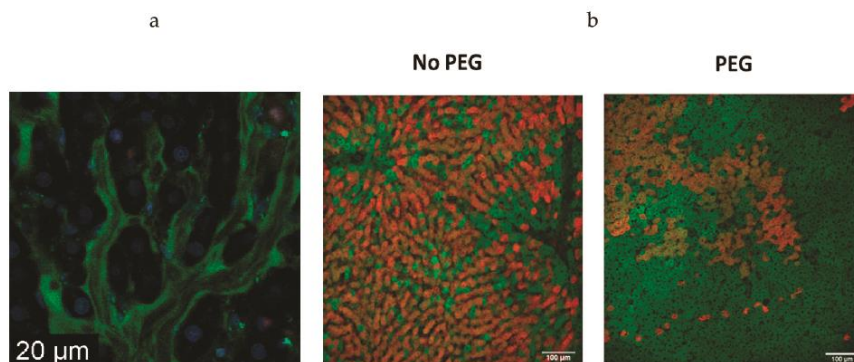

Figure 2. Electron microscopy imaging showing effects of PEG35 on rat liver. (**a**) Intravital microscopy of rat liver perfused with PEG35 covalently conjugated to FITC (green). Cell nuclei were labelled with intravenous injection of Hoescht 33, 342 (blue). PEG35 is deposited in the liver vascular bed. Male Sprague-Dawley rats were treated with PEG35 (10 mg/kg) and then subjected to 1 h ischemia followed by 2 h reperfusion. (**b**) Ex vivo two-photon imaging of liver grafts labelled with rhodamine 123 (green) and propidium iodide (red), showing polarized mitochondria and dead cells respectively. Liver grafts were worse preserved when they were rinsed with a solution without PEG35 (left) than with the same solution containing PEG (right). Images show better preservation of polarized mitochondria in PEG rinsed livers, as well as smaller size of cells and more compact structure, indicating that PEG, as oncotic agent, prevents cell swelling and interstitial edema.

Our recent investigations demonstrated the protective actions of intravenous PEG35 administration (10 mg/kg) in a rat model against the deleterious effects of liver IRI [25,27]. PEG35 administration was associated with NO generation and an increased protection of endothelial cell barrier, as previously evidenced in human lung endothelium by Chiang et al. [49], who reported beneficial changes due to actin rearrangements. This idea is supported by electron microscopy imaging findings, which reveal that PEG35 interacts with the luminal vascular bed when administered intravenously (Figure 2). This PEG35 deposition on the vascular endothelium could explain the subsequent activation of transduction mechanisms of protective cell signalling activation pathways, similar to those occurring in ischemic preconditioning, such as AMPK and eNOS activation in the rat liver [56,57]. All of them presumably are involved in the cytoskeleton rearrangement [19,49].

3. Endothelial Glycocalyx: Fluid Dynamics and PEG35 Effects

Additional factors to be considered in dynamic preservation are the physical characteristics of the perfusate, such as viscosity and shear stress, which can provoke both mechanotransduction or/and destruction of the glycocalyx (GCX) [42,58]. The GCX comprises the thin luminal sugar monolayer that covers the graft endothelia and is highly exposed to all circulating fluids, and it is damaged within human liver grafts during preservation [58–60].

The endothelial GCX comprises proteoglycans, glycoproteins and glycosaminoglycans. Due to its superficial location covering the endothelial cells (EC), the GCX affects the vascular permeability, mitigates blood cell-vessel wall interactions and plays a critical role in EC mechanosensing and transduction in the blood flow regulation [60–62].

The vasculo-protective role of GCX may be disrupted or modified by diverse pathophysiological conditions and clinical settings, such as the IRI occurring in liver transplantation (TX) [63]. The fluid dynamics (blood in the case of TX, and perfusate in the case of MP) are intrinsically related (to a greater or lesser extent) to shear stress, which in turn may induce the disruption or destruction of GCX. Shear stress is highly determined by the viscosity of the solution, which depends on the composition and the oncotic agent. Therefore, obtaining optimum shear stress associated with viscosity is a critical point for preserving GCX integrity and graft viability.

As reported by Schiefer et al. [63], the damage to the GCX is well-correlated with alterations in graft injury and function in clinical liver transplantation, and the assessment of some of its components, such as syndecan or heparan sulphate, can be used as a marker of GCX degradation.

As shear stress undermines many aspects of the GCX, it is not less important the mechanotransduction [58–62]. One of the main consequences of an adequate functioning of the GCX mechanotransduction is NO production [60]; therefore, mitigating GCX destruction is a necessary step to regulating NO production in order to maintain a good perfusion and an adequate preservation [60]. In previously reported studies, we demonstrated the relevance of NO induced by PEG35, which correlated with an enhanced preservation of the GCX during static preservation when comparing two solutions, IGL-1 and HTK [64].

With these considerations, and in accordance with the observations of Schiefer et al. [63], we corroborated that the GCX suffers an alteration during hypothermic preservation [64]. The improved preservation of the GCX, supported by our data, correlate with an increased presence of NO in the group where livers were preserved in 24 h SCS with a solution containing PEG35 (IGL-1) as compared to one without oncotic agent (HTK). In this case, the protective benefits of IGL-1 were presumably associated with the presence of the oncotic agent PEG35 [64].

In sum, the presence of PEG35 in perfusates for MP, such as HOPE, could favour a more efficient GCX integrity, which will be translated in an increased mechanotransduction capability and generation of endothelial NO. Overall, this contributes to a better endothelial barrier protection, as shown in Scheme 1. It is important to point out that the viscosity of UW or Belzer MPS solutions with HES is twice as much as IGL-1 with PEG35 (2.4 centipoise [cP] vs. 1.2 cP, respectively). Consequently, the use

of PEGs should be considered in order to confer the right viscosity and oncotic pressure features to the perfusate for the preservation of the GCX [19,44].
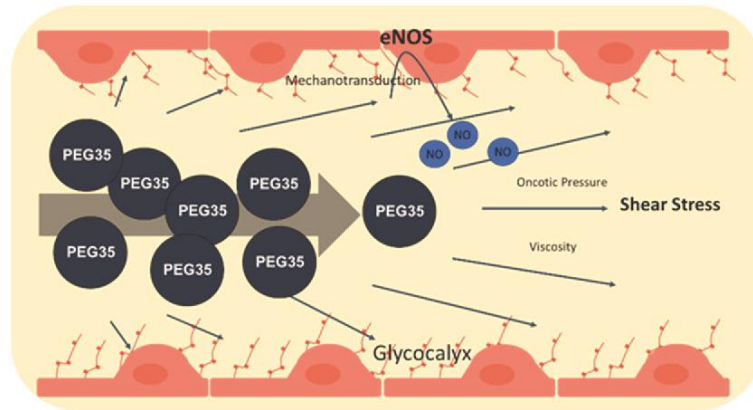
Scheme 1. Schematic depiction of proposed PEG35 mechanotransduction through its interaction with the glycocalyx due to its physical properties that affects the sheer stress in HOPE strategies. This results in an increase of nitric oxide (NO) production through an upregulation of eNOS [60,64].

4. HOPE and PEG35 Perfusates

Machine perfusion allows the dynamic perfusion of the organs and was developed decades ago. However, the difficulties to implement the logistics kept their impact to a low profile. Nowadays, with the advance in innovation, their design is more portable and efficient. HOPE is the modality that embraces a dynamic perfusion at the range of 4–11 °C with active oxygenation of the perfusate [36–39].

MP allows a continuous supply of oxygen and nutrients while flushing cellular waste products from the liver, thereby preventing the damage cascade build-up that occurs in SCS. Furthermore, the oxygen flow permits energy production through the mitochondrial electron transport chain, helping to restore cellular homeostasis and to prevent mitochondrial collapse [65]. In addition, this technique also enables organ viability to be monitored and makes pharmacologic interventions possible.

A complete stop of oxidative phosphorylation that occurs in SCS has practical consequences not only for the ability to limit the extent of hypoxia but also for reperfusion once normal conditions are re-established, as this stop hinders cells from reaching the previous levels of production due to the destruction of the mitochondria. However, the addition of oxygen in HOPE provides a way to keep certain levels of ATP production, which as a side effect implies a lower accumulation of ROS precursors derived from anaerobic metabolism (such as succinate), giving cells a better chance to return to normality. In this sense, the presence of PEG35 in the perfusate could confer mitochondrial protection during HOPE, as evidenced by our prior studies with PEG35 rinse solution and IGL-1 [19].

In a comparative study using a rodent model of DCD liver grafts, machine perfusion strategy proved to be more protective than SCS [66]. In addition, comparison among different machine perfusion approaches (e.g., warm vs. cold perfusion) showed that normothermic oxygenated perfusion failed to protect from lethal injury in grafts exposed to 1 h warm ischemia, with a concomitant activation of Kupffer- and endothelial cells [66]. On the other hand, HOPE prevented the development of lethal graft injury, probably by the downregulation of electron transfer rates, which hampers the initial oxidative stress. These results suggest a better outcome for the HOPE technique to rescue DCD livers [66,67].

Further, a 1 h HOPE treatment after SCS protected liver grafts from initial ROS and damage-associated molecular pattern (DAMPs) release after transplantation, alongside with decrease activation of inflammatory pathways [67]. This HOPE period was also appropriate for recovering ATP loading prior to reperfusion and for reducing cell death during reperfusion [68].

The composition of perfusion solutions for hepatic hypothermic oxygenated perfusion are identical to those used for SCS. As mentioned above, the main drawback of solutions containing HES (such

as Belzer MPS) is their high viscosity, which may lead to sinusoidal shear stress. To overcome these shortcomings, a new solution containing PEG35 instead of HES was developed, called Polysol [23,69]. As an oncotic agent, PEG35 exerts an oncotic pressure similar to HES but with a relatively lower viscosity and consequently less shear stress in the hepatic sinusoid. A better liver function and less liver damage was observed using the Polysol solution as compared to Belzer MPS [23,69]. In addition, 24 h of MP using Polysol showed a better preservation as compared to the same solution but with HES instead of PEG (Polysol-HES), highlighting the protective role of PEG. A lower molecular weight PEG, PEG20, supplemented to a Celsior solution offered a better protection of pig kidneys recovered after cardiac death using MP, as compared to Celsior without supplementation or to Belzer MPS [70]. Schlegel et al. [71] showed that endothelial cleaning or repair of the GCX represents an important mechanism of protection conferred to the liver by HOPE. The GCX can be cleaved by either enzymatic cleavage of the proteoglycan core proteins or direct oxidative stress from ROS underlying ischemia reperfusion injury (IRI), inducing endothelial permeability and edema [72]. As mentioned above, Lopez et al. [64] showed compelling evidence for the benefits of IGL-1 solution on steatotic livers, highlighted by the importance of GCX protection during SCS. It is known that PEG35, present in IGL-1 solution, prevents cell swelling and vascular endothelial damage through the stabilization of lipid membranes and by lowering membrane permeability. To deepen the findings of Lopez et al. [64] regarding the role played by GCX during hepatic IRI and its connection to PEG35, the shear stress should be also taken into account. HOPE seems like a valid strategy to investigate the shear stress inherent in IRI and its effect on the GCX integrity using a perfusate containing PEG35. Such a therapeutic approach of GCX protection could potentially enhance organ viability of marginal grafts and diminish the severity of IRI.

Based on these strong observations, our target was to ascertain whether PEG could have a relevant effect in protecting the liver during HOPE at the mitochondrial level. For this reason, we compared the only available perfusates Belzer MPS and a new one including PEG35 (IGL-2) (Table 2) for 1 h of HOPE after 7 h of SCS in the same solutions, using liver in Sprague Dawley rats [24]. Table 2 shows the composition of the two solutions tested. The comparative levels of mitochondrial damage (measured as GLDH activity) in both MP solutions revealed a significant prevention of mitochondrial damage IGL2 solution in HOPE conditions (Figure 3a), although no significant differences were found in transaminases levels (Figure 3b).

To measure the deleterious effects of IRI, other markers has been suggested, such as the mitochondrial enzyme ALDH2 [73] and glycocalyx [74]. We observed an increase in ALDH2 activation in liver grafts that had been subjected to HOPE followed by 1 h of normothermic reperfusion (unpublished data). This ALDH2 activation is a protective factor that would prevent lipoperoxidation subproducts, such as 4HNE, associated with IRI [73]. In this context, improvement of the mitochondrial status during HOPE is closely linked to the presence of PEG35 [75], and it could be a critical point for restoring the protective mitochondrial mechanisms and modulating succinate accumulation during cold preservation [76–78].

In the hypothermic oxygenated perfusion (HOPE), having a continuous oxygen delivery allows cells to maintain and restore the basal mitochondrial machinery as compared to the static preservation (no oxygen support) [36,38]. These HOPE benefits were more potentiated in IGL2-perfusate (containing PEG35) than in generic Belzer perfusate (containing HES). The better mitochondrial protection was concomitant with an increase in mitochondrial ALDH2 activity measured 1 h reperfusion after (unpublished data). In any case, the cold cellular oxygenation during HOPE using PEG35 perfusate is likely to help reduce accumulation of some metabolites (e.g., succinate) that are responsible for dysfunctional liver graft mitochondria as compared to static preservation [77,78]. Thus, the clearance of such metabolites by the dynamic flow observed in HOPE might be an important way to guarantee proper mitochondrial function during early normothermic reperfusion.

Table 2. Composition of IGL2 and Belzer MPS solutions.

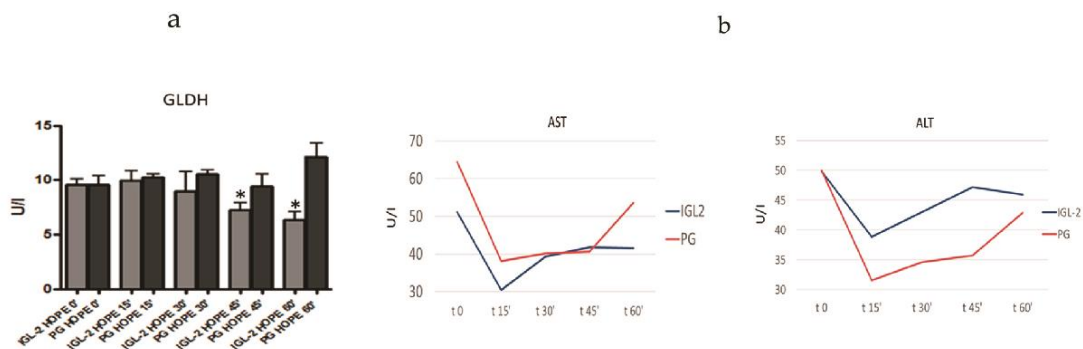| Preservation Solution	Belzer-MPS	IGL-2
Electrolytes (mmol/L)		
K +	25	25
Na +	120	125
Mg2 +	5	5
SO4 2-	5	5
Ca +	0.5	
Zn 2+		0.091
Buffers (mmol/L)		
Phosphate	25	25
HEPES	10	
Histidine		30
Impermeants (mmol/L)		
Mannitol	30	60
Lactobionic acid		80
Dextrose	10	
Ribose	5	
Gluconate	85	
Colloids (g/L)		
Hydroxyethyle starch	50	
Polyethylene glycol-35		5
Antioxydants (mmol/L)		
Glutathione	3	9
Metabolic precursors (mmol/L)		
Adenosine		5
Adenine	5	
NaNO2 (nmol/L)		50
pH	7.4	7.4
Osmolarity (mosmol/L)	320	320
Viscosity (cP)	2.4	1.4

a　　　　　　　　　　　　　　　　　　　　　b

Figure 3. Comparison of Belzer MPS and IGL-2 in HOPE. (**a**) Mitochondrial damage as GLDH activity in fatty livers preserved for 7 h at 4 °C and analyzed for 1 h (measured in 15 min intervals) of HOPE in IGL-2 solution (PEG35-enriched IGL-1 solution) and Perf-gen solution. (**b**) AST/ALT levels during HOPE. Significant differences were found in mitochondrial damage at 40 min and 60 min of HOPE between the two solutions. However, this did not affect AST/ALT levels during the same period. * $p < 0.05$ represent significant differences vs. Perf-gen group (PG-HOPE).

5. New Biomarkers for Dynamic Preservation

Widely known parameters of clinical relevance, such as transaminases and lactate, are indicative but not definitive for liver status. Therefore, another scope of this study could be the assessment of other markers that could bring a more accurate diagnosis of the status of the liver. For instance, it might be of interest to assess levels of syndecan and heparan sulphate, which would give us an insight of the

status of the GCX in HOPE strategies [63–68]. Further, it would be interesting to assess mitochondrial parameters besides ALDH2 [47], such as succinate [77,78], or others that have been recently linked both to lipoperoxidation and energetic levels, such as UCP1 and UCP2 [79–83], which could provide relevant information, especially when steatotic livers are used (Scheme 2). The assessment of these new proposed markers involved in different critical points of different crucial pathways brought together could strengthen the informative value of the most widely used, such as transaminases.
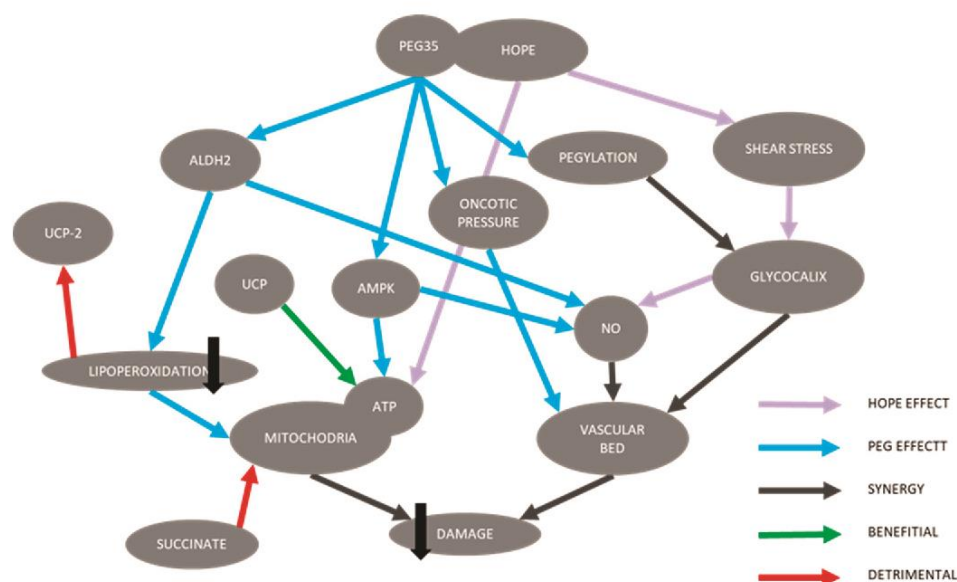
Scheme 2. Schematic representation of the working mechanisms of PEG35 and HOPE leading to improvement of the mitochondria and the vascular bed and thereby prevent damage. PEG35 upregulates ALDH2, which prevents lipoperoxidation. High levels of lipoperoxidation promotes UCP-2 formation. However, reduction of lipoperoxidation prevents the formation of ROS in the mitochondria. The upregulation of AMPK exerted by PEG35 has a double side effect: it promotes NO formation, which helps vasodilation, and prevents the energetic breakdown of the mitochondria. In this sense, upregulation of UCP also prevents this depletion of ATP. Furthermore, succinate has been reported as a subproduct of the anaerobic metabolism, making it is a reliable marker of the status of the mitochondria.

6. HOPE-COR-NMP and PEG35

Considering all the data presented above, there is a strong evidence that PEG35 inclusion in a perfusate for HOPE (such as IGL-2 [24]) could be a promising tool to be applied to other modalities of graft preservation, such as HOPE-COR-NMP, which was recently used in clinical settings [84].

PEG35 is a suitable candidate to increase preservation quality of liver grafts subjected to any modality of dynamic preservation, such as HOPE or combined methodologies such as HOPE-COR-NMP, due to three main points: 1) PEGs are stable, nontoxic, non-immunogenic and water-soluble molecules, as recognized by FDA [11]; 2) PEG35 confers oncotic and cytoprotective properties to be used for liver transplantation as defined by ELTR [9]; and 3) PEG35 is protective against IRI in experimental hypothermic, normothermic and hyperthermic conditions [26,27,85,86].

Given the promising results of PEG-based solutions in HOPE, it seems justified to explore its use in promising combination techniques such as HOPE-COR-NM, keeping in mind the physical characteristics of non-Newtonian fluids at different temperatures that make that the viscosity increases with the decrease in temperature. Therefore, as the viscosity of IGL-2 at 4 °C seems to be suitable for HOPE, increasing the temperature to subthermic and normothermic conditions will only lower the viscosity. Such levels of viscosity would not be a problem for normothermic MP. We therefore believe

that it is of great interest to assess PEG potential benefits in the mitochondria status, the GCX and the mechanotransduction processes inherent to graft preservation in any dynamic condition [6,23,24].

7. Concluding Remarks

This newly proposed perfusion strategy might have an especially relevant impact in those points where dynamic perfusion and PEG35 act in synergy in critical cell survival and graft integrity points, such as the mitochondria and the GCX. In this sense, any improved aspects during the ischemic time will positively affect the reperfusion phase, with an emphasize on rescuing marginal grafts, such as fatty livers for liver transplantation.

Along these lines, we report a new window of MP strategy improvements focused on the exploration of new perfusates with adequate physical characteristics that could confer both cytoprotection and mitochondrial restoring [75]. One tool to do so is to incorporate agents like PEG35 into HOPE perfusates; this is in contrast to HES, which is present in Belzer MPS solutions and which can cause a potential aggregation of resting red blood cells and endothelial damage due its high viscosity that increases shear stress. The use of PEG35 in perfusates could be a rational and potential option to be evaluated in promising strategies such as HOPE-COR-NMP [84] for rescuing liver grafts for transplantation.

More studies are needed to deepen our comprehension of the dynamic systems as compared to the static ones, and specifically their relationship with mitochondrial preservation, as well as the improvement of perfusates at both the molecular and physical levels. This is likely to represent one of the milestones of organ preservation in the near future, where the challenge is to take advantage of marginal organs, to increase donor pool for transplantation.

Abbreviations

4H NE	4-Hydroxynonenal
ALDH2	Aldehyde dehydrogenase-2
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
AST	Aspartate aminotransferase
Belzer-MPS	Belzer Machine Perfusion Solution
DAMPs	Damage associated molecular patterns
DCD	Donor after cardiac death
EC	Endothelial cells
ECD	Extended criteria donors
eNOS	Endothelial nitric oxide synthase
GCX	Glycocalyx
GLDH	Glutamate dehydrogenase
PEG	Polyethylene glycol
HES	Hydroxyethyl starch
HIF	Hypoxia inducible factor
HOPE	Hypothermic oxygenated perfusion
HTK	Histidine-tryptophan-ketoglutarate

i.v.	Intravenous
IGL-1	Institut Georges Lopez 1
IRI	Ischemia–reperfusion injury
MP	Machine perfusion
MPTP	Mitochondrial permeability transition pore
NO	Nitric oxide
ROS	Radical oxygen species
SCS	Static cold storage
SS	Shear stress
TX	Liver transplantation
UCP1	Uncoupling protein 1
UCP2	Uncoupling protein 2
UW	University of Wisconsin

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IGL-2 as unique solution for cold static preservation and machine perfusion in liver and mitochondrial protection.

Hypothermic static cold storage and machine perfusion strategies remain as the clinical standard for liver graft preservation. In the last few years, the protection of the mitochondrial function and subsequent energetic levels have emerged as key points for organ preservation. Nevertheless, the complex interactions between hepatic mitochondrial protection and its relationship with the preservation solutions/perfusates, has been poorly investigated.

In this study we highlight the use of IGL-2 as a distinctive solution for hepatic and mitochondrial protection. The use of IGL-2 solution for HOPE or SCS, instead of the standard solution, Belzer MP, introduces a new variety of perfusate that may possibly be used for livers grafts subjected to HOPE strategies, either alone or in combination with SCS strategies. Of note, besides protecting the mitochondrial integrity, the use of IGL-2 also avoids the mixture of different solutions/perfusates. As a result, it may reduce the operational logistics and the period prior transplantation, a critical factor when marginal organs, such as fatty liver grafts, are used for transplantation. In addition, this approach could also be extended to *ex vivo* liver splitting technique.

To sum up, we consider that the use of PEG35-containing solution, like IGL-2, would favor the protection of mitochondrial functions in a combined use of SCS and HOPE strategies. Moreover, it would also facilitate the logistics while avoiding the mixture of preservation solutions/perfusates for transplantation purposes. Nevertheless, more studies are needed to shed light in the mitochondrial protection induced by polyethylene glycols.

IGL-2 as a Unique Solution for Cold Static Preservation and Machine Perfusion in Liver and Mitochondrial Protection

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ABSTRACT

Hypothermic static cold storage and machine perfusion strategies remain the clinical standard of care for liver graft preservation. Recently, the protection of the mitochondrial function and the energetic levels derived from it has emerged as one of the key points for organ preservation. However, the complex interactions between liver mitochondrial protection and its relation with the use of solutions/perfusates has been poorly investigated. The use of an alternative IGL-2 solution to Belzer MPS one for hypothermic oxygenated perfusion (HOPE), as well as in static cold storage, introduce a new kind of perfusate to be used for liver grafts subjected to HOPE strategies, either alone or in combination with hypothermic static preservation strategies. IGL-2 not only protected mitochondrial integrity, but also avoided the mixture of different solutions/perfusates reducing. Thus, the operational logistics and times prior to transplantation, a critical factor when suboptimal organs such as donation after circulatory death or steatotic ones, are used for transplantation. The future challenges in graft preservation will go through (1) the improvement of the mitochondrial status and its energetic status during the ischemia and (2) the development of strategies to reduce ischemic times at low temperatures, which should translate in a better transplantation outcome.

INTRODUCTION

TRADITIONALLY, liver graft preservation strategies have been based in static cold storage (SCS) [1,2], but more recently, a variety of dynamic perfusion techniques using machine perfusion (MP) strategies in normothermic and hypothermic conditions using the oxygenated perfusion techniques (hypothermic oxygenated perfusion [HOPE]) [2—9] resulted in a promising tool to rescue marginal livers, such as the ones presenting with steatosis for transplantation purposes [4,10,11].

Since the first investigations on hepatic perfusion carried out by Guarrera et al [3] in 2010, the interest has grown in the field of MP, both for normothermic hepatic perfusion [7,8] and for HOPE (with less operative complexity than normothermic perfusion) [3]. Recent advances have made it a very promising strategy to increase the donor pool in the face of the pressing shortage of organs for transplantation [4,10,11].

It is well known that the HOPE benefits are tied to the oxygenation of the perfusate, which is responsible of maintaining the integrity and function of mitochondrial machinery [12], and this applies either by using HOPE itself or in combination with SCS using a commercial preservation [13]. Recent investigations have shown that the emerging interest of mitochondrial protection during hypothermic graft preservation and its energetic status is growing [14]. In this sense, the induced HOPE protection mechanisms, defined recently by Schlegel et al [12], are associated with the sustaining of the mitochondrial state that contributes to: (1) the prevention of energy breakdown with the subsequent sustaining of intracellular ATP levels; (2) the prevention of damage-associated molecular pattern formation; and (3) the induction of underlying mechanisms related to mitochondrial repair and endothelial protection. However, the relevance of the interactions between mitochondrial graft protection and preservation solutions/effluents would need to be considered further.

In this context, the relevance of mitochondrial consequences of organ preservation techniques in organ transplantation should be specially considered in future organ hypothermic preservation strategies, especially when new solute/effluents could be a useful tool to increase mitochondrial protection for the liver graft [14].

The perfusion solutions normally used for HOPE are a modification of Belzer's solution (Table 1) used for the static preservation of the graft [2,3], which on one hand contains hydroxyethyl starch (HES) as an oncotic agent, and on the other hand, shows a higher decreased K+ concentration than its analogue University of Wisconsin, among other components (Table 1). It is well reported that HES presence may lead to hyperaggregability of the red blood cells during static hypothermic preservation [15]. HES could also interfere with further HOPE strategies using Belzer MPS, where the presence of HES is responsible for increasing the viscosity of the perfusate during hypothermic perfusion vs IGL-2 (Table 1). This is especially relevant for steatotic liver grafts in which the fluid disturbances due to dynamic of fluids in HOPE [4] may destroy the luminal sugar thin layer covering liver endothelia, also known as glycocalyx [16,17]. However, the lower

Table 1. Composition of IGL-2 and Belzer MPS solutions

	Belzer-MPS	IGL-2
Electrolytes (mmol/L)		
K+	25	25
Na+	120	125
Mg2+	5	5
SO4 2-	5	5
Ca+		0.5
Zn2+		0.091
Buffers (mmol/L)		
Phosphate	25	25
HEPES		10
Histidine		30
Impermeants (mmol/L)		
Mannitol	30	60
Lactobionic acid		100
Dextrose	10	
Ribose	5	
Gluconate	85	
Colloids (g/L)		
Hydroxyethyl starch		50
Polyethylene glycol—35	5	
Antioxidants (mmol/L)		
Glutathione	3	9
Metabolic precursors (mmol/L)		
Adenosine		5
Adenine	5	
NaNO2 (nmol/L)		50
pH	7.4	7.4
Osmolarity (mosmol/L)	320	360
Viscosity (cP)	2.6	1.7

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

concentration of K+ in Belzer perfusate seems to be not relevant to affect to vascular resistance in hypothermic conditions given that it is well known that perfusates with physiologically low content prevented the vascular resistance increases when livers are subjected to cold perfusion [3,18].

Recently, we have proposed the use of IGL-2 solution as a good alternative to Belzer MPS for HOPE strategies alone or combined with cold static preservation [19,20] (Table 1). The substitution of HES by polyethylene glycol 35 (PEG35); as well as the presence of glutathione (among other components) constitute the main difference between IGL-2 and Belzer MPS; glutathione content in IGL-2 (9 mM) is responsible for a higher antioxidant capacity compared with Belzer MPS (3 mM glutathione), which is translated as an enhanced protection against radical oxygen species formation and their potential damage against mitochondria in hypothermic static preservation followed by HOPE strategies [19—20], where the transient oxygenation sustains the liver mitochondrial machinery at basal levels. We compared Sprague Dawley rats' liver grafts subjected 1h HOPE after 7 hours SCS in both solutions (Belzer MPS and IGL-2)[20]. No significant differences in transaminases (alanine transaminase/aspartate transaminase) were found. However, significant lower levels of glutamate dehydrogenase (as a mitochondrial damage marker), were found in the IGL-2 rats' group vs Belzer MP, which were concomitant with
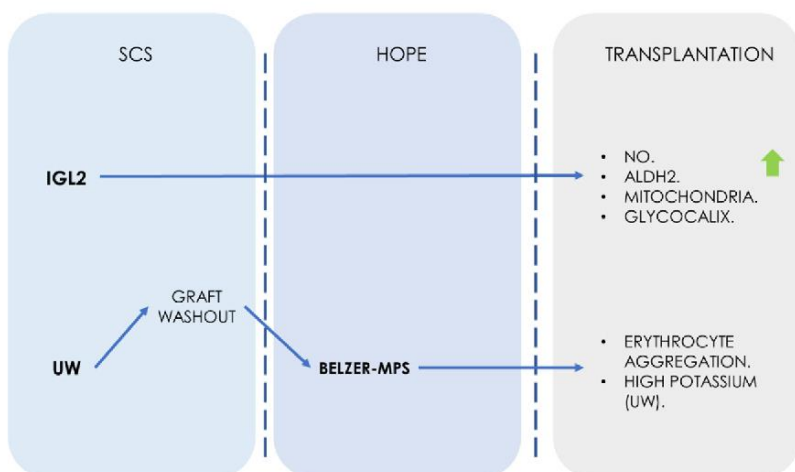
Scheme 1. IGL 2 Mechanisms of liver graft protection suggested for HOPE and hypothermic SCS preservation. The use of IGL-2 facilitates the logistics of using different solutions/perfusates besides favouring mitochondrial function, NO generation (vasodilation agent) and diminishing the disturbances associated with low viscosity that affect to endothelial glycocalyx.

higher levels of the mitochondrial enzyme aldehyde dehydrogenase 2 (ALDH2). These results are in agreement with the results of Schlegel et al [12], which confirm that the quality and protection of the mitochondria will greatly determine the capability of the graft to recover from ischemia-reperfusion injury insult, although further post-transplant studies are needed.

In addition, it is well known that in glutathione-based solutions, such as Belzer MPS and IGL-2, the presumed vs actual oxidation of glutathione over time is a key point that needs to be carefully overseen [21]. This is especially relevant when the hypothermic storage conditions of the preservation solution are not maintained properly and according to manufacturer instructions. To avoid the oxidation of the glutathione, additional factors such as high-quality package or the maintenance of the cold chain during transport are of utmost importance. However, these points are only valid if the initial quantity of glutathione is the optimal one, which is a differential point between original solutions and white brands. [22].

The use of IGL-2 in hypothermic preservation strategies also prevents the generation of aldehydes such as 4-hydroxynonenal through the activation of ALDH2 and its related protective mechanisms [13,19,20], contributing to HOPE benefits when PEG35 is used (Scheme 1). Scheme 1 summarizes the potential protective mechanisms of PEG35 solutions/perfusate in hypothermic static preservation [13] and HOPE [19,20] for liver transplantation purposes.

Scheme 1. IGL-2 mechanisms of liver graft protection suggested for HOPE and hypothermic SCS preservation. The use of IGL-2 facilitates the logistics of using different solutions/perfusates besides favoring mitochondrial function, nitric oxide generation (vasodilation agent), and diminishing the disturbances associated with low viscosity that affect to endothelial glycocalyx.

In accordance with the relevant investigations of Schlegel et al [12] and Horváth et al [14], we reported for the first time the benefits of using a novel IGL-2 solution for a combined use of SCS and HOPE strategies to rescue marginal livers, facilitating the logistics and avoiding the mixture of preservation solutions/perfusates for transplantation purposes. With this in mind, the use of a unique solution, such as IGL-2, for static and HOPE preservation strategies, could also be a useful tool in combination with "ex vivo" liver splitting and HOPE strategies, as recently reported by Mabrut et al [23].

In conclusion, the actual strategies used in liver graft hypothermic preservation suggest that the use of and unique preservation solution for the protection of mitochondrial functions should be considered as a priority in the actual studies of liver preservation solutions [24]. Future investigations on the mitochondrial protection induced by polyethylene glycols need to be explored in depth.

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Letter to the Editor

The Use of a Single, Novel Preservation Solution in Split Liver Transplantation and Hypothermic Oxygenated Machine Perfusion

In this letter we have drawn some considerations regarding the importance of use a single solution in split liver transplantation and HOPE. Split liver transplantation is a technique used to increase organ availability, which is also connected to prolonged cold ischemia and higher risks of postreperfusion bleeding and IRI. Consequently, partial liver grafts have been considered as marginal grafts, which also occurs in the case of fatty liver grafts.

In here, we comment the important work done by Mabrut *et al.,* which involves a surgical design that allows the combination of SCS and HOPE in the setting of split liver transplantation. We consider this report extremely relevant for the field, not only by reducing the cold ischemic period but also by diminishing the warm ischemic injury inherent to any surgical intervention. Additionally, this approach can also be extended to other types of marginal organs, including the fatty liver grafts, to improve their preservation.

Based on the study 1, 2 and now on study 3, we consider that there is still room to further reduce the injury associated with cold ischemia observed by Mabrut and colleagues. The use of the new IGL-2 solution showed decreased cold ischemic injury during SCS and HOPE preservation on fatty and non-fatty liver grafts. Moreover, the use of a unique solution when SCS and HOPE are combined may provide further benefit by simplifying the preservation period. Moreover, the use of PEG35-containing solution, such as IGL-2, may reduce the proaggregation effects observed in HES-containing solution, and support the mechanotransduction mechanisms inherent to HOPE, leading to glycocalyx protection and maintenance of its integrity.

Of note, our study also suggest that PEG35-containing solutions increase mitochondrial protection and promote protective autophagy during hypothermic preservation, culminating in the reduction of graft injury.

In conclusion, we strongly believe that the use of a unique PEG35-containing solution for split graft flushing, preservation and perfusion by HOPE should be considered for decreasing the detrimental effects of IRI. This approach would maximize the benefits of *ex vivo* splitting and the HOPE strategies reported by Mabrut and co-workers for split liver transplantation.

The Use of a Single, Novel Preservation Solution in Split Liver Transplantation and Hypothermic Oxygenated Machine Perfusion

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Split liver transplantation was introduced to increase organ availability, but its inherent prolonged cold ischemia raises the risks of ischemia/reperfusion injury (IRI). For this reason, the split partial liver grafts are considered to be marginal grafts, which also occur for fatty liver grafts. These organs are highly vulnerable to IRI, which can lead to different types of organ failure (such as small-for-size syndrome) after transplantation. Under these conditions, hypothermic oxygenated perfusion (HOPE) techniques constitute a promising strategy to minimize the deleterious effects of IRI, as shown by Muller et al[1] in the context of controlled donation after circulatory death. Besides, all trials using split liver grafts are excluded when applying HOPE. Thus, the communication by Mabrut et al[2] is highly relevant for the field.

Specifically, Mabrut et al[2] proposed a surgical design that allows cold static preservation to be combined with HOPE in the setting of split liver preservation. This approach not only reduces the time of cold ischemia (and therefore injury sustained due to it) but also reduces the warm ischemic injury inherent to surgical manipulation that occurs in normothermic conditions when conventional split techniques are used for liver transplantation. In our opinion, it is noteworthy that the HOPE application has been extended to different pools of organs, improving their preservation.

Here, we would like to highlight that there is still a window to further reduce cold ischemic injuries associated with surgical procedures, such as in split liver strategies. Based on our previous experimental works in rats, we have tested the benefits of a new preservation solution, for decreasing cold ischemic injuries during static and HOPE preservation using steatotic grafts and normal grafts.[3,4] In addition, the use of a unique solution for both static preservation and HOPE—and especially when they are combined—would provide further benefit by facilitating the whole preservation period. In our group experience, the use of this solution/perfusate for cold static and dynamic preservation, containing polyethylene glycol 35 rather than hydroxyethyl starch and an increased concentration of glutathione, improves preservation in both strategies (eg, cold static and dynamic preservation).[3,4] In addition, its use may prevent the proaggregating effects of hydroxyethyl starch and favor the mechanotransduction mechanisms inherent to HOPE, thereby protecting the glycocalyx and contributing to maintaining its integrity.[5] Moreover, this new preservation solution has a lower viscosity as compared with University of Wisconsin or Belzer-MPS solutions, which facilitates rinsing the organ and HOPE procedures.[3,5] This lower viscosity also helps to improve the glycocalyx preservation, which in turn promotes the microcirculation of the graft, as well as the generation of nitric oxide; notably, nitric oxide acts as a vasodilator, which is beneficial in steatotic grafts.[3] All of these effects would be consistent with the relevance of solution/perfusate associated with the modulation of inflammation events existing in liver graft preservation procedures,[3,4] which influence the graft outcome after transplantation. In addition, we would like to note that, presumably, the polyethylene-glycol-35–promoted mitochondrial protection and the induction of protective autophagy during

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hypothermic preservation are linked to the reduction of graft injury.[3-5]

In sum, we believe that the use of a single solution for split graft flushing, preservation, and perfusion by HOPE—such as Institut Georges Lopez 2—should be considered for reducing the deleterious effects of IRI and thereby maximizing the benefits of ex vivo liver splitting and the HOPE strategies reported by Mabrut et al[2] for split liver transplantation purposes.

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PEG35 as a preconditioning agent against hypoxia/reoxygenation injury

When compared to mitochondrial aerobic oxidation, glycolysis is an ineffective pathway of energy production. However, it plays a crucial role in sustaining basic life activities under ischemia/hypoxia conditions. In this way, the discovery of novel approaches to reduce the deleterious effects following ischemia/reperfusion is of utmost importance.

PEGs have caught attention by their widespread applications. PEGs are neutral, water-soluble nontoxic polymers with different properties and molecular weights. They have shown multiple benefits in graft preservation, such as edema prevention, antioxidant capacity and membrane stabilization. Several studies have reported PEG35 is associated with liver graft protection against cold ischemia insult. On study 1, we reported that the beneficial effects exerted by PEG35 on cold storage were mediated, at least in part, by the increased activity of the mitochondrial enzyme, ALDH2.

Besides its role in SCS and HOPE, PEG35 has also been reported to have a protective role when used as a preconditioning agent. Recently, our research group reported that PEG35 preconditioning ameliorates pancreatic inflammatory response in cerulein-induced acute pancreatitis both in *in vivo* and *in vitro* models. PEG35 was able to attenuate inflammation response and associated cell death in a dose dependent manner. High molecular weight PEGs (15-20 kDa) have been reported to protect cardiomyocytes from hypoxia/reoxygenation-induced cell death. The authors found that PEG pretreatment significantly decreased apoptosis associated to cyt *c* release as well as decreased intracellular ROS production. In the liver, PEG35 have also been shown to be a key player in warm IRI, displaying powerful hepatic protection. Nevertheless, the mechanisms underlying such protection have not been fully elucidated.

In this study a hypoxia reoxygenation model using HepG2 cells was established to evaluate the effects of PEG35 preconditioning. PEG35 preconditioning was able to preserve mitochondrial function by decreasing ROS excessive production, ATP depletion and recovering the mitochondrial membrane potential. Additionally, PEG35 increased levels of autophagy-related proteins and expression of genes involved in mitochondrial dynamics. In conclusion, PEG35 preconditioning effectively ameliorates hepatic hypoxia/reoxygenation injury through the enhancement of autophagy and mitochondrial quality control. Therefore, PEG35 could be useful as a potential pharmacological maneuver for attenuating hepatic IRI in clinical practice.

Article

PEG35 as a Preconditioning Agent against Hypoxia/Reoxygenation Injury

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Abstract: Pharmacological conditioning is a protective strategy against ischemia/reperfusion injury, which occurs during liver resection and transplantation. Polyethylene glycols have shown multiple benefits in cell and organ preservation, including antioxidant capacity, edema prevention and membrane stabilization. Recently, polyethylene glycol 35 kDa (PEG35) preconditioning resulted in decreased hepatic injury and protected the mitochondria in a rat model of cold ischemia. Thus, the study aimed to decipher the mechanisms underlying PEG35 preconditioning-induced protection against ischemia/reperfusion injury. A hypoxia/reoxygenation model using HepG2 cells was established to evaluate the effects of PEG35 preconditioning. Several parameters were assessed, including cell viability, mitochondrial membrane potential, ROS production, ATP levels, protein content and gene expression to investigate autophagy, mitochondrial biogenesis and dynamics. PEG35 preconditioning preserved the mitochondrial function by decreasing the excessive production of ROS and subsequent ATP depletion, as well as by recovering the membrane potential. Furthermore, PEG35 increased levels of autophagy-related proteins and the expression of genes involved in mitochondrial biogenesis and fusion. In conclusion, PEG35 preconditioning effectively ameliorates hepatic hypoxia/reoxygenation injury through the enhancement of autophagy and mitochondrial quality control. Therefore, PEG35 could be useful as a potential pharmacological tool for attenuating hepatic ischemia/reperfusion injury in clinical practice.

Keywords: polyethylene glycol 35; hypoxia/reoxygenation injury; mitochondria; autophagy

1. Introduction

Ischemia/reperfusion injury is a major hurdle in many clinical scenarios, including liver transplantation, hepatic resection and trauma settings [1,2]. Hepatic ischemia/reperfusion injury contributes to an increased rate of acute liver failure, graft rejection and chronic hepatic dysfunction, affecting liver surgery outcomes and patient rehabilitation [3,4]. The mechanisms of organ damage following ischemia/reperfusion have been widely studied, and involve the complex interactions of multiple pathways. Unfortunately, despite intensive research, effective therapeutic approaches for the prevention/treatment of ischemia/reperfusion injury are still clinically limited.

Mitochondria are crucial players in every single living eukaryote. In addition to their well-established role in energetic metabolism and ATP generation, mitochondria participate in many other physiological functions, including catabolic and anabolic processes, diverse signaling pathways, calcium homeostasis and cell death mechanisms [5]. Therefore, disruptions to these processes affect normal mitochondrial function, and disruptions have

been implicated in mitochondrial dysfunction. It is well established that mitochondria play a central role in ischemia/reperfusion injury [6]. The lack of oxygen observed during the ischemic period leads to a decrease in ATP levels, while the reestablishment of blood supply gives rise to ROS production, the activation of immune cells to promote inflammation and consequent cell death [5,7].

In physiological conditions, the maintenance of a healthy mitochondrial network is a determinant factor for cellular homeostasis and cell survival. After an ischemic event, if mitochondria remain functional enough to generate ATP, the tissue can recover and overcome the injury. Conversely, when the ischemic period is more aggressive, the subsequent restoration of blood flow and, significantly, oxygen compromises mitochondrial function, leading to an exacerbation of ROS generation. Thus, the clearance of dysfunctional mitochondria through selective degradation and their replacement by new ones via mitochondrial biogenesis, alongside changes in mitochondrial dynamics, have been suggested to be an effective quality control system to counteract hepatic ischemia/reperfusion injury and maintain mitochondrial function [8].

In the past few years, a sizeable body of literature has suggested that different drugs could play a beneficial role in avoiding ischemia/reperfusion-associated adverse effects and organ failure. Polyethylene glycols are non-toxic, neutral, water-soluble compounds approved by the Food and Drug Administration for their use in cosmetics, foods and drugs [9]. Their beneficial effects have been reported in different organs, including the liver, heart, kidney, intestine and pancreas [10–14]. In the liver, several studies have demonstrated the protective role that different molecular weight polyethylene glycols play during cold preservation. Polyethylene glycol 35 kDa (PEG35) has been associated with higher levels of mitochondrial aldehyde dehydrogenase 2 (ALDH2) and improved mitochondrial machinery and the subsequent diminishing of cold ischemic injury [15,16]. Furthermore, intravenous PEG35 pretreatment improved liver graft preservation and protected the mitochondria when cold ischemia was followed by warm reperfusion [17]. Besides their effects in cold storage, PEG35 has also shown hepatoprotection against warm ischemia/reperfusion injury [18]. PEG35 preconditioning efficiently reduced transaminases levels and maintained hepatocyte morphology, and also preserved mitochondrial membrane potential.

Based on these protective features, the aim of the present study was to assess the ability of PEG35 preconditioning to protect human hepatocytes submitted to hypoxia/reoxygenation. We also explore the possible protective molecular mechanisms involved in PEG35-mediated hepatoprotection.

2. Results

2.1. PEG35 Preconditioning Increases Cell Viability

To examine the effect of PEG35 preconditioning against hypoxia/reoxygenation injury, cell viability was assessed through the reduction of a yellow tetrazolium salt to purple formazan crystals (using an MTT assay, as described in the Materials and Methods section) (Figure 1). Firstly, it was confirmed that the two different concentrations of PEG35 used throughout the study were not noxious to the cells (Figure 1a).

The HepG2 pretreated with 5% PEG35 for 1 h and then subjected to 2 h of hypoxia followed by 2 h of reoxygenation demonstrated significantly higher cell viability compared to cells that did not receive PEG preconditioning (Figure 1b). Significantly, the protective effect of PEG35 was shown to be dose dependent, since 1% PEG35 demonstrated almost no protection against hypoxia/reoxygenation, similar to the results of the hypoxia/reoxygenation group. Strikingly, the 5% PEG35 + H/R group was able to protect HepG2 at similar levels to the control group.
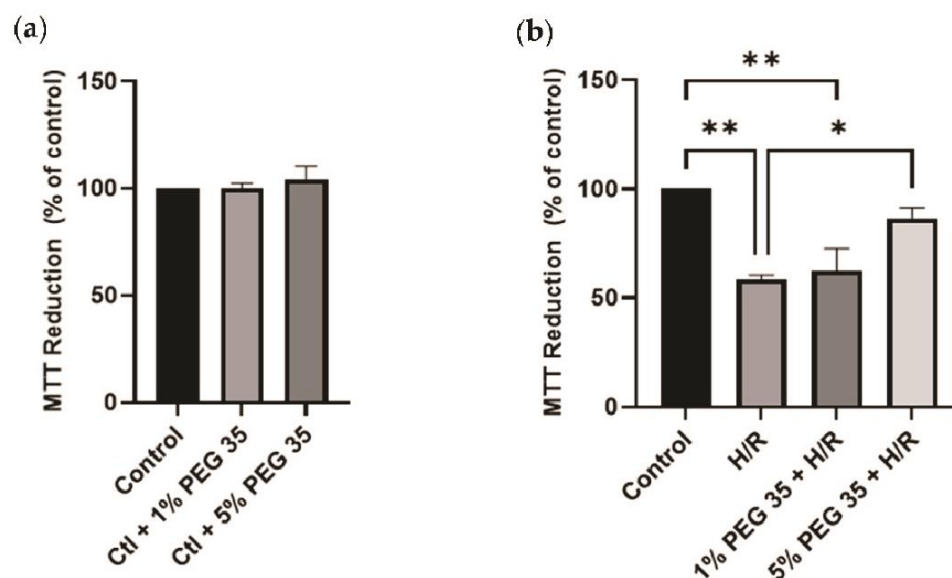
Figure 1. Effects of PEG35 on cell viability, in the absence (**a**) or presence (**b**) of hypoxia/reoxygenation (H/R). Cell viability rate determined via MTT assay. The values shown represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$; ** $p < 0.01$.

2.2. PEG35 Administration Increases Mitochondrial ALDH2 Content and Attenuates Oxidative Injury

PEG35 has been tightly linked to the mitochondrial enzyme ALDH2 when working against ischemia reperfusion injury in several studies [15,16]. Thus, we examined whether PEG35 treatment could increase ALDH2 levels. While in the control, hypoxia/reoxygenation and 1% PEG35 groups, the protein levels were similar, in the 5% PEG35 group there was a significant increase in ALDH2 content (Figure 2a). As ALDH2 is a key enzyme that functions against oxidative stress, we then investigate if its increased levels could reduce oxidative stress. As expected, compared to the control, hypoxia/reoxygenation significantly increased ROS production (Figure 2b). Moreover, in accordance with the increased ALDH2 content, the 5% PEG35 preconditioning significantly prevented the increase in ROS levels found in the hypoxia/reoxygenation group. In addition, we also evaluated the protein levels of one of the master regulators of antioxidant defense and a regulator of the expression of the mitochondrial antioxidant protein, nuclear factor-E2-related factor 2 (Nrf2) and manganese-dependent superoxide dismutase (MnSOD), respectively. The results revealed that hypoxia/reoxygenation treatment inhibited the expression of Nrf2, while the 5% PEG35 preconditioning seemed to reverse this tendency, but not in a significant way (Figure 2c). The expression of the MnSOD gene was decreased in the hypoxia/reoxygenation group, but it was recovered to control levels in the PEG35-treated groups (Figure 2d).
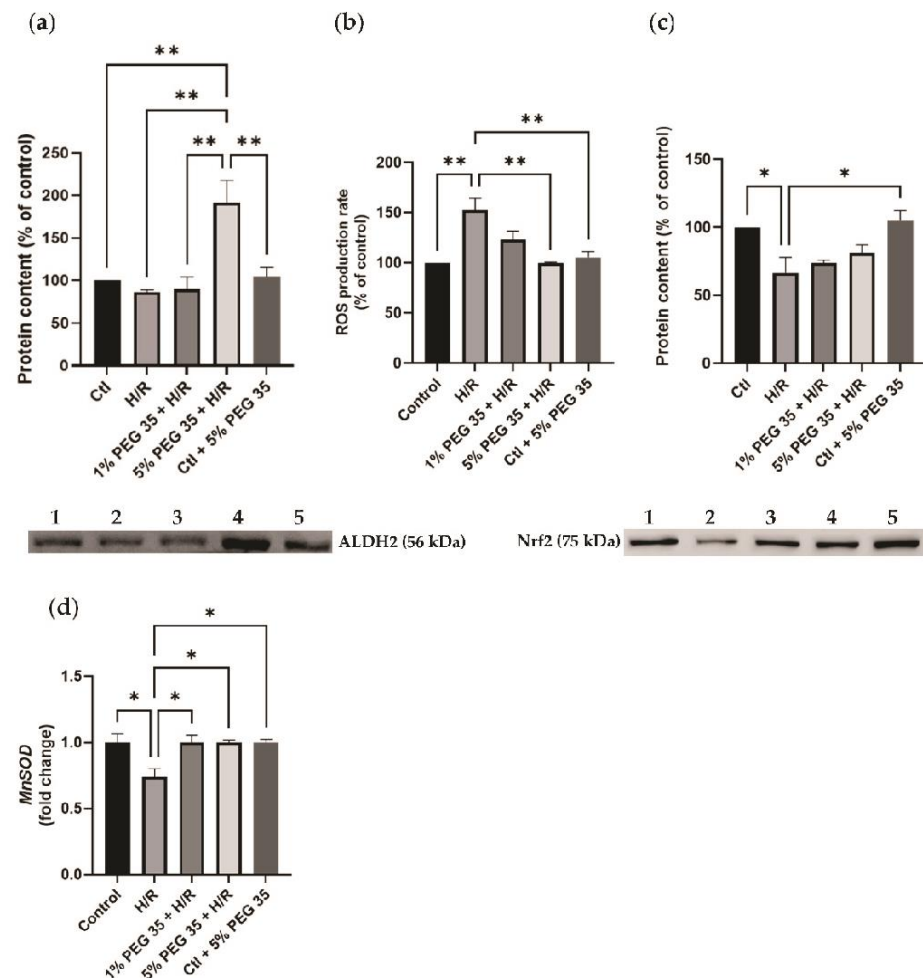
Figure 2. Effects of PEG 35 in mitochondrial ALDH2 levels and oxidative stress in HepG2 submitted to H/R. (**a**) Representative Western blot for ALDH2 and respective quantification for: 1-Ctl; 2-H/R; 3–1% PEG35 + H/R; 4–5% PEG35 + H/R; 5-Ctl + 5% PEG35. (**b**) ROS production rate measured fluorometrically as described in the Materials and Methods section. (**c**) Representative Western blot for Nrf2 and respective quantification for: 1-Ctl; 2-H/R; 3–1% PEG35 + H/R; 4–5% PEG35 + H/R; 5-Ctl + 5% PEG35. (**d**) Gene expression for MnSOD. The values shown represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$; ** $p < 0.01$.

2.3. PEG35 Alleviates Mitochondrial Damage Induced by Hypoxia/Reoxygenation Injury

The deleterious effects associated with hepatic ischemia/reperfusion injury are well known to cause mitochondrial dysfunction. To evaluate if PEG35 preconditioning-mediated hepatoprotection during hypoxia/reoxygenation injury is due to enhanced mitochondrial dysfunction, we assessed the mitochondrial membrane potential ($\Delta\Psi$). Figure 3a clearly shows a decrease in mitochondrial $\Delta\Psi$ in the cells submitted to hypoxia/reoxygenation, which was efficiently recovered in the presence of both PEG35 concentrations.

Mitochondria are the main source of ATP production, which is known to be compromised by ischemia/reperfusion injury. Thus, we next evaluated the ATP generation in all groups. As shown in Figure 3b, ATP content was decreased upon hypoxia/reoxygenation, but the administration of 5% PEG35 before hypoxia was able to significantly preserve mitochondrial ATP content.
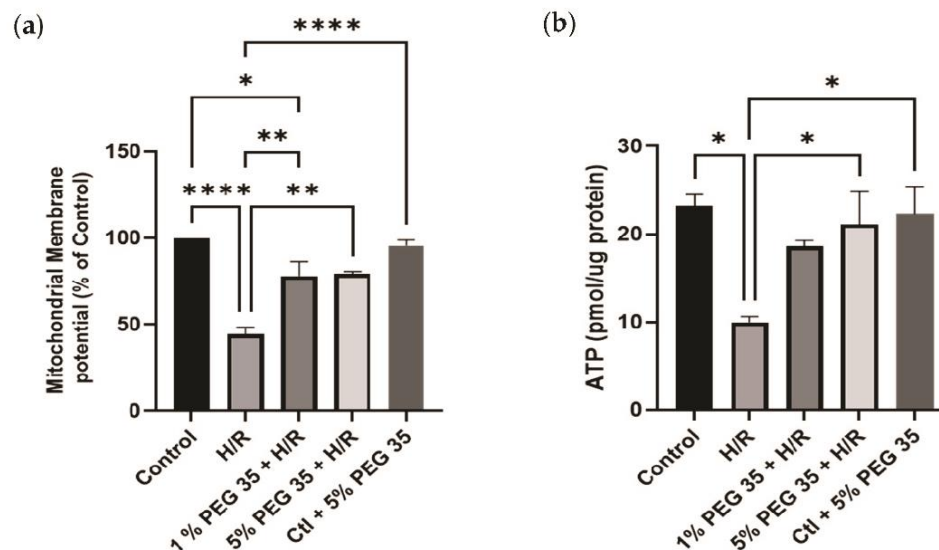
Figure 3. PEG35 effects on mitochondrial function. (**a**) Mitochondrial membrane potential ($\Delta\Psi$) was assessed using TMRM, as described in the Materials and Methods section. (**b**) ATP content in HepG2 cells. The values shown represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

2.4. PEG35 Attenuates Hypoxia/Reperfusion Injury through Enhanced Autophagy

Growing evidence has shown the cytoprotective role autophagy plays in maintaining mitochondrial function and cell survival following hepatic ischemia/reperfusion [19]. Therefore, we next investigated whether PEG35 preconditioning induces autophagy following hypoxia/reoxygenation injury. The levels of LC3-II, the active form of LC3, a protein involved in autophagosome formation, exhibited an increase in its content in the presence of 5% PEG35 when compared to the hypoxia/reoxygenation group (Figure 4a). In addition, the analysis of another autophagy marker, p62, showed that cells submitted to hypoxia/reoxygenation have a significant increase in p62 protein content, which was significantly prevented by PEG35 preconditioning (Figure 4b). The augmented LC3II/I ratio and decreased p62 content strongly suggest that, at least in part, PEG35 preconditioning may significantly alleviate hypoxia/reoxygenation injury via autophagy activation.
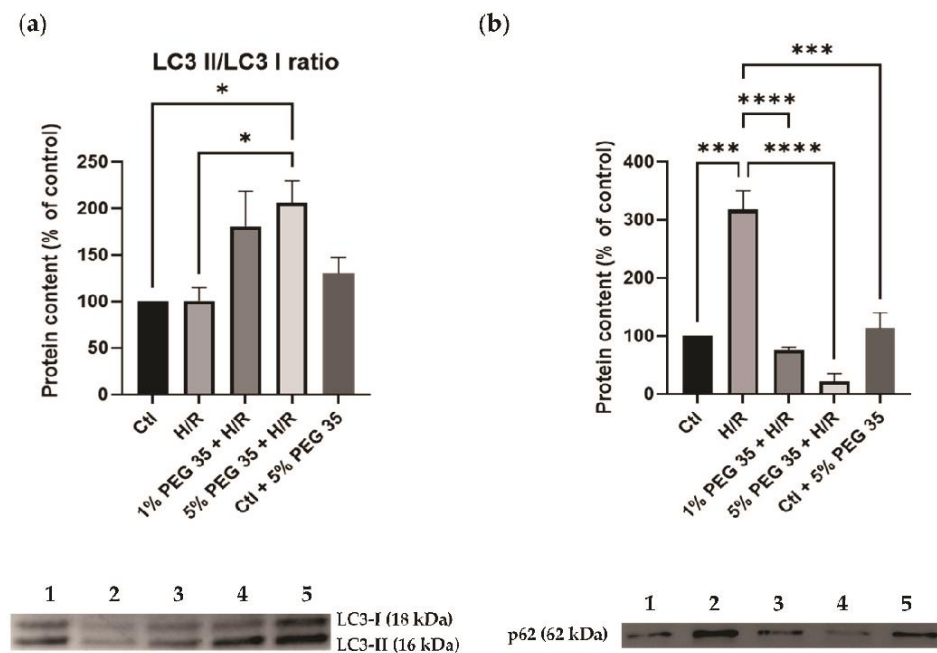
Figure 4. Autophagy-related protein markers in HepG2 cells. (**a**) Representative Western blot for LC3 I and LC3 II and respective LC3II/LC3I ratio quantification. (**b**) Representative Western blot for p62 and respective quantification for: 1-Ctl; 2-H/R; 3–1% PEG35 + H/R; 4–5% PEG35 + H/R; 5-Ctl + 5% PEG35. The values shown represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

2.5. PEG35 Restored Mitochondrial Biogenesis and Fusion–Fission Dynamics

Mitochondrial biogenesis is regulated by PGC-1α and the downstream nuclear respiratory factor 1 (NRF1) and transcription factor A (TFAM), controlling mitochondrial turnover, content and number to maintain the metabolic demands. Our study demonstrates a significantly increased expression of both genes coding for PGC-1α and NRF1 (*ppargc1a* and *nrf1*, respectively), upon 5% PEG35 preconditioning (Figure 5a). However, no differences were observed in the *tfam* expression.

Mitochondria are highly dynamic organelles regulated by fission and fusion events. As can be seen in Figure 5b, hypoxia/reoxygenation leads to the upregulation of genes coding for the mitochondrial fission 1 protein (Fis1) and dynamin-related protein 1 (Drp1) (*fis1* and *dnm1l*, respectively), both mitochondrial fission-related genes. Both PEG35 concentrations were able to attenuate this increased gene expression to control levels. In addition, 5% PEG35 preconditioning significantly upregulated the expression of the gene coding for optic atrophy 1 protein (*opa1*), a key mediator in mitochondrial fusion.

These results suggest that PEG35 preconditioning may protect against hypoxia/reoxygenation-induced mitochondrial dysfunction through the regulation of PGC-1α-mediated mitochondrial biogenesis and the balance of fusion–fission dynamics, resulting in the alleviation of hepatic hypoxia/reoxygenation injury.
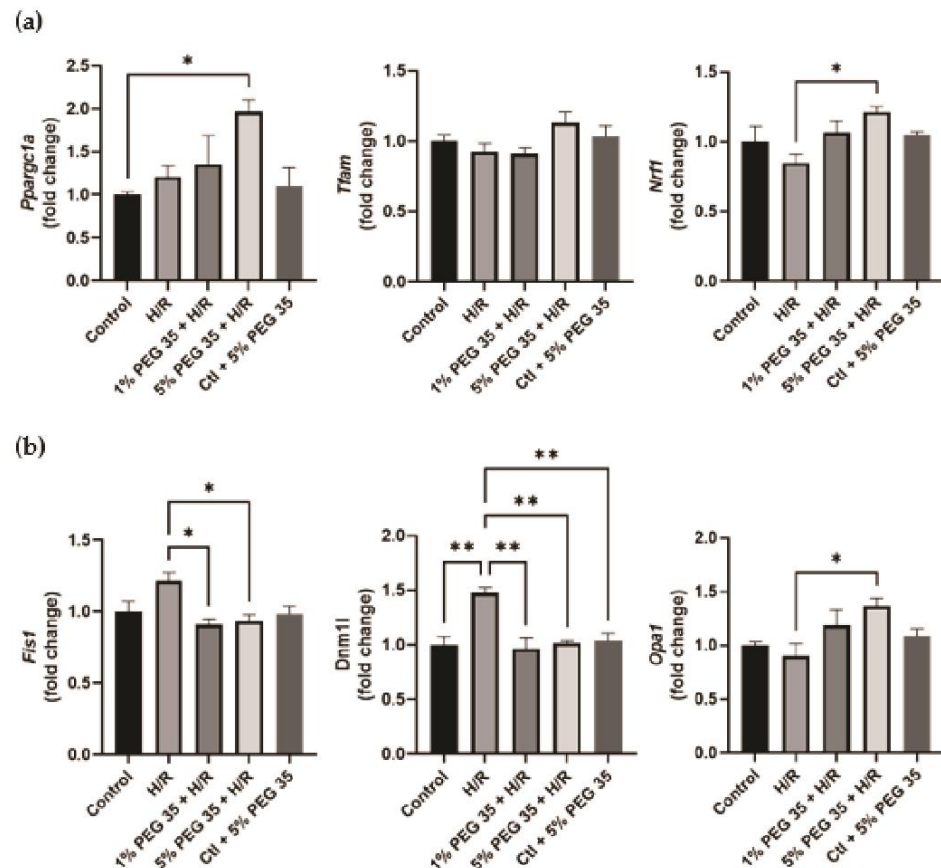
Figure 5. Effect of PEG35 preconditioning on mitochondrial biogenesis and fusion–fission dynamics during HRI. (**a**) Gene expression of proteins involved in mitochondrial biogenesis (*ppargc1a*, *tfam* and *nrf1*). (**b**) Gene expression of mitodynamics-involved proteins, dynamin-related protein 1 (dnm1l), fission protein 1 (*fis1*) and optic atrophy 1 (*opa1*). The values shown represent the mean ± SEM of 3 independent experiments. * $p < 0.05$; ** $p < 0.01$.

3. Discussion

Hepatic ischemia/reperfusion injury is the primary cause of liver damage after liver transplantation or hepatectomy. It is characterized by oxidative stress, inflammation and impaired autophagy and mitochondrial function, which leads to hepatocellular damage, contributing to organ failure. During the ischemic period, the deprivation of oxygen leads to the cessation of oxidative phosphorylation, causing ATP depletion, while in the reperfusion period, a burst of mitochondrial ROS production is considered to play a critical role in the organ damage associated with ischemia/reperfusion injury [5].

Several therapeutic strategies have been implemented to counteract the deleterious effects of ischemia/reperfusion injury, including those involving pharmacological conditioning. Polyethylene glycols are linear polymers of ethylene oxide with hydroxyl terminal groups that have been shown to provide beneficial effects by regulating cell survival pathways in different organs [20]. Nevertheless, the exact mechanism of the protective effects of PEG35 on ischemia/reperfusion-induced cellular injury is unclear.

Both in vitro [21] and in vivo [14] studies have demonstrated that PEG preconditioning can protect myocytes from ischemia/reperfusion injury-induced cell death. PEG35 administration has also been described as a protective strategy in other organs, such as the pancreas in an inflammation model [10]. To the best of our knowledge, this is the first study evaluating the effects of PEG35 preconditioning against hypoxia/reoxygenation-induced injury in HepG2 cells. In accordance with the above studies reporting that PEG induces

protection from ischemia/reperfusion injury-related cell death, we found that human hepatocytes pretreated with 5% PEG35 and then submitted to hypoxia/reoxygenation presented a higher cell viability compared to cells without PEG35 preconditioning (Figure 1b).

PEG35 has been associated with higher levels of the mitochondrial enzyme ALDH2 and enhanced mitochondrial machinery, culminating in decreased ischemic injury [15,16]. ALDH2 is located in the mitochondrial matrix and is abundantly expressed in numerous organs, including the liver, heart, brain, intestine and kidneys [22,23]. ALDH2 is mainly known for its detoxifying properties, conferring a protective shield against toxic agents, including acetaldehyde (alcohol metabolism), lipid peroxidation-originated products and ROS [15]. In our experimental model, we detected an increased ALDH2 content in the 5% PEG35 group, while in the other groups, the protein levels were similar (Figure 2a).

A pathological increase in ROS production is a hallmark of hepatic ischemia/reperfusion injury, driving hepatocyte death. As expected, the hypoxia/reoxygenation group showed an increase in ROS production, which was significantly reverted in the presence of 5% PEG35 (Figure 2b). This result is in accordance with the increased levels of ALDH2, and its ability to decrease oxidative stress, observed under 5% PEG preconditioning. We also assessed the protein content of Nrf2, which plays an important role in the cellular antioxidant response against multiple stress injury factors. The effective activation of Nrf2 is well known to lead to better outcomes following hypoxia/reoxygenation injury [24]. In the present study, we observed that hypoxia/reoxygenation diminishes the levels of Nrf2 but, despite a tendency to increase Nrf2 content, 5% PEG35 was not able to significantly reverse the hypoxia/reoxygenation effects (Figure 2c). In addition, the gene expression of MnSOD, an essential mitochondrial antioxidant enzyme, was shown to be downregulated upon hypoxia/reoxygenation and upregulated in both PEG35 groups (Figure 2d). PEG35 decreased the generation of ROS during hypoxia/reoxygenation and upregulated the expression of ALDH2 and MnSOD, indicating that PEG35 protected hepatocytes from hypoxia/reoxygenation injury via the upregulation of antioxidant enzymes.

Ischemia/reperfusion is well known to alter the energy metabolism due to the impairment of mitochondrial function [25,26]. The observed excessive ROS production during hypoxia/reoxygenation may lead to the attack of cellular membranes and subcellular organelles, leading to the decrease in mitochondrial membrane potential and consequent mitochondrial dysfunction. In this study we demonstrated that PEG35 preconditioning protected against the loss of mitochondrial function in human hepatocytes during hypoxia/reoxygenation (Figure 3a). Furthermore, the ATP content in HepG2 cells submitted to hypoxia/reoxygenation was found to be decreased (Figure 3b), which is in agreement with the lower mitochondrial membrane potential observed in the same group, suggesting the impairment of mitochondrial function. As expected by the observed recovering of mitochondrial $\Delta\Psi$, PEG35 preconditioning efficiently preserved the ATP content following hypoxia/reoxygenation. The results reported here show that PEG35 preconditioning may improve ischemia/reperfusion injury by preserving mitochondrial function and decreasing excessive ROS production and ATP depletion, as well as recovering the membrane potential.

Recently, Bardallo et al. [27] reported better liver protection against ischemic insult through the reduction of oxidative stress via ALDH2 upregulation and the enhancement of cytoprotective autophagy in a PEG35-dependent manner. Autophagy is a crucial process in the clearance of dysfunctional organelles, and it has been generally recognized as a protective process in response to various intra- and extracellular stimuli, including ischemia/reperfusion injury [28]. Enhancing autophagy ameliorates hepatic function by eliminating the dysfunctional mitochondria [4], while autophagy inhibition increases mitochondrial oxidative stress and triggers cell death during liver ischemia/reperfusion injury [29]. In accordance with this, Wang and colleagues reported that increasing autophagy alleviates hepatic injury and improves mitochondrial function against ischemia/reperfusion injury [30]. LC3 is a key player in the autophagic processes, which also includes mitophagy. During autophagic events, the soluble form (LC3-I) is converted into LC3-II, which is recruited to the autophagosome formation [31]. Furthermore, p62, also known as sequesto-

some 1, is another key protein involved in the autophagic process that targets specific cargoes for autophagy. Under normal conditions, basal autophagy clears p62 and its associated cargo. On the other hand, under conditions of decreased/deficient autophagy, p62 and its associated cargo accumulate in the cytoplasm [32]. In Figure 4a, we observed that PEG35 preconditioning increased the conversion of LC3-I into LC3-II. In agreement with this, HepG2 cells submitted to hypoxia/reoxygenation showed an increased accumulation of p62, while HepG2 cells exposed to PEG35 prior to hypoxia/reoxygenation presented a strong decrease in p62 content (Figure 4b). The increased levels of LC3 II and p62 degradation suggest an autophagy enhancement followed PEG35 preconditioning, which may contribute to the elimination of dysfunctional mitochondria during ischemia/reperfusion injury, leading to improved mitochondrial performance.

PGC-1α is a master regulator of mitochondrial biogenesis that enhances different transcription factors, such as NRF1 and TFAM, which control mitochondrial turnover, content and number to maintain the metabolic demands [4]. Previous studies reported enhanced mitochondrial functioning following hepatic ischemia/reperfusion injury through the stimulation of mitochondrial biogenesis via the induction of the PGC-1α/NRF1/TFAM pathway [4,33,34]. In accordance, in our study, 5% PEG preconditioning upregulated the expression of *ppargc1a* and *nrf1* (Figure 5a), indicating an enhancement of mitochondrial biogenesis.

Mitochondria are dynamic organelles, continuously dividing and elongating through frequent fusion and fission in response to cellular stress and consequent alterations in the intracellular environment [35]. Mitochondrial fusion and fission are two essential quality control mechanisms which favor the segregation and clearance of dysfunctional mitochondria to achieve homeostasis. Compelling evidence of the interplay between mitochondrial quality control and cell fate in different organs subject to ischemia/reperfusion has been gathered over the last few years [8]. Modifications to the regulators involved in fission or fusion processes, the loss of cristae integrity, alongside the inefficient removal of damaged mitochondria, have all been implicated to play a vital role in ischemia/reperfusion injury [36]. OPA1 is a crucial protein involved in mitochondrial fusion which may interact with various numbers of mitophagy receptor proteins in order to collaborate in mitochondrial dynamics and mitophagy [37,38]. OPA1 knockdown has been reported to exacerbate the deleterious effects provoked by ischemia/reperfusion [39,40]. Moreover, increased mitochondrial fusion and mitophagy through the AMPK-OPA1 signaling pathway was observed to protect against cardiac ischemia/reperfusion injury, whereas OPA1 knockout abolished the protective effects [41]. In here, we report an increased expression of *opa1* after 5% PEG35 preconditioning (Figure 5b). It has been demonstrated that the loss of mitochondrial membrane potential triggers OPA1 proteolysis and inhibits mitochondrial fusion [42,43]. As mentioned above, our results demonstrate that 5% PEG35 preconditioning increased mitochondrial membrane potential when compared to hypoxia/reoxygenation, which is in accordance with the effects of increased *opa1* upregulation.

Contrary to fusion, mitochondrial fission has been reported to be triggered by ischemia/reperfusion injury [44,45]. Mounting evidence confirms that the inhibition of mitochondrial fission might protect several tissues from ischemia/reperfusion injury [46]. Excessive mitochondrial fission has been related to mitochondrial fragmentation and the triggering of cell apoptosis [47]. Drp1 and Fis1 are essential proteins involved in the fission processes [36,48]. Bi et al. [49] have reported increased levels of Drp1 and Fis1 after hepatic ischemia/reperfusion.

Nevertheless, pharmacological postconditioning efficiently decreased the levels of these two mitochondrial fission-related proteins. Furthermore, the inhibition of Drp1 led to increased mitophagy and the consequent clearance of damaged mitochondria and the prevention of ROS production in cardiac ischemia/reperfusion injury [50]. In our study, cells submitted to hypoxia/reoxygenation significantly increased the expression levels of *dnm1l* and *fis1*, and this upregulation was attenuated by PEG35 preconditioning (Figure 5b). We understand that it is reasonable to assume that changes in mRNA expression will have

corresponding changes in protein levels. However, the correlation between proteins and their mRNA levels are sometimes poor [51,52].

Taken together, our findings suggest that PEG35 preconditioning regulates hypoxia/reoxygenation injury-induced imbalances in mitochondria dynamics by elevating mitochondrial fusion and diminishing mitochondrial fission.

In summary, in the present study we used an in vitro model to explore the possible protective molecular mechanisms of PEG35 preconditioning on diminishing hypoxia/reoxygenation injury. We observed that 5% PEG35 preconditioning presented a better outcome in most of the parameters analyzed when compared to 1% PEG35 preconditioning, showing that PEG35 protective effects are dose dependent. We demonstrated that 5% PEG35 preconditioning efficiently attenuates hepatic hypoxia/reoxygenation injury by alleviating mitochondrial dysfunction via the enhancement of autophagy and mitochondrial biogenesis and dynamics (Figure 6).
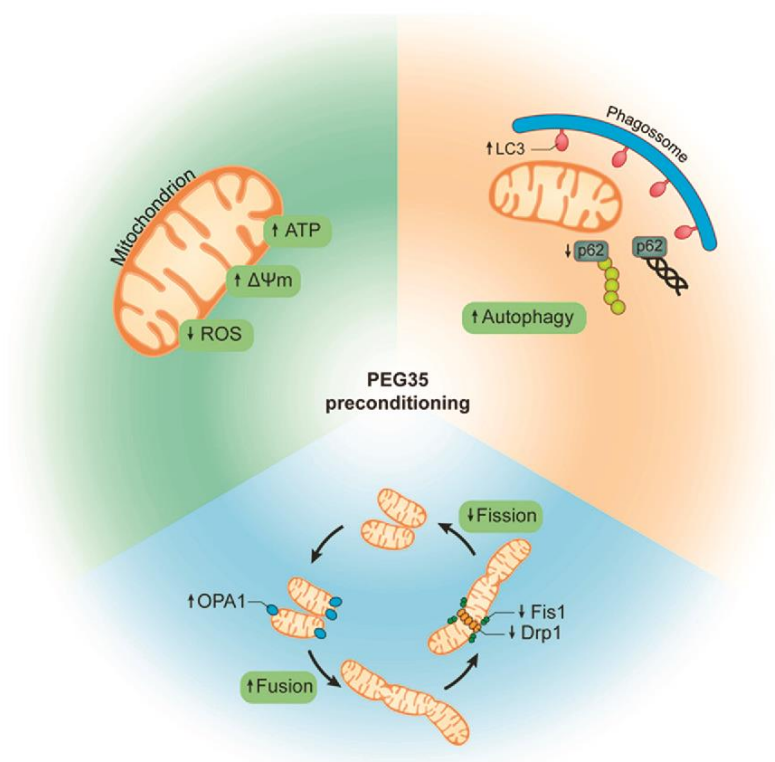
Figure 6. Schematic representation of PEG35-mediated protection following hypoxia/reoxygenation injury. PEG35 preconditioning alleviates mitochondrial dysfunction, including the recovery of mitochondrial membrane potential and ATP levels and the reduction of ROS production. In addition, PEG35 treatment suggests enhanced autophagy and a modulation of mitochondrial biogenesis via increased mitochondrial fusion and decreased mitochondrial fission following hypoxia/reoxygenation injury.

In conclusion, PEG35 preconditioning seems to be a viable strategy to confer hepatocellular protection against ischemia/reperfusion injury and, therefore, we suggest that PEG35 might be considered to be a suitable pharmacological preconditioning agent in liver surgery.

4. Materials and Methods

4.1. Cell Culture

HepG2 cells were cultured in 75 cm^2 flasks (Sarstedt, Nümbrecht, Germany) with 15 mL Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA),

supplemented with 1% antibiotic–antimycotic (penicillin/streptomycin/amphotericin B; Gibco, Waltham, MA, USA) and 10% fetal bovine serum (FBS; Invitrogen, Waltham, MA, USA) in a humidified 5% CO_2 atmosphere at 37 °C. When cells reached 70–90% confluence, they were detached with TrypLE Express (Gibco, Waltham, MA, USA) and subsequently counted using the trypan blue dye exclusion technique and plated in 12-well plates.

4.2. Cell Culture

HepG2 cells were placed in a hypoxia-mimetic solution (137 mM NaCl, 12 mM KCl, 0.9 mM CaCl2, 0.49 mM MgCl2, 4 mM HEPES, 20 mM lactate, 10 mM deoxyglucose, 0.75 mM sodium dithionite, pH 6.5) to induce hypoxia [53,54]. To attempt to simulate ischemic conditions, we used a hypoxia medium because it is a solution free of metabolic substrates. It was supplemented with lactate to simulate its accumulation due to anaerobic glycolysis, and deoxyglucose to inhibit glycolysis and further shut down cellular metabolism [55]. In addition, sodium dithionite is a powerful oxygen scavenger, and consequently leads to rapid oxygen depletion from the solution, as well as a rapid reversibility of its effects during the washout [56]. Following the hypoxia time, reoxygenation was induced via hypoxia medium exchange to complete the growth medium (DMEM).

For PEG35 preconditioning, cells were pretreated with PEG35 diluted in PBS for 1 h prior to the induction of hypoxia, at 2 different concentrations, 1% or 5% PEG35 (Figure 7).
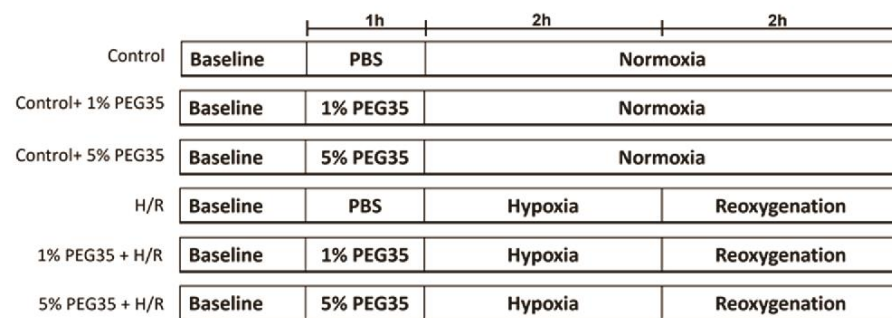
Figure 7. A schematic representation of the experimental protocol. HepG2 cells were randomly divided into the following groups: control, control treated with either 1% PEG35 (Ctl + 1% PEG35) or 5% PEG35 (Ctl + 5% PEG35), 2 h of hypoxia followed by 2 h of reoxygenation (H/R) and preconditioning with 1% PEG35 or 5% PEG35 prior to H/R, 1% PEG35 + H/R and 5% PEG35 + H/R, respectively.

4.3. MTT Assay

Cellular viability was determined through the evaluation of cellular reductive capacity with the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its insoluble formazan crystals, as previously described [57]. HepG2 cells were seeded in 12-well plates and allowed to attach for 1 day prior the assay. After the corresponding treatments, MTT solution (5 mg/mL in PBS) was added to the cells for 3 h. Afterwards, the incubation media were discarded, and formazan crystals were dissolved in isopropanol. A crystal-dissolved solution of each sample was quantified via spectrometry (540 nm) using a Victor plate reader (Perkin-Elmer, Waltham, MA, USA).

4.4. Measurement of Mitochondrial Membrane Potential ($\Delta\Psi m$)

HepG2 cells was measured with a fluorescent probe, tetramethylrhodamine methyl ester (TMRM), as described before [57]. Briefly, after the hypoxia reoxygenation protocol, cells were incubated with 6.6 μM TMRM for 15 min at 37 °C. Afterwards, cells were washed, and culture medium without FBS and phenol red was added. Fluorescence was assessed using the excitation and emission wavelengths of 485 and 590 nm, respectively.

4.5. Measurement of ROS Production

H2DCFDA oxidation to 2,7-dichlorofluorescein (DCF) by ROS was measured as an indicator of ROS accumulation, as before [58]. Briefly, cells were incubated under the same conditions described in the previous section. Then, cells were loaded with 50 µM H2DCFDA for 30 min at 37 °C, and washed and placed in a culture medium without FBS and phenol red. The fluorescence resulting from the formation of oxidized derivatives was monitored at an excitation wavelength 485 nm and an emission wavelength 538 nm for 10 min to calculate the rate of ROS formation.

4.6. ATP Content

HepG2 cells were cultured in 6-well plate under the same conditions described in the experimental protocol in Section 4.2. At the end of the treatment, the extraction of ATP from cells was performed as previously described [59]. Briefly, cells were washed and then scraped in PBS 1X at 37 °C. Cells were centrifuged at $1000 \times g$ for 3 min and the pellets were resuspended in 25 µL of KOH buffer (KOH 2.5 M, K2HPO4 1.5 M) and 75 µL of H_2O. After sonication and centrifugation at $18,000 \times g$ at 4 °C for 2 min, the pH of the supernatant was adjusted to 7 with KH_2PO_4 1 M, and the pellet was stored at -20 °C for protein quantification.

An ATP Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) was used to measure the ATP content in each sample, according to the supplier's instructions. Bioluminescence was measured using a Victor3 plate reader (PerkinElmer, Waltham, MA, USA).

4.7. Quantitative Real-Time PCR

Total RNA was extracted from HepG2 cells using the PureLink RNA Mini Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's recommendations. RNA was quantified with a Nanodrop One (Thermo-Fisher, Waltham, MA, USA) and 10 ng of RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA), according to manufacturer's instructions. Then, cDNA was diluted 1:10 and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used for qPCR reactions.

The expression level of target genes was calculated using the $2^{-\Delta\Delta Ct}$ transformation method [60] and normalized to the housekeeping gene 18S rRNA. The primers used are shown in the Table 1.

Table 1. Nucleotide sequences of primers used in qPCR.

Gene	Sequence	NCBI's Nucleotide Accession Number
Dnm1l	AAG AAC CAA CCA CAG GCA AC GTT CAC GGC ATG ACC TTT TT	NM_012062.4
Fis1	TTA TTT ACA CTC ATC CCA AAG C CTG TCC TTT CCC TGT TCT C	NM_016068.2
Mnsod	GGA AGC CAT CAA ACG TGA CT CTG ATT TGG ACA AGC AGC AA	NM_000636.2
Nrf1	GAA TTG CCA ACC ACG GTC AC GCG CCA TAG TGA CTG TAG CT	NM_005011.4
Opa1	CAG AAA GAT GAC AAA GGC ATT C GCA ATC ATT TCC AAC ACA CTA G	NM_015560.2
Ppargc1a	CCT TGC AGC ACA AGA AAA CA CTG CTT CGT CGT CAA AAA CA	NM_013261.3
Tfam	CCG AGG TGG TTT TCA TCT GT ACG CTG GGC AAT CTT TCT AA	NM_003201.2
18S rRNA	AAC GGC TAC CAC ATC CAA TTT TCG TCA CTA CCT CCC	NR_003286.2

4.8. Western Blot Analysis

After treatments, cells were washed twice and scraped in 1 mL of ice-cold PBS 1X. Cells were centrifuged for 3 min at $10,000 \times g$ at 4 °C and the pellets were resuspended in an ice-cold RIPA lysis buffer supplemented with protease inhibitors (Thermo Scientific). Lysates were sonicated and centrifuged for 10 min at $12,000 \times g$ at 4 °C. The protein concentration was quantified via the bicinchoninic acid assay and subsequently mixed with Laemmli buffer containing 8% β-mercaptoethanol and denatured at 80 °C for 5 min. Then, 50 µg of protein was separated using TGX Stain-Free polyacrylamide gels (Biorad), according to the manufacturer's recommendations. Gels were activated using a GelDoc EZ (Bio-Rad) and the proteins were subsequently transferred to a nitrocellulose membrane using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked for 2 h in 5% non-fat dry milk and incubated with the specific primary antibody at 4 °C overnight. Membranes were washed with TBS-T and incubated for 1 h with secondary antibodies. After washing the membranes with TBS-T, they were revealed using a ChemiDoc MP (BioRad). Total protein quantification of the respective lanes was used to perform blots normalization, following standard procedures [61,62]. Images were analyzed using Image Lab 6.1 Software (Bio-Rad). The antibodies used are listed in Table 2.

Table 2. Primary and secondary antibodies used in Western blot analysis.

Antibody	M_W (kDa)	Dilution	Supplier	Reference Number
		Primary antibodies		
ALDH2	56	1:1000	Abcam	ab194587
LC3	18, 16	1:1000	Sigma-Aldrich	L7543
Nrf2	75	1:1000	Millipore	ABE413
p62	62	1:100	Santa Cruz	sc-84618
		Secondary antibodies		
StarBright Blue 520, Goat Anti-Rabbit IgG	-	1:5000	Bio-Rad	12005870
StarBright Blue 700 Goat Anti-Rabbit IgG	-	1:5000	Bio-Rad	12004162

4.9. Statistical Analysis

Data are presented as mean ± S.E.M. A one-way ANOVA with Tukey's test was performed for the evaluation of statistical significance ($p < 0.05$). Statistical analysis was performed using GraphPad Prism 9.0.0 (GraphPad Software, San Diego, CA, USA).

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General Results

In the recent years, a sizeable body of data have suggested that different drugs could have a beneficial role in the prevention and/or damage reduction against IRI.

PEGs are non-toxic and water-soluble compounds polymers widely used in cosmetics, food, and drugs. In the last decades, increasing studies have been showing the protective effects of PEG both *in vivo* and *in vitro*, in several experimental setups.

The better protection against liver cold ischemia injury demonstrated by PEG35-containing solution made us try to understand the role that PEG35 plays both in static and dynamic preservation. In addition, since PEG35 preconditioning have been reported to ameliorates pancreatic inflammatory models as well as cardiomyocyte protection from hypoxia/reoxygenation-induced cell death, we wanted to investigate the mechanisms underlying PEG35 preconditioning-induced protection against IRI.

We based our study in *in vivo*, *ex vivo* and *in vitro* models, obtaining the following main results:

◊ The presence of PEG35 in IGL-1/IGL-2 solutions was determinant for protecting liver mitochondria and preserving the energy breakdown against ischemic insult in SCS.

◊ In the IGL-2 solution, the augmented PEG35 content and the higher antioxidant capacity prevented oxidative stress through ALDH2 upregulation as well as promoted cytoprotective autophagy.

◊ In dynamic preservation, PEG35-containing perfusates, such as the new IGL-2 solution, showed a better mitochondrial liver protection.

◊ Of note, besides protecting the mitochondrial integrity, the use of IGL-2 also avoids the mixture of different solutions/perfusates. Consequently, it may decrease the operational logistics and the period prior transplantation, a critical factor when marginal organs are involved.

◊ In a hypoxia/reoxygenation model using HepG2 cells, we observed that PEG35 preconditioning was able to preserve mitochondrial function by decreasing ROS excessive production, ATP depletion and recovering the mitochondrial membrane potential. Moreover, PEG35 increased levels of autophagy-related proteins and expression of genes involved in mitochondrial dynamics

Discussion

NAFLD is a widespread pathological condition characterized by a lipid accumulation in the hepatic parenchyma. Currently, NAFLD is the leading cause of liver disease in the American population, and, by 2030, it is expected to be the leading cause of liver transplant (Doycheva et al., 2017). Consequently, this may further aggravate the existing shortage of donor organs. Nowadays, in fact, to deal with the growing waiting lists for LT, many transplantation centers are compelled to adopt extended criteria for graft selection, moving the limit of acceptance for marginal livers, such as the steatotic livers. Nevertheless, it is well known that steatotic livers present higher susceptibility against IRI, and their use enhances primary failure and compromises graft outcome after transplant (Said, 2013).

IRI is a major obstacle in many clinical scenarios, including liver resection and transplantation. Prior transplantation into the recipient, the liver goes through a series of mandatory steps, including retrieval from the donor, washing, cold storage in a preservation solution and finally, rinsing. The cumulative injury resulting from the combined action of ischemia and cold preservation will render the graft more vulnerable after transplantation. Therefore, the discovery of new therapeutic strategies to minimize organ non-function after transplantation are extremely important, especially in the case of fatty liver grafts.

PEGs have been showing beneficial effects in different organs, including liver, kidney and heart (Giraud et al., 2018; Lopez et al., 2018; Xu et al., 2015). In the liver, several studies have reported the protective role of different molecular weight PEGs on cold preservation. PEG35 has been linked to increased ALDH2 levels and improved mitochondrial machinery, resulting in a reduction of cold ischemic injury (Panisello-Roselló, Alva, et al., 2018; Panisello-Roselló, Lopez, et al., 2018). Additionally, intravenous PEG35 preconditioning ameliorated liver graft preservation and protected mitochondria in cold ischemia followed by warm reperfusion (Bejaoui et al., 2015). When present in the rinse solution for graft washing out, PEG35 has also demonstrated hepatoprotection, including the generation of NO (Mohamed Amine Zaouali et al., 2014). The vasodilation properties of NO compensate the microcirculation disorders in fatty liver grafts, increasing graft preservation and revascularization (Abu-Amara et al., 2012).

PEG35 is the oncotic agent present in the IGL-1 solution and, alongside with the reversal Na^+/K^+ concentrations, is the main differential trait to the gold standard solution, UW solution (Ben Mosbah et al., 2006). In fact, IGL-1 solution has been considered a good alternative to UW solution according to the European Liver Transplant Registry (Adam et al., 2015). Therefore, one can suspect that the differences found between UW and IGL-1 solutions may be related to the presence/absence of the oncotic agent PEG35. This hypothesis is further supported by the fact that the presence or absence of PEG35 in rinse solutions for liver grafts protects against

mitochondrial injury (Pasut et al., 2016), and by the comparison between IGL-1 (with PEG35) and HTK (without PEG35) in fatty liver preservation (Panisello-Roselló, Verde, et al., 2018).

In the first part of this thesis our purposes were to explore in depth the importance of PEG35 in IGL-1 solution against cold IRI in fatty livers. Despite, the protective effects that PEG35 has been demonstrated, its specific role in IGL-1 solution on liver SCS has not been fully elucidated. The comparison between the conventional IGL-1 solution and a solution with the same composition, but without the oncotic agent PEG35 (IGL-0 solution), may shed some light into the mechanism underlying PEG35 cytoprotection. In addition, given the higher vulnerability of fatty liver to cold ischemia, we also aimed to enhance the graft outcome after preservation by using a new solution, IGL-2. The IGL-2 solution is quite similar to the IGL-1 solution, but it contains 5g/L of PEG35 and 9 mM of glutathione, instead of 1g/L and 3 mM, respectively, present in the IGL-1 solution.

In our study we observed a protective effect on fatty livers in the PEG35-cointaining solution, reflected by the reduced AST/ALT levels. In addition to the better prevention of liver injury shown by the levels of transaminases, PEG35 also led to a better mitochondrial protection as suggested by a lower GLDH content and the increased ATP content.

The relevance of mitochondria in IRI pathophysiology is well established for many years. Recently, the involvement of a liver mitochondrial enzyme, ALDH2, has been reported in hepatic IRI (Panisello-Roselló, Lopez, et al., 2018). Ethanol detoxification is known as the major role of ALDH2 (C. H. Chen et al., 2014), but ALDH2 has also been reported as an crucial player in the 4-HNE detoxification, an important and harmful sub-product of lipid peroxidation. Panisello-Roselló and co-workers observed that in liver preserved in IGL-1 solution, ALDH2 upregulation was increased when compared to liver preserved in UW and HTK solutions, which was associated with decreased transaminases levels, apoptosis and lipoperoxidation (Panisello-Roselló, Alva, et al., 2018). In addition, Alda-1 preconditioning, an ALDH2 activator, protected the liver against warm IRI by preventing oxidative stress (T. Zhang et al., 2018).

In our study, livers preserved in IGL-1 solution showed a higher ALDH2 activity and content and, consequently, a prevention of toxic aldehydes, 4-HNE and lipoperoxidation products, MDA, in accordance with the previous studies. Strikingly, the protection observed in the PEG35-containing solution contrasts with the injury found in the liver preserved in IGL-0. Of note, our work suggests that PEG35 may trigger the mitochondrial ALDH2 upregulation, which in turn protect mitochondria and cells from the damaging effect of toxic aldehydes. In accordance, IGL-2 solution which contains increased levels of PEG35, further prevented the oxidative stress via ALDH2 upregulation and promotion of cytoprotective autophagy. Indeed, IGl-1 solution led to increased ALDH2 levels than IGL-0 solution, and IGL-2 solution to even higher levels comparing

to IGL-1 solution, suggesting that ALDH2 increases with PEG35 in a concentration dependent manner.

However, we cannot rule out the importance of higher concentrations of glutathione present in the IGL-2 solution in the total antioxidant effects. The higher prevention of AOPP (oxidized proteins) and 4-HNE protein adducts formation observed in IGL-2 solution could be the results of the synergetic effect of increased PEG35 and glutathione levels.

Finally, we also observed increased expression of eNOS synthase and subsequent NO generation in PEG35-containing solutions. These findings support the hypothesis that ALDH2 might be involved in NO synthesis *in vivo* (C. H. Chen et al., 2014). NO can act as a scavenger by its interaction with ROS and thus, it may diminish the number of oxidizing elements. In addition, the vasodilatation properties of NO play a key role in preventing the microcirculatory disturbances that occur during revascularization, especially in steatotic livers which are more susceptible to dysfunctional microcirculation (Ramalho et al., 2006).

To sum up, the data showed in this study highlight that the oncotic agent PEG35 is a keystone element in the traditional IGL-1 solution to protect fatty liver grafts against cold ischemia insult. This protective action is mediated, at least in part, by increased ALDH2 activity, which prevents the formation of toxic aldehyde adducts and lipoperoxides. Furthermore, IGL-2 solution seems to be a suitable alternative for enhancing cold graft preservation strategies. The combined action of increased PEG35 and glutathione levels reinforce the protective mechanisms by modulating the redox state through mitochondrial ALDH2. These results suggest that IGL-2 could improve the preservation of fatty liver grafts when static or dynamic cold preservation are involved.

As mentioned above, the presence of PEG35 in SCS and rinse solution conferred a significant protection to the mitochondria compared to solution without PEG35. The maintenance of the mitochondrial function and ATP levels are key points in organ preservation. Recently, Horváth and colleagues have highlighted the importance of mitochondrial protection during hypothermic graft preservation and its energetic status (Horváth et al., 2021).

HOPE has been linked to the preservation of the mitochondrial state, which contributes to the prevention of energy breakdown and subsequent maintenance of intracellular ATP content (Schlegel et al., 2020). In addition, lower accumulation of ROS precursors derived from anaerobic metabolism has also been described in HOPE strategies, culminating in a better outcome after the unavoidable reperfusion.

Based on the strong observations described above, our main goal in the study 2 was to ascertain whether IGL-2 could have a relevant effect in protecting the liver during HOPE at the mitochondrial level. HOPE is a complementary tool which improves graft preservation by

combining the benefits of cold preservation conditions with the supply of oxygen to the organ in a perfusion system and it has been suggested as a promising tool to rescue marginal livers, such as the ones presenting with steatosis for transplantation purposes (Patrono et al., 2019). Recent advances in the field have made HOPE a very promising strategy to increase the donor pool in the face of the pressing shortage of organs for transplantation. A variation of the original UW solution, Belzer MPS, is the most commonly used perfusion solution for HOPE. However, this perfusate has been showing some limitations in liver machine perfusion due to its high viscosity which leads to an increased shear stress. In addition, the oncotic agent, HES, increases the hyper-aggregation of erythrocytes, making difficult a suitable rinsing and preservation during machine perfusion (Morariu et al., 2003). The presence of PEG35 as oncotic agent instead of HES and the higher glutathione concentration are the main differences between IGL-2 and Belzer MPS. These features confer to IGL-2 a higher antioxidant capacity, which is reflected in an improved protection against ROS production and their potential damage against mitochondria in SCS followed by HOPE strategies.

In our study, we compared non-fatty livers grafts subjected to 7 hours SCS followed by 1 hour HOPE in either Belzer MPS or IGL-2. Despite no significant differences were found in ALT/AST levels, a significant lower GLDH content (mitochondrial damage marker) was observed in the IGL-2 group.

IGL-2 solution seems to be a more efficient perfusate for dynamic oxygenated preservation over the currently used perfusates, Belzer-MPS and its genetics. Of note, IGL-2 not only protects mitochondrial integrity, but its use both in SCS and HOPE strategies facilitates the logistics by avoiding the mixture of different solutions/perfusates when both techniques are combined. In addition, PEG35-containing perfusates are likely to reduce the buildup of some metabolites responsible for mitochondrial dysfunction (Chouchani et al., 2014), leading to a proper mitochondrial function during early normothermic reperfusion.

In conclusion, IGL-2 solution shows promising benefits for a combined used of SCS and HOPE strategies to rescue marginal livers for transplantation purpose, although further investigations are needed.

In study 3, we stressed the importance of IGL-2 solution in SCS and HOPE as well as its involvement in mitochondrial protection. Recently, there has been an increasing interest in the mitochondrial protection during hypothermic graft preservation and its energetic status (Horváth et al., 2021). The benefits of HOPE are well known to be linked to the oxygenation of the perfusate, which in turn maintains the integrity and function of mitochondrial machinery (Schlegel et al., 2020). The maintenance of the mitochondrial state which occurs during HOPE, leads to the prevention of the energetic breakdown and subsequently, preservation of

intracellular ATP levels. In addition, HOPE also triggers mechanisms connected to mitochondrial repair and endothelial protection (Schlegel et al., 2020). Nevertheless, the relationship between mitochondrial protection and the preservation solutions needed further investigation.

The perfusion solution used in HOPE seems to have an important role in the outcome of liver graft preservation. As aforementioned, the standard perfusate, Belzer- MPS, contains HES which is well known to lead to hyperaggregation of red blood cells. In addition, HES presence also increases the viscosity of the perfusate during hypothermic perfusion which may lead to fluid disturbances due to fluid movement and subsequent destruction of the glycocalyx, the luminal sugar thin layer that covers the liver endothelia (Mathis et al., 2021). In study 2, we have proposed the use of the new PEG35-containing solution, IGL-2, as a good alternative to Belzer MPS for HOPE strategies alone or combined with SCS. Although further studies are need, the results obtained confirm that the mitochondrial quality and protection will, in great extent, determine the capability of the graft to recover from IRI insult, which are in agreement with the study of Schlegel and colleagues (Schlegel et al., 2020).

We suggested that the use of IGL-2 protects mitochondrial function, favors NO generation (vasodilation agent) and diminishes the disturbances that affects the endothelial glycocalyx due to its lower viscosity. In addition, we stressed the importance of using a novel IGL-2 solution for a combined used of SCS and HOPE strategies to rescue marginal organs, facilitating the logistics and avoiding the mixture of preservation solutions/perfusates for transplantation purposes.

Moreover, we complemented the study 3 with a letter to the editor, suggesting the value of using a single solution, such as IGL-2, in split liver transplantation and HOPE. We draw some considerations regarding the important work to the field performed by Mabrut and colleagues (Mabrut et al., 2021). In this study, the authors report an original technique of "ex vivo" graft splitting with concurrent HOPE. Split liver transplantation is used to increase organ availability. However, this technique is associated with prolonged cold ischemia and higher risks of postreperfusion bleeding and IRI (Ishii et al., 2021). Thus, partial liver grafts have been considered as marginal grafts, as in the case of fatty liver grafts.

Despite we consider the work done by Mabrut et al., extremely important to the field, not only by reducing the cold ischemic period but also by diminishing the warm ischemic injury inherent to any surgical intervention, based on our previous studies, we think there is still room to further reduce the injury associated with cold ischemia. The use of IGL-2 for both SCS and HOPE strategies could have the beneficial effects reported in our previous studies using marginal organs and consequently, it could also be an important asset in the case of "ex vivo" liver splitting and HOPE strategies.

Besides its role in SCS and HOPE, PEG35 has also been reported to have a protective role as a preconditioning agent. Pharmacological preconditioning relies on the administration of specific drugs mimicking the protective mechanisms and biologic effects of ischemic preconditioning, which is achieve by blood flow restriction for a small period (usually 10-15 min) followed by flow reestablishment by restrictor removal. There is still no definitive answer regarding the mechanisms underlying ischemic preconditioning, but mitochondrial function integrity, elevated mitophagy and prevention of apoptosis seem to be crucial events (Teodoro et al., 2022).

Recently, our research group reported that PEG35 preconditioning ameliorates pancreatic inflammatory response in cerulein-induced acute pancreatitis both in *in vivo* and *in vitro* models (Ferrero-Andrés, Panisello-Roselló, Roselló-Catafau, et al., 2020). PEG35 was able to attenuate inflammation response and associated cell death in a dose dependent manner. Moreover, high molecular weight PEGs (15-20 kDa) have been reported to protect cardiomyocytes from hypoxia/reoxygenation- induced cell death (Malhotra et al., 2011). The authors found that PEG pretreatment significantly decreased apoptosis associated to cyt *c* release as well as decreased intracellular ROS production. In the liver, PEG35 have also been shown to be a key player in warm IRI, displaying powerful hepatic protection by reducing transaminases levels and maintaining hepatocyte morphology as well as preserving mitochondrial membrane potential (Bejaoui et al., 2016). Nevertheless, the mechanisms underlying such protection have not been fully elucidated.

Therefore, based on these protective features, in the study 4 we aimed to assess the ability of PEG35 preconditioning to protect human hepatocytes submitted to hypoxia/reoxygenation and shed some light on the possible protective molecular mechanisms involved in PEG35-mediated hepatoprotection.

To our knowledge, this is the first study assessing the effects of PEG35 preconditioning against hypoxia/reoxygenation-induced injury in HepG2 cells. In accordance with the above studies reporting that PEG induces protection from IRI-cell death, we found that human hepatocytes pretreated with 5% PEG35 and then submitted to hypoxia/reoxygenation presented a higher cell viability compared to cell without PEG35 preconditioning.

As confirmed in the study 1, PEG35 is associated with higher levels of ALDH2 and enhanced mitochondrial machinery, culminating in decreased ischemic injury. In our experimental model, we observed an increased ALDH2 content in the 5% PEG35 group while in the other groups the protein levels were similar.

A burst of ROS production is considered to play a crucial role in the organ damage associated with IRI. As expected, the hypoxia/reoxygenation group revealed an increase in ROS production, which was significantly reverted in the presence of 5% PEG35. This result is in accordance with

the increased levels of ALDH2, and its ability to decrease oxidative stress, observed under PEG35 preconditioning. The results were also consistent with decreased gene expression of MnSOD, an essential mitochondrial antioxidant enzyme, upon hypoxia/reoxygenation and upregulation in both PEG35 groups. PEG35 preconditioning decreased the ROS production observed during hypoxia/reoxygenation and upregulated the expression of ALDH2 and MnSOD, indicating that PEG35 protected hepatocytes from hypoxia/reoxygenation injury by upregulation of antioxidant enzymes.

IRI is well known to alter the energy metabolism due to impairment of mitochondrial function (Anzell et al., 2018). The excessive ROS production found during hypoxia/reoxygenation may damage the cellular membranes and subcellular organelles, causing the decrease of mitochondrial membrane potential ($\Delta\Psi_m$) and consequent mitochondrial dysfunction. In this study we showed that PEG35 preconditioning protected against loss of $\Delta\Psi_m$ in human hepatocytes during hypoxia/reoxygenation.

The deprivation of oxygen during hypoxia is related to the cessation of OXPHOS and consequent ATP depletion. Accordingly, ATP content in HepG2 cells submitted to hypoxia/reoxygenation was found to be decreased, which is in agreement with the lower $\Delta\Psi_m$ observed in the same group, suggesting an impairment of mitochondrial function. As expected by the observed recovering of mitochondrial $\Delta\Psi_m$, PEG35 preconditioning efficiently preserved the ATP content followed hypoxia/reoxygenation. Thus, these results revealed that PEG35 preconditioning may improve IRI by reserve mitochondrial function through the decrease of excessive ROS production, ATP depletion as well as recovering the membrane potential.

In the study 1, we observed a better hepatic protection against ischemic insult by the reduction of oxidative stress through ALDH2 upregulation and enhancement of cytoprotective autophagy in a PEG35-dependent manner. Autophagy is a key process in the clearance of dysfunctional organelles, and it has been recognized as a protective process in response to various intra- and extracellular stimuli, including IRI (Hamacher-Brady et al., 2006).

Enhancing autophagy improves hepatic function by removing the dysfunctional mitochondria while autophagy inhibition increases mitochondrial oxidative stress and triggers cell death during liver IRI (Sun et al., 2013). Accordingly, Wang *et al.,* demonstrated that increasing autophagy alleviates hepatic injury and improves mitochondrial function against IRI (J.-H. Wang et al., 2012). LC3 is a key player in the autophagic process which also includes mitophagy. In the autophagic event, the soluble form (LC3-I) is converted into LC3-II, which is recruited to the autophagosome formation (Youle & Narendra, 2011). p62 is another key protein involved in autophagic process by targeting specific cargoes for autophagy. Under normal conditions, basal autophagy clears p62 and associated cargo. Conversely, under conditions of decreased/deficient

autophagy, p62 and associated cargo accumulates in the cytoplasm (Rusten & Stenmark, 2010). In our model, we observed an increased LC3-II/LC3-I ratio upon PEG35 preconditioning. In agreement, HepG2 cells submitted to hypoxia/reoxygenation revealed an increased accumulation of p62, while HepG2 cells pretreated PEG35 presented a strong decrease in p62 content. In that sense, the increased levels of LC3 II and p62 degradation suggest an autophagy enhancement followed PEG35 preconditioning, which may contribute to the elimination of dysfunctional mitochondria during IRI, leading to improved mitochondrial performance.

The maintenance of a healthy mitochondrial network is a key factor for cellular homeostasis and survival. Mitochondrial biogenesis as well as mitochondrial fission and fusion mediate cellular energetics and metabolic demands. PGC-1α is a master regulator of mitochondrial biogenesis that enhances different transcription factors, such as NRF1 and TFAM, which control mitochondrial turnover, content and number to maintain the metabolic demand (Dusabimana et al., 2019). Previous studies reported enhanced mitochondrial functioning following hepatic IRI via the stimulation of mitochondrial biogenesis through the induction of the PGC-1/NRF1/TFAM pathway (Joe et al., 2015; Shin & Lee, 2017). In accordance, in our study, 5% PEG35 preconditioning upregulated the expression of *ppargc1a* and *nrf1*, indicating an enhancement of mitochondrial biogenesis.

Mitochondrial fusion and fission are two essential quality control mechanisms which favor the segregation and clearance of dysfunctional mitochondria to achieve homeostasis (Youle & Van Der Bliek, 2012). Compelling evidence of the interplay between mitochondrial quality control and cell fate in different organs subject to ischemia/reperfusion has been gathered over the last few years (Zheng et al., 2020). OPA1 is a crucial protein involved in mitochondrial fusion which may interact with various numbers of mitophagy receptor proteins in order to collaborate in mitochondrial dynamics and mitophagy (Chen et al., 2016; Murakawa et al., 2015). OPA1 knockdown has been reported to aggravate the harmful effects provoked by IR (Guan et al., 2019; Le Page et al., 2016). Moreover, increased mitochondrial fusion and mitophagy through the AMPK-OPA1 signaling pathway was observed to protect against cardiac IRI, whereas OPA1 knockout abolished the protective effects (Zhang et al., 2019). In our models, we found an increased expression of *opa1* after 5% PEG35 preconditioning. It has been reported that the loss of $\Delta\Psi_m$ triggers OPA1 proteolysis and inhibits mitochondrial fusion (Griparic et al., 2007; Ishihara et al., 2006). As mentioned above, our results demonstrate that 5% PEG35 preconditioning increased $\Delta\Psi_m$ when compared to hypoxia/reoxygenation, which is in accordance with the effects of increased *opa1* upregulation.

On the other hand, mitochondrial fission has been demonstrated to be triggered by IR (H. Kim et al., 2011). Mounting evidence confirms that inhibition of mitochondrial fission may protect

several tissues from IRI (J. Huang et al., 2021), while excessive mitochondrial fission has been related to mitochondrial fragmentation and triggering of cell apoptosis (Toyama et al., 2016). Drp1 and Fis1 are essential proteins involved in the fission processes (Yu et al., 2020), and increased levels of Drp1 and Fis1 have been reported after hepatic IRI (Bi et al., 2019). Moreover, Drp1 inhibition led to increased mitophagy and subsequent removal of damaged mitochondria and prevention of ROS production in cardiac IRI (Bian et al., 2019). In our study, cells submitted to hypoxia/reoxygenation significantly increased the expression levels of Drp1 and Fis1 and this upregulation was attenuated by PEG35 preconditioning. Taken together, our findings suggest that PEG35 preconditioning regulates hypoxia/reoxygenation-induced imbalance in mitochondria dynamics by elevating mitochondrial fusion and diminishing mitochondrial fission. In conclusion, in the study 4 we established and used an *in vitro* model to explore the possible protective molecular mechanisms of PEG35 preconditioning on diminishing hypoxia/reoxygenation injury. We observed that 5% PEG35 preconditioning presented a better outcome in most of the parameters analyzed when compared to 1% PEG35 preconditioning, showing that PEG35 protective effects are dose dependent. We showed that 5% PEG35 preconditioning efficiently ameliorates hepatic hypoxia/reoxygenation injury by alleviating mitochondrial dysfunction through the enhancement of autophagy and mitochondrial biogenesis and dynamics.

Throughout all the 4 studies we have assessed the role of PEG35 in different circumstances. In the first study, we investigated the relevance of PEG35 in the IGL-1 solution in a context of SCS by using an IGL solution with and without PEG35 and a new solution containing more PEG35 and glutathione. With this approach, we demonstrated the importance of the oncotic agent PEG35 in protecting fatty liver grafts against cold preservation through the modulation of the redox state via mitochondrial ALDH2 and in combination with glutathione. In the second study, we suggested the use of IGL-2 solution as a good alternative to Belzer MPS for HOPE strategy. The combination of 5-fold increase in PEG35 concentration with an increased concentration of glutathione found in the IGL-2 solution constitutes an interesting tool for improving hypothermic liver preservation in static and/or dynamic approaches by offering both liver protection and mitochondrial restoration. In the third study, we have emphasized the benefits of using the novel IGL-2 solution for a combined approach of SCS and HOPE to rescue marginal livers, by facilitating the logistics and avoiding the mixture of preservation solutions/perfusates in a context of liver transplantation. In addition, we have also suggested that the protection of mitochondrial functions should be considered as a priority in the studies of liver preservation solutions.

Finally, after having assessed the importance of PEG35 in SCS and dynamic preservation, in the study 4 we explored the effects of PEG35 in warm IRI. PEG35 preconditioning effectively improved hepatic hypoxia/reoxygenation injury via the enhancement of autophagy and mitochondrial quality control. Thus, PEG35 seemed to be useful potential pharmacological tool for diminishing hepatic IRI in clinical practice.

Conclusions

The following conclusions can be highlighted as a summary of the present doctoral thesis:

- PEG35 is a key player in the preservation of fatty liver grafts against cold ischemia insult and the protective action is partly mediated by increased ALDH2 activity.

- The newly developed IGL-2 solution revealed a superior antioxidant capacity against the ischemic insult during graft preservation, presenting a reinforced hepatoprotection in hypothermic fatty liver preservation.

- IGL-2 is a suitable alternative to be used as perfusate for increasing cold graft preservation strategies when static cold preservation and HOPE are combined. In addition, eases the logistics and avoids the mixture of different solutions/perfusates, which might be interesting for rescuing fatty liver grafts as well as the combination of split liver transplantation with HOPE.

- Mitochondrial preservation should be a priority direction for a better graft protection when static and HOPE strategies are used in combination or alone.

- PEG35 preconditioning effectively ameliorates hepatic hypoxia/reoxygenation injury through the enhancement of autophagy and mitochondrial quality control. Therefore, PEG35 could be useful as a potential pharmacological tool for attenuating hepatic IRI in clinical practice.

Annexes

Glycocalyx as a Useful Marker of Endothelial Injury in Liver Transplantation: The Role of Preservation Solution

Arnau Panisello-Roselló, PhD,[1] Carlos Castro Benitez, PhD,[2] Alexandre Lopez, PhD,[2] Rui Teixeira da Silva, MS,[1] Joan Roselló-Catafau, PhD,[1] and René Adam, PhD[3]

The reported investigations from Schiefer et al,[1] on the role of glycocalyx (GC) in orthotopic liver transplantation, propose GC as a relevant injury marker to be used in this field. GC is a complex sugar monolayer, which covers the endothelium and is thus exposed and susceptible to alteration as a consequence of liver ischemia-reperfusion (IR) injury associated with orthotopic liver transplantation. The authors have used histidine-tryptophan-ketoglutarate (HTK) solution in their study. However, other preservation solutions (PSs) with different compositions (such as University of Wisconsin [UW], Celsior, and IGL-1 PS) are commercially available and should be tested. Extension of the study with use of these alternative PS could provide important insights into organ PS. The presence of an oncotic agent (such as hydroxyethyl starch in the UW solution or polyethylene glycol 35 [PEG35] in IGL-1 solution) differentiates UW and IGL-1 from HTK and Celsior solutions, which do not contain oncotic agents. This difference is relevant because oncotic pressure, controlled by the oncotic agent, minimizes interstitial edema, which could directly affect GC. However, the oncotic agent increases viscosity, which may in turn affect the protective performance of the PS. Balancing the limitation of edema against viscosity during organ preservation is critical to GC integrity and graft status.

In this context, our group reported that PEG35 used in a rinsing solution reduces liver graft damage after cold static preservation.[2] The comparison of 2 PS, HTK, and IGL-1, in a rat liver cold preservation model, showed that the presence of PEG35 in the IGL-1 PS was the determinant of GC protection.[3] These data support the concept that PEG35 reduces IR injury effects on the narrowing and the integrity of the GC.

GC protection is linked to the mechanical properties that PEG35 confers on PS because of its viscosity, such as shear stress and PEG35 interacting with the cells causing remodeling of the cell's actin cytoskeleton.[4] It is acknowledged that IR injury is maximized in fatty livers partly because of sinusoidal narrowing and is accentuated by both normothermic and hypothermic machine perfusion.[5] In addition, nitric oxide, a potent physiological vasodilator, is promoted by PEG35 through endothelial nitric oxide synthase activation.[2,3] Therefore, PEG35 should be considered as an important component in any dynamic preservation strategy, such as hypothermic oxygenated perfusion, to preserve GC integrity and improve liver preservation, especially when fatty liver grafts are used.[5] Thus, we support the relevance of the investigations reported by Schieffer et al, which point firmly, for the first time, to the utility of GC as a suitable marker of graft damage and preservation in orthotopic liver transplantation. Further investigation will be important to compare GC as an injury marker using different PS.

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Review

Shaping of Hepatic Ischemia/Reperfusion Events: The Crucial Role of Mitochondria

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Abstract: Hepatic ischemia reperfusion injury (HIRI) is a major hurdle in many clinical scenarios, including liver resection and transplantation. Various studies and countless surgical events have led to the observation of a strong correlation between HIRI induced by liver transplantation and early allograft-dysfunction development. The detrimental impact of HIRI has driven the pursuit of new ways to alleviate its adverse effects. At the core of HIRI lies mitochondrial dysfunction. Various studies, from both animal models and in clinical settings, have clearly shown that mitochondrial function is severely hampered by HIRI and that its preservation or restoration is a key indicator of successful organ recovery. Several strategies have been thus implemented throughout the years, targeting mitochondrial function. This work briefly discusses some the most utilized approaches, ranging from surgical practices to pharmacological interventions and highlights how novel strategies can be investigated and implemented by intricately discussing the way mitochondrial function is affected by HIRI.

Keywords: mitochondria; liver; ischemia/reperfusion; liver surgery; conditioning

1. Introduction

1.1. The Liver

The liver is an organ with dozens of functions in the body, ranging from the better-known bile production to assist digestion, to others that are referenced less often, such as its involvement in carbohydrate (production and storage of glycogen; release of glucose to circulation; gluconeogenesis to generate glucose from amino acids, lactate or glycerol), protein (most circulating proteins are produced by the liver, as are most amino acids and some hormones such as angiotensinogen; hormones and other circulating proteins are broken down in the liver) or lipid (production of cholesterol, lipogenesis and triglyceride synthesis) metabolism. Other functions involve the detoxification of xenobiotics and some heavy metals, serving as a blood reservoir, producing lymph, storing vitamins and metallic ions and promoting blood immune activity (by the liver native Kupffer cells, as well as a panoply of other immune cells) [1,2].

Given of its range of roles, it is unsurprising that liver transplantation is one of the most commonly performed organ transplants. Liver injury and failure is an increasingly common event [3] and, despite improvements in survivability and transplant success, there is still a tremendous unbalance between liver donations and necessities. This is further

aggravated by the fact that many (if not most) potential donors are barred from providing organs due to innate conditions, such as steatosis or steatohepatitis, cirrhosis, or cancer, just to name a few [4]. As such, novel therapeutic approaches and interventions that can increase both the pool of available donors as well as the restoration of homeostasis upon liver insult, both of which are urgently needed. One of the most common types of hepatic injury is that caused by the cut and eventual restoration of circulation, which is commonly known as ischemia and reperfusion.

1.2. Ischemia/Reperfusion

Hepatic ischemia-reperfusion injury (HIRI) is an accumulation of processes and events that revolve around cellular and organelle damage upon blood-flow restriction (ischemia), which is followed by a seemingly contradictory augmentation of injury upon the restoration of blood flow (reperfusion) [5]. HIRI is the main reason for complications and even mortality in the setting of hepatic surgery or transplantation [6,7]. Interestingly, despite intense investigation on the matter, the exact causes of HIRI are still unclear [8].

There are two main types of HIRI, depending on the setting of the organ at the time of ischemia, i.e., whether it is still inside the organism (warm) or outside, as is the case of transplantation (cold) [8,9]. Although in different induction settings, the pathophysiological events that take place are quite similar between the two types of HIRI [9]. While warm (normothermic, 22–25 °C) HIRI begins with hepatocyte injury and during different events such as trauma, surgery or other events that restrict blood flow, cold (ice temperature, i.e., 0–1 °C) HIRI only applies to a transplant setting, where the liver is harvested and transported in quasi-freezing temperatures in order to reduce tissue degradation. Regardless, both events result in an immune response triggered not by pathogens per se, but due to the release of pro-inflammatory signals [10], in what is known as sterile inflammation.

There are many processes involved in HIRI events, namely the metabolic shift towards an anaerobic metabolism, mitochondrial dysfunction and oxidative stress (i.e., overproduction of reactive oxygen species that overbear natural antioxidant defenses such as scavenger molecules and, more relevantly, antioxidative enzymes, which contributes to alterations in redox signaling and molecular injury [11]), calcium overload and the immune response, heavily mediated by Kupffer cell and other immune cell types' activation, such as infiltrating neutrophils and macrophages [12,13]. As such, given the central role of the liver in metabolic and energetic whole-body homeostasis, it comes as no surprise that HIRI causes a broad-range effect on the body. In fact, during the ischemic period, the lack of oxygen shifts the ATP-generating processes from aerobic respiration (i.e., oxidative phosphorylation, OXPHOS) towards anaerobic respiration, or glycolysis. However, given enough time, the lack of oxygen results in a shutdown of redox processes, severe reduction in ATP generation capacity, and parallel acidification of the cellular milieu, given the accumulation of lactic acid and ketone bodies, resulting in what is known as metabolic acidosis. Thus, the lower pH results in enzyme, organelle and even cellular injury [14]. As expected, once blood flow is restored (reperfusion), acidification is neutralized, which is paradoxically responsible for further injury due to the activation of pH-dependent proteases and phospholipases [15,16]. In parallel, the low O_2 pressure can also result in the elevation of cyclic AMP (cAMP) levels, resulting in the activation of cAMP-sensitive enzymes, with concomitant phosphorylation and perturbation of the function of key enzymes of the carbohydrate metabolism [17], which further contributes to the accumulation of acidic metabolites [8].

1.3. Mitochondrial Function and HIRI

Mitochondria are the essential players in the metabolism of eukaryotes. Virtually all of the ATP requirements of the nucleated cell derive from the processes taking place within mitochondria, i.e., the citric acid (Krebs) cycle and OXPHOS. While the Krebs cycle takes place exclusively on the mitochondrial matrix, taking in Acetyl-CoA to generate reducing equivalents (NADH and $FADH_2$), OXPHOS is a process that takes place mostly within the inner mitochondrial matrix (apart from the cytochrome *c* electron transport

in the intermembrane space), where electrons provided by the aforementioned reducing equivalents are transported in energetically favorable leaps along the respiratory chain (from Complexes I and II towards III and then IV) towards a molecule of oxygen, generating water. In tandem with the energetically favorable electronic transport, there is vectorial, the energetically unfavorable (against the concentration gradient) transport of protons from the matrix towards the intermembrane space, thus traversing the mostly proton-impermeable inner membrane. This generates an electrochemical gradient across this membrane (electrical due to the charge disparity, since protons are charged; chemical since protons are what make a solution acid), which is the storage of a tremendous potential energy. This energy is used to create a covalent bond between adenine diphosphate (ADP) and a molecule of ionic phosphate (Pi), generating adenine triphosphate (ATP), the cell's energetic currency. This phosphorylation of ADP takes place at the level of the ATP synthase or complex V, a protonic channel bound to a catalytic head [18].

However, given that OXPHOS heavily relies on electron transport in a biological setting, it is thus expected that some instability is present. In fact, most of the typical cell's reactive oxygen species (ROS) are produced in mitochondria simply as by-products of cellular respiration [19]. First considered as unwanted by-products of mitochondrial ATP generation, ROS are now ubiquitously understood as necessary, given their production does not exceed manageable levels, since mild oxidative stress is a fundamental modulator of redox signaling and the maintenance of adequate defenses and function [20]. However, it is true that excessive, prolonged ROS generation invariably results in oxidative stress, causing impaired mitochondrial function and exacerbated ROS generation, creating a snowball effect of oxidative stress that ultimately might lead to the cell's death. Mitochondria are particularly susceptible to oxidative stress, since several of its components are severely damaged by ROS, such as mitochondrial DNA (mtDNA), which are phospholipids from the membranes or proteic elements of various metabolic pathways. For example, thiol groups within Complex I of the respiratory chain are readily oxidized, resulting in elevated ROS generation due to the mishandling of electron transport [21]. Furthermore, cardiolipin, a hallmark phospholipid of the inner mitochondrial membrane and the element most responsible for this membrane's protonic impermeability (and, as such, for the membrane potential), is composed of highly unsaturated fatty acids, which are also prime targets for oxidation [22]. Furthermore, cardiolipin also has a regulatory role in the function of various enzymes, such as creatine kinase [23]. Metabolism is also obviously affected, and ß-oxidation is particularly susceptible, since increased acylation of proteins due to elevated matrix accumulation of acyl-CoA was found in ischemia/reperfusion events [24,25]. Another metabolic consequence is the depletion of NAD^+, an essential co-factor for numerous metabolic pathways (such as glycolysis and Krebs cycle, just to name a few) and important enzymes such as NAD^+-dependent sirtuins, deacetylases involved in cellular survival. Since OXPHOS is the major syphon for NADH, refreshing the NAD^+ pool, the drastic reduction in NADH consumption helps in this pool's exhaustion [25,26].

Paradoxically, hypoxic conditions appear to create a prime environment for ROS generation, since not all of the oxygen supply is removed, but the shift towards an anaerobic metabolism is a driver for ROS generation, particularly hydrogen peroxide [27]. Figure 1 illustrates this escalation of injury, where damage to mitochondrial function and integrity escalates to tissue damage and, eventually, to organ failure.
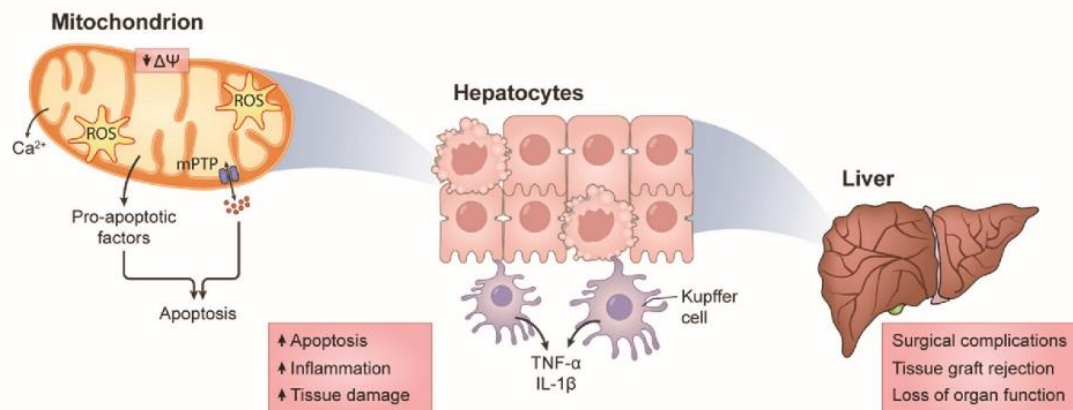
Figure 1. The upscaling of damage in HIRI. Severe compromise to mitochondrial function results in the exacerbated generation of reactive oxygen species (ROS), resulting in the activation of pro-apoptotic protocols such as the opening of the mitochondrial permeability transition pore (mPTP) and release of pro-apoptotic factors, such as ionic calcium (Ca^{2+}). While mitochondrial Ca^{2+} release is markedly lower when compared with other Ca^{2+} sources that could lead to elevated levels (for example, the endoplasmic reticulum or from extracellular sources), the damage to mitochondrial function and integrity will undoubtedly lead to the release of Ca^{2+} and other pro-apoptotic factors. Furthermore, these sources could initiate mitochondrial dysfunction, rather than mitochondrial injury per se. Regardless, given enough replication of these phenomena, cellular survival is at risk, which in turn is a marker for further damage, due to the activation of sterile inflammatory procedures. All these processes, if left unchecked, might result in tissue loss and, eventually, organ failure.

One way by which the cellular mitochondrial population responds and adapts to fluctuating metabolic and biophysics conditions is through the modulation of mitochondrial dynamics, i.e., through alterations in mitochondrial fusion, fission, degradation and biogenesis events. These alterations result in the modification of the mitochondrial bioenergetic capacity, not only through elevated OXPHOS elements' production, but also by more macro alterations, such as reticulation and volume [28,29]. In typical conditions, the mitochondrial network within a cell is found to be highly regulated to serve the cell's needs (for example, mitochondria are typically found in great numbers around the nucleus). However, if an elevated energetic output is necessary, mitochondria usually undertake fission and biogenesis protocols, to elevate organelles' numbers. In tandem, the fission process also allows for the isolation and removal, by intracellular degradation, of incompetent or damaged mitochondrial units or elements, in order to boost energetic production by the mitochondrial unit [30]. Thus, mitochondrial dynamics (i.e., the fluid shape, size and numbers) are paramount to a proper and efficient response to the ever-shifting metabolic environment and to reply to the sometimes quite different cellular necessities.

Of course, all metabolic effects deeply involve mitochondria, whereby virtually all of the cell's ATP needs are produced, given that oxygen is available. Concomitantly, the low O_2 pressure in ischemia all but impedes ATP generation by OXPHOS, which has immediate effects on various cellular processes, such as ion balance which, in turn, help the loss of mitochondrial membrane potential, the driving force for ATP generation in aerobiosis [15,31]. This in turn leads to the induction of what is known as the permeability transition (mPT), where smaller than 1.6 kDa (in high-conductance state) or up to 0.3 kDa (in low-conductance state) solutes can freely cross the mitochondrial membranes, including various pro-apoptotic factors [32,33], in the apparent formation of unspecific pores of dubious, possibly fluid, composition [34].

As expected, low O_2 pressure results in a decreased mitochondrial biogenesis, as hypoxia-sensitive elements such as hypoxia-inducible factors are negative regulators of the peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC-1α), the master regulator of mitochondrial biogenesis [35]. In a similar fashion, the other dynamism

fluxes are also perturbed. At the start of reperfusion, there is a drive for increased fission, due to elevated levels of dynamin-related protein 1 (Drp1) acting in harmony with other pro-apoptotic proteins such as Bax or Bak [36], a mechanism that appears to be heavily related to altered calcium metabolism (Ca^{2+} flux perturbation are discussed in more detail below). Conversely, Mitofusin protein 1 (Mfn1) and the optic atrophy protein 1 (Opa1), which are involved in the mitochondrial fusion processes, are downregulated by oxidative stress in ischemic events [37,38]. As for mitophagy, it is now ubiquitously accepted that this process is severely limited in HIRI, and that addressing it yields protection against pathogenicity [36,39,40].

As previously mentioned, one of the most affected ionic balances is the one pertaining to calcium (Ca^{2+}), a ubiquitous and powerful secondary messenger as well as a cofactor for various enzymes (including some involved in cellular processes of self-degradation). As expected, given these roles, its intracellular levels are highly regulated [41]. Along with the endoplasmic reticulum, mitochondria are the main site of Ca^{2+} storage for a quick and localized release upon specific signaling processes, typically by using the mitochondrial membrane potential ($\Delta\Psi$) as a token for Ca^{2+} accumulation against the gradient [42]. In ischemia, cytosolic Ca^{2+} levels increase (partly due to the diminished $\Delta\Psi$, as mentioned above), resulting in the activation of Ca^{2+}-sensitive enzymes (ex: calpains, calmodulin, protein kinase C, amongst many others), elevated oxidative stress and other deleterious events that typically result in cellular death by apoptosis [43,44].

Cellular death usually leads to the release, to the extracellular milieu, of pro-inflammatory factors that induce an immune response and overall localized aggravation of damage. In the liver, at the start (i.e., during hypoxia), Kupffer cells induce zonal injury via a combination of elevated ROS generation (generated primarily by white blood cells that are chemoattracted to the site such as polymorphonuclear neutrophils [45]) and release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β). These, in turn, lead to the attraction, migration, adhesion and chemotaxis of neutrophils, exacerbating the immune activity in the area [46,47]. However, this process further escalates, particularly after blood-flow restoration (reperfusion), partly due to the established organelle injury suffered, but also caused by the already undergoing cellular injury processes [48]. Of course, if mitochondrial function is compromised by these processes, a further escalation of injury is sure to ensue, and that is why the conservation of mitochondrial capacity and activity might tip the scale towards the survival of the cell against HIRI.

As an example of the responsibility of mitochondria in this survival is their aforementioned role in Ca^{2+} handling. As mentioned, mitochondria are not the only reservoir for Ca^{2+} storage, but simultaneously are one of the most important intracellular calcium storage locations, due to an exchange with membrane potential [49]. As such, the quick and efficient removal of Ca^{2+} from the cytosolic milieu is literally vital for the cell and, as such, mitochondria in prime conditions are mandatory for this effect, not just because of the membrane potential swap, but also because of the effects of Ca^{2+} within mitochondria. In fact, this ion's presence in the organelle's matrix is a major event in OXPHOS and ATP generation, since excess Ca^{2+} can oftentimes lead to mitochondrial rupture and the release of pro-apoptotic signals [49]. As such, mitochondria are a paramount player in intracellular Ca^{2+} metabolism, which is particularly relevant in non-excitable tissues that have poor endoplasmic reticulum (ER) Ca^{2+} clearance capacity and, since mitochondrial Ca^{2+} handling appears to be crucial not in homeostasis, but in situations of stress such as HIRI [42] further implicates mitochondrial function conservation as paramount for the successful recovery from HIRI. Unsurprisingly, mitochondria can be found in high numbers near Ca^{2+} release points, as to better control local ionic concentration, guaranteeing a localized Ca^{2+} effect, rather than a full cellular response, which is typically associated with events of apoptosis [49,50].

Finally, it has also been shown that mitochondria play a role in immune responses, being a central element in the metabolic transition of immune cells in proliferation as well as

in inflammatory signaling [51]. Since the inflammatory process typically requires sustained oxidative stress, it is unsurprising to consider that mitochondria are considered part of the process of inflammation. In fact, their role in mediating and, in some cases, initiating the inflammatory process, was completely unknown until very recently. Recent works have shown that mitochondria actively release some of its DNA molecules (mtDNA), which are powerful pro-inflammatory elements [52–54]. A reason for the apparent recognition of mtDNA as an antigen might rely on the mitochondrial bacterial origin, for mtDNA is markedly over methylated when compared with nuclear DNA [55]. Furthermore, unlike the commonly depicted bacterial-like circular DNA molecule, mtDNA is in fact tightly packed with proteins, one of which is the mitochondrial transcription factor A (TFAM), which various studies have identified as a potent immunostimulator [56,57]. Curiously, there also appears to be a role for oxidative stress in this matter, as only mtDNA with oxidized bases was capable of eliciting an immune response [58]. There are many other ways by which mtDNA can be a pro-inflammatory molecule, and this topic has been excellently revised in previous research [52,55].

NF-kB, a known pro-inflammatory cytokine, has also been shown to be activated by the loss of mitochondrial membrane integrity [52]. The NOD-, LRR-, or pyrin domain-containing protein 3 (NLRP3) inflammasome proteins are ROS-dependently localized to mitochondria [59,60]. In tandem, mitochondria also have several molecules recognized by the NLRP3 inflammasome, such as cardolipin or even the mitochondrial DNA [60–62]. Mitochondria further enhance this inflammasome's activity by the release of various activators (such as, for instance, cytochrome *c* or Smac), causing in turn the release of proinflammatory cytokines IL-1ß and IL-18 [60]. These pathways are apparently carefully preserved in various species, since they are fundamental following bacterial or viral infections [61,63]. However, this should be finely balanced, since these processes are also linked to highly inflammatory processes in pathologic conditions, such as HIRI [64]. There are also numerous evidences of mitochondrial roles in cancer and neurodegeneratory immunological responses (which have been extensively reviewed before [65]).

In summary, since mitochondria have a vital role in a panoply of cellular activities, it is easy to understand how mitochondrial dysfunction is intimately associated with pathological processes. As such, maintaining or restoring mitochondrial homeostasis is a widely studied and desired goal for countering various diseases, such as HIRI.

2. Surgical Approaches in HIRI Mitochondrial Function Compromise

In order to achieve the preservation of mitochondrial function, various surgical practices have been tested and utilized in a clinical setting for over a decade. They mostly rely on a form of damage priming, i.e., the introduction of small bouts of damage through mild, short, controlled events of removal and restoration of blood flow, in a manner that is now recognized as hormetic [30], a concept and phenomenon discussed further down. We discuss the most common ones, explaining their similarities and differences, and how they relate to mitochondrial function preservation.

2.1. Ischemic Preconditioning

Ischemic preconditioning (IPC) is a strategy that has shown promising results in the bench. To achieve hepatic IPC, a clamp or other means of blood flow restriction is used for a small period of time (usually 10–15 min) after which flow is restored by restrictor removal. After a proportional amount of time has passed, the procedure that will generate HIRI is then initiated [66]. While no definitive answers exist as to why IPC is a protective strategy against HIRI, some elements have been confirmed to be crucial, such as ATP levels and, thus, mitochondrial function integrity [67–70]. In tandem with preserved mitochondrial function and integrity, elevated mitophagy [71,72] and prevention of apoptosis are also key events [73,74]. In fact, not just mitophagy is important, but apparently also other cellular components' replacement is a part of the process [75,76]. However, some criticism about the efficiency of this approach exists, because some meta-analyses have concluded that IPC

is not always a guarantee of improved HIRI, since there is simply too much heterogeneity in the human population or, more clearly, in the patients studied [39,69].

It is impossible though to discuss IPC without the introduction of the concept of hormesis and, more specifically, mitohormesis. While previous work has discussed in great detail the intricacies of mitohormesis [30], it should suffice, for this works' purpose, to acknowledge that the mitohormetic process is one where a small injurious event leads to the fairly limited but definitely present injury in mitochondria, in particular in subsets or even subsections of the organelles. These are quickly identified and removed through various mitodynamic processes such as fission and mitophagy, and are replaced by newer, more competent units, thus contributing to a more resilient cell. This is far from a novel concept or even observation, as various reports on the matter have surfaced for at least two decades, involving both intracellular events [77,78] or even the liver's immune system [79], which is apparently also a critical participant.

2.2. Intermittent Clamping

A similar surgical procedure to IPC is known as intermittent clamping (IC). The main difference is the number of occlusion-flow restoration cycles and the timing of performance; whereby IPC is by default just one cycle before the HIRI event, IC consists of various cycles of flow occlusion and restoration, and these are not limited to just the pre-HIRI event. In fact, IC can be spread, even during the HIRI event [66]. In terms of hepatic HIRI, IC has been shown to be superior [80], inferior [81] and even similar to IPC [67]. These differences might fall prey to various causes, of which differences in intervention time is but one of them (different populations intervened, different medical backgrounds, etc.). However, surgery timings are possibly of the most important, for the authors of various studies have concluded that, for shorter interventions, IPC is superior as it improves transaminase levels and surgical complications but, for longer time frames, they are virtually the same procedure [60,63], for which mitochondrial function preservation and cellular ATP levels' maintenance are imperative [82].

A variation of this procedure is ischemic post-conditioning (IPostC), where the bouts of flow restriction/restoration are performed after the main ischemic event, so it acts more like an IC but between the phases of ischemia and reperfusion [83,84]. This method results in virtually the same results as pre-HIRI IC, albeit the lack of time-consuming flow restriction/restoration events to initiate the surgical procedure might be advantageous in certain settings where there is a rush to initiate the surgical event, such as, for instance, transplantation [85,86].

2.3. Remote Ischemic Preconditioning

Another surgical procedure to tackle HIRI is known as remote IPC (RIPC). This is a rather bizarre phenomenon whereby events of ischemia/reperfusion localized in distant organs results in improved liver resistance to HIRI. While much is yet to be understood about this phenomenon, it is undoubtedly a reality, as many works have reported quasi-unbelievable responses of the liver (and other organs as well) to remote IPC events. While the release of protective elements (what those are is still a hotly debated topic) is probably involved and can flow in the circulation, protecting distant tissues, mitochondrial-function protection in the remote tissue/organ is apparently a necessity for RIPC in the liver [87–91].

2.4. Machine Perfusion

A technique that has attracted much attention due to its potential for use in a particular setting (transplantation of less-than-optimal tissue samples) is hypothermic machine perfusion, HMP [92]. This method's intents are to allow for these otherwise rejected organ donations to be reconditioned and to achieve an extension of the preservation time window [86]. In this technique, the organ to be transplanted is perfused in a similar solution to the one used in normal, static cold (4 °C) storage, but with a faster flow. However, for obvious reasons, function testing is not possible, since the idea is to aggressively lower

the organ's temperature. A key modification to this protocol is to oxygenize the perfusion solution, which appears to prevent HIRI in these organs, since ROS generation and inflammation are reduced, while the ATP-generation capacity of mitochondria is salvaged [93]. While normothermic (i.e., core temperature) perfusion is both the more common method and results in lower levels of HIRI, allowing for increased graft viability (up to around 19 h), HMP has the significant advantage to help expand the pool of viable donors to otherwise unusable and certain immunorejection grafts [94].

2.5. Mitochondrial Transplantation

Finally, although it is not a surgical technique as classically defined, the transplantation of mitochondria is a surprisingly effective approach [95,96]. In fact, in HIRI studies, mitochondrial transplantation resulted in improved serum transaminase levels, decreased inflammatory markers and other success indicators, after mitochondria were injected directly into the spleen, from where they migrated towards the injured liver [94,97]. However, there are still many questions regarding this novel approach. It is not clear how mitochondria migrate to, enter the injured cell and start working to replace the damaged ones, but further studies on the matter will certainly help explain this matter.

3. Pharmacological Intervention for HIRI

It is well established that the mechanisms involved in HIRI are of a multifactorial nature, which entails complex signaling pathways. The modulation of protective/deleterious pathways using pharmacological interventions represents an attractive approach in this context, since it is theoretically cheaper and safer than surgical approaches.

Pharmacological preconditioning relies on the administration of specific drugs mimicking the protective mechanisms and biologic effects of IPC. Although the exact mechanism is still not fully understood, it seems that the protection can be achieved, at least in part, by the modulation of well characterized pathways, namely the Reperfusion Injury Salvage Kinase (RISK), the Survivor Activating Factor Enhancement (SAFE), the cyclic guanosine $3',5'$-monophosphate/ Protein Kinase G (cGMP/PKG) as well as a combination of others, including inflammatory, metabolic and mitochondrial factors (for an excellent revision on this matter, please consult [98]).

3.1. Mitochondrial Targeting for HIRI

3.1.1. Natural Compounds

The vast majority of the pharmacological interventions involve the administration of natural (i.e., melatonin [99]), mimetic (i.e., *N*-acetylcysteine, NAC [100]), or metabolism shifting agents (i.e., trimetazidine [101] or indirubin-$3'$-oxime [102]). Several studies have shown the potential role of melatonin in reducing IRI. Zhang and colleagues reported that melatonin diminished myocardial IRI through the improvement of mitochondrial fusion and mitophagy, as well as the activation of AMPK-OPA1 signaling pathways [103]. Melatonin was also found to decrease IRI via the upregulation of the mitochondrial sirtuin 3 and a subsequent reduction in oxidative stress and apoptosis [104]. In addition, melatonin conditioning also alleviated hepatic IRI via the suppression of the induction of mPT [105].

Based on the crucial role of ROS in HIRI pathophysiology, pharmacological interventions aiming to neutralize or modulate its production using antioxidants were one of the first pharmacological approaches attempted. NAC is a precursor of the synthesis of glutathione, the main endogenous ROS scavenger involved in the cellular protection against oxidative stress, as well as directly scavenging pro-oxidant agents with unpaired electrons [100]. Numerous studies have reported the hepatoprotective effects of NAC administration prior to HIRI, especially via the significant reduction of transaminase release [106] and decreased oxidative stress, resulting in diminished apoptosis and autophagy [107,108].

Trimetazidine (TMZ) is a piperazine derivative that has been used as an anti-ischemic agent. The prevention of ROS production, mitochondrial damage (such as protein and phospholipid oxidation, to name a few) and decreased ATP levels are thought to be im-

portant targets by which TMZ exerts its cytoprotective effect [109], for while TMZ is not an antioxidant per se, its action in metabolic modulation results in elevated antioxidant enzyme activity [110]. The presence of TMZ confers a reduction in hepatic injury and an improved fatty liver functionality after ischemia/reperfusion, through the activation of AMPK and subsequent increase of nitric oxide levels [111].

The pharmacological preconditioning with indirubin-3′-oxime was demonstrated to protect the liver against HIRI by preserving mitochondrial function and hepatic energetic balance [112]. This compound inhibits GSK-3β and, consequently, prevents cyclophilin D phosphorylation by GSK-3β. Since cyclophilin D serine residues' phosphorylation leads to mPT induction [112,113], its inhibition modulates the susceptibility to mPT induction, thus preserving mitochondrial function following HIRI.

3.1.2. Synthetic and Directed Agents
OXPHOS Elements Manipulation

Succinate accumulation has been heavily implied in HIRI [114]. By using succinate dehydrogenase (OXPHOS Complex II) inhibitors such as malonate, succinate accumulation is prevented and thus ischemic injury is reduced [114]. Similarly, Complex I inhibitors have been investigated. It is true that a wide panoply of mitochondrial-beneficial agents have Complex I inhibitory capacity (ex: Metformin, Berberine, to name a few) but those appear to rely on mitohormetic effects; here, other types of molecules such as MitoSNO (mitochondrial-specific S-nitrosating agent) or Amobarbital have proven protection in ischemia/reperfusion [115,116], effects that might depend on diminished pro-apoptotic factors' release for the prevention of retrograde electron flow and thus increased ROS generation [36].

Mitochondrially Targeted Antioxidants

Unlike the aforementioned NAC, which has a wider range of activity than just mitochondria, other antioxidants specifically designed to target mitochondria have already been tested for the same reasons. For instance, Coenzyme Q_{10} (or ubiquinol) is not only a member of the respiratory chain but also a potent antioxidant, which has proven to be able to prevent HIRI [117,118]. MitoQ and SkQ1 are other mitochondrial-specific antioxidant molecules, modelled after the widely known tetraphenylphosphonium (TPP^+), a molecule that freely traverses the inner mitochondrial membrane and, as such, this property was explored by various researchers to deliver antioxidant agents to the mitochondrial matrix [119,120]. Other classes of compounds, with different antioxidant roots and delivery alternatives exist and most have shown similarly effective results, such as Bendavia (a Szeto-Schiller peptide [121]), MitoGSH [122], or Euk-8 [123] although not all exactly on HIRI, but on other tissues/organs, which opens the possibility for more studies.

NAD^+ Metabolism

As previously mentioned, NAD^+ is an essential co-factor to many metabolic reactions, and in low abundance in HIRI. To further complicate matters, NAD^+ transport across biological membranes is a very complicated procedure, and oftentimes impossible although in recent years some transporters have been identified [124]. As such, most studies have focused on more mobile NAD+ biosynthesis precursors nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN). Their use boosts the cell's NAD^+ pool, a feature already explored in various works [125,126].

PPAR Agonists

Another strategy that is not immediately intuitive is the use of agonists of the nuclear receptor family of peroxisome proliferator-activated receptor. For instance, fibrates that directly activate PPARα, a commonly used antidiabetic class of drugs, have been shown to attenuate HIRI [127]. Even more striking is the fact that the PPARγ agonist pioglitazone

has very interesting anti HIRI potential [128]. It is far from clear how this class of molecules achieve these results, but so far, a purely antioxidant effect has been shown [36].

Mitochondrial Dynamics Modulators

As previously discussed, mitochondrial dynamics play a significant role in HIRI. As such, it was only logical to target these processes to discover potential clinical avenues. In fact, just by boosting mitochondrial biogenesis it was possible to prevent damage associated with HIRI. Various works on this matter used different strategies to achieve this (examples are by using the AMP-activated kinase, AMPK, agonist AICAR [129], or by using the recently identified hormone irisin [130], or even using natural compounds such as the flavonoid nobiletin [131]). Regardless of the strategy, the result was always the increased PGC-1α activity, thus increasing mitochondrial numbers and preventing the drop of ATP in HIRI.

If by increasing mitochondrial levels these works demonstrated a prevention of HIRI, the same can also be said when blocking mitochondrial fission. By targeting the dynamin-related protein 1 (Drp1) translocation to mitochondria with Mdivi-1, a quinazolinone that is commonly used to inhibit fission, Yu and colleagues were able to alleviate mitochondrial fragmentation and reduce apoptosis [132]. This reduction in mitochondrial fission was also found and associated in a previously mentioned work by Bi and collaborators [130]. Since mitochondrial fission is a hallmark of post-ischemic injury, the prevention of excessive fission appears to be a fairly efficient strategy to reduce HIRI.

However, not all mitochondria are unscathed in HIRI, and their removal and replacement by non-injured ones could be essential for surviving this phenomenon. The mechanistic target of rapamycin (mTOR) is a rapamycin-sensitive central regulator of the metabolism, and its activity has been negatively associated with mitophagy [133]. As such, it is unsurprising that rapamycin has been reported to boost mitophagy and substantially prevent HIRI [134]. Similarly, the silencing of the *park7* gene that codes for the protein deglycase DJ-1 also protects the liver against HIRI by boosting mitophagy [135]. Comparatively, the augmenter of liver regeneration (ALR) is an anti-apoptotic protein primarily found in mitochondria, which has been found to boost mitophagy by driving up the expression of mitofusin 2, protecting against HIRI [71]. Finally, boosting mitophagy with natural compounds is also analogously efficient, since the polyphenol pterostilbene has also been found to protect the liver against HIRI by upregulating the Parkin-PINK1 pathway of mitophagy [136].

mPT Inhibiton

Even though taking different routes, many of the pathways involved in HIRI culminate in critical events, and the most well recognized among them is the mPT pore opening. Aside from the already mentioned natural compounds whose activity results in mPT inhibition, several other agents that achieve this same goal were tested. In fact, the widely known mPT pore opening inhibitor Cyclosporin A has been known to protect the liver against HIRI for decades [137,138]. Other molecules, similar to Cyclosporin A in function but without some of its more undesirable side effects (such as immunosuppression) have also been tested, such as Sanglifehrin or NIM811 [139,140].

These are just a few examples of intense research on the field of mitochondrial-function manipulation to combat HIRI. However, no definitive pharmacological agent has proven to be a widespread, safe and effective clinical agent. Thus, the development of new, more efficient and safe pharmacological strategies aiming to improve mitochondrial function might be a viable approach to mitigate the hepatic damage underlying HIRI.

3.2. Mitochondrial Aldehyde Dehydrogenase 2 as a Therapeutical Target

Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial enzyme, mostly expressed in the liver, where it plays a pivotal role in ethanol metabolism [141]. In addition, ALDH2 is also involved in the clearance of toxic aldehydes originated from the lipoperoxida-

tion of mitochondrial and plasma membranes, mainly 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), under oxidative stress conditions.

Furthermore, Alda-1 is a well-known ALDH2 activator, and several reports have demonstrated a significant improvement against IRI in the presence of Alda-1 in various types of organs, including heart, brain, lung, kidney and intestine [142–144].

Recently, Li and colleagues reported the protective effect of Alda-1 against HIRI in mice [145]. The authors suggested that the protective mechanism was related to the clearance of reactive aldehydes (decreased accumulation of 4HNE and MDA) and autophagy enhancement by AMPK activation. These results suggest that Alda-1 pretreatment could increase ALDH2 activity, which in turn scavenges reactive aldehydes.

In accordance, Alda-1 pretreatment protected the liver in a rat model of HIRI, resulting in decreased hepatic enzyme release, oxidative stress and inflammation, through autophagy enhancement which might be dependent on the AKT/mTOR and AMPK signaling pathways [146]. Moreover, reduction in the liver mitochondrial damage and attenuation of hepatocyte apoptosis were also observed.

These results demonstrate that ALDH2 might be a possible target for pharmacological strategies in a context of HIRI in clinical practice.

3.3. Polyethylene Glycol: A New Promising Approach

Polyethylene glycols (PEGs) have shown multiple benefits in cell and organ preservation, including antioxidant capacity, edema prevention and plasma-membrane stabilization [147]. Besides being widely used as oncotic agents in preservation and rinse solution [148], a sizeable body of literature have been demonstrating the beneficial effects of PEGs against IRI by promoting the protection of mitochondria and cytoskeleton [98]. In addition, both in vitro and in vivo studies demonstrated that the high molecular weight PEGs can reduce cytokine production and neutrophil activation [149,150].

Furthermore, PEG35 has been linked to increased levels of ALDH2 and improved mitochondrial machinery, diminishing the ischemic injury [151,152]. Recently, Bardallo and colleagues demonstrated an improved hepatic protection against an ischemic insult by the reduction of oxidative stress through ALDH2 upregulation and the consequent promotion of cytoprotective autophagy in a PEG35-dependent manner [153].

PEG preconditioning was also reported to protect cardiomyocytes from hypoxia and reoxygenation-induced cell death. The protective effect is suggested to be linked to PEG's capacity to decrease ROS production and lipoperoxidation, which in turn leads to membrane stabilization and consequent maintenance of cell integrity and inhibition of apoptotic cell death. PEG is capable of sealing and progressively eliminate membrane disruptions [154]. Still, some PEG molecules can pass through the membrane openings and interact with the mitochondrial membrane, preventing the induction of mPT [155]. Consequently, there is a reduction in mitochondrial swelling, which allows for the maintenance of mitochondrial membrane potential and inhibition of cytochrome *c* (Cyt. *C*) release and subsequent apoptosis induction. Furthermore, PEG postconditioning improved myocardial protection in an in vivo model of rat hearts subjected to 1 hour of artery occlusion followed by either 48 hours or 4 weeks reperfusion [156]. The mortality within the first 24 hours had significantly better outcome in the presence of PEG, as 40% of the animals died in non-PEG group against only 10% in the PEG-treated rats.

In the liver, PEG35 preconditioning ameliorated hepatic injury and protected mitochondria in a rat model of cold ischemia followed by warm reperfusion (4 and 37 °C, respectively) [157]. The protective effect was associated with the stimulation of the pro-survival pathway via AKT phosphorylation as well as the activation of two important cytoprotective mediators, e-NOS and AMPK. Bejaoui and colleagues have also shown PEG35 hepatoprotective effects against warm HIRI. Additionally, PEG35 preconditioning efficiently decreased transaminase levels and maintained hepatocyte morphology as well as preserved mitochondrial membrane potential. The activation of the pro-survival kinase

Akt and the cytoprotective factor AMPK and the inhibition of apoptosis were the protective mechanisms correlated with PEG35 protection in this study [158].

In summary, PEG35 might be considered a suitable pharmacological agent against HIRI in a clinical setting. In fact, PEG is already in use in various formulations of hepatic preservation solutions and, more specifically, PEG35 is also showing fast-tracking potential [159].

4. HIRI and Transplantation

The advances in surgical techniques and immunosuppressive drugs have established liver transplantation as a standard mainstay curative therapy in end-stage liver disease. Organ transplantation is a complex procedure associated with multifactorial reasons which may end up in graft failure, such as donor factors (i.e., age, steatosis, or donation after death), organ retrieval and preservation (cold and warm ischemic times), and the transplantation procedure itself, including surgeon expertise as well as possible surgical complications [160].

The gap between the number of patients waiting for liver transplantation and the number of available organs has drastically increased (due to increased demand), leading to an urgent need for the expansion of the donor pool to match the growing need for organs. In this sense, the possible use of suboptimal or marginal organs as a viable approach to augment the number of available organs for transplantation has been highly encouraged. However, this expanded criteria donor (ECD) organs are well known for presenting a higher vulnerability against HIRI and their use increases primary nonfunction and compromises graft outcome after transplantation [161]. Therefore, the challenge in the transplantation community is look for new therapeutic strategies to minimize factors that overall render the organ non-functional and at the same time, increase the use of marginal livers for transplantation instead of discarding them.

The use of cold preservation solutions is the main technique in organ transplantation to maintain the morphological and functional integrity of the graft. It has the purpose of reducing, as much as possible, factors that compromise graft quality, especially the ones implicated in HIRI and the complications deriving from it. The composition of a preservation solution will dictate the quality and duration of graft preservation, through the prevention of energy depletion, acidosis, edema and oxidation, among others [162–165].

Throughout the transplantation process, the liver faces events of warm, cold and rewarming ischemia which lead to organ damage. Briefly, when the organ is retrieved from the donor and stored within the preservation solution under hypothermic (4 °C) conditions, it undergoes a period of cold ischemia. Once removed from cold storage, the graft is exposed to warm (22–25 °C) ischemia before the beginning of reperfusion into the recipient. Consequently, the cumulative injury resulting from the combined action of ischemia and cold preservation must be minimized in order to achieve the maximal possible recovery of the graft's function after liver transplantation.

Currently, there are two main strategies for organ preservation, namely static and dynamic. Static Cold Storage (SCS) consists of organ preservation at low temperatures (0–4 °C) to reduce metabolic activities and consequently cellular damage [166]. Hypothermia is well known to significantly decrease cellular metabolic rates and, on average, oxygen and glucose requirements decrease up to 8% for every degree of temperature lowered [167]. Consequently, cold storage intends to delay the depletion of ATP levels and slow down the deleterious processes associated with ischemia. In SCS, the organ is perfused with a cold solution and is afterwards statically stored in a container filled with the cold preservation solution while waiting for transplantation. Regarding dynamic perfusion, the retrieved organ is placed in a chamber, in which it is continuously perfused with either an oxygenated or non-oxygenated solution using a machine perfusion (MP) pump, as previously mentioned. The continuous perfusion allows for a better distribution of the preservation solution throughout the graft, as well as blood washout, continuous delivery of oxygen and nutrients and toxic metabolites clearance and, as a result, a better outcome [168,169].

In addition, this technique also allows for a real-time monitorization of the functional and biochemical performance of the graft as well as the possibility of the application of a pharmacologic agent [170]. MP can be performed under different temperatures, including Hypothermic Machine Perfusion (HMP), Normothermic Machine Perfusion (NMP), Subnormothermic Machine Perfusion (SNMP) and Hypothermic Oxygenated Perfusion (HOPE), as previously discussed [171].

4.1. Static Cold Preservation

The maintenance of organ viability during cold storage is of utmost importance to a successful outcome after liver transplantation. Preservation solutions were initially developed decades ago to minimize graft injury during cold storage. The first preservation solution was developed by Collins and co-workers in 1969 and intended to mimic the intracellular composition and protect the intracellular spaces during the onset of ischemia [172]. A few years later, the University of Wisconsin (UW) solution was developed and has since been considered the gold standard for hepatic preservation solutions. However, its high potassium concentration induces cellular depolarization as well as vasoconstriction, which impairs the organ perfusion during washout and reperfusion [173]. In addition, one of its components, the oncotic agent hydroxyethyl starch (HES), has been associated with red blood aggregation, macrophage invasion and tubular damage [174,175].

The replacement of HES for another oncotic agent (PEG20 kDa) in the UW solution was reported to improve rabbit-heart performance after 24 h preservation [176]. In addition, liver grafts stored in UW solution and then rinsed with a solution containing PEG35 showed reduced hepatic injury and improved liver function after reperfusion [148]. The better outcome was associated with the prevention of oxidative stress, mitochondrial damage and liver autophagy.

The beneficial effects of PEGs have been known for decades. Back in the 1970s, Daniel and Wakerley demonstrated increased cellular viability using PEG 20 kDa during the cold preservation of renal pig cells [177], whereas lower molecular-weight PEG (6 kDa) protected myocardium from cellular edema and membrane damage during preservation [178]. Since then, several studies have demonstrated the protective role of different molecular weight PEG during cold preservation using different animal models and the satisfying results obtained led the use of PEG to a clinical level. In France, a solution similar to UW solution was developed in the beginning of the century: the Institute Georges Lopez (IGL)-1 solution. Contrary to UW solution, IGL-1 is characterized by high sodium and low potassium concentrations and the presence of PEG35 as a colloid [142], and it has been considered as a suitable alternative to UW solution by the European Liver Transplant Registry [179] due to and its efficiency in the preservation of abdominal organ [180–182].

Liver grafts preserved in IGL-1 solution have been found to possess increased ALDH2 expression and activity, which in turn were associated with reduced mitochondrial damage and ATP breakdown prevention [152]. In accordance, the presence of PEG35 in the IGL-1 solution was reported to be a crucial agent in mitochondria preservation and in the reduction of hepatic injury, when compared liver graft preservation using IGL-1 and IGL-0 (same composition than IGL-1, but without the presence of PEG35) solutions [159]. Accumulating evidence demonstrates the efficacy of PEGs in upregulating cell-survival pathways in distinct tissues, resulting in mitochondria and cellular membrane protection and the prevention of ROS production and cell swelling [149,156,157,183,184]. Despite IGL-1's beneficial effects, the underlying protective mechanisms are rather complex and not completely understood. They may include cytoprotective mechanisms exerted, at least in part, by the upregulation of ALDH2 and subsequent activation of AMPK (cytoprotective factor), Nitric oxide (NO) generation (vasodilator agent), as well as the aforementioned mitochondrial protection, among others [152,185].

Recently, a new IGL solution (IGL-2) was designed, and the main differences to the previous IGL solution are a higher concentration of PEG35 and glutathione. In order to understand the direct role of PEG35, Bardallo and colleagues evaluated liver graft

preservation using 3 IGL solutions, IGL-0, IGL-1 and IGL2, containing 0 g/L, 1 g/L and 5 g/L of PEG35, respectively. The authors found that the presence of PEG35 seemed to be a major factor in preventing hepatic injury in a dose-dependent manner. In addition, a superior antioxidant capacity of IGL-2 solution against ischemic insult during liver graft preservation was also observed, most likely through ALDH2 upregulation and the subsequent promotion of cytoprotective autophagy [153]. The main characteristics and components of the discussed and more common preservation solutions are detailed in Table 1.

Table 1. Static Cold Storage and Dynamic preservation solutions compositions.

Components	UW	IGL-1	IGL-2	Celsior	Belzer-MPS
K^+ (mmol/L)	125	25	25	15	25
Na^+ (mmol/L)	27	125	125	100	120
Mg^{2+} (mmol/L)	5	5	5	13	5
SO_4^{2-} (mmol/L)	4	5	5	-	5
Ca^{2+} (mmol/L)	-	0.5	-	0.25	0.5
Cl^- (mmol/L)	-	-	-	40	-
Zn^{2+} (mmol/L)	-	-	0.091	-	-
Diphosphate (mmol/L)	25	25	25	-	25
HEPES (mmol/L)	-	-	-	-	10
Histidine (mmol/L)	-	-	30	30	-
Raffinose	-	30	-	-	-
Mannitol (mmol/L)	-	-	60	60	30
Lactobionic acid (mmol/L)	105	100	80	80	-
Dextrose (mmol/L)	-	-	-	-	10
Ribose (mmol/L)	-	-	-	-	5
Gluconate (mmol/L)	-	-	-	-	85
Hydroxyethyl starch (g/L)	50	-	-	-	50
Polyethylene glycol 35 (g/L)	-	1	5	-	-
Glutathione (mmol/L)	3	3	9	3	3
Allopurinol	-	1	-	-	-
Adenosine (mmol/L)	5	5	5	-	-
Glutamic acid (mmol/L)	-	-	-	20	-
Adenine (mmol/L)	-	-	-	-	5
$NaNO_2$ (nmol/L)	-	-	50	-	-
pH	7.4	7.4	7.4	7.4	7.4
Osmolarity (mosmol/L)	320	320	320	320	320

The concentration of some components may vary among manufacturers.

4.2. Dynamic PreservationRe

As previously mentioned, the need for organs is constantly increasing and thus, the use of novel techniques for optimizing suboptimal graft preservation is of utmost importance. Machine perfusion devices are not a novel technology, but were scarcely used for organ preservation, mainly due to logistical issues. Nowadays, with the advancement in innovation and technology, their design is more portable and efficient, and consequently, provides a more promising therapeutic strategy for graft preservation.

As mentioned above, oxygen and mitochondria play an important role during HIRI. Hepatic perfusion in HOPE benefits in great extent of the oxygenation of the perfusate, which is responsible for maintaining the integrity and function of the mitochondrial population [186]. In this review, we will only address the dynamic preservation under HOPE conditions. As for other modes of dynamic organ preservation (HMP, NMP, SNMP), an excellent work has been previously published [187].

MP demonstrated to be more protective than static cold storage in a comparison study of DCD (donation after circulatory death) liver grafts [188]. HOPE combines the benefits of cold preservation conditions with active oxygenation of the perfusate, enabling graft's mitochondria the capacity to produce and restore ATP to levels similar to before reperfusion, which significantly increase within the first hour of perfusion [189]. Moreover,

cold oxygenation also reduces mitochondrial ROS release, triggering less oxidative damage in mitochondria in early reperfusion [190]. Furthermore, the accumulation of some metabolites such as succinate, during the ischemic period have been reported to provoke mitochondrial dysfunction on several tissues [191]. Hence, the removal of such metabolites by the dynamic flow observed in HOPE might be an important way to increase the odds of a proper mitochondrial function during early normothermic reperfusion.

Kron and colleagues reported the protective effects of HOPE in fatty liver grafts in rats and humans [192]. SCS, followed by 1h HOPE treatment was found to protect the liver from initial ROS generation and DAMPs release after transplantation, as well as decreased activation of inflammatory pathways. Moreover, recovery of ATP levels prior to reperfusion and reduction of cell death during reperfusion have also been reported after the HOPE period [193].

Belzer Machine Perfusion Solution (Belzer MPS) and its generics, which are a variation of the original UW solution used in the SCS, are the most commonly used perfusion solution for HOPE. However, the use of Belzer MPS in liver machine perfusion has some limitations. For instance, given its high viscosity, Belzer MPS may lead to sinusoidal shear stress, which can induce the destruction of the glycocalyx of hepatocytes [194]. Glycocalyx comprises the thin luminal sugar monolayer that protects the graft endothelia and its damage has been related to graft injury and function in clinical liver transplantation [195,196].

As previously mentioned, HOPE has a proven effect in mitochondrial protection in dynamic preservation, while PEG35 has been demonstrated to protect mitochondria in static preservation. In this sense, the new IGL-2 solution has been proposed as the perfusate for HOPE. A comparison study using Belzer MPS and the new IGL-2 solution for 1h of HOPE after SCS in a rat model, revealed a significant prevention of mitochondrial damage in the IGL-2 HOPE conditions. The authors suggested that IGL-2 could be a suitable tool in HOPE strategies, especially for rescuing vulnerable liver grafts such as the steatotic ones for transplantation [159]. These procedures are represented in Figure 2.
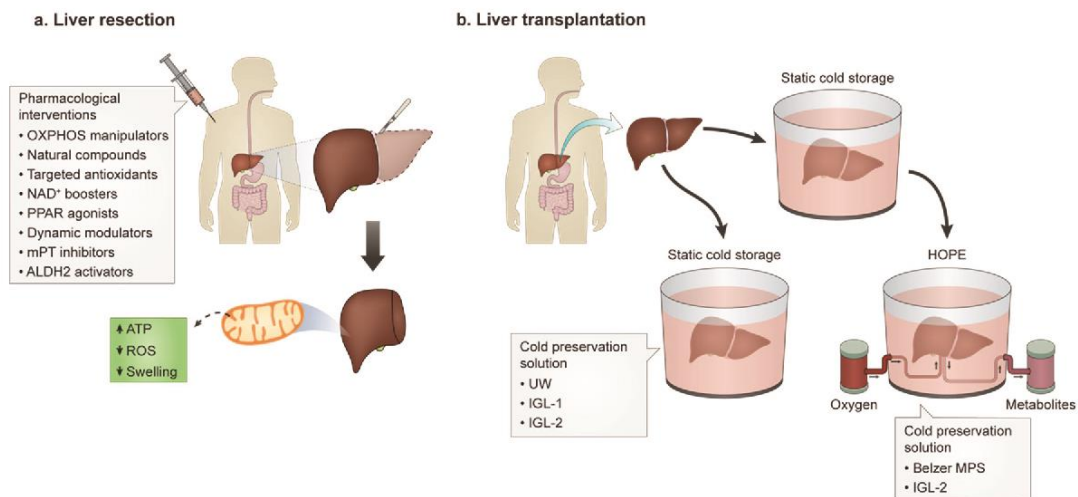
Figure 2. Interventions towards improved hepatic survival in liver surgery upon HIRI. (**a**) in liver surgeries where warm ischemia is required (for instance, in liver resection surgeries), preconditioning with mechanical (i.e., blood flow occlusion and restoration) or with pharmacological agents have been tested both in the bench and the bedside, with promising results. Of note is that, by directly or indirectly targeting mitochondria for function preservation, these strategies can make up for the difference in between failure or preservation and restoration of hepatic function, size and proficiency. (**b**) On the other hand, where cold ischemia is required (i.e., transplantation), new developments in preservation solutions have both increased the time window for which the organ is still in usable form, and also expanded the pool of organs that can be used, by virtue of the massively improved preservation protocols.

5. Concluding Remarks

It is now clear that many of the cellular and mitochondrial events in HIRI are also present in other organs/tissues subjected to a similar event. This is extremely encouraging and helpful, as it means that virtually all of the research conducted in this field of ischemia/reperfusion is highly transversal between organs, saving both time and resources [98]. From all of the explored literature, patterns of intervention begin to emerge, which have been mentioned in this work. Most strategies would thus benefit from a combination of the main goals of the works discussed here, ranging from reduced oxidative stress from the earliest possible time to the elevation of mitochondrial numbers, activity and resilience.

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Review

Liver Graft Hypothermic Static and Oxygenated Perfusion (HOPE) Strategies: A Mitochondrial Crossroads

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Abstract: Marginal liver grafts, such as steatotic livers and those from cardiac death donors, are highly vulnerable to ischemia–reperfusion injury that occurs in the complex route of the graft from "harvest to revascularization". Recently, several preservation methods have been developed to preserve liver grafts based on hypothermic static preservation and hypothermic oxygenated perfusion (HOPE) strategies, either combined or alone. However, their effects on mitochondrial functions and their relevance have not yet been fully investigated, especially if different preservation solutions/effluents are used. Ischemic liver graft damage is caused by oxygen deprivation conditions during cold storage that provoke alterations in mitochondrial integrity and function and energy metabolism breakdown. This review deals with the relevance of mitochondrial machinery in cold static preservation and how the mitochondrial respiration function through the accumulation of succinate at the end of cold ischemia is modulated by different preservation solutions such as IGL-2, HTK, and UW (gold-standard reference). IGL-2 increases mitochondrial integrity and function (ALDH2) when compared to UW and HTK. This mitochondrial protection by IGL-2 also extends to protective HOPE strategies when used as an effluent instead of Belzer MP. The transient oxygenation in HOPE sustains the mitochondrial machinery at basal levels and prevents, in part, the accumulation of energy metabolites such as succinate in contrast to those that occur in cold static preservation conditions. Additionally, several additives for combating oxygen deprivation and graft energy metabolism breakdown during hypothermic static preservation such as oxygen carriers, ozone, AMPK inducers, and mitochondrial UCP2 inhibitors, and whether they are or not to be combined with HOPE, are presented and discussed. Finally, we affirm that IGL-2 solution is suitable for protecting graft mitochondrial machinery and simplifying the complex logistics in clinical transplantation where traditional (static preservation) and innovative (HOPE) strategies may be combined. New mitochondrial markers are presented and discussed. The final goal is to take advantage of marginal livers to increase the pool of suitable organs and thereby shorten patient waiting lists at transplantation clinics.

Keywords: liver graft preservation; AMPK; succinate; ALDH2; glycocalyx

1. Introduction

In liver transplantation, graft quality is the key factor that determines procedural success and long-term survival and outcome, but this depends not only on its intrinsic performance but also on the time and quality of preservation and transportation from donor to recipient implantation. At Christmas in 1971, Belzer decided to ship a recovered

kidney in San Francisco (USA) to the Leiden transport for transplantation. After 37 h of in-flight preservation, the kidney was successfully transplanted into a 42-year-old truck driver with polycystic kidney disease, as planned [1,2].

Since the first investigations initiated by Belzer et al. [1,3–7], much progress has been made in the preservation of liver grafts [8–10]. The actual challenge in liver transplantation is organ scarcity and the pressing need for liver transplantation, which have led physicians to use marginal livers, such as steatotic livers, to increase the organ pool for transplantation [11–14]; however, its high vulnerability against ischemia and reperfusion injury [14–16] due to microcirculation exacerbated by fat accumulation in hepatic sinusoids could compromise graft viability and further outcomes with post-transplantation problems [17–19].

Cold static preservation is a mandatory step characterized by oxygen deprivation to the graft that provokes ischemic damage, leading to mitochondrial integrity changes and energy metabolism breakdown alterations that affect fatty liver graft integrity during cold storage in experimental settings. Fatty livers exhibit reduced tolerance against ischemic events, with further reduced ATP levels and greater injury levels when compared to nonsteatotic livers [20,21]. However, the cold ischemic time accepted for clinical purposes is 12 h [22,23], considered suitable against the apparition of "primary nonfunction", graft failure, and patient death, along with reduced long-term graft survival [22].

The mechanism of mitochondrial metabolic changes upon cold ischemia is relatively well known [24], as shown in Figure 1. During prolonged cold graft storage, the oxygen deprivation conditions allow diminishing the metabolic demands, and consequently, the mitochondrial respiration complexes are seriously altered, leading to succinate accumulation at the end of ischemia [24]. Succinate dehydrogenase (SDH) activity drives a significant portion of ROS generation, and it has been demonstrated that SDH inhibition by malonate is protective against reperfusion injuries. Notably, itaconate inhibits SDH in a dose-dependent manner, leading to succinate accumulation [25,26], and its exogenous administration modifies the host response to ischemia–reperfusion that is sufficient to suppress reperfusion-related injuries [24–28].

The accumulation of succinate is partly responsible for controlling graft injury after oxygenation [24–28], and consequently, the ability of itaconate to inhibit succinate dehydrogenase in complex II may also play a determining role, reducing the initial succinate metabolism after earlier oxygenation before reperfusion conditions (Figure 1) [24–28]. Moreover, the longer the ischemic period lasts, such as during liver graft cold storage, the more compromised the mitochondrial antioxidant electron transport chain (ETC) system, depleting substrates such as glutathione, which render the cells more susceptible to oxidative stress at reperfusion after graft revascularization.

With this in mind, it seems necessary to envisage new directions in graft cold static preservation strategies [8,29], possibly in combination with newer techniques developed, such as hypothermic oxygenated perfusion (HOPE) using machine devices [30,31], for better protection from harvest to revascularization. New protective strategies to increase graft protection are urgently needed to take advantage of marginal grafts and increase the donor pool to reduce waiting lists for liver transplantation. This review covers different aspects of hypothermic liver graft preservation as follows: cold ischemic insult and liver graft cold storage are discussed in Section 2; mitochondrial protection and organ preservation solutions in static cold storage are discussed in Section 3; new additives for improving cold static preservation are discussed in Section 4; HOPE, mitochondrial protection, glycocalyx preservation, and PEG35 effluents (IGL-2) are discussed in Section 5; and (5) some considerations and concluding remarks are presented in Section 6.
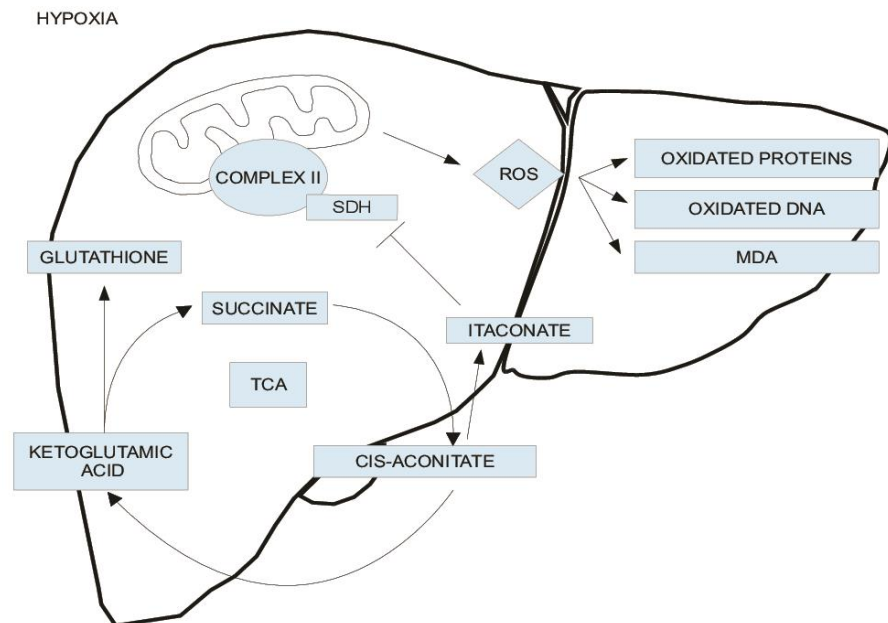
Figure 1. Intracellular mechanisms of ischemic injury. The lack of oxygen to the preserved graft during cold storage provokes a mitochondrial switch to anaerobic metabolism with the interruption of electron flow and mitochondria machinery, the accumulation of interacting energy metabolites succinate and itaconate, and rapid adenosine-triphosphate breakdown. These events that occur during graft cold preservation are modulated by organ preservation solutions. When oxygen is reintroduced under hypothermic oxygenated perfusion and/or with further normothermic conditions, mitochondria re-establish the electron flow with rapid and suitable consumption of the accumulation of succinate at the end of cold ischemia. Subsequent release of reactive oxygen species from complex I occurs. Mitochondria and energy breakdown appear, therefore, as the main targets not only to improve graft quality but also to identify a valid biomarker to predict the "graft status" after static cold storage in organ preservation solutions just before transplantation procedures.

2. Cold Ischemic Insult and Liver Graft Cold Storage

Crucial adverse consequences for liver graft during cold storage due to deprived oxygen conditions have led us to use organ preservation solutions such as UW, HTK, Celsior, and IGL-1 [8,29]. UW and IGL-1 solutions are characterized by an oncotic agent in their composition such as hydroxyethyl starch (HES) in UW and PEG35 in IGL-1 [8,29], in contrast to HTK and Celsior, which do not contain an oncotic agent in their formulations [8]. Moreover, IGL-2 showed higher concentrations of PEG35 and glutathione compared to IGL-1. In clinical transplantation, IGL-1, Celsior, and HTK were demonstrated to be suitable alternatives to UW (gold-standard reference), although some limitations for HTK were recently described in the European Liver Transplantation Register (ELTR) [32].

Since 2006, we have investigated the protective mechanisms exerted by polyethylene glycol 35 (PEG35) in IGL-1 solution, which was found suitable for fatty liver graft preservation [33]. In this line, the presence of PEG35 in IGL-1 [33] and rinse solution [34] was a determinant for protecting the graft against the deleterious effects accumulated from organ recovery and washout up to graft cold storage and the following reperfusion [33,34]. This confirms that PEG35 is a useful tool for preventing cold IRI associated with transplantation [35]. The benefits of PEG35 in rinse and IGL-1 solutions are mainly associated with the prevention of mitochondrial damage, activated protective cell signaling mechanisms such as AMPK (energy sensor), and nitric oxide generation through constitutive eNOS activation [33,34,36,37].

With this in mind, we recently proposed IGL-2 [38] in order to enhance fatty liver graft protection by preserving liver damage with mitochondrial integrity inherent in oxygen de-

privation conditions during static cold graft storage. As shown in Figure 2, IGL-2 protected liver injury more efficiently (measured as AST/ALT transaminases) and mitochondrial damage (measured as GLDH) than UW and HTK solutions.
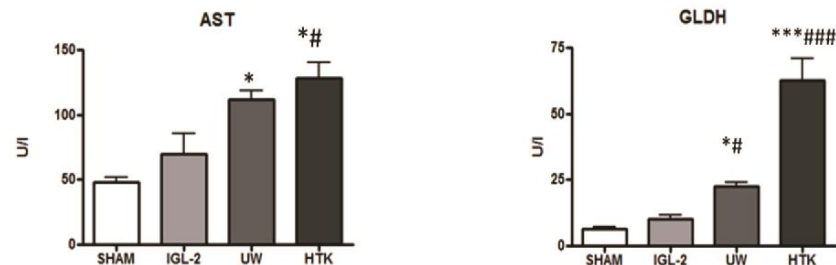
Figure 2. Liver injury and mitochondrial integrity of fatty liver graft preserved in IGL-2, UW, and HTK solutions (24 h; 4 °C). The bars represent the mean values $\pm$ SEM of each group (n = 4–6). Differences are shown comparing groups (* vs. Sham, # vs. IGL-2) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; three symbols indicate $p < 0.001$).

3. Mitochondrial Protection and Preservation Solutions in Static Cold Storage

ATP energy metabolism breakdown is inherent to oxygen deprivation conditions during graft cold storage. As shown in Figure 3, noticeable prevention of ATP breakdown after 24 h cold storage occurred when IGL-2 was compared to UW and HTK.
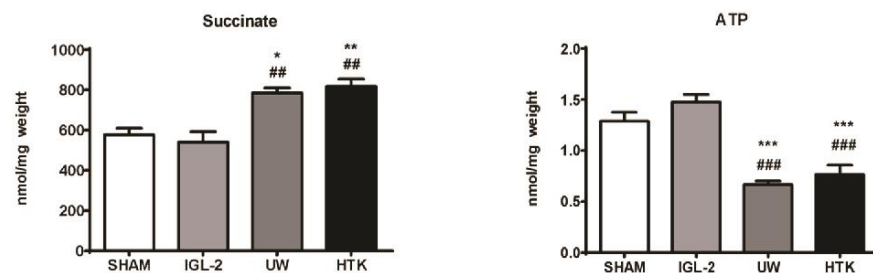
Figure 3. Succinate accumulation and ATP breakdown in fatty livers preserved in IGL-2, UW, and HTK solutions (24 h; 4 °C). The bars represent the mean values $\pm$ SEM of each group (n = 4–6). Differences are shown comparing groups (* vs. Sham, # vs. IGL-2) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

Moreover, ATP energy metabolism breakdown is associated with alterations in mitochondrial respiration complexes that occur as a consequence of oxygen deprivation, forcing the cell to adapt to anaerobic metabolism that leads to the accumulation of metabolites such as succinate (Figure 1), which will partly control graft damage when oxygen restoration occurs after reperfusion [24]. As shown in Figure 3, IGL-2 solution was capable of limiting succinate accumulation in a more efficient way compared to UW and HTK. The presence of PEG35 and glutathione in IGL-2 contributed to modulating succinate accumulation at the end of cold ischemia. Other metabolites such as itaconate, which accumulated at the same time, may partially contribute to inhibiting succinate dehydrogenase (SDH) during earlier stages of reperfusion [26–28]. Consequently, both succinate and itaconate levels accumulated at the end of the cold storage period will have an important role in determining the viability of the graft after revascularization.

It is well known that fatty livers show reduced tolerance to ischemic events with further reduced ATP levels and a greater injury level compared to nonsteatotic livers [21]. It has been discussed that mitochondrial uncoupling protein-2 (UCP-2), highly expressed in steatotic hepatocytes, may be responsible for the aforementioned higher fatty liver

sensitivity to ischemia [39]. UCP-2 acts as an inner-mitochondrial membrane proton carrier that uncouples ATP synthesis, facilitating proton leakage into the mitochondrial matrix and uncoupling mitochondrial respiration. Increased UCP-2 expression was associated with low ATP levels in hearts preserved in Celsior solution after static hypothermic preservation [40]. This would suggest that UCP-2 has a relevant role between mitochondrial machinery function and ATP preservation during cold graft storage [40]. The use of genipin, a UCP-2 inhibitor added to Celsior solution, would contribute to limiting ATP depletion and protecting fatty livers against cold ischemic insult during IRI [40]. UCP inhibitors, such as 2,4-dinitrophenol and others, have been proposed, but no clinical applications have been carried out [41,42].

Mitochondrial aldehyde dehydrogenase-2 (ALDH2) is best known for its critical detoxifying role in liver alcohol metabolism, but there is growing evidence that it also plays a role in IRI through the prevention of oxidative stress processes (oxygen free radicals and toxic 4-hydroxy-nonenal generation) [20,43]. PEG35 in rinse [34] and IGL-1/IGL-2 solutions [33,44] was a determinant for increasing mitochondrial protection and function, as well as preventing lipid peroxidation when compared to identical solutions with or without PEG35 [44]. The high antioxidant capacity of IGL-2 was corroborated by MDA and AOPP when compared to livers preserved in UW and HTK (Figure 4).
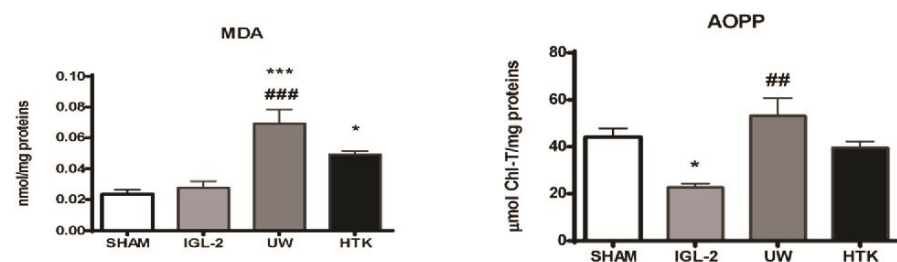
Figure 4. Lipid peroxidation (measured as MDA levels) and oxidized proteins (AOPP) in liver grafts in IGL-2, UW, and HTK solutions (24 h; 4 °C). The bars represent the mean values $\pm$ SEM of each group (n = 4–6). Differences are shown comparing groups (* vs. Sham, # vs. IGL-2) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

Although oxygen levels of the media embedding the organ (preservation solution) are very low, there is still an impairment of mitochondrial oxidative phosphorylation, leading to the generation of reactive oxygen species (ROS). In this sense, NO generation in IGL-1/IGL-2 solutions [33,38] could act, among many other functions, as an ROS scavenger in the lipoperoxidation process [44]. Interestingly, these involved cytoprotective mechanisms observed for PEG35 in IGL-1 and IGL-2 solutions were similar to those we proposed for liver ischemic preconditioning strategies in a rat model, where AMPK and NO are also well-known liver preconditioning cytoprotective factors [36,37].

With this in mind, we consider that preservation solutions can be considered preconditioning tools against IRI. This NO generation and its vasodilator properties are especially beneficial for steatotic liver grafts, in which fat accumulation in the sinusoidal space exacerbates microcirculation alterations [16,45].

4. New Additives for Improving Cold Static Preservation: Oxygen Carriers, Ozone, and AMPK Inducers

4.1. Oxygen Carriers: M-101

Static hypothermic preservation of liver grafts static hypothermic preservation implies a lack of oxygen, and its adverse consequences affect energy metabolism, blocking mitochondrial respiration, concluding with succinate accumulation. Under these circumstances, the use of a natural extracellular oxygen carrier added to the preservation solution could

help maintain the functionality of certain metabolic pathways, hence avoiding the nocive accumulation of succinate.

M-101 is a natural giant extracellular hemoglobin (Hb) extracted from the marine invertebrate *Arenicola marina* that was first used by Alix et al. as an additive to UW solution in pig liver transplantation [46]. The authors demonstrated that UW solution enriched with M-101 improved liver graft oxygenation when compared to simple UW solutions, but it did not reach the oxygenation level achieved with alternative dynamic hypothermic oxygenated machine perfusion (HOPE) strategies (with active flux of oxygen) [46]. This transient restoration of oxygenation achieved with M101 presumably contributes to maintaining liver mitochondrial machinery to function at basal levels, thus avoiding the accumulation of succinate during cold ischemia, according to Schlegel's mechanism [25]. Furthermore, the M-101 additive is also useful for fatty liver static preservation when combined with HOPE [47], but additional studies should investigate in depth its usefulness in maintaining graft quality when longer cold storage periods are applied, especially in vulnerable organs (DCD donors, pancreas, and fatty liver grafts).

4.2. Ozone

Ozone is a gas with antioxidant effects [48,49] that has been proposed as an additive in UW solution (gold-standard reference) to minimize cold ischemic damage during graft preservation in UW solution [50]. Ozone protection during cold storage modulates the XHD/XOD system, preserving adenosine storage and blocking the xanthine/xanthine oxidase pathway that promotes ROS generation [51]. Taking this into account, ozone persufflation strategies should be considered and further evaluated as a potential tool for hypothermic graft preservation strategies.

The deleterious oxidation consequences of oxygen generated by ozone conversion to oxygen during cold storage would be presumably even more negligible when glutathione and allopurinol are present in the composition of UW and IGL-2 solutions. Presumably, the persufflation of the liver graft before cold graft storage in ozonized UW solution could offer additional protection to preserve the liver graft.

4.3. Other Antioxidants

It is well known that glutathione is present in its reduced form and is a critical component often used in commercial UW and IGL-1 [8]. Reduced glutathione is a very labile component, and its auto-oxidation is accelerated by an increase in temperature and contact with oxygen [52]. With this in mind, we assume that long periods of storage at room temperature are not recommended for storing any preservation solutions containing glutathione that could affect the initial glutathione composition. [53]. This was one reason why we proposed increasing the content of glutathione in IGL-2 solution [44] vs. UW and IGL-1 [33]

In addition, well-known hydrosoluble antioxidants (including vitamin C, N-acetyl cysteine (NAC), alpha-tocopherol analog, and others) were used as additives to preservation solutions to reinforce their respective antioxidant features [54–57]. Further investigations are needed to explore new antioxidants for graft preservation purposes.

4.4. Adenosine Monophosphate Protein Kinase (AMPK) Inducers

Graft oxygenation is an alternative to solve the hypoxic conditions during cold storage; however, other studies focus on another of the main problems occurring during ischemia, such as the breakdown of energy metabolism and the dropping of ATP levels, which can be countered by the activation of the AMPK energy sensor [58–60]. In this line, AMPK inductors, such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) or metformin, have evidenced their benefits when added to UW solutions [61,62]. Other natural AMPK activators, such as 3,5,4′-trihydroxy-*trans*-stilbene (resveratrol) [63], could be good candidates as additives for preservation solutions in future approaches. This principle also applies to PEG35 solutions, in which there has been an increase in activated AMPK under

hypoxic conditions [64–66]. The mechanisms by which PEG35 activates AMPK in IGL-1 and rinse solutions remain to be investigated in depth.

5. HOPE, Mitochondrial and Glycocalyx Protection, and PEG35 Effluents (IGL-2)

The introduction of dynamic strategies by using machine devices for graft conservation, such as hypothermic oxygenated perfusion (HOPE), is a promising tool to not only increase graft quality for transplantation [67–72] but also to make useful marginal organs that were originally discarded for transplantation [31].

The use of transient and sustained oxygenation to the graft is the "key" that maintains the operational mitochondrial machinery at basal levels during HOPE, whose protective mechanisms were described by Schlegel and Cols [25]. Transient graft oxygenation during HOPE activates the mitochondrial machinery through mitochondrial complexes I and II, preventing preservation. Therefore, the importance of succinate metabolism by complex II/SDH during HOPE was shown to slowly re-establish a normal directed electron flow without significant ROS release, thereby slowing the breakdown of accumulated succinate (Figure 1) [24–27].

The mechanisms proposed by Schlegel et al. [25] also apply to static preservation, but in this case, the benefits of decreasing the accumulation of succinate can only be altered by preservation solutions, which can do so to a very limited extent. This is the case of IGL-2 solution, which better prevented succinate accumulation when compared to HTK and UW, the gold-standard reference (Figure 3), bringing the graft to more suitable conditions for the subsequent HOPE application. In clinical and experimental settings, the most used perfusate used in HOPE is modified UW solution, named Belzer MP, adapted for dynamic perfusion and maintaining HES as an oncotic agent in its formulation [25,67]. It is well known that HES can induce hyperaggregability of red blood cells [73], this being a limitation for graft washout and cold storage. This serious issue is not present with other oncotic agents such as PEG35 [74], which also confers more mitochondrial protection than HES.

Fluid dynamics constitutes one of the most differential features when HOPE and cold static preservation are compared. In any kind of dynamic perfusion, the perfusate applies pressure and shear stress that may involve the destruction of the superficial sugar thin layer covering the endothelium, known as "glycocalyx" [75,76]. The presence of PEG35 in perfusates (IGL-2 solution) contributes to favoring mechanotransduction processes inherent to fluid dynamics in HOPE, thereby also favoring better protection of the luminal glycocalyx [77]. Perfusates in a dynamic system, such as HOPE, can promote glycocalyx destruction, but this will greatly depend on factors such as viscosity, pressure, and other properties of the perfusate, which in turn depend on the components of the preservation solution, such as the oncotic agent, whose characteristics can be delimited by factors such as temperature [77].

Notably, PEG35 in IGL-2 lowers the viscosity in Belzer MPS currently used in HOPE by half [67]. The properties of PEG35 in effluents for HOPE and its effects in the organ justify a deep debate to change the paradigm on which is the better approach for dynamic preservation considering the aforementioned factors (high mitochondrial protection, diminished viscosity, no red blood cell hyperaggregability) that have been overlooked since the beginning of dynamic perfusion. Furthermore, PEG35 seems to be a more efficient agent for the protection of mitochondrial machinery and should be considered as a main strategy for hypothermic graft conservation in the complex route of the liver graft from "harvest to revascularization".

Recently, aldehyde dehydrogenase-2 has been described as a regulator of mitochondrial machinery during hypothermic machine perfusion to mitigate the deleterious effects of renal ischemia–reperfusion [78]. ALDH2 plays an important cytoprotective role in HMP, reducing the accumulation of 4-HNE and regulating the Akt/mTOR autophagy pathway in a similar way as in cold static preservation [79]. In addition, our previous observations in HOPE studies are consistent with better mitochondrial protection and increased ALDH2

in livers preserved for 7 h in IGL-2 after 1 h in HOPE and then 1 h of reperfusion with previous HOPE [67].

Taking this into account, mitochondrial GLDH and ALDH2 could be considered good indicators in HOPE to evaluate graft liver mitochondrial integrity and mitochondrial machinery function. This could also be presumably extended to cold static preservation enriched with oxygen inductors such as M-101, which could be combined with HOPE, as suggested by Alix et al. [46].

6. Some Considerations and Concluding Remarks

The challenge for the next decade is to develop technical strategies to rescue marginal organs. To do so, part of the effort should be aimed at mitochondrial protection and, therefore, at maintaining the mitochondrial machinery at basal levels to prevent energy breakdown and the subsequent generation of toxic metabolites, such as 4HNE. Certainly, HOPE is more efficient than static preservation because it provides the cell with an environment closer to physiological conditions, hence triggering less stress and coping mechanisms that inevitably lead to cell death. The continuous supply of oxygen, nutrients, and metabolic substrates for ATP generation and assuring the maintenance of mitochondrial machinery, which includes regulatory enzymes such as ALDH2, will in turn be proactive in the upregulation of other cytoprotective factors.

In HOPE, as mentioned above, one of the most common tools used is Belzer MP solution, containing an oncotic agent (HES) that provokes red blood cell aggregation [73], which can be fatal, and has some nonoptimal physical properties (high viscosity) that have been considered as a major factor of impact on shear stress occurring in the dynamic fluids system.

In this line, the use of PEG35 would be "killing two birds with one stone", avoiding red blood cell hyperaggregation and bringing the solution to more adequate parameters of viscosity in order not to damage the endothelial cells and their associated structures, such as glycocalyx, its application in the narrowed sinusoids of steatotic liver being especially interesting [16,45]. Furthermore, these benefits are not only applied at the physical level, as the use of IGL-2 confers higher mitochondrial protection and assures better conditions for the ALDH2 enzymatic function to prevent oxidative stress in hypothermic graft preservation [43,44].

The MP benefits of ALDH2 extend further, as demonstrated by Lin et al. [78], who evidenced that ALDH2 regulated autophagy via the Akt/mTOR pathway to mitigate renal ischemia–reperfusion injury and can activate the negative regulatory mammalian target of rapamycin (mTOR) pathway. We established similar protective autophagic mechanisms when IGL-2 and IGL-1 solutions were used [38,79]. Moreover, the fluid dynamics existing in HOPE may have adverse consequences due to the viscosity of the effluent generally used, Belzer MPS, leading to endothelial glycocalyx destruction and thus altering the induced transduction mechanisms involved in HOPE when compared to PEG35 solutions [77,80].

In this sense, the use of IGL-2/PEG35 solution would be a better alternative than Belzer MP/HES by two means: (1) protecting the glycocalyx due to reduced viscosity that produces less shear stress, which is especially relevant in narrowed sinusoids and peripheral microcirculation [77], and (2) as a consequence of a less damaged glycocalyx, mechanotransduction mechanisms are more preserved, promoting NO generation (with vasodilator properties), being, again, beneficial in narrowed sinusoids [45,77].

The scarcity of organs and the growing demand for liver transplantation have led to hospitals using marginal grafts for organ transplantation. For years, static preservation has been based on the principles of hypothermic and static storage; however, the limitation of oxygen supply is serious for graft survival, given that the conditions proposed for static preservation are far from physiological. The use of HOPE is very promising [67–72], as well as gas additives or hemoglobins such as M-101 that, combined with further HOPE, could be an alternative method to palliate the deficient oxygenation of the graft during static preservation conditions [46,47].

Further investigations should be carried out on the usefulness of oxygen carriers, such as M101, for transporting organs in countries with complex logistic operations that extend the static cold storage periods, since currently, the dynamic systems known to provide oxygen in a dynamic system (HMP/NMP) are not sufficiently developed/cheap enough to be applied at a real clinical level.

With all this in mind, we assume that new promising predictors may evaluate graft quality at the end of the cold storage/cold ischemia step such as glutamate dehydrogenase (mitochondrial damage), ALDH2 (mitochondrial function), succinate and itaconate accumulations, and flavin mononucleotide (FMN); in contrast to HOPE, succinate and itaconate metabolites have a predictive value at early oxygenation/reperfusion times, including FMN [24–27]. Moreover, we could logically assume that the use of a unique solution, such as IGL-2 [81], could be an efficient and interesting tool to protect graft mitochondrial machinery and avoid the cumulative damage induced by the mandatory complex processes before transplantation, including organ recovery and its earlier washout, subsequent graft cold storage, and additional washout to finally proceed with HOPE strategies [25,67–72]. Certainly, the suitable use of a unique solution such as IGL-2 would simplify the logistics of liver transplantation, including also the combination of split liver transplantation with HOPE [82,83].

Finally, it is clear that new markers are needed to assess graft quality. In a recent editorial, Schegel et al. [84] asked whether the current combination of biomarkers used in liver transplantation could be useful in predicting liver function or whether we are only measuring actual lesions. In this sense, the accumulation of energy metabolites such as succinate and itaconate metabolites at the end of ischemia, together with GLDH and mitochondrial ALDH2 and UCP2, including energy sensors such as activated AMPK, could be promising predictors for the evaluation of the graft at the end of cold storage prior to reoxygenation, a phase in which the role of those metabolites is unleashed and in which the harm of ROS is a major issue.

Summary

Mitochondrial preservation should be a prioritary direction for better conserving the graft when static and HOPE strategies are used in combination or alone. Promising markers such as GLHD, mitochondrial ALDH2, and accumulating succinate, itaconate, and flavin mononucleotide should be considered for future investigations in clinical transplantation. Glycocalyx and nitric oxide are altered in static preservation [80], but their use as predictors could be even more appropriate to HOPE strategies for evaluating the adverse effects of effluent dynamics and transduction mechanisms for future HOPE strategies, given the relevance of measuring glycocalyx alterations in clinical transplantation [85–88]. The benefit of a unique IGL-2 solution of static and dynamic HOPE preservation strategies is a topic that needs to be addressed for a simplified clinical procedure [81,83]. This will speed up current static and HOPE strategies in clinical liver transplantation, including liver split transplantation for HOPE application [82,83] and marginal livers. We assume that investigating in depth the underlying mitochondrial protection mechanisms [25,26] should be a priority direction for future approaches to liver hypothermic graft preservation strategies using PEG solutions. At this point, let us go back to 1989 when Belzer and Southard reported that the "mechanisms by which polyethylene glycols prevents cell swelling and thus maintains cell viability is not related to osmotic/oncotic properties but instead is apparently related to PEG/cell interactions that confer stability during hypothermia" [89].

Finally, due to their valuable insights, we now know that, in these interactions, the role of mitochondria, their integrity, and their function are determinants for hypothermic graft preservation, especially when marginal livers (steatotic and CDC) are used. In summary, Belzer and Southard [1,2] "left the door open for us" to return to the origins of preservation solutions and suggest new paradigms to increase liver cold graft preservation with PEG35 solutions for clinical transplantation purposes.

Abbreviations

4H NE	4-Hydroxynonenal
ALDH2	Aldehyde dehydrogenase-2
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
AST	Aspartate aminotransferase
Belzer MPS	Belzer Machine Perfusion Solution
DCD	Donor after cardiac death
eNOS	Endothelial nitric oxide synthase
GCX	Glycocalyx
GLDH	Glutamate dehydrogenase
PEG35	Polyethylene glycol 35
HES	Hydroxyethyl starch
HOPE	Hypothermic oxygenated perfusion
HTK	Histidine-tryptophan-ketoglutarate
IGL-1/IGL-2	Institut Georges Lopez 1/Institut Georges Lopez 2
IRI	Ischemia–reperfusion injury
MP	Machine perfusion
NO	Nitric oxide
ROS	Radical oxygen species
SCS	Static cold storage
TX	Liver transplantation
UCP2	Uncoupling protein-2
UW	University of Wisconsin

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