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Experimental Models in Liver Surgery

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1. Introduction

Ischemia-Reperfusion (I/R) injury is an important cause of liver damage occurring during surgical procedures including hepatic resections and liver transplantation (LT) [1-3]. The shortage of organs has led centers to expand their criteria for the acceptance of marginal grafts that exhibit poor tolerance to I/R [4]. Some of these include the use of organs from older donors and grafts such as small-for-size or steatotic livers. However, I/R injury is the underlying cause of graft dysfunction in marginal organs [4]. Indeed, the use of steatotic livers for transplantation is associated with an increased risk of primary nonfunction or dysfunction after surgery [5]. In addition, the occurrence of postoperative liver failure after hepatic resection in a steatotic liver exposed to normothermic ischemia has been reported [6]. A large number of factors and mediators play a part in liver I/R injury. The relationships between the signalling pathways involved are highly complex and it is not yet possible to describe, with absolute certainty, the events that occur between the beginning of reperfusion and the final outcome of either poor function or a non-functional liver graft. We will show that the mechanisms responsible for hepatic I/R injury depends on the experimental model used, who are valuable tool for understanding the physiopathology of hepatic I/R injury and discovering novel therapeutic targets and drugs. Several strategies to protect the liver from I/R injury have been developed in animal models and, some of these, might find their way into clinical practice. The species used for experimental investigation of hepatic I/R injury range from mice to pigs. The book chapter will discuss the numerous experimental models used to study the complexity of hepatic I/R injury, data reported in choice of the animal model, when selecting an animal species, the age, the sex, the degree of steatosis...etc. Thus, the different strengths and limitations of the different experimental models will be discussed. Also the standardized experimental conditions, such as anesthetic and analgesic procedures will be described. We also attempt to highlight the fact that the types of ischemia (cold and warm ischemia) play an important role in experimental liver surgery. The most

existing reviews concerning about mechanisms responsible of I/R does not make a distinction between cold and warm ischemia. We will discuss the different experimental models of normothermic ischemia including global hepatic ischemia with portocaval decompression, global liver ischemia with spleen transposition and partial liver ischemia. Among the different experimental models of cold hepatic I/R injury, we will described the different experimental models used, including a section on orthotopic liver transplantation (OLT) because it is a common yet and complex microsurgical technique. In an attempt to expand the size of the donor pool, the different surgical techniques including reduced-size liver transplantation (RSLT), split liver transplantation (SLT) and living donor liver transplantation (LDLT) will be mentioned in the book chapter. In line with this, the optimization of graft function and survival through the static organ preservation and machine perfusion will also discussed. Static organ preservation was a breakthrough and remains the conventional method of preservation. The machine perfusion has emerged as a suitable strategy for preserving liver grafts with promising data over the past decade, especially when marginal organs such as steatotic liver are used for transplantation. The strengths and disadvantages of the different types of machine perfusion (normothermic, hypothermic and subnormothermic machine perfusion) will be discussed. Furthermore some factors, including the duration and extent of hepatic ischemia, starvation, graft, age, and steatosis-which must be considered before the selection of an experimental model of hepatic I/R-will be mentioned. All of these factors contribute to enhancing liver susceptibility to I/R injury. In line with this, we will focused on the negative effects of ischemia on liver regeneration in both normal and marginal livers when they are subjected to liver surgery associated with hepatic resections or LT. The different experimental models of hepatic I/R in which both conditions-ischemia and resection- are present will be described.

2. Hepatic ischemia-reperfusion injury

Due to the complexity of hepatic I/R injury, the present review summarizes the established basic concepts of the mechanisms and cell types involved in this process (Fig. 1). The imbalance between nitric oxide (NO) and endothelin production, contributes to microcirculatory diseases associated with I/R. Concomitantly, the activation of Kupffer cells (KC) releases reactive oxygen species (ROS) and proinflammatory cytokines, including tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1) [7-9]. ROS can also derive from mitochondria and the xanthine dehydrogenase/xanthine oxidase (XDH/XOD) pathway in activated SEC and hepatocytes. Cytokines promote neutrophil activation and accumulation, thereby contributing to the progression of parenchymal injury by releasing ROS and proteases [7,10]. Capillary narrowing also contributes to hepatic neutrophil accumulation [11]. Besides, IL-1 and TNF- α recruit and activate CD4⁺ T-lymphocytes, which produce granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (INF- γ) and TNF- β . These cytokines amplify KC activation and TNF- α and IL-1 secretion and promote neutrophil recruitment and adherente into the liver sinusoids [12]. Platelet activating factor can prime neutrophils for ROS generation, whereas leukotriene B₄ (LTB₄) contributes to the amplification of the neutrophil response [7,10]. In addition, I/R initiates protein misfolding in the endoplasmic

reticulum (ER), which can activate a highly conserved unfolded protein response (UPR) signal transduction pathway. The UPR is characterized by coordinated activation of three ER transmembrane proteins, inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor (ATF)-6. If the damage is so severe that homeostasis cannot be restored, ER stress signal transduction pathways ultimately initiate apoptosis and necrosis [9]. In addition to the high ROS level-generating system found in liver grafts shows low levels of antioxidants such as glutathione (GSH) and superoxide dismutase (SOD) [1,9]. Alterations in the renin-angiotensin system (RAS), retinol binding protein 4 (RBP4), adiponectin and peroxisome proliferator activated receptor gamma (PPAR γ) contribute to oxidative stress. Toll like receptor (TLR4) signaling pathway is also responsible for the hepatic I/R damage. Myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon- β (TRIF) activate intracellular signaling cascades that ultimately trigger an inflammatory response [9,13].

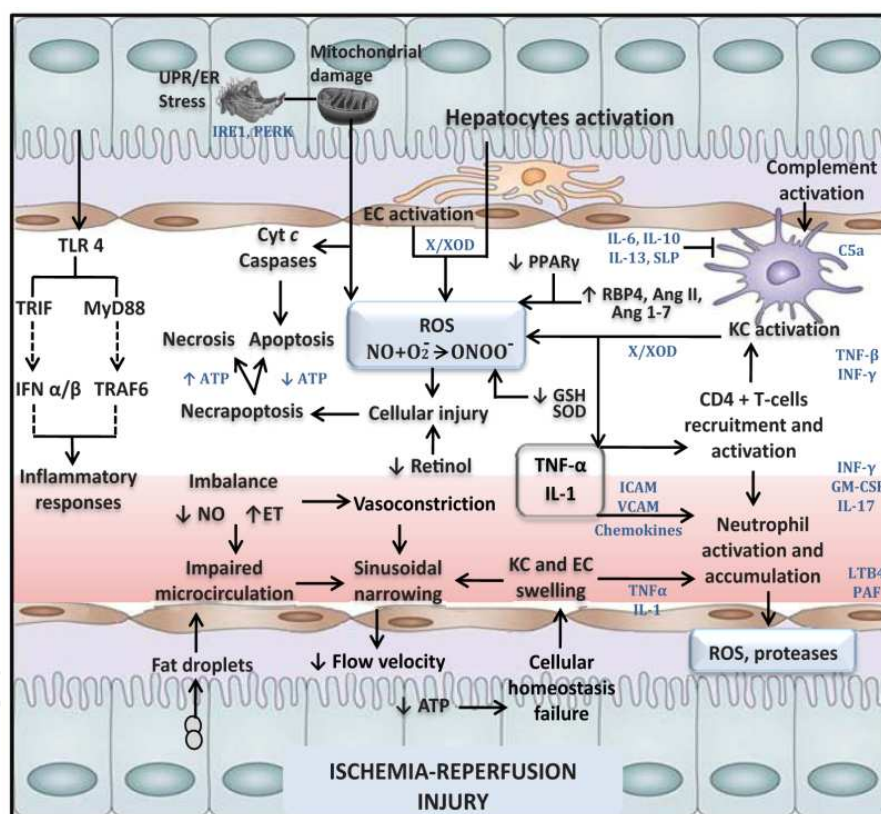


Figure 1. Mechanisms involved in hepatic ischemia-reperfusion injury. ATP, adenosine triphosphate; Cyt c: cytochrome c; EC, endothelial cell; ET, endothelin; GM-CSF, granulocyte-macrophage colony-stimulating factor; GSH, glutathione; ICAM, intracellular cell adhesion molecule; IFN α/β , interferon α/β ; IL, interleukin; INF, interferon; IRE1, inositol-requiring enzyme 1; KC, kupffer cell; LTB4, leucotriene B4; MyD88, myeloid differentiation primary response gene 88; NO, nitric oxide; ONOO $^-$, peroxynitrite; PAF, platelet activating factor; PPAR γ , peroxisome proliferator-activated receptor γ ; RBP4, retinol binding protein 4; Renin-Angiotensin system (RAS): Ang II and Ang 1-7, angiotensin; ROS, reactive oxygen species; SLP, secretory leukocyte protease inhibitor; SOD, superoxide dismutase; TLR4, toll-like receptor 4; TNF, tumor necrosis factor; TRAF6, TNF receptor-associated factor 6; TRIF, TIR-domain-containing adapter-inducing interferon- β ; UPR/ER, unfolded protein response/endoplasmic reticulum; VCAM, vascular cell adhesion molecule; X/XOD, xanthine/xanthine oxidase

3. Experimental models

Experimental surgery is an activity within the scientific development, offering a wide range of possibilities for the progress of medicine. As a discipline can be accessed from various branches of science and allows testing and development of surgical procedures and learning the scientific method, so that, working with laboratory animals has been and is required prelude to innovation and development of advances in clinical surgery. The reproduction and validation of experimental models has facilitated the extrapolation of the knowledge acquired to Medicine [16]. The animals used in research models have been divided into four groups: spontaneous, induced, negative and orphans. 1) The spontaneous or non-manipulated models are obtained by selection of inbred animals that express a variable or among populations in which a large number of animals that express variable; 2) Induced or manipulated models are obtained by an experimental challenge that can be classified into five groups: A. Administration of biologically active substances, eg., induction of steatosis after alcohol ingestion. B. Surgical manipulation, such as partial hepatectomy (PH) for the study of liver regeneration. C. Administration of modified diets, lack or surplus components, e.g., in the study of hyperlipidemia. D. Genetic manipulation and transgenic animals which produce special models that are being helpful in understanding mechanisms of pathogenesis and therapy. 3) The negative patterns are those in which a given variable does not develop. The interest is in studying the mechanisms that provide resistance. 4) Orphan models are those expressing an unknown variable in humans [16].

The speed of human studies is slow, the majority of human tissues are not routinely accessible for research purposes, and there is a very limited opportunity for interventional studies. Although scientific research has always relied on the use of cell cultures, information that is obtained through *in vitro* studies can be extrapolated to biomedical research only when analyzed within a complex organism with metabolic functioning. Therefore, one avenue holding tremendous potential in the search for therapies against I/R damage is the use of intact living systems, in which complex biological processes can be examined. There are many advantages of animal studies: large numbers of animals (especially rodents) can be bred and studied, interventional studies can be performed, and established and emerging tools for targeted manipulation of gene expression levels provide insight into the function of mediators in hepatic I/R injury.

Comparison of the results of animal studies and their extrapolation to human beings is feasible, but with limitations. Among the primary obstacles are differences in hypothermia and ischemia tolerance, differences in the anatomy of the livers of various species and subspecies, differences between and within the experimental models used, and differences in the modes of administration, dosage, and metabolic breakdown of the drugs under investigation. Thus, it is very important to choose the animal species and the experimental model and to standardize the protocol according to the clinical question under study.

Small and large animals have their own advantages and disadvantages but the ultimate choice of animal species depends essentially on the scientific problema in question. Small animals such as mice and rats are exceptionally useful because they are easy to manage,

present minimal logistical, financial, or ethical problems, and provide the potential for genetic alterations (e.g., transgenic and knockout animals). However, an important drawback is that the results of studies performed in small animals are of limited applicability to human beings due to their varying size and anatomy of the liver and their faster metabolism [17]. Large animals such as pigs, sheep, and dogs exhibit greater similarity in their anatomy and physiology to human beings. Thus, they are more suited for the study of problems of direct clinical relevance. However, their use is restricted by serious logistical and financial difficulties and often by ethical concerns. Furthermore, the technical possibilities of blood and tissue processing are extremely restricted because of the limited availability of immunological tools for use in large animal species [17].

Extensive data exist on liver anatomy in various species of animals, but a few examples of species variations will suffice to prove that caution is warranted in the extrapolation of this data to humans. Mice and rats each have 4 liver lobes: median (or middle), left, right, and caudate and all, except the left, are further subdivided into 2 or more parts. Human liver lobes can be subdivided into 9 segments based on the vascular and ductal branching patterns to the right and left sides. The hepatic lobes of the rat appear to have similar fundamental portal and hepatic venous systems, and thus segments, comparable to that of human liver. The vascular systems to or from lobes show individual variations in humans as well as in rats. In humans and other mammals, sinusoids drain only into the terminal hepatic veins whereas in the rat sinusoids enter the hepatic venous system at all levels of the hepatic venous tree. In rats, unlike humans, the sinusoids are supplied not only by the terminal portal venules but also directly from larger venous branches. In addition, rat livers lack the septal vein branches, which are present both in humans and pigs [18]. The presence of arterio-portal anastomosis is very frequent in rats but not in hamsters and humans. The rat is unique in possessing a perihilar biliary plexus, which is present from the large hilar portal tracts to smaller portal tracts. An equivalent, less developed structure exists in humans only in large portal tracts. The biliary system in pigs lacks this plexus altogether, but contains numerous side pouches throughout the course of the bile duct [18,19]. Mice and humans have a gall bladder, but not the rat. Significant difference is present among the species with respect to the extent of hepatic parenchymal innervation and the human has the most abundant supply of autonomic nerves in the intraparenchymal region [20]. Differences in hepatic cell types have been reported depending of species evaluated. For example, regarding to endothelial cells, rats have relatively higher fenestrae compared to some other species. Defenestration is thought to play a role in some liver diseases [18]. Intrinsic biochemical differences between the hepatocytes of the various species have been also reported. Rats and mice are extremely sensitive to the response of peroxisome proliferators, hamsters show a less marked response while primates and humans are insensitive or non-responsive [21]. There are two principle hypotheses to explain species differences in response to PPs: quantity of PPAR α and/or the quality of the PPAR α -mediated response [22].

When selecting an animal species, the age and sex of the animals should be considered. Depending on the duration of ischemia, young (35–50 g) and older rats (250–400 g) exhibit significant differences in their hepatic microcirculation [23]. A mature rat weighing more than 250 g (14–16 weeks old) is the most suitable because younger rats can present technical problems, whereas older rats are more prone to respiratory infections and fat accumulation. Sex selection also affects experimental results, as hormone levels in female animals are dependent on the estrous cycle, which certainly affects the ischemia tolerance of the liver. For instance, a study demonstrated that after normothermic liver ischemia, male rats were less sensitive to reperfusion injury than female rats.

Considering the relevancy of hepatic steatosis in surgery, experimental models of hepatic I/R injury in the presence of steatosis have been developed. However, the mechanisms involved in hepatic I/R injury, as it will be described in following sections, are different depending on the method used to induce steatosis. The different models of steatosis include 1) induced genetic models; 2) animals fed diets with high levels of saturated fat and/or carbohydrates and/or proteins; 3) animals fed diets deficient in methyl groups (choline, methionine, folates); and 4) animals fed modified high-fat diets (lower methionine and choline and higher-fat content).

The induction of I/R injury must be performed under standardized experimental conditions. Of primary importance are the conditions under which the animals are kept such as adequate acclimatization time, maintenance under climatized conditions with 12 hours light / 12 hours darkness, and standardized diets. The anesthetic method and postoperative analgesic regimen must also be standardized. When choosing the anesthetic and analgesic procedures, possible interactions with liver metabolism must be considered. Attention must be paid to adequate monitoring of blood pressure, heart rate, and body temperature.

4. Normothermic hepatic ischemia

4.1. Global hepatic ischemia with portocaval decompression

The model of global liver ischemia with portal decompression ideally simulates the clinical situation of warm ischemia after the Pringle maneuver for liver resection and LT. The first successful shunt operation in humans was performed by Vidal in 1903 [24]. Blakemore was one of the first workers to report successful portal-systemic anastomosis in rats working principally with endothelium-lined tubes [25]. Burnett et al., modified this technique to form a portocaval shunt [26]. In 1959 Bernstein and Cheiker developed the portosystemic shunt that conducted the portal blood after functional hepatectomy into one of the iliac veins [27]. In small animals, in addition to many other shunt techniques such as the portofemoral shunt and the mesentericocaval shunt via the jugular vein, in 1995, Spiegel et al., developed the splenocaval shunt [28] (Figure 2).

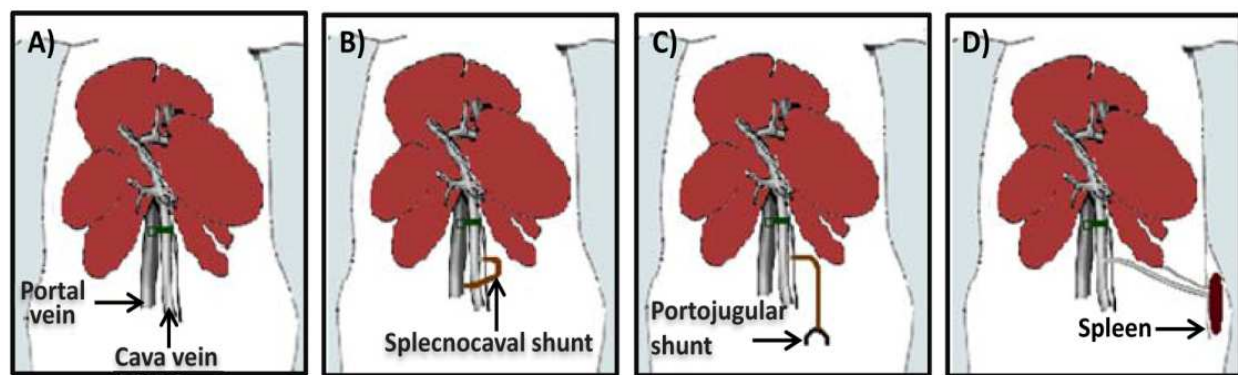


Figure 2. Models of global normothermic liver ischemia. A) Pringle-maneuver. B) Splenocaval shunt. C) Portojugular shunt. D) Spleen transposition.

4.2. Global liver ischemia with spleen transposition

Bengmark et al., developed this model in 1970 for the surgical treatment of portal hypertension [29]. In 1981 Meredith and Wade presented a rat model that by transposition of the spleen produced a portosystemic shunt in the anhepatic rat [30]. A small incision is made in the left hypochondrium. After transposition of the spleen into a subcutaneous pouch, adequate portosystemic anastomoses arise after two to three weeks (Figure 2). Reversal of blood flow in the splenic vein, induced by the transposition, stimulates angiogenesis. In the second step 2 weeks later, the surgeon performs a median laparotomy and temporary occlusion of the hepatoduodenal ligament. This decompression by spleen transposition does not require microsurgical technique and is therefore easy to perform. Two-to-three weeks postoperatively, the spleen will have been encapsulated without any signs of bleeding or inflammation. One disadvantage of this model is the long time lapse (3 weeks) until the formation of adequate portosystemic collaterals. Not until this point in time are the collaterals sufficiently large to take over portal vein flow completely. Furthermore, it is uncertain how the changes in hepatic inflow will react upon the collaterals [31].

4.3. Partial liver ischemia and liver regeneration

In 1982, Yamauchi et al., described a model of hepatic ischemia [32]. In this technique, ischemia is induced by occlusion of the hepatic artery, the portal vein, and the bile duct of the left and median lobes. An extracorporeal shunt is not necessary because blood flow continues through the right and caudal liver lobes. This model of 70% partial ischemia has been widely used in experimental studies of hepatic I/R [13,33]. Additionally, an experimental model of 30% partial liver ischemia has been used in which blood supply to the right lobe of the liver is interrupted by occlusion at the level of the hepatic artery and portal vein [34]. It is known that, in clinical situations, PH under I/R is usually performed to control bleeding during parenchymal dissection. *In vitro* studies, although they have proved helpful in disclosing the signal transmission pathways of various hepatocyte mitogens, need to be supplemented by *in vivo* studies with experimental animals so as to simulate the interactions

between the various cell populations of the liver. Different strategies have been adopted for the experimental induction of liver regeneration as follow below [35]. On the other hand, the use of an experimental model including both hepatic regeneration and I/R injury is advisable to simulate the clinical situation of selective or hemihepatic vascular occlusion for liver resections. In experimental model, after resection of left hepatic lobe, a microvascular clamp is placed across the portal triad supplying the median lobe (30%). Congestion of the bowel is avoided during the clamping period by preserving the portal flow through the right and caudate lobes. At the end of ischemia time, the right lobe and caudate lobes are resected, and reperfusion of the median lobe is achieved by releasing the clamp. This model of hepatic resection does not require any portal decompression and also fulfills certain important criteria such as reversibility, good reproducibility, and simple performance [36].

4.4. Other experimental models of liver regeneration – Regeneration after liver injury

There are large numbers of toxins that can cause liver damage and cell death in the liver parenchyma followed by liver regeneration. Carbon tetrachloride, d-galactosamine, ethanol, thioacetamide and acetaminophen are the hepatotoxins that have been most frequently employed to induce experimental liver regeneration in the hope of answering various questions [35]. In contrast to PH, these so-called hepatotoxic models of liver regeneration are easier to perform and of greater clinical relevance. Whereas PH leaves all the remaining hepatic acini intact, hepatotoxins can be used selectively to induce centrilobular or periportal necrotic lesions and can thus better simulate certain liver diseases. One serious weakness of toxin-induced liver regeneration is the poor reproducibility and standardisability of the models, because the local and systemic effects of the toxin depend on the dose, the mode of administration, the species of animals, their age and nutritional status and other factors, and the extent of the liver injury and the regeneration can vary accordingly. The regenerative response of the liver is often determined by the dose and mode of administration. Furthermore, the toxins can directly interfere with the cellular and molecular mechanisms of liver regeneration, e.g., by damaging membranes (interruption of the interaction between growth factors and membrane receptors), impairment of gene expression and protein synthesis, inflammatory reactions (increased production of cytokines and oxygen radicals) or activation of nonparenchymal cells [37]. Finally, in these toxic models the processes of liver injury and repair are closely interwoven, a fact that adds to the difficulties of investigating liver regeneration. It is therefore difficult to predict the extent of liver damage and liver regeneration and to avoid significant variability between individual experiments [35].

5. Liver transplantation

The development and implementation of different surgical techniques in LT have been based upon animal experimental studies. LT in larger laboratory animals such as dogs and pigs is technically easier. However, the rat has become the most important subject for experimental LT because of, among other factors, the availability of genetically defined animals [38]. The first experimental liver replacement with OLT was reported by Cannon in 1956,

but none of those dogs survived [39]. Surgical techniques for experimental OLT on pigs were started by Garnier et al., in 1965 [40]. OLT in mice is technically very difficult, even without reconstruction of the hepatic artery. By contrast, OLT in rats is technically accessible, producing more clinically relevant and reliable data [41]. The development of clinically relevant OLT models in rats [41] has advanced clinical knowledge in LT. These experimental models facilitate the study of new preservation methods, tolerance induction, rejection mechanisms, and novel immunosuppressor therapies [42].

The first model of OLT in the rat was described by Lee et al., in 1973 using hand-suture techniques [43]. This technique includes standard microvascular suture technique for venous anastomoses and a miniaturized extracorporeal portal-tojugular shunt ("microsuture model"). Rearterialization of the graft is performed by anastomosing the donor aorta end-to-side to the host aorta, and the donor bile duct is implanted into the duodenum [43]. Two years later, in 1975, Lee reported a modified model without hepatic artery reconstruction and temporal shunt of the portojugular venovenous bypass [44]. However, these models were not widely used due to the prolonged surgical time and technical demand. In 1979, Zimmermann introduced a microsuture model [45] that is similar to the simplified model of Lee [44]. He developed a new technique for bile duct reconstruction that preserves the sphincter of ampulla "splint technique". In the same year, Kamada and Calne [46] developed a cuff technique for anastomoses of portal vein and bile duct to simplify Lee's model and especially to shorten the anhepatic time and reduce biliary complications. With the cuff method being introduced by Kamada and Calne [46], OLT in rats without hepatic artery reconstruction became globally accepted [41]. Other models introduced by later investigators contain for the most part only a few modifications. In 1980 Miyata introduced the "three-cuff model" [47] with cuff technique for the three venous anastomoses. Bile duct anastomosis is performed by using the splint technique first described by Zimmermann [45], in which reestablishment of hepatic blood flow is not carried out. Anastomosis of the portal vein is done by the method of Kamada and Calne [46]. For connecting the bile duct, splint technique was used [47]. In 1982 Engemann [48] devised a microsuture model that corresponds closely to the model of Lee [43]. During the anhepatic time he dispensed with portosystemic bypass and used an aortic-celiac segment for rearterialization. This had been already prepared in the donor operation, and anastomosed end-to-side to the infrarenal aorta of the recipient. Bile duct anastomosis is performed using the splint technique [48]. Portal vein clamping causes a rise of endotoxin in the portal vein, which could lead to disturbances in hepatic microcirculation. Lee was the first to use a portosystemic shunt, but in further models it has not been established because the acceleration of the transplantation procedure by improved anastomotic techniques was expected to preclude the need for this complicated operative procedure [38]. Kitakado completed the "two-cuff model" in 1992 by developing a bioabsorbable material (synthesis of D, L-lactic acid and glycolic acid). Its *in vivo* degradation time is about 4 months when used for cuff anastomosis of portal vein and infrahepatic vein cava [49]. He established a longterm model in OLT in rat. This surgical procedure is usually performed according to the procedure described by Kamada and Calne [46]. After arterial and portal perfusion, the suprahepatic vena cava is dissected free from the diaphragmatic ring, and the intrathoracic vena cava is transected. The aorta is cut around the celiac axis to form

the aortic patch. Finally, the inferior vena cava, the portal vein, and the bile duct are cut, and the graft is placed in a cold preservation solution (Figure 3). OLT is then performed by suture or mechanical microvascular anastomoses. Sutured vascular anastomosis reduces the incidence of thrombosis but takes a long time to perform. Suprahepatic vena cava anastomosis is performed by the continuous suturing technique. Then, portal vein and infrahepatic vena cava anastomosis is performed in the same manner. Hepatic artery reconstruction in rat LT can prevent bile duct ischemia and preserve the structure of the liver [50]. Several techniques of rearterialization by suture have been proposed [50], the best being the aortic segment anastomosis technique. After rearterialization, the common bile duct is anastomosed. OLT by hand-sewn microanastomosis is a very useful method because this technique comes closest to the techniques used in human transplantation surgery. Alternatively, livers can be satisfactorily allografted in rats by using the rapid cuff-ligature technique for anastomosis [46]. In the simplified technique, the donor hepatic artery can be ligated because it will not be anastomosed [42].

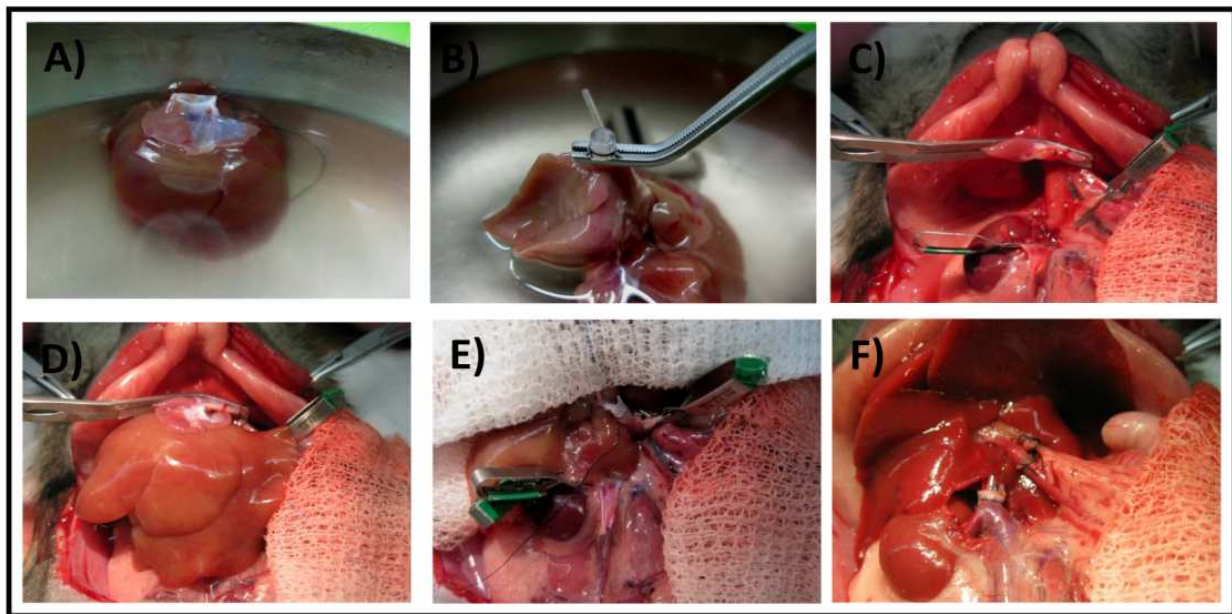


Figure 3. Liver transplantation procedure. A) Suprahepatic cava vein prepared for the anastomosis. B) Inferior vein cava cuff attachment. C) Anhepatic phase in the recipient rat. D) Anastomosis of suprahepatic cava vein by continuous suture. E) Portal vein anastomosis through the cuff method. F) Anastomosis of the bile duct.

6. Strategies to expand the size of the donor pool

In an attempt to expand the size of the donor pool, a number of surgical techniques have been developed over the past 15 years, including reduced-size liver transplantation (RSLT), split liver transplantation (SLT) and living donor liver transplantation (LDLT) [51]. For children and small adult recipients, RSLT has been developed to maximize the use of donor or-

gans. Bismuth and Houssin in 1984, transplanted the left lateral segment of the left liver lobe from a cadaveric donor into a small child and discarded the remainder of the donor liver [52]. Couinaud's anatomical classification permits the creation of partial liver allografts from either deceased or living donors. Couinaud's classification divides the liver into eight independent segments, each of which has its own vascular inflow, outflow, and biliary drainage [53]. Segments IV to VIII are used for adults, whereas left lateral lobes (Segments II and III) or left lobes (Segments II, III, and IV) are used for pediatric recipients. Bleeding, bilomas, and portal vein thrombosis are complications related to the procedure itself, which are associated with an increased number of re-operation. SLT, first performed in 1988, allows the division of the adult donor liver, together with its vascular and biliary structures, into two or more functional grafts, which can be transplanted into two or more recipients [54]. Liver splitting is performed either *ex situ* or *in situ*. So far, there is no consensus on which technique is superior because both techniques demonstrate similar patient and graft survival rates compared with whole liver grafting [54]. Biliary complications occur in 22% of recipients. In 1990, Broelsch et al., reported the first 20 series of LDLT in the USA [55]. In 1996, Lo et al., [56] performed the first successful LDLT using an extended right lobe from a living donor for an adult recipient. One of the benefits of reduced-size grafts from living donors is a graft of good quality with a short ischemic time, this latter being possible because live donor procurements can be electively timed with the recipient procedure. Conversely, the major concern over the application of LDLT for adults is graft-size disparity. Small grafts require posterior regeneration to restore the liver/body ratio. A small graft may result in malfunction or the small for size syndrome in which the recipient fails to sustain adequate metabolic function. It is well known that I/R significantly reduce liver regeneration after hepatectomy. Thus, the identification and subsequent modulation of mechanism that are involved in liver injury and regeneration might favor the recovery and functioning of the transplanted organ.

To mimic some of the pathophysiological events that occur during such clinical situations, several experimental models of RSLT have been developed. For example, OLT with the implantation of liver grafts that approximated 30%–70% of the normal mass of a rat liver has been performed. Graft size is important for normal liver function and host survival [51]. It has been reported that 100% of recipient rats that were implanted with 40%, 50%, 60%, or 70% of the liver survived regardless of the duration of preservation. This suggests that graft sizes of 40% or greater are sufficient to meet the metabolic demands of the recipients. The transplantation of a graft of 30% of the normal liver mass provides an extreme model of hepatic reduction that presumably stimulated a maximal regenerative response [51]. Three possibilities exist with respect to the timing of the graft reduction: in the donor before perfusion, in the container (*ex situ*), or in the recipient after reperfusion. If the reduction is done *in vivo* prior to the removal of the donor liver, then two concerns exist: 1) excessive bleeding might stimulate systemic responses that could alter the liver and 2) the immediate phase of the regeneration response could be initiated in the donor animal. The second choice, *ex situ* reduction, can be done without the risk of damaging the graft by manipulation or affecting anastomosis after reperfusion. Finally, resection of the graft after implantation in the recipient adds surgical stress and the risk of bleeding.

7. Modes of organ preservation and optimizing the graft

The ideal method of organ preservation should: 1) Reverse injury sustained during donor death and organ procurement; 2) Provide viability testing; 3) Prolong safe preservation time and 4) Improve the graft quality [57]. There are currently 2 modes of preservation methods for livers: static and dynamic (Figure 4). Simple cold storage is the main method for static storage while hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP) comprise some of the methods for dynamic preservation. Of these methods, only simple cold store is proved clinically for livers. The remaining methods are in various stages of pre-clinical and early clinical studies. Dynamic preservation methods require some dynamic movement of either fluid or gas to facilitate preservation. The advantage of these methods over simple cold storage is that they all have been shown to improve recovery of donor after cardiac death organs. These organs have the potential to increase the donor pool by 20–40%.

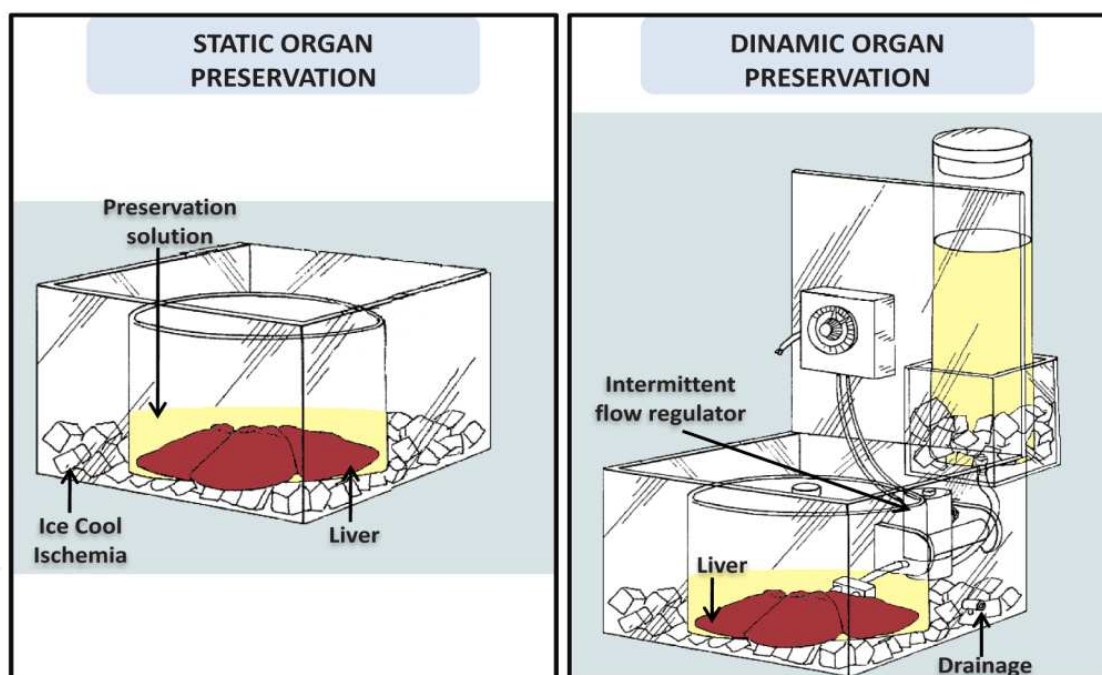


Figure 4. Illustrative modes of organ preservation. Static or dynamic organ preservation.

7.1. Static organ preservation

Static cold storage (SCS) is the most commonly used preservation method used for all organs. The principles underlying cold preservation are the slowing of metabolism (by cooling) and the reduction of cell swelling due to the composition of preservation solutions. The introduction of the University of Wisconsin (UW) solution by Belzer and Southard for SCS was a breakthrough and remains the conventional method of preservation. Reduction of metabolic activity (by cooling) is the major principle of organ preservation [57,58]. At the

moment the flow of oxygenated blood is terminated, the supply of oxygen, cofactors and nutrients stops and the accumulation of metabolic waste products begins. Although metabolism is slowed 1.5- to 2-fold for every 10°C drop in temperature, anaerobic metabolism continues, which leads to depletion of energy stores and concomitant build up of an acidotic milieu. Depletion of ATP causes loss of transcellular electrolyte gradients, influx of free calcium and the subsequent activation of phospholipases, and therefore is the main contributor for cell swelling and lysis. Ischaemia creates the basis for the subsequent production of toxic molecules after reperfusion, particularly reactive oxygen intermediates, the basis of the cascade of events that characterize the I/R injury. Even with the most effective preservation solutions, cold storage aggravates graft injury at the time of transplantation. This situation is due to two processes, one proportional to the duration of ischemia and the other specifically related to cooling [57]. Using this preservation method, however, organs undergo injury at several consecutive stages: warm ischemia prior to preservation, cold preservation injury, ischemic rewarming during surgical implantation and reperfusion injury. With the extension of criteria to include expanded criteria donor and donation after cardiac death organs, static preservation is associated with increased delayed graft function and graft loss. In organs retrieved from non-heart-beating donors (NHBD) -with an inevitable period of oxygen deprivation between cardiac arrest and organ perfusion – the deleterious effects of cold ischaemia are superimposed on the injury sustained during warm ischaemia [57]. Only a few studies have demonstrated the optimization of graft function and survival with modification of static preservation. It is doubtful that considerable improvements in organ preservation and especially in the rescue of marginal organs will be possible as long as the strategy is based on static principles [58]. In 1990s, Minor et al., developed a new method, called venous systemic oxygen persufflation (VSOP) to supply gaseous oxygen to livers during SCS preservation [59]. The oxygen was introduced into hepatic vasculature via the suprahepatic vena cava. This technique was employed on steatotic rat livers for 24 h, and resulting in improved preservation of mitochondria and sinusoidal endothelial linings, less KC activation and reduced hepatocellular enzyme release compared to SCS preservation. Recently, by assessing the enzyme release, energy storage, bile production, and cell death during isolated reperfusion, it was demonstrated that application of VSOP for 90 minutes may rescue the steatotic livers after extended (18 h) SCS preservation [60].

7.2. Machine perfusion

Machine liver perfusion is an alternative preservation method to SCS which can be further categorized based on the temperature employed and has emerged with promising data over the past decade because it has significant potential in graft preservation and optimization when the use of marginal organs is the objective. Machine perfusion involves pulsatile perfusion of the liver using a machine as opposed to SCS. This can be performed by perfusing the liver with a hypothermic perfusate or with a normothermic perfusate. There is experimental evidence in animal models that machine perfusion protects against liver I/R injury [61]. The safety and efficacy of machine perfusion compared to SCS to decrease liver I/R injury is yet to be assessed in humans by randomized controlled trials [61,62].

Compared with simple cold storage, machine perfusion confers many anticipated advantages such as the following: 1) provision of continuous circulation and better preservation of the microcirculation; 2) continuous nutrient and oxygen delivery; 3) removal of metabolic waste products and toxins; 4) opportunity to assess organ viability; 5) improved clinical outcomes via improved immediate graft function rates; 6) prolonged preservation time without increased preservation damage; 7) administration of cytoprotective and immunomodulating substances; and 8) lower graft dysfunction incidence, shorter hospital stays, and better graft survival rates [62].

7.3. Normothermic machine perfusion

In the first half of the 20th century, Alexis Carrel perfused different organs with normothermic, oxygenated serum and demonstrated viability for several days [63]. Actually, the first successful human LT carried out by Starzl [64], were transplanted after liver graft pretreatment by machine perfusion with diluted, hyperbaric oxygenated blood. Most perfusion circuits were assembled from standard cardiopulmonary bypass components. Principle constituents are a centrifugal pump, a membrane oxygenator and a heat exchanger. Other critical components of the perfusate include nutrition (glucose, insulin, aminoacids), drugs to prevent thrombosis or microcirculatory failure (heparin, prostacyclin) and agents to reduce cellular oedema, cholestasis and free radical injury [57]. Normothermic machine perfusion (NMP) provides a physiologically-relevant environment to the isolated donor organ, the quality of liver grafts can be manipulated more efficiently than those simply stored in an ice-box during SCS, because NMP maintains and mimics normal *in vivo* liver conditions and function during the entire period of preservation, thus avoiding hypothermia and hypoxia and minimizing preservation injury [58,62]. In contrast to cold storage preservation the concept of normothermic preservation is to maintain cellular metabolism. The underlying principle is the combination of continuous circulation of metabolic substrates for ATP regeneration and removal of waste products. There is accumulating evidence for the superiority of the more physiological approach of normothermia in association with an oxygenated blood-based perfusion solution [57].

Schön et al., [65] studied NMP to preserve pig livers for transplantation and to rescue them from warm ischemia in a model of donor after cardiac death. Short (5 h) or prolonged (20 h) NMP preservation is superior to SCS for normal and ischemically damaged livers, respectively [62]. The longest preservation of steatotic livers was the NMP preservation for 48 hours in a pig model by Jamisson et al., who employed blood containing additional insulin and vasodilators as perfusate, and observed a mild reduction of steatosis from 28% to 15%. The NMP circuit dually perfuses 1.5 L of autologous heparinized blood at physiological pressures, which allows hepatic blood flow autoregulation. Prostacyclin, taurocholic acid, and essential amino acids are infused continuously. Apart from logistics, one potential drawback of NMP is the mandatory use of oxygen carriers if blood is not available [62]. Perhaps the only weakness is that SCS prior to NMP revokes its beneficial effect. Therefore, immediately after cardiac asystole, normothermic perfusion in the donor should be installed, as described by Fondevila et al., [66] for the preservation of livers from uncontrolled donation after cardiac death. The use of

NHBDs as a source of liver grafts for transplantation has long been debated. The concept of normothermic recirculation in the context of NHBDs was first developed by Garcia-Valdecasas et al., [67]. With 4 h of NMP, hepatic damage incurred during 90 minutes of cardiac arrest can be reverted, achieving 100% graft survival after 5 days of postransplant follow-up. These results offer the hope that NMP will be able to increase the clinical applicability of NHBD LT over that offered by traditional cold storage [67].

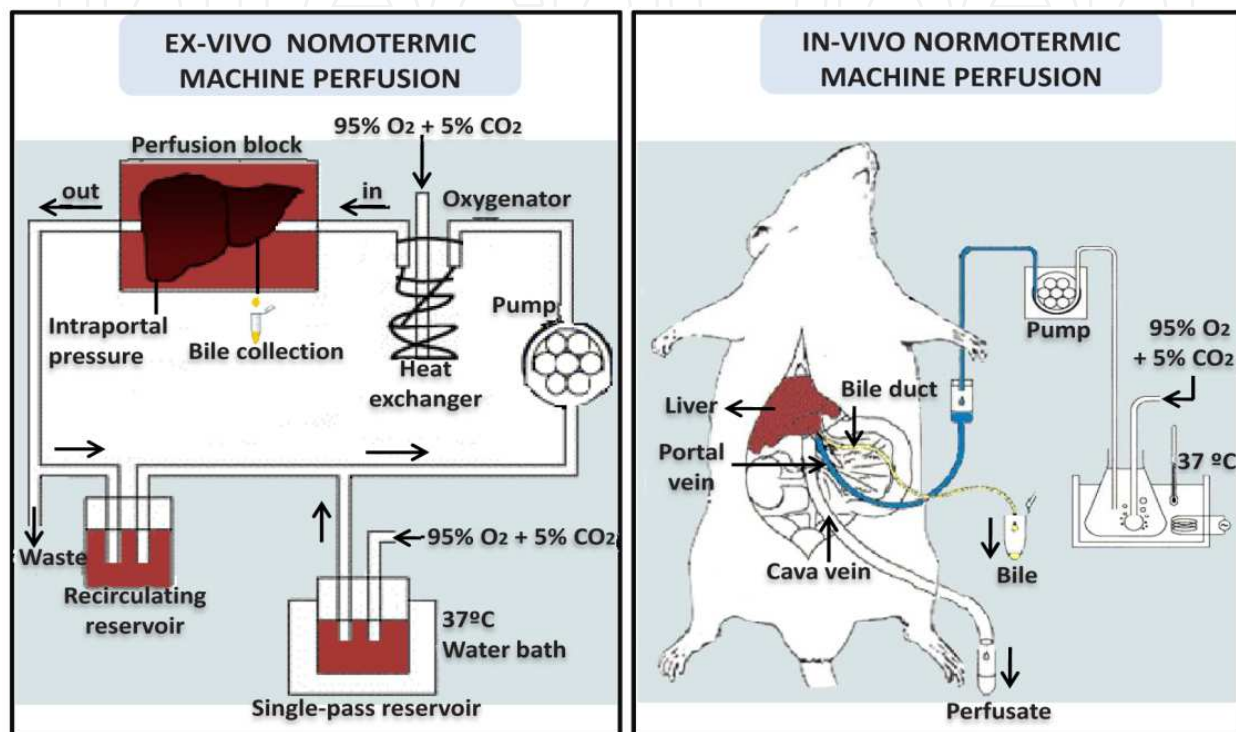


Figure 5. Esquematic illustration for *ex-vivo* and *in-vivo* normothermic machine perfusion

7.4. Hypothermic machine perfusion

For decades, cooling down organs to cold temperatures allowed successful organ transplantation within a limited period. The first and most prominent difference between SCS and (oxygenated) hypothermic machine perfusion (HMP) is the restoration of the tissue's energy charge and glycogen content while preventing ATP depletion [62]. In 1990, Pienaar et al., [68] reported that seven of eight dogs survived after LT with HMP preservation for 72 h and a similar outcome after 48 h of SCS. HMP is increasingly being used as an alternative method to SCS for the preservation of grafts obtained from nonoptimal donors. Indeed, several studies have reported a greater reduction in delayed graft function after HMP preservation than after SCS. Bessems et al., employed HMP preservation with UW-gluconate solution on steatotic rat livers for 24 h and alleviated I/R compared to SCS [69]. There is a substantial body of research, predominantly in rodents, demonstrating improved preservation by providing oxygen to livers [70]. Nevertheless, clear guidelines towards target values/ranges for

oxygen levels regarding the optimal duration of oxygenation during HMP are lacking. HMP can also be applied at the end of the cold storage period, which is attractive for logistical reasons. The disadvantage here is the time-dependent increase in vascular resistance, bearing the risk of damage to the sinusoidal endothelium [58].

7.5. Subnormothermic machine perfusion

Subnormothermic machine perfusion (SNMP) preservation lies between HMP and NMP, but it remained relatively unexplored until recently despite holding promising applications [71]. In an isolated rat liver perfusion model, SNMP enhanced the functional integrity of steatotic livers compared with SCS findings. Organ protecting properties mediated by decreasing the temperature to a 20–28°C have been observed previously. SNMP avoids some of the downsides of hypothermia while maintaining mitochondrial function and it may circumvent the logistical restraints of NMP [62]. Vairetti et al., preserved steatotic rat livers by SNMP (20°C) with Krebs-Henseleit solution for 6 hours and obtained reduced I/R damage compared to SCS [71].

8. Factors to be considered before the selection of an experimental model of hepatic I/R

Many investigators have used rodent models of warm (*in situ*) liver I/R to mimic some of the pathophysiological events that occur during LT. Although a great deal of useful information has been generated from these studies, an overriding question remains: Are the mechanisms responsible for transplant-mediated liver injury and dysfunction the same as those that have been reported for warm liver I/R injury? The answer is yes and no; that is, some of the mechanisms are similar, but many are dissimilar. It is important to make a distinction between the different types of ischemia, because there already is some controversy regarding the pathophysiological mechanisms depending on the type of ischemia (cold or normothermic), and it should be considered that the type of ischemia, the extent and time of ischemia, the type of liver submitted to I/R, and the presence of liver regeneration, all lead to differences in the pathophysiological mechanisms of hepatic I/R. These are discussed below to provide the reader with a guide to select the appropriate experimental model of hepatic I/R depending on the aims being pursued.

8.1. Relevance of the type of surgical procedure

The mechanisms responsible for hepatic I/R injury as well as the effects of pharmacological treatments are dependently of the liver surgical procedure. There is a range of potentially conflicting results with regard to the mechanisms responsible for ROS generation in liver I/R injury depending of the liver surgical procedure evaluated. XDH/XOD system is the main ROS generator in hepatocytes and LT-related lung damage [72]. However, results obtained in experimental models of the isolated perfused liver have underestimated the importance of the XDH/XOD system, and suggest that mitochondria could be the main source of ROS

[9]. In addition, studies by Metzger et al., in experimental models of normothermic hepatic ischemia showed that the increased vascular oxidant stress after 30 and 60 minutes of ischemia was attenuated by inactivation of KC but not by high dose of allopurinol in experimental models of normothermic hepatic ischemia [73].

It should be considered that the effectiveness of drugs on hepatic regeneration and damage could be different depending on the surgical conditions evaluated. Thus, gadolinium chloride treatment protected against hepatic damage in conditions of I/R without hepatectomy and improved liver regeneration after PH without I/R [74]. However, the same drug had injurious effects on hepatic damage and impaired liver regeneration in conditions of PH under I/R [75]. It should be also considered that the effectiveness of RAS blockers on hepatic regeneration and damage could be different depending on the surgical conditions evaluated. In conditions of PH under I/R, the AT1R antagonist for nonsteatotic livers and the AT1R and AT2R antagonists for steatotic ones improved regeneration in the remnant liver. The combination of AT1R and AT2R antagonists in steatotic livers showed stronger liver regeneration than either antagonist used separately and also provided the same protection against damage as that afforded by AT1R antagonist alone. However, the loss of protection of Ang II receptor antagonists against damage in conditions of PH under I/R (only AT1R antagonist protected steatotic liver against damage) compared with the study of I/R without hepatectomy (in which both Ang-II receptor antagonists reduced damage in both liver types) could be explained by the different surgical conditions. In the model of I/R without hepatectomy [33], the blood supply to the left and median liver lobes (70% hepatic mass) was interrupted, and the other hepatic lobes remained intact. However, in the conditions evaluated herein, only blood supply to the remnant liver (30% hepatic mass) was interrupted and the other hepatic lobes were excised. Compared with the study of I/R without hepatectomy [33], in PH under I/R, there are two main differences, the percentage of hepatic mass that is deprived of blood supply and hepatic resection. It is well known that the mechanisms of hepatic damage are different depending on the percentage of hepatic mass that is deprived of blood supply [76,77]. In addition, the inherent mechanisms of hepatic damage derived from the massive removal of hepatic mass should be considered. This may explain, at least partially, why the same drug, such as an Ang II receptor antagonist, may show differential effect on hepatic injury depending on surgical conditions [36]. In line with this, clinical and experimental studies revealed the injurious effects of NO on damage in the remnant liver in conditions of PH under I/R [36]. However NO protect against hepatic damage in an experimental model of I/R without PH [11]. In PH under I/R, Ang-II is an appropriate therapeutic target to protect steatotic livers against hepatic damage and regenerative failure. However, this target could be not appropriate in steatotic LT, since the results indicate a novel target for therapeutic interventions in LT within the RAS cascade, based on Ang 1-7, which could be specific for this type of liver. Indeed, Ang 1-7 receptor antagonist reduced necrotic cell death and increased survival in recipients transplanted with steatotic liver grafts [15].

The results, based on isolated perfused liver, indicated that the addition of epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-I) separately or in combination to UW reduced hepatic injury and improved function in both liver types. EGF increased IGF-I,

and both additives up-regulated AKT in both liver types. This was associated with glycogen synthase kinase-3 β (GSK3 β) inhibition in non-steatotic livers and PPAR γ over-expression in steatotic livers [78]. The benefits of EGF and IGF-I as additives in UW solution were also clearly seen in an experimental model of normothermic hepatic ischemia. However, the relationship between EGF and IGF-I was different dependently of the surgical procedure. Indeed, under these conditions, IGF-I increased EGF, thus protecting steatotic and non-steatotic livers against I/R damage. The beneficial role of EGF on hepatic I/R damage may be attributable to p38 inhibition in non-steatotic livers and to PPAR γ overexpression in steatotic livers [79].

PPAR α agonists as well as ischemic preconditioning (IP), through PPAR α , inhibited mitogen-activated protein kinase expression following I/R in steatotic livers undergoing normothermic hepatic ischemia. This in turn inhibited the accumulation of adiponectin in steatotic livers and reduced its negative effects on oxidative stress and hepatic injury [13]. In line with this, adiponectin silent small interfering RNA (siRNA) treatment decreased oxidative stress and hepatic injury in steatotic livers. However, another study by Man et al., 2006 [80] in small fatty grafts, adiponectin treatment exerted anti-inflammatory effects that down-regulated TNF α mRNA and vasoregulatory effects that improved the microcirculation. Adiponectin anti-inflammatory effects also include the activation of cell survival signaling via the phosphorylation of Akt and the stimulation of NO production. Additionally, the studies by Man et al., [80] showed the anti-obesity and proliferative properties of adiponectin in small fatty transplants. Taken together, the aforementioned data indicate that the action mechanisms of adiponectin depend on the surgical conditions. Thus, on the basis of the different results reported to date in hepatic I/R, it is difficult to discern whether we should aim to inhibit adiponectin, or administer adiponectin to protect steatotic livers against cold ischemia associated with transplantation. Moreover, the adiponectin data reported for these experimental models of hepatic I/R [13,80] should not be extrapolated to cadaveric organ transplantation. For small liver grafts (which are relatively common) and under conditions of warm ischemia, the periods of ischemia range from 40 to 60 minutes; this range may not be accurate for cadaveric donor LT.

RBP4 is an adipokine synthesized by the liver, whose known function is to transport retinol in circulation. However, the role of RBP4 in hepatic I/R could depend on the liver surgical procedure. Steatotic liver grafts were found to be more vulnerable to the down-regulation of RBP4. RBP4 treatment-through AMP-activated protein kinase (AMPK) induction- reduced PPAR γ over-expression, thus protecting steatotic liver grafts against I/R injury associated with transplantation. In terms of clinical application, therapies based on RBP4 treatment and PPAR γ antagonists might open new avenues for steatotic LT and improve the initial conditions of donor livers with low steatosis that are available for transplantation [81]. On the other hand, the effects of RBP4 could depend on the surgical conditions. Indeed, RBP4 administration not only failed to protect both liver types from damage and regenerative failure, it exacerbated the negative consequences of liver surgery in PH under I/R [82]. Under these conditions, RBP4 affected the mobilization of retinol from steatotic livers, revealing actions of RBP4 independent of simple retinol transport. The injurious effects of RBP4 were

not due to changes in retinol levels. Thus, strategies based on modulating RBP4 could be ineffective and possibly even harmful in both liver types in PH under I/R or surgical conditions including small-for-size LT.

8.2. Relevance of the duration of hepatic ischemia

The severity of hepatocyte damage depends on duration of ischemia. Depending on the objectives of the research, it is important to consider a specific ischemia duration. In other words, if you want to study the mechanisms involved in hepatic I/R injury or the protective mechanisms of a drug, it is more appropriate to use a duration of ischemia associated with high survival. If the purpose is to study the relevance of a drug in hepatic I/R injury, then it is advisable to assess survival, and, therefore, it is more adequate to use experimental models in which the ischemic period is associated with low survival. These observations are based on the following data reported in the literature. It appears that short periods (60 minutes) of warm ischemia result in reversible cell injury, in which liver oxygen consumption returns to control levels when oxygen is resupplied after ischemia. Reperfusion after more prolonged periods of warm ischemia (120-180 minutes) results in irreversible cell damage. These observations agree with a previous report on rat liver subjected to I/R, indicating a cellular endpoint for hepatocytes after 90 minutes of ischemia [83]. In human LT, a long ischemic period is a predicting factor for posttransplantation graft dysfunction, and some transplantation groups hesitate to transplant liver grafts preserved for more than 10 h. Some studies in experimental models of LT indicate that cold ischemia for 24 h induces low survival. However, LT, following shorter ischemic periods, may also result in primary organ dysfunction [72].

It is important to distinguish between the types of Ischemia (warm and cold) because there is already some controversy about the pathophysiological mechanisms of cold ischemia, which may depend, for example, on the time. The mechanisms of hepatic I/R injury are also different depending on the duration of hepatic ischemia. Along these lines, in the same experimental model of LT, XDH/XOD plays a crucial role in hepatic I/R injury only in conditions under which significant conversion of XDH to XOD occurs (80–90% of XOD) such as 16 h of cold ischemia. However, this ROS generation system does not appear to be crucial for shorter ischemic periods such as 6 h of cold ischemia [72]. Similarly, it should also be noted that oxidative stress in hepatocytes and the stimulatory state of KCs after I/R depend on the duration of ischemia and may also differ between ischemia at 4°C and that at 37°C, which probably leads to different developmental mechanisms of liver damage.

Our previous results indicate that PPAR α does not play a crucial role in I/R injury in non-steatotic livers. This contrasts with a study published by Okaya and Lentsch [84], in which the authors reported the benefits of PPAR α agonists on postischemic liver injury. Although the dose and pretreatment time of the PPAR α agonist WY-14643 were similar in both studies, Okaya and Lentsch reported an ischemic period of 90 minutes; ours was 60 minutes, which is the ischemic period currently used in liver surgery [3]. Thus, 60 minutes of ischemia seems to be insufficient to induce changes in PPAR α in nonsteatotic livers [13].

8.3. Relevance of the extent of hepatic ischemia

Another factor to consider before selecting the experimental model of hepatic I/R is the percentage of hepatic ischemia applied. The extent of hepatic injury as well as the hepatic I/R mechanisms, including the recovery of blood flow and energy charge during hepatic reperfusion is dependent on the extent of ischemia-whether total or partial (70%) hepatic ischemia is applied [36]. This fact could be explained by the stealing phenomenon. In contrast to 100% hepatic ischemia, during ischemia in the left and median lobes, the flow is shunted via the right lobes and following the release of the occlusion of the left and median lobes, a significant amount of shunting via the right lobes will continue during reperfusion until vascular resistance in the postischemic lobes decreases. This occurs because blood flows through the path of least resistance. The reasons for this may be cellular swelling endothelial, stasis, or other changes. Thus, the recovery of blood flow and hepatic perfusion of the preischemic lobe is later in the case of 70% hepatic ischemia than in 100% hepatic ischemia [76]. In line with these observations, the benefits of some drugs such as ATP-MgCl₂ were dependent on the extent of hepatic ischemia used [32,77].

8.4. Relevance of the type of liver submitted to I/R

A variety of clinical factors including starvation, graft age, and steatosis have been studied in different experimental models of hepatic I/R because of the relevance of these factors in clinical practice. These factors enhance liver susceptibility to I/R injury, further increasing the patient risks related to reperfusion injury.

8.4.1. Starvation

The pre-existent nutritional status is a major determinant of the hepatocyte injury associated with I/R. In clinical LT, starvation of the donor, due to prolonged intensive care unit hospitalization or the lack of adequate nutritional support, increases the incidence of hepatocellular injury and primary nonfunction [85]. Based on the nutritional state status, several experimental and clinical studies support the hypothesis that the availability of glycolytic substrates is important for maintenance of hepatic ATP levels during I/R. Fasting exacerbates I/R injury because the low content of glycogen stores results in more rapid ATP depletion during ischemia. In addition, fasting causes alterations in tissue antioxidant defenses, accelerates the conversion of XDH to XOD during hypoxia and induces mitochondrial alterations [85]. Caraceni et al., [86] have shown that mitochondrial damage is greatly enhanced by fasting which decreases the hepatic content of antioxidants and therefore sensitizes the mitochondrial to the injurious effects of ROS. Considering these observations, an artificial nutritional support may represent a new approach for the prevention of reperfusion injury in fasted livers. On the contrary, fasting has been reported to improve organ viability and survival [87], as it reduces phagocytosis and the generation of TNF- α [87]. To understand these apparent contradictory results, it is important to consider the different experimental conditions in these investigations. A beneficial effect of high glycogen content can mainly be expected under conditions of long preservation times and long periods of warm ischemia. Under these conditions, high metabolic reserves of the liver may attenuate ischemic cell in-

jury and preserve defense functions against cytotoxic mediators of KCs. Conversely, short ischemic periods require lower metabolic reserves, and the extent of KC activation can be the dominant factor in early graft injury.

8.4.2. Age

A number of distinct age-related alterations have been identified in the hepatic inflammatory response to hepatic I/R [88]. Under warm hepatic ischemia, mature adult mice had greatly increased neutrophil function, increased intracellular oxidant levels, and decreased mitochondrial function compared with the findings in young adult mice. These alterations contributed to the increased liver injury after I/R observed in mature adult mice compared with that in young adult mice. The results obtained in an experimental model of isolated perfused liver indicate that, during reperfusion, livers obtained from old rats generate a lower amount of oxyradicals than livers from young rats. This fact could be explained by the lower KC activity, the reduction of liver blood flow, and the impaired functions and structural alterations observed in the livers of old rats. In fact, in hepatocytes from mature adult mice, delayed activation of nuclear factor kappa B (NF κ B) in response to TNF- α and virtually no production of macrophage inflammatory protein 2 have been detected, which may be due to an age-related defect in hepatocytes [88].

8.4.3. Steatosis

The first step to minimize the adverse effects of I/R in steatotic livers is a full understanding of the mechanisms involved in I/R injury in these marginal organs. This can be achieved only with the selection of an appropriate method to induce steatosis in livers undergoing I/R. It is well known that the mechanisms involved in hepatic I/R injury are different depending on the type of liver (nonsteatotic versus steatotic livers). In addition to the impairment of microcirculation, mitochondrial ROS generation dramatically increases during reperfusion in steatotic livers [9,86]. Results obtained under warm hepatic ischemia indicate that apoptosis is the predominant form of hepatocyte death in the ischemic nonsteatotic liver, whereas the steatotic livers develop massive necrosis after an ischemic insult [9]. Steatotic livers differed from nonsteatotic livers in their response to the UPR and ER stress since IRE1 and PERK were weaker in the presence of steatosis [89]. Decreased ATP production and dysfunction of regulators of apoptosis, such that Bcl-2, Bcl-xL and Bax have been proposed to explain the failure of apoptosis in steatotic livers. Differences were also observed when we analyzed the role of the RAS, as the nonsteatotic grafts exhibited higher Ang-II levels than steatotic grafts whereas steatotic grafts exhibited higher Ang 1-7 levels [15]. In the context of I/R injury associated with LT, the axis ACE-Ang II-ATR and ACE2-Ang 1-7-Mas play a major role in nonsteatotic and steatotic grafts, respectively. From the point of view of clinical application, these findings may open up new possibilities for therapeutic interventions in LT within the RAS cascade, based on Ang 1-7 for steatotic livers and Ang II for non-steatotic ones [15]. Moreover, reduced RBP4 and increased PPAR γ levels were observed in steatotic livers compared to non-steatotic livers [81]. The vulnerability of steatotic livers subjected to

warm ischemia is also associated with increased adiponectin, oxidative stress, and IL-1 levels and a reduced ability to generate IL-10 and PPAR α [13,90].

It should be considered that there are differences in the mechanisms involved in hepatic I/R injury depending on the method used to induce steatosis. In contrast with other experimental models of steatosis, both dietary high fat and alcohol exposure induced the production of SOD/catalase-insensitive ROS, which may be involved in the mechanism of steatotic liver failure after OLT [9]. Neutrophils have been involved in the increased vulnerability of steatotic livers to I/R injury, especially in alcoholic steatotic livers. However, neutrophils do not account for the differentially greater injury in non-alcoholic steatotic livers during the early or late hours of reperfusion. Similarly, the role of TNF in the vulnerability of steatotic livers to I/R injury may be dependent on the type of steatosis [1,9].

8.5. Relevance of regeneration in experimental models of hepatic I/R

It is known that different experimental models trigger different responses when a common mechanism or the same drug is investigated. This situation is witnessed when analyzing liver injury in models of I/R with or without hepatectomy. This situation is illustrated by Ramalho et al., [36] regarding the loss of protection of Ang-II receptor antagonists against liver damage in conditions of PH under I/R compared with the study of I/R without hepatectomy, in which Ang-II receptor antagonists reduced hepatic damage. These different results could not be explained by differences in the dose or frequency of drug administration but rather by differences in surgical conditions (percentage of hepatic ischemia and the presence or absence of hepatectomy). In the model of I/R without hepatectomy [33], the blood supply to the left and median liver lobes (70% hepatic mass) was interrupted, and the other hepatic lobes remained intact. However, in PH under I/R, only blood supply to the remnant liver (30% hepatic mass) was interrupted and the other hepatic lobes were excised [36].

According to the cell type and experimental or pathologic conditions, TNF- α may stimulate cell death or it may induce hepatoprotective effects mediated by antioxidant, antiapoptotic, and other anti-stress mediators coupled with a pro-proliferative biologic response. For example, although the deleterious effect of the TNF- α in local and systemic damage associated with hepatic I/R in experimental models of normothermic hepatic ischemia is well established [91], this mediator is also a key factor in hepatic regeneration [92], an important process in RSLT and PH associated with hepatic resections [93]. These differential effects observed for TNF- α can also be extrapolated to transcription factors. It is well known that NF κ B can regulate various downstream pathways and thus has the potential to be both pro- and antiapoptotic [8]. Currently it is not clear whether the beneficial effects of NF κ B activation in protection against apoptosis or its detrimental proinflammatory role predominate in liver I/R [8]. Hepatic neutrophil recruitment and hepatocellular injury are significantly reduced if NF κ B activation is suppressed in mice following partial hepatic I/R. However, NF κ B activation is essential for hepatic regeneration after rat LT, and reduces apoptosis and hepatic I/R injury [94].

9. Strategies applied in experimental models of hepatic I/R

9.1. Pharmacological treatment and additives in preservation solution

Numerous experimental studies have focused on the developing *in vivo* pharmacological strategies aimed at inhibiting the harmful effects of I/R [9,72,89,90,95-99]. Some of these studies are summarized in Table 1. However, none of these treatments has managed to prevent hepatic I/R injury. A large number of ingredients-which have been introduced into UW solution in experimental models of hepatic cold ischemia [9,95,100-102] (Table 1). However, none of these modifications to the UW solution composition have found their way into routine clinical practice. Further studies will be required to elucidate whether the use of perfluorochemicals (PFC) in preservation solutions might improve the viability of liver grafts undergoing transplantation. PFC are hydrocarbons with high capacity for dissolving respiratory and other nonpolar gases. A negligible O₂-binding constant of PFC allows them to release O₂ more effectively than hemoglobin into the surrounding tissue (acts as an oxygen-supplying agent). PFC differs from hemoglobin preparations in that it is a totally synthetic compound formed on a liquid hydrocarbon base. Unlike hemoglobin, acidosis, alkalosis, and temperature seem to have no or little effect on the oxygen delivery of PFC, allowing this compound to be used effectively during cold storage of organs [103]. A recently study, used Oxycyte, a PFC added to UW solution can be beneficial after cardiac death liver graft preservation in a rat model [103]. However, their effects on reperfusion injury were not evaluated in that study. In fact, the possibility that preoxygenated PFC exacerbates the ROS during reperfusion should not be discarded since the use of gaseous oxygen applied to the livers during the storage period was only effective in improving hepatic viability upon reperfusion when antioxidants were added to the UW rinse solution [104].

It should be also considered that the inclusion of some components in the UW solution has been both advocated and criticized. Indeed, simplified variants of the UW solution in which some additive were omitted were demonstrated to have similar or even higher protective potential during cold liver storage. Another limitation of the UW solution is that some of its constituent compounds, including allopurinol do not offer very good protection because they are not present at a suitable concentration and encounter problems in reaching their site of action [9]. The possible side effects of some drugs may frequently limit their use in human LT. For example, idiosyncratic liver injury in humans is documented for chlorpromazine, pernicious systemic effects have been described for NO donors, allopurinol therapy can cause hematological changes and gadolinium can induce coagulation disorders. Some case reports of acute hepatotoxicity attributed to rosiglitazone have been published [105]. The development of therapeutic strategies that utilize the protective effect of heme oxygenase-1 induction is hampered by the fact that most pharmacological inducers of this enzyme perturb organ function by themselves [106].

Pharmacological treatment-derived difficulties must also be considered. In this regard, SOD and GSH exhibit inadequate delivery to intracellular sites of ROS action [9]. The administration of anti-TNF antibodies does not effectively protect against hepatic I/R injury,

and this finding has been related to the failure of complete TNF- α neutralization locally [11]. Although this also occurs in non-steatotic livers, modulating I/R injury in steatotic livers poses a greater problem. Differences in the action mechanisms between steatotic and non-steatotic livers mean that therapies that are effective in non-steatotic livers may prove useless in the presence of steatosis, and the effective drug dose may differ between the two liver types. Findings such as these must be considered when applying pharmacological strategies in the same manner to steatotic and non-steatotic livers because the effects may be very different. For example, caspase inhibition, a highly protective strategy in non-steatotic livers, had no effect on hepatocyte injury in steatotic livers [9]. Moreover, whereas in an LT experimental model, an NO donor reduced oxidative stress in non-steatotic livers, the same dose increased the vulnerability of steatotic grafts to I/R injury. Furthermore, there may be drugs that would only be effective in steatotic livers. This was the case of compounds such as cerulenin, which reduce UCP-2 expression in steatotic livers and carnitine [9].

Pharmacological Therapy – Warm Ischemia			
Species	Drug	Ischemic Time	Effect
Mice	Cerulenin (<i>Fatty acid synthase inhibitor</i>)	15 min	↓ UPC2, ↑ ATP
	Catalase and derivatives	30 min	↓ Oxidative stress
	Apocynin (<i>NAPH oxidase inhibitor</i>)		↓ Oxidative stress
	TBC-1269 (<i>Pan-selectin antagonist</i>)	90 min	↓ Inflammatory response, ERK ½
Rat Rat	Lisinopril (<i>ACE inhibitor</i>)	30 min	↓ Oxidative stress
	Ascorbate (<i>ROS scavenger</i>)		↓ Apoptosis
	Allopurinol (<i>XOD inhibitor</i>)	30 60 min	↓ Oxidative stress
	Melatonin (<i>Hormone</i>)	40 min	↓ IKK, JNK pathways
	SOD (<i>antioxidant</i>)	45 min	↓ Microcirculatory disturbances, leukocyte accumulation
	L-arginine (<i>NO precursor</i>)		↑ NO, ATP ↓ Neutrophil accumulation
	Tocopherol (<i>Antioxidante</i>)	45 90 min	↓ Microcirculatory disturbances, Lipid peroxidation, SEC damage
	IL-10	60 min	↓ IL-1, Oxidative stress
	Anti-ICAM-1		↓ Adherence of leukocytes in postsinusoidal venules
	Gabexate mesilate (<i>Protease inhibitor</i>)		↓ TNF- α , Leukocyte activation
	OP-2507 (<i>Analogue of prostacyclin</i>)		↓ Microcirculatory disturbance
	WY-14643 (<i>PPARα agonist</i>)		↓ Oxidative stress, Inflammatory cytokines

	n-3 PUFA		↓ Liver injury, Oxidative stress
	Glutathione (<i>Antioxidant</i>)	60 90 min	↓ Microcirculatory disturbances ↑ Detoxification of ROS
	Spermine NONOate (<i>NO donor</i>)		↓ IL-1α, Oxidative stress
	FK506 (<i>Immunosupressant</i>)		↓ TNF
	Rosiglitazone (<i>PPARα agonist</i>)		↑ Autophagy ↓ Cytokines
	AMPK activators	90 min	↑ NO, ATP
	Adenosine		↑ NO
	Anti-TNF antiserum		↓ TNF, Leukocyte accumulation
	α-Lipoic acid (<i>Antioxidant</i>)		↑ Liver regeneration, ↓ Apoptosis
Pharmacological Therapy – Warm Ischemia with Hepatectomy			
Species	Drug	Ischemic Time	Effect
Rat	Tauroursodeoxycholate (<i>Bile acid</i>)	60 min	↓ Endoplasmic reticulum stress
	Sirolimus (<i>Immunossupressant</i>)		↓ Linfocytes
	IL-1ra (<i>IL-1 receptor antagonist</i>)	90 min	↓ TNF, Oxidative stress
Dog	FK 3311 (<i>Cox-2 inhibitor</i>)	60 min	↓ Neutrophil infiltration, Cox-2
Pharmacological Therapy – Liver Trasplantation			
Species	Drug	Ischemic Time	Effect
Mice	Cerulenin (<i>fatty acid synthase inhibitor</i>)	80 min	↓ UPC2, ↑ ATP
Rat	FK 409 (<i>NO donor</i>)	80 min	↑ HSP, IL-10, ↓ SEC damage, IL-1
	CS1 peptides (<i>FN-α4β1 interac blocker</i>)	4 h	↓ Neutrophil and lymphocyte T infiltration, TNF-α, iNOS
	Tocopherol (<i>antioxidante</i>)	5 h	↓ Lipid peroxidation, SEC damage, Microcirculatory disturbance
	Hemin (<i>HO-1 inducer</i>)	6 h	↑ Bcl-2
	Cobalt-protoporphyrin IX (<i>HO-1 inducer</i>)		↓ Macrophages infiltration and T cells
	PSGL-1 (<i>P-selectin blocker</i>)		↓ Neutrophil infiltration, TNF-α, INFγ, iNOS
	Anti-TNF antiserum	6, 24 h	↓ TNF, Leukocyte accumulation
	SOD (<i>antioxidant</i>)	8 h	↓ Microcirculatory disturbance, Leukocyte acumulation
	Tauroursodeoxycholate (<i>Bile acid</i>)		↓ Endoplasmic reticulum stress
Allopurinol (<i>XOD inhibitor</i>)	8, 16 h	↓Oxidative stress	

	Z-DEVD-FMK (<i>caspase 3 and 7 inhibitor</i>)	16 h	↑ Microvascular perfusión, Bcl-2 ↓ Apoptosis
	L-arginine (<i>NO precursor</i>)	18 h	↑ NO, ATP, ↓ Neutrophil accumulation
	Treprostinil (<i>Prostacyclin analogue</i>)		↓ Liver injury, Platelet deposition, microcirculatory disturbance
	ANP (<i>vasodilating peptide</i>)	24 h	↑ PI3K/Akt, ↓ Apoptosis
	Bucillamine (<i>antioxidant</i>)		↓ Oxidative stress
	Chlorpromazine (<i>Ca²⁺ + channel antagonist</i>)		↑ ATP ↓ Mitochondrial dysfunction, Alterations in lipid metabolism
	sCR1 (<i>complement inhibitor</i>)		↓ Microcirculatory disturbance, Leukocyte adhesion
	Glutathione (<i>antioxidant</i>)		↓ Microcirculatory disturbance ↑ Detoxification of ROS
	N-acetylcysteine (<i>glutathione precursor</i>)		↓ Microcirculatory disturbance
	Anti-ICAM-1		↓ Adherence of leukocytes in postsinusoidal venules
	Glycine (<i>Kupfer cell modulator</i>)		↓ Neutrophil accumulation, TNF-α
	GdCl3 (<i>Kupffer cell blocker</i>)		↓ Neutrophil accumulation, TNF-α
	Cbz-Val-Phe methyl ester (<i>calpain inhibitor</i>)	24, 40h	↓ Calpain activation, SEC apoptotic
	EHNA (<i>adenosine deaminase inhibitor</i>)	24, 44 H	↑ Interstitial adenosine ↓ Microcirculatory disturbance, Leukocytes rolling
	CGS-21680 (<i>adenosine A2 receptor agonist</i>)	30 h	↑ cAMP, ↓ SEC Killing
	Sotrastaurin (<i>PKC Inhibitor</i>)		↓ Apoptosis, macrophage/neutrophil accumulation
	FR167653 (<i>IL-1β and TNF-α supressor</i>)	48 h	↓ TNF-α, IL-1α, Kupffer cell activation
	Doxorubicin (<i>Heat shock proteins inducer</i>)		↓ TNF-α, MIP-2, NKκB
Pig	Sodium ozagrel (<i>Thromboxane synthase inhibitor</i>)	8 h	↓ ET-1
Additives to UW solution – Liver Trasplantation			
Species	Drug	Ischemic Time	Effect
Mouse	Erythropoietin (<i>EPO</i>)	24 h	↓ Liver injury
Rat	Meloxicam (<i>COX-2 Inhibitor</i>)	1 h	↓ Apoptosis, Liver injury, Oxidative stress
	Simvastatin (<i>KLF2-inducer</i>)	1, 6, 16 h	↓ Inflammation, Liver injury, Oxidative stress,
	Tauroursodeoxycholate (<i>Bile acid</i>)	2 h	↓ Endoplasmic reticulum stress
	S-nitroso-N-acetylcysteine	2, 4, 6 h	↓ Liver injury

	LY294002 (<i>PI3K inhibitor</i>)	7, 9, 24 h	↓ Apoptosis
	8br-cAMP, 8br-cGMP (<i>nucleotide analogs</i>)	24 h	↓ TNF- α and neutrophil accumulation
	Ruthenium red (<i>mitochondrial Ca²⁺ uniporter inhibitor</i>)		↓ Mitochondrial dysfunction
	Melatonin (<i>Hormone</i>)		↓ Oxidative stress, Liver injury
	OP-4183 (<i>PGI₂ analogue</i>)		↓ Oxidative stress
	SAM (<i>ATP precursor</i>)		↓ Oxidative stress
	IDN-1965 (<i>caspase inhibitor</i>)	24, 30 h	↓ Apoptosis
	Pifithrin- α (<i>p53 inhibitor</i>)	24, 48 h	↓ Apoptosis
	Sodium nitroprusside (<i>NO donor</i>)		↓ Microcirculatory disturbances
	FR167653 (<i>p38 inhibitor</i>)	30 h	↓ Microcirculatory disturbances
	GSNO (<i>NO donor</i>)	48 h	↓ SEC damage
Dog	Trifluoperazine (<i>calmodulin inhibitor</i>)	24 h	↓ Microcirculatory disturbances
Pig	E5880 (<i>PAF antagonist</i>)	8 h	↓ Microcirculatory disturbances
	EGF, IGF-1, NGF- α	18 h	↑ ATP

Table 1. In vivo pharmacological therapy and additives in preservation solution in experimental models of warm hepatic ischemia (with or without hepatectomy) and liver transplantation

9.2. Gene therapy

Advances in molecular biology provide new opportunities to reduce liver I/R injury by using gene therapy. Genome manipulation can be achieved by: A) germ line manipulation (oocyte injections); B) stem cell transformation and reintroduction into embryos, and C) targeting specific cells or organs with vectors or viruses (gene transfer). The first 2 approaches include germ-line alterations and are neither feasible nor accepted by society. The third approach would lend to the treatment of individual patients with either acquired or congenital diseases [12]. In the last years, significant advances in gene therapy vectors have occurred. Gene transfer can be accomplished by direct injection of DNA into a target organ or tissue, transduction by recombinant viral vectors carrying a specific gene of interest, e.g., adenovirus (Ad) or retrovirus, transfection of cells by chemical methods (e.g., cationic liposomes), or stem cell transduction and reintroduction of genetically-altered cells back into embryos [107] (Table 2). Currently, researchers in gene transfer have focused efforts toward targeting vectors to specific cells or organs without loss of transduction ability [108,109], allowing high level gene transduction of the liver without affecting other organs [12,107].

		Genetic material	Packaging capacity	Duration of experiment	Integration into genome	Transduction of postmitotic cells
Recombinant viruses	Oncoretrovirus	RNA	9 kb	Long	Yes	Low
	Lentivirus	RNA	10 kb	Long	Yes	Low
	Foamy	RNA	12 kb	Long	No	High
	Herpes virus	DNA	>30 kb	Transient	No	High
	Adenovirus	DNA	30 kb	Transient	Rarely	Moderate
	AAV	DNA	4.6 kb	Long postmitotic tissues	Rarely	Moderate
	Oncoretrovirus	RNA	9 kb	Long	Yes	Low
	Lentivirus	RNA	10 kb	Long	Yes	Low
Non-viral methods	siRNA	RNA	No limitation	Transient	No	Zero
	DNA injection	DNA	No limitation	Transient	No	Zero
	Cationic liposomes	DNA	No limitation	Transient	No	Zero
	Stem cell transduction	DNA	No limitation	Transient	No	Zero

Table 2. Summary of gene therapy vectors commonly used.

Antiapoptotic Strategies (Bcl-2/Bcl-Xl, Bag-1 and caspases): Bcl-2 blocks apoptosis and necrosis and has been implicated in the prolongation of cell survival [110]. Given its functional importance in the cell death cascade, it constitutes one of the key targets for cytoprotective therapeutic manipulation for the regulation of apoptosis [110,111]. As demonstrated by Bilbao et al., [111] in a mouse hepatic I/R model, overexpression of Ad-mediated Bcl-2 gene significantly decreased hepatocyte apoptosis and necrosis, improved hepatic function, and prolonged survival as compared with controls. In addition, Bag-1 is a Bcl-2 binding protein resulting in a prolonged and stabilized antiapoptotic activity [112]. In addition, Bag-1 appears to exert an indirect silencing effect on TNF receptor R1 and hence suppresses the death receptor signal. A recent study by Sawitzki et al., [113] has demonstrated the cytoprotective effect of Ad-mediated Bag-1 gene transfer in rat liver I/R. Using a model of cold ischemia and OLT, Ad-Bag-1 transfer improved portal venous blood flow, increased bile production, and improved hepatic function with decreased neutrophil accumulation in the graft. Furthermore, Ad-mediated Bag-1 expression preserved hepatic architecture and reduced inflammation. The activation of T cells infiltrating the graft was inhibited, since decreased expression of TNF- α , CD25, IL-2, and IFN γ [107]. Caspase-8 is presumed to be the apex of the death-mediated apoptosis pathway, whereas caspase-3 belongs to the “effector” proteases in the apoptosis cascade. Contreras et al., demonstrated that inhibition of caspase-8 and caspase-3 by siRNA provided significant protection against warm hepatic I/R injury and decreased animal mortality. In addition, animals given siRNA caspase-8, or more significantly siRNA caspase-3, presented lower neutrophil infiltration and better histologic profiles [114].

Antioxidant therapy (SOD, HO-1, Ferritin): Oxidative stress can activate NF- κ B and the AP-1 pathway and induce expression of proinflammatory genes including cytokines, adhesion molecules, and chemokines leading to neutrophil-mediated inflammation [115-117]. To inhibit the burst of ROS or its effect on hepatocytes, several oxygen stress inhibitory proteins have been studied, e.g., SOD and catalase have been transfected by either adenovirus, liposomes or polyethylene-glycol [8,12,118]. Using partial hepatic I/R models, Ad-mediated MnSOD administration reduced liver tissue damage and activation of both NF- κ B and AP1 [119,120] when compared with lacZ-transduced controls. In another study, He et al., [121] demonstrated that SOD or catalase gene delivery by poly lipid nanoparticles injected via the portal vein 1 day prior to the warm I/R procedure resulted in high levels of the transgene enzyme activity in the liver, and markedly attenuated hepatic I/R injury [121]. However, results with NF κ B activation have been conflicting. Takahashi et al. reported that overexpression of I κ B, an NF κ B inhibitor (mediated by Ad-I κ B) resulted in partial protection in hepatic I/R injury [122]. Heme oxygenase 1 (HO-1) is a stress responsive protein and can be induced by various conditions such as hypoxia [12,107]. Several studies have shown that HO-1 exhibits potent cytoprotective effects after hepatic I/R [123,124]. In a cold ex-vivo rat liver perfusion model and a syngeneic liver transplant OLT model, treatment of genetically obese Zucker rats with Ad-HO-1 improved portal venous blood flow, increased bile production, and decreased hepatocyte injury [123]. Unlike in untreated rats, upregulation of HO-1 correlated with preserved hepatic architecture, improved liver function, and depressed infiltration by T cells and macrophages. Ad-mediated HO-1 gene overexpression increased survival of recipients from 40% to 80% [12,107]. Ad-HO-1 gene transfer decreased macrophage infiltration in the portal areas and inducible nitric oxide synthetase (iNOs) expression; it also increased the expression of antiapoptotic genes Bcl-2/Bcl-xl and Bag-1, as compared with controls [107]. Iron chelation is another approach to ameliorate the I/R injury cascade. Free iron has been shown to play a role in the formation of the free radicals through the Fenton reaction; these contribute to endothelial cell damage. Ferritin induction is a result of the action of HO-1 on the heme porphyrin causing the release of Fe²⁺. Ferritin can reduce the availability of intracellular free Fe²⁺, which can participate in free radical generation [125]. Studies by Ke et al., [107] demonstrated that overexpression of Ad vector carrying the ferritin heavy chain (H-ferritin) gene protects rat livers from I/R injury [126]. In these studies, the protective effect of H-ferritin was associated with the inhibition of endothelial cell and hepatocyte apoptosis. Evidence suggested that H-ferritin exerts an antiapoptotic role and may be used as a therapeutic measure to prevent I/R [107].

Immunoregulatory cytokines (IL-10 and IL-13) and IL-1 receptor antagonist (IL-1R): IL-13 regulates liver inflammatory I/R injury via the signal transducer and activator of transcription 6 (STAT6) pathway [127]. IL-10 induces antioxidant HO-1 gene expression in murine macrophages and exerts anti-inflammatory effects [128]. In recent studies, Ad-IL-13 gene transfer in cold ischemia models has shown powerful cytoprotective effects [129]. Gene transfer of IL-13 improved hepatic function, upregulated HO-1, and prevented hepatic apoptosis through the upregulation of Bcl-2/Bcl-xl [107]. The beneficial effects of IL-13 correlated with *in vivo* cross talk between innate TLR4 and adaptive Stat6 immunity [130]. In fact, using an experimental model of warm hepatic ischemia, Stat6-deficient mice with Ad-IL-13 failed to

improve hepatic function and hepatic histological features. Transfer of Ad-IL-13 increased anti-oxidant HO-1 expression and inhibited TLR4 activation in WT mice, whereas low HO-1 and enhanced TLR4 expression was shown in Stat6-deficient mice [107]. It has been demonstrated that the pro-inflammatory cytokine IL-1 plays a critical role in the pathophysiological response to I/R. Experimental results have shown that blockade of the IL-1R reduced TNF production and liver damage [131]. In a partial hepatic I/R model, gene transfer of Ad-mediated IL-1R antagonist prolonged animal survival and improved hepatic function while preserving the histological architecture. In addition, a marked decrease in production of proinflammatory cytokines such as IL-1, TNF- α , and IL-6 was present [107].

T-cell co-stimulation blockade: CD40-CD154. A number of studies have shown that CD4+ T lymphocytes play an important role as key cellular mediators in I/R injury mediated inflammatory responses. The CD40-CD154 co-stimulation pathway provides the essential second signal in the initiation and maintenance of T-cell-dependent immune responses [132]. Recent studies have demonstrated that CD40-CD154 is required for the mechanism of hepatic warm I/R injury [133]. In OLT, prolonged *in vivo* blockade of the CD40-CD154 interaction following pretreatment of liver isografts with Ad-CD40Ig exerted potent cytoprotection against I/R injury. Apoptosis was prevented and neutrophil accumulation was reduced. Evidence also demonstrated prevention of Th1-type cytokine (interferon γ (IFN- γ) and IL-2) upregulation and the local expression of antioxidant HO-1 and antiapoptotic Bcl-2/Bcl-xl genes were triggered [107].

Adipocytokine, sphingolipid and TLR4 regulation: Massip-Salcedo et al., [13] demonstrated though the systemic delivery of adiponectin in livers treated with adiponectin siRNA that steatotic livers by themselves can generate adiponectin as a consequence of I/R. This study reports evidence of the injurious effects of adiponectin in steatotic livers under warm ischemic conditions, and results suggest the clinical potential of gene therapy for I/R damage in steatotic livers by siRNA-mediated adiponectin gene silencing [13]. Products of sphingolipid metabolism are important second messengers that regulate a variety of cell processes including cell death, proliferation, and inflammation. Using a mice warm hepatic I/R model, Shi et al., demonstrated that SK2 knockdown by siRNA effectively prevented hepatocyte death [134]. Jiang et al., [135] reported a hepatocyte-specific delivery system for the treatment of liver I/R, using galactose-conjugated liposome nanoparticles (Gal-LipoNP). Hepatocyte-specific targeting was validated by selective *in vivo* delivery as observed by increased Gal-LipoNP accumulation and gene silencing in the liver. Gal-LipoNP TLR4 siRNA treatment reduced hepatic damage, neutrophil accumulation and the inflammatory cytokines IL-1 and TNF- α [135].

Advances in molecular biology have provided new opportunities to reduce liver I/R injury using gene therapy [9,12,13,96,114] (Table 3). However, the experimental data indicate that there are a number of problems inherent in gene therapy, such as vector toxicity, difficulties in increasing transfection efficiencies and protein expression at the appropriate time and site, and the problem of obtaining adequate mutants (in the case of NF κ B) due to the controversy regarding NF κ B activation [136]. Although non-viral vectors (such as naked DNA and liposomes) are likely to present fewer toxic or immunological problems, they suffer from in-

efficient gene transfer [136]. In addition, LT is an emergency procedure in most cases, which leaves very little time to pre-treat the donor with genetic approaches. Efforts to reduce the time between gene therapy and LT might open new venues for preventative gene therapy [12]. Currently, viral vectors hydrodynamic injection and cationic liposomes are the main methods for delivering siRNA *in vivo*. While viral vectors are associated with severe side effects, other methods require large volume and high injection speed, which are not clinically applicable [135]. Systemic administration of small interfering RNA (siRNA) may cause globally nonspecific targeting of all tissues, which impedes clinical use.

9.3. Cell therapy – Hepatocyte transplantation

The liver was among the first organs considered for strategies based on the transplantation of isolated cells. The first hepatocyte transplant was performed to treat the Gunn rat, the animal model for Crigler-Najjar syndrome, which is congenitally unable to conjugate bilirubin and consequently exhibits life long hyperbilirubinemia. The transplant resulted in a decreased plasma bilirubin concentration. Later, isolated hepatocytes were transplanted into rats with liver failure induced by dimethylnitrosamine. These experiments demonstrated that hepatocyte transplantation could potentially be used for the treatment of liver failure and innate defects of liver-based metabolism. More than 30 years later, these models are still used in work to improve hepatocyte engraftment and/or function [137].

Many studies have shown that hepatocytes transplanted into rodents via the spleen or the portal vasculature enter through portal vein branches and are entrapped in proximal hepatic sinusoids; consequently, the hepatocytes are distributed predominantly in periportal regions of the hepatic lobules. Transplanted hepatocytes cause both portal hypertension and transient I/R injury. The portal hypertension, in experimental animals at least, usually resolves within 2 to 3 hours with no obvious long-term detrimental effects, and microcirculatory abnormalities disappear within 12 hours. Numerous hepatocytes (up to 70% of transplanted cells) remain trapped in the portal spaces, and most of them are destroyed by the phagocytic responses of KC, which are activated shortly after deposition of hepatocytes in liver sinusoids [138]. The remaining cells translocate from sinusoids into the liver plates through a process involving disruption of the sinusoidal endothelium and release of vascular endothelial growth factor by both host and transplanted cells. In rodents, hepatic remodeling is complete within 3 to 7 days, and the engrafted cells become histologically indistinguishable from host cells. Transplantation of 2×10^7 hepatocytes in rats has led to the engraftment of about 0.5% of the transplanted cells in the recipient livers [139]. Only hepatocytes harboring a selective advantage for survival/proliferation can efficiently repopulate a recipient liver, and as a result, many repopulation strategies have been developed using approaches involving the induction of acute or chronic liver injury [137]. Despite decades of research, the processes and factors underlying cell engraftment and *in situ* proliferation are only partially understood, and a good understanding of these mechanisms is essential for the development of new and efficient treatments of human liver diseases. The prevention of early loss of transplanted cells would undoubtedly improve hepatocyte transplantation. First, it has been recently shown that cell-cell interactions between transplanted hepatocytes

and hepatic stellate cells modulate hepatocyte engraftment in rat livers. After cell transplantation, soluble signals activating hepatic stellate cells are rapidly induced along with early up-regulated expression of matrix metalloproteinases and their inhibitors [140]. Second, the interaction between integrin receptors and the extracellular matrix plays a role in cell engraftment. Third, hepatocytes express soluble and membrane-bound forms of tissue factor–dependent activation of coagulation and exert tissue factor–dependent hepatocyte-related procoagulant activity [137].

Gene	Specie	Ischemia	Vector	Effect
Bcl-2	Mouse	Warm ischemia	Adenovirus	↓ Apoptosis and Necrosis ↑ Survival
eNOS	Mouse	Warm ischemia	Adenovirus	↓ Liver injury
SOD	Mouse/Rat	Warm ischemia	Adenovirus	↓ Liver injury
IL-13	Mouse/Rat	Cold ischemia	Adenovirus	↓ Liver injury, Neutrophil infiltration, TLR4 activation, Apoptosis ↑ HO-1 expression, Survival
Bag-1	Rat	Cold ischemia	Adenovirus	↓ Liver injury, Neutrophil infiltration
CD40lg	Rat	Cold ischemia	Adenovirus	↓ Liver injury, Neutrophil accumulation, Apoptosis and Necrosis
IκB	Rat	Cold ischemia	Adenovirus	↓ Liver injury
HO-1	Rat	Cold ischemia	Adenovirus	↓ Liver injury, Macrophage infiltration, iNOS ↑ Survival
Ferritin	Rat	Cold ischemia	Adenovirus	↓ Liver injury, Apoptosis
IL-1R antagonist	Rat	Warm ischemia	Cationic liposomes	↓ Liver injury ↑ Survival
SOD	Mouse	Warm ischemia	Polyplexes	↓ Liver injury ↑ Antioxidative enzyme activity
Catalase	Mouse	Warm ischemia	Polyplexes	↓ Liver injury ↑ Antioxidative enzyme activity
SK2	Mouse	Warm ischemia	siRNA	↓ Liver injury, Apoptosis ↑ survival
Caspase-3	Mouse	Warm ischemia	siRNA	↓ Liver injury, Neutrophil infiltration
Caspase-8	Mouse	Warm ischemia	siRNA	↓ Liver injury, Neutrophil infiltration
TLR4	Mouse	Warm ischemia	siRNA	↓ Liver injury, Neutrophil infiltration, ROS, Inflammation
Adiponectin	Rat	Warm ischemia	siRNA	↓ Liver injury

Table 3. Summary of gene therapy using specific target genes in hepatic ischemia-reperfusion

In recent years, the development of different animal models has allowed significant progress in hepatocyte transplantation. In rats, the occlusion of portal branches of the two anterior liver lobes results in a regeneration response in the remaining nonoccluded lobes leading to their hypertrophy. This procedure, portal branch ligation, favors efficient retroviral trans-

duction of hepatocytes *in vivo*. Furthermore, hepatic tissue engineering using primary hepatocytes is an emerging therapeutic approach to liver diseases. Two recent studies reported engraftment of functional hepatocytes in a neovascularized subcutaneous cavity in mice. A method to manipulate uniform sheets of hepatic tissue allowing the formation, *in vivo*, of a 3-dimensional miniature liver system that maintained its biological function for several months has been also described [137,139]. In the view of clinical practice, treatment of fulminant hepatic failure patients by hepatocyte transplantation has been attempted by a number of investigators [141]. In one report, patients who received a hepatocyte transplant, one patient fully recovered and three were successfully bridged to OLT [141]. In a prospective study of five patients who were transplanted with cryopreserved human hepatocytes, three patients were successfully bridged to OLT [142]. Other reports have described clinical improvement and relatively longer survival in hepatocyte- transplanted patients [143] but poor final outcome has also been reported, possibly related to immunosuppression, inadequate number of transplanted cells, and limited engraftment time [137].

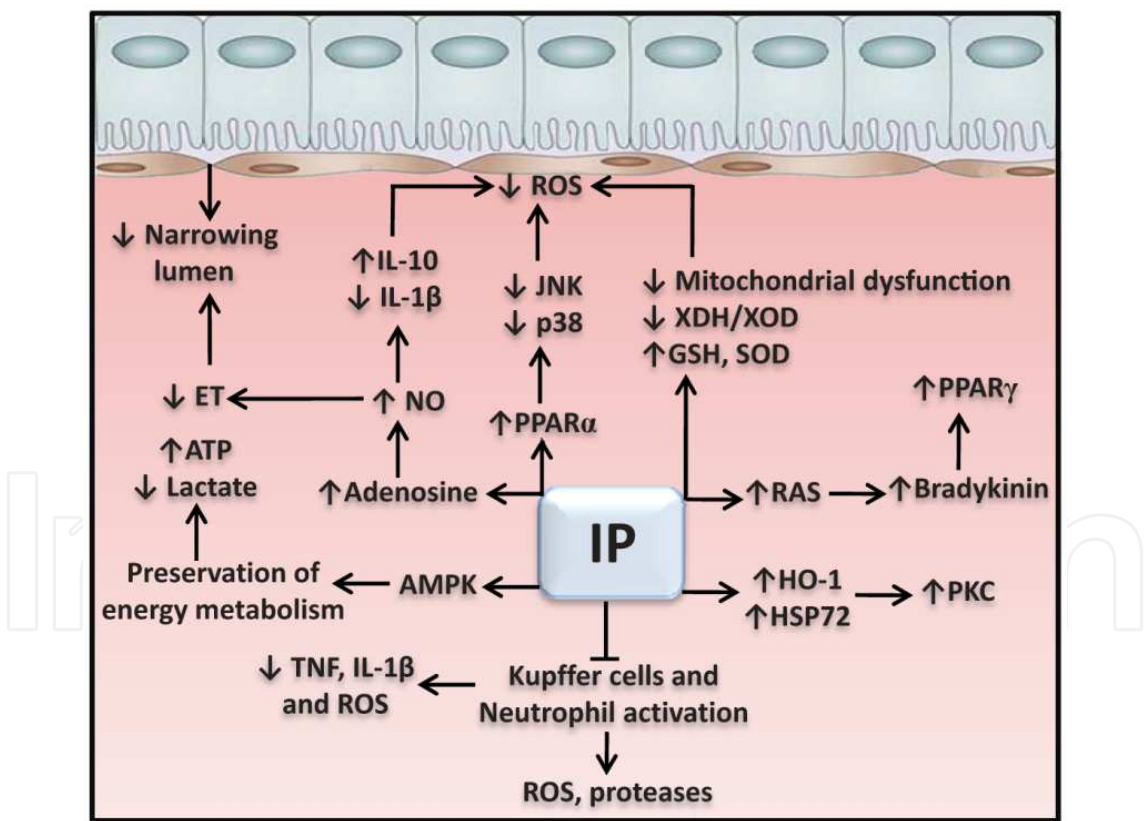


Figure 6. Mechanisms of Ischemic preconditioning in hepatic ischemia-reperfusion injury. AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; ET, endothelin; GSH, glutathione; HO-1, heme oxygenase 1; HSP72, heat shock protein 72; IL, interleukin; JNK, c-Jun N-terminal kinase; NO, nitric oxide; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; RAS, renin-angiotensin system; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; XDH/XOD, xanthine/xanthine oxidase

9.4. Surgical strategies

The response of hepatocyte to ischemia never ceases to surprise. In fact, contrary to what might be expected, the induction of consecutive periods of ischemia in the liver does not induce an additive effect in terms of hepatocyte lesions. Ischemic preconditioning (IP) based on brief periods of ischemia followed by a short interval of reperfusion prior to a prolonged ischemic stress protects the liver against I/R injury by regulating different cell types and multiple mechanisms such as energy metabolism, microcirculatory disturbances, leukocyte adhesion, KC activation, proinflammatory cytokine release, oxidative stress, apoptosis and necrosis [96] (Figure 6). This is an advantage in relation with the use of drugs that exerts its action on a specific mechanism. The benefits of IP observed in experimental models of hepatic warm and cold ischemia [96] prompted human trials of IP. To date, IP has been successfully applied in human liver resections in both steatotic and non-steatotic livers but unfortunately, it proved ineffective in elderly patients [144]. Preliminary clinical studies have reported the benefits of IP in LT [145,146]. IP may also have a role in the transplantation of small grafts whose pathophysiology overlaps with I/R injury. Additional randomized clinical studies are necessary to confirm whether this surgical strategy can be commonly used in clinical liver surgery.

10. Conclusion and perspectives

From the data obtained in experimental models of hepatic I/R, we can state that I/R injury is a multifaceted and intriguing phenomenon. The increasing use of marginal donors in major liver surgery and the fact that these organs are more susceptible to ischemia highlight the need for further research directed at the mechanisms of I/R injury. Machine perfusion has been criticized for its complicated logistics and for possibly damaging the organ and vital structures such as the endothelium. On the contrary, NMP fulfils all ideal organ preservation criteria by avoiding hypoxia and hypothermia. Responses to the strategies aimed at reducing hepatic I/R injury might depend on the surgical procedure, type of liver and percentage of hepatic ischemia. Further research is required to elucidate whether the pharmacological approaches presented in this review can be translated into liver surgery associated with hepatic resections and LT. Advances in molecular biology have provided new opportunities to reduce liver I/R injury using gene therapy. However, there are a number of problems inherent in gene therapy, such as vector toxicity and difficulties in increasing transfection. Liver-cell transplantation is at an early stage. Numerous approaches to isolating stem cells of hepatic or extrahepatic origin, including embryonic stem cells, are being developed. However, extensive work is still required to assess the number of cells that need to be expanded and differentiated, and the functionality of the different cell types needs to be carefully addressed in animal models. Surgical strategies such as IP affect multiple aspects of I/R injury, whereas pharmacological approaches often affect only a few mediators and might have systemic side effects.

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