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Ischemia Reperfusion Injury in Liver Transplantation: Cellular and Molecular mechanisms

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Abstract

Liver disease causing end organ failure is a growing cause of mortality. In most cases, the only therapy is liver transplantation. However liver transplantation is a complex undertaking and its success is dependent on a number of factors. In particular, liver transplantation is subject to the risks of ischemia-reperfusion injury (IRI). Liver IRI has significant effects on the function of a liver after transplantation. The cellular and molecular mechanisms governing IRI in liver transplantation are numerous. They involve multiple cells types such as liver sinusoidal endothelial cells, hepatocytes, Kupffer cells, neutrophils, and platelets acting via an interconnected network of molecular pathways such as activation of toll-like receptor signaling, alterations in micro-RNA expression, production of ROS, regulation of autophagy, and activation of hypoxia-inducible factors. Interestingly, the cellular and molecular events in liver IRI can be correlated with clinical risk factors for IRI in liver transplantation such as donor organ steatosis, ischemic times, donor age, and donor and recipient coagulopathy. Thus, understanding the relationship of the clinical risk factors for liver IRI to the cellular and molecular mechanisms that govern it are critical to higher levels of success after liver transplantation. This in turn will help in the discovery of therapeutics for IRI in liver transplantation--a process that will lead to improved outcomes for patients suffering from end stage liver disease.

Keywords

Ischemia-Reperfusion; Liver Transplantation; Therapeutics; Hypoxia Inducible Factors

Introduction:

Liver disease represents a growing cause of mortality worldwide. Up to 25% of the world's population has risk factors for liver disease including viral infection, alcohol abuse, and non-alcoholic steatohepatitis (NASH). The high prevalence of risk factors has contributed to the rising incidence of primary liver cancer, which causes more than 800,000 deaths per year.[1]

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In most instances, the only therapy for end-stage liver disease is liver transplantation. However, liver transplantation carries with it the risks of ischemia-reperfusion injury (IRI). IRI is defined as tissue injury that occurs when the blood supply to organs is interrupted and then returns.[2] Liver IRI has significant effects on liver function and causes systemic injury.[3] The liver-specific and systemic effects of IRI have important implications for the practice of liver transplantation.[4]

Patients awaiting liver transplant are at risk for IRI due to alterations in the balance between pro-coagulant and anti-coagulant processes. Patients with chronic liver injury are more susceptible to unregulated coagulation due to lack of matrix metalloproteinases, increased pro-thrombotic factors, and hypoalbuminemia.[5]

Organ procurement results in both cold and warm IRI. The severity of IRI in liver transplantation is affected by characteristics of the donor organ such as donor age, organ fat content, and brain death. Thus, organs at high risk for IRI, when put into recipients primed for ischemic injury, can lead to a potentially catastrophic scenario. Understanding IRI is, therefore, critical to success in liver transplantation.[6]

Liver IRI involves many cellular compartments. Liver sinusoidal endothelial cells and hepatocytes are targets of IRI-induced cell death. Early IRI-induced cell death is a result of metabolic disturbances and ATP depletion. Following reperfusion, neutrophils and macrophages are activated and accumulate in the liver. These cells exacerbate IRI through secretion of paracrine and autocrine signals such as reactive oxygen species (ROS) and inflammatory cytokines. Finally, hepatic stellate cells (HSC) become activated in IRI and promote long-term recovery from IRI, which can manifest as allograft fibrosis.[7,8]

The molecular pathways involved in IRI are similarly multifaceted. Release of inflammatory cytokines and chemokines results in activation of neutrophils and macrophages, promoting tissue destruction. Lipid peroxidation, ROS, and release of damage associated molecular patterns (DAMPS) during hepatocyte injury amplify the destructive activity of neutrophils and macrophages.[9,10]

Other molecular pathways regulate IRI after it is initiated. Autophagy can limit production of ROS. Hypoxia inducible factors (HIF) sense tissue hypoxia and help maintain neutrophil viability and promote macrophage activation. HIF are also critical in promoting tissue recovery through regulation of stem cell niches and matrix metalloproteinases (MMP).[11,12]

In short, hepatic IRI worsens the survival of patients needing liver transplants. It reduces the pool of organs available for transplant as some may suffer severe IRI if used. Those patients who experience severe IRI in their allografts have poor graft function and survival after liver transplantation. Thus, understanding the mechanisms of IRI in liver transplantation is critical to designing effective therapeutics. Effective therapeutics in turn will improve patient outcomes after liver transplantation and provide patients with liver disease a superior chance at survival (Figure 1).

Clinical Context of IRI in Liver Transplantation

Recipient Challenges—The risks of IRI in liver transplantation are present in the transplant recipient and the organ donor (Figure 2). Transplant recipients suffer from chronic liver injury, i.e. cirrhotics. Cirrhotics demonstrate altered coagulation profiles. Superficially, this appears to indicate impaired coagulation, but deeper investigation reveals a pro-thrombotic state.[13] Clinically this manifests as increased rates of deep venous and portal vein thrombosis in cirrhotics as compared to patients without liver disease.[14,15]

The pro-thrombotic profile of cirrhotics increases the risk of IRI before and after liver transplantation; up to 30% of cirrhotics may have thrombosis of the portal vein, the dominant blood supply to the liver. This may cause the patient awaiting a liver transplant to have IRI in their native liver, thus priming them for more severe IRI after liver transplantation.[16] In the post-operative period, the pro-thrombotic state of the cirrhotic patient is a risk factor for thrombosis of vessels to the newly transplanted liver. This likely contributes to the risk of hepatic artery thrombosis that occurs in 5–7% of patients after liver transplant.[17]

The mechanisms of this pro-thrombotic state are multifactorial. Blood flow in cirrhotics is more turbulent due to increased resistance within the liver parenchyma.[18] Liver cirrhosis leads to decreased production of the anti-coagulant proteins C and S.[19] Cirrhotics have chronic nutritional deficiency leading to low albumin levels. Low serum albumin is associated with an increased risk of thrombosis.[13]

Of particular interest in cirrhotics is the imbalance between von Willebrand factor (VWF) and ADAMTS13. In homeostasis, VWF in vascular endothelium promotes platelet aggregation and clotting while ADAMTS13, produced by HSC, cleaves the multimeric form of VWF inhibiting its activity.[20] Cirrhosis results in increased VWF in the hepatic sinusoids.[21] This, coupled with decreased ADAMTS13, leads to platelet aggregation and microthrombi formation in cirrhotic livers. Microthrombotic injury increases the risk of IRI in cirrhotic livers, especially at times of physiologic stress. This correlates with the observation that the imbalance of VWF and ADAMTS13 increases as the severity of liver disease increases.[22]

The impaired ratio also affects recipients post liver transplant. Donor livers release large amounts of multimeric VWF further depleting already low levels of ADAMTS13. In turn, this increases generation of micothrombi in the liver, contributing to hepatic IRI and increasing the risk of acute rejection.[23]

Patients with NASH have additional risk factors for thrombosis. NASH is correlated with obesity.[24] Obesity is associated with increased levels of plasminogen activator inhibitor-1.[25] Thus, patients who are transplanted for NASH may not only be at risk for microthombotic complications in the liver due to an altered VWF/ADAMTS13 ratio, but could also be at increased risk for macrothrombotic complications due to altered regulation of the coagulation cascade.[26]

Donor Challenges—Donor related risk factors contribute to the severity of IRI in liver transplantation (Figure 2). Liver steatosis, older donor age, prolonged ischemic time, and nature of organ recovery are risk factors for increased hepatic IRI. Severe IRI is the cause of increased primary non-function of these categories of liver allografts. Liver donors with these risk factors have been labeled as “marginal.” However, due to the high risk of mortality while awaiting a liver transplant, there is increased demand for using these “marginal” organs.[27]

Steatosis of the donor liver is a strong predictor of graft function after liver transplant. Patients transplanted with livers with greater than 30% macrosteatosis have higher transaminases, decreased synthetic function, and higher rates of non-function of the liver after transplant. These complications are a result of more severe IRI in steatotic livers.[28,29]

Prolonged cold ischemic time is also a risk factor for poor allograft function. Currently, donor livers are preserved cold in solutions that slow down cellular respiration. However, as ischemic time accumulates, ATP stores are slowly depleted leading to large deficits of metabolic precursors once normal levels of cellular respiration are restored at reperfusion. This leads to cell death. Prolonged cold ischemic time is an additive threat for severe IRI in steatotic livers.[30]

Due to limited supply of available organs from heart-beating donors, there has been an increase in the number organs recovered from non-heart beating donors (NHBD). These represent up to 15% of all allografts used for liver transplantation in the United States.[31] Livers from NHBD donors are subject to IRI from warm and cold ischemia. As opposed to heart-beating donors, NHBD have lack of blood flow prior to cold storage. This period of warm ischemia results in arrest of oxygen delivery, rapid depletion of ATP stores, blood stasis, and activation of the coagulation cascade.[32] NHBD donor livers demonstrate increased levels of ceramide18, a pro-apoptotic activator correlating with increased cell death from IRI. VWF is also elevated in NHBD donor livers indicating higher risk of microthrombi formation and loss of sinusoidal blood flow contributing to ongoing IRI during organ recovery.[33,34]

Increasing donor age is a risk factor for IRI in liver transplantation. Livers from donors over the age of 70 experience more injury at prolonged cold ischemic times as compared to livers from younger donors. Older livers have a higher chance of severe IRI due to smaller volumes and less total blood flow. At the molecular level, older donors have fewer and less functional mitochondria making them more susceptible to depletion of ATP during cold storage and warm reperfusion. This results in increased production of ROS and activators of cell death. Homeostatic regulation of this process is altered as older livers have a muted stress response as manifest by diminished amounts of heat-shock protein 70.[35–37]

Brain Death and IRI—Donor organs are recovered from persons with brain death. Brain death is an inflammatory state that primes organs during the recovery phase for more severe IRI during reperfusion. End-organs in persons with brain death show increased accumulation of neutrophils, increased platelet deposition, and activation of Fas-FasL dependent apoptotic pathways. IRI is further primed in brain death by increased production of ROS and pro-

inflammatory cytokines such as TNF-alpha. Finally, adhesion molecules such as ICAM-1 increase in expression along the vascular endothelium in response to brain death. This results in immune cell infiltration into tissues with concomitant end organ injury.[38]

The negative effects of brain death are amplified in marginal liver donors. Take, for example, steatotic livers. These livers suffer more severe IRI due to lipid peroxidation of the fat droplets in hepatocytes after reperfusion. This leads to increased production of ROS. Lipid accumulation also increases the amount of and metabolism of cholesterol in mitochondria in the liver leading to dysregulation of immune signaling pathways, such as those mediated by TLR4. This results in increased inflammatory cytokine production.[39–41]

Increased production of pro-inflammatory cytokines, such as IL-6 and TNF-alpha, activates the immune system. Activation of the immune system after IRI in steatotic livers leads to accumulation of neutrophils and macrophages at areas of fat deposition. Activation of these cell populations promotes production of ROS that exacerbate tissue damage.[29,30]

Thus, given that in brain death liver injury occurs via similar pathways as in IRI, it is no surprise steatotic livers have increased parenchymal injury in the setting of brain death as compared to non-steatotic livers. Mechanistically, as in IRI, this involves decrease in antioxidants, increase in ROS, alterations in TLR4 signaling, and reduction in heaptic microcirculation.[42,43] Although data in other marginal liver donors regarding the effects of brain death are limited it is likely that brain death primes them for more severe IRI, thus, negatively affecting outcomes after liver transplantation.

Cellular Mechanisms of Liver IRI

Liver Sinusoidal Endothelial cells—Liver sinusoidal endothelial cells (LSEC) line the vascular endothelium of the liver. They control vascular tone and thereby blood flow and delivery of nutrients and oxygen to hepatocytes. Expression of cell adhesion molecules on LSEC is upregulated in hepatic injury allowing them to play a key role in accumulation, activation, and modulation of the cellular response to hepatic IRI. Thus, normal LSEC function is important to protecting the liver from the disruptions in homeostasis after IRI.[44]

LSEC are highly susceptible to injury during static cold preservation.[45] Due to ATP depletion during cold storage, active transmembrane transport of ions is disrupted (Figure 3). This leads to cell swelling and mitochondrial dysfunction.[46] Intensity of LSEC injury is increased by lack of active blood flow across the sinusoids during static cold storage.[47]

Upon reperfusion, damaged LSEC incur further injury. ROS derived from lack of energy sources for cellular respiration quickly reduce free radical scavengers. Lack of NO production from the injured LSEC, depletion of NO stores and the unopposed activity of vasoconstrictors such as thromboxane A2 cause an increase of vascular tone and reduction of blood flow to hepatocytes.[48–50] Activated LSEC express vascular adhesion molecules such as P-selectin allowing for platelet adhesion and activation. Platelet adhesion to LSEC induces LSEC cell death as well as causing congestion and thrombus formation in the liver microcirculation.[51,52] The cumulative effect of these processes worsens IRI in transplant allografts (Figure 3).

Kupffer Cells—Kupffer Cells (KC) are resident liver macrophages derived from erythro-myeloid progenitors in the fetal liver.[53] During normal liver function, KC are central to a tolerogenic response as the liver is exposed to numerous circulating antigens. In homeostasis, KC scavenge circulating antigens passing through the liver, present them to T-cells, and induce expansion of tolerogenic T-cells. This leads to production of immunosuppressive cytokines such as IL-10.[54]

During liver injury, KC lose this tolerogenic profile and instead promote inflammation and injury.[55] During IRI in liver transplantation, injury to LSEC and hepatocytes releases damage-associated molecular pattern molecules (DAMPS) such as high mobility group box-1 (HMGB1), free fatty acids (FFA), and heat shock proteins (HSP). KC recognize these injury signals through expression of toll-like receptors 3, 4, and 9. Activation of TLR's, especially TLR4, drive cytokine production in KC toward an inflammatory phenotype.[56]

Pro-inflammatory cytokines secreted by KC during IRI include IL-1-beta, TNF-alpha, IFN-gamma, and IL-12. IL-1-beta and TNF-alpha induce upregulation of the adhesion molecules CD11b/CD18a (Mac-1) on neutrophils and intracellular adhesion molecule-1 (ICAM-1) on LSEC to promote neutrophil adhesion and extravasation into the liver parenchyma.[57] These same cytokines drive release of ROS from neutrophils causing further tissue injury.[58]

TNF-alpha secretion from KC in IRI induces the upregulation of P-selectin on LSEC to promote platelet adhesion and activation. It also promotes chemokine secretion from KC during liver injury.[59] Release of the chemokines CXCL1, −2, and −3, results in increased migration of neutrophils into the injured liver tissue (Figure 3). This amplifies recruitment and adhesion of neutrophils to LSEC and also promotes further activation of KC.[60]

Hepatocytes—Hepatocytes experience direct injury during IRI. (Figure 3). The respiratory chain in hepatocytes in IRI is disrupted due to lack of oxygen, impaired delivery of nutritional substrates, and depletion of residual ATP. Mitochondria deprived of O_2 and glucose cannot replenish ATP supplies. This causes accumulation of toxic substances such as lactate.[61] Further, active ionic transporters are disrupted leading to cell swelling and membrane damage.[62]

Although accumulation of ATP by-products such as AMP and activation of cAMP dependent protein kinase can protect against hepatic IRI, ultimately the disruption of cellular respiration results in production of ROS from mitochondria and tissue damage.[41] During reperfusion, reintroduction of O_2 does not restore normal respiratory chain function but rather exacerbates ROS production due to impaired mitochondrial function.[39] Injured hepatocytes go on to release DAMPS that usher in a self-perpetuating inflammatory cycle of which LSEC and KC are the central regulators.[63]

Neutrophils—If LSEC and KC are central regulators of IRI in the liver, neutrophils represent the main actor in causing injury. Neutrophils are rapidly recruited to the liver after reperfusion. IRI causes activation of complement which leads to production of the chemotactic agents C3a and C5a. Neutrophils respond to C3a and C5a by infiltrating the liver after reperfusion.[64]

Once in the liver, chemokines secreted by activated KC, such as CXCL1 and CXCL2 (human CXCL8) create a chemoattractant gradient that is detected by neutrophils. Chemotaxis driven by chemokines brings neutrophils into liver sinusoids.[65] Chemokines also bind to glycosoamino-glycans on the vascular surface of LSEC such that when neutrophils reach that location, chemokine-chemokine receptor interactions lead to activation of integrins and binding of neutrophils via the integrin Mac-1 to ICAM-1 on the sinusoidal endothelium.[66]

Once in the injured liver, neutrophils respond to the inflammatory signals therein. HMBG-1 and DNA released from damaged hepatocytes induces release of ROS from neutrophils.[67] DNA from damaged hepatocytes activates TLR9 on neutrophils enhancing their production of ROS and secretion of chemokines such as CXCL1 and 2. This is mediated by TLR9 via activation of NF-κB.[68,69]

Activation of TLR9 in neutrophils results in their rapid degranulation during liver injury. Expression of TLR9 at the cell surface of neutrophils as opposed to endosomal expression leads to faster binding of DNA fragments to TLR9. This accelerates signaling via TLR9.[70] Accelerated signaling via TLR9 amplifies tissue damage caused by neutrophils after hepatic IRI (Figure 3). Ongoing tissue damage creates a positive feedback loop in which neutrophil recruitment, migration, and tissue infiltration is promoted through further release of signals of tissue injury such as DAMPS, DNA, chemokines, and cytokines.[71] In liver transplantation, modulating neutrophil activity is likely to improve allograft function.

Platelets—Platelet aggregation during hepatic IRI represents an important mechanism by which IRI is potentiated after liver transplantation.[72] LSEC injury causes increased adhesion of platelets through upregulation of P-selectin. Increased platelet adhesion mechanically limits sinusoidal blood flow exacerbating ischemia; subsequent activation of other pathways amplifies this effect (Figure 3).[51,52]

LSEC express CD39 on their luminal surface. CD39 regulates the effect of ATP products on platelets. During homeostasis, CD39 cleaves ATP and ADP to AMP, limiting platelet activation. In IRI, as LSEC are injured, CD39 activity declines and ADP increases.[73] ADP is a potent inducer of platelet activation and aggregation. Activated platelets release a variety of cytokines which 1. Affect vascular tone—thomboxane A2 (TXA2) and serotonin, 2. Regulate local thrombosis—plasminogen activator inhibitor-1 (PAI-1), and 3. Induce fibrosis —transforming growth factor-beta (TGF-beta).[74–76]

This process is termed extravasated platelet aggregation (EPA).[72] LSEC injury induces vasoconstriction in the sinusoids.[44] Platelet adhesion and aggregation increases leading to platelet activation and migration into the subendothelial space. With platelet activation comes sinusoidal vasoconstriction via TXA2 and serotonin.[49,52] Thrombus degradation is inhibited by PAI-1; progression of platelet aggregation during EPA causes them to secrete TGF-beta.[75,76] Secretion of TGF-beta induces a transition from the acute phase of IRI toward chronic remodeling of the liver allograft. TGF-beta activates HSC which results in collagen deposition and the initiation of graft fibrosis.[77]

Hepatic Stellate Cells—HSC reside in the perisinusoidal space and are central regulators of hepatic fibrosis.[78] During homeostasis, HSC suppress excess inflammatory responses to hepatic injury. In this setting, HSC cause apoptosis of reactive T-cells, expand regulatory T-cell populations, and secrete antioxidants to scavenge ROS.[79,80] However, severe injury leads to constitutive activation of HSC with negative consequences for parenchymal recovery.[77]

TNF-alpha, NO, and IL-6 released during IRI from KC activate HSC. Activated HSC undergo transformation to a myofibroblast phenotype. Immediate consequences of HSC activation are release of MMP, chemokines, and inflammatory cytokines which lead to ECM destruction, ingress of platelets and neutrophils, and further activation of HSC.[8] HSC also contribute to endothelial vasoconstriction through secretion of endothelin-1 (ET-1).[81]

Long term consequences of HSC activation are deposition of ECM and fibrosis of the liver parenchyma.[77] As noted above, this process is initiated through EPA with TGF-beta being the stimulator of persistent HSC activity after IRI.[72] This affect is attenuated when the number of HSC in the liver are reduced in experimental models of IRI.[8,82]

Molecular Mechanisms of IRI

Autophagy—Autophagy is the process by which cells remove mis-folded, damaged, and over-age proteins and organelles through transport into lysosomes.[83] There is some contradictory data regarding the effects of autophagy in liver IRI but in the context of liver transplantation, the balance favors regulated autophagy as a protective mechanism to limit injury.[11,84]

IRI in warm ischemia leads to rapid depletion of ATP and oxygen. Mitochondria in hepatocytes are overwhelmed by ROS. Loss of substrates for oxidative phosphorylation leads to increased mitochondrial permeability, especially in the reperfusion phase.[85,86] This increases production of ROS and leads to further necrotic damage to hepatocytes.

Autophagy is protective in this setting as it leads to shuttling of damaged mitochondria into the autophagosome, limiting production of ROS.[87] Evidence of this pathway is seen when expression of autophagosome localizing protein Beclin 1 is impaired leading to increased hepatocyte death.[88,89] Overexpression of Beclin 1 or induction of autophagy through mTOR inhibitors such as rapamycin (in combination with delivery of ROS scavengers such as hydrogen sulfide) reduces IRI-induced hepatocyte death.[11,90]

The negative effects of older donor age and increased donor steatosis on hepatic IRI can be limited through upregulation of autophagy. Aging leads to decreased autophagy and increased hepatocyte injury after IRI. Activation of the PPAR-gamma nuclear receptor increases autophagy and limits hepatocyte damage after IRI. This effect is more pronounced in younger mice and diminished in older mice.[91] Similarly, induction of autophagy through the HMG-CoA reductase inhibitor simvastatin reduces IRI in steatotic livers.[45,92]

The benefits of simvastatin are likely mediated through LSEC. In LSEC, injury from IRI is ameliorated by upregulating autophagy. Treating LSEC subject to static cold storage with

simvastatin induces expression of the vasoprotective transcription factor KLF-2 (Kruppel-like factor 2). This prevents the arrest of autophagy that occurs during reperfusion and decreases LSEC death in IRI.[45,93]

As opposed to regulated autophagy, unregulated autophagy, may be detrimental to recovery from IRI. More severe hepatocyte necrosis is seen in correlation with increased autophagy as represented by higher levels of expression of the autophagosome related protein LC3-II.[94] Further, HMGB1 released during IRI is a competitive binder of Beclin 1 and its binding to Beclin 1 induces autophagy. The protective effects of reduction of HMGB1 secretion after IRI may relate to decreased autophagy when lower levels of HMGB1 are present.[95] Thus, although autophagy appears primarily protective in hepatic IRI and liver transplantation, these data suggest that manipulation of autophagy in liver transplantation should be undertaken with caution to avoid an unregulated response.

Hypoxia Inducible Factors—Hypoxia inducible factors (HIF) are heterodimeric transcription factors whose effects are directly tied to levels of oxygen present in tissue.[96] HIF are stabilized in the presence of hypoxia.[97] After hepatic IRI, stabilized HIF enter the nucleus and induce transcription of genes involved in cellular metabolism (*Glut-1, PDK-1*), angiogenesis (*VEGF, NOS*), and cytoprotection (*Hmox-1, HSP*) to ameliorate IRI induced hepatocyte injury and cell death.[98–100]

HIF have a constitutive beta subunit and a variant, oxygen sensing alpha subunit.[96] Of the three known HIF alpha isoforms, HIF1-alpha is the best characterized in terms of its role in hepatic IRI. Inhibitors of prolyl hydroxylation of HIF, which targets it for destruction, appear to mitigate IR injury by increasing the amount of activated HIF1-alpha.[98,101] Similarly, the positive effects of ischemic preconditioning on hepatic IRI appear to involve increased activation of HIF1-alpha.[102]

Data regarding the role of HIF2-alpha in hepatic IRI is limited. However, the growing amount of information regarding the role of HIF2-alpha in the development and homeostasis of the gastrointestinal tract may provide insight into its potential role in hepatic IRI.[103] HIF2-alpha has selective expression in the endothelium of the GI tract.[104] In macrophages, HIF2-alpha appears to be associated with an M2 phenotype. Reciprocally, Th2 type cytokines appear to increase HIF2-alpha expression.[105] In neutrophils, HIF2-alpha promotes survival of neutrophils. In inflammatory bowel disease models, increased HIF2-alpha expression leads to increased tissue inflammation caused by neutrophils. Suppression of HIF2-alpha causes apoptosis of neutrophils and decreased inflammation.[106]

HIF2-alpha also regulates stem cell niches. HIF2-alpha knockout mice are genetically lethal with defects in vascular and mitochondrial development.[107] In intestinal development, HIF2-alpha regulates stem cell niches through regulation of *Oct4* and its downstream targets *Sox2* and *Nanog*.[108,109] Finally, evidence from hemopoietic stem cell development demonstrates HIF2-alpha is important for robust pluripotency of those stem cell populations.[110]

Further speculation regarding the role of HIF2-alpha in hepatic IRI comes from study of other diseases affecting the GI tract. In inflammatory bowel disease HIF2-alpha has a role in

initiation and recovery from ischemic injury. Chronic activation of HIF2-alpha via NF-kappa B leads to colitis. Conversely, HIF2-alpha activation after ischemic injury from radiation leads to epithelial cell regeneration via VEGF pathways.[111,112]

Energy metabolism is an important part of initiation and recovery from IRI. Recent reports suggest HIF2-alpha regulates energy metabolism in the liver both in the context of insulin response and fatty acid metabolism.[9] HIF2-alpha is a necessary component of the insulin response mediated by VEGF in the liver. Fat deposition and steatohepatitis in the liver are increased in the absence of HIF2-alpha.[113,114]

Lastly, HIF2-alpha may also regulate long term recovery from IRI through control of expression of MMP and collagen prolyl hydroxylases. HIF2-alpha is the major transcriptional regulator of these genes.[115] As a corollary, activation of HIF2-alpha in the liver is sufficient to cause liver fibrosis.[116]

Based on these findings, one can speculate on the role of HIF2-alpha in hepatic IRI (Figure 4). Hypoxia in IRI would stabilize HIF2-alpha. HIF2-alpha expression would be increased in LSEC, KC, and neutrophils contributing to inflammation via increased vasoconstriction through decreased NO, enhanced production of cytokines, promotion of macrophage polarization, and enhanced survival of neutrophils. HIF2-alpha would also contribute to recovery from IRI by stabilizing the stem cell niche in hepatocytes. Finally, HIF2-alpha could affect the long-term architecture and function of the liver after IRI via remodeling of the ECM through regulation of MMP and collagen hydroxylases (Figure 4). Given the potential effects HIF2-alpha could have in heaptic IRI, further investigation in this area is warranted.

MicroRNA—MicroRNA's (miRNA) are single stranded, non-coding RNA's which modulate gene expression via translational repression of target mRNAs.[117] MiRNA expression has been linked to regulation of a number of cellular processes that affect hepatic IRI. These include promotion of inflammation, cellular regeneration, and autophagy.[118]

MiRNA 122 is the most abundant miRNA in the liver, representing 70% of the total.[119] In miRNA-122 knock out mice, there are increased levels of monocytes and neutrophils. Further, loss of miRNA-122 leads to increased expression of the chemokine CCL2, which promotes migration of macrophage and neutrophil to the liver.[120] After prolonged IRI, miRNA-122 levels are reduced in liver tissue but elevated in serum of patients with liver injury suggesting miRNA-122 is important in maintaining normal liver function and is released into the serum due to hepatocyte death after IRI.[118]

Other miRNAs upregulated in hepatic IRI include miRNA-34a, miRNA-17, and miRNA-155. MiRNA-34a appears to regulate the effect of ROS by decreasing expression of antioxidants after IRI and increasing apoptosis through upregulation of p53.[121] Increased miRNA-34a decreases expression of *Nrf2* a key molecule that promotes production of antioxidants. MiRNA-34a stabilizes and promotes increased expression of p53 resulting in increased liver injury after hepatic IRI, suggesting a multifaceted role for it in promotion and recovery from ischemic liver injury.[122,123]

MiRNA-17 is increased after hepatic IRI. It appears to regulate autophagy: increased miRNA-17 correlates with increased numbers of autophagosomes. Excess expression of miRNA-17 leads to unregulated autophagy and to decreased hepatocyte viability via increased release of caspases. MiRNA-17 controls autophagy through transcriptional regulation of STAT-3, a known inhibitor of autophagy. MiRNA-17 binds to the 3' UTR of *STAT-3* repressing its transcription.[124]

MiRNA-155 regulates the response of KC after IRI. MiRNA-155 deficient mice have less liver injury after IRI with decreased expression of CD80, CD86 and MHC class II on KC. Further, KC in these mice produced IL-10 which improved the viability of hepatocytes. Elimination of miRNA-155 negative KC led to increased injury after hepatic ischemia.[125]

Other miRNAs involved in apoptosis and regeneration show down- or up-regulation in expression respectively after hepatic IRI. Downregulated miRNAs involved in apoptosis include miRNA-200b and miRNA-183. Upregulated miRNAs involved in liver regeneration include miRNA-27a, −494, −1224, and −149.[126] These findings indicate an important role for miRNA in the regulation of hepatic IRI.

Reactive oxygen species and inflammatory cytokines—In the ischemic phase of IRI, reducing substances are consumed and anaerobic metabolism dominates. In reperfusion, oxygen is reintroduced and due to the need for reducing substances, excess oxygen free radicals are produced. Oxygen free radicals cause direct cellular damage and induce an inflammatory response. In liver IRI, they induce release of HMGB1 and activate NF-kappa-B.[127] This leads to production of a number of cytokines such as IL-1alpha and beta, IL-2, IL-3, IL-6, IL-8, and TNF-alpha and beta.[128] Production of cytokines and release of ROS, HMBG1 and DAMP from damaged hepatocytes leads to neutrophil recruitment and KC activation.[9] Thus ROS are critical initiators of the injury response (Figure 5).

This is relevant in donors livers which are steatotic or are from NHBD. In steatotic livers, excess fat deposited in the hepatocytes is a target for peroxidation.[129,130] Lipid peroxidation generates ROS and amplifies hepatic IRI.[131] First, lipid peroxidation depletes NAD+ and increases the NADH/NAD+ ratio to promote production of superoxide free radicals.[132] Second, to regulate this, mitochondria express mitochondrial uncoupling protein 2 (UCP2). UCP2 causes a leak in the proton gradient in cellular respiration to reduce ATP production. This has detrimental effects as rapid reduction of energy stores leads to further hepatocyte death due to loss of membrane integrity.[133]

In NHBD, the effects of ROS are more pronounced because of extended periods of warm ischemia. This leads to a rapid depletion of ATP and switch to anaerobic respiration. Subsequently, ROS production after reperfusion is greater and more pronounced leading to increased cellular injury.[33] These mechanisms help explain the clinical observation as to why steatotic allografts and those from NHBD are at higher risk for early allograft dysfunction (Figure 5).

Recent data also indicates that a broad class of metabolic signaling molecules associated but not exclusive to adipose tissue, i.e. adipokines, affect liver regeneration after IRI. Some of

the more well-known members of this family include IL-6, adiponectin, and leptin. In a comprehensive review, Peralta and colleagues examined the available data regarding adipokines and concluded that there was sufficient evidence to suggest that these molecules had a role in liver regeneration after IRI, particularly IL-6, but that more data in partial ischemia and steatotic liver injury models was required to clearly establish the positive or negative effects of the more than 600 adipokines known to date.[134]

Gut-Liver Axis of Injury—An emerging area of interest in liver IRI is the effect of the intestinal microbiome on liver injury. Clamping the portal circulation in murine models of liver IRI leads to impaired gut-barrier function and increased translocation of bacteria and bacterial debris into the portal circulation. This leads to activation of TLR4, promoting the inflammatory cascade after reperfusion and increasing liver injury. Removal of bacteria, abrogation of TLR4 signaling, and remote ischemic preconditioning all reduced the negative effects of the disruption of gut microbiome on liver IRI.[135] In particular, remote ischemic preconditioning restores gut barrier function and normal composition of microbiota in these models.[136] Thus, modulation of the gut-liver axis may provide new therapeutic avenues to treat IRI in liver transplantation.

Therapeutics for Hepatic IRI

Therapeutic strategies to limit IRI in liver transplantation generally fall into the following categories. 1. Reducing effects of ROS 2. Modulation of the cytokine response 3. Blocking activation of the immune system and 4. Improving organ preservation (Table 1).

Using free radical scavengers to limit IRI in liver transplantation is an elegant idea that unfortunately has not been clinically effective. Much effort has focused on the use of N-acetyl-cysteine (NAC), a glutathione precursor, as a free radical scavenger.[137] NAC use in acetaminophen overdose has shown promise, but its effectiveness in liver transplantation is less clear.[138] In NHBD, NAC was thought to be effective in reducing the injurious consequences of IRI on bile ducts, but this data has not been reproducible.[139,140] Use of free radical scavengers and antioxidants such as S-adenosyl-methionine and Vitamin E has shown no clinical value in liver transplantation.[141]

A complex network of cytokine expression underlies the response to hepatic IRI. Modulating the cytokine response would be a logical way to blunt the effects of hepatic IRI in liver transplantation. To this end, both blocking cytokines produced during IRI and administration of cytokines to counter those released during IRI have been proposed as therapeutic strategies.

Prostaglandins E-1 and I2 experimentally promote vasodilation of the liver microcirculation. Unfortunately, neither has shown promise in clinical trials. PGE-2 administration in liver transplantation has no effect on the rates of allograft dysfunction or patient survival.[142] Data for the effectiveness of PGI-2 is similarly lacking.[143]

TNF-alpha is a central cytokine in hepatic IRI. There are a number of anti-TNF-alpha biologic agents available for use. Unfortunately, none of these biologics has shown promise

in liver transplantation. This may be due to the fact that TNF-alpha is essential for regeneration of the liver after IRI and blocking it eliminates its beneficial effects.[144]

Blockade of the immune system has been practiced in liver transplantation for some time with the use of steroids as part of an immunosuppressive regimen. Steroids suppress transcription of cytokines in immune cells.[145] Steroids likely attenuate hepatic IRI, but the severity of IRI in steatotic, NHBD, and older livers, suggest steroids alone are not sufficient as a therapy.[146]

An intriguing direct strategy involves use of gadolinium chloride to inhibit KC activation. This drug has shown promise in animal models of liver transplantation where administration of gadolinium chloride reduced markers of liver injury and improved hepatic blood flow.[60,147] Unfortunately at this time no correlative human data exists.

Finally, strategies to improve organ preservation have gained favor in recent years. Initial attempts to precondition liver allografts prior to cold preservation with brief periods of ischemia were found not be successful.[148] However, the use of ex vivo perfusion has demonstrated reduction in liver injury.[149] The concept underlying hypothermic ex vivo perfusion is that flow through the liver sinusoids limits LSEC injury. In normothermic ex vivo perfusion, the liver is preserved under normal physiologic conditions. Ongoing clinical trials are attempting to answer whether this reduction in liver injury has benefits in terms of early and late graft function and patient survival. Further, there is interest in extending these trials to preserve marginal liver allografts (Table 1). Early data suggests that ex vivo perfusion would have substantial benefit in reducing IRI in this setting although the mechanisms for this are yet to be fully elucidated.[150]

Conclusion

Hepatic IRI represents a barrier to the use of organs available for transplant. The risk of early and late graft dysfunction of donor livers is directly related to hepatic IRI. Therefore understanding the mechanisms of hepatic IRI and development of therapeutics are of enormous importance in reducing the mortality of patients awaiting liver transplant. This will allow more organs to be used and reduce the dysfunction of those organs once transplanted.

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Abbreviations:

IRI	Ischemia Reperfusion Injury
DAMP	Damage associated molecular pattern
HIF	hypoxia inducible factor
MMP	matrix metalloproteinase

VWF	von Willebrand factor
NASH	non-alcoholic steatohepatitis
NHBD	non heart beating donor
LSEC	liver sinusoidal endothelial cells
KC	Kupffer cells
ROS	reactive oxygen species
HSC	hepatic stellate cell

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Key Points

IRI negatively affects allograft function after liver transplant

The cellular and molecular mechanisms of IRI in liver transplantation can be correlated with clinical risk factors for IRI in donors and recipients

Developing therapies to ameliorate IRI in liver transplantation is critical to improving patient survival

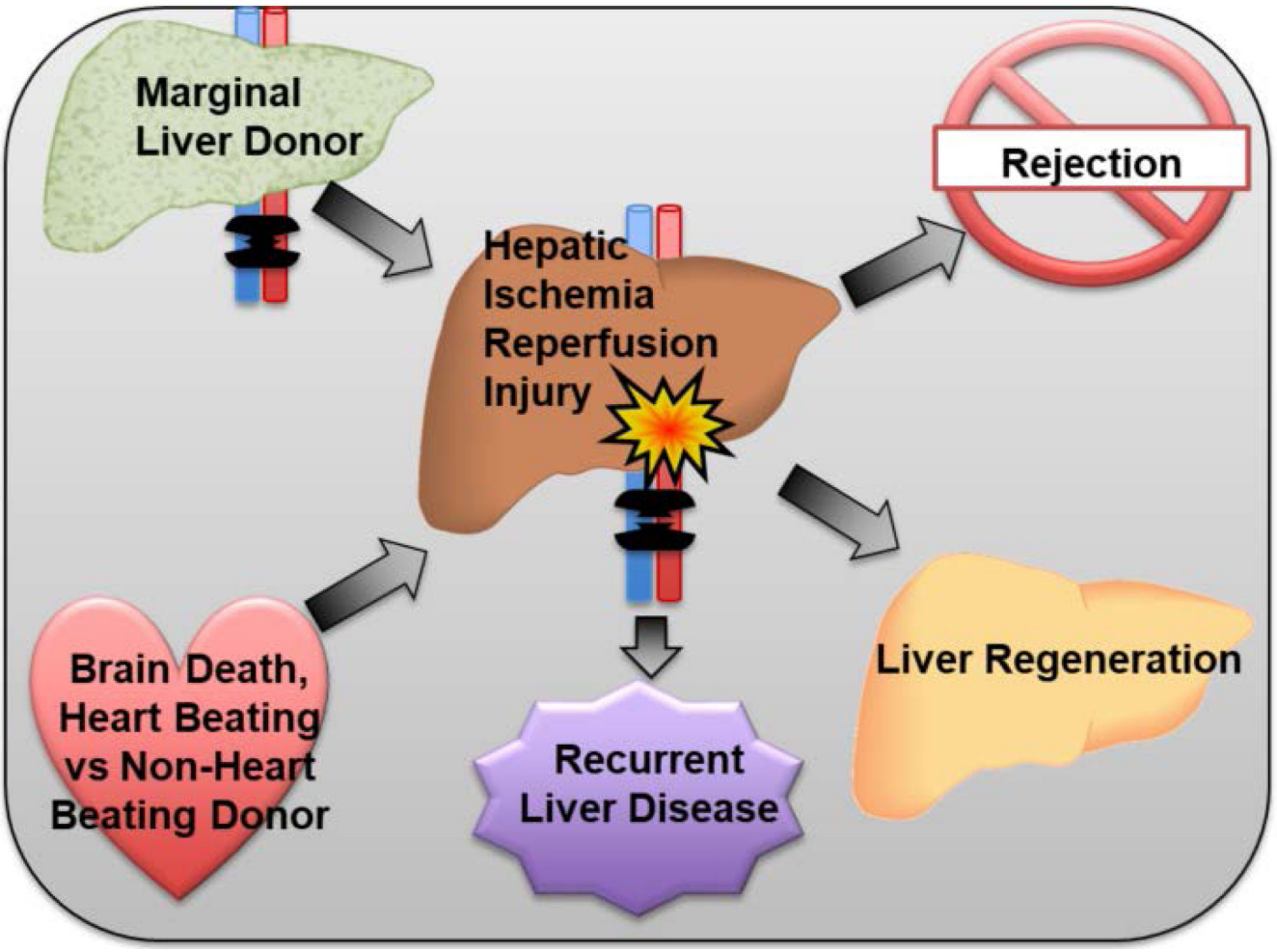

Figure 1: Risk factors and sequelae of hepatic IRI in liver transplant patients.

Factors such as use of marginal donor organs due to donor scarcity and critical donor hemodynamics, including NHBBD donors, can increase the risk of hepatic IRI. Higher levels of hepatic IRI consequently contribute to poor outcomes, including rejection, recurrence of liver disease, and liver regeneration.

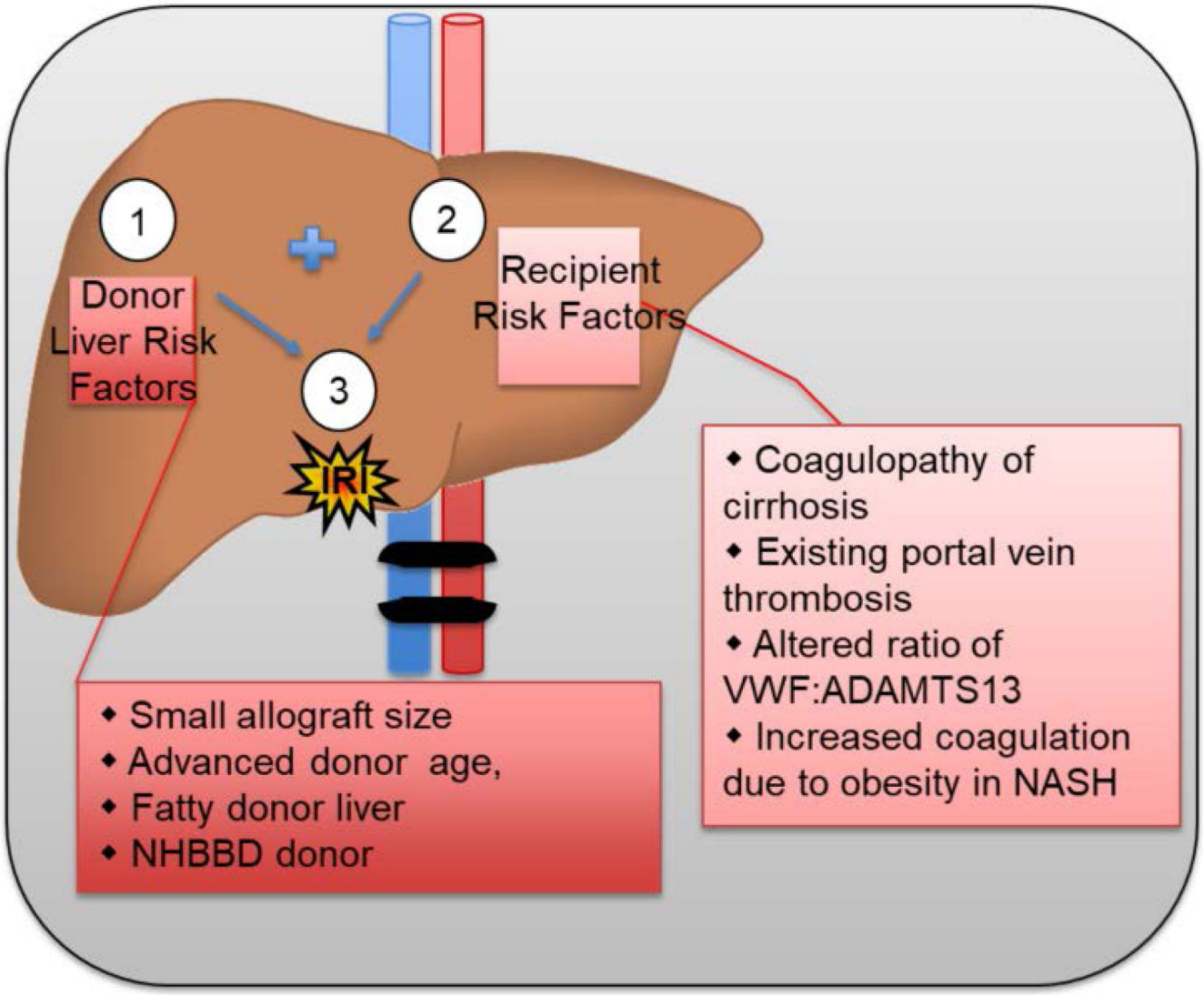

Figure 2: Surgical, Donor, and Recipient factors that contribute to risk of IRI.

(1) Risks of IRI inherent to liver transplantation include: organwide ischemia during clamping and resection of donor organ, cold storage, re-anastomosis and circulation of widespread inflammatory factors, increased ischemic time. (2) Donor Liver risk factors can increase the severity of IRI: small allograft, advanced age, especially age >70, donor fatty liver, especially macrosteatosis > 30%, cause of donor death, NHBBD donor, use of marginal organs due to donor scarcity. (3) Recipient risk factors can further increase the severity of IRI: cirrhosis or hepatic fibrosis induced coagulopathy, nutritional coagulopathy, portal vein or hepatic artery thrombosis, altered VWF:ADAMTS13 ratio, history of NASH.

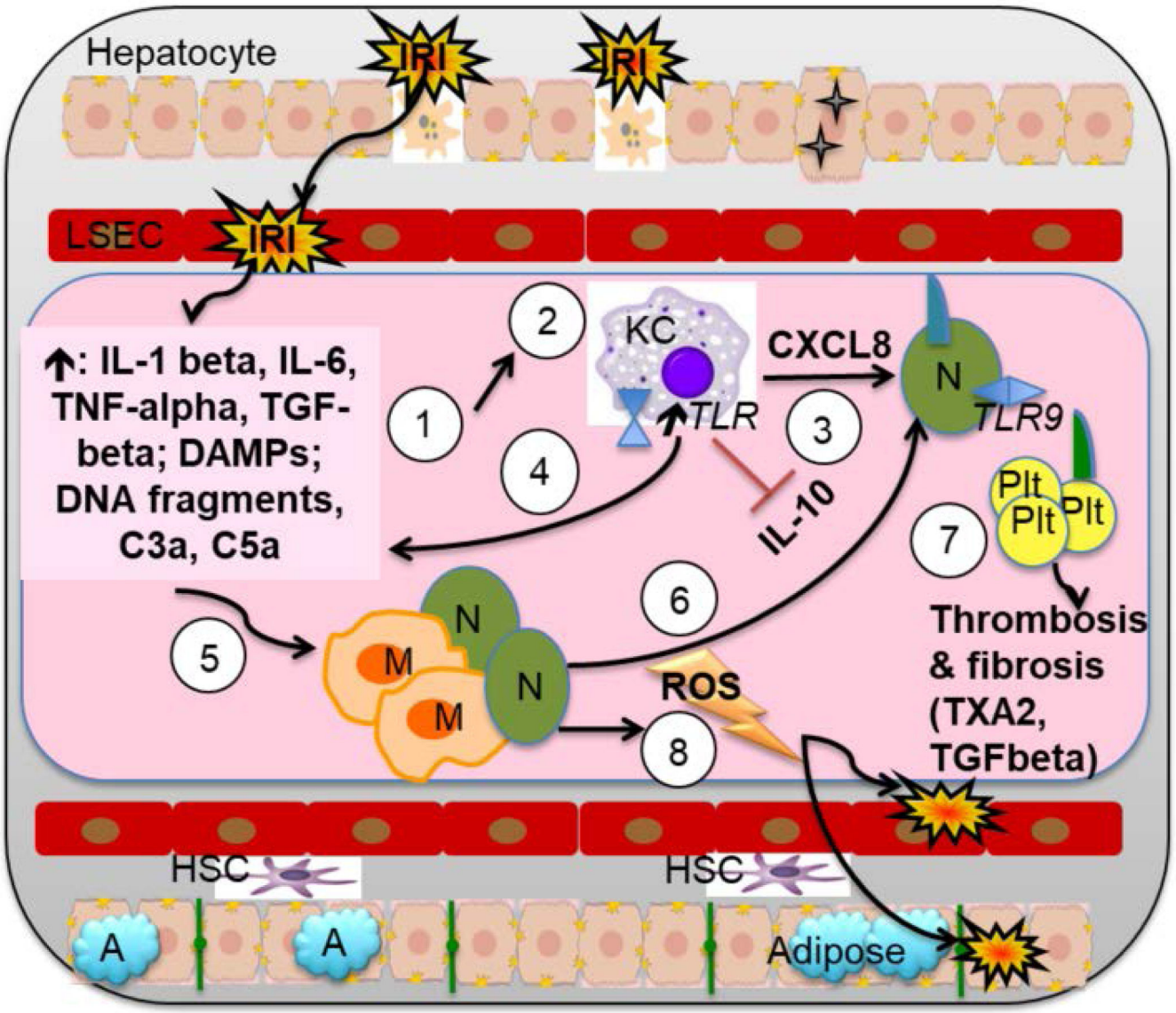

Figure 3: Cellular and molecular IRI pathway: A focus on the roles of Kupffer cells, macrophages, neutrophils, and platelets.

(1) IRI damage to LSECs and hepatocytes causes cell death and release of inflammatory cytokines IL-1 beta, IL-6, and TNF-alpha, TGF-beta, as well as DAMPS (HMGB1, FFA, and HSB), DNA fragments, and complement. (2) DAMPs cause KC to increase KC TLR activation, inhibit immunosuppressive IL-10 production, and increase KC cytokine production toward inflammatory phenotype. Inflammatory type KC release: (3) CXCL8, which amplifies recruitment and adhesion of neutrophils to LSEC; and (4) IL-1-beta, TNF-alpha, IFN-gamma, and IL-12. Together, the factors released from IRI damage to LSECs and hepatocytes, as well as those released by inflammatory KCs, (5) induce migration of neutrophils early in IRI and of macrophages late in IRI. (6) These factors also serve to promote further neutrophil adhesion and extravasation into the liver parenchyma via CD11b/CD18a on neutrophils and ICAM-1 on LSEC, while promoting platelet adhesion and activation via upregulation of P-selectin on LSEC. (7) Activated platelets release cytokines

that affect vascular tone (TXA2 and serotonin), regulate local thrombosis (PAI-1), and induce fibrosis (TGF beta). (8) Simultaneously, neutrophils and macrophages cause further tissue injury and cellular destruction, through release of ROS and other destructive factors.

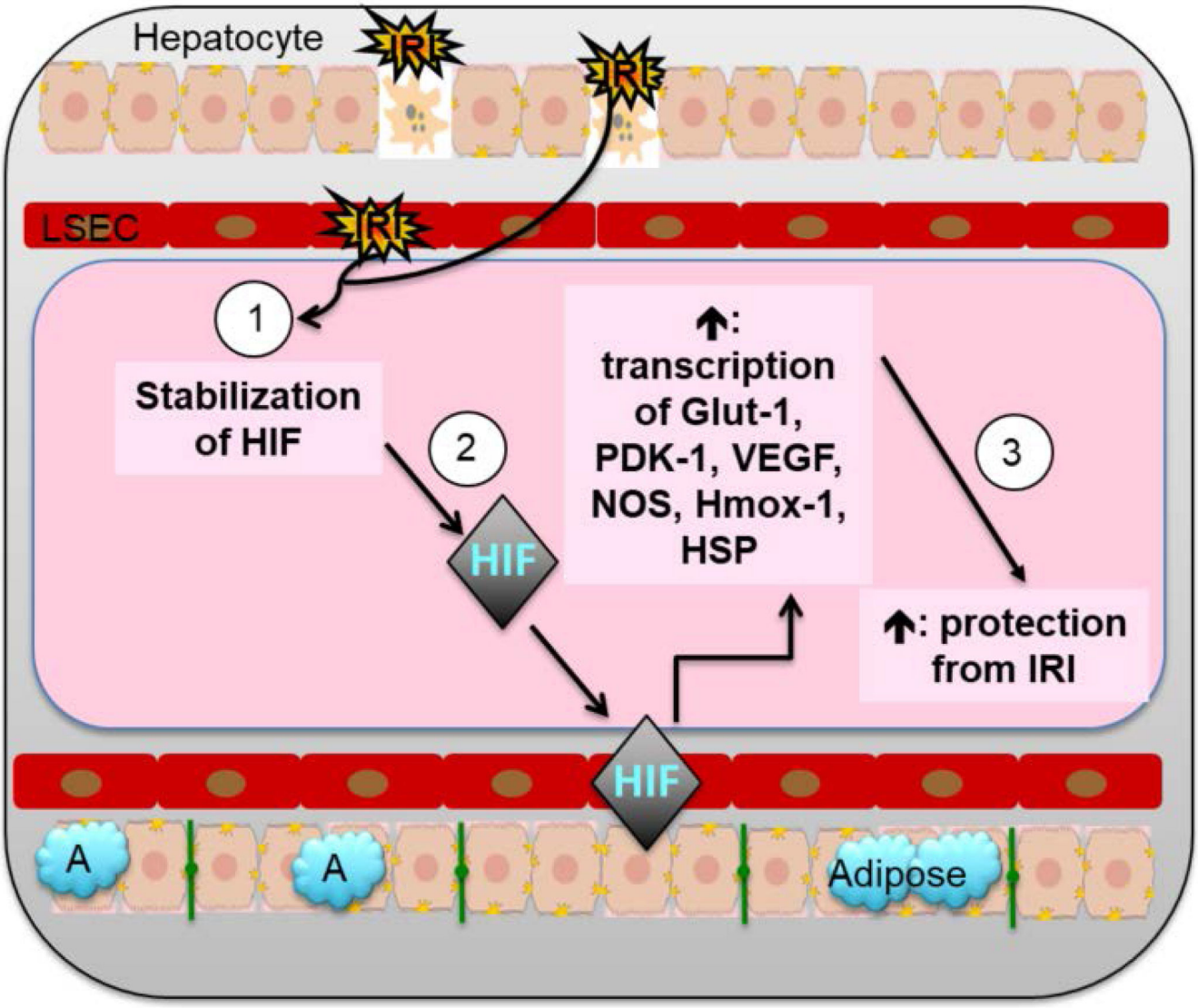

Figure 4: Cellular and molecular IRI pathway: A focus on the role of HIF.

(1*)* Hepatic IRI results in stabilization of HIF, which (2) enter the nucleus and induces transcription of genes in multiple pathways such as cellular metabolism (Glut-1, PDK-1), angiogenesis (VEGF, NOS), and cytoprotection (Hmox-1, HSP) to (3) ameliorate IRI induced hepatocyte injury and cell death.

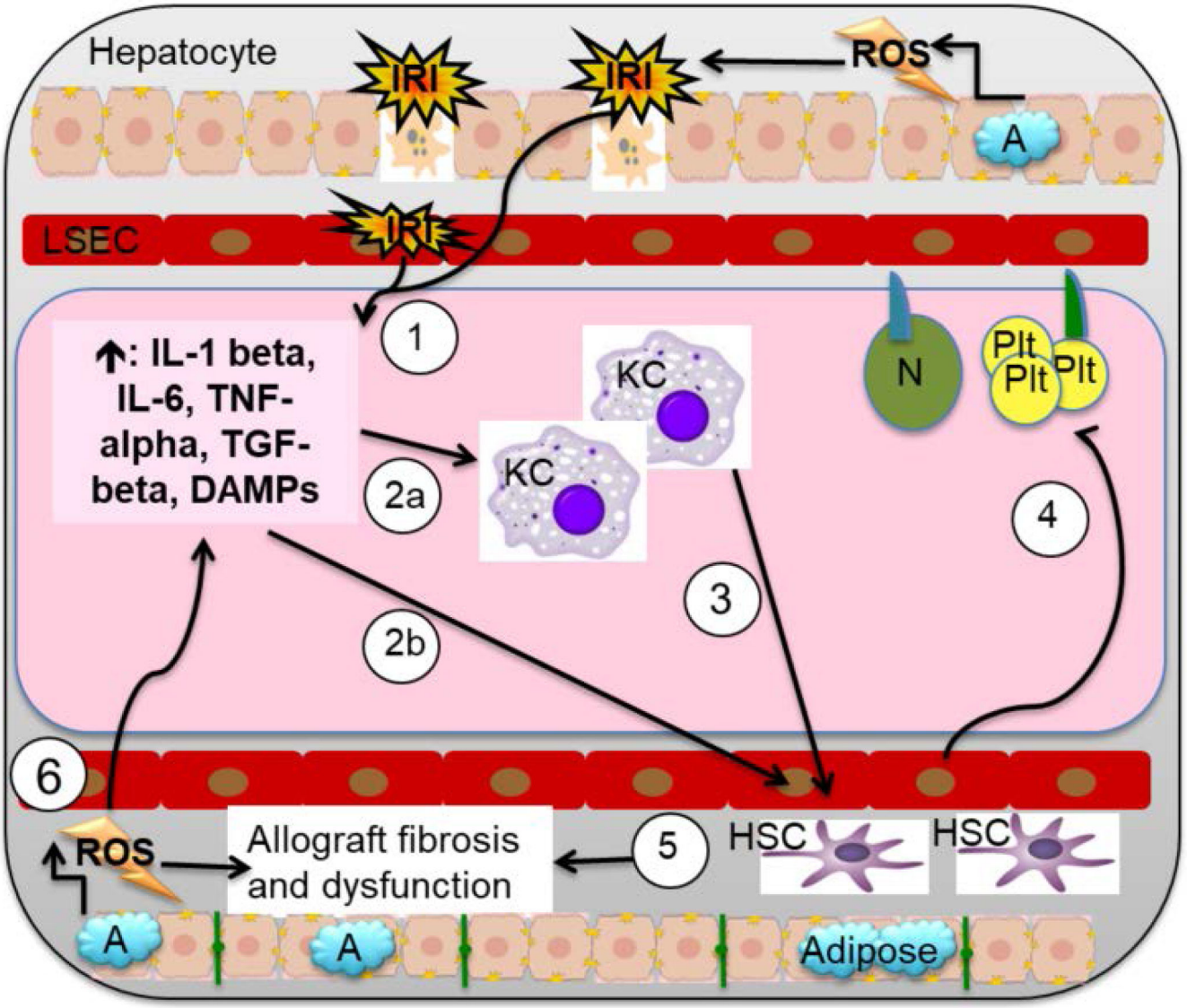

Figure 5: Cellular and molecular IRI pathway: A focus on the roles of HSC, adiposity, and ROS.

(1) IRI damage to hepatocytes and LSECs causes cell death and release of inflammatory cytokines IL-1 beta, IL-6, and TNF-alpha, DAMPS, HMGB1, TGF-beta, FFA, and HSB, as well as DNA fragments, which cause (2a) KC to lose tolerogenic profile and increasing cytokine production in KC toward inflammatory phenotype and (2b) hepatic stellate cell (HSC) activation. (3) KC activation is associated with increased KC cytokine production, which further recruits HSCs. The activation and recruitment of HSC to areas of IRI results in (4) ingress of platelets and neutrophils, (5) MMP release, ECM destruction, and endothelial vasoconstriction through ET-1. Together, this results in collagen deposition and graft fibrosis, increasing the risk of graft failure, morbidity, and mortality. (6) After organ reperfusion, lipid peroxidation due to high levels of steatosis causes release of ROS and DAMPs. This results in increased release of IL6 and TNF-alpha, resulting in further acute

liver injury, ineffective autophagy, early graft dysfunction or non-function, and increased long-term fibrosis.

Table 1.

Current Clinical Trials for Prevention of Ischemia Reperfusion Injury in Liver Transplantation

Trial Name	Intervention Type	Description	Sponsor
Efficacy and Safety Pilot Study of Reparixin for Early Allograft Dysfunction Prevention in Orthotopic Liver Transplantation Patients	Pharmacotherapy	Use of Reparixin (CXCL8/IL-8) inhibitor to prevent IR injury in liver transplantation	Dompé Farmaceutici S.p.A (multicenter)
Safety and Efficacy of Treprostinil in Ischemia and Reperfusion Injury in Adult Orthotopic Liver Transplantation	Pharmacotherapy	Use of Treprostinil (prostacyclin analogue) to prevent IR injury in liver transplantation	University of Pittsburgh
Omega 3 Lipid Emulsions and Liver Transplantation	Pharmacotherapy	Perioperative Omega 3 rich lipid infusions to prevent IR injury in living donor liver transplantation	Mansoura University
YSPSL for Prevention of Ischemic Reperfusion Injury in Patients Undergoing Cadaveric Orthotopic Liver Transplantation	Pharmacotherapy	P-selectin Ig infusion during reperfusion of liver allograft to prevent IR injury during liver transplantation	UCLA Medical Center
Intermittent Portal and Graft Purge in Living Donor Liver Transplantation	Ischemic Preconditioning	Intermittent portal clamping during reperfusion of living donor liver transplantation	Mansoura University
Liver Protection of RIPC in Pediatric Living Donor Liver Transplantation	Ischemic Preconditioning	Donor and Recipient distant (limb) ischemic preconditioning to reduce IR injury in pediatric liver transplantation	Renji Hospital Shanghai
Efficacy Evaluation of Normothermic Perfusion Machine Preservation in Liver Transplant Using Very Old Donors	Ex Vivo Perfusion	Ex Vivo normothermic perfusion of older/marginal liver allografts to prevent IR injury in liver transplantation	Azienda Ospedaliero, Universitaria Pisana
Hypothermic Oxygenated Perfusion (HOPE) of human liver allografts	Ex Vivo Perfusion	Hypothermic perfusion with oxygenated perfusate after cold storage to prevent IR Injury in liver transplantation	Universisty of Zurich (multicenter)
Dual Hypothermic Oxygenated Perfusion of DCD Liver Grafts (HOPE-DCD) in Preventing Biliary Complications After Transplantation	Ex Vivo Perfusion	Hypothermic perfusion with oxygenated perfusate via portal vein and aorta of DCD livers to prevent IR Injury in liver transplantation	University Medical Center Groningen (multicenter)
HOPE for Human Extended Criteria and Donation After Brain Death Donor (ECD-DBD) Liver Allografts	Ex Vivo Perfusion	Hypothermic perfusion with oxygenated perfusate after cold storage of ECD livers to prevent IR Injury in liver transplantation	University of Aachen (multicenter)
Using Ex-vivo Normothermic Machine Perfusion With the Organox Metra™ Device to Store Human Livers for Transplantation	Ex Vivo Perfusion	Normothermic machine perfusion of liver allografts after procurement and prior to implantation to prevent IR injury in liver transplantation	OrganOx (multicenter, Europe, United States and Canada)