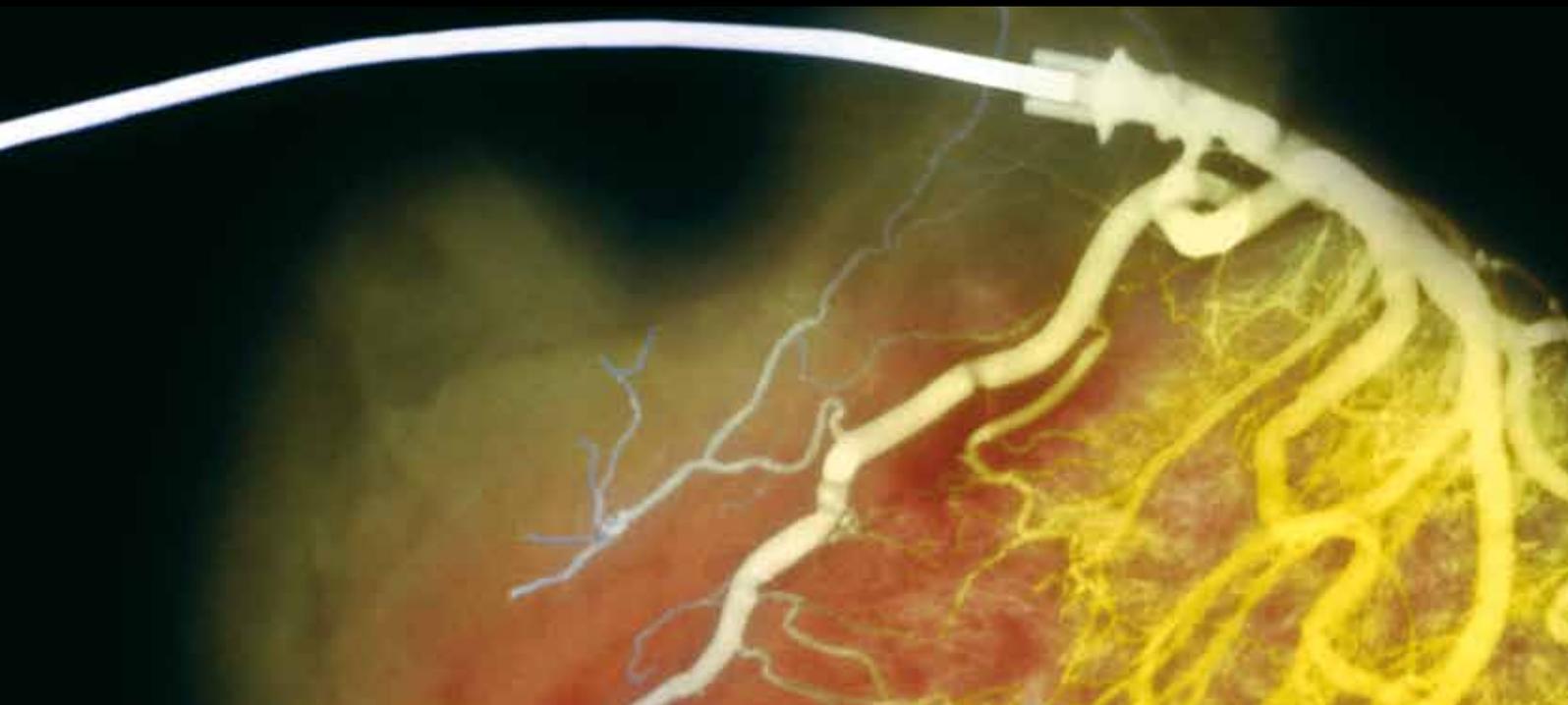


Ischemia/Reperfusion Injury, Its Mechanisms, and Prevention

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AND WOJCIECH ROWIŃSKI





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Guest Editors: Maciej Kosieradzki, Johann Pratschke,
Jerzy Kupiec-Węgliński, and Wojciech Rowiński



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Editorial

Ischemia/Reperfusion Injury, Its Mechanisms, and Prevention

Maciej Kosieradzki,¹ Johann Pratschke,² Jerzy Kupiec-Węgliński,³ and Wojciech Rowiński⁴

¹ Department of General and Transplantation Surgery, Medical University of Warsaw, 02-006 Warsaw, Poland

² Department of General Transplant and Surgery, Medical University of Innsbruck, 6020 Innsbruck, Austria

³ The Dumont-UCLA Transplant Center, David Geffen School of Medicine, Los Angeles, CA 90095, USA

⁴ Department of Surgery, University of Warmia and Mazury, 10-957 Olsztyn, Poland

Correspondence should be addressed to Maciej Kosieradzki, mkosieradzki@wp.pl

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Since the beginning of transplantation era, ischemia/reperfusion injury (IRI) has remained one of the most serious pitfalls of the procedure. Although thousands of scientific studies and analyses have been published, and mechanisms of the injury recognized and described, the most serious step forward to avoid the IRI was Geoffrey Collins' and Folkert Belzer's invention of two different intracellular-type solutions in 1960s, which allowed for safe renal preservation exceeding 24 hours. Since then, no such important improvement took place and prevention of IRI remains a holy grail of transplantation, especially in thoracic and sub-optimal abdominal organs (fatty livers, older donor kidneys). In this issue we present two studies examining various modalities to reduce both cold and warm ischemia. W. Li et al. showed significant improvement in posttransplantation liver function tests, histology, and reduction of apoptosis with administration of sodium nitrite either to preservation solution or directly to the recipient animal. What is more important is that protection was more evident with more pronounced injury, when liver cold ischemia time was extended to 18–24 hours. Although NO is believed to render protection only in specific concentrations, the authors have shown a dose-dependent effect in a 25–250 μM concentration range. In this edition also, M. G. W. van den Heuvel et al. present use of statins in a human model of approximately one hour warm ischemia and reperfusion during breast reconstruction with free cutaneous flap. Although numerous authors postulate usefulness of statins in attenuation of ischemia/reperfusion injury (IRI) [1, 2], employing dozens of pathways and mechanisms in rendering protection, in this study statins did not show any benefit

and rather deteriorated the results and increased risk of complications in skin flap warm ischemia and transposition.

Some agents can be administered in a gaseous form and A. Siriussawakul et al. give a nice overview of potential applications and proposed mechanisms of action of inhaled nitric oxide, carbon monoxide, and hydrogen sulfide in prevention of IRI and inflammation.

Liver is known to tolerate 12 hours of cold ischemia relatively well. However, exceeding preservation beyond this time leads to significant deterioration of both short and long term results of transplantation. This decline is mostly due to biliary pathology, as biliary tree is the most ischemia susceptible tissue of the liver. R. Cursio and J. Gugenheim give a nice overview of biliary complications after liver transplantation. They extensively discuss pathomechanism of ischemic type biliary lesions and the role of cold and warm ischemia as well as intrahepatic cholestasis, bile salts, and immune-dependent events in development of this phenomenon.

Posttransplant acute renal failure is mainly caused by ischemia and reperfusion. Although primary damage occurs mainly to tubular cells, microvasculature is also affected, especially with prolonged warm ischemia. This becomes clinically more sound with more and more organs retrieved after cardiac death. Patschan et al. discuss features and mechanism of endothelial cells' injury in renal IRI and potential therapeutic application of endothelial progenitor cells. With the focus of contemporary medicine on regenerative technologies this is definitely a "sexy" topic in IRI, regardless of whether peripheral blood-retrieved endothelial outgrowth cells are true progenitor cells or not. What is more

important, several authors mention antifibrogenic properties of such therapy, making it even more attractive in the field of renal transplantation.

A key player involved in inflammation, cellular apoptosis and survival in a transplanted organ is a pathway of mitogen activated protein kinases (MAPKs). Despite its essential role in recovery from ischemia, and some new inhibiting agents with potential therapeutic application in the pipeline, this pathway is rather poorly recognized by transplant physicians. We decided to publish a review by G. Vassalli et al., who give a concise description of MAPK pathway in ischemia/reperfusion injury and ischemic preconditioning of the heart. Bases of this knowledge are critical not only to surgeons involved in heart or other organ transplantation, but equally to any other physician treating patients for coronary disease or myocardial infarction.

*Maciej Kosieradzki
Johann Pratschke
Jerzy Kupiec-Węgliński
Wojciech Rowiński*

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Clinical Study

The Effect of Statins and Other Cardiovascular Medication on Ischemia-Reperfusion Damage in a Human DIEP Flap Model: Theoretical and Epidemiological Considerations

M. G. W. van den Heuvel,¹ A. Bast,² A. W. Ambergen,³ and R. R. W. J. van der Hulst¹

¹ Department of Plastic, Reconstructive and Hand Surgery, Maastricht University Medical Centre, P.O. Box 5800, 6202 AZ, Maastricht, The Netherlands

² Department of Pharmacology and Toxicology, Maastricht University Medical Centre, P.O. Box 616, 6200 MD, Maastricht, The Netherlands

³ Department of Methodology and Statistics, Maastricht University Medical Centre, P.O. Box 616, 6200 MD, Maastricht, The Netherlands

Correspondence should be addressed to M. G. W. van den Heuvel, m.vandenheuvel@maastrichtuniversity.nl

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Background. Statins and other cardiovascular medication possess antioxidant capacity. It was examined whether chronic use of these medications protects from the development of ischemia-reperfusion (I/R) related complications after DIEP (Deep Inferior Epigastric Perforator Free Flap) surgery. This paper contains a literature study on the antioxidant working mechanisms of these drugs. **Methods.** Medical information of 134 DIEP patients (173 flaps) was studied from their medical files. Patient and operative characteristics were registered, as well as I/R related complications. **Results.** Of the group that did not use statins, 16.3% developed complications versus 30.8% amongst patients that did use these drugs ($P = 0.29$). Amongst patients that chronically use other cardiovascular medication, 26.8% developed I/R related complications versus 14.4% of the patients without medication ($P = 0.10$). **Conclusions.** Chronic use of statins or other cardiovascular medication did not decrease the occurrence of I/R related complications after DIEP surgery. Therefore, research should be aimed at evaluating short-term pre-treatment with statins.

1. Introduction

Neutrophil influx and the formation of reactive oxygen species (ROS) play an important role in the initiation of ischemia-reperfusion (I/R) damage. An early survey of registered medication showed that many drugs possess antioxidant capacity, which might contribute to their pharmacological action [1]. Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are inhibitors of sterol synthesis [2]. They block the conversion of HMG-CoA to mevalonate, which is the rate-determining step in cholesterol production [3]. Statins display a pleiotropic activity including anti-atherosclerotic, antithrombotic, but also anti-inflammatory and neuroprotective effects [2, 4, 5]. They also exert antioxidant effects [4, 6]. All these effects seem to be independent of their cholesterol-lowering effect [7, 8]. Animal studies have

shown a protective effect by administration of statins previously to I/R. They demonstrated that pre-treatment with statins for several days, reduces myocardial injury after I/R [8–11]. Renal function and survival after renal I/R were improved after short-term statin pre-treatment in rats [12, 13]. Improved graft function and decreased renal inflammation were achieved after statin pre-treatment before renal transplantation [14]. Furthermore, atorvastatin treatment for 10 days significantly reduced infarct volume after cerebral ischemia [15].

Other cardiovascular drugs also possess antioxidant capacity, for example, ACE-inhibitors, calcium antagonists, antiarrhythmic drugs, and β -adrenoceptor antagonists [1, 16]. ACE inhibitors have shown to display cardioprotective effects through free radical scavenging [17, 18]. Furthermore, they

display anti-inflammatory effects. This is probably achieved by preventing leukocyte adhesion [19]. These beneficial effects were also demonstrated in I/R of rat liver [20]. Calcium antagonists have also demonstrated protective effects in I/R of the liver [21]. All these studies were carried out in animals; little research has been performed in humans in this area.

Administration of statins or other cardiovascular medication would be a safe and simple method to protect patients who are undergoing a medical procedure from the development of I/R damage. The Deep Inferior Epigastric Perforator Free Flap (DIEP) operation is featured by I/R. During this procedure, a tissue flap is dissected from the abdomen and transplanted to the patient's thorax. The tissue flap has to endure around 60 minutes of ischemia. Although this operation is usually successful, in 5–10% of the patients (partial) flap loss occurs. Flap failure after DIEP surgery is a traumatic event for the patient, who already battled against cancer. Flap failure represents another disappointment which leaves the patient with less reconstructive options and the knowledge of having to endure yet another operation. Therefore, much research is being performed in order to prevent the effects of I/R injury. In this study it was examined whether chronic use of statins or other cardiovascular medication protects from the development of I/R related complications after DIEP surgery.

2. Materials and Methods

2.1. Population. The sample consisted of women that had undergone breast amputation or mastectomy due to breast cancer or in order to prevent breast cancer and underwent breast reconstruction at the Department of Plastic and Reconstructive Surgery in the Maastricht University Medical Centre in The Netherlands between September 2004 and June 2009. Medical information of 134 DIEP patients, amongst whom 39 patients who underwent bilateral surgery, was studied from their medical files.

2.2. Medical Information. Of every patient, the medical file was studied for the following patient characteristics: age, length, weight, smoking, and the use of medication. Furthermore, I/R related complications after DIEP surgery were registered (reexploration, necrosis, and (partial) flap loss). Data were processed anonymously and per DIEP flap.

2.3. Statistics. With STATA random-effects logistic regression, two statistical models were calculated to answer both research questions. In each statistical model, the patient characteristics age, BMI, and smoking were included and integrated as covariates. For patients who underwent bilateral DIEP surgery, statistical correction was performed. Significance was considered present at $P \leq 0.05$, two tailed.

3. Results

In 13 of the 173 DIEP flaps, statins were used (7.51%) and in 41 flaps other cardiovascular drugs were taken (23.70%).

TABLE 1: patient characteristics per study group.

	All	Statin	Cardiovascular
N (flaps)	173	13	41
Age (yr)	47.67	58.31	53.93
BMI (kg ² /m ²)	27.17	28.40	28.60
Smoking (%)	12.72	0	2.44
Complication (%)	17.34	30.77	26.83

Patient characteristics are shown in Table 1. In 30 Flaps (17.34%) I/R related complications developed.

Statistical analysis showed that the number of I/R related complications after DIEP surgery had not decreased due to chronic use of statins. Of the group that did not use statins 16.3% developed complications versus 30.8% amongst patients that did use these drugs ($P = 0.29$). Furthermore, a nonsignificant increasing trend in the occurrence of complications appeared after chronic use of other cardiovascular medication; 14.4% of the patients without medication versus 26.8% of the patients using drugs ($P = 0.10$). Patient characteristics age, BMI, and smoking did not significantly influence the occurrence of complications in both regression models. In 22 out of 173 DIEP flaps, the patient smoked (12.72%).

4. Discussion

4.1. The DIEP Flap As a Model of I/R. In this study, the DIEP flap was used as a clinical, human model of I/R. It has the advantage of being visible and within reach, even after surgery. Therefore, this new model is well suited for analysing I/R in humans over time. Furthermore, duration of ischemia is relatively constant and patients are healthy, at the time of surgery there is no active disease. Another benefit that the DIEP model offers is that it is an autologous transplantation and there is no interference of donor incompatibility. Therefore, the isolated effect of an intervention can be studied.

4.2. Statins. Statins exert their actions through several mechanisms. Through inhibition of the mevalonate pathway, statins inhibit isoprenoid production [9]. Isoprenoids are responsible for posttranslational modification of many proteins, amongst which is Rho [9]. Rho plays an important role in inflammation by activating transcription factor nuclear factor- κ B and it also decreases endothelial production of nitric oxide (NO) [9]. By inhibiting the isoprenylation of Rho and Rho kinase, statins increase eNOS (endothelial nitric oxide synthase) mRNA stability and thereby NO production [3, 4, 7–9]. Statins may also directly activate eNOS through protein kinase Akt activation [8, 12, 13, 22]. Statins activate receptor tyrosine kinases and G-protein-coupled receptors, thereby activating phosphoinositol-3 kinase, which consequently activates the protein kinase Akt by phosphorylation [8]. Next, Akt causes eNOS to be phosphorylated and NO production to increase [9]. Increased availability of NO improves endothelium function and blood flow to the tissues [23].

Secondly, statin administration inhibits upregulation of adhesion molecules, like VCAM-1, ICAM-1 and P-selectin [9, 24]. Hereby, neutrophil rolling, adherence, and neutrophil influx are reduced [25, 26]. This decreased expression of adhesion molecules and PMN infiltration is thought to be regulated through NO release from the endothelium [9, 26–30]. However, how this occurs remains unclear. Some studies demonstrate that eNOS just functions as a trigger for initiating protection, while iNOS (inducible nitric oxide synthase) is the essential mediator in protection through pharmacological preconditioning and is upregulated after statin use [10, 11]. Other studies, on the other hand, show that statins decrease iNOS expression [24, 30].

Research showed that the protective effects of statin treatment could also be mediated by increased prostaglandin production, which is due to an upregulation of cyclooxygenase-2 and other prostaglandin synthases [10]. Cyclooxygenase-2 is the enzyme that catalyses the rate-limiting step in prostaglandin synthesis. Prostaglandins can have beneficial effects during I/R, like anti-inflammatory effects, vasodilation, and platelet disaggregation.

Furthermore, statins display antioxidant effects. They are exerted through many pathways, all resulting in decreased ROS production. First, they inhibit NADPH oxidase, thereby attenuating neutrophil respiratory burst [6]. Furthermore, statins cause S-nitrosylation of thioredoxin, thereby increasing its enzymatic activity and reducing intracellular ROS production [31]. Reduction in ROS production is also achieved by activation of the heme oxygenase-1 promoter in endothelial cells [32]. Heme oxygenases convert heme to biliverdin. Degradation products of heme have the capacity to decrease superoxide anion production [9]. Superoxide production can also be reduced by inhibiting tyrosine phosphorylation in activated neutrophils [33]. Finally, statins downregulate the aldose reductase pathway, which is involved in oxidative stress [14]. Aldose reductase competes with glutathione reductase for NADPH, causing a decrease in reduced glutathione content. Subsequently, the sorbitol metabolism produces NADH, which enables NADH oxidase to produce more ROS [14]. By inhibiting the aldose reductase pathway, statins thus reduce ROS production during I/R and they increase antioxidant capacity by restoring tissue glutathione levels [25].

4.3. Cardiovascular Medication. The protective effects of cardiovascular medication are also established through different mechanisms. Calcium antagonists, angiotensin II, and ACE-inhibitors increase blood flow during reperfusion either by vasodilation or through stimulation of angiogenesis [34]. Treatment with angiotensin II and captopril have been demonstrated to stimulate angiogenesis and thereby incline free flap viability and vascularity [35]. Through activation of the AT1 receptor, angiotensin II increases vascular endothelial growth factor expression by vascular smooth muscle and endothelial cells and directly stimulates endothelial cells to produce NO [35].

Furthermore, cardiovascular drugs can prevent ROS formation. First, calcium antagonists inhibit the influx of calcium during I/R [18]. In this process, the lack of ATP leads

to ATP-dependent calcium pump dysfunction, causing the intracellular calcium level to increase. This calcium overload triggers conversion of xanthine dehydrogenase to xanthine oxidase, causing the production of ROS [21]. Thus, inhibiting calcium influx prevents ROS formation. β -adrenoceptor antagonists also protect from I/R by attenuating calcium influx [16]. Secondly, cardiovascular drugs prevent autoxidation of catecholamines. The antihypertensive effect of ACE-inhibitors is related to inhibition of norepinephrine release from peripheral sympathetic neurons [17]. In the presence of oxygen and transition metals catecholamines could be autoxidized, leading to OH-radical formation [17]. ACE-inhibitors attenuate OH-radical production by decreasing the level of norepinephrine [17]. However, not all ACE inhibitors have radical scavenging properties. It is believed that only ACE inhibitors containing a SH-group in particular possess this capacity [17]. Enalapril, for example, is a non-SH-group containing ACE inhibitor and displayed no protective effects during I/R [18].

The last ROS preventing mechanism is attenuation of neutrophil accumulation during late reperfusion [34]. Just like statins, calcium antagonists can inhibit neutrophil influx, thereby decreasing ROS production from the respiratory burst.

Other working mechanisms of cardiovascular medication do not prevent ROS formation, but increase free radical scavenging properties. Some drugs do this by increasing the antioxidant reserve [18]. Captopril, for instance, increased SOD activity, but induced no changes in glutathione peroxidase and catalase enzyme activity [18]. However, it significantly attenuated lipid peroxidation [18]. Stobadine, a pyridindole derivative that displays cardioprotective and anti-arrhythmical effects, showed a protective effect during I/R by increasing the glutathione peroxidase activity and the total antioxidant capacity [36]. ACE inhibitors and calcium antagonists, especially the dihydropyridines, possess radical scavenging properties and prevent ROS formation. They decrease bradykinin degradation, stimulating eNOS to produce NO [19, 34]. NO may act as an antioxidant itself and also prevents activation of polymorphonuclear leukocytes, thereby decreasing the amount of ROS [19]. The increased bradykinin activity stimulates NO and prostacyclin production, causing vasodilation too [18].

4.4. Results of This Study. Whether chronic statin treatment offers protective effects in patients undergoing I/R-during surgical procedures, remains unclear. Although some studies demonstrated a decrease in C-reactive protein, plasma adhesion molecule levels and cytokine levels [29, 37–40], other studies could not confirm these findings [41, 42]. And whether these changes result in a clinical benefit, like a decrease in I/R related complications, is not clear either. Pascual et al. demonstrated a decrease in early complications after coronary artery bypass grafting, but these beneficial effects only occurred in patients with a positive troponin T status [41]. This beneficial effect might be caused by an effect on atherosclerotic plaques or cholesterol level rather than a general antioxidant or anti-inflammatory mechanism. Patti et al. and

the study of Pasceri and colleagues both demonstrated a decrease in postprocedural complications after statin use [42, 43]. However, this was achieved after short-term pre-treatment (7 days). An animal study showed that short-term statin administration could have protective effects, while these effects are absent after chronic statin treatment [44].

There is little evidence proving a clinical protective effect of chronic statin use. Our study could not demonstrate a beneficial effect either. Chronic use of statins did not decrease the occurrence of I/R related complications after DIEP surgery. Unfortunately, the group that endured statin pre-treatment was small (13 flaps). There are studies demonstrating evidence that *short-term* pre-treatment with statins could be effective in preventing I/R injury. Possibly the body adapts to chronic statin use, thereby compensating the beneficial effects of statins on I/R. Therefore, further research has to be aimed at evaluating the effects of short-term pre-treatment with statins before I/R.

The use of cardiovascular medication even showed a mild increase in the number of complications. In this category different types of drugs have been included. The effects of these drugs on the process of I/R are different. Some drugs might not have a protective effect at all, like non-SH-group containing ACE inhibitors. It is possible that a study in which the different types of cardiovascular drugs are studied separately, shows a different result. However, in our study no distinction was made because groups would be small due to the large variety of cardiovascular medications and because many patients use multiple types of drugs.

Furthermore, patients in this study that use statins or other cardiovascular drugs do this based on a medical indication. This means all patients in the statin and cardiovascular group suffer from hypertension or dyslipidemia, no patients displayed cardiovascular disease. Probably, the decreased vascular condition and perfusion of these patients is the reason for the increased risk of complications. The beneficial effects of these drugs as described in the literature are probably occurring, but are not strong enough to compensate for the deleterious vascular condition of these DIEP patients. The same accounts for the statin group in our study.

We had the idea to study whether a beneficial effect of chronic use of statins or other cardiovascular medication could be demonstrated from a medical file study in DIEP patients, but unfortunately this was not the case. The main disadvantage of this study is its observational, retrospective design. However, it demonstrated the probability that chronic treatment with statins and other cardiovascular medication influences the occurrence of I/R related complications and thereby gave important directions for further research. Because there is ample literature demonstrating evidence that *short-term* pre-treatment with statins could be effective in preventing I/R injury, future research should be aimed at short-term pre-treatment instead of chronic use. Ideally, this would be performed as an intervention study in which these drugs are administered for a short period to healthy patients undergoing I/R.

In conclusion, this study failed to demonstrate a protective effect from the chronic use of statins or other cardiovascular medication on the effects of I/R. However, there is

evidence demonstrating beneficial effects from short-term pre-treatment with statins. Further research in this area should therefore be focussed on short-term premedication with statins. The most important feature in this study is the use of the DIEP operation as a human model of I/R. In contrast to other models, this new, clinical model is well suited for analysing I/R in humans over time because the DIEP flap remains within reach after surgery and interference of donor incompatibility is avoided.

Disclosure

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Review Article

Medical Gases: A Novel Strategy for Attenuating Ischemia—Reperfusion Injury in Organ Transplantation?

Arunotai Siriussawakul,¹ Lucinda I. Chen,² and John D. Lang²

¹Department of Anesthesiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

²Department of Anesthesiology and Pain Medicine, University of Washington School of Medicine, 1959 NE Pacific Street, Seattle, WA 98195, USA

Correspondence should be addressed to John D. Lang, jdlang@uw.edu

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Ischemia reperfusion injury (IRI) is an inevitable clinical consequence in organ transplantation. It can lead to early graft nonfunction and contribute to acute and chronic graft rejection. Advanced molecular biology has revealed the highly complex nature of this phenomenon and few definitive therapies exist. This paper reviews factors involved in the pathophysiology of IRI and potential ways to attenuate it. In recent years, inhaled nitric oxide, carbon monoxide, and hydrogen sulfide have been increasingly explored as plausible novel medical gases that can attenuate IRI via multiple mechanisms, including microvascular vasorelaxation, reduced inflammation, and mitochondrial modulation. Here, we review recent advances in research utilizing inhaled nitric oxide, carbon monoxide, and hydrogen sulfide in animal and human studies of IRI and postulate on its future applications specific to solid organ transplantation.

1. Introduction

Organ transplantation is an established treatment that allows patients suffering from end-stage organ diseases to start living their life anew. In the United States, more than 100,000 patients are waiting for solid organ transplants, but less than 10% of patients have undergone the necessary transplantation. While the survival rate has increased substantially over the past decade, according to the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients, morbidity and mortality remains substantial. With the shortage of organs, it is clear that treatments need to be developed to optimize the quality of the organs that are available and to attenuate injury to transplanted organs.

Ischemia reperfusion injury (IRI) remains a critical clinical issue in organ transplantation. It can result in a higher incidence of acute and chronic rejection, as well as long-term morbidity and mortality [1, 2]. Ischemia is an inevitable event, starting with the cessation of arterial blood flow

after organ procurement, cold ischemic time of the organ being donated, and warm ischemic time of the recipient during the organ transplantation. Reestablishment of blood flow in transplant recipients results in reperfusion injury, which is characterized by oxidative stress and inflammation (Figure 1). Much interest has been shown recently, not only in microcirculatory flow disturbances, but also in the pathophysiology of IRI in terms of the intracellular and molecular mechanisms.

A class of signaling molecules called “gasotransmitters” has been investigated as a supplementary therapeutic agent during solid organ transplantation. These medical gases, nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H₂S), have traditionally been considered to be toxic and environmentally hazardous. However, numerous experimental animal and human studies of these agents have demonstrated protective effects against IRI. The aim of this review is to summarize the current understanding of medical gases on attenuation of the pathophysiology IRI in the setting of organ transplantation.

2. Ischemia and Reperfusion Injury in Organ Transplantation

Ischemia reperfusion injury is an occurrence where injury to an organ occurs during times of hypoxia and is amplified when blood flow (oxygen) is reestablished. Injury can be incurred during both phases of ischemia (warm and cold) [3]. Warm ischemia occurs at the time of organ procurement and reperfusion during transplantation when blood flow is restored. Cold ischemia occurs during the time of storage in preservation solution prior to the anticipated transplant [4].

Warm ischemia leads to the activation of a multitude of immunoinflammatory pathways culminating in cellular injury to a particular organ, but also systemically [5]. Warm IRI can be divided into early and late phases. The early phase occurs within the period of the first two hours of reperfusion and the late phase generally described as occurring within 6–48 hours after reperfusion. During the early phase, significant activation of immune cells occurs with resultant formation of reactive oxygen species (ROS). The late phase is characterized by neutrophil-mediated organ injury that occurs as a consequence of early phase consequences and net cumulative effect of the overlap of both phases [6].

More specifically, each organ is subjected to a particular sequence of inflammatory events leading to injury. For example in the liver, Kupffer cells, the resident macrophages appear to be instrumental in orchestrating injury during the early phase of IRI. These cells are major producers of ROS that not only leads to local injury but serves as substrate for other inflammatory reactions that inflict both local and systemic inflammatory injury. This includes cellular injury from lipid peroxidation, DNA damage, and enzyme denaturation. Generation of ROS not only leads to direct cellular injury but serves to activate other immune cell lines that hone in neutrophils. Cytokine and chemokine production occurs also during the early phase of IRI. For example, tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1) significantly increase early systemically in the serum just minutes after reperfusion. Interestingly, these cytokines along with others upregulate the production of other cytokines, chemokines, and adhesion molecules, all critical to the pattern of injury observed in the late phase of IRI [7].

Chemokines and adhesion molecules recruit neutrophils and other immune cells to the site of injury. Chemokines vital to this process include interleukin-8, and macrophage inhibitory proteins. Cells that release these molecules include resident macrophages, endothelial cells, and organ-specific parenchymal cells. Various adhesion molecules are required to the transmigration of the neutrophils journey via the bloodstream to the sites of ischemic organ injury. This is facilitated by the upregulated expression of selectins, integrins, and immunoglobulins, all which ultimately lead to the accrual of neutrophils and platelets within organ parenchyma resulting in late phase IRI [8].

Neutrophils themselves when primed by a proinflammatory stimulus are highly active and release proteases and other cytotoxic substances. Neutrophils are also responsible for the release of clinically significant concentrations of ROS through nicotinamide adenine dinucleotide phosphate

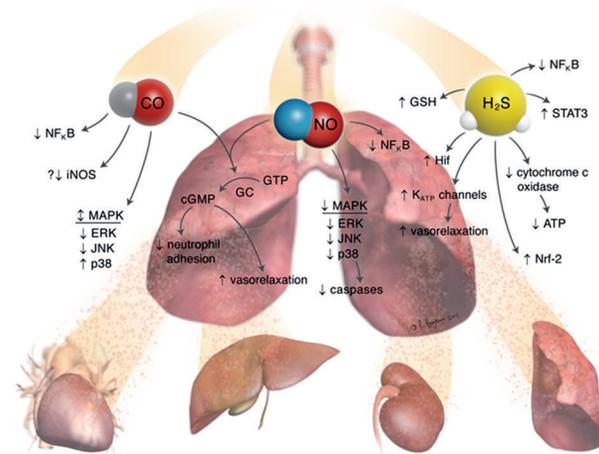


FIGURE 1: Preemptive treatment with inhaled nitric oxide, carbon monoxide and hydrogen sulfide can attenuate ischemia-reperfusion injury via modulation of a myriad of inflammatory, cellular and vascular mechanisms. GTP: guanosine triphosphate, GC: guanylate cyclase, cGMP: cyclic guanosine monophosphate, MAPK: mitogen-activated protein kinase, ERK: extracellular signal-regulated kinase, JNK: c-Jun N-terminal kinase, p38: a class of mitogen-activated complexes, iNOS: inducible nitric oxide synthase (iNOS), NFκB: nuclear factor kappa B, STAT-3: signal transducer and activator of transcription 3, ATP: adenosine triphosphate, Nrf-2: nuclear factor-like 2, Hif: hypoxia inducible factor, and GSH: glutathione.

hydrogen (NADPH) oxidase-dependent pathway. Activation of this enzyme system results in the predominant production of superoxide radical that in itself induces injury but also serves as substrate for a multitude of other proinflammatory reactions.

Not all of the pathways activated during the ischemia and reperfusion are injurious. Several pathways have been identified to limit injury. One of the most commonly referenced is the heme oxygenase (HO) system that is triggered due to the formation of ROS resulting in heme breakdown [9]. It is the heme breakdown products (a more neutral reduced iron), carbon monoxide (CO), and biliverdin that in their own dissimilar ways result in organ protection during IRI. The therapeutic utilization of CO will be discussed in more detail and is an obvious focus of this concise review.

Injury suffered during cold ischemia has been demonstrated to be different from warm and is directed toward injury to the endothelial cells and microcirculation as opposed to direct damage to the organ parenchymal cells. Endothelial cells seem to be more susceptible to cold storage compared to parenchymal cells [10]. Previous studies have shown that cell death is much greater in endothelial cells compared to parenchymal cells at the same time points. The injury appears to occur due to activation of apoptotic and necrotic pathways. In livers, the degree of injury has been shown to correlate with graft function following reperfusion. And overall, generalized inflammatory pathways involved in both early and late warm IRI seem to be enhanced with increasing cold storage times.

The pathogenesis of IRI is also significantly influenced by toll-like receptors (TLRs) (Figure 2) [11]. These receptors are transmembrane proteins which form the major pattern recognition receptors that transduce signals in response to diverse pathogen-associated molecular patterns (PAMPs). There are a variety of TLRs (TLR 1–7,9) with each of them recognizing distinct PAMPs with their activation leading to the initiation of innate and adaptive responses through the upregulation of cytokines, chemokines, and others immune cells. Once TLRs are ligated, they undergo conformational change and recruit cytoplasmic adapter proteins (Figure 2). The proximal adaptor proteins that mediate TLR signaling are myeloid-differentiation primary response gene 88 (MyD88). Downstream of MyD88, regulatory kinases are recruited, ultimately leading the activation of NF- κ B, ERK and/or activation of mitogen activation protein kinase pathways (MAPK). The net effect of activating these pathways enhanced production of inflammatory mediators thus injury.

Apoptosis or controlled program cell death is a major occurrence of IRI during organ transplantation [12]. Apoptosis pathways are activated and regulated between a multitude of signals. It has been demonstrated that organs commonly transplanted including liver, commonly incur allograft cellular apoptosis. During reperfusion both endothelial cells and parenchymal cells are susceptible to apoptosis. Many previous studies have depended on the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick and end labeling) assay to characterize apoptosis. This technique has been shown to stain any cell with DNA strand breaks irrespective of whether the mechanism of cell death was apoptosis or necrosis. Since the institution of more specific techniques to assist in identifying apoptotic cells, the absolute numbers of cells being affected by IRI during organ transplantation are probably not as high as previously thought. Many of the cells thought previously to have been apoptotic were probably necrotic. These necrotic cells probably lead to enhanced inflammation via activation of complement and generation of ROS. One important mediator specifically released by necrotic cells is high mobility group box 1 (HMGB1). HMGB1 is a nuclear factor bound to chromatin that during warm ischemia has been shown to bind to TLR4 and stimulate proinflammatory cytokine release. Previously, inhibition of HMGB1 attenuated cytokine release and reduced neutrophil infiltration in a model of liver IRI.

The previous paragraphs are a brief summary of the pathways involved in IRI and serve as to assist in understanding how the aforementioned inhaled medical gases may assist in attenuating IRI during organ transplantation.

3. Medical Gases and Organ Transplantation

The gases NO, CO, and H₂S have been recognized as important signaling molecules. They regulate vascular tone and ameliorate inflammatory effects. Traditionally, these gases have been regarded as toxic and lethal. However, many studies have established that these gases are safe and at lower concentrations possess benefits in attenuating IRI in the setting

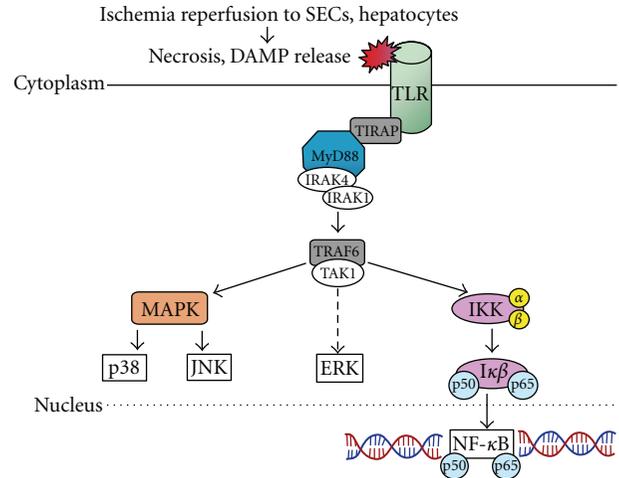


FIGURE 2: Toll-like receptor (TLR): mediated pathway during IRI. Ligation of the TLR leads to activation of upstream MyD88 and activation downstream NF- κ B, p38 and JNK pathways resulting in cellular injury. SEC: sinusoidal endothelial cells, DAMP: damage-associated molecular proteins [10].

organ transplantation. To date, there are an increasing number of reports on the role of medical gases in mitigating IRI.

4. NO and Organ Transplantation

Inhaled NO (iNO) was approved by the U. S. Food and Drug Administration in December 1999 for the treatment of persistent hypertension of the newborn. Over the last decade, the primary advantage of iNO has been its ability to selectively decrease pulmonary vascular resistance with minimal effects on systemic blood pressure. However, there is currently much interest in exploring its other benefits, including its antioxidant properties and cytoprotective abilities.

Nitric oxide was first identified as an endothelium-derived relaxing factor. Since then, it has been recognized for its effects as an antioxidant, an antiapoptotic agent, as well as its ability to inhibit adhesion molecules [13]. All of these properties contribute to its effectiveness in attenuating IRI. Nitrite (NO₂⁻), which is reduced to NO during ischemia, protects mitochondria from IRI by blocking complex I of the electron transport chain, thereby limiting ROS generation and cytochrome *c* release [14]. The cytoprotective effect of nitrite in cardiac and hepatic ischemia-reperfusion in mice demonstrates the potential for nitrite, in addition to inhaled NO, to be used as treatment to modulate IRI [15]. Head-to-head prospective comparisons assessing mechanism and clinically relevant endpoints should be encouraged.

The quality and viability of an organ to be transplanted is of primary concern. With thousands of patients on organ transplantation waiting lists, it is critical to enlarge the pool of usable organs and to more predictably mitigate IRI in the donor organ. The use of nonheart-beating donors (NHBDs) would expand the donor pool, but the additional cellular damage that is incurred during warm ischemia may render these organs unusable. If however, these otherwise poorer

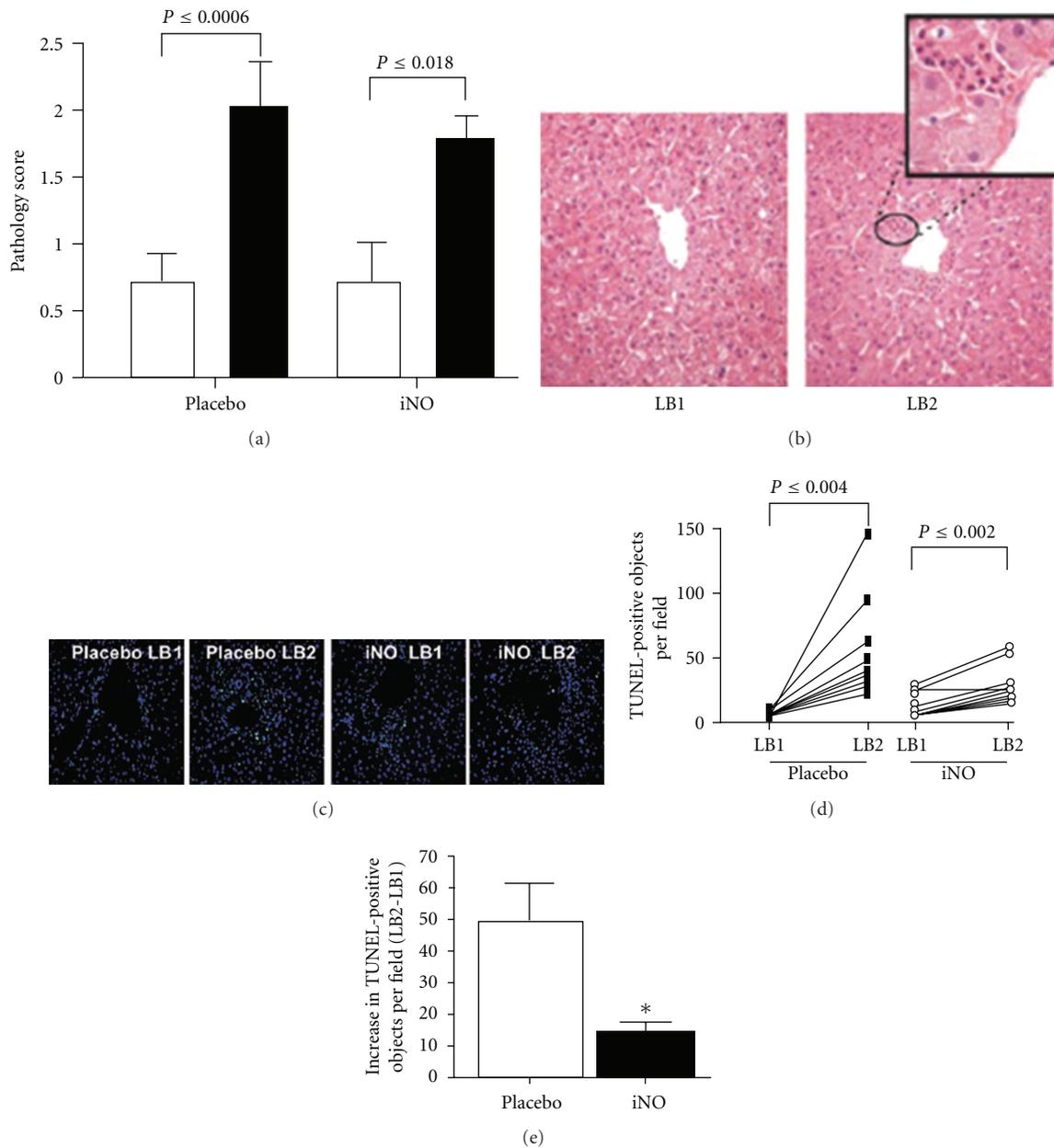


FIGURE 3: iNO decreases reperfusion-dependent hepatic cell death. (a) Histopathologic scoring of hepatic tissue samples before (white bars) and 1 hour after reperfusion (black bars). P -values represent significance calculated by paired t test. (b) Representative H&E-stained sections indicating increased injury in LB2. Original magnification $\times 25$. The circled area is shown at a higher magnification ($\times 100$) in the inset and shows increased PMN infiltration adjacent to the hepatic vein (zone 3). (c) Representative fluorescence micrographs showing changes in TUNEL-positive cells (green); blue staining: DAPI. Original magnification, $\times 25$. (d) Paired changes in TUNEL-positive objects in liver biopsies before (LB1) and 1 hour after reperfusion (LB2). P values represent significance calculated by paired t -test. (e) Average reperfusion-dependent increases in TUNEL-positive objects. * $P \leq 0.0005$ relative to placebo [17].

quality organs could be salvaged, more patients could receive transplants. In a rat model of lung transplantation using NBHDs, the treatment of donors with NO during ischemia, *ex vivo* perfusion, and after transplant resulted in better oxygenation and reduced pulmonary vascular resistance [16]. A similar NO treatment could easily be translated to human transplant patients in order to see an improvement in lung function.

Inhaled NO has been shown to be safe and efficacious in human trials. In a prospective, blinded, placebo-controlled study, 80 ppm of iNO was administered to 10 out of 20 patients undergoing orthotopic liver transplantation. Many advantages were reported in the iNO group, including reduced platelet transfusion, an improvement in the rate at which liver function was restored after transplantation, and a decrease in the length of hospital stay (Figure 3) [17]. A larger

trial enrolling 80 patients is currently underway in order to confirm these results. [Add in unpublished data].

Organ rejection is a critical concern with any transplant. Inhaled NO has also been used effectively in preventing allograft dysfunction. Treatment with iNO in lung transplant patients postoperatively was shown to improve oxygenation and decrease pulmonary artery pressure. No complications were associated with the use of NO, and the mortality rate was reduced [18]. Likewise, a retrospective study of patients who received iNO before or after transplantation also presented an improvement of overall respiratory functions [19]. However, in a randomized, placebo-controlled study of 84 patients who received NO or nitrogen following lung transplantation, no differences were found between the treatment and control groups [20]. In this latter study, iNO was administered at a concentration of 20 ppm while the other two studies started at 40 ppm and gradually reduced NO concentrations. Clearly, additional prospective randomized studies are necessary to further evaluate the most effective levels of iNO usage. But it is significant to note that no adverse side effects were associated with the use of inhaled NO in any of the clinical trials [17–20].

5. CO and Organ Transplantation

Carbon monoxide is a lethal gas when inhaled in high concentrations for long periods of time. CO avidly binds to hemoglobin and forms carboxyhemoglobin (COHb) with an affinity more than 200 times higher than of oxygen. As a result, the blood's oxygen-carrying capacity is impaired, which eventually causes tissue hypoxia. Because of their high metabolic rate, the brain and the heart are the most sensitive to CO exposure [21].

Humans and animals actually produce carbon monoxide endogenously [22]. In humans, CO arises from the action of microsomal heme oxygenase (HO) enzymes, which catalyze the conversion of heme into equimolar amounts of iron, biliverdin, and CO. HO has three isoforms: HO-1 (also known as heat shock protein 32), HO-2, and HO-3. Of the three isoforms, only HO-1 is inducible in response to a variety of cytokines and growth factors, as well as hypoxia and oxidative stress [23]. The CO that is produced by HO enzymes has multiple physiological effects, including vasodilation [24], anti-inflammatory, and antiapoptotic effects (Figure 4) [25, 26].

The known effects of carbon monoxide on the vasculature and on apoptosis make it an intriguing molecule to test in clinical models of IRI. Both inhaled CO and CO-releasing molecules (CO-RMs) have been shown to be effective in protecting rat hearts from IRI injury, resulting in less inflammation, less apoptosis, and less endothelial damage [27, 28]. Similarly, when rat livers were perfused with CO-supplemented blood or preserved in a CO-containing solution, they exhibited less hepatocyte histologic injury and a reduction in neutrophil extravasation, and they had an increased survival rate [29, 30]. There is also evidence that CO reduces delayed graft function in swine kidneys [31] and may prevent graft rejection [32].

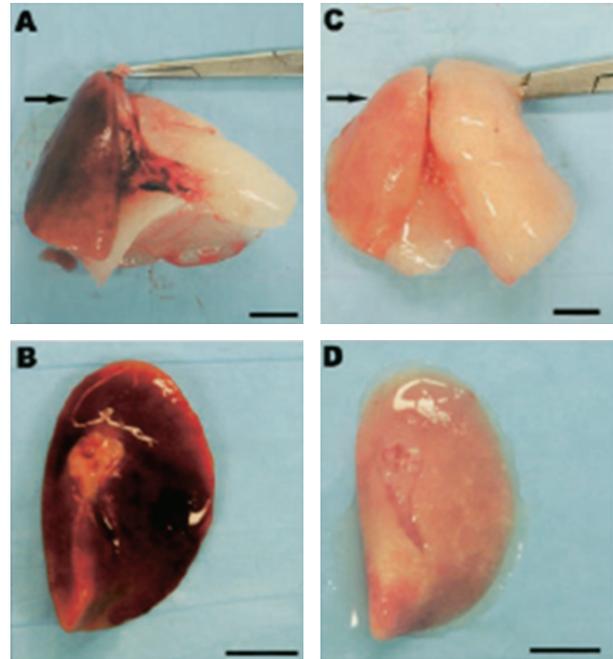


FIGURE 4: Gross anatomy of lungs from rats 6 days after transplantation (arrow in a, b) and lungs from rats 6 days after transplantation which received CO at 500 ppm. In (a) and (c), the arrow points to the left transplanted lung; the right lung is the remaining native lung. Note that in the absence of CO, transplanted lungs were noted to be markedly hyperemic (a) and (b), which was notably absent in the transplanted lung exposed to CO (c) and (d). Scale bar, 1 cm [26].

Despite the success of CO therapy in animal studies, the utility of CO as therapy in humans is uncertain. In unpublished data, CO in Phase I trials has been shown to be well tolerated with no significant adverse effects compared to placebo [33]. Further human clinical trials should be conducted cautiously in order to determine therapeutic doses and routes of administration. In fact, a Phase II trial is in progress that investigates the ability of CO to attenuate IRI in kidney transplantation with the focus of assessing the influence of delayed graft dysfunction.

6. H₂S and Organ Transplantation

Like NO and CO, hydrogen sulfide is primarily thought of as a toxic gas. Its reputation is well earned, for those who inhale it suffer from a range of effects, including mucosal irritation, memory loss, respiratory paralysis, and, sometimes, death. What is less known is that H₂S naturally occurs in mammalian tissue as a byproduct of cysteine metabolism. Its production from L cysteine is catalyzed by cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE), or 3-mercaptopyruvate sulfurtransferase (3MST). 3MST is the major H₂S-forming enzymes in the brain while CSE is a principle H₂S-forming enzyme in the cardiovascular system [34–36]. In other organs, such as the liver and the kidney, both enzymes contribute to H₂S production [37].

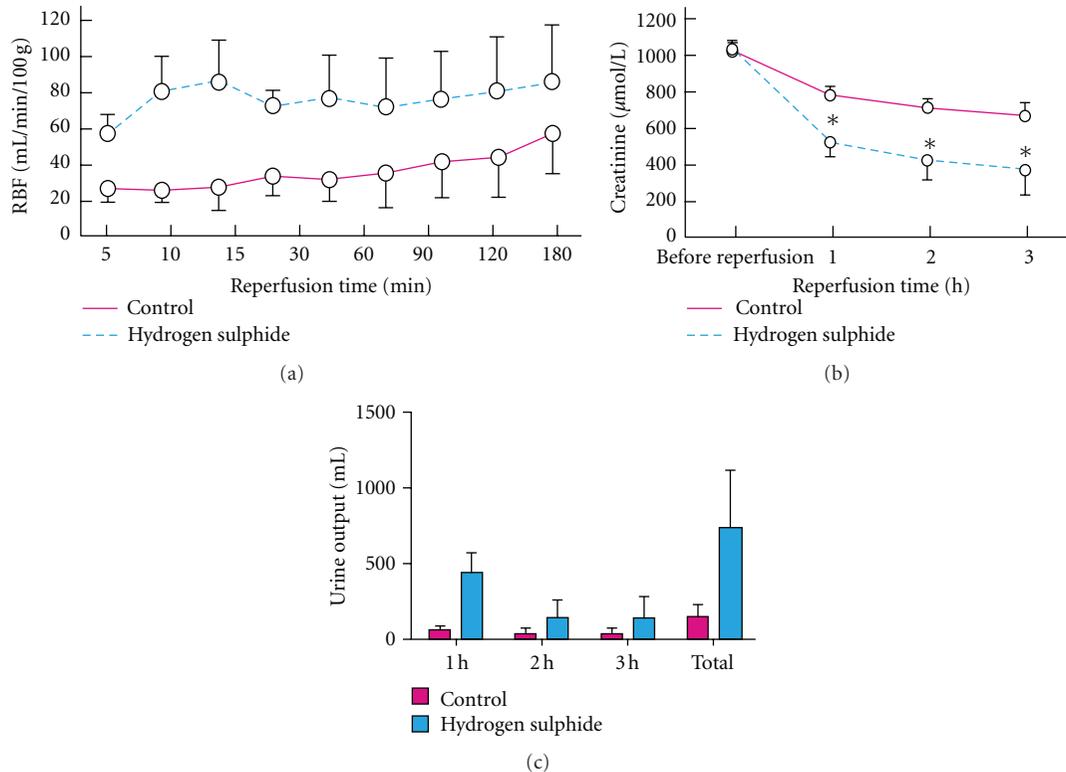


FIGURE 5: Indices of renal function were enhanced with hydrogen sulphide treatment. (a) Mean renal blood flow (RBF) over 3 hrs of reperfusion (b) Mean (s.d.) serum creatinine level over 3 hrs of reperfusion. * $P < 0.050$ versus control (Mann-Whitney U test) (c) Urine output at each hour of reperfusion [46].

The distinctive rotten-egg odor of H_2S gives no indication of its therapeutic potential. Nevertheless, endogenous and exogenous H_2S have been shown to reduce myocardial infarct size in various animal models when administered prior to reperfusion [38–42]. The cardioprotective effects are partially a result of the scavenging of hydrogen peroxide and free radicals [36, 43] and by the preservation of mitochondrial function [42]. H_2S also attenuates IRI by dilating blood vessels through the opening of ATP-sensitive K^+ (K_{ATP}) channels in vascular smooth muscle [39, 44]. This protection against ischemic damage has been demonstrated in the liver [45], and kidney [46, 47], as well.

With a low dose of hydrogen sulfide, mice enter a suspended-animation state with a reduced metabolic rate and lower core body temperature [48]. This artificial means of inducing a hypothermic-like state had no apparent adverse effects; thus, the potential exists to take advantage of this reduced metabolic usage to improve organ preservation. When an H_2S donor was added to the preservation solution in an *ex vivo* rat liver model, the damaging effects of cold storage were partially reversed [49]. In a mouse model of renal transplantation, treatment with H_2S during the hypoxic stage of IRI resulted in dramatically reduced renal damage (Figure 5) [49, 50]. The apparent mechanism for prevention of renal injury is by reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial electron transport chain [1, 50]. These data combined provide a compelling case for the further exploration of H_2S usage in the attenuation

and prevention of ischemia-reperfusion injury. Currently, there has not been any testing of H_2S in human subjects, but the data from animal models is intriguing, and clinical trials are necessary to validate the efficacy of H_2S .

7. Medical Gases Administered in Combination

The authors are unaware of studies performed in animals or in humans to determine the combined effects of the aforementioned gasotransmitters. It is unclear what effect combination therapy may elicit. However, both NO and CO are known to inhibit platelets by mediating a decrease in intraplatelet calcium via cGMP signaling. The effect of H_2S on platelets has not been elucidated, but it is also believed to be a platelet inhibitor [51]. A possible complication of the use of multiple gasotransmitters during transplantation could be increased bleeding or a need for platelet transfusion during surgery. It should be noted, though, that the use of NO as a single agent decreased the frequency of platelet transfusions during liver transplantation [20], so the possibility of increased bleeding with medical gas combination treatment may be quite small.

8. Conclusions and Perspectives for the Future

Administration of medical gases, specifically NO, CO, and H_2S , has been shown to mitigate IRI during organ

transplantation because these gases ameliorate oxidative stress, stimulate vascular smooth muscle cell relaxation, and reduce inflammation and apoptosis. From an optimistic viewpoint, these gases would decrease acute and chronic organ rejection, thereby increasing the quality of organ transplantation. Because there are currently no other therapies being utilized that increase the quality of transplanted organs, the further testing of these gases is crucial to improving the pool of potential donor organs. However, the treatments are in their infancy, and more clinical trials are needed to determine the indications, therapeutic doses, and optimal times of administration as well as adverse effects.

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Review Article

Role of Mitogen-Activated Protein Kinases in Myocardial Ischemia-Reperfusion Injury during Heart Transplantation

Giuseppe Vassalli,^{1,2} Giuseppina Milano,³ and Tiziano Moccetti¹

¹ *Fondazione CardioCentro Ticino, Via Tesserete, 6900 Lugano, Switzerland*

² *Department of Cardiology, University of Lausanne Medical Center, Av. du Bugnon, 1011 Lausanne, Switzerland*

³ *Department of Cardiovascular Surgery, University of Lausanne Medical Center, Av. du Bugnon, 1011 Lausanne, Switzerland*

Correspondence should be addressed to Giuseppe Vassalli, giuseppe.vassalli@cardiocentro.org

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In solid organ transplantation, ischemia/reperfusion (IR) injury during organ procurement, storage and reperfusion is an unavoidable detrimental event for the graft, as it amplifies graft inflammation and rejection. Intracellular mitogen-activated protein kinase (MAPK) signaling pathways regulate inflammation and cell survival during IR injury. The four best-characterized MAPK subfamilies are the c-Jun NH₂-terminal kinase (JNK), extracellular signal-regulated kinase-1/2 (ERK1/2), p38 MAPK, and big MAPK-1 (BMK1/ERK5). Here, we review the role of MAPK activation during myocardial IR injury as it occurs during heart transplantation. Most of our current knowledge regarding MAPK activation and cardioprotection comes from studies of preconditioning and postconditioning in nontransplanted hearts. JNK and p38 MAPK activation contributes to myocardial IR injury after prolonged hypothermic storage. p38 MAPK inhibition improves cardiac function after cold storage, rewarming and reperfusion. Small-molecule p38 MAPK inhibitors have been tested clinically in patients with chronic inflammatory diseases, but not in transplanted patients, so far. Organ transplantation offers the opportunity of starting a preconditioning treatment before organ procurement or during cold storage, thus modulating early events in IR injury. Future studies will need to evaluate combined strategies including p38 MAPK and/or JNK inhibition, ERK1/2 activation, pre- or postconditioning protocols, new storage solutions, and gentle reperfusion.

1. Introduction

Heart transplantation is the final therapeutic option for heart failure [1]. Over the past two decades, advances in immunosuppression and antimicrobial agents have improved outcomes after heart transplantation. An analysis of the UNOS database in 14,401 first-time orthotopic heart transplant recipients between the years 1999 and 2006 showed that the survival rate at 30 days, 1 year, and 5 years was 94%, 87%, and 75%, respectively, for the young group (<60 years of age) and 93%, 84%, and 69% for the older group [2]. Graft vasculopathy, a unique form of accelerated coronary artery disease, is a major cause of late graft failure [3]. The disease is characterized by intimal thickening mainly due to smooth muscle cell proliferation and fibrosis. Occlusive narrowing of the coronary vessels can develop within a few months and is not prevented by current treatments.

The pathogenesis of graft vasculopathy is complex and has been reviewed elsewhere [4–6]. The observation that, while graft coronary arteries develop lesions, the host's native arteries are spared suggests a major pathogenic role for immune rejection. Consistent with this, while hearts transplanted into a genetically different recipient are affected, those placed back in the original donor strain are spared [7]. Clinical data support a major role for chronic rejection in the development of graft vasculopathy and graft failure. Indeed, the degree of donor-recipient human leukocyte antigen (HLA) matching correlates significantly with graft survival [8–10]. Moreover, acute cellular rejection has been associated with an increased risk of developing graft vasculopathy [11–14].

Both the innate [15] and the adaptive immune system including B cells and antibody formation against graft antigens [16] play central roles in the development of graft

vasculopathy. Nonimmunological factors such as dyslipidemia, hypertension, drug toxicity, and infections also play contributory roles. Accordingly, the current paradigm is that graft vasculopathy results from repeated immune and nonimmune-mediated insults to graft coronary endothelium leading to endothelial inflammation and dysfunction, vascular cell proliferation, fibrosis, and intimal thickening.

Extended cold ischemic times during heart transplantation have been associated with increased risk of developing graft vasculopathy and failure both in animal models [17, 18] and in humans [19]. Moreover, prolonged times between donor brain death and organ retrieval have been associated with increased mortality in cardiac transplant recipients [20]. Graft coronary microvascular dysfunction after ischemia and reperfusion can culminate in primary graft failure or untreatable chronic rejection [21].

Cold ischemia stimulates the expression of inflammatory mediators acting as “danger signals” and amplifying tissue injury and graft rejection. Toll-like receptors (TLRs) play a central role in this regard [22]. Consistent with this, systemic administration of anti-TLR-2 antibody reduces neutrophil, macrophage, and T-lymphocyte infiltration in mouse hearts after ischemia and reperfusion [23]. Multiple strategies applied at the time of organ transplantation have a potential for limiting cold ischemic organ damage, reperfusion injury, and graft immunogenicity [24, 25].

2. Myocardial Ischemia/Reperfusion (IR) Injury

Early observations in animal models of myocardial infarction indicated that ischemic cell death progresses as a “wavefront” phenomenon correlated to the duration of ischemia [26], and that early reperfusion can salvage reversibly injured ischemic myocardium [27]. Subsequently, morphological changes appearing during reperfusion, including cardiomyocyte swelling and loss of sarcomeric organization, were recognized [28]. Moreover, interventions applied at the onset of reperfusion were still able to limit infarct size, suggesting a contributory role for reperfusion in lethal cell injury.

A comprehensive discussion of the molecular mechanisms of myocardial IR injury is beyond the scope of the present paper. These mechanisms have been reviewed elsewhere [29, 30]. It is possible here to briefly mention the role of mitochondria as both a source and a target of IR injury [31, 32]. Under normoxic conditions, mitochondria use oxygen to synthesize adenosine triphosphate (ATP). Sustained hypoxia leads to ATP depletion, acidosis, intracellular calcium accumulation, mitochondrial swelling, and cell death [30]. Cold ischemia exacerbates swelling via inhibition of the Na^+/K^+ ATPase. At reperfusion, calcium is taken up into the sarcoplasmic reticulum (SR) by the SR calcium ATPase. Calcium overload then leads to calcium release into the cytosol, cardiomyocyte hypercontracture, membrane disruption, and cell death [30].

During ischemia, mitochondria produce reactive oxygen species (ROS). An extra burst of ROS generation takes place at reperfusion. ROS mediates opening of the mitochondrial permeability transition pore (MPTP) leading

to increased inner mitochondrial membrane permeability, mitochondrial depolarization, ATP depletion, mitochondrial matrix swelling, outer mitochondrial membrane rupture, cytochrome *c* release, and apoptosis [30, 32]. In addition, ROS activates multiple molecular cascades of inflammation [33]. Proinflammatory cytokines, such as IL-1 and TNF α , and chemokines are produced within hours of reperfusion in allogeneic and syngeneic grafts alike. Chemokines mediate early migration of neutrophils and macrophages into the graft [34, 35]. Early T-cell reaction precedes alloantigen priming and induces graft necrosis [36, 37]. Inflammatory activation of graft endothelium [38], platelets, the coagulation cascade, and the complement system [39] plays important roles in early graft injury and subsequent graft vasculopathy.

A multitude of intracellular signal transduction pathways are activated during myocardial IR injury [29, 30]. Among them, mitogen-activated protein kinases (MAPKs) are key regulators of cell function and survival [40, 41]. The present paper aims to discuss the role of MAPK activation in myocardial IR injury and its potential implications for heart transplantation.

3. MAPK Subfamilies

The MAPK family includes four major serine/threonine protein kinase subfamilies. Each MAPK subfamily comprises successively acting kinases including an upstream MAPK kinase kinase, a MAPK kinase, and a MAPK (Figure 1) [40]. Distinct isoforms of a MAPK bind molecules with different affinities and can activate distinct signaling pathways. In response to a variety of stress stimuli, MAPKs convey extracellular signals to their intracellular targets, thereby regulating cell survival, function, growth, and differentiation [41]. The best characterized MAPK subfamilies are c-Jun NH₂-terminal kinases (JNKs), extracellular signal-regulated kinase-1/2 (ERK1/2, also known as p42/p44 MAPK), p38 MAPKs, and the big MAPK-1 (BMK1/ERK5). The role of each MAPK subfamily in myocardial IR injury is discussed in the next sections.

3.1. ERK1/2 Activation during Myocardial IR Injury. ERK1/2 was discovered as the first member of the MAPK family in 1990 [42]. This serine/threonine protein kinase is tyrosine-phosphorylated in response to various extracellular signals. We observed a ≈ 2 -fold increase in ERK1/2-specific in vitro kinase activity in isolated-perfused adult rat hearts subjected to 20 min of ischemia followed by 15 min of reperfusion [43]. Several studies support a protective role for the MEK1-ERK2 signaling pathway against IR injury [44–47]. Accordingly, this pathway has been identified as a central component of the so-called “Reperfusion Injury Salvage Kinase” (RISK) pathway [48].

3.2. JNK Activation during Myocardial IR Injury. JNK was discovered as the second member of the MAPK family in 1991 [49]. It is primarily activated by various cellular stresses

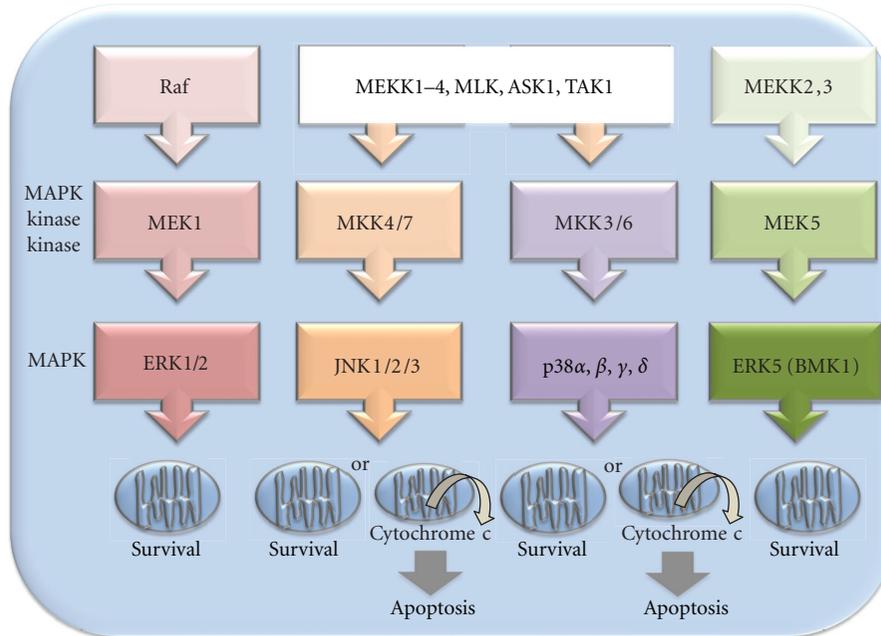


FIGURE 1: Schematic depicting the activation cascades of the four major MAPK subfamilies and corresponding effects on mitochondrial chromosome *c* release and apoptosis. ERK1/2 and ERK5/BMK1 have been associated with cell survival, whereas JNK and p38 MAPK have been predominantly associated with apoptosis.

such as heat, UV light, and cytokines. We observed a ≈ 6 -fold increase in JNK-specific in vitro kinase activity and a ≈ 2 -fold increase in phosphorylated c-Jun protein in nuclear extracts from isolated-perfused rat hearts subjected to 20 min of ischemia and 15 min of reperfusion [43]. JNK activation was increased during ischemia as well as reperfusion, in line with a limited number of previous studies [44, 50, 51]. In contrast, a larger number of studies reported JNK activation predominantly at reperfusion [52–56].

Dichotomous effects of JNK activation during IR injury including both cardioprotection [56–59] and myocardial damage [55, 60–64] have been reported. A potential mechanism of JNK-mediated protection is reactivation of Akt and enhanced cardiomyocyte survival after hypoxic injury [56]. Data in genetically modified mice show that JNK1/2 knockout mice and, paradoxically, transgenic mice overexpressing MKK7, the MAPK kinase upstream of JNK1/2, are each significantly protected from IR injury [65]. These findings illustrate the complexity of the biological effects of JNK activation.

A word of caution is warranted regarding the reliance on curcumin as a specific JNK inhibitor in early studies [66]. We therefore used a cell-penetrating peptide inhibitor of JNK, D-JNKI-1, as a more selective agent. In the isolated-perfused adult rat heart, D-JNKI-1 administered before the ischemic period selectively prevented JNK activation and improved post-ischemic cardiac function, cytochrome *c* release, caspase-3 activation, and apoptosis [43]. D-JNKI-1 administered at reperfusion failed to improve cardiac function but still prevented apoptosis. In vivo, D-JNKI-1 reduced myocardial infarct size by half after coronary artery

occlusion and reperfusion in rats [43]. D-JNKI-1 similarly reduced cerebral infarct size after common carotid artery occlusion and reperfusion in adult rats [67].

Inconsistent findings from previous studies regarding the role of JNK activation during IR injury likely reflect differences in the experimental models and JNK inhibitors used, as well as JNK isoform-specific effects. It has been shown that inhibition of JNK1 isoform, but not of JNK2 isoform, prevents apoptosis induced by IR injury in rat cardiomyocytes [61].

3.3. p38 MAPK Activation during Myocardial IR Injury. The p38 MAPK subfamily comprises 4 main isoforms, p38 α , p38 β , p38 γ , and p38 δ , of which p38 α and p38 γ are most abundantly expressed within the myocardium. The role of p38 MAPK activation during myocardial IR remains controversial [68–70]. We observed a ≈ 2 -fold increase in p38 MAPK-specific in vitro kinase activity in isolated-perfused rat hearts subjected to 20 min of ischemia and 15 min of reperfusion [43]. These results are in agreement with previous data [71]. p38 MAPK activation contributes to tissue injury induced by TNF α in response to hydrogen peroxide generated during reperfusion [33]. Moreover, p38 MAPK activation counteracts adenosine- or insulin-induced cardioprotection against IR injury [72, 73]. p38 MAPK inhibition limits infarct size and polymorphonuclear accumulation in mouse hearts subjected to IR injury [74]. Transgenic mice expressing a dominant-negative p38 α mutant or a dominant-negative mutant of MKK6, a MAPK kinase upstream of p38 MAPK, are each significantly protected from IR injury [75].

These data suggest a potential role for p38 α isoform as a mediator of myocardial IR injury.

Much of our current knowledge regarding cardioprotection comes from studies of preconditioning (PC) and postconditioning (PostC). Although a majority of these studies relate to nontransplanted hearts, they are relevant to heart transplantation.

4. Ischemic Preconditioning (IPC)

IPC was originally described as an experimental phenomenon whereby repeated episodes of brief, sublethal ischemia induced tolerance to a successive, prolonged period of lethal ischemia [30, 76, 77]. In the anesthetized dog, four 5 min periods of occlusion of the left coronary artery, interspersed with 5 min periods of rapid reflow, markedly attenuated infarct size after occlusion of the same artery for 40 min. Two distinct “windows” of IPC-mediated protection have been described [78, 79]. The first window of protection is induced within minutes, lasts for 1-2 h, is dependent on activation of MAPKs as well as of other signaling pathways, and attenuates infarct size but not contractile dysfunction nor myocardial stunning. The second window of protection takes place between 24 and 72 h after the triggering phase of IPC, requires synthesis of protective proteins within the heart, and limits cell death as well as contractile dysfunction [80]. IPC involves changes in energy metabolism, ionic homeostasis, and gene regulation as well as a decrease in ROS generation, neutrophil activation, and apoptosis [81]. Pharmacological agents such as opioids [82], inhalational anesthetics [83], adenosine, isoproterenol, and nitric oxide (NO) donors, [84] along with stress stimuli such as rapid cardiac pacing and thermal stress can precondition myocardial tissue to subsequent ischemia [30].

A comprehensive discussion of the molecular mechanisms of IPC is beyond the scope of the present paper. The interested reader is referred to recent reviews published elsewhere [30, 77, 85, 86]. It is possible here to merely mention a few molecular mechanisms. While the triggering phase of IPC requires NO and superoxide synthesis, IPC mitigates NO, superoxide, and peroxynitrite overproduction during subsequent IR [87]. Beside MAPKs, protein kinases activated by IPC include protein kinase C (PKC) isoforms [88, 89], phosphatidylinositol 3-kinase (PI3K) and its substrate kinase Akt [90, 91], receptor tyrosine kinases of the Src family [92, 93], the JAK/STAT pathway [94, 95], and glycogen synthase-3 β (GSK-3 β) [96]. The latter is a downstream kinase phosphorylated by other kinases such as ERK1/2 and Akt which has been implicated in cardioprotection including inhibition of MPTP opening at reperfusion. However, recent data suggest that decreased oxidative stress, rather than mitochondrial protein phosphorylation, is responsible for inhibition of MPTP opening in the context of IPC [97].

A number of studies have demonstrated MAPK activation during the triggering phase of IPC, at reperfusion, or both. In some cases, IPC has been associated with decreased MAPK activation during subsequent ischemia, suggesting a detrimental role for MAPK activation in this context. The

activation of the different MAPK subfamilies in preconditioned hearts is discussed in the next sections.

4.1. ERK1/2 Activation during IPC. Both in vitro and in vivo studies have demonstrated ERK1/2 activation and cardioprotection after IPC [98–101], which was abolished by an ERK1/2 inhibitor in a pig model of IR injury [99]. In addition, hypoxic PC [102, 103] as well as delayed hypoxic PC [104, 105], adenosine-induced PC [106] as well as adenosine-induced delayed PC [107], isoflurane/desflurane-induced PC [83, 108], metabolic PC [109], and opioid-induced delayed PC are associated with increased ERK1/2 activation [110]. Moreover, mitochondrial K_{ATP} channel openers activate ERK1/2 by an oxidant-dependent mechanism [111].

Several studies reported biphasic ERK1/2 activation during IPC [82, 83]. The first phase of activation takes place immediately after the PC stimulus, and the second phase of activation occurs at reperfusion. Blocking the first phase of activation prevents the second one [83]. In response to IPC, PKC ϵ induces the activation of ERK1/2 in the cytosol and its translocation to the nucleus, with increased activation of NF- κ B and AP-1 transcription factors and protection against cardiomyocyte apoptosis [101]. Another mechanism by which ERK1/2 can impart protection to hypoxic myocardium involves phosphorylation of hypoxia-inducible factor (HIF)-1 [104].

A small number of studies either reported ERK1/2 activation during IPC [90] or metabolic preconditioning [112] without a contribution of it to the observed protection, or failed to detect ERK1/2 activation during IPC [113, 114].

4.2. JNK Activation during IPC. Several studies documented increased JNK activation during the triggering phase of IPC [55, 98, 100, 101, 113, 115–117] or, less frequently, during the sustained ischemic period after the IPC stimulus [113] or during reperfusion [55, 117]. Some studies suggested a potential role for JNK as a mediator of IPC-induced protection [100, 116], but this was not confirmed by other reports [117, 118]. Decreased JNK activation was observed in preconditioned brains, kidneys, and hepatocytes [119–121], suggesting that JNK activation may contribute to IR injury in these tissues.

4.3. p38 MAPK Activation during IPC. Several studies reported increased p38 MAPK activation during the triggering phase of IPC and reperfusion [83, 113, 116, 117, 122–132]. A limited number of studies showed p38 MAPK activation during the sustained ischemic period after the IPC stimulus [133–135]. p38 MAPK activation has also been observed in hypoxic PC [136, 137] and delayed hypoxic PC [138] as well as in NO, [139], angiotensin II [140], or adenosine-induced PC [115, 141–143].

The role of p38 MAPK as a potential mediator of protection in the preconditioned heart remains controversial. A majority of studies showed p38 MAPK activation during the triggering phase of PC [85, 110, 116, 122, 123, 125, 127–129, 131–138, 140, 141, 143–145]. IPC appears to require p38 α

but not p38 β isoform activation [145]. Potential p38 MAPK-mediated protective mechanisms include phosphorylation of small heat shock protein (Hsp) 27, which stabilizes the actin cytoskeleton [146–148], and $\alpha\beta$ crystalline [124].

A distinct group of studies failed to support a contributory role for p38 MAPK activation in IPC [50, 110, 149–152], hypoxic PC [140, 153], NO-induced PC [154], delayed metabolic PC [109, 112], and opioid-induced delayed PC [110]. A third group of studies showed reduced p38 MAPK activation during the sustained ischemic period after the PC stimulus [140, 150, 152–154], suggesting a detrimental role for p38 MAPK activation in this setting. Consistent with this, numerous studies demonstrated that a p38 MAPK inhibitor applied during the sustained ischemic period can protect the myocardium against IR injury [44, 116, 125, 126, 140, 147, 149–153, 155, 156].

These inconsistent findings from different studies are difficult to reconcile; however, it should be considered that the mechanism of p38 MAPK activation can differ by circumstance [70], and that distinct p38 MAPK isoforms activate different signaling pathways. Increased p38 α isoform activation during sustained ischemia [50, 153] has been associated with cardiomyocyte apoptosis [157, 158], contractile dysfunction [158], and increased infarct size [159]. p38 MAPK has been shown to negatively regulate myocardial contractility [160–162].

4.4. IPC and BMK1/ERK5 Activation. The big MAP kinase 1 (BMK1/ERK5) pathway [163] is activated in the heart in response to IPC [164] and has been implicated as a potential mediator of cardioprotection [165]. BMK1/ERK5-induced phosphorylation of the mitochondrial protein BAD has been shown to attenuate endothelial cell and cardiomyocyte apoptosis [166–168]. Similarly, BMK1/ERK5 activation during cerebral IPC prevents apoptosis in the ischemic rat hippocampal CA1 region [169].

5. Remote Preconditioning (RPC)

RPC is a biological mechanism of interorgan protection against IR injury [170, 171]. Brief cycles of IR applied to a tissue remote from the heart, such as the small intestine [172] or the upper or lower limb [173], before the onset of myocardial ischemia limit myocardial infarct size. A comparison of RPC and IPC induced by occlusion of the superior mesenteric artery and the left coronary artery, respectively, in a rat model of myocardial IR injury showed a greater effect of IPC compared with RPC in terms of infarct size reduction [174]. In this study, IPC was associated with increased ERK1/2 and JNK1 activation but reduced p38 MAPK activation in the heart. In contrast, RPC triggered by occlusion of the superior mesenteric artery induced ERK1/2 and JNK1 activation in the small intestine without participation of MAPKs in the heart. Each of the applied ERK1/2, JNK, and p38 MAPK inhibitors abrogated RPC-mediated protection. An underlying mechanism may be PKC ϵ isoform activation in the heart via remote ischemia-induced transmitter release [175]. A distinct study showed

equivalent degrees of cardioprotection induced by IPC and RPC, while suggesting a role for bradykinin as a mediator of cardiac PC at a distance [176].

6. Postconditioning (PostC)

Ischemic PostC can be elicited by repetitive cycles of rapid reflow/reocclusion in the initial 2 min after release of a protracted coronary occlusion [29, 30, 177–181]. Because tissue injury is initiated within minutes of reperfusion, PostC must be applied at the onset of reperfusion [181]. PostC has limited infarct size in all species tested so far [177, 178, 182–184], including humans [185, 186]. The degree of PostC-mediated cardioprotection is comparable to that induced by IPC [177, 178, 186], or slightly lower than it [187]. PostC activates adenosine receptors and the NO/cGMP pathway [188, 189], mitochondrial K_{ATP} channels, PKC and protein kinase G (PKG) [190], and the RISK pathway including ERK1/2 [188] and PI3K/Akt [184, 189, 191]. In the rabbit model of myocardial IR injury, an ERK1/2 inhibitor abolished protection by brief episodes of coronary occlusion applied at reperfusion [188]. PostC has also been shown to reduce oxidative stress in a senescent mouse model [192] and to attenuate cardiomyocyte apoptosis after simulated ischemia via JNK and p38 MAPK inhibition [193]. Moreover, PostC has been shown to inhibit MPTP opening in the early minutes of reperfusion [194].

The RISK pathway is not the only cardioprotective pathway [195]. In mouse and rabbit hearts, protection after ischemic PostC was associated with increased activation of ERK, but not Akt [183, 196]. In pigs, ischemic PostC enhanced ERK and Akt activation during reperfusion without a decrease in infarct size [197]. A distinct study in anesthetized pigs demonstrated myocardial protection after PostC without an increase in Akt, ERK, and GSK-3 β phosphorylation and with no effect of PI3K or ERK1/2 blockade [198]. Gentle reperfusion likewise reduced infarct size in pigs without activation of the RISK pathway [199]. The so-called “Survivor Activating Factor Enhancement” (SAFE) pathway [200] which includes the JAK-STAT signaling pathway [94, 95], may be responsible for cardioprotection in the absence of activation of the RISK pathway.

Pharmacological stimuli including inhalational anesthetics can replace the ischemic PostC stimulus applied at the onset of reperfusion [201–203]. While myocardial protection after ischemic PostC is not enhanced by IPC [187], pharmacological PostC and IPC or pharmacological PC may have additive effects.

7. IPC, RPC, and PostC for Protection against Myocardial IR Injury in Humans

Recently, IPC, RPC, and PostC strategies for attenuating myocardial IR injury have been tested in clinical trials in nontransplanted patients [77]. Both IPC and pharmacological PC reduced myocardial IR injury in patients undergoing coronary artery bypass graft surgery [204–207]. In a randomized controlled trial, RPC triggered by a

simple noninvasive technique of four 5 min cycles of lower limb ischemia and reperfusion induced cardioprotection in children undergoing cardiac surgery for congenital heart disease [208]. In a distinct randomized controlled trial, RPC triggered by transient upper limb ischemia induced cardioprotection in adult patients undergoing coronary artery bypass graft surgery [209]. In the prospective randomized controlled cardiac remote ischemic preconditioning in coronary stenting (CRISP Stent) trial, RPC alleviated ischemic chest discomfort and myocardial injury during coronary stenting, while also reducing subsequent cardiovascular events [210]. In a randomised trial in patients with acute myocardial infarction undergoing angioplasty, ischemic RPC before hospital admission proved to be safe and appeared to salvage ischemic myocardium [211].

Ischemic PostC has been evaluated in patients with ST elevation myocardial infarction (STEMI) undergoing angioplasty [185]. Within the first minute after stent implantation, patients in the PostC group underwent four cycles of 1 min inflation and 1 min deflation of the coronary angioplasty balloon. Creatine kinase release, measured as a surrogate for infarct size, was significantly reduced by 36% in PostC versus control patients. Contractile function was still improved in the PostC group at 1 year following infarct [212]. Whether or not PostC protects against endothelial IR injury in humans remains unclear [213, 214].

To our knowledge, no data on IPC, RPC, or PostC in human heart transplantation have been published so far. Analogously, data on MAPK inhibitors in this setting are restricted to animal models, as discussed in the next section.

8. MAPK Inhibition in Experimental Heart Transplantation

ERK1/2, JNK, and p38 MAPK activation within cardiac grafts has been demonstrated in dogs [215]. MAPK activation can contribute to graft injury via multiple mechanisms including cytokine upregulation [216–219], immune cell activation, and apoptosis.

JNK promotes T-cell activation and differentiation. For instance, JNK and ERK1/2 have been shown to stimulate IL-2 production by Thy-1-activated mouse T lymphocytes *in vitro* [220]. JNK inhibition reduced histological rejection and improved graft survival in a rat model of heart transplantation [221].

p38 MAPK is involved in IL-2R signaling in T lymphocytes, while also stimulating cytokine release from human macrophages *in vitro* [222]. A p38 MAPK inhibitor administered at reperfusion improved functional recovery of rat hearts after prolonged hypothermic ischemia [223]. In a brain-dead donor model, a p38 MAPK inhibitor lowered systemic levels of proinflammatory cytokines while not affecting intracardiac cytokine levels [224]. Addition of a p38 MAPK inhibitor to the Celsior solution enhanced the viability of cardiac grafts from non-heart-beating donors in a canine model of heart transplantation [225]. Moreover, p38 MAPK blockade attenuated the release of proinflammatory IL-6 by human endothelial cells *in vitro* after cooling and rewarming

[226]. p38 MAPK inhibition similarly prevented endothelial adhesion molecule expression and polymorphonuclear accumulation after myocardial IR injury in rats [74]. p38 MAPK blockade markedly reduced vascular smooth muscle cell proliferation in aortic grafts and the development of graft vasculopathy [227]. Finally, addition of a p38 MAPK inhibitor to the Euro-Collins and University of Wisconsin solutions mitigated IR injury in lung [228] and liver [229] grafts, respectively, as well as in kidney grafts from non-heart-beating donors [230]. Thus, a p38 MAPK inhibitor applied during organ procurement and storage can protect the graft against IR injury.

9. PC and PostC in Experimental Heart Transplantation

The potential relevance of PC and PostC strategies to organ transplantation has been reviewed elsewhere [231–233]. Proof-of-principle studies in animal models have demonstrated that IPC can impart protection on cardiac grafts [234–236]. Pretreatment of rat hearts with an adenosine analog prior to harvesting and storage in the Euro-Collins solution for 8 hours improved functional recovery at reperfusion [237]. In another study, IPC combined with Na⁺/H⁺ antiporter inhibition improved cardiac function in rat hearts after 4 hours of storage at 4°C in Celsior solution and extracorporeal reperfusion [238]. K_{ATP} channel activation mimicked the protective effect of IPC in hearts after prolonged hypothermic storage [239–241]. However, one study showed IPC-induced cardioprotection after global ischemia, but not after cold cardioplegia [242]. Also, brain death completely abolished PC-mediated protection in ischemic rabbit hearts [243]. This finding might be explained by catecholamine storm after brain death, since norepinephrine injection before IPC abolished protection in the absence of brain death [244]. AMP-activated protein kinase (AMPK) is emerging as a target for PC in transplantation medicine [245].

PC induced by sildenafil administration to the donor 30 min before the onset of ischemia improved the function of cardiac grafts after 3 h of hypothermic cardioplegic arrest [246]. In contrast, PostC induced by sildenafil administration 5 min before reperfusion in the recipient was ineffective.

PKC δ inhibition improved cardiac contractile performance and coronary perfusion after cold cardioplegic arrest in isolated rat hearts [247]. This approach similarly attenuated heart transplant injury and graft coronary vasculopathy after prolonged organ ischemia [248]. Isoflurane as well as inhaled hydrogen or carbon monoxide has been shown to alter energy substrate metabolism to preserve mechanical function in isolated rat hearts after extended no-flow hypothermic storage [249, 250].

Ischemic RPC was tested in a pig model of orthotopic heart transplantation from brain-dead donors [251]. RPC of the recipient by four 5 min cycles of lower limb ischemia attenuated IR injury of the denervated donor heart via a K_{ATP} channel-dependent mechanism.

Ischemic PostC was tested in isolated working rat hearts after global total ischemia (4 h/4°C) and 45 min of reperfusion [252]. Three brief episodes of total global ischemia applied at the onset of reperfusion reduced myocardial injury and postischemic dysfunction. In another study, both PostC and remote PostC attenuated tissue damage in warm ischemic rat cardiac grafts [253].

The first clinical application of IPC in solid organ transplantation concerned liver transplantation [254]. Although IPC mitigated inflammatory responses [255], it was associated with initial poor function. It did neither improve nor compromise the outcome of cadaver liver transplantation [254].

10. Concluding Remarks and Perspectives

Proof-of-principle studies have provided evidence that therapeutic manipulation of the donor heart at the time of transplantation can mitigate graft injury, immunogenicity, and rejection. A possibility is that molecular events during the triggering phase of PC, which induce protection, can be applied to the donor heart before transplantation. A preconditioning drug (e.g., sildenafil) can be administered to the donor before organ retrieval and/or 5 min before reperfusion in the recipient [246]. The clinical efficacy of ischemic PostC in STEMI patients [185] suggests that this approach might be beneficial in heart-transplanted patients as well. A p38 MAPK inhibitor can be added to an organ preservation solution or administered at reperfusion [223, 225]. A p38 MAPK inhibitor administered to the recipient markedly inhibited the development of aortic graft vasculopathy in an experimental model [227]. Small-molecule inhibitors of p38 MAPK have been developed [256] and tested in initial clinical trials in patients with active rheumatoid arthritis or neuropathic pain [257, 258]. Further preclinical studies are needed, however, before these drugs can be tested in heart transplant recipients. In principle, extended p38 MAPK inhibitor administration during several weeks or months after transplantation might protect against graft vasculopathy.

Because distinct MAPK isoforms have different substrate affinities and functions [61, 145, 159], the precise identification of MAPK isoforms that contribute to IR injury would allow for the development of targeted therapies. Avoiding indiscriminate MAPK blockade is important because MAPK activates signaling pathways participating in host defense against infection and tumors.

Despite promising results obtained with MAPK inhibitors as well as PC and PostC in animal models, it should be noted that clinical trials of cardioprotective agents successfully tested in animal models have been largely negative so far [259]. However, a recent trial suggested a protective effect of cyclosporine, a MPTP opening inhibitor, against reperfusion injury in patients with acute myocardial infarction [260]. In transplantation medicine, MAPK inhibitors will need to be tested in combination with other PC and PostC strategies, as well as with improved organ preservation solutions and

reperfusion protocols (e.g., continuous myocardial perfusion and controlled initial reperfusion) [261–263].

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Review Article

Ischemia-Reperfusion Injury and Ischemic-Type Biliary Lesions following Liver Transplantation

Raffaele Cursio^{1,2} and Jean Gugenheim¹

¹ *Service de Chirurgie et Transplantation Hépatique, Hôpital P'Archet 2, Centre Hospitalier Universitaire de Nice, Université de Nice Sophia-Antipolis, 151 Route Saint Antoine de Ginestière, B.P. 3079, 06202 Nice Cedex 2, France*

² *INSERM U526, Equipe 2: Cell Death, Differentiation and Cancer, Centre Méditerranéen de Médecine Moléculaire, Bâtiment Universitaire ARCHIMED, Université de Nice Sophia-Antipolis, 151 Route Saint Antoine de Ginestière, B.P. 2 3194, 06204 Nice Cedex 3, France*

Correspondence should be addressed to Raffaele Cursio, cursio@unice.fr

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Ischemia-reperfusion (I-R) injury after liver transplantation (LT) induces intra- and/or extrahepatic nonanastomotic ischemic-type biliary lesions (ITBLs). Subsequent bile duct stricture is a significant cause of morbidity and even mortality in patients who underwent LT. Although the pathogenesis of ITBLs is multifactorial, there are three main interconnected mechanisms responsible for their formation: cold and warm I-R injury, injury induced by cytotoxic bile salts, and immunological-mediated injury. Cold and warm ischemic insult can induce direct injury to the cholangiocytes and/or damage to the arterioles of the peribiliary vascular plexus, which in turn leads to apoptosis and necrosis of the cholangiocytes. Liver grafts from suboptimal or extended-criteria donors are more susceptible to cold and warm I-R injury and develop more easily ITBLs than normal livers. This paper, focusing on liver I-R injury, reviews the risk factors and mechanisms leading to ITBLs following LT.

1. Introduction

After liver transplantation (LT), the incidence of biliary complications, which include a wide spectrum of functional and anatomical abnormalities varies from 10 to 30% [1–3]. These biliary complications lead to an increase of graft dysfunction and patient morbidity and in some cases even to graft loss [4] and retransplantation [5]. They are associated with an increased mortality rate (8 to 15%) [6].

Liver ischemia-reperfusion (I-R) injury during transplantation occurs at different periods [7]. The first, after liver explantation from the donor and storage on ice at 0° to 4°C, is a variable but generally long period of cold ischemia. The time of vascular anastomosis, when the liver is removed from ice until its implantation in the recipient, represents the second, relatively shorter period of warm I-R injury. In this period of ischemia, the liver warms slowly up to a temperature of 12.5°C during the realization of suprahepatic cava and portal vein anastomoses, and to a temperature of 34°C, once hepatic artery anastomosis is performed [8]. Now

the liver is fully revascularized and graft temperature stabilizes. Normothermic reperfusion of the implanted liver with the recipient's blood at 37°C delineates the third period.

Liver ischemia-reperfusion injury following LT causes up to 10% of early transplant failures and can lead to acute and chronic rejection [9]. Moreover, liver I-R injury is associated with intra- and/or extrahepatic nonanastomotic biliary strictures following liver transplantation [4, 10–13].

The ischemic injury itself, a localized process of cellular metabolic disturbances, results from glycogen consumption, lack of oxygen supply and adenosine triphosphate (ATP) depletion [14]. Reperfusion, which consists of initial phase injury (within 2 h after reperfusion) and late phase injury (6–48 hours after reperfusion), aggravates the cellular injuries caused by the ischemic period [9, 15–17].

Although all types of ischemia share common mechanisms cold ischemia of the liver is characterized mainly by injury to sinusoidal lining cells and disruption of the microcirculation, whereas warm ischemia results primarily in

Kupffer cell (KC)-derived cytotoxic molecule-mediated hepatocellular injury [17–19].

Liver I-R injury during transplantation involves necessarily the peribiliary plexus resulting in endothelial cell activation, which triggers a cascade of events leading to microvascular thrombosis, microcirculatory disturbances and again ischemia [10, 20]. Stricture formation, biliary apoptosis, necrosis, and cholangitis are the results and may even lead to progressive graft failure. Indeed, it seems that cholangiocytes are more sensible to the ischemic insult than the liver parenchyma [10].

2. Anatomy and Blood Supply of the Biliary System

The human biliary system is divided into extrahepatic and intrahepatic bile ducts and is lined by biliary epithelial cells (or cholangiocytes). The classical extrahepatic biliary anatomy consists of a right and left hepatic duct draining the right and left liver lobes, respectively [21–23]. The fusion of the right and left hepatic ducts gives rise to the common hepatic duct (choledochus) [21–23]. The intrahepatic bile ducts are further sub-divided into large and small bile ducts [24–26]. They represent that part of the biliary tree proximal to the confluence of the hepatic ducts [27] extending from the canals of Hering to the large extrahepatic ducts [24–26]. Small ductules that are lined by 4–5 cholangiocytes have a basement membrane, tight junctions between cells, and microvilli projecting into the bile duct lumen [25]. In larger bile ducts cholangiocytes too are progressively larger and more columnar in shape. Ten to twelve cholangiocytes line a larger bile duct [28, 29]. The vascular plexus of the biliary system is composed of branches arising directly from the right and left hepatic arteries (and accessory hepatic arteries when present) and their segmental branches and indirectly from the gastroduodenal artery via the arteries supplying the common bile duct [21–23]. This peribiliary vascular plexus is arranged around the extra- and intrahepatic biliary tree in normal liver [25]. The peribiliary vascular plexus delivers blood to the sinusoids both through lobular branches and through peribiliary branches into the portal vein [25]. In very small portal spaces a small capillary, representing the terminal branches of the hepatic artery, can continue the course of the arteriole and eventually run into the sinusoids [25]. In large portal spaces, the peribiliary vascular plexus is an anastomotic network between short collateral arterioles arising from the same arterial branches. Since the blood flows in the opposite direction to the bile (from the large towards the small vessels), the peribiliary vascular plexus presents a countercurrent stream [25, 30]. The veins draining the surface of the bile ducts follow closely the arterial plexus and drain into the marginal veins. The marginal veins end in subcapsular capillaries related to the hilum of the liver [31].

3. Classification of Biliary Complications Following LT

Bile duct strictures following LT have been classified as anastomotic strictures (ASs) and nonanastomotic biliary

strictures (NAS) [32]. ASs are isolated strictures at the site of the bile duct anastomosis (choledochocholedochostomy or choledochojejunostomy reconstruction), while NASs concern strictures located in both, the extrahepatic and intrahepatic biliary system of the liver graft [32]. NASs occur after hepatic artery thrombosis (HAT), but also with an open hepatic artery [32]. NASs with an open hepatic artery represent a separate entity and are generally referred to as ischemic-type biliary lesions (ITBLs) [32, 33]. ITBLs were also termed as “ischemic-type biliary complications or ITBC” [3], “ischemic cholangitis” [34] and “ischemic cholangiopathy” [35]. NAS were subclassified according to their etiology: (a) NAS secondary to microangiopathic injury (hepatic arterial thrombosis or stenosis), (b) ITBLs secondary to microangiopathic injury (preservation injury, prolonged cold and warm ischemia times, donation after cardiac death, and prolonged use of dopamine in the donor), and (c) ITBLs secondary to immunogenetic injury (ABO incompatibility, rejection, autoimmune hepatic disease, CMV infection, and chemokines polymorphisms) [36].

4. Incidence and Risk Factors of ITBLs Following LT

The incidence of ITBLs following LT is 5–15% [33]. This great variability may be partially due to the different definitions used for ITBLs. Although most ITBLs secondary to ischemic lesions occur within 1 year after the transplantation, their prevalence continues to increase with time after liver transplantation [5]. ITBLs appearing more than 1 year after transplantation are mainly related to immunological causes [5].

Risk factors involved in the development of ITBLs are old donor age [36, 37], prolonged cold and warm ischemia times [4, 10–12], non-heart-beating donors (NHBD) [38, 39], graft steatosis [40, 41], some graft perfusion methods [42], high viscosity of cold storage solutions [43, 44], prolonged use of dopamine in the donor [45], and posttransplant liver cytolysis and cholestasis due to I-R injury [46, 47]. In liver transplantation, the increasing gap between the number of patients awaiting an organ and the number of available organs has led to the use of extended-criteria donor (ECD) organs, including organs, which present risk factors mentioned above [48]. As livers from suboptimal donors or ECD are more susceptible to I-R and preservation injury, primary nonfunction (PNF), initial poor graft function (IPGF), delayed graft function (DFG), and also ITBLs are more frequent in these organs [4, 12, 37, 38, 49, 50].

5. Pathomechanisms of ITBLs Following LT

ITBLs following liver transplantation result in bile duct destruction and subsequent stricture formation; even a case of sequestration of the bile duct has been described [51]. As shown in Table 1, there are three main interconnected mechanisms causing ITBLs after LT: cold and warm I-R injury [4, 13, 39, 44, 46, 52], injury induced by cytotoxic bile salts [53–57], and immunological-mediated injury [4, 58–76].

TABLE 1: Pathomechanisms leading to ITBLs after liver transplantation.

Type of injury
Ischemia reperfusion related
(i) Warm ischemia in the donor
(ii) Prolonged cold ischemia time
(iii) Reperfusion injury
(iv) High viscosity of cold preservation solutions
(v) Warm ischemia during graft implantation
(vi) Microcirculatory disturbances in the peribiliary capillary plexus
Bile salts related
(i) Cytoprotective hydrophilic bile salts (decreased after liver transplantation)
(ii) Cytotoxic hydrophobic bile salts (accumulated after liver transplantation)
Insufficient flush out of bile from the bile ducts during liver transplantation
High biliary bile salt/phospholipid ratio after liver transplantation
Impaired vectorial bile duct secretion with intracellular accumulation of bile salts in cholangiocytes
Impaired biliary secretion of the protecting cholangiocyte factor HCO ₃ ⁻
Immune mediated
(i) ABO-incompatible liver transplantation
(ii) Acute rejection
(iii) Chronic rejection
(iv) Gender (female liver transplanted in male recipient)
(v) Cytomegalovirus (CMV) infection in the graft
(vi) Chemokine polymorphism in graft recipients (CC receptor 5 delta 32)
(vii) Preexisting autoimmune disease of the graft
Primary sclerosing cholangitis
Autoimmune hepatitis
(viii) Metalloproteinase (MMP) polymorphism in donor and recipient graft
MM P-2 genotype polymorphism

6. Cold and Warm I-R Injury and ITBLs Following LT (Figures 1 and 2)

Cold ischemic storage of the liver graft and its reperfusion produces injury to the biliary epithelium [2, 13] and is strongly associated with the development of biliary strictures including ITBLs [77].

As Kupffer cells are situated within the lumen of the sinusoid, they are in direct contact with the endothelial surface. From this position when activated by I-R, they release ROS, proinflammatory cytokines, such as tumor necrosis factor alpha (TNF α) and oxidants into the circulation as well as directing them to the endothelial layer and the underlying hepatocytes [78]. Although ROSs are essential to cell life at physiological levels, when overproduced they may be

responsible of IPGF after LT [79]. Increased production of ROS is associated to reduced basal levels of intracellular glutathione, a principal nonprotein thiol responsible for maintaining intracellular redox status and protecting cells against oxidative injury [80]. Glutathione has an important role in the prevention of cellular ischemia-related oxidative injury during liver preservation by reducing biliary tract cell ROS production [10]. Glutathione present in bile may prevent cholangiocyte injury by counteracting the cytotoxic effects of ROS within the biliary tract [2, 10]. Glutathione is also one of the major determinants of bile acid-independent bile flow [81]. In animals, impaired biliary excretion of glutathione may contribute to the decreased bile flow after cold ischemia [82]. Decreased biliary glutathione excretion is due to impaired transport across the canalicular membrane [82], but also to increased intrabiliary degradation by solubilized γ -glutamyltranspeptidase (GGT) [83]. The resulting lower biliary glutathione concentrations diminish the resistance of the cholangiocytes to oxidative stress provoked by I-R [83, 84] and induce cholangiocyte apoptosis [84] through the loss of the antiapoptotic protein B-cell CLL/lymphoma 2 protein (Bcl-2) [85]. Thus, glutathione depletion might explain the intense injury of bile ducts seen in LT [10, 11, 84, 86].

The late or subacute phase of I-R injury is a polymorphonuclear (PMN) leukocyte-dependent process in which the above described ROS generation is associated to cytokine and chemokine expression [78, 87, 88].

The epithelial-lining cells of the biliary system are not only exposed to proinflammatory mediators deriving from intrahepatic sources, but also to those deriving from extrahepatic sources via arterial circulation [89]. These inflammatory mediators promote the invasion of PMNs into the interstitium via the upregulation of adhesion molecules and formation of chemotactic agents [87, 90]. PMNs can penetrate the ductal basal membrane and thus contribute to bile duct injury [91]. Ductal cells are desquamated to the lumen of the bile duct and, together with PMNs, they appear in bile during the first few days after LT [92–94]. There is a clear relationship between postreperfusion hepatic biopsy findings (the degree of PMN infiltration and hepatocellular necrosis of the graft) and biliary complications after liver transplantation, including ITBLs [77].

PMNs and platelets synergistically exacerbate sinusoidal endothelial cell injury by induction of apoptosis during reperfusion. During cell anoxia, cholangiocytes are significantly more resistant to cell death than hepatocytes [10]. This is inverted after reoxygenation of the anoxic cells (which mimics tissue reperfusion), when hepatocytes are more resistant to cell death than cholangiocytes. The rate of ROS formation by cholangiocytes during reoxygenation is greater than in hepatocytes at this moment with concomitant low basal levels of the antioxidant glutathione in cholangiocytes [10]. These findings suggest that bile duct injury after LT is mainly caused during the reperfusion period [10]. Liver reoxygenation upregulates other apoptotic receptor expression than Fas and enhances apoptosis in human biliary epithelial cells [20]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) which binds TRAIL receptor1/death receptor 4 (DR4) and TRAIL receptor2/death

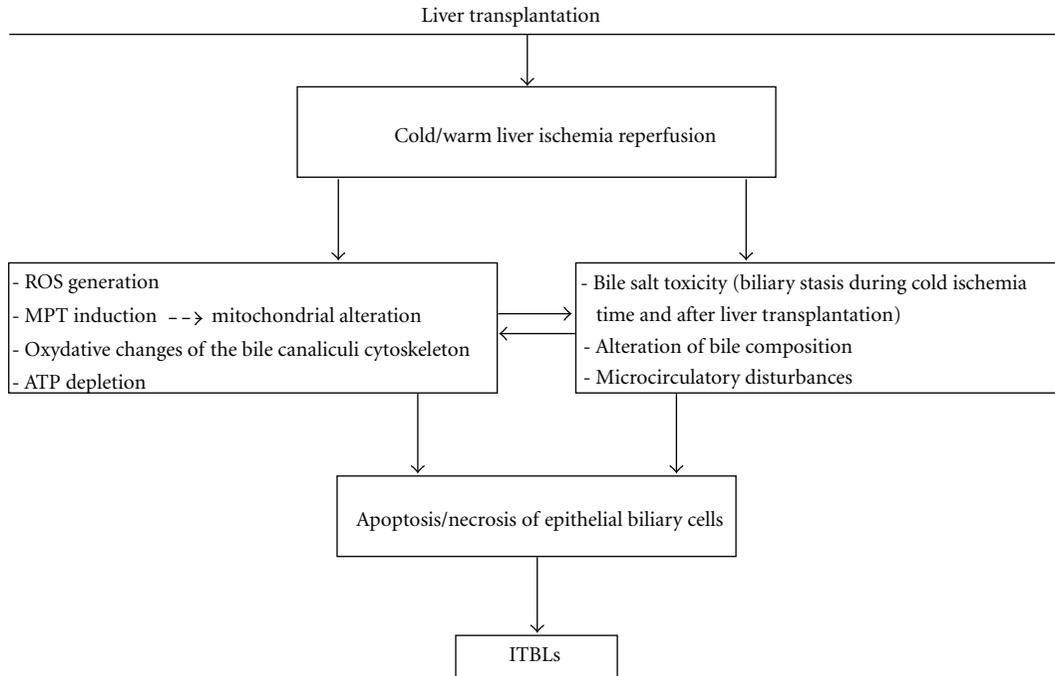


FIGURE 1: Cold and warm liver I-R leading to ITBLs following LT. ROS: reactive oxygen species; MPT: mitochondrial permeability transition; ATP: adenosine triphosphate.

receptor 5 (DR5) membrane death receptors can activate apoptosis [95]. Reoxygenation up-regulates DR4 and DR5 expression and enhances TRAIL-mediated apoptosis in human intrahepatic biliary epithelial cells [20].

Human bile epithelial cells [20] do not normally express DR5 [96], but during reoxygenation an even increased DR5 expression of cholangiocytes can be observed [20]. Reoxygenation increases also the activity of caspase-8 and caspase-3 in a TRAIL-dependent manner [20]. Some studies demonstrated an association of a longer warm ischemia time and a marked cholangiocyte apoptosis [20, 97]. Cholangiocyte apoptosis after cold and warm liver I-R is at least partly involved in the pathogenesis of ITBLs after LT [86].

7. Intrahepatic Cholestasis and Pathological Effects of Bile Salts Following LT

7.1. Intrahepatic Cholestasis. Bile formation requires the coordinated function of hepatocytes and intrahepatic cholangiocytes, which represent 2 to 5% of liver cells [98, 99]. Hepatocytes produce primary or hepatic bile, which percolates through the intrahepatic bile ducts. During this journey, cholangiocytes modify the bile via secretory and absorptive processes that provide additional bile water and alkalinity [100–102]. Cholestasis is an impairment of bile secretion, which results either from a functional defect at the level of hepatocytes (hepatocellular cholestasis) or from an impairment in bile secretion and flow at the level of bile ductules or ducts (ductular/ductal cholestasis) [103]. Intrahepatic cholestasis following liver transplantation is common and generally subclinical [104–107]. However, when severe, cholestasis may be associated with irreversible liver damage,

requiring retransplantation [104, 108]. One of the main causes of intrahepatic cholestasis after LT is cold and warm I-R injury [104, 109]. Under normal conditions, bile production requires an active vectorial secretion of biliary constituents from portal blood plasma into bile canaliculi [110]. An intact cytoskeleton is required for bile canalicular contraction, which is based on a pericanalicular web of contractile proteins, actin microfilaments, and cytokeratin intermediate filaments [111] acting as a pump to facilitate bile flow into the intrahepatic canalicular system [112, 113]. The bile canaliculus is one of the liver structures that is early damaged by I-R [105]. This oxidative stress-dependent structural damage contributes to perturbate the bile acid transport during ischemia. The resulting loss of microvilli and the canalicular atony, decrease the bile flow and lead to cholestasis [105–107]. The impairment of bile canaliculi structure following I-R, and postreperfusion biliary complications observed in patients undergoing LT, may be due to an altered reassociation of Ras GTPase-activating-like protein IQGAP1, a regulator molecule of bile canaliculi structure, with the endocytic machinery, particularly with the endocytic multimeric (AP-2) and monomeric (clathrin) adaptors (proteins that mediate the interactions between “address tickets” on cargo proteins and clathrin, as clathrin cannot bind directly to cargo or membranes) [114]. The maintenance of the hepatocyte bile secretion properties would then depend on their ability to rapidly rereform integral adherent junctions and maintain bile canaliculi structure upon reperfusion [114].

Although during 120 min of ischemia or ATP depletion, cell viability and integrity of tight junctions supported by adherent junctions in cholangiocytes were maintained, striking alterations in the secondary structure of their plasma

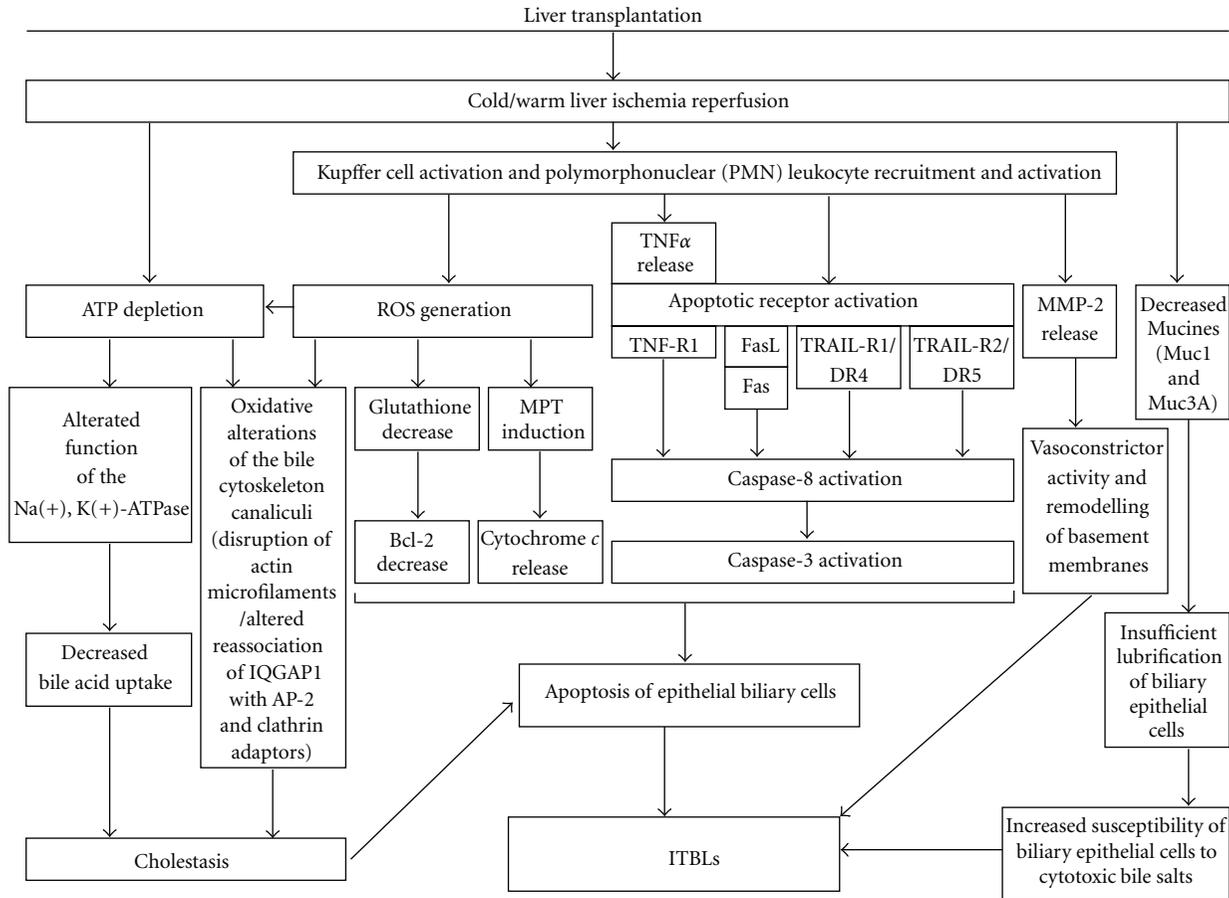


FIGURE 2: Role of Kupffer cells and PMNs in cold and warm ischemia leading to the development of ITBLs following LT. The epithelial lining of the biliary tree is exposed not only to proinflammatory mediators derived from extrahepatic sources, via arterial circulation, but also to proinflammatory mediators derived from intrahepatic sources, such as inflammatory cells or Kupffer cells. These inflammatory mediators promote the invasion of PMNs into the interstitium. PMNs then penetrate the ductal basal membrane and contribute to bile duct injury. Thus the main event injury seems to be activation of Kupffer cells and recruitment and activation of PMNs leading to apoptosis of epithelial biliary cells. PMN: polymorphonuclear neutrophils; ROS: reactive oxygen species; ATP: adenosine triphosphate; Na(+), K(+)-ATPase: sodium pump; TNF α : tumor necrosis factor alpha; TNF-R1: tumor necrosis factor receptor 1; MPT: mitochondrial permeability transition; Bcl-2: B-cell CLL/lymphoma 2 protein; IQGAP1: regulator molecule of bile canaliculi structure; AP-2: endocytic multimeric adaptor; Clathrin: endocytic monomeric adaptors; FasL: Fas ligand; TRAIL-R1/DR4: tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) which binds TRAIL-receptor1/death receptor 4 (DR4); TRAIL-R2/DR5: tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) which binds TRAIL-receptor2/death receptor 5 (DR5); Muc1: mucine 1; Muc3A: mucine 3A; MMP-2: metalloproteinase-2.

membrane, with decrease of the basolateral interdigitations and apical microvilli have been observed [115]. This reorganization of cholangiocyte membrane domains represents an early event in rat liver ischemia and contributes to impaired vectorial bile duct secretion and postischemic cholestasis [115].

During the ischemic phase failure of the sodium pump or Na(+), K(+)-ATPase [116] leads to intracellular accumulation of Na(+), edema, and swelling of Kupffer cells, sinusoidal endothelial cells, and hepatocytes [117]. Hepatocellular Na(+), K(+)-ATPase is an important driving force for bile secretion and has been localized in the basolateral plasma membrane domain [118, 119]. Bile acid uptake by the hepatocyte is a secondary active transport that is energized by the Na(+) gradient maintained by the Na(+), K(+)-ATPase. Thus, Na(+), K(+)-ATPase appears important in

coupling the energy from ATP to transport activity, resulting in so-called bile acid-dependent bile flow [120]. Decreased Na(+), K(+)-ATPase activity following cold and warm I-R results in apoptosis, necrosis, and shedding of biliary tract epithelial cells [121]. Reasons for alterations of Na(+), K(+)-ATPase activity after hypoxia and reoxygenation in the perfused rat liver [122] and after cold and warm I-R in human LT [123], may be direct alteration of the enzyme catalytic subunit and modification of its environment; ROS released from activated Kupffer cells, changes in ATP levels and in membrane lipid fluidity and ionic distribution may also contribute to Na(+), K(+)-ATPase activity disturbances [122]. Moreover, a marked delay of functional recovery in cultured biliary epithelial cells, which was provoked by ATP depletion, induced intrahepatic bile duct injury following I-R [124].

7.2. Pathological Effects of Bile Salts. Cholestasis induced by I-R injury is characterized by dilatation of bile canaliculi and loss of microvilli [105] and exposes hepatocytes and cholangiocytes to an elevated concentration of toxic bile acids [125].

Bile formation is an energy consuming process, which is regulated by specific transport proteins situated in the membrane of hepatocytes and cholangiocytes [126]. I-R can induce selective and/or temporary modification of the expression and function of some biliary transporters, leading to abnormal bile composition and to toxic injury to the cholangiocytes [110, 127], as well as to the hepatocytes [98].

The toxic bile composition early after LT, characterized by a low biliary phospholipid/bile salt ratio, is associated with histological signs of injury of the small bile ducts in the liver [51, 54, 56]. The most important apoptotic initiator in cholangiocytes is the Fas receptor/Fas ligand pathway [128]. Human cholangiocytes express Fas receptor [129]. Activated Fas receptor complexes on the plasma membrane cause caspase-8 activation and trigger apoptosis [128]. By liver I-R activated Kupffer cells can potentiate cholestatic injury through the synthesis of the proapoptotic Fas-independent receptor TRAIL [130]. Then, as in a vicious circle, during cholestasis bile acids themselves may initiate or aggravate hepatocellular damage [131]. Toxic hydrophobic bile acids retained in the hepatocytes during cholestasis initiate the generation of ROS metabolites from mitochondria, leading to lipid peroxidation and loss of cell viability [132–134]. The mitochondrial oxidative stress triggers the mitochondrial permeability transition (MPT), resulting in exaggerated mitochondrial cytochrome *c* release and apoptosis [135].

Biliary secretion of HCO_3^- prevents the uncontrolled membrane permeation of cytotoxic hydrophobic bile salts by maintaining an alkaline pH near the apical surface of hepatocytes and cholangiocytes [13, 136].

The cholangiocyte “protector” HCO_3^- secretion may be disturbed after LT, as I-R results in altered expression of the anion exchanger 2 (AE2) and of the cystic fibrosis transmembrane conductance regulator (CFTR) proteins, which regulate the biliary secretion [137].

Prolonged cold ischemia time during LT is associated to a downregulation of membrane-associated Mucine 1 (Muc1), 3A (Muc3A), and 5B (Muc5B) expression [138, 139]. Mucines are expressed on the apical membrane of the biliary epithelial cells and lubricate and protect these cells from diverse injuries, including injury by cytotoxic bile salts [140]. Decreased expression of Muc1 and Muc3A after LT may favour the development of ITBLs [138].

8. Immunologically Mediated ITBLs Following LT

8.1. ABO Incompatibility. In the past, liver transplantation across ABO blood group barriers has been discouraged because of multiple complications, particularly acute rejection and biliary complications [58]. However, organ shortage and new developed immunosuppressive agents decreasing humoral rejection have led to an increased use of ABO-incompatible liver for transplantation with acceptable results

concerning patient and graft survival rate [65, 141, 142]. Although in children, there is no obvious difference in the outcome of ABO-compatible LT and ABO-incompatible LT, in adults graft survival rate after ABO-incompatible LT is not so satisfactory [65]. Moreover, the incidence of ITBLs after ABO-incompatible LT in adults is much higher than in ABO-compatible LT [60, 65, 67].

ABO blood group antigens are expressed on both, bile duct epithelium and vascular endothelial cells [143, 144]. Donor ABH antigen expression up to 150 days after LT is associated with a high incidence of late, severe biliary strictures (82%), hepatic artery complications (24%), decreased graft survival (44%), and acute cellular rejection [60]. Persisting ABH antigen expression after ABO-incompatible LT is often the consequence of the vascular occlusion. Subsequent ischemic injuries caused by endothelial lesions increase the susceptibility to immunologic injury of biliary cells leading to ITBLs [62, 67, 145]. Preexisting primary sclerosing cholangitis and autoimmune hepatitis are also associated with a higher incidence of ITBLs [75, 76, 146].

8.2. Acute and Chronic Rejection. In liver allograft rejection, most tissue damage occurs as a consequence of direct cellular immunologic injury to the bile duct epithelium [147].

Acute cellular rejection, occurring generally within 90 days of LT, concerns 50 to 75% of liver allograft recipients [148]. The targets of activated lymphocytes are donor-derived bile duct epithelial cells and vascular endothelium [147]. Acute rejection is associated with lymphocytic cholangitis, a cytotoxic T-cell-mediated nonsuppurative destructive cholangitis of the small intrahepatic bile ducts that can induce cholestasis [147, 149]. Activated Kupffer cells migrate into the rejecting liver and release cytokines, resulting in the loss of the Na^+ , K^+ -ATPase activity, which plays an important role in bile secretion [119]. Tauroursodeoxycholic acid (TUDCA), a hydrophilic bile acid, can protect from cholestatic and hepatocellular injury by enhancing the secretory capacity of the cholestatic liver cells and by its cytoprotective action against hydrophobic bile salts [150–152]. During allograft rejection, the loss of Na^+ , K^+ -ATPase activity, a cotransporter for hepatocyte taurocholate uptake, leads to impaired secretion of TUDCA and results in subsequent cholestatic injury [119]. In patients presenting ITBLs following LT, the response of lymphocyte T helper 1 (Th1) was decreased, while the response of Th2 was increased [153]. Whether these immunological changes were induced by the damage of the bile ducts or occur as an additional damaging factor or are found as an epiphenomenon in patients with liver transplant dysfunction remains unclear.

Although some studies did not show that chronic graft rejection was a risk factor for development of ITBLs following LT [154, 155], others demonstrated the association of chronic allograft rejection and development of ITBLs [33, 46, 60, 66–68]. Chronic allograft rejection after LT also termed “ductopenic rejection”, is characterized by ischemic injury and paucity of bile ducts [148]. It occurs within the first year after LT with an incidence rate of 2 to 5% [156]. The zone of the central venous drainage or zone 3 of liver parenchyma is poorest in oxygen concentration and more sensitive

to ischemia. During cellular chronic rejection, progressive intimal and subintimal infiltration of second- and third-order branches of the hepatic artery with foam macrophages accompanied by foam cells or obliterative arteritis can result in arterial stenoses and ultimately in ischemic injury to interlobular bile ducts and hepatocytes of zone 3 [104, 157]. However, in the early stages of chronic rejection, the necroinflammatory lesions of interlobular bile ducts and hepatocytes, frequently associated with typical portal inflammatory infiltrates of acute cellular rejection, seem more likely to be the results of direct immune-mediated mechanisms [158].

8.3. Gender. Gender-mismatched liver transplant recipients had a higher likelihood of graft failure when compared with gender-matched liver transplant recipients [74]. In male recipients receiving female donor organs there is an increased risk of graft failure compared with a female recipient receiving a liver from a male donor [74]. Moreover a female to male donor/recipient match is associated with late occurrence of ITBLs [76].

8.4. Cytomegalovirus (CMV) Infection. The overall rate of CMV infection in liver transplant recipients varies from 30 to 50% [159, 160]. In CMV-infected rat liver allografts undergoing acute rejection there was a significant increase in portal inflammation and more severe bile duct injury compared with CMV-negative liver allografts [161]. In transplanted patients developing ITBLs during CMV infection, histological examination of specimens from bile duct strictures showed CMV inclusions [71]. CMV infection induces injury of endothelial cells of the peribiliary capillary plexus, with subsequent microthrombi formation and insufficient oxygenation of the biliary epithelium, ultimately leading to ischemic injury of bile duct cells and development of ITBLs [13, 72]. CMV infection can damage the bile duct cells in a direct manner by infecting directly biliary epithelial cells and in an indirect manner by immune attack evoked against infected biliary epithelial cells [13].

8.5. Chemokine Polymorphism CCR5 Delta 32. Loss-of-function mutation in the CC chemokine receptor 5 (CCR5delta32) has been associated with development of ITBLs following LT [61]. Functional changes in the immune system resulting from CCR-5 delta 32 mutation, which include impaired chemotaxis of regulatory T cells to the site of injury may be responsible [162]. Indeed, a greater risk of developing ITBLs after LT was observed in liver transplant recipients carrying CCR5-delta 32 polymorphism compared with CCR5 wild-type transplant recipients [13, 61, 73].

8.6. Metalloproteinase-2 (MMP-2) Polymorphism in Liver Graft Donor and Recipient. A large family of proteolytic enzymes involved in the degradation of extracellular matrix called matrix metalloproteinases (MMPs), secreted by Kupffer and stellate cells [163], are involved in the mechanisms of neutrophil infiltration and in the alteration of liver microcirculation due to the loss of the normal sinusoidal extracellular matrix following cold [164] and warm [165, 166] I-R injury.

The activation of one of these MMPs, the metalloproteinase-2 (MMP-2), also called Gelatinase-2, takes place at the cell surface, which confers to this unique MMP a pivotal role in cellular migration during processes requiring the remodelling of basement membranes, the thin extracellular matrices underlying endothelial and epithelial cells [167, 168].

MMPs are subject to complex regulation at multiple levels: gene transcription, proenzyme activation, and inhibition of activity by tissue inhibitors of matrix metalloproteinases (TIMPs) [167, 168]. At gene level, it has been demonstrated that several single nucleotide polymorphisms (SNPs) in the gene promoter regions of MMPs have an impact on the transcription rate into the cells [169, 170]. The SP C/T transition at position—1306 in the promoter of MMP-2, abolishes the single polymorphism 1 binding site and leads to decreased mRNA transcription and protein expression of MMP-2 [169, 170]. After LT, in association with the—1306 CT genotype of donor and recipient, the serum levels of MMP-2 were decreased in patients that developed ITBLs [75].

MMP-2 CT genotype in both, donor and recipient is strongly and independently related to the development of ITBLs within 4 years after LT [75]. The presence of the MMP-2 CT genotype in donor and/or recipient was found to increase the incidence of ITBLs incidence stepwise from 9% when absent, increasing to 16% when present in either donor or recipient, further increasing to 29% when present in both donor and recipient [75]. These findings indicate that a genetically determined reduced MMP-2 tissue remodelling contributes to the development of ITBLs after LT.

9. Extended Donor Criteria and ITBLs

9.1. Donor Age and ITBLs. As old livers are more susceptible to warm and cold I-R injury than young livers [117–172], donor age seems to be a relevant risk factor in the development of ITBLs after LT [4, 12, 36, 37, 76, 173].

The activation of peroxisome proliferators-activated receptor gamma (PPAR γ), which belongs to the hormone nuclear receptor superfamily [174], is significantly reduced in old mice compared to young mice [175]. During liver ischemia, its activation is suppressed [176]. In old mice, PPAR γ activation significantly improves liver I-R injury [176] by modulating inflammatory response and apoptosis [177].

Different from young livers, the initiation of apoptosis by nonparenchymal cells in older livers is increased and is driven by the enhanced release of TNF α [171]. TNF α is a cytokine mainly released by activated Kupffer cells following liver I-R [19, 178, 179]. Aging may directly affect Kupffer cells resulting in TNF α release [180, 181] and apoptosis [171]. Apoptosis is a highly regulated ATP-requiring form of cell death [182]. Despite decreased ATP levels and reduced hepatic mitochondrial function in older livers [117], apoptosis seems to be a predominant feature of liver cell death following ischemic injury in these old livers [171]. Lower ATP content in older liver does not directly affect the apoptotic cascade but facilitates the activation of apoptotic mediators and inhibits survival mechanisms [171].

9.2. Cold Ischemia Time and ITBLs. Prolonged cold ischemia time is an independent risk factor for liver preservation injury, even more so than donor age [183]. Cold graft preservation for more than 14 h has been associated with a two-fold increase in preservation injury, resulting in biliary strictures and decreased graft survival [183–185]. Accordingly, the risk of graft loss increases by 1% for each additional hour of cold ischemia [186]. Although several studies failed to show a correlation between the incidence of ITBLs and cold ischemia time [44, 68, 155, 173, 187, 188], in other studies this incidence was increased after a prolonged cold ischemia time [2, 12]. After a cold ischemia time less than 13 hours the percentage of ITBLs was 7%, whereas the percentage increased to 52% when the cold ischemia time was longer than 13 hours, and to 69% if it was longer than 15 hours [2, 12]. In a recent large retrospective study with an overall incidence of post liver transplantation ITBLs of 3.9% [12], 10 hours of cold ischemia time turned out to be the threshold that should not be exceeded in order to avoid ITBLs, [12].

9.3. NHBDs and ITBLs. In liver transplantation, the use of NHBDs has been introduced in order to expand the organ donor pool [189]. However, the addition of donor warm ischemia time to the subsequent cold preservation time and warm reperfusion injury negatively impacts graft function following LT [190].

Compared with donation after brain death (DBD), livers from NHBD inevitably sustain a period of warm ischemia from circulatory arrest until start of preservation, resulting in ischemic injury with higher risk of biliary complications including ITBLs [39]. Also the incidence of IPGE, PNF, acute and chronic rejection, and retransplantation is higher with NHBD [190–194].

In liver transplantation, the overall rate of biliary complications is 29% (range: 11%–53%) for NHBD and 17% (9%–22%) for DBD recipients [190]. The ITBL rate is 16% (8%–38%) for NHBD recipients and 3% (0%–8%) for DBD recipients [39, 52, 190, 192–195]. ITBLs occur within 30 days in NHBD and about 3 months after transplantation in DBD grafts [4].

In NHBD, low blood flow during the period of hypotension after tracheal extubation and no blood flow during the period between cardiac arrest and organ recovery result in formation of microthrombi that obstruct the capillaries and limit liver perfusion [196]. Inadequate flush of these capillaries leads to suboptimal cold preservation and subsequently to exacerbated ischemic injury [196]. In a pig NHBD model of liver transplantation, prolonged warm ischemia time resulted in a high biliary salt-to-phospholipid ratio, which contributes to the development of ITBLs [51].

9.4. Graft Steatosis and ITBLs. Steatosis of the liver is considered pathologic when the hepatic fat content, consisting mainly of triglycerides, exceeds 5% of the actual wet weight of the liver [197]. Hepatic steatosis is present in approximately 20% of liver donors, and 5–6% of cadaveric livers are discarded due to steatosis [198]. Liver steatosis is histologically classed as “macrovesicular” when the hepatocytes are distended by a single large fat vacuole that displaces

the nucleus to one side of the cell and as “microvesicular” when multiple small droplets finely are dispersed in the cytoplasm without nuclear displacement [197]. More than 30% of macrovesicular steatosis on donor liver biopsy is an independent risk factor for allograft loss at one year along with other elements of the donor risk index [199]. Early biliary complications seems to be associated with moderate macrovesicular steatosis [200, 201]. In a recent study, the time interval between portal and arterial reperfusion and macrovesicular steatosis of the graft of more than 25% revealed to be significant predictors of biliary complications [40, 41]. At Univariate analysis macrosteatosis of more of 25% of the graft is the only independent risk factor predicting biliary complications after liver transplantation [40, 41]. The increased susceptibility of the steatotic liver to I-R injury is due to the perturbation of both, blood flow microcirculation and changes in the cells [202]. Brain death of the liver donor may amplify the adverse effects of pre-existing steatosis by inducing hypotension, and reducing portal venous and hepatic microcirculation [203, 204].

10. Storage Solutions and Perfusion Methods of the Liver Graft and ITBLs

10.1. Graft Perfusion and ITBLs. Although approximately 75% of the total liver blood flow is provided by the portal vein, the hepatic artery supplies approximately 50% of the oxygen consumed by the liver in physiologic conditions [205]. There are two main methods for revascularization of the liver graft: sequential and simultaneous revascularization [42]. In the first method, sequential revascularization, the graft is first reperfused via either the portal vein or the hepatic artery (anterograde reperfusion), or via the inferior vena cava (IVC) (retrograde reperfusion) with subsequent reconstruction of the remaining vessels. In the second method, simultaneous revascularization, the graft is reperfused simultaneously via the portal vein and the hepatic artery. The sequence of graft reperfusion may be relevant for the development of ITBLs, particularly in grafts from ECD [42]. Liver transplantation standard technique involves initial blood perfusion by the portal vein to shorten the anhepatic period and graft rewarming *in situ*. In this period, the graft is exclusively perfused through the portal vein for at least 10 min until the realization of the hepatic arterial anastomosis [40]. The delay of rearterialization in sequential revascularization is associated with more pronounced microvascular disturbances and subsequent graft dysfunction [206]. Indeed rearterialization of the graft during liver transplantation causes an increased volumetric blood flow within the sinusoids called “reactive hyperemia” [207]. A long interval between portal and arterial reperfusion of the liver, in case of sequential revascularization, is associated to a higher incidence of biliary complications following DBD LT [40]. Simultaneous revascularization elicits a remarkable improvement in oxygen tension and maintenance of tissue ATP, compared to sequential revascularization [208]. The disadvantage of simultaneous revascularization is the prolongation of warm ischemia time and the anhepatic phase,

which can be detrimental to postoperative graft function and survival [184, 209].

Whether simultaneous revascularization is better than sequential revascularization remains unclear [42, 207, 210, 211].

In some retrospective studies, the incidence of ITBLs in patients who underwent simultaneous revascularization of the graft [45, 212] was lower compared to patients who had sequential revascularization [42, 211]. Particularly in a recent study, simultaneous revascularization resulted in a minor incidence of ITBLs compared to sequential revascularization (none versus 26%, resp.) [213], suggesting that simultaneous revascularization may be more suitable to protect the integrity of the intrahepatic biliary tree [213].

Retrograde perfusion of the liver graft via the vena cava, followed by antegrade sequential reperfusion of the portal vein and the hepatic artery, decreases liver I-R injury and IPGF [210]. However, on the biliary epithelium or other cells of the biliary tract retrograde reperfusion has detrimental effects with an increased risk of ITBLs [210]. Improvement in flushing the microscopic biliary vasculature and possibly preventing microvascular thrombosis in the biliary tree may be obtained by adding the high-pressure aortal perfusion technique to the main graft perfusion methods, [66] and additional arterial back-table pressure perfusion [36]. These graft perfusion methods seem to reduce the rate of ITBLs following LT [36, 66].

10.2. ITBLs: Importance of Portal Venous Blood Flow. The blood supply to the biliary tree is almost solely arterial, with no significant contribution from the portal vein in physiological conditions [31, 214, 215]. However, some support the hypothesis that the peribiliary vascular plexus is not only sustained by blood from the hepatic artery as traditionally reported, but also by blood from the portal vein [216]. The hepatic artery is in essence an end artery for the donor biliary tree, as collaterals from the lower extrahepatic biliary tree are interrupted in the process of liver procurement and transplantation. In case of hepatic artery thrombosis, new collateral vessels can form and limit additional biliary stricture formation [155]. As ITBLs occur in the absence of hepatic artery thrombosis, it has been suggested that the portal venous blood flow has an important impact on the pathogenesis of ITBLs after liver transplantation [216]. In a recent study, patients with partial portal vein thrombosis and intact hepatic arterial blood supply developed ITBLs in the hepatic segments affected by portal vein thrombosis [216]. In many cases of hepatic artery thrombosis it seems that the portal perfusion maintains hepatocytes [216]. Thus, the contribution of the portal blood flow to the biliary microcirculation is not negligible and a compromised portal venous blood supply can predispose to the development of ITBLs [216].

10.3. Static Cold Storage Solutions of the Graft and ITBLs. Liver preservation techniques do influence the graft quality [9]. Static and dynamic preservation are the two current methods of liver preservation in LT [217]. Static preservation means simple cold storage while dynamic preservation comprises hypothermic machine perfusion, normothermic

machine perfusion, and oxygen persufflation [217]. Until today, only static cold storage preservation is clinically approved for liver transplantation in humans [217].

Cold preservation injuries to the biliary tract of the donor liver were decreased by efficient flushing of the biliary tract in animals [218, 219] and in humans [220, 221]. In a recent study, an effective biliary flush reduced the effects of bile salt toxicity to the epithelium, reduced cell edema, prevented cell acidification, and provided adequate ATP precursor substances, resulting in reduction of biliary cold preservation injuries [222]. Generally, static cold storage UW solution is used for organ preservation [223], however the histidine-tryptophan-ketoglutarate (HTK) static preservation solution has started to compete with UW [224] and is now mainly used in deceased donor liver transplantation (DDLT) in Europe and North America and in living-related liver transplantation (LRLT) in Japan and Hongkong [43, 221, 225–233]. UW cold storage solution has more hepatocytoprotective effects than HTK cold storage solution [234–236], but its viscous nature may hinder an efficient flushing of the small bile duct capillaries, so residual bile can crystallize and obstruct capillary ducts, thus aggravating the cold ischemic insult to the epithelial biliary cells [237, 238]. HTK cold storage solution has the same viscosity as water and its average velocity is three times greater than UW solution under the same perfusion pressure [221]. The time of liver cooling with HTK cold storage solution is shorter and improves the perfusion of the biliary vascular plexus resulting in reduced biliary tract preservation injury [36, 220, 221, 239, 240]. Also the lack of macroaggregate formation of adenosine crystals and the absence of plastic byproducts in HTK solution, responsible for occlusion of small capillaries, which exacerbates small bile duct ischemia following reperfusion, contribute to the beneficial effects of HTK cold storage solution [237, 238]. HTK cold storage solution may be used particularly in livers with existing I-R injury, with high risk of I-R injury, or with biliary injury such as ECD organs [221, 223, 241]. A combined use of both cold storage solutions, HTK with its low viscosity and UW with its hepatocytoprotective effects, may have additional benefits for the biliary system [219, 242]. Thrombolytic agents as urokinase [243], which may help flushing the microscopic biliary vasculature, were employed to prevent microvascular thrombosis in the biliary tree [244].

As there are no standardized guidelines regarding the methods of liver graft perfusion in terms of solution type, amount of solution, route of perfusion, perfusion pressure, and the time of perfusion, adequately powered randomized clinical trials with long follow-up periods are needed to evaluate the long-term impact on warm and cold I-R injury and induction of ITBLs after LT.

11. Conclusion

The main pathomechanisms leading to ITBLs following LT are cold and warm I-R injury, stagnation of cytotoxic bile salts and changes in bile composition, and immunological mechanisms. These mechanisms are mutually connected, one inducing or reinforcing the other, that it may be difficult sometimes to settle the “culprit”. Besides, knowledge of these

mechanisms remains superficial and in the beginnings. Naturally, that goes too for the possibilities of ITBL prevention and treatment. Until sustained progressions are not made in the field of ITBL research, the only way to keep the incidence of ITBLs after LT as low as possible is to reduce as much as possible their risk factors.

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Research Article

The Hepatoprotective Effect of Sodium Nitrite on Cold Ischemia-Reperfusion Injury

Wei Li,^{1,2} Zihui Meng,^{1,2} Yuliang Liu,³ Rakesh P. Patel,⁴ and John D. Lang⁵

¹Division of Transplantation, Department of Surgery, University of Washington Medical Center, Seattle, WA 98195, USA

²Department of Hepato-Pancreatic Surgery, The Third Hospital of Bethune Medical College (China-Japan Union Hospital), Jilin University, Changchun, Jilin 130033, China

³Department of Surgery, The University of Alabama at Birmingham, Birmingham, AL 35294, USA

⁴Department of Pathology and Center for Free Radical Biology, The University of Alabama at Birmingham, Birmingham, AL 35294, USA

⁵Department of Anesthesiology & Pain Medicine, University of Washington Medical Center, P.O. Box 356540, Seattle, WA 98195, USA

Correspondence should be addressed to Wei Li, weili888@hotmail.com and John D. Lang, jdlang@uw.edu

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Liver ischemia-reperfusion injury is a major cause of primary graft non-function or initial function failure post-transplantation. In this study, we examined the effects of sodium nitrite supplementation on liver IRI in either Lactated Ringer's (LR) solution or University of Wisconsin (UW) solution. The syngeneic recipients of liver grafts were also treated with or without nitrite by intra-peritoneal injection. Liver AST and LDH release were significantly reduced in both nitrite-supplemented LR and UW preservation solutions compared to their controls. The protective effect of nitrite was more efficacious with longer cold preservation times. Liver histological examination demonstrated better preserved morphology and architecture with nitrite treatment. Hepatocellular apoptosis was significantly reduced in the nitrite-treated livers compared their controls. Moreover, liver grafts with extended cold preservation time of 12 to 24 hours demonstrated improved liver tissue histology and function post-reperfusion with either the nitrite-supplemented preservation solution or in nitrite-treated recipients. Interestingly, combined treatment of both the liver graft and recipient did not confer protection. Thus, nitrite treatment affords significant protection from cold ischemic and reperfusion injury to donor livers and improves liver graft acute function post-transplantation. The results from this study further support the potential for nitrite therapy to mitigate ischemia-reperfusion injury in solid organ transplantation.

1. Introduction

Liver ischemia-reperfusion injury (IRI) is a major cause of primary graft nonfunction or initial function failure posttransplantation, both of which can ultimately lead to acute and/or chronic rejection. Moreover, the occurrence of significant IRI in marginal liver donors serves to limit the number of organs available for transplantation. Therefore, insights into therapies targeted toward attenuating liver IRI should assist in thwarting liver graft primary nonfunction or poor function, reduce the episodes of acute and chronic graft rejection, extend the usage of marginal donors, and thus aid in reducing the donor organ shortage. Interestingly, to date, clinically translatable mechanisms of liver IR have not

yet been well characterized, resulting in a paucity of available therapies for prevention and treatment of liver IRI.

Nitric oxide (NO) is a free radical produced from L-arginine and is a versatile signaling mediator involved in a multitude of critical cellular events [1]. NO is also an important effector molecule produced by macrophage and dendritic cells (DCs) that is involved in immune regulation and host innate and adaptive immunity [2, 3]. NO has been found to attenuate liver IRI during transplantation through various mechanisms including reducing hepatocellular apoptosis and inflammatory tissue injury [4]. More recently, inhaled NO administered to human liver transplant recipients prior to transplantation and continued throughout the entire operative procedure significantly accelerated

restoration of liver function by decreasing hepatocyte apoptosis [5]. While the underlying mechanisms of inhaled NO on liver IRI are largely unknown, the possibility of the anion nitrite, an oxidative product of NO metabolism that is increased in the circulation with NO breathing, playing a role has been suggested. Consistent with this concept, sodium nitrite has been shown to limit acute IRI in both murine heart and liver of warm IR and is associated with decreased incidence of myocardial infarction and hepatocyte apoptosis [6]. Underlying this protection are biochemical pathways that couple ischemia to nitrite reduction to NO, which then can mediate cytoprotective effects by multiple possible mechanisms [7–10]. Similarly, nitrite-dependent protection has been observed in other models of ischemic tissue injury encompassing all major organ systems [11]. In this study, we critically examined the protective effects of nitrite administration on liver IRI in a cold murine IRI model using different preservation solutions. Our study demonstrates that nitrite supplementation of either Lactated Ringer's (LR) or University of Wisconsin's (UW) solutions significantly reduced liver cold IR injury and protected hepatocytes from apoptosis posttransplantation.

2. Materials and Methods

2.1. Animals. Male C57BL/10 (B10; H2^b) and C57BL/10 (C3H; H2^k) mice, 10–12 weeks of age (The Jackson Laboratory, Bar Harbor, ME), were maintained in a specific pathogen-free facility of the University of Washington Medical Center. The mice were provided with Purina rodent chow and tap water *ad libitum*. Animal care was in compliance with our institutional animal care and use of committee-approved protocol, and with the "Guide for the Care and the Use of Laboratory Animals" published by the National Institutes of Health.

2.2. Reagents. Sodium nitrite was purchased from Sigma-Aldrich (cat no. S-2252, St. Louis, MO). UW solution was obtained from Life Center Northwest (Bellevue, WA, manufactured by Duramed Pharmaceuticals, Inc, Cincinnati, OH), and LR solution was from Baxter Healthcare Corporation (Deerfield, IL). ApopTag Peroxidase In Situ Apoptosis Detection Kit was from Millipore Corporation (Billerica, MA).

2.3. Nitrite Treatment Protocol. The livers were flushed via the portal vein with 5 mL of either cold LR or UW solution containing 20 units/mL heparin with or without sodium nitrite supplementation of 25–250 μ M and then were procured and stored in a sterile container containing 2 mL (equal mouse total body blood volume) perfusion solution for different time periods, from 1 hour to 48 hours, respectively. AST and LDH enzyme release from the preservatives and the liver histology were examined at different time points. The time course curves were analyzed to determine the optimal concentration of nitrite. The livers preserved in the UW solution with or without nitrite supplementation were transplanted into syngeneic recipients after either 12 or 24 hours of cold preservation, respectively. The recipients

of syngeneic liver grafts also received either sodium nitrite 250 μ mol in 2 mL total blood volume final concentration or equal amounts of phosphorus buffer solution (PBS) by i.p. injection just prior to liver transplantation. Liver tissue was obtained for histology and apoptosis assays at 10 minutes and 3 hours, respectively.

2.4. Liver Enzyme Assays. Samples from the liver preservatives at 1, 6, and 12 hours in LR solution and 12, 24, and 48 hours in UW solution with the concentration range of nitrite from 0.25 μ M to 1000 μ M were assayed for ALT, AST, and LDH levels using a spectrophotometric method, respectively. The LR and UW solutions without anion nitrite supplementation were used as controls.

2.5. Liver Transplantation. Orthotopic liver transplantation (OLTx) with revascularization accomplished with a combination of suture and cuff techniques was performed between syngeneic strain combinations as described [3, 12]. Donor livers were preserved in the UW solution at extended times of 12 and 24 hours, with or without nitrite supplementation at 25 or 125 μ M, respectively. Liver biopsy was performed at 10 minutes and 3 hours postreperfusion, respectively.

2.6. Histological Analysis. Formalin-stored tissue specimens were embedded in paraffin and cut into 4 mm sections and then were examined by routine hematoxylin and eosin staining. Three samples from each group were analyzed under 10 high-power fields. The histology scores of liver tissue sections were determined by 2 independent persons in a blind manner according to the following scoring criteria: 0, no hepatocellular damage; (1) mild injury characterized by cytoplasmic vacuolization and focal nuclear pyknosis; (2) moderate injury with dilated sinusoids, cytosolic vacuolization, and blurring of intercellular borders; (3) moderate to severe injury with coagulative necrosis, abundant sinusoidal dilation, red blood cell extravasation into hepatic chords, and hypereosinophilia and margination of neutrophils; (4) severe necrosis with loss of hepatic architecture, disintegration of hepatic cords, hemorrhage, and neutrophil infiltration. Criteria to specifically evaluate peripheral mononuclear cell infiltration ((0) zero; (1) minimal; (2) mild; (3) moderate; (4) severe) were also used (Lang 2007).

2.7. TUNEL Staining. Apoptotic cells in frozen sections (4 μ m) were identified using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore Corporation, Billerica, MA) and followed according to the manufacturer's instructions, as described by (Li, 2008). Cryosections (4 μ m) were mounted on precleaned slides, air-dried overnight at room temperature (RT), and then fixed for 10 min at RT in 10% neutral buffered formalin (pH 7.4), followed by two washes (5 min each) in PBS. Endogenous peroxidase activity was quenched in 2% H₂O₂ before exposure to TdT enzyme at 37°C for 60 min. After washing in stop wash buffer (37°C, 30 min), anti-digoxigenin-peroxidase was added (RT; 30 min). AEC or DAB (ScyTek Laboratories, Inc, Logan, UT) was used for color development, and the sections were counterstained with hematoxylin. The numbers of

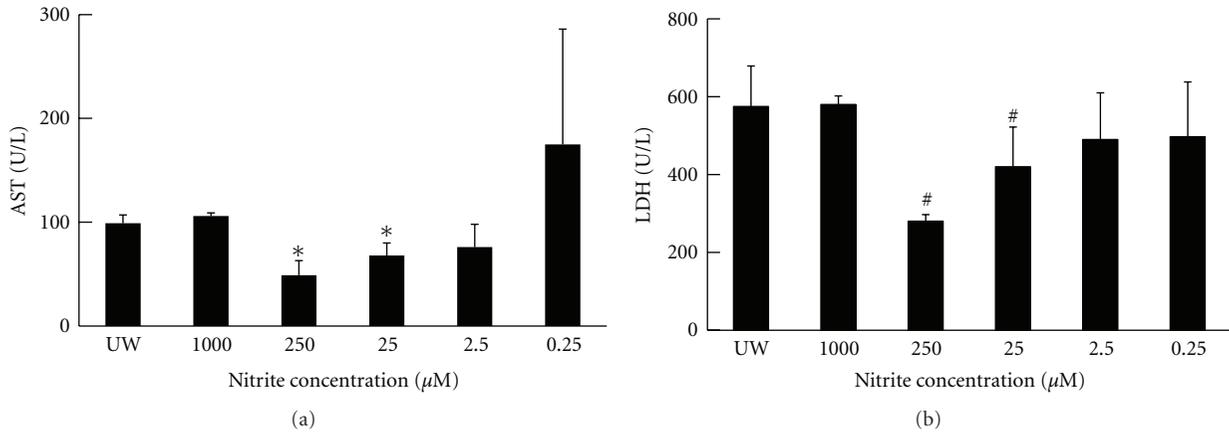


FIGURE 1: Nitrite-supplemented UW solution decreased hepatic graft enzyme release. UW solution supplemented with anion nitrite decreased hepatic graft enzyme release during 24 hours of cold ischemia. Solutions of 250 (AST— 68 ± 12 ; LDH— 420 ± 102) and 25 (AST— 38 ± 4 ; LDH— 280 ± 17) μM anion nitrite resulted in the greatest reduction. Data are expressed as mean \pm SE. # $P < 0.05$ for corresponding measurements.

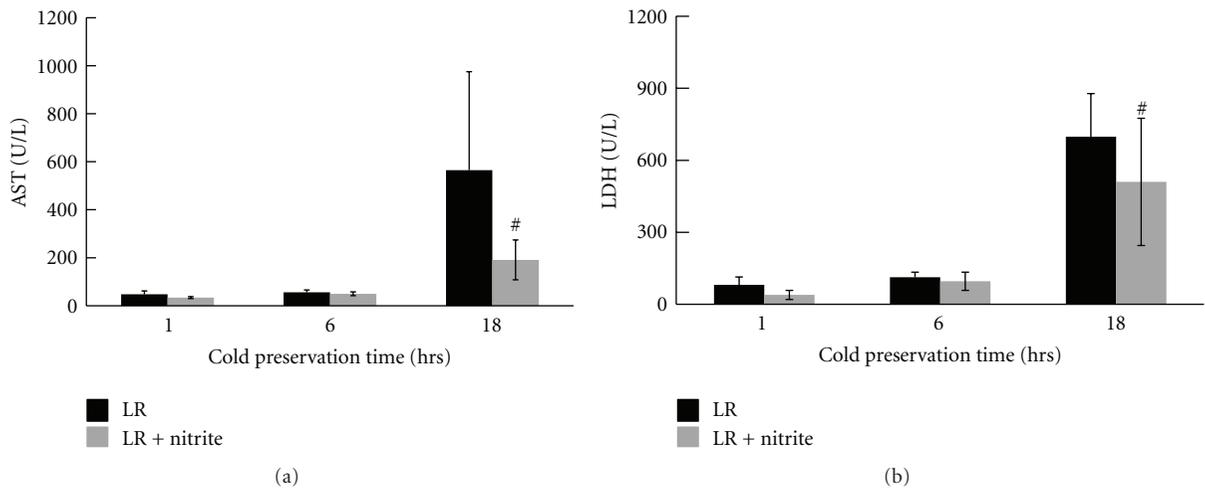


FIGURE 2: Nitrite-supplemented LR decreased graft enzyme release. LR supplemented with 25 μM anion nitrite significantly decreased enzyme release at 18 hours (AST— 599 ± 492 versus 178 ± 59 ; LDH— 692 ± 269 versus 498 ± 272). Data are expressed as mean \pm SE. # $P < 0.05$ for corresponding measurements.

apoptotic cells in liver sections were enumerated under light microscopy by numbers of apoptotic cells per 40 high-power fields in five sections per tissue per mouse (three mice per group).

2.8. Caspase-3 Activity Assay. A caspase-3 ELISA kit (BD Pharmingen, San Diego, CA) was used for *in vitro* determination of caspase-3 enzymatic activity in liver homogenates derived from mice in different groups. Briefly, supernatants were obtained from homogenized liver grafts with mammalian lysis buffer (Qiagen, Inc., Valencia, CA) including benzonase nuclease and protease inhibitor. Flat-bottom 96-well microtiter plates were coated with 100 μL /wells of diluted capture antibody (BD Pharmingen). Nonspecific binding sites were blocked with blocking buffer. Plates were rinsed, and standards and samples of diluted supernatant (100 μL each) were added, followed by the addition of

100 μL /well working detectors (diluted detector antibody and streptavidin HRP in blocking buffer). After washing, the plates were incubated for color development; the reaction was terminated with 50 μL of stopping solution. Plates were read at 450 nm in an ELISA reader. To determine the active caspase-3 concentration of samples, computer data reduction was employed, utilizing log-log regression analysis in a series of diluted caspase-3 standards.

2.9. Western Blot Analysis. The antiapoptosis Bcl-2 protein expression in the liver tissue was determined by western blot assay. Proteins (50 μg /sample) extracted from the liver tissue in SDS-loading buffer (50 mM Tris, pH 7.6, 10% glycerol, 1% SDS) were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The gel was then stained with Coomassie Blue to document protein loading. The

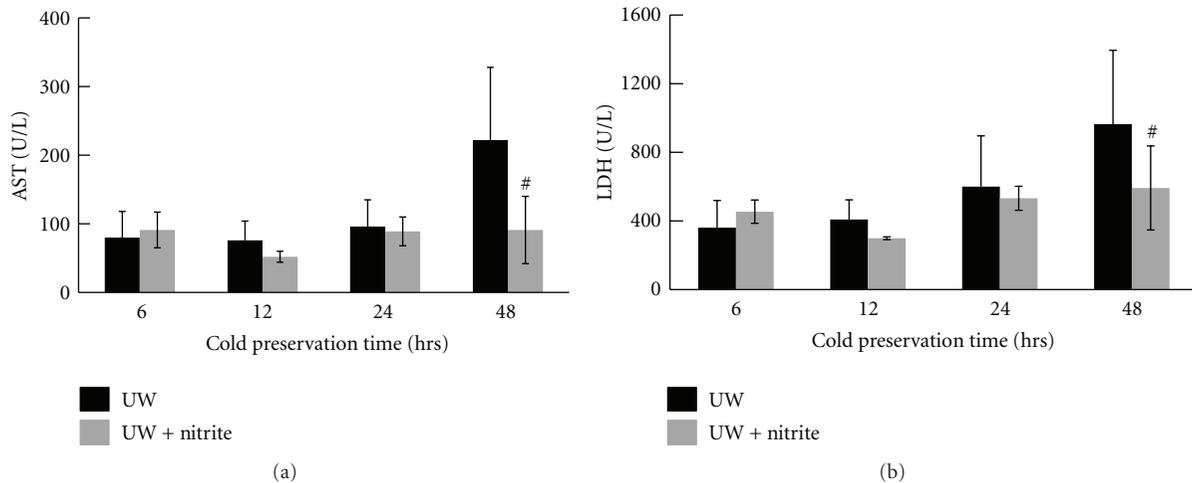


FIGURE 3: Nitrite-supplemented UW solution decreased graft enzyme release; UW solution supplemented with 25 μM anion nitrite significantly decreased enzyme release at 48 hours (AST—221 \pm .97 versus 76 \pm 40; LDH—991 \pm 420 versus 578 \pm 292). Data are expressed as mean \pm SE. # P < 0.05 for corresponding measurements.

membrane was blocked with 5% dry milk and 0.1% Tween 20 (Bio-Rad) in PBS. The membrane was subsequently incubated with the primary antibodies at 4°C overnight. The primary antibody was a mouse monoclonal antihuman Bcl-2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membranes were developed according to the Amersham-enhanced chemiluminescence protocol. The beta-actin was measured as a loading control.

3.10. Statistical Analysis. The comparisons were made using Student's *t*-test. Probability (*P*) values ≤ 0.05 were considered statistically significant. Data is expressed as mean \pm SE.

3. Results

3.1. The Protective Effects of Nitrite to Liver IRI Were Dose Dependent. To determine if nitrite supplementation could confer protection in the liver from cold ischemia injury, nitrite (0.25 μM –1000 μM) was added to UW solution. The liver enzymes AST and LDH were then measured from the preservation solutions at 24 hours. In contrast to the UW only control group, AST and LDH release was significantly reduced in livers supplemented with nitrite with maximal protection observed with 25 μM and 250 μM nitrite (AST—38 \pm 4; LDH—280 \pm 17) (Figure 1).

3.2. Nitrite Supplementation of LR or UW Solution Reduced Liver Damage from Extended Cold Ischemia Time. We examined whether nitrite supplementation of either LR or a preservative (UW) could protect the liver from cold ischemia injury. B6 mouse livers were perfused with 5 mL cold Ringer's solution containing heparin 20 units/mL with or without nitrite (25 μM) addition. Livers were then preserved in 5 mL of the above solution on ice for 1 hour, 6 hours, and 18 hours, respectively. AST and LDH levels were tested from the preservation solution from 3 livers pooled together in each group. AST was detected to have a slight increase after

1 and 6 hours of cold preservation and a significant increase after 18 hours of cold preservation in the LR control groups. The LDH level also increased significantly after 18 hours of cold preservation. Release of AST and LDH was significantly reduced in the nitrite-supplemented groups compared to LR only groups (Figure 2). Similarly, nitrite supplementation in the UW solution also reduced the release of liver AST and LDH significantly after 48 hours of cold preservation (Figure 3).

3.3. Nitrite Supplement in LR Solution Reduced Liver Damage from Extended Cold Ischemia Time. To examine the protective role of nitrite on hepatocytes during cold ischemia, liver samples were harvested from naïve B6 mice without perfusion, or from donor livers perfused and preserved with either LR solution only or LR plus anion nitrite supplementation at a concentration of 250 μM after 1 hour, 6 hours, and 18 hours-cold preservation, respectively. Histological examination on H & E staining section revealed hepatocyte swelling, increased cytoplasmic vacuolization, nuclear pyknosis, sinusoidal dilatation, and focal necrosis in the LR only group after 6 hours of cold preservation, and becoming more severe at 18 hours. In contrast, addition of 25 μM anion nitrite to the Ringer's preservation solution gave liver significant protection from cold ischemia injury during extended preservation period. The liver morphology remained essentially normal in those groups (Figure 4). Furthermore, livers preserved in the nitrite supplemented LR solution demonstrated a significant reduction of hepatocyte apoptosis after 18 hours of cold preservation (Figure 5). Similarly, nitrite supplementation of UW solution also reduced liver apoptosis after both 24 and 48 hours of cold preservation time (Figure 6).

3.4. Nitrite Modulates Caspase-3 and Bcl-2 Protein Expression. To determine the mechanisms by which nitrite decreases liver apoptosis under conditions of cold ischemia, caspase-3,

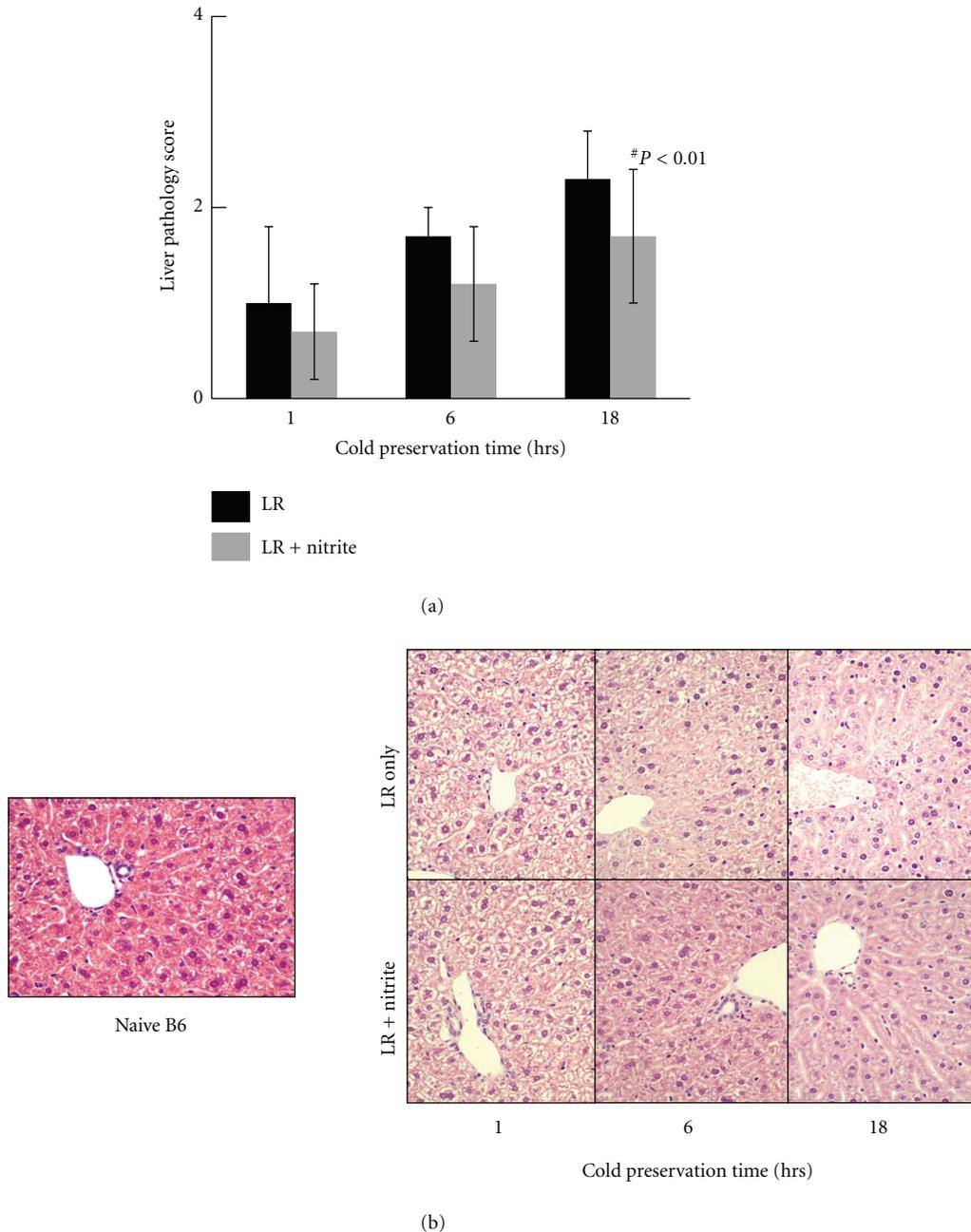
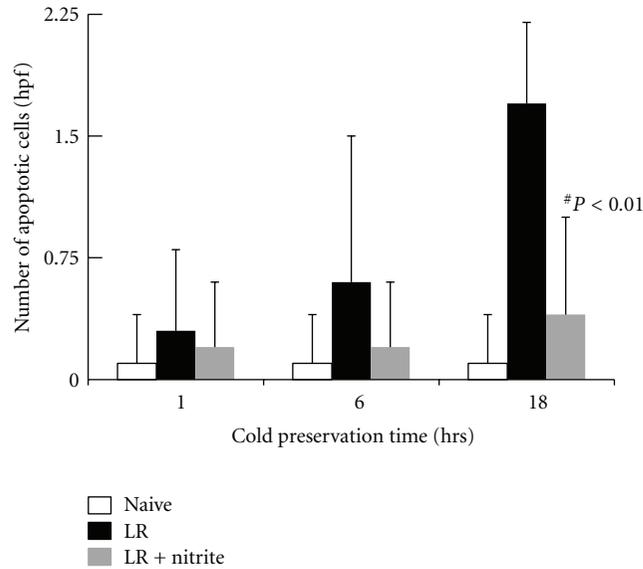


FIGURE 4: Liver pathology scores decreased with nitrite. Increased hepatocyte swelling, increased cytoplasmic vacuolization, nuclear pyknosis, sinusoidal dilatation, and focal necrosis in LR only group with maximal injury at 18 hrs are observed. These findings were significantly reduced at 18 hrs with $25 \mu\text{M}$ of anion nitrite (2.4 ± 0.8 versus 1.7 ± 0.7). Data are expressed as mean \pm SE. $\#P < 0.05$ for corresponding measurements.

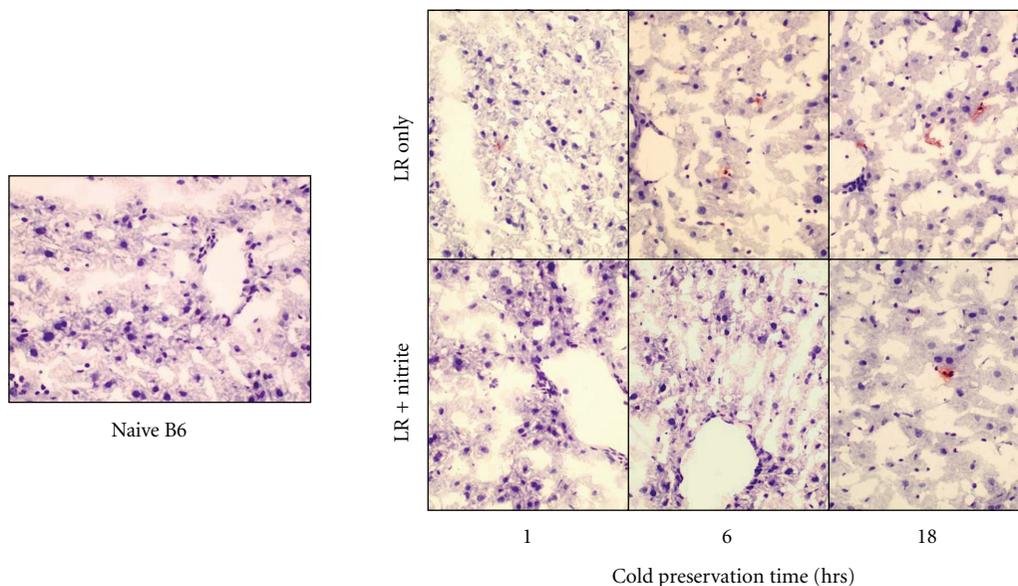
a cysteine protease critical for executing apoptosis, and Bcl-2, a family of proteins demonstrated to reduce apoptosis were measured after varying cold ischemia times (18 and 48 hours). Caspase-3 in livers supplemented with $25 \mu\text{M}$ nitrite in LR and UW solution during cold ischemia was significantly reduced at 18 (LR) and 48 hours (UW), respectively (Figure 7). The magnitude of caspase-3 reduction between the LR and UW preservation groups was similar (29.7 to 28.4%, resp.). Bcl-2 protein concentration determined by western blot analysis demonstrated a significant reduction

in both LR and UW preservation solution alone. However, concentrations of both caspase-3 and Bcl-2 were restored to near or back to baseline values with anion nitrite supplementation (Figure 8).

3.5. Nitrite Reduced Liver Reperfusion Injury after Extended Cold Preservation Time in UW Solution. To determine whether nitrite protected livers from IRI after liver transplantation, syngeneic liver transplantation from donor livers which were either preserved in UW solution plus $250 \mu\text{M}$



(a)



(b)

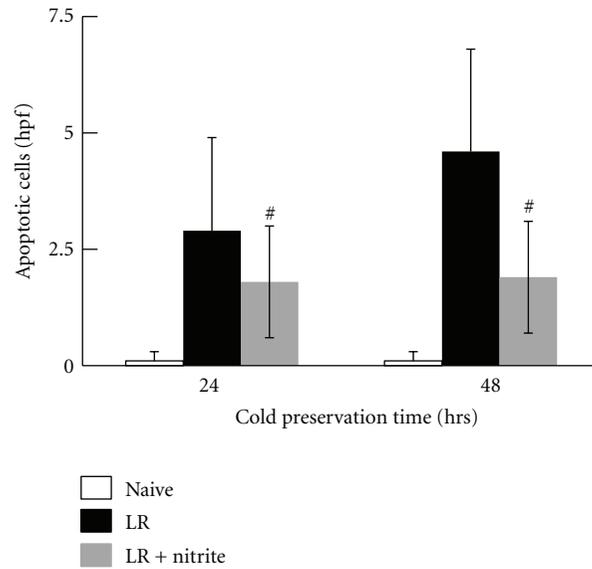
FIGURE 5: Hepatocellular apoptosis significantly decreased within LR-supplemented nitrite therapy. TUNEL evaluation revealed a significantly reduced number of hepatocellular apoptotic nuclei treated with nitrite at 18 hours (0.1 ± 0.3 versus 1.7 ± 0.4 versus 0.4 ± 0.5). Data are expressed as mean \pm SE. $^{\#}P < 0.05$ for corresponding measurements.

nitrite for 12 hours or recipients that received $250 \mu\text{M}$ i.p. nitrite treatments at the time of liver transplantation was performed. Liver enzyme release and apoptosis from the donated livers were examined 3 hours posttransplantation. Assessment of liver enzyme release revealed that nitrite supplementation in the preservation solution only and intraperitoneal treatment alone significantly decreased ALT and LDH release compared to the control group and the group receiving the combined treatment of both donor liver graft and recipient i.p. injection (Figure 9). Moreover,

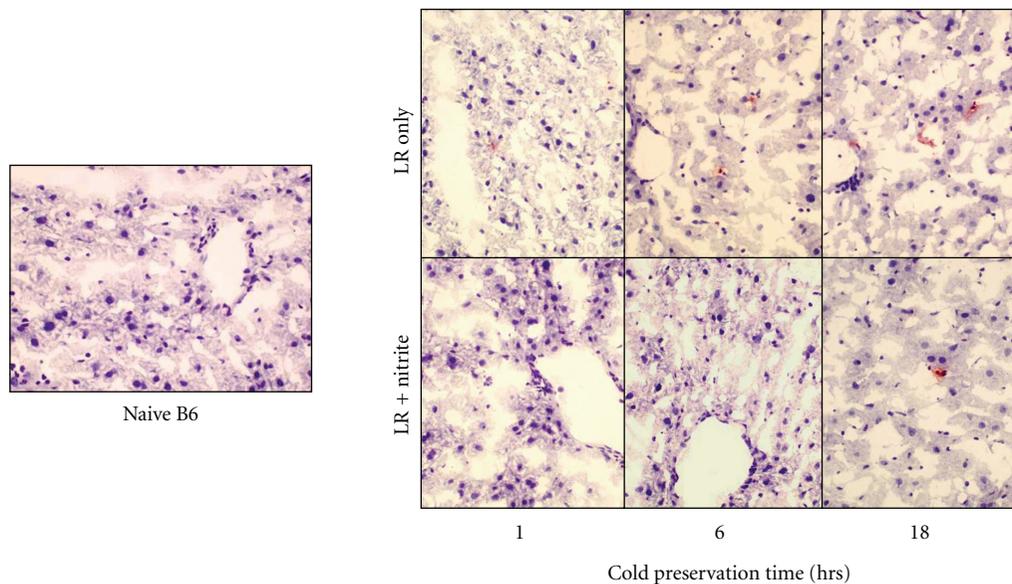
nitrite treatment of the donor liver only and recipient only significantly reduced hepatocyte apoptosis when compared to the control and combined treatment groups (Figure 10).

4. Discussion

Previous studies in animal models of warm IRI utilizing nitrite as an anti-inflammatory therapy have demonstrated significant benefit. In this study, we chose to supplement solutions with varying concentrations of nitrite under



(a)



(b)

FIGURE 6: Nitrite reduced hepatocellular apoptosis during cold ischemia. Hepatocellular apoptosis was significantly reduced with 250 μM of anion nitrite after 24 hours (0.2 ± 0.3 versus 2.9 ± 2.2 versus 1.7 ± 1.5) and 48 hours (0.2 ± 0.3 versus 4.3 ± 3 versus 1.8 ± 1.4) of cold ischemia. Data are expressed as mean \pm SE. $\#P < 0.05$ for corresponding measurements.

conditions of cold ischemia and to assess potential anti-inflammatory effects of nitrite therapy on murine hepatic graft function. Our results show that nitrite-supplemented solutions during cold ischemia decreased hepatocellular injury and apoptosis during both cold ischemia and 3 hours posttransplantation.

Previous studies have shown that nitrite dose versus therapeutic benefit exhibits a U-shaped relationship [6]. A

similar efficacy profile was observed with nitrite protection against cold-ischemia-mediated liver injury with 25–250 μM affording maximal protection. Previous *in vivo* studies demonstrated protection against IRI injury in the liver with $<10 \mu\text{M}$ nitrite concentrations [6, 12] suggesting that liver grafts subjected to cold ischemia (compared to warm IRI) warrant higher concentrations of nitrite as 25 μM consistently attenuated injury during cold ischemia alone,

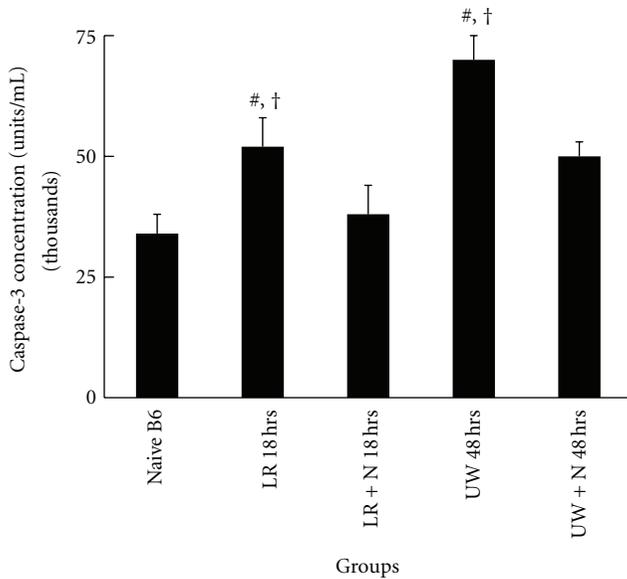
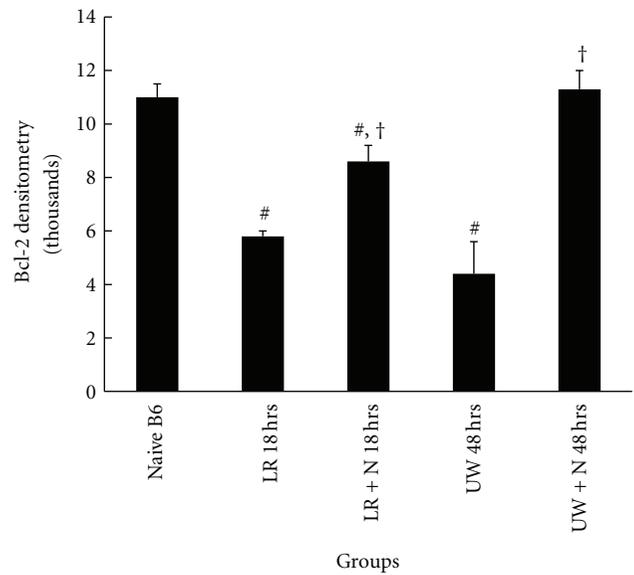


FIGURE 7: Caspase-3 concentrations decreased with nitrite supplemented solutions. Caspase-3 concentrations increased during cold preservation with both LR (18 hours) and UW solutions (48 hours) alone, but were significantly attenuated when the respective solutions were supplemented with 250 μ M of anion nitrite (LR—49.1 \pm 3.7 versus LR + nitrite—38.6 \pm 3.7; UW—71.4 \pm 4 versus UW + nitrite 50.4 \pm 3.1). Supplemented UW solution demonstrated the greatest attenuation. Data are expressed as mean \pm SE. [#] P < 0.05 for corresponding measurements that compared groups LR and UW only to native B6 and [†] P < 0.05 for corresponding measurements that compared groups LR and UW only to LR and UW groups treated with anion nitrite.

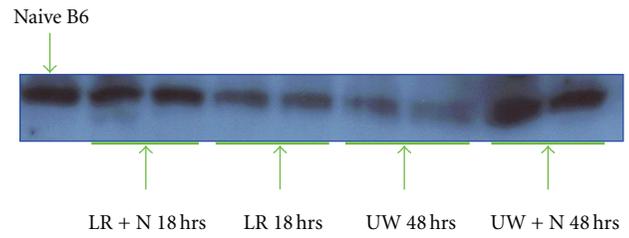
while 250 μ M anion nitrite was beneficial when administered to either the graft alone or recipient animal alone in animals 3 hours posttransplantation. Attenuation of cold ischemic injury by nitric oxide implicates the sinusoidal endothelial cells as a principal culprit as the injury orchestrator

Hepatic enzyme release significantly decreased when supplemented with nitrite demonstrating a more robust protective effective effect with UW compared to Ringer's solution as would be expected. The maximal protective effect peaked at 48 hours with the use of UW solution compared to 18 hours with Ringer's solution. Nitrite treatment to only the liver graft or recipient mouse was superior in reducing hepatic enzyme release compared to combination treatment of both graft and recipient. Interestingly, in this series of liver transplantation studies, the supplementation or administration of 250 μ M anion nitrite was required to exert a significant protective effect compared to 25 μ M used only during cold ischemia. The mechanisms underlying why the effective doses varied by 10-fold remain unclear.

Histological injury per standardized scoring system demonstrated reduced injury in liver grafts treated with nitrite. The injury was maximal at 18 hours, although not severe in either group. This observation is consistent with previous data corroborating the fact that net and type of



(a)



(b)

FIGURE 8: Nitrite-supplemented solutions mitigated reductions in Bcl-2 concentrations. Bcl-2 protein (pooled from 3 livers per condition) expression decreased during cold preservation with both LR (18 hours) and UW solutions (48 hours) alone but significantly increased when the respective solutions were supplemented with 250 μ M of anion nitrite (LR—5.9 \pm 0.6 versus LR + nitrite—8.8 \pm 1; UW—4.4 \pm 1.3 versus UW + nitrite—10.8 \pm 1.1). Supplemented UW solution demonstrated the greatest increase in protein expression. Data are expressed as mean \pm SE. [#] P < 0.05 for corresponding measurements.

injury exists between cold ischemia compared to warm ischemia [13].

Hepatocellular apoptosis decreased both with nitrite-supplemented Ringer's and UW solutions at 18 and 48 hours, respectively. There was less apoptosis during cold ischemia as compared to cold ischemia plus warm ischemia reperfusion. Nitrite treatment to only the liver graft or recipient mouse was more efficacious in reducing apoptosis compared to combination treatment of both graft and recipient. One possibility explaining this observation is that the observed "protective" concentrations of nitrite may have been exceeded leading to loss of protection per the U-shaped dose-response relationship. Further studies are required to determine optimal nitrite dosing and timing strategies to prevent liver injury during cold ischemia and posttransplantation. Liver grafts exposed to cold ischemia displayed only decreased caspase-3 and increased Bcl-2 with

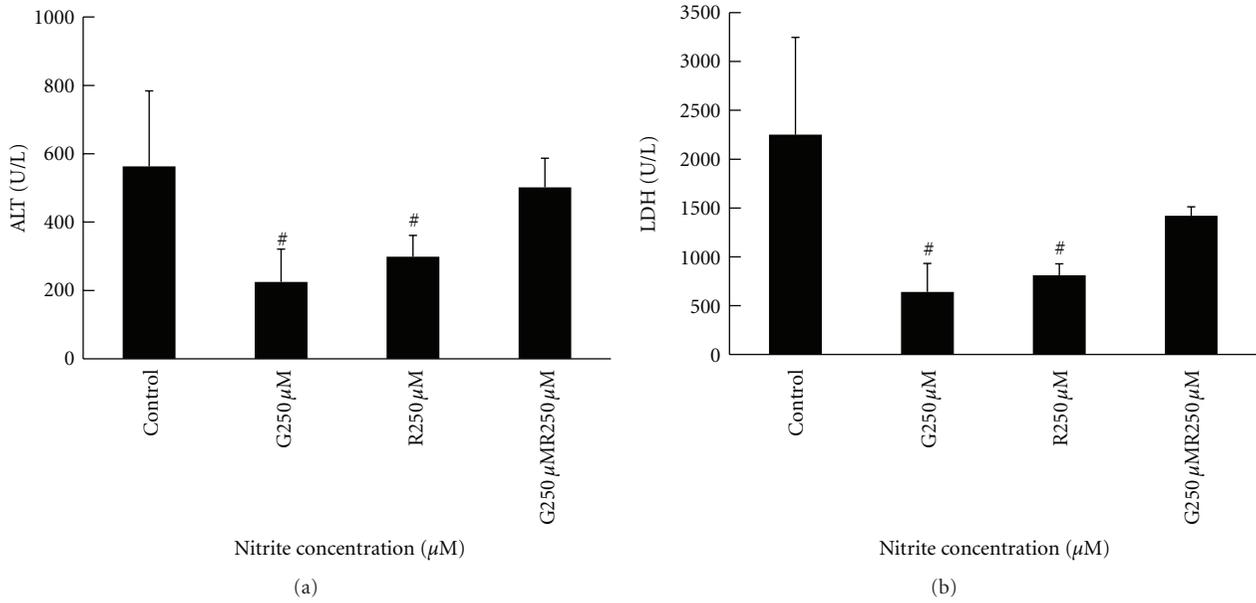


FIGURE 9: Nitrite administration to either the donor liver graft or recipient decreased hepatic enzyme release posttransplantation. Serum enzymes in only the nitrite-treated graft and recipient only groups decreased in syngeneic recipients 3 hours posttransplantation (ALT—580 ± 118 versus 210 ± 90 versus 229 ± 41 versus 493 ± 92; LDH—2228 ± 1019 versus 652 ± 380 versus 810 ± 182 versus 1375 ± 202). Data are expressed as mean ± SE. #P < 0.05 for corresponding measurements.

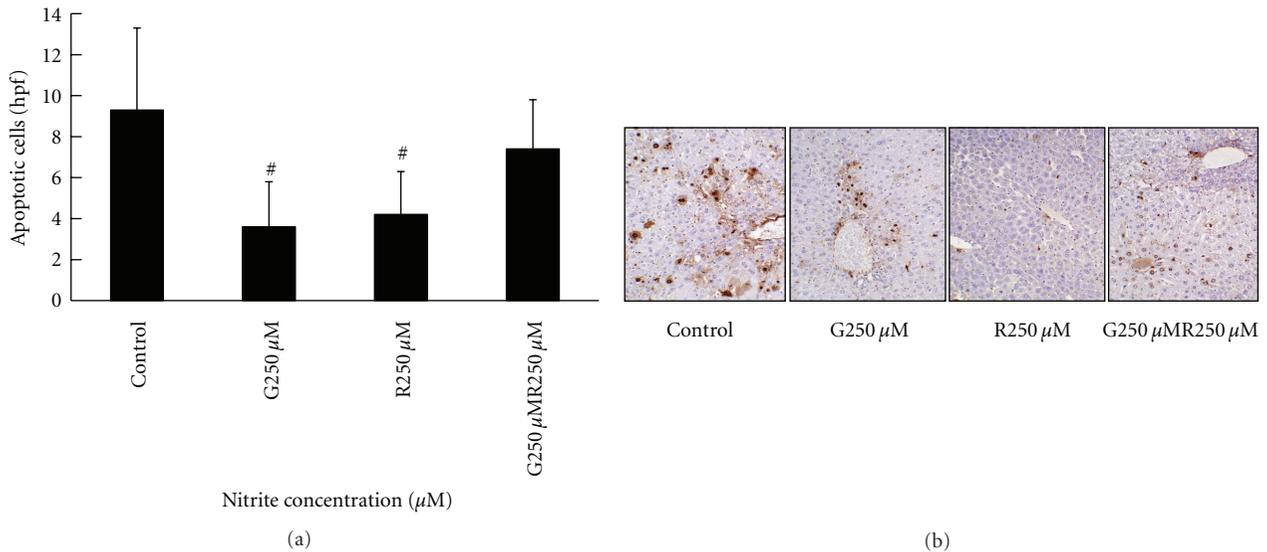


FIGURE 10: Nitrite reduced hepatocellular apoptosis in the donor liver graft and in the recipient after liver transplantation. Hepatocellular apoptosis was significantly reduced by anion nitrite in the donor liver graft and recipient only groups compared to control and combined treatment groups 3 hours posttransplantation (9.8 ± 4.1 versus 3.9 ± 2.4 versus 4.1 ± 2.6 versus 7 ± 3.9). Data are expressed as mean ± SE. #P < 0.05 for corresponding measurements.

both Ringer’s and UW’s solutions supplemented with nitrite. The magnitude of change in both groups while divergent was enhanced with anion nitrite-supplemented UW solution.

Despite the study limitations that include a small sample size, no measurements of tissue concentrations of NO, and very short followup of the animals posttransplantation, in aggregate, our study demonstrated a hepatoprotective effect of nitrite as evidenced by reductions in liver enzymes,

reduced pathology score, and reduced hepatocellular apoptosis with either LR or UW preservation solutions. The preservation solution, UW, afforded extended hepatoprotection compared to LR and nitrite. Liver grafts harvested from transplanted animals were observed to have reduced injury when only the graft or animal received nitrite supplementation compared to both graft and animal being supplemented with anion nitrite.

Acknowledgment

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Review Article

Inflammation and Microvasculopathy in Renal Ischemia Reperfusion Injury

Daniel Patschan, S. Patschan, and G. A. Müller

Abteilung für Nephrologie und Rheumatologie, Universitätsklinikum Göttingen, Robert-Koch-Straße 40, 37077 Göttingen, Germany

Correspondence should be addressed to Daniel Patschan, d.patschan@gmail.com

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Acute renal failure (ARF) severely worsens prognosis of hospitalized patients. The most frequent cause of intrarenal ARF is transient or prolonged renal hypoperfusion (ischemia). Ischemia primarily affects the function and structure of tubular epithelial cells, which, in severe cases, is characterized by epithelial cell necrosis. Nevertheless, ischemia does not exclusively lead to alterations of epithelial cells but also causes interstitial inflammation and interstitial microvasculopathy. Both inflammation and microvasculopathy are particularly important in terms of postischemic kidney repair. Postischemic microvasculopathy is characterized by endothelial cell swelling with subsequent microvascular occlusion. Thus, reperfusion is inhibited (no-reflow phenomenon). Such endothelial cell dysfunction offers new therapeutic perspectives in ischemic ARF. Newer observations point towards the role of the so-called endothelial progenitor cells (EPCs) in the treatment of ARF. Systemic administration of EPCs to mice with bilateral renal ischemia mitigates postischemic endothelial cell dysfunction and protects animals from acute renal failure.

1. Introduction

Acute renal failure (ARF) severely worsens prognosis of hospitalized patients. Approximately 1–5% of all patients treated in the hospital develop ARF [1]. The clinical significance of ARF results from its high mortality, which still today ranges from 30 to 70% [2]. ARF is categorized depending on its primary cause. Prerenal failure results from transient renal hypoperfusion. It is a functional response of a structurally intact kidney to hypoperfusion [3]. While postrenal ARF is caused by urinary tract obstruction with or without subsequent damage of renal tissue, intrinsic or intrarenal ARF is caused by diseases that either affect the glomeruli, the vasculature, the interstitium, or the tubules. The difference between prerenal and intrarenal failure due to hypoperfusion lies in the presence of structural tubular damage in the latter. The most frequent cause of intrarenal ARF in hospitalized patients is transient or prolonged renal hypoperfusion (ischemia reperfusion injury—IRI) [4–6]. Renal IRI is of particular importance in the setting of kidney transplantation

[7]. Ischemia primarily affects the function and structure of tubular epithelial cells, which, in severe cases, is characterized by epithelial cell necrosis [8]. Nevertheless, IRI does not exclusively lead to alterations of epithelial cell function and structure but also causes interstitial inflammation and interstitial microvasculopathy (Figure 1). These alterations can delay restoration of renal function which potentially worsens prognosis of patients with ischemic ARF [3]. Postischemic microvasculopathy is characterized by endothelial cell swelling, leading to prolonged ischemia even if the primary cause of hypoperfusion has been eliminated [9, 10]. Such *no-reflow phenomenon* has also been described in other organs [11]. In recent years, it has become more and more evident that by targeting postischemic renal microvasculopathy, kidney function can partially or completely be preserved [9, 10]. First investigations were performed by the group of Goligorsky [12]. Immunoincompetent rats with renal IRI were injected with mature endothelial cells from humans (human umbilical vein endothelial cells—HUVECs). Animals were not only protected from ARF, but histological

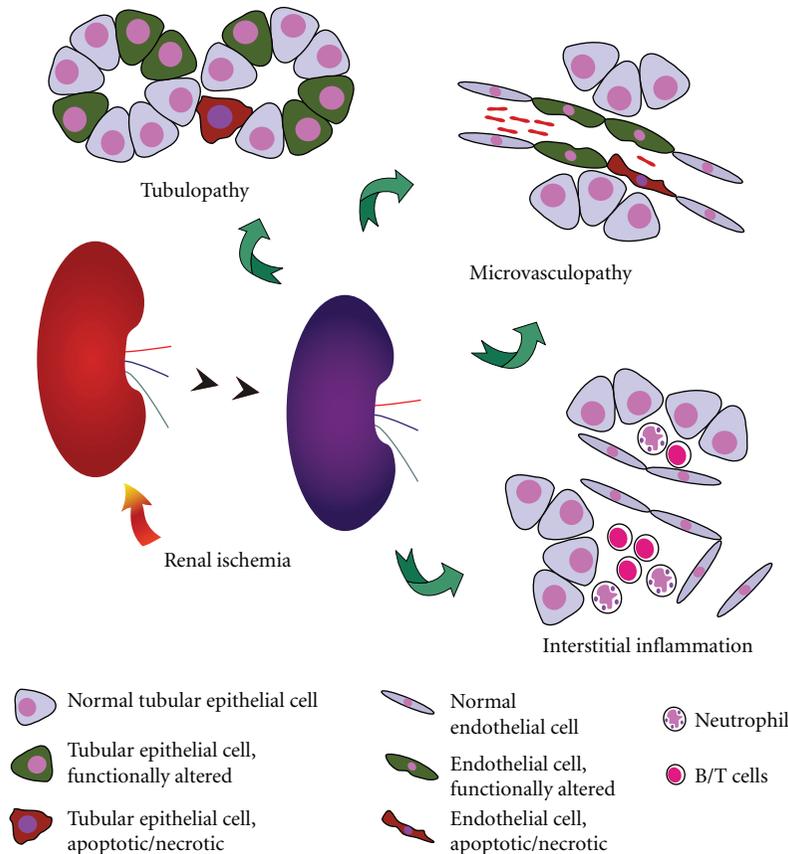


FIGURE 1: Acute ischemic renal failure is characterized by tubular epithelial cell dysfunction and damage. Posts ischemic restoration of kidney function and structure critically depends on both interstitial inflammation and peritubular microvasculopathy. The latter causes ongoing renal hypoperfusion even if ischemia has been corrected.

analysis showed direct incorporation of HUVECs into the endothelial cell layer within the renal microvasculature [12]. In subsequent years, comparable therapeutic effects were demonstrated for so-called endothelial progenitor cells (EPCs). In this paper, we will summarize the current knowledge on posts ischemic interstitial inflammation and microvasculopathy, and we will discuss therapeutic strategies to target microvasculopathy in acute ischemic renal failure.

2. Interstitial Inflammation

During the last 15 years, our knowledge of posts ischemic inflammation in the kidney has significantly been increased. The inflammatory process is initiated by both endothelial and tubular cell dysfunction. A number of different proinflammatory/immunomodulatory cytokines, such as IL-1, -6, and -8, TGF- β , TNF- α , and MCP-1 (monocyte chemoattractant protein-1), are released into the renal tissue and the circulation, respectively [13, 14]. Serum levels of IL-6 have been shown to indicate a higher risk of death in patients with acute kidney injury (AKI) [15]. Recently, Toll-like receptor 4 (TLR 4) has been shown to play an essential role in posts ischemic renal IL-6 production [16]. In this study, leukocytes from TLR 4 knockout mice (TLR 4 (-/-)) infiltrated kidneys of TLR 4-expressing animals (TLR 4 (+/+)), but there

was almost no impairment of renal function. In addition, only leukocytes from TLR 4 (+/+) mice produced IL-6 in response to high-mobility group protein B1 (HMGB1). The renoprotective consequences of TLR 4 inactivation have also been documented by Zhang and colleagues [17]. Earlier studies showed that TLR 2 represents an important regulator of the proinflammatory response as well. Renal tubular epithelial cells displayed increased TLR 2 expression after acute ischemia, and TLR 2 inactivation protected mice from AKI [18]. TLR 2 (-/-) mice did not only show decreased intrarenal expression of MCP-1, TNF- α , IL-6, and IL-1, but tissue infiltration by neutrophils was also markedly reduced.

Posts ischemic tissue infiltration by certain populations of inflammatory cells is a hallmark in renal IRI. Neutrophils, macrophages, natural killer cells, and different subtypes of T cells home to the interstitial space where they modulate the inflammatory response [3, 19–24]. The earliest population of cells that accumulate within peritubular capillaries, the interstitium, and to some extent within tubules are neutrophils [3]. Nevertheless, the clinical significance of neutrophil infiltration can be doubted or at least remains a matter of debate, although blockade of neutrophil function partially protects animals from AKI in some models [20]. Depletion of macrophages also ameliorates renal function [21] in ischemic AKI. However, macrophage activity critically depends on the

function of T cells. The data on the role of T cells in renal IRI are conflicting. Nu/nu-mice, which neither produce CD4⁺ nor CD8⁺ T cells, displayed higher ischemia tolerance as compared to wild-type animals [23]. Selective reconstitution of CD4⁺ T cells significantly worsened renal function after ischemia. Comparable renoprotective effects were observed in Rag-1 knockout (KO) mice. Such animals have small lymphoid organs that do not contain mature B and T cells [25]. The recombination activating genes-1 and -2 are required for somatic assembly of both the B- and T-cell receptors [26]. Ischemia protection in Rag-1 KO mice was reversed if the animals were adoptively transferred with WT CD4⁺ cells. These effects critically depended on the cells' ability to produce Interferon- γ [24]. Nevertheless, it is doubtful that CD4⁺ T cells exclusively mediate deleterious effects on renal function. Recently, Lee and colleagues investigated the role of CD4⁺/CD25⁺ (regulatory) T cells in cisplatin-induced AKI [27]. Nu/nu-mice showed increased survival, reduced tubular epithelial cell damage, and decreased tissue levels of TNF- α after administration of CD4⁺/CD25⁺ T cells. Comparable data were published by Yokota and colleagues [28]. Depending on their respective phenotype, CD4⁺ T cells were shown to either act protective or deleterious in the setting of IRI. Such modulatory actions partly depended on the balance between INF- γ and IL-4 production [28]. In summary, the role of T cells in AKI is rather complex, and it can be assumed that individual biological properties of the different subsets of T-cells are fundamentally important in the process of kidney repair after ischemia. While T cell-mediated effects on postischemic kidney repair and function seem to require the presence of the cells in the kidney, this is not mandatory with B cells. B-cell depletion has been shown to partly protect from ischemia-induced structural damage, although neutrophil and T-cell infiltrates were not diminished [29]. Interestingly, susceptibility to ischemia could be reestablished by serum transfer from wild-type animals. Transfer of B cells in contrast was not associated with decreased ischemia tolerance. Whether these effects were exclusively related to antibodies remains unclear [29].

Natural killer T-cells (NKT cells) belong to the T cell family of lymphocytes. They express cell surface marker molecules of conventional T cells, but in addition they are positive for NK1.1, also known as NKR-P1.9 (natural killer cell receptor P1.9) [30, 31]. The T-cell receptor of NKT cells recognizes glycolipids presented by the class I-like molecule CD1d [30]. NKT cells can produce a diverse group of cytokines in response to antigen recognition which amplifies the activity of dendritic cells, conventional T cells, regulatory T cells, other NKT cells, and B cells, respectively [30]. In an elegant study, Li and colleagues [32] investigated the role of NKT cells in murine IRI. Renal ischemia of 30 minutes was followed by NKT cell and neutrophil accumulation in the kidney and by significantly increased IFN- γ tissue levels. Inhibition of NKT cell activity by cell depletion decreased numbers of IFN-producing neutrophils in the kidney and protected mice from AKI [32].

Another hallmark of IRI-associated inflammation is activation of the complement system [3, 33]. The complement cascade is represented by more than 30 plasma proteins

which predominantly are produced by the liver. They are critically involved in the innate immune response. Complement activation can occur by binding of C1q to the Fc fragment of antibodies (classical pathway) and, on the other hand, the cascade is permanently activated by spontaneous degradation of complement factor C3 (alternative pathway) [34]. Factor C5a has been shown to play an important role in IRI-induced kidney inflammation [33]. As a matter of fact, the C5a receptor is expressed by tubular epithelial cells and by certain interstitial macrophages, respectively [35]. IRI increases C5a receptor expression in the proximal tubule [36]. C5a acts as potent chemoattractant which results in the recruitment of neutrophils, monocytes, and T cells [3]. Administration of anti C5a mAb has been shown to block neutrophil and macrophage invasion after renal ischemia and to protect mice from renal dysfunction [36]. Although complement activation in murine IRI is predominantly mediated by the alternative pathway [37], a recent study identified the classical pathway to be the essential complement activator in human IRI [38]. Hypoxia treatment of proximal tubular epithelial cells from humans (PTEC) induced significant complement activation. By using C1q-depleted serum or by blocking C1q with antibodies, the classical pathway was shown to be critically responsible for initiating the cascade. Furthermore, this process was mediated by IgM which binds to PTEC in response to hypoxia [38].

The consequences of interstitial inflammation for the treatment of renal IRI are currently investigated. IL-10, which acts as an anti-inflammatory mediator, protected against ischemic AKI by inhibiting Th1 cell function [39]. Comparable renoprotective effects were observed with the administration of anti-IL-6 mAb. This approach significantly inhibited production of proinflammatory mediators and tissue infiltration by neutrophils [40]. Very interesting results are related to effects of α -melanocyte-stimulating hormone or melatonin. The hormone, also known as N-acetyl-5-methoxytryptamine, is produced by the pineal gland, and it is released into the circulation in a circadian manner [41]. It had once been discussed to act as key regulator in sleep-wake rhythm [42]. Although this concept has been modified in recent years, the hormone has been shown to be involved in a number of other physiological events, namely, the detoxification of free radicals [9]. In addition, melatonin can inhibit activation of proinflammatory genes which cause renal injury after ischemia. In this setting, it even augments anti-ischemic actions of erythropoietin and protects mice from AKI-associated lung injury [43].

If such strategies will be established in human AKI one day remains speculative at the moment, but one has to be aware of the fact that renal IRI is neither an exclusive tubular nor vascular disease but also a severe inflammatory process. Postischemic renal inflammation may also contribute to microvasculopathy in IRI, which will be the topic of the next section.

3. Microvasculopathy in Renal IRI

Microvasculopathy significantly contributes to ongoing postischemic kidney dysfunction. Despite the fact that renal

hypoperfusion mainly causes functional and structural alterations of the tubular epithelium, studies performed in recent years pointed toward the role of postischemic endothelial cell dysfunction (ED) in peritubular capillaries as an important perpetuating factor of prolonged kidney malfunction [12, 44].

First evidence for the role of postischemic ED in acute ischemic kidney injury came from studies performed in the early 1970s [45]. Rats undergoing renal artery clamping displayed swelling of all cellular elements in the kidney which caused persistent renal hypoperfusion even after reperfusion. Swelling of endothelial cells significantly contributed to such *no reflow*. Extracellular fluid expansion, induced by hypertonic mannitol solution, partly prevented swelling of endothelial cells and thus protected from (post)ischemic renal damage. Hence, cell swelling had been identified as pathogenetic factor in tissue ischemia [46]. As a matter of fact, postischemic ED has to be considered as global cellular dysfunction syndrome, characterized, in addition to cell swelling, by increased paracellular and transcellular endothelial permeability and by increased endothelial expression of different types of cell adhesion molecules. Among those are P- and E-selectin and ICAM-1, respectively. The two selectins and ICAM-1 mediate leukocyte-endothelial interactions, a prerequisite for transvascular leukocyte migration. Inhibition of the selectins and of ICAM-1 has been shown to reduce renal injury in IRI [47]. Postischemic ED does not exclusively perpetuate acute kidney dysfunction but also worsens long-term outcome of renal IRI [48]. Studies performed in 2001 showed permanent damage of peritubular capillaries after acute renal ischemia [49].

Taken together, these observations pointed toward a new therapeutic approach in ischemic AKI: targeting of postischemic ED [50]. First evidence for the viability of such treatment came from studies performed by the group of Goligorsky [12, 44]. Systemic injections of mature endothelial cells from humans (HUVECs—human umbilical vein endothelial cells) into immunoincompetent nude rats protected the animals from ischemic kidney damage. *In vivo* microscopic analysis showed postischemic endothelial cell swelling within the peritubular capillary network, and, in addition, showed that complete normalization of microvascular tissue perfusion occurred as late as 24 hours after ischemia. In this setting, systemic administration of HUVECs markedly inhibited swelling of endothelial cells and promoted a faster functional and structural recovery of the organ. Histologically, injected cells had partly been incorporated into the endothelial layer of small blood vessels surrounding the tubular integrity. These studies showed for the first time that targeting of postischemic ED by the administration of cells of the endothelial lineage is a true option in the treatment of acute ischemic kidney injury. Although such therapeutic approach is promising, a number of questions remain. The most relevant question is related to the source of cells of endothelial origin. If endothelial-type cells are supposed to be administered in acute kidney injury, they must become available within a short period of time. The next problem is related to the immunological acceptance of exogenously injected cells. In an optimal setting, cells would rapidly be isolated from

the recipient in order to become available for immediate systemic administration if necessary. A possible alternative may be represented by the so-called endothelial progenitor cells (EPCs) which will be reviewed in the last section.

4. Endothelial Progenitor Cells (EPCs) in Renal IRI

EPCs were described for the first time in 1997 [51]. Cells expressing CD34 were isolated from human umbilical vein blood and, after several days of culturing, systemically injected into immunoincompetent animals with ischemic lesions of the lower extremities. This measure did not only improve postischemic blood flow, but microscopic analysis showed direct incorporation of injected cells into the endothelial layer of blood vessels within the reperfused tissue. For many years, it has been assumed that EPCs are more or less exclusively derived from pluripotent hematopoietic stem cells in the bone marrow. Meanwhile, this concept has significantly been modified. According to the current literature on EPC biology at least two major populations of EPCs have to be differentiated [52–55]. The first and by far more in detail analyzed population is represented by so-called *endothelial cell-like cells* (EC-like cells) or *early endothelial outgrowth cells* (eEOCs) [9]. Early endothelial outgrowth cells develop from hematopoietic stem cells in the bone marrow and they express both, endothelial and hematopoietic cell marker molecules [55]. In addition, they are capable of differentiating into cells of the hematopoietic lineage. Culturing eEOCs from mononuclear blood cells takes 5–7 days and it has reproducibly been shown that the cells can act anti-ischemic in different experimental situations. A number of studies evaluated the diagnostic and therapeutic value of eEOCs in ischemic heart disease [56–58]. Although initial studies by Asahara et al. [51] suggested that EPC-mediated vascular repair results from direct cell incorporation into the endothelial layer of small blood vessels, newer concepts favor indirect mechanisms to be responsible for vasoprotection. Thus, EPCs home into the postischemic tissue where they release different proangiogenic mediators such as VEGF (vascular endothelial growth factor), HGF (hepatocyte growth factor), and IGF-1 (insulin-like growth factor-1), respectively [59]. These substances promote a faster recovery of damaged endothelial cells [9, 60]. The second EPC-subpopulation is represented by the so-called *late endothelial outgrowth cells* (lEOCs) or *endothelial colony-forming cells* (ECFCs) [54, 55]. Late outgrowth endothelial cells can also be cultured from mononuclear blood cells, but in contrast to eEOCs, they are not capable to differentiate into cells of the hematopoietic lineage [9]. *In vitro* studies showed significantly stronger formation of vessel-like structures with lEOCs than with eEOCs. In a newer review paper by Yoder and Ingram [54], it has even been questioned if lEOCs are true *progenitors* of endothelial cells since they share a number of characteristics with mature endothelial cells. The only difference between mature endothelial cells and lEOCs lies in the higher proliferative potential of the latter. Nevertheless, proangiogenic or anti-ischemic effects have been shown for both cell types,

eEOCs and lEOCs. The vast majority of studies performed over the last 14 years investigated the role of eEOCs.

Acute ischemic renal failure is characterized by severe endothelial dysfunction [10, 12]. With regard to the promising studies by Brodsky et al. [12], the role of eEOCs in the treatment of acute ischemic renal failure was investigated for the first time about 6 years ago [61]. Early endothelial outgrowth cells were significantly mobilized by acute kidney ischemia and ischemic preconditioning of the animals induced eEOC homing into the postischemic tissue. Mononuclear cells isolated from such kidneys protected recipient animals from renal failure which was the proof-of-principle that eEOCs can serve as therapeutic tool in iAKI. Meanwhile, different strategies to increase renoprotective effects of eEOCs in iAKI have been established. In 2009, the substance 8-pCPT-2'-O-Me-cAMP (Epac-1 Ac) has been shown to augment the efficiency of an eEOC-based therapeutic regimen in iAKI [62]. Epac-1 Ac induced redistribution of β 1-integrins towards the plasma membrane of eEOCs which increased eEOC homing into the renal tissue. Newer and yet unpublished data indicate significantly eEOC stimulating effects of the hormone melatonin. Melatonin-pretreated eEOCs showed higher renoprotective potential than untreated cells. The responsible mechanisms were not related to increased homing of the cells, but melatonin acted antiapoptotic, stimulated VEGF secretion by the cells, and increased migration of eEOCs *in vitro*. In addition, supernatant from melatonin-treated eEOCs induced faster migration of mature endothelial cells.

Together, these data clearly show that eEOCs are a reliable option to ameliorate the short-term prognosis of iAKI. Current own investigations focus on the process of fibrogenesis after acute kidney ischemia. The mid- to long-term prognosis of iAKI critically depends on the amount of postischemic renal fibrosis [63]. It has recently been shown that EPCs inhibit renal fibrogenesis in two models of chronic renal failure [64, 65]. It seems most likely that eEOCs can be employed as antifibrotic therapeutic tool in iAKI and as a matter of fact, first own and also yet unpublished observations seem to confirm this theory.

Nevertheless, a number of questions still need to be answered before eEOCs will finally become usable for treating patients with iAKI. The most significant problem is still related to the time frame which is needed for the cells to be isolated. Further investigations will have to be performed in order to optimize the process of cell enrichment for therapeutic administration.

5. Conclusion

Acute ischemic renal failure (iARF) is the most frequent type of acute renal failure in hospitalized patients. Although renal hypoperfusion primarily affects the function and structure of the tubular epithelium, alterations of the microvasculature and inflammatory processes within the interstitial space are of particular importance with regard to postischemic restoration of kidney function. Postischemic microvasculopathy, which in severe cases is characterized by obstruction of the

peritubular vasculature, can potentially serve as therapeutic target in acute ischemic renal failure. Early endothelial outgrowth cells (eEOCs) are potent inhibitors of postischemic microvasculopathy in murine iARF, and systemic administration of the cells protects mice from acute renal failure after ischemia.

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