GSH Attenuates Organ Injury and Improves Function after Transplantation of Fatty Livers


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Key Words
Fatty liver · Glutathione · Organ injury · Transplantation

Abstract
Ischemia-reperfusion injury (IRI) is increased after transplantation of steatotic livers. Since those livers are increasingly used for transplantation, protective strategies must be developed. Reactive oxygen species (ROS) play a key role in hepatic IRI. In lean organs, glutathione (GSH) is an efficient scavenger of ROS, diminishing IRI. The aim of this study was to evaluate whether GSH also protects steatotic allografts from IRI following transplantation. Fatty or lean livers were explanted from 10-week-old obese or lean Zucker rats and preserved (obese 4 h, lean 24 h) in hypothermic University of Wisconsin solution. Arterialized liver transplantation was then performed in lean syngeneic Zucker rats. Recipients of fatty livers were treated with GSH (200 μmol/h/kg) or saline during reperfusion (2 h, n = 5). Parameters of hepatocellular damage and bile flow were measured. Transplantation of steatotic livers enhanced early reperfusion injury compared to lean organs as measured by increased aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase plasma levels. Bile flow was also reduced in steatotic grafts. Intravenous administration of GSH effectively decreased liver damage in fatty allografts and resulted in improved bile flow. Intravenous application of GSH effectively reduces early IRI in steatotic allografts and improves recovery of these marginal donor organs following transplantation.

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elevated plasma liver enzyme levels and delayed organ function [3, 4]. Zucker rats homozygotic for a leptin-receptor gene defect (fa/fa) develop macrovesicular hepatic steatosis. Studies have described increased reperfusion injury in fa/fa Zucker rats compared to animals with healthy livers following warm and cold ischemia [5, 6]. Therefore, fa/fa Zucker rats may represent a suitable animal model of IRI following transplantation of non-alcoholic fatty livers. Therefore, those rats may represent a suitable animal model of reperfusion injury following transplantation of non-alcoholic fatty livers.

Although the precise sequence of events leading to IRI in liver transplants has not been completely described, the generation of reactive oxygen species (ROS) and disturbances of the hepatic microcirculation may play key roles in the mechanisms following reperfusion injury [7, 8]. Administration of the antioxidant glutathione (GSH) has been shown to attenuate ischemia-reperfusion injury following warm ischemia or liver transplantation in healthy organs [9, 10]. However, the effect of GSH on hepatocellular damage caused by reperfusion of transplanted fatty livers remains largely unknown. Recent work has demonstrated differences in the cellular and humoral response in healthy and diseased livers following exposure to ischemia and reperfusion [11].

The purpose of the present study is to test the efficacy of post-ischemic intravenous GSH administration in preventing early reperfusion injury after transplantation of fatty livers. Despite promising results in several experimental studies, no agent has made its way into clinical practice [12]. This discrepancy between experimental studies and patient treatment may be in part due to the frequent use of healthy grafts in many animal studies [13, 14]. This is not representative of the clinical situation, in which organ shortage often necessitates the use of grafts of poor quality (i.e. steatotic grafts) from extended criteria donors. Thus, the results of our study may provide a novel and clinically relevant therapeutic approach for the protection of steatotic organs, thereby increasing the pool of transplantable livers.

**Methods**

**Animals**

Male syngeneic Zucker rats (Charles River Wiga, Sulzfeld, Germany) aged 10–14 weeks were used in this study. Animals were housed in a temperature- and humidity-controlled room under a constant 12-hour light/dark cycle. Rats with a homozygote point mutation of codon 269 in the leptine receptor gene (fa/fa) develop massive obesity. In contrast, animals with a heterozygote defect of this gene (fa/–) remain lean. Donors (body weight: fa/fa 478 ± 63 g, fa/– 345 ± 72 g) and recipients (fa/– 318 ± 22 g) had free access to water and rat chow (standard diet; Altromin, Lage, Germany). Recipient rats were fasted 12 h prior to donor operation and liver transplantation. All procedures were carried out in accordance with the guidelines of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The institutional animal care and use committee of the Government of Upper Bavaria and the Ludwig-Maximilians-University (Munich, Germany) approved this project.

**Experimental Groups**

Male Zucker rats were randomly assigned to three groups (n = 5/group; table 1). In group 1 (fa/– veh) livers were explanted from lean male Zucker rats and subjected to 24 h of cold ischemia (4°C) in University of Wisconsin solution. Orthotopic arterialized liver transplantation was then performed on lean male Zucker rats (fa/–). During reperfusion, 6 ml of saline 0.9% (vehicle) were infused continuously at a rate of 3 ml/h starting 20 min before declamping of the portal vein and hepatic artery. In group 2 (fa/fa veh) and 3 (fa/fa GSH) steatotic livers were explanted from obese male Zucker rats (homozygote, fa/fa). After a cold ischemia period of 4 h these livers were transplanted into lean Zucker rats (fa/–). This reduction in ischemic time from 24 h in lean organs to 4 h in steatotic grafts was applied based on previous studies by Amersi et al. [6]. During reperfusion, animals in group 2 received 6 ml saline (vehicle) by continuous infusion at a rate of 3 ml/h (vehicle), whereas animals in group 3 received GSH (Tationil 600, Roche, Italy) at a concentration of 200 μmol/h/kg dissolved in 6 ml saline. Reperfusion time was 120 min in all groups.

**Surgical Procedures**

Donor and recipient procedures were performed under spontaneous ether inhalation. For continuous monitoring of mean arterial blood pressure and for substitution of plasma volume, left carotid artery and jugular vein were cannulated with polyethylene catheters. Body temperature was kept between 36.5 and 37.5°C using a heating pad. Donor livers were preserved by retrograde aortal flush with 15 ml of University of Wisconsin solution and stored at 4°C for 24 h (group 1) or 4 h (group 2/3). Before implantation, livers were rinsed with 10 ml of Ringer’s lactate solution at 4°C via portal vein (hydrostatic pressure: 10 cm H2O). Orthotopic liver transplantation was performed according to the cuff technique described by Kamada and Calne [15]. Grafts were rearterIALIZED and simultaneously reperfused via portal vein and
hepatic artery as described by Post et al. [16]. Portal clamping times never exceeded 20 min. Five minutes after declamping, all rats received 0.5 ml of albumin (5%) and 0.5 ml of sodium bicarbonate to maintain blood pressure and physiological pH values. After 120 min of reperfusion, animals were sacrificed and liver weight was determined.

**Quantification of Bile Synthesis**
To quantify liver function during the reperfusion period, bile flow was quantified as previously described [17]. The common bile duct was cannulated with a polyethylene tube during donor surgery. Cumulative bile flow (µl/g/min) was calculated from total bile volume secreted between start of reperfusion and end of experiment (120 min).

**Determination of GSH and GSSG**
As opposed to other plasma parameters, determination of GSH (reduced GSH prior to oxidation, administered form) and GSSG (oxidized form of GSH following radical scavenging or spontaneous oxidation) in plasma requires a separate isolation procedure as described previously by Jaeschke et al. [18]. A total of 500 µl of whole blood is required for determination of total GSH (GSH total = sum of GSH and GSSG). For GSSG analysis, an aliquot (200 µl) of blood was mixed immediately with 200 µl of 10 mmol/l N-ethylmaleimide (NEM) in 100 mmol/l phosphate buffer (pH 6.5) containing 17.5 mmol/l EDTA. The remaining blood was centrifuged for 1 min. An aliquot (100 µl) of plasma was pipetted into 100 µl sulfosalicylic acid (5%) for determination of total GSH. To separate GSSG from NEM and NEM-GSH, an aliquot of NEM-treated plasma was passed through a Sep-PakC18 cartridge (Waters, Framingham, Mass., USA) followed by 1 ml of 100 mmol/l phosphate buffer (pH 7.5). GSSG in the eluates and total GSH in acidified plasma samples was determined by an enzymatic test as described previously [19]. GSH plasma concentrations were calculated as difference between total GSH and GSSG.

**Plasma Collection and Storage**
Whole blood was obtained via arterial catheter line (approximately 1.5 ml) in microcentrifuge tubes (Microtainer, Becton Dickinson, Rutherford, N.J., USA) and centrifuged at 16,000 g for 15 min at 4°C. Plasma was separated, placed in pyrogen-free microcentrifuge tubes, immediately frozen, and stored (−80°C) until assayed for alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH).

**Assessment of Serum Aminotransferases**
Serum aminotransferases were used as established markers of hepatic injury. AST and ALT were measured 2 h after reperfusion with a kinetic UV test using a serum multiple analyzer (Olympus AU 2700) [20].

**Assessment of Lactate Dehydrogenase**
Systemic cellular damage was determined by assaying serum levels of LDH 2 h after reperfusion with a kinetic UV test using a serum multiple analyzer (Olympus AU 2700) [21].

**Histology**
Representative histological sections (HE staining) of fatty livers were examined 2 h after reperfusion.

**Results**

**Plasma GSH and GSSG**
Plasma GSH was determined in rats receiving steatotic organs and treated with vehicle or GSH at time of reperfusion (t = 0 min) and 15, 30, 60, and 120 min thereafter (fig. 1). GSH-treated rats displayed systemic peak GSH levels 15 min after reperfusion. Plasma GSH levels were significantly increased in GSH treated rats throughout the whole experiment compared to vehicle-treated animals (p < 0.05).

Plasma GSSG levels were significantly elevated 60 and 120 min after reperfusion in GSH-treated animals compared to non-treated rats (p < 0.05; fig. 2).

**Plasma Aminotransferases (ALT/AST)**
Alanine Aminotransferase
ALT levels were significantly increased in recipients of steatotic livers compared to recipients of lean livers after
reperfusion (p < 0.05; fig. 3). In recipients of steatotic grafts, administration of GSH significantly reduced plasma ALT levels compared to administration of vehicle (p < 0.05).

Aspartate Aminotransferase
Plasma AST levels were significantly enhanced in vehicle-treated recipients of fatty livers compared to rats receiving lean organs (p < 0.05; fig. 4). In recipients of steatotic livers, treatment with GSH was associated with a significant reduction of AST compared to administration of vehicle (p < 0.05).

Lactate Dehydrogenase
LDH, a non-organ-specific marker of IRI, was significantly higher in recipients of fatty livers treated with vehicle compared to recipients of lean livers following liver transplantation (p < 0.05; fig. 5). In recipients of steatotic livers, the administration of GSH significantly reduced serum LDH levels compared to the administration of vehicle (p < 0.05).

Bile Flow
Two hours after reperfusion, cumulative bile flow (a sensitive marker of liver function) was significantly diminished in steatotic livers receiving vehicle compared to lean grafts (p < 0.05; fig. 6). GSH treatment, however, significantly increased bile flow in steatotic grafts compared to treatment with vehicle (p < 0.05).

Histology
Histological sections (HE staining) demonstrated a 50–80% degree of steatosis (data not shown). Two hours after liver transplantation, no alterations between the groups and due to IRI were evident.

Discussion
The critical organ shortage for liver transplantation has resulted in the routine acceptance of steatotic organs. In the general population, steatosis occurs with a prevalence of 13–50% [1, 2]. Steatotic livers substantially contribute to the donor pool, thereby partially compensating for organ shortage. Clinical and experimental studies have shown that steatotic livers are more susceptible to IRI than lean livers [3, 4, 22–25]. This results in increased plasma aminotransferases and diminished liver function in the early postoperative phase following transplantation [3, 4]. Other risk factors, including cold ischemia time and donor age [26, 27], have been shown to exhibit additive detrimental effects on organ function and survival following liver transplantation. Steatosis is a particularly relevant risk factor for IRI due to its increasing prevalence. Moreover, in contrast to risk factors such as cold ischemia time, steatosis cannot be affected by changing allocation procedures. Nonetheless, future experimental studies should consider other risk factors beside steatosis to better mimic the clinical situation.

Due to the clinical importance of steatosis for outcome following liver transplantation, animal models of steatosis have been established [2]. Genetically obese Zucker rats have been shown to develop liver steatosis mimicking non-alcoholic fatty liver disease [2, 28]. Interestingly, the pathophysiological mechanisms associated with reperfusion injury after orthotopic liver transplantation have been shown to be different between lean and steatotic Zucker rats [11]. Lean rats tend to develop hepatic apoptosis following ischemia-reperfusion whereas necrosis is the predominant form of cell death in steatotic organs [11]. Moreover, steatotic livers exhibit an increased production of reactive oxygen species (ROS) following warm and cold ischemia and reperfusion [5, 29]. These results collectively suggest that steatosis has to be considered when investigating new therapeutic strategies in liver transplantation.
The present data indicate that steatotic livers harvested from genetically obese Zucker rats develop significantly more organ injury compared to lean allografts. Interestingly, increased organ injury following transplantation of fatty organs was evident despite a reduction in cold ischemia time from 24 h in lean to 4 h in steatotic organs. These findings are supported by previous results demonstrating increased organ damage and diminished organ function in fatty compared to lean organs following IRI [30, 31]. Cold ischemia time of 24 h for healthy donor organs was based on previous studies, demonstrating significant IRI in recipient animals [13, 17]. For stea-
totic organs, cold ischemia time was substantially reduced to avoid deleterious organ damage [7].

In healthy organs, continuous administration of GSH protects liver tissue from reperfusion injury after warm and cold ischemia [9, 10, 32]. Endogenous GSH is concentrated in the intracellular space [33]. It reacts with oxidants released during reperfusion resulting in formation of oxidized GSH (GSSG) [34]. Its effect on ischemia-reperfusion injury in steatotic grafts, however, remains unknown. In the present study, GSH was administered at a dosage of 200 μmol/kg body weight/h to provide steatotic organs with supraphysiological GSH levels. This dosage has been shown to ameliorate IRI following transplantation of lean liver allografts [30] and resulted in peak plasma GSH levels below the limit of toxicity in humans (500 μM) [35]. As has been found in lean organs, the administration of GSH at the beginning of reperfusion significantly decreased early liver injury (plasma amino-transferase levels) and ameliorated liver function (bile flow) in steatotic grafts.

Determination of the exact mechanisms which are responsible for the beneficial effects of GSH in steatotic organs was beyond the scope of our study. Nonetheless, GSSG levels, the oxidized form of GSH, were increased in GSH-treated recipients of fatty grafts. This suggests that intravenously applied GSH was mostly oxidized. Oxidation of GSH is known to be associated with detoxification of detrimental ROS [36, 37]. In addition, the metabolism of GSH produces glycine [35], which also has protective properties against liver reperfusion injury [14]. It has been proposed that the GSH-induced reduction of ROS is associated with improved sinusoidal perfusion, significant decrease of leukocytes sticking to sinusoids, as well as prevention of sinus endothelial cell injury in lean organs [10, 32]. Steatotic livers are characterized by decreased sinusoidal blood flow due to swollen hepatocytes which may induce chronic hypoxia [38] and ATP depletion [39]. Moreover, ischemia-reperfusion injury in fatty organs is associated with enhanced leukocyte adhesion and microcirculatory failure, potentially based on an increased ROS release [23, 25, 29, 40]. These studies collectively suggest that GSH may also decrease ROS in fatty organs following cold storage and transplantation, thereby improving microcirculation and preventing endothelial damage, at least during the early reperfusion period which was of particular interest in this study.

In summary, the present study demonstrated increased IRI in transplanted, fatty allografts. Continuous posts ischemic infusion of GSH, a ROS scavenger, ameliorated early hepatocellular injury and improved liver function in fatty organs. The protective effects of GSH were associated with an increase in oxidized plasma GSSG, indicating detoxification of ROS by GSH. Since no severe side effects of GSH administration have been reported, this peptide has significant potential to be a useful and safe means of improving the function of steatotic organs following liver transplantation in humans. A prospective randomized trial is required to verify these promising experimental results in the clinical arena.

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References

Glutathione Reduces Graft Injury


