• BASIC RESEARCH •

Intravenous administration of glutathione protects parenchymal and non-parenchymal liver cells against reperfusion injury following rat liver transplantation

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Abstract

AIM: To investigated the effects of intravenous administration of the antioxidant glutathione (GSH) on reperfusion injury following liver transplantation.

METHODS: Livers of male Lewis rats were transplanted after 24 h of hypothermic preservation in University of Wisconsin solution in a syngeneic setting. During a 2-h reperfusion period either saline (controls, $n=8$) or GSH (50 or 100 µmol/(h·kg), $n=5$ each) was continuously administered via the jugular vein.

RESULTS: Two hours after starting reperfusion plasma ALT increased to 1 457±281 U/L (mean±SE) in controls but to only 908±187 U/L ($P<0.05$) in animals treated with 100 µmol GSH/(h·kg). No protection was conveyed by 50 µmol GSH/(h·kg). Cytoprotection was confirmed by morphological findings on electron microscopy: GSH treatment prevented detachment of sinusoidal endothelial cells (SEC) as well as loss of microvilli and mitochondrial swelling of hepatocytes. Accordingly, postischemic bile flow increased 2-fold. Intravital fluorescence microscopy revealed a nearly complete restoration of sinusoidal blood flow and a significant reduction of leukocyte adherence to sinusoids and postsinusoidal venules. Following infusion of 50 µmol and 100 µmol GSH/(h·kg), plasma GSH increased to 65±7 mol/L and 97±18 mol/L, but to only 20±3 mol/L in untreated recipients. Furthermore, plasma glutathione disulfide (GSSG) increased to 7.5±1.0 mol/L in animals treated with 100 µmol/(h·kg) GSH but did not raise levels of untreated controls (1.8±0.5 mol/L) following infusion of 50 µmol GSH/(h·kg) (2.2±0.2 mol/L).

CONCLUSION: Plasma GSH levels above a critical level may act as a "sink" for ROS produced in the hepatic vasculature

during reperfusion of liver grafts. Therefore, GSH can be considered a candidate antioxidant for the prevention of reperfusion injury after liver transplantation, in particular since it has a low toxicity in humans.

INTRODUCTION

A decade after the introduction of the University of Wisconsin (UW) solution for cold preservation of solid organs, hepatic preservation and reperfusion injury is still a serious problem which contributes to primary nonfunction and dysfunction of the liver allograft[1-3]. Years of research in liver preservation have established the superiority of the UW formulation over other solutions. Although hepatocytes and sinusoidal endothelial cells are injured during cold preservation in UW solution they remain alive even after periods of ischemia well beyond the limit of organ viability[2-4]. Hepatocyte and SEC death occurs rapidly on oxygenated reperfusion[2,4] and the extent of SEC death has been shown to be a critical factor influencing graft survival in various animal models[5-7]. However, therapeutic strategies which reduce reperfusion injury to hepatocytes and SEC during liver transplantation remain to be established.

There is substantial evidence that activation of Kupffer cells (KC), the generation of reactive oxygen species (ROS) and disturbance of the hepatic microcirculation contribute to reperfusion injury[8-11]. During the reperfusion, activated KC produce mediators of inflammation, including tumor necrosis factor α, interleukins and chemokines, and release ROS into the sinusoidal space[3,8]. The resulting vascular oxidant stress has been discovered as potential mechanism of vasoconstriction and leukocyte adherence in the liver[12-16]. This may lead to disturbance of the hepatic microcirculation, ultimately resulting in hypoxic cell injury. Furthermore, KC-derived ROS could activate redox-sensitive transcription factors such as nuclear factor (NF)-κB and activator protein-1 (AP-1) in endothelial cells and hepatocytes, thereby activating proinflammatory genes and adding to the hepatic damage[17,18].

Thus, ROS can be considered as signal molecules which trigger several pivotal mechanisms of reperfusion injury. A large number of investigations using antioxidant interventions support this hypothesis[10]. However, the clinical relevance of many antioxidants is limited by side-effects or high cost which may explain the lack of established antioxidative interventions to prevent hepatic reperfusion injury[3]. Therefore, recent studies investigated the therapeutic potential of the endogenous

antioxidant *glutathione (GSH)*, in particular since it has a low toxicity in humans[19]. Furthermore, GSH is able to react spontaneously with nearly all oxidants formed during inflammation, which results in the formation of oxidized glutathione (GSSG)[20-22]. Previous studies demonstrated that GSH released through the GSH transporter of hepatocytes may act as an endogenous defense system against KC – and leukocyte – derived ROS, thus protecting the hepatic vasculature from damage by inflammatory cells[8]. Therefore, interventions increasing plasma GSH levels should confer protection against reperfusion injury. Accordingly, treatment of cold preserved livers with GSH upon reperfusion prevented damage to hepatocytes in the model of isolated rat liver perfusion[23]. These *in vitro* findings suggest that GSH, as a candidate drug, could prevent reperfusion injury of the liver allograft. Consequently, future studies need to be directed toward characterization of the protective potential of GSH treatment *in vivo* which may have important implications for an application in the clinical setting.

Therefore, the purpose of this study was to investigate whether intravenous administration of GSH protects the rat liver against reperfusion injury during liver transplantation. In particular, the aims of the current study are: (1) to investigate effects of intravenously applied GSH on hepatocyte and SEC damage; (2) to examine the effect of GSH treatment on disturbances of the hepatic microcirculation, and (3) to determine the functional significance of changes of the extracellular antioxidant capacity.

MATERIALS AND METHODS

Animals and preparation
Syngeneic, male Lewis rats (donors: 207 ± 12 g; recipients: 276 ± 18 g body mass) were purchased from Charles River Wiga, Sulzfeld, Germany and housed in a temperature- and humidity-controlled room under a constant 12-h light/dark cycle. Animals had free access to water and rat chow (standard diet, Altromin, Germany). All experiments were performed with rats fasted 12 h prior to donor and recipient operations. All studies were performed with the permission of the government authorities and in accordance with the German Legislation on Laboratory Animal Experiments.

Donor and recipient operations were performed under spontaneous ether inhalation. The left carotid artery was cannulated with a polyethylene catheter for continous monitoring of the mean arterial blood pressure and heart rate during the operation. Another catheter was inserted into the jugular vein for substitution of plasma volume and injection of fluorescent compounds as well as for continuous infusion of glutathione during reperfusion. Body temperature was kept between 36.5 ℃ and 37.5 ℃ by means of a heating pad. Donor livers were preserved by retrograde aortal flush with 10 mL UW-solution and stored at 4 ℃ for 24 h. Prior to the implantation procedure, the livers were rinsed with warm Ringer's lactate solution (10 mL) via the portal vein at a hydrostatic pressure of 10 cm H_2O. Orthotopic liver transplantation was performed using a modified cuff technique[24]. Differing from the original technique, grafted livers were rearterialized and simultaneously reperfused through the portal vein and hepatic artery[25]. Portal clamping time was less than 20 min in all experiments.

The common bile duct of the graft was cannulated with a PE-tube and bile was collected in Eppendorf cups. Plasma samples (500 μL) were obtained in the recipient before hepatectomy and 60 and 120 min after reperfusion of the transplanted liver. The volume of the blood drawn was replaced by saline. Five minutes after starting reperfusion, all rats received 0.5 mL of albumin (5%) and 0.5 mL sodium bicarbonate in order to maintain blood pressure and physiological pH. To avoid major fluid loss and drying of the liver, possibly affecting microhemodynamics the abdominal cavity was covered with Saran wrap throughout the operation. After 120 min of reperfusion, experiments were terminated and the liver weight was determined.

Experimental groups
Two intervention groups (*n*=5 each) were compared with the control group (*n*=8). In control animals, saline was infused intravenously (2 mL/h) during reperfusion of the grafted liver, starting 20 min before declamping of the portal vein and hepatic artery. In contrast, intervention groups received GSH via the jugular vein, starting 20 min prior to reperfusion until the end of the two hour reperfusion period (total infusion time 140 min). GSH (Tationil 600®, Roche Pharmaceuticals/Italy) was administered at 50 or 100 μmol/(h· kg) (*n*=5 each) by continuous infusion (2 mL/h) of stock solutions (0.24 mol/L and 0.48 mol/L), using a micro-infusion pump (SP100i, WPI, Aston, UK). Two sham groups underwent laparotomy and intravital microscopy without hepatic ischemia in the absence (*n*=6) or presence of intravenous GSH infusion (100 μmol GSH/(h· kg), *n*=5) for 140 min.

In-vivo fluorescence microscopy (IVM)
In vivo fluorescence microscopic studies were performed 30 min after revascularization under stable hemodynamic conditions, using a modified Leitz-Orthoplan microscope combined with epi-illumination technique[26,27]. For assessment of microvascular liver perfusion and leukocyte-endothelial interaction the left liver lobe was exteriorized on a stage. For visualization of microcirculatory disturbances, sodium fluorescein (1 μmol/kg; Merck AG, Darmstadt, Germany) and rhodamin 6G (0.1 μmol/kg; Merck AG, Darmstadt, Germany) were injected intravenously for fluorescent staining of hepatocytes (negative contrast for plasma) and leukocytes, respectively. Quantification of microhemodynamic parameters was performed offline by frame-to-frame analysis of the videotaped images in a blinded fashion. Lobular perfusion and leukocyte adherence were analyzed by scanning a region of 10 randomly selected acinar areas and postsinusoidal venules, respectively. *Non – perfused sinusoids* were estimated by counting the number of continuously perfused and non - perfused sinusoids and were expressed as the percentage of all sinusoids visible in a pre-defined area. *Permanent adherence (sticking) of leukocytes to the sinusoidal endothelium* was quantified by counting the number of permanently attached leukocytes (at least for 20 s) within the 3 different segments of sinusoids. *Leukocyte sticking in postsinusoidal venules* was determined by the quantity of leukocytes attached for at least 20 s to the surface of postsinusoidal venules. Furthermore, *temporary adherence of leukocytes (rolling)* was assessed as the number of transiently attached leukocytes along the endothelial surface of postsinusoidal venules during an observation period of 20 s.

Determination of Kupffer cell function
KC function was assessed by determination of their particle phagocytosis as described earlier[28,29]. At the end of IVM analysis, plain fluorescent latex beads in isotonic saline solution were injected as bolus (3×10^8/kg) through the carotid catheter (diameter 1.1 μm, Polysciences Inc., Warrington, PA, USA). Phagocytic activity was then analyzed successively during the first 5 min after injection in 10 randomly selected liver lobules in each experiment. Adherence of latex particles was quantified by counting the number of beads moving in sinusoids as a percentage of all beads visible in the field during an observation period of 10 s. Beads in presinusoidal and postsinusoidal venules were not included in analysis.

Analytical methods

Blood samples of 500 µL were collected for determination of total glutathione (sum of GSH and GSSG) and GSSG. For GSSG analysis, an aliqot (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[30]. The remaining blood was centrifuged at full speed for 1 min. An aliquot (100 µL) of plasma was pipetted into 100 µL sulfosalicylic acid (5%) for determination of total glutathione. To separate GSSG from NEM and NEM-GSH adducts, an aliquot of NEM treated plasma was passed through a Sep-PakC$_{18}$ cartridge (Waters, Framingham, MA, USA) followed by 1 mL of 100 mmol/L phosphate buffer (pH 7.5). GSSG in the eluates and total glutathione in acidified plasma samples was determined by an enzymatic test as described previously[31]. GSH plasma concentrations were calculated as the difference between total glutathione and GSSG.

Serum aminotransferases

Serum aminotransferases were used as established markers of hepatic injury. Aspartarte aminotransferase (AST) and alanine aminotransferase (ALT) were measured 2 h after reperfusion using a serum multiple analyzer (Hitachi 917, Roche Germany).

Transmission electron microscopy

At the end of the experiments, perfusion-fixation of hepatic tissue was performed using a 50 g/L glutaraldehyde/40 g/L paraformaldehyde mixture in phosphate buffer (pH 7.4) via the hepatic artery at a constant pressure of approximately 80 mmHg. Specimens were cut from both the right and left liver lobes and blocks were diced into 1 mm cubes. Samples were stored in fixative for 2 to 3 d prior to further processing. Specimens were postfixed with osmium tetroxide, dehydrated in graded alcohol and embedded in Epon 812. Ultrathin sections were cut and contrasted with uranyl acetate and lead citrate for electron microscopy[11].

Statistical analysis

All data are expressed as mean±SE. Statistical differences between groups were calculated using paired or unpaired Student's t test for randomly distributed data and the Mann-Whitney U test for nonparametric data following analysis of variance (ANOVA). Differences were considered significant at $P<0.05$.
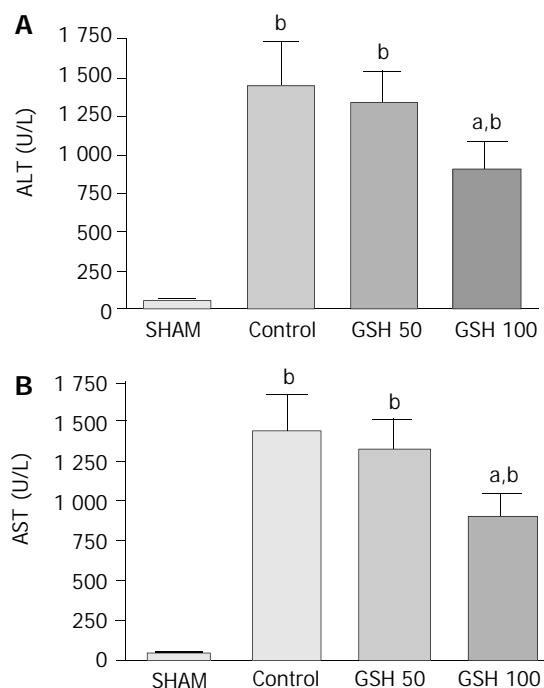
RESULTS

Cell injury and liver function after liver transplantation

Injury of parenchymal and non-parenchymal liver cells after liver transplantation was assessed by the release of ALT and AST as well as by electron microscopic analysis at 120 min of reperfusion. Parenchymal cell damage in untreated animals was indicated by a 25- and 40-fold increase of ALT and AST serum levels, respectively, when compared with sham- operated animals (Figure 1). Continuous intravenous administration of 100 µmol GSH/(h· kg) during reperfusion significantly ($P<0.05$) reduced ALT and AST levels by almost 40% whereas infusion of 50 µmol GSH/(h· kg) had no effect (Figure 1).

Transmission electron microphotographs of the untreated liver grafts after 120 min of reperfusion showed alterations of hepatocytes, including cell edema, a substantial loss of microvilli and generalized swelling of mitochondria (Figure 2A,B). Furthermore, non- parenchymal injury was evident by various degrees of SEC–detachment from the perisinusoidal matrix plate or even complete loss of the sinusoidal endothelial lining (Figure 2 A,B). Consequently, the space of Dissé was enlarged in control livers. In contrast, treatment of animals with 100 µmol/(h· kg) GSH was effective in protecting hepatocytes with only minimal loss of microvilli of normally shaped parenchymal cells and only sporadic mitochondrial swelling. Moreover, GSH prevented SEC detachment, thus resulting in normal spaces of Dissé (Figure 2 C,D).

Function of liver allografts was estimated by recovery of bile flow. Bile flow of sham-operated animals remained constant during the observation period of 150 min and was comparable to bile flow rates of donor livers before ischemia (Figure 3). After transplantation of control livers, bile flow returned to only 45% of baseline values. Intravenous infusion of 50 µmol GSH/(h· kg) had no effect on the postischemic bile flow whereas 100 µmol GSH/(h· kg) significantly ($P<0.05$) increased bile flow during the complete reperfusion period (Figure 3).


Figure 1 ALT (A) and AST (B) plasma levels after liver transplantation. Livers preserved for 24 h in UW solution at +4 ℃ before transplantation. Compared to untreated livers ($n=8$) administration of 100 µmol GSH∕(h· kg) ($n=5$), but not of 50 µmol GSH∕(h· kg) ($n=5$), resulted in a significant decrease of plasma ALT and AST levels at 2 h of reperfusion. Results are mean±SE. [b]$P<0.001$ *vs* sham- operated animals ($n=5$); [a]$P<0.05$ *vs* untreated livers.

Kupffer cell function after liver transplantation

Latex particle phagocytosis by KC reflects their function under various conditions. In agreement with earlier observations, rapid clearance of intra-arterially administered fluorescent latex particles was observed in sham-operated animals. Five minutes after injection only 8.1±2.5% particles were freely movable in the sinusoids. This rate was not influenced by liver transplantation as indicated by 11.2±1.0% of all visible beads, freely movable in the sinusoids of untreated animals. Neither GSH treatment with 50 µmol/(h· kg) nor with 100 µmol/(h· kg) affected phagocytosis of latex particles, resulting in 12.0±1.1% and 10.5±2.0% of beads moving in sinusoids at 5 min upon bolus injection, respectively. Since KC represents approximately 90% of the phagocytic capacity in the liver, these results suggest unaltered KC function after reperfusion of untreated and GSH- treated liver grafts.
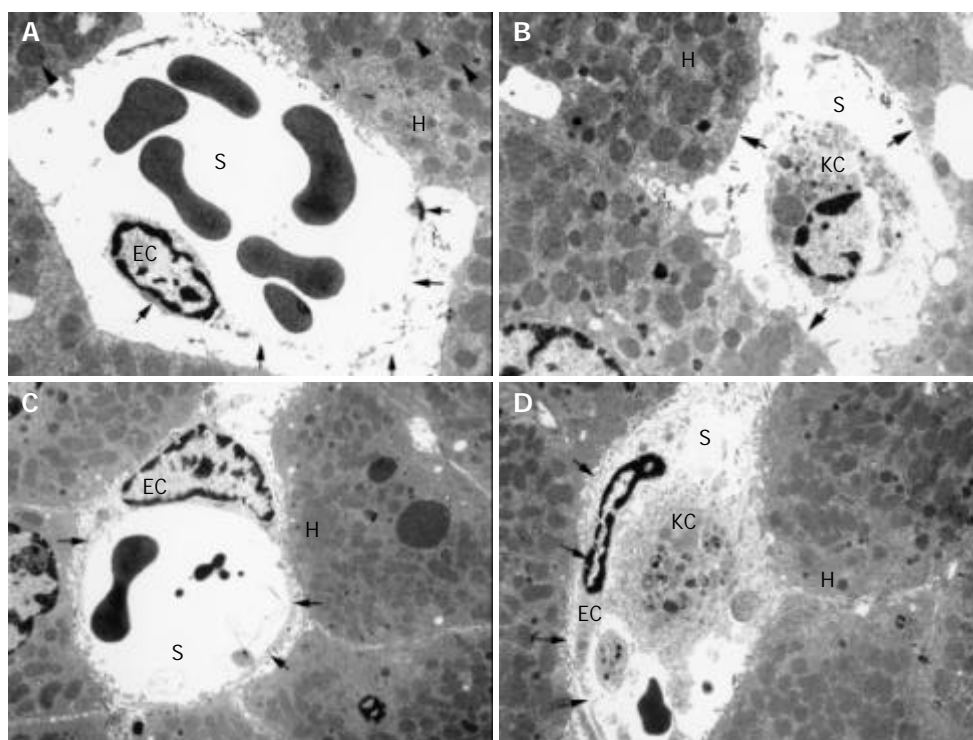
Figure 2 Transmission electron microphotographs of untreated and GSH- treated liver allografts. Arrow-heads indicate mito-chondrial swelling of hepatocytes in untreated livers (A) which was almost absent in allografts treated with 100 µmol GSH/(h· kg) during reperfusion (C). Arrows demonstrate the loss of hepatocyte microvilli in untreated controls (B) whereas GSH administration preserved microvilli in nearly all hepatocyte membranes (D). As indicated by open arrows detachment of SEC (A) as well as its complete loss (B) was evident in untreated livers. GSH- treatment preserved sinusoidal endothelial lining with a normal space of Dissé (C,D). Letters indicate: H, hepatocyte; KC, Kupffer cell; EC, endothelial cell; S, sinusoidal lumen. Bars represent either 1.7 µm (B) or 2.5 µm (A,C,D).
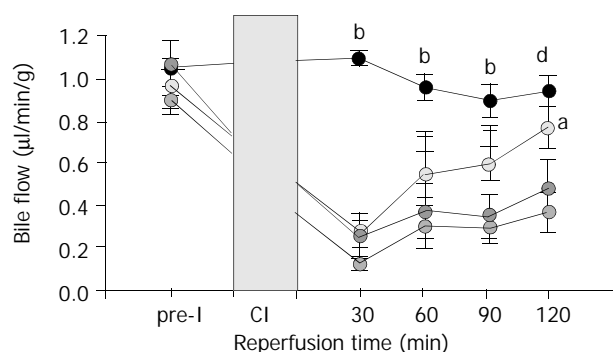
Figure 3 Effect of GSH treatment on bile flow after liver transplantation. Animals were treated with 50 µmol/(h· kg) GSH (◯, *n*=5) or 100 µmol/(h· kg) GSH (◕, *n*=5) and compared with untreated controls (◑, *n*=8) and a sham- operated group (●, *n*=5). Results are mean±SE. [b]*P*<0.001 *vs* untreated or GSH-treated livers; [d]*P*<0.01 *vs* 100 µmol GSH/(h· kg); [a]*P*<0.05 *vs* un-treated controls.

Effect of GSH treatment on the microcirculation of liver allografts

Compared with sham-operated animals, substantial disturbances of the microcirculation were observed in untreated liver allografts (Figure 4). Thirty minutes after starting reperfusion almost 25% of the sinusoids were not perfused. Intravenous infusion of 50 as well as 100 µmol GSH/(h· kg) prevented this no – reflow phenomenon, thereby improving hepatic perfusion (Figure 4 A).

Furthermore, intravital microscopy revealed considerable leukocyte sticking within sinusoids and postsinusoidal venules in untreated liver allografts (Figure 4 B,C). A significant

reduction (*P*<0.001) of stagnant leukocytes in sinusoids was found in both treatment groups (Figure 4 B). Comparable to acinar leukocyte sticking, permanent adherence of leukocytes to postsinusoidal venules was reduced by 57% and 69% (*P*<0.001) when animals received 50 or 100 µmol GSH/(h· kg), respectively (Figure 4 C). Temporary adherence of leukocytes (rolling) in postsinusoidal venules is supposed to be the initiating event in the sequence to permanent adherence to the vascular endothelium. However rolling of leukocytes was slightly increased by GSH treatment (Figure 4E).

Effect of intravenous GSH infusion on plasma GSH and GSSG levels

Sixty minutes after starting reperfusion plasma GSH levels of untreated animals increased 2-fold when compared to sham-operated animals (Figure 5). This was accompanied by a 3-fold increase of plasma GSSG (Figure 5). Infusion of 50 µmol GSH/(h· kg) resulted in a 6-fold increase of plasma GSH whereas GSSG did not exceed levels of untreated controls. In contrast, 100 µmol GSH/(h· kg) elevated plasma GSH as well as GSSG by up to 9-fold above values obtained in the sham-group (Figure 5). Moreover, plasma GSSG increased to significant higher levels when compared with untreated or GSH – treated (50 µmol/(h· kg)) animals. In order to estimate the role of spontaneous oxidation of intravenously applied GSH a second group of sham-operated animals was treated with 100 µmoL GSH/(h· kg). This resulted in marked increase of plasma GSH to 93±10 mol/L which was similar to that observed in liver allografts (97±18 mol/L). However, plasma GSSG increased to only 3.0±0.9 in contrast to 7.5±0.9 mol/L in GSH-treated liver allografts. These findings clearly indicate enhanced oxidation of intravenously applied GSH at the cytoprotective dose of 100 µmol/(h· kg).
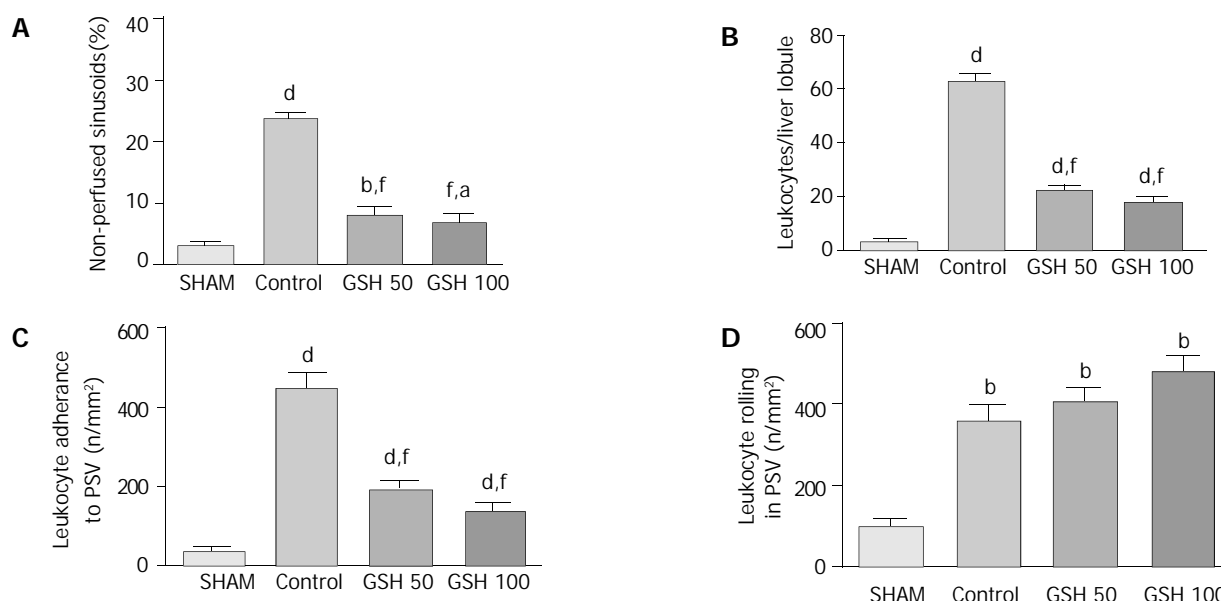
Figure 4 Impact of GSH treatment on the hepatic microcirculation after liver transplantation. Data from *in vivo* microscopy were obtained 30 min after starting reperfusion comparing untreated controls ($n=8$) with GSH- treated allografts (50 µmol/(h· kg) and 100 µmol/(h· kg); each $n=5$) as well as to a sham group ($n=5$). (A) Acinar perfusion failure is indicated as the percentage of non-perfused sinusoids (no reflow). (B) Leukocyte-adhesion (sticking) to sinusoids and (C) to postsinusoidal venules. (D) Temporary attachment of leukocytes (rolling) to the endothelium of postsinusoidal venules (PSV). Results are mean±SE. [a]$P<0.05$, [d]$P<0.01$ and [b]$P<0.001$ *vs* sham group. [f]$P<0.001$ *vs* control group.
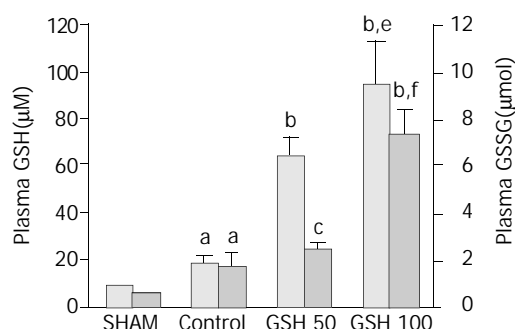
Figure 5 Plasma GSH- and GSSG concentrations following liver transplantation. Compared to sham-operated animals ($n=5$) a significant increase of reduced and oxidized glutathione was determined upon reperfusion of untreated grafts ($n=8$). Intravenous infusion of GSH at rates of 50 µmol/(h· kg) ($n=5$) and 100 µmol/(h· kg) ($n=5$) resulted in a dose- dependent increase of plasma GSH concentrations. The corresponding GSSG levels showed only a slight increase after administration of 50 µmol GSH/(h· kg), but increased markedly following infusion of 100 µmol GSH/(h· kg). Mean±SE. [a]$P<0.05$ *vs* sham; [b]$P<0.001$ and [c]$P<0.05$ *vs* control group; [e]$P<0.05$ *vs* GSH 50 and [f]$P<0.01$ *vs* GSH 50.

DISCUSSION

Recently, we have found that treatment of cold preserved livers with GSH upon reperfusion prevented reperfusion injury in the model of isolated rat liver perfusion[23]. Due to several limitations of this experimental model, such as the absence of plasma antioxidants and the lack of leukocytes[32,33], the potential protective effect of GSH treatment during liver transplantation remained to be defined. We also found that intravenous GSH treatment during liver transplantation prevents reperfusion injury of the liver allograft.

We obtained the following main results: (1) increase of the vascular antioxidant capacity by intravenous infusion of GSH causes a significant reduction of injury to hepatocytes and SEC as well as of (2) microcirculatory disturbances in liver allografts, and (3) Administration of GSH does not affect

KC function but enhances detoxification of KC-derived ROS as indicated by a marked increase of plasma GSSG.

Prevention of reperfusion injury to liver allografts by GSH
Intravenous administration of GSH at a dose of 100 µmol/h/kg during the reperfusion phase of liver transplantation significantly reduced ALT and AST release by approximately 40% indicating the protection of the liver allografts. This contention is supported by distinct signs of parenchymal and non-parenchymal cell injury under electron microscopy already after 2 h of re-establishing blood flow. Untreated livers showed a complete detachment of SEC from the perisinusoidal matrix which in part determines graft viability[4-7]. In contrast, detachment of SEC was dramatically attenuated in GSH-treated livers, indicating preservation of the sinusoidal architecture. Furthermore, GSH treatment markedly reduced mitochondrial swelling and the loss of microvilli in hepatocytes. These cytoprotective effects were accompanied by a marked improvement of postischemic liver function as assessed by a 2.5-fold increase of bile flow. Because postischemic GSH treatment can only protect cells which are not already seriously damaged before the onset of reperfusion, our results clearly demonstrate prevention of reperfusion injury to parenchymal and non-parenchymal liver cells by GSH.

Prevention of reperfusion injury by GSH depended on the GSH dose: 100 µmol/(h· kg) but not 50 mmol/(h· kg) showed protection. Based on steady state GSH plasma concentrations following GSH infusion, a concentration of approximately 100 mol/L appears to be pivotal for cytoprotection. In view of these findings, GSH seems as ideal and presumably safe candidate drug for prevention of reperfusion injury of the liver allograft since plasma GSH levels up to 500 mol/L showed no toxicity in humans[19].

Mechanisms of GSH – mediated protection
Effect on Kupffer cell function There is extensive experimental evidence for KC activation as a central pathomechanism of reperfusion injury of the liver after warm or cold ischemia[8,9]. To assess KC function we used the tool of intravital microscopic

analysis of latex particle phagocytosis[28,29]. In agreement with earlier studies[29] particle phagocytosis was not affected by cold preservation indicating functionally intact Kupffer cells in liver allografts. Furthermore, clearance of latex particles was not affected by postischemic administration of GSH. These results argue against a suppression of KC function as a potential protective mechanism of GSH action. Thus, potential hazards of other protective approaches interfering with vital host-defense function of KC (*e.g.* calcium channel blocker, KC-depleting agents;[8,34,35]) would be avoided by GSH treatment.

Detoxification of ROS in the hepatic vasculature Intravenous administration of GSH results in the degradation of GSH to its amino acids which are the again available for GSH synthesis in various organs including the liver[36]. However several studies demonstrated no relevant increase of the hepatic GSH content 2 h after starting high-dose GSH infusion[22,23]. These findings suggest that GSH effects are related rather to the increase in the extracellular antioxidant capacity than to influences on intracellular GSH. In line with this interpretation we obtained evidence for an extracellular mechanism of GSH-mediated cytoprotection.

The increase in plasma GSSG observed during reperfusion of untreated and GSH-treated livers was associated with a marked increase in plasma GSH. This parallel increase in GSH and GSSG suggests that GSSG resulted from extracellular oxidation rather than from intrahepatic generation of GSSG[37,38]. Accordingly, previous studies with the blood – free perfused rat liver found only a very small release of GSSG into the perfusate during reperfusion[39]. Furthermore, there was no evidence for a relevant increase of intracellular GSSG formation[8,39]. Thus, plasma GSSG formation observed during reperfusion of liver allografts is likely to take place extracellularly. This could be due to spontaneous as well as reactive oxygen species-related oxidation of GSH. The results of the present study argue against a predominant role for spontaneous GSH oxidation. First, intravenous infusion of 50 μmol GSH/(h· kg) during reperfusion of liver allografts resulted in a several fold increase of plasma GSH whereas plasma GSSG did not exceed the levels of untreated controls. Second, a comparable increase of plasma GSH was observed when sham-operated or transplanted animals were treated with 100 μmol GSH/(h· kg). In contrast, a 2.5-fold higher increase of plasma GSSG was determined in transplanted rats. This difference clearly indicates that spontaneous oxidation of intravenously applied GSH contributes only in part to the increase in plasma GSSG. Based on these findings oxidation of GSH by ROS appears as a major determinant of plasma GSSG formation following liver transplantation. Consequently, the marked increase of plasma GSSG during infusion of the protective GSH dose of 100 μmol/(h· kg) most likely reflects an enhanced detoxification of ROS in the hepatic vasculature.

Activated KC have been identified as potential source of extracellular ROS formation and GSH oxidation during reperfusion after warm hepatic ischemia[8,40]. Accordingly, there was no evidence for plasma GSSG formation during reperfusion of KC-depleted liver allografts[41]. These findings suggest that intravenously applied GSH protects liver allografts against the vascular oxidant stress produced by KC during reperfusion. This interpretation is further substantiated by the observation that externally added GSH and catalase protected leukocyte- free perfused rat livers against oxidant cell injury following selective KC activation[42,43]. In this experimental model cytoprotection was achieved by vascular GSH concentrations of 100 mol/L, but not by 50 mol/L. The same vascular concentrations of GSH were achieved in the present study. Again, protection of liver allografts was only evident at plasma GSH concentrations of approximately 100 mol/L and was associated with a considerable extra-formation of plasma GSSG. These findings suggest that plasma GSH above a critical level may act as a "sink" for ROS produced by KC during reperfusion. This could be due to non-enzymatic reactions between GSH and various ROS which strongly depend on the GSH concentration[29,44,45].

Prevention of microcirculatory perfusion failure and SEC injury GSH of both doses remarkably improved the hepatic microcirculation. In particular, the number of perfused sinusoids increased to that observed in non-ischemic livers indicating prevention of the no-reflow phenomenon. This could be related to the counteraction of ROS-mediated mechanisms of vasoconstriction and leukocyte adherence to sinusoids and postsinusoidal venules[12-16]. In accordance with the proposed stepwise process of leukocyte-endothelial interaction from leukocyte rolling as an initial event to permanent attachment of leukocytes as the second step[46], our results indicate GSH as an inhibitor of the second step: treatment with GSH prevented sticking but slightly increased the rolling of leukocytes. Similar effects can be achieved by the administration of superoxide dismutase[12,13,16], suggesting superoxide anion ($O_2^{-\bullet}$) as the contributing mediator of leukocyte adherence. Therefore, it seems likely that GSH prevents leukocyte adherence through reaction with $O_2^{-\bullet}$ or inhibition of $O_2^{-\bullet}$ - mediated mechanisms of leukocyte adherence. Protection of leukocyte adherence by 50 μmoL GSH/ (h· kg) without evidence for additional GSSG formation does not argue against this concept because the reaction of GSH with $O_2^{-\bullet}$ can result in the formation of glutathione sulfonate[44].

However, the antioxidative potential of GSH does not rule out beneficial effects through non-antioxidative properties. Accumulating evidence indicates that detachment of SEC from the perisinusoidal matrix plate represents a critical component of preservation injury and postischemic microcirculatory failure[5,47,48]. It has been proposed that SEC damage occurs independent of ROS formation because several antioxidants failed to prevent SEC injury in the liver allograft[6]. SEC detachment is caused by various matrix metalloproteinases (MMPs) released into the extracellular compartment during cold liver preservation. In the present study attachment of SEC to their matrix was impressively preserved by GSH and could contribute to the improvement of the hepatic microcirculation. Recent experiments demonstrated the inhibition of MMPS by GSH *in vitro*. These findings suggest that MMPs were possible targets of GSH in liver allografts but this requires further investigations.

In summary, the present study demonstrates a dose-dependent prevention of reperfusion injury in rat liver allografts by postischemic intravenous administration of GSH. Treatment of recipient animals with GSH prevented reperfusion injury to SEC as well as to hepatocytes, markedly improved the hepatic microcirculation and preserved postischemic liver function. GSH-mediated protection of liver allografts was associated with an increased formation of plasma GSSG providing evidence for an accelerated detoxification of ROS by intravenously applied GSH. Therefore, intravenous administration of GSH appears to be a candidate therapy for the prevention of ROS-related reperfusion injury after liver transplantation, in particular since it has a low toxicity in humans and seems cost-effective.

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