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ORIGINAL ARTICLE

Danshen protects liver grafts from ischemia/reperfusion injury in experimental liver transplantation in rats

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Summary

Reperfusion injury remains one of the major problems in transplantation. Free radicals and disturbance of microcirculation are the supposed main contributors. Recent evidence shows that Danshen, a traditional Chinese drug used in vascular diseases, can scavenge radicals and improve microcirculation. This study investigates its effect on liver transplantation (LTx). Before organ recovery, female Sprague-Dawley rats (210–240 g) received intravenous Danshen or the same volume of Ringer solution as control. LTx was performed after 1 h of cold storage. Microperfusion, leukocyte-endothelium interaction and latex-bead phagocytosis were evaluated with *in vivo* microscopy. Survival, transaminases and histology were assessed. Immunohistology was used for TNF-α levels. ANOVA and Fisher's exact test were employed for statistical analyses as appropriate. Survival increased from 60% in controls to 100% ($P < 0.05$). AST and LDH decreased from 3969 ± 1255 U/l and 15444 ± 5148 U/l in controls to 1236 ± 410 U/l and 5039 ± 1594 U/l, respectively ($P < 0.05$). *In vivo* microscopy revealed decreased leukocyte-adherence and increased blood flow velocity in sinusoidal zones after administration of Danshen ($P < 0.05$), while latex-bead phagocytosis was found in 60% of controls ($P < 0.05$). The TNF-α index decreased from 2.08 ± 0.09 in controls to 1.09 ± 0.09 ($P < 0.05$). This study clearly demonstrates hepatoprotective effects after experimental LTx, which can be explained via anti-oxidative effects, improved microcirculation and decreased Kupffer cell activation.

Introduction

Ischemia/reperfusion injury (IRI) is one of the main contributors to poor initial graft function, primary dysfunction (PDF) and primary nonfunction (PNF) after liver transplantation (LTx) [1]. Because of increased usage of marginal grafts, PDF and PNF may become more common [2,3]. Therefore, protocols have to be developed that ameliorate IRI after LTx.

Activated Kupffer cells, disturbance of hepatic microcirculation and cellular interaction between platelets, leukocytes and endothelial lining cells are some of the suspected mechanisms involved in the development of IRI [4–9]. Most substances that have been demonstrated to ameliorate IRI under experimental conditions could not be introduced in clinical routine because of potential toxicity or being otherwise inapplicable [10,11]. Danshen (DS), a traditional Chinese drug which has been approved by the Chinese National Drug Administration as a standard treatment for diseases such as stroke, myocardial infarction, occlusive vasculitis and atherosclerosis has been available as a standardized substance for more than

a decade now in eastern Asia. DS is derived from the dried root or rhizome of Salvia miltiorrhizae Bunge. Extensive experimental research has proven that DS improves circulatory status and eliminates blood stasis. Anti-oxidative and anti-inflammatory effects as well as inhibition of platelet aggregation and protection of liver microsomes, hepatocytes and erythrocytes have been described [12–16].

Thus, this study was designed to investigate the effect of DS on survival, microcirculation, leukocyte-endothelial interaction, Kupffer cell activation and laboratory findings after experimental LTx.

Materials and methods

Experimental design and treatment

Female Sprague-Dawley rats (210–240 g) were used as organ donors and also as recipients. The animals were housed at the veterinary care facility of the University of Heidelberg Medical Center (Heidelberg, Germany), maintained in a 12 hours:12 hours light:dark cycle, and had access to water and standard rat chow (ssniff R/M-H; ssniff Spezialdiäten, Soest, Germany) *ad libitum*. All experiments were approved by the animal care committee of the University of Heidelberg Medical Center. German regulations for the care and use of laboratory animals were followed at all times.

For anesthesia, Narcoren® 20 mg/kg body weight (100 ml Narcoren includes Pentobarbital-Natrium 16.0 g, Benzylalkohol 3.0 g; Merial GmbH, Hallbergmoos, Germany), and Ketanest® 100 mg/kg body weight (Esketaminhydrochlorid; Parke-Davis GmbH, Berlin, Germany) were used. Ten minutes before organ harvest, donors were randomly divided in two groups. One group received 1.5 ml standardized DS-extract [1.5 mg/ml, Shanghai Tongyong Medical Company lt. (Z20027936, Nr. 030214, Shanghai, China)] via inferior vena cava (IVC). To allow identification of effects that can be attributed exclusively to Danshen, the same volume of Ringer solution was used as a control with no anti-oxidative properties. After organ harvest, liver grafts were stored for 1 h in Ringer solution at 4 °C. Subsequently, LTx was performed. The time to homogenous blood distribution in the transplanted liver [time to red (TTR)] was measured. After complete reperfusion, *in vivo* microscopy (IVM) was performed and intrahepatic microcirculation and leukocyte-endothelial interaction was assessed. Eight hours after transplantation, samples of blood and liver tissue were taken.

To prevent any influence on the other parameters, individual groups of animals have been used exclusively for each parameter, i.e. control and experimental groups for blood sampling, tissue sampling, survival, and *in vivo* microscopy. In each of these groups, the number of animals (n) was at least 10.

Survival

Survival of 1 week was considered to be permanent [17]. Thus, survival was compared 1 week after LTx.

Harvest procedure

Surgical dissection, including separating the organ from ligaments and cannulation of the bile duct was standardized and performed by the same surgeon for all animals. After dissection, livers were perfused *in situ* until blood free with 10 ml of cold Ringer solution via the portal vein. After explantation, cuffs were attached to the infrahepatic vena cava and portal vein. Splints were inserted into the bile duct and hepatic artery as described elsewhere [18].

Transplantation

Orthotopic LTx was performed with arterialization as described elsewhere [18]. Briefly, after rinsing the grafts with 10 ml of Ringer solution (18 °C), the suprahepatic vena cava was connected with a running 7/0 Prolene® suture (Ethicon GmbH & Co. KG, Norderstedt, Germany). Cuffs were inserted into the corresponding vessels, and the bile duct and hepatic artery were anastomosed over an intraluminal polyethylene splint. Clamping time of the portal vein was 16–20 min. The whole procedure required less than 40 min [18].

Enzyme assays

Eight hours after transplantation, blood samples were collected from the IVC and serum was obtained by centrifugation. Samples were stored at −20 °C until analysis. AST and LDH levels were determined by standard enzymatic methods [19].

Histology

Eight hours after LTx, organs were fixed by perfusion with 5% paraformaldehyde in Krebs-Henseleit bicarbonate buffer (118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl and 1.3 mM $CaCl_2$, pH 7.6). After paraffinizing, cuttings were processed for light microscopy by standard hematoxylin and eosin staining. Liver damage was assessed as described elsewhere by estimation of the proportion of necrotic to non-necrotic areas [4]. Briefly, five viewfields were selected randomly at 20× magnification from at least four different sections per sample. Sections were evaluated by a point counting method using an ordinal

scale as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophilic infiltration. To determine the percentage of necrotic cells, 40 sections were evaluated per slide. To estimate vascular leukocytic infiltration, leukocytes per viewfield were counted at 40× magnification and stratified on a scale for each hepatic zone and graded from 0 to 4: grade 1, <10 leukocytes (focal infiltration); grade 2, 10–20 leukocytes (mild infiltration); grade 3, 21–50 leukocytes; grade 4, >50 leukocytes. Mean values were calculated both for indices of liver damage and leukocytic infiltration.

In vivo fluorescence microscopy

Polypropylene catheters (Braun, Melsungen, Germany) were placed in the left jugular vein and carotid artery to enable i.v. application of contrast media, volume replacement and blood pressure monitoring respectively. During IVM, central body temperature of the rats was monitored and kept between 35.8 and 36.5 °C. The hepatic microcirculation was observed *in vivo* at the upper surface of the left liver lobe with a modified inverted Leitz-Orthoplan microscope (Leitz, Wetzlar, Germany) and epi-illumination technique as described elsewhere [20]. FITC-labeled erythrocytes were used to asses liver microcirculation (3 μmol/kg body weight; FITC Isomer 1; Sigma, Steinheim, Germany). Simultaneously, rhodamine 6G (0.05 mmol/kg body weight; Sigma) was given i.v. to enable leukocyte staining.

Sinusoidal perfusion was analysed immediately after reperfusion and the perfusion rate was calculated as $\frac{N_G+0.5\times N_I}{N_T}$, where N_G represents the number of well-perfused sinusoids, N_I the number of irregularly perfused sinusoids and N_T the total number of analysed sinusoids [20].

Erythrocyte velocity and mobility were determined by manual tracing of erythrocyte paths on video images. A computer-based morphometry system was used to measure start, end and distance covered on digitized images [21,22].

Blood flow was calculated from red blood cell velocity and diameter measurements described by Uhlmann *et al.* [21].

Moreover, sinusoidal perfusion as well as temporary (rollers) and permanent adherent leukocytes (stickers) in 10–15 randomly selected acini and at least five venules were observed in each animal. Rollers were defined as leukocytes moving along the vessel wall of postsinusoidal venules with a velocity of less than 30% of central blood flow velocity and stickers were defined as leukocytes adhering for more than 20 s to the endothelium. Leukocyte velocity within postsinusoidal venules was assessed as a mean value from 10 measurements (analysed in five venules) in each animal.

Latex-bead phagocytosis was assessed at the end of *in vivo* microscopy and used as a surrogate parameter for Kupffer cell activation. Fluorescent latex beads (3×10^8 beads/kg body weight, diameter = 1.1 μm; Polysciences Inc., Warrington, PA, USA) were infused into the left jugular vein through the polyethylene catheter. Ten randomly selected microscopic fields were analysed successively within the first 3 min after injection. The number of latex beads-positive Kupffer cells was counted per microscopic field and the mean value was calculated.

Immunohistochemistry

Eight hours after transplantation, livers were explanted, shock-frozen in liquid nitrogen and stored at −80 °C for later analysis. Immunohistochemistry for TNF-α was performed on cryosections (6 μm) of liver tissue. Monoclonal anti-TNF-α antibody (R&D Systems, Minneapolis, MN, USA) at dilution 1:100 and LSAB kit (DAKO, Carpinteria, CA, USA) was used. TNF-α positive cells were assessed in 10 microscopic areas per slide and slides were evaluated semi-quantitatively with scores from 0 to 4 corresponding to the percentage of stained cells: 0 corresponding to 0%; 1 to less than 5%; 2 to 5–20%; 3 to 20–40% and 4 to more than 40% of cells [23].

Statistics

Fisher's exact test and analysis of variance (ANOVA) were used as appropriate. $P < 0.05$ was considered significant. Data are represented as mean values ± SEM.

Results

General data

Blood pressure (104 ± 2 mmHg), hematocrit (46.4 ± 2.3%) and body temperature (36.0 ± 0.2 °C) were comparable in both groups during all experimental phases.

Survival

Control survived in 60% of cases. After administration of DS, survival increased to 100% ($n = 10$ animals/group, $P < 0.05$) (Fig. 1). Survival rates did not change during the second week of observation.

Liver injury

Eight hours after transplantation, AST and LDH levels were decreased after Danshen (median 1009 U/l; IQR: 876–1497

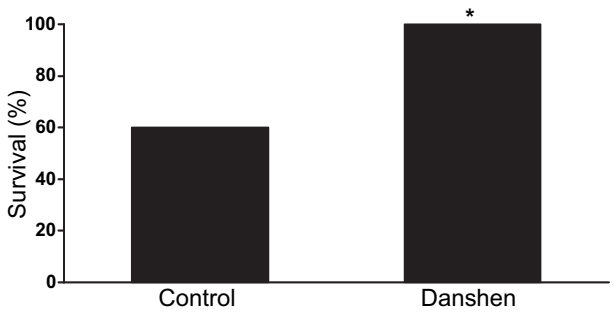

Figure 1 Survival. Seven days after liver transplantation, survival was compared. More than 7 days survival was considered to be permanent. Values are percentages of surviving animals after 7 days (*$P < 0.05$ by Fisher's exact test, $n = 10$ per group).

and 3154 U/l; IQR: 1257–5512) to approximately one-third of control values (median 2084 U/l; IQR: 1757–6308 and 17 633 U/l; IQR: 7650–18 661) (Fig. 2a and b).

Leukocyte-endothelium interaction

Leukocyte accumulation was observed in sinusoids in all subacinar zones and in postsinusoidal venules after LTx in all animals (Table 1, Fig. 3a and b). After DS, the number of permanent adherent leukocytes decreased in both sinusoids and venules from 107 ± 8/mm^2 and 650 ± 83/mm^2 in controls to 15 ± 3/mm^2 and 108 ± 20/mm^2 (Table 1, Fig. 3a and b; $P < 0.05$). Separate analysis of subacinar zones revealed a significant increase of stickers in controls from zone 1 to zone 3 after transplantation. Similar effects were observed on rollers in venules (Table 1, Fig. 4; $P < 0.05$).

Sinusoidal perfusion

Sinusoidal perfusion was significantly increased in DS-treated grafts (Table 2; $P < 0.05$). Moreover, DS decreased the number of nonperfused sinusoids within perfused acini to 40% of the level of controls (Table 2; $P < 0.05$).

While blood flow and RBC velocity decreased after reperfusion in all sinusoids of controls, DS markedly increased blood flow and velocity of RBCs in different sinusoidal zones by 40% and 20% respectively (Table 2). In addition, DS significantly expanded the diameter of capillary vessels in all sinusoidal zones (Table 2).

TTR decreased from 142 ± 44 s in controls to 61 ± 19 s in DS-treated animals ($P > 0.05$).

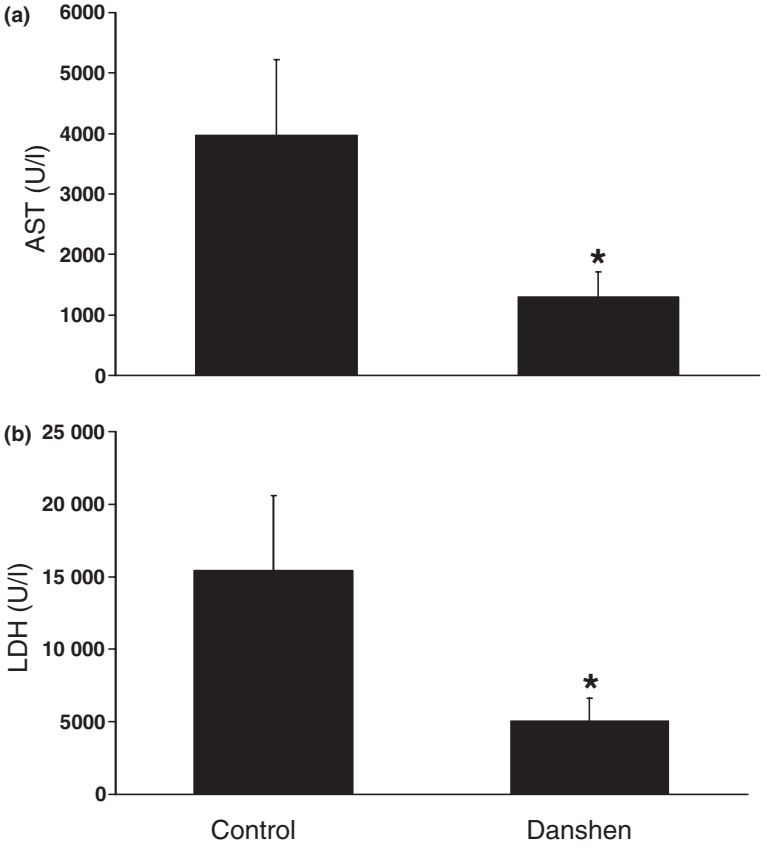

Figure 2 Effects of Danshen on (a) AST and (b) LDH after liver transplantation. Eight hours after transplantation, blood was collected from the inferior vena cava and serum levels of AST and LDH were detected by standard laboratory methods. Values are mean ± SEM (*$P < 0.05$ by two-way ANOVA with Student-Newman-Keuls *post hoc* test, $n = 10$ per group).

Table 1. Leukocyte-endothelium interaction in control and Danshen-treated animals.

Parameter	Control	Danshen
Stickers in sinusoids (n/mm^2)	107.14 ± 7.58	14.96 ± 3.40*
Pericentral	117.75 ± 16.00	17.42 ± 5.42*
Midzonal	90.86 ± 13.39	20.38 ± 4.08*
Periportal	72.07 ± 14.71	20.37 ± 4.07*
Stickers in venules (n/mm^2)	650.22 ± 82.73	108.43 ± 20.22*
Rollers in venules (n/mm^2)	185.79 ± 42.38	22.51 ± 13.67*

Values are mean ± SEM; *$P < 0.05$ for controls versus Danshen.

Phagocytosis of latex-beads

The number of latex beads phagocytized by Kupffer cells was compared between groups and used as a parameter indicating the activation of these cells. DS significantly reduced phagocytosis after transplantation to 60% of the level of controls ($P < 0.05$; Fig. 5).

TNF-α

TNF-α levels were detected from liver tissue taken 8 h after reperfusion. TNF-α expression of DS-treated grafts was 57% of control levels (Table 3; Fig. 6).

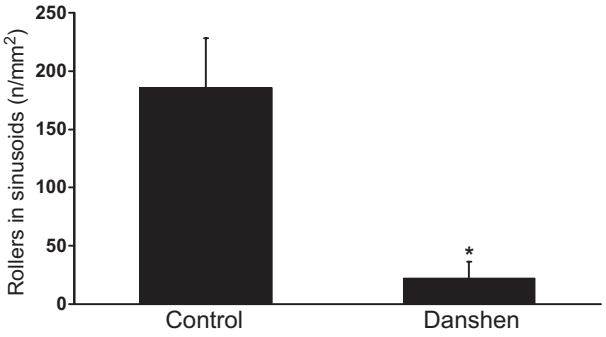

Figure 4 Transient leukocyte adherence in venules. Leukocyte adherence immediately after reperfusion was compared in venules. Rollers were defined as leukocytes moving along the wall of postsinusoidal venules with a velocity of less than 30% of central blood flow. Rollers were counted in at least five venules per each animal. Values are mean ± SEM (*$P < 0.05$ by two-way ANOVA with Student-Newman-Keuls *post hoc* test, $n = 8$ per group).

Histology

Inflammatory response after reperfusion as estimated by leukocytic infiltration index decreased significantly (2.2 ± 0.03 in controls and 1.1 ± 0.06 after DS, $P < 0.05$; Table 3). Necrosis and liver damage were assessed with H&E-stained liver tissue 8 h after reperfusion. Mean

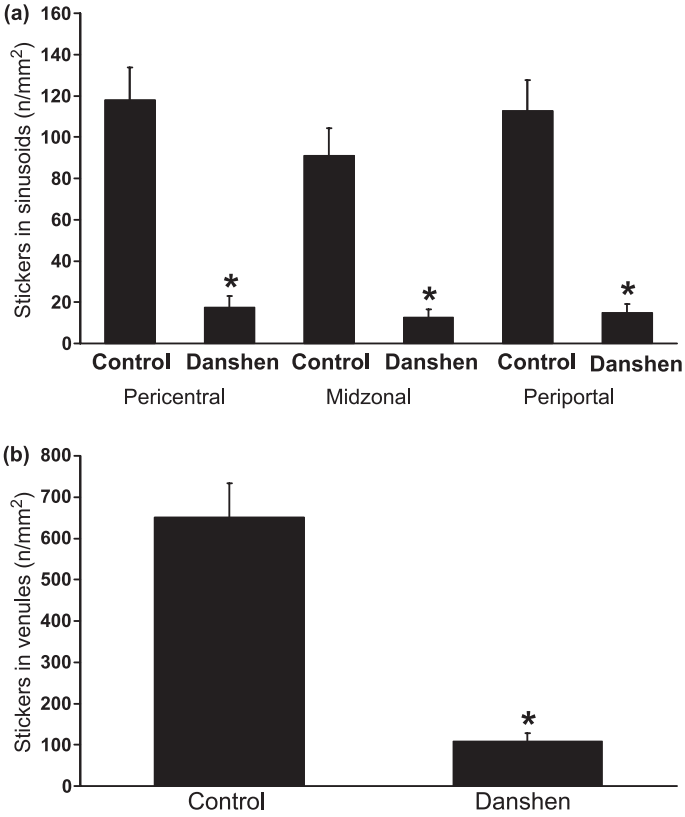

Figure 3 Permanent leukocyte adherence in (a) venules and (b) hepatic sinusoids. Acinar zones have been stratified to pericentral, midzonal and periportal areas. Leukocyte adherence immediately after reperfusion was compared. Stickers were defined as leukocytes adhering for more than 20 s to the endothelium and were counted in 10–15 randomly selected acini and at least five venules in each animal. Values are mean ± SEM (*$P < 0.05$ by two-way ANOVA with Student-Newman-Keuls *post hoc* test, $n = 8$ per group).

Table 2. Hepatic microcirculation in control and Danshen-treated animals.

Parameter	Control	Danshen
Sinusoidal perfusion		
Perfusion index	0.94 ± 0.02	0.98 ± 0.06*
Nonperfusion sinusoidal	4.7 ± 1.5	1.9 ± 0.06*
Sinusoidal speed (mm/s)		
Periportal	0.21 ± 0.01	0.31 ± 0.02*
Midzonal	0.29 ± 0.01	0.40 ± 0.02*
Pericentral	0.43 ± 0.02	0.57 ± 0.03*
Sinusoidal diameter (μm)		
Periportal	8.35 ± 0.11	10.62 ± 0.13*
Midzonal	9.85 ± 0.11	13.46 ± 0.16*
Pericentral	10.88 ± 0.12	14.66 ± 0.18*
Sinusoidal flow [$mm^2/s \times 10^{-6}$]		
Periportal	11.52 ± 1.34	28.86 ± 5.38*
Midzonal	22.80 ± 2.57	59.05 ± 8.32*
Pericentral	40.65 ± 4.82	100.74 ± 13.99*

Values are mean ± SEM; *$P < 0.05$ for controls versus Danshen.

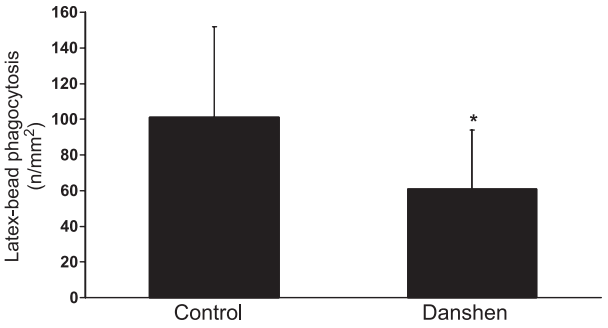

Figure 5 Latex-bead phagocytosis. Latex-bead phagocytosis at the end of *in vivo* microscopy. Fluorescent latex beads were infused into the left jugular vein. Ten randomly selected microscopic fields were analysed within the first 3 min after injection. The number of latex beads positive Kupffer cells was counted per microscopic field and the mean value was calculated. Values are mean ± SEM (*$P < 0.05$ by two-way ANOVA with Student-Newman-Keuls *post hoc* test, $n = 8$ per group).

values of necrosis and liver damage index were 22.5 ± 2.4% and 2.8 ± 0.12 in controls and 10.1 ± 1.2% and 1.4 ± 0.05 after DS respectively ($P < 0.05$; Table 3).

Discussion

Danshen (DS), long used in traditional Chinese medicine, has been introduced in the 1960s as a standardized extract. Since then, DS has been widely used in clinical settings in China as an effective remedy for cerebrovascular disorders, angina pectoris and hypertension with only minor side-effects [24]. Its components provide a wide spectrum of anti-oxidant activity.

Despite high standardization, individual doses of compounds may vary. Therefore, beneficial effects of DS may differ even when different batches from the same manufacturer are used. Further, it should be addressed before any clinical study on this promising substance that some of DS' compounds may alter the efficiency of other drugs through cytochrome-peroxidase-dependent or other pathways [25]. While because of the once-only application to the donor before organ harvest this may not be relevant for immunosuppressive regimens applied to the recipient, there may be other interactions which influence organ function.

Tanshinone and tanshinol derivatives protect against lipid peroxidation *in vitro* and *in vivo* [14,26–28]. Three of the water-soluble components, salvianolic acid A, salvianolic acid B and rosmarinic acid, inhibit NADPH, vitamin C- and Fe (II)-cysteine-induced lipid peroxidation in microsomes and the production of superoxide in a xanthine oxidase system [14,29]. As a radical scavenger, DS improves microcirculation and reduces injury [14].

Table 3. Necrosis and semi-quantitative histology indices.

Parameter	Control	Danshen
Leukocyte infiltration index	2.2 ± 0.03	1.1 ± 0.06*
Liver damage index	2.8 ± 0.12	1.4 ± 0.05*
TNF-α index	2.08 ± 0.09	1.19 ± 0.09*
Necrosis (%)	22.5 ± 2.4	10.1 ± 1.2*

Values are mean ± SEM; *$P < 0.05$ for controls versus Danshen.

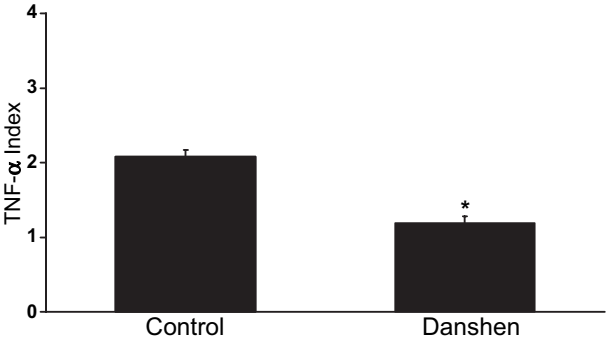

Figure 6 TNF α-Index after transplantation. TNF α-expression was graded on a scale from 0 to 4 by using microscopic criteria as described in Materials and methods. Values are mean ± SEM (*$P < 0.05$ by two-way ANOVA with Student-Newman-Keuls *post hoc* test, $n = 8$ per group).

Most recently, DS has been shown to protect kidney grafts from IRI; however, mechanisms involved in the development of IRI are not identical based on underlying cellular and biochemical cascades [1,29].

This study clearly shows that DS improves graft survival and reduces hepatic injury after experimental LTx.

LTx is the only available curative therapy in a number of different diseases, e.g. acute liver failure, metabolic diseases and end-stage liver disease. One of the major problems in LTx is IRI, which may be the main contributor to organ dysfunction or graft nonfunction. IRI is characterized by multiple pathophysiologic reactions and involvement of different cells. The pivotal role of disturbed microcirculation, adhesion of leukocytes to the endothelium and Kupffer cell activation in the development of reperfusion injury with subsequent graft failure has been demonstrated in recent studies [30–37].

Danshen effectively reduces liver injury by improving hepatic microcirculation (Table 2) and decreasing leukocyte-endothelial interactions (Table 1; Fig. 3a and b). A survival of 60% was in controls as a standard because of cold storage in Ringers. Further, gentle organ manipulation must be discussed as another factor for poor graft and overall survival after experimental LTx as demonstrated elsewhere [8,9,17,33,34,38,39]. To protect the graft at the earliest time point possible during the process of LTx, the donors underwent preconditioning with Danshen prior to organ harvest.

Graft survival in DS-treated animals increased from 60% in controls to 100% in DS-treated animals (Fig. 1; $n = 10$ animals/group; $P < 0.05$). Necrosis as a direct marker of tissue damage and leukocytic infiltration as a marker of inflammatory reactions decreased significantly in DS-treated animals after liver reperfusion (Table 3). Kupffer cell activation, directly and indirectly assessed by TNF-α index and latex-bead phagocytosis, respectively, was significantly reduced as compared with controls (Table 3, Fig. 5).

Danshen improves liver microcirculation after LTx

Microcirculatory disturbances are pivotal contributors to early graft failure and graft nonfunction after LTx [4,40]. Our present study shows that DS significantly improves microcirculation after LTx (Table 2). TTR, as an easily assessable but inaccurate marker, was decreased.

Danshen effectively scavenges oxygen radicals which are generated after reperfusion by the xanthine oxidase system, leukocytes and Kupffer cells [41,42]. Oxygen radicals attack the cell membrane and rupture the DNA and also lead to the generation of many inflammatory mediators, including platelet activating factor (PAF) and thromboxane A2 (TXA2) amongst others, which also are hazardous to the cell [43]. Oxygen radicals up-regulate P-selectin, activate leukocytes and Kupffer cells and lead to disturbed microcirculation, which again results in the production of more oxygen radicals and thus forms a self-perpetuating cycle [44]. Kupffer cell- and neutrophil-induced oxidative stress plays an important role in vascular and parenchymal cell injury during reperfusion. Sinusoidal microcirculation during the reperfusion period is impaired mainly by excessive formation and action of vasoconstrictors. The counteracting influence of constrictors and dilators can be protective, when maintained in balance; inevitable local imbalances of mediators can result in focal ischemia, thus propagating injury. Superoxides originating from the radicals further contribute to the imbalance [14,45,46].

Indeed, it is shown here that DS significantly increases the perfusion of hepatic sinusoids after LTx (Table 2).

Danshen prevents leukocyte-adherence

Leukocytes contribute to hepatic injury by adhering to sinusoids after reperfusion. Neutrophils are a major source of oxygen radicals and also generate various proteases and hypochlorous acid. These mediators induce and subsequently increase ICAM-1 expression on endothelial cells and hepatocytes. ICAM-1, an important transendothelial migration factor, leads to activation and adherence of neutrophils. DS minimizes the generation of free radicals and inflammatory mediators and also scavenges them, hence attenuating the leukocyte-endothelial interactions and exhibiting a protective effect [14]. In other studies, it has been shown that DS can decrease PMN-EC adhesion rate and the levels of CD11a/CD18, CD11b/CD18 and TNF-α in case of endotoxemia and thus improve microcirculation [47]. Other authors have shown that Danshen reduces the concentration of plasma epinephrine and IL-8 [48].

Kupffer cell activation

Kupffer cells are the major source of eicosanoids in the liver [49]. Moreover, activated Kupffer cells release proteases and tumor necrosis factor (TNF-α) [50]. Inactivation of these cells effectively reduces reperfusion injury [38]. Both immunohistochemical data and phagocytosis of latex beads as a direct marker of Kupffer cell activation confirmed that DS reduces Kupffer cell activation after LTx (Table 3, Figs 5 and 6). Reduced TNF-α levels after DS point to decreased activation of Kupffer cells, but Kupffer cells may not be the only cells involved in decreased TNF-α levels in reperfused livers. Considering the *in vivo* microscopy results, it can be proposed and evidenced that DS prevents activation of Kupffer cells, thereby minimizing reperfusion injury after transplantation. The mechanisms involve scavenging of oxygen radicals, improved microcirculation, reduced generation of free radicals and inflammatory mediators, and direct suppression of Kupffer cell activation. Findings from an *in vitro* model suggest that DS protects hepatocytes from peroxidation injury [51].

In our *in vivo* model, necrosis – which can be considered as a global marker of reperfusion injury – and leukocytic infiltration were significantly and effectively decreased by DS while survival was improved from 60% in controls to 100% in DS-treated animals (Table 3, Fig. 1). Our findings from *in vivo* microscopy and immunohistochemistry support the inhibition of Kupffer cell activation as a possible and important mechanism (Table 3, Figs 5 and 6). Other mechanisms such as cytokine regulation and direct scavenging of oxygen radicals most likely have synergistic effects on the inhibition of Kupffer cells.

While other protective substances are available, we have chosen to compare DS to Ringer solution. In our opinion, not using any experimental or clinically used other substance with anti-oxidative effects eliminates any possible other modulating effect on IRI; thus, our findings can clearly be attributed to DS. Further, the concept of donor preconditioning most recently has been demonstrated in a model of kidney transplantation with Danshen [29]. Data presented here clearly demonstrate that Danshen given to donors is also beneficial for LTx.

Conclusion

Danshen significantly increases survival after rat LTx and effectively scavenges oxygen radicals and improves intrahepatic microcirculation. Leukocyte-endothelium interaction is significantly decreased in DS-treated animals. Phagocytosis of latex beads by Kupffer cells and TNF-α levels are significantly decreased by DS.

Clinical implications

In eastern Asia, DS has been extensively used in traditional medicine. As a standardized extract, DS has been in clinical use for several decades now. Toxic effects in humans have not been reported for therapeutic doses yet. This study clearly demonstrates that DS increases survival after LTx and improves hepatic microcirculation while endothelium-leukocyte-interaction and Kupffer cell activation are decreased. Thus, DS could be beneficial in human LTx; further experiments must be performed to prove the safety of this promising substance before clinical trials may be started.

Authorship

RL, XG, MK, JL and PS: performed research/study. XG, HB, GDA and PS: wrote the paper. TL and RL: contributed important reagents. HB and MMG: analysed data. MWB: reviewed the manuscript and study protocol. PS: designed research/study.

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