RESEARCH ARTICLE

Green tea extract ameliorates reperfusion injury to rat livers after warm ischemia in a dose-dependent manner

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Scope: Polyphenolic constituents of green tea (*Camellia sinensis*) have been shown to be potent scavengers of reactive oxygen species (ROS). Thus, this study was designed to assess its effects after liver ischemia–reperfusion.

Methods and results: Fasted Sprague–Dawley rats were gavaged with different concentrations of green tea extract (GTE) 2 h before 90 min of warm ischemia of the left lateral liver lobe (30% of liver). Controls were given the same volume of Ringer's solution. A preparation of pentobarbital sodium (intraperitoneal) and ketamine (intramuscular) was used for anesthesia. After reperfusion, transaminases, liver histology, hepatic microcirculation, and both phagocytosis of latex bead particles as well as the expression of tumor necrosis alpha (TNF-α) to index cellular activation were investigated. Furthermore, the expression of superoxide dismutase (Mn-SOD) was assessed. After 90 min of warm ischemia aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) increased dramatically to 1946 ± 272/3244 ± 757 U/L, 1680 ± 134/2080 ± 379 U/L, and 7857 ± 1851/2036 ± 1193 U/L at 2/6 h, respectively. GTE (200 mg/kgbody weight) significantly prevented this increase in a dose-dependent manner by 21–51% at 2 h and 29–34% at 6 h, respectively. Histology confirmed the protective effects while both TNF-α expression and phagocytosis of latex beads by Kupffer cells (KCs) were significantly reduced. GTE intake significantly increased the expression of manganese superoxide dismutase. In vivo microscopy revealed improved acinar and sinusoidal perfusion after GTE.

Conclusion: Preconditioning with a single oral dose of GTE ameliorates ischemia–reperfusion injury in liver. Decreased cellular activation and improved microcirculation are the proposed mechanisms.

Keywords:
Green tea extract / Ischemia / Liver / Microcirculation / Reperfusion

1 Introduction

Ischemia/reperfusion injury (IRI) is the major cause of liver dysfunction or failure after a variety of clinical conditions including liver surgery (Pringle maneuver, total and intermittent vascular exclusion, organ manipulation for resection) and liver transplantation. An excessive inflammatory response is clearly recognized as a key pathophysiological mechanism of IRI [1–4]; there is strong evidence that acti-

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Abbreviations: ALT, alanine aminotransferase; **AST,** aminotransferase; **BW,** body weight; **EGCG,** (−)-epigallocatechin-3-gallate; **GTE,** green tea extract; **IRI,** ischemia/reperfusion injury; **KC,** Kupffer cell; **LDH,** lactate dehydrogenase; **Mn-SOD,** manganese superoxide dismutase; **PMN,** polymorphonuclear leukocyte neutrophil; **ROS,** reactive oxygen species; **TNF-α,** tumor necrosis factor-α

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vated Kupffer cells (KCs) play a pivotal role [5, 6]. Once activated, KCs release reactive oxygen species (ROS), initiate a proinflammatory cascade, and the recruitment and activation of inflammatory cells, leading to microcirculatory disturbance and liver injury [5–11]. Because of the complex pathophysiology of IRI, it is difficult to design protective strategies to target single effector or mechanisms; several approaches have been proposed but none has attained the level of clinical routine yet [12–14].

Previous studies have shown that polyphenolic components of the higher plants possess antioxidant properties including scavenging of superoxide and hydroxyl radicals [15–18], reduce lipid peroxyl radicals [19], and inhibit lipid peroxidation [20, 21]. Recently, the catechin-containing polyphenolic extracts of green tea (*Camellia sinensis*), with (−)-epigallocatechin-3-gallate (EGCG) being the highest in concentration, have been shown to be hepatoprotective [22–25] including protecting livers from IRI [26]. This was followed by publications of adverse event case reports involving green tea products [27] rendering both the optimal protective dosage of green tea extract (GTE) and its exact mechanism of action in vivo still unclear. Thus, this study was designed to find a protective dosage of GTE in hepatic IRI as well as further investigating its mechanism of action on liver including the hepatic microcirculation through in vivo microscopy.

2 Materials and methods

2.1 Animals

Adult female Sprague–Dawley rats (200–220 g) were allowed free access to standard laboratory chow (ssniff R/M-H, ssniff Spezialdiäten, Soest, Germany) and tap water before experiments. All experimental procedures were reviewed and approved by the responsible authority (Regierungspräsidium Karlsruhe, Baden-Württemberg, Germany) (file number 35-9185.81/G-92/04) according to the animal welfare legislation (§ 8 Abs. 1 Tierschutzgesetz (TierSchG) dated May 18, 2006, (BGBI. I S. 1206)) and were performed according to institutional guidelines at the Ruprecht-Karls University of Heidelberg.

2.2 Surgical procedure

Rats were anesthetized with a combination of an intraperitoneal injection of Pentobarbital sodium (Narcoren®, 20 mg/kg, Merial GmbH, Hallbergmoos, Germany), and intramuscular injection of ketamine (Ketanest®, 100 mg/kg, Parke-Davis GmbH, Berlin, Germany). Body temperature was kept constant at 37°C with a warming pad. Polypropylene catheters (Braun, Melsungen, Germany) were placed in the left carotid artery and right jugular vein for monitoring the hemodynamic parameters and intravenous infusions, respectively. Rats were fasted overnight and were randomized into four groups of 10 animals each: control, GTE 50, GTE 100, and GTE 200. Experimental groups were given an oral dose (gavage) of either 5 mL Ringer solution (control) or different concentrations of a decaffeinated catechin GTE (Sunphenon® 90LB, Taiyo International, Yokkaichi, MIE, Japan) with polyphenol content of ≥94% of the dry matter dissolved in the same volume of Ringer solution. GTE had an EGCG content of 48.5% of the dry matter measured through high-performance liquid chromatography (HPLC). Other catechins included: (−)-epigallocatechin (EGC) 18.1%, (−)-epicatechin (EC) 9.7%, (−)-epicatechin gallate (ECG) 9.5%, (+)-catechin (C) 1.6%, and (+)-gallocatechin (GC) 1.6%. GTE 50, GTE 100, and GTE 200 groups received 50, 100, and 200 mg/kg of rat body weight (BW) GTE, respectively 2 h before inducing warm ischemia. Subsequently, in both controls and GTE-treated animals a midline incision was performed, and the left lateral liver lobe (30% of liver) was clamped with a Yasargil clamp (Aesculap, Tübingen, Germany) for 90 min to induce warm ischemia. After 90 min, the liver lobe was reperfused and blood was taken at 0, 2, and 6 h post-reperfusion for transaminases. In vivo microscopy was done 3 h after reperfusion. Six hours after reperfusion liver tissue samples were taken for histology and immunohistochemistry (Fig. 1).

2.3 Serum transaminases

Blood was drawn right after reperfusion as well as 2 and 6 h after reperfusion. After centrifugation, serum was stored at −20°C until analysis. Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were determined with standard enzymatic methods [28].

2.4 Histology

Livers were fixed by perfusion with 5% paraformaldehyde in Krebs–Henseleit bicarbonate buffer (118 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 4.7 mmol/L KCl, and 1.3 mmol/L $CaCl_2$) at pH 7.6, embedded in paraffin, and processed for light microscopy (hematoxylin and eosin (H&E) staining) 6 h after warm ischemia. In order to assess the histomorphological changes 40 areas of 0.15 mm^2 were evaluated per slide with a point counting method as described previously [29]: grade 0, minimal or no evidence of injury; grade 1, mild injury including 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 neutrophil infiltration. To describe leukocyte infiltration into the

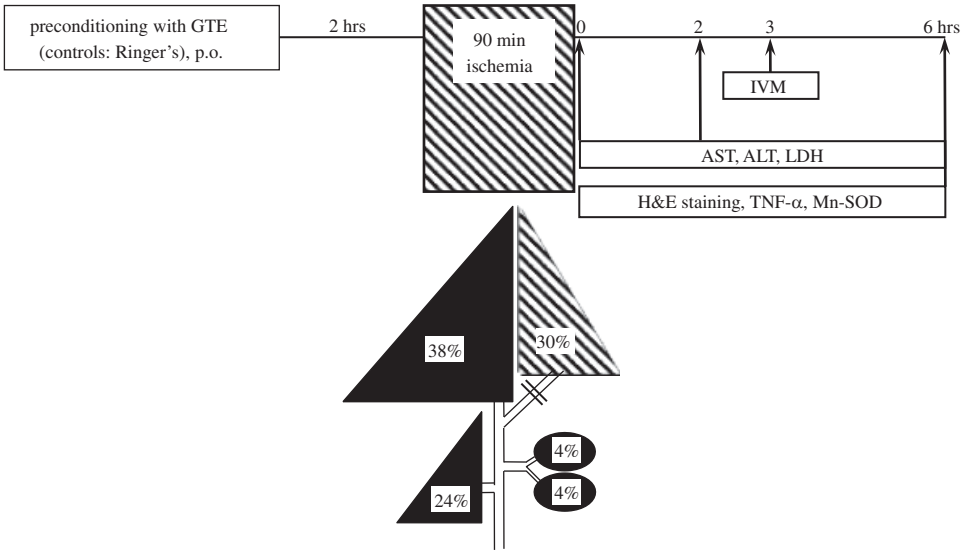

Figure 1. Experimental design. Fasted rats were gavaged with different concentrations of GTE 2 h before 90 min of warm ischemia of the left lateral liver lobe (30% of liver). Controls were given the same volume of GTE-vehicle (Ringer's solution). After reperfusion, transaminases, liver histology (hematoxylin and eosin staining), in vivo microscopy (IVM), and the expression of tumor necrosis alpha (TNF-α) and Mn-SOD were assessed as described in Section 2; p.o.: orally; hrs: hours; GTE: green tea extract; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase.

hepatic tissue, a scale from 1 to 4 was used: grade 1, <10 leukocytes/field (focal infiltration); grade 2, 10–20 leukocytes/field (mild infiltration); grade 3, 21–50 leukocytes/field; grade 4, >50 leukocytes/field.

2.5 In vivo fluorescence microscopy

The left lobe was exteriorized on a mechanical plate and in vivo microscopy was performed 3 h after reperfusion with a fluorescence microscope (Leica GmbH, Wetzlar, Germany). Two fluorescence filter blocks were used: I3 (excitation filter 450–490; emission filter LP520) and N2.1 (excitation filter 515–560; emission filter LP580). The microcirculatory images were transmitted by a video camera (CF 8/1, Kappa GmbH, Gleichen, Germany) to a monitor (PVM-1440, Sony, Tokyo, Japan) and recorded on a video recorder (AG-7350-E, Panasonic, Osaka, Japan) for later offline evaluation. FITC-labeled erythrocytes (3 μmol/kgBW; FITC Isomer 1, Sigma, Germany) were used to assess sinusoidal perfusion and erythrocyte velocity in both sinusoids and central venules. At the end of in vivo microscopy, intraarterial injection of fluorescent latex beads (3×10^8 beads/kg of BW; diameter = 1.1 μm; Polysciences, Warrington, PA, USA) was performed to study their phagocytosis by KCs.

2.6 Quantitative offline video analysis

The evaluation of hepatic microperfusion was performed offline with special software (Capimage, Zeitl GmbH, Heidelberg, Germany). RBC velocity within the central venules was measured as the median value of 10 determinations (analyzed in five venules) in each animal. Blood flow in cubic millimeters per second was calculated as $\pi \times (0.5 \times \text{microvascular diameter})\ 2 \times$ RBC velocity [30]. The number of latex bead-positive KCs was counted per square millimeter within 300 s starting 10 s after the end of their injection as described previously [31].

2.7 Immunohistochemistry

Paraffin sections from liver tissue obtained 6 h after reperfusion were deparaffinized in xylene and rehydrated with graded ethanol. Antigen retrieval was performed via microwave pre-treatment in EDTA-buffer (pH 9.0), 3 times for 5 min. The specimens were then cooled and treated with 30% hydrogen peroxide (H_2O_2) in phosphate-buffered saline (PBS) – final H_2O_2 concentration: 1% – to block endogenous peroxidases. Non-specific antibody-binding was blocked by normal rabbit serum. Sections were incubated with rabbit polyclonal anti-manganese superoxide dismutase (Mn-SOD) antibody (Dako, Hamburg, Germany) at a 1:500 dilution and rabbit polyclonal anti-mouse tumor necrosis factor-alpha (TNF-α) antibody (Biosource Europe, Nivelles, Belgium) at the dilution of 1:50. After incubation, secondary biotinylated polyclonal mouse anti rabbit immunoglobulins (Dako) at a dilution of 1:200 was applied for 1 h followed by streptavidin–biotin complex. Positive hepatocytes for immunohistochemistry were counted in 10 microscopic fields per slide and slides were evaluated by the semiquantitative technique, relating the score of 0–4 points to the fraction of stained cells: scale 0, 0% cells; 1, $<5\%$ cells; 2, 5–20% cells; 3, >20–40% cells; 4, $>40\%$ positive cells [32]. Histological and immunohistochemical assessments were done by a pathologist (MK) who was blinded to samples.

2.8 Statistics

Mean values $\pm$ SEM were compared using one-way ANOVA with Students–Newman–Keuls post-hoc test for parametric data. Differences in histological grading of injury as well as

in immunohistochemical staining were tested by ANOVA on ranks. $p<0.05$ was selected prior to the investigation as the criterion for significance of differences between groups.

3 Results

3.1 Liver injury

After 90 min of warm ischemia AST, ALT, and LDH increased dramatically in controls. Although the application of 50 mg/kg BW of GTE and further increase to 100 mg/kg could lower the liver enzymes, this was not significantly different from controls (Fig. 2). Further increasing the GTE dose to 200 mg/kg BW significantly diminished the enzymatic surge after reperfusion compared to controls by 21–51% at 2 h and 29–34% at 6 h, respectively (Fig. 2). In parallel, controls showed focal necrosis and ischemic degeneration of hepatocytes mainly located in the central venous area 6 h after reperfusion. GTE 200 significantly reduced all detected pathology including index of liver damage, index of leukocyte infiltration, and percentage of necrosis (Table 1). Since AST, ALT, and LDH served in our study as screening markers for liver injury, no further investigation (histology, immunohistochemistry, and in vivo microscopy) was done in GTE 50 and GTE 100.

3.2 Immunohistochemistry

Immunohistochemical analysis of sections obtained 6 h after reperfusion indicated positive staining for TNF-α and Mn-SOD. GTE 200 reduced the number of positively stained hepatocytes for TNF-α compared with controls (2 ± 0.05 versus 2.3 ± 0.07, $p<0.05$) (Fig. 3, A1–A3). The expression of Mn-SOD on hepatocytes was higher in GTE 200 compared with controls (1.8 ± 0.06 versus 1.4 ± 0.05, $p<0.05$) (Fig. 3, B1–B3).

3.3 In vivo microscopy

Diameter, erythrocyte velocity, and blood flow were significantly higher in GTE 200 compared with controls both in sinusoids and central venules 3 h after reperfusion (Fig. 4).

3.4 Phagocytosis of latex beads

The phagocytosis of latex beads was monitored to index KC activation. Warm ischemia and subsequent reperfusion significantly increased the degree of latex beads adherence in controls counted per square millimeter within 300 s starting at 10 s after the end of their injection. GTE 200 reduced the number of latex bead-positive KCs per square millimeter compared with controls (10 ± 1 versus 16 ± 2 per square millimeter, $p<0.05$) (Fig. 3, C1–C3).

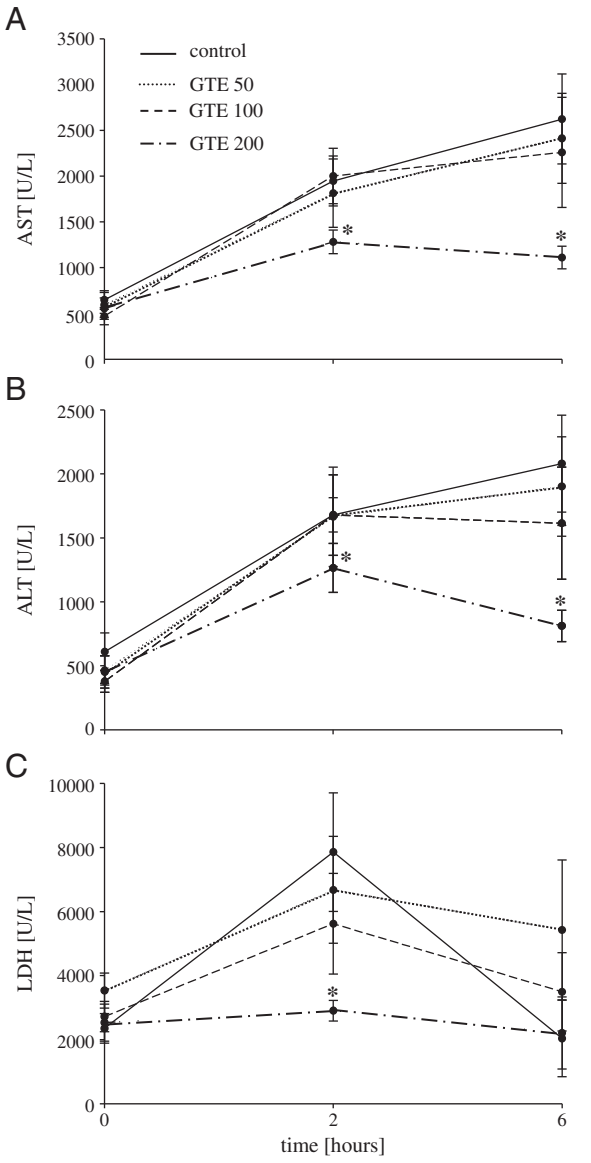

Figure 2. Dose-dependent *effects of GTE on enzyme profile after reperfusion.* Right after reperfusion as well as 2 and 6 h after reperfusion AST (A), ALT (B), and LDH (C) were determined as described in Section 2. GTE 50 and 100 reduced the values of AST, ALT, and LDH compared with controls. This reduction was, however, not significant. Further increase in GTE to 200 mg/kg BW could significantly reduce the values compared with controls. Values are mean ± SEM ($p<0.05$ by one-way ANOVA with Students–Newman–Keuls post-hoc test, $n=10$ per group); *, $p<0.05$ for comparison to controls; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; GTE 50–200: GTE 50–200 mg/kg of animalBW.

Table 1. GTE decreased liver injury, leukocyte infiltration, and necrosis

Parameter	Control	GTE 200
Index of liver damage	2.1±0.2	1.2±0.1[a)]
Index of leukocyte infiltration	2.1±0.1	1.4±0.1[a)]
Necrosis (%)	18.6±1.4	9.4±0.8[a)]

Conditions as described in Section 2; $n = 10$ in each group; values 6 h after warm ischemia/reperfusion to index liver damage (index of histological injury), inflammatory response (index of leukocyte infiltration), and percentage of necrosis. Values are mean±SEM ($p < 0.05$ by one-way ANOVA on ranks).
a) $p < 0.05$ for comparison to controls; GTE 200: green tea extract 200 mg/kg animal BW.

4 Discussion

In the recent years, polyphenolic compounds in GTE have been shown to possess antioxidative properties, thus becoming a potential candidate for the experimental pharmacologic tackling of IRI in liver warm ischemia [33] as well as a variety of other hepatic pathologic conditions including ethanol-induced fatty livers [34], small-for-size liver grafts [35], and cholestasis-induced liver fibrosis [36]. On the other hand, several clinical reports have been published in the medical literature describing patients presenting with marked hepatotoxicity attributable to the consumption of supplements containing GTE [37–41]. While EGCG has been considered the most potent polyphenol of GTE [42],

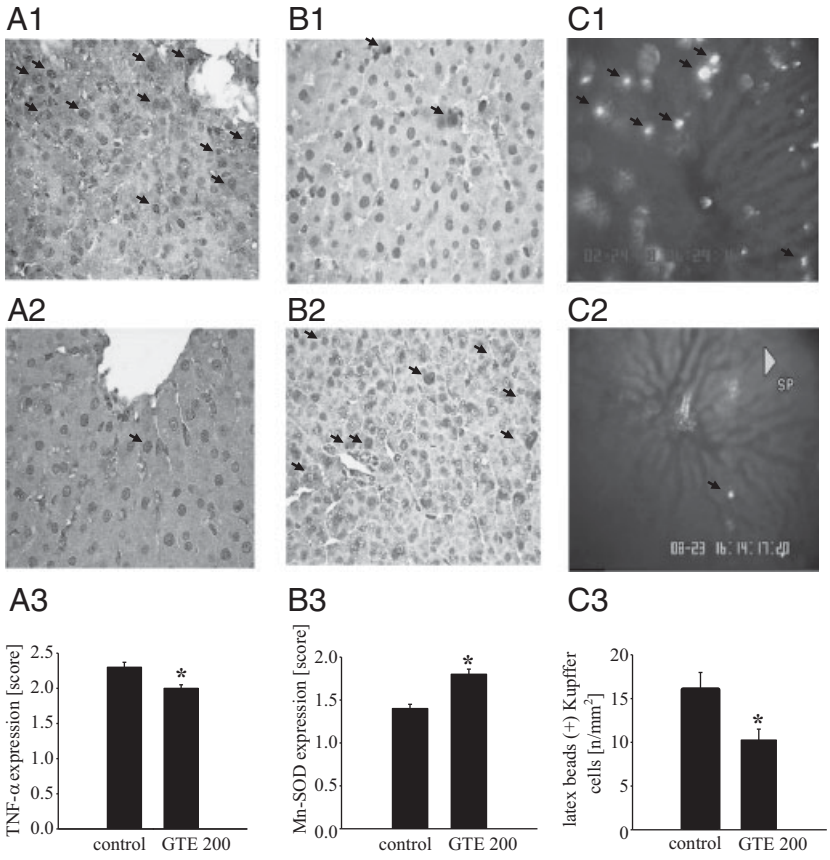

Figure 3. Immunohistochemistry and latex bead phagocytosis. Conditions as described in Section 2. A1, A2: Immunohistochemistry for TNF-α; 6 h after reperfusion, tissue was collected and processed for immunohistochemical analysis with light microscopy. The intensity of TNF-α expression (brown staining, black arrows) in hepatocytes was significantly higher in controls (A1) compared to GTE 200 (A2). Quantitative assessment of TNF-α expression was used for statistical analysis. The expression index of TNF-α was graded according to particular immunohistochemical findings graded with a scale from 0 to 4 as described in Section 2 (A3). B1, B2: Immunohistochemistry for Mn-SOD; the intensity of Mn-SOD expression (brown staining, black arrows) in hepatocytes was significantly higher in GTE 200 (B2) compared with controls (B1). Pictures depict typical pattern of staining for TNF-α and Mn-SOD (original magnification: × 400). Quantitative assessment of Mn-SOD expression is shown in B3. C1, C2: Phagocytosis of latex beads; the number of latex bead-positive KCs was counted per square millimeter. The high number of beads taken up by KCs (black arrows) in control animals suggests their more intense activation (C1). This was significantly reduced by GTE 200 (C2). C3 depicts the quantitative analysis of latex bead phagocytosis. TNF-α: tumor necrosis factor-alpha; Mn-SOD: manganese superoxide dismutase; GTE 200: GTE 200 mg/kg of animalBW.

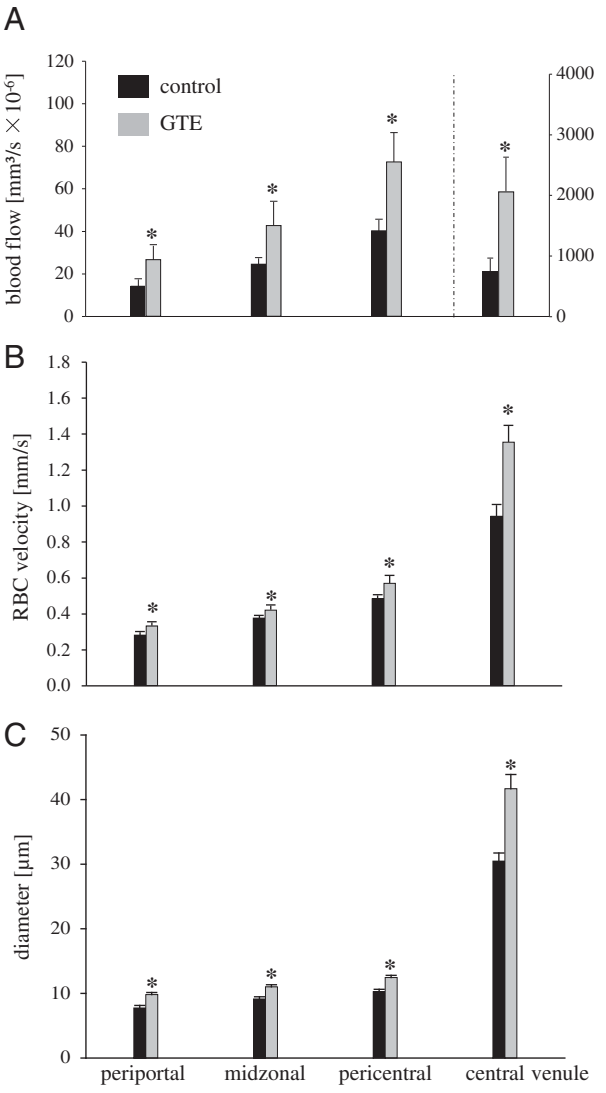

Figure 4. In vivo microscopy of the left lateral lobe was performed 3 h after reperfusion. Sinusoids were divided into three zones (periportal, midzonal, and pericentral). Blood flow (A), RBC velocity (B), and microvascular diameter (C) was significantly higher in GTE 200 compared with controls both in sinusoids and central venules. Right vertical axis in A corresponds blood flow in central venule ($mm^3/s \times 10^{-6}$) which is much higher than in sinusoids and is therefore depicted separately. Plotted values are mean ± SEM ($p < 0.05$ by one-way ANOVA with Students–Newman–Keuls post-hoc test, $n = 8$); *, $p < 0.05$ for comparison with controls; RBC: red blood cells.

others have shown it to be as 10 times more cytotoxic to isolated rat hepatocytes in vitro than other minor catechins [43]. Studies in beagle dogs have indicated the potential for toxic EGCG plasma Cmax concentrations (comparable to lethal concentrations in vitro) at high levels of green tea consumption under fasting conditions [44]. Accordingly, the no-observed adverse effect level (NOAEL) of a GTE preparation with an 80% content of EGCG was determined to be 500 mg preparation/kg BW/day for fed dogs and 40 mg/kg BW/day (equivalent to 32 mg/kg BW/day EGCG) for fasted dogs [45]. In our study, fasted rats received a single dose of 200 mg/kg BW GTE (equivalent to ≥ 90 mg/kg BW EGCG), far below the abovementioned dosages, not only produced no adverse effects but also reduced detrimental effects of warm ischemia developing after reperfusion in vivo and proved indeed to be beneficial.

The pathophysiology of the hepatic IRI includes a number of contributing mechanisms. It is well established that no-flow ischemia and reperfusion activates KCs [46, 47] with subsequent generation of ROS and production of proinflammatory cytokines such as TNF-α. While the former can directly cause lipid peroxidation and damage cell membrane macromolecules, the latter amplifies further KC activation and leads to systemic activation of polymorphonuclear leukocyte neutrophils (PMNs), expression of endothelial adhesion molecules with subsequent sinusoidal PMN infiltration causing microcirculatory disturbances and further ischemic injury [2–4, 48, 49]. The endpoint of IRI is the death of hepatocytes and endothelial cells characterized by swelling of cells and subcellular structures, release of cell contents and enzymes, eosinophilia, pyknosis, karyolysis, and further induction of inflammation [50]. Here, GTE 200 proved to be quite effective in reducing the release of transaminases as well as focal necrosis and ischemic degeneration of hepatocytes including index of liver damage, index of leukocyte infiltration, and percentage of necrosis after IRI. Indeed, GTE 200 decreased both the phagocytosis of latex beads and the release of TNF-α following reperfusion. Since it has already been demonstrated that latex beads are phagocytized exclusively by KCs [51] – this finding was further documented by means of electron micrographs [52] –, GTE 200 most likely blunted KC activation. Further, the administration of GTE 200 was associated with an increased expression of Mn-SOD. It has been shown that blocking the hepatic ROS production by overexpression of Mn-SOD almost completely prevented hepatic c-Jun *N*-terminal kinase activation and injury [53]. Similarly, data presented here clearly indicate that GTE 200 can upregulate Mn-SOD, thus enhancing the hepatic antioxidative capacity to remove ROS and protect cells against lipid peroxidation. Direct administration of SOD enzymes to tackle IRI has been shown to be therapeutically ineffective due to their extremely short half-lives. Moreover, clinical administration of large amount of these proteins, which have been further undergone chemical modifications (e.g. glycosylation) in order to prolong their half-lives, has been associated with immunological complications resulting in their withdrawal from patient use [54]. In this regard, GTE may have some potential benefits of upregulating SOD without classical side effects. Finally, in vivo microscopy documented that GTE 200 improves the hepatic microcirculation. This positive effect can be attributed to the KC inhibitory effect of GTE 200. As previously stated, KCs generate cytokines like TNF-α and IL-1 upon activation and

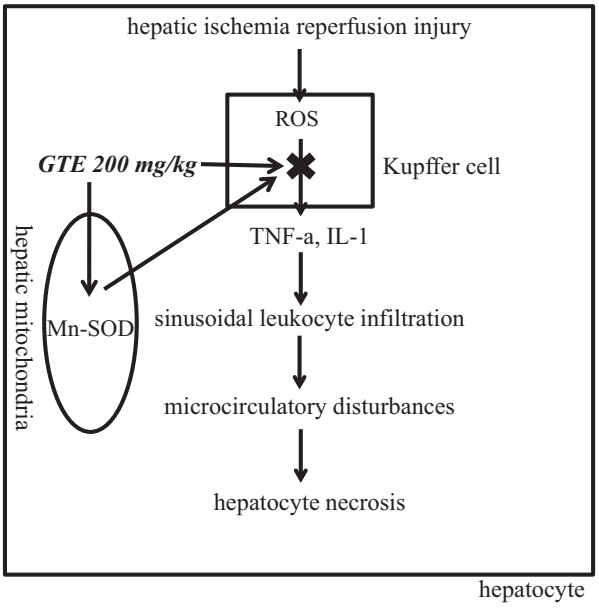

Figure 5. Proposed mechanistic pathways for GTE. Preconditioning with a single oral dose of GTE 200 mg/kg BW ameliorates ischemia–reperfusion injury in liver. GTE directly prevents KCs from activation and subsequently tackles the generation of ROS and production of proinflammatory cytokines such as TNF-α and IL-1. This in turn reduces the systemic activation of PMNs and less expression of endothelial adhesion molecules with subsequent reduced sinusoidal leukocyte infiltration and microcirculatory disturbances. The endpoint is the reduction of ischemic injury and the death of hepatocytes. In parallel, GTE may upregulate the expression of Mn-SOD, thus enhancing the hepatic antioxidative capacity to block ROS.

further regulate the proinflammatory genes in hepatocytes and endothelial cells. Proinflammatory cytokines, together with complement factors and platelet activating factor upregulate adhesion molecules [55] that are critical for neutrophil adherence and neutrophil-mediated liver injury [56]. In our experiment, an increasing percentage of adherent latex beads in controls was followed by microcirculatory disturbances (reduced sinusoidal and central venular diameter, erythrocyte velocity, and blood flow) which was ameliorated through the administration of GTE 200.

Figure 5 outlines the mechanistic pathways through which GTE may play a role in ameliorating hepatic IRI.

In summary, this study clearly demonstrates that a single oral dose of GTE 200 in overnight fasted rats reduces detrimental effects of warm ischemia developing after reperfusion in vivo most likely via mechanisms including inactivation of KCs with subsequently decreased oxidative stress and TNF-α release, and improved hepatic microcirculation. GTE can therefore be viewed as an attractive modality for tackling hepatic IRI. However, its potential therapeutic efficacy warrants the investigation in large animal models and clinical trials.

R. L. and A. N. did the experiments; A. N. wrote the manuscript. M. K. reviewed the histological part of the study. H. S. and S. B. supported the study regarding the experimental product and the design. M. Z. was consulted regarding the biochemical measurements. M. W. B. and P. S. supported the design of the study with their knowledge and experience. Further, P. S. conceived and designed the study based on his experimental experience. The manuscript has been seen and approved by all authors listed above. The authors thank Dylan P. Rahe for editing the manuscript as native speaker. This work is supported in parts by an unrestricted educational grant from Fresenius Kabi, Germany.

The authors have declared no conflict of interest.

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