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ORIGINAL CONTRIBUTION

Plant polyphenols attenuate hepatic injury after hemorrhage/resuscitation by inhibition of apoptosis, oxidative stress, and inflammation via NF-kappaB in rats

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Abstract
Purpose Oxidative stress and inflammation contribute to hepatic injury after hemorrhage/resuscitation (H/R). Natural plant polyphenols, i.e., green tea extract (GTE) possess high anti-oxidant and anti-inflammatory activities in various models of acute inflammation. However, possible protective effects and feasible mechanisms by which plant polyphenols modulate pro-inflammatory, apoptotic, and oxidant signaling after H/R in the liver remain unknown. Therefore, we investigated the effects of GTE and its impact on the activation of NF-kappaB in the pathogenesis of hepatic injury induced by H/R.
Methods Twenty-four female LEWIS rats (180–250 g) were fed a standard chow (ctrl) or a diet containing 0.1% polyphenolic extracts (GTE) from *Camellia sinensis* starting 5 days before H/R. Rats were hemorrhaged to a mean arterial pressure of 30 ± 2 mmHg for 60 min and resuscitated (H/R and GTE H/R groups). Control groups (sham, ctrl, and GTE) underwent surgical procedures without H/R. Two hours after resuscitation, tissues were harvested.
Results Plasma alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) increased 3.5-fold and fourfold, respectively, in vehicle-treated rats as compared to GTE-fed rats. Histopathological analysis revealed significantly decreased hepatic necrosis and apoptosis in GTE-fed rats after H/R. Real-time PCR showed that GTE diminished gene expression of pro-apoptotic caspase-8 and Bax, while anti-apoptotic Bcl-2 was increased after H/R. Hepatic oxidative (4-hydroxynonenal) and nitrosative (3-nitrotyrosine) stress as well as systemic IL-6 level and hepatic IL-6 mRNA were markedly reduced in GTE-fed rats compared with controls after H/R. Plant polyphenols also decreased the activation of both JNK and NFκB.
Conclusions Taken together, GTE application blunts hepatic damage, apoptotic, oxidative, and pro-inflammatory changes after H/R. These results underline the important roles of JNK and NF-kappaB in inflammatory processes after H/R and the beneficial impact of plant polyphenols in preventing their activation.

Keywords Plant polyphenols · Green tea extract · NF-kappaB · Hemorrhagic shock · Liver · Apoptosis · In vivo

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Introduction

Trauma is one of the leading causes of deaths worldwide, while blood loss remains the leading contributor to mortality after trauma [1, 2]. The pathophysiology of resuscitated blood loss includes the development of the systemic inflammatory response syndrome (SIRS), a dysfunctional inflammatory response after injury that often leads to dysfunction or even failure of multiple organs [3, 4]. Hemorrhagic shock (HS) and subsequent resuscitation (H/R) compromise the integrity of several organs at the level of microcirculation. This resulting tissue injury is mediated in part by excessive neutrophil activation and

subsequent release of inflammatory cytokines. Clinical data support an association of high activation state of polymorphnuclear leukocytes (PMNL) with SIRS [5, 6]. H/R "prime" or "pre-activate" PMNL leading to enhanced functional responsiveness of those cells to pro-inflammatory cytokines, which is expressed by increased production of reactive oxygen (ROS) and nitrogen (RNS) species [7, 8]. ROS and RNS, derived from hepatocytes, activated macrophages and the infiltrating neutrophils induce lipid peroxidation of cellular membranes, oxidation of DNA, protein nitrosylation, necrosis and apoptosis, and therefore play a crucial role in the induction and in the progression of liver injury after H/R [9–11]. Moreover, H/R is accompanied by enhanced hepatic and serum IL-6 levels [12]. IL-6-deficient mice were protected from pro-inflammatory changes, hepatic necrosis, enhanced nuclear factor-kappaB (NF-kappaB) activity, and organ damage as observed in wild-type mice after H/R [13].

NF-kappaB is activated by hypoxia, ROS, and/or cytokines [14, 15]. The induction of NF-kappaB by cytokines is mediated at least partially by mitogen-activated protein kinases, such as c-Jun NH2-terminal kinase (JNK), extracellular signal regulated kinase (ERK), p38 and PI3K/AKT [16]. Several studies associated NF-kappaB activation with upregulated gene expression of pro-inflammatory mediators and H/R-induced liver injury [17–19]. IkappaB proteins regulate and inactivate NF-kappaB via masking the nuclear localization signal (NLS) of NF-kappaB, thus preventing its nuclear translocation [20]. IkappaB is activated by phosphorylation of its regulatory N-terminus on serine 32 and 36, thereby inducing its dissociation from NF-kappaB and conjugation with ubiquitin. Ubiquitination leads to subsequent degradation of the NF-kappaB inhibitory unit with the following activation of the transcription factor (TF) NF-kappaB, its translocation to the nucleus, binding to DNA, and induction of gene transcription [20].

Green tea (GT; from *Camellia sinensis*) contains high levels of polyphenols such as catechin and catechin gallates that have remarkable scavenging effects on free radicals [21, 22]. In accordance with these effects, the intake of GT in human is associated with a lower incidence of diseases such as coronary artery disease and myocardial infarction [23, 24]. A recent, population-based prospective cohort study (40,530 people) reported that green tea consumption was associated with reduced mortality from cardiovascular disease and even cancer mortality [23]. In several models of acute inflammation, such as ischemia/reperfusion or liver transplantation in mice, GTE reduced hepatic injury due to its anti-oxidative properties, such as decreased free radical adduct formation and its anti-inflammatory properties, such as reduced expressions of TNF-alpha mRNA and protein [25–27].

Accordingly, this study was to evaluate the effects and underlying mechanisms of GTE impact on livers damaged after H/R.

Materials and methods

Animals and experimental model

Twenty-four female LEWIS rats (180–250 g) were obtained from Harlan (Borchen, Germany). Five days prior H/R, rats received daily a diet containing 0.1% polyphenolic extracts from *C. sinensis* (GTE, Sunphenon 90LB kindly provided by Heinz Schneider, HealthEcon AG, Basel, Switzerland) or regular chow (ctrl). After an overnight fast, rats were anesthetized with Isofluran (1.5%), and the right carotid artery, the right femoral artery, and the left jugular vein were cannulated with polyethylene tubing. Then, shock was induced over 5 min by withdrawing blood from the right carotid artery into a heparinized syringe (10 U) to a mean arterial blood pressure (MABP) of 30–32 mm Hg. Systemic blood pressure was monitored in the right femoral artery using a blood pressure analyzer (BPA 400, Digi-Med, Louisville, KY). Constant pressure was maintained by further withdrawal of small volumes of blood as necessary for 60 min. Then, rats were resuscitated by transfusion of 60% of the shed blood and twice the shed blood volume of Ringer's lactate solution with a syringe pump over 30 min via the left jugular vein. The MABP was monitored until all experimental procedures until wound closure were concluded. After the end of resuscitation, catheters were removed, the vessels were occluded and the wounds were closed. Two hours after the end of resuscitation, the animals were re-anesthetized. The cava was punctured, blood was collected, and tissue was harvested. For each rat, the two right dorsal liver lobes were snap-frozen in liquid nitrogen. The remaining liver was flushed with normal saline, infused and fixed with 10% buffered formalin through the portal vein, embedded in paraffin and subsequently sectioned and stained with hematoxylin-eosin. Sham-operated animals underwent the same surgical procedures, but hemorrhage was not carried out (ctrl and GTE, each group $n = 6$). Two hours after wound closure in sham-operated animals, they were killed. Body temperature was measured in the colon with a thermocouple and maintained at 37 °C throughout the experiment with a heating pad. Animal protocols were approved by the Veterinary Department of the Regional Council in Darmstadt, Germany.

Green tea extracts

GTE, SunphenonLB used in this study contain high levels of polyphenols consisting mostly of catechin and catechin gallates including epicatechin, gallocatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (EGCG). SunphenonLB contains >80% polyphenols and >80% catechins, which contain >40% EGCG, that possesses the highest reducing potential among catechins (Taiyo Kagaku Co., Ltd., Yokkaichi, Mie, Japan).

Examination of tissue injury

Plasma was stored at −80 °C for later analysis of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) using the Vitros 250 device (Ortho-Clinical Diagnostics, Neckargemünd, Germany). Histological damage was determined by an independent examiner who allocated the hematoxylin-eosin stained liver sections to the various experimental groups in a blinded manner as published previously [13].

In situ assessment of caspase activity, DNA fragmentation, and apoptotic cell death

DNA cleavage during apoptosis results in DNA strand breaks. These are identified by labeling free 3'OH termini with modified nucleotides in an enzymatic reaction involving terminal deoxynucleotidyl transferase (TdT) using a commercially available kit (in situ Cell Death Detection Kit, Roche Molecular Biochemicals, Mannheim, Germany). Paraffin-embedded liver tissue was sectioned (7 μm), deparaffinized, rehydrated, and stained by the TUNEL (TdT-mediated dUTP nick end labeling) method according to the manufacturer's instructions (In situ Cell Death Detection Kit, TMR red, Roche Applied Science). Additional staining of a neo-epitope of cytokeratin 18, a caspase-mediated early cleavage product during apoptosis, was performed by use of the monoclonal antibody anti M30 (M30 CytoDEATH Fluorescein, Roche Applied Science). Using fluorescence microscopy, positive cells were counted in 10 randomly selected fields per tissue sample (200X magnification) in a blinded manner.

Detection of 4-hydroxynonenal

The staining of 4-hydroxynonenal (4-HNE) was performed as described previously using a rabbit antibody against 4-HNE (Alpha Diagnostics International, Biotrend, Cologne, Germany) [28]. Sections were counterstained with hematoxylin. The immunostained tissue sections were captured at 400X and analyzed in a blinded manner. The extent of labeling in the liver lobule was defined as the percentage of the field area within a preset color range determined by the software (Adobe Photoshop 7.0). Data from each tissue section (10 fields per section) were pooled to determine mean values, as described previously [29].

Detection of 3-nitrotyrosine

The staining of 3-nitrotyrosine (3-NT) was performed as described previously by using a mouse antibody against 3-NT (HyCult Biotechnology, Uden, the Netherlands) [28]. Sections were counterstained with hematoxylin, captured at 400X, and analyzed in a blinded manner. The extent of labeling in the liver lobule was defined as the percentage of the field area within a present color range determined by the software (Adobe Photoshop 7.0). Data from each tissue section (10 fields per section) were pooled to determine mean values, as described previously [29].

Quantification of plasma IL-6 levels

Concentrations of plasma IL-6 were determined using a Quantikine Rat IL-6 ELISA kit of R&D Systems according to manufacturer's instructions (Wiesbaden-Nordenstadt, Germany). The ELISA 96-well micro titer plates were analyzed using a microplate reader Bio-Tek Ceres UV900C (Bio-Tek, Winooski, VT, USA).

Ribonucleic acid (RNA) isolation, semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA of snap-frozen liver lobes was isolated using the RNeasy-system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The residual amounts of DNA remaining were removed using the RNase-Free DNase Set according to the manufacturer's instructions (Qiagen, Hilden, Germany). The RNA was stored immediately at −80 °C. Quality and amount of the RNA were determined photometrically using the NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, DE, USA).

RNA was subsequently used for qRT-PCR. In brief, 100 ng of total hepatic RNA were reversely transcribed using the Affinity script QPCR-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. To determine the mRNA expression of IL-6, Bax, Bcl-2, and Casp 8, qRT-PCR was carried out on a Stratagene MX3005p QPCR system (Stratagene) using gene-specific primers for rat Il6 (NM_012589, UniGene#: Rn.9873, Cat#: PPR06483A), rat Bax (NM_017059, UniGene#: Rn.10668, Cat#: PPR06496B), rat Bcl2 (NM_016993, UniGene#: Rn.9996, Cat#: PPR06577A), and rat Casp8 (NM_022277, UniGene#: Rn.54474, Cat#: PPR06555A) purchased from SABiosciences (SuperArray, Frederick, MD, USA). As reference gene, the expression of *GAPDH* with rat Gapdh (NM_017008, UniGene#: Rn.91450, Cat#: PPR06557A, SABiosciences, SuperArray, Frederick, MD, USA) was measured. Sequences of these primers are not available. PCR was set up with 1X RT^2 SYBR Green/Rox qPCR Master mix (SABiosciences) in a 25 μL volume according to manufacturer's instructions. A two-step amplification protocol consisting of initial denaturation at 95 °C for 10 min followed by 40 cycles with 15 s denaturation at 95 °C and 60 s annealing/extension at 60 °C was chosen. A melting-curve analysis was applied to control the specificity of amplification products.

Relative expression of each target gene's mRNA level was then calculated using the comparative threshold-cycle (CT) method ($2^{-\Delta\Delta CT}$ method). In brief, the amount of target mRNA in each sample was first normalized to the amount of *GAPDH* mRNA, to give ΔCT and then to a calibrator consisting of samples obtained from the sham_ctrl group. The relative mRNA expression of target genes is presented as fold increase calculated in relation to sham_ctrl after normalization to *GAPDH*.

Western blot

Liver proteins were extracted and Western blot was performed as described previously [30]. Phosphorylated and non-phosphorylated JNK were detected by mouse antibodies against JNK-PAN/SAPK1 and JNK/SAPK (pT183/pY185; BD Pharmingen, Heidelberg, Germany). IkappaKalpha and phosphorylated IkappaBalpha were detected using rabbit monoclonal IKK alpha [Y463] and rabbit polyclonal IKB alpha (phosho S32 + S36) antibodies (Abcam, Cambridge, UK). Determination of β-actin with anti-β-actin antibody (Sigma, Taufkirchen, Germany) served as a loading control. Proteins were detected with ECL™ western blot detection reagents (GE Healthcare, Munich, Germany). Films were digitized, and the integrated density of individual bands was determined using the software *Multianalyst* (Biorad, Munich, Germany). By densitometric measurements using the same software, the amount of protein expression was normalized to β-actin. The activation state of each protein analyzed was calculated as the ratio of phosphorylated and total (phosphorylated plus non-phosphorylated) protein values of densitometry results in percent (phospho/total*100).

Statistical analysis

Differences between groups were determined by one-way analysis of variance (ANOVA) using a multiple comparison procedure (Student–Newman–Keuls *post hoc*). Changes in target gene expressions were analyzed by Wilcoxon matched-pair analysis followed by Bonferroni correction. A p value of less than 0.05 was considered significant. Data are given as mean ± standard error of the mean. All statistical analyses were performed employing GraphPad Prism 5 (Graphpad Software, Inc., San Diego, CA).

Results

Hemodynamic characteristics of hemorrhage and resuscitation

To evaluate potential effects of GTE on the MABP in our model, we measured MABP in vehicle and GTE-fed rats before and after hemorrhage as well as during resuscitation. GTE did not influence blood pressure at any time point evaluated (Tab. 1). The amount of blood removed to induce and maintain hypotension was comparable in both groups (19 ± 1 and 18 ± 1 mL/kg body weight in vehicle and GTE-fed rats, respectively). GTE had no effects on the total food intake of rats as compared to animals fed with normal ctrl chow (data not shown).

Lipid peroxidation and protein nitrosylation after hemorrhage and resuscitation

Hepatic oxidative stress was evaluated by immunohistological staining 4-HNE, indicating lipid peroxidation. At 2 h after resuscitation, 4-HNE staining in liver sections (H/R) was increased significantly as compared to sham-operated rats (ctrl) (51 ± 1 and 27 ± 1%, respectively, $P < 0.001$, Fig. 1a, b, e). Hepatic 4-HNE after H/R was significantly reduced in rats fed with GTE-chow (GTE H/R) as compared to H/R group (39 ± 2% and 51 ± 1, respectively, $P < 0.001$, Fig. 1e).

Hepatic nitrosative stress was evaluated by immunhistological staining of 3-NT indicating nitrosylation of protein tyrosine residues. Following H/R, hepatic 3-NT staining was significantly increased as compared to sham-operated ctrl rats (57 ± 1 and 36 ± 2%, respectively, $P < 0.01$, Fig. 1c, d, f). The amount of nitrotyrosine after H/R was significantly reduced in rats receiving GTE-chow compared with the control group after H/R (39 ± 3% and 57 ± 1, $P < 0.01$, Fig. 1f). These results indicate that GTE attenuated both significantly, hepatic oxidative and nitrosative stress after H/R.

Cell damage after hemorrhage and resuscitation in the liver

Plasma ALT, a marker of hepatocellular damage, increased significantly to 1,295 ± 149 IU/L at 2 h after H/R as compared with 93 ± 11 IU/L after sham operation (ctrl) ($P < 0.001$, Fig. 2e). GTE dimished significantly the ALT release by 71% after H/R (368 ± 48 IU/L, $P < 0.01$, Fig. 2e) as compared to H/R ctrl group (1,295 ± 149 IU/L), but was still significantly elevated compared with both sham groups (ctrl sham: 93 ± 11 IU/L and GTE sham: 71 ± 10 IU/L, respectively, $P < 0.05$). GTE did not affect ALT levels after sham operation as compared to ctrl sham group (Fig. 2e). LDH, an indicator of general cell damage, was significantly elevated after H/R in the ctrl group to 5,058 ± 1,172 IU/L as compared to sham-operated ctrl rats (324 ± 68 IU/L, $P < 0.05$). GTE reduced significantly LDH release after H/R by 76% (GTE H/R: 1,237 ± 453 IU/L and ctrl H/R: 5,058 ± 1,172 IU/L, respectively,

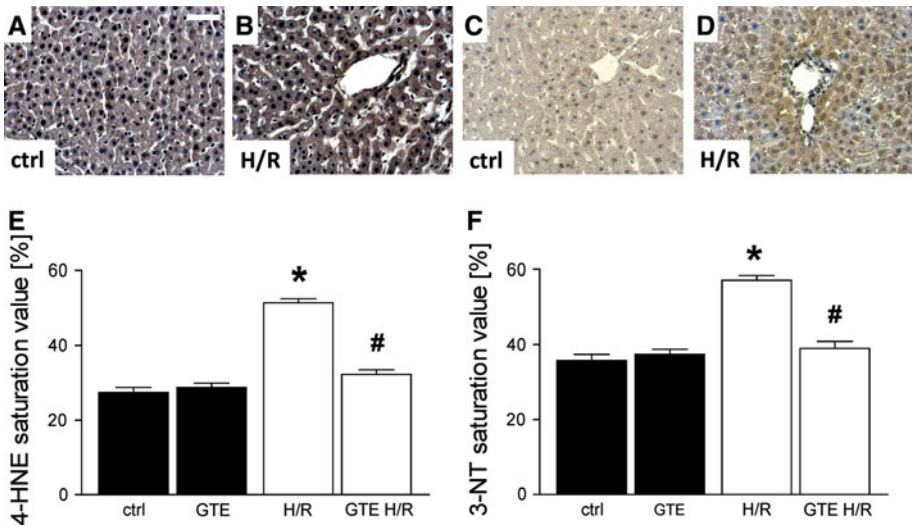

Fig. 1 GTE decreases lipid peroxidation and protein nitrosylation after hemorrhage and resuscitation. Immunohistological staining for 4-HNE (**a** and **b**) reveals areas of lipid peroxidation and immunohistology for 3-NT (**c** and **d**) reveals areas of protein nitrosylation, respectively. 4-HNE stained, representative liver sections from ctrl rats are given in **a** (*standard chow*) and **b** after H/R. 3-NT stained, representative liver sections from ctrl rats are given in **c** for ctrl and **d** for rats undergoing H/R; *bar* is 80 μm. The percentage of 4-HNE (**e**) and 3-NT (**f**) positive cells (*staining*) was semi-quantified in liver sections 2 h after resuscitation. Ctrl, standard chow + sham procedure; GTE, GTE-chow + sham procedure; H/R, standard chow + H/R; GTE H/R, GTE-chow + H/R (Newman-Keuls, $^{*}P < 0.05$ vs. both sham groups, $^{\#}P < 0.05$ ctrl H/R vs. GTE H/R group, $n = 6$)

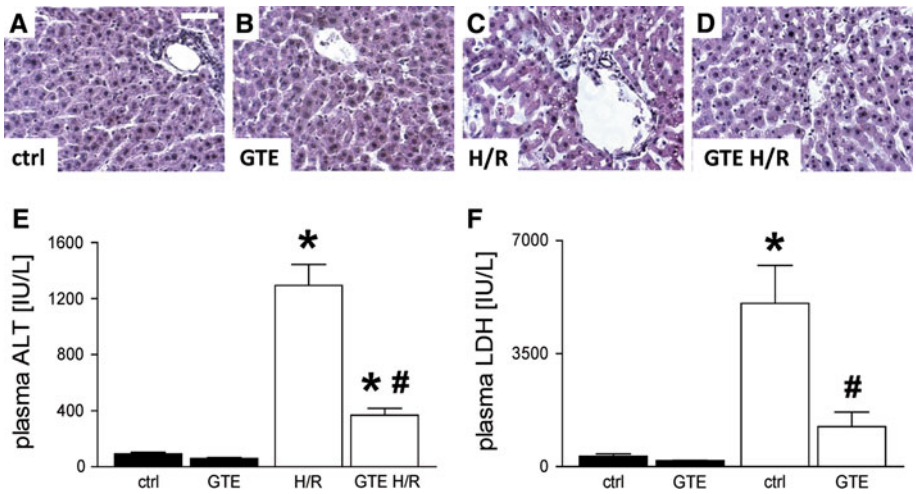

Fig. 2 GTE reduces histological liver necrosis (**a–d**) and plasma ALT (**e**) and LDH (**f**) levels assessed at 2 h after resuscitation. Representative hematoxylin and eosin stained liver lobes (A, ctrl, standard chow + sham procedure; B, GTE, GTE-chow + sham procedure; C, H/R, standard chow + H/R; and D, GTE H/R: GTE-chow + H/R) are shown; bar is 80 μm (Newman-Keuls, $^{*}P < 0.05$ vs. both sham groups, $^{\#}P < 0.05$ ctrl H/R vs. GTE H/R group, $n = 6$)

$P < 0.001$, Fig. 2f). GTE did not affect LDH levels after sham operation as compared to ctrl sham group (Fig. 2f).

Histopathological changes in hepatic tissue after hemorrhage and resuscitation

H/R induced large confluent areas of coagulative necrosis as indicated by cellular enlargement and dissolution (Fig. 2c). These changes were not detected after sham operation, neither in GTE nor in ctrl group (Fig. 2a–b). GTE-chow decreased hepatic necrosis after H/R (Fig. 2d) as compared to H/R group (Fig. 2c).

The specific differentiation between apoptotic and necrotic changes was assessed by TUNEL assay (reveals necrosis and apoptosis, Fig. 3, left row, red) combined with immunohistological staining for a neo-epitope of cytokeratin 18 (reveals apoptosis, Fig. 3, right row, green) using the monoclonal antibody M30 (31, 32). Livers of rats receiving normal chow demonstrated increased number of TUNEL as well as M30 positive, apoptotic hepatocytes after H/R as compared to sham-operated rats (quantified data not shown, $P < 0.05$, Fig. 3). GTE decreased the number of TUNEL positive as well as M30 positive cells (quantified data not shown, $P < 0.05$, Fig. 3) at 2 h after

resuscitation. Hence, GTE decreased hepatic necrosis as well as apoptosis after H/R.

Hepatic gene expression of pro- and anti-apoptotic genes after hemorrhage and resuscitation—Bax, Bcl 2 and Casp 8

The semi-quantitative real-time PCR showed a significant increase in Bax after H/R (100 ± 32) compared with GTE sham group (2 ± 1, $P < 0.05$) and the GTE H/R group (30 ± 10, $P < 0.05$, Fig. 4a). The transcription of Bax was also significantly enhanced in the GTE H/R group compared with the GTE sham group ($P < 0.05$), but significantly decreased compared with the ctrl H/R group ($P < 0.05$).

The mRNA expression of the anti-apoptotic gene Bcl 2 was not significantly increased after H/R in the control group (3 ± 1), whereas Bcl 2 mRNA expression was significantly enhanced in GTE-fed rats after H/R (8 ± 1) compared with sham-treated GTE group (1 ± 1) and the H/R group (3 ± 1, $P < 0.05$, Fig. 4b).

The transcription of Caspase 8 was significantly increased after H/R (11 ± 4) compared with both the GTE sham group (1 ± 1, $P < 0.05$) and the GTE H/R group (4 ± 2, $P < 0.05$, Fig. 4c).

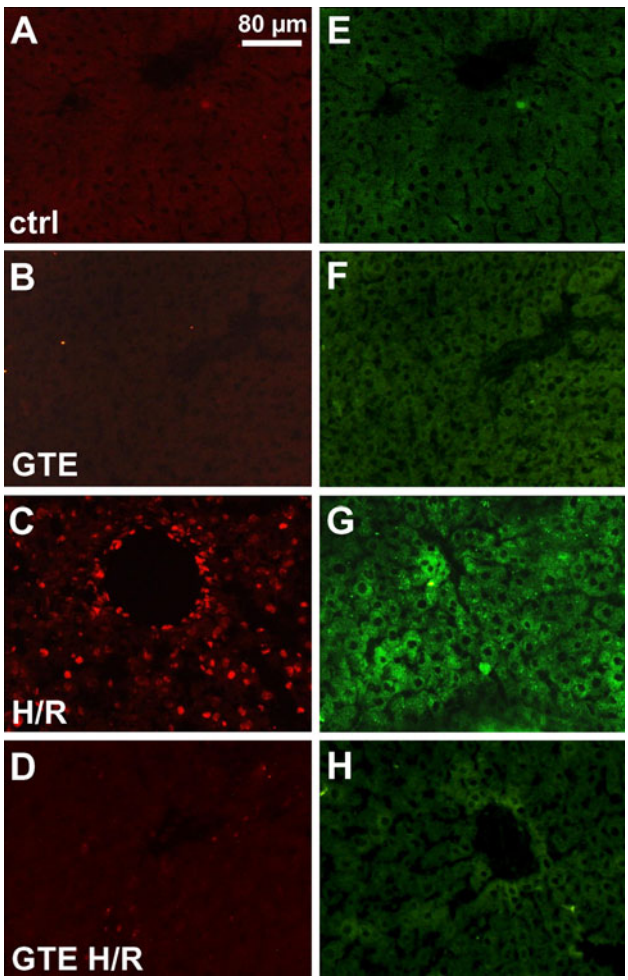

Fig. 3 GTE reduces apoptosis after hemorrhage and resuscitation. Apoptosis was assessed by TUNEL assay (*left row, red*) and immunohistological staining of the caspase cleavage product cytokeratin 18 (*right row, green*). TUNEL stained, representative liver sections from sham-operated rats are given in **a** (standard chow, ctrl) and **b** (GTE-chow, GTE). M30 stained, representative liver sections from sham-operated rats are given in **e** (ctrl) and **f** (GTE). TUNEL stained, representative liver sections from rats after H/R are given in **c** (*standard chow*, H/R) and **d** (GTE-chow, GTE H/R). M30 stained, representative liver sections from rats undergoing H/R are given in **g** (H/R) and **h** (GTE H/R); *bar* is 80 µm

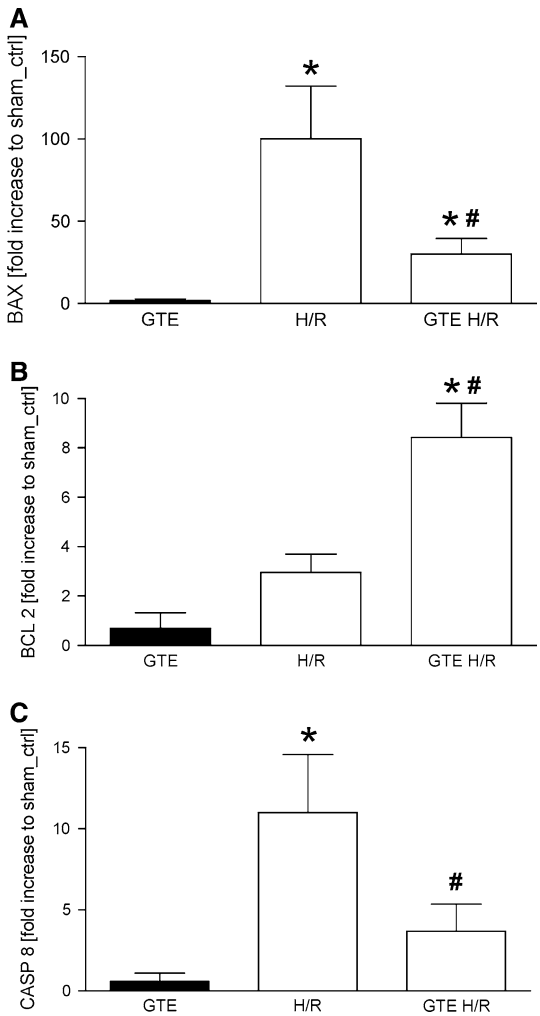

Fig. 4 Effects of GTE on hepatic gene expression of Bax, Bcl 2 and Caspase 8 at 2 h after resuscitation. Hepatic gene expressions of Bax (**a**), Bcl 2 (**b**) and Caspase 8 (**c**) were analyzed. After normalization to GAPDH expression, gene expression was measured as fold increase compared with sham-operated ctrl group (GTE: GTE-chow + sham procedure, H/R: standard chow + H/R, GTE H/R: GTE-chow + H/R; Wilcoxon, $^{*}P < 0.05$ vs. sham group, $^{\#}P < 0.05$ ctrl H/R vs. GTE H/R group, $n = 6$)

Systemic and local IL-6 changes after hemorrhage and resuscitation

Hemorrhage followed by resuscitation induces a systemic immune response, which was determined at 2 h after H/R by plasma IL-6 levels. Compared with sham ctrl group (14 ± 4 pg/mL) and sham GTE group (16 ± 5 pg/mL), plasma IL-6 levels increased significantly in rats fed with standard chow after H/R (1,509 ± 389 pg/mL, $P < 0.05$, Fig. 5a). This increase after H/R in the ctrl group was significantly reduced in the GTE H/R group (492 ± 135 pg/mL, $P < 0.05$, Fig. 5a). Plasma IL-6 levels did not differ between ctrl and GTE groups after sham operation (14 ± 4, and 16 ± 5 pg/mL, respectively, Fig. 5a).

The real-time PCR showed an increase in IL-6 mRNA expression at 2 h after resuscitation in liver samples obtained from ctrl H/R animals (12 ± 3) compared with both sham groups ($P < 0.05$, Fig. 5b). Administration of GTE attenuated significantly the increase in IL-6 gene expression in the GTE H/R group (3 ± 2) compared with the ctrl H/R group (12 ± 3, $P < 0.05$, Fig. 5b).

Western blot analysis of the inhibitory effects of GTE on NF-kappaB and JNK after hemorrhagic shock and resuscitation

To analyze the mechanism influenced by GTE, detections of non-phosphorylated JNK and IkappaKalpha as well as phosphorylated JNK and IkappaBalpha were performed by Western blot in liver homogenates collected at 2 h after resuscitation. Densitometric analysis of relative protein expression related to β-actin content and subsequent ratio of phosphorylated to total protein indicated an increase in phosphorylated JNK expression after H/R to 68 ± 3% compared with 46 ± 3% in sham-operated ctrl rats ($P < 0.05$, Fig. 6a, b). GTE administration reduced significantly the increase in JNK phosphorylation after H/R compared with ctrl H/R group (GTE H/R: 46 ± 7% and ctrl H/R: 68 ± 3%, respectively, $P < 0.05$, Fig. 6a, b). In parallel, the expression of JNK was significantly reduced after H/R in the ctrl group compared with both the sham groups and the GTE H/R group ($P < 0.05$, Fig. 6a, b).

Protein phosphorylation of the downstream inhibitor kappaB (IkappaB) of the IkappaB kinase alpha (IkappaK-alpha) was significantly enhanced after H/R in the ctrl H/R group (74 ± 8%) compared with 25 ± 3% in sham ctrl, 22 ± 2% in sham GTE group, and 30 ± 9% in GTE H/R group, respectively ($P < 0.05$, Fig. 6a, b, Table 1).

These results indicate that H/R-induced JNK and NF-kappaB activation effects strongly inhibited by GTE administration before H/R.

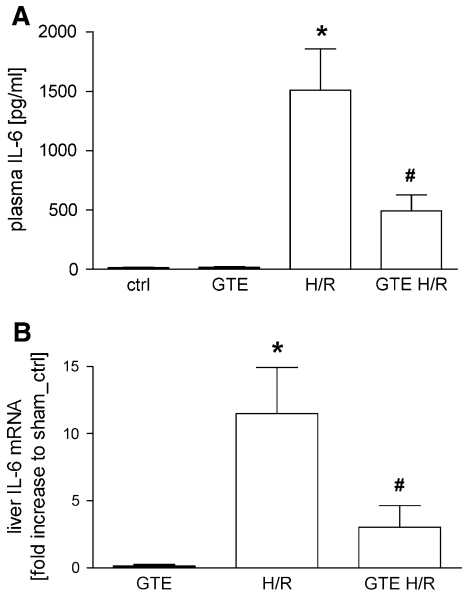

Fig. 5 GTE decreases plasma IL-6 levels (**a**) and hepatic IL-6 gene expression (**b**) at 2 h after resuscitation. Ctrl: standard chow + sham procedure, GTE: GTE-chow + sham procedure, H/R: standard chow + H/R, GTE H/R: GTE-chow + H/R (Newman-Keuls, $^{*}P < 0.05$ vs. both sham groups, $^{\#}P < 0.05$ ctrl H/R vs. GTE H/R group, $n = 6$)

Discussion

In the present study, we examined the effects of GTE on liver injury induced by hemorrhage followed by resuscitation and also provide molecular evidence for elucidation the function of GTE serving as potential anti-apoptotic and anti-inflammatory drug. GTE administration attenuates H/R-induced hepatic injury, apoptosis, synthesis and release of pro-inflammatory cytokines, oxidative and nitrosative stress (Figs. 1–5). These beneficial effects were associated with decreased phosphorylation of JNK and the NF-kappaB inhibitory unit IkappaBalpha as well as increased IkappaB-alpha expression after H/R, indicating that GTE may exert its beneficial effects via JNK and NF-kappaB inhibition (Fig. 6).

Formation of free radicals occurs in a variety of forms of hypoxia-induced liver injuries [25, 31]. Additionally, activated macrophages and neutrophils also produce ROS and RNS, predominantly during the reperfusion phase, leading to increased liver injury in several ischemia/reperfusion (I/R) models [32, 33]. Hepatic injury after I/R and H/R is associated with oxidative and nitrosative stress and characterized by lipid peroxidation, thereby damaging of cellular membranes, proteins, and DNA resulting in cellular injury [25, 28, 34]. Besides hepatocellular necrosis, apoptosis occurs in hepatic warm I/R partly due to the release of reactive oxygen species from activated neutrophils and macrophages and is associated with NADPH-oxidase-dependent peroxynitrite production after H/R [35].

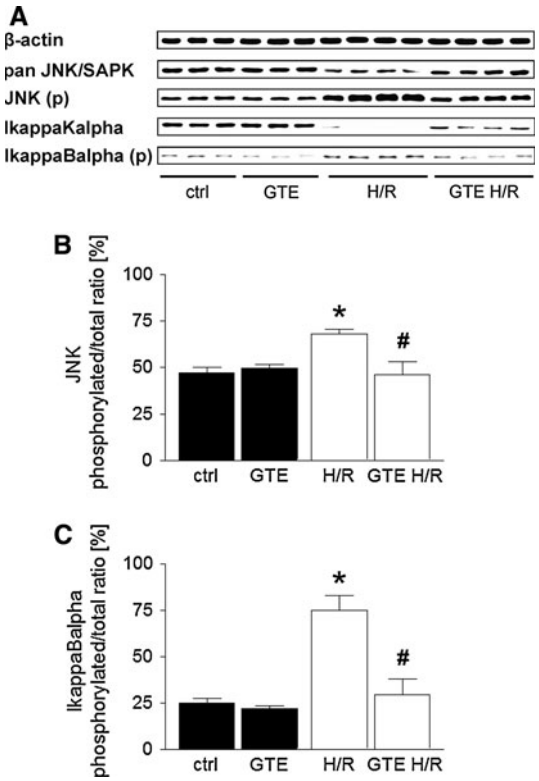

Fig. 6 GTE modifies the expression of JNK and IkappaKalpha as well as JNK and IkappaBalpha phosphorylation after hemorrhage and resuscitation. Ctrl, standard chow + sham procedure; GTE, GTE-chow + sham procedure; H/R, standard chow + H/R; GTE H/R, GTE-chow + H/R. **a**: *lane 1–6* were liver protein extracts from rats after sham operation (ctrl: *lane 1–3* and GTE: *lane 4–6*) or hemorrhage/resuscitation (H/R: *lanes 7–10* and GTE H/R: *lane 11–14*). **b** represents the ratio of phosphorylated JNK and total protein, **c** represents the ratio of phosphorylated IkappaBalpha and total protein, both respectively after densitometric measurements and normalization to β-actin (Newman-Keuls, $^{*}P < 0.05$ vs. both sham groups, $^{\#}P < 0.05$ ctrl H/R vs. GTE H/R group, $n = 6$, representative gel from 3 experiments is shown)

Table 1 Arterial pressure before and after hemorrhage and during resuscitation

	Ctrl [mm Hg]	GTE [mm Hg]	H/R [mm Hg]	GTE H/R [mm Hg]
During sham procedure	93 ± 3	92 ± 2	–	–
Before shock	–	–	91 ± 2	92 ± 2
Shock	–	–	31 ± 2	31 ± 2
At 15 min resuscitation	–	–	65 ± 7	66 ± 5
End of resuscitation	–	–	91 ± 3	91 ± 2

Mean arterial blood pressure in mm Hg was measured during sham procedure, before hemorrhagic shock, and at different times during the hemorrhage/resuscitation protocol; ctrl, standard chow + sham procedure; GTE, GTE-chow + sham procedure; H/R, standard chow + H/R; GTE H/R, GTE-chow + H/R ($n = 6$)

In support of this hypothesis, liver injury (ALT, necrosis, and apoptosis) is associated with increased formation of free radicals in our model of H/R (Figs. 1–4).

Green tea extracts from *C. sinensis* contain high concentrations of natural polyphenols that exert beneficial effects in models of hepatic I/R, liver transplantation, and other oxidative stress models due to their high scavenging activity on active free radicals. In a prospective, population-based study GT consumption even reduced mortality from cardiovascular diseases and cancer [23]. Consistent with these reports, in this study, we show that GTE (Sunphenon90LB) has potent anti-oxidative (4-HNE, Fig. 1) and anti-nitrosative (3-NT, Fig. 1) effects on H/R-induced oxidative and nitrosative stress in the liver, which are associated with only moderate hepatic injury.

Environmental stress as well as a wide variety of pro- and anti-inflammatory cytokines activate JNK, a stress activated protein kinase (SAPK) [36, 37]. With regard to the liver, JNK activation promotes organ damage in models of I/R, rat liver transplantation, partial hepatectomy, and H/R [12, 30, 36, 38, 39]. Furthermore, JNK activation is linked to the expression of several inflammatory genes, production of pro-inflammatory molecules, such as IL-6 and apoptosis after H/R and hypoxia [40–42]. Our previous studies demonstrated that inhibition of JNK had a beneficial effect on liver after H/R [12, 30]. However, data from this study indicate that JNK is activated after H/R and that GTE consumption prior to H/R reduced markedly JNK phosphorylation and hepatic injury (Fig. 6).

Oxidative stress also activates NF-kappaB, a transcription factor that consists of p65 and p50 subunits [43]. Several studies reported that reactive oxygen species directly induce NF-kappaB activation and cytokine production [44, 45]. Inhibitory proteins of the IkappaB family are bound to NF-kappaB and prevent its translocation to the nucleus. The phosphorylation of IkappaB leads to dissociation from NF-kappaB and subsequent degradation [46]. The activated NF-kappaB complex translocates to the nucleus, binds DNA, and induces the expression of pro-inflammatory mediators, which are involved in the pathogenesis of liver damage after H/R, such as IL-6, TNF-alpha, ICAM-1, endothelin 1, and heme oxygenase-1 [19, 47, 48]. NF-kappaB is activated in the lung, heart and liver after H/R [17, 49]. Consistent with these findings, we detect an activation of the NF-kappaB inhibitory IkappaBalpha unit in the liver after H/R, and low expression of its activator IkappaKalpha underlines IkappaBalpha phosphorylation (Fig. 6). In parallel to previously indicated results, activated NF-kappaB was accompanied by enhanced plasma IL-6 levels and hepatic IL-6 mRNA expression after H/R (Figs. 5, 6). The deleterious effects of IL-6 were demonstrated by IL-6 knockout mice undergoing

H/R that were protected from pro-inflammatory changes and hepatic injury [13]. Adjacent to anti-oxidative effects, green tea has shown remarkable anti-inflammatory and inhibitory effects on NF-kappaB, thus liver protection after hepatic I/R [25]. In line with these results, GTE consumption had an anti-inflammatory effect associated with decreased liver tissue damage that may be caused at least partly by NF-kappaB inactivation after H/R in our model.

Liver apoptosis occurs during H/R and is also associated with the release of ROS [50, 51]. Furthermore, prevention of increased caspase activity has been associated with reduced hepatic injury after H/R in rats [52, 53]. Hepatocellular necrosis also attributes to cellular disruption after H/R [12, 54]. JNK activation represents an important pro-apoptotic and pro-necrotic mechanism in tissues exposed to oxidative and/or nitrosative stress [30, 55]. JNK inhibition diminished apoptosis and brain injury after I/R as well as apoptosis and liver injury after H/R [12, 56]. Furthermore, NF-kappaB is known to protect against apoptosis via transcription of specific inhibitory proteins [57, 58]. Other studies report that NF-kappaB is an integral part of apoptosis and tissue injury, notably under conditions of I/R in rats [59]. Reduced hepatocellular apoptosis and necrosis after H/R by GTE were accompanied by reduced JNK phosphorylation and NF-kappaB activation (Figs. 3, 6). GTE significantly reduced hepatic gene expression of the pro-apoptotic Bax and caspase-8 and enhanced gene expression of the anti-apoptotic gene Bcl 2 after H/R (Fig. 4). Based on the present data, we cannot rule the possibility that GTE reduces hepatic injury via NF-kappaB and/or JNK inhibition by non-anti-oxidant mechanism. However, consistent with previous reports, GTE most likely inhibits JNK and NF-kappaB activation by scavenging ROS and RNS.

In conclusion, activation of NF-kappaB and JNK play decisive roles in the pathogenesis of hepatic injury after H/R by induction of pro-apoptotic gene expression, oxidative and nitrosative stress, apoptosis and necrosis, pro-inflammatory changes, and reduction of anti-apoptotic gene expression. GTE reduced the expression of pro-apoptotic genes, both types of cell death, apoptosis and necrosis, pro-inflammatory changes, oxidative and nitrosative stress, and enhanced the expression of anti-apoptotic Bcl 2 gene via inhibition of Nf-kappaB and JNK activation leading to highly improved hepatic morphology after H/R.

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Conflict of interest None.

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