



Epigallocatechin-3-gallate protects against hepatic ischaemia–reperfusion injury by reducing oxidative stress and apoptotic cell death

Eunyoung Tak¹, Gil-Chun Park²,
Seok-Hwan Kim², Dae Young Jun¹,
Jooyoung Lee¹, Shin Hwang²,
Gi-Won Song² and Sung-Gyu Lee²

Abstract

Objective: To investigate the protective effects of epigallocatechin-3-gallate (EGCG), a major polyphenol source in green tea, against hepatic ischaemia–reperfusion injury in mice.

Methods: The partial hepatic ischaemia–reperfusion injury model was created by employing the hanging-weight method in C57BL/6 male mice. EGCG (50 mg/kg) was administered via an intraperitoneal injection 45 min before performing the reperfusion. A number of markers of inflammation, oxidative stress, apoptosis and liver injury were measured after the ischaemia–reperfusion injury had been induced.

Results: The treatment groups were: sham-operated (Sham, $n = 10$), hepatic ischaemia–reperfusion injury (IR, $n = 10$), and EGCG with ischaemia–reperfusion injury (EGCG-treated IR, $n = 10$). Hepatic ischaemia–reperfusion injury increased the levels of biochemical and histological markers of liver injury, increased the levels of malondialdehyde, reduced the glutathione/oxidized glutathione ratio, increased the levels of oxidative stress and lipid peroxidation markers, decreased B-cell lymphoma 2 levels, and increased the levels of Bax, cytochrome c, cleaved caspase-3, and cleaved caspase-9. Pretreatment with EGCG ameliorated all of these changes.

Conclusion: The antioxidant and antiapoptotic effects of EGCG protected against hepatic ischaemia–reperfusion injury in mice.

¹Asan Institute for Life Sciences, Asan Medical Centre, University of Ulsan College of Medicine, Seoul, Republic of Korea

²Department of Surgery, Division of Liver Transplantation and Hepatobiliary Surgery, Asan Medical Centre, University of Ulsan College of Medicine, Seoul, Republic of Korea

Corresponding author:

Gi-Won Song, Department of Surgery, Division of Liver Transplantation and Hepatobiliary Surgery, Asan Medical Centre, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 138-736, Republic of Korea.

Email: drsong71@amc.seoul.kr



Keywords

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Introduction

Hepatic ischaemia–reperfusion injury is the major cause of liver injury, which is of clinical concern, especially following diverse hepatectomy and living donor liver transplantation.¹ Ischaemia–reperfusion injury, which involves complex inflammatory pathways, is a significant cause of liver damage and an important factor in a variety of diseases, injuries, and following transplantation surgery.^{2,3} As liver ischaemia ends and reperfusion begins, oxidative stress is one of the first changes to take place and it regulates an oxidative stress-mediated apoptosis signalling pathway.^{4–6} Hepatic oxidative stress and inflammation are important factors involved in the development of acute hepatic injury.⁷ Damage to DNA bases is usually caused by reactive oxygen species (ROS), which include hydroxyl, the superoxide anion, and hydrogen peroxide.^{8,9} All ROS respond to varying degrees at the initiation of reperfusion as reduced electron carrier molecules of hypoxic cells donate electrons to oxygen during reperfusion.¹⁰ Therefore, effective therapeutic strategies for hepatic ischaemia–reperfusion injury are urgently needed. During the early period of reperfusion, damage is induced by signalling molecules, specifically proinflammatory chemokines and cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)- α , whereas neutrophil-mediated cytotoxicity is the main cause of hepatic injury during the late period.^{11,12}

Epigallocatechin-3-gallate (EGCG) is the major polyphenol found in green tea.^{13,14} It has a defensive effect against chronic inflammation and other diseases due to its anti-oxidative effects.^{15–19} Furthermore, EGCG

has been shown to be an effective treatment for the high levels of fat, cholesterol, and alcohol uptake that induce chronic liver injury, liver fibrosis and fatty hepatic disease in murine models.^{20–22} However, the protective role of EGCG on hepatic ischaemia–reperfusion injury and the specific molecular mechanisms related to oxidative stress remain unclear.^{23,24}

The aim of this current study was to investigate the protective effects of EGCG against hepatic ischaemia–reperfusion injury in mice. Additionally, the study sought to clarify the molecular signalling mechanisms involved in regulating liver ischaemia–reperfusion injury, particularly the mechanisms related to oxidative stresses signalling and apoptosis.

Materials and methods

Murine model of hepatic ischaemia–reperfusion injury

Six-week old male C57BL/6 male mice were purchased from Joongang Laboratory Animals (Seoul, Republic of Korea) and were housed under constant temperature (22–23°C) and normal humidity (40–60%) with a 12 h/12 h light/dark cycle. Mice (body weight range 20–30 g) were supplied with standard chow diet and reverse osmosis purified water. After 4 weeks, the partial hepatic ischaemia–reperfusion injury model was created by employing the hanging-weight method as described previously.²⁵ In the partial hepatic ischaemia–reperfusion injury model mice, approximately 70% of the liver including the left lobe was exposed to 45 min of hepatic ischaemia followed by 6 h of reperfusion. All animal surgery and

experimental protocols were approved by and performed in accordance with the Republic of Korea and Asan Medical Centre Institution Animal Care and Use Committee Guidelines regarding the use of living animals (no. 2015-02-052).

Administration of EGCG

Epigallocatechin-3-gallate was diluted in saline according to the supplier's instructions (Sigma-Aldrich, St Louis, MO, USA). In order to determine the dose of EGCG to use in the hepatic ischaemia–reperfusion injury experiments, a pilot study was undertaken in which EGCG was administered at a range of doses (10 mg/kg, 20 mg/kg, 50 mg/kg, 80 mg/kg, and 100 mg/kg; $n=8$ for each group). Each dose of EGCG was administered intraperitoneally just before performing the partial hepatic ischaemia injury for 45 min. After 6 h of reperfusion, whole blood samples and the damaged livers were obtained from the mice. In order to produce serum, the whole blood samples were kept at room temperature (22–23°C) for 30–60 min to enable coagulation and then centrifuged at 2000 g for 30 min at 4°C to separate the serum. The clear supernatant was collected and stored at –80°C until use. Liver histology, hepatic malondialdehyde (MDA), hepatic glutathione (GSH):oxidized GSH (GSSG) ratio, serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were assessed and significant improvements in all results were not observed until the mice were pretreated with at least 50 mg/kg (data not shown). As this pilot study demonstrated that 50 mg/kg was the most efficient dose, 50 mg/kg EGCG was administered via an intraperitoneal injection 45 min before performing the reperfusion for the remainder of the hepatic ischaemia–reperfusion injury experiments. For the current study, the mice were separated into the following three groups: sham-operated group ($n=10$), hepatic

ischaemia–reperfusion injury group ($n=10$), and EGCG with ischaemia–reperfusion injury group ($n=10$). All surgical procedures were performed on mice anaesthetized by intraperitoneal injection of Zoletil 50 (0.15 ml kg^{-1} ; Virbac SA, Carros, France) and Rompun (0.3 ml kg^{-1} ; Bayer Korea, Ansan, Republic of Korea) mixture. The mice in the sham-operated group underwent anaesthesia, laparotomy and suturing only.

Lipid peroxidation and glutathione tests

The thiobarbituric acid levels for reactive substances in the hepatic tissues were measured at a wavelength of 532 nm to measure the MDA level, which is the last product of lipid peroxidation. All reagents and specimens were prepared according to the manufacturer's instructions for a lipid peroxidation (MDA) assay kit (catalogue no. ab118970; Abcam, Cambridge, UK) and the optical density was assessed using a SunriseTM microplate reader (Tecan, Männedorf, Switzerland). The GSH level was measured using yeast-GSH reductase, 2-nitrobenzoic acid and nicotinamide adenine dinucleotide phosphate, at a wavelength of 412 nm using the SunriseTM microplate reader. The same protocol was employed to measure the level of GSSG in the presence of 2-vinylpyridine; then, the GSH:GSSG ratio was calculated. All procedures were undertaken in accordance with the manufacturer's instructions for the glutathione assay kit (catalogue no. 703002; Cayman Chemical Company, Ann Arbor, MI, USA).

Liver function test

Whole blood samples were collected from the inferior vena cava of the mice and were kept at room temperature (22–23°C) for 30–60 min to enable coagulation. Clotted blood specimens were centrifuged at 2000 g for 30 min at 4°C to separate the serum. The clear supernatant was collected and stored at

–80°C until use. The levels of AST and ALT were analysed using a Hitachi 7180 automated biochemistry analyser (Hitachi, Tokyo, Japan) according to the manufacturer's instructions.

Transcriptional analyses

Total RNA was isolated from 45–50 mg of murine liver tissue after 6 h of reperfusion using QIAzol lysis solution and then separated into its mRNA components following the manufacturer's instructions (QIAGEN Korea, Seoul, Republic of Korea). cDNA was generated from the mRNA using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The transcript levels were identified using real-time reverse transcription–polymerase chain reaction (RT–PCR) using the LightCycler® 480 system (Roche Diagnostics, Mannheim, Germany) with AccuPower® 2X Greenstar qPCR Master Mix (Bioneer Corporation, Daejeon, Republic of Korea). IL-6 (Mm_Il6_1_SG, catalogue no. QT00098875), TNF- α (Mm_Tnf_1_SG, catalogue no. QT00104006), IL-1 β (Mm_Il1b_2_SG, catalogue no. QT01048355), IL-10 (Mm_Il10_1_SG, catalogue no. QT00106169) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm_Gapdh_3_SG, catalogue no. QT01658692) primers were purchased from QIAGEN (QuantiTect Primer Assay, product no. 249900; Valencia, CA, USA). Primer sequences used for PCR were as follows: heme oxygenase-1 (HO-1) sense 5'-CACGCATATACCCGCTACCT-3', antisense 5'-CCAGGTGTTTCATTTCGAGCA-3', producing a PCR amplification product of 174 base pairs (bp); thioredoxin reductase-1 (TRXr-1) sense 5'-CAGTTCGTCCCAACGAAAAT-3', antisense 5'-GCACATTGGTCTGCTCTTA-3', producing a PCR amplification product of 232 bp; nitric oxide synthase-2 (NOS-2) sense 5'-CTCACCCCGTCCTTGAGTA-3', antisense 5'-GGTCG

CTTTGACTCTCTTGG-3', producing a PCR amplification product of 171 bp; heat shock protein (Hsp) 60 sense 5'-CGTTGCCAATAACACAAACG-3', antisense 5'-CTTCAGGGGTTGTCACAGGT-3', producing a PCR amplification product of 200 bp; and Hsp70 sense 5'-TGGTGTGACGAAGATGAAG-3', antisense 5'-AGGTCTGAAGATGAGCACGTT-3', producing a PCR amplification product of 235 bp. The primers were synthesized by Bioneer Corporation. The cycling programme involved preliminary denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s, followed by a final elongation step at 72°C for 5 min. The relative mRNA levels of each gene were normalized to GAPDH as described previously.²⁶

Western blot analyses

Protein samples were extracted from approximately 70 mg of frozen liver tissue. For the whole lysate extraction, RIPA Lysis and Extraction Buffer (Biosesang, Sengnam, Republic of Korea) supplemented with a protease inhibitor cocktail (complete Mini, ethylenediaminetetra-acetic acid-free; Roche Diagnostics) was used according to the manufacturer's instructions. The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Tissue protein lysates containing the same amount of protein were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and were transferred onto a nitrocellulose membrane using Trans-Blot® Turbo™ Transfer System (catalogue no. 1704155, Bio-Rad) and RTA Mini Nitrocellulose Transfer Kit (catalogue no. 1704270, Bio-Rad). For blocking, the nitrocellulose membrane was incubated for 1 h with 5% skimmed milk (AppliChem, Cheshire, CT, USA) in Tris-buffered saline with Tween-20 (TBST; pH 7.6; 20 mM Tris-HCl, 150 mM NaCl and

0.1 % Tween 20) at room temperature (22–23°C). Then, the nitrocellulose membrane was incubated with the appropriate primary antibody overnight at 4°C, followed by incubation with a secondary antibody for 1 h at room temperature. All primary and secondary antibodies were diluted in TBST (pH 7.6). Primary antibodies against HO-1 (1:1000 dilution; catalogue no. ADI-SPA-895, Enzo Life Sciences, Exeter, UK), TRXr-1 (1:1000 dilution; catalogue no. 6925, Cell Signaling Technology, Danvers, MA, USA), NOS-2 (1:1000 dilution; catalogue no. BS1186, Bioworld Technology, St Louis Park, MN, USA), Hsp 70 (1:2000 dilution; catalogue no. 610608, BD Biosciences, San Jose, CA, USA) and Hsp60 (1:2000 dilution; catalogue no. 611562, BD Biosciences) were used in order to detect oxidative stress marker proteins. Primary antibodies against hydroxynonenal (HNE, 1:1000 dilution; catalogue no. LS-C68183, LifeSpan Biosciences, Seattle, WA, USA), MDA (1:1000 dilution; catalogue no. ALX-210-879, Enzo Life Sciences), carbonyl reductase 1 (CBR1, 1:1000 dilution; catalogue no. ab174852, Abcam), and acrolein (1:1000 dilution; catalogue no. LS-C63521, LifeSpan Biosciences) were used to detect lipid peroxidation marker proteins. Primary antibodies against aldehyde dehydrogenase (ALDH, 1:1000 dilution; catalogue no. SAB2501484, Sigma-Aldrich) and aldose reductase (AR, 1:1000 dilution; catalogue no. SAB2100091, Sigma-Aldrich) were used to detect CBR1 downstream signalling proteins. Primary antibodies against B-cell lymphoma 2 (Bcl-2, 1:1000 dilution; catalogue no. sc-7382, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-2 Associated X Protein (Bax, 1:1000 dilution; sc-7480, Santa Cruz Biotechnology), cleaved caspase-3 (1:1000 dilution; catalogue no. 9664, Cell Signaling Technology), cleaved caspase-9 (1:1000 dilution; catalogue no. 9508, Cell Signaling

Technology) and cytochrome c (1:1000 dilution; catalogue no. BS1089; Bioworld Technology) were used to detect apoptotic signalling proteins. Primary antibodies against actin (1:20 000 dilution; catalogue no. A3854, Sigma-Aldrich) were used as an internal control. The nitrocellulose membranes treated with primary antibodies against HO-1, TRXr-1, NOS-2, HNE, MDA, CBR1, acrolein, AR, cleaved caspase-3 and cytochrome c were washed three times with TBST (pH 7.6) for 10 min at room temperature and incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10 000 dilution; catalogue no. sc-2004; Santa Cruz Biotechnology) diluted in TBST (pH 7.6) for 1 h at room temperature. The nitrocellulose membranes treated with primary antibodies against Hsp60, Hsp70, Bcl-2, Bax, cleaved caspase-9 and actin were incubated in HRP-conjugated goat anti-mouse antibody (1:10 000 dilution; catalogue no. sc-2005, Santa Cruz Biotechnology) after being washed three times with TBST (pH 7.6) for 10 min at room temperature. The nitrocellulose membrane treated with primary antibodies against ALDH was treated with rabbit anti-goat IgG-peroxidase antibody (1:30 000 dilution; catalogue no. A5420, Sigma-Aldrich) after being washed three times with TBST (pH 7.6) for 10 min at room temperature. After incubating with the secondary antibodies, the membranes were washed five times with TBST (pH 7.6) for 30 min at room temperature. Chemiluminescence reagent SuperSignal™ West Femto Substrate (Thermo Fisher Scientific) was used to develop the membranes according the manufacturer's instruction. An Image Quant LAS-4000 imaging system (GE Healthcare Life Sciences, Piscataway, NJ, USA) was used to capture the chemiluminescent images in accordance with the equipment instructions.

Liver histology

The hepatic tissue samples were harvested after 6 h of reperfusion. The samples were fixed in 4% paraformaldehyde (pH 7.4; Sigma-Aldrich) and then embedded in paraffin wax for 24 h. The paraffin blocks were cut into 3 μ m-thick sections. Liver sections were deparaffinized with xylene, serially rehydrated in a series of graded ethanol (100% to 80%) and stained with Mayer's haematoxylin (Sigma-Aldrich) and eosin Y (Sigma-Aldrich) solutions. Then the sections were dehydrated with serial solutions of graded ethanol (80% to 100%) and xylene. The sections were mounted on coverslips with Histomount mounting solution (Life Technologies, Carlsbad, CA, USA). Morphological changes to the liver tissues were observed using a light microscope ($\times 200$; Axioskop 2 plus with AxioCam; Carl Zeiss, Thornwood, NY, USA). The tissue sections were scored for liver injury using the Suzuki scoring method (0–4) in order to determine the severity of sinusoidal congestion, vacuolization of the cytoplasm, and parenchymal cell necrosis.²⁷ Up to eight sections for each liver sample were studied ($n = 5$ –8 for each condition) by a pathologist who was blind to the experimental group.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 6.0 software for Windows XP (GraphPad Software, La Jolla, CA, USA). The liver injury scores from the histological analysis are presented as median and range. All other data are presented as the mean \pm SD of five to eight animals per group. For the Western blot analyses, the experiments were repeated at least three times. Statistical analyses were performed using one-way analysis of variance followed by Bonferroni's multiple

comparison test. A value of $P < 0.05$ was considered statistically significant.

Results

Mice with hepatic ischaemia–reperfusion injury (IR group) showed higher levels of markers of liver injury compared with age-, sex- and weight-matched mice that had been pretreated with 50 mg/kg EGCG (EGCG-treated IR group), including significantly increased serum AST and ALT levels (Figures 1a and 1b) ($P < 0.05$ for both comparisons), significantly increased mRNA transcript levels of inflammatory markers (IL-6, TNF- α and IL-1 β ; Figures 1c–1f) ($P < 0.05$ for all comparisons) and greater histological evidence of liver injury (Figures 1g and 1h). Hepatic reperfusion injury (IR group) was associated with a significant reduction in the level of IL-10 compared with the EGCG-treated mice (EGCG-treated IR group) ($P < 0.05$).

To examine the levels of hepatic ischaemia–reperfusion injury in each experimental group, lipid peroxidation and GSH component tests were undertaken on the injured hepatic tissue samples. As shown in Figure 2a, the EGCG-treated mice (EGCG-treated IR group) had significantly higher MDA levels compared with the sham-operated group (Sham group) ($P < 0.05$). The MDA level in EGCG-treated mice (EGCG-treated IR group) was significantly reduced compared with that in mice with hepatic ischaemia–reperfusion injury (IR group) ($P < 0.05$). The GSH:GSSG ratio in the mice with ischaemia–reperfusion injury (IR group) was significantly lower compared with the ratio in the sham-operated mice (Sham group) and in the EGCG-treated mice (EGCG-treated IR group) ($P < 0.05$ for both comparisons) (Figure 2b).

The results of the Western blot analyses of the protein levels of antioxidant enzymes

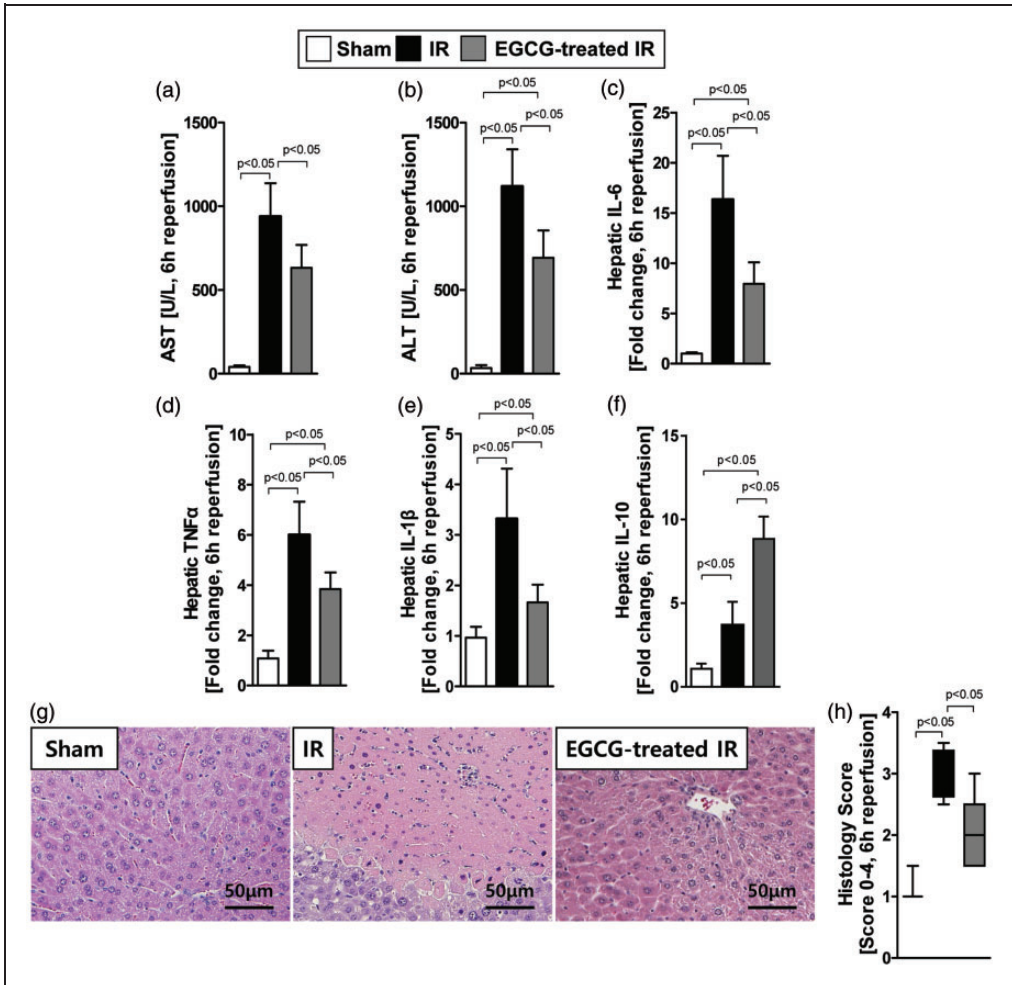


Figure 1. Investigations into the effects of pretreatment with 50 mg/kg epigallocatechin-3-gallate (EGCG) prior to hepatic ischaemia–reperfusion injury on biochemical and histological makers of liver injury in mice. The three treatment groups were as follows: sham-operated group (Sham, $n = 10$), hepatic ischaemia–reperfusion injury group (IR, $n = 10$), and EGCG with ischaemia–reperfusion injury group (EGCG-treated IR, $n = 10$). (a) Serum aspartate aminotransferase (AST) levels. Data presented as mean \pm SD of five to eight animals per group; (b) Serum alanine aminotransferase (ALT) levels. Data presented as mean \pm SD of five to eight animals per group; (c–f) Results of real-time reverse transcription–polymerase chain reaction analysis of the mRNA levels in samples of mouse liver after hepatic ischaemia–reperfusion injury for the following markers of liver injury: (c) interleukin(IL)-6, (d) tumour necrosis factor (TNF)- α , (e) IL-1 β , and (f) IL-10. Data presented as mean \pm SD of five to eight animals per group; (g) Representative photomicrographs showing liver sections stained with haematoxylin and eosin. The hepatic ischaemia–reperfusion injury group (IR) showed inflammatory cell infiltration in the liver. Scale bar: 50 μ m; (h) Quantification of the liver injury measured using the Suzuki histological scoring index (0–4). Data are presented as median (50th percentile), box (25th to 75th percentile) and whisker (minimum to maximum). $P < 0.05$, one-way analysis of variance followed by Bonferroni's multiple comparison test. The colour version of this figure is available at: <http://imr.sagepub.com>.

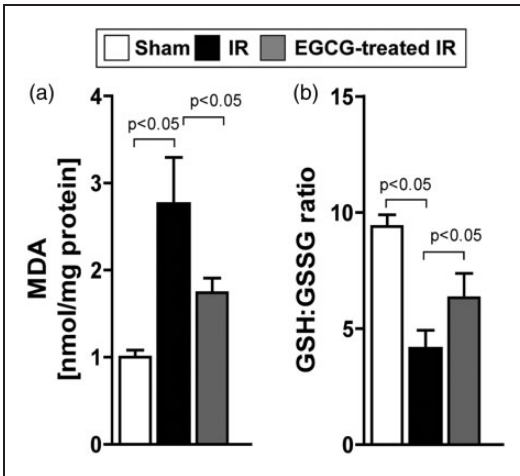


Figure 2. Investigations into the effects of pre-treatment with 50 mg/kg epigallocatechin-3-gallate (EGCG) prior to hepatic ischaemia–reperfusion injury on lipid peroxidation and glutathione (GSH)/oxidized glutathione (GSSG) ratio in mice. The three treatment groups were as follows: sham-operated group (Sham, $n = 10$), hepatic ischaemia–reperfusion injury group (IR, $n = 10$), and EGCG with ischaemia–reperfusion injury group (EGCG-treated IR, $n = 10$). (a) Malondialdehyde (MDA) levels. Data presented as mean \pm SD of five to eight animals per group; (b) GSH/GSSG ratio. Data presented as mean \pm SD of five to eight animals per group. $P < 0.05$, one-way analysis of variance followed by Bonferroni's multiple comparison test.

are shown in Figure 3a. The levels of the oxidative stress markers were increased in the mice with hepatic ischaemia–reperfusion injury (IR group) compared with the sham-operated mice (Sham group) and the EGCG-treated mice (EGCG-treated IR group). The lipid peroxidation proteins were increased in the mice with hepatic ischaemia–reperfusion injury (IR group) compared with the sham-operated mice (Sham group) in an effort to overcome hepatic injury (Figure 3a). In contrast, the levels of HNE, MDA, CBR1, and acrolein were decreased in the EGCG-treated mice with ischaemia–reperfusion injury (EGCG-

treated IR group) compared with the mice with hepatic ischaemia–reperfusion injury (IR group). Similarly, the levels of ALDH and AR were higher in the mice with hepatic ischaemia–reperfusion injury (IR group) compared with the EGCG-treated mice with ischaemia–reperfusion injury (EGCG-treated IR group). The results of RT–PCR of the mRNA levels of five oxidative stress markers confirmed the findings of the Western blot analyses (Figure 3b).

The results of the Western blot analyses of the protein levels of Bcl-2, an anti-apoptotic protein, showed that it was significantly downregulated in the mice with ischaemia–reperfusion injury (IR group) compared with the sham-operated mice (Sham group) ($P < 0.05$) (Figures 4a and 4b). The EGCG-treated mice (EGCG-treated IR group) had levels of Bcl-2 comparable with the sham-operated mice (Sham group) suggesting that they had less liver injury as a result of activating the antiapoptotic signalling pathway. The results of the Western blot analyses of the protein levels of the proapoptotic protein Bax showed that the mice with ischaemia–reperfusion injury (IR group) had significantly higher levels compared with the sham-operated mice (Sham group) and the EGCG-treated mice (EGCG-treated IR group) ($P < 0.05$ for both comparisons). Mice with ischaemia–reperfusion injury (IR group) had significantly upregulated levels of cleaved caspase-3 and cleaved caspase-9 compared with the sham-operated mice (Sham group) and the EGCG-treated mice (EGCG-treated IR group) ($P < 0.05$ for all comparisons) (Figures 4c and 4d). Mice with ischaemia–reperfusion injury (IR group) had significantly upregulated levels of cytochrome c compared with the sham-operated mice (Sham group) and the EGCG-treated mice (EGCG-treated IR group) ($P < 0.05$ for both comparisons) (Figures 4e and 4f).

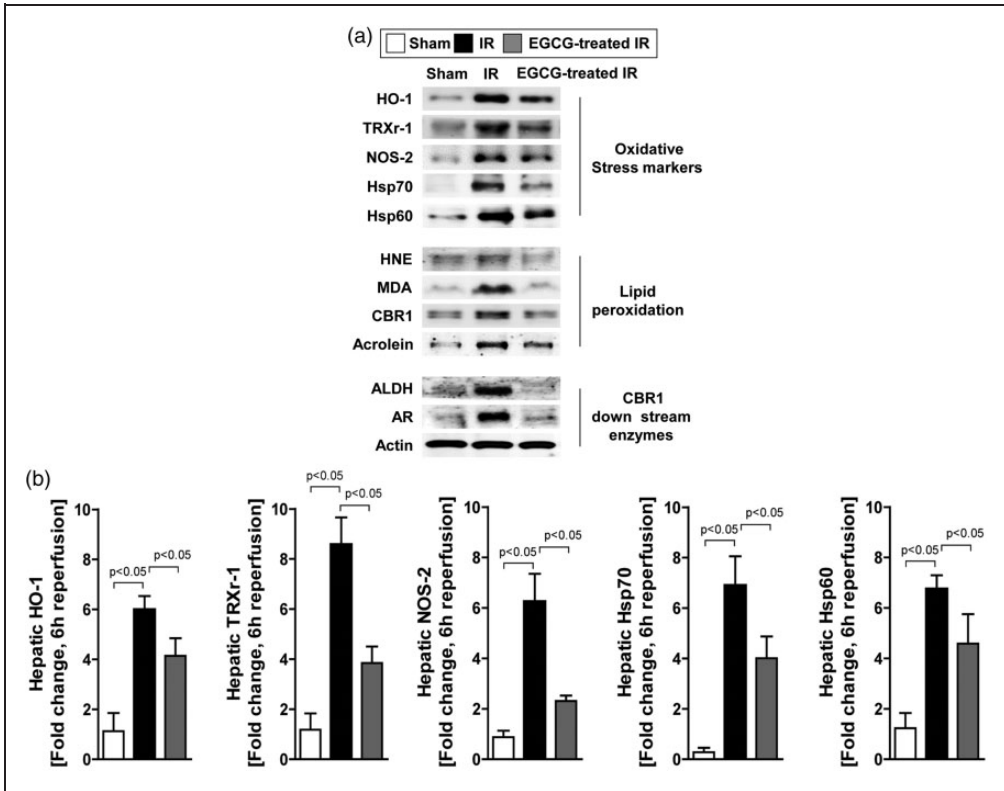


Figure 3. Western blot and reverse transcription–polymerase chain reaction (RT–PCR) analyses to investigate the effects of pretreatment with 50 mg/kg epigallocatechin-3-gallate (EGCG) prior to hepatic ischaemia–reperfusion injury on antioxidant enzyme protein and mRNA levels in mice. The three treatment groups were as follows: sham-operated group (Sham, $n = 10$), hepatic ischaemia–reperfusion injury group (IR, $n = 10$), and EGCG with ischaemia–reperfusion injury group (EGCG-treated IR, $n = 10$). (a) Representative Western blots showing oxidative stress markers, lipid peroxidation markers, and carbonyl reductase I (CBR1) down-stream enzymes after hepatic ischaemia–reperfusion injury. β -actin was used as a loading control; (b) Results of RT–PCR analysis of the mRNA levels of oxidative stress markers. The control housekeeping gene was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are presented as mean \pm SD. $P < 0.05$, one-way analysis of variance followed by Bonferroni's multiple comparison test. HO-1, heme oxygenase-1; TRXr-1, thioredoxin reductase-1; NOS-2, nitric oxide synthase-2; Hsp70, heat shock protein 70; Hsp60, heat shock protein 60; HNE, hydroxynonenal; MDA, malondialdehyde; ALDH, aldehyde dehydrogenase; AR, aldose reductase.

Discussion

The biological functions of EGCG have been studied previously, including its anti-inflammatory, antioxidative stress, and tumour-suppressing effects.^{28,29} However, the most important function of EGCG is its antioxidant effect.³⁰ During ischaemia–

reperfusion injury, ROS are highly involved in the development of tissue damage.³¹ In the early stage of ischaemia, the singlet O_2 concentration is consistent with a low oxygen supply and the PO_2 is low enough to keep the oxygen-dependent lipid peroxidation reactions occurring at undetectable rates.³² Free radicals can also induce lipid

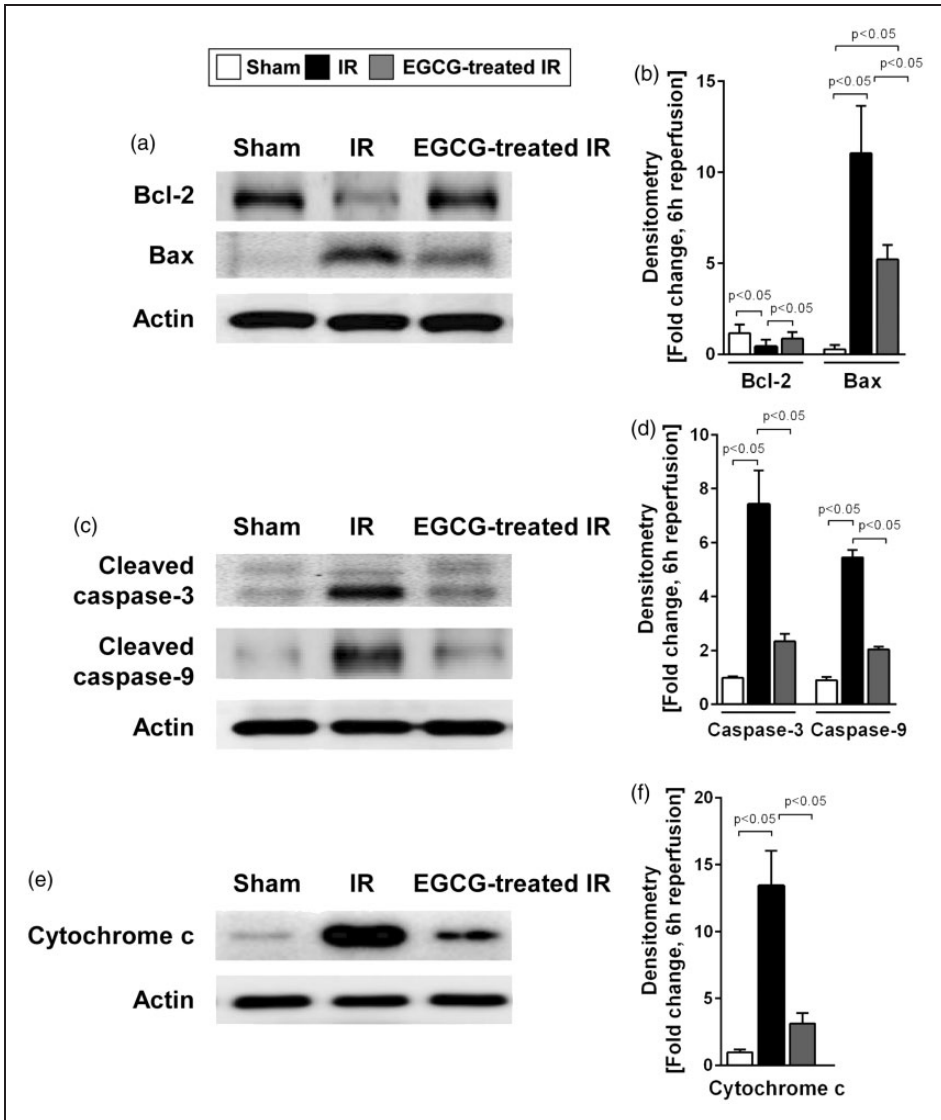


Figure 4. Western blot analyses to investigate the effects of pretreatment with 50 mg/kg epigallocatechin-3-gallate (EGCG) prior to hepatic ischaemia–reperfusion injury on the levels of B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax), cleaved caspase-3, cleaved caspase-9 and cytochrome c in mice. The three treatment groups were as follows: sham-operated group (Sham, $n = 10$), hepatic ischaemia–reperfusion injury group (IR, $n = 10$), and EGCG with ischaemia–reperfusion injury group (EGCG-treated IR, $n = 10$). (a) Representative Western blots showing Bcl-2 and Bax protein levels. β -actin was used as a loading control; (b) Densitometric results of Bcl-2 and Bax protein levels; (c) Representative Western blots showing cleaved caspase-3 and cleaved caspase-9 protein levels. β -actin was used as a loading control; (d) Densitometric results of cleaved caspase-3 and cleaved caspase-9 protein levels; (e) Representative Western blots showing cytochrome c protein levels. β -actin was used as a loading control; (f) Densitometric results of cytochrome c. For b, d and f, data are presented as mean \pm SD; $P < 0.05$, one-way analysis of variance followed by Bonferroni's multiple comparison test.

peroxidation in the cell phospholipid bilayer, which can destroy the cytosol and other organelles.^{5,33} The severity of oxidative stress and cell damage depends on the duration of the ischaemia.^{25,34} Reperfusion increases the steady state concentration of singlet O₂ and decreases both the concentration of chain-breaker antioxidants and antioxidant enzymes with a slight decrease in the oxygen uptake by tissues.³² Critically, the antioxidant functions of EGCG can reduce ischaemia–reperfusion injury via activating antioxidant protection signalling.³⁵ In addition, EGCG has been shown to be an effective treatment for chronic liver disease, liver fibrosis, hepatic inflammation, alcohol-induced liver disease, and fatty liver.^{7,36–38} Despite this body of evidence and to the best of our knowledge, the potential protective roles of EGCG against hepatic ischaemia–reperfusion injury has not been investigated previously.

In the present study, hepatic ischaemia–reperfusion injury in a mouse model was characterized by the upregulation of the proinflammatory cytokines IL-6, IL-1 β , and TNF- α . In contrast, levels of the anti-inflammatory cytokine IL-10 were significantly decreased following hepatic ischaemia–reperfusion injury. Administration of EGCG reduced the levels of the proinflammatory cytokines and increased the level of IL-10. In this current study, EGCG treatment effectively reduced the markers of hepatic ischaemia–reperfusion injury, namely the GSH:GSSG ratio, showing that EGCG protects against oxidative stress by decreasing GSH following hepatic ischaemia–reperfusion injury. EGCG administration also reduced the amount of lipid peroxidation following hepatic ischaemia–reperfusion injury. As these current data demonstrated, the upregulation of the oxidative stress markers, including HO-1, TRXR-1, NOS-2, Hsp70, and Hsp60, played an important role in protecting the liver from oxidative stress-induced damage.

These upregulated oxidative enzymes are able to protect against hepatic ischaemia–reperfusion injury by regulating oxidative stresses and free radical reactions.³⁹

These current data also showed that mice treated with EGCG had increased levels of antioxidant enzymes (i.e. HO-1 and TRXR-1) following hepatic ischaemia–reperfusion injury. The increase in the serum levels of AST and ALT was a strong indicator of the occurrence of oxidative stress damage in mice following hepatic ischaemia–reperfusion injury. Thus, the current findings suggest that the administration of EGCG reduces the oxidative stress damage that occurs following hepatic ischaemia–reperfusion injury.

Oxidative stress has been shown to regulate the apoptotic signalling pathway in cells.⁴⁰ Research has shown that many factors are associated with oxidative stress-related cellular destruction, including members of the Bcl-2 family,^{41–43} poly (ADP-ribose) polymerase (PARP),^{44,45} cleaved caspase-3,⁴⁶ and cytochrome c.^{47,48} In particular, within the Bcl-2 family are antiapoptotic molecules that inhibit Ca²⁺ release from the endoplasmic reticulum,⁴⁹ and lipid peroxidation from free radicals.⁴² In contrast, Bax, another Bcl-2 family member, initiates cellular apoptosis through mitochondrial-related apoptosis.⁵⁰ Together, Bcl-2 and B-cell lymphoma extra large (Bcl-xL) can create a heterodimer that can repress the proapoptotic function of Bax.^{51,52} Physiologically, equal amounts of the Bcl-2 and Bax proteins exist in the cell.⁵³ The present study hypothesized that EGCG administration would reduce hepatic ischaemia–reperfusion injury via Bcl-2-dependent mechanisms. Hepatic ischaemia–reperfusion injury in mice resulted in a downregulation of Bcl-2 protein levels and an upregulation of Bax protein levels in the present study. These effects were reduced following the administration of EGCG. In addition, PARP, caspase-3, and caspase-9 have been shown to be important cellular molecules involved in

apoptotic signaling.^{54,55} Increases in PARP, caspase-3 and caspase-9 have been shown in other cells and the induction of caspase-3 and caspase-9 has been demonstrated in hepatic ischaemia–reperfusion injury, indicating that caspase-related cellular apoptosis signalling is harmful in ischaemia–reperfusion induced tissue damage.⁵⁶ In the present study, the levels of cleaved caspase-3 and cleaved caspase-9 protein increased in response to ischaemia–reperfusion injury and EGCG administration significantly reduced the levels of these two caspases. These present data suggest that EGCG administration reduces hepatic ischaemia–reperfusion-related cell apoptosis, possibly by decreasing cleaved caspase-3 and cleaved caspase-9 levels. Thus, the levels of cleaved caspase-3, cleaved caspase-9, and Bcl-2 proteins appear to be restored to normal levels by administering EGCG.

Proapoptotic proteins from the mitochondria result in mitochondrial membrane collapse, membrane depolarization, and the release of proapoptotic proteins from the mitochondria into the cytoplasm.⁵⁷ Therefore, the release of mitochondrial cytochrome c activates a signalling cascade for apoptosis. In the current study, the level of cytochrome c in the cytosol was upregulated by hepatic ischaemia–reperfusion injury and EGCG treatment decreased the hepatic ischaemia–reperfusion-induced increases in cytochrome c.

In summary, this present study demonstrated that EGCG treatment reduced the extent of hepatic ischaemia–reperfusion injury by decreasing oxidative stress-induced damage and cellular apoptosis. Hence, EGCG has the potential to be used as a therapeutic agent for hepatic ischaemia–reperfusion injury.

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Declaration of conflicting of interests

The authors declare that there are no conflicts of interest.

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References

1. Fayed NA, Sayed EI, Saleh SM, et al. Effect of dexmedetomidine on hepatic ischaemia–reperfusion injury in the setting of adult living donor liver transplantation. *Clin Transplant* 2016; 30: 470–482.
2. Wanner GA, Ertel W, Müller P, et al. Liver ischemia and reperfusion induces a systemic inflammatory response through Kupffer cell activation. *Shock* 1996; 5: 34–40.
3. Jaeschke H. Molecular mechanisms of hepatic ischemia–reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol* 2003; 284: G15–G26.
4. Marshall KM, He S, Zhong Z, et al. Dissecting the complement pathway in hepatic injury and regeneration with a novel protective strategy. *J Exp Med* 2014; 211: 1793–1805.
5. Lemasters JJ and Thurman RG. Reperfusion injury after liver preservation for transplantation. *Annu Rev Pharmacol Toxicol* 1997; 37: 327–338.
6. Mukhopadhyay P, Horváth B, Zsengeller Z, et al. Mitochondrial reactive oxygen species generation triggers inflammatory response and tissue injury associated with hepatic ischemia–reperfusion: therapeutic potential of mitochondrially targeted antioxidants. *Free Radic Biol Med* 2012; 53: 1123–1138.

7. Karamese M, Guvendi B, Karamese SA, et al. The protective effects of epigallocatechin gallate on lipopolysaccharide-induced hepatotoxicity: an in vitro study on Hep3B cells. *Iran J Basic Med Sci* 2016; 19: 483–489.
8. Cooke MS, Evans MD, Dizdaroglu M, et al. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J* 2003; 17: 1195–214.
9. Unal B, Ozcan F, Tuzcu H, et al. Inhibition of neutral sphingomyelinase decreases elevated levels of nitrative and oxidative stress markers in liver ischemia–reperfusion injury. *Redox Rep* 2016; 1–13. [Epub ahead of print].
10. Quesnelle KM, Bystrom PV and Toledo-Pereyra LH. Molecular responses to ischemia and reperfusion in the liver. *Arch Toxicol* 2015; 89: 651–657.
11. Jaeschke H and Woolbright BL. Current strategies to minimize hepatic ischaemia–reperfusion injury by targeting reactive oxygen species. *Transplant Rev (Orlando)* 2012; 26: 103–114.
12. Kojima Y, Suzuki S, Tsuchiya Y, et al. Regulation of pro-inflammatory and anti-inflammatory cytokine responses by Kupffer cells in endotoxin-enhanced reperfusion injury after total hepatic ischaemia. *Transpl Int* 2003; 16: 231–240.
13. Nam NH. Naturally occurring NF-kappaB inhibitors. *Mini Rev Med Chem* 2006; 6: 945–951.
14. Cabrera C, Artacho R and Giménez R. Beneficial effects of green tea—a review. *J Am Coll Nutr* 2006; 25: 79–99.
15. Morris J, Fang Y, De Mukhopdhyay K, et al. Natural agents used in chemoprevention of aerodigestive and GI cancers. *Curr Pharmacol Rep* 2016; 2: 11–20.
16. Wu H, Xin Y, Xu C, et al. Capecitabine combined with (-)-epigallocatechin-3-gallate inhibits angiogenesis and tumor growth in nude mice with gastric cancer xenografts. *Exp Ther Med* 2012; 3: 650–654.
17. Zeng X and Tan X. Epigallocatechin-3-gallate and zinc provide anti-apoptotic protection against hypoxia/reoxygenation injury in H9c2 rat cardiac myoblast cells. *Mol Med Rep* 2015; 12: 1850–1856.
18. Shi J, Liu F, Zhang W, et al. Epigallocatechin-3-gallate inhibits nicotine-induced migration and invasion by the suppression of angiogenesis and epithelial-mesenchymal transition in non-small cell lung cancer cells. *Oncol Rep* 2015; 33: 2972–2980.
19. Lee JC, Chung LC, Chen YJ, et al. Upregulation of B-cell translocation gene 2 by epigallocatechin-3-gallate via p38 and ERK signaling blocks cell proliferation in human oral squamous cell carcinoma cells. *Cancer Lett* 2015; 360: 310–318.
20. Tipoe GL, Leung TM, Liong EC, et al. Epigallocatechin-3-gallate (EGCG) reduces liver inflammation, oxidative stress and fibrosis in carbon tetrachloride (CCl₄)-induced liver injury in mice. *Toxicology* 2010; 273: 45–52.
21. Shen K, Feng X, Su R, et al. Epigallocatechin 3-gallate ameliorates bile duct ligation induced liver injury in mice by modulation of mitochondrial oxidative stress and inflammation. *PLoS ONE* 2015; 10: e0126278.
22. Xiao J, Ho CT, Liong EC, et al. Epigallocatechin gallate attenuates fibrosis, oxidative stress, and inflammation in non-alcoholic fatty liver disease rat model through TGF/SMAD, PI3 K/Akt/FoxO1, and NF-kappa B pathways. *Eur J Nutr* 2014; 53: 187–199.
23. Suzuki M, Tabuchi M, Ikeda M, et al. Protective effects of green tea catechins on cerebral ischemic damage. *Med Sci Monit* 2004; 10: BR166–BR174.
24. Aneja R, Hake PW, Burroughs TJ, et al. Epigallocatechin, a green tea polyphenol, attenuates myocardial ischaemia reperfusion injury in rats. *Mol Med* 2004; 10: 55–62.
25. Zimmerman M, Tak E, Kaplan M, et al. Use of a hanging-weight system for liver ischaemia in mice. *J Vis Exp* 2012; 7: e2550.
26. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402–408.
27. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, et al. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and

- cyclosporine. *Transplantation* 1993; 55: 1265–1272.
28. Chowdhury A, Sarkar J, Chakraborti T, et al. Protective role of epigallocatechin-3-gallate in health and disease: a perspective. *Biomed Pharmacother* 2016; 78: 50–59.
 29. Ahmad N, Feyes DK, Nieminen AL, et al. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Natl Cancer Inst* 1997; 89: 1881–1886.
 30. Namal Senanayake SPJ. Green tea extract: Chemistry, antioxidant properties and food applications—A review. *J Funct Foods* 2013; 5: 1529–1541.
 31. Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, et al. Factors in the pathophysiology of the liver ischemia-reperfusion injury. *J Surg Res* 2008; 147: 153–159.
 32. González-Flecha B, Cutrin JC and Boveris A. Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. *J Clin Invest* 1993; 91: 456–464.
 33. Johnson MK and Loo G. Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutat Res* 2000; 459: 211–218.
 34. Tu Q, Xiong Y, Fan L, et al. Peroxiredoxin 6 attenuates ischemia- and hypoxia-induced liver damage of brain-dead donors. *Mol Med Rep* 2016; 13: 753–761.
 35. Oliveira MR, Nabavi SF, Daglia M, et al. Epigallocatechin gallate and mitochondria – A story of life and death. *Pharmacol Res* 2016; 104: 70–85.
 36. Yu DK, Zhang CX, Zhao SS, et al. The anti-fibrotic effects of epigallocatechin-3-gallate in bile duct-ligated cholestatic rats and human hepatic stellate LX-2 cells are mediated by the PI3K/Akt/smad pathway. *Acta Pharmacol Sin* 2015; 36: 473–482.
 37. Ding RB, Tian K, Huang LL, et al. Herbal medicines for the prevention of alcoholic liver disease: a review. *J Ethnopharmacol* 2012; 144: 457–465.
 38. Gan L, Meng ZJ, Xiong RB, et al. Green tea polyphenol epigallocatechin-3-gallate ameliorates insulin resistance in non-alcoholic fatty liver disease mice. *Acta Pharmacol Sin* 2015; 36: 597–605.
 39. Shen SQ, Zhang Y, Xiang JJ and Xiong CL. Protective effect of curcumin against liver warm ischemia/reperfusion injury in rat model is associated with regulation of heat shock protein and antioxidant enzymes. *World J Gastroenterol* 2007; 13: 1953–1961.
 40. Chandra J, Samali A and Orrenius S. Triggering and modulation of apoptosis by oxidative stress. *Free Radic Biol Med* 2000; 29: 323–333.
 41. Susnow N, Zeng L, Margineantu D, et al. Bcl-2 family proteins as regulators of oxidative stress. *Semin Cancer Biol* 2009; 19: 42–49.
 42. Hockenbery DM, Oltvai ZN, Yin XM, et al. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 1993; 75: 241–251.
 43. Adams JM and Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998; 281: 1322–1326.
 44. Yu SW, Wang H, Poitras MF, et al. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* 2002; 297: 259–263.
 45. Soldani C and Scovassi AI. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. *Apoptosis* 2002; 7: 321–328.
 46. Ricci JE, Gottlieb RA and Green DR. Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *J Cell Biol* 2003; 160: 65–75.
 47. Kluck RM, Bossy-Wetzell E, Green DR, et al. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997; 275: 1132–1136.
 48. Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997; 275: 1129–1132.
 49. He H, Lam M, McCormick TS, et al. Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl-2. *J Cell Biol* 1997; 138: 1219–1228.
 50. Wolter KG, Hsu YT, Smith CL, et al. Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 1997; 139: 1281–1292.
 51. Oltvai ZN, Millman CL and Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a

- conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993; 74: 609–619.
52. Shimizu S, Eguchi Y, Kosaka H, et al. Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. *Nature* 1995; 374: 811–813.
53. Chao DT and Korsmeyer SJ. BCL-2 family: regulators of cell death. *Annu Rev Immunol* 1998; 16: 395–419.
54. Soldani C and Scovassi AL. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. *Apoptosis* 2002; 7: 321–328.
55. Mancini M, Nicholson DW, Roy S, et al. The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. *J Cell Biol* 1998; 140: 1485–1495.
56. Jaeschke H and Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. *Gastroenterology* 2003; 125: 1246–1257.
57. Luo X, Budihardjo I, Zou H, et al. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998; 94: 481–490.