Epigallocatechin 3-Gallate Ameliorates Bile Duct Ligation Induced Liver Injury in Mice by Modulation of Mitochondrial Oxidative Stress and Inflammation

Kezhen Shen¹,²*, Xiaowen Feng¹,², Rong Su¹,², Haiyang Xie¹,², Lin Zhou¹,², Shusen Zheng¹,²,³*

¹ Key Laboratory of Combined Multi-organ Transplantation, Ministry of Public Health, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, China, ² Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, China, ³ Division of Hepatobiliary and Pancreatic Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China

☯ These authors contributed equally to this work.

* shusenzheng@zju.edu.cn

Abstract

Cholestatic liver fibrosis was achieved by bile duct ligation (BDL) in mice. Liver injury associated with BDL for 15 days included significant reactive oxygen/nitrogen species generation, liver inflammation, cell death and fibrosis. Administration of Epigallocatechin 3-Gallate (EGCG) in animals reduced liver fibrosis involving parenchymal cells in BDL model. EGCG attenuated BDL-induced gene expression of pro-fibrotic markers (Collagen, Fibronectin, alpha 2 smooth muscle actin or SMA and connective tissue growth factor or CTGF), mitochondrial oxidative stress, cell death marker (DNA fragmentation and PARP activity), NFκB activity and pro-inflammatory cytokines (TNFα, MIP1α, IL1β, and MIP2). EGCG also improved BDL induced damages of mitochondrial electron transport chain complexes and antioxidant defense enzymes such as glutathione peroxidase and manganese superoxide dismutase. EGCG also attenuated hydrogen peroxide induced cell death in hepatocytes in vitro and alleviate stellate cells mediated fibrosis through TIMP1, SMA, Collagen 1 and Fibronectin in vitro. In conclusion, the reactive oxygen/nitrogen species generated from mitochondria plays critical pathogenetic role in the progression of liver inflammation and fibrosis and this study indicate that EGCG might be beneficial for reducing liver inflammation and fibrosis.

Introduction

Metabolic liver disease, chronic alcohol drinking and viral hepatitis are major causative agents for chronic liver damage. Chronic liver damage leads to liver inflammation followed by fibrosis and cirrhosis, which is life threatening if not treated [1]. Chronic cholestatic liver disease with
massive fibrosis is one of the most common occurrences after liver transplantation [2]. The most common treatment is ursodeoxycholic acid but it does not prevent fibrosis [3]. Antioxidants, inhibitors of cell death pathways and diet are tested against experimental animal models [4–7].

Epigallocatechin-3-Gallate (EGCG) is major component of green tea and has been used in traditional medicine in China [8, 9]. EGCG is a polyphenol and is a type of catechin. It is an ester of epigallocatechin and gallic acid. The beneficial effect of EGCG has been reported in many liver disease model in animals such as ischemia/reperfusion injury, fatty liver, alcoholic liver damage and cancer [10–14]. Recently, clinical studies with EGCG dietary supplement demonstrated improved liver function in obese [15].

Liver fibrosis is manifested by significant deposition of extracellular matrix and stellate cells play a major role in the process [16]. Fibroblasts derive from hepatocytes also contribute to fibrosis [17]. The specific role of myofibroblast and endothelial cells in developing liver fibrosis has also been reported [18, 19]. Thus, liver fibrosis is a complex process involving different cell types with specific role. The most important trigger for fibrosis is chronic inflammation and inflammatory cells plays a an orchestrated network with liver cell types to develop conducive environment for fibrosis [20].

Hepatocytes are most sensitive cells among liver cells when exposed to toxic agents such as bile acids. The major cause of damage is mediated by ROS and leads to apoptosis. In liver fibrosis, damaged hepatocytes induce trigger signal by releasing cytokines which promote macrophage/kupffer cells and lymphocyte recruitment [21]. It is also proposed that fibroblast in fibrotic liver is mainly generated from those hepatocytes. In addition to that, hepatocytes also release paracrine molecules (such as fibroblast growth factor) which lead to stellate cell activation. Activated stellate cells with altered morphology secrete pro-inflammatory cytokines, induce adhesion molecules and generate extracellular matrix [22]. Activated HSC also converted to myofibroblastic phenotype which have contractile capability and differentiate into collagen producing cells and expressing myogenic and fibrotic markers such as smooth muscle actin, TGF-β etc.

In this study we investigated the role of EGCG in liver inflammation and fibrosis using in vivo mice model of bile duct ligation. We also provided evidence that EGCG selectively reduced mitochondrial injury by modulating oxidative damage and antioxidant defense. We also demonstrated that EGCG reduced hydrogen peroxide induced cell death in hepatocytes and attenuated production of fibrotic markers from stellate cells in vitro.

Method

Ethics Statement of the study

This study was performed under “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health. All protocols were approved by Animal Ethics Review Committees of Zhejiang University. All efforts were made to minimize suffering.

Animal Experiments

Male inbred C57BL/6 (H2b) mice at 6–8 weeks old were purchased from the Animal Research Institution of Zhejiang Province (Hangzhou, China). Mice were housed under a standard SPF environment with a 12h dark-light cycle and free access to water and food. All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Review Committees of Zhejiang University.
Bile duct ligation procedure

Bile duct ligation (BDL) surgeries were performed under anesthesia. The anesthesia was performed by intraperitoneal injection of xylazine at 10mg/kg and ketamine at 100mg/kg which allows mice to stay deep anesthetized condition for over 2 hours when the surgeries were carried out. The surgical area was shaved. The area was then be prepped by scrubbing the skin 3 times with povidone-iodine alternating with 70% ethanol. These survival surgeries involve a midline laparotomy (about 15mm) and bile duct was ligated with silk thread at two position as described earlier [4] and wounds were closed. In sham-operated control (refereed as control), the common bile duct was not ligated. Post-operative analgesic administration e.g. ketoprofen 5 mg/kg in sterile isotonic fluids, SQ, was given daily for at least 2 days after surgery The mice were sacrificed under 5% isoflurane anesthesia followed by cervical dislocation at 15 days after the surgery as described earlier [23, 24].

All mice were monitored every day for distress or pain. We have observed a death rate of 12% in 15 days BDL, which is in consistence with earlier report [25]. No adverse effect was observed. To test the effect of EGCG on BDL-induced hepatic fibrosis, mice received one dose of pre-operative (2 hour) IP injection of EGCG and alternate day postoperative IP injection of EGCG (30mg/kg/day) for two weeks. A total of 24 mice were performed with bile duct ligation where 12 of them treated with EGCG. For sham control and EGCG treatment we used 10 mice/group.

Isolation of Mitochondria from fresh tissue

All procedure of liver tissues was performed at on ice to minimize mitochondrial-membrane and protein degradation. Mitochondria were isolated using commercial tissue mitochondria isolation Kit (Pierce, USA). Liver tissue from experimental animals were harvested and immersed in isotonic buffer provided with kit. Mitochondrial pellets were suspended in 500 μl of RIPA buffer, and protein concentration was determined by using Bio Rad protein assay kit. The values obtained were corrected for BSA as standard.

Mitochondrial protein 3-nitrotyrosine (3-NT) content

Hepatic 3-NT levels were determined with ELISA kit from Hycult biotechnology, Cell sciences, Canton, USA.

Picro-Sirius Red staining

Quantitative determination of hepatic fibrosis was performed with Picro Sirius Red Stain kit (Abcam Company Ltd. China) and followed manufacturer’s instruction. The amount of red staining, marker for collagen staining was analyses from histological image using ImageJ software (NIH, USA). Images were analyzed from six random 100X fields from each animal and averaged.

RNA isolation and Quantitative Real-time-PCR

RNA isolation was carried out Trizol reagents (Life Technologies, USA) according to previously published method [26]. Superscript III (Life Technologies, USA) was used for reverse transcription and Syber green (TaKaRa, China) method was employed for Real-time PCR. Real-time PCR were carried out in ABI 7500 instrument. Fold change was calculated as described earlier [27].The gene specific primers were purchased from Qiagen (USA).
Mitochondrial complex I, II and IV activities

Activities of mitochondrial complex I, complex II, and complex IV were performed using Microplates assay kits according to the manufacturer’s instructions. (Mitosciences, USA) Activities were calculated based on the manufacturer’s instruction on kinetic data and expressed as fold change.

Quantification of MnSOD activity from mitochondrial fraction

MnSOD activity was determined from mitochondrial fraction using SOD activity kit (Enzo Life Sciences International, Inc., USA). SOD activity was determined from percent inhibition of the rate of WST-1-formazan formation with colorimetric readout at 450 nm. A kinetic assay was performed according to manufacturer’s instruction. SOD activity is expressed as fold change compared to sham control.

Glutathione Peroxidase Assay from mitochondrial fraction

Mitochondrial glutathione peroxidase was measured using Glutathione Peroxidase Assay Kit (Abcam Company Ltd. China) according to manufacturer’s instruction. Glutathione peroxidase reduces cumene hydroperoxide while oxidizing GSH to GSSG. The generated GSSG is reduced to GSH with consumption of NADPH by GR. The decrease of NADPH (measured at 340 nm) is proportional to Glutathione Peroxidase activity. The activity was displayed as fold change compared to sham control.

Western blot analysis

The protein content of of mitochondrial and cytosolic fractions was determined according to the Bradford method. Equal amounts of protein were loaded onto SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Western blots were performed by using antibodies directed against prohibitin (1:500, Abcam Company Ltd. China) and beta-actin (1:2000, Abcam Company Ltd. China), Secondary antibodies were purchased from FazendoMedia (Beijing, China).

Hepatic PARP activity

PARP activities in the liver tissue were performed using commercial assay kit (Trevigen Inc.) according to instruction provided. The quantitative values were expressed as fold change compared to sham control.

NFkB p65 binding ELISA

Nuclear lysates were assessed to quantify relative nuclear p65 transcript binding using kit from Abcam (China). In short, a specific double stranded DNA sequence containing the NFkB response element is immobilized onto the wells of a 96 well plate for ELISA analysis of p65 binding as detected by antibody binding and HRP conjugate detection. Absorbance was measured at 450nm. Both positive and non-specific binding controls were included.

Primary hepatocyte isolation and culture

Hepatocytes from mice were isolated based on standard method as published earlier [28]. Hepatocytes were treated with one ROS component hydrogen peroxide (HP) for 24 hours at 5mM. EGCG was added at 10μM concentration. Cells were analyzed by flow cytometry for
early apoptotic marker Annexin V APC and cell death dye Sytox green (Life Technologies, USA).

**Stellate Cells isolation and culture**

Mouse stellate cells were isolated based on earlier published method [29]. Stellate cells were grown in 6 well culture plates for 8 days. EGCG were added at 10μM every day after adding fresh media. RNA was isolated from fresh (Day0) or after 8 days(Day8) and real-time PCR were performed as described before.

**Statistical Analysis**

All data were presented as the means ± SEMs. Multiple comparisons (Tukeys) were performed using one way ANOVA using Graph Pad Prism software (USA). A p value less than 0.05 was considered statistically significant in all analyses.

**Results and Discussion**

**EGCG attenuates BDL induced liver fibrosis in mice**

Chronic liver injury was achieved at 15 days after BDL procedure. BDL induced significant fibrosis as evident from picrosirius red staining of collagen (Fig 1A). Treatment with EGCG on alternate day schedule at 30mg/kg dose reduced fibrosis. Quantitative measurement demonstrated that there is about 16 fold increase in fibrotic area due to BDL and EGCG reduced the fibrotic area up to 37% (Fig 1B). Further analyses of the images at higher magnification demonstrated fibrosis and necrosis of damaged parenchymal cells (Fig 2). We also examined expression of four key genes associated with fibrosis by real-time PCR All four genes namely collagen I, Fibronectin, SMA and CTGF were induced at mRNA level to 7.9 fold, 6.2 fold, 7.7 fold and 8.8 fold respectively in BDL model as expected (Fig 3A–3D). Treatment with

![Fig 1. Effect of EGCG on BDL induced liver fibrosis in mice. BDL caused significant liver fibrosis as measured by Picrosirius Red staining (Panel A) and quantified by Image J (Panel B). BDL resulted in severe liver fibrosis which was attenuated by EGCG treatment. Results are mean ± S.E.M. n = 6/group. * p<0.05 versus control; and # p<0.05 versus BDL.](https://doi.org/10.1371/journal.pone.0126278.g001)
EGCG reduced BDL induced gene expression of collagen I, Fibronectin, SMA and CTGF to 62%, 54%, 48% and 70% of BDL level respectively. BDL is a well-established chronic liver injury model and is widely used [30]. BDL induces hepatocyte progenitor cells proliferation (such as biliary epithelial cells, oval cells) with associated portal inflammation and fibrosis [31, 32]. These processes lead to obstructive jaundice in two weeks and cirrhosis in 4 to 6 weeks [33]. The justification behind 15 days endpoint after BDL in this study was to investigate fibrosis as well as reduced mortality in mice was also considered [25]. This time point has been used earlier [4, 24]. The polyphenolic compound EGCG is the major catechin found in green tea and is thought to impart many of the health benefits [34]. The EGCG dose used for this animal study has been reported earlier and does not have any negative effect on liver or other vital organ [35–37]. In a recent human studies, bioavailability was between 1.6% at a low dose (75 mg/kg body weight) and 13.9% at higher doses (250 mg/kg and 400 mg/kg) when provided orally to healthy volunteers [38]. Protective effect of EGCG from BDL induced fibrosis demonstrated beneficial effect. EGCG shown to improve liver function in other liver injury model [10, 12–14] and reduced fibrosis in acute CCl₄ liver injury [39].
EGCG attenuates BDL induced mitochondrial oxidative stress by modulating mitochondrial antioxidant defense in mice

To understand the role of mitochondrial oxidative stress, mitochondria were isolated from liver and oxidative/nitrative markers were analyzed. HNE protein adducts from mitochondrial fraction increased significantly in liver due to BDL (3.2 fold) and EGCG treatment reduced significantly to 41% (Fig 4B). Mitochondrial protein nitration is also increased to 2.8 fold and EGCG attenuated 37% from BDL with vehicle group (Fig 4A). We also demonstrated the purity of mitochondrial fractions and separated cytosolic fraction from the same set of samples by western blot analyses using mitochondrial marker prohibitin and cytoplasmic market beta-actin (Fig 4C). BDL induced HNE or nitrated protein might be relevant for mitochondrial dysfunction. Oxidized or nitrated modified mitochondrial proteins significantly affect mitochondrial function [40–42]. Targets of protein nitration in mitochondria include key enzymes and electron transport chain complexes and this process is mediated by peroxynitrite [43, 44].
modification of mitochondrial proteins has been reported earlier and is mediated by lipid peroxidation [45].

Oxidative stress in mitochondria is modulated by its antioxidant defense mechanism. The balance of pro-oxidant and anti-oxidant in mitochondria is important for mitochondrial function in liver. We examined two anti-oxidant enzymes Glutathione Peroxidase and Manganese Superoxide Dismutase (Fig 5). BDL leads to 31% decrease in Glutathione Peroxidase activity in liver mitochondria and EGCG attenuated that reduced activity to control level. Activity of Manganese Superoxide Dismutase also reduced 24% compared to control group and restored to normal level by EGCG treatment. EGCG did not have any effect when administered alone (Fig 5). Loss of these enzyme activities may be contributed to oxidative modification, which was shown earlier [46–48]. The protective role of EGCG against mitochondrial oxidative stress might be due to its antioxidant capacity and it has been shown recently that EGCG can accumulate in mitochondria [49]. EGCG demonstrates mitochondrial protection in other tissue injury models [37, 50–54]. Many other similar plant polyphenol also have similar role in protecting against tissue injury [55–57].

Fig 4. Effect of EGCG on BDL induced mitochondrial oxidative/nitrative stress in mice liver. BDL caused significant increase in mitochondrial oxidative and nitrative stress as measured by protein nitration and HNE protein adducts using commercial ELISA kits and these are footprints for nitrative and oxidative stress respectively. BDL induced nitrative stress (Panel A) and oxidative stresses (Panel B) in mitochondria were attenuated by EGCG treatment. Results are mean ± S.E.M. n = 6/group. * p<0.05 versus control; and # p<0.05 versus BDL. Western blot analyses for mitochondrial fraction and cytoplasmic fractions of same six samples for prohibitin (mitochondrial marker) and β-actin (cytoplasmic marker).
EGCG improves BDL impaired mitochondrial membrane complex activities in mice

Mitochondria are the source of power in cells and electron transport chain is essential component for mitochondrial function. Here, we also examined electron transport chain complex activities from isolated mitochondria of liver. BDL procedure reduced complex I, complex II and complex IV activities to 49%, 39% and 42% respectively (Fig 6). Administration of EGCG
Fig 6. Effect of EGCG on BDL induced decrease of mitochondrial membrane complex activities in mice liver. BDL caused decrease in mitochondrial membrane complexes as measured from enzyme activities of electron transport chain complex I (Panel A), complex II (Panel B) and complex IV (Panel C). BDL mediated decrease of their activities in mitochondria were restored to control level by EGCG treatment. Results are mean ± S.E.M. n = 6/group. * p<0.05 versus control; and # p<0.05 versus BDL.

doi:10.1371/journal.pone.0126278.g006
during BDL increased complex I activity to 52%, complex II to 40% and complex IV to 38%. Thus EGCG administration significantly attenuated BDL induced mitochondrial dysfunction in liver. Electron transport chain complexes are also prone to oxidative modification and that leads its loss of activity [58, 59]. Selective accumulation of EGCG in mitochondria and its anti-oxidant properties is one of the mechanisms of protection from BDL injury in liver.

**EGCG attenuates BDL induced cell death pathway in mice liver**

BDL for two weeks induces significant cell death in liver [60, 61]. We also observed similar trend with cell death marker DNA fragmentation and PARP activity. BDL induced 3 fold increase in DNA fragmentation and EGCG attenuated 30% of BDL induced DNA fragmentation (Fig 7A). DNA fragmentation is predominantly apoptotic cell death marker. We also investigated another cell death marker PARP activity which is primarily known for necrotic pathway but also plays role in apoptotic pathway [62–64]. Chronic liver injury by BDL leads to 3.1 fold increase in PARP activity and EGCG administration attenuated BDL induced PARP activity to 40% (Fig 7B). In liver injury both apoptosis and necrosis play crucial role [65, 66]. PARP is a key mediator of liver fibrosis [4]. EGCG has been shown to be anti-apoptotic and it inhibits cell death [67, 68]. The major response to BDL induced cell death in liver is inflammatory trigger. The inflammatory response is mediated by neutrophil and other leucocyte accumulation [33, 69]. To understand the role of EGCG in BDL induced inflammation, we also looked the inflammatory cytokine and their master regulator NFκB.

**EGCG attenuates BDL induced NFB activation and pro-inflammatory cytokine**

BDL induced fibrosis is mediated by inflammation and NFκB plays key role in these inflammatory diseases [70]. We have measured NFκB activity from nuclear fraction of liver homogenate. NFκB activity was increased (2.8 fold) in BDL group and the increased activity is reduced (upto 39%) by administration of EGCG during two weeks period (Fig 8). The modulation of NFκB activity by EGCG in cell line, immune cells and cardiac model has been reported earlier [71–74].

We also investigated pro-inflammatory cytokines TNFα, MIP1α, IL1β and MIP2. Real-time PCR analyses demonstrated BDL induced TNFα, MIP1α, IL1β and MIP2 mRNA level to 5.9 fold, 3.8 fold, 5.1 fold and 3.9 fold respectively (Fig 8B–8E). Administration of EGCG reduced BDL induced pro-inflammatory cytokines to 60.3%, 37%, 47.4% and 41% (TNFα, MIP1α, IL1β and MIP2 respectively). These pro-inflammatory cytokine are key mediator of inflammatory cell infiltration, associated oxidative burst and cell death. Recently EGCG demonstrate to modulate inflammatory response via PI3K/Akt/mTOR pathway [75].

**Mechanistic role of parenchymal cells in EGCG mediated anti-fibrotic action**

To address the role parenchymal cells in EGCG mediated protection against fibrosis, we used isolated primary hepatocytes and stellate cells from mice and performed in vitro experiments. The initial response of accumulated bile acids is to generate significant oxidative stress and other toxic compounds, which damage sensitive hepatocytes. We want to understand the role of EGCG in reactive oxygen species induced hepatocyte cell death. EGCG treatment significantly reduced hydrogen peroxide(HP) induced hepatocyte cell death in vitro (Fig 9). It is well known that reactive oxygen species (ROS) exposure to hepatocytes followed by cell death plays
critical role in liver fibrosis [48, 76]. EGCG significantly attenuated ROS mediated cell death and thus contributing anti-fibrotic properties.

In addition to hepatocytes, we also examine the effect of EGCG on stellate cell mediated pro-fibrotic gene expression. After receiving many paracrine signal molecules including cytokines, stellate cells activated and generate profibrotic molecules which leads to regulate the generation of extracellular matrix in mice liver. In primary culture of stellate cells, it was observed that stellate cells are activated spontaneously when cultures for 7 days or more [77]. Our aim is

**Fig 7. Effect of EGCG on BDL induced cell death markers DNA fragmentation and PARP activity in mice liver.** BDL caused significant increase in liver DNA fragmentation (Panel A) and PARP activity (Panel B). BDL induced increases in DNA fragmentation and PARP activity were attenuated by EGCG treatment. Results are mean ± S.E.M. n = 6/group. * p<0.05 versus control; and # p<0.05 versus BDL.

doi:10.1371/journal.pone.0126278.g007
to understand the role of EGCG in the activation of stellate cell process and its effect on profibrotic marker production. We observed that TIMP-1, metallopeptidase inhibitor, is upregulated during collagen deposition in stellate cells (Fig 10A). However, treatment with EGCG significantly reduced upregulated TIMP-1. TIMP-1 is a powerful inhibitor of enzymes that degrade matrix molecule and shown plays critical role in mouse fibrosis [78]. In liver fibrosis, it is important that upregulation of extracellular matrix protein (ECM) and matrix metalloproteinas (MMPs) mediated proteolytic degradation of ECM [79]. The real-time PCR analyses demonstrated that EGCG treatment at 10 μM significantly reduced pro-fibrotic genes such as TNFα, MIP1α, IL1β, and MIP2.

Fig 8. Effect of EGCG on BDL induced NFκB activation and pro-inflammatory cytokines in mice liver. BDL induced NFκB activation as measured from nuclear fraction using commercial kit (Panel A) and the activity was reduced by treatment with EGCG. BDL induced pro-inflammatory cytokines as measured by Real-time PCR analyses for TNFα (Panel B), MIP1α (Panel C), IL1β (Panel D) and MIP2 (Panel E). BDL induced increases in pro-inflammatory cytokines were attenuated by EGCG treatment. Results are mean ± S. E.M. n = 6/group.* p<0.05 versus control; and # p<0.05 versus BDL.

doi:10.1371/journal.pone.0126278.g008
SMA, collagen1 and fibronectin (Fig 10B–10D). Stellate cells are one of the major player in progression of liver fibrosis [21].

Liver fibrosis is a complex process involving various parenchymal, non parenchymal and infiltrating immune cells. Protecting liver fibrosis or its reversal is a challenging task. Our data demonstrated that EGCG conferred significant protection and did not protect completely. This limitation of the study might be due to many factors including EGCG’s bioavailability, accumulation in liver and targeting other cell types. In addition to that multiple pathways are contributing factors including apoptotic pathway, renin angiotensin pathway, β-Catenin signaling, autophagy, angiogenesis, metabolic reprogramming and many others. It is difficult to understand that a single molecule EGCG can target all of pathways. However, hepatocyte cell death inhibition and preventing fibrotic marker from stellate cells by EGCG are very important contributions in protecting liver.

**Conclusion**

The basis of hepatic fibrogenesis is caused by pre-existing and renewed liver injury. The fibrogenesis is a complex process involving liver hepatocytes, stellate cells, kupffer cells and infiltrating leukocytes. The current study shows that administration of EGCG was effective in attenuating BDL induced liver injury and fibrosis. The EGCG specifically attenuated of ROS induced hepatocyte cell death and down regulated pro-fibrotic gene expression in stellate cells.
The protective effect of EGCG may be due to its ability to modulate mitochondrial oxidative/nitrative stress, NFκB activation and the inflammatory process (Fig 11). The detailed mechanism of action of EGCG in BDL induced liver fibrosis will require future studies with mitochondrial accumulation of EGCG and its mechanism of NFκB regulation will allow us to better understand the effects of EGCG on liver fibrogenesis.

**Author Contributions**

Conceived and designed the experiments: KS SZ. Performed the experiments: KS XF RS. Analyzed the data: KS HX. Contributed reagents/materials/analysis tools: HX LZ. Wrote the paper: KS SZ.
Fig 11. Schematic diagrams of protection mechanisms for EGCG in BDL induced liver injury. EGCG inhibit BDL induced mitochondrial ROS (Reactive Oxygen Species) generation which caused cell death. BDL induced cell death and NFkB activation leads to pro-inflammatory response with cytokines (TNFα, MIP1α, IL1β and MIP2). These process leads to leukocytes infiltration with additional burst of oxidative stress. EGCG also neutralize these pro-inflammatory cytokines. This multilevel interference by EGCG leads to reduced inflammation and cell death, thus protecting against BDL induced liver injury.

doi:10.1371/journal.pone.0126278.g011

References

1. Lim YS, Kim WR. The global impact of hepatic fibrosis and end-stage liver disease. Clin Liver Dis. 2008; 12(4):733–46. Epub 2008/11/06. doi: 10.1016/j.cld.2008.07.007 [pii]. PMID: 18984463.

2. Starzl TE, Demetris AJ, Van Thiel D. Liver transplantation (1). N Engl J Med. 1989; 321(15):1014–22. Epub 1989/10/12. doi: 10.1056/NEJM198910123211505 [pii]. PMID: 2674716; PubMed Central PMCID: PMC3091023.

3. Stiehl A, Rudolph G, Raedisch R, Moller B, Hopf U, Lotterer E, et al. Ursodeoxycholic acid-induced changes of plasma and urinary bile acids in patients with primary biliary cirrhosis. Hepatology. 1990; 12(3 Pt 1):492–7. Epub 1990/09/01. doi: S0270-9139(90)00223-3 [pii]. PMID: 2401455.

4. Mukhopadhyay P, Rajesh M, Cao Z, Horvath B, Park O, Wang H, et al. Poly (ADP-ribose) polymerase-1 is a key mediator of liver inflammation and fibrosis. Hepatology. 2014; 59(5):1998–2009. Epub 2013/10/04. doi: 10.1002/hep.26763 [pii]. PMID: 24089324; PubMed Central PMCID: PMC3975736.

5. Pastor A, Collado PS, Almar M, Gonzalez-Gallego J. Antioxidant enzyme status in biliary obstructed rats: effects of N-acetylcysteine. J Hepatol. 1997; 27(2):363–70. Epub 1997/08/01. doi: S0168-8278(97)08183-3 [pii]. PMID: 9288612.

6. Jiao K, Sun Q, Chen B, Li S, Lu J. Vitamin K1 attenuates bile duct ligation-induced liver fibrosis in rats. Scand J Gastroenterol. 2014; 49(8):715–21. Epub 2014/04/20. doi: 10.3109/00365521.2014.899618 [pii]. PMID: 24742111.
7. Wang Y, Cheng M, Zhang B, Nie F, Jiang H. Dietary supplementation of blueberry juice enhances hepatic expression of metallothionein and attenuates liver fibrosis in rats. PLoS One. 2013; 8(3):e58659. Epub 2013/04/05. doi: 10.1371/journal.pone.0058659 PONE-D-12-33490 [pii]. PMID: 23554912; PubMed Central PMCID: PMC3595269.

8. Chacko SM, Thambi PT, Kuttan R, Nishigaki I. Beneficial effects of green tea: a literature review. Chin Med. 2010; 5:13. Epub 2010/04/08. doi: 10.1186/1749-8546-5-13 1749-8546-5-13 [pii]. PMID: 20370896; PubMed Central PMCID: PMC2855614.

9. Zhu Y, Wang S, Lin F, Li Q, Xu A. The therapeutic effects of EGCG on vitiligo. Fitoterapia. 2014, Epub 2014/08/17. S0367-326X(14)00221-4 [pii] doi: 10.1016/j.fitote.2014.08.007 PMID: 25128425.

10. Maruyama T, Murata S, Nakayama K, Sano N, Ogawa K, Nowatari T, et al. (-)-Epigallocatechin-3-gallate suppresses liver metastasis of human colorectal cancer. Oncol Rep. 2014; 31(2):625–33. Epub 2013/12/18. doi: 10.3892/or.2013.2925 PMID: 24337301.

11. Ren Y, Deng F, Zhu H, Wan W, Ye J, Luo B. Effect of epigallocatechin-3-gallate on iron overload in mice with alcoholic liver disease. Mol Biol Rep. 2011; 38(2):879–86. Epub 2010/05/22. doi: 10.1007/s11033-010-0180-5 PMID: 20490691.

12. Giakoustidis DE, Giakoustidis AE, Iliadis S, Koliakou K, Antoniadis N, Kontos N, et al. Attenuation of liver ischemia/reperfusion induced apoptosis by epigallocatechin-3-gallate via down-regulation of NF-kappaB and c-Jun expression. J Surg Res. 2010; 159(2):720–8. Epub 2009/04/28. doi: 10.1016/j.jss.2008.08.038 S0022-4804(08)00582-9 [pii]. PMID: 19394642.

13. Bose M, Lambert JD, Ju J, Reuhl KR, Shapses SA, Yang CS. The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat fed mice. J Nutr. 2008; 138(9):1677–83. Epub 2008/08/22. doi: 10.1093/jn/nxs1677 PMID: 18716169; PubMed Central PMCID: PMC2586893.

14. Kaviarasas S, Ramarmurthy N, Gunasekaran P, Varalakshmi E, Anuradha CV. Epigallocatechin-3-gallate(-)protects Chang liver cells against ethanol-induced cytotoxicity and apoptosis. Basic Clin Pharmacol Toxicol. 2007; 100(3):151–6. Epub 2007/02/21. PTO036 [pii]. doi: 10.1111/j.1742-7843.2006.00036.x PMID: 17309517.

15. Mielgo-Ayuso J, Barrenechea L, Alcorta P, Larrarte E, Margareto J, Labayen I. Effects of dietary supplementation with epigallocatechin-3-gallate on weight loss, energy homeostasis, cardometabolic risk factors and liver function in obese women: randomised, double-blind, placebo-controlled clinical trial. Br J Nutr. 2011; 111(7):1263–71. Epub 2013/12/05. doi: 10.1017/S0007114513003784 [pii] PMID: 24299662.

16. Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem. 2000; 275(4):2247–50. Epub 2000/01/25. PMID: 10644669.

17. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore M, et al. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem. 2007; 282(32):23337–47. Epub 2007/06/15. M700194200 [pii] doi: 10.1074/jbc.M700194200 PMID: 17562716.

18. Forbes SJ, Russo FP, Roy V, Burra P, Rugge M, Wright NA, et al. A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. Gastroenterology. 2004; 126(4):955–63. Epub 2004/04/02. doi: 10.1053/j.gastro.2004.01.001 PMID: 15057733.

19. Rieder H, Armbrust T, Meyer zum Buschenfelde KH, Ramadori G. Contribution of sinusoidal endothelial liver cells to liver fibrosis: expression of transforming growth factor-beta 1 receptors and modulation of plasmin-generating enzymes by transforming growth factor-beta 1. Hepatology. 1993; 18(4):937–44. Epub 1993/10/01. doi: 10.1002/hup.1350180410 PMID: 8327093.

20. Czaja AJ. Hepatic inflammation and progressive liver fibrosis in chronic liver disease. World J Gastroenterol. 2014; 20(10):3251–32. Epub 2014/03/15. doi: 10.3748/wjg.v20.i10.2515 PMID: 24627588; PubMed Central PMCID: PMC3949261.

21. You H, Sun YM. [Cellular mechanism in the fibrogenesis of liver fibrosis]. Zhonghua gan zang bing za zhi = Zhonghua ganzangbing zazhi = Chinese journal of hepatology. 2012; 20(8):563–4. PMID: 23227528.

22. Puche JE, Saiman Y, Friedman SL. Hepatic stellate cells and liver fibrosis. Comprehensive Physiology. 2013; 3(4):1473–92. doi: 10.1002/cphy.c120035 PMID: 24265236.

23. Soroka CJ, Lee JM, Azzaroli F, Boyer JL. Cellular localization and up-regulation of multidrug resistance-associated protein 3 in hepatocytes and cholangiocytes during obstructive cholestasis in rat liver. Hepatology. 2001; 33(4):783–91. Epub 2001/04/03. S0270-9139(01)81416-6 [pii] doi: 10.1053/jhep.2001.23501 PMID: 11268340.

24. Bohan A, Chen WS, Denson LA, Held MA, Boyer JL. Tumor necrosis factor alpha-dependent up-regulation of Lrh-1 and Mrp3(Abcc3) reduces liver injury in obstructive cholestasis. J Biol Chem. 2003; 278(38):36688–98. Epub 2003/07/03. doi: 10.1074/jbc.M304011200 M304011200 [pii]. PMID: 12837754.
25. Alaishe SM, Torres M, Ferlito M, Sun CC, De Maio A. The severity of cholestatic injury is modulated by the genetic background. Shock. 2005; 24(5):412–6. Epub 2005/10/26. doi: 10.1097/01.shk.0000180263.08180.0a [pii]. PMID: 16247325.

26. Zhang Q, Yan S, Tian Y, Ding Y, Yao J, Chen H, et al. Ischemic preconditioning improves liver tolerance to congestion-reperfusion injury in mice. J Surg Res. 2014; 189(1):152–8. doi: 10.1016/j.jss.2014.01.061 PMID: 24589179.

27. Pan H, Shen K, Wang X, Meng H, Wang C, Jin B. Protective effect of metalloporphyrins against cisplatin-induced kidney injury in mice. PLoS One. 2014; 9(1):e86057. Epub 2014/01/24. doi: 10.1371/journal.pone.0086057 PONE-D-13-28896 [pii]. PMID: 24454954; PubMed Central PMCID: PMC43891880.

28. Cui Y, Hosui A, Sun R, Shen K, Gavrilova O, Chen W, et al. Loss of signal transducer and activator of transcription 5 leads to hepatosteatosis and impaired liver regeneration. Hepatology. 2007; 46(2):504–13. doi: 10.1002/hep.21713 PMID: 17640041.

29. Jeong WI, Park O, Radaeva S, Gao B. STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity. Hepatology. 2006; 44(6):1441–51. doi: 10.1002/hep.21419 PMID: 17133483.

30. Marques TG, Chaib E, da Fonseca JH, Lourenco AC, Silva FD, Ribeiro MA Jr., et al. Review of experimental models for inducing hepatic cirrhosis by bile duct ligation and carbon tetrachloride injection. Acta Cir Bras. 2012; 27(8):589–94. Epub 2012/08/02. doi: S0102-86502012000800013 [pii]. PMID: 22850713.

31. Alison M. Liver stem cells: a two compartment system. Curr Opin Cell Biol. 1998; 10(6):710–8. Epub 1999/02/23. doi: S0955-0674(98)80111-7 [pii]. PMID: 9914178.

32. Olynyk JK, Yeoh GC, Ramm GA, Clarke SL, Hall PM, Britton RS, et al. Gadolinium chloride suppresses hepatic oval cell proliferation in rats with biliary obstruction. Am J Pathol. 1998; 152(2):347–52. Epub 1998/02/18. PMID: 9466559; PubMed Central PMCID: PMC1857969.

33. Georgiev P, Joychum W, Heinrich S, Jang JH, Nocito A, Dahm F, et al. Characterization of time-related changes after experimental bile duct ligation. Br J Surg. 2008; 95(5):646–56. Epub 2008/01/16. doi: 10.1002/bjs.6050 PMID: 18196571.

34. Nagle DG, Ferreira D, Zhou YD. Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives. Phytochemistry. 2006; 67(17):1849–55. Epub 2006/08/01. S0031-9422(06)00353-0 [pii] doi: 10.1016/j.phytochem.2006.06.020 PMID: 16876833; PubMed Central PMCID: PMC2903211.

35. Lambert JD, Kennett MJ, Sang S, Reuhl KR, Ju J, Yang CS. Hepatotoxicity of high oral dose (-)-epigallocatechin-3-gallate in mice. Food Chem Toxicol. 2010; 48(1):409–16. Epub 2009/11/04. doi: 10.1016/j.fct.2009.09.0491-8 [pii]. PMID: 19883714; PubMed Central PMCID: PMC2905152.

36. Mochizuki M, Hasegawa N. (-)-Epigallocatechin-3-gallate reduces experimental colonic injury in rats by regulating macrophage and mast cell. Phytother Res. 2010; 24 Suppl 1:S120–2. Epub 2009/06/24. doi: 10.1002/ptr.2862 PMID: 19548282.

37. Devika PT, Prince PS. Preventive effect of (-)epigallocatechin-gallate (EGCG) on lysosomal enzymes in heart and subcellular fractions in isoproterenol-induced myocardial infarcted Wistar rats. Chem Biol Interact. 2008; 172(3):245–52. Epub 2008/02/26. doi: 10.1016/j.cbi.2008.01.003 S0009-2797(08)00055-0 [pii] PMID: 18294627.

38. Ullmann U, Haller J, Decourt JP, Girault N, Girault J, Richard-Caudron AS, et al. A single ascending dose study of epigallocatechin gallate in healthy volunteers. J Int Med Res. 2003; 31(2):88–101. Epub 2003/05/23. PMID: 12760312.

39. Tipoe GL, Leung TM, Liong EC, Lau TY, Fung ML, Nanji AA. Epigallocatechin-3-gallate (EGCG) reduces liver inflammation, oxidative stress and fibrosis in carbon tetrachloride (CCl4)-induced liver injury in mice. Toxicology. 2010; 273(1–3):45–52. Epub 2010/05/05. doi: 10.1016/j.tox.2010.04.014 S0300-483X(10)00187-3 [pii] PMID: 20438794.

40. Zhao Y, Miriyala S, Miao L, Milov M, Schnell D, Dhar SK, et al. Redox proteomic identification of HNE-bound mitochondrial proteins in cardiac tissues reveals a systemic effect on energy metabolism after doxorubicin treatment. Free Radic Biol Med. 2014; 72:55–65. Epub 2014/03/19. doi: 10.1016/j.freeradbiomed.2014.03.001 S0891-5849(14)00107-5 [pii]. PMID: 24632380; PubMed Central PMCID: PMC4053505.

41. Garcia-Ruiz I, Solis-Munoz P, Fernandez-Moreira D, Grau M, Colina F, Munoz-Yague T, et al. High-fat diet decreases activity of the oxidative phosphorylation complexes and causes nonalcoholic steatohepatitis in mice. Dis Model Mech. 2014; 7(11):1287–96. Epub 2014/09/28. doi: 10.1242/dmm.016766 dmm.016766 [pii]. PMID: 25281569; PubMed Central PMCID: PMC4213732.

42. Abdelmegeed MA, Jang S, Banerjee A, Hardwick JP, Song BJ. Robust protein nitration contributes to acetaminophen-induced mitochondrial dysfunction and acute liver injury. Free Radic Biol Med. 2013;
61. Wagner M, Fickert P, Zoller G, Fuchsbiicher A, Silbert D, Tsybrovskyy O, et al. Role of farnesoid X receptor in determining hepatic ABC transporter expression and liver injury in bile duct-ligated mice. Gastroenterology. 2003; 125(3):825–38. Epub 2003/09/02. doi: S0016508503010680 [pii]. PMID: 12949728.

62. Ivana Scovassi A, Dieidrich M. Modulation of poly(ADP-ribosylation) in apoptotic cells. Biochem Pharmacol. 2004; 68(6):1041–7. Epub 2004/08/18. doi: 10.1016/j.bcp.2004.04.023 S0006295204003594 [pii]. PMID: 15313399.

63. Aredia F, Scovassi AI. Poly(ADP-ribose): A signaling molecule in different paradigms of cell death. Biochem Pharmacol. 2014. Epub 2014/07/01. S0006-2952(14)00364-5 [pii]. doi:10.1016/j.bcp.2014.06.021 PMID: 24976902.

64. Virag L, Robaszkiewicz A, Rodriguez-Vargas JM, Oliver FJ. Poly(ADP-ribose) signaling in cell death. Mol Aspects Med. 2013; 34(6):1153–67. Epub 2013/02/19. doi: 10.1016/j.mam.2013.01.007 S0989-2997(13)00008-3 [pii]. PMID: 23416893.

65. Jaeschke H, Gujral JS, Bajt ML. Apoptosis and necrosis in liver disease. Liver Int. 2004; 24(2):85–9. Epub 2004/04/14. doi: 10.101111.1478-3231.2004.00906.x LIV906 [pii]. PMID: 15078470.

66. Nanji AA, Hiller-Sturmhofel S. Apoptosis and necrosis: two types of cell death in alcoholic liver disease. Alcohol Health Res World. 1997; 21(4):325–30. Epub 1997/01/01. PMID: 15706744.

67. Siegeln MD, Habel A, Gaiser T. Epigallocatechin-3-gallate (EGCG) downregulates PEA15 and thereby augments TRAIL-mediated apoptosis in malignant glioma. Neurosci Lett. 2008; 448(1):161–5. Epub 2008/10/25. doi: 10.1016/j.neulet.2008.08.039 S03043908(08)01391-8 [pii]. PMID: 18948169.

68. Thomas F, Patel S, Holly JM, Persad R, Bahl A, Perks CM. Dihydrotestosterone sensitises LNCaP cells to death induced by epigallocatechin-3-Gallate (EGCG) or an IGF-I receptor inhibitor. Prostate. 2009; 69(2):219–24. Epub 2008/10/23. doi: 10.1002/pros.20073 PMID: 18942120.

69. Gujral JS, Farhood A, Bajt ML, Jaeschke H. Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. Hepatology. 2003; 38(2):355–63. Epub 2003/07/29. doi: 10.1053/ jhep.2003.50341 S0270913903005779 [pii]. PMID: 12883479.

70. Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest. 2001; 107(1):7–11. Epub 2000/10/03. doi: 10.1172/JCI11830 PMID: 11134171; PubMed Central PMCID: PMC198552.

71. Sen T, Moulik S, Dutta A, Choudhury PR, Banerji A, Das S, et al. Multifunctional effect of epigallocatechin-3-galate (EGCG) in downregulation of gelatinase-A (MMP-2) in human breast cancer cell line MCF-7. Life Sci. 2009; 84(7–8):194–204. Epub 2008/12/25. doi: 10.1016/j.lfs.2008.11.018 S0024-3205(08)00489-X [pii]. PMID: 19105967.

72. Harakeh S, Diab-Assaf M, Azar R, Hassan HM, Tayeb S, Abou-El-Ardat K, et al. Epigallocatechin-3-gallate inhibits tax-dependent activation of nuclear factor kappa B and of matrix metalloproteinase 9 in human T-cell lymphotropic virus-1 positive leukemia cells. Asian Pac J Cancer Prev. 2014; 15(3):1219–25. Epub 2014/03/13. PMID: 24606444.

73. Babu PV, Liu D. Green tea catechins and cardiovascular health: an update. Curr Med Chem. 2008; 15(73).

74. Peairs A, Dai R, Gan L, Shimp S, Rylander MN, Li L, et al. Epigallocatechin-3-gallate (EGCG) attenuates inflammation in MRL/lpr mouse mesangial cells. Cell Mol Immunol. 2010; 7(2):123–32. Epub 2010/02/09. doi: 10.1016/cmim.2010.01.025 PMID: 20401894.

75. Duarte S, Baber J, Fuji T, Coito AJ. Matrix metalloproteinases in liver injury, repair and fibrosis. Matrix Biology. Journal of the International Society for Matrix Biology. 2015. doi: 10.1016/j.matbio.2015.01.004 PMID: 25599939.