The protective effects of epigallocatechin gallate on lipopolysaccharide-induced hepatotoxicity: an *in vitro* study on Hep3B cells

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ARTICLE INFO	ABSTRACT
	Objective(s): In the present study, our aim was to investigate the possible protective effects of epigallocatechin gallate (EGCG) on lipopolysaccharide (LPS)-induced hepatotoxicity by using Hep3B human hepatoma cells. Specifically, the study examines the role of some proinflammatory markers and oxidative damage as possible mechanisms of LPS-associated cytotoxicity. Consequently, the hepatocellular carcinoma cell line Hep3B was chosen as a model for investigation of LPS toxicity and the effect of EGCG on LPS-exposed cells. ***Materials and Methods:*** The Hep3B human hepatoma cells were used for this study. The cytotoxic effects of chemicals (EGCG and LPS), AST and ALT levels, SOD and CAT activities, GSH-Px level and TNF-alpha and IL-6 levels were detected by using different biochemical and molecular methods. LPS and EGCG were applied to cells at various times and doses. ***Results:*** The highest treatment dose of EGCG (400 μM) led to a dramatic decrease in SOD level and increase in CAT and GSH levels. Additionally, the highest dose of EGCG also led to a dramatic increase in TNF-alpha and IL-6 levels. On the other hand, effective doses of EGCG (200 and 100 μM) normalized all related parameters levels. ***Conclusion:*** LPS caused hepatotoxicity, but interestingly, a high dose of EGCG was found to be a cytotoxic agent in this study. However, other two doses of EGCG led to a decrease in both inflammatory cytokine levels and antioxidant enzyme levels. Further studies should examine the effect of EGCG on secondary cellular signaling pathways.

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Introduction

Acute liver failure, frequently results from hepatitis virus infection, induction of drugs and toxins, or hepatic ischemia-reperfusion injury, is an important dramatic clinical syndrome. It can also be caused by apoptosis and necrosis in liver. Liver oxidative stress and inflammation are significant determinants in the physiopathology of acute liver failure. Pro-inflammatory cytokines and reactive oxygen species have key roles in the mentioned liver failure (1).

Additionally, cytokines are one of the most important regulators of host responses to infection, immune responses, and inflammation. Some cytokines make the disease worse (pro-inflammatory), while others act to reduce inflammation (anti-inflammatory).

The well-known examples of pro-inflammatory cytokines are interleukin-6 (IL-6) and tumor necrosis factor (TNF) (2). On the other hand, oxidative stress is described as a disturbance in the balance between the production of reactive oxygen species and antioxidant defenses. In current literature, most claim that a close relationship exists between oxidative stress and inflammation (3, 4).

Intestinal bacteria and their metabolites cause over-activation of the immune system when liver function is severely damaged, which leads to hepatocyte necrosis (5). Endotoxic lipopolysaccharide (LPS) is a cell component in gram-negative bacteria. It plays a key role in the pathogenesis of gram-negative bacterial infections. Exposure to large amounts or high doses of

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LPS under certain conditions may contribute to sepsis associated with fever, circulatory shock, and injury to several organs, including the liver (6). The commonly accepted strategy used for prevention of LPS-based damage is the reduction of reactive metabolites and the use of antioxidants (7).

Epigallocatechin-3-gallate (EGCG), a major catechin isolated from green tea, is known to be a potential antioxidant. It has been used to treat a variety of diseases such as diabetes, cancer, cardiovascular disease, and nephrotoxicity (8-10). EGCG suppresses the migration and adhesion of multiple cell types, and it possesses excellent chemopreventive properties. EGCG plays anti-inflammatory and anti-oxidant roles in the elimination of cellular damage (11). It has also shown cancer preventive activities both *in vitro* and *in vivo*. More recently, reports have demonstrated the potential of EGCG to generate oxidative stress *in vitro* (12).

Therefore, the aim of this study was to investigate the alternative protective effects of EGCG on LPS-induced hepatotoxicity by using Hep3B human hepatoma cells. Specifically, the study examines the role of some proinflammatory markers and oxidative damage as possible mechanisms of LPS-associated cytotoxicity. Consequently, the hepatocellular carcinoma cell line Hep3B was chosen as a model for investigation of LPS toxicity and the effect of EGCG on LPS-exposed cells.

Material and Methods

Cell Culture and Treatment

The human hepatoma-derived cell line Hep3B, was purchased from the American Type Culture Collection (ATCC, USA), was cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with a 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution (Gibco, Pittsburgh, USA) at 37°C in a humidified atmosphere of 5% CO_2. Subconfluent cultures were detached with Trypsin/EDTA (Gibco, Pittsburgh, USA) and counted with trypan blue (Sigma-Aldrich, USA) counting method, and cells were seeded in six-well plates in 2 ml of growth medium. Next, Epigallocatechin gallate (EGCG) (Santa Cruz Biotechnology, CA, USA) and a single dose application (100 ng/ml) of LPS (Sigma-Aldrich, USA) were conducted on related groups in the appropriate doses.

The EGCG doses were 400 μM, 200 μM, 100 μM, and 50 μM, respectively. After incubation, the culture media were removed. The cells were then washed with Dulbecco's phosphate-buffered saline (dPBS) (Sigma-Aldrich, USA), and a(3-([4, 5-dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Sigma-Aldrich, USA) was performed. Optimal doses of EGCG and LPS were determined by the MTT assay, cells were seeded in 96-well plates in 100 μl of growth medium, and the optimum doses were applied. After incubation and centrifugation, the pellet and supernatant were stored for molecular methods.

MTT assay

Cell viability was determined by using the MTT assay. Hep3B hepatoma cells were seeded into 96-well plates at 2 x 10^4 cells per well and treated with EGCG and LPS. Next, 10 μl of MTT was added to each well and incubated for 4 hr at 37 $^\circ$C. The medium was then removed, and 150 μl of dimethyl sulfoxide (DMSO) was added to solubilize the MTT formazan. The absorbance of converted dye by living cells was measured at the wavelength of 570 nm. All experiments were done in duplicate in at least three cultures.

Quantitative real-time PCR (Q-PCR) analysis

The total RNA from Hep3B cells was isolated using a Qiagen RNA isolation kit with an automated device (Qiacube, Qiagen, Germany) according to the manufacturer's protocol. The cDNA synthesis was performed by using the first strand synthesis kit (Qiagen, Germany). Real-time PCR was done using TNF-alpha, IL-6, and human beta-actin (Actb) reference gene hybrid probes with Roche COBAS z480 (Roche, Basel, Switzerland). The values for specific genes were normalized by using beta-actin reference gene. In each PCR run, the cDNA samples were amplified in triplicate. Primer sequences of references genes and all other genes are displayed in Table 1. The relative quantification of the mRNAs was performed using the $\Delta\Delta$CT method.

Biochemical Examination

Cells were incubated, the culture supernatant was collected, and the concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined for each culture using an automatic biochemical analyzer. The cells were centrifuged at 600 g for 10 min at 4 °C to remove nuclear fractions, and the remaining separated supernatant was recentrifuged at 10,000 g for 20 min at 4 °C to collect the mitochondrial fraction (pellet) including peroxisomes for a catalase activity (CAT) assay. Superoxide dismutase (SOD) activity was detected and inhibition of the formation of nicotinamide adenine dinucleotide phosphate (NADPH)-phenazine methosulphate nitroblue tetrazolium formazan was measured spectrophoto-metrically at 560 nm. The SOD and CAT activities in the culture media were assayed with SOD and CAT assay kits (Enzo, Telluride, USA) according to the manufacturers' instructions.

Table 1. PCR primer sequences of TNF-alpha, IL-6 and β-actin

Primer	Forward	Reverse
TF-alpha	5'-CCAGGAGAAAGTCAGCCTCCT-3'	5'-TCATACCAGGGCTTGAGCTCA-3'
IL-6	5'-CGAAAGTCAACTCCATCTGCC-3'	5'-GGCAACTGGCTGGAAGTCTCT-3'
β-actin	5'- GCAAGCAGGAGTATGACGAG-3'	5'- CAAATAAAGCCATGCCAATC-3'

GSH-Px activity detection by enzyme-linked immunosorbent assay (ELISA)

The activity of Glutathione peroxidase (GSH-Px) in the culture medium was determined with Human GSH-Px ELISA Kit (Elabscience Biotechnology Co., Ltd, USA) according to the manufacturers' instructions. GSH-Px activity was assayed using a method based on the reaction between glutathione remaining after the action of GSH-Px and 5,5'-dithiobid-2-nitrobenzoic acid to form a complex that absorbs maximally at 412 nm.

Statistical analysis

All parameters recorded during sampling and laboratory findings were entered and stored in Microsoft-Excel. The data were analyzed using the IBM Statistical Package for Social Sciences (SPSS) version 20.0 statistical software (IBM, NY, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Duncan's Multiple Range tests (DMRT). All *P*-values were based on a two-sided test of statistical significance and significance was accepted at the level of *P* < 0.05.

Results

Different doses of EGCG and high dose of LPS were applied to Hep3B cells. The cytotoxic effects of chemicals (EGCG and LPS), AST and ALT levels, SOD and CAT activities, GSH-Px, TNF-alpha, and IL-6 levels were determined by using different biochemical and molecular methods.

The MTT assay shows cell viability. According to the MTT assay results of both 24 and 48 hr applications, the lower cell viability rate was produced by the LPS application. At that point, there was a significant difference between the LPS application group and all other experimental groups in terms of cell viability (*P<0.05*). However, all doses of EGCG led to an increase in cell viability at different levels in the LPS-induced hepatotoxicity groups (Figure 1).

In this study, the liver enzyme levels (AST and ALT) were checked to determine whether or not LPS lead to hepatotoxicity. Both ALT and AST enzyme levels were highest in the LPS and LPS+EGCG400 μM groups. There were significant differences between the control group, LPS, and LPS+EGCG400 μM groups (*P*<0.05). Normalization of AST and ALT levels was detected in EGCG 200, 100, and 50 μM treatment groups. The enzyme levels were reduced in appropriate doses of EGCG (Figure 2).
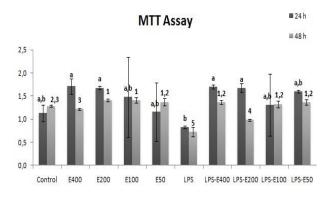

Figure 1. Cell viability of all experimental groups detected by MTT assay
a and b show the significant differences among 24 hr treated experimental groups (*P*<0.05)
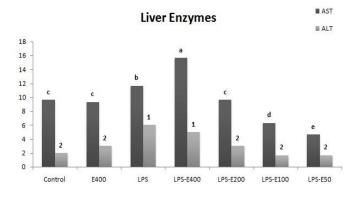1–5 show the significant differences among 48 hr treated experimental groups (*P*<0.05)

Figure 2. The effects of LPS and EGCG treatment on AST and ALT enzyme levels
a–e show the significant differences among experimental groups in AST enzyme levels (*P*<0.05)
1 and 2 show the significant differences among experimental groups in ALT enzyme levels (*P*<0.05)

Some anti-oxidant system parameters such as SOD, CAT, and GSH-Px were detected by biochemical methods as shown in Figures 3, 4, and 5. SOD is an essential antioxidant enzyme that defends cells against potentially damaging superoxide radicals. The highest treatment dose, 400 μM of EGCG, lead to a dramatic decrease in SOD level of Hep3B cells, and there were significant differences in the control group versus the EGCG400 and control vs. LPS+EGCG400 groups (*P*<0.05). However, other treatment doses of EGCG normalized the SOD level as seen in Figure 3. On the other hand, catalase is also an antioxidant enzyme which catalyzes the conversion of H_2O_2 to water and

oxygen. The results were nearly the same as SOD activity results. When the highest dose of EGCG was applied to LPS-induced Hep3B cells, the CAT activity level surpassed the control group's level. However, other doses of EGCG showed a possible treatment effect on LPS-induced hepatotoxicity. They reduced the CAT activity level in contrast to EGCG400 μM application (Figure 4). Finally, GSH-Px enzymes catalyzed the reduction of hydroperoxides to water and the respective alcohols by oxidizing GSH to oxidized glutathione (GSSG) (13, 14).

When GSH-Px levels were evaluated in the present study, there were statistical differences (*P<0.05*) between the control and other experimental groups. LPS led to an increase in GSH-Px levels. As with SOD and CAT activity levels, the highest dose of EGCG led to a significant increase in LPS-induced hepatotoxicity (Figure 5).

Initially, it was seen that LPS caused an increase in both TNF-alpha and IL-6 levels and led to inflammation. At that point, attempts were made to treat and normalize these levels with various doses of EGCG. However, the highest dose of EGCG also led to a dramatic increase in these cytokine levels (Figure 6). TNF-alpha and IL-6 levels increased 5-fold and 2-fold in the LPS administration group, respectively. Additionally, this fold change was interesting in high dose EGCG treatment group. It was understood that high dose EGCG treatment caused serious changes in some crucial parameters. TNF-alpha and IL-6 levels increased 36-fold and 14-fold in the high dose EGCG+LPS group, respectively. Other treatment doses of EGCG were found to be beneficial doses because they normalized these cytokine levels after LPS-induced hepatotoxicity.
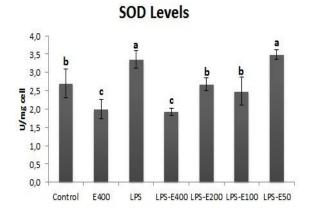

Figure 4. The effects of LPS and EGCG treatment on CAT enzyme activity in Hep3B cells
a–f show the significant differences among experimental groups in CAT enzyme activity (*P*<0.05)

Figure 5. The effects of LPS and EGCG treatment on GSH-Px enzyme activity in Hep3B cells
a–d show the significant differences among experimental groups in GSH-Px enzyme activity (*P*<0.05)

Figure 3. The effects of LPS and EGCG treatment on SOD enzyme activity in Hep3B cells
a–c show the significant differences among experimental groups in SOD enzyme activity (*P*<0.05)
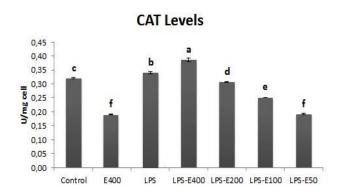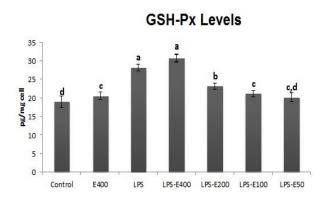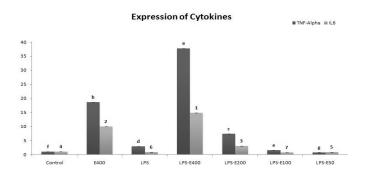

Figure 6. TNF-alpha and IL-6 mRNA expression levels of all experimental groups
a–g show the significant differences among experimental groups in TNF-alpha mRNA expression levels (*P*<0.05)
1–7 show the significant differences among experimental groups in IL-6 mRNA expression levels (*P*<0.05)

Discussion

Sepsis is a systemic inflammatory response capable of triggering endotoxic shock. Infection, especially gram-negative bacteria, is the most common cause of sepsis, and LPS found in such bacteria is an endotoxin that has been implicated in the pathogenesis of infection and as a cause of septic shock. LPS has serious effects on several organs, including the liver. It also can lead to endotoxic shock and death. LPS-induced hepatotoxicity is characterized by disturbed intracellular redox balance and excessive reactive oxygen species (ROS) accumulation, leading to liver injury (15).

Furthermore, human hepatoblastoma Hep3B cells have been used in many toxicity studies to screen for hepatotoxic compounds (16).

These cells have low levels of phase I cytochrome P450 enzymes when compared with primary hepatocytes, but they have normal levels of phase II enzymes (17, 18). Some studies have indicated that hepatocellular carcinoma cells present a valuable *in vitro* model for hepatotoxicity studies (18-20). This study evaluated the possible role of EGCG in LPS-induced hepatotoxicity using Hep3B cells. For this reason, MTT assay, AST and ALT liver enzyme levels, GSH level, SOD and CAT activities, TNF-alpha and IL-6 levels were investigated by different methods in this study.

In current literature, there are few published studies about the anti-inflammatory effects of EGCG on various cells and organs in either animal or *in vitro* models. However, limited studies have examined the relationship between EGCG and LPS-stimulated hepatotoxicity. The goal of our study was to investigate both the anti-oxidant and anti-inflammatory effects of EGCG on LPS-induced hepatotoxicity under *in vitro* conditions on Hep3B cells. Initially, it was seen that a high dose EGCG is also a toxic agent for hepatocytes as it negatively affects all parameters in Hep3B cells. A 400 μM EGCG treatment leads to an increase in the levels of CAT, GSH-Px, AST, ALT, TNF-alpha, and IL-6, while it decreases the level of SOD. So, a high dose of EGCG treatment is a toxic dose. However, other doses of EGCG treatment may be as effective, especially doses of 100 and 200 μM doses.

When MTT results were considered, it was seen that LPS and a high dose of EGCG caused a serious reduction in the level of cell viability. Most *in vitro* studies have shown that LPS is a serious cytotoxic agent especially on hepatocytes (18). However, a study confirms our hypothesis that a high dose of EGCG (400 μM) is cytotoxic on cell viability (21). The other two doses of EGCG (200 and 100 μM) were the most effective doses of EGCG treatment used in this study. These doses normalized nearly all the parameters and cell viability. As in this study, Kim *et al* showed that the protection by

EGCG is most effective when cells are exposed to EGCG for short periods at 100 or 200μM concentrations (22).

Evaluation of several biochemical parameters, including enzymes, can be helpful in identifying damage to target tissues, as well as identifying the health status of the host. In addition, AST and ALT are the most important enzymes acting as transaminases involved in amino acid metabolism (23). For this reason, liver AST and ALT enzymes are considered relevant stress indicators, and they were used to determine liver toxicity in this study. As seen in Figure 2, LPS causes an increase in both AST and ALT levels in Hep3B cells. Additionally, a high dose of EGCG exacerbated the increase of liver enzymes. Previous studies have shown that LPS can lead to a dramatic increase in AST, ALP, and ALT levels (5, 24, 25).

Oxidative stress is a system made up of a group of antioxidant enzymes and non-enzymatic antioxidant substances capable of neutralizing free radicals and preventing an excess production of ROS (26). SOD, GSH-Px, and catalase enzymes are the first line of cellular defense against oxidative stress. In our study, LPS+EGCG400 treatment caused a serious increase in some of these enzyme levels. These increases are an expected result when the toxicity potential of LPS and high dose EGCG are suspected. However, as expected, effective doses of EGCG treatment normalized these enzyme levels. Study showed that non-lethal toxic doses of EGCG treatment markedly decrease the levels of SOD, catalase, and glutathione peroxidase. It also showed that EGCG also significantly suppresses mRNA levels of hepatic SOD and CAT levels (27). Moreover, most scientific studies have proven that LPS leads to an increase in oxidative stress parameters (18).

Oxidative stress mediated tissue damage can be reversed by the SOD enzyme and glutathione (GSH). The action of these two antioxidant parameters prevents the cytotoxic effects of toxic free radicals. CAT is also an anti-oxidant enzyme that reacts with hydrogen peroxide to produce water and molecular oxygen. The currently accepted knowledge supports our anti-oxidant data that EGCG can affect the antioxidant enzyme levels and prevent oxidative damage.

In addition, cytokines play an important role in many cellular processes such as immunity, inflammation, cell proliferation, differentiation, and cell death. Among the inflammatory networks, TNF-α, IL-1β, and IL-6 are known to be the most important inflammatory mediators involved in the initiation of acute inflammation. TNF-alpha has been linked to increased oxidative stress and is known to activate other inflammatory cells (28, 29). Also, it is the earliest and primary endogenous mediator for the process of inflammatory responses and has been linked to increased oxidative stress (28). According to literature,

it has been shown that IL-6 triggers some cellular signaling pathways and induces some pro-apoptotic proteins such as B-cell CLL/lymphoma-2 (Bcl-2) (30).

In the present study, only LPS administration, only high dose EGCG treatment, and LPS+EGCG400 administration led to a serious increase in both the TNF-alpha and IL-6 levels and also caused acute inflammation. However, effective doses of EGCG were also used in an attempt to normalize and reduced cytokine levels. A previous study has concluded that the anti-inflammatory mechanism of green tea polyphenols contributes to down-regulation of TNF-alpha gene expression (5).

Consequently, there exists a close relationship between oxidative stress and inflammation. Increases in oxidative stress can increase the production of inflammatory cytokines and in turn, an increase in inflammatory cytokines can induce the production of radicals. Oxidative stress increases cytokine production via various mechanisms (4). Most related studies have proven this relationship and are recorded in current literature. For example, Chen *et al* examined the possible protective effects of EGCG in mastitis rats. It was found that EGCG inhibits the LPS-induced inflammatory response and normalizes anti-oxidant enzyme levels (29). Another study (31) claimed that EGCG could inhibit IL-6 and nuclear factor-kappa-light-chain-enhancer of activated B cell (NF-kB) activities in human gingival fibroblasts. A similar study (32) showed that some EGCG derivates suppress the LPS-induced production of nitric oxide and pro-inflammatory cytokines in macrophages. Another study, performed in gingival epithelial cells, showed that EGCG inhibited inflammatory cytokine secretion and also suggested that it may be a novel adjunctive therapeutic agent (33). Liu *et al* (5) used LPS-induced L0 hepatocytes in their study to examine the effects of EGCG on liver cells. They showed that pre-treatment of EGCG reduces the production of TNF-alpha and nitric oxide production in a dose-dependent manner.

Conclusion

In conclusion, LPS caused hepatotoxicity, but interestingly, a high dose of EGCG was found to be a cytotoxic agent in this study. However, other two doses of EGCG led to a decrease in both inflammatory cytokine levels and antioxidant enzyme levels in LPS-induced Hep3B cells. To better understand the molecular mechanisms of the anti-inflammatory and anti-oxidant effects of EGCG, further studies should examine the effect of EGCG on secondary cellular signaling pathways such as MAPK in LPS-stimulated hepatocytes because the ERK1/2 and p38 are the major members of MAPK family and are associated with cellular oxidative stress, inflammation, proliferation, and migration.

Finally, the protective effect of EGCG in LPS-enhanced Hep3B cells is associated with the inhibition of inflammatory mediators, including TNF-alpha and IL-6, and may affect the antioxidant enzyme levels. Taking everything into account, EGCG may be seen as a promising agent for the treatment of hepatic over-active inflammatory response.

Conflict of interest

The authors have no conflict of interest.

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