

Effect of Bioflavonoid Quercetin on Endotoxin-Induced Hepatotoxicity and Oxidative Stress in Rat Liver

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ABSTRACT

Septicaemia caused by gram-negative pathogens is a dangerous infection which is associated with high incidence of liver dysfunction. The severe and acute hepatotoxicity is presumably due to massive release of endotoxin into systemic circulation after bacterial killing. The direct toxic effect of endotoxin is probably due to the increased production of reactive oxygen intermediates as O_2^- , peroxides and nitric oxide. Quercetin (QT) and its sugar conjugates are the most abundantly-distributed bioflavonoids in plant kingdom and has potent antioxidant properties. The present study is aimed at investigating effect of QT in salvaging endotoxin-induced hepatic dysfunction and oxidative stress in rat liver. Hepatotoxicity was induced by administrating lipopolysaccharide (LPS), in a single dose of 1 mg/kg intraperitoneally to the rats. Liver enzymes (alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST)), total bilirubin and total protein were measured in serum. Oxidative stress in liver tissue homogenates was estimated by measuring thiobarbituric acid reactive substances (TBARS), glutathione content (GSH) and superoxide dismutase (SOD). LPS induced a marked hepatic dysfunction evident by rise in serum levels of ALT, AST and decrease in total bilirubin ($p < 0.05$). TBARS levels were significantly increased whereas GSH and SOD levels decreased in the liver homogenates of LPS-treated rats. Chronic treatment of QT successfully attenuated these effects of LPS. In conclusion, these findings suggest that QT attenuates LPS-induced hepatotoxicity possibly by preventing cytotoxic effects of NO, oxygen free radicals and cytokines.

Keywords: *Flavonoids, Lipid peroxidation, Lipopolysaccharide, Septicemia*

Despite advances in antimicrobial therapy and medical support, septic shock remains a major cause of morbidity and mortality among hospitalised patients. Septicaemia caused by gram-negative pathogens is a dangerous complication in intensive care units (ICUs) and is associated with high incidence of liver dysfunction. The severe and acute hepatotoxicity is presumably due to massive release of endotoxin or lipopolysaccharide (LPS) into systemic circulation after bacterial killing [1,2]. The use of potent antibiotics has fuelled interest in developing therapeutic strategies that neutralize or inhibit the key toxins and mediators in plasma responsible for LPS-induced hepatotoxicity. The direct toxic effects of endotoxin to variety of organs are due to the increased production of reactive oxygen intermediates as O_2^- , peroxides and nitric oxide [3,4]. The indirect cytotoxic effects are mediated through the action of endogenous mediators such as pro-

inflammatory cytokines (tumor necrosis factor alpha (TNF- α), interleukins 1 (IL-1), IL-6) produced and released by activated macrophages and neutrophils. LPS-induced increase in lipid peroxidation, which is an index of oxidative stress, has been described in several studies and is, reported to be both time- and dose-dependent. Yoshikawa et al. [5] observed enhanced lipid peroxidation in rats as early as 45 minutes after LPS infusion (100 mg/kg b.w.) in many tissues including liver, small intestine, stomach and abdominal aorta. Hepatic levels of malonaldehyde (MDA, a product of lipid peroxidation) increased five-fold within 16 hours after LPS administration (15 mg/kg b.w.) and 3.4 fold 8 hours after 30 mg/kg LPS administration [6]. Several studies have shown that LPS can cause reduced glutathione (GSH) depletion in liver in a dose-dependent manner [7] which is thought to be secondary to enhanced efflux of GSH from the liver or acute

depression of liver GSH synthesis. It has been found that precursor of GSH synthesis, N-acetylcysteine (NAC) protects against LPS toxicity and the inhibitor of GSH synthesis, DL-buthionine-SR-sulfoxime has the opposite effect. Sewerynek et al. [8] have shown an increase in GSSG levels after LPS injection, which is decreased by the antioxidant melatonin.

Flavonoids are groups of low molecular weight polyphenolic compounds that are ubiquitous in nature. Quercetin (QT) and its sugar conjugates are the most abundantly-distributed bioflavonoids and represent the largest proportion of flavonols in the plant kingdom. QT has potent antioxidant property and was found to be effective in various models of oxidative stress [9-12]. In one study, QT protected rat brain from LPS-induced shock by attenuating lipid peroxidation and nitric oxide generation [13]. Various *in vitro* and animal studies demonstrated inhibition of degranulation from mast cells, basophils and neutrophils by QT. It has been found that QT inhibit JNK/SAPK, its substrate C-Jun and iNOS transcription, in part, by suppressing activation of the transcription factor AP-1 (activator protein) [14]. Besides, it also modulates the activity of inflammatory mediators by inhibiting NF κ B [15]. QT can inhibit various tyrosine and serine/threonine kinases *in vitro*, including mitogen activated protein kinases MAPKs [16]. It is demonstrated that decreased cytokine secretion from LPS-treated macrophages is due to QT and other flavonoids being potent inhibitors of protein tyrosine kinases [17,18]. Hence it will be worthwhile to investigate the pro-modulatory effects of natural antioxidants in the endotoxin-mediated hepatotoxicity. Due to strong implication of cytokines and reactive oxygen species in endotoxin-induced hepatic damage, the present study is aimed at investigating effect of QT in salvaging endotoxin-induced hepatic dysfunction and oxidative stress in rat liver.

MATERIALS AND METHODS

Animals

Male wistar rats (150g-200g), bred in the central animal house of Panjab University (Chandigarh, India) were used. The animals were housed under standard conditions of light and dark cycle with free access to food (Hindustan Lever Products, Kolkata, India) and water. The experimental protocols were approved by the Institutional Ethical Committee of Panjab University, Chandigarh.

Drugs

Quercetin (QT) (Sigma, St. Louis, MO, USA) was prepared in dimethyl sulfoxide (DMSO) solution and Lipopolysaccharide (LPS) (serotype E. coli 0111:B4) (Fluka) was prepared in pyrogen free normal saline. The drug solutions were made freshly at the beginning of each experiment.

Experimental Groups and Protocol

Induction of Liver damage

Lipopolysaccharide, prepared in saline, was injected on 15th day in a single dose of 1 mg/kg intraperitoneally to the rats, which were being administered QT daily for

14 days. This particular dose of LPS has been shown to induce hepatic damage in a study done in our lab [19].

Treatment Schedule

At the beginning of the experiment, rats were divided into the following four groups, 6-7 animals each, and treated for two weeks:

1) The animals were treated with vehicle of QT and then challenged with saline on 15th day. This group served as control group.

2) The animals were treated with equivalent volume of vehicle of QT and then challenged with LPS on 15th day.

3) The animals were treated with QT (25 mg/kg, i.p.) for 14 days and then challenged with saline on 15th day.

4) The animals received QT (25 mg/kg, i.p) for 14 days and challenged with LPS on 15th day.

The dose of QT was selected on the basis of previous studies done in our lab [9].

Assessment of Liver Function

After 6 hours of LPS injection, all the animals were sacrificed; blood collected and their livers were harvested. Serum was separated from the blood. Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were estimated by International Federation of Clinical Chemistry (ERBA test kits). Total bilirubin was estimated by Diazo method of Pearlman and Lee [20] (ERBA test kits). Total protein was also estimated by Biuret method (ERBA test kits). All the readings were taken on a semi-autoanalyzer (Erba Chem-5 plus, Transasia, India)

Assessment of Oxidative Stress

Post mitochondrial supernatant preparation (PMS)

On the day of biochemical estimation, livers were rinsed with cold isotonic saline, carefully dried on filter papers and weighed. The entire liver was then minced properly and the homogenate was prepared with 10% (w/v) cold phosphate-buffered saline (0.1 mol/l, pH 7.4) using a homogenizer. The homogenates were centrifuged at 800 g for 5 minutes at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 g for 20 minutes at 4°C to get the post mitochondrial supernatant, which was used to assay superoxide dismutase (SOD) activity.

Estimation of lipid peroxidation

Lipid peroxidation was assayed in the form of thiobarbituric acid reacting substances (TBARS) by method of Wills [21]. The amount of malondialdehyde formed was measured by acid heating reaction with thiobarbituric acid at 532 nm using Erba Chem 5 Plus (Transasia, India). The results were calculated as nmol of malondialdehyde per mg protein using the molar extinction coefficient of chromophore ($1.56 \times 10^5 \text{ mol}^{-1} \text{ cm}^{-1}$) for which protein content of tissue was calculated according to the Biuret method [22]. The results were expressed as % of vehicle control.

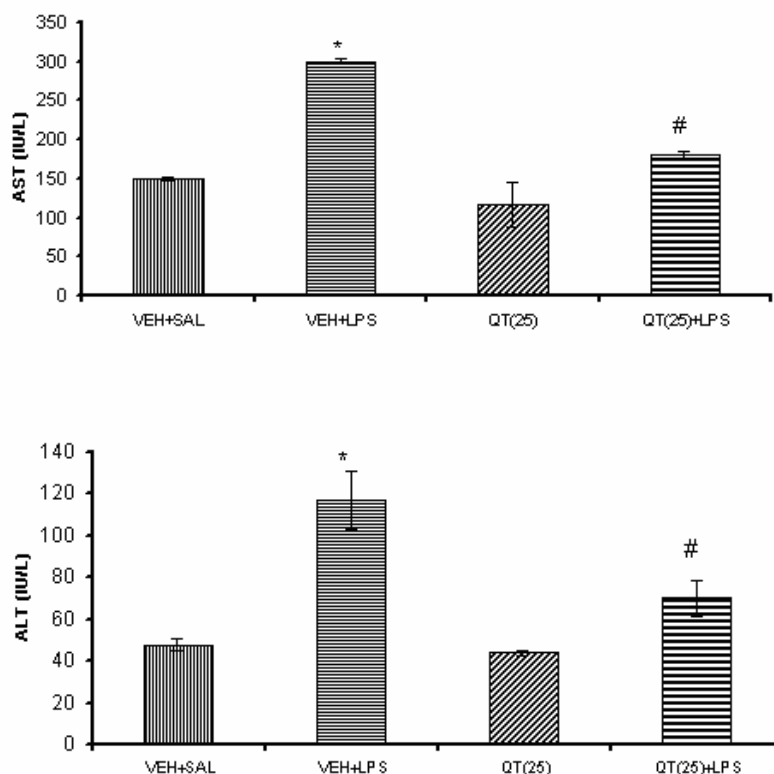


Fig 1. Effect of chronic QT (quercetin) administration on liver aspartate aminotransferase (AST) (upper panel) and alanine aminotransferase (ALT) (lower panel) after LPS treatment. Values are expressed as mean \pm S.E.M. * p <0.05 as compared to vehicle and saline treated group. # p <0.05 as compared to vehicle and LPS challenged rats. VEH + SAL means group receiving vehicle of QT and then challenged with saline, VEH + LPS means group receiving vehicle of QT and then challenged with LPS, QT(25) means group receiving QT (25 mg/kg, i.p.) and challenged with saline. QT(25) + LPS means group receiving QT (25 mg/kg, i.p.) and challenged with LPS.

Estimation of reduced glutathione

Reduced GSH in the liver was estimated by method of Jollow [23]. About 1.0 ml of tissue homogenate (10%) was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4°C for at least 1 hr and then subjected to centrifugation at 1200 g for 15 min at 4°C. The assay mixture contained 0.1 ml aliquot and 2.7 ml of phosphate buffer (0.1M pH 7.4) to which was added 0.05 ml of DTNB (5-5'-dithio bis- 2 nitrobenzoic acid). The yellow color developed was read immediately at 412 nm using an Erba Chem 5 Plus (Transasia, India) semiauto-analyzer. The results were calculated as nmol of GSH per mg protein and expressed as % of vehicle control.

Estimation of SOD

SOD activity was assayed by the method of Kono *et al.* [24]. The assay system consisted of EDTA 0.1mM, sodium carbonate 50 mM and 96 mM of nitro blue tetrazolium (NBT). In a cuvette, 2 ml of above mixture, 0.05 ml hydroxylamine and 0.05 ml of PMS were taken and the auto-oxidation of hydroxylamine was observed

by measuring the absorbance at 560 nm. The results were calculated as units/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 100%.

Statistical Analysis

Results were expressed as mean \pm SEM. The inter-group variation was measured by one way analysis of variance (ANOVA) followed by Fischer's LSD test. Statistical significance was considered at p < 0.05. The statistical analysis was done using the Jandel Sigma Stat Statistical Software version 2.0.

RESULTS

Effect of QT on LPS-induced alteration in liver enzymes (ALT & AST).

LPS induced a marked hepatic dysfunction evident by rise in serum levels of ALT and AST (Fig 1). QT *per se* had no effect on liver enzyme levels; however, it significantly attenuated LPS-induced rise in serum concentration of liver enzymes.

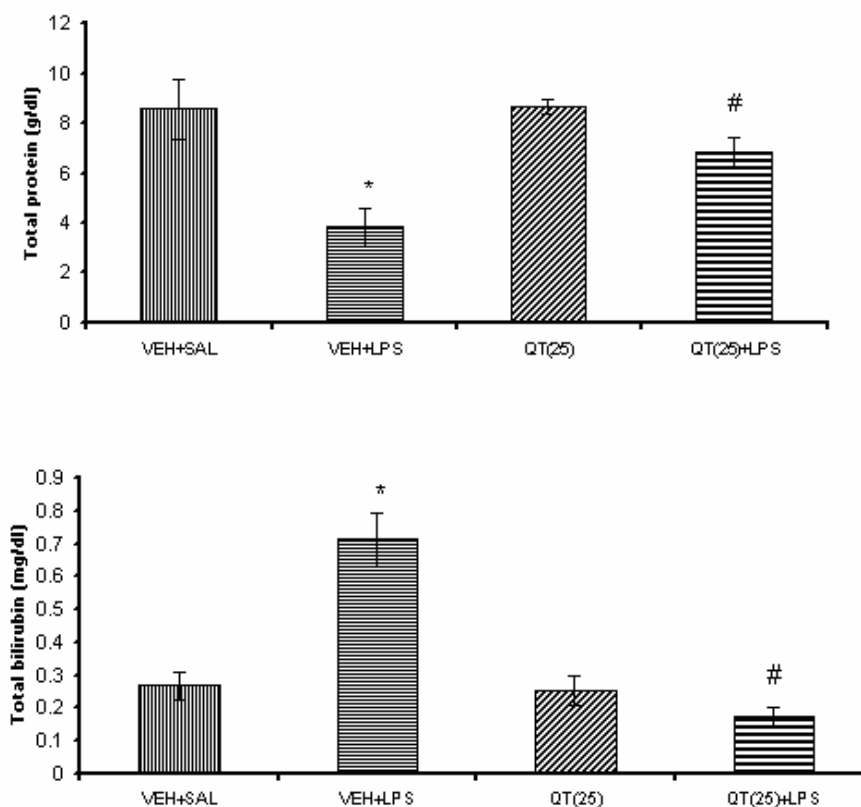


Fig 2. Effect of chronic QT (quercetin) administration on total protein (upper panel) and total bilirubin (lower panel) after LPS treatment. Values are expressed as mean \pm S.E.M. * $p < 0.05$ as compared to vehicle and saline treated group. # $p < 0.05$ as compared to vehicle and LPS challenged rats. VEH+SAL means group receiving vehicle of QT and then challenged with saline, VEH + LPS means group receiving vehicle of QT and then challenged with LPS, QT(25) means group receiving QT (25 mg/kg, i.p.) and challenged with saline. QT(25) + LPS means group receiving QT (25 mg/kg, i.p.) and challenged with LPS.

Effect of QT on LPS-induced alteration in total bilirubin and total protein

LPS led to a dramatic increase in total bilirubin (Fig 2, lower panel) while a significant decrease in total protein (Fig 2, upper panel) was seen, depicting marked liver damage. Chronic treatment with QT significantly attenuated the LPS-induced increase in bilirubin and decrease in total protein.

Effect of QT on LPS-induced Oxidative stress in liver

Effect of QT on hepatic TBARS levels

LPS caused a significant increase in lipid peroxidation as indicated by a marked increase in liver TBARS levels as compared to control group. Chronic administration of QT significantly attenuated LPS-induced increase in liver TBARS levels (Fig 3, upper panel).

Effect of QT on liver GSH levels in LPS-treated rats

LPS caused a significant decrease in hepatic GSH levels. Chronic administration of QT significantly attenuated this effect (Fig 3, lower panel). The QT treated *per se* group did not show any significant changes in GSH levels as compared with vehicle-treated control group.

Effect of QT on hepatic SOD levels in LPS-treated rats

LPS markedly decreased liver SOD (Fig 4) levels indicating oxidative stress as compared with control group. Chronic QT treatment significantly attenuated the decrease in SOD in LPS-challenged rats. *QT per se* had no effect on liver SOD levels.

DISCUSSION

LPS-induced oxidative stress has been described in several studies [25]. Chronic administration of QT for 14 days before the onset of endotoxin intoxication markedly attenuated LPS-induced hepatic dysfunction and accompanying oxidative stress in liver. It relieved hepatic dysfunction as measured by the levels of serum hepatic enzymes, total bilirubin and total protein. This observation is in line with our previous study, which showed that hesperidin produced a beneficial effect on LPS-induced hepatotoxicity [19]. LPS is a potent stimulator of nitric oxide (NO) as it is reported that endotoxaemia for 6 hours resulted in a 4.5 fold rise in the serum levels of nitrite, an end product of NO. The increased levels of NO after endotoxin challenge can react with O_2^- leading to formation of the peroxynitrite anion (ONOO⁻) which oxidizes sulphhydryl groups and generates $\cdot OH$ [26]. This overproduction of

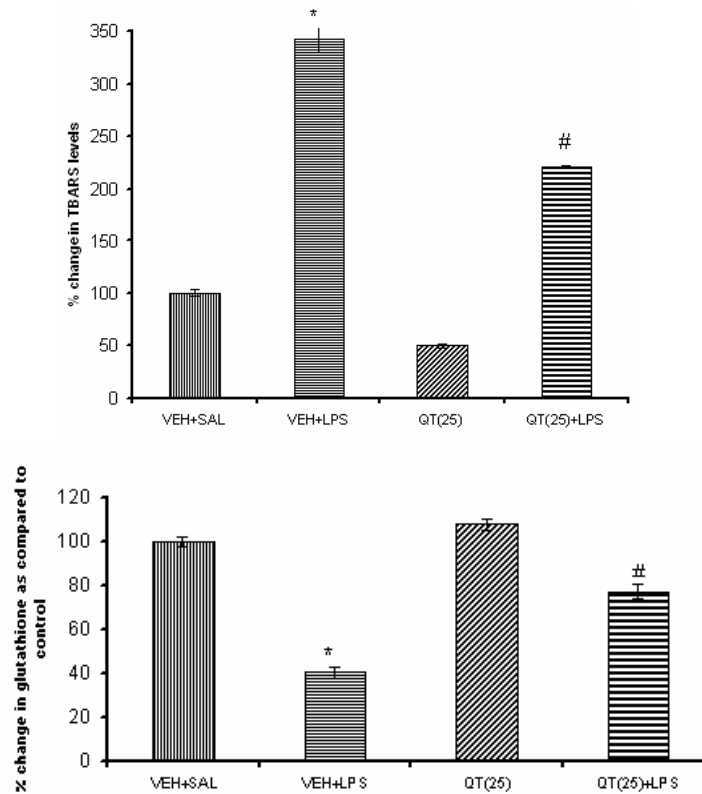


Fig 3. Effect of chronic QT (quercetin) administration on liver thiobarbituric acid reactive substances (TBARS) (upper panel) and reduced glutathione content (GSH) (lower panel) after LPS treatment. Values are expressed as mean \pm S.E.M. * p <0.05 as compared to vehicle and saline treated group. # p <0.05 as compared to vehicle and LPS-challenged rats. VEH + SAL means group receiving vehicle of QT and then challenged with saline, VEH + LPS means group receiving vehicle of QT and then challenged with LPS, QT(25) means group receiving QT (25 mg/kg, i.p.) and challenged with saline. QT(25) + LPS means group receiving QT (25 mg/kg, i.p.) and challenged with LPS.

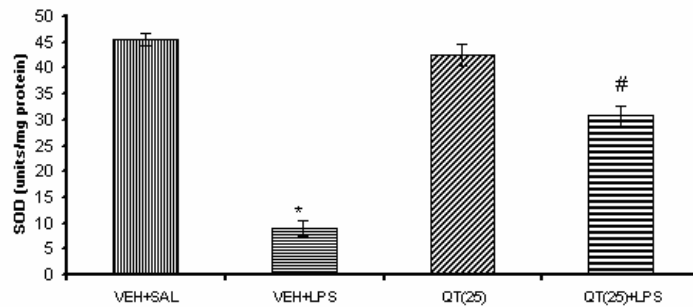


Fig 4. Effect of chronic QT (quercetin) administration on superoxide dismutase (SOD) activity after LPS treatment. Values are expressed as mean \pm S.E.M. * p <0.05 as compared to vehicle and saline treated group. # p <0.05 as compared to vehicle and LPS challenged rats. VEH + SAL means group receiving vehicle of QT and then challenged with saline, VEH + LPS means group receiving vehicle of QT and then challenged with LPS, QT(25) means group receiving QT (25 mg/kg, i.p.) and challenged with saline. QT (25) + LPS means group receiving QT (25 mg/kg, i.p.) and challenged with LPS.

free radicals induces oxidative damage that can be measured by the increase in lipid peroxidative products and decrease in reduced glutathione content. Besides, induction of iNOS activity in hepatocytes and kupffer cells results in: 1) a reduction in the synthesis of proteins, prostaglandins and IL-6 [27], 2) Inhibition of mitochondrial respiratory chain enzymes, possibly due to the formation of peroxynitrite [28,29]. QT is reported to dose-dependently inhibit the production of NO and TNF- α in RAW cells treated with LPS [30]. Biochemical studies revealed the inhibition of nitric oxide production by quercetin in endotoxin/cytokine-

stimulated microglia [31]. Similarly, the inhibitory effects of QT on tumor necrosis factor and interleukin-1 β have been studied by Zhang [32].

Phagocytosis by neutrophils in response to LPS is accompanied by a dramatic increase in oxygen consumption with an abundant production of reactive oxygen intermediates such as the superoxide anion and various oxidising species (H₂O₂, \cdot OH). The highly reactive oxygen metabolites along with other mediators elaborated by neutrophils and macrophages promote liver and other tissue damage [33]. These cytotoxic mediators are generated sequentially starting with

superoxide anion production by a membrane-bound NADPH oxidase activity [34]. QT is reported to inhibit activation of NADPH oxidase localised in the plasma membrane [35]. In one study, QT decreased the susceptibility of neutrophils to proinflammatory factors like LPS [36]. Quercetin was found to be a potent inhibitor of human neutrophil degranulation and superoxide anion production [37,38] at concentrations that inhibited the phosphorylation of neutrophil proteins accompanying neutrophil activation by phorbol myristate acetate (PMA), suggesting thereby that the phosphorylation of neutrophil protein is an important intracellular event associated with neutrophil activation [37].

In conclusion, the data presented here suggests that pre-treatment with QT attenuates LPS-induced hepatotoxicity and oxidative stress possibly by preventing cytotoxic effect of oxygen free radicals as there are many studies depicting beneficial effect of quercetin on oxidative stress induced by LPS [13,31,36]. Further, these findings also warrant a study on the hepatoprotective effect of quercetin due to inhibition of cytokines produced by LPS during infection.

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