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Original Article

Thymoquinone protects the rat kidneys against renal fibrosis

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Abstract

Thymoquinone (TQ) is the main active ingredient of Nigella sativa seeds with various pharmacological effects. The aim of this study was to investigate the effect of TQ on renal fibrosis and permeability and oxidative stress status in lipopolysaccharide (LPS)-induced inflammation in male rats. Eighty male Wistar rats were divided into 5 groups as follow: control (received normal saline), LPS (1 mg/kg/day), and LPS+TQ (by doses of 2, 5 and 10 mg/kg/day). After three weeks, the biochemical parameters such as blood urea nitrogen (BUN) and creatinine in serum samples, oxidative stress markers including malondialdehyde (MDA), total thiol groups, superoxide dismutase (SOD) and catalase (CAT) activities in renal tissue homogenate and renal permeability (evaluated by Evan's blue dye method) were measured and renal fibrosis was evaluated, histologically using Masson's trichrome staining. LPS administration induced renal fibrosis (1.49 ± 0.08 vs. 7.15 ± 0.18%) and significantly increased renal permeability (6.03 ± 1.05 vs. 13.5 ± 1.04 µg evans blue(EB)/g tissue), serum BUN and creatinine levels and oxidative stress marker (MDA) ($P < 0.05$), while, it reduced anti-oxidative markers including total thiol group, SOD and CAT activities ($P < 0.05$). Administration of TQ significantly improved these alterations which were dose-dependent in oxidative stress markers, renal permeability (TQ 2, 5 and 10 mg/kg: 10.7 ± 0.3, 9.2 ± 1.4 and 11.5 ± 0.6 µg EB/g tissue; respectively) and fibrosis (TQ 2, 5 and 10 mg/kg: 6.09 ± 0.7, 4.26 ± 0.14 and 2.52 ± 0.08%; respectively). In conclusion, administration of TQ reduced renal fibrosis and permeability and improved oxidative stress status. Thus, TQ can be considered in conditions accompanied with chronic inflammation at least as a part of treatment strategy.

Keywords: Thymoquinone; Lipopolysaccharide; Renal; Fibrosis; Permeability

INTRODUCTION

Chronic kidney disease (CKD) is defined as functional or structural abnormalities of one or both kidneys presenting for an extended period of time whose main cause is unknown but pathological diagnosis is often indicative of renal fibrosis (1). Fibrosis is a procedure of typical wound healing and repair that is enacted because of damage to keep up the original tissue design and functional integrity (1). However, prolonged chronic damaging stimuli may bring about deregulation of ordinary processes and result in an excess deposition of extracellular matrix (ECM) (2). Most of the time, tissue damage and injured cells are replaced by cells of a similar sort and/or fibrous tissue after inflammatory reaction (2).

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Inflammation is a risk factor for renal fibrosis (1). It is also specified that the activation of reactive oxygen species (ROS) may lead to renal fibrosis (1). In laboratory studies, bacterial endotoxin is used for induction of inflammation models. The bacterial endotoxin in the wall of negative gram bacteria is lipopolysaccharide (LPS) (3). LPS also induces a pro-oxidant effect and an increase in generation of ROS and development of organ failure because of sepsis involvement of ROS production and inflammatory cytokines (4). It is reported that subclinical inflammation is associated with increased oxidative stress (5).

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Oxidative stress, ROS and antioxidant immune deficiency are involved in inflammation and renal fibrosis, thus, drugs that reduce the severity of oxidative stress can be useful in the prevention of injury or improvement of the symptoms.

Nigella sativa is a flowering annual plant and is native to the southwest Asia with many useful compounds (6). Thymoquinone (TQ) is the main active ingredient of *N. sativa* seeds (30-48%), which has various pharmacological effects such as antioxidant, anti-inflammatory, antiproliferative, antifibrotic activities and protect heart, liver and kidneys (6). In addition, it can diminish the nephrotoxic complication of some drugs such as gentamicin, cisplatin and doxorubicin (7). Therefore, according to the evidences that approved the beneficial effects of TQ and its nontoxic effects on renal tissue, in this study, we attempted to determine whether the TQ would diminish LPS-induced renal fibrosis in male rats.

MATERIALS AND METHODS

Animals and treatments

Eighty male Wistar rats weighing 225 ± 25 g were taken from the institute of experimental animals of the Mashhad University of Medical Sciences. The ethical committee of Mashhad University of Medical Sciences approved the experimental protocol (code: 950832). The animals were housed under standard conditions (20-25 ºC and 12 h light/dark cycle) and had free access to tap water and chow. The rats were randomized into five experimental groups (n = 16 in each group): (1) control; (2) LPS; (3-5) LPS+TQ with three doses of TQ at 2, 5 and 10 mg/kg/day.

Chemicals

LPS from *Escherichia coli* serotype, 5,5´-dithiobis-2-nitrobenzoic acid (DTNB), and 2-isopropyl-5-methyl-p-benzoquinone (TQ) were purchased from Sigma Co. (USA) 2-thiobarbituric acid (TBA), hydrochloric acid (HCl), ethylene diamine tetraacetic acid (EDTA), trichloro acetic acid (TCA), Evans Blue (EB), diethyl ether and formamide were purchased from Merck (Germany).

Solvents

LPS was dissolved in cold saline. TQ was dissolved in ethanol and diluted with normal saline (final concentrations 2.5%). The EB was dissolved in distilled water (8,9).

Protocol design

For induction of chronic inflammation, LPS-treated groups received LPS 1 mg/kg/day, intraperitoneally (i.p.) for three weeks (8). Control group received 0.9% saline instead of LPS. In TQ-treated groups, TQ was administered by three doses of 2, 5 and 10 mg/kg/day; i.p.) 30 min before starting administration of LPS for three weeks (9).

Organ harvest

After three weeks, blood samples were taken and separated serums were kept at -70 °C for further analysis. Half of the animals in each group were sacrificed and their kidneys were removed and washed with saline. Then, the right kidneys were homogenized with phosphate buffer solution for determination of tissue oxidative and antioxidative indicators. The left kidneys were fixed in 10% formalin for histopathological evaluations.

Renal permeability measurement

In half of the animals in each group, permeability of the renal tissues was evaluated by EB dye that binds to albumin. For this purpose, the animals were anesthetized with urethane and EB dye (20 mg/mL; 100 µL) was injected via tail vein before sacrifice. After 20 min, the kidneys were isolated, cleaned from connective tissues, cut from longitudinal section, washed in normal saline, and weighed out. Then, they were put in formamide solution for 48 h at laboratory temperature. The optical concentration of the solution was measured spectrophotometrically at 620 nm. Data were expressed as µg EB/g of wet tissue weight (9,10).

Serum creatinine and blood urea nitrogen concentration measurement

Serum creatinine and blood urea nitrogen (BUN) levels were measured by creatinine and BUN measurement kits (Pars Azmoon Company, Iran).

Measurement of serum and tissue levels of IL-1β

The specific rat ELISA kits (eBioscience Co, San Diego, CA, USA) were used for the measurement of serum and heart homogenate IL-1βaccording to the manufacturer instructions.

Malondialdehyde measurement

The malondialdehyde (MDA) concentrations of the renal homogenates were measured using the thiobarbituric acid method. In with the presence of thiobarbituric acid, MDA forms a pink chromogen compound with maximal absorbance at 532 nm. The results were expressed as nmol/mg protein (11).

Total sulfhydryl groups measurement

Total thiol groups were evaluated based on Ellman method (12) using following equation:

Total thiol concentration (mM) = (A2-A1-B) × (1.07/0.05) 13.6

Superoxide dismutase enzyme activity

Superoxide dismutase (SOD) activity was determined by colorimetric assay (13). One unit of SOD was defined as the amount of enzyme required to inhibit the rate of MTT reduction by 50%. The absorbance of solution was read at 570 nm.

Catalase activity

The catalase (CAT) activity was assessed by measuring the initial rate of H_2O_2 disappearance at 240 nm. It is based on the assessment of the rate constant of hydrogen peroxide decomposition.

The CAT activity was calculated using the extinction coefficient of 40 mM^{-1} cm^{-1} for H_2O_2 (14).

Histological analysis

Paraffin-embedded tissues were deparaffinized, dehydrated and stained with haematoxylin and eosin (H&E) for morphological analysis and also stained with Masson's trichrome for renal fibrosis evaluation. The slide were checked under light microscope with magnification of 400 and percent of collagen content was evaluated with Image J software (15).

Statistical analysis

All data are expressed as means ± standard error (SE). The analysis of data was performed using the SPSS software version 20 (SPSS, Inc, Chicago, IL). All data were compared by one way ANOVA followed by LSD post hoc test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Renal permeability

Results showed that renal tissue permeability of LPS receiving group was higher than control animals ($P < 0.001$). All doses of TQ decreased renal permeability compared to LPS group, which was significant at doses of 2 and 5 mg/kg/day of TQ ($P < 0.05$ and $P < 0.01$, respectively). The permeability of renal tissue was reduced by middle dose of TQ which was not statistically different form control group, while in low and high dose of TQ, there were significant differences compare to control level ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 1).

Serum creatinine and BUN concentration

Serum BUN and creatinine levels in LPS group were higher than control ($P < 0.001$ and $P < 0.01$ respectively, Fig. 2). Treatment with three different doses of TQ decreased serum BUN (Fig. 2A) and creatinine (Fig. 2B), dose dependently.

Oxidative and antioxidative markers in renal tissue

LPS increased renal MDA concentration and reduced total thiol concentrations compared to the control group (Fig. 3A and B; $P < 0.001$). Treatment by TQ significantly reduced MDA (Fig. 3A; $P < 0.001$) and raised total thiol concentrations in renal tissues (Fig. 3B; $P < 0.001$), dose-dependently.

Our results also showed that SOD concentration in renal tissues of LPS group was lower than that of control group ($P < 0.001$, Fig. 3C) which was significantly increased after TQ administration. This increase in tissue homogenate SOD was statistically significant in high doses of TQ compare to control ($P < 0.001$, Fig. 3D).

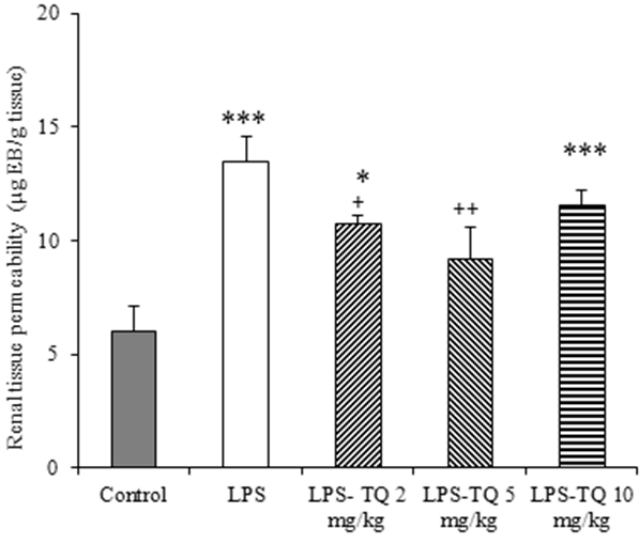

Fig. 1. Renal tissue permeability in experimental groups. LPS-treated group exhibited higher permeability than the control group and administration of TQ significantly reduced permeability in renal tissue especially by its middle dose. ***$P < 0.001$ and **$P < 0.01$ compared to control group; $^{++}P < 0.01$ and $^{+}P < 0.05$ compared to LPS-treated group. n = 6 in each group. LPS, lipopolysaccharide; TQ, thymoquinone.

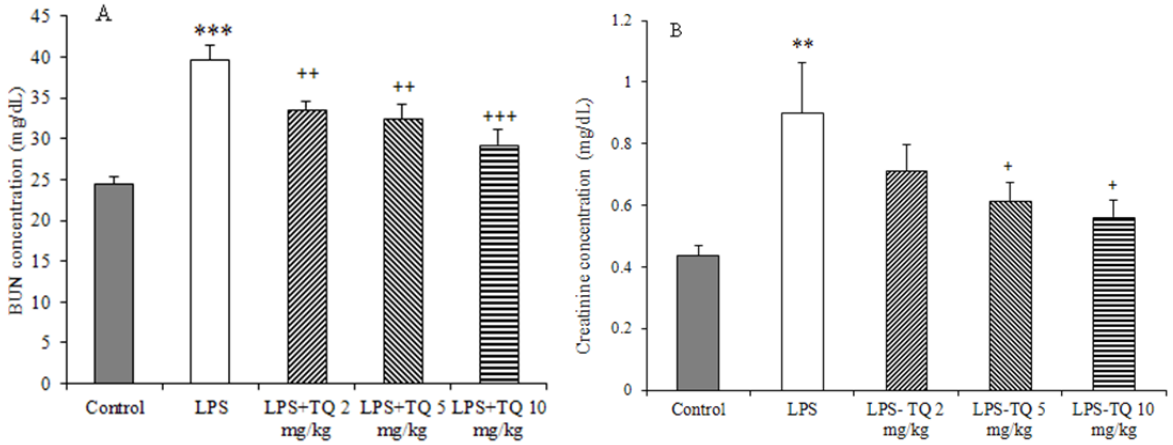

Fig. 2. (A) BUN and (B) creatinine concentration in experimental groups, LPS-treated group had higher BUN and creatinine levels than the control group and administration of TQ significantly reduced these parameters especially by the highest dose. ***$P < 0.001$ compared to control group; $^{+++}P < 0.001$; $^{++}P < 0.01$ compared to LPS-treated group; ***$P < 0.001$ and *$P < 0.05$ compared to control group; $^{+}P < 0.05$ compared to LPS-treated group. n = 10 in each group. LPS, lipopolysaccharide; TQ, thymoquinone; BUN, blood urea nitrogen.

Histopathological assessment

Histopathological changes of the renal sections stained with hematoxylin and eosine (H&E) stain are shown in Fig. 4. In the control group, renal tissue sections had a normal morphology (Fig. 4A).

Histologic examination of the kidneys exposed to LPS showed a distinctive pattern exhibiting degeneration of tubular architecture and infiltration of inflammatory cells which was reversed by different doses of TQ (Fig. 4B to 4D). Administration of LPS increased fibrotic tissue and collagen deposition in renal tissue compared to the control group (Fig. 5 A&B). All doses of TQ decreased renal fibrosis (Fig. 5C to 5E). Furthermore, the increase in percent of collagen content in LPS group was significant compared to that of the control group (Fig. 5F) and administration of TQ decreased percent of collagen content in renal tissue in a dose-dependent manner ($P < 0.05$).

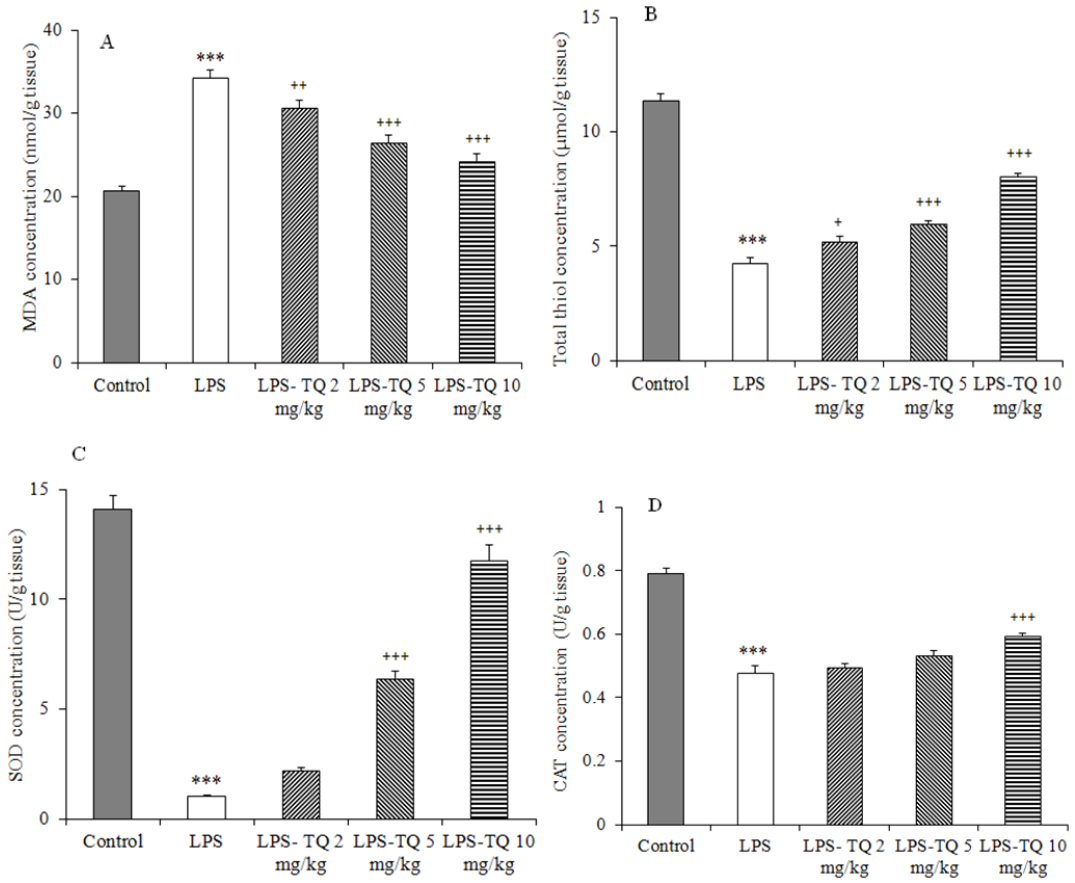

Fig. 3. Renal concentrations of oxidative/antioxidative markers showed decreased oxidative stress and increased antioxidative stress markers in tissue homogenates**.** (A) Renal MDA concentration $^{***}P < 0.001$ and $^{**}P < 0.01$ compared to the control group; $^{+++}P < 0.001$ and $^{++}P < 0.01$ compared to LPS-treated group. (B) Total thiol concentration in renal tissues, $^{***}P < 0.001$ compared to control group; $^{+++}P < 0.001$ and $^{+}P < 0.05$ compared to LPS-treated group. (C) SOD activity in renal tissues, $^{***}P < 0.001$ compared to control group; $^{+++}P < 0.001$ compared to LPS-treated group. (D) Catalase activity in renal tissue, $^{***}P < 0.001$ compared to the control group; $^{+++}P < 0.001$ compared to LPS-treated group. n = 10 in each group. MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; LPS, lipopolysaccharide; TQ, thymoquinone.

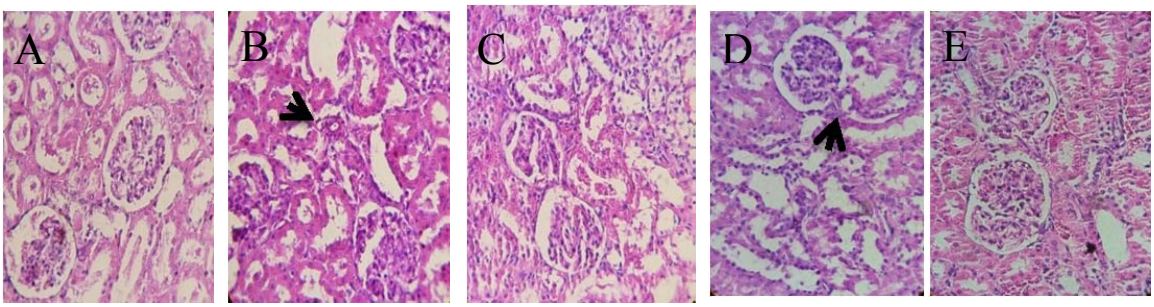

Fig. 4. Histopathological assessment by hematoxylin and eosine staining. The light micrograph of renal tissue stained by hematoxylin and eosine. A; Control group with normal architecture (×40), B; LPS-treated group showing infiltration of inflammatory cells (arrows) and degeneration of tubular architecture. (C to E) TQ-treated groups, 2 mg/kg, 5 mg/kg, and 10 mg/kg, respectively, n = 6 in each group. LPS, lipopolysaccharide; TQ, thymoquinone.

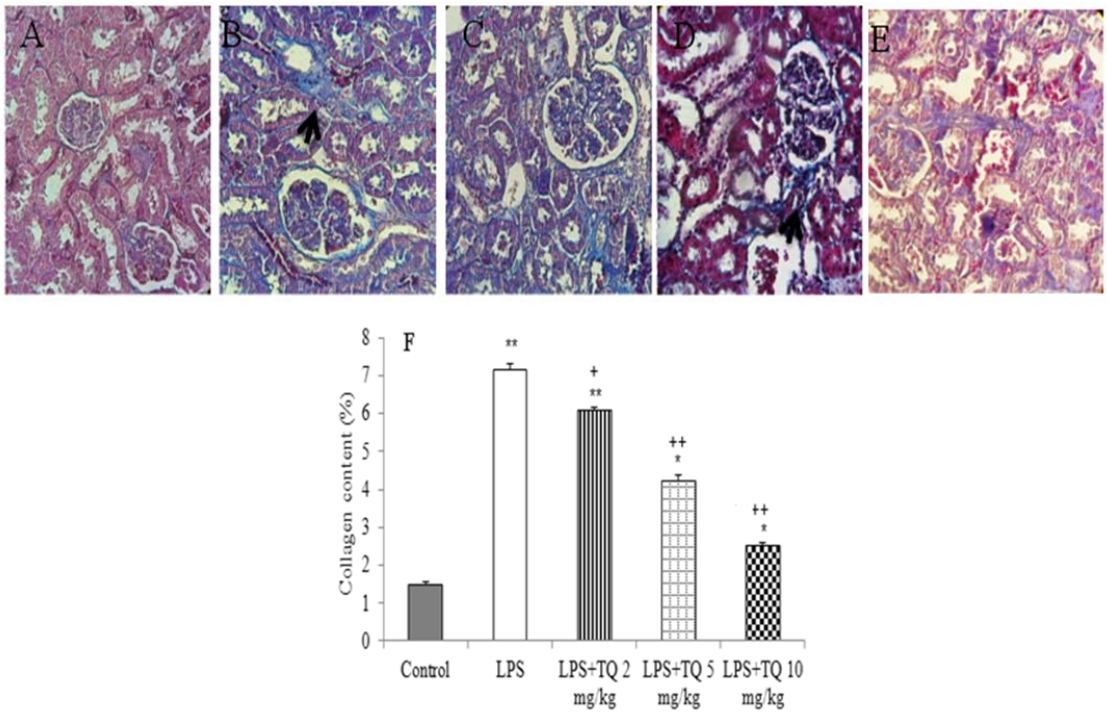

Fig. 5. Histopathological assessment by Masson's trichrome staining. Masson trichrome staining of renal tissue of (A) control and (B) More collagen deposition in LPS-treated group is observed. Blue color and black arrows demonstrates collagen fibers. Administration of TQ decreased renal fibrosis (C to E), 2 mg/kg, 5 mg/kg, and 10 mg/kg, respectively. (F) Renal fibrosis shows higher collagen content (%) in LPS-treated group compared to control which decreased dose-dependently by TQ. **P* < 0.05 compared to control; ***P* < 0.01 compare to control. $^{+}P < 0.05$ compared to LPS and LPS + TQ treated with 5 mg/kg; $^{++}P < 0.05$ compared to LPS and LPS + TQ 2 mg/kg. n = 6 in each group. LPS, lipopolysaccharide; TQ, thymoquinone.

DISCUSSION

It is believed that beneficial effects of *N. sativa* seeds are attributed to its quinone compounds including TQ, dithymoquinone and thymohydroquinone. Since TQ is the active ingredient of *N. sativa* oil, this hypothesis that pharmacological effects of *N. sativa* oil is due to TQ becomes more robust (16). Despite the underlying cause, it is believed that oxidative stress has a basic role in the pathophysiology of chronic kidney disease and the propagation of renal fibrosis (17,18). In the present study, we found that in terms of exposure to LPS, renal fibrosis occurs as a primary effect. We already reported that administration of *N. sativa* improved fibrosis in myocardial tissue (19). In this study, we examined the effect of TQ on fibrosis and permeability of renal tissue and oxidative indicators in subclinical LPS administration. The results of previous studies indicated that LPS injection increased the level of MDA and decreased the levels of glutathione (GH) (20). Some studies suggest that treatment with LPS, decreased glutathione peroxidase, catalase and superoxide dismutase (21). Based on our observations, subclinical injection of LPS significantly increased MDA, decreased total thiol groups and SOD and CAT activities in the renal tissue. Also administration of all doses of TQ in LPS groups reduced levels of MDA and

increased anti-oxidative indicators. It is reported that TQ increased expression and activity of antioxidant enzymes such as glutathione catalase, SOD, glutathione peroxidase and glutathione reductase, and also reduced expression of nitric oxide synthase and decreased lipid peroxidation resulting in reduced free radicals and oxidative stress (22). TQ also protects different organs against free radicals such as cardiac toxicity induced by doxorubicin, tetrachloride-induced liver toxicity and cisplatin-induced nephropathy (23). TQ could invert the diminishing in decreased glutathione, glutathione peroxidase and CAT levels in the renal tissue of gentamicin-treated rats (24). These propose that TQ is a radical scavenger with a potential role in the prevention and/or treatment of oxidative damage.

In the present study, we showed that chronic administration of LPS increased serum BUN and creatinine levels. Previous studies demonstrated that renal function parameters were improved by TQ treatment. In accordance to our results, TQ diminishes serum BUN and creatinine in cisplatin-induced nephrotoxicity in rats (25).

The first tissue barriers in the circulation of LPS is lining of the host vascular endothelial cells (26). In this study, we showed that renal tissue permeability in LPS animals was significantly higher than that of the control group, and administration of TQ especially by dose of 5 mg/kg in LPS group reduced renal tissue permeability. There are few studies which reported the effect of TQ on vascular permeability, however, in a study to find the effect of TQ on airway-induced hypersensitivity and permeability, administration of TQ by dose of 8 mg/kg (i.p.) (which is very close to the doses we used in the present study) prevented the pathological changes and permeability that occurred in response to LPS (27). It was shown that TQ improves endothelial function through inhibition of oxidative stress and impact on angiotensin system (28). NFκB plays an important role in endothelial response to inflammatory stress (29) and it is possible that TQ improves LPS-induced endothelial damages via strong antioxidant activity and ability to inhibit NFκB.

Pro-fibrotic growth factors and chemokines from activated epithelial and endothelial cells, as well as infiltrating leukocytes are involved in renal fibrosis. Interstitial fibroblasts play a crucial role in extracellular matrix homeostasis in the normal kidney (30). In the present study, we found that the higher interstitial fibrosis in renal tissue in LPS animals was improved by TQ. The renal fibrosis at least in unilateral ureteral obstruction model is induced by activation of TLR4, the LPS receptor (31). On the other hand, the beneficial effects of TQ on tissue fibrosis has been shown in bleomycin-induced pulmonary fibrosis (32), and liver damage due to bile duct obstruction suggesting that it acts through inhibiting the NFκB.

CONCLUSION

LPS-induced renal fibrosis was improved by TQ administration in a dose-dependent manner. The improvement in oxidative/ antioxidative balances after TQ administration might be responsible for reduced renal fibrosis and permeability. More studies are needed to clarify its exact mechanism.

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