

## Effect of Thymoquinone on Oxidative Stress in *Escherichia coli*-Induced Pyelonephritis in Rats

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### ABSTRACT

**BACKGROUND:** Recurrent urinary tract infections are important in children and adults with diabetes mellitus and/or incontinence due to risk of pyelonephritis (PYN) and renal damage. There is a positive correlation released free radicals during PYN and renal damage. Experimental studies showed that antioxidant agents improve renal damage when used immediately after bacterial inoculation.

**OBJECTIVE:** The aim of the present study was to evaluate whether treatment by thymoquinone (TQ) before or during *Escherichia coli* inoculation prevents oxidative damage in acute pyelonephritis (PYN) in an ascending obstructive rat model.

**METHODS:** In this study, 42 Wistar rats were grouped as follows: control, PYN (24, 48, and 72 hours), and TQ-PYN (24, 48, and 72 hours). *E. coli* ( $1 \times 10^9$  colony forming units) was inoculated into the bladder via urethral catheterization in both the PYN and TQ groups. TQ injections were performed 24 hours before bacteria inoculation and repeated at 24-hour intervals during the indicated time at a dose of 10 mg/kg body weight intraperitoneally in TQ groups.

**RESULTS:** Superoxide dismutase activity was statistically lower in the TQ-PYN-48 and -72 groups than the PYN-48 and -72 groups ( $P < 0.001$ ,  $P = 0.004$ , respectively). Catalase activity was significantly higher in PYN-24, -48, and -72 groups than the control group ( $P < 0.001$ ). In addition, there was a significant difference between the TQ-PYN-24, -48, and -72 groups and PYN groups in terms of glutathione peroxidase activity ( $P < 0.001$ ,  $P = 0.026$ ,  $P = 0.046$ , respectively). When the TQ-PYN-72 group was compared with the PYN-72 group, malondialdehyde levels were significantly lower in the TQ-PYN-72 group than in the PYN-72 group ( $P = 0.033$ ). A histologic examination also confirmed the protective effect of TQ. In statistical analysis of histopathologic findings, there were significant differ-

ences between the PYN-24 and TQ-PYN-24, PYN-48 and TQ-PYN-48, and PYN-72 and TQ-PYN-72 groups ( $P = 0.008$ ,  $P < 0.001$ ,  $P < 0.001$ , respectively).

**CONCLUSIONS:** The results indicate that TQ administration attenuated the oxidative damage that occurred in PYN and, therefore, could be used as a supportive agent to protect the kidneys from oxidative damage caused by PYN. (*Curr Ther Res Clin Exp.* 2011;72:204–215) © 2011 Elsevier HS Journals, Inc. All rights reserved.

**KEY WORDS:** kidney, oxidative stress, pyelonephritis, thymoquinone, Wistar rats.

## INTRODUCTION

Urinary tract infection is one of the most common diseases encountered in medical practice. *Escherichia coli* is the most frequent cause of urinary tract infection. Clinical manifestations of these infections range in severity from cystitis to acute or chronic pyelonephritis (PYN). If not treated properly, permanent renal damage is possible.<sup>1</sup>

Renal scarring is the worst result of PYN. This event depends on the inflammatory damage followed by a bacterial infection in the kidneys. In experimental and clinical studies, it has been reported that PYN should be treated urgently with antibiotics, such as aminoglycosides or  $\beta$ -lactam antibiotics, to prevent renal scar formation. A late start of antibiotic treatment can lead to renal scarring.<sup>2–5</sup>

Recurrent urinary tract infections are important in children and adults with diabetes mellitus and/or incontinence due to the risk of PYN and renal damage.<sup>6,7</sup> There is a positive correlation between acute PYN and renal failure secondary to chronic scarring due to the released free radicals during PYN.<sup>8</sup> In experimental studies regarding rat models of PYN, antioxidant agents such as vitamins A, E, and pentoxifylline improved *E. coli*-induced renal scar formation when used immediately after bacterial inoculation.<sup>9,10</sup>

*Nigella sativa* was shown to have many therapeutic effects, including immunomodulatory,<sup>11</sup> antibacterial,<sup>12</sup> antidiabetic,<sup>13,14</sup> antihistaminic, and antioxidant.<sup>15</sup> Thymoquinone (TQ; 2-isopropyl-5-methyl-1,4-ben-zoquinone) is the main active component of the volatile oil of the black seed (*Nigella sativa* L.) and may have a protective effect against leukocytes, membrane lipid peroxidation, and cisplatin-induced nephrotoxicity.<sup>16,17</sup> However, the therapeutic or preventive effect of TQ on *E. coli*-induced acute PYN remains unknown.

The aim of the present study was to investigate whether treatment by TQ before or during *E. coli* inoculation prevents oxidative damage in acute PYN in an ascending obstructive rat model.

## MATERIALS AND METHODS

### ANIMALS AND EXPERIMENTAL INFECTION

A total of 42 male Wistar albino rats weighing 200 to 250 g (average body weight of groups, 236 [19] g) and averaging 12 weeks of age were obtained from the Laboratory Animal Production Unit of Gaziantep University (Gaziantep, Turkey). They were kept in an environment with controlled temperature (21 [2]°C), humidity

(55%–60%), and photoperiod (12:12-hour light–dark cycle) for 1 week before the start of the experiment. A commercial balanced diet (Gaziantep Yem Sanayi, Gaziantep, Turkey) and tap water were provided ad libitum. Experimental procedures were approved by Mustafa Kemal University's Local Ethics Committee for the use and care of laboratory animals.

The rats were divided into 7 groups to be equal to the average body weights (6 animals in each group). In the control group ( $n = 6$ ), saline was given to the rats intraperitoneally and into the urinary bladder via urethral catheterization at once; they were euthanized 24 hours later. In the PYN groups ( $n = 18$ ), bacterial inoculation was introduced into the bladder of the rats at 24 hours after intraperitoneal sterile saline injection. The rats were followed at 24 (PYN-24,  $n = 6$ ), 48 (PYN-48,  $n = 6$ ), and 72 (PYN-72,  $n = 6$ ) hours in terms of PYN development. Sterile saline injection was repeated each day. The rats were euthanized at the end of follow-up. In the TQ-PYN groups ( $n = 18$ ), bacterial inoculation was introduced into the bladder of the rats 24 hours after the intraperitoneal TQ injection. The rats were followed at 24 (TQ-PYN-24,  $n = 6$ ), 48 (TQ-PYN-48,  $n = 6$ ), and 72 (TQ-PYN-72,  $n = 6$ ) hours in terms of PYN development. TQ injection was repeated each day. The rats were euthanized at the end of follow-up.

The rats were anesthetized with ether. The urethra was catheterized with a sterile 22-gauge angiocatheter sheath and the bladder was emptied, and 0.4 mL of bacterial solution ( $1 \times 10^9$  colony forming units, all of the groups except the control group) or sterile saline solution (control group) was instilled into the bladder via urethra. The angiocatheter was removed and the urethra was occluded with collodium. The collodium was removed by acetone 4 hours after the bacterial inoculation. TQ injections were performed at a dose of 10 mg/kg/d body weight intraperitoneally in the TQ-PYN groups.<sup>18</sup> Under ether anesthesia, animals were euthanized by intracardiac puncture for blood sampling at the indicated times (24, 48, and 72 hours) after bacterial inoculation. Both kidneys were removed aseptically, and one of them was stored in a 10% formalin solution for blind histopathologic evaluation, whereas the other was stored ( $-30^\circ\text{C}$ ) for blind biochemical analysis.

#### BACTERIAL STRAIN

*E. coli* strain ATCC 25922 was used to induce PYN. This *E. coli* strain was grown in trypticase soy broth at  $37^\circ\text{C}$  overnight. Organisms were harvested and diluted to a final concentration of approximately  $10^{10}$  organisms per milliliter in  $1 \times$  phosphate buffered saline as determined by optical density at 600 nm in accordance with the method of Bennett et al.<sup>10</sup> The solution containing  $10^{10}$  bacteria/mL was diluted 4 times in sterile saline solution. Bacterial suspensions were administered to the rats on the day they were prepared. The rats were anesthetized with ether. The urethra was catheterized with a sterile 22-gauge angiocatheter sheath and the bladder was emptied, and 0.4 mL bacterial solution ( $1 \times 10^9$  colony forming units) or sterile saline solution was instilled. The angiocatheter was removed and the urethra was

occluded with collodium. The collodium was removed by acetone 4 hours after bacterial inoculation.

#### **TISSUE HOMOGENIZATION AND PROTEIN DETERMINATION**

The kidney tissue samples were stored at  $-30^{\circ}\text{C}$  until assayed for kidney tissue malondialdehyde (MDA) levels, and catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activities. The kidney tissues were homogenized (for 2 minutes at 5000 rpm) in 4 volumes of ice-cold Tris-hydrogen chloride buffer (50 mmol, pH 7.4) using a glass Teflon homogenizer (Ultra Turrax IKA T10 Basic, Staufen, Germany). MDA and protein levels were measured at homogenate. The homogenate was then centrifuged at 5000g for 60 minutes to remove debris. The supernatant fluid was collected, analyses of CAT and GSH-Px activities were conducted, and protein concentration was measured. The supernatant solutions were used for the assay, mixed with an equal volume of an ethanol/chloroform (5/3, volume per volume [v/v]). After centrifugation at 5000g for 30 minutes, the clear upper layer (the ethanol phase) was collected and used in the analysis of SOD activity and protein assays. All preparation procedures were carried out at  $+4^{\circ}\text{C}$ .

#### **DETERMINATION OF MALONDIALDEHYDE LEVELS**

The thiobarbituric acid reactive (TBA) substances level was determined by a method based on the reaction with TBA at  $90^{\circ}\text{C}$  to  $100^{\circ}\text{C}$ .<sup>19</sup> In the TBA test reaction, MDA or MDA-like substances (ie, the byproduct of the lipid peroxidation process of the polyunsaturated fatty acid) and TBA reacted together, producing a pink pigment with an absorption maximum of 532 nm. The reaction was performed at pH 2 to 3 at  $90^{\circ}\text{C}$  for 15 minutes. The sample was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 minutes. After cooling, the absorbance was read at 532 nm (Ultra Spec Plus, Pharmacia LKB Biochrom Ltd, Cambridge, United Kingdom). The results were expressed as nanomoles per gram wet tissue by reference to a standard curve prepared from measurements made with a standard solution (1,1,3,3-tetramethoxypropane).

#### **DETERMINATION OF CATALASE ACTIVITY**

CAT (Enzyme Commission Number [EC] 1.11.1.6) activity was measured according to the method of Aebi.<sup>20</sup> The principle of the assay is based on the determination of the rate constant  $k$  (dimension:  $\text{s}^{-1}$ ,  $k$ ) of hydrogen peroxide decomposition. By measuring the absorbance changes per minute, the rate constant of the enzyme was determined. Activities were expressed as  $k$  (rate constant) per gram of protein.

#### **DETERMINATION OF GLUTATHIONE PEROXIDASE ACTIVITY**

GSH-Px (EC 1.6.4.2) activity was measured using the method of Paglia and Valentine.<sup>21</sup> The enzymatic reaction was initiated by the addition of hydrogen peroxide to the reaction mixture containing GSH, nicotinamide adenosine dinucleotide phosphate, and glutathione reductase. The change in the absorbance at 340 nm

was monitored by a spectrophotometer. The activity was expressed as units per gram protein.

#### **DETERMINATION OF PROTEIN LEVEL**

Protein measurements were performed in the tissue homogenate in accordance with the method of Lowry et al.<sup>22</sup>

#### **DETERMINATION OF SUPEROXIDE DISMUTASE ACTIVITY**

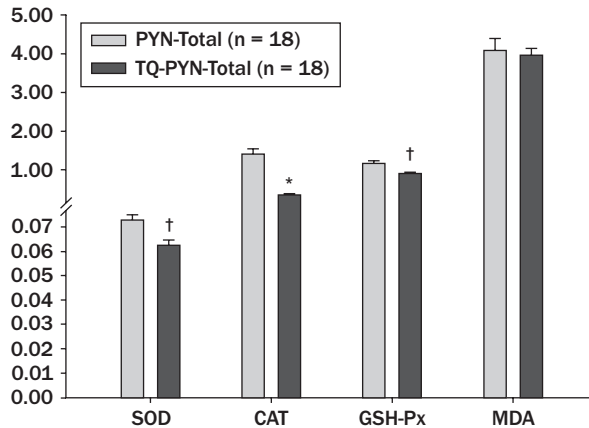
The principle of the total SOD (EC 1.15.1.1) activity method is based, briefly, on the inhibition of nitroblue tetrazolium reduction by  $O_2^-$  generated by a xanthine/xanthine oxidase system.<sup>23</sup> Activity was assessed in the ethanol phase of the serum after 1.0 mL of an ethanol/chloroform mixture (5/3, v/v) was added to the same volume of the serum and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the nitroblue tetrazolium reduction rate. The SOD activity was expressed as unit per gram protein.

#### **HISTOLOGIC EXAMINATION**

For light microscopic evaluation, renal samples were fixed in 10% neutral buffered formalin and dehydrated in a graded alcohol series from 70% to 100%. The tissue samples were then embedded in paraffin. Sections of 4 to 6  $\mu\text{m}$  were prepared from paraffin blocks using a rotary microtome. These sections were then stained with hematoxylin and eosin and photographed using an Olympus CX-41 photomicroscope (Olympus Corporation, Tokyo, Japan) powered with an Olympus DP20 digital camera (Olympus Corporation, Tokyo, Japan). All sections of the kidney samples were examined by a histologist blinded to the group in which the rats were randomized for characteristic histologic changes, including tubular epithelial alterations (vacuolization, atrophy, and cellular desquamation) and glomerular pathology (collapse, edema). To obtain a histologic score, histopathologic changes were graded according to cortical involvement and the severity of the lesions, modified from Karahan et al.<sup>24</sup> and Celik et al.<sup>25</sup>

#### **STATISTICAL ANALYSIS**

Biochemical data were analyzed using a commercially available statistics software package (SPSS for Windows 15.0; SPSS Inc, Chicago, Illinois). Distribution of the groups was analyzed using 1-sample Kolmogorov-Smirnov test. The groups showed normal distributions for the level of MDA and the activities of CAT, SOD, and GSH-Px, so parametric statistical methods were used to analyze the data. A 1-way ANOVA test was performed, and post hoc multiple comparisons were made using least-squares differences. The results are presented as mean (SEM);  $P < 0.05$  was considered statistically significant. One-way ANOVA and post hoc multiple comparisons by least-square difference test were used to analyze the histopathologic changes.



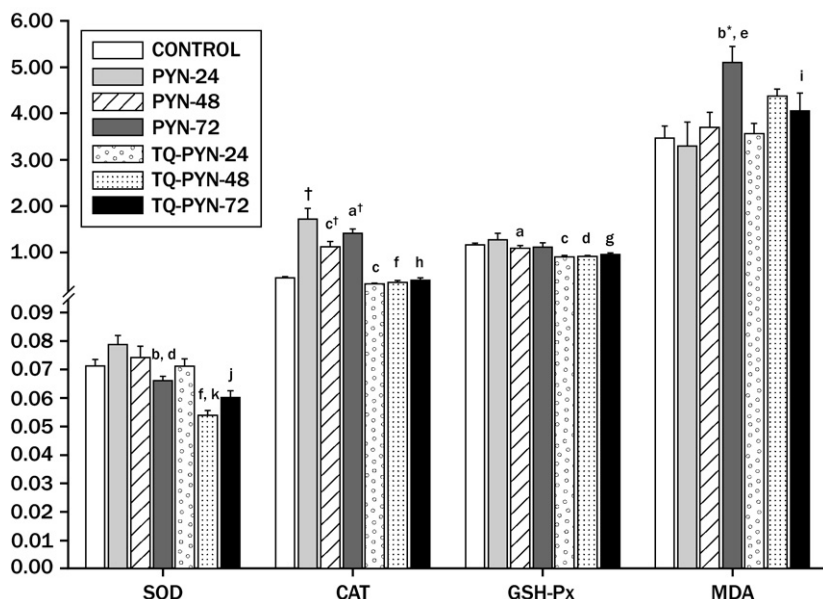
**Figure 1.** Malondialdehyde (MDA) levels and superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activity in kidney homogenates. Results are presented as mean (SEM). Student *t* test was used to test for differences between groups. \* $P < 0.01$ ; † $P < 0.001$  compared with pyelonephritis (PYN) total group. TQ = thymoquinone.

## RESULTS

When the total PYN group (24 + 48 + 72 hours) was compared with the total TQ-PYN group (24 + 48 + 72 hours), serum SOD, CAT, and GSH-Px activities were statistically lower in the TQ-PYN group than in the total PYN group ( $P < 0.001$ ,  $P = 0.002$ , and  $P < 0.001$ , respectively). However, serum MDA levels were not significant ( $P > 0.05$ ) (Figure 1).

In addition, control, PYN, and TQ-PYN groups were compared for aspects of serum SOD, CAT, GSH-Px activity, and MDA levels. There was no statistical difference between control and PYN groups in terms of SOD activity ( $P > 0.05$ ). However, SOD activity was statistically lower in the TQ-PYN-48 and -72 groups than the PYN-48 and -72 groups ( $P < 0.001$ ,  $P = 0.004$ , respectively). CAT activity was significantly higher in the PYN-24, -48, and -72 groups than in the control group ( $P < 0.001$ ). However, there was no significant increase in the TQ groups ( $P > 0.05$ ). GSH-Px activity was not statistically different between the control group and PYN groups. However, there was a significant difference between TQ-PYN-24, -48, and -72 groups and PYN groups in terms of GSH-Px activity ( $P < 0.001$ ,  $P = 0.026$ , and  $P = 0.046$ , respectively). MDA levels were statistically higher in the PYN-72 group than in the control group ( $P = 0.002$ ). When the TQ-PYN-72 group was compared with the PYN-72 group, MDA levels were significantly lower in the TQ-PYN-72 group than the PYN-72 group ( $P = 0.033$ ) (Figure 2).

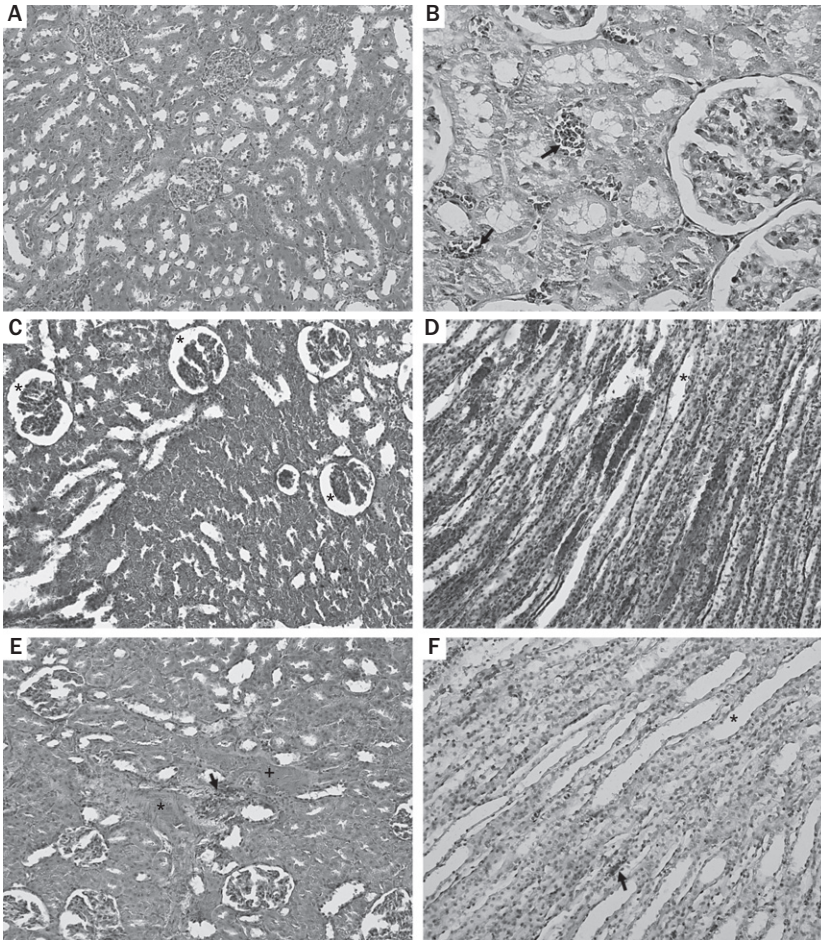
The control group showed normal renal architecture in the histologic examination (Figure 3A), but there were neutrophil casts in the tubuli and signs of tubular degeneration such as vacuolization in the tubular epithelium in group 2 (Figure 3B). PYN caused diffuse damage in the glomeruli (Figure 3C) and the



**Figure 2.** Malondialdehyde (MDA) levels and superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activity in kidney homogenates. Results are presented as means (SEM). One-way ANOVA and post hoc by least-square difference was used to test for differences between groups. \* $P < 0.01$ ; † $P < 0.001$  compared with control group. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$  compared with pyelonephritis (PYN)-24 group. <sup>d</sup> $P < 0.05$ , <sup>e</sup> $P < 0.01$ , <sup>f</sup> $P < 0.001$  compared with PYN-48 group. <sup>g</sup> $P < 0.05$ , <sup>h</sup> $P < 0.001$  compared with PYN-72 group. <sup>i</sup> $P < 0.05$ , <sup>j</sup> $P < 0.01$ , <sup>k</sup> $P < 0.001$  compared with thymoquinone (TQ)-PYN-24 group.

medullar region in 48 hours. Extensive vascular congestion, tubular degeneration, and dilatation were noticed in the medulla (Figure 3D). The most severe findings were noted in group 4. Hyalinization with fibrosis occurred in the cortical area (Figure 3E). TQ application reversed the damage in renal tissues, to some extent, in the TQ groups. Cortical lesions, such as glomerular shrinkage, cellular casts, or vacuolization in the tubular epithelium, were fewer in these groups (data not shown). Microscopic examination also showed mild tubular degeneration, dilatation, and hemorrhage in the medullar region (Figure 3F). In statistical analysis of histopathologic findings, there were significant differences between PYN-24 and TQ-24, PYN-48 and TQ-48, and PYN-72 and TQ-72 groups ( $P = 0.008$ ,  $P < 0.001$ , and  $P < 0.001$ , respectively). In addition, when we combined all PYN subgroups into one PYN group and all TQ subgroups into one TQ group and compared the two, we obtained a significant difference between the two groups, as shown in Table ( $P < 0.001$ ).





**Figure 3.** (A) Normal glomerular and tubular structure (hematoxylin and eosin [HE]  $\times 100$ ). (B) Neutrophil casts (arrow) (HE  $\times 400$ ). (C) Diffuse glomerular shrinkage (asterisk) (HE  $\times 200$ ). (D) Extensive vascular congestion, tubular degeneration, and dilatation (asterisk) (HE  $\times 200$ ). (E) Interstitial fibrosis (asterisk), cellular casts (arrow), hyalinization (plus sign), and degenerated glomeruli (HE  $\times 200$ ). (F) Mild tubular degeneration, dilatation (asterisk), and hemorrhage (arrow) (HE  $\times 200$ ).

## DISCUSSION

Infection of the kidney is one of the most common serious conditions in humans, particularly in childhood. *E. coli* may often cause cystitis or PYN. The activities of CAT, SOD, and GSH-Px, and lipid peroxidation in *E. coli*-induced PYN were evaluated in an experimental rat model.<sup>26</sup>



**Table. Grading levels of inflammation in pyelonephritis (PYN)- and thymoquinone (TQ)-treated groups as determined by histopathologic examination.**

Grade	Histopathologic Changes
Grade 0	No pathologic changes
Grade I	Slight degenerative changes in tubuli and glomeruli with cortical involvement <25%
Grade II	Mild degenerative changes with cortical involvement of 25%–50%
Grade III	Tubular and glomerular necrosis at different foci throughout the cortex with cortical involvement of 50%–75%
Grade IV	Extensive and marked necrosis throughout the cortex with cortical involvement >75%

Between Group Comparisons	P
Control vs PYN-24	<0.001
Control vs PYN-48	<0.001
Control vs PYN-72	<0.001
PYN-24 vs TQ-PYN-24	0.008
PYN-48 vs TQ-PYN-48	<0.001
PYN-72 vs TQ-PYN-72	<0.001
PYN total vs TQ-PYN total)	<0.001

One-way ANOVA and post hoc test by least-square difference test was used to analyze the histopathologic changes.

Some studies demonstrated that TQ shows a potent superoxide radical scavenger effect, with scavenging power being as effective as SOD against superoxide. Badary et al<sup>27</sup> evaluated the antioxidant and pro-oxidant effects of TQ in vitro. Their results showed that TQ is a very active superoxide anion scavenging agent. Nagi et al<sup>28</sup> reported a protective effect of TQ with respect to doxorubicin-induced cardiotoxicity in rats. Their results suggested that the peroral administration of TQ provided potent superoxide radical scavenger activity. In this study, there were no increases in the SOD and GSH-Px enzyme activity when the control group was compared with the PYN groups, whereas CAT activity for the PYN groups was significantly higher than that for the control group. Significant attenuates in antioxidant enzyme activities were observed in the TQ groups versus the control and PYN groups. It was thought that these attenuated antioxidant enzyme activities might be the consequence of the direct scavenger activity of TQ in kidney tissue with *E. coli*-induced PYN.

Another parameter that has been used widely in demonstrating oxidative injury is MDA,<sup>29</sup> a product of lipid peroxidation. In a study that carried out isolated rat kidney homogenates, Celik et al<sup>25</sup> reported that *E. coli*-induced PYN caused oxidative injury. In the present study, there was a statistically significant rise in MDA activity in rat renal tissue with PYN. This increase in MDA levels demonstrated that *E. coli*-induced PYN led to lipid peroxidation followed naturally by oxidative injury in

the renal tissues. According to early studies, TQ decreased the level of lipid peroxidation products such as MDA. Abdelmeguid et al<sup>30</sup> observed a significant decrease in the levels of MDA in the TQ groups in rats made diabetic with streptozotocin.

Treatment with TQ showed a therapeutic and protective effect by attenuating oxidative stress. Fouda et al<sup>31</sup> reported that the maximal protection offered by TQ treatment was particularly noticeable 48 and 72 hours after administration of the toxic agent at the time when histologic damage, apoptotic events, and proliferative reactions reached their maximum. There was a similar result in our study. MDA levels significantly attenuated in TQ-72 hour treatment versus the PYN-72 hour group. This result suggests that TQ might be used in *E. coli*-induced PYN. In addition, the significant attenuate in the MDA level in the TQ-72 group showed that TQ might affect the MDA levels over a longer period, such as 48 to 72 hours.

In the present study, a histologic examination also confirmed the protective effect of TQ in kidney tissue with *E. coli*-induced PYN. Microscopic examination revealed a better renal histology in the TQ-administered group. Complete improvement was not seen, but both cortical and medullary lesions were reversed with TQ application. Fewer hemorrhagic areas and inflammatory cells and better glomerular and tubular architecture were also observed. The protective effect of TQ has been shown in various pathologic conditions. For example, Fouda et al<sup>31</sup> demonstrated that treatment of rats with TQ reduced the number of superoxide-positive cells by 70% and offered imperative protection from mercury chloride-induced renal damage. Kanter et al<sup>32</sup> showed that TQ therapy caused renal morphologic and functional improvement after streptozotocin-induced diabetes in rats. Also, Sayed-Ahmet et al<sup>33</sup> reported that peroral TQ supplementation completely prevented an increase in nephrotoxicity indexes and histopathologic lesions induced by gentamicin. Although various toxic or injuring agents were used in those studies, TQ provided a significant improvement in renal lesions.

#### STUDY LIMITATIONS

There are some limitations to the present study. First, we did not measure plasma levels of lipid peroxidation. In the present study, our aim was to investigate oxidative injury in tissue and histopathologic evaluation. However, we did not use different TQ doses and longer duration of the drug. Also, we did not study other parameters, such as the nitrogen oxide, carbonyl, and sulfidryl group, and 8-hydroxy guanosine in terms of oxidative injury. These parameters were important for understanding the efficacy of the drugs on oxidative injury.

#### CONCLUSION

To our knowledge, this is the first report demonstrating a protective effect of TQ against *E. coli*-induced renal oxidative damage in a rat model of lower tract obstruction based on histopathologic and biochemical findings. This protective effect could be crucial in adult patients with diabetes and urinary incontinence and pediatric patients with recurrent urinary tract infections, because PYN may cause permanent renal damage in these patients.

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The authors have indicated that they have no conflicts of interest regarding the content of this article.

Dr. Evirgen was responsible for the general organization and writing. Dr. Gökçe was responsible for the study design and writing. Dr. Hasan Ozturk was responsible for the Biochemical analysis. Mr. Nacar was responsible for the histological study, Dr. Onlen was responsible for the literature search and hypothesis formation. Drs. Ozer and Koksaldi Motoer were responsible for the data collection and analysis.

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