Protective Effect of *Nigella Sativa* on Renal Reperfusion Injury in Rat

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**Introduction.** This study was designed to investigate the effect of *Nigella sativa* (NS), in reperfusion-induced renal injury in rats.

**Materials and Methods.** A total of 24 male Sprague-Dawley rats were divided into 3 groups of controls and rats that underwent ischemia-reperfusion with and without pretreatment with NS. A rat model of renal reperfusion injury was induced by 45-minute occlusion of the bilateral renal pedicles and 24-hour reperfusion. In the NS group, a single dose NS (400 mg/kg orally) was administered by gastric gavage.

**Results.** Renal reperfusion caused severe histopathological injury such as tubular damage, atrophy dilatation, loss of brush border, and hydropic epithelial cell degenerations. Treatment with NS significantly attenuated the severity of reperfusion injury and significantly lowered tubulointerstitial damage score as compared with the reperfusion group. When kidney sections were stained with anti-proliferating-cell nuclear antigen antibody, nuclear factor kappaB p65 antibody, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, there was a clear increase in the number of positive cells in the reperfusion group in the renal cortical tissues. However, there was a significant reduction in the number of stain-positive cells in kidney tissue from the NS group. Treatment of renal reperfusion injury with NS decreased the elevated tissue malondialdehyde levels and increased the reduced activities of the enzymatic antioxidants glutathione peroxidase and catalase.

**Conclusions.** Pretreatment with NS has a protective effect against renal damage induced by renal reperfusion. This protective effect is possibly due to its ability to inhibit reperfusion-induced renal damage, apoptosis, and cell proliferation.

**INTRODUCTION**

Reperfusion injury in the kidney is a complex pathophysiologic process that occurs in the context of cardiac arrest with recovery, transplantation, heminephrectomy, and vascular surgery, which is a common cause of renal cell death, kidney failure, delayed graft function, and kidney allograft rejection.¹² The mechanisms underlying reperfusion injury to the kidneys are likely multifactorial and interdependent, involving hypoxia, free radical damage, and inflammatory responses.³ Ischemic injury to the kidneys leads to acute kidney failure (AKF), associated with high mortality.⁴ Acute kidney failure produced by ischemia and reflow is a clinical and experimental syndrome characterized by major reductions in glomerular filtration rate, extensive tubular damage, tubular cell necrosis, glomerular injury, and signs...
of tubular obstruction with cell debris. Although many factors are involved in ischemia-reperfusion-induced kidney damage, reactive oxygen species (ROS) play a major role in the pathogenesis of reperfusion injury. Reactive oxygen species are capable of reacting with proteins, lipids, and nucleic acids, leading to lipid peroxidation in biological membranes, which in turn impacts enzymatic processes such as ion pump activity and damages DNA, thereby inhibiting transcription and repair.

Mounting evidence now indicates that apoptosis is the major mechanism of early tubule cell death in contemporary clinical AKF. During recent years, several animal models of ischemic acute kidney injury consistently and unequivocally demonstrated the presence of apoptotic tubule cells using a variety of sensitive assays. Importantly, this now has been confirmed by several investigators in human models of acute kidney injury. Nevertheless, controversies still exist regarding the contribution of apoptosis to the syndrome of AKF.

Many studies of plants and drugs illustrate their protective antioxidant and anti-inflammatory effects on reperfusion injury. For example *Urtica dioica*, ginger, and garlic juice have a protective effect against kidney damage induced by renal ischemia-reperfusion. The black seed, *Nigella sativa* (NS), from the family of Ranunculaceae has been shown to contain more than 30% of fixed oil and 0.4% to 0.45% wt/wt of volatile oil. The volatile oil has been shown to contain 18.4% to 24% thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) and 46% monoterpenes such as \(\alpha\)-cymene and \(\alpha\)-piene. Recently, clinical and animal studies have shown that the extracts of the black seeds have many therapeutic effects such as bronchodilative, immunomodilative, antibacterial, hypotensive, antidiabetic, hepatoprotective, gastroprotective, antihistaminic, antioxidative, and neuroprotective effects. In spite of these studies, there is little available information on the effect of NS on renal reperfusion injury. The aim of this study was, therefore, to investigate the effect of NS on reperfusion-induced renal injury in rats.

**MATERIALS AND METHODS**

**Animals**

Thirty-two healthy male Sprague-Dawley rats, weighing 250 g to 300 g, averaging 10 to 12 weeks old were utilized in this study. The animals were purchased from Trakya University Animal Care and Research Unit. The rats were fed on a standard rat chow and tap water ad libitum. In the windowless animal quarter automatic temperature (22 ± 2°C) and lighting controls was performed (light on at 7:00 AM and off at 9:00 PM; 14-hour-light-10-hour-dark cycle). Humidity ranged from 50% to 55%. All animals received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health. The study was approved by the Institutional Animal Ethical Committee of Trakya University, Edirne, Turkey.

**Plant Materials and Extraction Procedure**

The NS seeds were purchased from a local herb store in Eskisehir, Turkey. Sample specimens have been kept at the Plant, Drug and Scientific Researches Center, Anatolian University Faculty of Pharmacy, Eskisehir, Turkey, for future reference. The seeds of NS were powdered in a mixer. Twenty grams of the powdered seeds was added to 400 mL of distilled water, and the extraction was carried out by steam distillation. The distillation process was continued until about 200 mL of distillate was collected. The distillate was extracted 3 times with chloroform. Moisture was removed by anhydrous sodium sulphate and the resultant extract was evaporated using a 40°C water bath, leading to the appearance of the volatile oil. Five hundred milligrams of the volatile oil was dissolved in 1 mL of dimethyl sulfoxide; then, 9 mL of normal saline was added to yield a concentration of 50 mg of volatile oil per 1 mL of the solution.

**Experimental Groups**

The rats were randomly allotted into one of the three experimental groups: control, reperfusion, and reperfusion plus NS; each group contained 8 animals. The animals in the third group were pretreated with NS orally (400 mg/kg) with a single dose, 3 days before reperfusion as described for the reperfusion groups.

**Technique of Ischemia-Reperfusion**

Rats in the reperfusion and NS groups were anaesthetized with intramuscular injection of xylazine, 10 mg/kg, and ketamine, 50 mg/kg. The abdominal region was shaved and cleansed.
with povidone iodine. Laparotomy was performed as midline incision. Room temperature was 25°C during the procedure and the body temperature of the rats were about 35°C to 37°C throughout the surgery. They were heated by light and warm pads. A midline incision was made and ischemia was induced by bilateral renal pedicle clamping for 45 minutes with smooth vascular clamps under sterile conditions. After the clamps were removed, the kidney was inspected for restoration of blood flow. The abdomen was then closed in 2 layers and the animals were allowed to recover. Twenty-four hours after reperfusion the animals were re-anaesthetized with xylazine, 10 mg/kg, and ketamine, 50 mg/kg, and the abdominal wall was reopened. After bilateral nephrectomies were carried out, the right and left kidneys were stored in Bouin solution for histological examination.

**Histopathologic Evaluation**

The renal tissues were individually immersed in Bouin fixative, dehydrated in alcohol, and embedded in paraffin. Sections of 5-μm thickness were obtained, deparaffinized, and stained with hematoxylin-eosin. The renal tissue was examined and evaluated in random order under blindfold conditions with standard light microscopy. Tubulointerstitial injury was defined as tubular atrophy, dilatation, loss of brush border, cellular infiltration, and widening of the interstitium. The degree of glomerular and tubulointerstitial damage in the cortex was determined using a semiquantitative graded scale, where zero was no abnormality; 1, minimal damage (involvement of < 25% of cortex); 2, mild damage (involvement of 25% to 50% of cortex); 3, moderate damage (involvement of 50% to 75% of cortex), and 4, severe damage (involvement of > 75% of cortex). The glomerular matrix was semiquantified in 50 randomly selected glomeruli. Glomerular injury scores and tubulointerstitial damage were generated using the following formula:

\[
(0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4) / 50
\]

These analyses were performed in 2 sections from each animal at ×400 magnification in at least 10 different regions for each section.

**Immunohistochemistry**

Immunohistochemical reactions were performed according to the ABC technique described by Hsu and coworkers. The procedure involved the following steps: (1) endogenous peroxidase activity was inhibited by 3% hydrogen peroxide in distilled water for 30 minutes; (2) the sections were washed in distilled water for 10 minutes; (3) nonspecific binding of antibodies was blocked by incubation with normal goat serum (DAKO X 0907, Carpinteria, CA) with PBS, diluted 1:4; (4) the sections were incubated with specific mouse monoclonal anti-proliferating-cell nuclear antigen (PCNA) antibody (Cat No MS-106-B, Thermo LabVision, USA) and specific rabbit polyclonal nuclear factor kappaB (NFkB) p65 antibody (ChIP Grade [ab7970], USA), diluted 1:50 for 1 hour, and then at room temperature; (5) the sections were washed in phosphate-buffered saline 3 × 3 minutes; (6) the sections were incubated with biotinylated anti-mouse immunoglobulin G (DAKO LSAB 2 Kit); (7) the sections were washed in phosphate-buffered saline 3×3 min; (8) the sections were incubated with ABC complex (DAKO LSAB 2 Kit); (9) the sections were washed in phosphate-buffered saline 3 × 3 minutes; (10) peroxidase was detected with an aminoethylcarbazole substrate kit (AEC kit; Zymed Laboratories); (11) the sections were washed in tap water for 10 minutes and then dehydrated; (12) the nuclei were stained with hematoxylin; and (13) the sections were mounted in DAKO paramount.

**TUNEL Assay**

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method, which detects fragmentation of DNA in the nucleus during apoptotic cell death in situ, was employed using an apoptosis detection kit (TdT-Fragel DNA Fragmentation Detection Kit, Cat No QIA33, Calbiochem, USA). All reagents listed below are from the kit and were prepared following the manufacturer’s instructions. Five-millimeter-thick renal sections were deparaffinized in xylene and rehydrated through a graded ethanol series as described previously. They were then incubated with 20 mg/mL proteinase K for 20 minutes and rinsed in tris-buffered saline. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. Sections were then incubated with equilibration buffer for 10 to 30 minutes and then TdT-enzyme, in a humidified atmosphere at 37°C, for 90 minutes. They were subsequently
put into pre-warmed working strength stop/wash buffer at room temperature for 10 minutes and incubated with blocking buffer for 30 minutes. Each step was separated by thorough washes in tris-buffered saline. Labeling was revealed using diaminobenzidine tetrahydrochloride, counter staining was performed using methyl green, and sections were dehydrated, cleared, and mounted.

Proliferating-cell nuclear antigen, NFkB p65, and TUNEL staining was scored in a semiquantitative manner in order to determine the numbers of the positive staining were also recorded as absent (–), a few (±), few (+), medium (++), high (+++), and very high (++++). These analyses were performed in 2 sections from each animal at × 400 magnification in at least 10 different regions for each section.

Biochemical Analyses
The malondialdehyde content of homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances. Three milliliters of 1% phosphoric acid and 1 mL of 0.6% thiobarbituric acid solution were added to 0.5 mL of plasma pipetted into a tube. The mixture was heated in boiling water for 45 minutes. After cooling, the color was extracted into 4 mL of n-butanol. The absorbance was measured in spectrophotometer (Shimadzu UV-1601, Japan) with 532 nm. The amounts of lipid peroxides were calculated as thiobarbituric acid reactive substances of lipid peroxidation. The results were expressed as nanomole per gram wet tissue (nmol/g wet tissue) according to a standard graph which was prepared from the measurements done with a standard solution (1,1,3,3-tetramethoxypropane). Protein measurements were made at all stages according to the Lowry method.

Glutathione peroxidase activity was measured by the method of Paglia and Valentine. The enzymatic reaction in the tube, which is containing following items: nicotinamide adenine dinucleotide phosphate, reduced glutathione (GSH), sodium azide, and glutathione reductase, was initiated by addition of hydrogen peroxide and the change in absorbance at 340 nm was monitored by a spectrophotometer.

Catalase activity was determined according to Aebi method, which is based on the determination of the rate (s^-1; k) of hydrogen peroxide decomposition at 240 nm.

Statistical Analysis
All statistical analyses were carried out using the SPSS software (Statistical Package for the Social Sciences, version 11.0, SPSS Inc, Chicago, IL, USA). All continuous variables were presented as mean ± standard deviations. Differences in measured parameters among the three groups were analyzed with the nonparametric Kruskal-Wallis test. Dual comparisons between groups exhibiting significant values were evaluated with a Mann-Whitney U test. These differences were considered significant when P value was less than .05.

RESULTS
Histopathologic Findings
Normal structure of the renal cortical tissues was observed in the control rats (Figure 1, Left). Histology indicated the kidneys from the
reperfusion group had severe tubular damage in some proximal and distal tubules, as evidenced by widespread tubular atrophy, dilatation, loss of brush border, and hydropic epithelial cell degenerations. In addition, there were renal corpuscle atrophy, glomerular shrinkage, markedly focal mononuclear cell infiltrations, and widening of the interstitium in the renal cortical tissues at 24-hour reperfusion (Figure 1, Middle). In the NS group, the severity of degenerative changes in the tubules and glomerules of renal cortex were less than in the reperfusion group. Tubular atrophy, dilatation, loss of brush border, and hydropic epithelial cell degenerations decreased (Figure 1, Right).

Table 1 shows that there was a significantly higher renal tubulointerstitial damage and glomerular injury score in the reperfusion group than the control group. The NS group had a significantly lower tubulointerstitial damage score than the reperfusion group.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Control Group</th>
<th>Reperfusion Group</th>
<th>Nigella Sativa Group</th>
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</thead>
<tbody>
<tr>
<td>Tubulointerstitial damage</td>
<td>0.10 ± 0.01</td>
<td>3.28 ± 0.16†</td>
<td>1.59 ± 0.09‡</td>
</tr>
<tr>
<td>Glomerular injury score</td>
<td>0.11 ± 0.02</td>
<td>1.60 ± 0.05†</td>
<td>0.90 ± 0.03‡</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± standard deviation.
†P < .001 compared to the control groups
‡P < .01 compared to the reperfusion group

Immunohistochemical Findings

The PCNA-positive cells were weakly detected in the renal cortical tissues of the control rats (Figure 2, Left). However, the signal density of positive cells was significantly higher in the reperfusion group (Figure 2, Middle). Treatment of NS markedly decreased the positivity of PCNA in the renal cortical tissues (Figure 2 Right; Table 2).

The NFkB p65-positivity was weakly detected in the cytoplasm of renal tubule epithelial cells of the control rats (Figure 3, Left). However, the signal density of positive cells was significantly higher in the reperfusion group (Figure 3, Middle). Treatment of NS markedly decreased the positivity of NFkB p65 in the renal cortical tissues (Figure 3, Right; Table 2).

TUNEL Findings

The number of TUNEL-positive cells in the control group was negligible (Figure 4, Left). When kidney sections were TUNEL stained, there was a clear increase in the number of positive cells in the reperfusion group in the renal cortical tissues.
Treatment of NS markedly reduced the reactivity and the number of TUNEL-positive cells (Figure 4, Right; Table 2).

Biochemical Findings

In the reperfusion group, tissue catalase activity and GSH-peroxidase levels were significantly decreased and malondialdehyde levels were increased compared with the control group. Treatment with NS led to significant increases in the mean tissue catalase activity and GSH-peroxidase levels. Moreover, NS significantly decreased malondialdehyde levels, which have been induced by ischemia-reperfusion. The values of the tissue malondialdehyde and GSH-peroxidase levels with catalase activities are shown in Table 3.

DISCUSSION

Reperfusion injury in the kidney is a complex pathophysiologic process that occurs in the context of cardiac arrest with recovery, transplantation, heminephrectomy, and vascular surgery, which is a common cause of renal cell death, kidney failure, delayed graft function, and kidney allograft dysfunction.
The mechanisms underlying reperfusion damage to the kidneys are likely multifactorial and interdependent, including hypoxia, free radical damage, and inflammatory responses.

The AKF produced by ischemia and reflow is a clinical and experimental syndrome characterized by major reductions in glomerular filtration rate, extensive tubular damage, tubular cell necrosis, glomerular injury, and signs of tubular obstruction with cell debris. Acute kidney failure due to ischemia is a complex syndrome involving renal vasoconstriction, extensive tubular damage, tubular cell necrosis, glomerular filtration failure, and glomerular injury. Studies in animals have revealed a number of factors that could contribute to the injury associated with ischemic ARF. In the present study, we found better histopathological structure in the kidney of the rats with reperfusion that received NS as prophylaxis compared to the reperfusion only group. In the reperfusion group, the kidneys showed tubular atrophy, dilatation, loss of brush border, and hydropic epithelial cell degenerations. In addition, there were renal corpuscle atrophy, glomerular shrinkage, markedly focal mononuclear cell infiltrations, and widening of the interstitium in the renal cortical tissues at 24-hour reperfusion. In accordance with the previous plant studies, our results also support treatment with NS prevented the injury and showed normal glomeruli.

Proliferation was determined by immunohistochemical staining for the PCNA, which is expressed when cells are going into DNA synthesis. This PCNA is expressed when cells are going into DNA synthesis (cell division, DNA repair, and apoptosis). After an ischemic insult, damaged cells are desquamated, after which the dedifferentiated proximal tubular cells proliferate and migrate into denuded areas of the basement membrane to establish new epithelium. Pursuant to our results, PCNA-positive cells were strongly detected in the renal cortical tissues of the control rats. However, the signal density of positive cells was significantly higher in the reperfusion group. Treatment of NS markedly increased the reactivity of PCNA in the renal cortical tissues.

The nuclear factor NFκB pathway has long been considered a prototypical pro-inflammatory signaling pathway, largely based on the role of NFκB in the expression of pro-inflammatory genes including cytokines, chemokines, and adhesion molecules. It can be activated by a variety of pathogenic stimuli, including ischemia-reperfusion. A number of clinical and experimental studies have demonstrated that NFκB, a transcription factor, plays a central role in renal pathology. Activation of NF-κB was reported to occur during ischemia and reached its peak after 15 minutes of reperfusion. Pretreatment of rats with NFκB inhibitors not only prevented NFκB activation induced by reperfusion injury, but also inhibited monocyte chemoattractant protein 1 mRNA expression, suggesting that NFκB plays a major role in the initiation of inflammation in reperfusion injury. The transcription factor p65 is a member of the NFκB family, and most NFκB target genes are regulated by p65. Spandou and colleagues reported that renal reperfusion injury led to the activation of NFκB, which suggested that it might be a marker of injury linked to the pathophysiology of a variety of renal disorders, including reperfusion injury, and that its inhibition prevented in vivo cell apoptosis associated with renal the injury. Therefore, inhibition of the permanent activation of NFκB protects renal tissue from ischemic injury. In the present study, NFκB p65-positive cells were weakly detected in the renal cortical tissues of the control rats. However, the signal density of positive cells was significantly higher in the reperfusion group. Treatment of NS markedly increased the reactivity of NFκB p65 in the renal cortical tissues.

Recent studies demonstrated that tubular cell apoptosis is a primary and major contributor to the pathophysiology of renal reperfusion injury and determined the outcome of the renal damage. According to Kim and associates, the renal tubular epithelium is at least partially due to apoptotic cell death after renal reperfusion injury. Prevention of cell apoptosis has been demonstrated to reduce ischemic damage in many organ systems. The importance of apoptotic changes in ischemia-reperfusion models had been shown in the investigations of Kim and associates, who showed that in the reperfusion group, significantly higher numbers of apoptotic cells were found than in the normal controls, and the apoptotic cell count increased in the reperfusion group of mice. In our study, renal tubular epithelial cell apoptosis was quantified by the TUNEL assay. When kidney sections were TUNEL stained, there was a markedly increase in the number of positive
cells in the reperfusion group of rats in the renal cortical tissues. We demonstrated that preventing apoptosis appeared to be associated with the protective effect of NS on renal reperfusion injury. Most of the tubular and glomerular damages occur during the reperfusion phase following ischemia, and generation of ROS. Reactive oxygen species are capable of reacting with lipids, proteins and nucleic acids leading to lipid peroxidation, impairments of enzymatic processes and DNA damages. The accumulation of ROS and reduction in antioxidant enzyme activities lead to damage in cellular components such as lipids and proteins. Malondialdehyde frequently used to show the involvement of free radicals in cell damage is one of the final products of lipid peroxidation. In our study, malondialdehyde levels were significantly increased in the reperfusion group compared to the control group. Treatment with NS significantly inhibits malondialdehyde production, implying a reduction in lipid peroxidation and cellular injury that protects the kidney against reperfusion-induced oxidative damage.

The cell natural protective system against the devastating actions of ROS includes the protective enzymes superoxide dismutase, catalase, and the antioxidant molecule, GSH. Superoxide radicals formed by reperfusion injury are converted into hydrogen peroxide, either spontaneously (in pH 4.8) or by dismutation with the superoxide dismutase enzyme (especially, in neutral and alkaline pH). Hydrogen peroxide is then converted to water by either catalase or GSH-peroxidase. Several investigations have demonstrated the beneficial effects of antioxidants, which prevents ROS generation and ROS scavenging molecules in renal reperfusion injury. Our observation, the amelioration of catalase, GSH-peroxidase enzyme activities and malondialdehyde levels, finds that NS protects the kidney against reperfusion injury.

CONCLUSIONS

This study provides strong evidence that NS has a protective effect against proximal tubule damage after reperfusion injury in the rats’ kidney. Our results strongly suggest potential clinical benefits of NS in patients undergoing partial nephrectomy or kidney transplantation. However, further investigations are needed to establish the feasibility and efficacy of NS in clinical reperfusion settings.

CONFICT OF INTEREST

None declared.

REFERENCES


