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Effect of ground black seeds (*Nigella sativa* L.) on renal tubular cell apoptosis induced by ischemia/reperfusion injury in the rats

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ABSTRACT

Objective(s): The aim of this study was to evaluate the effects of ground black seeds on renal tubular cell apoptosis following ischemia/reperfusion (I/R) injury in rats.
Materials and Methods: Forty male Wistar rats were randomly allocated into 5 equal groups including Sham, I/R model and three I/R+ black seeds (5, 10 and 20%)-treated groups. I/R groups' kidneys were subjected to 60 min of ischemia followed by 24 h of reperfusion. Microscopically, apoptosis of tubular cells was assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) method.
Results: The apoptotic cells of renal tubules were increased significantly (P<0.01) in I/R group than those in sham operation group. The TUNEL positive cells in black seeds (10% and 20%) treatment groups decreased significantly (P<0.05).
Conclusion: Inhibition of apoptosis may be responsible for the protective effects of black seeds in rats with renal I/R injury.

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Introduction

Ischemia of kidney is a common problem during kidney transplantation, partial nephrectomy, cardiopulmonary bypass, or hydronephrosis leading to renal dysfunction and injury (1).

Reperfusion of renal ischemia initiates the complex cellular events that results in renal injury and the eventual death of renal cells due to necrosis and then apoptosis (2). Reactive oxygen species (ROS) are considered to be the principal components involved in the pathophysiological tissue alterations observed during renal ischemia/reperfusion (IR) (1).

ROS are involved in induction of apoptosis in renal cells and impairing renal function (3).

Antioxidant therapy has been well documented to help the improvement of organ functions and to prevent apoptosis (4). Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical disease. More attention has been paid to the protective effects of natural antioxidants. *Nigella sativa* L. from the Ranunculaceae family is commonly known as Black seed in traditional medicine. In many diseases, such as cirrhosis, hepatitis and chemical poisoning in which free radicals are produced; the antioxidant properties of *N. sativa* can be very useful (5). Also, previous studies demonstrated anti-apoptotic effects of *N. sativa* (6).

In this study, we aimed to investigate whether Black seeds can decrease renal tubular epithelial cell apoptosis-induced renal ischemia/reperfusion.

Materials and Methods

Chemicals: All chemicals used in this study were of analytical grade and obtained from Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

Animals: A total of forty male Wistar rats (about 180 to 200 g body weight) obtained from Pasteur Institute of Iran were selected for the study and were acclimatized to their environment for one week prior to experimentation. Animals randomly allocated into five groups of eight animals in each group, including Sham operation group, IR group and three I/R+ black seed (5, 10 and 20%)-treatment groups. Investigations using experimental animals were conducted in accordance with the internationally accepted principles for laboratory animal use and

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care as found in the United States guidelines (United States National Institutes for Health publication no. 85-23, revised in 1985), and also the protocol was approved by Ethical Committee on Animal Care of Islamic Azad University of Tabriz branch, Iran. The animals were housed under standard environmental conditions (23±1˚C, with 55±5% humidity and a 12 hr light/12 hr dark cycle) and maintained with free access to water and a standard laboratory diet *ad libitum*. Three weeks before the experiment, the diet of I/R+ black seed-treatment animals were supplemented with ground black seed (*N. sativa* L.) and these three groups of rats received different percentages (5, 10 and 20%) of ground black seed in their standard diet. The experiments and surgical procedures performed in the different groups of animals are described below. A previous study showed that supplementation of *N. sativa* up to the dose of 1 g/kg supplemented for a period of 28 days resulted no changes in liver enzymes level and did not cause any toxicity effect on the liver function (7).

Surgery and experimental design

Animals were anesthetized by intraperitoneal injection of ketamine-xylazine (50 mg/kg and 10 mg/kg, respectively). Surgery was carried out on an operating table that was equipped with automated electronic heater (Model THM-100, Indus Instruments, Houston, TX), and was set to maintain a core temperature of 37 °C to guard against hypothermia.

The abdominal area was prepared with povidone iodine. Autoclave-sterilized surgical instruments were used for the procedure. A laparotomy was performed with a vertical midline incision. Renal arteries were exposed by blunt dissection. Bilateral renal artery occlusion was carried out for 60 min using a nontraumatic vascular clamp to create complete renal ischemia (8). The clamp was removed later to allow restoration of blood flow to the kidney. Additionally, sham-operated rats underwent a simple laparotomy under identical conditions and served as the operation controls. After removing the clamp, the abdomen was closed in 2 layers and the animal was returned to its cage. In all groups, the animals were kept in the cages for 24 hr. After that, the rats were slaughtered by decapitation. Blood samples were obtained from abdominal aorta into dried tubes and maintained about 15 min in lab temperature and then centrifuged at 4˚C, 1,000 g for 15 min to collect sera. Serum samples were stored at 20˚C until analysis. After sacrificing the animals, the kidney was quickly harvested and a portion of the kidney was fixed in 10% neutral-buffered formalin solution for histopathologic evaluations.

Kidney function study: Serum creatinine (Scr), blood urea nitrogen (BUN), and uric acid levels, as renal functional parameters (9), were measured by commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using semiautomatic analyzer.

Microscopic studies: For detection of apoptotic cells, apoptotic index was examined by the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) method. *In situ* cell death detection kit POD (ISCDD, Boehringer Mannheim, Germany) was used to detect the apoptotic cells. The procedures were according to the protocol of the kit. Briefly, the sections were deparaffinized, rehydrated, and washed in distilled water (DW). The tissues were digested with 20 g/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 min. Endogenous peroxidase activity was blocked by incubating it in 3 mL/L hydrogen peroxide/methanol in PBS at 37°C for 30 min. The sections then were incubated with terminal deoxynucleotidyltransferase at 37°C for 60 min, and dioxigenin-conjugatddUTP was added to the 3-OH ends of fragmented DNA. Anti-digoxigenin antibody peroxidase was applied to the sections to detect the labeled nucleotides. The sections were stained with DAB (Diaminobenzidine) and counterstained slightly with hematoxylin. For each paraffin section, three fields were randomly selected and the frequency of TUNEL-positive cells was estimated at ×200 magnification. TUNEL-positive staining was observed in nuclei and nuclear fragments with the morphological characteristics of apoptosis in the I/R rats kidneys. The identification of stained apoptotic bodies was confirmed by specific morphological criteria including nuclear condensation, cytoplasmic compaction and detachment from neighboring cells (10).

Statistical analysis

The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), version 17.0, was used for statistical analysis. All data were presented as mean ± SEM. Before statistical analysis, all variables were checked for normality and homogeneity of variance by using the Kolmogorov-Smirnoff and Levene tests, respectively. The data obtained were tested by ANOVA followed by Tukey'*spost hoc* multiple comparison test. $P<0.05$ was considered statistically significant.

Results

Kidney function study

Table 1 shows the effects of black seed on the serum levels of markers of kidney function (Serum creatinine, blood urea nitrogen and uric acid). The serum concentrations of creatinine, urea and uric acid in the

Table 1. Effect of black seed (Bs) pretreatment on serum creatinine, blood urea nitrogen and uric acid levels in experimental rats

Group	Scr (mg/dl)	BUN (mg/dl)	Uric Acid (mg/dl)
Sham	1.55 ± 0.09	61.32 ± 5.8	0.79 ± 0.11
I/R model	4.23 ± 0.21[a]	142.95 ± 12.75[a]	1.82 ± 0.18[a]
I/R+Bs 5%	3.72 ± 0.18	131.87 ± 9.24	1.71 ± 0.14
I/R+Bs 10%	2.84 ± 0.11[b]	102. 66 ± 7.32[b]	1.32 ± 0.12[b]
I/R+Bs 20%	1.95 ± 0.08[c]	72.64 ± 4.3[c]	0.92 ± 0.15[c]

[a]: $P<0.001$ compared with sham group; [b]: $P<0.05$, [c]: $P<0.01$ Compared with I/R group

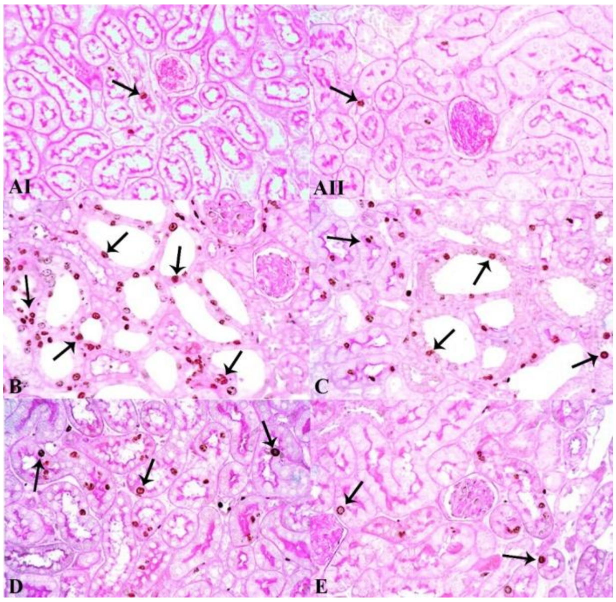

Figure 1.*In situ* end-labeling of the apoptotic bodies in kidney sections of Sham operation rats, I/R model rat and I/R+Back seed treated rats at 24 hr after reperfusion.TUNEL-stained nuclei are marked by arrows. Hematoxylin counterstaining. (Original magnification ×250)

I/R group were found to be significantly(P<0.001)higher than those in the sham rats, suggesting a significant degree of glomerular dysfunction mediated by renal I/R. Pretreatment with 10 and 20% of black seed dose-dependently decreased serum creatinine (P<0.05 and P<0.01, respectively), urea (P<0.05 and P<0.01, respectively), and uric acid (P<0.05 and P<0.01, respectively) levels compared with the reperfusion group, although these levels were still significantly higher than the sham control. Treatment with black seed 5% led to a non-significantly reductions in the mentioned serum biochemical parameters.

Apoptotic cell death: In the sham operated group, few TUNEL-positive cells were observed (Figure 1AI, AII). In ischemia-reperfusion group, a significant increase in the number of TUNEL-positive cells were observed at 24 hr reperfusion after ischemia, predominantly located at the proximal tubules of the cortex and distal tubules of the outer medulla. These cells demonstrated characteristic morphologies, e.g. shrunken and condensed nuclei (Figure 1B). Black seed at higher doses (10% and 20%) significantly attenuated TUNEL-positive cells (Figure 1D and E) as compared to the renal ischemia-reperfusion group (Figure 1B), and low dose treatment group (Figure 1C) did not demonstrate significant protection. Quantitative analyses of TUNEL-positive cells in sections of kidneys is shown in Figure 2.

Discussion

The results of our study indicated that pretreatment with black seed (*N. sativa* L.) had preventive effects on renal reperfusion injury (I/R) of the kidney, as evidenced by functional parameters

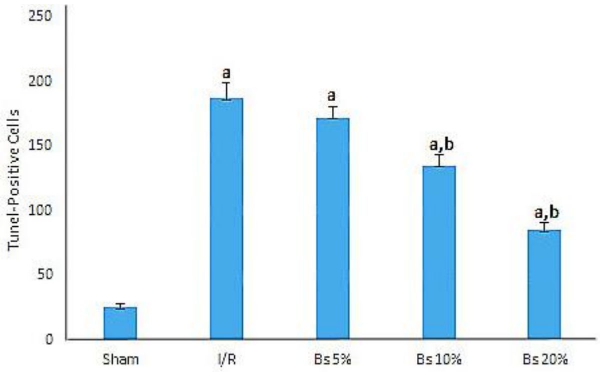

Figure 2. Quantitative analyses of TUNEL-positive cells. All values displayed are mean±SEM from eight independent animals (n=8). [a] P<0.01 versus sham control; [b] P<0.05 versus 24 hr after 60 min of ischemia group

and histological examination. These results were consistent with the findings reported by Bayrak*et al*, who investigated the effects of *N. sativa* on I/R-induced renal injury in the rats (11).

In this study, animals with reperfusion injury displayed renal injury that was characterized by deterioration in kidney function, increase of serum urea, uric acid and creatinine; and apoptosis of renal tubular cells.

These histopathological changes are in line with the findings reported by Ahmadiasl*et al*, who observed the renoprotective action of Erythropoietin and Melatonin against I/R injury in the rats (1).

In this study, serum creatinine, urea and uric acid as functional parameters were measured according to Bhalodia*et al,* in 2009 (9). Kidney excretes the metabolic wastes including urea, uric acid and creatinine and other ions. Plasma levels of urea, uric acid and creatinine are significant markers of renal dysfunction and reflect a decline in the glomerular filtration rate (12). In this study, serum creatinine, urea and uric acid levels in I/R rats were significantly higher than those in sham operated rats. This indicates that renal dysfunction occurred after I/R operation.

However, our results showed that with black seed pre-treatment in the reperfusion rats, functional and histopathological changes were reversed, dose dependently. This demonstrates that pre-treatment with black seed was helpful in preventing IR-induced renal dysfunction in a dose dependent manner.

Extensive studies to elucidate the mechanisms by which apoptosis is induced and transduced have resulted in the generally accepted theory that intrinsic and extrinsic mechanisms (the receptor-dependent pathway) are involved (13). The extrinsic apoptotic pathway is initiated by the activation of a death receptor, such as the TNF-receptor and Fas, and caspase-8 (13). Zhang *et al* data highlight the pivotal role of caspase-3 in the execution of ischemia-induced apoptosis (14). Caspase-3 inhibitors can prevent delayed cell death after ischemia (15). Thus, caspase-3 is a key step in the execution process of apoptosis, and its inhibition can block apoptotic cell death. Extrinsic apoptotic signal pathway results in activation of executioner caspase-3.

Our results demonstrated that *N. sativa*, protected kidney tissue against ischemia/reperfusion injury which may be due to antioxidant and anti-apoptotic effects of *N. sativa*.

It is assumed that these probable anti-apoptogenic effects of *N. sativa* may be mediated by one or more of the following mechanisms: Antioxidant activity, immunomedulatory action and genoprotective effects (6).

Conclusion

In summary, our results clearly demonstrated that antioxidant activity of black seed could protect kidney against ischemic injury via decreasing the number of apoptotic renal epithelial tubular cells, which provides a novel and effective strategy for prevention of the renal ischemic/reperfusion injury. In general, herbal medicines are complex mixtures of different compounds that often act in a synergistic fashion and exert their full beneficial effect as total extracts or fresh and intact plant. Though there is a widespread belief that herbal remedies are safe, their therapeutic use has been shown to alter liver and kidney functions. Therefore, future studies and trials to establish efficacy and optimum dosage of this plant are essential.

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