

# *Nigella sativa* oil and thymoquinone ameliorate albuminuria and renal extracellular matrix accumulation in the experimental diabetic rats

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**Abstract. – OBJECTIVE:** Increasing evidence suggests that *Nigella sativa* oil (NSO) and its principal bioactive constituents, thymoquinone (TQ), exhibit antioxidant, antihyperglycemic and renoprotective effects in streptozotocin (STZ)-induced diabetes in rats. However, the potential molecular mechanisms by which NSO and TQ may exert their actions in the diabetic kidney are still poorly characterized. This study was designed to investigate the effect of NSO and TQ treatment on the albuminuria, podocyte injury and the complex systems controlling the extracellular matrix proteins accumulation and angiogenesis in the STZ-induced model of diabetic nephropathy.

**MATERIALS AND METHODS:** Adult female Wistar rats were divided into four experimental groups (control, untreated STZ-diabetic, and NSO or TQ treated STZ-diabetic rats). The treated rats received 2 mL/kg NSO or 50 mg/kg TQ via oral gavage once a day for 10 weeks.

**RESULTS:** The results showed that the albuminuria and the kidney weight/body weight ratio were increased in the diabetic rats compared with the control animals and they were significantly ameliorated by the treatment with NSO or TQ. The real-time PCR showed that the NSO and TQ treatment prevented diabetes-induced down-regulation of mRNA expression of the podocyte-specific marker (podocin) as well as the mRNA overexpressions of collagen IV, transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) and vascular endothelial growth factor-A (VEGF-A) in the diabetic kidney. These results were also confirmed by immunohistochemistry.

**CONCLUSIONS:** NSO and TQ treatment decreased albuminuria in the experimental models of the diabetic nephropathy by the preservation of the podocyte function; along with the suppression of enhanced extracellular matrix gene expression through interfering with TGF- $\beta_1$  production and angiogenesis.

*Key Words:*

*Nigella sativa* oil, Thymoquinone, Diabetic nephropathy, Albuminuria.

## Introduction

Among all diabetes mellitus complications, the diabetic nephropathy is a common microvascular complication estimated to affect 40% of patients with diabetes<sup>1</sup>. Microalbuminuria and a decrease in creatinine clearance and glomerular filtration rate are the early clinical signs of the diabetic nephropathy<sup>1,2</sup>. A variety of structural alterations is involved in the progression of the diabetic nephropathy, including glomerular and tubular hypertrophy, thickening of basement membranes, and progressive mesangial expansion<sup>2</sup>. End-stage diabetic kidneys are commonly characterized by irreversible structural renal changes such as, glomerulosclerosis and tubulointerstitial fibrosis<sup>2</sup>.

*Nigella sativa* (plant family of Ranunculaceae), is ripe fruit that contains minute black seeds, known as black seed or black cumin<sup>3,4</sup>. *Nigella sativa* seeds contain an essential oil (0.4-2.5%), fixed oil (36-48%), alkaloids, saponin and proteins<sup>4</sup>. *Nigella sativa* oil (NSO) was shown to contain thymoquinone (TQ) which is the main bioactive component (27.8-57.0%) of the essential oil of the black seed<sup>3</sup>. Toxicity studies on laboratory animals have reported that NSO and TQ are quite safe, mainly when given orally<sup>3,4</sup>. Consequently, NSO and TQ have been used as anti-inflammatory, antioxidant and anticancer therapeutic agents<sup>4,6</sup>.

It has been shown that TQ protects the proximal tubular epithelial cells against tubular injury induced by angiotensin II<sup>7</sup>. In the gentamicin-induced nephrotoxicity, *Nigella sativa* and TQ supplementation prevent the development of gentamicin-induced acute renal toxicity failure<sup>8,9</sup>. Furthermore, NSO improved renal function and attenuated oxidative stress induced by chronic cyclosporine A treatment<sup>10</sup>. Recent studies suggest that TQ treatment exerts a therapeutic renoprotective effect in streptozotocin (STZ) induced diabetes in rats by decreasing oxidative stress and the improvement of renal morphology and function<sup>11,12,13</sup>. Findings from those studies support the concept that NSO and TQ exerted a renal protective effect in renal disease models. However, further preclinical research regarding the beneficial effect of NSO and TQ treatment on the molecular mechanisms accounts for diabetic nephropathy is required to specify their usefulness as an effective therapy in this disease. Therefore, the aim of this report was to investigate the effects of NSO and TQ treatment on the albuminuria, podocyte injury and the complex systems controlling the extracellular matrix proteins accumulation and angiogenesis in the STZ-induced model of the diabetic nephropathy.

## Materials and Methods

### *Animals*

All experimental procedures were approved by the Committee of Animal Ethics at Yarmouk University; Irbid-Jordan. Female adult Wistar rats, 55-60 days old and weighing approximately 200 g were maintained in the animal house of Yarmouk University under the standard conditions of a 12-light-dark cycle, temperature at 23 ± 1°C. The animals were fed a standard rodent chow and tap water ad libitum.

### *Induction of Diabetes and Experimental Protocols*

After an overnight fast, diabetes mellitus was induced in rats by intra-peritoneal injecting a freshly prepared STZ (Sigma-Aldrich, St Louis, MO, USA; 55 mg/kg; dissolved in 0.1 M acetate buffer; pH 4.5). A control group of rats received citrate buffer only. Once serum glucose is higher than 300 mg/dl, the rats were randomly divided into four treatment groups (n = 5-6 per group): (1) control (non-diabetic, ND), (2) diabetic (D), (3) diabetic treated with 50 mg/kg TQ (D+TQ,

Sigma-Aldrich, St Louis, MO, USA), administered orally and (4) diabetic which were treated orally with 2 mL/kg NSO (D+NSO). The dosage of TQ (50 mg/kg) and NSO (2 mL/kg) based on previous studies<sup>11,14</sup>. The ND group received the vehicle only (2 mL/kg corn oil). Body weight was recorded weekly and the dose of administration was adjusted according to the recorded body weight measurements.

After being treated for 10 weeks and one day before sacrifice, the rats were placed in metabolic cages and urine was collected for 24 hrs for the analysis of urine albumin concentration and the urine volume. Then, the animals were weighed and anesthetized with ether. Blood samples were collected and the right kidney was removed and then transferred into RNAlater solution (Sigma-Aldrich, St Louis, MO, USA) for real-time PCR analysis. The left kidney was fixed with 4% paraformaldehyde for immunohistochemical analysis.

### *Measurements of Blood Glucose and Urinary Albumin Excretion*

The blood glucose level was determined by glucometer (Accu-Chek Performa, Roche Diagnostics, Indianapolis, IN, USA). Urine samples were centrifuged at 4°C and 2,000 rpm for 10 min and the supernatant was used to measure the urinary albumin excretion rate (UAE) using albumin rat ELISA kit (Abcam, Cambridge, UK).

### *Immunohistochemistry*

Immunostaining for podocin, transforming growth factor-β1 (TGF-β<sub>1</sub>), collagen IV and vascular endothelial growth factor-A (VEGF-A) was performed using 1:100 dilution of primary antibodies against podocin (Abcam, Cambridge, UK; Cat.No. ab50339), TGF-β<sub>1</sub> (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat.No.sc-146), collagen IV (Abcam, Cambridge, UK; Cat.No. ab6586) and VEGF-A (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat.No. sc-7269) as previously described<sup>15</sup>.

### *Real-time RT-PCR Analysis*

RNAlater conserved kidney tissues were homogenized and the total RNA was extracted using RNeasy mini tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer instructions. cDNA was synthesized from the total RNA (0.5 µg) using oligo-(dT)15 primer in a 20-µl reaction according to the manufacturer's instructions (iNtRON, Biotechnology, Sungnam, South Korea).

**Table I.** Sequences of primers used for quantitative real time RT-PCR.

Gene	GenBank accession	Forward (5-3)	Reverse (5-3)
β-Actin	NM_031144	CCTAGACTTCGAGCAAGAGA	TCCATACCCAGGAAGGAAG
podocin	NM_130828.2	ATTCCGACTGGGACATCT	GTTACCACCTCATGGAAAGG
TGF-β <sub>1</sub>	NM_021578	CGTACACAGCAGTTCTTCTCT	ATGACATGAACCGACCCTTC
Collagen IV	NM_001135759	AGCACCCCTTTTCTGATCCATAG	TTTACTACTCCGACACCCATAC
VEGF-A	NM_031836	TTTCCCTTTCCTCGAACTGAT	ACGTCACTATGCAGATCATGC

*Abbreviations:* TGF-β<sub>1</sub>: transforming growth factor-β<sub>1</sub>; VEGF-A: vascular endothelial growth factor-A.

The real-time RT-PCR was performed on Li-neGene 9600 Real-Time PCR system (Bioer Technology Co, Bingjiang, China), using the KAPA SYBR® FAST Universal 2X qPCR master mix (KAPA Biosystem, Boston, MA, USA). Primers were designed and synthesized by IDT (Integrated DNA Technologies, INC., IA; Table I). The cycling parameters were: 95°C for 3 min and 45 cycles of 95°C for 3 s and 60°C for 20 s. The relative gene expression levels were determined by the CT method as described by Livak and Schmittgen<sup>16</sup>. The levels of genes expression were expressed as the normalized ratio of gene expression relative to β-actin mRNA.

**Statistical Analysis**

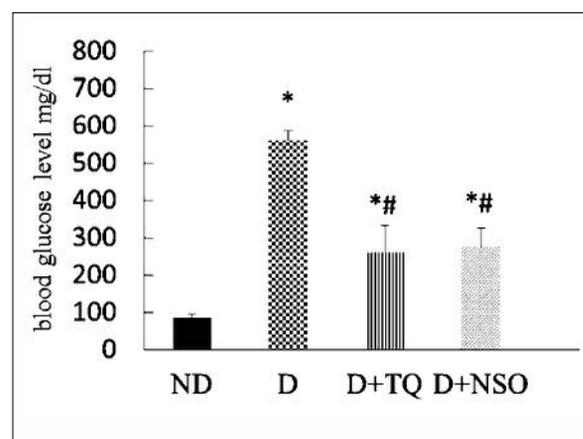
Statistical analyses of data were performed using the SPSS version 14.0 for Windows (SPSS Inc., Chicago, IL, USA). All data will be expressed as means ± SEM. One-way analysis of variance (ANOVA) was used to identify differences between the groups followed by LSD post-hoc test analysis. p-value < 0.05 was considered as statistically significant difference.

**Results**

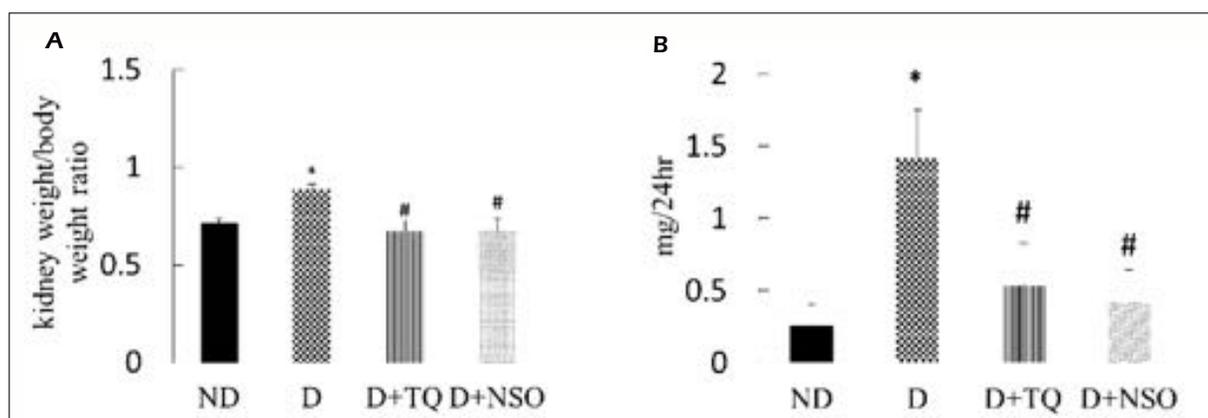
Blood glucose level was elevated in all of the diabetic rats compared with the control rats (Figure 1; *p* < 0.05). Treatment of diabetic rats with NSO or TQ significantly reduced blood glucose level compared with that in untreated diabetic rats. To evaluate the renal hypertrophy and renal functional parameters, ratio of kidney weight/body weight and UAE were measured, respectively (Figure 2). Kidney weight/body weight ratio was increased in the untreated diabetic rats compared with the control rats (Figure 2A; *p* < 0.05). The treatment of diabetic rats with NSO or TQ reduced significantly the kidney weight/body weight ratio compared with the dia-

betic rats. Diabetes was associated with an increase in UAE compared to the diabetic control rats. However, UAE was significantly lower in diabetic rats treated with NSO or TQ (Figure 2B; *p* < 0.05).

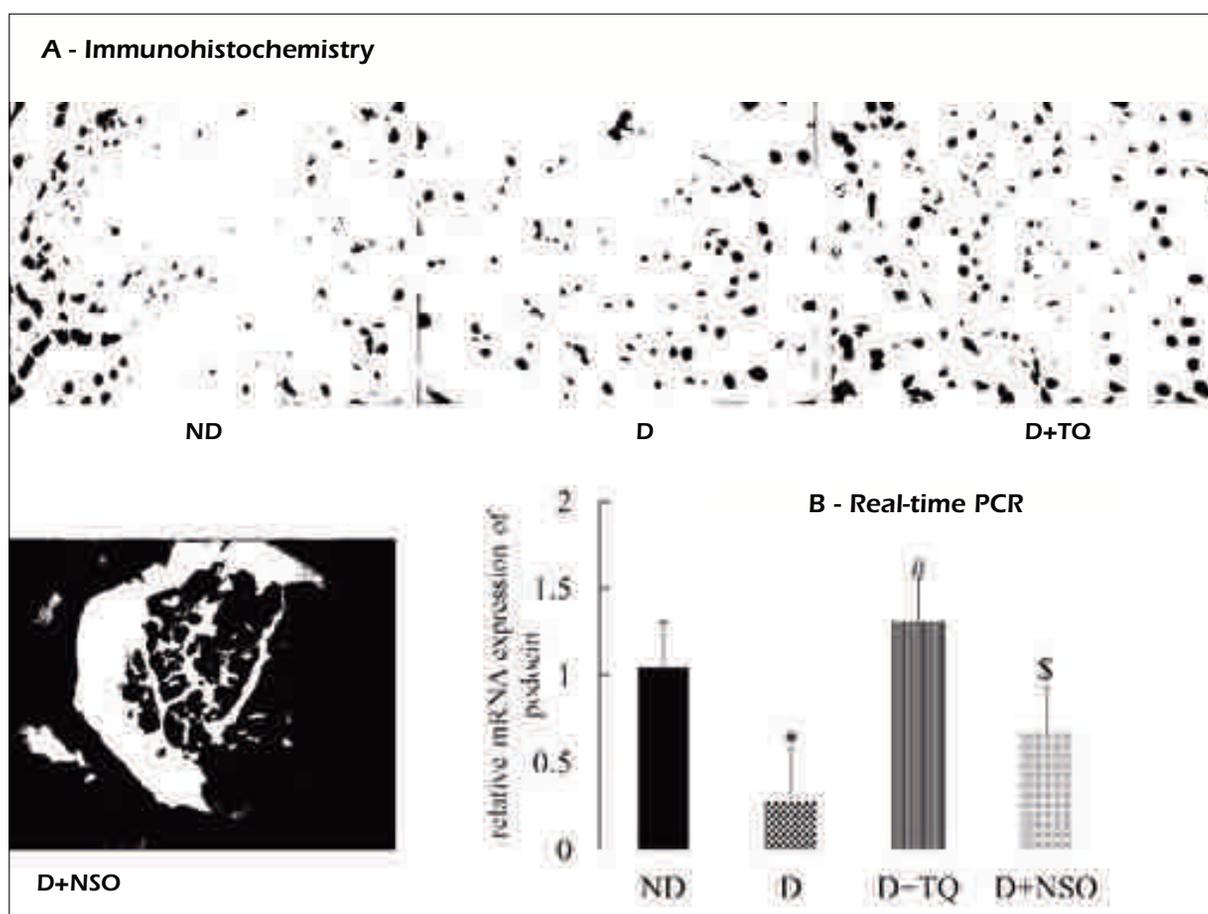
In attempt to clarify the mechanism of the beneficial effect of NSO and TQ treatment on diabetes-induced proteinuria in the present study, we assessed the extent of podocyte damage by monitoring the protein expression of slit diaphragm protein (podocin). The mRNA expression and the immunostaining intensity of podocin were lower in the glomeruli of the D group when compared to the control ND group (Figures 3A and 3B; *p* < 0.05). In the NSO and TQ treatment groups, however; the expression and distribution of podocin were completely (D+TQ group) and partially (D+NSO group) recovered (Figures 3A and 3B). Collectively, these results strongly suggest that restoration of podocin expression mediated the reno-protective effect of NSO and TQ.



**Figure 1.** Effect of NSO and TQ treatment on blood glucose level in control and diabetic rats. Data represent the mean ± SEM. \**p* < 0.05 compared to the ND group. \*\**p* < 0.05 compared to D group. *Abbreviations:* NSO, *Nigella sativa* oil; TQ, thymoquinone; ND, non-diabetic; D, diabetic.



**Figure 2.** Effect of NSO and TQ treatment on **(A)**, kidney weight/body weight ratio and **(B)**, urinary albumin excretion in control and diabetic rats. Data represent the mean  $\pm$  SEM. \* $p < 0.05$  compared to the ND group. # $p < 0.05$  compared to D group. Abbreviations: NSO, *Nigella sativa* oil; TQ, thymoquinone; ND, non-diabetic; D, diabetic.



**Figure 3.** Immunohistochemistry and real-time PCR detect that NSO and TQ treatment restores the podocin expression in the diabetic kidney. **A**, Immunohistochemical stain of the kidney sections (hematoxylin staining; magnification,  $\times 400$ ) shows that the podocin immunostaining (*brown staining*) in the glomeruli was much stronger in the ND group compared with the D group. NSO and TQ treatment inhibited the decrease in the podocin immunostaining in the NSO and TQ groups. **B**, Podocin mRNA expression by real-time PCR. Data represent the mean  $\pm$  SEM. \* $p < 0.05$  compared to the ND group. # $p < 0.05$  compared to the D group. § $p < 0.10$  compared to the D group. Abbreviations: NSO, *Nigella sativa* oil; TQ, thymoquinone; ND, non-diabetic; D, diabetic.







betic nephropathy<sup>25</sup>. Given the evidence above, targeting angiogenic pathways may prevent the progression of the diabetic nephropathy. In the present study, we demonstrated that NSO and TQ treatment could attenuate the diabetic nephropathy via inhibiting the angiogenesis related factor VEGF-A.

Finally and as expected, the observed renoprotection effect of NSO and TQ treatment was associated with a decrease in glycemic levels that could be explained by beta-pancreatic islet regeneration<sup>26,27</sup>. Hyperglycemia is a key factor in inducing TGF- $\beta$  in the diabetic kidney<sup>19</sup>. In addition, high glucose treated podocyte cells exhibit reductions in podocin and nephrin expression indicating podocytes damage<sup>28</sup>. Therefore, the reno-protective effect of NSO and TQ treatment in the current study could be mediated, at least in part, by lowering the blood glucose level in the diabetic rats.

## Conclusions

This study demonstrated that NSO and TQ treatment decreased albuminuria in the experimental models of diabetic nephropathy by the preservation of podocyte function; along with the suppression of enhanced extracellular matrix gene expression through interfering with TGF- $\beta_1$  production and angiogenesis. Although our report is an experimental study of diabetic nephropathy in rats, we provide a proof of concept evidence that NSO and TQ may be effective in controlling the diabetic nephropathy in humans.

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## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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