Effects of *Nigella sativa* Oil and Thymoquinone on Renal Oxidative Stress and Apoptosis Rate in Streptozotocin-Diabetic Rats

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Abstract: The effects of *Nigella sativa* oil (NSO) and thymoquinone (TQ) on diabetes, renal oxidative stress, lipid peroxidation and apoptosis rate were investigated in streptozotocin (STZ)-diabetic rats. Forty five Sprague Dawley rats were randomly distributed into 5 groups (n=9): healthy control; diabetic control; diabetic given NSO (1ml/kg); diabetic given TQ (3 mg/kg) and diabetic co-administered with NSO and TQ, orally for 4 weeks. Diabetes was induced by a dose of STZ (50 mg/kg, i.p.) in fasted rats. Blood samples were collected for biochemical analysis. Kidneys were removed for determination of tissue antioxidant capacity, lipid peroxidation and apoptosis rate. The results showed that oral co-administration of NSO and TQ significantly increased serum insulin and decreased serum levels of glucose, glycosylated hemoglobin (HbA1c), urea, creatinine and alkaline phosphatase in diabetic treated rats. Treatment with NSO and TQ significantly increased activities of antioxidant enzymes (SOD, GPx and CAT), increased serum level of reduced glutathione (GSH) and decreased lipid peroxidation and apoptosis index. In conclusion, NSO and TQ exhibit good antidiabetic and antioxidant effects; improve renal function and reduce both malondialdehyde (MDA) and apoptosis rate in diabetic rats. Therefore, NSO and TQ may be beneficial for diabetes mellitus associated with nephropathy due to oxidative stress.

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1. Introduction

Diabetes mellitus (DM) represents one of the greatest threats to modern global health and affects millions of people worldwide. DM is a chronic and progressive metabolic disorder characterized by hyperglycemia resulting from deficiency in insulin secretion, insulin action, or both **(Balakumar *et al.*, 2009 and Luis-Rodríguez *et al.*, 2012).** The increased extra- and intracellular glucose concentrations result in oxidative stress, which seems to be due mainly to increased production of reactive oxygen species (ROS) and free radicals with a sharp reduction in antioxidant body defenses **(Hayoz *et al.*, 1998)**. Free radicals are continuously produced during normal physiologic processes and attack macromolecules including proteins, lipids, and DNA, so causing tissue injury. It has been widely accepted that oxidative stress plays a key role in the onset and development of diabetes complications, notably nephropathy **(Bonnefont *et al.*, 2000)**. Several mechanisms seem to be involved in the generation of oxidative stress in experimental animals and patients. These mechanisms include glucose auto-oxidation, peroxidation or glycation of proteins, lipids, and DNA. Oxidative stress can arise from a number of different sources, whether disease state or lifestyle, including episodes of ketosis, sleep restriction, and excessive nutrient intake **(Rains and Jain, 2011)**.

Streptozotocin (STZ), an antibiotic produced by *Streptomyces achromagens*, is the most commonly used agent to induce experimental diabetes **(Rakieten *et al.*, 1963)** in rats and mice because of its ability to target selectively and destroy insulin producing β-cells of pancreatic islets. The diabetogenic action of STZ has been ascribed to the increase in intracellular methylation reactions **(Wilson *et al.*, 1984)**.

During the past decades, some approaches such as diet control, exercise, use of antidiabetic drugs and insulin therapy have been provided to reduce diabetes complications. Because of adverse effects of antidiabetic drugs, there is a great need to search for natural agents from medicinal plants with negligible adverse effects which would improve diabetic patient's health problems.

Nigella sativa (*N. sativa*, also known black seed, Family *Ranunculaceae*) seeds have been used an important nutritional flavoring agent and used traditionally as natural remedy for many ailments, notably for treating diabetes in ancient medicine. Previous studies reported that *N. sativa* seeds have been shown to possess antitumor **(Worthen *et al.*, 1998)**; immunostimulant **(Haq *et al.*, 1999)**;

antioxidant **(Burits and Bucar, 2000)**, and antidiabetic **(Meral *et al*., 2001)** properties. The oil of *N. sativa* exhibits nephroprotective **(Ali, 2004)** and analgesic and anti-inflammatory **(Houghton *et al.*, 1995)** effects in rats. Most of *N. sativa* properties have been attributed mainly to the quinone constituents of which thymoquinone, the main bioactive ingredient of the volatile oil isolated from *N. sativa* seeds **(Aboutabl *et al.*, 1986)**. Thymoquinone was reported to possess strong antioxidant **(Houghton *et al.*, 1995)**, neuroprotective **(Abdulhakaeem *et al.*, 2006)**, anti-inflammatory **(Ragheb *et al.*, 2009)**, and anticancer **(Randhawa and Alghamdi, 2011)** effects.

The present study was designed to investigate the effects of *Nigella sativa* oil (NSO), thymoquinone (TQ) and their combination on diabetes, renal oxidative stress, lipid peroxidation and apoptosis rate in streptozotocin-diabetic rats.

2. Material and Methods

2.1. *Nigella sativa* oil and thymoquinone

Nigella sativa oil was purchased from local Agricultural Seeds and Herbs Co., Egypt. Thymoquinone is 2-isopropyl-5-methyl-1, 4-benzoquinone. It was obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

2.2. Chemicals and diagnostic kits

Streptozotocin was purchased from Fluka Company (St Louis, MO, USA). Nicotinamide, other chemicals and commercial diagnostic kits were purchased from El-Gomhoryia Company for Chemicals, Cairo. Egypt.

2.3. Rats

Forty five adult male rats of Sprague Dawley strain weighing 285-295 g body weight and 14-16 weeks old were used in this study. The rats were obtained from the Laboratory Animal Farm, Helwan, Egypt. The animals were housed individually under hygienic conditions in plastic cages. The rats were kept at a room temperature of 25 ± 2°C with relative humidity of 50–60% and on 12 hrs light/12 hrs dark cycles. The experiment was carried out according to guidelines for animal experimentation approved by the Institutional Animal Care and Use Committee, National Research Centre, Animal Care Unit, Dokki, Egypt.

2.4. Preparation of basal diet

Basal diet was prepared using AIN 93 according to **Reeves *et al.* (1993)**. It consists of 20 % protein, 10 % sucrose, 4.7% fat, 2% choline chloride, 1% vitamin mixture, 3.5 % salt mixture and 5% fibers (cellulose). The remainder was corn starch up to 100 %.

2.5. Induction of diabetes

After 12hr fasting period, the rats were injected with nicotinamide (110 mg/kg, i.p.) followed (15 minutes later) by streptozotocin injection (50 mg/kg, i.p.). Nicotinamide preserves the pancreatic β-cells (up to 40%) from STZ cytotoxicity and produces diabetes similar to human diabetes mellitus **(Masiello *et al.*, 1998)**. To prevent from the fatal hypoglycemic effect of insulin release, 10 % glucose solution was provided for the rats for 2 days. After 48 hrs, the blood glucose (BG) levels were measured and rats with BG level higher than 250 mg/dl were considered as diabetic.

2.6. Experiment and grouping of rats

Forty five Sprague Dawley rats were used in this study and randomly distributed into 5 groups of 9 animals each. Group (1) was negative control and rats of the other 4 groups were injected intraperitoneally with streptozotocin (STZ) in a single dose of 50 mg/kg for induction of diabetes as previously mentioned. Group (2) was kept as a positive control and groups (3), (4) and (5) were given orally using stomach tube *Nigella sativa* oil (1ml/kg), thymoquinone (3mg/kg) and their combination respectively, daily for 4 weeks. Blood samples were withdrawn from portal hepatic vein into clean dry centrifuge tubes and centrifuged to separate the serum which used for biochemical analysis. The kidneys were removed on ice bag and kept at 4 ^{o}C in a refrigerator till further use.

2.7. Biochemical measurements

Levels of blood glucose **(Siest *et al.*, 1981)**; glycosylated hemoglobin **(Roghani and Baluchnejadmojarad, 2010)**; urea nitrogen **(Patton and Crouch, 1977)**; uric acid **(Fossati *et al.*, 1980)**; creatinine **(Husdan and Rapoport, 1969)** and alkaline phosphatase enzyme **(Roy, 1970)** were measured spectrophotometrically using chemical kits. Serum insulin was determined by ELISA test using rat insulin kit (Millipore, Carlsbad, USA) as described by **Yallow and Bauman (1983)**.

2.8. Lipid peroxidation and tissue antioxidant

Kidney tissue homogenate was prepared and used for measurement of lipid peroxidation and oxidative stress markers and quantification of apoptosis. One gram of kidney tissue was washed in ice-cold 0.9% NaCl and homogenized in ice-cold 1.15% solution of potassium chloride in 50 mM potassium phosphate buffer solution (pH 7.4) to yield 10% (W/V) homogenate. Homogenization was performed using Sonicator, 4710 Ultrasonic Homogenizer (Cole-Parmer Instrument Co., USA). Kidney homogenate was centrifuged at 1400 rpm for 10 min at 4°C. The supernatant was used for determination of contents of reduced glutathione (GSH) as described by **Beutler and Kelly (1963)** and lipid peroxide (MDA) using thiobarbituric acid reaction according to **Ohkawa *et al.* (1979)**. Activities of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were determined in renal tissue according to **Kakkar *et al.* (1984)**, **Paglia and Valentine (1967)** and **Sinha (1972)**, respectively.

2.9. Quantification of apoptosis

DNA fragmentation was used as a measure of apoptosis rate according to **Kamalay *et al.* (1990)**. The renal supernatant containing the cytoplasmic protein fraction was used for determination of programmed cell death rate. Cayman protein determination kit (Roche, Germany) was used to quantitate protein concentrations. Cell death detection ELISA kit (Roche, Germany) was used to quantitatively detect the cytosolic histone-associated DNA fragmentation, based on the manufacturer's instructions. Renal cytoplasmic extract (25 µl) was used as an antigen source in a sandwich ELISA using a Dynex MRX plate reader. The change in color was measured at a wavelength of 405 nm and the optical density reading was normalized to the total amount of cytoplasmic protein in the sample and data were recorded as apoptotic index (OD/mg protein).

2.10. Statistical analysis

Data were expressed as mean ± SD. Differences between the experimental groups were analyzed by one-way ANOVA test using computerized SPSS program followed by Duncan's multiple range test (**Snedecor and Cochran (1986)**.

3. Results

The results of this study revealed that single intraperitoneal injection of streptozotocin in a single dose of 50 mg/kg to rats caused a significant (P <0.05) increase in blood glucose (hyperglycemia) and glycosylated hemoglobin (HbA1c) and decrease in serum insulin levels when compared to healthy control rats. Oral administration of *Nigella sativa* oil (NSO), thymoquinone (TQ) and their combination significantly (P <0.05) decreased blood glucose and HbA1c levels and increased insulin levels in the serum when compared to diabetic control rats as recorded in Table (1).

Table 1. Effects of *Nigella sativa* oil (NSO) and thymoquinone (TQ) on serum levels of blood glucose (BG), glycosylated hemoglobin (HbA1c) and insulin in streptozotocin-diabetic rats.

Parameters Groups	BG (mg/dl)	HbA1c (% Hb)	Insulin (ng/ml)
Group (1) Healthy control	264 ± 17.0^{c}	7.46 ± 0.12^{c}	2.60 ± 0.15^{a}
Group (2) Diabetic control	325 ± 19.0^{a}	27.61 ± 0.22^{a}	0.88 ± 0.17^{c}
Group (3) NSO (1ml/kg)	295 ± 10.0^{b}	18.12 ± 0.25^{b}	1.70 ± 0.24^{b}
Group (4) TQ(3 mg/kg)	283 ± 13.0^{b}	12.20 ± 0.17^{b}	1.94 ± 0.16^{b}
Group (5) NSO + TQ	270 ± 12.0^{b}	10.86 ± 0.19^{b}	2.10 ± 0.19^{b}

Means ± SD with different letters superscripts in the same column are significant at P < 0.05 using one way ANOVA test. n= 9 rats/group.

Data recorded in Table (2) show that experimental diabetes induced by streptozotocin (STZ) in rats produced significant (P <0.05) increases in blood urea nitrogen (BUN), creatinine (Cr) and alkaline phosphatase (ALP) enzyme levels as compared to healthy control rats. Oral administration of *Nigella sativa* oil (NSO), thymoquinone (TQ) and their combination to diabetic rats significantly (P <0.05) normalized the elevated serum levels of BUN, Cr and ALP when compared to diabetic control rats.

As shown in Table (3) intraperitoneal injection of streptozotocin in a single dose of 50 mg/kg to rats significantly (P < 0.05) decreased the content of reduced glutathione (GSH) and increased content of lipid peroxide malondialdehyde (MDA) in renal tissues when compared to healthy control rats. Oral administration of *Nigella sativa* oil (NSO), thymoquinone (TQ) and their co-administration to diabetic rats normalized renal GSH and MDA contents when compared to diabetic control rats.

Data in Table (4) show that streptozotocin-diabetic rats had significant (P < 0.05) decreases in the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in renal tissue as compared to healthy control rats. Oral administration of *Nigella sativa* oil (NSO), thymoquinone (TQ) and their co-administration to diabetic rats significantly (P < 0.05) increased activities of SOD, GPx and CAT antioxidant enzymes when compared to diabetic control rats.

Table 2. Effects of *Nigella sativa* oil (NSO) and thymoquinone (TQ) on levels of blood urea nitrogen (BUN), creatinine (Cr), uric acid (UA) and alkaline phosphatase (ALP) enzyme in streptozotocin-diabetic rats.

Parameters Groups	BUN (mg/dl)	UA (mg/dl)	Cr (mg/dl)	ALP (U/dl)
Group (1) Healthy control	32.90±2.34[d]	2.11±0.02[a]	0.69±0.02[d]	57.5±3.55[c]
Group (2) Diabetic control	72.46±2.64[a]	2.18±0.06[a]	1.73±0.03[a]	82.6±4.21[a]
Group (3) NSO (1ml/kg)	55.74±3.66[b]	2.11±0.04[a]	1.35±0.04[b]	67.5±5.03[b]
Group (4) TQ (3 mg/kg)	48.43±2.28[c]	2.12±0.07[a]	1.23±0.02[b]	65.8±4.22[b]
Group (5) NSO + TQ	43.55±3.76[c]	2.18±0.02[a]	0.88±0.02[c]	61.3±3.44[b]

Means ± SD with different letter superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test and those with similar letter superscripts are not significant. n= 9 rats/group.

Table 3. Effects of *Nigella sativa* oil (NSO) and thymoquinone (TQ) on contents reduced glutathione (GSH) and lipid peroxide malondialdehyde (MDA) in renal tissue of streptozotocin-diabetic rats.

Parameters Groups	GSH (nmol/min/mg protein)	MDA (nmol/min/mg protein)
Group (1) Healthy control	24.43 ± 3.24[a]	0.29 ± 0.05[c]
Group (2) Diabetic control	12.50 ± 2.88[d]	0.85 ± 0.03[a]
Group (3) NSO (1ml/kg)	16.74 ± 4.66[b]	0.52 ± 0.04[b]
Group (4) TQ (3 mg/kg)	18.95 ± 3.18[c]	0.45 ± 0.07[b]
Group (5) NSO + TQ	22.25 ± 3.33[c]	0.36 ±0.02 [b]

Means ± SD with different letter superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test. n= 9 rats/group.

Table 4. Effects of *Nigella sativa* oil (NSO) and thymoquinone (TQ) on activities antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in renal tissue of streptozotocin-diabetic rats.

Parameters Groups	SOD (U/mg protein)	GPx (nmol/min/mg protein)	CAT (nmol/min/mg protein)
Group (1) Healthy control	56.70 ± 1.24[a]	0.60 ± 0.02[a]	0.182 ± 0.001a
Group (2) Diabetic control	36.50 ± 2.88[d]	0.13 ± 0.04[d]	0.136 ± 0.003[d]
Group (3) NSO (1ml/kg)	42.74 ± 2.46[b]	0.25 ± 0.03[b]	0.142 ± 0.002[b]
Group (4) TQ (3 mg/kg)	46.95 ± 3.58[c]	0.28 ± 0.01[b]	0.153 ± 0.001[b]
Group (5) NSO + TQ	53.25 ± 3.73[c]	0.45 ± 0.01[c]	0.172 ± 0.002[c]

Means ± SD with different letter superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test. n= 9 rats/group. Unit of GPx= nmol of GSH utilized/min/mg protein. Unit of CAT= nmol of H_2O_2 utilized/min/mg protein.

As shown in Table (5) the apoptosis rate in renal tissue of diabetic rats was significantly ($P < 0.05$) higher than that in healthy control rats. DNA fragmentation in diabetic rats was 75 % versus to 10 % in healthy control rats. Oral administration of *Nigella sativa* oil (NSO), thymoquinone (TQ) and their co-administration to diabetic rats significantly ($P < 0.05$) decreased the apoptotic index. DNA fragmentations in renal tissue of diabetic rats orally given NSO, TQ and their co-administration were 60.0, 55.0 and 45.0%, respectively.

Table (5): Effects of *Nigella sativa* oil (NSO) and thymoquinone (TQ) and their combination on apoptosis index and DNA fragmentation in renal tissue of streptozotocin - diabetic rats.

Parameters Groups	Apoptosis index (OD/mg protein)	DNA fragmentation (%)
Group (1) Healthy control	0.10 ± 0.001^{e}	10.0
Group (2) Diabetic control	0.75 ± 0.004^{a}	75.0
Group (3) NSO (1ml/kg)	0.60 ± 0.003^{b}	60.0
Group (4) TQ (3 mg/kg)	0.55 ± 0.002^{c}	55.0
Group (5) NSO + TQ	0.45 ± 0.001^{d}	45.0

Means ± SD with different letter superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test. n= 9 rats/group. OD = Optical density measured at 405 nm

4. Discussion

The effects of *Nigella sativa* oil (NSO), thymoquinone (TQ) and their combination on diabetes, renal oxidative stress, lipid peroxidation and apoptosis rate were investigated in streptozotocin-diabetic rats.

In the current study, the results revealed that streptozotocin (STZ) caused hyperglycemia, increased glycosylated hemoglobin (HbA1c) and decreased insulin serum levels. STZ also elevated serum biomarkers of kidney function, increased lipid peroxidation and apoptosis rate and caused oxidative stress in renal tissue. These findings were partially similar with those reported by **Abdelmeguid *et al.* (2010)** who found that STZ-diabetes increased pancreatic tissue malondialdehyde (MDA) and serum glucose levels, decreased serum insulin and SOD levels and impaired the kidney function. **Palsamy and Subramanian (2008)** concluded that kidney malfunction induced by chronic hyperglycemia, in turn, reduces plasma proteins, enhances proteinurea, accumulates urea and creatinine and accelerates weight loss. However, **Gu *et al.* (1997)** reported that high doses of STZ produced experimental model of Type 1 diabetes which characterized by depletion of pancreatic β-cells in mice.

Oxidation of glucose is one of the mechanisms involved in pathogenesis of diabetes complications **(Maritim *et al.*, 2003).** Glucose oxidation enhances glycation of proteins such as hemoglobin (and produces HbA1c) and antioxidant enzymes which in turn, can reduce their activities for detoxification of reactive oxygen/nitrogen-free radicals and lead to lipids, proteins and DNA peroxidation and finally programmed cell death **(Rains and Jain, 2011)**. The concentration of HbA1c is considered as a good marker for diagnosis and prognosis of diabetes complications.

The results of the present study demonstrated that *Nigella sativa* oil (NSO), thymoquinone (TQ) and their combination has been shown to possess hypoglycemic and antidiabetic effects that evident by reductions of blood glucose and glycosylated hemoglobin (HbA1c) and elevation of insulin levels. These results are in agreement with those reported by **Meral *et al*. (2001) and Abdelmeguid *et al.* (2010)** who found that NSO and TQ exhibit hypoglycemic effect. The later authors concluded that NSO and TQ protect against STZ-diabetes by decreasing oxidative stress, thus preserving pancreatic β-cell integrity. The observed hypoglycemic effect could be due to amelioration of β-cell ultrastructure, thus leading to increased insulin levels.

Oral administration of *Nigella sativa* oil (NSO), thymoquinone (TQ) and their combination to diabetic rats improved kidney function as evident by lowering the elevated serum levels of blood urea nitrogen, creatinine and alkaline phosphatase enzyme. The nephroprotective activity of NSO and TQ was previously reported by **Ali (2004)** who found that *Nigella sativa* oil produced a protective effect against gentamicin induced-nephrotoxicity in rats.

The current study demonstrated that *Nigella sativa* oil (NSO), thymoquinone (TQ) and their combination

induced a high antioxidant effect as evident by amelioration of oxidative stress biomarkers in kidney tissues of diabetic rats. The antioxidant activities of NSO and TQ agreed with those obtained by **Houghton *et al.* (1995) and Burits and Bucar (2000)**. Dismutation of superoxide radicals (the most abundant reactive oxygen radical produced in the cells) to hydrogen peroxide is the first step in detoxification of reactive oxygen/nitrogen species. Hydrogen peroxide is then metabolized into water by the activities of CAT and GPx enzymes. Moreover, reduced glutathione (GSH), a substrate for GPx activity, is a major intracellular antioxidant molecule and acts as a direct free radical scavenger **(Palsamy and Subramanian, 2010).** Antioxidant machinery impairment duo to glycation of antioxidant enzymes and other proteins has been previously reported **(Palsamy and Subramanian, 2010).** On the other hand, **Davi *et al.* (2005)** found that activation of polyol pathway and consumption of NADH, GSH availability for efficient function of GPx reduced in diabetes mellitus. Data of the present showed that NSO and TQ and their combination attenuated antioxidant machinery impairment in STZ-diabetic rats as evident from increased activities of antioxidant enzymes (SOD, GPx and CAT) and content of GSH in renal tissue. These results are in line with obtained results by **Abdelmeguid *et al.* (2010)** who suggested N. sativa and thymoquinone ameliorated most of toxic effects of STZ and may be useful in the treatment of diabetes and in the protection of pancreatic β-cells against oxidative stress. The antioxidant properties of NSO and TQ may be accomplished directly or indirectly through reducing blood glucose level.

Many of above mentioned hyperglycemia-induced pathways get together to elevate the level of NF-κB, a proinflammatory master key, which activates proinflammatory cytokines gene expressions and apoptosis cascade **(Kern, 2007 and Palsamy and Subramanian, 2010).** The present study showed that renal apoptosis rate was higher in diabetic rats than in healthy control rats. This result was in agreement with the previous studies of **Kern (2007) and Barber *et al.* (2011)** who recorded that renal apoptosis rate in STZ-diabetic rats was significantly higher than in normal controls.

In conclusion, *Nigella sativa* oil (NSO), thymoquinone (TQ) and their combination exhibit antihyperglycemic effect, leading to reduction in serum level of glycosylated hemoglobin (HbA1c) in streptozotocin-diabetic rats. NSO and TQ induce good antioxidant activity, improve biomarkers of kidney function and reduce both lipid peroxidation and apoptosis rate in renal tissue. It is possible that NSO and TQ can improve kidney cellular function through reducing oxidative stress which in turn, reduces diabetes-induced hyperglycemia and its related complications, notably nephropathy. This study suggests that NSO and TQ may be considered as beneficial natural agents for the treatment of diabetes mellitus associated with nephropathy due to oxidative stress.

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