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Effects of *Nigella sativa* oil on ovarian volume, oxidant systems, XIAP and NF-kB expression in an experimental model of diabetes

H. N. Seflek, S. Kalkan, G. Cuce, I. Kilinc, and M. E. Sozen

*Departments of Histology and Embryology, Necmettin Erbakan University Meram Medical Faculty, Konya, Turkey; Department of Biochemistry, Necmettin Erbakan University Meram Medical Faculty, Konya, Turkey; Department of Histology and Embryology, Faculty of Medicine, Alanya Alaaddin Keykubat University, Antalya, Turkey*

**ABSTRACT**

We investigated the effects of *Nigella sativa* oil on ovary volume, nuclear factor-kappaB (NF-kB), X-linked inhibitor of apoptosis protein (XIAP) expression, and serum malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant status (TAS) and total oxidant status (TOS) levels in diabetic rats. We divided 21 adult female rats into three groups: controls, diabetics and diabetics + *N. sativa* oil. The diabetics + *N. sativa* oil group was given 0.2 mg/kg/day *N. sativa* oil 6 days/week for 4 weeks. NF-kB and XIAP expression was assessed in ovarian sections using immunohistochemistry. The right and left ovary volumes were calculated using stereology. We also measured serum MDA, SOD, TAS and TOS levels. We found that *N. sativa* oil reduced hyperglycemia, but not to control levels. *N. sativa* oil also exhibited antioxidant properties as demonstrated by reduced serum TOS and MDA levels, and increased SOD and TAS levels compared to controls. We found no significant difference in total ovarian volume, XIAP or NF-kB expression among the groups, which may be due to the short study period. Our findings suggest that *N. sativa* oil may be useful for reducing blood glucose levels and elevated oxidant activity in diabetic patients.

**KEYWORDS**

Antioxidants; apoptosis; diabetes; *Nigella sativa* oil; ovary; oxidant; stereology

**CONTACT** G. Cuce gokhancuce@gmail.com, Histology and Embryology, Necmettin Erbakan University Meram Medical Faculty, Morphology Building, First Floor, Konya, Turkey © 2019 The Biological Stain Commission

Diabetes mellitus is a major health problem. (Cuce et al. 2011). Diabetes accelerates the production of reactive oxygen species (ROS) and causes oxidative chemical modifications of lipids, DNA and proteins in various tissues (Osawa and Kato 2005). Tissue oxidative stress and damage due to diabetes play important roles in the development of complications of diabetes (Baynes and Thorpe 1999). Chronic hyperglycemia can affect every system in the body (Amaral et al. 2008) including the female reproductive system. ROS can damage the oocyte (Agarwal et al. 2005), and hypercholesterolemia caused by hyperglycemia (Young et al. 1988) may alter steroid metabolism (McLean et al. 1996). Oxidative stress affects the regulation of α and β estrogen receptors in vitro (Agarwal et al. 2005). Increases in atretic follicles, blood glucose levels, ovarian volume, and hyperemia and thickening of the germinal epithelium have been reported in diabetic rats (Farhad et al. 2013). Diabetic animals also exhibit increased apoptosis of granulosa cells (Chang et al. 2005). Increased oxidative stress in diabetic patients may be associated with reduced cellular antioxidant defenses (Rochette et al. 2014); antioxidant dietary supplementation may decrease cellular oxidative stress-dependent changes in diabetics (Abdelmeguid et al. 2010).

*Nigella sativa* has been reported to exhibit antitumor, anticancer (Salomi et al. 1989, 1992), antifungal (Islam et al. 1989), antimicrobial (Hanafy and Hatem 1991) and antidiabetic (Abdelmeguid et al. 2010; Sultan et al. 2014) effects. The biological activity of *N. sativa* seeds has been reported to be due to thymoquinone, the main component of the essential oil (Ali and Blunden 2003). X-linked inhibitor of apoptosis protein (XIAP) is an anti-apoptotic gene and member of the inhibitors of apoptosis (IAP) family. XIAP regulates apoptic signals by interacting with caspases-3, -7 and -9, and is a well-known anti-apoptotic factor in the ovaries (Phillipps and Hurst 2012).

NF-kB is a dimeric protein subfamily within the Rel family. It causes rapid expression of genes involved in early immune and inflammatory responses. NF-kB-responsive genes include pro-inflammatory cytokines, colony stimulating factor, inducible enzymes, chemokines, adhesion molecules and receptor genes (Barnes 1997). NF-kB has been reported to play a role in apoptosis associated with mitochondrial Bax and TdT (Pavlova et al. 2011). There is little information in the literature concerning ovarian volume and XIAP expression in diabetic ovaries; therefore, we investigated the effects of...
**N. sativa** oil on these characteristics in diabetic ovaries. We also compared plasma antioxidant and oxidant values.

**Material and methods**

Our study was conducted with the approval of Necmettin Erbakan University Center for Experimental and Applied Medical Sciences Research Animals Ethics Board (decision 2013/206, December 31 2013).

**Animals**

We used 21 4-month-old, 250–300 g adult female Wistar albino rats. Rats were housed at a maximum of five rats/cage at 20 °C, 30–40% relative humidity and 12 h light:12 h dark periods. Food and water were available *ad libitum*. The animals were divided into three groups of seven and subjected to their respective treatments.

We used streptozotocin (STZ; Sigma Aldrich, St. Louis, MO) to induce experimental diabetes. A single 50 mg/kg dose of STZ dissolved in 0.1 M sodium citrate-buffered solution, pH 4.50, was injected intraperitoneally (i.p.) to induce diabetes. Blood glucose levels were monitored using a glucose measuring device (eBSensor; Visgeneer, Hsinchu, Taiwan) for 3 days following the injection; all rats with blood glucose values >270 mg/dl were considered diabetic (Cuce et al. 2011). Blood was collected from the tail vein using a 23–25 gauge needle. Blood was transferred to the glucose meter.

**N. sativa** oil (Origo Nutrition, Gaziantep, Turkey) was prepared in a cold press machine. The specific gravity is 0.91–0.92 at 25 °C and the oil contains B complex vitamins, oleic acid (C18:1) 24.4%, linoleic acid (C18:2) 30.3%, linolenic acid (C18:3) 0.3%, palmitic acid (C16:0) 13.1%, stearic acid (C18:0) 4.2%, miristic acid 0.3%, miristoleic acid 0.1%, palmitoleic acid 0.4%, heptadecanoic acid 0.1%, heptadecenoic acid 0.1%, arachidic acid 0.3%, gadoleic acid 0.5%, erusik acid 0.1% and lignoceric acid 0.4%.

Group 1 (nondiabetic control) was injected i.p. with 0.2 mg/kg/day physiological saline 6 days/week for 30 days. Group 2 (diabetes group) consisted of diabetic animals injected i.p with 0.2 mg/kg/day physiological saline solution 6 days/week for 30 days. Group 3 (diabetes + oil) consistent of diabetic animals injected i.p. with 0.2 mg/kg/day **N. sativa** oil 6 days/week for 30 days (Abdelmeguid et al. 2010). Blood glucose levels were measured at the end of the experiment.

The right and left ovaries of all animals were excised under deep ketamine-xylazine anesthesia and fixed in 10% formalin. The tissues were dehydrated through 70 (2 h), 85 (1 h), 95 (1 h) and 100% (2 h) alcohol. After clearing in two baths of xylene (2 h each), the ovaries were embedded in paraffin. Sections were cut at 5 µm for stereology and immunohistochemistry. Slide preparation and histological assessments were performed in the Necmettin Erbakan University Meram Medical Faculty Department of Histology and Embryology.

**Immunohistochemistry**

For the immunohistochemistry studies, NF-κB (NF-κB p65; 0465R; Bioss-antibodies, Woburn, MA,) and XIAP (XIAP B1RC4, Bioss-antibodies) antibodies were used, both diluted 1:200. Paraffin was removed from 5 µm sections using xylene for 30 min. Sections were placed in Super Block (ScyTek Laboratories, Logan, UT), 10 min, washed with PBS, 5 min, incubated with primary antibody, 60 min, washed with PBS, 5 min, then incubated with secondary antibody, 20 min, followed by washing in PBS, 5 min. Streptavidin-peroxidase was added, 20 min, followed by washing in PBS, 5 min. ABC chromogen was added, 15 min, then washed with distilled water, 5 min, counterstained with Mayer’s hematoxylin, 5 min, followed by washing in tap water, 3 min, and rinsing in distilled water, 10 sec. We evaluated staining using the following criteria (Cuce et al. 2011): 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining.

**Stereology**

Right and left ovary volumes for all rats were measured using stereology. Slides were deparaffinized in two xylene baths for 2 h each and two paraffin baths for 2 h each, then processed and embedded in paraffin as above. Sections were cut at 5 µm and mounted on slides, then stained with hematoxylin and eosin (H & E). The Cavalieri estimator was used to measure ovary volumes. Sections for analysis were selected using a systematic random sampling principle. First, a sample from the first 40 sections was selected randomly, then every 40th section following this first section was selected. This process was repeated until the end of the tissue sample; 11–19 serial sections were obtained in this way. The distance between two samples (t) was set at 200 µm. Samples from every ovary were imaged using a x 4 objective and the images from the samples were combined and uploaded to the computer. Samples from every ovary were imaged using a x 4 objective and the images from the samples were combined and uploaded to the computer.

**Planimetric and volume calculations**

Planimetric calculations for the ovary were performed using the point counting method (Bolat et al. 2013). Serial slide images from each ovary were assessed using...
ImageJ software (LOCI, University of Wisconsin, Madison, WI) and calibrated. Then 0.3 mm² grids from each point were assigned randomly using the grid function of the software (Figure 1). The points that touched ovary tissue were counted and recorded. This process was repeated three times and the mean of the three results was used to determine the total number of points. Ovary volumes were calculated using the formula (Cuco et al. 2015):

\[ V = t \times a(p) \times \Sigma p \]

where \( V \) is the ovary volume, \( t \) is the distance between two slides used for analysis (120 µm), \( a(p) \) is the area represented by each point in the grids (0.3 mm²), and \( \Sigma p \) is the number of points touching ovary tissue.

The coefficient of error for volumetric calculations by the Cavalieri estimator indicates the reliability of the tests. It is preferable that this value be \( \leq 10\% \) (Gundersen and Jensen 1987; Gundersen et al. 1999; García-Finana et al. 2003). Several methods have been used to calculate the coefficient of error for stereological research (Ohm et al. 1997; Gundersen et al. 1999; García-Finana et al. 2003). We calculated coefficients of error using the method reported by Gundersen et al. (1999).

**Biochemistry**

Blood, 4–6 ml, from all animals was collected in an anticoagulant tube under ketamine-xylazine anesthesia. Blood was collected by intracardiac puncture using a 19–21 gauge cannula. The blood samples were centrifuged at 1,500 x g for 10 min and the separated serum was stored at −80 °C until analysis.

We assessed lipid oxidation by measuring malondialdehyde (MDA) (Bio Vision, Mountain View, CA) levels, superoxide dismutase (SOD) (Cayman Chemical, Ann Arbor, MI) enzyme activity, total antioxidant status (TAS) (Rel Assay®, Diagnostics kits, Mega Tıp, Gaziantep, Turkey) and total oxidant status (TOS) (Rel Assay®, Diagnostics kits,) using commercially available kits and reagents. All analyses were performed at the Necmettin Erbakan University Meram Medical Faculty Department of Biochemistry. All assays were calibrated appropriately. We used a Bio-Rad microplate absorbance reader (Bio-Rad, Hercules, CA).

**Statistical analysis**

Biochemical characteristics, ovary volumes and blood glucose levels were compared using one-way analysis of variance (ANOVA) with Duncan’s test.

The expression of IXAP and NF-κB in the oocytes and granulosa cells was evaluated by Kruskal Wallis test for the right ovaries. If there was a difference between the groups, the difference was investigated by forming binary groups with Man Whitney U Test. Values for \( p \leq 0.05 \) were considered significant. The resulting means ± SE were converted to graphics. For XIAP expression for primordial, primary, secondary and Graafian follicles, eight graphics were obtained (four graphics for oocytes, four graphics for granulosa cells) for the right ovary. Eight graphics also were obtained for the left ovary. We obtained 16 graphics for XIAP expression and 16 graphics for NF-κB expression.

**Results**

**Ovary volume and blood glucose levels**

We found no statistically significant difference in volume between right and left ovaries among the groups (Figure 2). Hyperglycemia increased significantly in group 2 compared to group 1. *N. sativa* oil significantly decreased blood glucose level caused by diabetes in group 3 compared to group 2 (Figure 2).

**Immunohistochemistry**

We found that XIAP expression increased in the oocytes and granulosa cells of Graafian follicles in group 2 compared to the other groups, but the differences were not statistically significant. XIAP expression in granulosa...
cells also increased in the primordial, primary and secondary follicles (Figure 4).

XIAP staining for oocytes and granulosa cells from four follicles was analyzed for all three groups and 16 graphics were obtained. In 12 of the graphs, XIAP expression was greater in diabetic group than the other groups, but no statistically significant difference was found (Figure 4).

NF-κB expression increased significantly only in primary follicles from oocytes of the left ovary between groups 2 and 3 (p < 0.05). No other significant differences in NF-κB expression levels among oocytes or granulosa cells were observed among the groups of right or left ovaries (Figure 5). XIAP and NF-κB expression was observed in all follicles at varying levels (Figures 4 and 5).
Biochemistry

TAS and SOD levels in the serum decreased in rats with diabetes, but increased in diabetic rats treated with *N. sativa* oil; the differences among all three groups were significant. Hyperglycemia increased TAS and SOD levels in group 2 compared to group

![Figure 4. XIAP synthesis and expression in granulosa cells and oocyte cytoplasm. Primary (a) and secondary (b) follicles from group 1. Scale bars = 100 μm a) and 50 μm b). Secondary (c) and primordial (d) follicles and a large atretic follicle from group 2. Red arrows, primordial follicles without oocytes; green arrows, expression in the epithelium of follicles. Scale bars = 100 μm c) and 50 μm d). Primordial, primary and secondary follicles from group 3 (e). Scale bar = 100 μm. Primordial, primary and secondary follicles from group 3 (f) Green arrows, expression in the follicle epithelium; yellow arrows, expression in the nucleus. Scale bar = 50 μm. P, primordial follicle; P1, primary follicle; S, secondary follicle.

![Biochemistry](image-url)
and *N. sativa* oil decreased significantly TAS and SOD levels in group 3 compared to group 2. By contrast, TOS and MDA values increased for group 2, but decreased for group 3. TAS, SOD, TOS and MDA; differences were significant among the three groups (Figure 3).

Figure 5. NF-κB synthesis and expression. Primary follicle from group 1 (A). Yellow arrows, expression in oocyte cytoplasm; green arrows, expression in epithelial cells of primary follicles. Scale bar = 100 μm. Graafian and secondary follicles from group 1 (B); yellow arrows, expression in oocyte nucleus. Scale bar = 500 μm. Primordial and secondary follicles from group 2 (C). Scale bar = 50 μm. Graafian and secondary follicles from group 2 (D). Scale bar = 500 μm. Primary follicles from group 3 (E). Yellow arrows, expression in oocyte cytoplasm; green arrows, expression in epithelial cells of primary follicle. Scale bar = 50 μm. Secondary follicles from group 3 (F). Scale bar = 500 μm. Different levels of expression can be seen in granulosa cells and the color intensity of oocyte cytoplasm can be distinguished. PI, primordial; PY, primary; S, secondary; G, Graafian follicles. Comparison of NF-κB expression among groups: *p < 0.05 compared to group 2.
Discussion

Ovarian volume and number of follicles increase in women with type 1 diabetes (Codner et al. 2006). Multiple insulin doses can cause hyperinsulinemia, which may stimulate the development of antral follicles and increase the sensitivity of granulosa cells to FSH so that the number and volume of ovarian follicles increase (Fulghesu et al. 1997). Similar increases have been reported in animal models. For example, induced diabetes for 14 weeks caused increased total volumes of the right ovary, cortex and medulla in rats (Mehrjani et al. 2009). Also, induced diabetes for 12 weeks significantly increased the total volume of the right ovary of rats (Farhad et al. 2013). Hyperandrogenism, polycystic ovary morphology and polycystic ovary syndrome are observed increasingly in diabetes mellitus type 1, which is thought to be associated with intensive insulin therapy in humans (Codner et al. 2006) and rats (Poretsky et al. 1992). Ovarian volumes may increase even without exogenous insulin (Mehrjani et al. 2009; Farhad et al. 2013). We found no increase in ovarian volume, which may be because we did not administer insulin or because the 4 week duration of diabetes was too short to cause increased ovarian volume or both.

Synthesis of XIAP in preantral and early antral follicles of healthy female rats is low, but it increases with follicular maturation. The fate of these follicles, either ovulation or atresia, was determined at the preantral and early antral follicle stages. XIAP is regulated by gonadotropins and is essential for normal follicular development (Li et al. 1998). We found both XIAP and NF-κB expression in developing follicles of the control group. XIAP is a potent caspase inhibitor (Deveraux et al. 1998). High concentrations of blood glucose can alter apoptosis in peripheral mononuclear blood cells by decreasing synthesis of Fas and Bax, and increasing XIAP gene expression in diabetic children (Valencia et al. 2012). Arroba et al. (2007) reported that the expression of XIAP in rat anterior pituitary did not change significantly during 4 weeks of diabetes, but increased significantly by 6 weeks of diabetes. We found that XIAP and NF-κB expressions were higher in untreated diabetic ovaries than in the control or N. sativa oil treatment groups, but the increases were not statistically significant. We observed neither increased ovarian volume nor significant increase in the expression of XIAP and NF-κB. One explanation for these results may be the short duration of diabetes.

FSH-induced XIAP expression occurs by the NF-κB pathway with activation of phosphatidylinositol 3-kinase rather than the “classical” IκB kinase pathway (Wang et al. 2002). We found no apparent relation between XIAP and NF-κB expression, however.

NF-κB is expressed in the cytoplasm of many cells from Drosophila to humans. When NF-κB is activated, it translocates to the nucleus. It may regulate the expression of nearly 200 genes associated with immunity, inflammation and growth (Aggarwal 2004). We observed expression of NF-κB in the cytoplasm of oocytes and granulosa cells, and in the oocyte nucleus of only a few primordial follicles.

Hyperglycemia increases production of ROS from the mitochondrial electron transport chain (Nishikawa et al. 2007). Lipid peroxide levels are elevated in diabetic rats treated with STZ (Singab et al. 2005) and MDA is the most common breakdown product of lipid oxidation (Schutt et al. 2003). Oxidative stress is due imbalance between oxidants and antioxidants (Valko et al. 2007). Oxidative stress plays a role in the pathogenesis of endometriosis, tubal factor infertility and unexplained infertility (Agarwal et al. 2005), and it is associated also with polycystic ovary syndrome (Palacio et al. 2006).

We found that MDA levels in the serum were elevated in the diabetic group and decreased in the N. sativa oil treated group. SOD enzyme levels, which participate in protecting tissues against oxidative damage (Kakkar et al. 1995), were increased in the N. sativa oil treated group. Kaleem et al. (2006) reported that N. sativa seeds exhibited anti-diabetic activity and may be useful for controlling complications of diabetes owing to their antioxidant properties in diabetic rats. N. sativa significantly increases SOD levels in the liver and kidney of normal rats (Kaleem et al. 2006). N. sativa also lowers blood glucose levels and increases serum SOD levels (Abdelmeguid et al. 2010).

We found that at the end of our 30 day experiment, N. sativa oil reduced hyperglycemia in diabetic rats, but did not restore normal levels. Antioxidant properties were demonstrated by lowered serum TOS and MDA values and increased SOD and TAS values. Significant differences in total ovarian volume were not observed and no significant relation was observed between XIAP and NF-κB expression, although this may have been due to the short duration of diabetes. N. sativa oil may be useful for reducing blood glucose levels and the effects of oxidants in diabetic patients.

Disclosure statement

No potential conflict of interest was reported by the authors.
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