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Original Research Article

The protective properties of hydro-alcoholic extract of Nigella sativa on male reproductive system in type 2 diabetes rat

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ABSTRACT

Male infertility related to diabetes is under investigation to find a proper therapeutic strategy with minimal side effects. Using *Nigella sativa* as a supplementary agent could be helpful. This study was designed to know *Nigella sativa* effect on hormone profiles and histology of diabetes type II model. 40 male rats (250-300 g) were divided into four groups which diabetic rats received 200 and 400 mg/kg extract of *Nigella sativa* for 90 days. The male hormones profiles, weight and the histopathological configuration were analyzed at end of study. *Nigella sativa* improved the hormones profile in experimental groups. Also, the number of germinal epithelium increased in experimental groups following *Nigella sativa* administration. Although, therapeutic effects of *Nigella sativa* are promising; several preclinical and clinical trials are necessary to optimize its proper dose and time in remedy of male infertility.

***Keywords*:** Diabetes, Nigella sativa, male hormone, spermatogenesis, infertility

INTRODUCTION

Diabetes as a health system threat is the main reason for blindness, renal failure, cardiovascular diseases and nervous system disturbances. It is being predicted that diabetes will be the 7th leading cause of mortality in 2030. Its prevalence has been increased incountries with low and moderate income including Iran [1,2]. On the other hand, non-insulin-dependent or Type 2 diabetes is increasing around the world due to people lifestyle such as consuming high calorie food, absence of physical activity, stressfull life and obesity [3]. The symptoms are similar with diabetes mellitus but the age of occurance decreased and the its prevalence increased among children. It is a priority for health system; however, therapeutic strategies are not efficient in this disease because of several Type 2 diabetes mellitus related disturbances such as renal and cardiovascular [4,5].

Note that, diabetes effects on reproductive systems specially on men's fertility aptitude because of its high sensitivity [6]. It could disturb the spermatogenesis through changes in seminiferous tubules epithelium and hormonal profiles. In addition, it leads to problems in ejaculation and sexual appetency and the glucose metabolism and homeostasis have been disrupted in diabetes. Also the sperm quality including motility, viability, morphology and chromatin integrity and function are decreased dramatically [6].

It is suggested that high production of ROS causes pathologic alternations in male reproductive system in diabetic patients [7]. Appropriate level of ROS has no effect on sperm's functionality; however, at high level, it disturbs the integrity of sperm and leads to abnormal spermogram in diabetic patients. Maintaining the balance between oxidant and antioxidant in the cells is the key point in normal spermatogenesis process. Therefore, in clinic, male fertilization ability can be improved by reducing seminal plasma ROS levels [8-10]. So, it is rational to use antioxidants to address this problem [11].

Applications of herbal medicine which are rich in antioxidants are promising and cost effective. In addition, the side effects and the harm on the cells can be ignored [12-14].

One of these herbal plants is *Nigella sativa* L. (Ranunculaceae) also known [14] as black seed or black cumin and Shouneez in Iran. Its pharmacological properties are described in literature and are effective in cancer, immunological diseases, inflammation and osteoporosis. In addition, it is analgesic, antimicrobial and antiallergic [13]. It is full

of ingredients such as carbohydrates, antioxidants, eight of the nine essential amino acids, fat, vitamins and mineral elements [15]. Four types of alkaloids including vitamin B1, B2, A1 and A2 were extracted from *Nigella sativa*. It is believed that thymoquinone (TQ), dithymquinone (DTQ), nigellone, thymohydroquinone (THQ), and thymol (THY) are its main active ingredients [16].

Of note, it is reported that *Nigella sativa* has protective effect on male's reproductive system and increases the spermatogenesis and systems morphologically and functionally. Thymoquinone, one of the most important component of *Nigella Sativa* can improve male fertility parameters via increase antioxidant defense [15,17]. Therefore, considering high prevalence of diabetes in male population and risk of infertility, the present study aims to study the protective effects of *Nigella sativa* on testis and spermatogenesis in rat diabetes model.

MATERIALS AND METHODS

Nigella sativa seeds Processing (Plant materials and extraction procedure)

The seeds of *Nagila. sativa* were purchased from a local herb market and the taxonomic identification of the seeds was confirmed by a senior plant taxonomist. The extract was prepared according to WHO protocol CG-04 [32]. Briefly, for the preparation of an alcoholic extract, the seeds were dried, powdered and then subjected to Soxhelet apparatus for extraction with 50 % ethanol. The extract obtained was filtered and then evaporated to a dry under reduced pressure which yielded about 8.5 % of solid residue [18].

Animal model of diabetes

Forty adult male wistar rats with 200-250 g weight were purchased from the Pasteur Institute of Iran. Rats were kept under standard conditions for 1 week for adaption to the new environment (ambient temperature of 21±2 °C and ambient light with 12h darkness and 12 h of lightness). The rats randomly are divided to four groups: Group 1: control; Group 2: diabetic rats (received 10 % Fructose for induction of diabetes); Group 3, 4: diabetic rats that received the hydro-alcoholic extract of *Nigella sativa* (200 & 400 mg/kg) [18]. The Rats were fed with gavage for 90 days. Experimental groups 2,3 and 4 received 10 % fructose for induction of diabetes in first 30 days, and then rats in groups 3 and 4 received 200 & 400 mg/kg hydro-alcoholic extract of *Nigella sativa*.

Blood sampling and testis weight measurement

After 90-days, the rats were anesthetized and then sacrificed with diethyl ether. The blood samples were taken directly from their hearts, and then the left testis were removed and weighted.

Testis histopathological evaluation

In order to examine the testis histological changes, the left testis was fixed for 7 days in a Modified Davidsons Fluid (MDF) solution containing 30 % formaldehyde, 15 % ethanol, 5 % glacial acetic acid and 50 % distilled water. Then the testis was washed with PBS solution and was cut into smaller pieces. The MDF-fixed testis were dehydrated, and embedded in paraffin.

5µm sections were prepared and at least 5 slides from each testis were stained for histopathological evaluation. Heiden Hain Azan method was used for tissue samples staining. The process was done according to the routine tissue staining methods. In order to evaluate testis tissue histopathological changes, 4 slices were assessed from each animal. 5 microscopic fields from each slice were observed at 400× magnification using a standard light microscope (Olympus, Japan). Spermatogonia, Sertoli and Leydig cells were observed by the light microscope and their images were prepared by a digital camera (BX 51 Japan) calibrated with a hemocytometer slide share attached to the microscope. Germinal epithelium area, seminiferous tubules diameter and number of seminiferous tubules and interstitial cells were measured using Image J 1.44P software (National institute of Health, USA).

Serum hormones concentration

Immediately after anesthetizing, the rats' blood samples were taken directly from their heart to determine the serum concentration of testosterone, LH and FSH at end of the study. Then, the blood samples were centrifuged at 3000 rpm for 20 min. Hormones levels were measured using an ELISA kits (Abcam, USA). All reagents, standards and samples were prepared according to the instructions presented in the kit.

Statistical analysis

The raw data was used to calculate the mean and standard deviation between the experimental and control groups with a significant level of 0.05 in SPSS software version 18. ANOVA and Tukey's test were used to compare the parameters (testicular weight, testosterone, LH and FSH concentrations and number of spermatogonies, leydig and sertoli cells). First Non-parametric test (Kruskal-wallis H) was used to compare the histopathological grades (diameter of cells and the area of germinal epithelium), moreover if test had significant error the non-parametric test

(Mann-whitney U) with modification of pairwise comparisons (To control the first type error) was used. The significant difference was considered to be at 0.05 level. Kolmogorov-smirnov test was used to determine the normal distribution of data.

RESULTS

The weight of different groups was recorded at the end of this study. It is shown that there were not a statistically significant difference between the testis groups in terms of weight (p=0.676) (Table 1). Hormones profiles in different groups were evaluated as well. Our findings indicate significant differences in serum testosterone level in the groups. However, no significant difference was observed between control and experimental group 1 and 2 (p=0.37, p=0.26, respectively). 400mg/Kg N. sativa seeds extract changed significantly the serum testosterone level compared to control group (p<0.05).

Table 1. Comparison of testis weight and serum hormone concentrations in the control and diabetic groups treated by N. sativa L

Groups / **Parameters**	**Control** mean± SD	Experimental 1 mean± SD	Experimental 2 mean± SD	**Experimental 3** mean± SD	**P value**
Testicle weight (g)	1.175 ± 0.532	1.098±0.363	1.114 ± 0.439	1.297 ± 0.163	0.676
Testosterone (ng/ml)	5.097 ± 1.231	4.326±0.787 [a,d]	5.981±1.041c [b]	8.149±1.114 [c]	0.0001
LH(ng/ml)	0.249±0.052	0.213±0.067	0.273±0.074	0.256±0.071	0.072
FSH(ng/ ml)	0.222±0.055	0.194±0.038[d]	0.238±0.034	0.278±0.052[c]	0.0002

[a:] P<0.05 experimental 1 compared with Experimental 2;[b:] P<0.05 experimental 2 compared with Experimental 3;[c:] P<0.05 control compared with Experimental 3; [d:] P<0.05 experimental 1 compared with Experimental 3

Table 2. Comparison of number of testis cells in different groups treated with N. sativa L

Groups / Parameter	Control mean± SD	Experimental 1 mean± SD	Experimental 2 mean± SD	Experimental 3 mean± SD	P value
N of Spermatogonia	41.586±13.080	36.809±17.193 [b]	50.742±7.521	66.602±7.508[a]	P<0.0001
N of Primary spermatocyte	66.602±7.508	56.856±8.274[b, c]	78.604±7.691[d]	90.796±6.190 [a]	P<0.0001
N of spermatid	66.214±9.232	56.893±8.433 [c]	76.486±8.762 [d , f]	92.156±5.318 [a]	P<0.0001
Sertoli cell number	22/053±7/292	21/188±5/504	23/777±8/017	23/937±7/913	P=0/795
Leydig cell number	16/117±3/810	15/617±5/575	17/579±8/039	17/980±9/274	P=0/852

[a:] P<0.05 control compared with Experimental 3;[b:] P<0.05 experimental1 compared with Experimental 3;[c:] P<0.05 experimental1 compared with Experimental 2;[d:] P<0.05 experimental2 compared with Experimental 3;[f:] P<0.05 control compared with Experimental 2.

In addition, our findings revealed that N. sativa seeds have significantly increased the plasma concentration of FSH in comparison with the control group (P=0.002) (Table 1). Moreover, significant lower values of this parameter were not significant in diabetic group compared with the control groups (P=0.53) (Table 1). Of noted, N. sativa seeds extract did not significantly change in serum LH level in (P=0.072) in experimental groups.

The spermatogenesis was evaluated in germinal epithelium with counting of spermatogonia. It was shown a significant difference between the mean of spermatogonies numbers in all groups

(P=0.0001). This difference was detected between control and experimental groups 3 (P=0.004) and experimental groups 1 and 3 (p<0.0001) (Table 2). On the other hand, the number of primary spermatocyte in experimental group 2 and 3 were statistically increased compared to control and diabetic groups (P<0.0001). As shown in table2 the number of primary spermatocyte in diabetic group were decreased (P=0.005). In addition, our results showed a significant difference between number of spermatids between control and diabetic rats (P=0.035), and experimental group 2 and 3 (P=0.001). More interestingly, the number of sertoli and leydig cells increased in groups received N. sativa seeds extract but not significantly (P=0.79, P=0.85) (Figure 1).

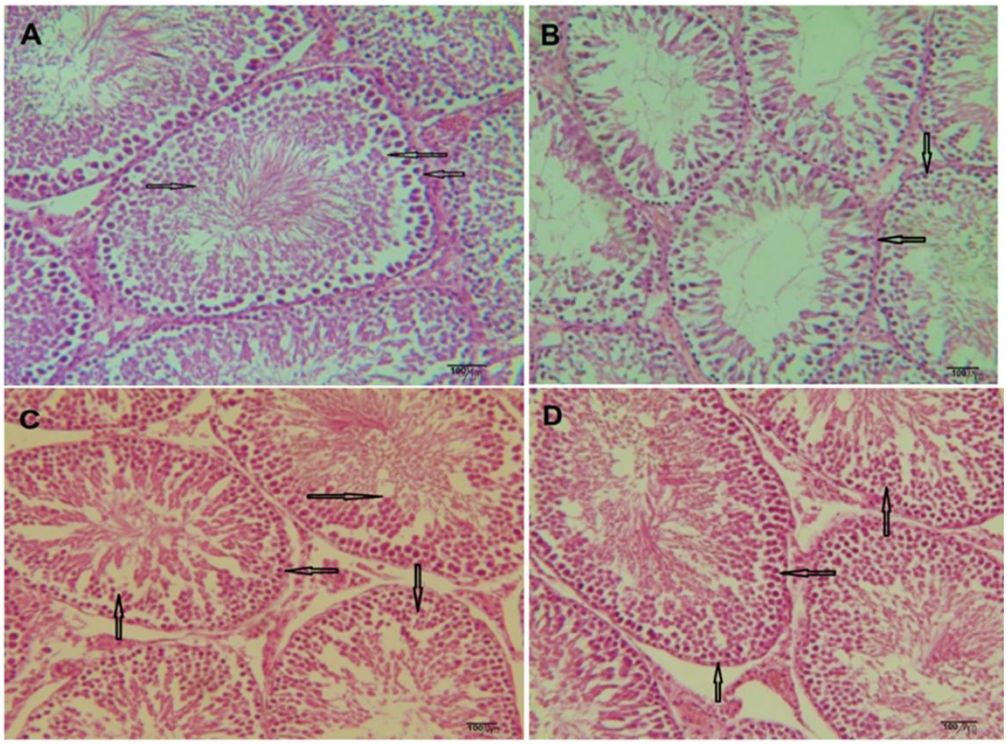

Figure 1. Microscopic image of testicular tissue sections in different groups, (400x Magnification) (Scale bar: 100 microns) (Staining method: Heiden Hain Azan). **A**: Control group (received distilled water). **B**: Experimental group 1 (received 10 % Fructose), the number of spermatogonies, spermatids and primary spermatocytes has reduced.**C**: Experimental group 2 (received 10% Fructose and 200 mg/kg extract of Nigella sativa): the number of spermatogonies, spermatids and primary spermatocytes has increased. **D**: Experimental group 2 (received 10 % Fructose and 400 mg/kg extract of Nigella sativa): the number of spermatogonies, spermatids and primary spermatocytes has improved similar to control group.

DISCUSSION

Supplementary medicine helps to improve the fertility in men and female. In our study, *Nigella sativa* with its several nourishment materials not only enhanced the hormone profiles in rat male testis but also improved the spermatogenesis and seminiferous tubules epithelium proliferation after 90 days of treatment. Administration of dietary antioxidants may maintain the testis blood barrier and protect the cells from reactive oxygen [19]. They also support the sperm during their development, migration and appropriate function.

Nigella sativa has antioxidants profiles and thiol components protect the sperms from oxidative stress [15]. The weight of the testis in our study were increased in insulin resistant group III when received 400mg/kg for 60 days. Like or study it is reported that 200 and 300 mg/ml *Nigella sativa* increased the weight of testis and another male reproductive system glands in diabetic and normal rats[15,20]. It the other two experimental groups did not experienced significant weight gain due to low concentration of the *Nigella sativa.* It is suggested that the *Nigella sativa* extract acts as a dose dependent manner. The weight of the testis is androgen dependent so that in group III with significant increase in testosterone level, the testis weight increased significantly.

The spermatogenesis may be disturbed in situations of insulin resistance disease. The studies demonstrate that the molecules such as ROS disturb the endocrine system attack to the biomolecules such as DNA and proteins [21]. It is rational to imagine that the germinal epithelium including spermatogonia, primary and secondary or spermatids are living in a stressful harsh environment. It has been reported that *Nigella sativa* through increasing insulin levels and stimulation of leydig cells may increase the level of serum testosterone [22]. There is also report indicating that *Nigella sativa* oil can improve sperm parameters in infertile men due to inactivate free radicals [21].

Using complementary medicine with a variety of vitamins and antioxidants may improve the sperm quality and increase the hormones gonadotropins, testosterone and their receptors. Therefore, application of these molecules including *Nigella sativa* is promising to help with infertility. It is reported that *Nigella sativa* has more than 100 constituents. One of its main molecules is Thymoquinone (TQ) [23]. Shikhbahaei

et al showed that TQ could increase and protect the seminiferous tubules cells against side effects of Methotrexate [24]. It increased the quality of sperm parameters. It is suggested that TQ is protective even in the female reproductive system when exposed to ROS [25]. TQ increases the sperm quality and parameters in the animals with fat diet. It means that TQ could be used in obese cases with infertility [26]. Researchers treated the Sterptozotocine -induced diabetes rat with *Nigella sativa* seed and Thymoquinone. They found that LH and testosterone hormones increased after this treatment [17]. In our study use of different doses of *Nigella sativa* also improved hormone profiles in diabetic rats (P=0.0001).

Fortunately, there are well-known researches that confirm the protective and therapeutic properties of active component of *Nigella sativa*. Now this is Thymoquinone is knocking at the door of clinical trial [21,27]. It is interesting to know *Nigella sativa* not only acts as cytoprotective agent but also is a spermicide. In a study, the authors tried to know spermicidal effect of *Nigella sativa* component TQ as the result .They found that it will be used instead of Nonoxynol-9 a nonionic surfactant to avoid unwanted pregnancies [28].

CONCLUSION

Our results showed *Nigella sativa* can improve male reproductive system parameters in the experimental groups such as: the number of spermatogonia, spermatids, sertoli and leydig cells. Although, therapeutic effects of *Nigella sativa* are promising, several preclinical and clinical trials are necessary to optimize its proper dose and time in remedy of male infertility.

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