



Research Article

Effects of Thymoquinone on Sperm Parameters, Apoptosis, Testosterone Level, and Oxidative Stress in a Mouse Model of D-Galactose-Induced Aging

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ABSTRACT

Background: The aging process is accompanied by low secretion of sex hormones and testicular apoptosis. The antioxidant properties of thymoquinone (TQ) may prevent the effects of aging. Therefore, in the present study, the effects of different doses of TQ were investigated on sperm parameters, testosterone level, apoptosis, and oxidative stress in a mouse model of D-galactose-induced aging.

Methods: In this experimental study, 30 adult male mice were randomly divided into 5 groups. The control group did not receive any injections, while the D-galactose group received an intraperitoneal injection of 300 mg/kg of D-galactose for 42 days. The TQ1-TQ3 groups received intraperitoneal injections of 5, 2.5, and 1.25 mg/kg of TQ plus D-galactose, respectively for 14 days (from the 1st to the 14th day of the experiment). The morphometric analysis, testicular apoptosis examination, and sperm analysis were performed, and testosterone level, total antioxidant capacity, and malondialdehyde level were evaluated on day 42 of the experiment. Data were analyzed using SPSS.

Results: Administration of TQ in the TQ1 group caused a significant difference in sperm parameters, compared to the D-galactose group ($P < 0.05$). The lowest amount of positive tunnel cells was related to 5 mg/kg of TQ and the highest to 2.5 mg/kg of TQ. There was no significant difference in the parameters of seminal vesicles, epididymis, prostate, and testis between the groups ($P > 0.05$). The malondialdehyde level were decreased in the TQ1-TQ3 groups, compared to the D-galactose group ($P < 0.001$). On the other hand, the total antioxidant capacity was increased significantly in the TQ1 group, compared to the D-galactose group ($P < 0.001$).

Conclusion: Administration of 5 mg of TQ for 14 days improved sperm quality and biochemical parameters, while reducing apoptotic cells of the testes in a mouse model of aging.

Introduction

Aging is a gradual biological process, associated with biochemical and morphological changes in biological systems.¹⁻³ This process causes changes in the capacity of organs and tissues. Various factors, such as environmental conditions, nutrition, and lifestyle, are important in the process of aging. Among different theories proposed for aging, production of free radicals and induction of oxidative stress in tissues are among the most important ones.¹⁻³

Nigella sativa L. (black seed), a herbal medicine from the Ranunculaceae family, is native to Southwest Asia.

Seeds of this plant are used as spices in traditional medicine. In addition, seeds had been subject of interest in pharmacological studies and several therapeutic and antioxidant activities. Thymoquinone (TQ) is the main active ingredient of *Nigella sativa*, which is responsible for most of the biological activity of this plant. In various studies, antioxidant, antiischemic, antiinflammatory, analgesic, anticonvulsant, and anticoagulant effects of TQ have been mentioned.⁴⁻⁷ In addition, studies showed that black seeds could have positive effects on sperm parameters, leydig cells,

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reproductive organs, and sex hormones in infertile men.^{8,9}

In a previous study, administration of diosgenin mildly improved sperm motility in a D-galactose-induced aging model.¹⁰ Also, administration of cysteine for 75 weeks improved sperm parameters in old rats, compared to the control group.¹¹ The results of some other studies showed that a certain dose of *Silybum marianum* extract could reduce inflammatory factors and lipid peroxidation and increase sex hormones.¹² Considering the importance of aging process and its impact on various biological systems in the body, study of factors having protective effects on this process seems to be of necessity.

According to our search, no study has been performed so far on the effects of TQ on sperm parameters, testicular structure, and sex hormones in the elderly. Therefore, the present study investigated the effects of TQ on sperm parameters, testosterone level, apoptosis, and oxidant and antioxidant levels in an aging model on mouse induced by D-galactose, which is one of the most accepted animal models of aging.^{13,14}

Materials and Methods

Animals

In this experimental study, after obtaining approval from the Ethics Committee of Mashhad University of Medical Sciences (code, 941314), a total of 30 adult male BALB/c mice (weight, 30-35 g) were purchased from the animal house of the faculty of medicine and maintained under standard conditions (room temperature, 22±2°C; humidity, 50±5%) in a 12:12 h light/dark cycle. The animals had free access to water and food during the study and were randomly divided into 5 groups: control, D-galactose, TQ1, TQ2, and TQ3.

The control group did not receive any injections, while the D-galactose group intraperitoneally received 300 mg/kg of D-galactose for 42 days.¹⁵ In addition, the TQ1-TQ3 groups received D-galactose intraperitoneally plus 5, 2.5, and 1.25 mg/kg of TQ (Sigma, USA), respectively for 14 days (from the 1st to the 14th day of the experiment). Subsequent analyses were performed 42 days after the initiation of injections.

Sperm analysis

In order to evaluate sperm parameters, tail of the epididymis was isolated. The crushed pieces were placed in normal saline and added to a CO₂ incubator for 30 to 45 minutes. After removal from the incubator, sperm motility, count, and morphology were evaluated, based on the World Health Organization (WHO) guidelines using a Neubauer hemocytometer. In addition, sperm viability was evaluated via eosin B staining.¹⁶

Evaluation of apoptosis in the testis

The *in situ* cell death detection kit, POD (Roche Inc.), was used in this study (No., 5301968). Following a routine process, serial sections with a thickness of 5 µm were placed on a poly-lysine slide. Then paraffin degradation,

the tissue was placed in descending grades of alcohol and incubated with H₂O₂ plus methanol. Subsequent washing with protein kinase, 50 µL of tunnel dye solution was incubated at 4°C for 24 hours. After washing with phosphate-buffered saline, the convertor-POD solution was added. Following that, diaminobenzidine (DAB) was added. Next washing with water, it was passed through ascending grades of alcohol, as well as xylene, and coverslipped.

Evaluation of positive tunnel cells in tissue sections

The numerical density of positive tunnel cells was calculated as frame per unit area. The tissue sections of different groups were imaged with an optical magnifier (Olympus Optical Microscope BX51, Japan) at 40× magnification. The numerical density of positive tunnel cells was counted in the unit area. The mean number of different cell types (spermatogonia, primary spermatocytes, and spermatids) was calculated per unit area of sections, using the following formula:

$$N_A = \frac{\sum \bar{Q}}{a/f \cdot \sum p} \quad \text{Eq.(1)}$$

where N_A is the number of cells per unit area, $\sum Q$ is the total number of counted cells, a/f denotes the surface area of each counting frame, and $\sum p$ refers to the total number of acceptable frames colliding with the cross section.

Thickness of the germinal epithelium

After examining the tissue sections from different groups at 10× magnification, the mean height of the germinal epithelium was determined using the following formulas:

$$H = \frac{V_V}{S_V} \quad \text{Eq. (2)}$$

$$V_V = \frac{\sum_{i=1}^n P(x)}{\sum_{i=1}^n P(\text{total})} \quad \text{Eq. (3)}$$

$$S_V = 2 \times \frac{\sum_{i=1}^n l_i}{l/p \times \sum_{i=1}^n p_i} \quad \text{Eq. (4)}$$

where $\sum I$ is the total collision point of the probe line with the luminal level of the germinal epithelium, $\sum p$ denotes the total points of contact with the testicular tissue, and l/p is the length of the probe line on the actual scale of the tissue.^{17,18}

Testicular volume and indices of reproductive organs

The testicles, epididymis, prostate, and seminal vesicles were weighed, divided by the animal weight, and multiplied by 100 to determine the percentage. In order to calculate the testicular mass, the following formula was used:

$$V = \frac{(w_2 - w_1)}{\delta} \quad \text{Eq.(5)}$$

where W_2 is the testicular weight, W_1 is the dry weight of the testicle, and δ is the density of normal saline solution.

Table 1. Comparison sperm parameters in experimental groups.

Group	Viability rate (%)	Motility rate (%)	Sperm count (million/ ml)	Abnormal morphology rate (%)
Control	87.16 ± 4.26	75.33 ± 3.77	4.63 ± 0.37	15.34 ± 5.35
D-galactose	70.16 ± 3.71*	49.33 ± 6.28*	3.85 ± 0.18*	32.17 ± 4.87*
TQ1	76.00 ± 4.04*	64.00 ± 3.22*^	4.25 ± 0.18*^	26.00 ± 4.93
TQ2	69.83 ± 1.32*	56.00 ± 4.73*	3.85 ± 0.18*#	32.00 ± 4.42*
TQ3	69.50 ± 6.53*	55.33 ± 6.25*#	3.78 ± 0.11#	29.5 ± 5.95*

Control group received no injection

D-galactose group received 300 mg/kg d-galactose for 42 days

Experimental group1 received 300 mg/kg d-galactose + 5 mg/kg thymoquinone

Experimental group2 received 300 mg/kg d-galactose + 2.5 mg/kg thymoquinone

Experimental group3 received 300 mg/kg d-galactose + 1.25 mg/kg thymoquinone

* (p<0.001): Significant difference with control group within same column by tukey post-hoc test

(p<0.05): Significant difference with experimental1 group within same column

^ (p<0.05): Significant difference with d-galactose group within same column

Measurement of testosterone level

After obtaining blood samples from the heart, blood serum was separated and maintained at -20°C. The level of testosterone was measured via enzyme-linked immunosorbent assay (ELISA; Elsys 2010, England) and testosterone ELISA kit (cat number: ab174569; Abcam). The electrochemiluminescence (ECL) tests were randomized for controlled results.

Measurement of total antioxidant capacity (TAC)

The amount of antioxidants were measured using the ferric-reducing ability of plasma (FRAP) assay. Nearly 1.5 mL of FRAP solution was completely verified with 50 µL of the standard and unidentified samples. After incubation for 10 minutes, optical absorption was read at a wavelength of 593 nm, and FRAP values were calculated as µmol per liter.²⁰

Measurement of malondialdehyde (MDA) level

The testicular tissue was homogenized by a homogenizer. Then, 1 mL of homogeneous tissue solution was mixed with 2 mL of thiobarbituric acid (TBA), trichloroacetic acid, and hydrogen chloride (HCL) solution. Following that, it was placed in a boiling water bath for 45 minutes. After cooling down, it was centrifuged at 1000 rpm for 10 minutes, and absorption was read at 535 nm, using a spectrophotometer. The concentration of MDA is presented in nmol/g.¹⁹

Data analysis

Data were entered into SPSS version 16 and expressed as percentage and mean±standard deviation. The normal distribution of data was evaluated by Kolmogorov–Smirnov test. Data were quantitative and normally distributed. Analysis of variance (ANOVA) and Tukey’s tests were performed to compare the groups. The significance level was considered as P<0.05.

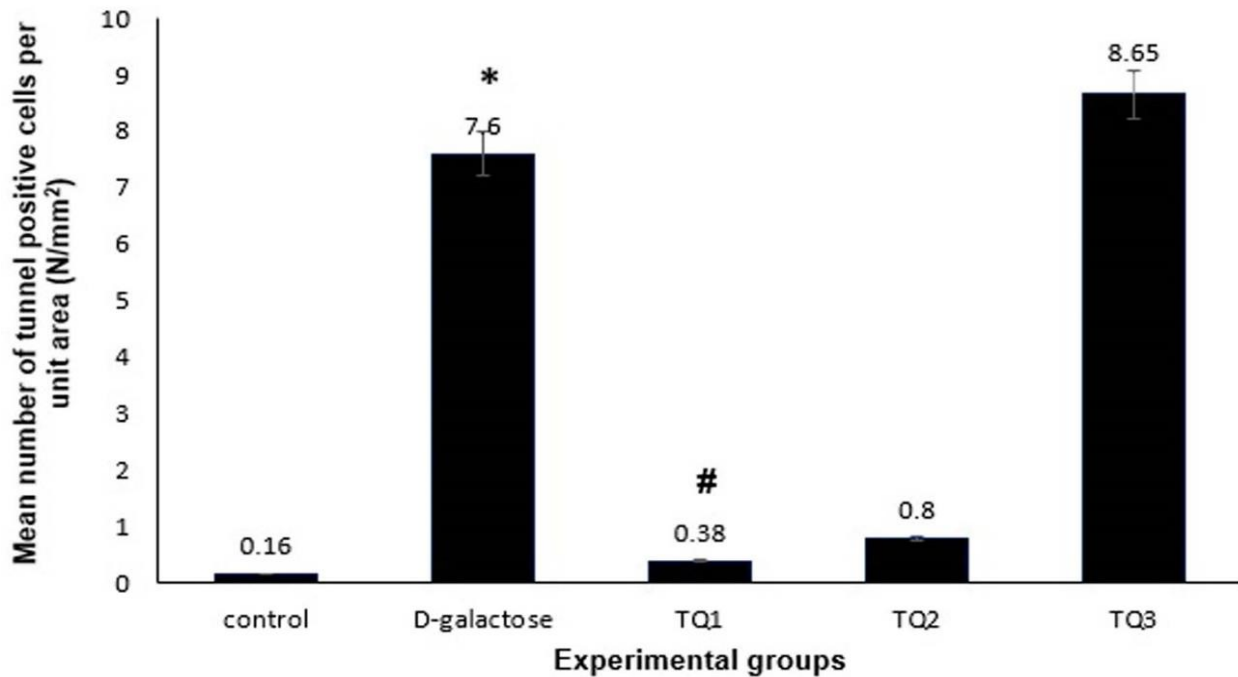


Figure 1. Mean number of positive tunnel cells in different groups

* (p<0.05): Significant difference with control group

(p<0.05): Significant difference with D-galactose group

Results

Sperm analysis

According to Table 1, the mean sperm count was 4.63 ± 0.37 million/mL in the control group, while it was significantly decreased in the D-galactose group after receiving 300 mg/kg of D-galactose (4.63 ± 0.37 vs. 3.85 ± 0.18). Administration of 5 mg/kg of TQ caused an increase in sperm count in comparison with the D-galactose group ($P < 0.05$); however, the increase was not significant, compared to the controls ($P > 0.05$).

Sperm count in the TQ1 group was increased significantly in comparison with the TQ2 and TQ3 groups ($P < 0.05$). The lowest sperm motility was observed in the D-galactose group (49.33%), while the highest rate of sperm motility was reported in the TQ1 group (64%). Sperm motility was also significantly different in the TQ3, D-galactose, and control groups ($P < 0.05$). Administration of 5 mg/kg of TQ, resulted the highest viability rate (76%) and normal sperm morphology (74%). However, the difference was not significant comparing with the control group ($P > 0.05$).

As presented in Figure 1, the mean number of positive tunnel cells in the D-galactose group was significantly higher than the control group. Administration of TQ in the TQ1-TQ3 groups reduced the number of positive tunnel cells in comparison with the D-galactose group (Figure 2).

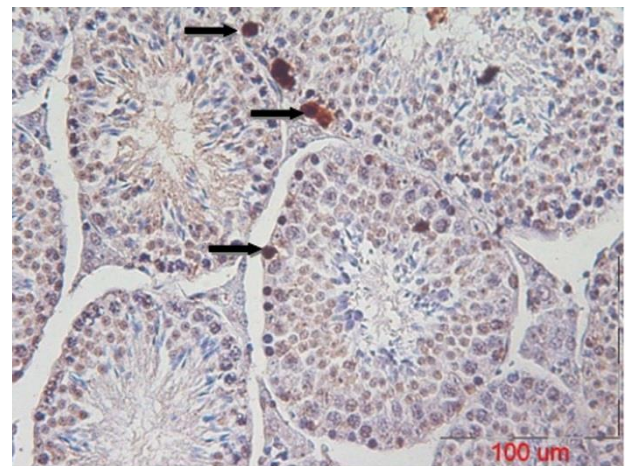
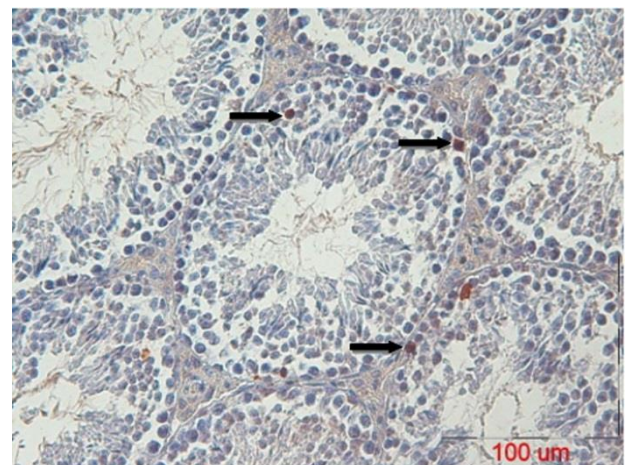
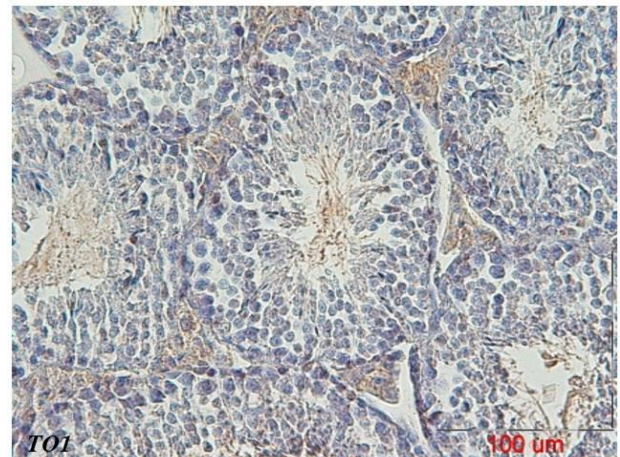
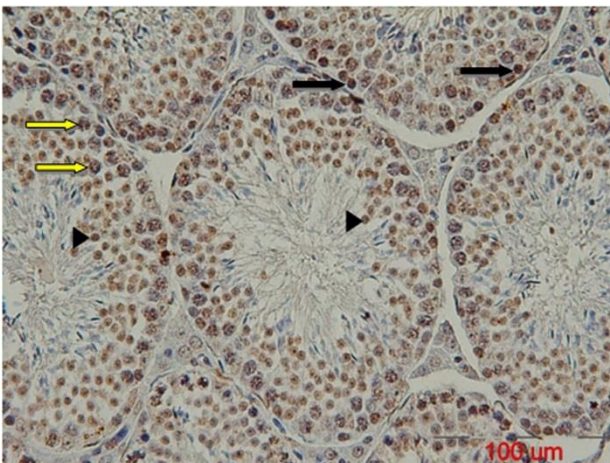
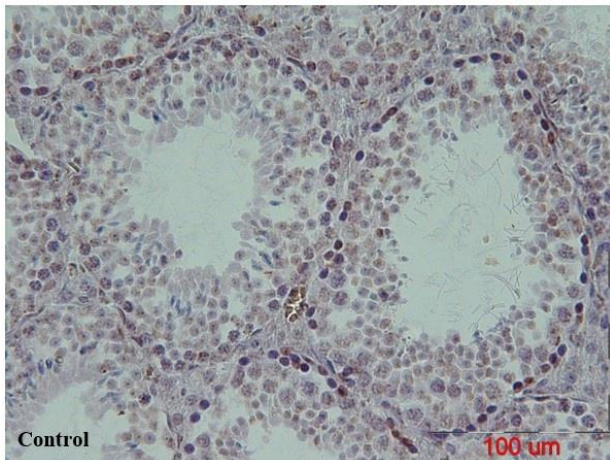


Figure 2. Transverse section of seminiferous tubules in which apoptotic cells are examined by the tunnel kit. The black arrow shows the spermatogonia, the yellow arrow the primary spermatocyte and the tip of the arrow shows the spermatoid. Magnification is 400 x.

Control group received no injection

D-galactose group received 300 mg/kg d-galactose for 42 days

Experimental group1 received 300 mg/kg d-galactose + 5 mg/kg thymoquinone

Experimental group2 received 300 mg/kg d-galactose + 2.5 mg/kg thymoquinone

Experimental group3 received 300 mg/kg d-galactose + 1.25 mg/kg thymoquinone

The lowest number of positive tunnel cells was related to 5 mg/kg of TQ and the highest to 2.5 mg/kg of TQ. Significant differences were observed between the D-galactose and TQ1 groups ($P < 0.05$).

Morphometric results

The morphometric results of reproductive organs are summarized in Table 2. As can be seen, thickness of the germinal epithelium decreased in the D-galactose group, compared to the controls, although it was not statistically significant ($P > 0.05$). Among the study groups, the greatest mean thickness of the germinal epithelium was reported in the group receiving 5 mg/kg of TQ, while the lowest thickness was attributed to the group receiving 1.25 mg/kg of TQ. There was no significant difference in the parameters of seminal vesicles, epididymis, prostate, and testis between the groups ($P > 0.05$).

Testosterone level, MDA level, and TAC

Table 3 presents the effects of different doses of TQ on testosterone level and serum biochemical parameters in male mice. The level of testosterone was significantly lower in the D-galactose group, compared to the controls ($P < 0.001$). On the other hand, testosterone levels were significantly higher in the TQ1 and TQ2 groups, compared to the D-galactose group ($P < 0.001$). In addition, testosterone level was higher in the TQ3 group, compared to the D-galactose group; however, the difference was not statistically significant ($P > 0.05$).

The MDA level was significantly lower in the TQ1-TQ3 groups, compared to the D-galactose group ($P < 0.001$). On the other hand, the MDA level was significantly higher in the TQ1-TQ3 groups, compared to the controls ($P < 0.001$). The MDA level showed a significant reduction in the TQ1-TQ3 groups, compared to the D-galactose group ($P < 0.01$). Moreover, TAC showed a significant reduction in the TQ1-TQ3 groups, compared to the controls ($P < 0.001$). On the other hand, TAC showed a significant increase in the TQ1 group, compared to the D-galactose ($P < 0.001$), TQ2 ($P < 0.01$), and TQ3 ($P < 0.01$) groups.

Discussion

Administration of TQ in the TQ1 group caused a significant difference in sperm parameters, compared to the D-galactose group. The lowest number of positive tunnel cells was related to 5 mg/kg of TQ and the highest to 2.5 mg/kg of TQ. There was no significant difference in testicular volume, epididymal index, seminal vesicles, prostate, and testes between the groups. However, the level of testosterone was significantly lower in the D-galactose and TQ3 groups, compared to the controls. The MDA level were decreased in the TQ1-TQ3 groups, compared to the D-galactose group, while TAC significantly was increased in the TQ1 group versus the D-galactose group.

Table 2. Effect of thymoquinone on thickness of germinal epithelium, volume of testis, index of testis, epididymis, vesicle seminal and prostate.

Group	Prostate index	Vesicle seminal index	Epididymis index	Testis index	Testis volume (mm ³)	Thickness of germinal epithelium (μm)
Control	0.07 ± 0.02	0.07 ± 0.03	0.23 ± 0.05	0.11 ± 0.00	0.11 ± 0.00	62.32 ± 12.86
D-galactose	0.13 ± 0.02	0.13 ± 0.07	0.32 ± 0.03	0.13 ± 0.01	0.13 ± 0.01	39.85 ± 7.55
TQ1	0.06 ± 0.05	0.06 ± 0.02	0.27 ± 0.14	0.12 ± 0.01	0.12 ± 0.19	65.38 ± 13.59
TQ2	0.06 ± 0.04	0.06 ± 0.02	0.34 ± 0.13	0.12 ± 0.01	0.12 ± 0.14	49.76 ± 19.11
TQ3	0.09 ± 0.03	0.45 ± 0.23	0.16 ± 0.00	0.33 ± 0.05	0.12 ± 0.01	43.38 ± 25.82

Control group received no injection

D-galactose group received 300 mg/kg d-galactose for 42 days

Experimental group1 received 300 mg/kg d-galactose + 5 mg/kg thymoquinone

Experimental group2 received 300 mg/kg d-galactose + 2.5 mg/kg thymoquinone

Experimental group3 received 300 mg/kg d-galactose + 1.25 mg/kg thymoquinone

($p > 0.05$): No significant difference between groups by ANOVA and tukey post-hoc test

Table 3. Comparison level of testosterone hormone, malonaldehyde, and total antioxidant capacity in testis of experimental groups.

Group	Total antioxidant capacity (μmol/L)	MDA (nanomol/g tissue)	Testosterone hormone (ng/m)
Control	44.87 ± 7.00	5.79 ± 0.27	8.06 ± 0.44 ^b
D-galactose	20.04 ± 2.46 ^a	15.20 ± 1.01 ^a	0.6 ± 0.17
TQ1	30.97 ± 1.08 ^{ab}	7.71 ± 0.33 ^{ab}	8 ± 6.3 1.92 ^b
TQ2	23.59 ± 1.42 ^a	9.80 ± 0.79 ^{ab}	7.00 ± 1.60 ^b
TQ3	20.06 ± 1.95 ^{ac}	12.16 ± 0.39 ^{ab}	3.80 ± 0.18 ^{acd}

Control group received no injection

D-galactose group received 300 mg/kg d-galactose for 42 days

Experimental group1 received 300 mg/kg d-galactose + 5 mg/kg thymoquinone

Experimental group2 received 300 mg/kg d-galactose + 2.5 mg/kg thymoquinone

Experimental group3 received 300 mg/kg d-galactose + 1.25 mg/kg thymoquinone

TAC: Total antioxidant capacity MDA: Malonaldehyde

$p \leq 0.001$ a: Significant difference with control group within same column by ANOVA and tukey post-hoc test

$p < 0.05$ b: Significant difference with d-galactose group within same column

$p \leq 0.001$ c: Significant difference with experimental 1 group within same column

$p \leq 0.001$ d: Significant difference with experimental 2 group within same column

Testosterone hormone was detected in blood serum. MDA and TAC were found in the tissue.

Parandin et al. reported that administration of 200 and 400 mg/kg of black seed extract for 60 days increased fertility, gonadotropin level, and testosterone level in male rats. In addition, genital weight, sperm motility, sperm viability rate, and sperm count improved after treatment.²¹ In this regard, Mahdavi et al. conducted a systematic review on the effects of black seed and TQ on male infertility. They concluded that black seed could have positive effects on sperm and semen parameters, leydig cells, reproductive organs, and sex hormones.²²

In the mentioned studies, the positive effects of black seeds on male fertility were reported. In the present study, these positive effects were observed in an aging mouse model. TQ administration at a dose of 5 mg/kg improved sperm parameters and increased the thickness of the germinal epithelium, compared to the D-galactose group; the lowest number of positive tunnel cells was related to 5 mg/kg of TQ. There was no significant difference in the reproductive organ index between the groups and the controls; however, the level of testosterone was significantly higher in the D-galactose group, compared to the controls.

To the best of our knowledge, there is no report regarding the effects of black seeds or TQ on the spermatogenesis of aging in male mice or humans. In this regard, Gokce et al. reported that administration of 10 mg/kg of TQ significantly improved sperm quality and testicular structure of mice after methotrexate-induced testicular injury.²³ Additionally, Alyoussef et al. examined the effects of 50 and 25 mg of TQ in mice for 85 days after toxication with sodium nitrite. The results showed that TQ improved sperm motility, testosterone level, luteinizing hormones, and follicle-stimulating hormones.²⁴

Moreover, Awadalla et al. investigated the effects of black seed on oxidative stress in adult rats. The results showed that administration of 0.5 mg/kg of black seeds improved the histopathology of testis after 21 weeks of treatment.²⁵ In another study, the effects of 50 mg/kg of TQ were investigated on damage to mitochondria, endoplasmic reticulum, and sertoli cells in mice, and TQ could reduce damage in testicular tissues.²⁶

In this study, use of aging mice was preferred to an animal model of aging; however, it was not possible due to restrictions in storage conditions. In addition, use of transgenic mice was not possible. It seems that administration of TQ improved testicular damage due to aging via reducing oxidative stress and increasing the testosterone level. Another possible mechanism was that TQ may affect genes involved in apoptosis, thereby contributing to changes in gene expression or reducing apoptosis. In addition, TQ influenced the level of MDA, as well as the antioxidant system, and reduced oxidative stress.

In future studies, investigation of the exact mechanism of TQ treatment is recommended in aging mouse models. It is also suggested to prescribe TQ for older men. It should be noted that the effects of black seed or TQ administration on spermatogenesis were not investigated

in old subjects in our study. Therefore, it is recommended to conduct future studies on groups with normal saline injections to evaluate the effects of stress and injection in mice.

Conclusion

Administration of 5 mg of TQ for 14 days improved sperm quality and biochemical parameters and reduced apoptotic cells of the testis in a mouse model of aging. The level of testosterone was significantly higher in the TQ groups, compared to the D-galactose group. Based on the results, it seems that black seed or TQ is suitable for the treatment of infertility. However, further research is necessary to determine the effects of these compounds on infertile men, especially older men.

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Conflict of Interests

The authors claim that there is no conflict of interest.

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