



Research Article

Effects of Bene (*Pistacia atlantica*) on Histopathology of Testis, Sperm Chromatin Quality and Stress Oxidative in Busulfan-Induced Infertile Mice

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ABSTRACT

Background: Some plants stimulate spermatogenesis and increase fertility, while some cause spermatogenesis arrest. So far, the effects of bene (*Pistacia atlantica*) on male fertility have not been studied. The aim of this study was to investigate the effects of bene on sperm parameters, testicular histopathology, sperm quality, and oxidative stress in busulfan-induced infertile mice.

Methods: Thirty-five male BALB/c mice were randomly assigned to control, sham, busulfan, bene, and bene + busulfan groups. The busulfan group received 10 mg/kg as a single dose and intraperitoneally. The bene group received pellets containing 10% of bene. Another group received 10 mg/kg busulfan and was fed with pellet containing 10% bene. Then, sperms, sperm chromatin quality, testicular histopathology, and oxidative stress levels were studied on the 35th day of the experiment.

Results: Busulfan injection resulted in a significant reduction in sperm parameters compared to the control group ($p < 0.001$); it decreased after bene administration ($p < 0.001$). In addition, in the group treated with bene, the sperm count with damaged DNA was reduced and the level of malondialdehyde decreased compared to the busulfan group. A significant increase was observed in the mean level of superoxide dismutase and catalase enzymes in the bene + busulfan group compared to the busulfan group ($p < 0.001$). The histopathological improvement of the testis was observed in the bene + busulfan group.

Conclusion: The administration of 10 mg/kg of bene powder for 35 days reduced the oxidative stress, improved testicular histopathology, sperm chromatin quality, and sperm parameters in the infertile mice model.

Introduction

Infertility is one of the important issues in medical science. A couple being unable to achieve pregnancy after one year of intercourse without the use of contraceptives can be considered to be showing signs of infertility.¹ In 40% of couples, infertility is related to men, and as sperm disorders are the causes of male infertility; the importance of correcting these disorders is necessary to help the fertility of the couples.¹ In recent years, treatment with medicinal herbs with the lowest cost and side effects has been considered.² Some plants stimulate spermatogenesis and increase fertility, while some cause spermatogenesis arrest.²

Pistacia atlantica is a species of pistachio, which has strong antioxidant properties due to the presence of phenolic compounds (phenolic acids, flavonoids and tannins).³⁻⁶ Bene can be found on the Mediterranean

coast, Turkey, Iran, Central Asian countries, and Africa. This plant grows extensively in Iran between Kurdistan and the Fars Provinces. Bene is used in treating throat infection, kidney stones, asthma, as well as in the treatment of hypertension, coughing, stomachache, and jaundice.³⁻⁶ In addition, it has antibacterial, anti-fungal, and anti-parasitic properties.⁷⁻⁹ Rigane et al. reported that bene has high levels of tocopherol and beta-carotene, which produce its high antioxidant properties. In addition, the presence of steroids, terpenes, anthocyanins, tannins, coumarins, proteins, amino acids, flavonoids, phytosterols and carbohydrates were confirmed in bene.^{10,11} Gholami et al., in 2016, investigated the effect of bene on colitis in rats. The results showed that the administration of bene improved the intestinal histopathology and decreased the oxidative stress marker level compared to the control group.¹² In 2017, Heidarian

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et al. evaluated the effects of hydroalcoholic extracts of different types of bene on gentamicin induced nephrotoxicity in rats.¹³ The results showed that the administration of bene increased catalase, superoxide dismutase, vitamin C, and improved histopathology of the kidney. It caused a reduction in urea, uric acid, creatinine, and malondialdehyde levels.¹³ Tolooei et al. reported the protective effects of bene on the erythrocyte membrane, oxidative stress, and hepatocyte cells in rats treated with CCl₄. Bene reduced free radicals and prevented liver damage by reducing the level of MDA and increasing the level of superoxide dismutase and catalase enzymes.¹⁴ Our investigation showed that no study had been done so far on the effects of *pistacia atlantica* on male fertility. Hence, the aim of this study was to investigate the effects of bene on sperm parameters, testicular histopathology, sperm quality, and oxidative stress in busulfan-induced infertile mice.

Methods and Materials

After receiving the approval of the Ethics Committee of Mashhad University of Medical Sciences (code IR.MUMS.fm.REC.1397.96), 35 BALB/c male rats were randomly assigned to control, sham, busulfan, bene, and bene + busulfan groups. The control group received no injections. The sham group received a single dose of busulfan solvent (DMSO), intraperitoneally. The busulfan group received a single dose of 10 mg/kg intraperitoneally.¹⁵

The bene group received a diet (pellet) containing 10% bene. Another group received 10 mg/kg busulfan and was fed with pellet containing 10% bene based on pilot study. Then, sperms, sperm chromatin quality, testicular histopathology, and oxidative stress levels were analyzed on the 35th day the experiment.

Sperm analysis

To evaluate the sperm parameters, the epididymis was isolated and its crushed pieces were placed in phosphate buffer saline and put in a CO₂ incubator for 30 minutes. Then, the sperm count, motility, percentage of normal morphology, and sperm viability were investigated according to the WHO guidelines using neubar.¹⁶

Histopathological examination

After opening the abdominal cavity, it was placed in a fixative solution, and dehydration and clearing were performed by passing alcohol and xylene, respectively. Then, the tissues were molded and cut and stained with hematoxylin-eosin. The sample was examined after staining by a pathologist with an optical microscope.¹⁷

Evaluation of the sperm chromatin quality

After fixing the sperms, samples were stained with 0.19% of acridine orange solution in a citrate phosphate buffer with pH = 2.5 for 10 minutes. Then, it was examined using a fluorescence microscope and a suitable filter. By counting 200 sperm per slide, the percentage of sperm with natural DNA (green), with denatured DNA (red), and intermediate state (yellow) were recorded.

Measuring malondialdehyde levels

At first, the TBA solution was prepared. For this purpose, 15 g of Trichloroacetic acid (TCA), 2 ml of hydrogen chloride (10 Normal) and 375 mg thiobarbituric acid were mixed with 100 CC of distilled water. Then, it was placed under a temperature of 50 °C for 2 hours to completely dissolve, and then a 10% homogenous solution was prepared from the tissue using 1.5% potassium chloride. One ml of a homogeneous tissue solution with 2 ml of the reagent TBA was mixed, and then the solution was heated at 50 min. 3 cc from the TBA reagent added to blank tube. After cooling, 25 µl of hydrogen chloride (pH = 1.6-1.7) was added to tubes. A 1.5 mL solution of n-butanol was added to each tube. Then, tubes were centrifuged at 1000 rpm for 10 minutes. The supernatant was removed from the deposited sediment, and absorbance was read using the spectrophotometer at 532 nm and then the malondialdehyde (MDA) concentration was expressed in terms of nmol / gram of testicular tissue.¹⁸

Measuring thiol levels

Tris-EDTA buffer was prepared at a concentration of 10 mM. 3 g of tris with 0.05 g EDTA and 100 ml of distilled water mixed. pH was reached 8.2 by adding hydrogen chloride and placing in a refrigerator. One mL of Tris-EDTA buffer was added to 50 µl of homogenized testicular tissue and read at 412 nm using a spectrophotometer (A1). The absorbance of the DTNB solution (5, 5'-dithiobis-2-nitrobenzoic acid) was considered as Blank (B). To prepare DTNB, 0.0396 g DTNB was mixed with methanol. The DTNB solution should be used as soon as it is ready. Then, 20 µl of the DTNB reagent was added to the solution and after 10 minutes incubation at room temperature, the absorption was re-evaluated (A2).

The following formula was used to calculate the concentration of thiol per µmol /g.¹⁸

$$\text{Thiol} = A_2 - A_1 - B \times \frac{1.07}{0.05 \times 13.6} \quad \text{Eq. (1)}$$

Measuring superoxide dismutase enzyme levels

First, 10 mM of phosphate buffer was prepared with the pH of 7. 0.024 g KH₂PO₄ was distilled in 100 ml of water, then 0.144 g of Na₂HPO₄ was dispensed in 100 ml of distilled water; then, 0.8 g of NaCl was dispensed in 100 ml of distilled water. 200 µl 1 mM EDTA was used, and these four solutions were combined and considered as the phosphate buffer. 0.005 g MTT [3-(4, 5-dimethyl-thiazol-2-yl) 2, 5-diphenyl tetrazolium bromide] was mixed with 1 ml of deionized water. Then 100 µl of it was mixed with 900 µl of deionized water. 0.001 g of pyrogallol was mixed with 10 ml of deionized water and stored in a dark container. To test 60 µL of the sample, + 15 µl of pyrogallol and + 6 µl of MTT were mixed and the microplate was placed in the dark for 5 minutes. After that, 150 µl of DMSO was added to the wells and the absorbance of the solution obtained at 570 nm wavelength was measured using ELISA and was reported in international unit/ grams of tissue.¹⁸

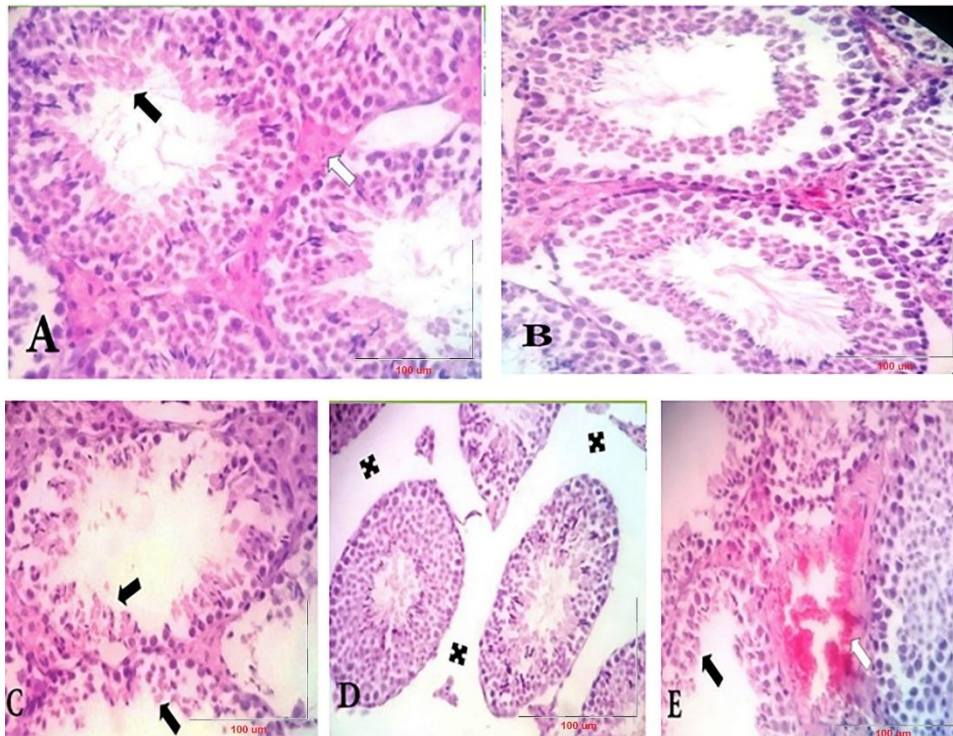
Table 1. The effect of bene powder administration on sperm parameters in different groups.

Group	Sperm count (million/ mL)	Normal morphology rate (%)	Sperm viability (%)	Motility Sperm (%)
Control	4.50 ± 0.30	88.35 ± 4.13	88.76 ± 3.12	79.72 ± 5.82
Sham	4.33 ± 0.37	84.61 ± 3.54#+	85.86 ± 4.84#+	78.17 ± 6.69#+
Busulfan	4.20 ± 0.62	68.30 ± 5.78*	59.69 ± 3.12*	50.13 ± 5.79*
Bene	4.56 ± 0.33	88.50 ± 3.20#+	89.50 ± 3.20#+	80.87 ± 6.03#+
Bene + Busulfan	4.27 ± 0.47	77.62 ± 2.61*#	75.00 ± 2.87*#	65.00 ± 2.87*#

* (p<0.001): Significant difference with control group

(p<0.001): Significant difference with busulfan group

+ (p<0.001): Significant difference with bene + busulfan

**Figure 1.** Transverse sections of seminiferous tubules in different groups using hematoxylin and eosin staining; Magnification × 400. A. Control group, B. sham group, C. busulfan group, D. bene group, E. bene + busulfan group.

Measuring catalase enzyme levels

First, 10 mM of phosphate buffer was prepared with the pH of 7.0. 0.24 g KH_2PO_4 was distilled in 100 ml of water; then, 0.144 g of Na_2HPO_4 was dispensed in 100 ml of distilled water and then 0.8 g NaCl was dispensed in 100 ml of distilled water. 200 μl 1 mM EDTA was used, and these four solutions were combined and considered as the phosphate buffer. 100 μl of H_2O_2 hydrogen peroxide and 30% to 900 μl of phosphate buffer was added with the pH of 7 and covered with foil (solution 2). 80 μl of the solution 2 was added to 2.5 ml of phosphate buffer with the pH of 7 (solution 3). In cuvette, 40 μl of homogenized tissue was diluted 1 to 10 with 650 μl of the solution 3. The absorption changes were immediately read at 240 nm by the spectrophotometer (A1). After 2 minutes, the absorbance was read (A2).¹⁸

$$\text{CAT} = (A1 - A2) \times 0.73 / (0.0436 \times 0.08) \quad \text{Eq. (2)}$$

Statistical analysis

Data was presented as the percentage and mean ± standard deviation. SPSS software, ANOVA, and TUKEY post-test were used for data analysis.

Results

Sperm parameters

According to Table 1, the mean value of sperm motility was 79.72 ± 5.82 in the control group, whereas, after receiving 10 mg/kg of busulfan, sperm motility decreased significantly (50.13 ± 5.79). The sperm motility was significantly increased in the sham, bene and the bene + busulfan groups compared to the busulfan group ($p < 0.001$). Statistical analysis showed a significant increase in sperm motility in the bene group compared to the bene + busulfan ($p < 0.001$). Busulfan injection caused a significant decrease in the percentage of the normal sperm morphology and viability compared to the control group ($p < 0.001$). Bene administration reversed this decrease and caused a significant increase in normal sperm morphology and the viability rate compared to that of the busulfan group ($p < 0.001$). Statistical analysis showed a significant increase in the percentage of viability and the normal morphology of sperm in the bene group compared with the bene + busulfan group ($p < 0.001$).

Testicular histopathology

Histological examination of the busulfan treated mice revealed remarkable reduction in the thickness of germinal epithelium, leydig cell hyperplasia, edema, and congestion (Figure 1).

The histology of seminiferous tubules was normal in the bene group. Mild edema and congestion was observed in some seminiferous tubules. Bene + busulfan treated rats showed edema, congestion, as well as little decrease in the thickness of germ cell epithelium in some seminiferous tubules (Figure 1).

Black arrow shows seminiferous tubules and white arrow shows leydig cells in control group. Arrow shows remarkable reduction in the thickness of germinal epithelium in busulfan group. Quadrate arrow shows mild edema between normal seminiferous tubules in bene group. Black arrow shows little reduction in the thickness of germinal epithelium in some seminiferous tubules and white arrow shows congestion in bene + busulfan group.

Sperm chromatin quality

Figure 2 shows the images of sperm DNA damage in different groups with acridine orange staining. The percentage of sperms with damaged DNA in the control group was 3.3 ± 1.52 , whereas, after receiving 10 mg/kg of bene powder, sperms with damaged DNA decreased significantly (44.66 ± 4.50 versus 25.33 ± 4.50). The percentage of sperm with damaged DNA in the bene group (3.66 ± 2.08) was significantly lower than that of the bene+ busulfan group ($p < 0.001$).

Thiol and malondialdehyde levels

Figure 3 shows the levels of thiol and malondialdehyde in testicular tissues of the different groups. Statistical analysis showed a significant decrease in the mean thiol level in the busulfan group compared to the control ($p < 0.001$), sham ($p < 0.001$) and bene group ($p < 0.001$). In addition, a significant decrease was observed between the mean level of thiol in the bene + busulfan group compared to the control ($p < 0.001$), sham ($p < 0.001$) and bene groups ($p < 0.001$). A significant increase was observed in the mean level of malondialdehyde in the busulfan group compared to the control ($p < 0.001$), sham ($p < 0.001$), bene ($p < 0.001$) and bene + busulfan groups ($p = 0.016$). In addition, a significant increase was observed in the mean level of malondialdehyde in the bene + busulfan group compared to the control ($p < 0.001$), sham ($p < 0.001$) and bene groups ($p < 0.001$).

Superoxide dismutase and catalase levels

Figure 4 shows the values of superoxide dismutase and catalase enzymes in different experimental groups in unit/g. Statistical analysis showed a significant decrease in the mean level of superoxide dismutase and catalase enzymes in the busulfan group compared to the control ($p < 0.001$), sham ($p < 0.001$), bene ($p < 0.001$) and bene + busulfan groups ($p < 0.001$).

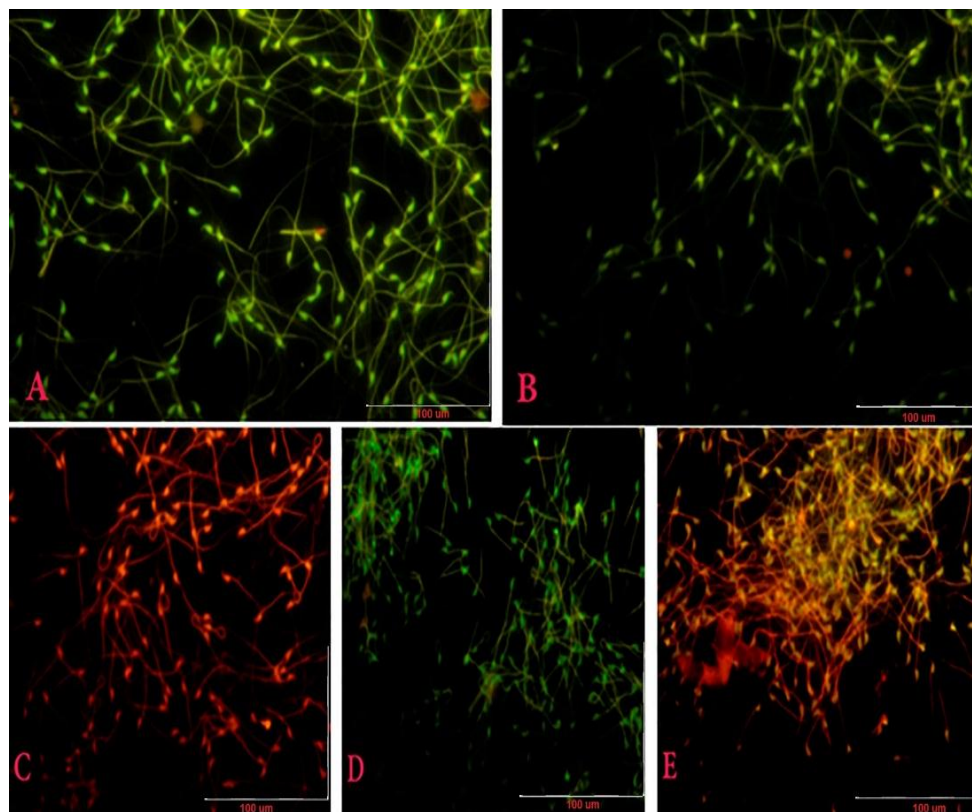


Figure 2. Images (A-E) show sperms in the control, sham, busulfan, bene, and bene+ busulfan groups, respectively. Identification of sperm DNA damage by acridine orange staining; Sperms with healthy DNA are green, the sperms with intermediate damage are shown in yellow color and the denatured DNA in red; Magnification $\times 400$

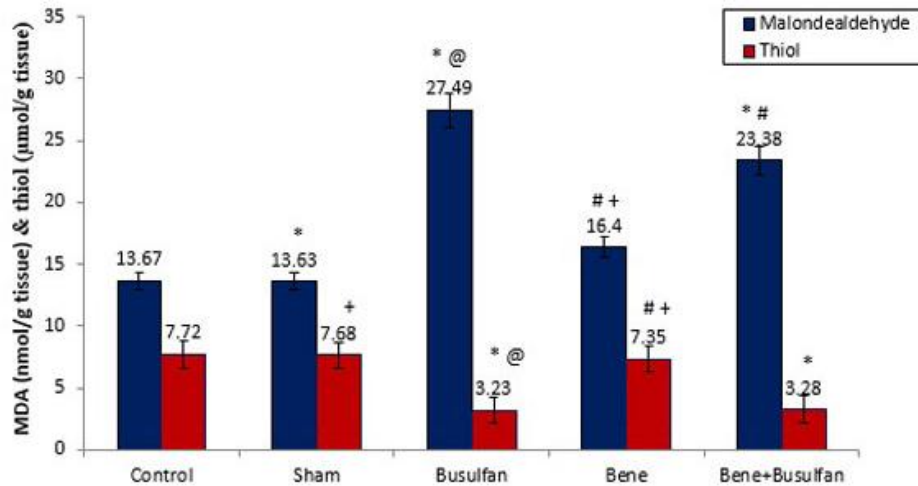


Figure 3. The effect of the administration of bene powder on the thiol and malondialdehyde levels in testis tissue of different groups
 * ($p < 0.001$): Significant difference with control group
 # ($p < 0.001$): Significant difference with busulfan group
 + ($p < 0.001$): Significant difference with bene + busulfan
 @ ($p < 0.001$): Significant difference with sham group

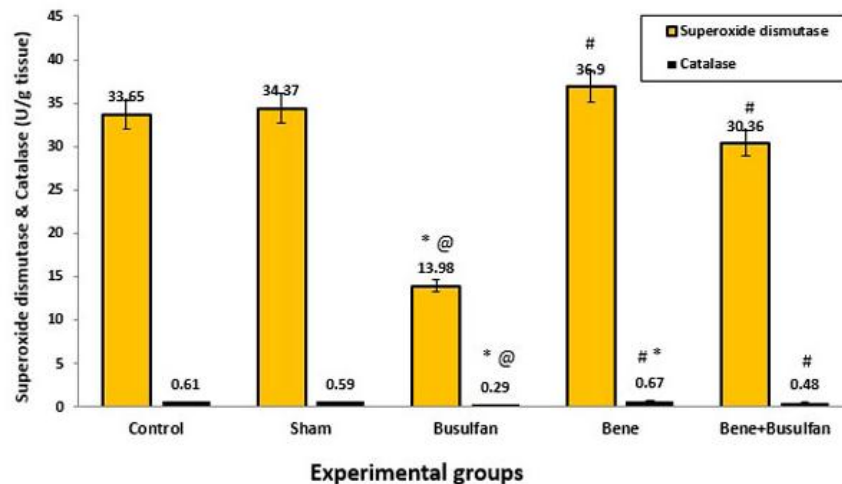


Figure 4. The effect of the administration of bene powder on the level of superoxide dismutase and catalase enzymes on testicular tissues of different groups
 * ($p < 0.001$): Significant difference with control group
 # ($p < 0.001$): Significant difference with busulfan group
 + ($p < 0.001$): Significant difference with bene + busulfan
 @ ($p < 0.001$): Significant difference with sham group

Discussion

The findings of the present study showed that receiving busulfan significantly reduced sperm parameters compared to the control group. Bene administration compensates this reduction. Studies have shown that reactive oxygen species have a negative effect on sperm count, motility, and morphology that can lead to sperm weakness and eventually infertility. The action mechanism of free-radical involves lipid peroxidation of the plasma membrane of the sperm, which is due to the presence of a large amount of unsaturated fatty acid, which is susceptible to oxidative damage and this can affect motility, fluidity of the membrane and fertilization ability. In addition, free radicals damage the axoneme of sperm, accelerate ATP consumption, and disrupt the

function of mitochondrial DNA.^{19,20} Since reducing the level of semen antioxidants or increasing oxidants in infertile men occurs, it is not surprising that dietary supplements or antioxidants play a major role in treating male infertility. These compounds protect sperm, prevent the maturation of immature sperm, and increase sperm motility.²¹

In the present study, tissue lesions in testicular tissue were clearly observed in the busulfan group. Busulfan, with the production of free radicals and oxidative stress, can have a destructive effect on testicular tissue. Therefore, it is likely that histopathological changes in the seminiferous tube will result from induction of oxidative stress. Since in the present study, bene as a potent antioxidant in the bene + busulfan group, could significantly compensate for

the destructive effects of testicular histopathologic changes, the possibility of inducing oxidative stress on testicular tissue has been shown to be more strong. Gholami et al. also studied the effects of bene on colitis in rats. Their results showed that the administration of bene improved the histopathology of the intestine (necrosis, tissue edema, and neutrophil infiltration) compared to the control group. In addition, the level of oxidative stress marker (myeloperoxidase) decreased.¹²

The selection of sperm in infertility centers is based on the appearance and sperm motility, which does not guarantee the health of the sperm genome. Therefore, sperm DNA damage is important. In this study, the quality of DNA of sperm was evaluated by acridine orange staining. In this method, the sensitivity of the sperm DNA to acid denaturation is measured by evaluating the color of the fluorescence of the acridine orange from green to red. The protective effect mechanism of bene is mainly related to polyphenolic compounds, gallic acid, quercetin, and luteolin, which plays an important role in protecting the sperm membrane against free radicals and oxidative stress phenomena.²² Rigane et al. reported (2017) that the bene had high levels of tocopherol and beta-carotene, which produces its high antioxidant capacity. In addition, the presence of steroidal, terpenoid, anthocyanin, tannin, coumarin, protein, amino acids, flavonoids, phytosterols and carbohydrates in the bene plant was reported.^{10,11}

Toloei et al., (2015), investigated the protective effects of bene extracts on hepatotoxicity in rats. In this study, rats were divided into four groups: olive oil, olive oil + bene, CCl₄ + bene powder, and CCl₄ + olive oil. The results showed that bene could reduce the amount of free radicals and prevent liver damage as an antioxidant by decreasing MDA and increasing the antioxidant level of superoxide dismutase and catalase enzymes.¹⁴

Consistent with our results, Heidarian et al. evaluated the effects of hydroalcoholic extracts of bene on gentamicin induced nephrotoxicity in rats.¹³ Their results showed that the administration of bene increased catalase, superoxide dismutase, vitamin C, and improved the histopathology of the liver. It caused a reduction in urea, uric acid, creatinine and malondialdehyde levels.¹³ Consistent with these studies, our results showed that 10 mg/kg of bene reduced the level of malondialdehyde and increased the level of superoxide dismutase and catalase enzymes. In addition, the testicular histopathology improved. In our study, the effects of bene were dose-dependent indicating that the duration of treatment and the selective dose of antioxidants are very because of the treatment.^{23,24}

It would have been better if this study investigated the potential of the mitochondrial membrane and the rate of cell apoptosis in testicular tissue, and it is recommended for future studies. In addition, it is recommended to study the effects of the other parts of bene, such as the crust and leaf on fertility rate.

Conclusion

The administration of 10 mg/kg of bene powder for 35 days reduced oxidative stress, improved testicular

histopathology, sperm chromatin quality, and sperm parameters in the infertile mice model.

Acknowledgments

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Conflict of interests

The authors claim that there is no conflict of interest.

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