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ORIGINAL ARTICLE

The Effect of CoQ10 on Testicular Tissue in Rats Treating with Busulfan: Sperm Quality and Histological Changes

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Abstract

Objective- Busulfan (Bus) is a chemotherapy drug that is widely used for cancer treatment. The protective effect of CoQ₁₀ evaluated on testis and sperm parameters after busulfan treatment.

Design- Experimental Study

Animals- Thirty tow adult male Wistar rats

Procedures- In this experimental study 32 adult male Wistar rats have randomly divided into four groups: Control group received normal saline (0.1 mL, daily, intraperitoneally). Sham group received a single dose of busulfan 10 mg/kg, IP. Positive control group received 0.1 mL CoQ₁₀ (10 mg/kg, daily, IP). The treatment group received busulfan along with CoQ₁₀ (10 mg/kg, daily, IP). All procedures were continued for 35 days. For histomorphometric analyses, the thickness of testicular capsule, the germinal epithelium height and the diameter of the seminiferous tubules were measured. Semen analysis was used for the assessment of sperm parameters.

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Results- Histomorphometric analyses showed the thickness of testicular capsule was increased in busulfan groups ($P<0.05$). Compared to the control group, germinal epithelium height was decreased significantly in Sham and treatment groups ($P<0.05$). There was a significant decrease in seminiferous tubules diameter in busulfan and treatment groups compared to the control group. In Sham group, the sperm count, motility, viability, nuclear maturation and sperm morphology were significantly decreased compared to control and CoQ₁₀ treated groups ($P<0.05$). The percentage of sperm with DNA damage in the CoQ₁₀ treated group was significantly increased compared to Sham groups ($P<0.05$).

Conclusion and Clinical Relevance- Administration of CoQ₁₀ in busulfan-treated animals improved histological and sperm quality.

1. Introduction

Testicular cell transplantation has been widely used to investigate the biology of spermatogonial stem cells, production of transgenic animals and restoration of fertility in different species. One critical step in successful transplantation is preparation of the recipient testes. The most widely used method for removing endogenous germ cells from the testes of wild-type animals and creating space for donor stem cell engraftment is treatment with a sublethal dose of busulfan.¹ However, the sterilizing dose of busulfan is species specific.² Busulfan [CH₃SO₂O(CH₂)₄OSO₂CH₃] is a bifunctional chemotherapeutic and cytostatic agent, readily absorbed from the gastro-intestinal tract and rapidly disappears from blood with a half-life of 2 to 3 hours. The drug is extensively metabolized and excreted in the urine almost entirely as sulphurcontaining metabolites. Busulfan has many side effects on different body organs such as bladder, liver, skin, nervous system and gonadal function and other alkylating agents, it is also potentially carcinogenic and teratogenic.^{3,4} Mammalian spermatogenesis *in vivo* is a long and complex process that originates with and depends on spermatogonial stem cells. Unlike other chemicals that destroy differentiated spermatogonia, busulfan is a potent agent that preferentially destroys spermatogonial stem cells.^{5,6} In a clinical setting, an individual undergoing chemotherapy treatment often produces prolonged and sometimes irreversible depression of sperm counts in humans so are well known to affect fertility in the male.⁷ Adverse effects of busulfan on the animals' health have been mentioned in the spermatogonial transplantation literature, but have not been quantified.^{8,9} Busulfan-treated adult rat are prepared with little difficulty and provide reliable recipients that allow quantitative assessment of stem cell activity in different donor testis cell populations. However, restoration of fertility in busulfan-treated adult rat is inefficient, perhaps because of damage to the testicular environment caused by the ablative therapy.¹⁰

Coenzyme Q₁₀ (CoQ₁₀) is an essential component for electron transport in oxidative phosphorylation of

mitochondria.^{11,12} It is a potent antioxidant, a membrane stabilizer, and cofactor in the production of adenosine triphosphate by oxidative phosphorylation. Coenzyme Q₁₀ has been widely applied in food supplements and cosmetics in Japan, the U.S., and many other countries. In recent years, frequency of studies involving CoQ₁₀ has increased in both basic and clinical research areas.¹¹⁻¹³ Several studies have demonstrated the protective effect of CoQ₁₀ in various forms of tissue injury.^{11,14} However, no study has investigated the effect of CoQ₁₀ on sperm quality parameters.

The present study aimed to assess the effect of CoQ₁₀ on testicular tissue in rats treating with busulfan. The assessments were based on sperm quality parameters and histological samples

2. Materials and Methods

Procedures and animal grouping

In this study, 32 healthy male rats were kept up in standard conditions of temperature 22 ± 2 °C, 30 to 60% humidity and the light period of 14 hr light and 10 hr of darkness. Animals were randomly divided into four groups of 8 animals each: Control group received normal saline (0.1 mL, daily, IP). Sham group received a single dose of busulfan (SIGMA-USA-ALDRICH B-2635) 10 mg/kg, IP.¹⁵ Positive control group received 0.1 mL CoQ₁₀ (10 mg/kg, IP).¹⁶ Treatment group received a single dose of busulfan (SIGMA-USA-ALDRICH B-2635) 10 mg/kg, IP and 0.1 mL CoQ₁₀ (10 mg/kg, IP). All procedures were continued for 35 days.¹⁷

Histological analyses

The left testicles were fixed in 10% formal saline for 72 hours, after which the samples were dehydrated, cleared, and embedded in paraffin. Paraffin sections were prepared (6-7µm in thickness) and stained with hematoxylin and eosin (H&E) for histomorphometry analyses with an Olympus light microscope (BH-2 model) and

photographed with a digital camera (Dino-Eye-AM-7023), then analyzed with Dino Capture 2.0 software for morphometric analysis. We measured the thickness of testicular capsule, the germinal epithelium height and the diameter of the seminiferous tubules.

Sperm sampling

After 35 days, rats were anesthetized and euthanized with 80 mg kg⁻¹ ketamine (Alfasan, Woerden, The Netherlands) and the abdominal skin was aseptically prepped. The epididymis was removed and transferred to Petri dish 6 cm containing medium 1 mL of human tubal fluid medium (HTF; Sigma-Aldrich, St. Louis, USA) and 4 mL⁻¹ of bovine serum albumin (BSA; Sigma, St. Louis, USA) that previously its temperature was balanced by incubator (5% CO₂, 37 °C) and then, by making a few incision in the epididymis and 30 min incubation at 37 °C in 5% CO₂, spermatozoa were released from epididymis.

Sperm count

For sperm counting at 1:20 dilution from sperm samples were prepared. For this purpose 10µL of the sperms were added to 190µL of distilled water, and then 10µL of the diluted sperm was dropped on a Neubauer slide and the average number of sperms were counted.¹⁸

Sperm motility

The medium (10mL) of containing sperm was placed on the Neubauer slide and under a light microscope with a magnification of 20× the percentage of sperm motility was evaluated.

Sperms viability

Semen sample (20µL) of was placed onto a clean slide and then 20µL of eosin solution was added to it, after 30 sec, 20µL of nigrosin solution was added. Then, from the desired solution, smear was prepared and after drying slides and using a light microscope with 40× magnification percentage of alive sperm (colorless) and dead sperm (red color in head) were determined.¹⁹

DNA strand damage

The semen samples were washed three times with phosphate buffered saline (PBS) and after removal of the

supernatant, the sediment was achieved by using PBS to a final concentration. Then, smears were prepared from the medium containing sperm and after drying in a laboratory environment, for 30 min, was placed into in acetone - ethanol (1:1) container. Acridine orange staining solution for 7 min and after the final drying in a dark place, the slides were examined using an immunofluorescence microscope (Model 466300; Carl Zeiss, Jena, Germany) with 100× objective magnification and the results were reported as percentage.^{20,21}

Sperm chromatin condensation

Similar to the above procedure, after fixing slides in ethanol - acetone solution and drying in air, they were placed in aniline blue staining solution for 7 min and after drying in air, were observed by light microscope in magnification of 100×. In this staining method, immature sperms with the proportion of histone protein in the nucleus become the color of dark blue gray and mature sperms showed pale.²²

Sperm morphology

In this process, two staining methods, aniline blue and eosin-nigrosin were used. Sperms that appeared abnormal by aniline blue staining were counted and results were expressed as percentage and using eosin-nigrosin staining, spermatozoa containing cytoplasmic debris were counted as immature sperms.²³

3. Statistical analysis

The data were analyzed by SPSS (Version 20; SPSS Inc., Chicago, USA) and two way ANOVA and Bonferroni test were used. A *p*-value less than 0.05 were considered significant. Data are presented as mean ± standard error (SE).

4. Results

Histological findings

The data for histomorphometric analyses are presented in table1. Testicular tissues showed the increased subscapular edema in animals with busulfan. Thickness of testicular capsule was similar between control groups and Q10

groups which were significantly increased in busulfan groups ($P < 0.05$). Compared to the control group, germinal epithelium height was decreased significantly in Sham and treatment groups ($P < 0.05$). There was no significant difference in germinal epithelium height between Positive control and Control groups. There was a significant decrease in seminiferous tubules diameter in groups which received busulfan compared to the control group (Table 1, Fig 3).

Table 1. Average of histomorphometric analyses for thickness of testicular capsule (TTC), seminiferous tubules diameter and germinal epithelium height (GEH) in different groups. All data are presented in Mean \pm SE.

	Control	Sham	Treatment	Positive Control
Testicular capsule (μm)	31.80 \pm 5.46 ^a	156.57 \pm 28.45 ^b	169.83 \pm 22.23 ^b	49.55 \pm 12.56 ^a
Tubules (μm)	262.62 \pm 4.96 ^a	163.23 \pm 16.08 ^b	177.07 \pm 21.29 ^b	269.08 \pm 25.79 ^a
Germinal epithelium height (μm)	77.11 \pm 10.26 ^a	21.89 \pm 5.22 ^b	26.52 \pm 5.23 ^b	69.84 \pm 11.23 ^a

a,b: indicate significant differences ($P < 0.05$) between marked data in the same row.

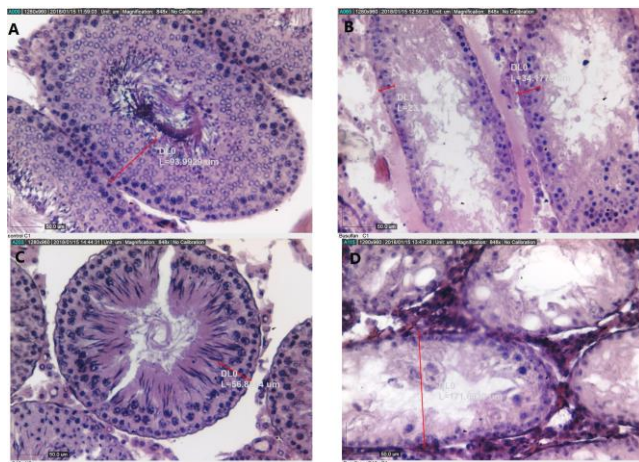


Fig 3. Representative micrographs of the rat testis sections in experimental groups. A) Control, B) Sham, C) Positive Control and D) Treatment (H&E staining). Scale bar: 50 μm .

Sperm count

The results showed a significant difference between average number of sperms in Treatment group compared to Sham groups ($P < 0.05$) (Table 2).

Table 2. Different parameters of sperm quality. Data are expressed as mean \pm SE

Groups	Sperm count ($\times 10^6/\text{mL}$)	Motility (%)	Viability (%)	Normal Sperms (%)
Control	56.5 \pm 2.73	62.9 \pm 3.07	69.3 \pm 2.65	81.6 \pm 4.19
Sham	30.7 \pm 2.79	36.7 \pm 2.39	41.6 \pm 2.34	61.2 \pm 2.79
Positive control	67.6 \pm 2.76	68.3 \pm 3.47	77.5 \pm 3.31	88.7 \pm 6.24
Treatment	42.3 \pm 2.47*	42.6 \pm 2.70	50.3 \pm 2.53	68.3 \pm 2.27*

*The mean difference is significant at the .05 level versus Sham group.

Sperm morphology

Sperm with normal morphology was calculated. Results showed a significant difference in Treatment group compared to the Sham groups ($p < 0.05$), (Table 2).

Sperm motility

The results for mean percentage of motile sperms in the studied groups indicated a significant difference in the Treatment group compared to the Sham group ($P < 0.05$) (Table 2).

Sperms viability

Results of live sperms using eosin-nigrosine staining indicated a significant difference in sperm viability in Treatment group compared to the Sham group ($P < 0.05$) (Table 2).

DNA integrity

Sperms observed with green nuclei were normal, and sperms with yellow, orange to red nucleus depending on the intensive of damage, recognized as sperms with DNA damage (Fig. 1). A significant difference in the mean percentage of sperm with damaged DNA was observed in treatment group compared to the Sham group ($P < 0.05$) (Table 3).

Table 3. Sperm chromatin condensation and DNA damage in experimental groups. Data are expressed as mean± SE

Groups	DNA DAMAGE ^{ab}	SPERM CHROMATIN CONDENSATION ^a
Control	2.05±0.07	2.09±0.05
Sham	35.70±2.37	40.28±2.62
Positive control	2.06±0.03	2.03 ±0.07
Treatment	15.30±2.17*	17.60±2.71

*The mean difference is significant at the .05 level versus Sham group. ^aAniline blue positive and ^{ab}Acridine orange positive

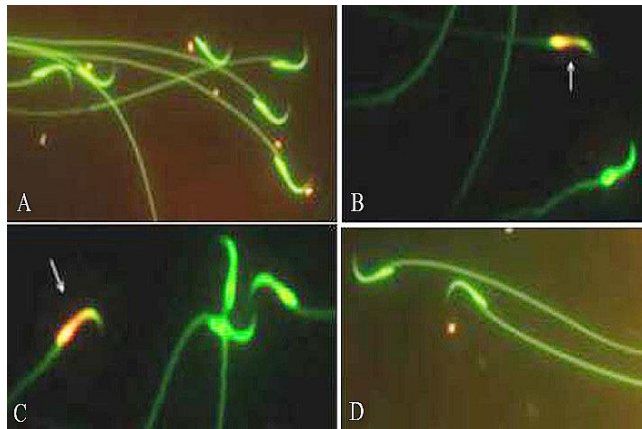


Fig1. Fluorescent image of spermatozoa in experimental groups. A) Control, B) Sham, C) Positive Control and D) Treatment. Intact DNA marked with light green appearance and sperm with damaged DNA presented with light yellow appearance (Acridine orange, 1000×).

Sperm chromatin condensation (Sperm maturity)

After aniline-blue staining the mean numbers of immature sperms in all groups were calculated (Fig 2). The mean percentage of immature sperms in treatment group was significantly different from that of the Sham groups ($P<0.05$), (Table 3).

5. Discussion

In the present study, we have determined the extent of degeneration in rats' testis structure and epididymis sperms parameters following busulfan administration. The

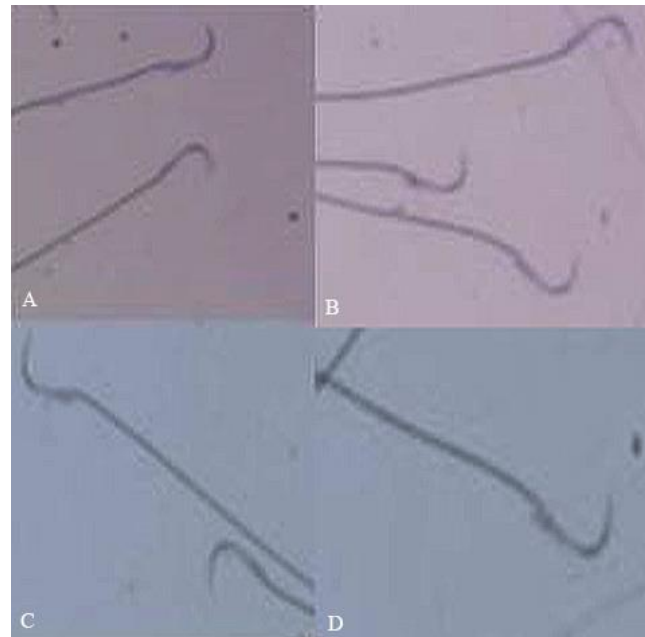


Fig 2. Chromatin condensation in experimental groups. A) Control, B) Sham, C) Positive Control and D) Treatment. Sperms with mature nucleus are stained with pale blue head and immature nucleus of sperm is stained with dark blue head (Aniline blue, 1000×).

obtained data would provide information, which potentially improve the clinical and biotechnological application of busulfan. We have used different doses as well as different time points of drug administration to evaluate the effects and side-effects of busulfan on spermatogenesis, since the length of time from initiation of stem cell division to formation of spermatozoa in rat is around 56 days,²³ the chosen period of time (5 and 8 weeks) provided sufficient time to monitor the potential recovery of spermatogenesis in surviving stem cells in busulfan treated animals. According to Nagano et al,²⁴ it took approximately 4 weeks after busulfan treatment that the cells in the process of differentiation are cleared from the lumen of the seminiferous tubules. The later finding was the main rational reason to choose 5-week time frame as the start time to monitor the effects of the drug on spermatogenesis. The 50 mg/kg dose of busulfan was lethal for rat probably due to the cytotoxic effects of the drug on the hematopoietic system.²⁵ Previous studies also demonstrated that the mortality rate increases from 0 to approximately 50% for doses of 20-40 mg/kg of busulfan.²⁶ The epididymal sperm count data indicated that animals in all busulfan treated groups were infertile (sperm numbers $<6.5 \times 10^5 \pm 9 \times 10^3$ cell/ml), except for the 10 mg/kg dose group. High-dose administration eliminates sperms more significantly in epididymal lumen and makes

animals permanently sterile and administration at low doses reduces the number of stem cells and spermatogenesis recovered from the surviving stem cells. The duration of sterility following busulfan treatments is dependent on the extent of stem cell depletion.²⁷ In addition to sperm count or seminiferous tubule type, testis weight, as demonstrated previously,²⁸ and its diameters (longitudinal and cross section) are good predictors of fertility status. Our data also revealed that the testes weight and diameters also were affected by busulfan, and can be used as further parameters to evaluate the extent of infertility in animal models. Our findings were in agreement with those of others who demonstrated that testicular masses went from a maximum to a minimum value, when doses of 20 mg/kg of busulfan administered in rat.²⁶ Histological evaluation suggested that the higher testis weights and diameters from controls and 10 mg/kg dose group could be attributed to higher levels of spermatogenesis. Eight weeks after busulfan treatment, the increase in weight of the testis was most apparent in animals treated at a dose of 10 mg/kg suggesting extensive regeneration of spermatogenesis at this dose. It seems that busulfan did not affect capsular thickness, because at high dose which testis more affected by drug, capsular thickness increased and vice versa. After busulfan administration, no or very little testicular spermatozoa were released in the epididymal lumen, however 5 weeks after drug administration animal showed some degree of spermatogenesis restoration. Nevertheless, the animals were still infertile based on their sperm counts. This finding was in agreement with the finding of others.^{28,29} According to the previous report by Karashima et al,³⁰ administration of busulfan seems to produce only a non-permanent testicular injury, and the drug-induced injury was somehow reversible. There are, however, some variations between the studies, and delays of 1–2 weeks have been reported for some degree of restoration.^{31,32} As previously reported, rat that received a lower dosage of busulfan may have some tubule repopulation from endogenous stem cells.²⁷ Germ cell count in the groups treated with busulfan after 8 weeks, showed a considerable recovery of spermatogenesis. However some tubules were still devoid of germinal cells, especially in the groups that received higher doses of busulfan. As described by previous studies, busulfan known to cause a transient loss of A1 spermatogonia, so surviving stem cells can regenerate to show spermatogenesis.²⁹⁻³³ Regeneration of surviving stem cells was reflected in the epididymal sperm numbers changes and other recovery observed during regeneration. Unlike other chemicals that destroy

differentiated spermatogonia, busulfan is a potent agent that preferentially kills spermatogonial stem cells of several species. However, at higher doses, the drug would kill differentiated progenies of spermatogonia causing depletion in spermatocytes and spermatids as well. Following the depletion of differentiating spermatogonia, the ratio between surviving stem cells and differentiating cells could be significantly varied on basis of the dosage, which may affect the self-renewal property of surviving stem cells, as suggested previously.²⁸

The finding of the present study showed that busulfan decrease germinal epithelium height in rats. There were sloughing and disorganization of spermatogenic cells with their exfoliation in seminiferous tubules lumen. Thus, the reduction in thickness of the seminiferous tubules epithelium could be due to degenerative effects of the busulfan.³⁴ Recent researches revealed that busulfan caused adverse effects on reproductive performance of male rat including sperm viability, motility and density impairment.³⁵ Others showed that busulfan produced free radicals, which directly affected DNA. The DNA destruction results in chromosomal abnormalities and dominant lethal mutations in sperm.³⁶ Busulfan inhibit the spermatogenesis process, especially by oxidative damage which increases the level of ck-18, a surface marker on sertoli cells. The elevation of this marker has caused spermatogenesis disorder and infertility and reduced sperm motility by reducing flagella length.³⁷ Our findings also revealed that, busulfan had increased capsular thickness, that was most likely due to inflammation from the administration of busulfan.³⁸

Coenzyme Q₁₀ was first introduced as an ethical drug for heart failure patients in Japan and other nations. Coenzyme Q₁₀, which functions endogenously in the mitochondrial electron transport chain, can be ingested to scavenge free radicals and contribute to antioxidant defenses in vivo.³⁹ In recent years, the role of CoQ₁₀ in disease prevention and treatment has been intensely investigated.⁴⁰ The successful results of CoQ₁₀ administration in different organ systems led us to attempt such treatment with a model of busulfan induced testicular disorders.

Both the bioenergetic and the antioxidant role of CoQ₁₀ suggest a possible involvement in sperm biochemistry and male infertility. CoQ₁₀ can be quantified in seminal fluid, where its concentration correlates with sperm count and motility.⁴¹ It was found that distribution of CoQ₁₀ between sperm cells and seminal plasma was altered in varicocele patients, who also presented a higher level of oxidative stress and lower total antioxidant capacity. The redox status of CoQ₁₀ in seminal fluid was also determined: an

inverse correlation was found between ubiquinol/ubiquinone ratio and hydroperoxide levels and between this ratio and the percentage of abnormal sperm forms. Subsequently, CoQ₁₀ was administered to a group of idiopathic asthenozoospermic infertile patients. Treatment led to a significant increase in the concentration of CoQ₁₀, both in seminal plasma and sperm cells, and improvement in sperm motility.⁴²

Adverse effects of busulfan on the animals' health have been mentioned in the spermatogonial transplantation.⁴³ In a recent study, it has been demonstrated that CoQ₁₀ improves semen quality.⁴⁴ Administration of CoQ₁₀ in busulfan treated animals improved sperm quality parameters and also DAN damage and chromatin condensation indices. The results from the present study indicated that CoQ₁₀ administration after busulfan administration could be of benefit to improve sperm indices in rat.

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Conflicts of interest

There was no conflict of interest regarding our results. The current manuscript is a part of thesis for post-graduate degree, which was confirmed previously in Deputy Committee of Urmia University.

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چکیده

تأثیر کو آنزیم کیو ۱۰ بر روی بافت بیضه تحت درمان با بوسولفان

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هدف: بوسولفان یک داروی شیمی‌درمانی است که به‌طور گسترده‌ای برای درمان سرطان مورد استفاده قرار می‌گیرد؛ اما تجویز بوسولفان ممکن است منجر به عقیمی موقت و یا دائمی در بیماران مرد گردد. اثرات محافظتی کو آنزیم کیو بر روی پارامترهای اسپرم متعاقب تجویز بوسولفان مورد ارزیابی قرار گرفت

روش: در این مطالعه تجربی تعداد ۳۲ موش صحرایی نر بالغ و بیستار حیوانات به‌طور تصادفی به چهار گروه شش‌تایی تقسیم شدند: گروه کنترل محلول سرم فیزیولوژی به میزان یک‌دهم میلی‌لیتر در روز به‌طور داخل صفاقی دریافت کردند. در گروه شش حیوانات تک دز بوسولفان با غلظت ۱۰ میلی‌گرم بر کیلوگرم به‌طور داخل صفاقی دریافت کردند. گروه کنترل مثبت محلول حاوی کو آنزیم کیو با دز ۵ میلی‌گرم بر کیلوگرم به‌طور داخل صفاقی دریافت کردند. درمان به مدت ۳۵ روز ادامه پیدا کرد. در این مطالعه ضخامت کپسول بیضه، قطر لوله‌های منی‌ساز و ارتفاع اپیتلیوم زایگر اندازه‌گیری شد. همچنین، آنالیز مایع منی برای ارزیابی پارامترهای اسپرم مورد ارزیابی قرار گرفت.

نتایج: در گروه شش، ضخامت کپسول بیضه افزایش نشان داد و از قطر لوله‌های منی‌ساز و ارتفاع اپیتلیوم زایگر در مقایسه با گروه کنترل کاهش نشان داد. شمارش اسپرم، حرکت، زنده‌مانی، بلوغ هسته‌ای و ریخت‌شناسی اسپرم به میزان معنی‌داری در مقایسه با گروه کنترل کاهش یافته بود ($P < 0.05$). در گروه درمان شده با کو آنزیم کیو پارامترهای مذکور در مقایسه با گروه شش افزایش پیدا کرده بود ($P < 0.05$). درصد اسپرم‌های با DNA تخریب‌شده در گروه درمان شده با کو آنزیم کیو میزان معنی‌داری در مقایسه با گروه شش افزایش یافته بود ($P < 0.05$).

نتیجه‌گیری و کاربرد بالینی: تجویز کو آنزیم کیو در حیوانات تحت درمان با بوسولفان پارامترهای بافت‌شناسی و کیفیت پارامترهای اسپرم و میزان آسیب DNA بهبود بخشید. نتایج مطالعه حاضر نشان داد که تجویز کو آنزیم کیو در حیوانات تحت درمان با بوسولفان می‌تواند شاخص‌های بافت‌شناسی و اسپرم را در موش صحرایی بهبود بخشد.

کلمات کلیدی: اسپرم، کو آنزیم کیو ۱۰، بوسولفان، بیضه، باروری، کیفیت اسپرم