RESEARCH ARTICLE

Busulfan Treatment Effects on Testicular Tissue and Serum Levels of Anti-Mullerian Hormone and Testosterone in Adult Mice

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Abstract

BACKGROUND: Busulfan, a chemotherapeutic drug, is an alkylating antineoplastic agent that belongs to the class of alkyl sulfonates and has some side effects on fertility. This research was aimed to investigate the effects of busulfan on testicular tissue and serum levels of anti-Mullerian hormone (AMH) and testosterone in adult mice.

METHODS: Eighteen adult male Balb/C mice were collected randomly and were assigned in three groups including; control, azoospermia and spontaneous recovery. The groups, except for control group, received two injections of busulfan (10 mg/kg) intraperitoneally with 21-days interval in order to induce azoospermia. Thirty-five and 140 days after the last injection, the effects of busulfan on testicular tissue were evaluated by histologic, histomorphometric and hormonal changes. AMH and testosterone were measured by enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) kits, respectively.

RESULTS: Hormonal analyses showed no significant differences in AMH and testosterone serum levels. Histologic and histomorphometric evaluation showed disrupted spermatogenesis in azoospermia group, and restoration of spermatogenesis spontaneously in spontaneous recovery group.

CONCLUSION: Busulfan at doses was used had no effect on AMH and testosterone serum levels. Busulfan can also induce azoospermia on a temporary basis however, in long term, spontaneous recovery can occur. The results indicated that some side effects are reversible and may depend on the dose applied.

KEYWORDS: Busulfan, anti-Mullerian hormone, testosterone, mouse, testis

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Introduction

While infertility has increased up to 50% since 1955, it is now one of the most crucial medical fields of research. Application of chemotherapy and radiotherapy in the treatment of malignant diseases may lead to temporary or permanent infertility. The antimitotic antineoplastic and DNA alkylating agent busulfan (1,4-butanediol dimethanesulphonate) has long been used in the chronic myeloid leukemia treatment and has also used before the stem cells transplantation of many other cancers being used.(1) Busulfan affects slowly proliferating, and non-proliferating cells.(2) Busulfan has also been applied to induce azoospermia in mice, and to study fertility restoration and recovery kinetics of spermatogonial stem cells (SSCs).(3) It is shown that busulfan in association with other alkylating agents or just alone, can cause SSC

death (4), prolonged azoospermia (5), and consequently infertility or subfertility in males (6). Busulfan induces DNA alkylation in sperm that cause to DNA-DNA and DNA-protein cross-links.(7) While alkylating agents may also affect Sertoli cells (8), some recent studies reported that busulfan treatment had not caused degeneration of Sertoli cells.

Cytostatic and cytotoxic effects of busulfan exert on cells that are at the G1 phase. The cells would be killed during mitosis, while the ones in S or G2 phase will be killed in next mitosis.(4) Even though, the effects of busulfan on spermatogenesis can be irreversible, there have been just few studies dedicated to busulfan side effects on testis structure, and fertility indices in animal models.(5) Administrating Busulfan would kill several species of SSCs, which results in male infertility. While its administration to pregnant animals, leads to producing germ-cell-free gonads in the offspring.(9)

AMH is a 140-kDa homodimeric glycoprotein which is a member of transforming growth factor-β (TGF-β) super family.(10) In both mouse and human, during fetal development, AMH is one of the first genes to be activated in Sertoli cells.(11) Inhibin B is another factor that is regulated by meiotic germ cells, and it regulates follicle-stimulatig hormone (FSH) secretion through negative feedback mechanism. AMH with inhibin B and FSH, is an important index of Sertoli cell function. It is reported that after chemotherapy, serum and testicular AMH levels is increased in both mouse and human. This conclusion could be drawn that lack of germ cells, followed by exogenous damage, results in dematuration or dedifferentiation of Sertoli cells. In adult males it is revealed that AMH affect Sertoli cells and leydig cells in autocrine and paracrine manners, respectively. AMH can inhibit differentiation of leydig cells, and spermatogenesis and may also be involved in sperm motility. Although, spermatogenesis is induced by FSH and testosterone, AMH level would be reduced; hence the hormones may have a conflicting effect in AMH regulation.(10) Some study shown that busulfan treatment not affected on leydig cells and serum concentration of LH and FSH not changed.

As it was showed earlier for older male rats, prenatal exposure to busulfan reduces the plasma level of testosterone. After busulfan induction, a significant decrease in total diameter of seminiferous tubules per testis was observed which conforms to previous studies.

The aim of this study was evaluation of the effects of busulfan on testicular tissue and serum levels of AMH and testosterone in adult mice.

Methods

Animals

Eighteen adult male Balb/C mice (10 weeks old and weighing 30±2 g) were adopted in the study. The animals were assigned into two busulfan-treated groups (including azoospermia group and spontaneous recovery group, n=6 in each group), and one control group (n=6). They were provided from Shiraz Medical Sciences Research Center and Experimental Animal House and were let to adapt to the new place condition for one week. Mice were maintained in the institute's animal house in standard hard bottom polypropylene cages at 23ºC±2ºC, 12:12 hours light/dark cycle and had free access to laboratory chow and tap water throughout the study. The animal experiments were performed with conform to the animal protection guidelines approved by the Ethics Committee for Experimental Animal Use at Shiraz Branch, Islamic Azad University, Shiraz, Iran (Ethical code: IR.Miau 13952009).

Experimental Protocol

The azoospermia and spontaneous recovery groups were intraperitoneally injected with 10 mg/kg^{-1} body weight of busulfan (Busilvex, Pierre Fabre Medicament, Boulogne, France) in two doses with 21-days interval. The control group received no injection of busulfan. The busulfan dose was determined based on those of previous studies using rat and hamster, in which disrupted spermatogenesis and induced azoospermia were reported using this dose.(12) The duration of spermatogenesis in mouse is approximately 35 days, with four cycles of 8.6 days.(13) At day 35 (approximately 4 cycles) and 140 (approximately 16 cycles) after the last injection, the animals of azoospermia and spontaneous recovery groups were anesthetized with ether, respectively and the blood samples were taken and then were euthanized for testis removal. The same process was done for the control group too.

Histologic and Histomorphometric Analysis

After blood sample collection, the mice testes were removed and were fixed in 10% formalin solution (Sigma-Aldrich, St. Louis, MO, USA) and were embedded in paraffin. Five sections of five-micron thickness were prepared, and stained with hematoxylin and eosin, and were observed under a light microscope (Nikon, Tokyo, Japan). Accord to Panahi, *et al.*, 10 round and roughly round seminiferous tubules within a section, were measured using Dinocapture 2.0 software (Dino-Eye, San-Chung,

Taiwan) with 100x magnification.(12) Then, nine histomorphometric parameters were measured randomly, which include total, lumen and cellular diameters of seminiferous tubules, luminal, cellular and cross sectional areas of seminiferous tubules, numerical density and number of profiles per unit area and its spermatogenesis index.

The total diameter (D) was calculated based on mean value of two diameters (D_1 and D_2) at right angles:

$$D= (D_1+D_2)/2$$

In the same manner, the lumen diameter (L) was achieved based on mean value of two diameters (L_1 and L_2) at right angles of luminal space: $L=(L_1+L_2)/2$. The cellular diameter (C_d) was calculated based on following equation: $C_d=D-L/2$. The Luminal (L_a), cellular (C_a), and cross sectional areas (A_c) of seminiferous tubules were achieved accord to following equations, respectively in which π is the mathematical constant equal to 3.142: $L_a=\pi L^2/4$, $C_a=\pi C_d^2$, $A_c=\pi D^2/4$. And equation $N_v=N_A/(D+T)$ represents the numerical density (N_v) of seminiferous tubules which is the number of profiles per unit volume of testis. Where N_A is the number of profiles per unit area, D is total diameter of seminiferous tubules and T stands for the mean thickness of the section.

Values 0-7 were attributed to the spermatogenesis index according to spermatogenic potential of the testis including number of cell layers, types of cells and also the late spermatids presented in the seminiferous tubules. The following illustrates each value: 0 = germ cell absence; 1 = only spermatogonia presence; 2 = presence of both spermatogonia and spermatocytes; 3 = spermatogonia, spermatocytes, and with < 50 late spermatids per tubule; 4 = spermatogonia, spermatocytes, and with 50–74 late spermatids per tubule; 5 = spermatogonia, spermatocytes, and with 75–99 late spermatids per tubule; 6 = spermatogonia, spermatocytes, and with 100–149 late spermatids per tubule; 7 = presence of all cell types and with ≥ 150 late spermatids per tubule.(12)

AMH and Testosterone Assay

The blood samples were directly collected from the heart and then centrifuged at 400 g for 20 minutes and stored at -80°C until the day of the analysis. AMH and testosterone were measured by enzyme-linked immunosorbent assay (ELISA) (BT Lab, China) and radioimmunoassay (RIA) (Isotopes Ltd, Hungary) kits, respectively.

Statistical Analysis

Statistical analyses of data were done using SPSS v.24 (SPSS Inc., Chicago, IL). For data normalization, Kolmogorov-Smirnov test was performed; then, ANOVA and Tukey post-hoc test were done to determine whether there were differences among all groups. Mann-Whitney U test was done to compare the spermatogenesis index of seminiferous tubules between groups. Comparisons were made between control and treated groups within the same period. Data are presented as means ± SEM (GraphPad Prism 5.01, Inc., San Diego, CA, USA). Differences were considered to be statistically significant at the 95% confidence level ($p<0.05$).

Results

Histologic and Histomorphometric Findings

Histological characteristics of seminiferous tubules were compared between control and busulfan-treated groups using light microscope (Figure 1). Meanwhile, the comparison of mean and standard error of histomorphometric indices of seminiferous tubules in the control, azoospermia and spontaneous recovery groups in mice have been shown in Figure 2.

Based on the histomorphometric findings, full spermatogenic activity was detected in control and spontaneous recovery groups. In azoospermia group, decreased thickness of germinal epithelium, range and vacuolar space on the basement membrane of the seminiferous tubules were observed. Sertoli cells were visible and spermatogenesis was completely destroyed. In addition, according to histomorphometric findings the total, lumen and cellular diameters, luminal, cellular and cross-sectional areas and spermatogenesis index in the seminiferous tubules of azoospermia group were less than control and spontaneous recovery groups ($p<0.05$). But, the number of tubules and numerical density in the seminiferous tubules of azoospermia group were more than control and spontaneous recovery groups ($p<0.05$). the total, lumen and cellular diameters, luminal, cellular and cross sectional areas, number of tubules and numerical density and spermatogenesis index in the seminiferous tubules of spontaneous recovery group had no significant difference compared to the control group ($p>0.05$).

AMH and Testosterone Levels

Figure 3 indicates comparison of mean and standard error of AMH and testosterone levels in busulfan treatment and

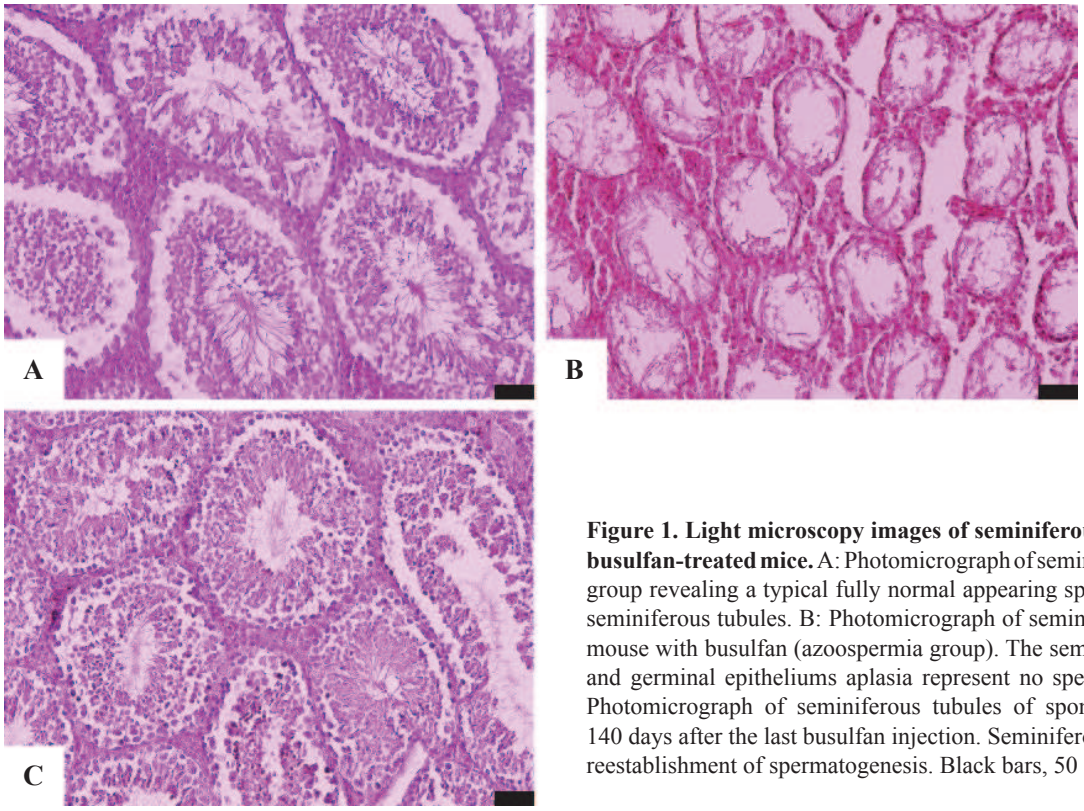

Figure 1. Light microscopy images of seminiferous tubule in control and busulfan-treated mice. A: Photomicrograph of seminiferous tubules of control group revealing a typical fully normal appearing spermatogenesis activity in seminiferous tubules. B: Photomicrograph of seminiferous tubules of treated mouse with busulfan (azoospermia group). The seminiferous tubular atrophy and germinal epitheliums aplasia represent no spermatogenetic activity. C: Photomicrograph of seminiferous tubules of spontaneous recovery group 140 days after the last busulfan injection. Seminiferous epitheliums represent reestablishment of spermatogenesis. Black bars, 50 µm (H&E staining).

control groups. Results analyses showed no significant differences in AMH and testosterone serum levels (p>0.05).

Discussion

Busulfan known as a strong agent that preferentially kills several species SSCs and when it intoxicates the cells in G1 phase, inhibits the next mitosis.(14) However busulfan has no effects on DNA synthesis, but it has side effects on various organs, including the bladder, liver, skin, gonadal function and, nervous system and is potentially mutagenic and carcinogenic.(15) In despite of the tremendous usefulness in biotechnology and chronic diseases therapy, its exact effects on the testis structure and epididymal-spermatozoa parameters have not been well studied.(16)

Recent studies demonstrated that chemotherapy drugs such as busulfan can cause testicular damage as manifested by oligospermia, reduced testicular volume and apoptotic cell death on testicular germinal epithelium.(17) Unlike other chemicals primarily, busulfan destroys SSCs. But, other chemicals except of busulfan kill differentiated spermatogonia.(18) In a study by Hosseini, *et al.*, busulfan decreases body and testes weight.(16) Zheng Wei, *et al.,* showed that there was a direct relationship between germinal cells number and testis weight in primates.(19) In another study by Bucci, *et al.*, demonstrated that chromosomal abnormalities and dominant lethal mutations in sperm caused by busulfan (4).

Variation of mice testicular histopathology and spermatogenesis potency in response to two injections of busulfan were investigated in present study. Our results demonstrated that there were no significant differences in AMH and testosterone serum levels. Disrupt spermatogenesis was observed in induced azoospermia and restoration of spermatogenesis spontaneously had already been done.

Moisan, *et al.*, showed when 20 mg/kg of busulfan doses administrated in mice, testicular masses went from a maximum to a minimum value. Histological evaluation suggested that the higher testis weights and diameters could be attributed to higher spermatogenesis levels from controls and 10 mg/kg dose group. Eight weeks after busulfan treatment, the increase in testis weight was most clear in treated animals at a 10 mg/kg dose suggesting extensive regeneration of spermatogenesis at mentioned dose. Probably busulfan had no effect on capsular thickness, because at high dose which testis more affected by drug,

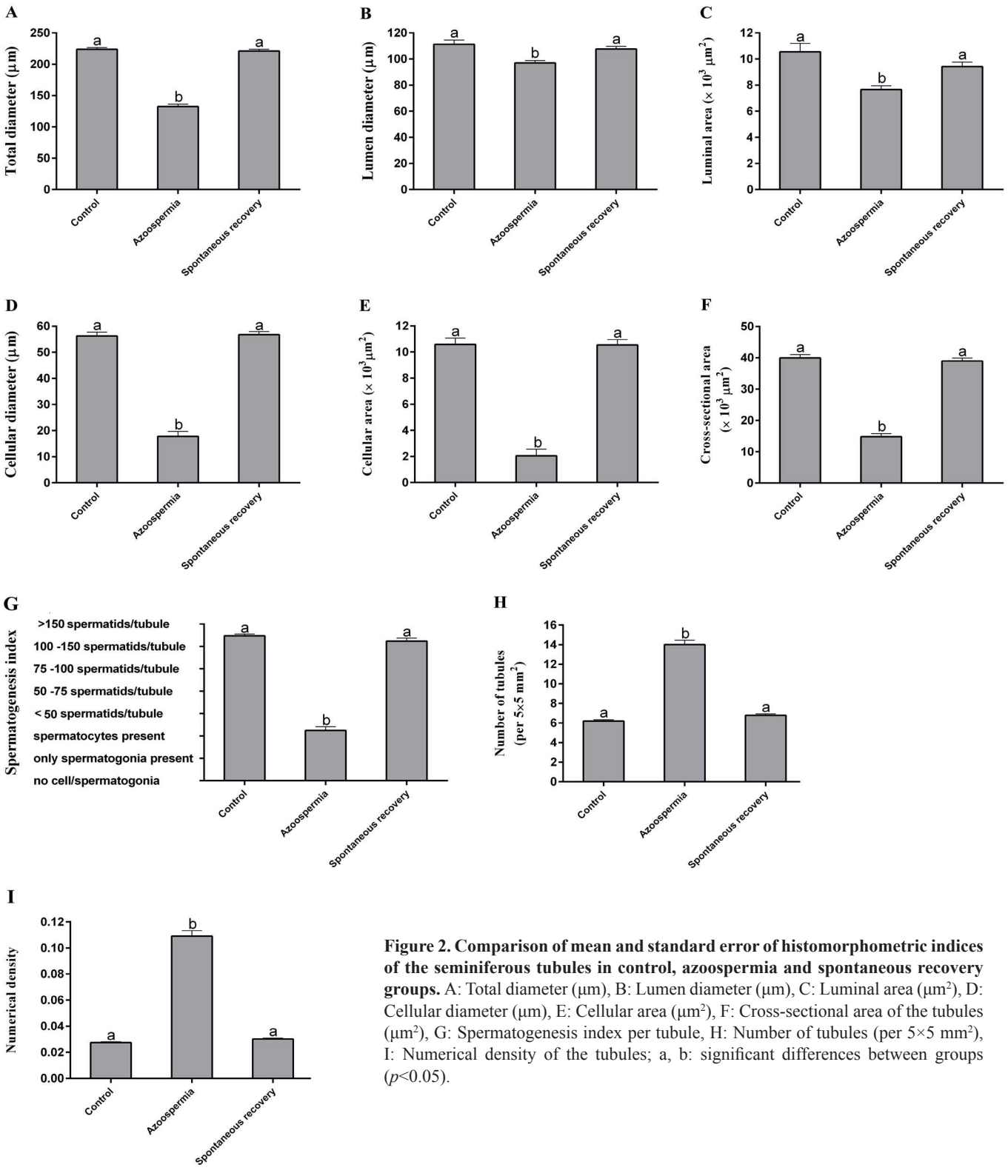

Figure 2. Comparison of mean and standard error of histomorphometric indices of the seminiferous tubules in control, azoospermia and spontaneous recovery groups. A: Total diameter (µm), B: Lumen diameter (µm), C: Luminal area (µm²), D: Cellular diameter (µm), E: Cellular area (µm²), F: Cross-sectional area of the tubules (µm²), G: Spermatogenesis index per tubule, H: Number of tubules (per 5×5 mm²), I: Numerical density of the tubules; a, b: significant differences between groups (p<0.05).

capsular thickness increased and *vice versa*.(20) According to the previous report by Karashima, *et al.,* (21), busulfan administration seems to produce only a non-permanent testicular injury, and the drug induced injury was somehow reversible. Delays of 1–2 weeks have been reported for some degree of restoration in different studies.(22) In another study, Panahi, *et al.*, found that two doses of busulfan injection with 21-days interval induced azoospermia 35

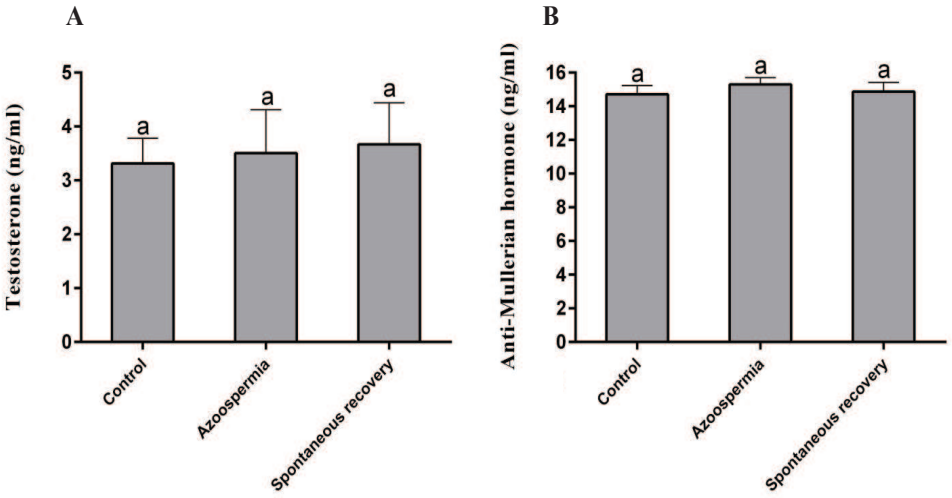

Figure 3. Comparison of mean and standard error of testosterone (A) and AMH (B) serum levels in control, azoospermia and spontaneous recovery groups in mouse. a: no significant difference between groups (p>0.05).

days after the last injection in rat.(12) Anjamrooz, *et al.*, in 2007 reported that intraperitoneal injection of busulfan at 20, 30, 40 and 50 mg/kg doses in rat could induce infertility after 4 weeks. So, a high dose of busulfan could eliminate sperms more significantly in epididymal lumen and permanently make the animals sterile while administration of a low dose resulted into a reduction in the number of germ cells. In mice, a dose of 30 mg/kg was found to be an optimal dose for treatment with busulfan to deplete the host germ cells and cause the lowest mortality in animals.(23) Anjamrooz results confirm the results of present study. So the mentioned dose is suitable to make azoospermia animals and stem cell transplantation, according to the spontaneous recovery.

Conclusion

Present study assigns detailed information on the busulfan effects on testicular tissue and serum levels of AMH and testosterone in adult mice. Used dosage of Busulfan prescription has no effect on AMH and testosterone serum levels but it is caused to destruction of the germinal epithelium and major reduction of histomorphometric indices of seminiferous tubules. In the other hand busulfan dosage injection can cause temporary azoospermia which is a good way to provide animal models in cell therapy research. Also, presented study indicates that spermatogenesis can occur spontaneously after busulfan therapy. So it can be used as a biomarker in spermatogenesis disorders and fertility in cancer patients.

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