Pharmacophore

Pharmacophore
an International Journal
ISSN 2229-5402

HISTOMORPHOMETRIC AND HISTOLOGICAL CHANGES OF TESTIS IN BUSULFAN-INDUCED OLIGOSPERMIA AND AZOOSPERMIA INFERTILITY

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ARTICLE INFO

ABSTRACT

Aim: The objective of the study was to determine the histological and hormonal changes in busulfan-induced oligospermia and azoospermia.

Materials and Methods: Eighteen BALB/c mice were divided into three equalgroups. Oligospermic group received a dose of 10 mg/kg busulfanintraperitoneally, the azoospermic group received a second dose of busulfan similarly, 21 days after the first injection and the control groupreceived no busulfan.All animals were assessed for histologic, histomorphometric and hormonal changes of testosterone and anti-Mullerian after 35 days.

Results: In an azoospermic group, the luminal diameter and area significantly increased in comparison to oligospermic animals and this increase was less than the control group. In an azoospermic group, cellular and total diameters and cellular and cross-sectional areas significantly decreased in comparison to oligospermic group and this decrease was less than the control group. Numerical density and tubular count per area unit in the azoospermic group were significantly more than the oligospermic group and in both groups were more than the control group. In mice receiving busulfan, spermatogenesis index significantly decreased when compared to the control group. In an oligispermic group, the testosterone levelwassignificantly more than other groups while anit-Mullerian level showed no changes.

Discussion: Histomorphometric, histological and hormonal assessment were introduced as prominent variables to confirm successful induction of oligo- and azoospermia,while these findings can be added to the literature and open a new window for future interventions in treatment of male infertility.

Introduction

Worldwide, a significant proportion of the population is subject to malignant diseases such as cancer, while surgery and the combination of chemo and radiation therapy have been introduced as current treatment measures for malignant diseases[1].On the other hand, malignant diseases were shown to affect gonadal activity through hormonal changes, even spermatogenesis may be affected more by cytotoxic drug side effects [2].Busulfan, 1,4 bis [metanesulfonyl-y] butane, with registered names of Myleran® and Busilvex® is a chemotherapeutic agent which is often used for the treatment of chronic myeloid leukemia[3] and is also used before bone marrow and stem cells transplantation of many other cancers[4,5].

Busulfan can inhabit cell division by sticking on one of DNA strands [6]. Therefore, any organs, tissues, and cells with high division activities such as spermatogonia are more sensitive to side effects of busulfan administration[7]. In mice, busulfan therapy can lead to an increase in apoptosis in testicular tissue and a decrease in testicular weight [8]. The administered dose

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and times of busulfan use can affect the recovery of spermatogenesis. A single dose of busulfan was demonstrated to influence fertility and lead to a permanent sterilization in experimental mice and result into long-term morphological damages of sperm lineages [9]. Busulfan was also shown to affect male germ cells and a depletion in these cells by loss c-Kit/SCF signaling, not by P53 or Fas/Fals dependent mechanisms [8].

Hamster and rat were previously introduced as azoospermia and oligospermia models using busulfan[10,11]. The required dose to sterilizevaries in different species. A doses of 40-100 mg/kg of busulfan in pigs [12] and two doses of10-15 mg/kg in rats [10,11,13,14],a dose of 4-12 mg/kg in coyotes [15] and in mice, 40-44 mg/kg in one injection [16-18] were successful protocols for induction of infertility.Anti-Mullerian hormone (AMH) is secreted by Sertoli cells during male fetal development and has also an important role in the regulation of Leydig cell and testosterone biosynthesis [19, 20]. Leydig cells are responsible for secretion of testosterone, which is an important hormone in male fertility and spermatogenesis [21].Busulfan was shown to decrease testosterone level after administration of a dose of 40 mg/kg of busulfan [22].There are several studies on busulfan side effects regarding spermatogenesis and fertility denoting to various therapeutic methods too. The current study was carried out to assess changes of the testis in busulfan-induced oligospermia and azoospermia using histomorphometry and histological evaluation.

Materials and methods

Animals

In this experimental study, eighteen male adult BALB/c mice (30±5 g) purchased from Comparative and Experimental Medicine Center, Shiraz University of Medical Sciences were enrolled in the study. All animals were in a controlled condition regarding temperature (22±2°C), humidity (55±5%) and lightening (12hr light/dark; from 07:00 a.m. to 19 p.m.). They had free access to feeding and drinking.

Induction of oligospermia and azoospermia

The mice were randomly assigned into three equal groups of oligospermia, azoospermia, and control. In oligospermia group, a single dose of busulfan (10 mg/kg, Busilvex®; Pierre Fabre Medicament Boulogne, France) was injected intraperitoneally and after 35 days to remove the testes for histomorphometric and histological evaluations, the mice were sacrificed. In azoospermia group, two doses of busulfan with 21 days interval was injected and then the testes were identically assessed on day 35 after the second injection. In control group, no busulfan was administered and their sacrifice and assessments were similar to other groups. On the day of sampling, sacrifice was undertaken using ether and cervical dislocation. For histomorphometric and histological evaluations, the removed testes were fixed in 10% formalin buffer solution.

Histomorphometric assessment

After fixation and alcohol dehydration of testicular tissue, it was embedded in paraffin and for tissue section, five horizontal cross-sections were conducted as described by Panahi et al.[10]. The sections were 5-µm in thickness and staining was conducted using hematoxylin-eosin (H&E) and were visualized using a light microscope (Model CX21, Olympus, Tokyo, Japan). The presence of spermatogonia, spermatocytes and spermatids were evaluated in all tubules. The inner, outer and total diameters were determinedin all tubules conducting 10 circular transverse sections from different regions of the tubules. The tubules' diameter was assessed in transverse sections by Dinocapture software (Version 2.0,Dino-Eye, San-Chung, Taiwan). By taking the average of two diameters of D1 and D2 at right angles, the average diameter of seminiferous tubules (D) was clarified (Figure1).

By use of diameter data, the luminal, cellular (germinal epithelium) and cross sectional areas were determined. In the seminiferous tubules, the cross-sectional area (Ac) was evaluated using the equation of $A_C = \pi D^2/4$; where D was considered as the mean diameter of tubules and π was regarded as equivalent to 3.142. The seminiferous tubulecount per unit area was also determined. The sections of seminiferous tubules per unit area were defined by use of an unbiased counting frame (Figure 2). In seminiferous tubules, numerical density (Nv) was considered as number of tubules per unit volume and it was assessedby the following equation: $N_V = N_A/D+T$[10], where, N_A denotes to the number of profiles per unit area, the tubule mean diameter was represented by D, and T revealed the mean thickness of the section.

The spermatogenesis index included the presence of spermatogenic cells throughout all testicular tissue compromising the types of cells, the number of cell layers, and the presence of late spermatids in the tubules. Therefore, the number of late spermatids if available were counted and then seminiferous tubules were classified based on the scale of 0–6 regarding spermatogenic potential, while the spermatogenesis index was modified[10]. The scale of 0 to 6 was considered as follows:0, without spermatogenic cells; 1, only spermatogonia was present;2, spermatogonia and spermatocytes were seen;3, spermatogonia, spermatocytes, and early spermatids noted as<50 late spermatids per tubule;4, all cell types were visible, and 50-100 late spermatids per tubule were present;5, all cell types were noticed and 100-150 late spermatids per tubule were noted; and6, all cell types were seen and up to >150 late spermatids per tubule were observed.

Testosterone and anti-Mullerian hormonal (AMH) assay

A blood sample was provided from the heart. Centrifugation at 400 g for 20 minutes was undertaken to provide the serum while later were stored at -80°C to be used for further analysis. To determine serum testosterone and AMH levels, testosterone was evaluated in all serum samples of the animals using testosterone radioimmunoassay kit (Institute of Isotopes Ltd. Budapest. Hungary. Catalog No: RK-61MACE040614) and measurement of serum AMH levels was done with the mouse anti-Mullerianhormone (AMH) ELISA kit (Bioassay Technology Laboratory- Shanghai, China. Catalog No: E1096Mo).

Statistical analysis

All data were presented as means and standard error (SE). Kolmogorov-Smirnov test was performed to compare the data. Statistical analysis was done with one-way ANOVA and Tukey post hoc test by SPSS software (Statistical Package for the Social Sciences for Windows, version 20, SPSS Inc., Chicago, Illinois, USA). The Mann-Whitney U test was used to assess spermatogenesis index. AP-value of less than 0.05 was regarded statistically significant.

Ethical consideration

The study received approval from Ethics Committee of Islamic Azad University for working with laboratory animals based on Iran Veterinary Organization regulations.

Results

Histopathologic findings

The control group compromised tubules with thin basement membrane and tunica propria, as well as normal germinal epithelium denoting to a proper order from spermatogonia to spermatocytes together with groups of spermatids and mature spermatozoa. Sertoli cells were compressed between the germinal cells and were not easily seen (Figure3A).

In oligospermia group, a hypospermatogenic state was noted. A decline was seen in all stages of cellularity in the germinal epithelium. The numbers of germ cells (spermatogonia, spermatocytes, and spermatids) revealed a decreasing trend, but, Leydig cells were normal. In addition, in some tubules maturation was arrested and histological assessment denoted to orders from spermatogonia to spermatids stages (Figure3B).

In the azoospermia group, only Sertoli cells were present and a complete germ cell aplasia was noted. The basement membranes and tunica propria were not thickened appreciably, and tubules showed a reduction in diameter, and only Sertoli cells were visible without any other spermatogenic cells (Figure3C).

Histomorphometric findings

The azoospermic group showed an increase in luminal diameter while the luminal area in seminiferous tubules was more than oligospermic group (p<0.001; Figure4A, D) and in control group, they were more than oligospermic and azoospermic groups (p<0.001).The cellular area and diameter in seminiferous tubules of the azoospermic group were less than the oligospermic and control groups (p<0.001 and p<0.001 respectively; Figure 4B, E). In an oligospermic group,the cellular area and diameter of the tubules showed a decline when compared to the control group (p=0.05 and p=0.03, respectively).In the azoospermic group, the total diameter and cross-sectional area of the tubules revealed a decrease in comparison to the oligospermic group (p<0.001 and p<0.001, respectively; Figure4C, F) and the control groups (p<0.001 and p<0.001, respectively).

In azoospermic and oligospermic groups, the total diameter and cross-sectional area of the tubules were less than the control group (p<0.001 and p<0.001, respectively).The spermatogenesis index of somniferous tubules in the azoospermic group was not different from the oligospermic group (p>0.05) and the index in both treatment groups was less than the control group (p<0.001; Figure4G). In the azoospermic group, the number of tubules per unit area and numerical density of tubules were more than the oligospermic group (p<0.001 and p<0.001 respectively; Figure4H,I).Both indices in both treatment groups were more than the control group (p<0.001 and p<0.001, respectively).

Hormonal assay

The testosterone level in the oligospermic group was significantly more than azoospermic and control groups (p<0.05; Figure 5A), while AMH level did not show any changes in all groups (p>0.05; Figure 5B).

Discussion

Administration of anticancer and alkylating agents in male patients was demonstrated to be accompanied by thickening of basement membrane, and germinal epithelium aplasia in the seminiferous tubules, which may lead to azoospermia[23].Busulfan as alkylating agent was shown to damage the germinal epithelium and cause azoospermia or oligospermia [24]. Busulfan as an anticancer and alkylating agent preferentially destroys spermatogonia; however, it has no effects on DNA synthesis.

Nevertheless, it inhibits the next mitosis when it intoxicates the cells in the G1 phase and inhibits DNA replication and leads to cell apoptosis [25],while prevention of DNA replication in cells by busulfan cannot be recovered[22]. After treatment with busulfan, the level of Stra 8, MAK mRNAs, and RAD51 which play a role in DNA damage repair, was found to decrease[8]. Therefore, busulfan as an alkylating agent was demonstrated to induce apoptosis in germ cells[26].After using busulfan, some morphological damages were also visible [9].

In our study, one injection of busulfan (10 mg/kg) was shown to induce oligospermia and two injections with a 21-day interval to induce azoospermia, 35 days after the last injection. Similar to our findings, Anjamrooz et al. [27] in rat demonstrated that intraperitoneal injection of different doses of busulfan (20, 30, 40, and 50 mg/kg) could induce infertility. A high dose of busulfan could remove sperms more significantly in the epididymal lumen and permanently make the animals infertile while administration of a low dose leads to a decrease in the number of germ cells. Several researchers revealed that busulfan therapy can induce long-term azoospermia even all spermatogonial cells did not disappear [28- 29]confirming our results.

Identical to our study, spermatogonia recovery in rats were previously followed morphologically after receipt of two doses of busulfan,21days after the second busulfan injection showing that most of the spermatogonial cells were absent [13]. Also in rats, two injections of busulfan with 21days interval revealed a decrease in spermatogenesis [11, 30] that confirms our data too. After chemotherapy, CK-18 is responsible for the increase in Sertoli cells, whereas in normal spermatogenesis after maturity, the marker is not present in these cells. Inactivation of Sertoli cells after chemotherapy canexplain the disruption of spermatogenesis and finally infertility[31].Sertoli cells were shown to be important in the development of germ cells and their support, thus any changes in these cells can disrupt spermatogenesis [18].

It was shown that busulfan treatment could distribute Sertoli cell vimentin filaments playing an important role in spermatogenesis [32]. It was shown that use of 30 mg/kg of busulfan can be an optimal dose to deplete the endogenous spermatogenesis and cause the lowest mortality in the animals [33]. It has been shown that local irradiation of the tissue can result in depletion of germ cells similar to the busulfan at doses of 50–55 mg/kg [34] too. Topically irradiated testes were found to be considered as an alternative to busulfan treatment; nevertheless, despite side effects and lethal effects, busulfan is still a good choice for induction of oligoseprmia and azoospermia when transplantation of spermatogonial stem cells are followed [11].Our results are in agreement with above mentioned studies, demonstrating that one does of busulfan could induce oligospermia and two injections with 21-days interval could induce azoospermia in BALB/c mice.

The testosterone level in the busulfan-induced oligospermic group was shown to be more than the control group. This change may be due to the effect of chemo- and radiotherapy reported to be associated with significant gonadal damagesin males [35].

Busulfan was previously shown to increase luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels [36], while two doses of busulfan(10 and then 15 mg/kg with 14 days interval) could elevate the testosterone level [37]. When spermatogenesis is impaired by chemical or irradiation agents, the LH level is usually associated with an increase [38] and the high level of LH can lead to a rise in vascular permeability [39].Also, increased level of LH was demonstrated to elevate the testosterone concentration confirming our findings related to busulfan group with an elevated testosterone level in attempts to recover depletion due to busulfan injection [37].

Conclusion

According to the results of this study, one interaperitoneal injection of 10 mg/kg busulfan could successfully induce oligospermia and two injections with a 21-day interval induce azoospermia in BALB/c mice after 35 days post-last injection. Histomorphometric, histological and hormonal assessment in BALB/c mice were introduced as prominent variables to confirm successful induction of oligo- and azoospermia, while these findings can be added to the literature and open a new window for future interventions in treatment of male infertility.

Competing interests

The authors declare that they have no competing interests.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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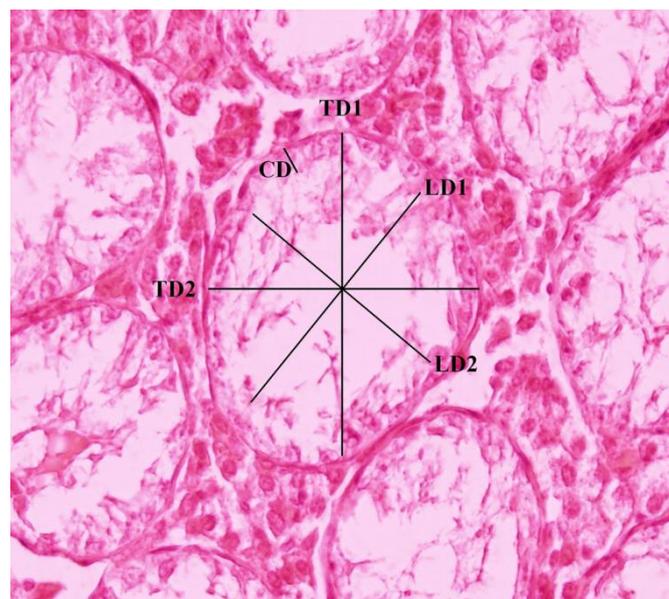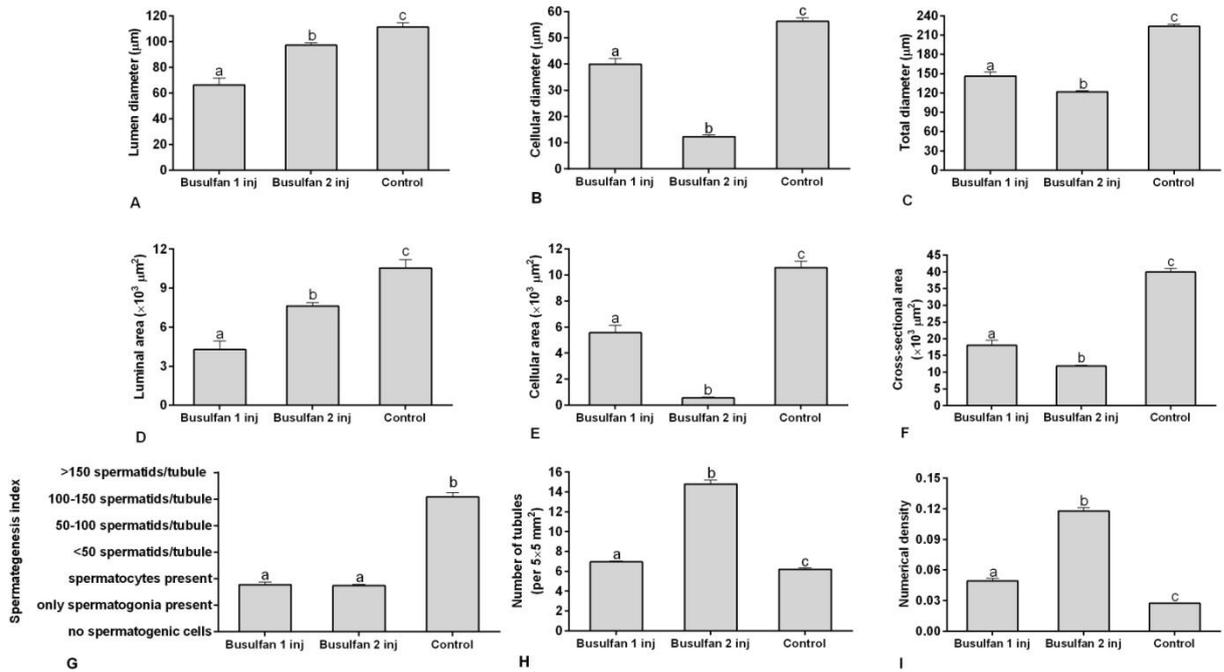
Figure1. Lumen and total diameter of seminiferous tubules in mouse,azoospermia group.TD1 and 2: Total diameter, LD1 and 2: Lumen diameter, CD: Cellular diameter (x100, H&E staining).
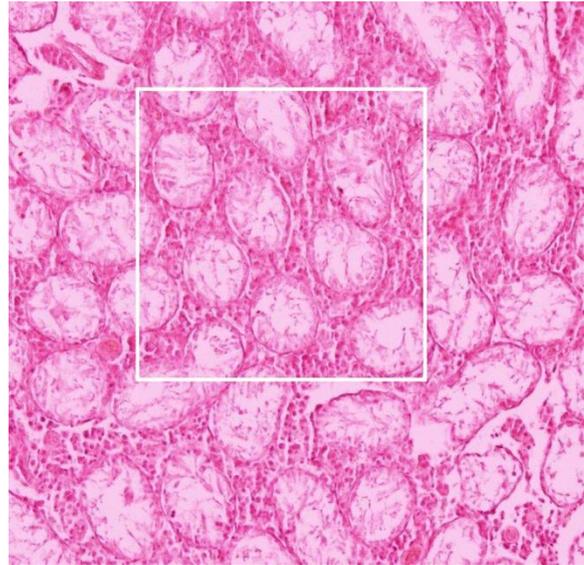
Figure2. In azoospermia group, the number of tubules per unit area was determined by unbiased counting frame (0.5×0.5 mm^2, x40, H&E staining).
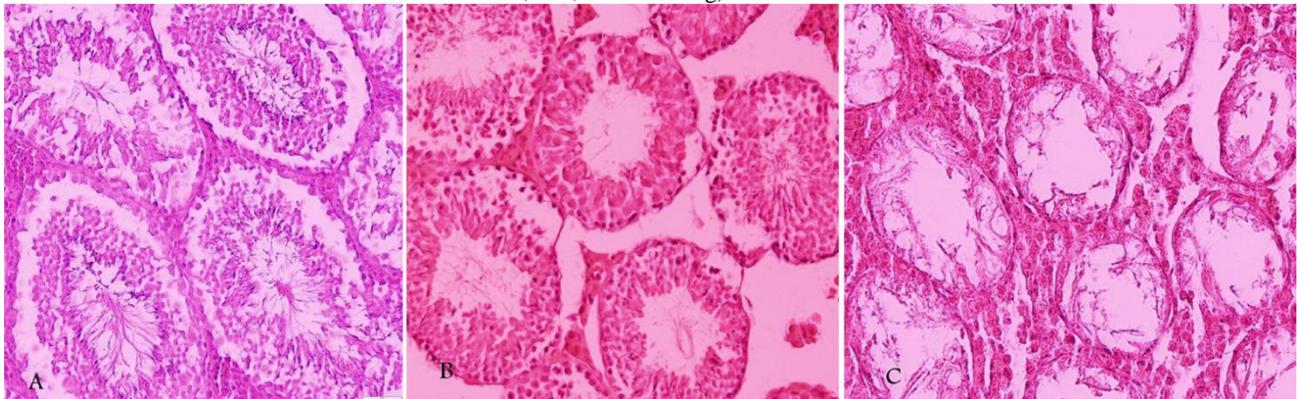

Figure3. Seminiferous tubules in different groups. Control group (a), oligospermia group (b), and azoospermia group(c) (x100, H&E staining).

Figure4. Mean and standard error of stereological indices of seminiferous tubules in mice with single dose, double doses busulfan injection and control groups.**A)** Lumen diameter (μm), **B)** Cellular diameter (μm), **C)**Total diameter (μm), **D)** Luminal area (μm^2), **E)** Cellular area (μm^2), **F)** Cross sectional area of the tubule (μm^2), **G)**Spermatogenesis index**, H)** Number of seminiferous tubules per unit area of testicular tissue, **I)** Numerical density of the seminiferous tubules.[a,b,c]Different superscript letters show significant differences between groups (p<0.05).
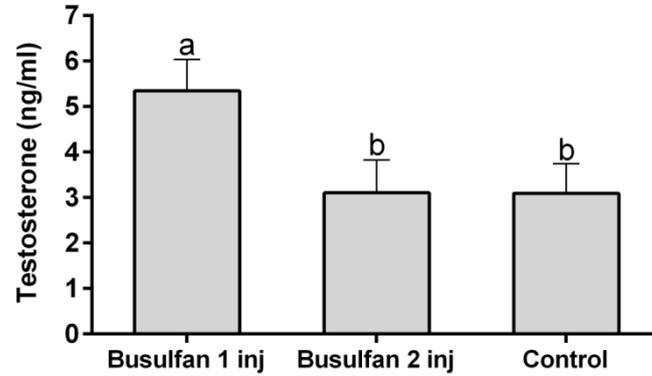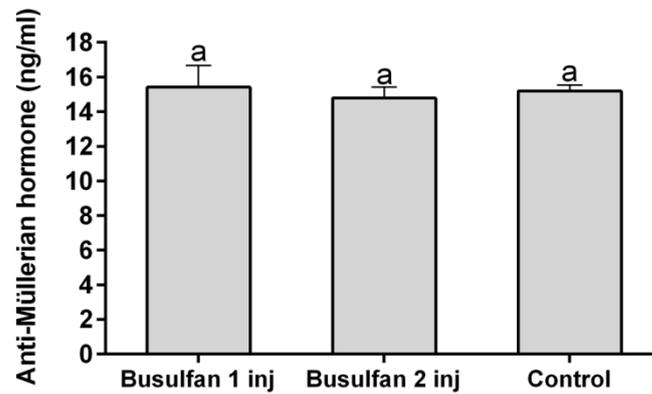
Figure 5. Comparison of mean serum testosterone **(A)**, and anti-mullerian hormone **(B)** in mice with single dose, double doses of busulfan injection and controlgroups.[a,b,c]Different superscript letters show significant differences between groups (p<0.05).