

Stereological evaluation of testes in busulfan-induced infertility of hamster

Mohadeseh Panahi · Negar Karimaghai · Farhad Rahmanifar · Amin Tamadon · Akbar Vahdati · Davood Mehrabani · Omid Koochi-Hosseiniabadi · Masood Sepehrimanesh

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Abstract Cytotoxic medications can influence spermatogenesis at least temporarily and in some cases permanently. Busulfan is a cytotoxic bifunctional alkylating agent which inhibits cell division by sticking to DNA strands. This study aims to stereologically evaluate the testes in busulfan-induced infertility of azoospermic albino hamsters. Eighteen male adult albino hamsters were randomly divided into 3 equal groups. The first group received just one dose of busulfan (10 mg/kg, intraperitoneally, Busilvex[®], France), the second group received two doses of busulfan with 21-days interval, and the control group left identically without any busulfan therapy. On day 35 postinjection, the testes of all animals were removed for histological evaluation and also for stereological indices. Lumen, cellular and total diameters, luminal, cellular and cross-sectional areas, number of tubules per unit area of testis, numerical density of the tubules, and spermatogenesis index in hamsters that received

two doses of busulfan were significantly more than the hamsters receiving one dose of busulfan and the control group. Two doses of busulfan injected with 21-days interval could properly induce azoospermia in the experimental hamster model.

Keywords Stereology · Testis · Infertility · Busulfan · Hamster

Introduction

Infertility is the inability to conceive due to low semen quality. Although, malignant diseases might influence gonadal function through hormonal alterations and metabolic conditions, but, the negative effects of cytotoxic drugs on spermatogenesis has been extensively investigated (Dohle 2010). It is clear that the cytotoxic therapy influences spermatogenesis at least temporarily and in some cases permanently (Wallace et al. 2005).

Busulfan with registered names of Myleran[®] and Busilvex[®] is a cytotoxic bifunctional alkylating agent, which inhibits cell division by sticking to the DNA strands (Iwamoto et al. 2004). It is used as a chemotherapeutic agent in a low dose during a long period for treatment of chronic myeloid leukemia (Suttrop and Millot 2010) or in a higher dose before bone marrow or stem cell transplantation for other types of cancer (Krivoy et al. 2008; Le Bourgeois et al. 2013; Nieto et al. 2012). It has been demonstrated that busulfan-treated mice exhibited a marked increase in apoptosis and a decrease in testicular weight (Choi et al. 2004). It must be remembered that recovery of spermatogenesis depends on the drugs used and the administered dose. A single dose of busulfan can permanently sterilize mice at non-lethal doses and can cause long-term morphological damage in sperms produced by surviving spermatogonia (Bucci and Meistrich 1987).

It was shown that an increased depletion of male germ cells happens in busulfan-treated mouse which is mediated by loss of c-kit, a protein that is found on the surface of many different types

M. Panahi · N. Karimaghai · A. Tamadon · D. Mehrabani (✉)
Stem Cells and Transgenic Technology Research Center, Shiraz
University of Medical Sciences, Shiraz, Iran
e-mail: davood_mehrabani@yahoo.com

D. Mehrabani
e-mail: mehrabad@sums.ac.ir

F. Rahmanifar
Department of Basic Sciences, School of Veterinary Medicine, Shiraz
University, Shiraz, Iran

A. Vahdati (✉)
Department of Physiology, Fars Science and Research Branch,
Islamic Azad University, Shiraz, Iran
e-mail: avahdatim@yahoo.com

O. Koochi-Hosseiniabadi
Laboratory Animal Center, Shiraz University of Medical Sciences,
Shiraz, Iran

M. Sepehrimanesh
Gastroenterohepatology Research Center, Shiraz University of
Medical Sciences, Shiraz, Iran

of cells binding to a substance called stem cell factor (c-kit/SCF) signaling, but not by p53- or *Fas/FasL*-dependent mechanisms (Choi et al. 2004). The reports revealed that busulfan is dose- and time-dependent which can diminish spermiogenesis and results into a delay in meiosis, but the Leydig cells stay unchanged and the weight of the seminal vesicles and serum LH and FSH do not alter versus a control group (Krause 1975). There are very few reports on busulfan side effects in testicular architecture and in relation to fertility indices. Therefore, this study was undertaken to evaluate histological changes in testicular tissue in response to a single dose or the two doses of busulfan injected in male hamster in comparison to the control group.

Materials and methods

Animals

Eighteen adult male albino hamsters (95 ± 5 g) from the Laboratory Animal Center of Shiraz University of Medical Sciences,

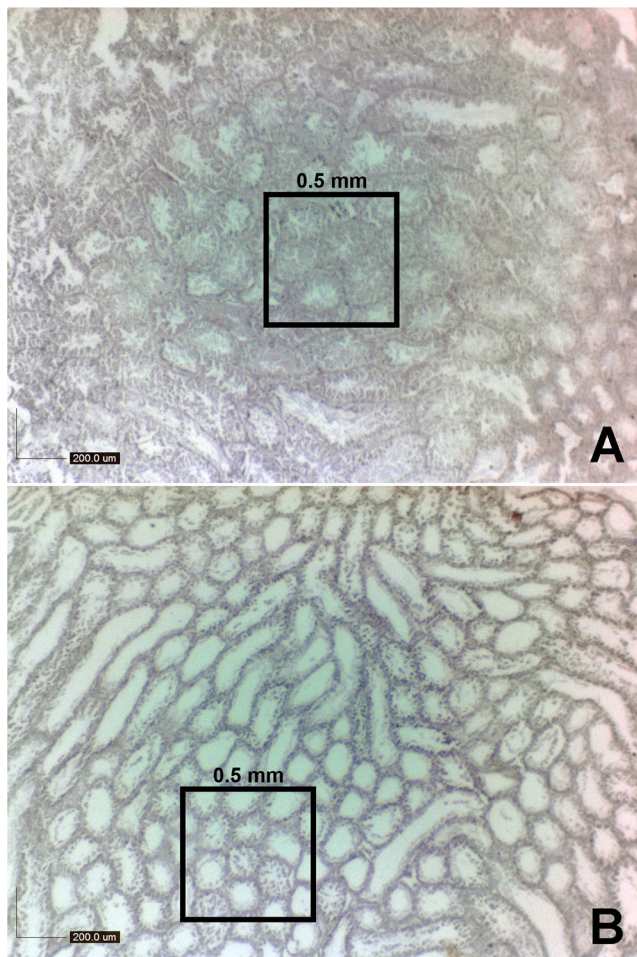


Fig. 1 The number of profiles of seminiferous tubules in different groups. Control group (a); injection of two doses of busulfan into the testes per unit area (b). Scale bar is 200 μ m (hematoxylin and eosin staining)

Shiraz, Iran were enrolled. They were housed in cages under controlled temperature (22 ± 2 °C) and lighting (14:10 h light/dark; lighting from 07:00 a.m. to 21:00 p.m.) with free access to food pellets diet and water. The animals were kept in accordance with regulations and recommendations of the animal care committee of Shiraz University of Medical Sciences.

Busulfan therapy and sampling

The animals were randomly assigned into three equal groups. The first group received one dose of busulfan (10 mg/kg, intraperitoneally, Busilvex[®], Pierre Fabre Medicament,

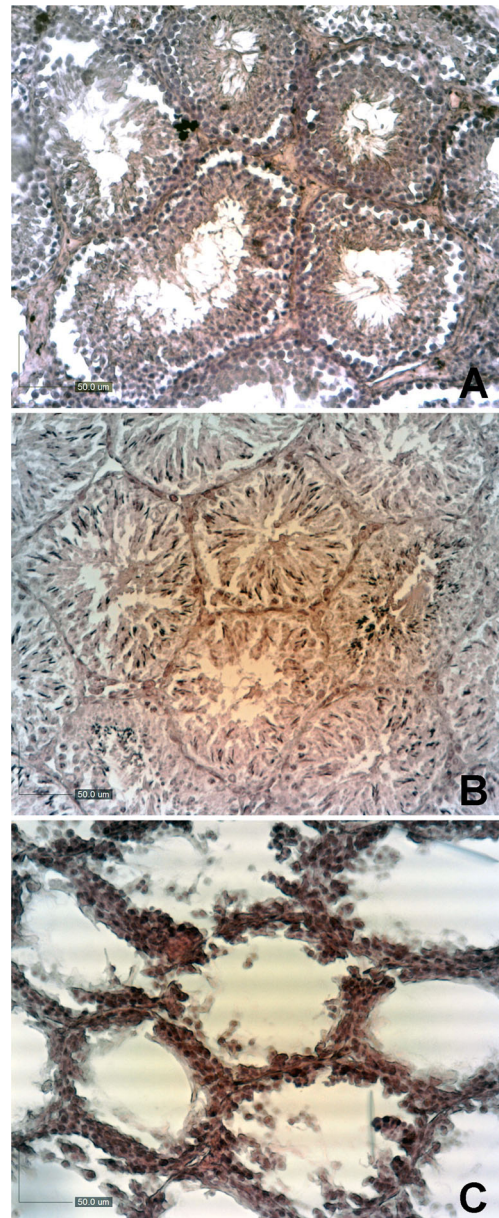


Fig. 2 Seminiferous tubules in different groups. Control group (a), injection of one dose of busulfan (b), and injection of two doses of busulfan into the testes (c). Scale bar is 50 μ m (hematoxylin and eosin staining)

Boulogne, France) while their testes were removed on day 35 postinjection for histological evaluation. The second group received two doses of busulfan with 21-days interval, and their testes were removed identically on day 35 after the second injection. The third group was considered as control group and their testes were similarly removed. On the day of sampling, the animals were euthanized with ether and their testes were collected and fixed in a 10 % formalin buffer

solution. After fixation, the testes were embedded in paraffin, and histological sections were made from each block. The 5- μm thickness sections were stained with hematoxylin–eosin.

Stereological analysis

For each testis, five vertical sections were provided from the polar and the equatorial regions. In a cross-section, all tubules

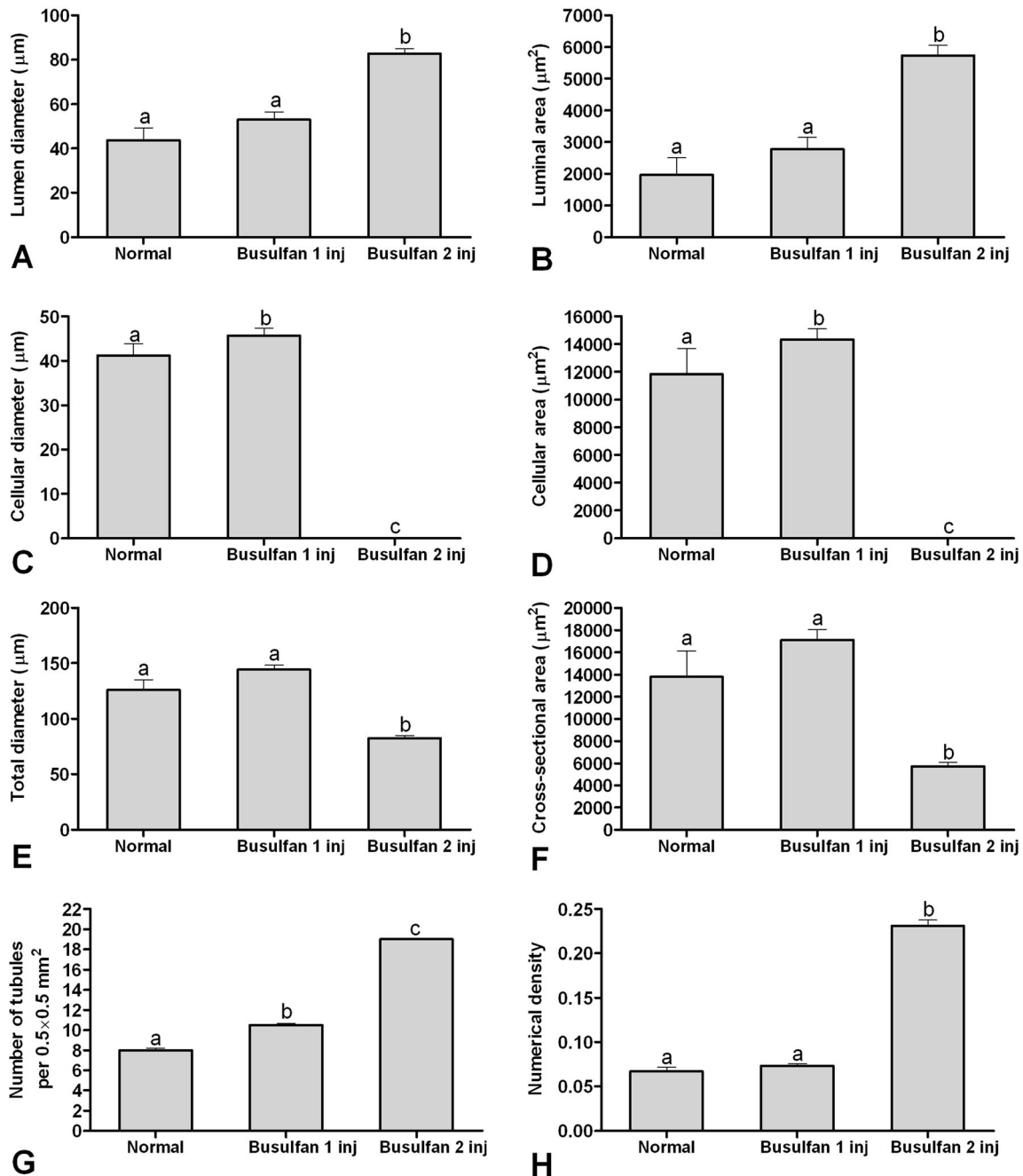


Fig. 3 Mean and standard error of stereological indices of seminiferous tubules in different groups of control, one dose and two doses of treatment with busulfan. **a** Lumen diameter (μm), **b** luminal area (μm^2), **c** cellular diameter (μm), **d** cellular area (μm^2), **e** total diameter (μm), **f** cross-

sectional area of the tubule (μm^2), **g** number of seminiferous tubules per unit area of testis, and **h** numerical density of the seminiferous tubules. **a, b, c** Different superscript letters show significant differences between groups

were evaluated for the presence of spermatogonia, spermatocytes, and spermatids. Ten identical circular transverse sections were undertaken in tubules, each in a different region of the testis using a systematic random scheme to determine the stereological indices (Gundersen and Jensen 1987; Whitsett et al. 1984). The mean seminiferous tubule diameter (D) was derived by taking the average of two diameters, D_1 and D_2 , at right angles. Cross-sectional area (A_c) of the seminiferous tubules was determined using the equation $A_c = \pi D^2/4$, where π is equivalent to 3.142 and D is the mean diameter of seminiferous tubules. The number of profiles of seminiferous tubules per unit area (N_A , Fig. 1) was determined using the unbiased counting frame proposed by Gundersen (1977). Numerical density (N_v) was defined as the number of profiles per unit volume using the modified Floderus equation: $N_v = N_A/(D+T)$ (Gilliland et al. 2001), where N_A is the number of profiles per unit area, D is the mean diameter of the seminiferous tubule, and T is the average thickness of the section.

A testis was rated for its spermatogenic potential (modified spermatogenic index) on a modified scale of 0 to 5 (Chang et al. 2002). The index was based on the appearance of the spermatogenic cells throughout the testis and included the number of cell layers, types of cells, and the presence of late spermatids in the seminiferous tubules. The index and criteria were as follows: 0, no spermatogenic cells; 1, only spermatogonia present; 2, spermatogonia and spermatocytes present; 3, spermatogonia, spermatocytes, and round (early) spermatids present with <50 late spermatids per tubule; 4, all cell types present, up to 50–100 late spermatids per tubule; and 5, all cell types present and >100 late spermatids per tubule.

Statistical analysis

Means and standard error (SE) of the data of stereological indices of seminiferous tubules were subjected to Kolmogorov–Smirnov test of normality and analyzed by one-way ANOVA (SPSS for Windows, version 11.5, SPSS Inc, Chicago, Illinois), and post hoc test was performed by LSD test. The spermatogenesis index of seminiferous tubules was compared using Mann–Whitney U test. The P value of less than 0.05 was considered to be statistically significant. Group means

and their standard error were reported in the text and graphs (GraphPad Prism version 5.01 for Windows, GraphPad software Inc., San Diego, CA, USA).

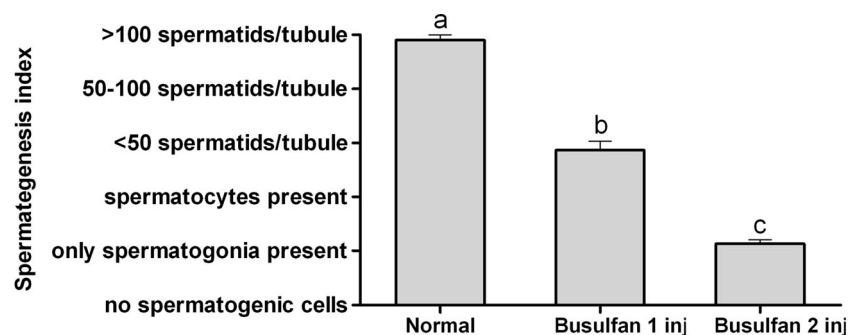
Results

Lumen diameter and luminal area of the seminiferous tubules in hamsters receiving two doses of busulfan were more than the hamsters in group receiving one dose of busulfan ($P < 0.001$ and $P < 0.001$, respectively) and control group ($P < 0.001$ and $P < 0.001$, respectively; Figs. 2a–c and 3a, b). Cellular diameter and cellular area of the seminiferous tubules in hamsters that received two doses of busulfan were less than the hamsters in the group receiving one dose of busulfan ($P < 0.001$ and $P < 0.001$, respectively) and the control group ($P < 0.001$ and $P < 0.001$, respectively; Fig. 3c, d). Moreover, the mean of cellular diameter and cellular area of the hamster that received one dose of busulfan were more than the control group ($P = 0.05$ and $P = 0.03$, respectively). An increase was noticed in cellular diameter and cellular area of the one dose busulfan-injected group while detachment and scattering of cells increased in cellular layer of seminiferous tubules after first injection of busulfan (Fig. 2b).

The total diameter and cross-sectional area of the seminiferous tubules in hamsters that received two doses of busulfan were less than the animals that received one dose of busulfan injection ($P < 0.001$ and $P < 0.001$, respectively) and the control group ($P < 0.001$ and $P < 0.001$, respectively; Fig. 3e, f). Moreover, the mean of cellular diameter and cross-sectional area of the tubules in the group that received one dose of busulfan were more than the control group ($P = 0.01$ and $P = 0.03$, respectively).

The number of seminiferous tubules per unit area of testis and numerical density of the seminiferous tubules in hamsters that received two doses of busulfan were more than those animals that received one dose of busulfan ($P < 0.001$ and $P < 0.001$, respectively). The index in both busulfan-treated groups was more than the control group ($P < 0.001$ and $P < 0.001$, respectively; Fig. 3g, h). The spermatogenesis index

Fig. 4 Mean and standard error of spermatogenesis index of seminiferous tubules in different groups of control, one dose and two doses of treatment with busulfan. ^{a, b, c} Different superscript letters show significant differences between groups



of seminiferous tubules in hamsters receiving two doses of busulfan was less than those that received one dose of busulfan ($P < 0.001$) and the index in both busulfan-treated groups was less than the control group ($P < 0.001$ and $P < 0.001$, respectively; Fig. 4).

Discussion

In the current study, histomorphological changes of male hamster testicular tissue in response to busulfan administration were determined. Our findings revealed that injection of two doses of busulfan could induce more damages in testicular tissue than injection of one dose of busulfan and the control group. Treatment with busulfan was shown to effectively destruct spermatogonial stem cells in several species with no effect on DNA synthesis; however, it could inhibit the next mitosis due to intoxication of the cells in the G1 phase (de Rooij and Vergouwen 1990; Kramer and De Rooij 1969). Also, different doses of busulfan were administered in different animal species to prepare the recipient before an experimental spermatogonial transplantation (Wang et al. 2010).

It was demonstrated that 20 days after a second intraperitoneal injection of busulfan, the testes lost most of their spermatogenic cells (Jiang 1998). Panahi et al. (2015) found that two doses of busulfan injection with 21-days interval induced azoospermia 35 days after the last injection in rat. This model can be used for stem cell transplantation that is carried out by our group in future studies. Anjamrooz et al. (2007) reported that intraperitoneal injection of busulfan at doses of 20, 30, 40, and 50 mg/kg in rat could induce infertility after 4 weeks with a sperm count of less than $6.5(\pm 0.09) \times 10^5$ cell per ml. 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 dosage 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 (Wang et al. 2010).

Several therapeutic measures are undertaken in malignancies including surgery, chemical, and radiotherapies. Irradiation schedule can result into depletion of an endogenous spermatogenesis similar treatment with busulfan at doses of 50–55 mg/kg (Zhang et al. 2006). These findings can direct researchers to this fact that local treatment of testicular tissue cannot endure the systemic toxicity of the drug. However, despite adverse and lethal effects of busulfan, this drug is still used to prepare an animal model for further cell transplantation because the drug can cause a successful depletion in host germ cells and allow efficient colonization of the donor spermatogonial stem cells.

In conclusion, the administration of busulfan for cancer therapy can have long-term consequences such as reduced fertility and sometimes sterility. Our findings showed that injection of two doses of busulfan with 21-days interval could properly induce a hamster animal model for azoospermia with comparable correlated stereological indices of seminiferous tubules 35 days after the last injection. This model can be used for stem cell transplantation in future studies.

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Conflict of interest There is no conflict of interest.

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