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Assessment of Morphological and Functional Changes in the Mouse Testis and Epididymal Sperms Following Busulfan Treatment

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ABSTRCT

Background: Busulfan, a cytotoxic drug, which is currently used as a chemotherapeutic agent, has many side effects on different body organs. In this research, the effects of busulfan on sperm parameters and microstructure of mouse testis were investigated. **Methods:** Busulfan was injected intrapretoneally at 10, 20, 30, 40 and 50 mg/kg and testes were removed after 4, 6 and 8 weeks, weighed and processed for light microscopic examination. Transverse and cross section diameters of testes, seminiferous tubules diameters, percentage of different types of tubules, epithelium thickness, spermatogenic cell numbers and capsule thickness as well as the sperm parameters in epididymis were measured. **Results:** There was a significant decline in sperm numbers and marked changes in testes structures. Almost 8 weeks after the injection of drug, some of the changes are reversed. Accordingly, the changes in percent of normal tubules without sperm, abnormal tubules and capsule thickness were increased until 6 weeks of drug administration, the changes declined thereafter. **Conclusion:** In general, busulfan caused a decrease in all analyzed parameters (except capsule thickness, normal tubules without sperm and abnormal tubules), probably due to the arrest of spermatogenesis. Our results also revealed that some of the changes are reversible and dose dependent. *Iran. Biomed. J. 11 (1): 15-22, 2007*

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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 various species. One critical step in successful transplantation is the 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 [$CH_3SO_2O(CH_2)_4OSO_2 CH_3$] 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 sulphur-containing metabolites. Busulfan has many side effects on different body organs such as bladder, liver, skin, nervous system and gonadal function and other alkilating 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 kills 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

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literature, but have not been quantified [8, 9]. Busulfan-treated adult mice 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 mice is inefficient, perhaps because of damage to the testicular environment caused by the ablative therapy [1, 10]. Despite the potential usefulness of busulfan in clinic and biotechnology, its exact effect on testis microstructure and epididymal sperm parameters has not well investigated yet. Therefore in this study, we are reporting the effects of different doses of busulfan on sperm parameters and testis microstructure of mouse. Our data reveals wide effects of busulfan on testis structure and morphology as well as sperm parameters in a dose-dependent and somehow reversible fashion.

MATERIALS AND METHODS

Animals. Adult NMRI mice at 6-8 weeks of age were purchased from Razi Vaccine & Serum Research Institute (Karaj, Iran). Animals were weighed and then given a single i.p. injection of busulfan (Sigma B2635, UK) with a wide range of doses: 10, 20, 30, 40 and 50 mg/kg. The low and high doses were selected based on pilot study in mice. Busulfan treated and control animals were sacrificed by cervical dislocation 4, 6 and 8 weeks after injection of busulfan. Following cervical dislocation, left testis and both epididymis were recovered, the testes weighed and fixed in Bouin's fixative, dehydrated and embedded in paraffin. Then, 5-µm serial microscopic sections were prepared and at least 5 slides from each testis stained with hematoxylin and eosin for histological assessment. In each experiment, at least 5 animals were received busulfan and analyzed for each dose and time point. All animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Tarbiat Modarres University (Tehran, Iran).

Sperm parameters assessment. The epididymis was placed in 1 ml PBS (pH 7.4) and minced into small pieces before being incubated at 37°C for 30 min. Sperm parameters were monitored by light microscopy. Sperms viability was assessed by determining the percentage of sperms excluding vital dye (25% eosin solution). Briefly, 7 µl of eosin solution was added to 20 µl of cell suspension after incubation and mixed thoroughly. Motility of sperm was also assessed by determining the percentage of motile sperm. Finally, sperm numbers was also calculated with a hemocytometer count and compared in both busulfan-treated and control groups.

Morphometrical analysis of testis. An ocular grid was used to measure the diameters of longitudinal and cross sections of the testes. Also for each testis, in 100 randomly selected tubular profiles that were round or nearly round, percentage of 3 types of tubules (normal seminiferous tubule with sperm (type 1), normal seminiferous tubules without sperm (type 2) and abnormal seminiferous tubule (type 3) as well as the diameters of the tubules and epithelium thickness were measured under light microscopy. Capsule thickness was also measured. Using a 441-intersection grid, volume density of spermatogonial cells, spermatocytes and spermatids in seminiferous tubules were determined. The location and morphology of the cells within the seminiferous tubules were used to identity them. An estimate of each parameter was performed by examining 20 fields in 5 histological sections from each testis.

Statistical analysis. The results were analyzed by performing ANOVA and Tukey's tests, with $P<0.05$ considered as statistically significant. The standard deviation (SD) was also calculated for each value.

RESULTS

Analyses of sperm parameters in epididymis following busulfan administration. As the results show, there is a significant decline in sperm viability and motility rates (Table 1) as well as a decline in sperms counts (Table 2) following busulfan administration. Also, the effects of busulfan are dose-dependent, with maximum effects seen at high doses (30 and 40 mg/kg). The viability of sperms decreased at all tested doses of the drug, however, the 10 mg/ml group showed the minimal effect, and the decline in sperm viability in the 6^{th} week group was more obvious than the 4^{th} and the 8^{th} week groups (Table 1). There was no sperm viability or motility at 40 mg/kg dose of the drug, but there was small viability and motility rate only 8 weeks after administration of 30 mg/kg of the drug. Accordingly, some of the effects of other doses of

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Table 1. Viability and motility rates (%) of sperms aspirated from epididymis after busulfan treatment*.

Sperm parameters	Viability rate (mean ± SD)			Motility rate (mean ± SD)		
Time of assessment	The 4th w	The 6th w	The 8th w	The 4th w	The 6th w	The 8th w
Control	77.0 ± 5.4	79.0 ± 6.6	78.0 ± 7.3	72.0 ± 6.2	70.0 ± 5.1	74.0 ± 7.3
10 (mg/kg)	60.5 ± 5[a,c]	38.6 ± 3.9[a,c]	54.3 ± 7. 5[a]	56.0 ± 5.5[a,c]	35.5 ± 2.7[a,c]	50.0 ± 2[a]
20 (mg/kg)	24.8 ± 2[a,b,c]	0[a,b,c]	31.2 ± 2.6[a,b]	15.8 ± 2[a,b]	0[a,b,c]	17.5 ± 2.5[a,b]
30 (mg/kg)	0[a,b]	0[a,c]	8.2 ± 1[a,b]	0[a]	0[a,c]	3. 6 ± 0.2[a,b]
40 (mg/kg)	0[a]	0[a]	0[a]	0[a]	0[a]	0[a,b]

*, The replication of experiences was at least 5 times for all groups; [a,] Significant difference versus control group in the same column, $P<0.05$; [b,] Significant difference versus lower dose of busulfan in the same column, $P<0.05$; [c,] Significant difference versus next time in the same row, $P<0.05$; SD, Standard deviation.

Table 2. Sperm count per ml in epididymis after busulfan treatment*.

Time of assessment	Sperm count (mean ± SD) × 10^6		
	The 4th w	The 6th w	The 8th w
Control	4.30 ± 0.05	4.50 ± 0.06	4.20 ± 0.07
10 (mg/kg)	1.80 ± 0.03[a,c]	0.70 ± 0.03[a, c]	1.50 ± 0.05[a]
20 (mg/kg)	0.04 ± 0.02[a, b, c]	0.01 ± 0.0[a, b, c]	0.30 ± 0.04[a, b]
30 (mg/kg)	0.01 ± 0.0[a, b, c]	0[a, b, c]	0.20 ± 0.1[a]
40 (mg/kg)	0[a, b]	0[a,c]	0.01 ± 0.01[a]

For legend, refer to Table 1.

busulfan (10 and 20 mg/kg) are reversible and came back after 8 weeks. Similar observation was made for the effect of the drug on sperm motility rate. However, the effect of busulfan on sperm motility was more sever and the effect of all doses group, including 10 mg/kg dose, was statistically significant (Table 1).

The number of sperms per ml aspirated from both epididymides was determined by hemocytometer counts. As presented in Table 2, all doses of busulfan significantly decreased the number of sperms in semen. We obtained $<6.5 \times 10^5 \pm 9 \times 10^3$cell/ml sperm/ml at 20, 30 and 40 mg/kg doses of the drug. At 10 mg/kg dose, however, sperms number was significantly decreased but didn't reach the critical number of $<6.5 \times 10^5 \pm 9 \times 10^3$ cell/ml [11]. Similar to our previous observations, the effect of busulfan on sperms number is dose-dependent and somehow reversible (Table 2).

Analyses of morphological changes of mouse testis following busulfan treatment. As the data show, the lethal dose of busulfan is 50 mg/kg in mice. Testicular weights in the busulfan-treated groups were significantly less than the ones in the control group. At lower dose (10 mg/kg), busulfan treated testes were heavier than other doses. There was not any significant difference between animal treated with busulfan at 20, 30 and 40 mg/kg. The decrease in the weight of testes in animals that received the drug was nearly dose-dependent (Table 3). The same effects were observed when we measured testis diameters at both cross and longitudinal sections of the testes. Both testis diameters were decreased in busulfan treated groups (Table 4).

Table 3. Testicular weights(***mg***) in the busulfan-treated and untreated groups *

Time of assessment	Testicular weights (mean ± SD)		
	The 4th w	The 6th w	The 8th w
Control	112.4±8.7	110.3±6.9	114.2±9.1
10 (mg/kg)	96.4±9.8[c]	69.4±5.5[a]	74.2±3.5[a]
20 (mg/kg)	39±4.5[a,b]	35±3.9[a,b]	41.6±4.1[a,b]
30 (mg/kg)	35.7±3.8[a]	32.6±3.2[a]	37±3.3[a]
40 (mg/kg)	33.9±3.2[a]	29±2.1[a]	32.5±3.7[a]

For legend, refer to Table 1.

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Table 4. Testis diameters (**µm**) after busulfan treatment*.

Sperm parameters	Cross section diameter (mean ± SD) ×10^3			Longitudinal diameter (mean ± SD) ×10^3		
Time of assessment	The 4th w	The 6th w	The 8th w	The 4th w	The 6th w	The 8th w
Control	3.3 ± 0.1	3.3 ± 0.2	3.2 ± 0.2	4.4 ± 0.1	4.5 ± 0.2	4.4 ± 0.2
10 (mg/kg)	3.2 ± 0.1^{c}	2.8 ± 0.09^{a}	2.7 ± 0.08^{a}	4.4 ± 0.2	3.7 ± 0.2^{a}	3.8 ± 0.2
20 (mg/kg)	2.6 ± 0.3 ^{a,b}	2.2 ± 0.1^{a,b}	2.4 ± 0.08^{a,b}	3.6 ± 0.1^{a,b,c}	3.0 ± 0.2^{a,b}	3.2 ± 0.1^{a,b}
30 (mg/kg)	2.4 ± 0.1^{a}	2.2 ± 0.1^{a}	2.5 ± 0.1^{a}	3.1 ± 0.2^{a,b}	2.8 ± 0.1^{a}	3.3 ± 0.2^{a}
40 (mg/kg)	2.2 ± 0.09^{a}	2.1 ± 0.03^{a}	2.2 ± 0.06^{a,b}	3.0 ± 0.1^{a}	2.5 ± 0.1^{a}	2.9 ± 0.1^{a}

For legend, refer to Table 1.

Histological examinations of the testes in both control and busulfan-treated groups revealed that capsular thickness was increased in busulfan-treated animals (Fig. 1). Accordingly, the diameter of seminiferous tubules was significantly decreased in all doses of busulfan treatment except for the 10 mg/kg dose at the 4th week group, where no apparent effect of busulfan on the seminiferous epithelium was observed. Measuring the seminiferous tubules diameters at different time groups showed that the maximum effects of lower doses (10 and 20 mg/kg) was evident after 6 weeks of drug administration. In contrast, in high doses groups (30 and 40 mg/kg), the changes was evident 4 weeks after busulfan administration (Fig. 2).

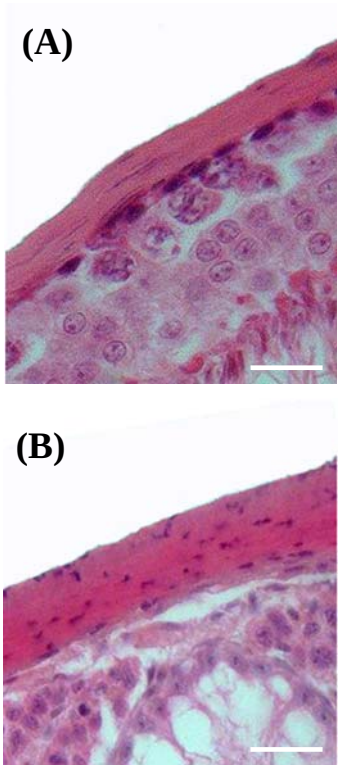

Fig. 1. Histological examination of the capsular thickness in mouse testes following busulfan treatment. A and B capsular thickness in control and busulfan treated groups respectively. (Bar A=50 µm, B=30µm).

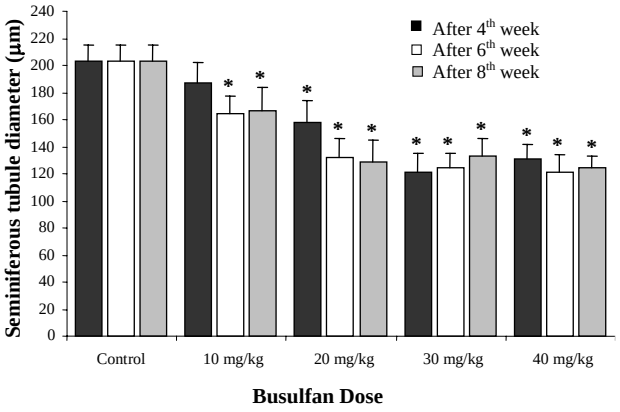

Fig. 2. Seminiferous tubules diameter (**µm**) of the mouse testes in the control and busulfan treated groups. *Significant difference versus control group, P<0.05.

We also determined the percentage of the 3 types of seminiferous tubules in the cross sections of testes in both control and busulfan treated groups. According to the results, except for the animals treated with 10 mg/kg busulfan, in all other doses of the drug, only less than 15% of the tubules contains spermatozoa. The maximum number of abnormal seminiferous tubules was seen at the 6th week and toward the 8th week the number of normal tubules increased. The relative number of different types of tubules demonstrated that normal tubules decreased and abnormal tubules increased in a dose-dependent fashion. Histological analysis of the testes in high doses groups (20, 30 and 40 mg/kg) indicated that busulfan-treated mice had more tubules with no spermatogenesis than did the controls and 10 mg/kg busulfan after 4 weeks (Fig. 3). Further histological analysis of animals testes treated with busulfan at 20, 30 and 40 mg/kg revealed that most tubules contained no apparent spermatozoa and devoid of germ cells. These tubules contained only a single, basal row of Sertoli cells nuclei mostly at the 4th and 6th week groups. The remaining tubular content was eosinophilic and acellular in animals treated with busulfan especially at high dose, and regeneration

occurred in a few tubules: some of these tubules show active spermatogenesis (Table 5 and Fig. 3). On the other hand, regeneration of complete spermatogenesis was more significant in animals which were given the drug at 10 mg/kg, by 8 weeks after drug administration, on average 68% of tubules showing spermatogenesis. At the same time, 57% of seminiferous tubules also showed apparently normal spermatogenesis with mature spermatozoa. Measuring the epithelium thickness at different time groups showed that the maximum effects of lower doses (10 and 20 mg/kg) was evident after 6 weeks of drug administration. In contrast, in high doses groups (30 and 40 mg/kg), the changes was evident as soon as 4 weeks after busulfan administration. The regeneration of epithelium was more significant after 8 weeks of drug administration at 20 and 30 mg/kg (Fig. 4).

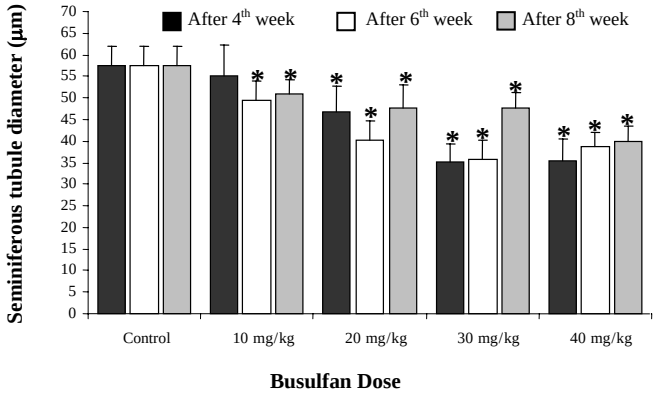

Fig. 4. Seminiferous epithelium thickness (µm) of the mouse testes in the control and busulfan treated groups. * Significant difference versus control group. P<0.05

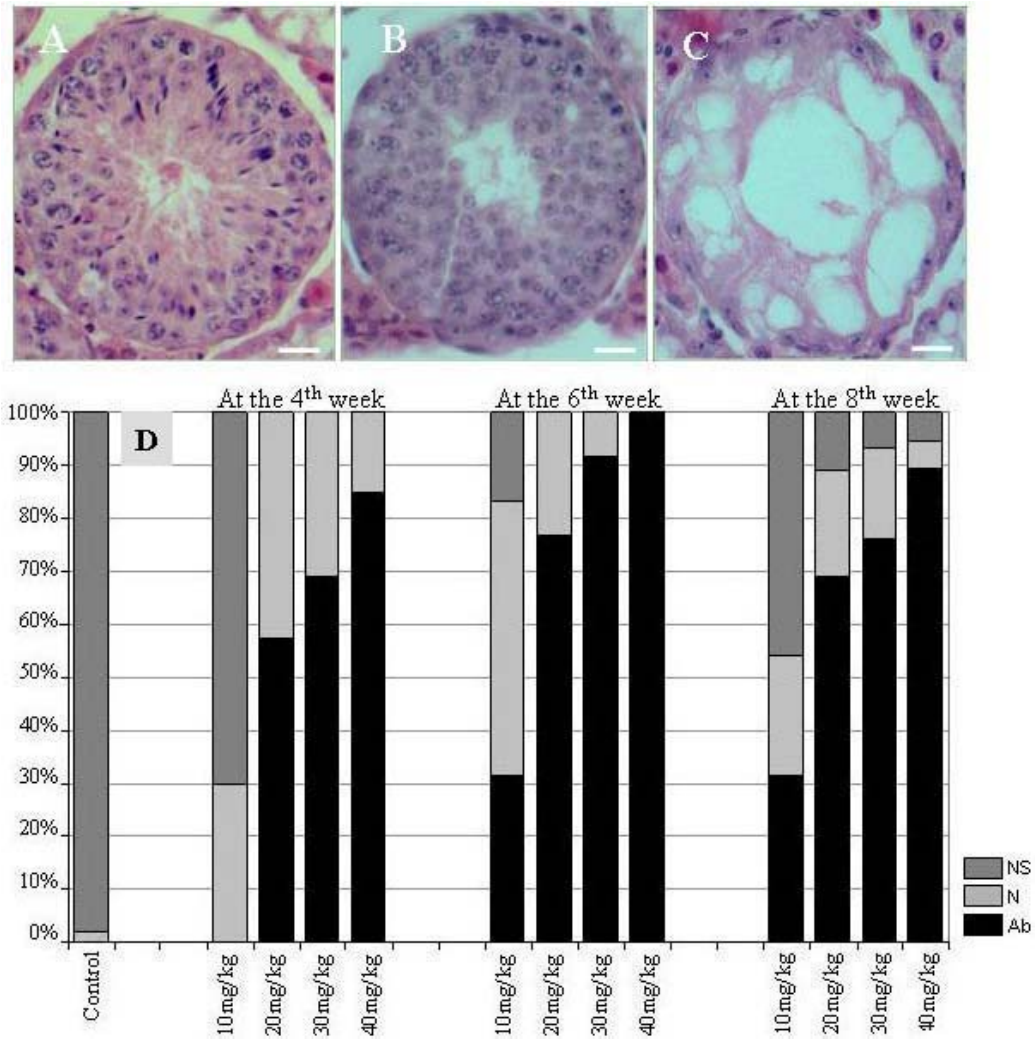

Fig. 3. Histological examination and percentage of 3 type seminiferous tubules cross section in mouse testes following busulfan treatment. **(A)**, normal seminiferous tubule with sperm (NS); **(B)**, normal seminiferous tubule without sperm (N); **(C)**, abnormal seminiferous tubule (Ab); **(D)**, percentage of tubule types in busulfan treated and control groups, (Bar A & B = 50 µm, C = 30µm).

Table 5. Cell count per $(mm)^3$ in seminiferous tubules* (mean ± SD) $\times 10^4$.

Time of extract		Control	10 mg/kg	20 mg/kg	30 mg/kg	40 mg/kg
4th week	Spg	18.6 ± 2.1	14.6 ± 2.2[c]	2.4 ± 0.3[a,b,c]	0[a,b]	0[a]
	Spc	14.3 ± 2.0	13.0 ± 1.9	8.2 ± 1.2[a,b,c]	0[a,b]	0[a]
	Spt	33.7 ± 3.8	33.0 ± 2.8[c]	0[a,b]	0[a]	0[a]
6th week	Spg	16.9 ± 2.5	10.0 ± 1.4[a,b]	0[a,b,c]	0[a,c]	0[a,c]
	Spc	13.4 ± 1.8	13.4 ± 2.0[c]	0[a,b,c]	0[a,c]	0[a,c]
	Spt	32.5 ± 5.1	23.5 ± 2.3[a]	0[a,b,c]	0[a,c]	0[a,c]
8th week	Spg	18.5 ± 2.9	13.0 ± 2.0[a]	9.0 ± 1.8[a,b]	8.6 ± 0.5[a]	3.4 ± 1.0[a,b]
	Spc	12.8 ± 1.5	9.1 ± 1.0[a]	6.5 ± 1.0[a,b]	5.5 ± 0.4[a]	2.2 ± 0.4[a,b]
	Spt	33.7 ± 4.1	22.0 ± 2.1	16.0 ± 1.7[a,b]	14.0 ± 2.0[a]	7.0 ± 1.1[a,b]

*, The replication of experiences was at least 5 times for all groups. a: Significant difference versus control group in the same row, $P<0.05$; [b], Significant difference versus lower dose of busulfan in the same row, $P<0.05$; [c,] Significant difference versus next time in the same column, $P<0.05$. Spg, Spermatogonia; Spc, Spermatocyte; Spt, Spermatid; SD, Standard deviation.

Busulfan treatment also resulted in a significant dose-dependent decrease in the number of spermatogenic cells (spermatogonia, spermatocyte and spermatid) in the seminiferous tubules. The maximum effects of busulfan were seen in the 6th week groups, when seminiferous tubules were mostly vacant of germ cells in all groups except for the 10 mg/kg dose group. The spermatocyte count revealed that in the lowest administrated dose (10 mg/kg) of the drug, a significant effect was seen very late (the 8th week group), but in other doses (20, 30 and 40 mg/kg), the significant effect was seen as early as the 4th week. However, a comparative count of spermatids at different time groups suggests that the effect of busulfan is dose-dependent and reversible. During the 4th and 6th week after busulfan treatment for high doses (30 and 40 mg/kg) and during the 6th week for 20 mg/kg dose, the seminiferous tubules solely contained Sertoli cells (Table 5 and Fig. 3 C). The 50 mg/kg busulfan treatment group was omitted from the analyses due to the small number of surviving individuals and high variability in the results.

DISCUSION

In the present study, we have determined the extent of degeneration in mouse testis structure and epididymal sperms parameters following busulfan administration. The 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 mice is around 35 days [12], the chosen period of time (6 and 8 weeks) provided sufficient time to monitor the potential recovery of spermatogenesis in surviving stem cells in busulfan treated animals. Also, according to Nagano *et al.* [13], 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 the 4 weeks 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 mice probably due to the cytotoxic effects of the drug on the hematopoietic system [14]. Previous studies also demonstrated that the mortality rate increases from 0 to approximately 50% for doses of 20-40 mg/kg of busulfan [2]. 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

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treatments is dependent on the extent of stem cell depletion [15]. In addition to sperm count or seminiferous tubule type, testis weight, as demonstrated previously [6], and it's 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 are in agreement with those of Moisan *et al.* [2] who demonstrated that testicular masses went from a maximum to a minimum value, when doses of 20 mg/kg of busulfan administrated in mice. 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 on capsular thickness, because at high dose which testis more affected by drug, capsular thickness increased and vice versa.

Our data also revealed that the effects of busulfan are, to some extent, dose-dependent and reversible. After busulfan administration at doses 20, 30 and 40 mg/kg, no or very little testicular spermatozoa were released in the epididymal lumen, however 8 weeks after drug administration animal showed some degree of spermatogenesis restoration. Nevertheless, the animals were still infertile based on their sperm counts. This finding is in agreement with the one in Kanatsu-Shinohara *et al.* [6] and Fouchecourt *et al.* [16] reports.

According to the previous report by Karashima *et al.* [17], 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 [18, 19].

In this research, histological examination of seminiferous tubule showed reversible spermatogenic cells loss. As previously reported, mice that received a lower dosage of busulfan may have some tubule repopulation from endogenous stem cells [19]. 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 [16, 20]. Regeneration of surviving stem cells was reflected in the epididymal sperm numbers changes and other recovery observed during regeneration. Busulfan treated adult testis showed that the seminiferous tubule are smaller in diameter probably this change was resulted from spermatogenic cell loss [21].

We have also shown that abnormal seminiferous tubules had only a single layer of cells attached to the tubular basal lamina. This finding is in agreement with the previous report documenting the degenerative effects of busulfan on spermatogenesis in pigeons and coyotes [22]. In general, approximately 15% of the seminiferous tubules must contain complete spermatogenesis for a mouse to be fertile [11], so all the tested groups in our study are considered infertile, except for the 10 mg/kg dose group. On the other hand, regeneration of complete spermatogenesis was more significant in animals receiving the drug at lower doses (10 mg/kg), there could be concordance between our finding and those reported previously by Kanatsu-Shinohara *et al.* [6]. Interestingly, the decrease of tubular diameters caused by busulfan treatment was not significant improved as late as 8 weeks after drug administration; probably due to the fact that the regeneration of stem cells is a very slow process [12]. The reversion showed itself only at epithelium thickness level for all dose except at 40 mg/kg; because repopulated tubules were not significant at this dose [23]. At higher doses, tubular cross-sections showed only one or two spermatogonia or none; therefore a significant proportion of spermatogonial stem cells were eliminated at these doses. These findings confirm the results of an earlier study in which it was shown that these doses of busulfan kills all types of A spermatogonia [2, 20]. 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 [6].

In conclusion, the present study provides detailed information on the effects of busulfan on many

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testicular and epididymal parameters. The information has the potential to modify and improve the administration of busulfan to increase its efficiency to make a reliable infertile recipient animal for germ cell transplantation technology, in one hand, and also to decrease its numerous side-effects in the clinical application of the drug, on the other hand.

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