

# Research Paper: Effects of Different Doses and Time-Dependency of Busulfan on Testes Parameters and Spermatogenesis in a Rat Model: A Quantitative Stereological Study



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## ABSTRACT

**Objectives:** The current study aimed at evaluating testis parameters and spermatogenesis changes in male rats administrated by different busulfan doses and time to construct a subfertile animal model by stereological methods.

**Materials & Methods:** In the present study, 150 male Wistar rats randomly divided into 5 groups. All experimental groups were treated by different concentrations of busulfan (0.0, 2.5, 5, 10, and 15 mg/kg). Rats were sacrificed 1, 15, and 30 days after busulfan treatment. The tissue processing was done for stereological study and the results were analyzed by the one-way ANOVA followed by the Duncan test.

**Results:** The most stereological parameters such as testes weight and volume, tubules volume density, interstitial tissue ( $P < 0.05$ ), and germinal epithelium ( $P < 0.01$ ) were significantly reduced by busulfan treatment. Also, at different busulfan doses, the number of spermatogenic cells including spermatogonia ( $P < 0.05$ ), spermatocyte, round and elongated spermatid, and the Sertoli and Leydig cells ( $P < 0.01$ ) significantly decreased, compared with those of the control group. The decline was more obvious in higher busulfan doses and time (from the day 15 to 30) ( $P < 0.05$ ).

**Conclusion:** Most of testicular stereological parameters reduced during 15 days onwards after busulfan treatment in a dose-dependent manner.

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## 1. Introduction

**S**permatogenesis, as a complex process, may be affected by some factors such as toxic substances and drugs, which leads to male infertility [1]. Chemotherapeutic agents are considerably used for the treatment of young patients with cancer, which negatively influence the fertility status by damaging the spermatogenesis in seminiferous tubules and result in subfertility such as oligo or azoospermia in young people during the fertile life [2, 3]. On the other hand, some impairments such as congenital abnormalities, ductus deferens, epididymis atrophy, bilateral vasectomy, sperm maturation deficiency, testes atrophy, and spermatogenesis problems due to some chemical agents can cause azoospermia [4, 5].

Busulfan, a chemotherapeutic agent as alkyl sulfonate (1,4 butanediol dimethanesulfonate), is used in cancer therapy for leukemia, ovarian cancer, and before bone marrow transplantation [6-10]. Previous studies reported that the administration of this drug during pregnancy in female rats led to insufficiency of gonadal function and reduction in testicular somatic and germinal cells of their offsprings [11, 12]. Panahi et al. (2015) showed that testicular stereological parameters reduced in rats treated with double doses of 10 mg/kg busulfan in a 21-day treatment course, compared with a single dose of busulfan and untreated groups [13]. Several studies reported that using busulfan with or without cyclophosphamide caused gonadal function and endocrine deficiency [14-18]. Although a histological and morphological study on seminiferous tubules treated with different doses of busulfan showed the sperm chromosomal abnormalities and lethal mutation [19], quantitative or stereological assessment was not performed.

Although a quantitative study including morphological analysis can give a good criteria and accurate results [20, 21], to the authors' best knowledge there was no published report on the stereological and quantitative study on dose- and time-dependency of busulfan on testis parameters. Thus in the present study, the volume density of seminiferous tubules along with interstitial tissue of testis and spermatogenic cells number were evaluated 3 times on male rats treated with different single doses of busulfan to determine the most effective dose and time of the drug using new stereological methods.

## 2. Materials and Methods

In the present randomized study, 150 adult male Wistar rats (weighed 180 to 200 g) were used. All the pro-

cedures employed in the present study were approved by Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran. The rats were maintained under the standard conditions including constant temperature (22±2°C) and 12:12 hours light: dark cycle. Also, the food and water were available ad libitum. The animals were randomly divided into 5 groups, the control group was untreated and the others as experimental groups were intraperitoneally administered by 2.5, 5, 10, and 15 mg/kg of busulfan (Pierre Fabre, France).

Animals in each group were scarified using 10% ketamine and 2% xylazine (Alfasan, Netherland) on the 1st, 15th, 30th days after busulfan treatment and afterwards their testes were removed and weighed. The primary volume (V1) of testis was recorded using the Archimedes principle as immersion technique [22], and then, testicular tissue was fixed in 4% formaldehyde in buffered solution. Isotropic Uniform Random sections (IUR) were required to obtain systematically random sampling in order to estimate some stereological parameters and shrinkages in testicular tissue during the tissue processing and staining. Therefore, the orientator method was applied. A circle was punched out from a testis slab by a trocar, and then, the area and vertical diameters of the circular piece of the testis were measured [23-26].

All tissue processing procedures were done by the tissue processor machine (Slee, MTP 1100020, Germany). All slabs and circular pieces of testicular tissue were embedded in paraffin blocks. Blocks were sectioned in 5- and 20-µm thicknesses and stained by the Heiden-Hain Azan-Trichrome and Hematoxylin-Eosin (H&E). The stereological parameters were conducted as follows:

The amount of shrinkage in each testis was calculated by the following equation:

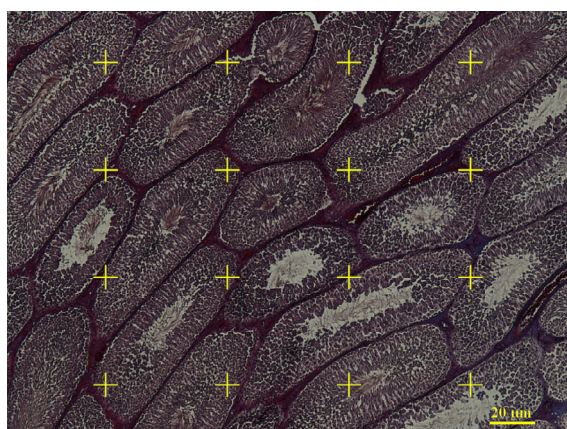
$$\text{Volume of shrinkage} = 1 - \left( \frac{\text{Area of the circular piece after processing}}{\text{Area of the circular piece before processing}} \right)^{1.5}$$

After that, the final volume of testis was measured as follows:

$$V_{\text{Final}} = V_{\text{Primary}} \times (1 - \text{shrinkage})$$

Then, the volume density "Vv (structure/testis)" of the seminiferous tubules, interstitial tissue and, germinal epithelium was assessed by a grid of points and following formula:

$$V_V = \frac{\Sigma P_{\text{structure}}}{\Sigma P_{\text{total}}}$$



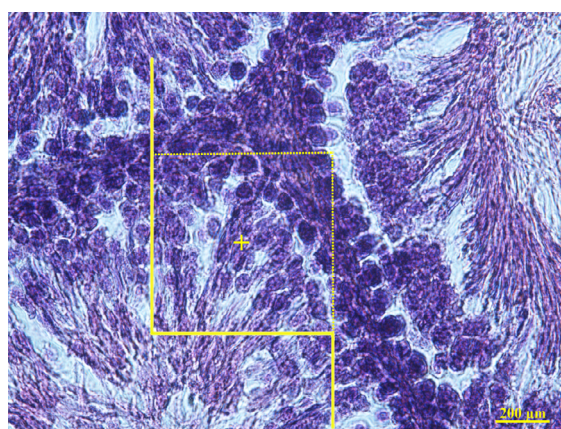
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**Figure 1.** Estimation of volume density using the point counting method

Where the “ $\sum P_{structure}$ ” is the number of points overlaid the profiles of the tubules or interstitial tissue and “ $\sum P_{total}$ ” is the number of points overlaid the testis. The total volume was obtained by multiplying the density in the final testis volume (Figure 1). Number of points hitting the interstitial tissue or epithelium was divided by the total number of points hitting the section.

### Estimating the number of germinal epithelium cells

The numerical density and the total number of cells including spermatogonia, spermatocyte, round and elongated spermatid, and the Sertoli and Leydig cells were estimated using the optical dissector method, using 20- $\mu$ m thickness sections. An unbiased counting frame was used to estimate the numerical density of cells using the optical dissector method [27]. Cell was counted if its nucleolus was inside the frame and not touched the forbidden lines (Figure 2). To have an unbiased counting, a guard zone was considered. Then, the first 5- $\mu$ m was ignored and next 10- $\mu$ m thickness section was selected and the cells were counted. The z-axis movement (depth) was measured by a microcator (MT12, Germany).



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**Figure 2.** The numerical density of cells was estimate using the optical dissector method. Cell was counted if its nucleolus was inside the frame and not touched the forbidden lines.

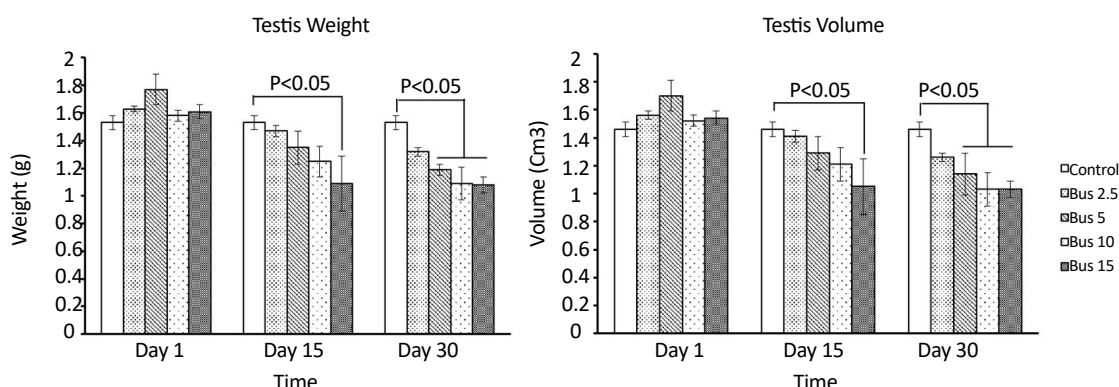
The numerical density ( $N_v$ ) or the number of cells in the unit was estimated using the following formula:

$$N_v = \frac{\sum Q}{\sum P \times h \times a(frame)} = \frac{t}{BA}$$

where “Q” is the number of cells counted with dissector per section; “h” is the height of the dissector (here was 10  $\mu$ m), “a/fram” is the area per frame, “P” is the number of frame points fall in the region of interest on the sections, “t” is the mean of the section thickness measured on all areas of the field in each microscopic slide through the z axis, “BA” is the microtome block advance (here was 20  $\mu$ m).

### Statistical analysis

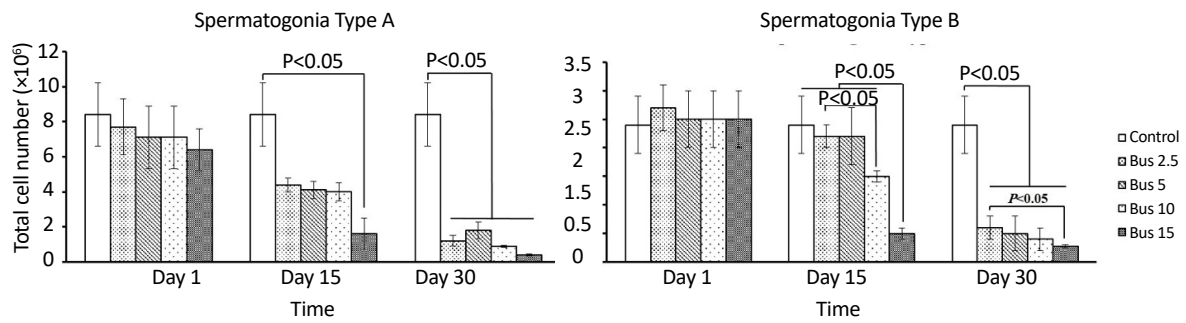
Before any statistical analysis, the normality of data was assessed. All data among the treatment groups and different days were analyzed by the one-way ANOVA with SPSS version 22. Treatment and day differences were compared by the Duncan multiple comparison post hoc test. Data were expressed as mean  $\pm$  standard deviation (SD).



**Figure 3.** Effect of busulfan treatment on testis weight and volume

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**Figure 4.** Effects of busulfan treatment on the spermatogonia types A and B

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### 3. Results

#### Testes weight and volume

The means weight and volume of testes in the experimental groups with different single doses of busulfan (5, 10, and 15 mg/kg) decreased in comparison with those of the control group. The significant reductions were observed after the day 30 at 5 and 10 mg/kg doses, and after 15 days at 15 mg/kg, compared with the control group ( $P < 0.05$ , Figure 3). The reduction in testes volume and weight at all busulfan doses was time-dependent because the changes were significant in the days 15 and 30 after the busulfan treatment, compared with the first day ( $P < 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 10/\text{group}$ ). Bus 2.5, 5, 10, and 15 indicated busulfan 2.5, 5, 10, and 15 mg/kg doses.

#### Seminiferous tubules and interstitial tissue volume

Means of seminiferous tubules volume revealed a significant reduction after 15 ( $P < 0.05$ ) and 30 ( $P < 0.01$ ) days at 15 mg/kg, and after 30 ( $P < 0.05$ ) days at 10 mg/kg of busulfan treatment, compared with those of the control group. All the busulfan doses (except 2.5 mg/kg

in the day 15) showed a significant reduction in seminiferous tubules volume, compared with the day 1 after injection ( $P < 0.05$ , Table 1). Interstitial tissue volume at 15 mg/kg in the day 15, and all busulfan doses in the day 30, significantly reduced compared with those of the control group ( $P < 0.05$ ). Interstitial tissue volume at 10 and 15 mg/kg doses in the day 30 significantly reduced, compared with that of the day 1 ( $P < 0.01$ ).

#### Germinal epithelium volume

The germinal epithelium volume of testes at 2.5, 5, and 15 mg/kg busulfan doses significantly decreased after 15 days, but after 30 days showed a significant reduction only at 10 and 15 mg/kg doses ( $P < 0.01$ , Table 1). Also, 2.5, 10, and 15 mg/kg busulfan doses (except 10 mg/kg in the day 15) showed significant declines, compared with the day 1 of treatment ( $P < 0.05$ ).

#### Types A and B of spermatogonia number

The number of types A and B spermatogonia showed a significant reduction in all the busulfan treatments, compared with those of the control group on the day 30

**Table 1.** Effect of different doses of busulfan on the seminiferous tubules, interstitial tissue, and germinal epithelium volume

Histomorphometrical Parameters	Time (Day)	Control	Busulfan (mg/kg)			
			2.5	5	10	15
Total tubule volume (mm <sup>3</sup> )	1		746.5 $\pm$ 11.3	802.0 $\pm$ 46.6	743.8 $\pm$ 26.1	737.2 $\pm$ 17.3
	15	689.3 $\pm$ 24.9	685.5 $\pm$ 22.0	615.0 $\pm$ 51.2 <sup>a</sup>	569.6 $\pm$ 57.3 <sup>a</sup>	531.2 $\pm$ 104.8 <sup>a*</sup>
	30		620.1 $\pm$ 18.2 <sup>a</sup>	563.8 $\pm$ 68.0 <sup>a</sup>	495.8 $\pm$ 56.3 <sup>a*</sup>	504.1 $\pm$ 30.9 <sup>a*</sup>
Total interstitial volume (mm <sup>3</sup> )	1		64.5 $\pm$ 11.2	82.1 $\pm$ 19.5	60.6 $\pm$ 11.7	64.1 $\pm$ 15.4
	15	69.9 $\pm$ 12.3	48.3 $\pm$ 5.3	55.9 $\pm$ 15.3	53.7 $\pm$ 11.9	24.4 $\pm$ 7.6*
	30		36.8 $\pm$ 5.6*	31.4 $\pm$ 10.2*	28.6 $\pm$ 4.5 <sup>a*</sup>	24.1 $\pm$ 2.3 <sup>**a</sup>
Total epithelial volume (mm <sup>3</sup> )	1		600.0 $\pm$ 89.6	568.1 $\pm$ 102.4	406.1 $\pm$ 32.1	398.6 $\pm$ 7.6
	15	485.0 $\pm$ 48.9	352.3 $\pm$ 15.1 <sup>a*</sup>	331.8 $\pm$ 27.4*	356.8 $\pm$ 51.5	198.8 $\pm$ 45.1 <sup>**a</sup>
	30		299.5 $\pm$ 60.1 <sup>a</sup>	342.1 $\pm$ 161.0	171.1 $\pm$ 20.88 <sup>a*</sup>	171.1 $\pm$ 12.8 <sup>**a</sup>

\*:  $P < 0.05$ , the Duncan test, control vs. busulfan 2.5 mg/kg or 5 mg/kg or 10 mg/kg or 15 mg/kg

†:  $P < 0.01$ , the Duncan test, busulfan 2.5 mg/kg vs. 15 mg/kg

a:  $P < 0.05$ , the Duncan test, day 1 vs. day 15 or 30

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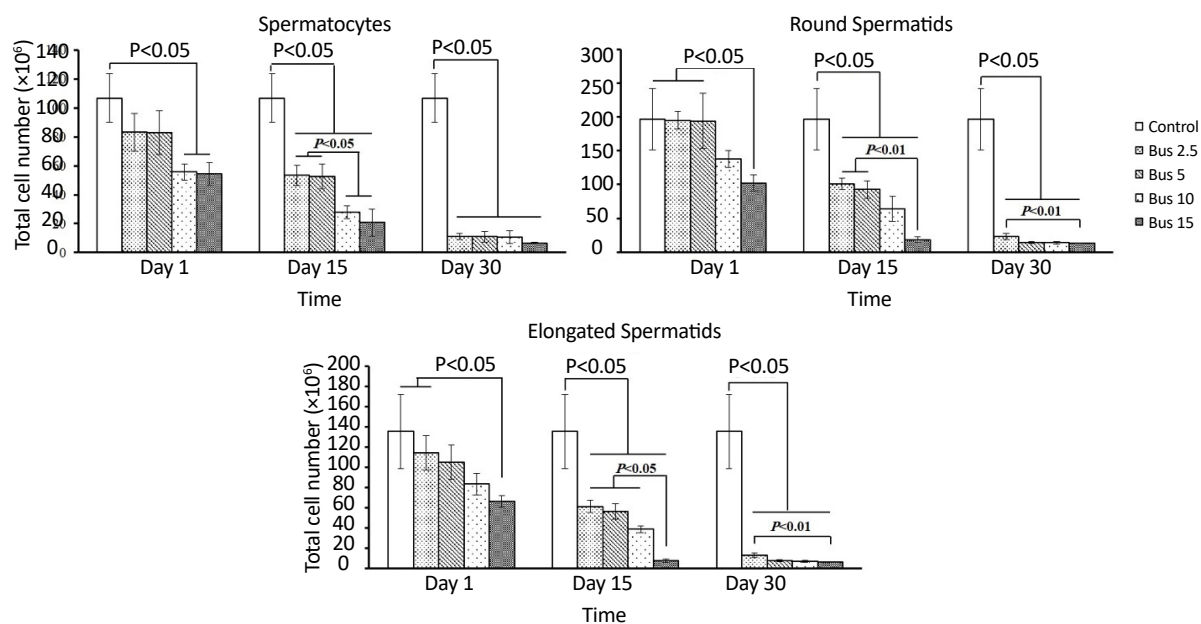


Figure 5. Effect of busulfan treatment on spermatocytes, elongated and round spermatids

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( $P < 0.05$ ). Although at 10 and 15 mg/kg of busulfan number of type A declined after 15 days of injection, compared with that of the control group ( $P < 0.05$ ); a depletion was observed at 15 mg/kg busulfan in the number of type B compared with other busulfan doses at the same time ( $P < 0.05$ , Figure 4). The 15 mg/kg treated group showed that the reductions in days 15 and 30 at 2.5, 5, 10 mg/kg doses occurred after the day 30, compared with that of the day 1 of treatment ( $P < 0.05$ ). Values are expressed as mean  $\pm$  SD ( $n = 10$ /group). Bus 2.5, 5, 10, and 15 indicated busulfan at 2.5, 5, 10, and 15 mg/kg doses.

### Spermatocyte number

The number of spermatocytes 1 day after busulfan injection significantly decreased at 10 and 15 mg/kg doses compared with the control group, but after 15 and 30 days post busulfan treatment all groups showed a diminishment in spermatocytes compared with the control group and after 1 day of injection ( $P < 0.05$ , Figure 5).

### Round spermatid number

The number of round spermatids significantly decreased in a time-dependent manner ( $P < 0.05$ ). In the 15 mg/kg busulfan group also, the number of round spermatids significantly decreased after 1 ( $P < 0.01$ ) and 15 days ( $P < 0.05$ ) of busulfan injection compared with the control as well as 2.5 and 5 mg/kg busulfan doses. The round spermatids number in all groups showed a significant reduction after 15 and 30 days of injection compared with the day 1 of treatment ( $P < 0.05$ , Figure 5).

### Elongated spermatid number

In the all treated groups, the elongated spermatids significantly reduced in a time-dependent manner in 15 and 30 days of treatment compared with that of the control group ( $P < 0.05$ ). Although these cells after 1 day of 15 mg/kg busulfan injection showed a significant decline compared with both control and 2.5 mg/kg groups ( $P < 0.05$ ), in the days 15 and 30, elongated spermatids significantly decreased at the same dose of busulfan compared with other doses ( $P < 0.05$ , Figure 5). Also, after 15 and 30 days of busulfan treatment, a significant reduction was observed in all groups compared with the day 1 of treatment ( $P < 0.05$ ). Values are expressed as mean  $\pm$  SD ( $n = 10$ /group). Bus 2.5, 5, 10, and 15 indicated busulfan at 2.5, 5, 10, and 15 mg/kg doses.

### Sertoli cells number

The Sertoli cells in both 10 and 15 mg/kg busulfan groups in the days 15 and 30 showed a significant decrease compared with those of the control group ( $P < 0.01$ , Table 2). In a time-dependent manner, the 2.5, 10, and 15 mg/kg busulfan groups showed a reduction in the Sertoli cells compared with the day 1 of treatment ( $P < 0.05$ ).

### The Leydig cells number

The Leydig cells in 5, 10, and 15 mg/kg busulfan groups in the day 15 and at all doses in the day 30 showed a significant reduction compared with those of the control group ( $P < 0.01$ ). They also showed a significant dimin-

**Table 2.** Effect of busulfan treatment on the sertoli and leydig cells number

Cell Type	Time (Day)	Control	Busulfan (mg/kg)			
			2.5	5	10	15
The sertoli cells ( $\times 10^6$ )	1		19.9 $\pm$ 2.8	19.7 $\pm$ 4.7	19.1 $\pm$ 2.7	18.1 $\pm$ 3.7
	15	20.6 $\pm$ 4.4	11.9 $\pm$ 1.9	13.1 $\pm$ 3.4	7.0 $\pm$ 1.4 <sup>*a</sup>	6.4 $\pm$ 1.6 <sup>**a</sup>
	30		10.1 $\pm$ 3.1a	10.2 $\pm$ 3.4	5.4 $\pm$ 2.4 <sup>**a</sup>	3.6 $\pm$ 0.8 <sup>**a</sup>
The leydig cells ( $\times 10^6$ )	1		20.48 $\pm$ 8.49	19.55 $\pm$ 6.58	19.23 $\pm$ 4.78	19.56 $\pm$ 8.01
	15	23.18 $\pm$ 4.14	10.75 $\pm$ 3.80	7.77 $\pm$ 2.22 <sup>*</sup>	7.11 $\pm$ 2.28 <sup>*a</sup>	1.83 $\pm$ 1.07 <sup>**a</sup>
	30		5.49 $\pm$ 0.47 <sup>*a</sup>	1.45 $\pm$ 0.51 <sup>**a</sup>	0.52 $\pm$ 0.12 <sup>**a</sup>	0.39 $\pm$ 0.08 <sup>**a</sup>

\*: P<0.05, the Duncan test, control vs. busulfan 2.5 mg/kg or 5 mg/kg or 10 mg/kg or 15 mg/kg

†: P<0.01, the Duncan test, busulfan 2.5 mg/kg vs. 15 mg/kg

a: P<0.05, the Duncan test, day 1 vs. days 15 or 30

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ishment in a time-dependent manner compared with the day 1 of treatment (P<0.01).

#### 4. Discussion

Male infertility, a fairly common disorder, approximately occurs in 1 per 20 males [28]. Using animals, as a subfertile platform, can improve knowledge of its causes and mechanisms. Busulfan, one of the chemotherapy agents, is used in the leukemia patients, which induces some degree of sterilization [19]. The present study evaluated the effects of different busulfan doses on fertility parameters in male rats at different post treatment intervals to find the most effective dose and time to create subfertile model. Results of the current study showed that most of the stereological and morphometrical parameters including testes weight and volumes, volume density of seminiferous tubules, interstitial tissue and germinal epithelium reduced significantly at 5, 10, and 15 mg/kg busulfan.

The germinal epithelium cells including spermatogonia, spermatocyte, round and elongate spermatid as well as supporting Sertoli and Leydig cells also significantly declined at all doses of busulfan. It was demonstrated that busulfan by producing free radicals can destruct the genome and cause chromosomal abnormalities and dominant lethal mutations in sperm [19], in addition, busulfan by elevating the level of immature Sertoli cell marker cytokeratin 18, can induce spermatogenic impairment and cause infertility [29].

Along with increasing the dose of busulfan, the severity and extent of damages also increased. The 15 mg/kg dose of busulfan has fast effect (the day 1 after injection) on subfertility in comparison with other doses that was in agreement with the results of Kanatsu-shinohara et al. (2003) showing that the 15 mg/kg dose was effective for depletion of the spermatogonial stem cells in a subfertile animal model [30].

Results of the current study also showed that although the lowest dose of busulfan required more time to induce its effects on cells number, in the case of volumes (except for interstitial volume), no significant effect was observed even after the day 30. Thirty days after the administration of a single dose at 5 mg/kg busulfan, the most evaluated parameters were similar to the higher doses. Thus, at least 5 mg/kg busulfan is required to show negative effects of busulfan on rat male fertility in long term. Previous study revealed that a single intraperitoneal dose of busulfan (40 mg/kg) led to the reduction of male germ cells and testis weight. Also, they showed that apoptosis was increased in a time-dependent manner [31].

Another study indicated that busulfan is a time-dependent agent and can decline spermatogenesis by delay in meiosis; the results were consistent with those of the 2 aforementioned studies. Also, they mentioned that the Leydig cells not being affected by busulfan in a time-dependent manner [32]; the results were inconsistent with those of the current study and may be attributed to different doses and intervals between the 2 studies.

#### 5. Conclusion

The stereological and quantitative results of the present study suggested that 15 mg/kg of busulfan was required for the fast induction of subfertility in male rats. Also, data of the current study indicated that at least 5 mg/kg of busulfan was necessary for the occurrence of subfertility effects after 30 days. The data may be helpful for future studies to find the most effective dose and time for constructing a subfertile animal model.

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### Conflict of Interest

All authors indicated no potential conflicts of interest.

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