Busulfan induces apoptotic and cytotoxic effects on testis and epididymal sperm of adult male mouse following low dose treatment

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Key words: Apoptosis, Busulfan, Epididymal Sperm, Seminiferous tubule, Sperm viability.

http://dx.doi.org/10.12692/ijb/6.5.70-78

Abstract

At adequate concentrations busulfan selectively attacks the dividing spermatogonia and spermatocytes. But, there is not enough information about side effects of low dose busulfan. The aim of this study was to assess changes that occur on seminiferous tubules morphology and epididymal sperm of adult male mouse following treatment with low dose busulfan. So, adult male NMRI mice (25–35 g) were assigned in three groups including; Group 1: Control, Group 2; treated with busulfan for 21 days (0.06 mg/kg/day) and Group 3: treated with busulfan for 21 days (0.8 mg/kg/day). The effects of busulfan on seminiferous tubules morphology and epididymal sperm were evaluated by light microscopy, Computer–Aided Sperm Analysis (CASA), MTT assay and flowcytometry. The results showed that busulfan caused significant germinal epithelium destruction in treated mice versus control. Also, busulfan had significant cytotoxic and apoptotic effects in epididymal sperm of treated mice. The percentages of early apoptotic, late apoptotic and necrotic sperms in groups 2 and 3 demonstrated significant increase versus control. Also, analysis of sperm parameters and sperm viability using CASA and MTT assay demonstrated significant differences between control and busulfan treated mice. In conclusion, chemotherapy with low dose busulfan causes significant changes on sperm viability, sperm parameters and morphology of seminiferous tubules. All side effects of busulfan are dose dependent and they are considerable in mice treated with 0.8 mg/kg busulfan. Hence, the treatment with low dose busulfan may reduce toxicity of chemotherapy.

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Introduction
Busulfan \(\text{CH}_3\text{SO}_2\text{O} \ (\text{CH}_2)_4\text{OSO}_2 \text{CH}_3\) has been widely used for the treatment of patients with chronic myelogenous leukemia and prior to bone marrow transplantation (Down and Ploemacher, 1993; Buggia et al., 1994). The treatment plan for busulfan depends on the type of cancer (Perry, 2012).

It seems that busulfan has various long term or late effects and its genotoxicity may be one reason accounting for these effects (Blasiak et al., 1999; Blasiak et al., 2000). In addition, recent studies showed long-term effects of busulfan on spermatogenesis (Temitope et al., 2011).

This chemotherapeutic agent affects different body organs such as gonads and also increases percentage of germ cell apoptosis of various mammalian (Choi et al., 2004; Vaisheva et al., 2007; Mohammad-Ghasemi et al., 2009). Male germ cell apoptosis is a persistent effect of busulfan that reported extensively in mouse and murine (Mohammad-Ghasemi et al., 2009).

Busulfan acts as an alkylating agent and primarily targets slowly proliferating or non-proliferating cells (Wenzhi et al., 2011). Also, busulfan induces DNA alkylation leading to DNA-DNA and DNA-protein cross-links in sperm (Iwamoto et al., 2004; Mertins et al., 2004).

Mohammad-Ghasemi et al (2009) reported that busulfan administration in a different single dose of 10, 20 and 40 mg/kg induces side effect on male reproductive system (Mohammad-Ghasemi et al., 2009).

Also, there are many reports about side effects of chemotherapeutic agents on normal tissue. But, they studied side effects of high or lethal doses of these agents on reproductive system (Choi et al., 2004; Mohammad-Ghasemi et al., 2009). In the other hand, there is no strong study which determined various data about effects of chemotherapeutic agents on spermatogenesis.

In addition, the study of underlying mechanisms that anticancer agents induced cytotoxicity is necessary for reducing side effect of chemotherapy. Hence, our present study is to evaluate busulfan mediated damage on epididymal sperm and testis tissue of adult male NMRI mice following low dose treatment.

Methods and materials
Experimental animals
Adult NMRI male mice \((n = 30)\) were included in the present study. The mice were 8 weeks old and weighed 25-35 g each. They were purchased from Ahwaz Medical Sciences Research Center and Experimental Animal House (Ahwaz, Jondishapour University of Medical Sciences, Iran) and were allowed to adapt themselves to the new conditions for one week.

The animals were housed in temperature controlled rooms \((25^\circ\text{C})\) with constant humidity \((40-70\%)\) and 12 h light/ dark cycle prior to experimental protocols. They were provided rat chow (Pars, Tehran, Iran) and water at libitum. All animals used were cared for according to the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (National Institutes of Health publication No. 86-23).

Treatment protocol
Busulfan (Sigma, St. Louis, MO) was dissolved into DMSO \((<0.2 \%)\) (Sigma, St. Louis, MO) and diluted with sterile deionized water \((1 : 1)\) at room temperature to provide final concentrations of 0.06 and 0.8 mg/kg. Dosage of busulfan was chosen based on previous studies (Fernandez et al., 2002; Bruce et al., 2011). Busulfan was daily prepared and freshly used during the experiment.

Male adult NMRI mice were divided into three groups of ten each. Group 1 (control) was administered DMSO for 21 days, groups 2 and 3 were given respectively 0.06 and 0.8 mg/kg busulfan for 21 days intraperitoneally. At the end of the experiment, animals were anesthetized using 80 mg/kg ketamine hydrochloride and 10 mg/kg xylazine, killed by
decapitation and the left testis and epididymis were removed.

Collection of sperm
Cauda-epididymis was dissected; several longitudinal incisions were made on its distal end and placed into a 60 mm tissue culture dish containing warmed 1 ml PBS (pH 7.4). Then the tissue was removed and the sperm suspension was used for Computer–Aided Sperm Analyzer, MTT assay and flowcytometry.

Histopathology and Light microscopy
The left testes were fixed in 10% formalin and embedded in paraffin. Five-micron thick sections were prepared and stained with Hematoxylin and Eosin (H&E). The specimens were examined under Olympus/3H light microscope-Japan.

This analysis examined morphologically whether busulfan induced cell depletion and destruction on seminiferous tubule of mice that treated with low dose busulfan.

The severity of seminiferous tubule destruction was determined according to two factors; germinal epithelium depletion and existence of sperm in seminiferous tubule. So, we determined four types of seminiferous tubules; type 1) normal germinal epithelium with sperm, type 2) germinal epithelium depletion and with sperm, type 3) germinal epithelium depletion and without sperm, type 4) empty seminiferous tubule. The tubular diameter of the seminiferous tubule epithelium was using image analyzer Leica (DMLB) and Leica Qwin software.

Sperm parameters
Sperm parameters including sperm count and sperm abnormality were monitored by CASA. Also, sperm parameters were compared in both control and busulfan treated groups.

Assessment of Cell Viability: MTT assay
MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) reduction assay is a method that validates the viability of an active cell. Dehydrogenase in mitochondria converts yellow colored insoluble tetrazolium salt to purple colored water-soluble formazan. Sperm also have mitochondria in the midpiece; therefore sperm viability could be evaluated by MTT reduction assay (Byum et al., 2008).

Recent studies showed that HAM’S F10+25mM HEPES (Gibco) is the most suitable media for the sperm MTT viability assay (Nasr-Esfahani et al., 2002). Thus routinely MTT (Loba Chemie, India) was dissolved in HAM’S F10+25mM HEPES at 0.5 mg/mL and then pH was adjusted to 7.4–7.45. BSA was added to MTT solution at 10% concentration. MTT solution was kept at 4°C in the dark for a maximum of 1 week. For routine MTT assay 50 µl of washed sperm (1.5-2.5 millions) was added to 450 µl of MTT solution, which was warmed to 37°C, 30 min before addition of sperm.

The absorbance was measured with a microtiter plate reader (Bio-Tek SX2, Winooski, VT, USA) at a test wavelength of 570 nm and a reference wavelength of 690 nm. The optical density (OD) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Results were expressed as percentage of control.

FITC Annexin V/PI Assay; flowcytometry
Flowcytometry measures apoptosis and phosphatidylserine (PS) translocation across the plasma membranes (Anzar et al., 2002; Hossain et al., 2011).

FITC Annexin V/Dead Cell Apoptosis Kit (Catalog no. V13242, Invitrogen) was used to detect the translocation of PS and type of cell death on epididymal sperm of control and busulfan-treated mice.

Sperm sample was centrifuged (500 × g, 10 min and 25°C). The pellet was resuspended in 1X Annexin-binding buffer. 5 µL of FITC Annexin V (Component A) and 1 µL of the 100 μg/mL PI working solution were added to each 100 µL of cell suspension and
incubated at room temperature for 15 minutes. After the incubation period, 400 μL of 1X Annexin-binding buffer was added to tube, mixed gently and the sample kept on ice. As soon as possible, the stained cells were analyzed by flow cytometry and the fluorescence emission was measured at 530 nm and > 575 nm. Flowcytometric evaluation was conducted within 5 min.

**Statistical analysis**

Data analyses were done using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA). The results were expressed as mean ± SD. The statistical significances were analyzed by analysis of variance (ANOVA) and Tukey post-hoc analyses. In all cases, P<0.05 was considered significant.

**Results**

*Seminiferous tubule morphology*

Morphological appearance of seminiferous tubule was compared between control and treated groups using light microscope (Fig. 1).

**Table 1.** The results of sperm count, normal morphology and sperm pathology of control and busulfan treated groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sperm Count×10⁶</th>
<th>Normal Morphology%</th>
<th>Head Pathology%</th>
<th>Neck pathology%</th>
<th>Tail Pathology%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.13 ± 1.2 a</td>
<td>81.7 ± 6.9 a</td>
<td>11.1</td>
<td>12.3</td>
<td>5.3</td>
</tr>
<tr>
<td>Group 2</td>
<td>7.3 ± 0.61 b</td>
<td>68.9 ± 7.1 a</td>
<td>22.7</td>
<td>13.6</td>
<td>16.1</td>
</tr>
<tr>
<td>Group 3</td>
<td>2.5 ± 0.4 c</td>
<td>21.5 ± 3.4 c</td>
<td>50.5</td>
<td>20.3</td>
<td>14.2</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences between groups based one-way ANOVA (P < 0.05).

Microscopic observations of seminiferous tubule demonstrated significant changes on germinal epithelium of busulfan treated mice (groups 2 and 3). In these groups, germinal epithelium showed high level of destruction. Percentage of type 3 seminiferous tubule (with destroyed germinal epithelium and no sperm) in groups 2 and 3 were about 21.9 ± 5.8% and 68.2 ± 7.3%, respectively (Fig. 2A). In addition, diameter of seminiferous tubule decreased remarkably in treated groups with low dose busulfan. Diameter of seminiferous tubules in control, groups 2 and 3 were 838.5 ± 43.9, 795.4 ± 31.4 and 756.7 ± 29.6 μm, respectively (fig. 2B). But, all seminiferous tubules in control (group 1) were normal (type 1).

**Table 2.** The percentages of early apoptotic, late apoptotic and necrotic sperm in control and busulfan treated mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Early Apoptotic Sperms %</th>
<th>Late Apoptotic Sperms %</th>
<th>Necrotic Sperms %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.8 ± 0.5 a</td>
<td>1.2 ± 0.3 a</td>
<td>0.3 ± 0.1 a</td>
</tr>
<tr>
<td>Group 2</td>
<td>11.8 ± 0.9 b</td>
<td>8.2 ± 1.5 b</td>
<td>2.1 ± 0.5 b</td>
</tr>
<tr>
<td>Group 3</td>
<td>27.6 ± 2 c</td>
<td>19.1 ± 3.1 c</td>
<td>3.2 ± 0.9 b</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences between groups based one-way ANOVA (P < 0.05).

*Sperm parameters*

The results showed remarkable differences on sperm count, normal morphology and sperm pathology (abnormality) between control and treated groups (Tab. 1). Percentages of sperm count (Fig. 3) and morphologically normal sperm (Fig. 4A) were lower in busulfan treated groups versus control. Also, sperm pathology increased in treated group compared with control (Tab. 1). Group 3 demonstrated highest percentage of head and neck pathologies. But, group 2 had more tail pathology between 3 groups. In addition, teratospermia was remarkable in treated groups in comparison with control (Fig. 4B).
Assessment of Cell Viability: MTT assay

MTT assay was used to examine the cytotoxicity of busulfan on epididymal sperm viability of control and treated mice. Busulfan treated groups showed high level of sperm cell death. Our results determined significant differences on sperm viability of treated groups in comparison with untreated mice (Fig. 5). Percentages of sperm viability were 72.4 ± 9.6% and 57.5 ± 5.2% in groups 2 and 3, respectively. Sperm viability decreased in treated groups notably. Also, this reduction was dose dependent.

![Fig. 1. Light microscopy images of seminiferous tubule in control and busulfan treated mice. A) Seminiferous tubules with normal germinal epithelium. B) Slight decrease of germ cell population and normal lumen in group 2. C) Atrophy of seminiferous tubule with widening of lumen, depletion of germinal epithelium and massive germ cell loss in group 3 (Arrows are showing germinal epithelium depletion).](image)

FITC Annexin V/PI Assay; flow cytometry

Flow cytometry separated the population of sperms into four groups: live cells showed only a low level of fluorescence, necrotic cells showed red fluorescence, early apoptotic cells showed green fluorescence and late apoptotic cells showed both red and green fluorescence (Fig. 6). Sperms were incubated with FITC Annexin V in a buffer which contained propidium iodide (PI) and analyzed by flow cytometry. Untreated cells were primarily FITC Annexin V and PI negative, indicating that they were viable and not undergoing apoptosis. There were two populations of cells (bottom panels): Cells that were viable and not undergoing apoptosis or necrosis (FITC Annexin V and PI negative) and cells undergoing apoptosis (FITC Annexin V positive and PI negative). The population of sperms was observed to be FITC Annexin V and PI positive, indicating that they were in end stage apoptosis or already dead. Also, a population of sperms was shown to be FITC Annexin V negative and PI positive, they underwent necrosis (Fig. 6).

The results illustrated remarkable differences between busulfan treated mice and control (Tab. 2). The percentages of early apoptotic, late apoptotic and necrotic sperms in groups 2 and 3 increased significantly versus control (Fig. 7).

Discussion

Recent studies demonstrated that chemotherapy drugs such as busulfan can cause testicular damage as manifested by reduced testicular volume, oligozoospermia and apoptotic cell death on testicular germinal epithelium (Mohammad-Ghasemi et al., 2009). Busulfan unlike other chemicals primarily destroys spermatogonial stem cells. But, other chemicals except of busulfan kill differentiated spermatogonia (Kanatsu-Shinohara et al., 2003; Anjamrooz et al., 2007; Kawashima et al., 2009). A single intraperitoneal injection of 10, 20, 30, 40 and 50 mg/kg of busulfan revealed the deletion of the spermatogenic cells and induces permanently infertility in several species of animals (Anjamrooz et al., 2007; Kawashima et al., 2009). Also, the duration of busulfan induced infertility is dependent on the
extent of stem cell depletion (Kanatsu-Shinohara et al., 2003). Agarwa et al (2003) suggested that high level of sperm DNA damage can be seen following a single dose of chemotherapeutic drugs, which may persist for several months after cessation of their use (Agarwa et al., 2003; Mohammad-Ghasemi et al., 2009).

Fig. 2. Analysis of germinal epithelium depletion and seminiferous tubule diameter in control and busulfan treated mice. A) Percentages of germinal epithelium depletion in treated groups showed significant changes in comparison with control, B) There was no significant change on seminiferous tubule diameter of group 2 in comparison with control. But, group 3 revealed significant decrease versus control. Different letters indicate significant differences between groups at P< 0.05.

Whereas previous researches which were shown destructive effects of high single dose of busulfan on testicular germinal epithelium structure, present study revealed busulfan-mediated side effects on seminiferous tubule morphology following low dose chemotherapy.

In addition, MTT assay demonstrated lower sperm viability in treated groups in comparison with control. The mice that received 0.8 mg/kg busulfan (for 21 days) had lower sperm viability versus control and treated mice with 0.06 mg/kg busulfan (for 21 days). Furthermore, apoptotic and necrotic cell deaths increased on epididyimal sperms of busulfan treated groups compared with control (p< 0.05). The level of apoptotic sperm was interestingly in treated groups (groups 2 and 3), but, the level of necrotic sperm was not notable in these groups. The extent of apoptotic sperm in group 3 may be related to dose-dependent effects of busulfan.

Fig. 3. Analysis of sperm count (×10⁶/ml) in control and busulfan treated mice. There were significant decline on sperm count of groups 2 (0.06 mg/kg/day busulfan for 21 days) and 3 (0.8 mg/kg/day busulfan for 21 days) in comparison with control. Different letters indicate significant differences between group based one-way ANOVA (P< 0.05). Bu: Busulfan.

percentages of morphologically normal sperm in treated groups was notable. Although there was no remarkable change between control and group 2, but, group 3 showed significant changes compared with control. B) There were remarkable differences in the percentage of sperm pathology between control and busulfan treated mice. Group 3 showed the highest level of sperm pathology and the lowest level of normal morphology versus control and group 2. Different letters indicate significant differences between groups based one-way ANOVA (P< 0.05). Bu: Busulfan.

In the other hand, our flowcytometric results strongly emphasized high level of DNA damage on epididyimal sperm of treated mice with low dose busulfan. DNA fragmentation as an important feature of apoptosis (Nasimi and Roohi, 2012) was remarkable in treated groups with low dose busulfan.
Fig. 4. The percentages of morphologically normal sperm and sperm pathology in control and busulfan treated mice. A) Difference in the Also, the high levels of sperm abnormality following treatment with low dose busulfan were due to cytotoxicity of chemotherapy on spermatogenesis and confirmed the results of flowcytometry and MTT assay.

Fig. 5. MTT assay evaluated sperm viability in control and busulfan treated mice. Sperm viability decreased significantly in groups 2 and 3 versus control. Different letters indicate significant differences between groups based one-way ANOVA (P< 0.05). Bu: busulfan.

Fig. 6. The flowcytometric profile of epididymal sperm in control and busulfan treated mice that labeled with Annexin V and PI. A) Necrotic cells labeled with PI but not with Annexin V-FITC. B) Late apoptotic cells with significant green and red fluorescence. C) Nonfluorescent, fully viable cells. D) Early Apoptotic cells labeled with Annexin V-FITC but not with PI.

Fig. 7. The flowcytometric results of epididymal sperm in control and busulfan treated mice. The percentages of early apoptotic, late apoptotic and necrotic sperm demonstrated significant changes between control and busulfan treated mice. Different letters indicate significant differences between groups based one-way ANOVA (P < 0.05). Bu: Busulfan.

The results of sperm parameters, MTT assay and flowcytometry revealed cytotoxic, genotoxic and apoptotic effects of low dose busulfan on epididymal sperm of busulfan treated mice. But, these cytotoxicity and genotoxicity were lower than high dose busulfan.
So, there are clear reasons that show chemotherapy harms most organs of body, but our observations illustrate that treatment with low dose busulfan has lower cytotoxicity and genotoxicity in normal cell.

Acknowledgments
This work was supported by the grant from Fars Science and Research Branch, Islamic Azad University, Fars, Iran.

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