

Original Article

Busulfan induces oxidative stress- and Bcl-2 family gene-related apoptosis in epididymal sperm and testis of adult male mice

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Abstract

Introduction: Busulfan as a chemotherapeutic agent causes testicular germinal epithelium depletion and cytotoxicity in germ cells. The aim of this study was to assess antioxidant status, reactive oxygen species (ROS) generation and apoptosis-related genetic markers of adult male mouse sperm following busulfan treatment.

Materials and Methods: Forty adult NMRI mice (30 ± 5 g) were divided into two groups. Control and busulfan treated group were administered with 100 μ L dimethyl sulfoxide and 3.2 mg/kg/day busulfan for 4 days, respectively. The superoxide dismutase and glutathione peroxidase assays were used for analyzing antioxidant status. Then, the levels of Bcl-2 family gene expression, lipid peroxidation and cytotoxicity were evaluated by Real-Time PCR, thiobarbituric and lactate dehydrogenase assays, respectively.

Results: The results showed significant decrease on antioxidant status, increase on lipid peroxidation and lactate dehydrogenase in epididymal sperm and testis of busulfan treated mice in comparison with control ($P < 0.05$). Real Time PCR demonstrated significantly increased-Bax gene expression and decreased-Bcl-2 gene expression in epididymal sperm of treated group ($P < 0.05$).

Conclusion: The high levels of lipid peroxidation and lactate dehydrogenase revealed increased-ROS and severe cytotoxicity in epididymal sperm and testis tissue following busulfan treatment at clinical dose. The oxidative stress and increased-ROS may induce Bcl-2 family gene expression-related apoptosis following busulfan therapy in normal cells.

Keywords:

Apoptosis;
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Introduction

Busulfan as a representative chemotherapeutic agent is used for the treatment of chronic myeloid leukemia

(Probin *et al.*, 2007; Suriaprabha *et al.*, 2012) and myeloablation prior to bone marrow transplantation (Probin *et al.*, 2007). Busulfan produces several side effects in normal tissue (Probin *et al.*, 2007; Suriaprabha *et al.*, 2012) such as cellular senescence in bone

marrow hematopoietic cells and human diploid fibroblasts (WI38 cells) (Meng *et al.*, 2003; Probin *et al.*, 2006). Busulfan as a potent reproductive toxic agent induces DNA fragmentation in epididymal or ejaculated sperm that can result in activation of the p53 (a tumor suppressor gene) pathway (Evenson and Wixon, 2006). The p53 pathway is one of the common apoptosis-mediated pathways that is induced by DNA damage following irradiation and chemotherapy (Probin *et al.*, 2007; Nasimi and Roohi, 2012; Vahdati *et al.*, 2015). The reactions/interactions between DNA and Reactive Oxygen Species (ROS) causes oxidative stress which leads to DNA damage (Suriapraba *et al.*, 2012). ROS can act as primary DNA damaging agents (Evenson and Wixon, 2006) and DNA damage is an early marker of apoptosis (Evenson and Wixon, 2006; Nasimi and Roohi, 2012). So, apoptotic events and ROS can increase DNA fragmentation (Evenson and Wixon, 2006).

The air pollution is related to sperm DNA damage and poor semen quality that may be due to increased ROS (Evenson and Wixon, 2006). Levels of ROS are negatively associated with the quality of sperm, sperm viability and male fertility (Choudhary *et al.*, 2010). In normal conditions, there are several antioxidant mechanisms in seminal plasma and inside the spermatozoa that are able to inhibit ROS activity, but, abnormal conditions such as smoking and air pollution can increase ROS generation and induce DNA damaged-related apoptosis in spermatozoa (Evenson and Wixon, 2006).

Probin *et al.* (2007) presented busulfan-related hematopoietic cell senescence via extracellular signal-regulated kinase (Erk) and p38 mitogen-activated protein kinase (p38 MAPK). They showed that ROS can contribute to the induction of DNA damage and DNA damage induces senescence via p38 MAPK pathway in normal hematopoietic cell (Meng *et al.*, 2003; Nasimi and Roohi, 2012). ROS are capable of activating various signal transduction pathways including the Erk-p38 MAPK cascade (Torres, 2003; Esposito *et al.*, 2004). Also, the Erk-p38 MAPK cascade mediates senescence (Esposito *et al.*, 2004) and apoptosis (Nasimi and Roohi, 2012). According to the recent studies busulfan can stimulate oxidative stress via GSH depletion and induces thioredoxin

reductase inhibition in normal human diploid WI38 fibroblasts (Probin *et al.*, 2006; Probin *et al.*, 2007).

Moreover, alterations on expression of Bcl-2 family members that is induced by anticancer drug treatment are potent factors for trigger or simply facilitate apoptosis in tumors (Probin *et al.*, 2006; Nasimi and Roohi, 2012). Apoptosis as a main type of programmed cell death can exert itself via various proteins which are regulated by Bcl-2 family members (Probin *et al.*, 2006; Nasimi and Roohi, 2012). The pro-apoptotic (Bax, Bad, Bid, and Bcl-xs) and anti-apoptotic (Bcl-2, Bcl-xl, and Bcl-w) members of Bcl-2 family can activate or inhibit the apoptotic pathways, respectively (Nasimi and Roohi, 2012; Kontos *et al.*, 2013).

On the other hand, the development of chemoprotection is not only for raising the effectiveness of cancer treatment but also for studying the underlying mechanisms of anticancer agents-induced cytotoxicity (Suriapraba *et al.*, 2012). There are different studies that indicate cytotoxicity and histopathology of chemotherapeutic agents in normal cells (Vahdati *et al.*, 2015). Most of recent studies used high single doses (including 20, 30 and 40 mg/kg) of these drugs (Mohammad-Ghasemi *et al.*, 2008; Mohammad-Ghasemi *et al.*, 2009).

Previous works on studying the adverse effects of busulfan have been done in doses higher than the ones that were clinically used. Based on our knowledge there is not enough information about the effects of busulfan on ROS production and apoptosis gene-expression alteration in sperm and testis of mammalian. Most of recent studies are about histological and cytotoxic effects of busulfan on reproductive tract of male (Mohammad-Ghasemi *et al.*, 2008; Dehghani *et al.*, 2013; Ahar *et al.*, 2014). In the present study, the effect of busulfan on the levels of oxidative stress markers and ROS generation in sperm and testis, and apoptosis-related gene expression (Bcl-2 family) in sperm of adult NMRI male mice were analyzed.

Materials and methods

Animals

Forty sexually-matured male NMRI mice with mean body weight of 30 ± 5 g were used. They were obtained

from Ahwaz Medical Sciences Research Center and Experimental Animal House (Ahwaz, Jondishapour University of Medical Sciences, Iran). The animals were housed in a temperature- and humidity-controlled room ($25 \pm 2^{\circ}\text{C}$ and $55 \pm 10\%$, respectively) on a 12 h light/dark cycle with free access to food and water. All procedures were performed 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).

Busulfan preparation

Busulfan (Sigma, St. Louis, MO) was dissolved into dimethyl sulfoxide (DMSO, $<0.2\%$, Sigma, St. Louis, MO) and diluted with sterile deionized water (1:1) at room temperature. Busulfan was daily prepared and freshly used during the experiment. Dosage of busulfan was chosen based on previous studies (Fernandez *et al.*, 2002; Chabner and Longo, 2011).

Treatment protocol

The mice were divided into two groups as follows:

Group 1- Control mice ($n=20$) were i.p. administered 100 $\mu\text{L/day}$ DMSO for 4 days.

Group 2- Treated group ($n=20$) were i.p. received 3.2 mg/kg/day busulfan for 4 days.

Sample collection

Male NMRI mice anesthetized by 80 mg/kg ketamine hydrochloride and 10 mg/kg xylazine, killed using decapitation. The left testis with left epididymis were immediately removed. The testis was stored at -80°C . Also, the epididymis was cut and the cauda was placed into a 60 mm dish with 1 mL of warmed phosphate buffer saline (PBS, pH 7.4). Several cuts were made in the cauda epididymis with scissors and dish was incubated at 37°C for 30 min. Then, the tissue was removed. The sperm suspensions were washed in PBS (pH 7.4) and were centrifuged. The pellets were frozen at -80°C . Subsequently, the frozen samples were used for determination the levels of lipid peroxidation and the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and Lactate Dehydrogenase (LDH). Then, the levels of Bcl-2 and Bax mRNA expression were analyzed in frozen sperm using Real-Time PCR.

Preparation of homogenate testis and sperm

The frozen left testes from all groups were washed in PBS (pH 7.4) and were homogenized with an ultrasonic homogenizer in 400 μL cold PBS (pH 7.4). Then, the frozen pellets of sperm were washed with PBS (pH 7.4) and were homogenized by vortexing shaking in 200 μL cold PBS (pH 7.4).

Lipid peroxidation (MDA) assay

Samples were lysed in 50 mM Tris buffer containing 0.5% Triton X-100, pH 8.0. The protein concentration was determined using Bradford method. Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) as described previously (Mihara and Uchiama, 1978). Briefly, 0.2 ml sample homogenate added to 0.8 ml of a solution containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid, and 0.25 N HCL. The protein precipitated and was removed by centrifugation at $5000 \times g$ for 5 min. The supernatants were transferred to glass test tubes containing 0.02% (w/v) butylated hydroxytoluene and heated for 15 min at 100°C in a boiling water bath, cooled and centrifuged at $2000 \times g$ for 5 min to remove precipitant. The absorbance at 532 nm was determined using spectrophotometer (Bio-Tek, Winooski, VT, USA). malondialdehyde levels were expressed as nmol/mg protein.

The activities of SOD and GPX

The activity of SOD was measured with a commercial kit (RANSOD kit, Randox Com, UK). In this method, xanthine and xanthine oxidase are employed to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The activity of SOD in sample was determined by the degree of inhibition of this reaction, as one unit of SOD corresponded to 50% inhibition of INT reduction under assay condition. Value was calculated using absorption at 412 nm for SOD and expressed as U/mg protein.

The activity of GPx was measured by a commercial kit (RANSEL kit, Randox Com, UK) based on the method of Paglia and Valentine (1967). The GPx present in the

sample catalysis the oxidation of GSH by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The absorbance was measured at 340 nm and the enzyme activity was expressed as units/mg of protein.

LDH release assay

LDH activity was measured using LDH Assay Kit (Zist Chem, Iran) according to manufacturer's protocol. Briefly, 1000 μ l R₁ (phosphate buffer pyruvate) was added to 10 μ l of sample and control (incubated at 37 °C for 5min). Then, 250 μ l R₂ (NADH) was added to sample and control. The absorbance at 340 nm was determined using spectrophotometer (Bio-Tek, Winooski, VT, USA). Results were expressed as percentage of control.

RNA extraction and Real-Time PCR

Total RNA was extracted from epididymal sperm of adult male mouse by RNeasy plus mini (QIAGEN), according to manufacturer's catalog. RNA concentration was measured the absorbance at 260 nm (A₂₆₀) using Nano Drop (Thermo Scientific, Nanodrop, Wilmington, DE). 200 ng RNA used for generated cDNA using the Revert Aid First Strand cDNA Synthesis kit (Fermentas, USA) and amplified by Real-Time PCR by using a Quanti Tect SYBR Green kit (QIAGEN, Germany) and the ABI Step One Plus Real-Time PCR instrument (Applied Biosystems). Primers list are shown in the Table 1. Control mixture consisted of PCR mixture without cDNA. Thermal cycler was programmed s: 40 cycles with initial denaturation at 95°C for 15 sec, 60°C for 30 sec and 72° C for 30 seconds.

Statistical analysis

Data were analyzed using independent t-test (SPSS 16.0 software package, SPSS Inc., Chicago, IL, USA). All experimental data were presented as the mean \pm SD. The level of significance for all tests was set at P<0.05.

Results

Evaluation of lipid peroxidation: TBA assay

The levels of lipid peroxidation and ROS activity in sperm and testis tissue of adult mice were analyzed by thiobarbituric (TBA) assay (Table 2). Sperm samples showed lower levels of lipid peroxidation versus homogenized testis.

The results confirmed high levels of lipid peroxidation in sperm samples of treated group (3.2 mg/kg/day busulfan for 4 days) in comparison with control (p<0.05) (Table 2). Also, lipid peroxidation was increased significantly in testis of busulfan treated mice versus control (p<0.05) (Table 2).

The study of SOD activity

Superoxide dismutase is one of the most important antioxidant that recent researches indicated as having a main role on inhibition of oxidative stress. The SOD assay was used for analyzing the level of SOD in sperm and testis tissue of control and treated animals (Table 3).

The busulfan treated mice were demonstrated significantly decreased SOD levels insperm and testis versus untreated mice (p<0.05) (Table 3).

The study of GPx activity

The levels of GPx activity as an important marker of the oxidative stress were evaluated by GPx assay in sperm and testis of control and busulfan treated mice (Table 3). The lowest level of GPx activity was shown in sperm and testis of busulfan treated group (Table 3).

The evaluation of cytotoxicity: LDH assay

The busulfan mediated cytotoxicity in sperm and testis of treated mice was analyzed by LDH assay (Table 2). The high levels of LDH presented extreme damage and cell death. The level of Lactate Dehydrogenase was increased significantly in sperm and testis of treated mice with busulfan (Table 2).

Table 1: Primer sequences were used for Real-Time PCR (5' to 3'). All primers were designed by PRIMER EXPRESS software (Applied Biosystems, USA).

Gene references	Primers	Genes
NM007527	F: CGAGCTGATCAGAACCATCA R: GAAAAATGCCTTTCCCTTC	Bax
NM009741	F: TAAGCTGTACAGAGGGGCT R: TGAAGAGTTCCTCCACCACC	Bcl-2
NM_001256799.2	F: AGTTCAACGGCACAGTCAAG R: TACTCAGCACCAGCATCACC	GAPDH

Table 2: The levels of malondialdehyde and Lactate Dehydrogenase activity in epididymal sperm and testis tissue of control and busulfan treated mice. The high levels of lipid peroxidation and Lactate Dehydrogenase revealed in busulfan treated group.

Groups	Thiobarbituric acid (U/mg protein)		Lactate Dehydrogenase (U/mg protein)	
	Sperm	Testis	Sperm	Testis
Control	1.63 ± 0.22 ^a	0.75 ± 0.06 ^a	17.05 ± 1.29 ^a	11.98 ± 2.33 ^a
Busulfan treated mice	2.2 ± 0.04 ^b	3.9 ± 0.10 ^b	43.51 ± 5.55 ^b	39.9 ± 3.79 ^b

Significant differences at $p < 0.05$ are shown by different letters in each column.

Table 3: The levels of superoxide dismutase (SOD) and Glutathione peroxidase (Gpx) activities in epididymal sperm and testis tissue of control and busulfan treated mice. These results showed busulfan-mediated decrease on the activities of SOD and Gpx.

Groups	Superoxide dismutase (U/mg protein)		Glutathione Peroxidase (U/mg protein)	
	Sperm	Testis	Sperm	Testis
Control	27.71 ± 2.19 ^a	44.24 ± 2.44 ^a	45.24 ± 2.93 ^a	46.15 ± 12.93 ^a
Busulfan treated mice	15.81 ± 3.78 ^b	18.47 ± 1.59 ^b	23.33 ± 7.58 ^b	29.38 ± 6.25 ^b

Significant differences at $p < 0.05$ are shown by different letters in each column.

Apoptosis gene expression: Bcl-2 family gene expression

The levels of Bcl-2 family gene expression as a main regulatory factors of apoptosis, was studied in sperm of control and treated mice. The results of Real-Time showed changes in expression of bax and Bcl-2. Bax as a pro-apoptotic protein increased significantly in sperm of busulfan treated animals (Figure 1A). Also, the expression of Bcl-2 as anti-apoptotic protein decreased significantly in sperm of busulfan treated group (Figure 1B).

Discussion

This study confirmed the results of recent researches/investigations that showed busulfan-induced apoptosis in spermatozoa (Mohammad-Ghasemi *et al.*, 2008; Mohammad-Ghasemi *et al.*, 2009) and germ cells (Choi *et al.*, 2004; Nieto *et al.*, 2012) of mammals. Although, these studies used high doses of busulfan at 20 (Mohammad-Ghasemi *et al.*, 2008; Kawashima *et al.*, 2009) and 40 mg/kg, but, this finding demonstrated that standard or lower clinical doses of busulfan (3.2mg/kg/days for 4 days or final

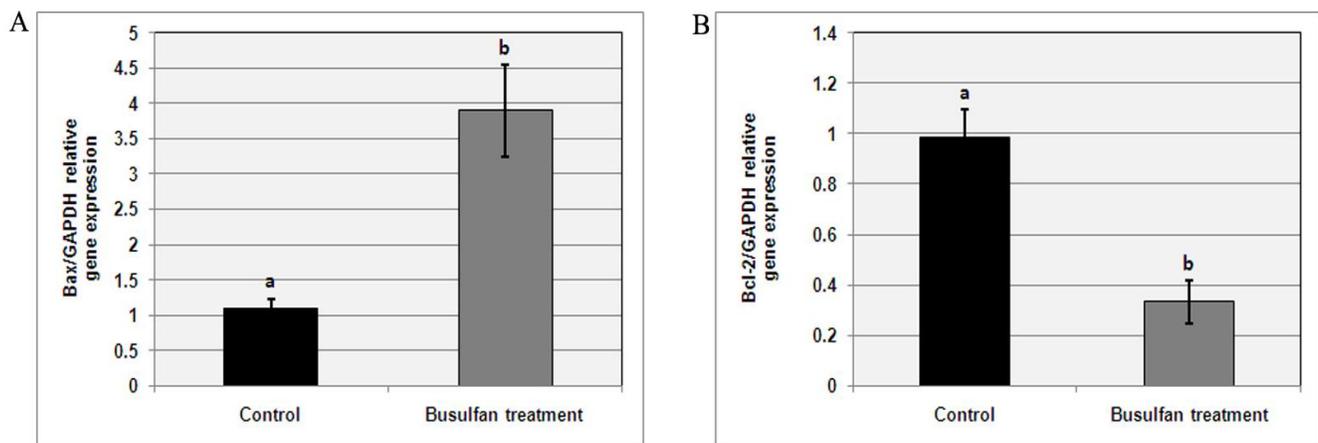


Fig.1. The study of Bax and Bcl-2 genes expression in epididymal sperm of control and busulfan treated mice. A) Bax gene expression was significantly increased in sperm of busulfan treated mice versus control. B) There was significant decrease of Bcl-2 gene expression in sperm of busulfan treated mice compared to control. Significant differences at $p < 0.05$ are shown by different letters in each column.

dose of 12.8 mg/kg) can also increase apoptosis in germ cells and especially adult sperm cells of mammals. Probin *et al.* (2007) reported that busulfan has the ability to increase ROS production in part by depletion of intracellular GSH and inducing senescence via Erk-p38 MAPK in human fibroblast cells (Probin *et al.*, 2007).

Reactive Oxygen Species in a normal range are produced routinely as a result of cellular activities. But, the levels of these cytotoxic agents increase in abnormal conditions such as air pollution and cigarette smoke (Evenson and Wixon, 2006; Birben *et al.*, 2012). Busulfan significantly mediated decrease on the levels of enzymatic antioxidants including GPx and SOD, and increase on the level of lipid peroxidation in sperm and testis tissue of treated mice. The results of GPx and SOD assays demonstrated increased-oxidative stress and high levels of lipid peroxidation and showed increased-ROS generation in testis and epididymal sperm of busulfan treated animals. Also, the imbalance between oxidants and antioxidants when the shift was in a favor of oxidants caused oxidative stress in the epididymal sperm and testis of busulfan treated mice.

The results of LDH assay illustrated severe cytotoxicity in sperm and testis of mice that received 3.2 mg/kg/4days busulfan. The extreme changes of Bcl-2 family gene expression level as the main regulatory markers of apoptosis showed busulfan-mediated apoptosis in epididymal sperm of treated mice. This fact corroborated Bcl-2 family gene-induced apoptosis

in normal tissue following busulfan administration.

Recent research showed ROS ability to regulate the phosphorylation and ubiquitination of Bcl-2 family of proteins and change of the activity of these apoptotic markers (Kontos *et al.*, 2013). Our results confirmed that decreased- Bcl-2 gene expression and increased-Bax gene expression were induced by ROS. Also, the high levels of lipid peroxide caused increased-ROS in epididymal sperm of treated mice with busulfan.

Bairey *et al.* (1999) found a similar association for Bcl-2 family members and LDH in Patients with Diffuse Large B-Cell Lymphomas. They illustrated 57% of patients with bax positivity and elevated LDH levels (Bairey *et al.*, 1999). We recognized high levels of LDH activity associated with bax positivity and low levels of Bcl-2 in sperm of busulfan treated mice.

In normal cells, Bax as a pro-apoptotic protein has monomeric structure and commonly located in cytosol. The death signals can activate Bax via translocation to the mitochondria where it becomes an integral membrane protein and cross-linkable as a homodimer (Wolter *et al.*, 1997; Gross *et al.*, 1998; Nasimi and Roohi, 2012). Bax was capable of killing sperms despite the presence of survival factor such as Bcl-2. Because, normally the presence of Bcl-2 as an anti-apoptotic molecule can inhibit the activation of Bax following a death stimulation (Dev *et al.*, 1999).

Over-expression of Bcl-2 as an upstream marker protein from intrinsic pathway of apoptosis was associated with decreasing in the activities of GPx and

SOD. The Bcl-2 gene expression (as an anti-apoptotic protein) decreased and Bax gene expression (as a pro-apoptotic protein) increased in epididymal sperm of busulfan treated mice. These results corroborate and are compatible with the observed oxidative stress-related effects in normal organs and especially male reproductive system of patients undergoing chemotherapy. So, busulfan-mediated apoptosis in sperm and testis was related to increased-oxidative stress, increased-ROS generation and changes on Bcl-2 family gene expression.

In conclusion, the above findings support the contention/claim that busulfan-related oxidative stress and bcl-2 family gene expression play main roles during apoptosis in sperm cells and can lead to increased cytotoxicity in germ cells of adult mice. These results reveal that ROS can induce apoptosis in epididymal sperm by increasing pro-apoptotic protein levels and decreasing anti-apoptotic protein expression following busulfan treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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