Kafkas Univ Vet Fak Derg
25 (5): 709-716, 2019
DOI: 10.9775/kvfd.2019.21726

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Amelioration Effects of Vitamin E, Melatonin, L-carnitine, and Atorvastatin, on Destructive Effects of Busulfan in the Testes of Male Rats: A Gene Expression Evaluation

Forutan SALEHINEZHAD [1] Hamidreza ESHRAGHI [1] Ali KADIVAR [2,3]
Sadegh SHIRIAN [4,5] Ahmad ASGHARI [6] Ehsan AALI [7] Najmeh DAVOODIAN [3]

[1] Department of Veterinary Basic Science, Science and Research Branch, Islamic Azad University, Tehran, IRAN
[2] Department of Clinical Science, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, IRAN
[3] Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, IRAN
[4] Department of Pathology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, IRAN
[5] Biotechnology Research Institute, Shahrekord University, Shahrekord, IRAN
[6] Department of Veterinary Clinical Science, Science and Research Branch, Islamic Azad University, Tehran, IRAN
[7] Department of Pharmacology, School of Medicine, Qazvin University of Medical Sciences, Qazvin, IRAN

Article ID: KVFD-2019-21726 Received: 11.01.2019 Accepted: 06.05.2019 Published Online: 06.05.2019

How to Cite This Article

Salehinezhad F, Eshraghi H, Kadivar A, Shirian S, Asghari A, Aali E, Davoodian N: Amelioration effects of vitamin E, melatonin, L-carnitine, and atorvastatin, on destructive effects of busulfan in the testes of male rats: A gene expression evaluation. *Kafkas Univ Vet Fak Derg,* 25 (5): 709-716, 2019. DOI: 10.9775/kvfd.2019.21726

Abstract

According to toxicity of various types of cancer treatments on different kind of cells with high division activities such as germ cells, antioxidants may protect these cells in testes against the toxic effects of the chemotherapeutic drugs. For this purpose, 24 h after busulfan treatment, 30 adult male wistar-rats were divided to six groups. Intra-peritoneally administrations of normal saline in control group and DMSO (as a busulfan solvent) in DMSO group were performed daily for 6 weeks beside the treatment contain vitamin E (Vit-E group), L-carnitine and melatonin (LM group), atorvastatin and melatonin (AM group), atorvastatin, L-carnitine, and melatonin (ALM group). After decapitation and removal of the testes, molecular evaluations were performed by the relative abundance measurement of *DAZL*, *Bcl2*, and *Casp3* transcripts. The results of this study exhibited high level of expression of DAZL in Vit-E treated rats compared to control counterparts (P<0.01). The expression level of *Bcl2* is significantly down-regulated in LM (P<0.008), and ALM groups (P<0.001), and the relative abundance of *Casp3* transcripts was significantly lower in AM (P<0.001) and ALM (P<0.007) than that of control group. As well as, there was significant high expression of this gene in Vit E-treated rats compared to the rats of control group. In conclusion, busulfan destructive effects were moderated with Vit-E administration through regulation of the expression of *DAZL*. The other antioxidants used in different combinations had not amelioration effects on spermatogenesis in busulfan-induced male rats, though the positive effects of some of these antioxidants on apoptosis reduction.

Keywords: *Rat, Busulfan, Vitamin E, Melatonin, L-carnitine, Atorvastatin, DAZL, Bcl2, Casp3*

Erkek Rat Testislerinde Busulfan Kaynaklı Hasara Karşı Vitamin E, Melatonin, L-karnitin ve Atorvastatin'in Koruyucu Etkisi: Gen Ekspresyonunun Değerlendirilmesi

Öz

Çeşitli kanser ilaçlarının toksisitelerine bağlı olarak eşey hücreleri gibi yüksek bölünme kapasitesine sahip çeşitli hücrelerde meydana gelen hasara karşı antioksidanlar koruyucu olabilir ve bu durum testis dokusunda kemoterapötik ilaçlarla oluşan hasarda etkili olabilir. Bu amaçla, 24 saat busulfan uygulaması sonucunda 30 ergin erkek Wistar rat altı gruba ayrıldı. İntraperitoneal olarak 6 hafta süresince DMSO grubuna fizyolojik tuzlu su ve DMSO (busulfan çözücüsü olarak) uygulandı. Diğer gruplarda busulfan uygulamasına ilave olarak E (Vit-E grubu), L-karnitin ve melatonin (LM grubu), atorvastatin ve melatonin (AM grubu), atorvastatin, L-karnitin ve melatonin (ALM grubu) uygulamaları gerçekleştirildi. Hayvanlarda dekapitasyonu takiben testis dokuları alındı ve *DAZL, Bcl2, ve Casp3* miktarları karşılaştırmalı olarak moleküler açıdan değerlendirildi. Çalışma sonucu, Vit-E uygulanan ratlarda DAZL ekspresyonunun kontrol grubuna oranla daha fazla olduğu gözlemlendi (P<0.01). LM (P<0.008) ve ALM (P<0.001) gruplarında *Bcl2* ekspresyonu aşağı yönde regule edilirken, AM (P<0.001) ve ALM (P<0.007) gruplarında *Casp3* ekspresyonu anlamlı derecede control grubuna oranla daha azdı. Kontrol ile karşılaştırıldığında Vit-E uygulanan grupta *Casp3* ekspresyonunun anlamlı derecede yüksek olduğu belirlendi. Sonuç olarak, busulfan kaynaklı hasarın Vit-E uygulaması sonucu *DAZL* ekspresyonu yolu ile normalize edildiği tespit edildi. Busulfan ile erkek ratlarda spermatogenez üzerine oluşturulan hasara karşı çalışmada uygulanan diğer antioksidanların hasarı azaltmada etkili olmadığı ancak bazılarının apoptozis üzerine etkisinin olduğu belirlendi.

Anahtar sözcükler: *Rat, Busulfan, Vitamin E, Melatonin, L-karnitin, Atorvastatin, DAZL, Bcl2, Casp3*

İletişim (Correspondence)
+98 917 7323202
kadivar.ali@sku.ac.ir

INTRODUCTION

Alkylating agents as chemotherapeutic drugs are often used to treat cancer and increase the survival rates of patients. Alkylating agents, affect cell division by adhesion to one strands of the DNA [1]. Therefore, cells or tissues with high division activities such as germ cells and testes are more susceptible to these agents' side effects. Spermato-genesis makes round haploid spermatids through the reductive divisions of meiosis [2]. It is clear that certain chemo-therapeutic drugs specially alkylating agents influence spermatogenesis at least temporarily and in some cases permanently. Single doses of busulfan as an alkylating agent can permanently sterilize rats at non-lethal doses and cause long-term morphological damage to sperm produced by surviving spermatogonia [3]. Busulfan toxicity occurs by several different mechanisms, including reactive oxygen species (ROS) formation and protein damage (oxidation) [4]. ROS are directly involved in precarious oxidative damage of cellular macromolecules such as proteins and nucleic acids in germ cells, which can lead to cell death [5]. Indeed, the overproduction of ROS and the consequent oxidative stress has a critical role in inhibition of development of germ cells. Forasmuch as, the rats treated with Busulfan exhibited a defined increase in apoptosis, using cytoprotective and anti-apoptotic agents such as antioxidants can neutralize both ROS and apoptosis procedure caused by them [6]. ROS are scavenged by some antioxidants such as L-carnitine, Vitamin E (α-tocopherol), and Atorvastatin via their interfering with the lipid peroxidation chain reaction. Another small molecule antioxidant, Melatonin, which can detoxify highly reactive hydroxyl free radicals (OH) is involved in the detoxification of free radicals [7]. Co-administration of these components also change produced ROS profile, prevented lipid peroxidation and improved antioxidant status, synergistically [8]. Therefore, strategies that effectively preserve fertility during the course of cancer treatment especially through the decrease in apoptosis should be developed.

In this study all mentioned antioxidants, singly or in different combinations, were used to amelioration busulfan side effects in treated male rats and then the expression amount of deleted in azoospermia-like *(DAZL)* as a gene related to spermatogenesis resumption and B-cell lymphoma 2 *(Bcl2)* and Caspase 3 *(Casp3)* as the genes related to apoptosis was measured.

DAZL is a gene cluster which gets deletions in at least 10% of males with oligozoospermia or azoospermia [9].This gene is a DAZ autosomal homologue. As a result, *DAZL* has always been seen as a promising candidate for male infertility. *DAZL* plays an important role in the spermatogenic processes.

Bcl2 gene is an anti-apoptotic member of *Bcl2* Family, regulators of the cellular life-or-death switch, that prevents cytochrome C release, and hence caspase-9 activation and subsequently several other caspases, independently of mitochondrial damage [10]. Besides, caspase-3 is a cysteine protease that is activated early in a sequence of events associated with apoptosis [11].

The aim of the present study was reduction the amount of apoptosis and subsequently improves the spermato-genesis and preserves fertility of azoospermic animal model using busulfan in rat, with a focus on related molecular pathways.

MATERIAL and METHODS

Animals

Thirty adult male wistar-Rats (2-3 months old and 200-250 g) were purchased from Faculty of Veterinary Medicine of Shahrekord University and were housed for 2 weeks at the animal lab of Veterinary Clinic under the Standard laboratory conditions (12 h dark and 12 h light cycle, temperature of 23±3°C, and 50±5% humidity) for adaptability of rats to new living environment. Animal cages were kept clean, and commercial food (pellet) and water were provided *ad libitum*.

Experimental Design

In total, all rats were treated with two doses of 25 and 10 mg/kg busulfan with 14 days interval and after 24 h were randomly assigned to the following groups: Sham group that treated with DMSO as busulfan solvent, treated with busulfan (control group), and treatment groups including: treated with 100 mg/kg vitamin E (Vit E group), treated with 100 mg/kg L-carnitine and 1 mg/kg melatonin (LM group), treated with 20 mg/kg atorvastatin and 1 mg/kg melatonin (AM group), treated with 20 mg/kg atorvastatin, 100 mg/kg L-carnitine, and 1 mg/kg Melatonin (ALM group). Each group assigned by 5 rats. In all groups all administrations were performed intra-peritoneally daily for 6 weeks. Busulfan dose was assigned based on previous studies that established the toxic effect of busulfan on rat testes [12]. The atorvastatin [13], L-carnitine [13], melatonin [14] and Vit E [15] doses were also selected based on previous reports demonstrating their anti-oxidative effect. This study was approved by the Institutional Ethics Committee for Animal Experimentation and was conducted in accordance with the international guidelines [16].

Organ Removal and Tissue Processing

Animals were killed by decapitation under ether anesthesia, and testes of the animals were removed. One testis was used for sperm collection and the other one was preserved in -80°C freezer until molecular evaluations.

Sperm Collection and Evaluation

Sperm was collected from tail of left epididymis for all the

experimental rats. The left epididymis was immediately minced with scalpels and placed in pre-warmed microtube, containing 1 mL of Human Tubal Fluid (HTF) medium without BSA and placed in a 37°C incubator for 10 min. For sperm counting, 50 µL of the solution was diluted 10 times with 0.9% saline and a drop was transferred into chamber of Neubauer hemocytometer. Sperm counted under a standard optical microscope in order to determine the number of spermatozoa.

After 10 min of incubation, sperm motility was assessed by putting one drop of the on a warmed microscope slide. A cover slip was placed and at least 5 microscopic fields was observed at 400-fold magnifications. The percentage of nonmotile sperm was recorded for each rat.

Sperm viability was assessed with eosin–nigrosin staining. For this, 10 µL of sperm suspension were mixed with equal volume of eosin-nigrosin on a warmed microscope slide and a thin smear was prepared. Samples were observed under light microscope at a magnification of 1000×. At least 100 sperm were evaluated to discriminate death sperm (red stained) of live (not stained).

Molecular Evaluation

Total RNA Extraction, DNAase Treatment and cDNA Synthesis: To extract total RNA, tissues of testes (100 mg) were mechanical fragmented with a scalpel. Half mL RNX-PLUS solution (Sinaclon Bioscience, Karaj, Iran) containing phenol and guanidine was added and placed at room temperature for 5 min to homogenized samples. After adding 120 µL chloroform, the mixture was centrifuged at 8.000 g and 4°C for 5 min. The upper aqueous phase of supernatant was separated, and after addition of 400 µL isopropanol (100%), it was centrifuged (8000 g, 4°C, 5 min) and the RNA pellet was washed with 80% ethanol. Upon centrifugation at the same condition, the pellet was suspended in DEPC-treated water. To remove genomic DNA contamination, the extracted RNA was treated by RNase-free DNase I and its buffer (Sinaclon Bioscience, Karaj, Iran) and incubated at 37°C for 30 min. The reaction was stopped by EDTA at 65°C for 10 min, and the amount and quality of RNA were determined by spectrophotometry (Micro-volume Spectrophotometer System, Nano Mabna Iranian, Tehran, Iran). Only RNA of sufficient purity, having an absorbance ratio (A260/280) between 1.8 and 2.2 was considered for synthesis of cDNA.

Shortly after extraction, total RNA (1 µg) was reversely transcribed into cDNA (less than 2 h) in a 5 µL reaction volume using the Easy cDNA synthesis kit, offered by the manufacturer (Pars tous biotechnology, Mashhad, Iran). The thermal program for cDNA synthesis included the following 3 steps: 25°C for 10 min (activation of the reverse transcriptase), 47°C for 60 min (reverse transcription), and 85°C for 5 min (inactivation of the reverse transcriptase). The synthesized cDNA was then stored at -20°C.

Quantitative Real-Time PCR: Real-time PCR was performed in two replicates for each sample (Rotor Gene Q 6000, Qiagen, USA). Primer sequences, the GenBank accession numbers, the size of amplified products, and annealing temperature of each primer are shown in *Table 1*. Half µL DNase I treated cDNA was added to 5 µL SYBR Premix Ex Taq II Mix and 0.5 µM of each specific primer in a total volume of 10 µL. The PCR program was comprised of 45 cycles of 94°C for 5 s (denaturation), and 54-60°C for 30 s (annealing & extension; *Table 1)* and 72°C for 30 s.

Considering the selection of an appropriate housekeeping gene as a reference gene for normalization, there are several studies demonstrating that *Actb* is highly reliable reference genes among the other genes used for RT-qPCR normalization and analysis of relative gene expression in the mouse testes [17].

Melt curve analysis was conducted to confirm the specificity of each product. The no-template control and no-reverse transcriptase control were considered to check contamination of the PCR reagents. Data were analyzed using LinReg PCR software version 2012.0 (USA), to give the threshold cycle number (Ct). Mean efficiency values (E) for each gene were also determined from the amplification profiles of individual samples using the same software [7]. The following formula was applied to determine the relative gene expression in tissue testes of treated rat compared to the control group [18,19].

Statistical Analysis

The differences in relative abundance of gene expression

Table 1. *Sequence and annealing temperature of primers used for Real Time-PCR*

Gene	Gene Bank Accession No.	Primer Sequence (5′-3′)	Product Size (bp)	Annealing Temp (°C)	Reference
Casp3	NM_012922.2	F-GGACCTGTGGACCTGAAAAA R-GCATGCCATATCATCGTCAG	159	54	[20]
Bcl2	NM_016993.1	F-GGTGAACTGGGGGAGGATTG R-GCATGCTGGGGCCATATAGT	197	60	[20]
DAZL	NM_001109414.1	F-TCTTCATCAGCAACCACCAG R-GACAAATCCATAGCCCTTCG	195	60	[12]
B-Actin (Actb)	NM_031144.3	F- CACCCGCGAGTACAACCTTC R- GAAGCCGGCCTTGCACAT	127	60	Designed by writers

between groups were analyzed using one sample t-test student after ArcSin transformation with SPSS software version 20.0.0 (IBM Corp.; USA). Data were expressed as mean±SEM. Differences were considered significant at P<0.05.

RESULTS

The results of sperm evaluation showed that the mean percent of live and non-motile sperm and the sperm concentration were not different significantly between treatment groups. The difference was only significant between control and sham for all three parameters *(Table 2)*.

The results of gene expression were presented by relative comparison of all of treatments groups with control group. The relative abundance (RA) of transcripts in tissue of treated rat testes has been shown in *Fig. 1A-B* and *Fig. 2*. As shown in *Fig. 1A*, the expression level of *Bcl2* is significantly down regulated in those rats received both L-carnitine and melatonin (LM) (P<0.008), and those that received all three antioxidants, Atorvastatin, L-carnitine and melatonin (ALM) (P<0.001) in comparison with control group. No significant difference was observed between each of other treatment groups and control group in *Bcl2* expression.

According to *Fig. 1B*, the relative abundance of *Casp3* transcripts was significantly lower in AM (P<0.001) and ALM (P<0.007) than control group. As well as, there was significant high expression of this gene in Vit E-treated rats compared to the rats of control group. There was no significant difference between each of other groups (DMSO, and LM) and control group.

Table 2. *The mean±SE percent of live and non-motile sperm and the sperm concentration*

Sperm Characteristics in Treatment Groups		N	Mean	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Live sperm (%)	Sham	8	67±1.52[a]	63.40	70.60
	ALM	8	52±1.92[ab]	47.46	56.54
	vitamin E	8	50.25±2.6[ab]	44.09	56.41
	Control	8	24.75±2.23[b]	19.47	30.03
	Total	32	48.5±2.9	42.58	54.42
Sperm count	Sham	8	564.38±15.63[a]	527.40	601.35
	ALM	8	461.63±18.78[ab]	417.21	506.04
	vitamin E	8	424.38±11.7[ab]	389.93	458.82
	Control	8	225±23.28[b]	197.35	252.65
	Total	32	418.84±131.72	371.35	466.34
Non-motile sperm	Sham	8	18.88±1.31[a]	15.76	21.99
	ALM	8	28±1.45[ab]	24.57	31.43
	vitamin E	8	31.13±1.7[ab]	27.09	35.16
	Control	8	34.5±3.15[b]	27.05	41.95
	Total	32	28.13±1.42	25.22	31.03

ALM: *atorvastatin, L-carnitine, and melatonin group,* ***Sham:*** *treated with DMSO as busulfan solvent*

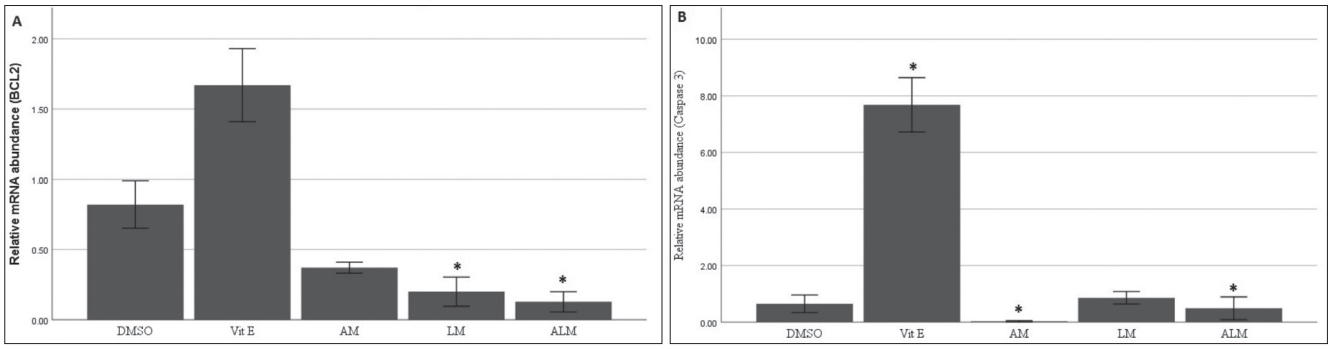


Fig 1. Relative abundance of *Bcl2* (A) and *Casp3* (B) transcripts in rat testes tissue derived from treated rats with busulfan solvent (DMSO), Vitamin E (Vit E), L-carnitine and Melatonin (LM), atorvastatin and melatonin (AM), atorvastatin, L-carnitine, and Melatonin (ALM) compared to control group (busulfun treated rats). All reactions were normalized for β-Actin mRNA expression. Values with superscripts "*" refers to significant (P<0.05) differences in relative transcript abundance of each group compared to control group

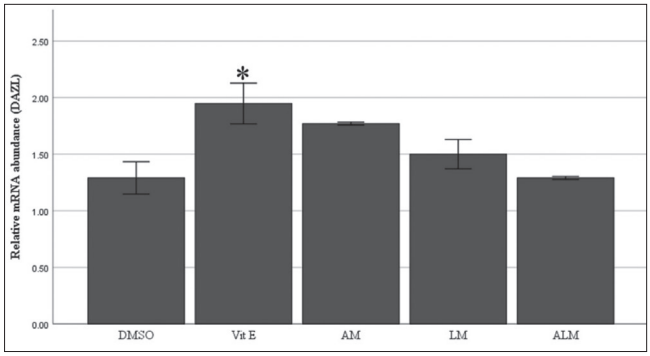


Fig 2. Relative abundance of DAZL transcripts in rat testes tissue derived from treated rats with busulfan solvent (DMSO), Vitamin E (Vit E), L-carnitine and Melatonin (LM), atorvastatin and melatonin (AM), atorvastatin, L-carnitine, and Melatonin (ALM) compared to control group (busulfun treated rats). All reactions were normalized for β-Actin mRNA expression. Values with superscripts "*" refers to significant (P<0.05) differences in relative transcript abundance of each group compared to control group

Table 3. *Mean±SEM of relative abundance of Bcl2, Casp3, DAZL mRNA in treatment groups in comparison with control group*

Groups	*Bcl2*	*Casp3*	*DAZL*
DMSO	0.82±0.13	0.65±0.24	1.29±0.11
Vit E	1.67±0.20	7.68±0.74*	1.97±0.08*
AM	0.37±0.03	0.03±0.02*	1.77±0.01
LM	0.2±0.08*	0.86±0.17	1.50±0.10
ALM	0.13±0.05*	0.49±0.31*	1.29±0.01

All reactions were normalized for β-Actin mRNA expression. "" refers to significant (P<0.05) differences in relative transcript abundance of each group compared to control group*

Deleted in azoospermia-like, exhibited high level of expression in Vit E treated rats compared to control counterparts (P<0.01) and no differences were found between each of other treatment groups (DMSO, AM, LM, and ALM) and control group *(Fig. 2)*.

The relative abundance of *Bcl2*, *Casp3*, and *DAZL* transcripts in rat testes tissue derived from treated rats in all treatment groups compared to control group (Busulfun treated rats) are briefly shown in *Table 3*.

DISCUSSION

One of the most important problems in the life of couples' is infertility and it's complications and the causes of some infertilities are related to men. The most common cause of male infertility is inability to produce sufficient numbers of active and healthy sperms [21]. Many factors can affect sperm production and infertility risks. Among these factors are using chemotherapy drugs for cancer, antibiotics, toxic substances, s, stress, pesticides, radiation, vitamin deficiency, and air pollution. These factors can reduce sperm concentration with generation of free radicals and oxidation of germ cells in the testes [21].

Busulfan, as a chemotherapy drug, is a bifunctional alkylating agent and is used for the treatment of various malignant diseases, such as polycythemia vera and chronic myelogenous leukemia [3]. Busulfan is a highly cytotoxic and genotoxic agent [3] that can induce various adverse effects, both acute and chronic, such as DNA damage and subsequently activates apoptosis or senescence in a cell type-dependent manner probably due to oxidative stress [4] in several biological organs such as hematologic [22], nervous [23] and reproductive organs [24]. It has been confirmed that chemotherapy with busulfan can induce apoptosis in sperm [6] and increase ROS generation and resulting in death of spermatozoa [25]. As well as, accurance of teratospermia, the presence of spermatozoa with abnormal morphology in semen is the other possible result of busulfan treatment in mice [3]. These studies showed that busulfan is involved in the arrest of spermatogenesis, though some of the changes are reversible and dose dependent. In this study two dose of 25 and 10 mg/kg busulfan with 14 days interval were used which can destruct the spermatogenic process in rat as was shown in other studies [3,26,27].

Antioxidants neutralize free radicals and subsequently the oxidative reactions caused by them. The dietary antioxidants may be beneficial in reducing the lipid peroxidation and DNA damage in sperm during the treatment with busulfan [28]. Different antioxidants have examined in challenging procedures to reduce the adverse effects of ROS on rat spermatozoa [16,29].

Here, we studied the protective effect of vitamin E, melatonin, L-carnitine, and atorvastatin treatment, alone and in different combinations, against busulfan-mediated sperm damage in mice. Our results demonstrated that perform all treatments 48 h after the beginning of chemotherapy with busulfan and daily administration of antioxidants for 8 weeks can slightly reduce busulfan-mediated destructions.

To confirm the role of busulfan in germ cells destruction and apoptosis, respectively, *DAZL* activation and anti-apoptotic molecule *Bcl2* as well as activation of *Casp3* were measured in testes tissue after busulfan treatment.

Consistent with busulfan-induced increased mitochondrial membrane depolarization, significantly increased expression of *Bcl2*, was observed in testes tissue treated with vitamin E compared to other antioxidants-treated rats, suggesting busulfan-induced apoptosis is moderated with Vitamin E administration through regulation of the expression of *Bcl2*, but did not lead to subsequent decrease caspase-3 activation.

Vitamin E (α-tocopherol) is an organic fat soluble compound located generally in cell membranes. This dominant antioxidant extinguishes superoxide anions and free hydroxyl radicals thereby reducing lipid peroxidation initiated by

ROS in plasma membranes [30] and thus protects the cell membrane from ROS-induced damages. This antioxidant reduces testicular tissue damages cause by cytotoxic agents through increasing the expression of antioxidant related genes. In the male reproductive tract, the antioxidant property of this vitamin in inhibition of destructive effects of free radicals in testes [31] and sperm [32] was confirmed. As well as, some studies showed vitamin E is efficient in protecting testes from induced damage by oxidative stress and mitigation this damage can be achieved by vitamin E treatment [33].

It was expected high expression of *Bcl2* reduces *Casp3* expression after administration of Vitamin E. But, it should be considered that there are at least two principal pathways for activating *Casp3*. The more ancient, which is induced by diverse intracellular stresses, including cytokine deprivation and genotoxic damage, is regulated by *Bcl2* and its relatives. Progression through the pathway usually leads to the activation of *Casp9* and subsequently *Casp3*. A more-recently evolved pathway is triggered when 'death receptors' on the plasma membrane engaged by cognate ligands of the tumour-necrosis factor (TNF) family, recruit *Casp8* through bind the adaptor protein FAS-associated death domain (FADD) (also called MORT1). This pathway eventually increase *Casp3* [10]. Therefore, high expression of *Casp3* in vitamin E-treated rats may be due to activation of second mentioned pathway.

Among the groups, Vitamin E more moderated spermatogenesis resumption as showed in increasing *DAZL* gene expression. This study was the first study in directly determination of the effect of vitamin E on *DAZL* expression as a worthy candidate for male infertility that plays an important role in the spermatogenesis.

However, a number of studies suggested that supplementation with oral antioxidants such as vitamin E and carnitine could improve the sperm quality in infertile patients which can certainly be through the stimulation of related gene expression such as *DAZL*.

However, we observed lower *Casp3* gene expression in other treatments, especially in those rats treated with both AM and ALM. When combination groups were compared to each other, it was defined that down-regulation of *Casp3* mRNA in AM and ALM groups might be more due to the presence of melatonin, and on the other hand, high expression of this gene in LM in comparison of AM group and then ALM group was created by L-carnitine. This is consistent with Fan et al.[34] study that expressed mRNA and protein levels caspase-8 was increased by L-carnitine treatment in hepa1c1c7 mouse cancer cells. As well as, it was noted that despite the inhibition effect of L-carnitine on the activity of recombinant caspases 3 in Jurkat cells (a human T lymphocyte cells line), its long-chain fatty acid derivative palmitoylcarnitine increases the activity of all the caspases. It was suggested that reversed effect of palmitoylcarnitine on the inhibition of caspase activity by carnitine, may be regulated in part by the balance of palmitoylcarnitine and carnitine under physiological conditions [35].

Many studies demonstrated that melatonin has an anti-apoptotic effect in somatic and germ cells [36-38]. Melatonin has been reported to be protective in male reproductive health, which readily crosses the blood-testes barrier and has a very low toxicity [39]. Studies have investigated the use of melatonin to relieve the side effects of environmental toxins and chemotherapy drugs during spermatogenesis [36,40]. However, few systematic studies have investigated whether melatonin employs a protective role in the psychological stress-induced impairment of spermatogenesis as well as the mechanisms by which melatonin mitigates the damage in testes.

Many studies have found that L-carnitine and its derivatives can optimize sperm motion parameters [41,42]. On the other hand, other studies failed to detect significant increases in sperm concentration following L-carnitine treatment [43-45]. The relatively small doses and short duration of treatment employed may be the main reason why no substantial increases were detected. By the large, it has be found that L-carnitine further improves sperm motility and chromatin quality via antioxidant properties, the enhanced glucose uptake by sperm [46], and long chain fatty acids transportation across the inner membrane of the mitochondria for use in metabolism [47]. On the other hand, its effects in reducing some of the side effects of busulfan on the testes is lower than other used compounds [25]. This maybe the reason of low expression of *DAZL* in the L-carnitine-contained groups in comparison the other groups.

In this study the lowest expression of *Bcl2* was seen in ALM group. The main reason of this event can be atorvastatin. Atorvastatin improves the lipid profile, lipid oxidation, and oxidative/antioxidative status. These positive effects may be attributed to the antioxidant properties of statins. It has been suggested that statins increase apoptosis and change levels of *Bcl2* family members (e.g., *Bax* increasing and *Bcl2* reduction). Several reports found that statins reduce levels of the anti-apoptotic protein *Bcl2*, and increase apoptosis and cell death [48-50]. Though, there is evidence that statins increase *Bcl2* abundance which would desirable and in some instances reduce apoptosis and cell death [51,52].

Generally at high statin concentrations apoptosis is increased, and *Bcl2* expression levels and cell viability are reduced [53]. The mechanisms for the statin-induced reduction of Bcl2 protein levels have not been forthcoming. Statins reduce cholesterol, protein prenylation, and two isoprenoids FPP (farnesyl pyrophosphate) and GGPP (geranylgeranyl pyrophosphate but how those reductions trigger a weakening of the anti-apoptotic protein Bcl2 and increase abundance of pro-apoptotic proteins such as Bax and Bim is not understood. There is evidence that statins has

function outside of the mevalonate pathway. Statins for example bind to a heterodimeric glycoprotein, lymphocyte function-associated antigen-1 (LFA-1) which is a member of the β2 integrin family [54]. Directly related to the subject of statins, these compounds increase *Bcl2* gene expression and protein levels, which do not involve the mevalonate pathway.

It can be concluded that, according to the antiapoptotic effects of these antioxidants, vitamin E retrieved spermatogenesis potential of *busulfan*-induced infertile male rats better than the other antioxidants used in different combinations. It was approved by increasing *DAZL* gene expression, a worthy candidate for male infertility evaluation.

Acknowledgment

The authors would like to thank Islamic Azad University, Science and Research Branch, Tehran, and Shahrekord University for their supports. We also thank Behnam Bakhtiarimoghadam for keeping rats and injection of drugs in treatment groups.

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