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Expression of Bax and Bcl-2 Apoptotic Regulatory Proteins in Melphalan-induced Spermatogenic Dysfunction

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Abstract

The adverse effect of Melphalan (MEP), an anti-cancer compound in the male reproductive system, was investigated. In this study, 1, 3, or 5 mg/kg/bwt intraperitoneal doses of MEP were administered daily for 28 days. Half of the mice were sacrificed after 24 hours of the last treatment, and the other half were sacrificed 28 days after withdrawal of treatment. Glutathione peroxidase (GPX), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), reactive oxygen species (ROS) and lipid peroxidation (LPO) levels of testicular tissue were evaluated. The induction of apoptosis was assessed by investigating the level of CASP-3 and CASP-9 activities in spermatogenic cells. CASP- 9, Bax and BCL-2 expression were checked by real-time PCR and western blotting. Testis weight in MEP-treated mice was significantly decreased. There was a significant decrease in sperm parameters, GSH GPx, CAT, and SOD levels, with a concomitant increase in ROS and MDA levels confirming the generation of oxidative stress. MEP-treatment leads to the increased expression of CASP-9, BAX, with decreased BCL-2 protein levels in a dose-dependent manner. No significant recovery after 28 days of withdrawal of the treatment was observed. The results indicate that MEP- treatment leads to testicular damage due to the formation of oxidative stress and induction of caspase-dependent apoptosis.

Keywords: Apoptotic proteins, Melphalan, Oxidative stress, Reactive Oxygen species, Testis.

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Introduction

Melphalan (MEP), a chemotherapy drug belonging to the class of nitrogen mustard alkylating agents, exerts its anti-cancer effect by a process called alkylation, which damages the deoxyribonucleic acid (DNA) of cells, preventing them from dividing and ultimately leads to cell death. Since cancer cells, in general, divide faster and with less error-correcting than healthy cells, cancer cells are more sensitive to this damage. In this way, melphalan slows or stops the growth of cancer cells in the body. Melphalan is used in the cure of multiple myeloma, ovarian cancer and occasionally malignant melanoma. The most frequent dose-limiting toxicity is dose-related, cumulative myelosuppression.[1] Irradiation and chemotherapy induce the p53 pathway, which is one of the common apoptosis-mediated pathways by DNA damage.[2] The series of interactions that occur between DNA and Reactive Oxygen Species (ROS) generates oxidative stress, which leads to DNA damage.[3] An important primary DNA damaging agents are ROS,[4] and an early marker of apoptosis is DNA damage.[2,4] Apoptotic events and ROS have been reported to have the capacity to increase the fragmentation of DNA.[4] ROS levels have been reported to be negatively associated with the quality of sperm, sperm viability, and male fertility.[3,5]

Under normal testicular conditions, there are several anti-oxidant mechanisms in seminal plasma and inside the spermatozoa that can curb ROS activity, but abnormal conditions mediated by some chemotherapy drugs can increase ROS generation and induce DNA damaged-related apoptosis in spermatozoa.[4] ROS has the capacity of activating various signal transduction pathways, including the Erk-p38 MAPK cascade.[3,6,7] The Erk-p38 MAPK cascade mediates Senescence[7] and apoptosis.[2] Bcl-2 family proteins have been reported to play a pivotal role in the induction of caspases activation and in the regulation of apoptosis, but their functions are not yet entirely defined.[8,9] As Bcl-2 proteins are involved in the control of apoptosis upstream of caspase-3 activation and any other irreversible cellular damage, they might be important in deciding whether a cell will live or die.[9,10]

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Moreover, alterations on the expression of Bcl-2 family members that is induced by anti-cancer drug treatment can trigger apoptosis in tumors.[2] Apoptosis a tightly regulated process of cell death, dependent on the expression of cell-intrinsic suicide machinery and are regulated by the family of Bcl-2 family.[10,11] The pro-apoptotic (Bax) members of the Bcl-2 family can trigger the apoptotic pathways, and anti-apoptotic (Bcl-2) can inhibit the apoptotic pathways.[2,12] Based on the literature available, there is not enough information about the effects of melphalan on ROS production and apoptosis gene expression alteration in testis of mammalian. Most of recent studies are about histological and cytotoxic effects of melphalan on the reproductive tract of a female.[1] The present study, therefore, investigated the effect of melphalan on oxidative stress biomarkers enzyme activities and ROS generation in testicular tissue. The effect on the apoptosis-related gene and protein expression (Bcl-2 family) in testes mice were also examined.

Materials And Methods

Animal Preparation

Male Swiss albino mice weighing about 20 g were obtained from the Laboratory Animal Division of the University of Ibadan. The animals were maintained under standard conditions of humidity (50 ± 5%),

temperature (25 ± 2°C) and dark and light cycles (12 hours each) with free access to food and water. Mice were divided into eight groups of ten animals each and treated intraperitoneally. Groups II, III, and IV received 1, 3, 5mg/kg melphalan respectively, and group one received the vehicle and served as control were sacrificed 24 hours after the last treatment while groups V, VI, VII and VIII were sacrificed 28 days after withdrawal of treatment.

Testes Weight and Testicular Tissue Sample Preparation

Mice were weighed daily prior to the administration of MEP. At autopsy, testis was removed, blotted free of blood and adhering tissues and weighed. Testis tissue was immediately removed, weighed and washed with ice-cold saline solution, blotted free of excess moisture, deep-frozen in liquid nitrogen and stored at –20°C until preparation of cytosolic fractions.

Biochemical Analysis

Testis homogenate (10%, w/v) was prepared in chilled 100 Mm Tris-HCL buffer (pH 7.4) and the homogenate was used to measure GPx, GR, GSH, GST, CAT, and SOD activities by using spectrophotometry method. Protein content in the samples was estimated, according to Lowry *et al.* All the parameters were expressed per mg protein.

Detection of Apoptosis

Preparation of Spermatogenic Cells

Testis was removed and homogenized in 15 mL tube containing 10 mL ice-cold 1X PBS buffer (pH 7.4) and the contents were incubated for 40 minutes at 37°C with vigorous shaking. Then, the tubes were placed on ice and incubated to allow the spermatogenic cells to settle. The supernatant was discarded, and cells were washed twice in 10 mL of PBS twice.

Analysis of Apoptosis Using Flow Cytometer

Annexin V-fluorescein isothiocyanate/propidium iodide is a sensitive and precise quantification method for detecting very early stages by flow cytometer analysis. Briefly, testis tissue was homogenized in PBS by centrifugation at 1500 x g for 5 minutes each. Cells were washed and re-suspended with phosphate-buffered saline (PBS). Fluorescein isothiocyanate-conjugated annexin V and PI (BD Biosciences Pharminge USA) were added at the manufacturer's recommended concentrations to 0.1mL aliquots containing cells. Cells were incubated for 15 minutes at 25°C in the dark. Cell suspensions were diluted with 0.4 mL of binding buffer and then analyzed by flow cytometry within 1-hour. Flow cytometry was performed on Model- FACS Calibur flow cytometer (BD, San Jose, CA, USA) using Cell Quest Pro software (BD).

Effect on CASP-3 and CASP-9 Activities

Spermatogenic cells were used for the detection of CASP- 3 and 9 activities by using colorimetric assay kits according to the manufacturer's protocol. Briefly, spermatogenic cells were washed with 1X PBS buffer 2 times at 500 x g for 5 minutes the pellet was dissolved in the extraction buffered and processed for activity assay.

RNA Isolation and cDNA Preparation

Total RNA was isolated from mice testis using TRIzol reagent (Invitrogen USA). A total of 100 mg of testes tissue from different treated groups was homogenized in 1 mL of TRIzol reagent and was kept at room temperature for 5 minutes, after which 200 µL chloroform was added. This was mixed vigorously for about 15 sec, and the homogenates were then kept at room temperature for 10 minutes followed by centrifugation at 13,500 rpm for 15 minutes at 4°C. The upper colorless aqueous phase containing RNA was collected. To precipitate RNA, isopropanol was added, mixed, and kept at room temperature for 10 minutes. Thereafter, samples were spun at 13,500 rpm for 10 minutes at 4°C. RNA precipitate obtained was washed by adding 75% ice-cold ethanol and spinning at 7500×*g* for 5 minutes at 4°C. After removing the ethanol, the RNA pellet was briefly air-dried and then dissolved in DEPC-treated water. RNA concentration and purity were determined at an optical density of 260/280 using nanodrop (Quawell UV-VIS spectrophotometer Q5000). cDNA was prepared according to supplier protocol (Invitrogen, USA).

Determination of mRNA Level Using Real-time RT-PCR

Real-time PCR analysis was performed according to the supplier's instruction (Roche Diagnostics Germany). The components of the reaction were SYBR Green PCR Master Mix, cDNA template, forward primer and reversed primers and nuclease-free water. PCR reactions were performed in Light Cycler ®480 (Roche Diagnostics Ltd Germany). For each primer pair, a melting curve analysis was performed according to the manufacturer's instruction. The program, in brief, was an initial incubation of 50°C for 2 minutes, and 95°C for 10 minutes was followed by 40 cycles at 95°C for 15 seconds 60°C for 60 seconds. Experiments were carried out in triplicates and Cp values were calculated. β-actin was used as an internal control to normalized ratios between the samples. Relative changes in mRNA level between control and treated groups were calculated by using $2^{-\Delta\Delta CT}$ (Table 1).

Western Blotting

Proteins were isolated from testis tissue by using a modified protocol of Ghribi *et al.* (2001). Testis tissues were homogenized in urea lysis buffer. Cellular debris was spun down at 20,000 x g for 30 minutes at 4°C, and supernatants were used as whole

Table 1: Sequences of primers used for real-time quantitative PCR

Name		*Sequence*	*Accession number*
BCL-2	Sense	CTGGGAGATCAAAATCGACCC	NM_013863
	Antisense	GCTGAAGATGCAGTGTCCTTAG	
BAX	Sense	TCCCACATAACTCCCTCGACA	NM_026669
	Antisense	GTGTGTGACCACATGGACATAG	
CASP-9	Sense	TCC TAC ATC GAG ACC TTG	NM_015733
	Antisense	AAG TCC CTT TCG CAG AAA CAG	
β-Actin	Sense	AAGAGTCCCTCATACTACAGGTG	NM_008905
	Antisense	CACACTTCAAGGTCCCGAATC	

protein extract. Isolated proteins were quantified using Bradford reagent. A 50 µg proteins from each sample was separated on 15% SDS-PAGE and transferred to nitrocellulose membrane using a semi-dry electro-blotting apparatus (GE Health, UK). The transfer was examined by Ponceaus stain and washed in distilled water until the stain disappeared. The membrane was incubated in 5% non–fat dried milk at 4°C overnight. The blocked membrane was washed with 0.1% PBST and probed with primary antibodies. After primary antibody incubation, further washing was done in 0.05% PBST. The membrane was incubated in HRP conjugated secondary antibody and rewashed three times. Enhanced chemiluminescent detection reagent was used to develop the blots. The expression levels of proteins detected by immunoblotting were quantitated using the program IMAGE (National Institutes of Health, USA) for the integrated density of each band. Quantitative Western blot data were calculated from the densitometric analysis of bands with the NIH Image J software. The values were normalized to β-actin, which served as a loading control.

Statistical Analysis

All statistical comparisons between the groups were made using analysis of variance (ANOVA) by Prism statistics software. Results were presented as Mean ± SD (n = 5). Values of $p < 0.05$ were considered as statistically significant.

Results

Effects of MEP Treatment on Testis Weights

The weight of testis was significantly reduced following MEP treatment for 28 days. No sign of significant recovery was seen after 28 days withdrawal of MEP treatment (Table 2).

MEP Induces Oxidative Stress

The activities of glutathione-related enzymes such as GPx, GR, and GST in the testis tissue of both the control and experimental animals are given in Table 2. The activities of GPx, GR, and GST were significantly lowered ($p < 0.05$). Also, MEP depletion of reduced glutathione (GSH) was recorded following MEP treatment. Levels of SOD and CAT in the testis of the animal treated with MEP, especially at 5 mg/kg were significantly lowered compared to control mice ($p < 0.05$) with a simultaneous increased level of lipid peroxidation. These results suggest a generation of oxidative stress (Table 3).

Effect of MEP on CASP-3 and CASP-9 Activities

Activation of CASP-3 and 9 activities was seen after 28 days MEP treatment in dose dose-dependent pattern (Figure 1). No significant recovery was seen after 28 days of withdrawal of MEP treatment. This indicates that MEP mediated a cascaded series of molecular events that led to the induction of apoptosis.

MEP Induces Apoptosis in Primary Spermatogenic Cells

MEP treatments result in an increased percentage of Annexin V-positive in groups II, III, and IV that received 1, 3, and 5 mg/kg respectively in a dose-dependent pattern (Figure 2). The ability of MEP to induce apoptosis in the spermatogenic cells after the withdrawal of the treatment was also noticeable. Increased necrosis of spermatogenic cells exhibiting 35.15, 50.15, and 59.31% following MEP treatment at 1 mg/kg, 3 mg/kg, and 5 mg/kg respectively, was also seen after the addition of propidium iodide. A significant increase following withdrawal after 28 days of MEP treatments was also seen (29.32, 40.92, and 48.84%) as compared to the controls.

Table 2: Effects of MEP on Testes weight

Treatment and doses (mg/kg body weight/day	*Testes/body weight (%)*
Group I (Control)	6.60 ± 0.28
Group II (1mg/kg)	5.33 ± 0.25
Group III (3mg/kg)	4.8 ± 0.29*
Group IV (5mg/kg)	3.93 ± 0.19**
Group V (Control)	6.26 ± 0.28
Group VI (1mg/kg)	5.05 ± 0.21
Group VII (3mg/kg)	4.9 ± 0.10*
Group VIII(5mg/kg)	4.10 ± 0.14**

The data are expressed as mean ± SD (n = 5). *, **, and *** indicate significant difference as compared to controls at ($p < 0.05$), ($p < 0.01$), and ($p < 0.001$) respectively.

Table 3: Effect of MEP treatment on biochemical enzymes.

Treatment and doses (mg/kg)	*GPx (U/mg protein)*	*GR (nM/min/ mg protein)*	*GST (nmol/min/ mg protein)*	*GSH µM/ mg protein)*	*LPO (nmol/min/ mg protein)*	*SOD (U/mg/protein)*	*CAT (µmol/min/ mg protein)*
Group I (Control)	2.14 ± 0.15	0.12 ± 0.01	1.20 ± 0.14	0.83 ± 0.04*	0.45 ± 0.03	2.02 ± 0.18	24.39 ± 0.89
Group II (1mg/kg)	1.59 ± 0.13	0.09 ± 0.01	1.00 ± 0.12	0.47 ± 0.03**	0.78 ± 0.06**	1.47 ± 0.14	20.08 ± 2.40
Group III (3mg/kg)	1.09 ± 0.13**	0.05 ±0.03**	0.67 ± 0.06	0.25 ± 0.05	0.98 ± 0.03**	0.91 ± 0.09**	18.20 ± 3.10**
Group IV (5mg/kg)	0.78 ± 0.15***	0.03 ± 0.01*	0.38 ± 0.02***	0.19 ± 0.01	1.75 ± 0.12**	0.56 ± 0.09**	15.47 ± 1.70***
Group V (Control)	1.80 ± 0.09	0.11 ± 0.01	1.11 ± 0.17	0.76 ± 0.05***	0.42 ± 0.03	1.80 ± 0.10	27.67 ± 2.92
Group VI (1mg/kg)	1.12 ± 0.09*	0.08 ± 0.01	0.89 ± 0.20	0.58 ± 0.07**	0.82 ± 0.10**	1.58 ± 0.13*	22.85 ± 1.59*
Group VII (3mg/kg)	0.91 ± 0.10**	0.05 ± 0.00**	0.66 ± 0.14	0.51 ± 0.04	1.17 ± 0.14***	1.29 ± 0.08**	18.86 ± 1.93**
Group VIII(5mg/kg)	0.73 ± 0.10**	0.03 ± 0.00*	0.37 ± 0.03*	0.25 ± 0.02	1.43 ± 0.16***	1.00 ± 0.07***	16.61 ±2.42***

The data are expressed as mean ± SD (n = 5). *, **, and *** indicate significant difference as compared to controls at ($p < 0.05$), ($p < 0.01$), and ($p < 0.001$) respectively.

Effect on BAX, CASP-9, and BCL-2 mRNA expression

MEP treatment at doses 3 and 5 mg/kg results in the marked increase in the gene expression level of Bax and CASP-9. The expressions of these genes were also detected 28 days after withdrawal of the treatment. However, reduced expression of anti-apoptotic gene BCL-2 was seen in all the treated groups. Statistical analysis indicates that increased levels of both apoptotic genes were significantly different from mice in control groups (Figure 3).

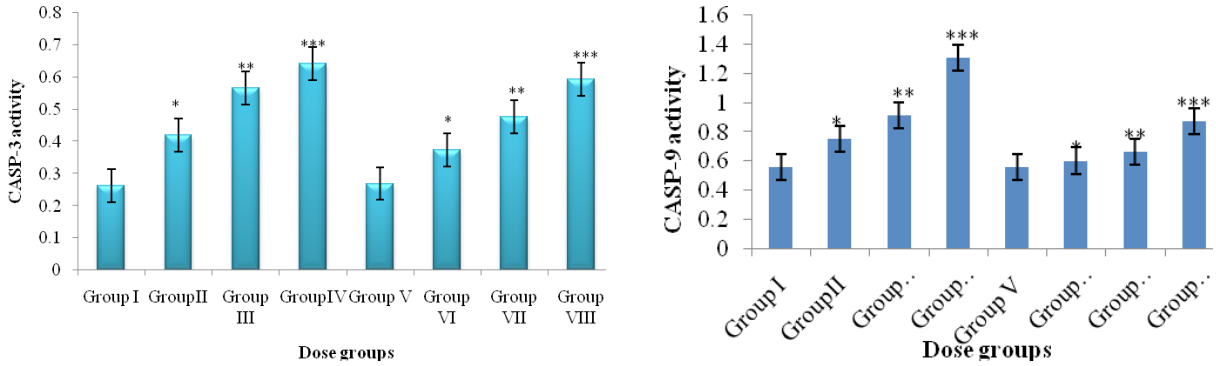


Figure 1: Effect of MEP on CASP-3 and CASP-9 activities of spermatogenic cells after 28 days MEP treatment and following 28 days of withdrawal of treatment. Note: The data are expressed as mean ± SD (n = 5). *, **, and *** indicate significant difference as compared to controls at ($p < 0.05$), ($p < 0.01$), and ($p < 0.001$), respectively.

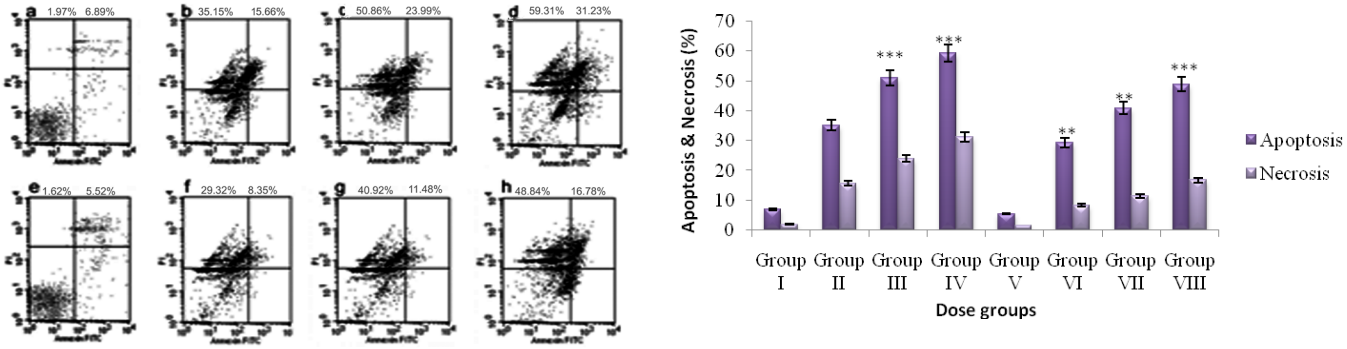


Figure 2: Shows numbers of apoptotic cells in the sample. Note: The data are expressed as mean ± SD (n = 5). *, **, and *** indicate significant difference as compared to controls at ($p < 0.05$), ($p < 0.01$), and ($p < 0.001$), respectively.

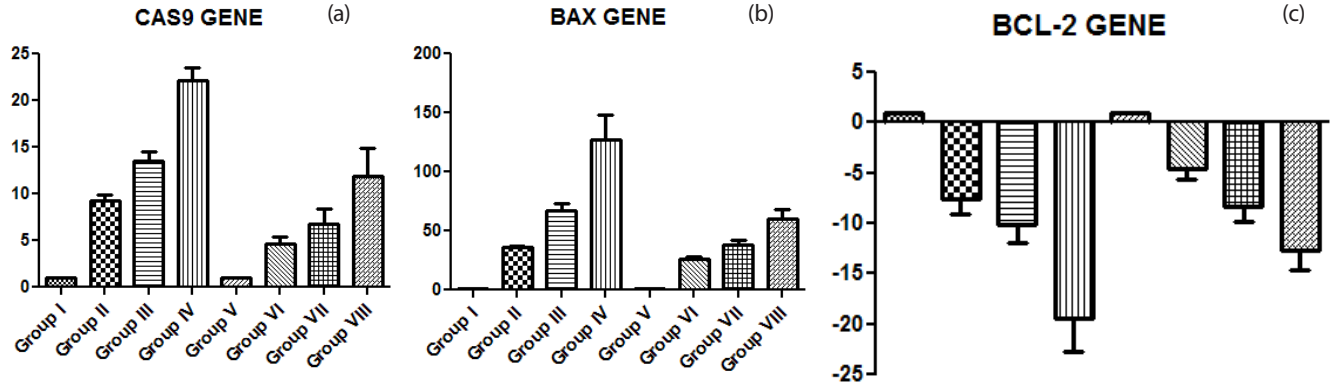
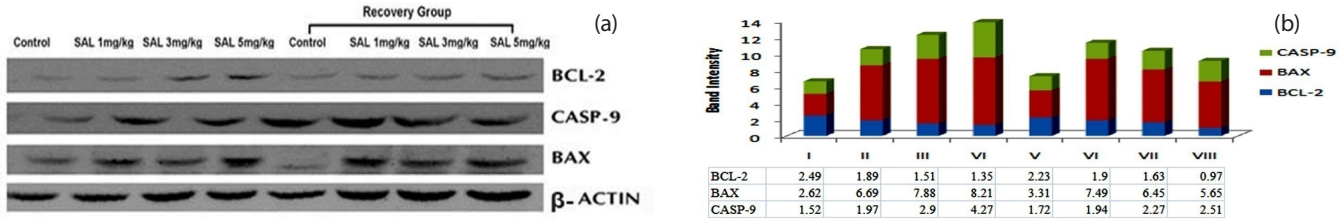


Figure 3: Bar chat represents the fold change of CASP-9, BAX, and BCL-2 genes in testes of mice treated with MEP for 28days and following 28 days withdrawal of the treatment. The data are expressed as mean ± SD (n = 5). *, **, and *** indicate significant difference as compared to controls at ($p < 0.05$), ($p < 0.01$), and ($p < 0.001$), respectively.



	I	II	III	VI	V	VI	VII	VIII
BCL-2	2.49	1.89	1.51	1.35	2.23	1.9	1.63	0.97
BAX	2.62	6.69	7.88	8.21	3.31	7.49	6.45	5.65
CASP-9	1.52	1.97	2.9	4.27	1.72	1.94	2.27	2.51

Figure 4 (a to b): Effect of MEP on CASP-9, BAX, and BCL-2 protein expression. The data are expressed as mean ± SD (n = 5). *, **, and *** indicate significant difference as compared to controls at ($p < 0.05$), ($p < 0.01$), and ($p < 0.001$), respectively.

Effect of MEP on the Expression of BAX, BCL-2 and CASP-9 Proteins

Melphalan lead to 4 fold change increased expression of Bax and CASP- 9 proteins. Reduced BCL-2 expression in a dose-dependent manner within total hepatic cell was also observed (Figures 4a and b).

Discussion

The present study demonstrated that treatment with melphalan resulted in spermatogenic dysfunctions, such as the deterioration of seminiferous tubules and the loss of spermatogenic cells. Reactive Oxygen Species are produced in a normal range routinely as a result of cellular activities and they are regulated by the anti-oxidant, anti-oxidant defense system to curb oxidative stress. Melphalan significantly meditated decrease in the levels of enzymatic anti-oxidants, anti-oxidants including GPx, Cat, GR, GSH, GST and SOD, and an increased level of lipid peroxidation in sperm and testis tissue of treated mice. In this present study, signs of oxidative stress were indicated by increased MDA activity in the testis of the MEP-treated mice. The drug-treated mice also showed a defective anti-oxidant, anti-oxidant response as noticed from diminished activity of anti-oxidant, anti-oxidant enzymes such as CAT, SOD, GPx and the non-enzymatic anti-oxidant, anti-oxidant GSH. The pro-apoptotic Bax and anti-apoptotic Bcl-2 are two extremely important regulatory molecules involved in cell death, and the ratio of Bax/Bcl-2 is an important factor that decides whether apoptosis will occur in cells. The MEP-treated mice had increased level of Bax (therefore, increased ratio of Bax/Bcl-2) and decreased Bcl-2 expression in their testis tissues causing a significant change from the normal equilibrium of Bax/Bcl-2. The significant changes of Bcl-2 family gene expression level as the main regulatory markers of apoptosis showed melphalan mediated apoptosis in epididymal sperm of treated mice. This fact confirms Bcl-2 family gene-induced apoptosis in normal tissue following melphalan administration. Recent reports showed ROS ability to regulate the phosphorylation and ubiquitination of Bcl-2 family of proteins and change of the activity of these apoptotic markers.[3,12] Our results (increased MDA level and decreased GPx, Cat, GR, GSH, GST and SOD activities in the testis tissues of MEP-treated mice) corroborated with recent report describing that increased production of free radicals leads to apoptotic death by deceased- Bcl-2 gene expression and increased Bax gene expression.3 In normal cells, Bax as a pro-apoptotic protein has monomeric structure and commonly located in the 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.[2,3,16,17] Normally, the presence of Bcl-2 as an anti-apoptotic molecule can inhibit the activation of Bax following a death signal[3,] but we noticed that Bax had the capacity of killing sperms despite the presence of Bcl-2. 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 melphalan treated mice. These results are in alignment with the observed oxidative stress-related effects in normal organs and especially male reproductive system of patients undergoing chemotherapy,[3] as one of the mechanisms by which chemotherapy drugs has been reported to function is by induction of apoptosis. So, MEP-mediated apoptosis in sperm and testis was as a result of increased-ROS generation which resulted in oxidative stress and changes in expression of Bax and Bcl-2 Apoptotic Regulatory Proteins. In view of the present observations as well as the above-cited reports, it is relevant to conclude that oxidative stress induced by melphalan treatment and expression of Bax and bcl-2 family gene following treatment with melphalan play a major role during apoptosis in sperm cells and can lead to spermatogenic dysfunction of adult mice. These results likewise confirm that ROS can induce apoptosis in epididymal sperm by increasing pro-apoptotic protein levels and decreasing anti-apoptotic protein expression following melphalan treatment.

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