

Mitomycin-C Induced Genotoxic Effect in Lymphocytes and Histological Alterations in Testes of Male Albino Mice

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ABSTRACT

Background: mitomycin-C (MC) is an anti-cancer drug against several tumor types, including colon, breast and head and neck. In this demonstration, the genotoxic effects of mitomycin-C on DNA content and testicular tissue of male albino mice *Mus musculus* were studied. **Materials & Methods:** mitomycin-C treated animal was injected intraperitoneally with tested doses of mitomycin-C single time at the first day of the experiment. Comet assay was used to detect the DNA damage in mice lymphocytes and the mean of total comet score was increased by dose and time among all treated groups. **Results:** the histological alterations caused in the testis of mice after mitomycin-C treatment displayed variable changes in both the seminiferous tubules and the interstitial tissue. Changes in seminiferous tubules were represented by hypoplasia of the germinal epithelium and spermatogenic arrest at various stages of spermatogenesis. The most prominent changes reported in the intertubular tissue were represented by the presence of a homogeneous and intensely eosinophilic ground substance in the interstitial areas, congestion of blood vessels as well as haemorrhage. The histological changes were also significantly increased by time and dose.

Key words: mitomycin-C, chemotherapy, comet assay, DNA, testicular histopathological alterations.

INTRODUCTION

Cytotoxic drugs are a unique therapeutic class of fundamental importance in current antineoplastic chemotherapy. They belong to different chemical and chemotherapeutic classes. Many of them are genotoxic, carcinogenic and teratogenic when tested *in vivo* and *in vitro* [1, 2]. Mitomycin-C, also known as mutamycin or mitomycin it is a commonly used as a chemotherapeutic agent, it is a naturally occurring antibiotic, originally has isolated from the Gram negative bacteria *Streptomyces Caespitosus* which has been shown to have antitumor activity and growth inhibitor [3, 4]. Mitomycin-C was found to combine with DNA in tumor cells and to inhibit replication of DNA by cross-linking to double strand DNA synthesis [5]; mitomycin-C or metabolites of mitomycin-C alkylate the DNA molecule [6]. So that mitomycin-C has been used for solving several medical problems as neoplasia, tissue transplantation and it has beneficial in the treatment of many types of solid tumors and hematologic malignancies. According to Hawkins, (1991) [7] cytotoxic drugs may increase the risk of predispose to malignancy and increase mutations in offspring; cytotoxic drugs that used in cancer treatment interrupt nucleic acid and protein synthesis. Several studies have shown that mitomycin-C to be cytotoxic, teratogenic and

mutagenic [8-10]. It produces sister-chromatid exchange in bone marrow

and testis of rats and mice. Also, mitomycin-C induces sister-chromatid exchange in human lymphocytes following *in vivo* treatment [11, 12]. Mitomycin-C induces chromosomal aberration, dominant lethal mutation and DNA damage in the spermatogonia. Also mitomycin-C may have caused the spermatogenic apoptosis [10, 13]. Since mitomycin-C is still in use as a chemotherapeutic agent in the treatment of cancer, so this study was performed in order to evaluate the harmful effect of different doses of mitomycin-C on male albino mice.

MATERIALS AND METHODOUS

I- MATERIALS:

1-Animals:

Fifteen mature male mice (CD1) of six to eight weeks old with an average body weight (28 ± 2 g.) were used in current investigation. Mice were apparently normal, healthy, were kept in animal houses under suitable conditions during the whole period of experiment and were fed on standard rodent pellet diet and supplied with water. Animal care and use was approved by the relevant ethics committee and guide line for the care and use of experimental animals.

2-Drug:

Mitomycin-C drug (Kyowa, Hakko Kirin, D-00208, Japan), the doses were converted from human dose to mice dose by using multiplication factors for dose conversion between different species by **Paget and Barnes, (1964)** ^[14].

3-Experimental design:

Twenty five mature male mice (CD1) were divided into five groups 5 mice/group. The first group served as control was injected intraperitoneally with (physiological saline solution 1ml/kg b.wt.) while the second group was treated with mitomycin-C (3 mg/kg b.wt.) for one week, group (3) was treated with mitomycin-C (3 mg/kg b.wt.) for two weeks, group (4) was treated with mitomycin-C (6 mg/kg b.wt.) for one week and group (5) was treated with mitomycin-C (6 mg/kg b.wt.) for two weeks. Each mitomycin-C treated animal was intraperitoneally injected single time at the first day of the experiment. After one week four animals from each group were chosen for collection of samples and then the experiment completed to two weeks.

II- METHOUDS:

1-Comet assay:

About 1-2 ml of mouse blood sample was isolated essentially and the comet assay was carried out under alkaline conditions, as described by **Singh et al.,(1988)** ^[15]. Lymphocytes cells were isolated according to **Boyum, (1968)** ^[16] from whole blood by ficoll separating solution (seromed). From the lymphocytes suspension 5 μ L were mixed with 95 μ L of %0.5low melting agarose (LMA) to prepare the final cell/agarose suspension. Base slides were coated with 1% normal melting agarose (NMA). A second layer of cell/agarose suspension (80 μ L) was poured over the base slides. Immediately, the coverslips were placed on the agarose layer followed by a third layer of agarose (80 μ L LMA) and these slides were placed in lysis buffer for 24 h. Subsequently, the slides were rinsed three times with distilled water and incubated in fresh alkaline buffer (pH>13) for 30 min. Electrophoresis of DNA was performed for 30 min at 300 mA and 25 V (0.90 V/cm). The slides were neutralized with 0.4 M Tris (pH 7.5) and then stained with ethidium bromide (50 μ g/mL). In this work images of comet assay are visualized by the digital camera fitted fluorescent microscope and classified according to the degree of damage after migration through electrophoresis. Cells were visually scored into comet classes according to tail size: class 0 = no tail; class 1 = tail shorter than the diameter of the

head (nucleus); class 2 = tail length 1 to 2x the diameter of the head; and class 3 = tail longer than 2x the diameter of the head. Comets with no heads and those with nearly all the DNA in the tail, or with a very wide tail, were excluded from the evaluation because they probably represented dead cells ^[17, 18].

Three parallel replicate slides per sample were analyzed. The total score for 100 comets was obtained by multiplying the number of cells in each class by the damage class, ranging from 0 (all undamaged) to 300 (all maximally damaged).

2-Histological preparation of the testis:

Animals were sacrificed and testis were fixed in Bouin's fluid for 24 hours and washed in 70 % alcohol containing few drops of saturated lithium carbonate to remove the excess of picric acid. The specimens were dehydrated in ascending series of ethyl alcohol, and then they were preserved for clearing in xylene. The specimens, was then embedded in three changes of pure paraffin wax (58-60° C) in an oven, each for 30 minutes, serial transverse section of 4-6 microns were cut, mount on clean slides and kept to dry for 24 hours. The slides were hydrated in descending grades of ethanol and then stained in haematoxylin and eosin and mounted in DPX. Images of histology were visualized by the digital camera fitted light microscope with magnification power ranged from 280x to 660x.

3-Statistical analysis:

The statistical analysis of the obtained data was revised by SPSS 16 for windows (2007). Each treatment group was compared with the control group with independent samples T- test.

RESULTS

1-Comet assay:

In the current study the extent and distribution of DNA damage indicated by the comet assay was evaluated by examining at least 100 randomly selected and non-overlapping cells on the slides per animal. These cells were visually scored into comet classes according to tail size: class 0 = no tail; class 1 = tail shorter than the diameter of the head (nucleus); class 2 = tail length 1 to 2x the diameter of the head; and class 3 = tail longer than 2x the diameter of the head. Comets with no heads and those with nearly all the DNA in the tail, or with a very wide tail, were excluded from the evaluation because they probably represented dead cells. The total score for 100 comets was obtained by

multiplying the number of cells in each class by the damage class, ranging from 0 (all undamaged) to 300 (all maximally damaged).

In this study the mean of comet score was gradually increased by time and dose in all groups treated with mitomycin-C than the corresponding control. Images of comet assay are visualized by the digital camera fitted fluorescent microscope and classified according to the degree of damage after migration through electrophoresis. **Figure (1)** reveals an intact DNA in control group; while **(Fig.2, 3, 4 and 5)** for groups (2), (3), (4) and (5) respectively, reveals a gradually increase of DNA damage clarified by a slightly pointed end due to the migration of fragmented DNA through electrophoresis (tailed).

The comet assay results of this study were summarized in **(Table 1)** as (mean \pm SD) of comet score for control and all treated groups. Comet score was (0.67 \pm 0.47) in control, while it was (12.33 \pm 0.74) in Group 2, (15 \pm 0.82) in Group 3, (22 \pm 0.82) in Group 4, and (27 \pm 0.82) in Group 5. Comet class data also was shown in **(Table 1)** most cells examined were undamaged comet class 0 cells, a few were comet class 1 cells showing minor damage, and a very few were comet class 2 and 3 showing a large amount of damage. In this respect, statistical analysis indicated a significant increase ($p < 0.005$) in comet score of group 2 (3mg/kg b.wt./one week). While, comet score was highly significant increased ($p < 0.001$) in group 3 (3mg/kg b.wt./two weeks), group 4 (6mg/kg b.wt./one week), and group 5 (6mg/kg b.wt./two weeks) compared to the corresponding control. **Figure (6)** showed comet score marked changes in mice of treated groups as compared to the corresponding mean control value. It is evident that the mitomycin-C administration was accompanied by a disturbance in the DNA content reflected by the gradual increase in the DNA migration in dose and time dependent.

2-Histopathological observations:

Examinations of transverse sections of control testis of adult mouse **(Fig. 7)** showed that the testis is composed of a large number of seminiferous tubules, which appear as rounded or oval structure. it is covered with a thick fibrous connective tissue, the tunica albuginea. Each tubule is surrounded by a thin basement membrane covered externally by a fibrous connective tissue. In the spaces between the seminiferous tubules, there is an interstitial tissue

stroma, consisting of clumps of interstitial cells of Leydig cells. Each seminiferous tubule is lined by a germinal epithelium surrounding a central lumen and lying on a thin basement membrane covered externally by a fibrous connective tissue. The epithelium consists of spermatogenic cells and Sertoli cells. The spermatogenic cells include the successive stages of spermatogenesis i.e .spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. These are regularly arranged so that the newly formed spermatogonia are next to the basement membrane, while the advanced spermatogenic stages are very close to the lumina. Treatment with mitomycin-C (3 mg/kg b.wt.) after one week **(Fig. 8)** showed the least histopathological lesions than other treated groups, animals treated with this dose revealed that some tubules were pathologically altered whereas others were not affected, the tunica albuginea was ruptured, several vacuoles scattered among the remaining spermatogenic cells, abnormal multinucleated giant cells were occasionally detected particularly in the spermatocytes and the spermatogonia and primary spermatocytes undergoing cellular degeneration and necrosis. While, After two weeks of treatment with the same dose of mitomycin-C (3mg/kg b.wt.) the histological preparations revealed more histopathological lesions in the testicular tissue **(Fig. 9)**, it demonstrate that most of the spermatogonia and spermatocytes were undergoing necrosis; their nuclei showed pyknosis and exfoliated germ cells are accumulated in the tubular lumina and cytoplasmic mass was also illustrated in tubular lumina.

Treatment with mitomycin-C (6 mg/kg b.wt.) after only one week showed more histopathological lesions in Testicular tissue compared to previous groups as shown in **(Fig.10)** Changes in the affected seminiferous tubules comprised of destruction the normal pattern of the germinal epithelium which showed various degrees of degeneration and Spermatogenesis showed maturation arrest and most of the seminiferous tubules were devoid of mature sperms. Beside, Congestion of blood vessel and oedema with marked haemolysis in the interstitial spaces was observed. While, treatment with mitomycin-C (6 mg/kg b.wt.) after two weeks revealed the highest histopathological lesions in the testicular tissue compared to those in the previous groups **(Figs. 11,**

12). The action of 6 mg/kg b.wt. of mitomycin-C after two weeks resulted in the occurrence of hemorrhage and several large vacuoles in the germinal epithelium in the majority of the seminiferous tubules (Fig. 11). Also, most tubules showed germ cell hypoplasia, in which the spermatogenic cells are reduced to few discrete layers, so the lumina of the tubules appeared wide as compared to control tubules (Fig. 12). In addition, most of the spermatogonia had pyknotic nuclei; lost their reticular pattern and were darkly stained (Fig. 12) and a noticeable increase in the intertubular spaces.

DISCUSSION

Comet assay or single cell gel electrophoresis (SCGE) is a well-established, simple, cost-effective and sensitive method to detect very low levels of single strand breaks or DNA damage. Ostling and Johnson, (1984)^[19], Singh *et al.*, (1988)^[15], Martinez *et al.*, (2006)^[20] and pan *et al.*, (2007)^[21] they suggested that the modified comet assay could prove to be a convenient and sensitive biomonitoring tool for individuals occupationally or voluntarily exposed to tinner inhalation.

The current investigation proved that mitomycin-C increased the number of total comet score in lymphocytes among different experimental groups as compared with the corresponding controls. This result can be explained according to Erickson *et al.*, (1980)^[22], Pan *et al.*, (1986)^[23], Gibson *et al.*, (1986)^[24] and Powis, (1989)^[25] who declared that mitomycin-C is an alkylating agent that forming DNA interstrand crosslink which block DNA activity. While, Bradley and Erickson, (1981)^[26], Fisher *et al.*,^[27] and Koedrith and Seo, (2011)^[28] explained that DNA damage by mitomycin-C was due to generation of reactive oxygen species (ROS) as a result of metabolism of mitomycin-C. Excessive levels of ROS lead to the damage of proteins, lipids, and DNA^[29-31]. Schlade *et al.*, (2002)^[32] added that interstrand cross-links caused by mitomycin-C greatly distort the DNA structure and cause catastrophic consequences for a cell if unrepaired. The result of current work revealed that mitomycin-C administration increased the number of total comet score in dose and time dependent compared to corresponding controls. This result is in accordance with Pfuhrer and Wolf, (1996)^[33] who found that mitomycin-C led to an increase in DNA migration at concentrations of 100–800µM in human whole blood cultures. Klaude *et al.*, (1996)

^[34] and Abdullah (2014)^[35] added that mitomycin-C induced cross-links take at least 24 hours to repair completely and after their removal, cells displayed damage comparable to that shown by control cells This was supported by data on the role of inter strand cross-links in the reduction in free DNA fragments, mainly detected under alkaline conditions.

The results of the present study showed that mitomycin-C treatment caused different histopathological alterations in the testes of mice as compared with the control group this can be explained according to Creasy, (2001)^[36] who demonstrated that blood flow and fluid balance are targets for cytotoxic agents, and because the seminiferous tubules are a vascular, so all oxygen and nutrients have to pass through the interstitial space, then through the peritubular myoid cells, and finally through the Sertoli cells to reach the germ cells. This places them on the boundary of hypoxia, which appears another reason for degeneration of seminiferous tubule cells then loss of germ cells. However, the severity of damages is influenced by the concentration of mitomycin-C. Histological results of current investigation observed that atrophy of spermatogenic cells are more commonly found in the seminiferous tubules of mitomycin-C treated animals. It was apparent in most of the seminiferous tubules as pyknosis, karyorrhexis, karyolysis and nuclear fragmentation with variation in the staining intensity, These findings are in agreement with Sabik and Abd El-Rahman, (2009)^[37]. Also, the result of the present study showed that loss of germ cells can be accompanied by cytoplasmic vacuolization of germ cells. As indicated by Mitchell and Cortran, (1997)^[38] and Wu *et al.*, (2008)^[39] cytoplasmic vacuolation is an indication of cell necrosis, and such cellular vacuolation was most probably a cellular defense mechanism against injurious substances^[40]. Exfoliation or sloughing of germ cells is one of histological observation in the current study this can be explained According to Creasy and Foster, (1991)^[41] they stated that premature exfoliation or sloughing of germ cells is presumably due to loss of the tenuous contact between Sertoli and germ cells. Hence, the lateral processes of Sertoli cell separating the germ cells are retracted leaving the cells in direct opposition to one another; this is rapidly followed by exfoliation of the germ cells into the tubular lumen and subsequent loss. In the present investigation, numerous giant cells were

noticed in the seminiferous tubules of mice treated with mitomycin-C. This confirms what has been shown by **El-Alfy *et al.*, (2013b)** [42] on mammalian cell lines treated with mitomycin-C. **Pedrazzoli *et al.*, (2011)** [43] explained the formation of giant cells on the bases of the fusion of more than one cell together due to the change in the physical condition. Non-nucleated masses of eosinophilic substances were also observed in the present material especially in the lumina of seminiferous tubules. In this respect, **Selvakumar *et al.*, (2006)** [44] and **Rezvanfar *et al.*, (2008)** [45] presumed that the masses of eosinophilic material in the lumina of seminiferous tubules represent central debris. Also the results showed that mitomycin-C causes an increase in the interstitial spaces associated with moderate congestion of intertubular blood vessels with accumulations of edematous fluid and fibrinous materials in these spaces. This can be explained according to **Nuyts *et al.*, (1994)** [46] who reported that mitomycin-C caused damage to the vascular endothelium of the testis and this resulted in the contraction of vascular endothelial cells with loosening of their tight junctions.

In conclusion, it can be stated that mitomycin-C treatment has profound deleterious impacts on the DNA content and testicular tissue of male albino mice.

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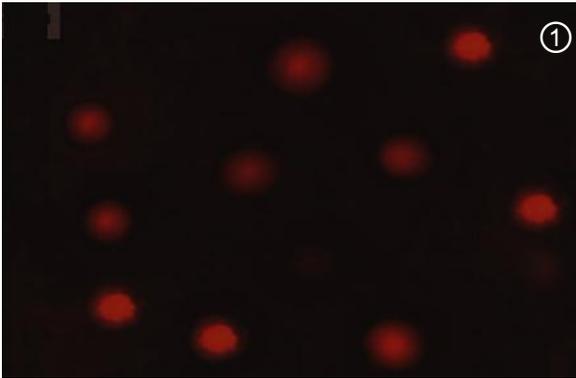


Figure1: Photomicrograph of comet images of mouse lymphocytes of control group had comets with very short tails or without any.

X: 1500

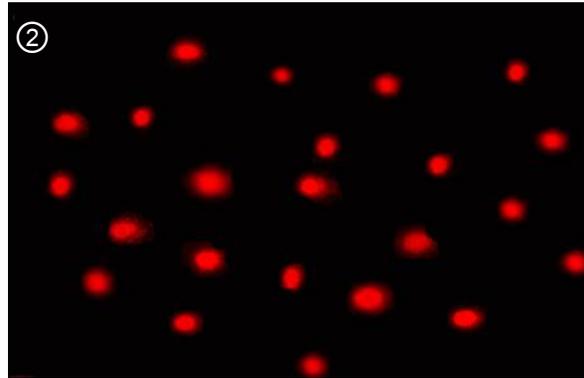


Figure2: Photomicrograph of comet images of mouse lymphocytes treated with mitomycin-C (3mg/kg b.wt.) after one week showing lymphocyte comets with untypical shapes.

X: 1500

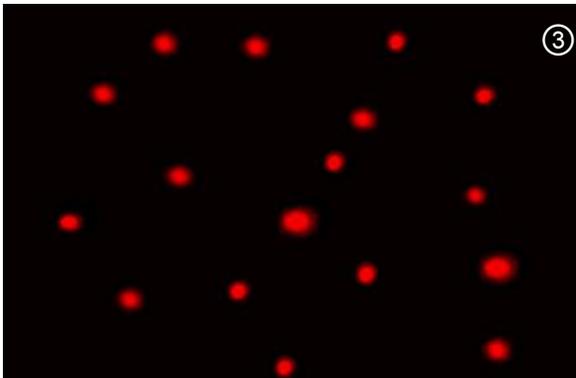


Figure 3: Photomicrograph of comet images of mouse lymphocytes treated with mitomycin-C (3mg/kg b.wt.) after two weeks showing most comets had short tails containing a high fraction of DNA.

X: 1500

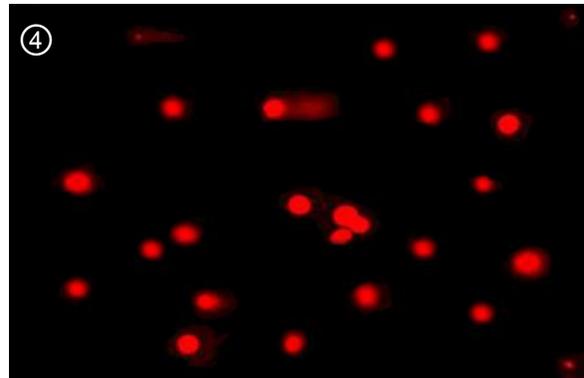


Figure 4: Photomicrograph of comet images of mouse lymphocytes treated with mitomycin-C (6mg/kg b.wt.) after one week showing untypical comet shapes without DNA fragments.

X: 1500

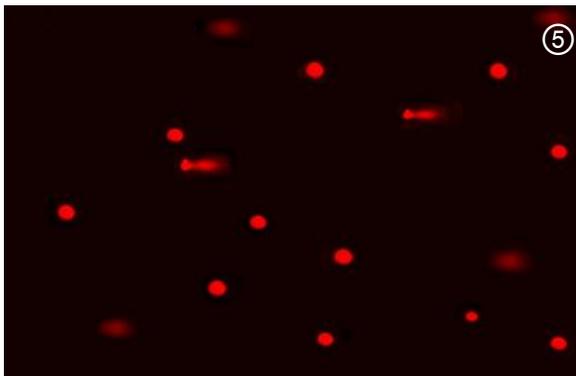


Figure 5: Photomicrograph of comet images of mouse lymphocytes treated with mitomycin-C (6mg/kg b.wt.) after two weeks showing some comets had long tails containing a low fraction of highly fragmented DNA.

X: 1500

Mitomycin-C Induced Genetoxic Effect in Lymphocytes...

Groups	Dose (mg/kg)	Time/ Week	Total scored cells / mice	Comet class and scored				Class total	Total comet score	Total comet score (mean ± SD)
				0	1	2	3			
Control			100	96	3	1	0	4	5	6±0.47
			100	96	2	2	0	4	6	
			100	95	3	2	0	5	7	
2	3	1	100	91	6	3	0	9	12	12.33±0.74*
			100	90	8	2	0	10	12	
			100	90	7	3	0	10	13	
3	3	2	100	88	9	3	0	12	15	15±0.82**
			100	89	7	3	1	11	19	
			100	90	6	4	0	10	14	
4	6	1	100	86	7	5	2	14	23	22±0.82**
			100	85	10	4	1	15	21	
			100	84	11	4	1	16	21	
5	6	2	100	82	11	5	2	18	27	27±0.82**
			100	81	12	5	2	19	28	
			100	80	15	4	1	20	26	

Table 1: The mean and standard deviation of the comet score of peripheral blood leukocytes of male albino mice *Mus musculus* of control and treated groups.

** Highly significant ($P < 0.001$), ** Highly significant ($P < 0.001$)

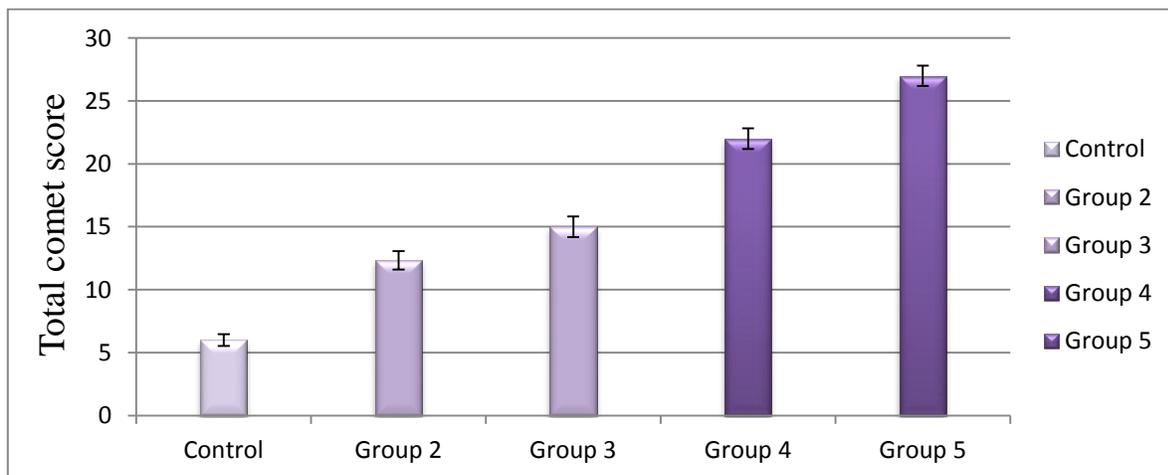


Figure 6: Histogram represents the relationship between the mean ± SD of Comet score of male albino mice in control and all treated groups.

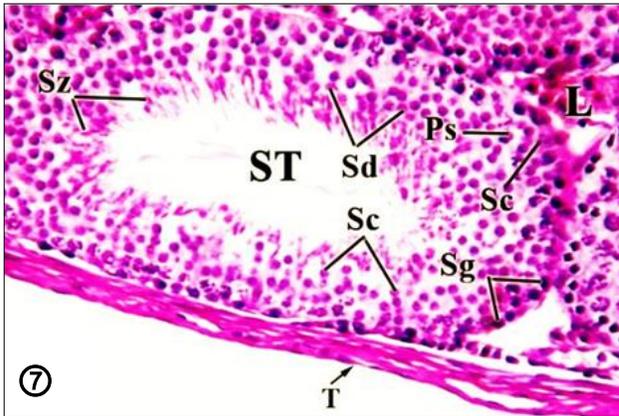


Figure 7: Photomicrograph of T.S. of the testis of control mouse showing tunica albuginea (T), seminiferous tubule (ST), spermatogonia (Sg), primary spermatocytes (Ps), secondary spermatocyte (Sc), spermatid (Sd) and interstitial tissue (L).
X: 400

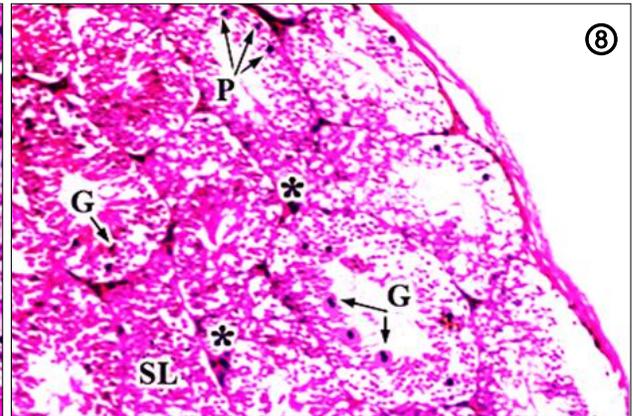


Figure 8: Photomicrograph of T.S. of the testis of mouse treated with mitomycin-C (3 mg/kg b.wt.) after one week showing maturation arrest at spermatid stage and spermatids are sloughed (SL) off into the lumen of tubules. Notice giant cell (G), nuclear pyknosis of germ cells, and pyknosis (*) of cells of interstitial tissue.
X: 280

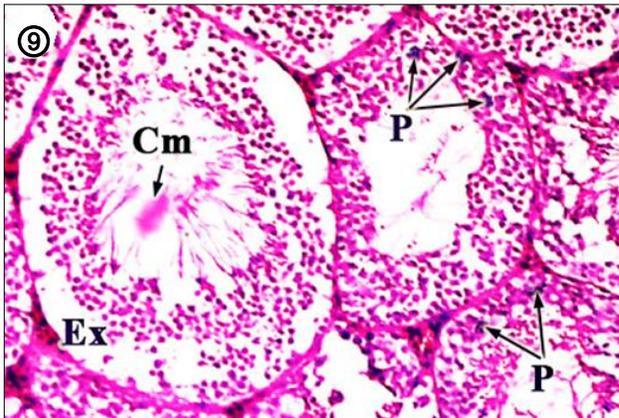


Figure 9: Photomicrograph of T.S. of the testis of mouse treated with mitomycin-C (3 mg/kg b.wt.) after two weeks showing accumulation of exfoliated (Ex) germ cells within the lumen of tubule. Notice cytoplasmic mass (Cm) and nuclear pyknosis (P) in other tubules.
X: 400

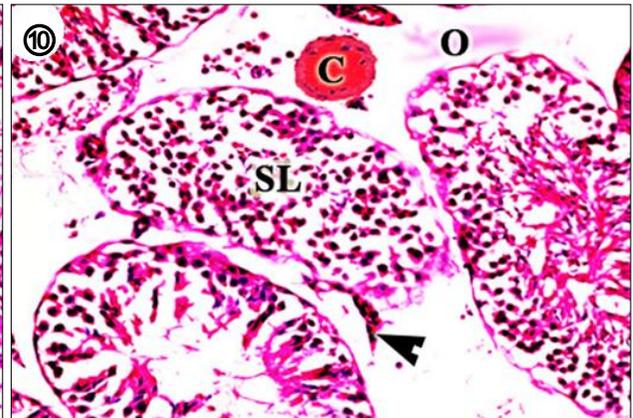


Figure 10: Photomicrograph of T.S. of the testis of mouse treated with mitomycin-C (6 mg/kg b.wt.) after one week showing sloughed tubule (SL), hypoplasia of interstitial tissue (arrows heads), congestion (C) of blood vessel and oedema (O).
X: 400

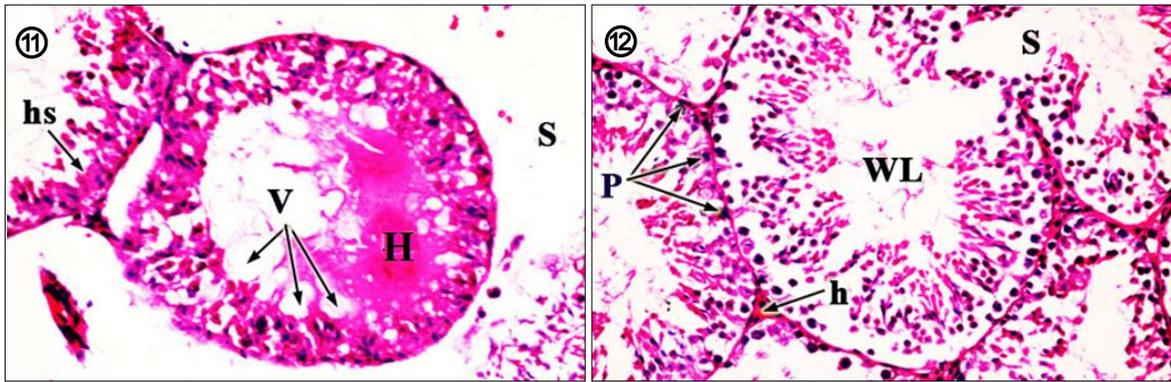


Figure 11: Photomicrograph of T.S. of the testis of mouse treated with mitomycin-C (6 mg/kg b.wt.) after two weeks showing seminiferous tubules with hypoplasia (h) of germinal epithelium, hemorrhage (H) and vacuoles (V). Notice nuclear pyknosis (P) and large space (S) among tubules. **X: 400**

Figure 12: Photomicrograph of T.S. of the testis of mouse treated with mitomycin-C (6 mg/kg b.wt.) after two weeks showing seminiferous tubules with wide lumen (WL), hypoplasia (h) in its germinal epithelium, hemorrhage (H) and vacuoles (V). Notice nuclear pyknosis (P) and large space (S) among tubules. **X: 400**