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Evaluation of Male-Mediated Reproductive Toxicity Induced by Polyethylene Glycol in Mice

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Abstract: In the present work polyethylene glycol (PEG6000) was given orally by gavage to male mice at three different doses 50, 100 and 200 mg/kg/day for consecutive 5 days per week up to six successive weeks. 24hr of treatment, half number of the mice from all experimental groups was sacrificed. Epididymis and testes were collected to evaluate the reproductive toxicity of PEG6000. The other half of treated male mice was mated with untreated virgin female mice to evaluate male-mediated genotoxicity of PEG6000 on fetuses. The results illustrated that PEG6000 decreased sperm motility and count in a dose-dependent manner. However, PEG6000 resulted in an increased fraction of sperm with abnormal morphology or abnormal chromatin integrity measured by toluidine blue and acridine orange. Besides, PEG6000 exhibited an increase in the occurrence of chromosomal aberrations in spermatocytes of treated male and in liver cells of their fetuses. Furthermore, testicular alternation and reduction of DNA nuclear content in spermatogonia and spermatocytes were observed in PEG6000 treated mice. In conclusion, chronic exposure of PEG6000 induced male reproductive impairment which correlated with genetic damage of fetuses generated from mating treated paternal mice with untreated female mice.

Key words: Polyethylene Glycol · Mouse · Sperm Morphology · Sperm Chromatin Integrity · Histopathology

INTRODUCTION

Polyethylene glycol is a polyether compound with many applications from industrial manufacturing to medicine. The structure of PEG is also known as polyethylene oxide or polyoxyethylene, depending on its molecular weight [1]. PEGs of different molecular weights (200-10000) are widely used in many cosmetic and pharmaceutical preparations. In the pharmaceutical industry, they are used as vehicles for drugs and as ointment bases, capsules, tablet and pill binders, suppositories liquid prescriptions and in veterinary drugs, including topical, ophthalmic, oral and rectal preparations [2]. PEGs are utilized as a laxative agent for treatment of constipation [3]. Further applications include use as ingredients in soaps and detergents, in the textile and leather industry, in plastics and resins, in the paper industry, in printing, in the ceramics and glass industry, in the rubber, petroleum, mining and metal industries, for wood preservation and as chemical intermediates [1]. Millions of tons of PEGs are involved in industrial process worldwide every year which of most of them have to be discharged as waste water after industrial utilization [4]. PEG6000 is permitted as food additives in various foods according to the European Parliament and Council Directive No. 95/2/EC of 20 February1995. Our previous experiments exhibited that PEG6000 has been induced DNA damage in mouse bone marrow cells using comet assay and micronucleus assay [5]. There is copious evidence of male mediated developmental toxicity in experimental models and some evidence showing a sensitivity of the male germ line to these transmissible effects. Putative mechanism have been proposed including direct action such as contamination through the seminal fluid and both genetic and epigenetic pathways including germ cell mutation, induction of germ–line genomic instability, suppression of germ cell apoptosis or interference with genomic imprinting [6] The body of knowledge regarding PEG6000 reproductive toxicity has been limited and based largely upon knowledge of similar

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compounds. Therefore, the objective of this study is to provide information regarding reproductive toxicity of PEG6000 in mice.

MATERIALS AND METHODS

Chemicals: PEG6000 was purchased from Al-Gomhoria pharmaceutical Industry, Cairo, Egypt. Cyclophosphamide monohydrate (CP) was manufactured by Baxter oncology GmbH, Frankfurt-Germany. PEG6000 and CP were dissolved in distilled water.

Animals: Adult BALB/c mice of both sexes *(Mus Musculus*), 6–8 weeks old and weighing approximately 25–30 g, were obtained from Animal House of National Research Center, Dokki, Egypt. The animals were acclimated for a period of two weeks before the beginning of the experiments. Mice were housed in stainless-steel cages, maintained in a room designed for control of temperature, humidity and light cycle. Mice were fed standard granulated diet with tap water supplied with *ad libitum*. The study protocol was approved by the Ethics Committee for Animal Care of National Research Center, Dokki, Egypt.

Treatment Schedule: Male mice were randomly assigned to five experimental groups of ten mice per group. Control group: mice were given distilled water. Positive control group: mice were given intraperitoneally CP at single dose of 20 mg/kg. PEG6000 groups: Three groups of mice were administrated orally PEG6000 at three doses 50, 100 or 200 mg/kg/day for consecutive 5 days per weeks up to six successive weeks.

Experimental Design: At the end of treatment schedule half number of the mice from each experimental group was sacrificed by dislocation of neck vertebra. Testes and epididymis were collected for different assays. Fifty untreated virgin female mice (10 female/group) were cohabited with other half of male mice from all groups for two weeks. Each treated male mouse was housed in a separate cage with two virgin untreated females of the same strain. The day of sperm positive vaginal smear was considered as day‘0’ of gestation. Pregnant female was removed from the male’s cage and placed in her own individual cage. The dams were killed by cervical dislocation on day 15-17 of gestation and fetuses' livers were collected for mitotic chromosome aberrations analysis.

Reproductive Toxicity Evaluation Epididymal Sperm Motility, Count and Morphology: The cauda epididymides were cut in a pre-warmed saline solution at 37°C and minced with scissors and placed in 37°C incubator for 5 min. To determine sperm motility, one drop of sperm suspension was placed on warmed microscope slide, covered with cover-slip and observed under a standard optical microscope at 400X magnification. A minimum of five microscopic fields were assessed to determine the percentage of motile sperm on at least 200 sperm for each animal. To count sperm, 5 µl of sperm suspension was diluted with 95µl saline solution. The diluted suspension was transferred into hemocytometer and sperm were counted under light microscope and data were expressed as the number of sperm per ml. For analysis of sperm morphology, a drop of sperm suspension was smeared onto cleaned slides, allowed to air dry and stained with 1% aqueous solution of eosin Y. At least 1000 sperm per each mouse were examined under light microscope at 1000X magnification. Sperm abnormalities were recorded according to Wyrobek and Bruce [7].

Sperm Chromatin Integrity Assay

Toluidine Blue Assay: The assay was performed according to standard protocol described by Erenpreisa *et al.* [8]. Briefly, air-dried sperm smears were fixed in 96% ethanol-acetone (1:1) at 4°C for 30 min. The slides were staining with 0.05% TB in 50% Mcilvaine citrate phosphate buffer for 10 min. Following staining, the slides were gently rinsed in a stream of distilled water and air dried again. A total 1000 spermatozoa per animal was examined under light microscope at 1000X magnification. Chromatin quality of sperm was assessed in following criteria: light blue (good chromatin) and dark blue (abnormal chromatin).

Acridine Orange Assay: The assay was performed according to the standard protocol described by Martins *et al.* [9]. Briefly, air-dried smears were fixed for 1 hr in Carnoy’s solution at 4°C and allowed to dry. The smears were incubated in 1 N HCl at 75°C for 15 min then stained with AO dye (0.2 mg/ml) for 10 min. Slides were washed with distilled water and air-dried. At least 1000 spermatozoa per mouse were analyzed under fluorescence microscope (490/530 nm excitation/barrier filter) at 1000X magnification. Two types of staining patterns were considered in sperm head; green spermatozoa (double-stranded DNA), yellow or orange spermatozoa (single-stranded DNA).

Meiotic Chromosome Aberrations Analysis: Chromosomes from primary spermatocytes were prepared according to technique of Evans *et al*. [10]. Briefly, the tunica of the testis was removed and the tubules were transferred to a small Petri dish containing isotonic solution (2.2% sodium citrate) and teased out with curved forceps on a piece of mesh. The cell suspension was transferred into a conical tube and centrifuged at 1000 rpm for 10 min at room temperature. The supernatant was removed and the pellets were re-suspended in hypotonic solution (1.1% trisodium citrate) for 20 min at 37°C and fixed in cold Carnoy's fixative. The cells were centrifuged and re-suspended in a small volume of Carnoy's fixative. To prepare slides, 3–5 drops of the fixed cell suspension were dropped on a clean slide and air-dried. The slides were stained in 10% Giemsa solution in phosphate buffer (pH 6.8) for 5 min. A total 100 well spread diakinesis-metaphase I cells per mice were examined under light microscope at 1000X magnification.

Fetal Chromosome Aberrations Analysis: Chromosomes from fetal liver were prepared according to Romagnano *et al.* [11]. Fetal liver tissue was placed in a conical centrifuge tube with 2ml RPMI-1640 medium and dissociated manually using Pasteur pipette. The liver sample was incubated with 0.02ml 0.05% colchicine at 37°C for 90 min. The samples were centrifuged for 5 min and then incubated in 2 ml of a prewarmed hypotonic KCL solution (0.56%) for 15 min at 37° C. Cells were fixed twice Carnoy's fixative and slides were prepared and air-dried, stained with 10% Giemsa in phosphate buffer (pH 6.8). Good-quality metaphases (n = 100) per each fetus were examined under light microscope at 2000X magnification.

Histological and Histochemical Study: Right testis from each mouse were excised and fixed in 10% formal saline followed by dehydration in ascending grades of alcohol, clearing in xylene and embedding in paraffin wax. Paraffin sections (5 ìm thicknesses) were stained with hematoxylin and eosin (H&E) for the histological examination and Feulgen method for demonstration DNA content [12]. The DNA contents were elucidated from Feulgen stained sections subjected to measurement of optical density using Image Pro Plus image analysis software (Media Cybernetics Inc, 2002).

Statistical Analysis: Statistical analysis was performed using SPSS for Windows (Version 11). Data were compared by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for multiple comparisons. Statistical significance was set at $p \leq 0.05$. Pearson's correlation coefficient (r) was calculated in order to evaluate correlations between the different parameters analyzed. The percentage of abnormal chromatin integrity was calculated according to Erenpreisa *et al.* [8] using the following formula

$$\left[\frac{Abnormal\ chromatin\ integrity}{Good + abnormal\ chromatin\ integrity} \times 100\right]$$

RESULTS

Routine Sperm Analysis: Sperm analysis showed that PEG6000 exhibited significant dose dependent decrease ($p= 0.01$) in the percentage of sperm motility and count when compared to negative control group (Table 1). However, such reduction in motility and number of sperm was more in the positive control value. As shown in Table (2), the three tested doses of PEG6000 induced significant increase in the frequencies of abnormal sperm morphology in both head and tail regions when compared with negative control. The increase in sperm abnormalities in PEG6000-treated mice was dose-dependent manner but lower than the positive control value. Sperm with amorphous head shape and coiled tail represent a major part of sperm abnormalities induced after oral repeated treatment with different doses of PEG6000. They reached their maximum percentages 9.20% and 22.20% respectively after treatment with the highest tested dose of PEG6000. Sperm with small and big head were observed only in positive control.

Sperm Chromatin Integrity: As shown in Table 1, the percentage of dark blue sperm (abnormal chromatin integrity) was significantly increased after repeated oral treatment with all doses of PEG6000 when compared with negative and positive control groups. It reached its maximum 16.36±0.14 at the highest tested dose of PEG6000 compared with 2.58±0.15 for the negative control. Such percentages represent 6.34 fold increases compared with control group. These data suggested that PEG6000 has prominent impact on chromatin proteins inhibition especially protamine.

Staining treated sperm with AO exhibited that PEG6000 induced significant increase in the denaturation DNA strand (red spermatozoa) with dose-dependent relationship. The proportion of red spermatozoa reached 8.06 ±0.21, 10.90±0.23 and 16.36±0.14 at the three different doses 50, 100 and 200 mg/kg, respectively compared with

Table 1: Effect of treatment with PEG6000 on mouse sperm motility, sperm count and chromatin integrity

	Sperm motility		Total sperm count × 10^6		Sperm with abnormal chromatin integrity (Mean% ± SE)	
Treatment groups	Mean ± SE	%	Mean ± SE	%	AO orange sperm	TB dark sperm
I-Negative control	82.00 ±1.23 [a]	82	549.60 ±13.96 [a]	78.5	2.58±0.15 [e]	2.66 ±0.051 [e]
II-Positive control	52.00 ±1.23 [e]	52	196.40 ±4.29 [e]	28.05	25.90±0.19 [a]	27.6±0.93 [a]
III-PEG-6000						
50	72.00 ±1.23 [b]	72	432.20 ±8.49 [b]	61.74	7.44±0.35 [d]	8.06 ±0.21 [d]
100	68.00 ±1.23 [c]	68	354.60 ±21.39 [c]	50.65	10.62±0.30 [c]	11.90±0.23 [c]
200	64.00 ±1.87 [d]	64	270.20 ±12.59 [d]	38.6	16.36±0.14 [b]	18.46±0.28 [b]

5000 sperm were examined for chromatin integrity in 5 mice per each experimental group

Mean values followed with different superscript letters within the same column are significantly different from one another (P= 0.05).

Table 2: Effect of treatment with PEG6000 on the mouse sperm plasma membrane integrity

	Type of head abnormalities							
Treatment groups	Amorphous	Hookless	Banana	Forked	Small	Big	Coiled tail	Total
I-Negative control	0.60±0.25 [e]	2.20±0.20 [c]	0.00±0.00 [b]	0.00±0.00 [b]	0.00 ±0.00 [b]	0.00 ±0.00 [b]	2.60±0.25 [e]	5.40±0.25 [e]
II-Positive control	12.80 ±1.07 [a]	9.40±0.87 [a]	1.20 ±0.37 [a]	2.80 ±0.49 [a]	1.80±0.49 [a]	1.00±1.03 [a]	27.00±1.76 [a]	56.00±3.98 [a]
III-PEG-6000								
50	5.00±0.32 [d]	4.60 ±0.25 [c]	0.40±0.25 [ab]	0 .40±0.25 [b]	0.00±0.00 [b]	0.00±0.00 [b]	10.20±0.86 [d]	20.60±0.51 [d]
100	7.20±0.37 [c]	7.40±0.40 [b]	0.60±0.25 [ab]	0.40±0.25 [b]	0.00±0.00 [b]	0.00±0.00 [b]	15.20±0.66 [c]	30.80±0.86 [c]
200	9.20±0.37 [b]	8.20±0.37 [b]	1.20±0.37 [a]	0.80±0.37 [b]	0.00±0.00 [b]	0.00 ±0.00 [b]	22.20±.860 [b]	41.40±1.21 [b]

5000 sperm were examined in 5 mice per each experimental group

Data were expressed as mean% ± S.E. Mean values followed with different superscript letters within the same column are significantly different from one another ($P \leq 0.05$)

Table 3: Effect of treatment with PEG6000 on mouse meiotic chromosomal aberrations

	Structural aberrations					Numerical aberrations		
Treatment groups	X-Y Uni.	Auto.Uni	X-Y Break	Fragment	Chain	Aneuploidy	Polyploidy	Total
I-Negative control	3.00±0.45 [c]	1.80±0.20 [d]	0.20±0.20 [a]	0.40±0.25 [a]	0.00±0.00 [b]	0.00±0.00 [c]	0.00±0.00 [b]	5.4± 0.250[e]
II-Positive control	6.60±1.03 [ab]	9.00±0.84 [a]	0.80±0.20 [a]	2.00±1.05 [b]	0.60±0.25 [a]	2.60±0.40 [a]	1.20±0.37 [a]	22.80±0.58 [a]
III-PEG6000								
50	5.80 ±0.80 [b]	3.00 ±0.55 [cd]	0.40±0.25 [a]	0.40±0.24 [a]	0.00+0.00 [b]	0.00+0.00 [c]	0.00±0.00 [b]	9.60±0.98 [d]
100	7.00 ±1.04 [ab]	4.20 ±0.66 [bc]	0.60±0.25 [a]	0.60[a]±0.40 [a]	0.20±0.20 [ab]	0.80±0.37 [bc]	0.20±0.20 [b]	13.60±0.93 [c]
200	8.60±0.68 [a]	5.60 ±0.51 [b]	0.80±0.37 [a]	0.80±0.37 [a]	0.40 ±0.25 [ab]	1.20±0.49 [b]	0.40±0.20 [b]	17.80±0.58 [b]

500 metaphases were examined in 5 mice per each experimental group. Auto = Autosomal; Uni= Univalent

Data were expressed as mean % ± S.E. Values within the same column followed by different letters are significantly different form one another ($p \leq 0.05$).

Table 4: Frequencies of different types of chromosomal aberrations induced in fetal liver generated from paternal mice treated with PEG6000

	Metaphases with different types of chromosomal aberrations									Abnormal metaphases	
	Structural aberrations						Numerical aberrations				
Treatment groups	Ch. Gap	Chro. gap	Ch. Break	C.T	Del. &Fr.	Total	Prediploidy	Polyploidy	Total	Including gaps	Excluding gaps
I-Negative control	0.12±0.07 [d]	0.00±0.00 [d]	0.12±0.07 [c]	0.12±0.07 [d]	0.00±0.00 [c]	0.36±0.11 [e]	0.16±0.08 [d]	0.00±0.00 [d]	0.16±0.08 [d]	0.48±0.12 [e]	0.40±0.10 [e]
II-Positive control	5.04±0.23 [a]	0.80±0.41 [a]	4.72±0.16 [a]	4.12±0.19 [a]	1.60±0.21 [a]	16.28±0.40 [a]	1.84±0.14 [a]	1.64±0.10 [a]	3.48±0.15 [a]	19.72±0.38 [a]	13.88±0.40 [a]
III-PEG6000											
50	0.72±0.09 [c]	0.20±0.08 [cd]	0.56±0.10 [c]	1.24±0.15 [c]	0.36±0.10 [c]	3.28±0.24 [d]	0.56±0.10 [c]	0.44±0.10 [c]	1.00±0.15 [c]	4.28±0.30 [d]	3.16±0.21 [d]
100	1.64±0.10 [b]	0.40±0.10 [bc]	1.00±0.12 [b]	1.60±0.10 [c]	0.92±0.13 [b]	5.60±0.22 [c]	1.32±0.14 [b]	1.04±0.14 [b]	2.32±0.19 [b]	7.92±0.24 [c]	5.88±0.23 [c]
200	1.80±0.08 [b]	0.56±0.10 [b]	1.20±0.14 [d]	2.08±0.11 [b]	1.24±0.13 [ab]	6.88±0.27 [b]	1.84±0.08 [a]	1.48±0.10 [a]	3.32±0.11 [a]	10.16±0.29 [b]	7.84 ±0.24 [b]

Data were expressed as mean% ± S.E. Values within the same column follow by different superscript letters are significantly different from one another ($P \leq 0.05$). Ch=chromatid; Chro=chromosomal; C.T= Centromeric attenuation; Del=Deletion; Fr=Fragment 25 fetus were analysis for chromosomal aberrations per each experimental group. 100 metaphases were examined per each fetus

Table 5: Effect of treatment with PEG6000 on DNA content in testes of male mice

	Control	50mg/kg PEG	100mg/kg PEG	200mg/kg PEG
Spermatogonial DNA	0.432+0.004 d	0.193+0.007 a	0.275+0.003 b	0.300+0.003 c
Spermatocytes DNA	0.341+0.003 d	0.180+0.005 a	0.254+0.002 b	0.273+0.003 c

Data were expressed as mean% ± S.E. Values within the same column follow by different superscript letters are significantly different from one another (P= 0.05).

2.66±0.051 for control group (Table 1). Such percentages represent 3.03, 4.09 and 6.15 fold increases at three doses of PEG6000 when compared with the value of control group, respectively. This result implies that PEG6000 has effect on disturbance of oxidation process of thiols of cyteines in protamine molecules leading to abnormal sperm with poor disulphide bonds.

The proportion of spermatozoa with abnormal chromatin conformation detected by AO was strongly correlated with the proportion of spermatozoa detected by TB (r=0.972, p≤0.0001). Pearson's correlation analysis showed positive significant correlations between abnormal sperm morphology and abnormal chromatin integrity detected by TB and AO. TB assay was strongly correlated with abnormal sperm morphology in head (r= 0.911, p≤0.0001) and tail (0.941, p≤0.0001) regions. Furthermore, AO was highly correlated with abnormal sperm morphology in head (r= 0.941, p≤0.0001) and tail (0.954, p≤0.0001) regions.

Meiotic Chromosome Aberrations (CAs) Analysis: All the tested doses of PEG6000 induced significant increase in the incidence of CAs in mouse spermatocytes with dose dependent relationship (Table 3). Such proportions were arisen from dramatically increase in frequency of X-Y and autosomal univalents with all doses of PEG6000. Metaphases with translocations in the form of chain IV were significant increase by two highest doses of PEG6000. However, metaphases with fragments and X-Y breaks were insignificantly increased with all three doses of PEG6000. Estimation of aneuploidy depends on number of hyperhaploid metaphases, while hypohaploids were completely excluded because an artificial loss of a single chromosome may be occur during preparation. The two highest doses of PEG6000 were induced a statistically significant and insignificant increase in metaphases with hyperhaploid and polyploidy respectively compared with controls group. It is noteworthy that, some types of aberrations including chain, aneuploidy, polyploidy were not recorded at low dose of PEG6000 and control group. Pearson's correlation analysis illustrated high significant positive correlation between meiotic CAs and total abnormal sperm morphology induced in PEG6000 (r=0.877, p≤ 0.001).

Fetal Chromosome Aberrations (CAs) Analysis: Analysis of mitotic CAs from fetuses generated from mating untreated female mice and paternal mice treated with PEG6000 showed that PEG6000 caused precocious increase in the occurrence of CAs with dose-dependent relationship even after excluding gaps (Table 4). All types of numerical and structural CAs were significantly increased with all three doses of PEG6000 when compared with negative control group. However, such increase was lower than the value of the positive control group.

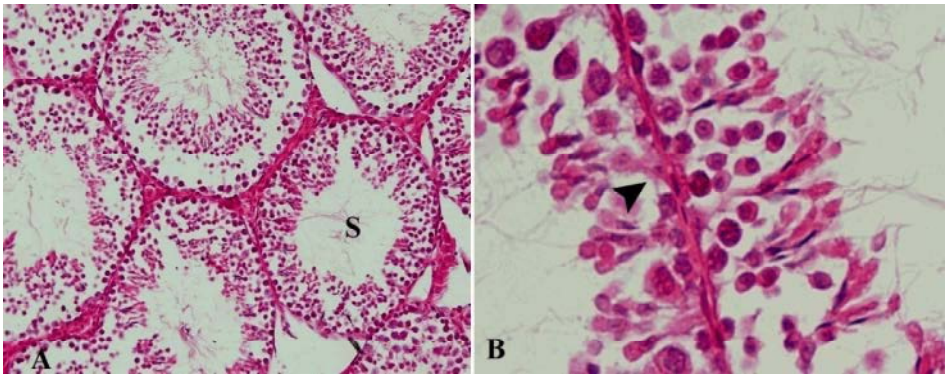

Fig. 1: Section of control testis of mice showing normal architecture of testis, seminiferous tubules (S) show a clear lumen and all cell types are represented including sertoli cells (arrow head), spermatogonia, primary spermatocytes and spermatids (Hx& E A, ×200; B, ×500).

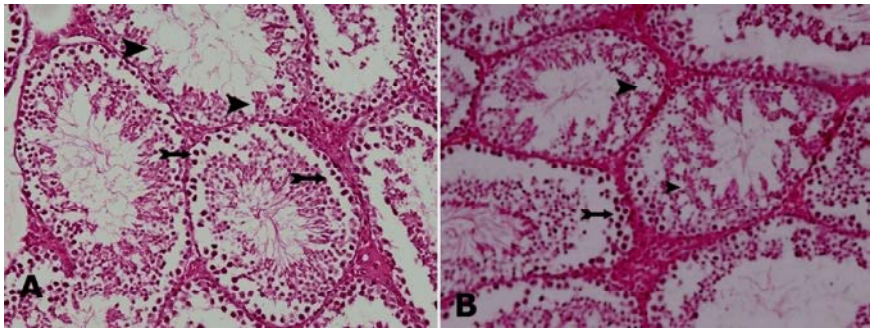

Fig. 2: Section of testis treated with 50 ml/Kg PEG6000 showing (A):degeneration in some seminiferous tubules, detachment of spermatogenic cells and vacuolation areas (area lacking of spermatogenic activity; arrow head). B: pyknosis in spermatogenic cells (arrow), depletion of germ cells layers (A-B Hx & E. x200).

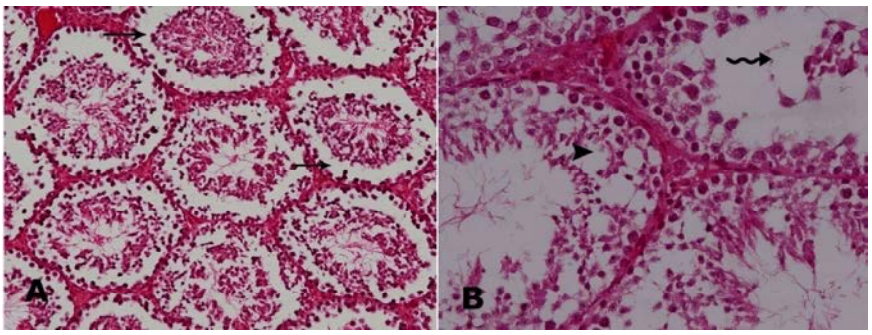

Fig. 3: Section of testis treated with 100 ml/Kg PEG600 showing (A): marked degeneration, necrosis of germ cells (arrows), sloughing of germ cells into tubular lumen. (B): higher magnification of another section showing depletion of long spermatids (weave arrow) and the presence of gaps (arrow head) inbetween the spermatogenic cells (Hx. & E. X 200 & 400).

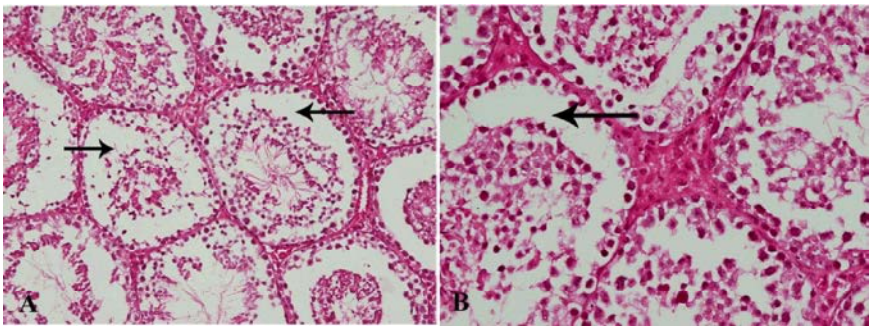

Fig. 4: Section of testis mice treated with 200 mg/kg PEG6000 showing (A): extensive exfoliation of germ cells into tubular lumen (arrow), while the germ cells degeneration, vacuolation, disorganization were generally seen. B-higher magnification of the same section showing depletion of most germ cells layers and long spermatids (Hx & E x 200 & 400).

Histological and Histochemical Study: Histological examination of testes from control mice revealed normal spermatogenesis, with nearly all tubules showing evidence of endogenous spermatogenesis (Figure 1). On the other hand, testicular degeneration and atrophy were observed in the testis of groups treated with PEG600 with a dose dependent manner. Testis of dose of 50mg/k PEG 6000 revealed degeneration in some seminiferous tubules, detachment of spermatogenic cells and cytoplasmic vacuolation, pyknosis in spermatogenic cell nuclei and sings of depletion of germ cells layers (Figure 2). The testis of mice treated with 100mg/kg bw appeared marked degeneration, necrosis of germ cells, sloughing of spermatogenic cells into tubular lumen, depletion of long spermatids and the presence of gaps between the spermatogenic cells (Figure 3). Also,200mg/kg of PEG600

treated mice showed extensive exfoliation of germ cells into tubular lumen, while disorganization of spermatogenic layers, vacuolation and germ cells degeneration were still present (Figure 4).

The mean value of DNA per nucleus was measured by image analyzer and the results was expressed as optical density values (Table 5). The mean value for nuclear DNA content of spermatogonial nuclei was significantly lower in all PEG600 treated groups (0.300 ±0.003,0.275 ±0.003 and 0.193±0.00715, respectively) than control (0.432±0.004). Also the mean value for nuclear DNA content of spermatocytes nuclei were significantly lower in all treated groups compared to control group.

DISCUSSION

The present study clearly illustrated that repeated oral administration of PEG6000 for six weeks caused marked reduction in number and motility of sperm and obvious increase in occurrence of sperm with abnormal morphology and abnormal chromatin integrity. In addition, dramatically increase in the incidence of CAs in mouse spermatocytes and fetal liver generated from mating untreated female mice with treated paternal mice with PEG6000. Histological examination revealed that PEG6000 caused testicular alteration that characterized by seminiferous tubules degeneration, detachment of spermatogenic cells, cytoplasmic vacuolation, necrosis and pyknosis of germ cells, sloughing of spermatogenic cells into tubular lumen, depletion of long spermatids and extensive exfoliation of germ cells into tubular lumen. These findings may be attributed to accumulation of PEG6000 in testis that causes detrimental effects to the male reproductive performance [13-15]. The observed effects are attributed to the mode of action of PEG6000. The precise mechanisms of PEG6000 remain unknown. However, the reproductive toxicity of PEG6000 is probably due to mode of action of one or more of its metabolites rather than to the parent compound. This view supported with the earlier studies reported that ethylene gylycols (EGs) are biologically activated by enzyme alcohol dehydrogenase into alkoxyacetic acid metabolites that interfered with the formation of nucleic acids [16]. Thus, toxicity may be more apparent in tissues undergoing rapid cell proliferation, such as during spermatogenesis, embryogenesis and fetal development [17]. Similar results were obtained when the animals exposed to ethylene glycol monoethyl ether (EGME) showed testicular atrophy, abnormal sperm morphology, degeneration of late spermatocytes and spermatids, decreased numbers of sperm and increased numbers of immature germ cells, decreased sperm motility, peritubular membrane damage and germinal epithelial distortion of the seminiferous epithelium [18].

In the current report, the reduction in sperm count may be reflected impact of PEG6000 on spermatogenesis causing severe erosion and necrosis of the germinal epithelium of the testes [13]. Testicular atrophy and degeneration of germinal epithelium causing oligo or azoospermia has been recognized consequence of treatment male rat with Ethylene glycol monomethyl ether, EGME [19]. Additionally, occupational exposure for male workers to EGs derivative caused an increased prevalence of oligospermia and azzospermia and an increased odds ratio for a lower sperm count per ejaculate [20, 21]. The reduction in sperm motility in PEG6000-treated mice might occur as a consequence of endogenous factors like the machinery for motility (flagellum) and alterations in the available energy and oxidative damages. Similar finding were reported that induction of free radical by EG may be affected on sperm motility by peroxidation of polyunsaturated fatty acid in sperm or by destruction of sperm mitochondria which is responsible for the energy production necessary to maintain spermatozoa movement in some species [22, 23]. Recently, oral administration of EGME at 600 mg/kg/daily for 5 weeks significantly decreased total and progressive motility of rat spermatozoa [24].

Cytochemical experiments were undertaken to validate impact of PEG6000 on sperm plasma membrane and chromatin integrity. Sperm analysis revealed that the proportion of sperm with abnormal morphology or abnormal chromatin integrity detected by TB and AO were dramatically increased in PEG6000 treated mice. These finding suggested that PEG6000 has prominent effect on deficiency of protamines and disturbance the formation of disulphide bonds which responsible for tight packaging of sperm chromatin. This view is explained by the fact that sperm chromatin condensation during the final steps of spermatogenesis in mammals is a multistep process that includes the sequential replacement of the chromatin proteins (especially histones) by protamines, allowing a different structural organization to take place in the sperm nucleus [25]. Protamine is rich in cysteine and also other basic amino acids. During the epididymal passage, the thiols of cysteines in protamine are oxidized to disulfide bonds (S-S). This type of organization provides sperm with a highly condensed nucleus (rich in S-S) and also protects sperm DNA against the enzymatic

attack of nucleases and polymerases. Due to the tight packaging afforded by the protamines, any modification or absence of these proteins leads to an anomaly in the packaging process of sperm nucleus and influence sperm quality and fertilizing capacity [26, 27]. Furthermore, abnormal sperm chromatin structure is thought to arise from many sources including deficiencies in recombination during spermatogenesis, absence of protamine, failure in epididymal maturation and chromatin stability during ejaculation [27, 28]. It is remarkable that, the high correlations between AO and TB assays suggested that the two assays were similarly efficacious in identifying chromatin alternations. Erenpreiss *et al.* [29] found high correlation between the primary sperm morphological abnormalities and sperm chromatin alterations. The substitution of histones by protamines that occurs during spermiogenesis is responsible for the high sperm chromatin compactation [7, 29]. Interestingly, the percentage of total sperm with abnormal morphology was higher than the percentage of sperm with abnormal chromatin integrity which determined by AO or TB. This finding demonstrated that abnormalities in chromatin condensation can influence the head morphology in different ways, although chromatin abnormalities are not always followed by evident morphological alterations. Similarly, morphological alterations in the head were not always followed by abnormalities in chromatin condensation [30]. This implies sperm head with abnormal chromatin was evaluated as normal morphology sperm while head sperm with abnormal morphology were appeared as sperm with normal chromatin. Meanwhile, coiled tail and chromatin alterations detected by TB or AO had significant positive correlations. This finding confirmed that final chromatin compaction occurs in the epididymus, where secondary sperm defects [31].

Meiotic CAs analysis of PEG6000-treated mice revealed significant increase in the frequency of CAs in mouse spermatocytes. This is implied that PEG6000 has destructive effect on genetic material in germ cells. These observations are validated by histochemical findings since PEG6000 caused reduction of the nuclear DNA content in spermatogonia and spermatocytes. Previous studies have shown that administration of EGME to the rats resulted in testicular damage with the spermatocyte being the primary cellular site for toxicity [32]. However, Foster *et al.* [33] reported that Sertoli and Leydig cells, spermatogonia, prepachytene spermatocytes and spermatids were unaffected by EGME administration from 250 to 1000 mg/kg for 11 days apart from partial maturation depletion of early spermatid stage. The differences in these data may be due to differences in treatment duration, dose and mode of administration and/or to species differences. Aneugenic activity of PEG6000 was illustrated by increase the incidence of numerical CAs in mouse spermatocytes. This implies that PEG6000 interacts with tubulin subunits to prevent microtubule assembly, inducing abnormal chromosome segregation in dividing cells and causing aneuploidy. In accordance with our observations, earlier studies demonstrated that inhalation exposure of rats to 300 ppm EGME for 3 days resulted in degenerative changes in spermatocytes of pachytene and meiotic division at spermatogenic stage in rats [34]. Such degenerative pachytene or meiotic spermatocytes were associated with discontinued chromosomal microtubules with deposition of electron-dense chromatin material and chromatin clumping around synaptonemal complexes [34]. Furthermore, it has been reported that EGEE treatment significantly decreased populations of haploid cells and increased diploid and tetraploid cells in spermatozoa at an oral gavage dose of 400 mg/kg b.w in adult rats [35]. Unassociated univalents, particularly X-Y univalents are the most frequently observed than other type of structural CAs in PEG6000 treated mice indicating its genotoxic effect in germ cells. Such effect is due to the prominent effect of PEG6000 on the cohesion of two homologous chromosomes forming unassociated univalent. It has been documented that, homologous chromosomes are physically held together as bivalents by crossover events and cohesion between the DNA molecules. The gradual loss of these physical connections between homologous chromosomes contributes to the high rates of unassociated univalent [36].

Comparing meiotic CAs with abnormal sperm morphology proved significant positive correlation between them. This correlation confirmed that induction of meiotic CAs by PEG6000 should have trigger structural changes in cell organelles involved in head and tail formation, leading to a significant increase in sperm abnormalities. Bagchi *et al.* [37] reported that, EGME caused alternations in testicular DNA, by interfering either with the integrity of the DNA itself and/or with the expression of the genome and therefore, the differentiation of sperm during spermatogenesis was altered, resulting in induced sperm abnormalities.

In the current work, the observed CAs in fetal livers generated from paternal mice exposed to PEG6000 is attributed to induction DNA damage in male germ cells that are kept unrepaired and transmitted to the next generation [38]. Additional support for this view is

provided by Sakkas *et al.* [39] who reported that sperm with protamine deficiency produce premature chromatin condensation that is the cause of failure in fertilization and embryo development. Besides, the majority of de novo structural CAs in fetuses and newborns are considered being of paternal origin that is of sperm origin [40]. In addition, polyploidy and aneuploidy were the major CAs observed in mouse embryo exposed to cryoprotectant EG during vitrification [41].

We concluded that the male chronic exposure to PEG6000 caused impairment of the reproductive functions in male mice. PEG6000 reproductive toxicity revealed to its potential male-mediated fetotoxicity. This is clearly of great concern for possible polyethylene glycols impact on human health.

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