

## Cytotoxicity and Genotoxicity of Depo-Provera<sup>®</sup>, Medroxyprogesterone Acetate (DMPA) in Female Rats

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**Abstract:** Depo-Provera<sup>®</sup>; Medroxyprogesterone Acetate (DMPA) is a long-acting, injectable contraceptive agent that is currently used by 30 million women in 90 countries. This study aimed to investigate the effect of DMPA on Deoxyribonucleic acid (DNA) and bone marrow chromosomes of female rats. Sixty mature healthy female rats (*Rattus norvegicus*) with body weight ranged from 130 – 200 g were intramuscularly injected with DMPA (150 and 300 mg) equivalent to rat doses (2.7 or 5.4 mg/rat/ week) and sacrificed after two and three months of the treatment. DNA was extracted from liver tissue samples using SS-phenol /chloroform, quantified by spectrophotometer and separated electrophoretically on agarose gel. Gels were photographed and images were analyzed using RFLP software version for Windows. Bone marrow cells were flushed out and collected for chromosomal preparations and examined for chromosomal aberrations and mitotic index. Analysis of variance test was carried out was adopted for assessment of significant changes occurring among groups. Results indicated that, DMPA induced decrease in the concentration of DNA as well as DNA damage manifested in fragmentation and adduct formation. Also, DMPA induced various chromosomal aberrations as polyploidy, centric fusion, centromeric attenuation, deletion, end to end association, endomitosis, fragment and ring-like chromosome. In conclusion, DMPA has a genotoxic potential and must be administered in the lowest possible, effective and acceptable doses to minimize any potential risk..

**Key words:** DMPA • Mitotic Index and Chromosomal aberrations • DNA

### INTRODUCTION

Depot Medroxyprogesterone Acetate (DMPA) is a highly effective contraceptive method. It has been used as a contraceptive agent by millions of women in more than 90 countries since 1967 and was approved for use in several developing countries in 1992. In Egypt, the majority of the Egyptian women who are using contraception use DMPA as their birth control method. The risk of chromosomal damage in relation to the use of contraceptives was evaluated in several trials by many authors [47, 48].

Progestins have been recently classified by the International Agency for Research on Cancer [1] as a possible carcinogene to humans. Significant abnormalities were observed in the babies from mothers taking contraceptives (Conovid) (4.6% abnormal cells in tested babies versus 2.0% in controls) [2]. Progestins (Mestranol

and Noiethinodrel) induced increased concentration of the nucleic acids extracted from uterus and liver in sexually mature female-rats [3]. Chromosomal abnormalities were recorded in 33,551 abortions and births of women using contraceptive [4]. Cyproterone acetate (CPA) induced hyperplasia of the rat liver by decreasing liver DNA content by about 25% within a few days of treatment [5]. Moreover, a highly significant increase of chromosomal breakage was observed in peripheral blood lymphocytes of women using different types of hormonal contraceptives (HC) for various periods of time [6]. No significant differences were found in evaluating numerical anomalies of chromosomes in peripheral lymphocytes of healthy women using contraceptives, but highly significant differences were detected in structural aberrations and in structural aberrations without gaps [7]. On the other hand, Herzog *et al.* [8] reported no indications for a mutagenic potential of DMPA. While,

Mitogenic, tumorigenic and induction of DNA-adducts and DNA-repair synthesis in rat liver increased at high Cyproterone acetate (CPA) doses were found [9]. Moreover, estrogens with or without progesterone increased sister-chromatid exchange (SCE) frequency during hormone replacement therapy in postmenopausal women with an evidence for the increased potential for malignancies [10]. Furthermore, DMPA induced progestin-dependent ductal metastatic mammary tumors with high levels of estrogen receptor (ER) and progesterone receptor (PR) in female BALB/c mice [11]. DMPA induced increase in p53 gene expression and induction of apoptosis in a breast cancer cell line [12, 13]. DNA adducts formation were induced in primary cultures of human hepatocytes by Cyproterone acetate, indicating that human liver cells have the capacity to activate Cyproterone acetate to genotoxic intermediates [14]. Excessive exposure to synthetic or endogenous estrogens has been associated with the development of cytotoxic/carcinogenic effects in breast cancer cells [15]. Cyproterone acetate, is activated to a reactive species by the liver and forms DNA adducts and elicits DNA repair in hepatocytes from both rats and humans [16]. In the last decade evidence has shown that a synthetic drug of this family, has similar effect in humans of both genders but markedly higher in female than in male rats [17,18]. Also, Siddique and Afzal [19] reported genotoxic and cytotoxic effect of DMPA and cyproterone acetate in human peripheral blood cultures *in vitro* and which were confirmed by another studies [20-22]. Genotoxicity potential of endogenous and synthetic sex hormones, were detected by the alkaline Comet assay, with no genotoxicity induced by the physiological level of the female sex hormones and oral contraceptives [23]. Although, breaks and gaps were seen at all time intervals, chromatid breaks were found to be more common than chromosome breaks as has been observed in case of certain other steroidal drugs [24, 25]. This indicates that the drug introduces replication errors or breaks replicated chromatids. Recently, the presence of DNA adducts have been reported in several genotoxic studies with many members of progestine family like Megestrol acetate, Cyprrterone acetate and Chlormadinon acetate [26]. Also, apoptosis was observed in human endometrial endothelial cells (HEECs) incubated with progesterone, levonorgestrel and medroxyprogesterone acetate [27]. So, the current study aimed to investigate the effect of DMPA on Deoxyribonucleic acid (DNA) and bone marrow chromosomes of rats.

## MATERIALS AND METHODS

The present study was carried out using mature female rats (*Rattus norvigcus*) with an average body weight of 130 – 200 g. Rats were apparently normal and healthy. They were housed in animal houses under regular periods of dark and light i.e. (12-hrs dark and 12 -hrs light) at room temperature. Animals were fed on standard rodent pellet diet with free access to water. These animals were divided into two major groups named two and three month groups. Each major group was divided into three minor groups, the first was considered a control group and the last two groups were treated groups. These animals were intramuscularly injected with DMPA (2.7 or 5.4 mg/ rat/ week). The doses were converted from human dose to rat dose by using multiplication factors for dose conversion between different species by Paget and Barnes [28]. Nucleic acid (DNA) was extracted from liver tissue samples using SS-phenol / chloroform, determined by spectrophotometer and electrophoretically on agarose gel separated according to Leonard *et al.* [29]as modified by Chen *et Al.* [30]. DNA gels were photographed using Polaroid Camera supplemented with hoods of variable size and Polaroid films model (667). After photography the images were analyzed using RFLP software version for Windows. Bone marrow cells were flushed out and collected for chromosomal preparations and examined for chromosomal aberrations and mitotic index according to the method of Nicholas *et al.* [31]. Analysis of variance (ANOVA) and assessment of significant changes occurring among groups were carried out according to the method of Bailly [32] and reviewed using SPSS ver12 software.

## RESULTS

**Mitotic Index:** DMPA at doses of 150 and 300 mg equivalent doses for two and three months induced cytotoxic effects on the chromosomes of bone marrow cells of treated adult female rats (maximum ~ - 62.33 %) in the treated groups which was manifested by marked reduction( $P = 0.01$ ) in mitotic index. when compared with the control group (Tables 1&2).

**Chromosomal Aberrations:** Examining the metaphase of bone marrow cells of DMPA- treated female rats revealed marked increases ( $P = 0.01$ ) in numerical aberrations as polyploidy and structural aberrations manifested in centric fusion (maximum ~ + 93.80 %),

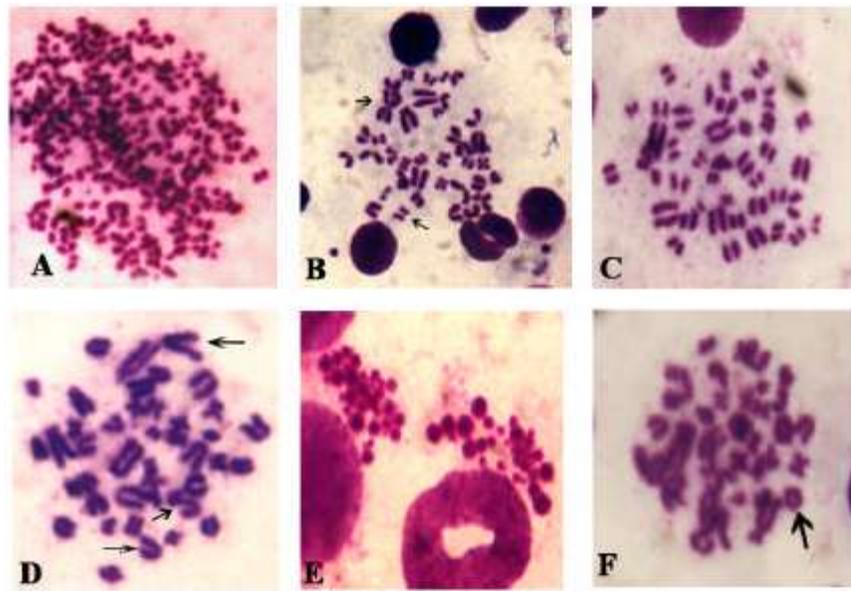


Plate 1: Chromosomal aberrations induced in bone marrow cells of rats after two and three months of DMPA treatment. A- Polyploidy, B- End to End association and centromeric attenuation, C- Centromeric attenuation, D- Chromatid delamination and end to end association, E- Stickiness of chromosomes and F- Ring-like chromosome.

Table 1: Chromosomal aberrations induced in bone marrow cells of rats after two month of treatment

Chromosomal Aberration	GI (Control)	GII		GIII		
		Mean $\pm$ SD	%	Mean $\pm$ SD	%	
N.A. Polyploidy	0.00 $\pm$ 0.00	1.82 $\pm$ 0.17	00.00	2.50 $\pm$ 0.28	00.00	
Structural aberrations	C.F.	1.30 $\pm$ 0.40	4.60** $\pm$ 1.80	+ 71.73 %	6.36** $\pm$ 1.21	+ 79.55 %
	C.A.	1.43 $\pm$ 0.52	9.38** $\pm$ 0.36	+ 84.75 %	15.20** $\pm$ 0.25	+ 90.59 %
	Deletion	1.88 $\pm$ 0.60	6.30** $\pm$ 0.72	+ 70.15 %	9.12** $\pm$ 0.19	+ 79.38 %
	E.T.E.	1.90 $\pm$ 0.10	11.00** $\pm$ 0.81	+ 82.72 %	14.90** $\pm$ 1.82	+ 87.24 %
	Stickiness	1.90 $\pm$ 1.20	6.40** $\pm$ 1.50	+ 70.31 %	8.12** $\pm$ 0.85	+ 76.60 %
	Ring	1.40 $\pm$ 0.20	5.40** $\pm$ 0.28	+ 74.07 %	7.21** $\pm$ 0.27	+ 80.58 %
Mitotic Index	44.57 $\pm$ 3.26	34.32** $\pm$ 2.28	- 22.99 %	28.17** $\pm$ 0.13	- 57.40 %	
Total damage cells	11.41 $\pm$ 2.52	55.10** $\pm$ 4.28	+ 79.29 %	75.81** $\pm$ 6.74	+ 84.94 %	
Cells with multiple aberrations	0.90 $\pm$ 0.12	9.78** $\pm$ 0.18	+90.79 %	12.10** $\pm$ 0.17	+92.56 %	

Data expressed as: mean $\pm$ standard deviation, \*= significant % = Percentage of change from control (GI), N.A= numerical aberration  
\*\*= highly significant and n.s.= non significant. C.F.= Centric Fusion, C.A.= Centromeric Attenuation, E.T.E.= End to end association.

centromeric attenuation (maximum  $\sim$  + 96.19 %), deletion (maximum  $\sim$  +91.95 %), end to end (maximum  $\sim$  +93.70 %), Stickiness (maximum  $\sim$  + 92.82 %) and ring-shaped chromosomes (maximum  $\sim$  + 92.70 %). When compared with the control group. (Tables 1,2; Plate 1).

Counting the number of bone marrow cells with two and more types of aberrations revealed increases (P = 0.01) in the number of cells with two and more types of aberrations (maximum  $\sim$  +92.56 %). Also, the examined bone marrow cells indicated increased (P = 0.01) number of

total damage cells (maximum  $\sim$  + 93.86 %) when compared with the control group.

**Liver DNA Concentration ( $\mu$ g/ $\mu$ L):** The data obtained from treated animals at two and three months, with 150 and 300 mg equivalent doses of DMPA led to a dose-related decrease in the concentration of DNA (maximum  $\sim$  - 60.00 %). These decreases were statistically significant (P = 0.01) when compared with the control (Table 3).

Table 2: Chromosomal aberrations induced in bone marrow cells of rats after three month of treatment

Chromosomal Aberration	GIV (Control)	GV		GVI		
		Mean±SD	%	Mean±SD	%	
N.A. Polyploidy	0.00±0.00	1.96±0.41	00.00	2.40±0.17	00.00	
Structural aberrations	C.F.	0.70±0.10	8.78**±0.61	+ 91.95 %	11.30**±1.14	+ 93.80 %
	C.A.	0.40±0.12	8.70**±0.21	+ 95.40 %	10.50**±0.61	+ 96.19 %
	Deletion	0.70±0.11	6.60**±0.13	+ 89.39 %	8.70**±0.12	+ 91.95 %
	E.T.E.	0.68±0.17	9.30**±1.41	+ 92.68 %	10.80**±0.66	+ 93.70 %
	Stickiness	0.87±0.12	9.22**±0.32	+90.56 %	12.13**±1.36	+ 92.82 %
Ring	0.70±0.12	7.14**±0.27	+ 90.19 %	9.60**±1.24	+ 92.70 %	
Mitotic Index	42.50±0.26	34.15**±0.16	- 24.45 %	26.18**±0.13	- 62.33 %	
Total damage cells	4.68±0.44	58.84**±4.32	+ 92.04%	76.33**±3.29	+ 93.86 %	
Cells with multiple aberrations	2.13±0.10	15.25**±1.20	+ 86.03 %	20.21**±1.70	+89.81 %	

Data expressed as: mean±standard deviation. \*= significant % = Percentage of change from control (GI), N.A= numerical aberration  
\*\*= highly significant and n.s.= non significant. C.F.= Centric Fusion, C.A.= Centromeric Attenuation, E.T.E.= End to end association.

Table 3: Effect of DMPA on the liver DNA Concentration ( $\mu\text{g}/\mu\text{l}$ ) of the treated female rats in comparison with the control

Groups	Mean±SD	Range	95% C.L.	%
GI	1.89±0.03	1.75 – 2.00	1.83 – 1.96	100 %
GII	1.33**±0.06	1.07 – 1.67	1.24 – 1.51	- 42.10 %
GIII	0.89**±0.03	0.78 – 1.12	0.88 – 1.01	- 52.91 %
GIV	1.60±0.02	1.58 – 1.75	1.59 – 1.68	100 %
GV	1.00**±0.03	0.83 – 1.16	0.93 – 1.06	- 60.00 %
GVI	0.73**±0.06	0.55 – 1.12	0.69 – 0.96	- 54.37 %

Where: SD = Standard Deviation. % = Percentage of change from control \*\* = Highly Significant.  
(-) = Decreased from control. 95 % C.L. = 95% Confidence Limits.

Table 4: Molecular weights (bp) and Area percentage (A %) of separated DNA Bands of control and treated rat groups with DMPA for two and three months

Bands	Ladder MW	Control MW	GII MW	GIII MW	GV MW	GVI MW
1	10000	9343	10000	10149	10299	13.06
2	8000	--	7639	5216	--	40.08
3	6000	--	5147	3676	--	46.86
4	5000	--	4529	--	--	--
No. of bands	4	1	4	3	1	3

Where: MW = molecular weight in (bp), A% area percentage, (GII&GIII) treated groups for two months. While, (GV&GVI) treated groups for three months

**Electrophoretic Assay:** DMPA induced several discomfitures in the pattern of DNA bands separated on agarose, these discomfitures represented by fragmentation and smearing. The number of DNA bands isolated from treated rats at two and three month ranged between 1 - 4 bands reflecting the ability of DMPA to induce DNA damage (Table 4).

**Cluster Analysis of DNA**

**Similarity Based on Multigraph:** Similarity index between control and treated groups profile using multigraph is illustrated in Figure 1. The profiles from treated groups (GII, GIII, GV & GVI) showing sever decrease in profile similarity to the control profile which ranged between 0.00 – 46.75 % and reflecting the effect of the DMPA on the DNA pattern.

Referring to similarity based on band matching of DNA bands dendrogram was used to assess this relationship between the resulted bands of the control and treated groups. Dendrogram based on simple band matching revealed that, there is no similarity between the control group and the resulted bands of all treated groups (GII, GIII, GV & GVI) (similarity index = 0.00) as shown in Figure 2. While, similarity index between GIII&GVI (66.67 %), GIII,GVI&GII (42.86 %) and GII, GIII,GVI&GV( 30.00 %).

Also, the dendrogram based on band matching and amount of DNA in bands revealed that there is no similarity between control group and the resulted bands of all treated groups (GII, GIII, GV & GVI) (similarity index = 0.00) as shown in Figure 3. While, similarity index between GII, GIII, GVI & GV reached 9.14 %.

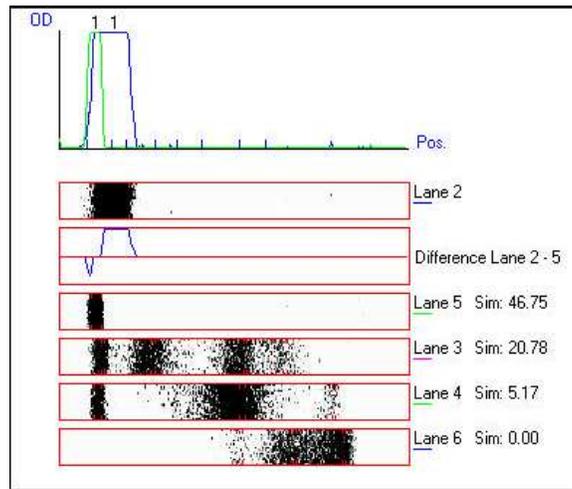


Fig. 1: Multigraph showing the relationship between DNA bands from both treated and control female rats with DMPA for two and three months. Where: Lane 2 = Control, Lane 3 = GII, Lane 4 = GIII, Lane 5 = GV and Lane 6 = GVI

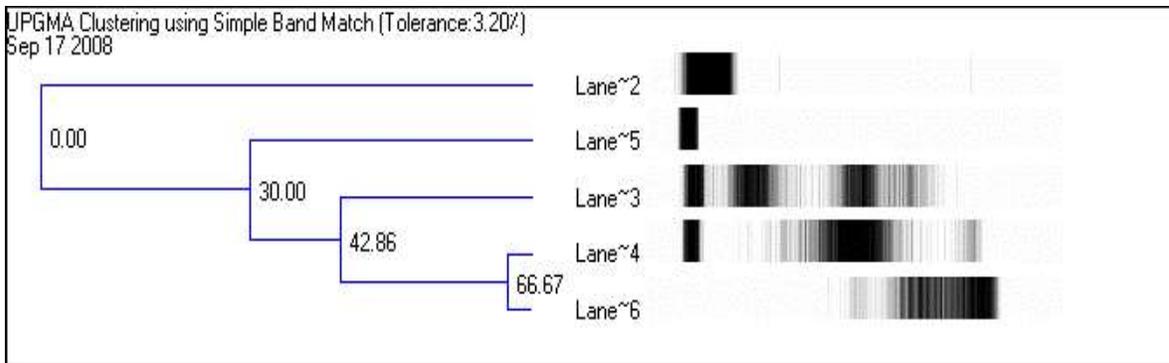


Fig. 2: Dendrogram showing the relationship between DNA bands from both treated and control female rats with DMPA for two and three months. Where: Lane 2 = Control, Lane 3 = GII, Lane 4 = GIII, Lane 5 = GV and Lane 6 = GVI

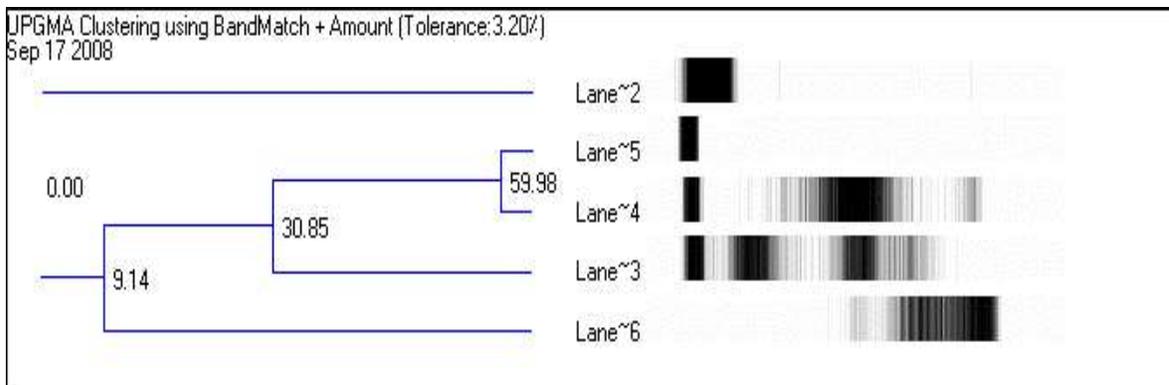


Fig. 3: Dendrogram showing the relationship between DNA bands from both treated and control female rats with DMPA for two and three months. Where: Lane 2 = Control, Lane 3 = GII, Lane 4 = GIII, Lane 5 = GV and Lane 6 = GVI

## DISCUSSION

DMPA induced cytotoxic effects on the chromosomes of bone marrow cells of treated female rats which were manifested by the marked reduction in mitotic index as well as induced various pictures of chromosomal aberrations. Most of the examined metaphases appeared with more than one type aberrations. Also, DMPA induced both reduction in the concentration and damage of DNA. These results were in agreement with these findings by IARC [1], Bishun *et al.* [2], Bursch *et al.* [5], Pinto [6], Fridrichova [7], Herzog *et al.* [8], Krebs *et al.* [9], Kayikcioglu *et al.* [10], Horita *et al.* [12], Chen *et al.* [30], Cavalieri *et al.* [33], Deml *et al.* [34] and Ivett & Tice [35].

Progestins are carcinogenic to humans on the basis of sufficient or limited evidence for carcinogenicity in experimental animals and inadequate evidence for carcinogenicity in humans. The mechanism(s) by which progestins induce tumor development is still unclear; induction of cell proliferation that enhances cancer incidence by stimulating the growth of genetically altered preneoplastic cells and induction of mono-oxygenases leading to increased chemical conversion of procarcinogens into ultimate carcinogens are the favored mechanisms [36-39].

The steroid has been shown to induce DNA repair synthesis in rat and human hepatocytes and to form DNA adducts in rat liver cells [40]. Formation of DNA adducts has also been shown *in vivo* in rat liver. In female rats DNA adducts have been observed at low doses of the steroid drug, which are in the range of the therapeutic doses used in women. DNA adduct formed due to binding of steroid hormone to DNA nitrogenous bases as adenine, guanine, thiamine and cytosine, as this takes place between liver DNA of rats treated by synthetic steroid cyproterone acetate (CPA) which was bound to guanine base. DNA adduct formation is considered to be a critical event in the multistage process of tumorigenesis. The progestogens cyproterone acetate (CPA) and MPA or their metabolites were DNA reactive in male and female rat liver cells, since the involvement of the steroid molecule into DNA binding has only been demonstrated as reported by Feser *et al.* [41]. On the other hand, Norgestrel (contraceptive drug- synthetic progestins) was found to be highly effective in inducing chromosomal aberrations in human lymphocytes *in vitro* [42].

Among the various aberrations observed, gaps, breaks and terminal deletions were more frequent and they increased significantly at higher concentrations.

Aberration frequencies increased with increase in treatment duration, in contrast to the observations for certain progestins in mouse bone marrow. Breaks and gaps were seen at all time intervals, chromatid breaks were found to be more common than chromosome breaks as has been observed in case of certain other steroidal drugs. This indicates that the drug introduces replication errors or breaks replicated chromatids. Another mechanism clarifying the mechanism of the genotoxicity of progestational contraceptive was explained by Brambilla and Martelli [16]. They were discussed the metabolic activation of cyproterone acetate in the liver of female rats is demonstrated by the presence of reactive metabolites in the bile; it involves the reduction of the keto group in the 3-position followed by sulfonation of the hydroxysteroid; the resulting sulfo-conjugate is most likely unstable and is thought to generate a reactive carbonium ion. This activation pathway is consistent with the markedly greater genotoxic effect displayed by cyproterone acetate in female rats in which the activity of the hydroxysteroid sulfotransferase is higher than in males.

In contradiction with our results, Schuppler and Günzel [43] and Reimann *et al.* [44] stated that; due to the generally negative responses obtained with the routinely employed standard genotoxicity assays namely the *Salmonella*/microsome test, in V79 cells, the chromosomal aberrations assay in human lymphocytes and the mouse bone marrow micronucleus test, DMPA is considered as non-genotoxic chemicals acting as tumor promoters.

Recently, there are a lot of publications [20,21,45,46] which confirm the current results. These authors reported genotoxicity and cytotoxicity of DMPA and CA in human blood cultures *in vitro*. Also, genotoxicity of the female sex hormones and oral contraceptives were detected by the alkaline Comet assay [23]. Chromosomal breaks, gaps chromatid breaks and chromosome breaks has been observed in case of certain other steroidal drugs [24,25].

Finally, not only progestins treatment induced formation of DNA adducts as reported by Siddique and Afzal [26] but also, Choksuchat *et al.* [27] observed apoptosis in human endometrial endothelial cells (HEECs) incubated with progesterone, levonorgestrel and medroxyprogesterone acetate.

The present study concluded that Medroxyprogesterone acetate could induced reduction in the mitotic activity and several types of chromosomal aberrations in the bone marrow cells of mice, which carry permanent mutation, to the offspring and causes either structural or behavioral malformations.

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