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Genotoxic Effect of Methotrexate on Bone Marrow Chromosomes and DNA of Male Albino Mice (*Mus musculus*)

Nagla Zaky Ibrahim El –Alfy[1], Mahmoud Fathy Mahmoud[1], Amany Ibrahim Alqosaibi[2] and Sally Ramadan Gabr El-Ashry[1]

[1]Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Cairo, Egypt, [2]Biology Department, Science College, University of Dammam, Dammam, Saudi Arabia

* Corresponding Author: Mahmoud Fathy Mahmoud, Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Egypt Mahmoudfathy76@yahoo.com

ABSTRACT

Aim of the work-Methotrexate (MTX), a structural analogue of folic acid, is an antineoplastic and antirheumatic agent which is used in a variety of clinical schedules and combination therapy regimens in man. Material and methods- Sixty mice of nearly the same age were randomly categorized into four groups (one control and three treated groups with different doses of methotrexate). Mice of the treated groups 1, 2 and 3 were intraperitoneally injected with a single dose of methotrexate (2.5, 5 or 10 mg/kg b. wt. respectively) at the first day of the experiment. All the control and the treated animals were sacrificed after 24, 48 or 72 hour by cervical dislocation post treatment. Results-Methotrexate treatment induced structural and numerical chromosomal aberrations in male mice bone marrow cells which were significantly increased ($P< 0.001$) by dose and time. Structural aberrations were chromosomal gap, fragment, break, centromeric attenuation, deletion, centric fusion, ring formation, end to end association and beaded chromosomes. Numerical aberration was polyploidy. Also, methotrexate treatment decreased the mitotic index in bone marrow cells of all the treated mice in comparison with the control group by increasing dose and time of treatment. Comet assay results indicated that treatment with methotrexate significantly increased ($P< 0.001$) DNA damage in the blood leukocytes in dose and time dependent manner. Conclusion- It can be concluded that methotrexate induced genetic damage on the chromosomes and DNA content of male albino mice even after single treatment with low doses .

Keywords: Methotrexate, Mice, Chromosomes, DNA, Comet assay.

INTRODUCTION

Cancers are a group of diseases characterized by uncontrolled cell growth and spread [1]. Methotrexate (MTX), is one of the widely used antineoplastic drug and a well known immunosuppressant introduced for therapeutic use since 1950s [2]. It is used against a broad range of neoplastic disorders including acute lymphoblastic leukaemia, non-Hodgkin's lymphoma, breast cancer and testicular tumors [3]. Further, it is effective for the treatment of psoriasis, rheumatoid arthritis and different immune suppressive conditions [4]. It is also one of the drugs of choice in the new regimen combination treatment against rheumatoid arthritis and for several tumors [5]. It was proved that high dose of MTX regimens can be used against primary central nervous system lymphomas as well as liver cholestatic disorders [6]. The basic principle of therapeutic efficacy of MTX is due to the inhibition of dihydrofolate reductase, a key enzyme in the folic acid metabolism, which converts dihydrofolic acid to tetrahydrofolic acid. The perturbation in the folic acid metabolism leads to depletion of nucleotide precursors like thymidylates and purines, which in turn inhibits DNA, RNA and protein synthesis. MTX also inhibits thymidylate synthase and the transport of reduced folates into the cell [7]. MTX was found to be a clastogenic agent in tumor cells and in cultured mammalian cells [8, 9]. The carcinogenicity, mutagenicity, teratogenicity and embryo lethality of MTX in different test systems have been studied and reviewed and the data have been listed in the genetic activity

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profile (GAP) database [10]. **Mardini** and **Record** [11] reported that the most serious side effect of MTX therapy is hepatic toxicity. **Dadhania *et al.*** [12] reported that MTX increased the intestinal toxicity in rat that assessed by evaluating different parameters of oxidative stress and DNA damage. **Padmanabhan *et al.*** [13] investigated that MTX decreased the sperm count and increased the frequency of sperms with abnormal head. **Del Campo *et al.*** [14] proved that MTX is also a potent teratogen. **Belur*et al.*** [15] proved the hematologic and myelo -suppressive effects of MTX. **Pellizzer *et al.*** [16] examined the developmentally toxic effects of MTX in a variety of other vertebrate model organisms such as mice, rats and rabbits. **Kasahara *et al.*** [17] reported that MTX is considered as a weak clastogen as it induced chromosomal aberrations in bone marrow of mouse only after multiple treatments. The aim of this study is to investigate the genotoxic effect of methotrexate on bone marrow chromosomes and DNA content of male albino mice.

MATERIAL AND METHODS

Animals:

Sixty mature male mice (*Mus musculus*) of nearly the same age (16-18 weeks old) with an average body weight (24 ± 2 g), obtained from the closed colony of **Theodor Bilharz Research Institute, Cairo**, were individually weighed and randomly categorized into four groups so that there were no statistically significant differences among group body weight means. Each group consisted of fifteen mice. Mice were apparently normal and healthy. They kept in animal house under suitable conditions during the whole period of the experiment. Animals were fed on standard rodent pellet diet and supplied with water. These animals were divided into four groups. One group served as the control group (injected intraperitoneally with 1 ml/kg distilled water) and the other three groups (group1, group2 and group3) served as the treated groups. Mice of group1, group2 and group3 were injected intraperitoneally with three different doses of methotrexate drug (2.5, 5 or 10 mg/kg b.wt. respectively, once on the first day of the experiment). All the control and the treated animals were sacrificed by cervical dislocation after 24, 48 and 72 hr of treatment for collection of samples.

Chemicals:

The drug used in the present investigation was methotrexate (MTX) the active substance in amethopterin .Its chemical structure is: L-glutamic acid, N-(4-{2, 4-diamino-6-pteridinyl) methyl)-nmethylamino} benzoyl with empirical formula $C_{20}H_{22}N_8O_5$, in the form of solution for injection (vial 50 mg MTX/ 2 ml distilled water) produced by Orion Pharma, Orion Corporation, Espoo, Finland. Appropriate methotrexate solutions of the different concentrations were prepared by dilution with distilled water, stored at or below 25°C and protected from light. Its chemical characteristics are represented by the following structure [18] as shown in figure 1.

Chromosomal Aberration Assay:

Bone marrow chromosome preparations were carried out according to the method of **Preston *et al.*** [19].

Mitotic index (MI):

It was calculated by counting the number of dividing cells among at least 1000 cells prepared for metaphase spreads of each group (five animals per each group) and expressed in percentage.

Comet assay :

Immediately after decapitation of animals, 1ml of blood was collected in an Eppendorf tube containing 100µl of 10% EDTA. The blood samples were stored in a dark box at 4ºC until use. Peripheral blood leukocytes were isolated by centrifugation (30 min at 1300xg) in Ficoll-Paque density gradient (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). After centrifugation, the leukocytes were aspirated and washed twice by phosphate-buffered saline at pH 7.4 (PBS). The comet assay was performed according to the method of **Singh *et al.*** [20] with modifications according to **Blasiak *et al.*** [21].

Statistical analysis:

The statistical software package, SPSS version 16.0 for windows was used for all statistical analysis. The study of chromosomal aberrations and data of comet assay were analyzed and expressed as mean and the

standard deviation (mean ± SD). Each treated group was compared to the control group with independent samples t- test. Histograms of cytogenetic data were drawn using Excel 2010. The result was considered to be significant when P is less than or equal to 0.05 or highly significant when P is less than or equal to 0.001.

RESULTS

Chromosomal Aberration Assay:

The diploid number of the control male mouse *Mus musculus* is 2n=38+XY. The chromosomes of the mouse are telocentric and their lengths are forming a continuous series. By using classical techniques the majority of the mouse chromosomes could be identified easily in figure 2. The chromosomes are subdivided into five distinct groups according to their lengths. Each group is made up of chromosomes having similar lengths figure 3. In the present study, 500 well spread metaphases were examined in each group to show the effects of the three doses of methotrexate drug at 24, 48 and 72 hour post treatment on the bone marrow cells respectively. There were two types of chromosomal aberrations; the first one is structural aberrations and the second is numerical aberrations. The current results indicated that the three doses of methotrexate (2.5, 5 and 10 mg/kg b.wt.) induced both structural and numerical aberrations in the chromosomes of the bone marrow cells of the mouse *Mus musculus*. The incidence of structural and numerical chromosomal aberrations were significantly increased ($P<$ 0.001) over the control group table 2, figure 17.

The results clearly indicated that there was a gradual significant increase in the mean of various types of chromosomal aberrations with increasing the dose and time intervals table 1 and figure 16. Structural chromosomal aberrations that were induced in the chromosomes of the bone marrow cells of *Mus musculus* by the treatment with three different doses of methotrexate were centromeric attenuation (Ca) figures 5, 6, 10, 13 and 14, centric fusion (Cf) figure 4, ring form chromosome (R) figures 6, 8 and 11 and end to end association (Ee) figures 5, 6, 11 and 13, chromosomal gaps (Chg) figures 6 and 12, chromatid gap (Cg) figures 8, 9, 11 and 14, beaded chromosomes (Bch) figure 7 chromatid deletion (D) figures 4, 6, 8, 9, 11, 12, 13 and 14 and chromatid fragmentation (F) figures 6, 8, 9, 11, 12 and 13. Numerical aberration that was induced after the treatment with the tested doses of methotrexate is in the form of polyploidy figure 15. The mean of chromosome and chromatid gaps, fragment, deletion and centromeric attenuation were higher as compared to other chromosomal aberrations as shown in figure 17 and table 2.

At 24 hrs administration of methotrexate, the total mean of chromosomal aberrations in the treated mice were increased to 21.2±5.13, 31±4.13 and 50.4±5.7 when compared to controls 10.8±3.53 respectively after administration of 2.5, 5, 10 mg/kg b.wt. of methotrexate. At 48 hrs of administration the total mean of chromosomal aberrations in the treated mice were 27±6.16, 46.2±6.89 and 67.4±6.7 respectively when compared to the control which reached 10.8±3.53 for the various doses of methotrexate with 2.5, 5, 10 mg/kg b.wt. of methotrexate. At 72 hrs administration of methotrexate the total mean of chromosomal aberrations in the treated mice increased to 44.2±6.13, 70±5.06, 98.8±8.52 respectively when compared to the control which reached 10.8±3.53 after the administration of 2.5, 5 and 10 mg/kg b.wt. of methotrexate .

The mitotic index:

In the present study, the mitotic index was calculated and expressed in percentage to evaluate the effect of methotrexate treatment on cellular proliferation, as shown in table 4 and figure 19 methotrexate administration resulted in a reduction in the percent of dividing cells. The reduction was proportional to both the dose and the period after adminstration

Comet Assay:

In the present study the extent and distribution of DNA damage in the blood leukocytes was assessed using the comet assay by counting the damaged cells out of 100 cells on the slides for each animal. The percentages of DNA damage were illustrated in table 3 as mean ± SD and graphically showed in figure 18. Table 3 showed a highly significant increase ($P<$ 0.001) in the mean ± SD of DNA damage

percentages in all the three treated groups after 72 hr of MTX treatment and also in group **3** of mice treated with 10 mg/kg b.wt. MTX after 24 and 48 hr of treatment as compared to the control group .

The mean ± SD of DNA damage in the treated mice was highly significant ($P< 0.001$) and increased to 45±7.07, 62.5±3.54, and 72.5±3.54 respectively after 72 hr of treatment with 2.5, 5 and 10 mg/kg MTX b.wt. as compared to 4±1.41 in the control group. Images of single cell gel electrophoresis were classified according to the degree of damage after migration through electrophoresis and visualized by the digital camera fitted fluorescent microscope as shown in figures. It revealed an intact DNA in the control group figure 20; while a high degree of DNA damage clarified by a slightly pointed end due to the migration of fragmented DNA through electrophoresis (tailed) was presented in figures 21, 22 and 23. Figure 18 showed that there were marked variations in the mean of DNA damage in all the three methotrexate treated groups as compared to the corresponding control values. Also, it indicated that there was a gradual increase in the DNA damage with increasing the dose and time.

DISCUSSION

The present results of chromosomal aberration assay showed that the incidence of aberrant metaphases with chromosomal and chromatid aberrations were significantly increased ($P< 0.001$) in mice bone marrow cells after 24, 48 and 72 hour of treatment with the three treated doses of methotrexate 2.5, 5 and 10 mg/kg b.wt. respectively. These results are in agreement with the reports of **Alam *et al.*** [22] that methotrexate treatment increased the mean of chromosomal aberrations and percentage of aberrant metaphases in mouse bone marrow. **Choudhury *et al.*** [23] recorded that the number of aberrations are not linear with the increase in the doses of methtrexate, in spite of that the present study showed that the increase of the mean of chromosomal aberrations in bone marrow cells of treated animals over that of the control animals was dose and time dependent and statistically highly significant ($P< 0.001$). The results of the current study showed that methotrexate treatment induced certain types of structural aberrations which were chromosomal gap, fragment, break, centromeric attenuation, deletion, centric fusion, ring formation, end to end association and beaded chromosomes. Also, MTX treatment caused numerical aberration in the form of polyploidy. This result agreed with study of **Branda *et al.*** [24] ;they reported that methotrexate caused deficiency of folates by interfering in the folate metabolism. The deficiency of folates may lead to demethylation of heterochromatin causing structural centromere defects that could induce abnormal distribution of replicated chromosomes during nuclear division such as centromeric attenuation, deletion, centric fusion, chromosomal gap, ring formation, fragment and segmented chromosomes. Because aneuploidy of chromosomes 17 and 21 is often observed in breast cancer and leukaemia and increased risk for these cancers was associated with folate deficiency [25]. **Sutherland** [26] observed that folate deficient medium or methotrexate treatment is standard methods of eliciting the expression of fragile chromosomes sites in human cells which have been implicated as sources of chromosome aberrations. Also, current results indicated that the mean of chromosome and chromatid gaps, fragment, deletion and centromeric attenuation were higher as compared to other chromosomal aberrations. This result can be explained according to **Stampfer *et al.*** [27] who confirmed that folic acid deficiency caused fragments in mice chromosomes and enhances tumor development. Also, **Henning *et al.*** [28] demonstrated that the deficiency of folate and niacine produced fragments in cultured human lymphocytes which would enhance the development of liver cancer in rats fed a diet deficient in methionine and choline. **Alonso** and **varela** [29] demonstrated that low folate intake or high alcohol consumption due to deletion and may negate some of the protective effects. **Nguyen *et al.*** [30] postulated the relationship between the folate deficiency and increasing of the percentage of fragments. **Kasahara *et al.*** [17] reported that methotrexate is considered as weak clastogen as it induced chromosomal aberrations in bone marrow of mouse only after multiple

treatments. It was reportedly clastogenic in human bone marrow (in patients) [31] but was nonclastogenic in human lymphocytes *in vivo* [32]. **Donya** and **Aly** [18] indicated that it is a potent inducer of chromosomal aberrations in mouse somatic and germ cells, MTX are found statistically highly significant ($P< 0.001$) after the treatment with the doses of 5 and 10 mg/kg b.wt. of methotrexate, in single treatment and were enhanced by multiple treatments. However, the present study showed that methotrexate induced chromosomal aberrations per 500 metaphases in bone marrow cells of mice after a single treatment with the three doses (2.5, 5 and 10 mg/ kg) and at 24 hr, 48 hr and 72 hr post-treatment. These induced aberrations were statistically significant ($P< 0.001$) and highly significant ($P< 0.05$). This is in agreement with the previous investigation of **Choudhury *et al.*** [33] who found that the induced chromosomal aberrations (excluding gaps) per hundred metaphases recorded from bone marrow cells of mice at 24 hr post-treatment with the three different doses (2, 10 and 20 mg/kg) of MTX were statistically highly significant ($P< 0.001$). Thus, this indicates that methotrexate was highly clastogenic in mouse bone marrow even after a low dose single treatment. The results of the present study showed that the maximum chromosome aberrations were recorded at 72 hr post-treatment with 10 mg MTX/kg b.wt. This is in agreement with the result of **Alam *et al.*** [22] ; they observed that methotrexate treatment caused a significant increase in chromosomal abnormalities and the frequency of aberrant cells and they observed also that the types of aberrations were structural chromosomal aberrations including chromatid breaks, fragments, gaps and deletions besides centromiric attenuation, endomitosis, centric fusion and end to end association. Also, numerical chromosome aberrations were observed in the form of hypoploidy, hyperploidy and polyploidy.

In the present study, we examined also effect of methotrexate on DNA in the peripheral blood leukocytes by using comet assay as it is a sensitive, simple method to detect very low levels of DNA damage [34]. The Comet assay was generally very sensitive in assessing genotoxic damage, making it a good biomarker of induced DNA damage [35]. Therefore, it has been widely used in studies on DNA repair, genetic toxicology, radiation, pollution and ageing [36]. **Martinez *et al.*** [37] suggested that the modified comet assay proved to be a convenient and sensitive biomonitoring tool for individuals occupationally or voluntary exposed to tinner inhalation. The data obtained from the present study clearly showed a highly significant increase ($P< 0.001$) in the mean ± SD of DNA damage in all the three treated groups at 72 hr of administration of methotrexate and also in group **3** of mice treated with 10 mg/kg methotrexate at 24 and 48 hr administration as compared to the controls. We demonstrated that the effect of methotrexate on DNA content was dose and time dependent. This result can be explained by the study of **Genestier *et al.*** [38] .They reported that the deficiency of folates caused by methotrexate treatment was often associated with genotoxic damage like strand breaks, chromosomal abnormalities, defective DNA repair, increased somatic mutation rates , inhibition of cell proliferation, disruption of cell cycle and induction of apoptosis in susceptible cells. Also, **Hassab El-Nabi** and **Elhassaneen** [39] indicated that methotrexate induced apoptosis (DNA fragmentation) in liver, spleen and thymus of rat after 24 hr of treatment with 90 and 150 mg MTX/ kg b.wt. and the intensity of DNA fragmentation increased with dose dependent manner. A number of investigators have examined the genotoxic effect of methotrexate on DNA content by using comet assay. **Kopjar** and **Garaj-Vrhovac** [40] used the alkaline comet assay to evaluate the genotoxicity towards peripheral lymphocytes of medical personnel regularly handling various antineoplastic drugs, including cyclophosphamide, vincristine, vinblastine, cis-platinum, 5-fluorouracil, bleomycin, MTX and adriamycin. Comet assay results of the study of **Kushwaha *et al.*** [41] indicated also significant DNA damage ($P< 0.05$) in different organs as liver, lung, heart, brain, kidney and bone marrow cells induced by MTX as compared to the control group after 28 days repeated oral doses treatment (0.5, 1 and 2 mg/kg).

Padmanabhan *et al.* [42] assessed DNA damage and the cytotoxicity of methotrexate in the germ cells using the sperm comet, halo and TUNEL assay in testis. The sperm comet assay in the study of **Padmanabhan *et al.*** [42] revealed the genotoxicity of MTX as observed by an increase in the main parameters of the comet DNA damage analysis.

According to this study, we can conclude that methotrexate is highly clastogenic, mutagenic and cytotoxic drug. It had very harmful effect on chromosomes and DNA of male *Mus musculus*even after low doses and single treatment.

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Table 1- The relationship between the mean of total chromosomal aberrations of all treated groups with methotrexate (Group 1, 2 and 3) after 24, 48 and 72 hour of treatment.

	24 hrs	48 hrs	72 hrs
Control	10.8	10.8	10.8
Group 1	21.2	27	44.2
Group 2	31	46.2	70
Group 3	50.4	67.4	98.8

Table 2- The relationship between mean of total chromosomal aberrations of male albino mice in the control group and each of the treated groups with methotrexate (groups 1, 2 and 3).

Groups	Ca	Chg&Cg	Cf	R	Bch	Ee	D	F	Po	Total
Control	1.8	2.6	0.4	1.8	0.2	0	1.6	2.4	0	10.8
Group 1	5.33	8.67	0.73	3.1	0.73	0.8	5.06	5.93	0.4	30.78
Group 2	8.13	15.27	2.27	4.6	1.8	1.27	6	8	1.67	49.08
Group 3	10.13	20.2	4	6.2	1.93	2.06	10.8	14.2	2.6	72.18

Table 3- The Mean and standard deviation of DNA damage as revealed by comet assay of peripheral blood leukocytes of the control and treated male Albino mice ***Mus musculus***.

Groups	Dose (mg/kg)	Time (hour)	DNA damage (mean ± SD)
Control			4±1.41
Group 1	2.5	24	9±1.41
		48	12.5±3.54
		72	45±7.07**
Group 2	5	24	14±5.66
		48	17.5±3.54
		72	62.5±3.54**
Group 3	10	24	18.5±2.12**
		48	22.5±3.54**
		72	72.5±3.54**

*Significant. ($P< 0.05$)** Highly significant. ($P< 0.001$)

Table 4- Mitotic index (MI) in 1000 bone marrow cells for each experimental group and its percentage after 24, 48 and 72 hour of treatment with 2.5, 5 and 10 mg/kg b.wt. of methotrexate and the control group.

Groups	Dose (mg/kg)	Time/hour	Score of divided cells / total cells (1000)	Percentages of mitotic index
Control			180	18%
Group 1	2.5	24 hr	140	14%
		48 hr	126	12.6%
		72 hr	112	11.2%
Group 2	5	24 hr	104	10.4%
		48 hr	92	9.2%
		72 hr	88	8.8%
Group 3	10	24 hr	50	5%
		48 hr	38	3.8%
		72 hr	12	1.2%

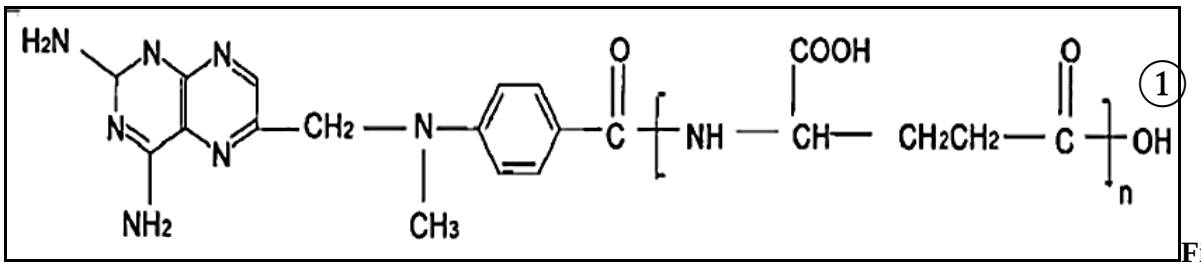

Figure 1: The chemical structure of methotrexate

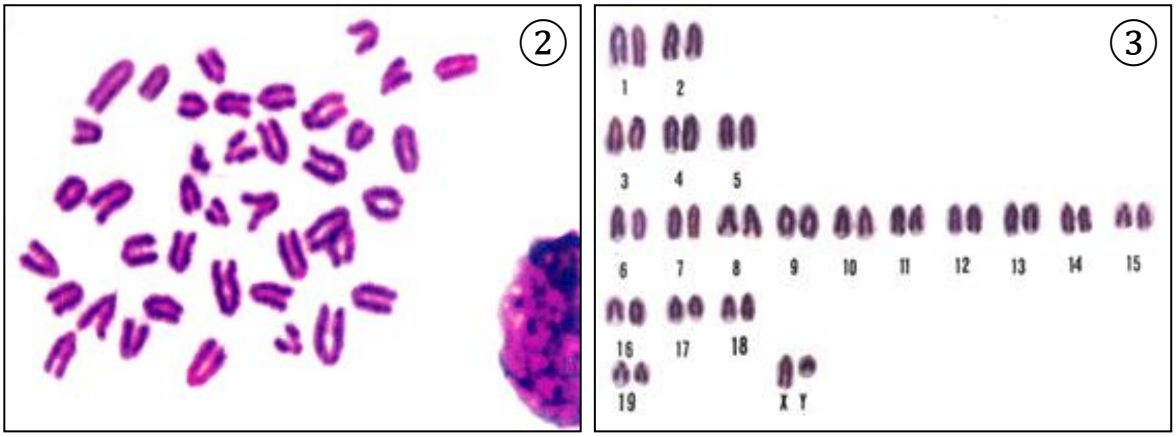

Figure 2- Photomicrograph of metaphase chromosomes of the control male albino mouse *Mus musculus* showing the diploid chromosome number 2n=38+XY. All chromosomes are telocentric. (**X: 2500)**

Figure 3- The karyotype of male albino mouse *Mus musculus*.

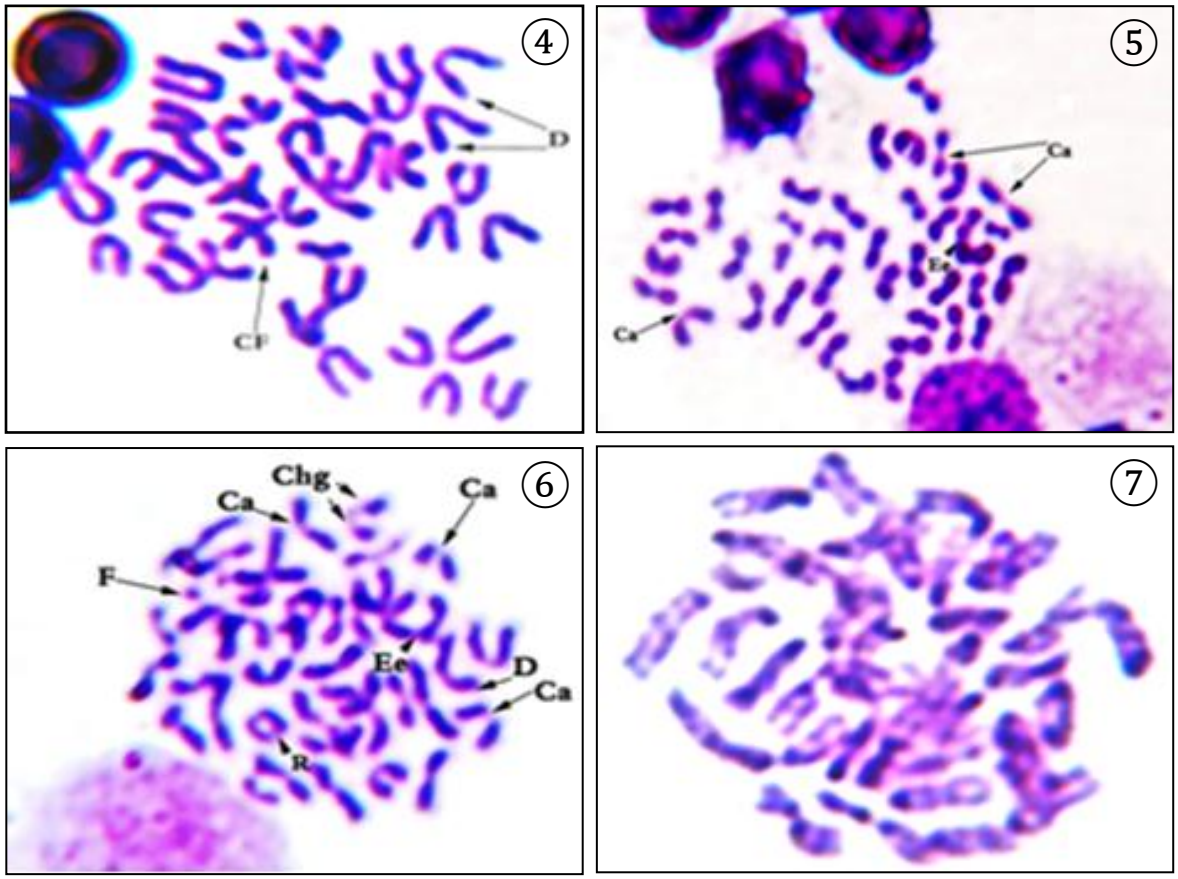

Figure 4- Photomicrograph of metaphase chromosomes of male albino mouse *Mus musculus* after 24 hours of treatment with methotrexate (2.5 mg/kg b.wt.) showing centric fusion (CF) and deletion (D).
(X: 2500)

Figure 5- Photomicrograph of metaphase chromosomes of male Albino mouse *Mus musculus* after 24 hours of treatment with methotrexate (5 mg/kg b.wt.) showing centromeric attenuation (Ca) and end to end association (Ee).
(**X: 2500)**

Figure 6- Photomicrograph of metaphase chromosomes of male albino mouse *Mus musculus* after 24 hours of treatment with methotrexate (10 mg/kg b.wt.) showing ring chromosome (R), deletion (D), centromeric attenuation (Ca), end to end association (Ee), chromosomal gap (Chg) and fragment (F). (**X: 2500)**

Figure 7- Photomicrograph of metaphase chromosomes of male Albino mouse *Mus musculus* after 24 hours of treatment with methotrexate (10 mg/kg b.wt.) showing beaded chromosomes (Bch). (**X: 2500)**

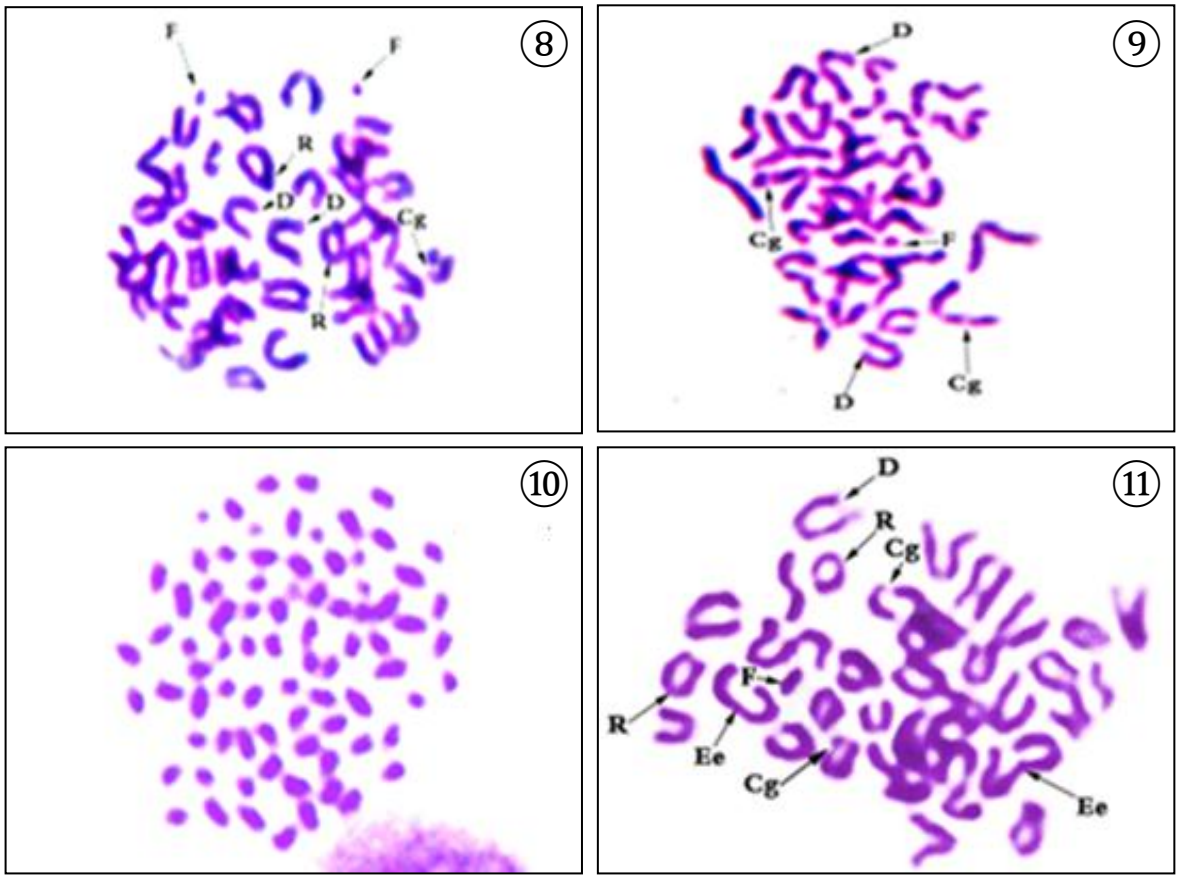

Figure 8- Photomicrograph of metaphase chromosomes of male albino mouse *Mus musculus* after 48 hours of treatment with methotrexate (2.5 mg/kg b.wt.) showing fragment (F), chromatid gap (Cg), deletion (D) and ring (R).

(**X: 2500)**

Figure 9- Photomicrograph of metaphase chromosomes of male albino mouse *Mus musculus* after 48 hours of treatment with methotrexate (5 mg/kg b.wt.) showing fragment (F), chromatid gap (Cg) and deletion (D). (**X: 2500)**

Figure 10- Photomicrograph of metaphase chromosomes of male albino mouse *Mus musculus* after 48 hours of treatment with methotrexate (10 mg/kg b.wt.) showing centromeric attenuation (Ca), it looks like polyploidy. (**X: 2500)**

Figure 11- Photomicrograph of metaphase chromosomes of male albino mouse *Mus musculus* treated with methotrexate (10 mg/kg b.wt.) for 48 hours showing end to end association (Ee), fragment (F), chromatid gap (Cg), deletion (D) and ring chromosome (R).
(**X: 2500)**

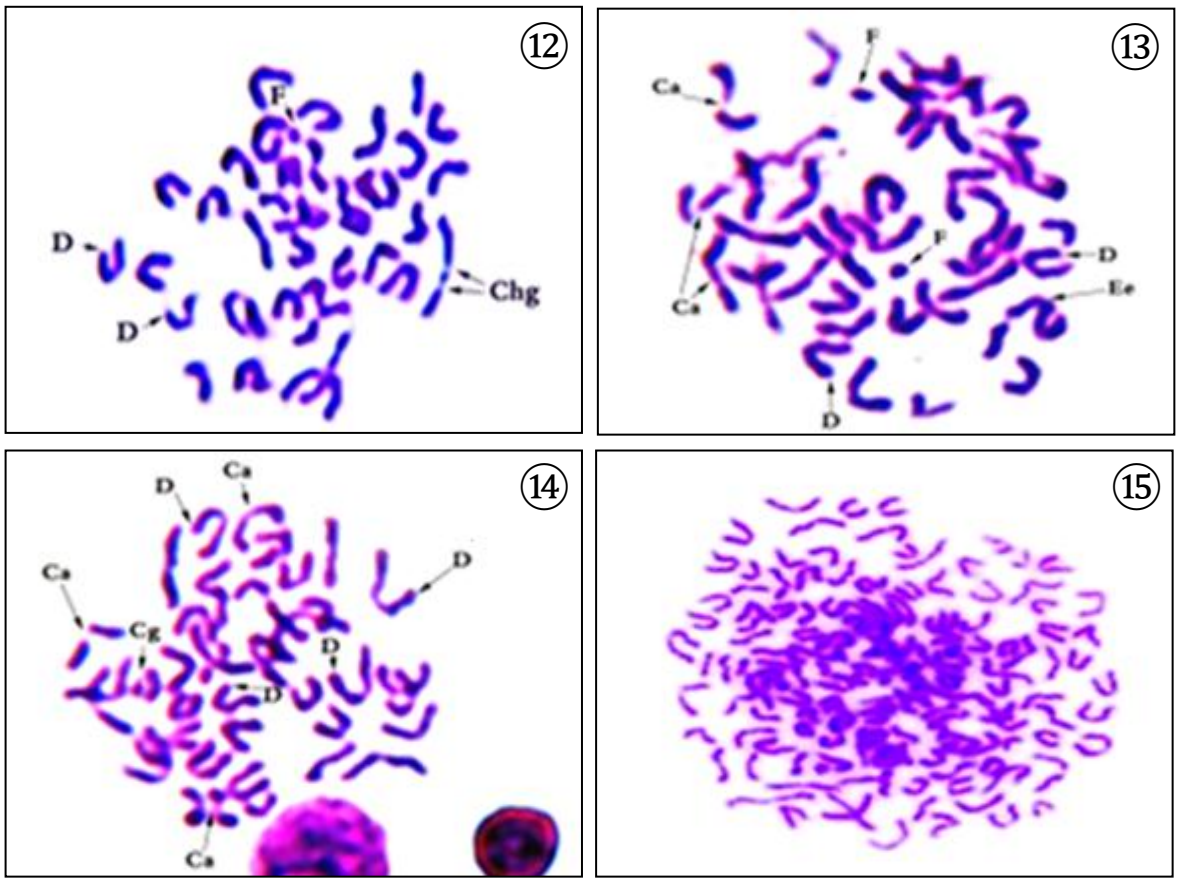

Figure 12- Photomicrograph of metaphase chromosomes of male albino mouse *Mus musculus* treated with methotrexate (2.5 mg/kg b.wt.) for 72 hours showing fragment (F), chromosomal gap (Chg) and deletion (D). (**X: 2500)**

Figure 13- Photomicrograph of metaphase chromosomes of male albino mouse *Mus musculus* treated with methotrexate (5 mg/kg b.wt.) for 72 hours showing centromeric attenuation (Ca), fragment (F), end to end association (Ee) and deletion (D).
(**X: 2500)**

Figure 14- Photomicrograph of metaphase chromosomes of male Albino mouse *Mus musculus* after 72 hours of treatment with methotrexate (10 mg/kg b.wt.) showing chromatid gap (Cg), centromeric attenuation (Ca) and deletion (D).
(**X: 2500)**

Figure 15- Photomicrograph of metaphase chromosomes of male albino mouse *Mus musculus* treated with methotrexate (10 mg/kg b.wt.) after 72 hours of treatment showing polyploidy (Po). (**X: 2500)**

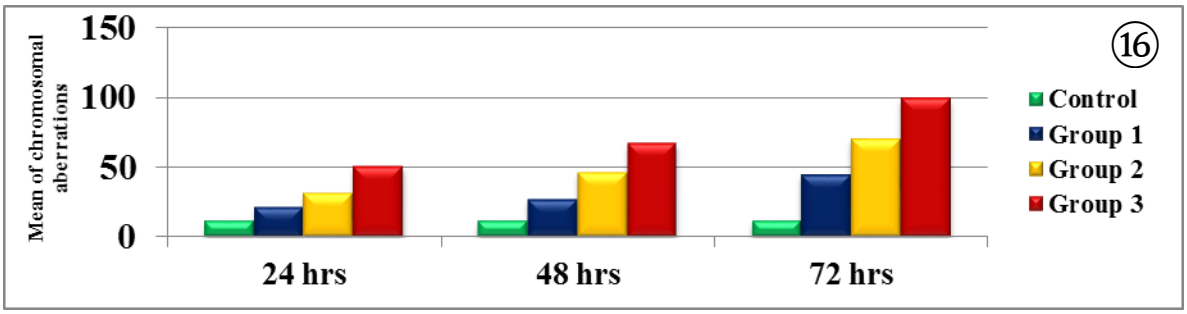

Figure 16- Histogram represents the relationship between the mean of total chromosomal aberrations of all treated groups with methotrexate (group 1, 2 and 3) after 24, 48 and 72 hour of treatment.

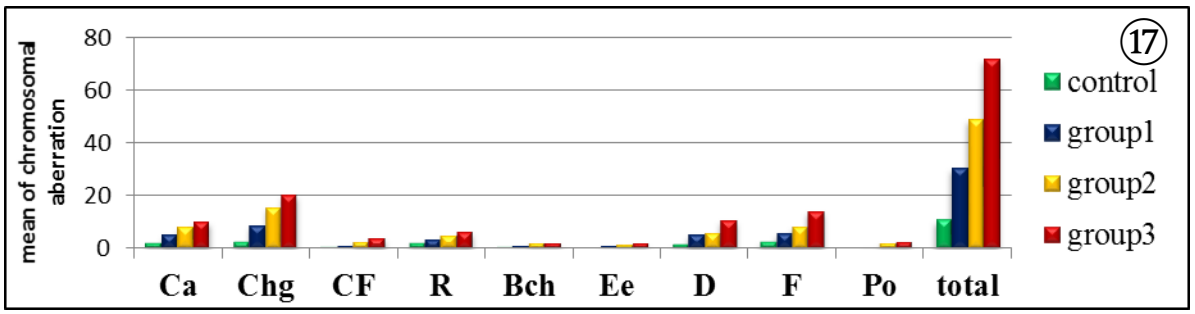

Figure 17- Histogram represents the relationship between the mean of chromosomal aberrations of male albino mice in all the treated groups with methotrexate (group 1, 2 and 3) after 72 hours of treatment

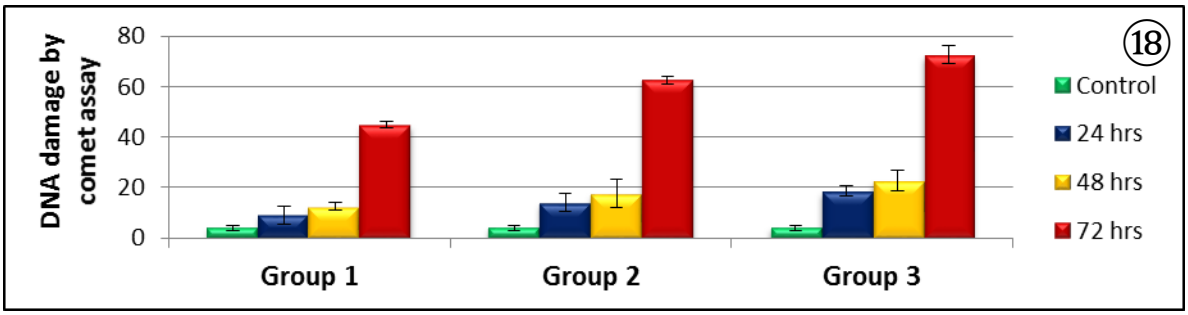

Figure 18- Histogram represents the relationship between the mean ± SD of DNA damage in peripheral blood leukocytes of male Albino mice by using comet assay in the control and all the treated groups with methotrexate after 24, 48 and 72 hour of treatment.

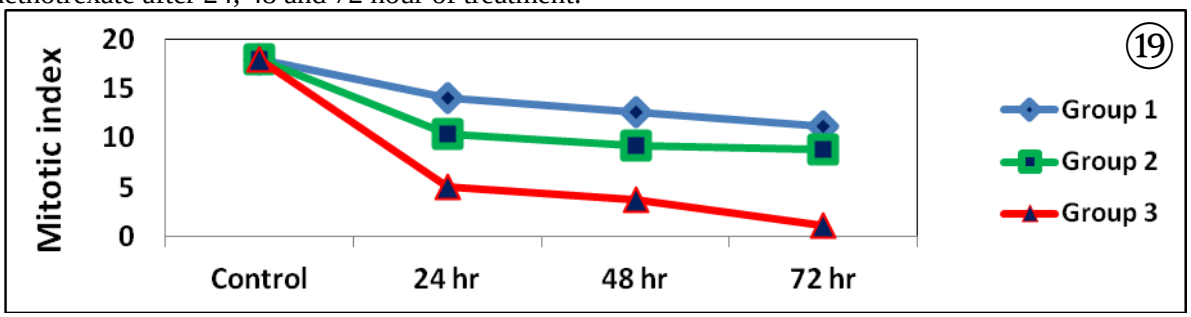

Figure 19- Chart represents the percentages of mitotic indices (MI) in all methotrexate treated groups and the control group after 24, 48 and 72 hour of treatment, as calculated in **table 3.**

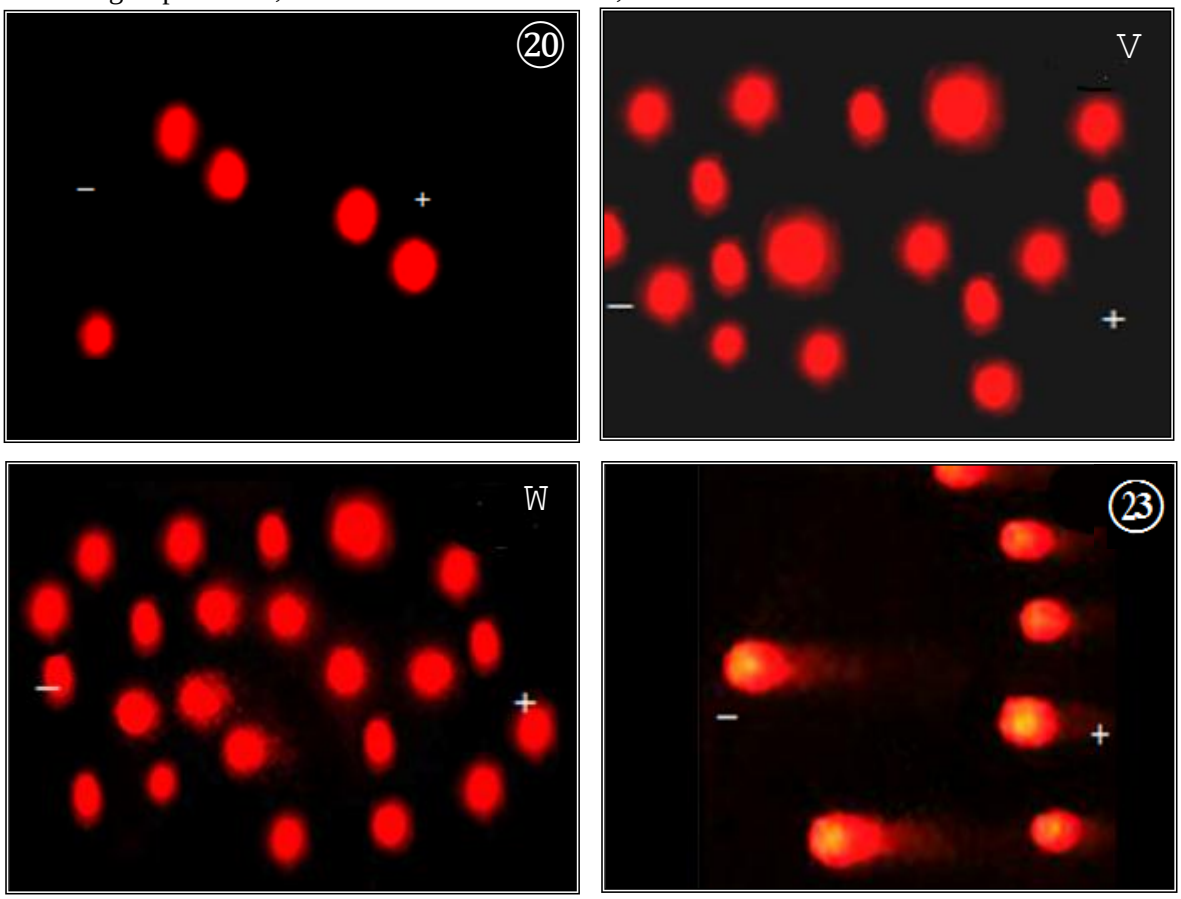

Figure 20- Photomicrographs of comet assay of mouse lymphocytes in the control group showing no or minimum DNA migration. (**X: 1500)**

Figure 21- Photomicrographs of comet assay of mouse lymphocytes of group 1 which was treated with 2.5 mg/kg b.wt. of methotrexate after 72 hr of treatment showing slight DNA migration (short tails) in the lymphocytes of mice . (**X: 1500)**

Figure22- Photomicrographs of comet assay of mouse lymphocytes of group 2 which was treated with 5 mg/kg b.wt. of methotrexate after 72 hr of treatment showing extensive DNA migration (long tails). (**X: 1500)**

Figure 23-Photomicrographs of comet assay of mouse lymphocytes of group 3 which was treated with 10 mg/kg b.wt. of methotrexate after 72 hr of treatment showing the most extensive DNA migration (very long tails). (**X: 1500)**

The symbols "-"and"+" represent cathode and anode, respectively, during the electrophoresis.