

An Analysis of Micronuclei and DNA Damage Induced by Methotrexate Treatment of Male Albino Mice

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ABSTRACT

Background: Methotrexate is an antineoplastic, antipsoriatic and antirheumatic agent belongs to the group of antimetabolites and inhibits folic acid metabolism. **Materials and methods:** To investigate its possible effect, sixty male mice were randomly assigned to one of four groups (one control and three treated groups with different doses of methotrexate). Mice of groups 1, 2 and 3 were intraperitoneally injected with 2.5, 5 and 10 mg /kg b.wt. methotrexate respectively. All the control and treated animals were sacrificed at 24, 48 and 72 hour by cervical dislocation post treatment. **Results:** Micronucleus assay results showed that methotrexate treatment induced genotoxicity in bone marrow cells, the number of micronucleated polychromatic erythrocytes (MNPCEs) and the ratio of polychromatic erythrocytes / normochromatic erythrocytes was gradually increased significantly ($P < 0.001$) by increasing dose and time of treatment in methotrexate treated groups in comparison with the control group. An analysis of randomly amplified polymorphism DNA-polymerase chain reaction (RAPD-PCR) showed different ranges of DNA modifications in the treated groups after 24, 48 and 72 hour of treatment in comparison with the control group. Results of this study indicate that methotrexate treatment induced cytotoxic and genotoxic effect on bone marrow cells and DNA content of male albino mice even after a low dose and single treatment. **Conclusion:** Therefore, the therapeutic uses of methotrexate should be restricted to a very narrow range border.

Keywords: DNA, Mice, Methotrexate, Micronuclei, RAPD-PCR.

INTRODUCTION

Chemotherapy is one of the most effective methods for cancer treatment, but it is often associated with several short and long term toxicities^[1]. Methotrexate (MTX), is one of the widely used antineoplastic drug and it is a well known immunosuppressant introduced for therapeutic use in the 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]. The basic principle of therapeutic efficacy of methotrexate is due to the inhibition of dihydrofolate reductase (DHER), a key enzyme in the folic acid (FA) metabolism, which converts dihydrofolic acid to tetrahydrofolic acid^[4]. 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. Methotrexate also inhibits thymidylate synthase and the transport of reduced folates into the cell^[5,6].

The induction of statistically significant number of chromosomal aberrations and micronuclei (MN) in mice by a single intraperitoneal treatment with three different doses of methotrexate (2, 10 &

20 mg / kg b.wt.) indicated it's highly clastogenic effect^[7]. Other *in vivo* studies indicated that micronuclei induction was enhanced after repeated treatments of methotrexate compared to a single treatment in male mice^[8,9].

Goodman and **Polisson**^[10] demonstrated that methotrexate caused damage to the small intestine leading to nausea, vomiting, diarrhea, stomatitis, decreased absorption and gastrointestinal ulceration in patients. **Dadhania et al.**^[11] reported that it increased the intestinal toxicity in rat that assessed by evaluating different parameters of oxidative stress and DNA damage.

Padmanabhan et al.^[12] investigated that methotrexate decreased the sperm count and increased the frequency of sperms with abnormal head. **Del Campo et al.**^[13] proved that methotrexate is also a potent teratogen^[13]. **Belur et al.** proved the hematologic and myelosuppressive effects of methotrexate^[14].

This study aimed to detect the genotoxic effects of MTX on mouse bone marrow cells using analysis of micronuclei and on DNA using

RAPD-PCR analysis to investigate the clastogenic and genotoxic effect of methotrexate in male albino mice even after a low dose and single treatment.

MATERIALS AND METHODS

Animals- Sixty mature male mice (CD1) of nearly the same age (16-18 weeks old) with an average body weight 22-26 g (mean 24 ± 2 g) were obtained from the closed colony of **Theodor Bilharz Research Institute**, Cairo. They were individually weighed and randomly assigned to one of four groups. Each group was consisted of fifteen mice. These animals were categorized into four groups. One group served as the control group (injected intraperitoneally with 0.1 mL distilled water) and the other three groups (group1, 2 and 3) served as the treated groups. Mice of groups1, 2 and 3 were treated intraperitoneally with three different doses of methotrexate drug (2.5, 5 and 10 mg/kg b.wt. respectively). The doses were converted from human dose to mice dose by using multiplication factors for dose conversion between different species by **Paget & Barnes** [15]. All the control and treated animals were sacrificed by cervical dislocation at 24, 48 and 72 hr after treatment with methotrexate for collection of samples.

Chemicals- The drug used in the present investigation is methotrexate. MTX produced by Orion Pharma, Orion Corporation, Espoo, Finland. Appropriate methotrexate solutions of different concentrations were prepared by dilution with distilled water, stored at or below 25°C and protected from light.

Micronucleus test- In the present study Schmid's [16] standard procedure was followed however with slight modification. Instead of fetal calf serum, 5% bovine albumin from (**National Research Center, Giza, Egypt**) was used as suspending medium to collect the bone marrow [17]. Polychromatic erythrocytes (PCEs), normochromatic erythrocytes (NCEs) and micronucleated polychromatic erythrocytes (MNPCEs) were scored manually using a light microscope with 40X objective and a hand held counter.

RAPD Profiles and Data Analysis- Mice tail samples of each group were collected after 24, 48 and 72 hour of methotrexate treatment and their genomic DNA was extracted from muscle tissues according to the salt chloroform method of **Lagoda et al.** [18].

After checking the genomic DNA, each five DNA samples were mixed in bulked sample [19]. A set of 7 random primers (10 nucleotides for each primer) synthesized by **Operon**

Biotechnologies, Inc. Germany were used in this study as shown in **Table 1**. The PCR mixture and amplification conditions were prepared according to the method of **Williams et al.** [20].

Only reproducible and clear amplification bands were scored. The marked changes observed in RAPD profiles as disappearance and/or appearance of bands in comparison with the untreated control treatments (+/- bands) were evaluated.

Statistical analysis- The statistical software package, SPSS for windows version 16.0 was used for all the statistical analysis. Each treated group was compared to the control group with independent samples T- test. Histograms of cytogenetic data were drawn using Excel 2007. The result was considered to be significant when *P* is less than 0.05 and highly significant when *P* is less than 0.001.

RESULTS

Results of micronucleus test:

In the present study, 2000 polychromatic erythrocytes (PCEs) and corresponding normochromatic erythrocytes (NCEs) were scored per animal (3 animals for each group = 6000 PCEs) to show the effects of the three doses of methotrexate drug at 24, 48 and 72 hr after treatment. The PCEs were stained light blue to gray and NCEs were stained light pink to light yellow (**Figs. 1-11**).

Micronucleus study was conducted based on the frequency of incidence of micronucleated polychromatic erythrocytes (MNPCEs) out of the total 2000 PCEs for each animal. Polychromic erythrocytes with micronuclei (MNPCEs) had small nucleus (dark blue in color) as a residual hereditary material remained after erythropoiesis process (**Figs. 2-10**).

The result of polychromic erythrocytes with micronuclei (MNPCEs) was summarized in **Table 2** as mean \pm SD of MNPCEs score for the control and the three treated groups with 2.5, 5 and 10 mg / kg b.wt. of methotrexate and at 24, 48 and 72 hr post treatment.

Table 2 showed a highly significant increase ($p < 0.001$) in the mean of MNPCEs in the three methotrexate treated groups when compared to the control group. Also, it showed that after 24 hr of treatment with 2.5, 5 and 10 mg/kg b.wt. methotrexate the mean \pm SD of MNPCEs was highly significant ($p < 0.001$) and increased by 53.5 %, 78.9 % and 84.2 % respectively; more than that of the control group, then after 48 hr of treatment with 2.5, 5 and 10 mg/kg b.wt. methotrexate . MNPCEs mean was highly

significant ($p < 0.001$) increased by 76.6 %, 92.7% and 96.3%, respectively more than that of the control group. After 72 hr of treatment with 2.5, 5 and 10 mg/kg b.wt. methotrexate, mean \pm SD of MNPCEs was highly significant ($p < 0.001$) increased by 88.1%, 95.3% and 96.8%, respectively more than that of the control group. Cytotoxicity was assessed by measuring the ratio of total polychromatic erythrocytes (PCEs) to total normochromatic erythrocytes (PCE / NCE) of the control and all the treated groups.

According to micronucleus assay protocol, two thousand polychromatic erythrocytes (PCEs) were screened manually per animal. The number of PCEs was 6000 for each group. The number of corresponding normochromatic erythrocytes (NCEs) was screened to calculate the ratio of PCE / NCE for each group. **Table 2** showed that the number of normochromatic erythrocytes (NCEs) decreased by dose and time in all the treated groups than the corresponding control group. The present result of cytotoxicity was summarized in **Table 2** as mean \pm SD of PCE / NCE for the control and all the treated groups.

Table 2 showed a highly significant ($p < 0.001$) dose and time dependant increase in cytotoxicity of bone marrow cells (PCE / NCE ratio) in all the methotrexate treated groups when compared to the control group.

24 hr post treatment with 2.5, 5 and 10 mg / kg b.wt. MTX, the mean of PCE / NCE was highly significantly ($p < 0.001$) increased by 3.3 %, 29.09 % and 39.6 % respectively more than that of the control group. 48 hr post treatment with 2.5, 5 and 10 mg/kg b.wt. methotrexate, the mean of PCE/NCE ratio was highly significant ($p < 0.001$) increased by 17.02 %, 29.5 % and 51.04 % respectively more than that of the control group. 72 hr post treatment with 2.5, 5 and 10 mg / kg b.wt. methotrexate the mean of PCE / NCE ratio was highly significant ($p < 0.001$) increased by 24.5 %, 38.09 % and 57.2 % respectively more than that of the control group. The current results indicated that the greater increase in the bone marrow cytotoxicity (PCE / NCE ratio) occurred after 72 hour of treatment with 10 mg / kg b.wt. methotrexate.

Results of randomly amplified polymorphic DNA (RAPD) analysis:

Seven random 10-mer primers were tested to perform the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis to assess the level of DNA damage in the control and treated mice with 2.5, 5 and 10 mg/kg b.wt. methotrexate. Different ranges of DNA modifications were observed in the treated groups in comparison with the control one.

Modifications of the RAPD patterns were due to structural changes occurred as a result of DNA damage which induced by methotrexate treatment. The changes occurred in RAPD profiles included variation in band density (Bd) as well as gain of new bands (+) and/or loss of normal bands (-) following methotrexate treatment.

Only three primers (primer OPA-07, primer OPA-10 and primer OPC-07) were most informative and they produced the most distinguishable banding profiles between the amplified samples of each group after RAPD assays was shown in **figs. 11-13**.

The maximum number of bands observed was eight by primer OPA-07, ten by primer OPA-10 and eight by primer OPC-07. The molecular size of PCR products generated by the three primers (primer OPA-07, primer OPA-10 and primer OPC-07) ranged from 187 to 997 bp, 186 to 1254 bp. and 175 to 1026 bp respectively.

As shown in **fig. 11** (primer OPA-07), there were no common bands between the control and treated samples of *Mus musculus*; while the characteristic individual bands were that of relative front (Rf) 0.856 with molecular size (MS) 187 bp for the control group and that of R f 0.716 with MS 487 bp for the group 1 (after 24 hr of treatment with 2.5 mg/kg b.wt. methotrexate). The number of bands differed from the control to the treated samples (3, 3, 4, 5, 3, 3, 3, 3, 3 and 5) for the control, group 1 after 24 hr of treatment, group 1 after 48 hr of treatment, group 1 after 72 hr of treatment, group 2 after 24 hr of treatment, group2 after 48 hr of treatment, group 2 after 72 hr of treatment, group 3 after 24 hr of treatment, group 3 after 48 hr of treatment and group 3 after 72 hr of treatment respectively.

As shown in **fig. 12** (primer OPA-10), there was one common band between the control and the MTX-treated samples of *Mus musculus* with Rf 0.575 against MS of 397 bp; while the characteristic individual bands were that of Rf 0.508 with MS 537 bp for the control, Rf 0.373 with MS 994 bp for the group 1 after 24 hr of treatment, Rf 0.44 with MS 732 bp and Rf 0.746 with MS 186 bp for the group 3 after 48 hr of treatment. The number of bands differed from the control to the treated samples (5, 4, 4, 3, 3, 2, 2, 3, 3 and 2) for the control group, group1 after 24 hr of treatment, group 1 after 48 hr of treatment, group 1 after 72 hr of treatment, group 2 after 24 hr of treatment, group 2 after 48 hr of treatment, group 2 after 72 hr of treatment, group 3 after 24 hr of treatment, group 3 after 48 hr of

treatment and group 3 after 72 hr of treatment respectively.

As shown in **fig. 13** (primer OPC-07), there was no common band between the control and the MTX-treated samples of *Mus musculus*; while the characteristic individual bands were that of Rf 0.621 with MS 1026 bp for group 2 after 48 hr of treatment, Rf 0.643 with MS 932 bp for the group 3 after 48 hr of treatment, Rf 0.73 with MS 626 bp and Rf 0.776 with MS 499 bp for the group 3 after 48 hr of treatment. The number of bands differed from the control to the treated samples (2, 3, 2, 2, 0, 2, 0, 0, 5 and 1) for the control, group 1 after 24 hr of treatment, group 1 after 48 hr of treatment, group 1 after 72 hr of treatment, group 2 after 24 hr of treatment, group 2 after 48 hr of treatment, group 2 after 72 hr of treatment, group 3 after 24 hr of treatment, group 3 after 48 hr of treatment and group 3 after 72 hr of treatment respectively.

DISCUSSIONS

Results of the current study showed a highly significant increase ($p < 0.001$) in the mean of micronucleated polychromatic erythrocytes (MNPCEs) in all the three treated groups with methotrexate when compared to the control group.

The present investigation was confirmed by following data from literature. Methotrexate produced a significant genetic damage which was proved by the increased incidence of chromosomal aberration and micronuclei formation in human as well as in animal model [21]. Significantly higher micronuclei frequencies in patients with acute lymphoblastic leukemia after the treatment with anti-leukemic agent (vincristine, methotrexate, daunomycin, prednisone and asparaginase) were reported by **Acar *et al.*** [22]. The previous result of **Novakovic *et al.*** [23] suggested that intratumoral application of methotrexate therapy by using sub endometrial injection into the myoma in patients with myoma uteri significantly increased the frequency of micronuclei in human peripheral blood lymphocytes.

The present study indicated that even after a low dose single treatment, methotrexate induced statistically significant ($P < 0.001$) increase in the mean of micronucleated polychromatic erythrocytes (MNPCE) in bone marrow of the treated mice when compared to that of the control group. This finding disagreed with the results of **Kasahara *et al.*** [24] who considered methotrexate as a weak inducer of MN and indicated that level of micronuclei and chromosomal aberrations in mice bone marrow

cells was far higher after multiple treatments than in single MTX treatments.

The current results of cytotoxicity test showed also that there was a highly significant dose and time dependant increase ($p < 0.001$) in the erythropoietic cytotoxicity (PCE / NCE ratio) in all the three treated groups with methotrexate when compared to the control group. **Suzuki *et al.*** [25] reported that evaluation of erythropoietic cytotoxicity was a key component of safety assessment in new drug development and polychromatic erythrocyte (PCE) counts in peripheral blood were the most popular and convenient method of monitoring erythropoiesis. The occurrence of fewer immature erythrocytes (PCE) relative mature or normochromatic erythrocytes (NCE) was considered to be an indicator of mutagen induced cytotoxicity.

The present result of bone marrow cytotoxicity showed that at 72 hr post treatment with 2.5, 5 and 10 mg / kg b.wt. methotrexate, the mean of PCE / NCE ratio (1.55, 1.89 and 2.74) was highly significantly ($p < 0.001$) increased by 24.5 %, 38.09 % and 57.2 % respectively more than that of the control group (1.17).

This finding is confirmed by **Kojima *et al.*** [26] who observed a decrease in the haemoglobin concentration, haematocrit and mature erythrocyte count in both male and female rats which received oral dose (0.45 mg / kg) of methotrexate. **Chan and Cronstein** [27] confirmed also that the anti-proliferative effects of methotrexate were sufficient to explain many side effects of MTX such as bone marrow cells suppression, alopecia and stomatitis. **Shimada *et al.*** [28] reported that erythrocytes with micronuclei were captured and destroyed by the spleen quickly.

In the present study, different ranges of DNA modifications were observed in the methotrexate treated groups in comparison with the control one by using RAPD technique. **Swaileh *et al.*** [29] reported that randomly amplified polymorphic DNA (RAPD) analysis was a rapid and sensitive technique and applicable to any organism without prior information of the nucleotide sequence used for the genetic characterization of populations, evolutionary studies and the detection of genetic alterations and instability.

The present investigation clearly demonstrated that methotrexate had a very lethal effect on DNA content of cells of the treated mice. This result is in accordance with that of **Rawtani and Agrawal** [30] who reported that methotrexate had a direct or indirect effect on various molecular targets which influenced DNA replication and

cell proliferation by integrating between the spaces between adjacent

Also, **Lee *et al.*** ^[31] used reverse transcription polymerase chain reaction (RT-PCR); the results showed that GART, HPRT1, TYMS, and CTPS genes (important purine and pyrimidine biosynthesis-related genes) altered their expression levels by methotrexate. Alteration of these genes might lead to methotrexate-induced teratogenicity, which caused by imbalance of nucleotide biosynthesis.

The present result confirmed that of **Aggarwal *et al.*** ^[32] which indicated that methotrexate is a folic acid analog with an amino group substituted for the hydroxyl group at the C4 position on the pyridine ring. The structural similarity of MTX with folic acid helped it to compete with the normal substrate folic acid for the binding site on the dihydrofolate reductase (DHFR) which is the key enzyme involved in the synthesis of essential DNA precursors like thymidylates and purines, then inhibition of DHFR led to an imbalance in the nucleotide pools and thereby perturbed the DNA synthesis.

The present investigation showed that the changes occurred in RAPD patterns of the control and the methotrexate treated groups as a result of DNA damage followed methotrexate treatment included variation in band density (Bd) as well as gain of new bands (+) and / or loss of normal bands (-).

These findings were explained by **Liu *et al.*** ^[33] who suggested that lost bands were likely to be due to one or a combination of the following events: (1) changes in oligonucleotide priming sites due mainly to genomic rearrangements and less likely to point mutations; (2) DNA damage in the primer binding sites; and (3) interactions of DNA polymerase in the test organism with damaged DNA. On the other hand, the appearance of new DNA bands occurred because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural changes or because some changes in the DNA sequence have occurred due to mutations, large deletions, and / or homologous recombination ^[34].

In the present study, RAPD profiles showed marked variations between the control and the methotrexate treated mice with apparent changes in the number and size of amplified DNA fragments for different primers. The number of bands generated by using the three primers (OPA-07, OPA-20 and OPC-07) differed from the control to the treated samples.

The changes in the number of DNA bands in the RAPD-PCR profiles were associated with

alterations of genetic material which caused by methotrexate treatment which might be due to different types of mutations including rearrangements, additions or deletions that occur in the DNA region of amplification in sequences flanked by the priming sites or within the priming site itself ^[35]. Comet assay results of the study of **El-Alfy *et al.*** ^[36] showed a highly significant increase ($p < 0.001$) in the mean \pm SD of DNA damage in all 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 controls. **In conclusion**, results of the present study indicated that methotrexate is highly clastogenic and cytotoxic as it induced clear harmful damage on bone marrow cells and DNA content of male albino mice even after a low dose and single treatment. Therefore, the therapeutic uses of methotrexate should be restricted to the least possible number of patients. The supplementation with folic acid in methotrexate treated patients may help to minimize its genotoxicity.

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Table 1- List of primers code and sequences.

| Primer code | Primer sequence |
|-------------|-----------------|
| OP-A07 | GAAACGGGTG |
| OP-A10 | GTGATCGCAG |
| OP-A14 | TCTGTGCTGG |
| OP-B20 | GGACCCTTAC |
| OP-C05 | GATGACCGCC |
| OP-C07 | GTCCCGACGA |
| OP-E07 | AGATGCAGCC |

Table 2- The mean and standard deviation of polychromatic erythrocytes (PCEs), micronucleated polychromatic erythrocytes (MNPCEs), normochromatic erythrocytes (NCEs) and cytotoxicity in bone marrow of male albino mice *Mus musculus* of the control group and treated groups.

| Group | Dose (mg/kg) | Time/ hour | Total Scored cells/Mouse | Total MNPCEs | Micronuclei | Total NCEs | Cytotoxicity |
|---------|--------------|------------|--------------------------|--------------|--------------------|------------|------------------|
| | | | | | MNPCEs (Mean ± SD) | | PCEs/ NCEs |
| Control | | | 6000/3 | 13 | 0.217 ± 0.02887 | 5098 | 1.17± 0.00330 |
| 1 | 2.5 | 24 | 6000/3 | 28 | 0.467 ± 0.02887** | 4970 | 1.21± 0.00379** |
| | | 48 | 6000/3 | 56 | 0.93 ± 0.07638** | 4259 | 1.41± 0.00306** |
| | | 72 | 6000/3 | 110 | 1.83 ± 0.02887** | 3860 | 1.55± 0.00863** |
| 2 | 5 | 24 | 6000/3 | 62 | 1.03± 0.07638** | 3623 | 1.65 ± 0.00379** |
| | | 48 | 6000/3 | 180 | 3.00± 0.10000** | 3600 | 1.66 ± 0.00721** |
| | | 72 | 6000/3 | 277 | 4.67± 0.12583** | 3164 | 1.89 ± 0.01000** |
| 3 | 10 | 24 | 6000/3 | 83 | 1.38± 0.02887** | 3077 | 1.94± 0.00577** |
| | | 48 | 6000/3 | 354 | 5.90± 0.10000** | 2500 | 2.39± 0.01528** |
| | | 72 | 6000/3 | 410 | 6.83 ± 0.05774** | 2190 | 2.74 ± 0.02646** |

* Significant (P < 0.05)

** Highly significant (P < 0.001)

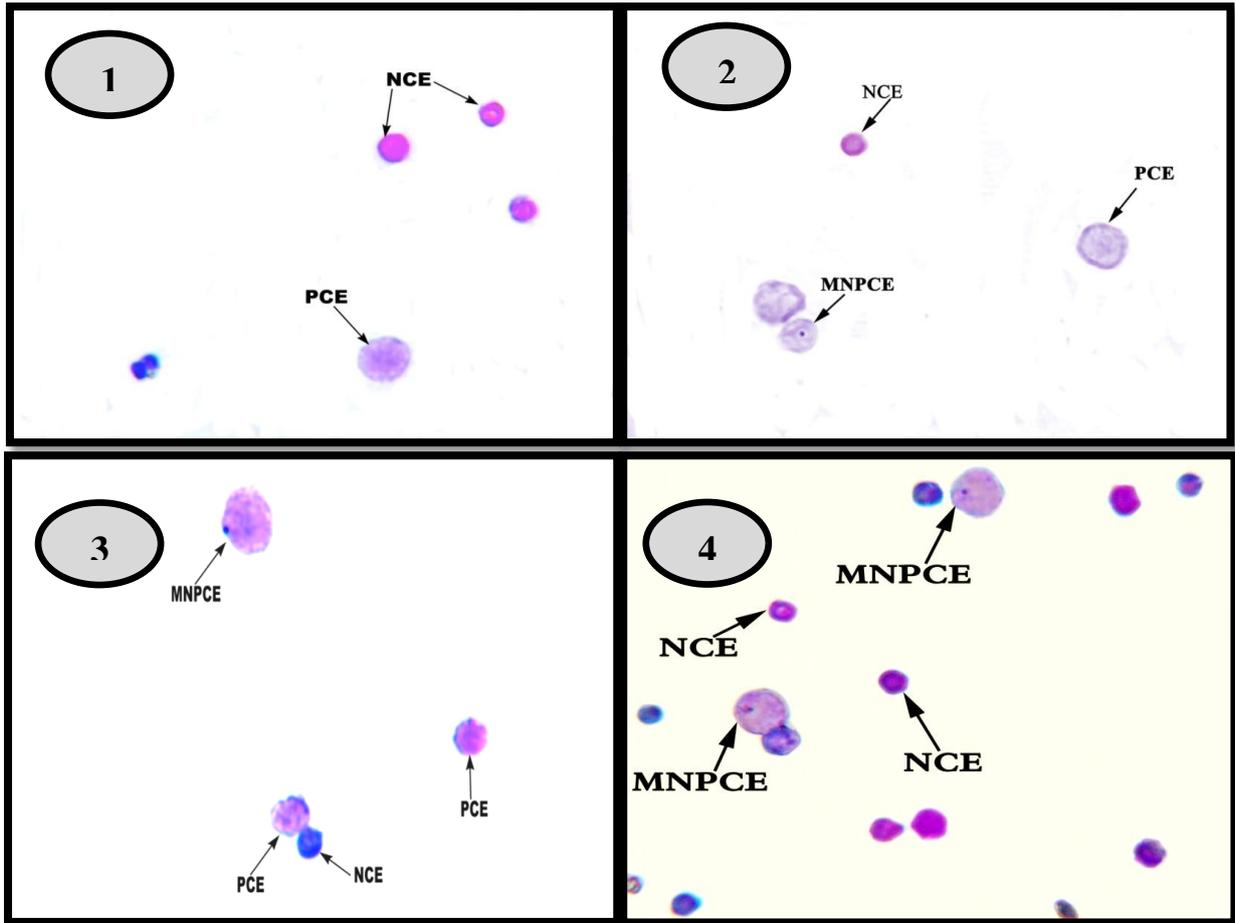


Figure 1- Photomicrograph of bone marrow smear of the control male albino mouse *Mus musculus* showing polychromic erythrocyte (PCE) and normochromatic erythrocytes (NCEs), X1500.

Figure 2- Photomicrograph of bone marrow smear of male albino mouse *Mus musculus* after 24 hours of treatment with methotrexate (2.5 mg/kg b.wt.) showing polychromic erythrocytes (PCEs), micronucleated polychromatic erythrocytes (MNPCEs) and normochromatic erythrocyte (NCE), X1500.

Figure 3- Photomicrograph of bone marrow smear of male albino mouse *Mus musculus* after 24 hours of treatment with methotrexate (5 mg/kg b.wt.) showing polychromic erythrocytes (PCEs), micronucleated polychromatic erythrocytes (MNPCEs) and normochromatic erythrocyte (NCE), X1500.

Figure 4- Photomicrograph of bone marrow smear of male albino mouse *Mus musculus* after 24 hours of treatment with methotrexate (10 mg/kg b.wt.) showing micronucleated polychromatic erythrocytes (MNPCE) and normochromatic erythrocytes (NCE), X1500.

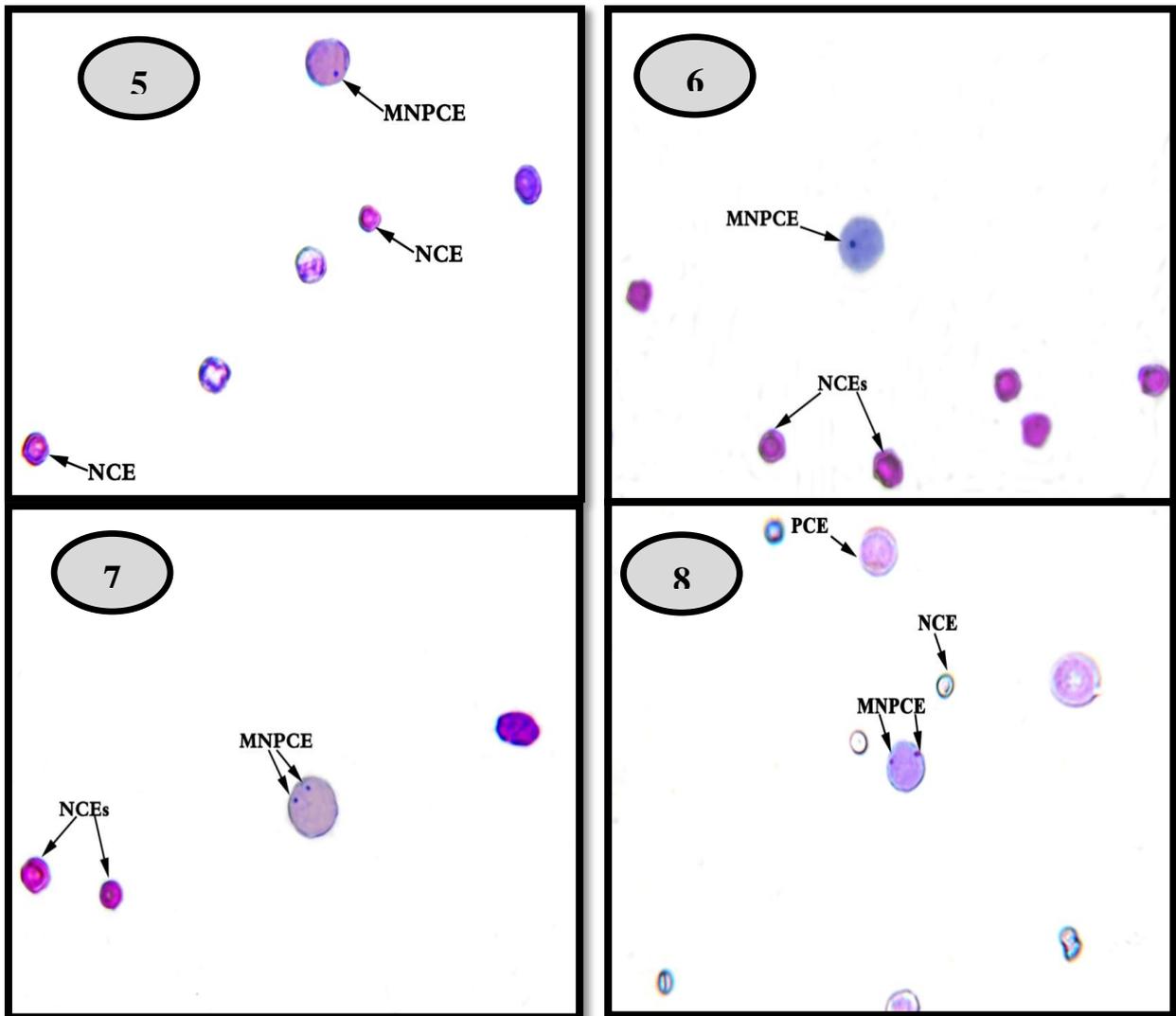


Figure 5- Photomicrograph of bone marrow smear of male albino mouse *Mus musculus* after 48 hours of treatment with methotrexate (2.5 mg/kg b.wt.) showing polychromatic erythrocyte with micronucleus (MNPCEs) and normochromatic erythrocytes (NCEs), X1500.

Figure 6- Photomicrograph of bone marrow smear of male albino mouse *Mus musculus* after 48 hours of treatment with methotrexate (5 mg/kg b.wt.) showing polychromatic erythrocyte with micronucleus (MNPCEs) and normochromatic erythrocyte (NCE), X1500.

Figure 7- Photomicrograph of bone marrow smear of male albino mouse *Mus musculus* after 48 hours of treatment with methotrexate (10 mg/kg b.wt.) showing polychromatic erythrocyte with micronuclei (MNPCE) and normochromatic erythrocytes (NCEs), X1500.

Figure 8- Photomicrograph of bone marrow smear of male albino mouse *Mus musculus* after 72 hours of treatment with methotrexate (2.5 mg/kg b.wt.) showing micronucleated polychromatic erythrocyte (MNPCE), polychromatic erythrocyte (PCE) and normochromatic erythrocyte (NCE), X1500.

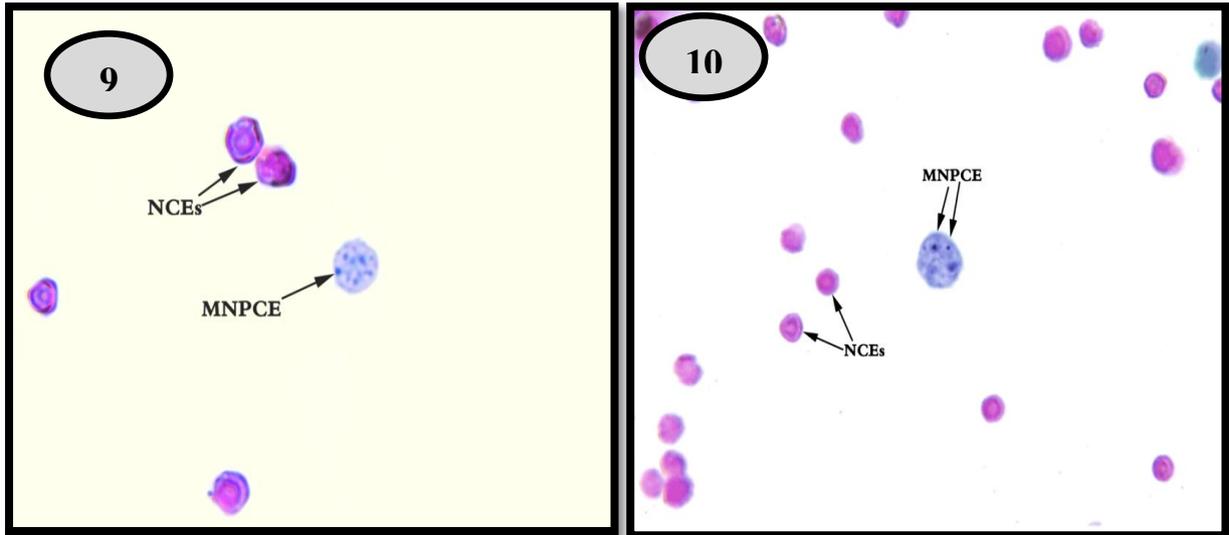


Figure 9- Photomicrograph of bone marrow smear of male albino mouse *Mus musculus* after 72 hours of treatment with methotrexate (5 mg/kg b.wt.) showing micronucleated polychromatic erythrocyte (MNPCE) and normochromatic erythrocytes (NCEs), X1500.

Figure 10- Photomicrograph of bone marrow smear of male albino mouse *Mus musculus* after 72 hours of treatment with methotrexate (10 mg/kg b.wt.) showing micronucleated polychromatic erythrocyte (MNPCE) and Normochromatic erythrocytes (NCEs), X1500.

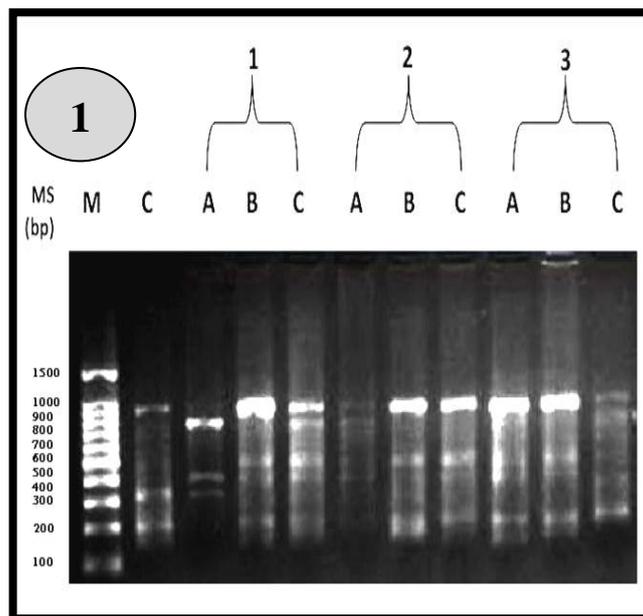


Figure 11- RAPD-PCR banding patterns of mice of the control group, the treated group 1, the treated group 2 and the treated group 3 using primer OPA-07.(Lane (M): DNA marker, Lane (C): the control group, Lane (1A): group 1 after 24 hr of treatment, Lane (1B): group 1 after 48 hr of treatment, Lane (1C): group 1 after 72 hr of treatment, Lane (2A): group 2 after 24 hr of treatment, Lane (2B): group 2 after 48 hr of treatment, Lane (2C): group 2 after 72 hr of treatment, Lane (3A): group 3 after 24 hr of treatment, Lane (3B): group 3 after 48 hr of treatment, Lane (3C): group 3 after 72 hr of treatment).

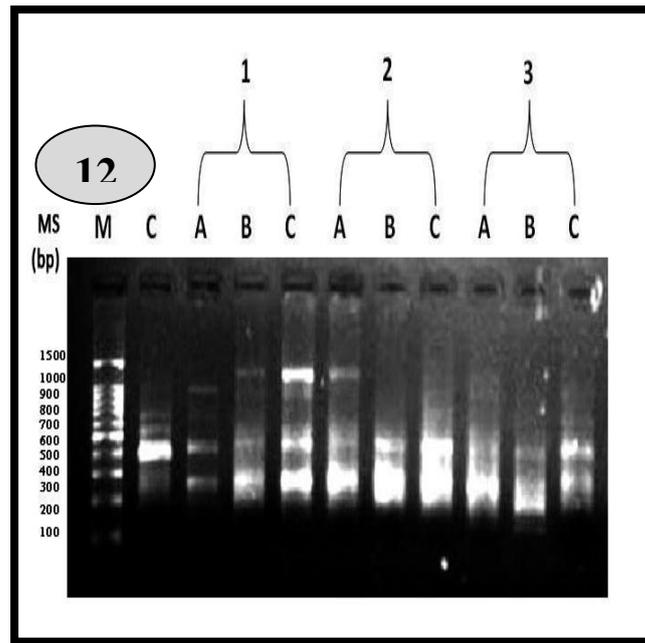


Figure 12- RAPD-PCR banding patterns of mice of the control group, the treated group (1), the treated group (2) and the treated group (3) using primer OPA-10. (Lane (M): DNA marker, Lane (C): the control group, Lane (1A): group 1 after 24 hr of treatment, Lane (1B): group 1 after 48 hr of treatment, Lane (1C): group 1 after 72 hr of treatment, Lane (2A): group 2 after 24 hr of treatment, Lane (2B): group 2 after 48 hr of treatment, Lane (2C): group 2 after 72 hr of treatment, Lane (3A): group 3 after 24 hr of treatment, Lane (3B): group 3 after 48 hr of treatment, Lane (3C): group 3 after 72 hr of treatment).

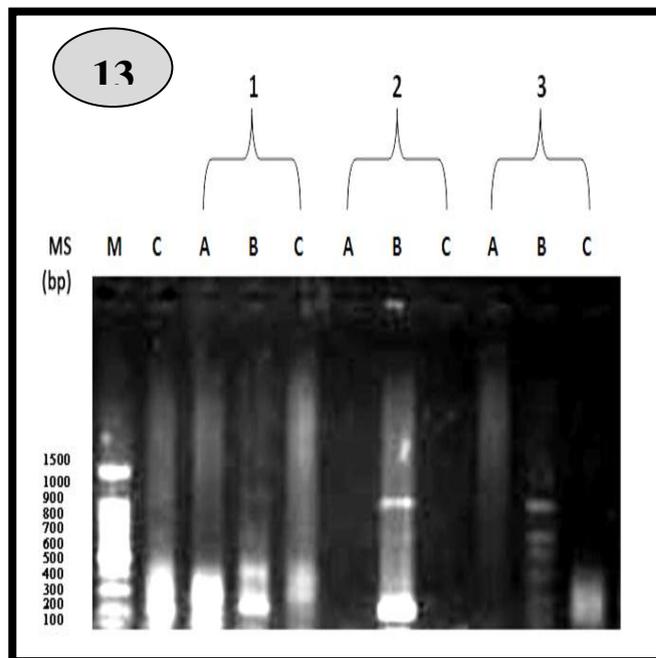


Figure 13- RAPD-PCR banding patterns of mice of the control group, the treated group 1, the treated group 2 and the treated group 3 using primer OPC-07. (Lane (M): DNA marker, Lane (C): the control group, Lane (1A): group 1 after 24 hr of treatment, Lane (1B): group 1 after 48 hr of treatment, Lane (1C): group 1 after 72 hr of treatment, Lane (2A): group 2 after 24 hr of treatment, Lane (2B): group 2 after 48 hr of treatment, Lane (2C): group 2 after 72 hr of treatment, Lane (3A): group 3 after 24 hr of treatment, Lane (3B): group 3 after 48 hr of treatment, Lane (3C): group 3 after 72 hr of treatment).