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Effect of Methotrexate and Omega-3 Combination on Cytogenetic Changes of Bone Marrow and Some Enzymatic Antioxidants: An Experimental Study

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ABSTRACT

Objective: To assess the effect of methotrexate and omega-3 combination on cytogenetic changes of bone marrow and activities of some enzymatic antioxidants.

Methods: Fifty-six mature male Wistar rats were divided into two experimental groups and a control group. The first experimental group was sub-divided into three sub-groups depending on the concentration of methotrexate (MTX): X1 (0.05 mg/kg MTX), X2 (0.125 mg/kg MTX) and X3 (0.250 mg/kg MTX), which were given intraperitoneally on a weekly basis for eight weeks. The second experimental group (MTX and omega-3 group) was also sub-divided into three sub-groups (Y1, Y2 and Y3), which were injected intraperitoneally with 0.05, 0.125 and 0.25 mg/kg MTX, respectively, weekly for eight weeks accompanied by the oral administration of 300 mg/kg omega-3. The rats of the control group were given distilled water. The enzymatic activity of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) were measured in the sera of rats. In addition, the mitotic index (MI) and chromosomal aberrations of bone marrow were also studied.

Results: MTX resulted in a significant decrease in the activities of CAT, SOD and GR compared to the controls. It also increased the MI and chromosomal aberrations of rat bone marrows. On the other hand, omega-3 significantly increased the activities of the investigated enzymatic antioxidants and reduced the MI and chromosomal aberrations in treated mice when given in combination with MTX.

Conclusions: MTX has a genotoxic effect on the bone marrow by increasing the MI and all types of chromosomal aberrations and decreasing the enzymatic activity of CAT, SOD and GR. The addition of omega-3 can lead to a protective effect by reducing the toxic and mutagenic effects of MTX.

Keywords: Methotrexate, Omega-3, Antioxidant, Wistar rat, Chromosomal aberration, Mitotic index

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1. Introduction

Methotrexate (MTX) is a folic acid antagonist because of their chemical similarity (1). Vezmar et al. (2) showed that MTX affects the synthesis of nucleic acids deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) by interfering with the biosynthesis of thymine and purines. It also directly affects the rapidly dividing and intact cells, especially those in the mucous membranes of the mouth, intestine and bone marrow (3).

Omega-3 is a type of unsaturated fats, which are classified as essential fatty acids that cannot be manufactured by the body and should be taken with food (4). Sources of omega-3 include fish oils, such as salmon, sardines and tuna, as well as soybeans, walnuts, raisins and linseed, almonds and olive oils (5). Omega-3 is used in the prevention of a number of diseases such as rheumatoid arthritis, ulcerative colitis, asthma, atherosclerosis, cancer, and cardiovascular diseases (6). A large amount of evidence indicates that omega-3 fatty acids have significant health benefits, including anti-inflammatory and antioxidant properties besides their effect on blood cholesterol levels (7). Antioxidants retard the oxidation process by different mechanisms such as the removal of free radicals (8).

Enzymatic antioxidants include catalase (CAT), which is the first line of defense in the cell that removes hydrogen peroxide formed during biological processes by converting it into an aldehyde, and superoxide dismutase (SOD). There are three major families of SOD enzymes: manganese SOD (Mn-SOD) in the mitochondria and peroxisomes, iron SOD (Fe-SOD) in prokaryote cells and copper/zinc SOD (Cu-Zn SOD) in the cytoplasm of eukaryote cells (9). Therefore, changes in the metal co-factors (manganese, iron, copper and zinc) can alter the effectiveness of SOD and may lead to diseases as a result of oxidative stress (10). Glutathione reductase (GR) is also an enzymatic antioxidant that converts the oxidized glutathione to the reduced glutathione in the presence of NADPH, which is oxidized to NADP (11). Therefore, the aim of the present study was to assess the effects of MTX and omega-3 on the cytogenetic changes of bone marrow as well as the activities of CAT, SOD and GR enzymatic antioxidants in male rats.

2. Methods

2.1. Laboratory animals and experimental design

Fifty-six mature male Wistar rats (*Rattus norvegicus*), aged 10–12 weeks old and weighing 250–300 gm, were used in the present study. The rats were kept in separate cages, with natural 13- hour light and 11-hour dark periods in a contamination-free environment with a controlled temperature (28.0 ± 1.0°C). In addition, rats were maintained on a standard diet and tap water *ad libitum.*

The rats were randomly allocated to two experimental groups and a control group. The first experimental group (MTX group) included 24 rats injected intraperitoneally with different MTX dilutions with distilled water (12). It was sub-divided into three sub-groups (eight rats per sub-group) according to MTX concentration as follows: X1 (0.05 mg/kg MTX), X2 (0.125mg/kg MTX) and X3 (0.25 mg/kg MTX). All rats were given a single dose of the specified MTX concentration weekly for eight weeks. The second experimental group (MTX and omega-3 group) included 24 rats allocated to three sub-groups (Y1, Y2 and Y3), which were injected intraperitoneally with 0.05, 0.125 and 0.25 mg/kg MTX, respectively, weekly for eight weeks accompanied by the oral administration of 300 mg/kg omega-3. The control group included eight rats that were intraperitoneally injected with distilled wa-

ter and given a single dose of distilled water orally weekly for eight weeks.

2.2. Blood collection and processing

After the end of the dosing period, 5 ml of blood were withdrawn from the heart (by cardiac puncture) using a 5 cc disposable syringe. The collected blood was immediately poured into a clean sterile screw-capped tube (plain tube) and left for coagulation in a water bath at 37°C for 15 minutes. After coagulation of blood, the plain tube was centrifuged for 5 minutes at 1500 rpm. Then the samples were stored at -20°C for subsequent analysis.

2.3. Measurement of the activity of antioxidant enzymes

The antioxidant activities of CAT, SOD and GR were measured using enzyme-linked immunosorbent assay kits purchased from Kamiya Biomedical Company (Seattle, WA, US), according to the manufacturer's instructions.

2.4. Cytogenetic study of bone marrow

Rats were killed by cervical dislocation, and their hip bones were cleaned from surrounding muscles and then dissected by cutting both ends of the bone. Five milliliters of physiological buffered saline were injected inside the bone to withdraw bone marrow into a test tube. Tubes were centrifuged at 2000 rpm/10 minutes. The supernatant was then removed, and 10 ml of KCL solution (0.075 M) were added to the sediment. The mixture was then incubated at 37 °C in a water bath for 30 minutes, with shaking from time to time. The tubes were then centrifuged at 2000rpm/10 minutes to remove the supernatant. However, 5 ml of a freshly prepared fixative solution (methanol: glacial acetic acid 1:3) were added gradually in the form of droplets into the inner wall of the tube with constant mixing. After that, the tubes were placed at 4 °C for half an hour to fix the cells. This process was repeated for three times, and the cells were then suspended in 2 ml of the fixative solution. The tubes were centrifuged at 2000 rpm for 5 minutes, and the supernatant was then removed while the cells were re-suspended in 1-2 ml of cold fixative solution. After shaking the tubes, 4–5 drops were then taken from each tube onto a clean slide from a height of about three feet to provide an opportunity for the cells and nuclei to spread well.

The slides were stained with acridine orange solution (0.01%) for 4–5 minutes, incubated in Sorensen's buffer (0.06M, pH 6.5) for a minute. and then examined using a fluorescence microscope Olympus BX 51 America at a wavelength of 450–500 nm (13, 14).

A total of 1000 cells were examined, and both dividing and non-dividing cells were calculated (13). Mitotic index (MI) was calculated according to the following formula (13): *MI= No. of dividing cells / 1000 × 100*

2.5. Analysis of chromosomal aberrations of bone marrow cells

A total of 1000 dividing cells were examined on the stained slides under a fluorescence microscope at a wavelength of 45–500 nm. The examined cells were at the first metaphase of the mitotic division, where chromosomal aberrations are clear and can be easily seen (13).

2.6. Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS®) software, version 9.1 (Cary, NC, USA) (15). Effects were expressed as mean ± standard error (SE) and statistically compared using a completely randomized design analysis of variance and least significant differences. Differences at *P* values <5 were considered statistically significant.

3. Results

3.1. Effects of MTX and MTX-omega-3 combination on antioxidant enzymatic activities

Table (1) shows significantly lower SOD activities among rats treated with MTX or MTX-omega-3 compared to controls. Moreover, sera of rats receiving relatively high doses of MTX (sub-groups X2 and X3) showed the lowest enzymatic activities of 4.29 ± 0.01 IU and 3.93 ± 0.11 IU, respectively. On the other hand, CAT activity differed significantly between treated and control rats as well as among treated rats themselves, In this respect, the controls showed the highest activity of 39.38 ±0.02 IU, while those receiving the highest MTX concentration, either alone or in combination with omega-3 (sub-groups X3 and Y3), showed the lowest activities of 30.97 ± 0.03 IU and 32.12 ± 0.06 IU, respectively.

Regarding GR activity, control rats showed a higher activity of 53.09 ± 0.05 IU compared to treated ones; however, the differences in GR activities in rats given low doses of MTX, either alone or in combination with omega-3 (sub-groups X1 and Y1), were not statistically significant. On the other hand, rats in sub-groups X3 and Y3 showed the lowest GR activities of 34.59 ± 0.63 IU and 37.15 ± 0.01, respectively, with statistically significant differences from other sub-groups.

3.2. Effects of MTX and MTX-omega-3 combination on mitotic index of bone marrow cells

Figure (1) shows a significant decrease in the MI in all treated groups compared to control. In addition, there was a reverse association between MTX concentration and MI, where rats treated with the highest dose of MTX (sub-group X3) showed a significant decrease in MI compared to all other treated rat sub-groups. In addition, rats in sub-groups treated with MTX and omega-3 (sub-groups Y1, Y2 and Y3) showed a significant increase in MI compared to their counterpart rats receiving MTX only.

Table 1. Activity of antioxidant enzymes in rats treated with MTX and MTX-omega-3

Group	**Enzymatic activity** (mean ± SE)		
	SOD (IU)	CAT (IU)	GR (µmol)
Control	6.41±0.02 ^{a}	39.38±0.02 ^{a}	53.09±0.05 ^{a}
X1 (0.05 mg MTX/ kg)	5.33±0.01 ^{b}	37.81±0.01 ^{c}	51.12±0.06 ^{a}
Y1 (0.05 mg MTX + 300 mg omega-3/ kg)	6.08±0.04 ^{a}	38.40±0.02 ^{b}	51.97±0.03 ^{a}
X2 (0.125 mg MTX/ kg)	4.29±0.01 ^{cd}	33.13±0.01 ^{e}	42.34±0.03 ^{b}
Y2 (0.125 mg MTX + 300 mg omega-3/ kg)	4.99±0.40 ^{b}	36.68±0.02 ^{d}	43.02±3.04 ^{b}
X3 (0.25 mg MTX/ kg)	3.93±0.11 ^{d}	30.97±0.03 ^{g}	34.59±0.63 ^{c}
Y3 (0.25 mg MTX + 300 mg omega-3/ kg)	4.47±0.02 ^{c}	32.12±0.06 ^{f}	37.15±0.01 ^{c}

SE, Standard error; IU, international unit; SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase; *statistically significant at $P < 0.05$; **statistically significant at $P < 0.01$. Means with different letters within the same column showed a statistically significant difference.

3.3. Effects of MTX and MTX-omega-3 combination on chromosomal aberrations of bone marrow cells

Rats receiving higher concentrations of MTX (sub-group X3) showed a significant increase in all types of chromosomal aberrations, i.e., chromatid gaps, chromosome gaps, chromatid breaks, chromosome breaks, deletions and simple fragments (Figure 2 and Table 2) than those of the control group or other treated sub-groups. All rats treated with MTX-omega-3 combination showed a significant decrease in almost all types of chromosomal aberrations compared to their counterpart rats receiving MTX alone (Table 2).

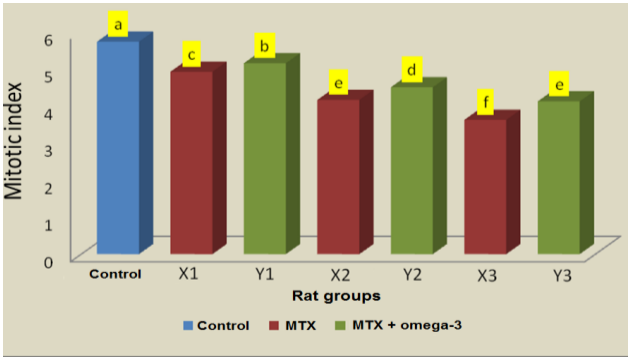

Figure 1. Effect of MTX and MTX-omega-3 on the MI of bone marrow cells of treated rats compared to the controls. The groups X1 (0.05 MTX), X2 (0.125 MTX) and X3 (0.250 MTX) were compared to the control group, while the groups Y1 (0.05 MTX+ omega-3), Y2 (0.125 MTX+ omega-3) and Y3 (0.25 MTX+ omega-3) were compared to X1, X2 and X3, respectively.

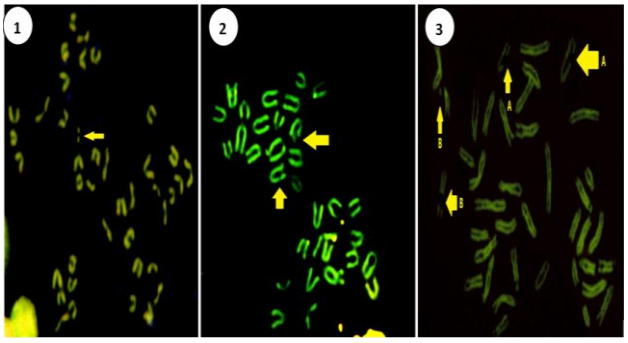

Figure 2. Effect of MTX and MTX-omega-3 on chromosomal aberration as seen under fluorescence microscope after staining with acridine orange: (1) a simple fragment; (2) a chromatid gap; (3) a chromosomal gap (A) and a chromosomal break (B).

The present experiment reveals that the addition of omega-3 to MTX alleviates its effects on the activities of the antioxidant enzymes CAT, SOD and GR, and decreases the MI as well as all types of chromosomal aberrations in the bone marrow cells. Daham et al. (16) showed that the decline in antioxidants associated with chemotherapy is attributed to the increase in lipid peroxidation caused by these kinds of drugs, which increase the level of free radicals. In addition, Weijl et al. (17) showed that some chemotherapeutic drugs have a negative effect on the antioxidant levels such as GR, whose activity decreases as a result of its involvement in many cellular processes such as cell defenses against the toxicity of some compounds. Al-Dalawy et al. (18) found that the decrease in the level of SOD is an evidence of its increased activity due to the increased release of free radicals.

MTX causes an increase in the release of free radicals, including the OH radical that causes direct damage to DNA (16). Al-Helaly (19) showed that the amount of food taken has an effect on antioxidants, where nutritional deficiency decreases the antioxidant levels, thus increasing free radicals that cause damage to DNA.

4. Discussion

Table 2. Chromosomal aberrations of bone marrow cells in rats treated with MTX and MTX-omega-3

Group	Type of chromosomal aberration (mean ± SE)						
	Chromatid gap	**Chromosome Gap**	**Chromatid breaks**	**Chromosome breaks**	**Deletion**	**Simple Fragments**	**Chromosomal aberration (%)**
Control	1.33±0.33 ^{e}	0.00±0.00 ^{e}	1.67±0.33 ^{c}	0.33±0.15 ^{c}	0.00±0.00	0.67±0.33 ^{cd}	0.04±0.005 ^{f}
X1	2.75±0.47 ^{cd}	1.50±0.28 ^{cd}	2.50±0.64 ^{bc}	1.00±0.41 ^{bc}	0.50±0.28 ^{bc}	0.75±0.25 ^{bcd}	0.09±0.02 ^{de}
Y1	1.75±0.47 ^{de}	0.75±0.25 ^{de}	1.50±0.28 ^{c}	1.00±0.00 ^{bc}	0.75±0.25 ^{abc}	0.75±0.25 ^{abc}	0.065±0.005 ^{ef}
X2	4.67±0.33 ^{b}	2.67±0.33 ^{ab}	2.67±0.33 ^{bc}	1.67±0.33 ^{ab}	0.67±0.33 ^{abc}	1.67±0.33 ^{ab}	0.14±0.006 ^{bc}
Y2	3.00±0.00 ^{c}	2.00±0.00 ^{bc}	3.00±0.057 ^{bc}	1.33±0.33 ^{b}	0.67±0.33 ^{abc}	0.33±0.15 ^{d}	0.106±0.003 ^{cd}
X3	6.80±0.37 ^{a}	3.00±0.31 ^{a}	4.60±0.74 ^{a}	2.40±0.24 ^{a}	1.40±0.24 ^{a}	1.80±0.37 ^{a}	0.20±0.017 ^{a}
Y3	5.60±0.40 ^{ab}	2.40±0.24 ^{ab}	3.60±0.24 ^{ab}	1.80±0.20 ^{ab}	1.20±0.20 ^{ab}	1.40±0.24 ^{abc}	0.16±0.003 ^{b}
LSD	1.231**	0.814**	0.602**	0.841**	0.774*	0.941**	3.499*

SE, Standard error; * statistically significant at P < 0.05; ** statistically significant at *P* < 0.01. Means with different letters within the same column showed a statistically significant difference. X1 (0.05 mg MTX/ kg); X2 (0.125 mg MTX/ kg); X3 (0.25 mg MTX/ kg); Y1 (0.05 mg MTX + 300 mg omega-3/ kg); Y2 (0.125 mg MTX + 300 mg omega-3/ kg); Y3 (0.25 mg MTX + 300 mg omega-3/ kg).

In the present study, the intraperitoneal administration of MTX to rats also caused a decrease in the MI of bone marrow and a significant increase in the rate of abnormal chromosomal aberration compared to the control rats. This finding is consistent with those reported previously (20, 21). The effect of MTX can be attributed to its ability to interfere with the genetic material, leading to the appearance of toxic and mutagenic consequences. Rushworth et al. (22) reported that MTX leads to a lack of dihydrofolate reductase, which is the key to the growth and cell division processes. This, in turn, leads to a reduction of the nucleotides involved in the building of DNA and, therefore, to a stop or obstruction of the repair mechanisms of the damaged DNA. In addition, Wong and Choi (23) concluded that MTX inhibits the action of enzymes controlling the purine metabolism, which leads to the accumulation of adenosine in addition to the damage of the molecule itself and to the occurrence of chromosomal aberrations.

Jafer et al. (24) reported the ability of MTX to induce chromosomal aberration in humans or animals by preventing the repair of DNA and affecting the proteins found in chromosomes. These findings were also confirmed by Hussain et al. (25), who found that MTX causes an increase in chromosomal aberrations. In the present study, the MI showed a significant increase in rat subgroups treated with MTX-omega-3 combination, but there was a decrease in the rate of chromosomal aberration, which confirms the role of omega-3 unsaturated fatty acids in protecting the cell from the impact of free radicals (26, 27). Attia and Nasr (28) reported the antioxidant effect of omega-3, which was attributed to the reduction in lipid peroxidation and the increase in SOD and CAT or the stimulation of GR. It is noteworthy that GR leads to the synthesis of reduced glutathione, which is important in the defense of the cell against toxic substances and the prevention of the occurrence of mutations (29).

5. Conclusions

MTX significantly decreases the activity of enzymatic antioxidants, reduce the MI and increase the chromosomal aberrations of all types in bone marrow. This gives further evidence on the genotoxic effects of MTX on the bone marrow. On the other hand, omega-3 shows a protective effect by reducing the toxic and mutagenic effects of MTX.

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Authors' contributions

INA, MMA and ASM contributed to the study design and analyzed data. All authors contributed to the manuscript drafting and revising and approved the final submission.

Competing interests

The authors declare that they have no competing interests associated with this article.

Ethical approval

The ethical clearance of this study was obtained from the Ethics Committee of the College of Science, University of Anbar (Reference No. A. D. 51 in 30/8/2015).

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