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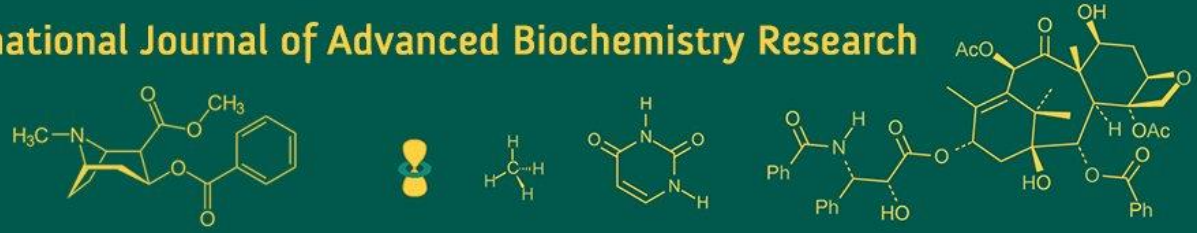

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Evaluated some of side effects of methotrexate's administration through Freund's complete adjuvant induced arthritis in rat model

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Abstract
The present study aimed to evaluate some of side effects of methotrexate adminstration (MTX) in the treatment of complete Freund's adjuvant (CFA) induced arthritis. Thirty Wistar male rats weighing 180-200 g were divided in 3 groups and arthritis was induced in them by using Complete Freund's Adjuvant, except in group I. In group II after the induction of arthritis no treatment was given. Group III received methotrexate as an anti-inflammatory medicine. After RA induction, all the animals received CFA near the site of tibio-tarsal joint subcutaneously. The clinical features of the adjuvant induced arthritis like difficulty in movement and edema in joint appeared 3 days after inoculation of adjuvant. The onset of inflammation was explosive occurring 13-15 days post inoculation with a peak onset at day15. The rats in group III received treatment with MTX subcutaneously from day 15 to day 30. At the end of experiment, blood collection was done for liver, kidney functions and heamatological observations. The present study demonstrated significant elevation in liver functions (ALT and AST) compared with normal control rats on the other hand there was increase in kidney functions but not significant. In contrast there was depletion in RBCs cont and Hb level.

Keywords: Evaluated, methotrexate's administration, Freund's complete adjuvant

Introduction
The term rheumatoid arthritis (RA) was described early by Garrod in 1859 [1]. It is an auto immune disease associated with chronic inflammation, described via pain, swelling and redness of the affected joints, stiffness of the surrounding muscles that causes damage of the cartilages and bones with substantial loss of functioning and mobility [2]. RA affecting about 0.25-1% of the general people around the world [3]. Occurrence rates of RA are greater in females than males (two to three fold), with a trail observing peaks in onset of disease at younger females (55 to 64 years) in comparison with males (75 to 84 years) [4]. The spectrum and disease development of rheumatoid arthritis is controlled via several changes in genetic, environmental and immune factors [5, 6].
Methotrexate should be applied early in protocol of RA treatment for the patients soon after the onset of RA [7, 8]. Methotrexate or cyclosporine A or anti- cytokine therapies have side effects according to the European League against Rheumatism (EULAR) and American College of Rheumatology (ACR). Disease-modifying antirheumatic drugs (DMARDs) and glucocorticoids cocktails administered as the alternates when the curative effect of initial prescription fails [9]. It is now widely used in the treatment of psoriatic arthritis, rheumatoid arthritis, acute lymphoblastic leukemia, ectopic pregnancy and inflammatory bowel diseases such as Crohn's disease and ulcerative colitis [10].

Materials and Methods
30 adult male albino Wistar rats (180-200 g) were purchased from the Veterinary Animal Unit, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. The animals were housed in plastic cages and kept at room temperature (30±5°C; 12-hr light/darkness cycle) in the animal house of the Zoology Department, Faculty of Science, Port Said University, Port Said, Egypt. They were fed with standard pellet diet and water. The rats were adapted to laboratory conditions for 7 days before starting of the experiment. All procedures of the experiment were carried out by authorized Supervisors.
The rats were randomly divided into 3 groups (10 rats for each group) according to the following design:

Group I: Negative control group, and was injected subcutaneously three time weekly only with isotonic saline solution (0.9% Nacl) from the 15th day till until the end of the study period**.**

RA was induced in the other two groups (groups II, III) by subcutaneous injection of a single dose 0.1 ml of complete Freund's adjuvant (CFA) in the footpad of the left hind paw**.** All treatments were started on day 15 after CFA injection unless the clinical signs of arthritis were clearly observed (Makhlouf *et al.*, 2013).

Group II: Arthritic group (positive control), left untreated and was injected subcutaneously three times weekly only with isotonic saline solution (0.9% Nacl) from the 15th day, after induction of RA, till 30th day.

Group III: Methotrexate (MTX) treated group that was treated subcutaneously with MTX (purchased from Orion Pharma, Espoo, Finland) three times weekly at a dose 0.3 mg/kg [11]. Blood samples were collected from retro-orbital sinus via heparinized capillary tubes, on day 30 from the beginning of experiment under diethyl ether anesthesia into two tubes. The first tube contained special anticoagulant for hematological assays. The second plain gel tube for separation of serum. Then the tubes were centrifuged at 5000 R.P.M for 10 min and the top yellow serum layer was pipetted off then stored at -10ºC until used.

I) Biochemical Assays

i) Liver Functions: Determination of serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST)

ALT or AST activity was determined by the kinetic method as described by Tietz [12] using available commercial kit which was purchased from EGY- CHEM for Lab Technology (Badr city, industrial Area, Cairo, Egypt).

Procedure

1. 100 µl of sample and 1.0 ml working solution (R1: 9 volumes + R2: 1 volume) were added to dry test tubes.
2. The tubes were mixed and read at λ 340 nm after 1, 2, 3 minutes.
3. The change in mean absorbance per minute (ΔA/min) was determined.

Calculation

The activity of ALT or AST was calculated using the following formula:

ALT or (AST) (U/L) = ΔA/min Sample × 1746

ii) Kidney functions

Determination of serum creatinine

The concentration of creatinine was determined by fixed rate kineticmethod as described by Henry [13] using available commercial kit which was purchased from EGY- CHEM for Lab Technology (Badr city, industrial Area, Cairo, Egypt).

Procedure

1. 1.0 ml of working solution (R1: 1 volume + R2: 1 volume) was added to all tubes.
2. 100 µl of sample and 100 µl of standard were added to sample tube and standard tube.
3. All tubes were mixed well, the initial absorbance (A1) of the standard and specimen were read at wavelength 492 nm, and then after exactly 2 minutes, the absorbance (A2) of both standard and specimen were read again.

Calculation

[A2 - A1 = $A_{specimen}$ or $A_{standard}$]

The level of creatinine in sample was calculated using the following equation

$$\text{Creatinine concentration (mg/dl)} = \frac{(A)\text{Sample}}{(A)\text{Standard}} \times 2.0$$

Where, 2 is the standard creatinine concentration.

Determination of serum urea:

The level of urea was determined by a colorimetric method as described by Chaney and Marbach [14] using available commercial kit which was purchased from EGY- CHEM for lab technology (Badr city, industrial Area, Cairo, Egypt).

Procedure

1. 50 µl of reagent 2 and 1.0 ml of reagent 3 were added to blank tube.
2. 50 µl of reagent 2, 1.0 ml of reagent 3 and 10 µl of sample were added to sample tube.
3. 50 µl of reagent 2, 1.0 ml of reagent 3 and 10 µl of reagent 1(standard) were added to standard tube.
4. All tubes were mixed well.
5. After incubation for 3 min. at 37ºC and then 200 µl of reagent 4 was added to all tubes.
6. After incubation 10 min at room temperature, the absorbance of sample and standard tubes was read atλ578nm. Against blank.

Calculation

The level of urea in sample was calculated using the following equation: $\text{Urea (mg/dl)} = \frac{\mathbf{(A)Sample}}{\mathbf{(A)Standard}} \times 50$

Where, 50 is the standard urea concentration.

II) Hematological parameters: (Complete blood picture)

Determination of hematological parameters was completed by using cell counting equipment (Humacount, Germany**)** according to Buttarello and Plebani [15]. Hematological assays includes hemoglobin (Hb), erythrocyte (RBCs) counts, leucocytic (WBCs) count and platelets count.

IV) Statistical analysis

The result values were expressed as means ± standard error (SE) for 8rats in each group. Tabulation and graphics were designed using Microsoft Excel XP software. Data were statistically analyzed using Statistical Package for Social Science (SPSS) version 18, software. One-way analysis of variance (ANOVA) test was performed to statistical analysis for determining the statistical significant differences between means of different groups. Data were considered statistically significant when the P values were ≤0.05**.**

Results

I) Biochemical assays

i) Liver function tests

The effect of subcutaneous injection of MTX on serum liver enzymes AST and ALT at day 30 of the experiment was illustrated in table (1) and figure (1). There was significant increase in serum AST and ALT in MTX treated group compared with arthritic group (P >0.01).

Table 1: Serum AST and ALT activities (U/L) in control, arthritic and MTX treated group at day 30 of the experiment.

Groups\ parameters	Control	Arthritic	MTX
AST (U/L) MEAN ±SE)	22±1.5	32.2±2.6	63.2±3.0*
% Of change compared with arthritic group			96.2%
ALT (U/L) MEAN ±SE)	20.5±2.05	31.3±2.5	60.9±3.3*
% Of change compared with arthritic group			94.5%

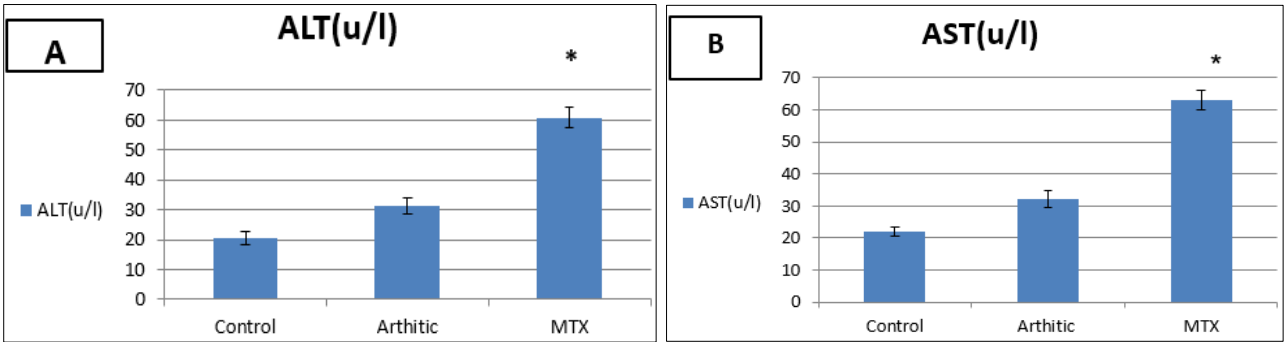

Fig A and B: ALT and AST activities in control, arthritic and MTX treated group respectively.
***** Represents significant difference between MTX treated group and non treated arthritic group.

ii) Kidney function parameters

Effect of MTX treatment on serum kidney creatinine and urea concentrations: As shown in table (2) and figure (2), there were no significant (P>0.05) changes in creatinine and urea levels between control group, non-treated arthritic group and MTX treated groups.

Table 2: Effect of MTX treatment on serum creatinine and urea (mg/ dl) concentrations.

Groups\Paramet	Control	Arthritic	MTX
Creatinine(mg/dl) M ±SE	0.45±0.03	0.43±0.02	0.51±0.03
% Of change compared with arthritic group			18.6%
Urea (mg/dl) (M ±SE)	43±3.4	54±3.2	79±2.5
% Of change compared with arthritic group			46.2%

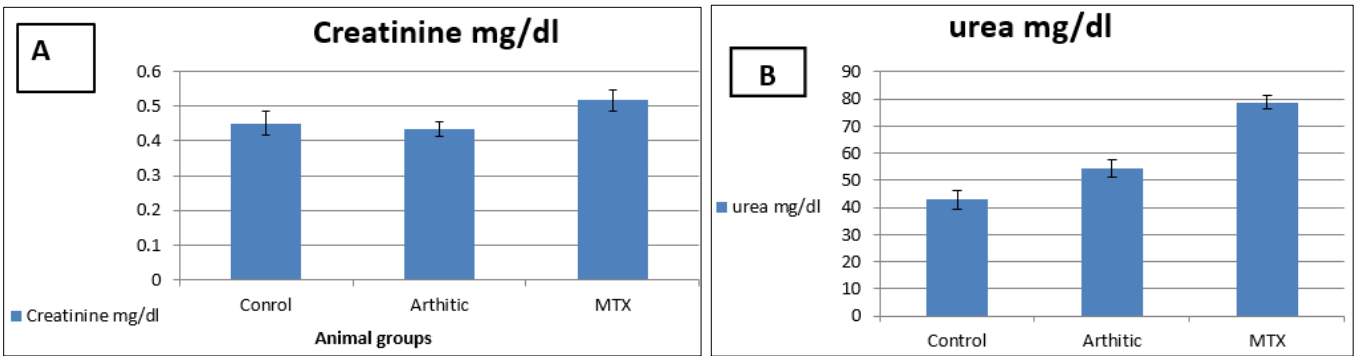

Fig 2 (A and B): Serum concentration of creatinine and in control, arthritic and MTX treated group respectively.

II) Hematological assays (Complete blood picture)

i) Hemoglobin level and RBCs count

Effect of SV treatment on red blood cells (RBC`s) count and hemoglobin (Hb) level. Data in table (3) and figure (3) revealed that there were no significant differences in RBCs count and Hb level between non treated arthritic group and normal control group (P=1.0), (P=0.8) respectively. Conversely, there was significant decrease (P< 0.001) in RBCs count and Hb level between MTX treated group and non-treated arthritic group.

Table 3: Effect of MTX on red blood cells (RBCs x10^6/ml) count and Hb-g/dl level.

Groups\Parameters	Control	Arthritic	MTX
BCs x10^6 (M±SE)	7.71±0.36	7.75±0.35	4.18±0.67*
% of change compared with arthritic			46%-
Hb-g/dl (M±SE)	13.0±0.58	12.1±0.33	7.7±1.09*
% of change compared with arthritic			36.3%-

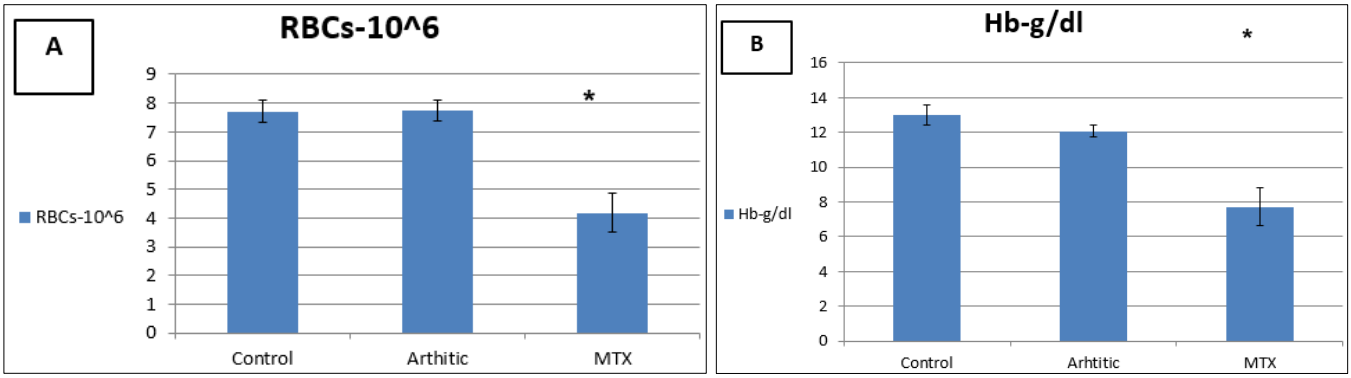

Fig 3 A and B: RBCs count and Hb level in control, arthritic and MTX treated group**.**
***** Represents significant difference between non treated arthritic group and MTX treated group.

ii) Platelets (PLTx10^3/ml) and white blood cells (WBCs x10^3/ml) count.

As shown in table (4) and figure (4), there were no significant differences in platelets count between arthritic group and normal control (P> 0.05)also, there was no significant changes in platelets count between MTX treated group compared to arthritic group (P> 0.05). There was significant increase in WBCs count in non-treated arthritic group compared with normal control group (P<0.05)**.** Conversely, there was no significant difference in WBCs count between MTX treated groups compared to arthritic group (*P*> 0.05).

Table 4: Effect of MTX treatment on platelets and white blood cells (WBCs x103/ml) count.

Groups\Parameter	Control	Arthritic	MTX
Platelets (10^3/ml) (M±SE)	388±56	572±54	900±213
% of change compared with arthritic			57%
WBCs x103 (M±SE)	11.8±1.91	6.68± 0.90	9.5 ± 0.35
% of change compared with arthritic			19%-

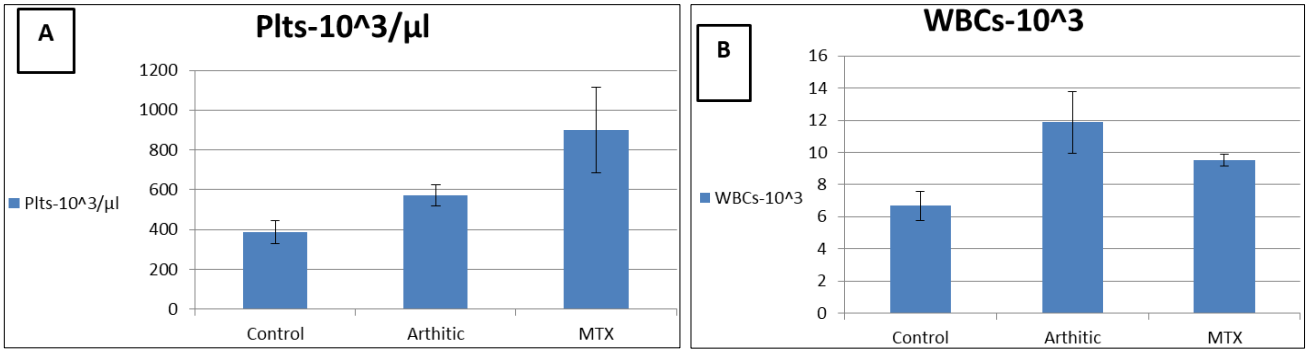

Fig 4: Platelets and WBCs count in control, arthritic and MTX treated groups.
***** Represents significant difference between non treated arthritic group and normal control group**.**

Discussion

Methotrexate (MTX), a folic acid antagonist, is widely used as a cytotoxic chemotherapeutic agent for the treatment of breast cancer, lymphoma, osteosarcoma, head and neck cancers, and also in the therapy of non-oncologic disorders such as psoriasis and rheumatic diseases [16]. MTX is considered as first choice therapy among the DMARDs for the treatment of RA [17]. Its favorable efficacy and toxicity profile results in a comparatively low withdrawal rate. Nevertheless, the toxicity spectrum in RA patients is widespread with respect to symptoms and intensity, including serious osteopenia and sometimes fatal cytopenia, pneumonitis, hematologic disorders and liver disease [18]. In the present work, liver enzymes level was significantly increased in MTX treatment group. This elevation of enzymes is considered as a side effect during MTX treatment period. This finding was in agreement with Gilani, Khan [19] and Kasper, Fauci [20] who reported that the most serious side effects of MTX therapy is liver toxicity, where mild hepatitis, cholestasis, fatty changes, fibrosis and cirrhosis have been reported in patients receiving MTX for rheumatoid treatment. Mitochondrial dysfunction and generation of ROS have been correlated with administration of MTX accompanied with clinical hepatotoxicity [21]. MTX hepatotoxicity could be also as a result of increase permeability, damage or necrosis of hepatocytes [22].

The risk of severe toxicity is increased about 4-fold in patients with renal impairment. Concomitant use of other potentially nephrotoxic drugs, including NSAIDs, and preexisting renal insufficiency are additional risk factors. Therefore, although direct toxicity of low dose MTX has not been proven, the dosage regimen should be adjusted in patients with severe renal impairment [18]. Since more than 90 % of MTX is excreted via the kidneys, nephrotoxicity is one of the important reasons for restricting its use [23]. High-dose MTX produces a wide clinical range of damages in the kidneys varying from subclinical tubulopathy to acute renal failure [24]. Reactive oxygen radical overproduction and neutrophil infiltration are among the significant causes of MTX-related renal toxicity [25].

In the present study**,** RBCs count was in the normal range values in all groups except for MTX treated group, there was a significant decrease in RBCs count and Hb level after treatment. MTX, an immunomodulator drug, has many commonly reported hematological adverse effects such as leukopenia, pancytopenia, anemia, megaloblastic anemia [19]. MTX is retained within the cells as polyglutamates which inhibit folate metabolism then blocking the enzymes dihydrofolic acid reductase and thymidylate synthase, thereby inhibiting synthesis of purines and pyrimidines and decreasing DNA and RNA synthesis [26, 27]. Recent studies also reported that MTX treatment increases marrow adiposity in both short and long term of MTX chemotherapy [28, 29].

Conclusion

Methotrexate is widely used as a cytotoxic chemotherapeutic agent for the treatment of cancer and rheumatoid arthritis. The most serious side effects of MTX therapy is liver toxicity and depletion in Hb content where, severe anemia reported in arthritic rats which receiving MTX**.** So we recommended for patients to take liver supports as Silymarin and folic acid supplementation to diminish severe side effects caused by MTX administration.

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