Some Histogenetic Aspects of Methotrexate Associated with Juvenile Idiopathic Arthritis (JIA) Treatment Doses

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Abstract

A total of (36) mice in the age of (1-3) weeks were selected to evaluate the methotrexate effect on some histogenetic aspects of kidneys. Treatment lasted for three months, and results showed marked hemorrhage, cortical tubular basophilia, necroses, and degeneration in the autopsied organ of animals with low oral hydration. Besides, changes in the DNA methylation pattern were noticed in the RAPD-PCR profile upon analysis with agarose gel electrophoresis.

Keywords*: Methotrexate, Kidney, DNA methylation, JIA, RAPD-PCR.*

Introduction

Methotrexate (MTX) is an antimetabolic agent and a structural analog that affects the metabolism of folic acid and inhibits cell proliferation [1&2]. This drug came into the clinical application as an advanced antineoplastic drug in 1948, due to its indirect effects on DNA synthesis [3]. It is a well-known immunosuppressive agent widely used for the treatment of many rheumatologic, dermatologic, and hematologic diseases successfully [3-6]. In children, methotrexate is the cornerstone for the treatment of juvenile idiopathic arthritis (JIA), the most common chronic rheumatic disease of childhood, and the major cause of disability [6-8]. It acts by inhibiting dihydrofolate reductase, which reverts DNA hypomethylation and reducing cell proliferation [8-10]. Methotrexate dose is usually eliminated by renal excretion, which increases the risk of renal damage and nephrotoxicity that sometimes develops as a side effect due to inadequate oral hydration and increased urine acidity [11&12]. Moreover, it was found that gene dysregulation associated with variability in DNA methylation might contribute to the initiation of cancer diseases [3-5]. Thus, many perturbations raised considering the side effects of using MTX as the cornerstone for prolonged treatment of JIA[11-13].

In accordance, this study aimed to investigate the side effects of methotrexate treatment associated with the treatment doses of juvenile idiopathic arthritis on the kidney tissue and DNA methylation pattern of white mice as a model.

Methods

2.1. Study animals

Healthy mice of *Mus musculus* strain obtained from the Association of Genetic & Environmental Resource Conservation (AGERC)/Iraq during 2019. Mice aged (1-3) weeks and weighed (20-23 gm) were housed and fed according to the procedure mentioned by Ref. [14].

2.2. Experimental design and Methotrexate administration

All animal studies conducted were according to the Guide of Experimental Animals [15]. Intraperitoneal injections of Methotrexate brand of (FRESENIUS KABI/ USA) used in the administration protocol summarized in (Table 1) according to the procedure cited in Ref[16].

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Table 1:- Methotrexate administration protocol of the study

Treatment Groups *n*=12 mice /GP 1:1 male to female ratio	MTX Dose mg/m^2 BSA/Week	Water Intake ml/day	Water Intake (L / 3 months) Study Interval	Total MTX concentration mg/m^2
$_{Gm}1$	10	100	8.4	120
G_{m2}	10	300	25.2	120
$_{G}C$	0	300	25.2	0

*MTX doses were calculated to bodyweight Index according to the FDA protocols cited in Ref.[17]

Four mice of both sexes per treatment group were autopsied at the end of each month after anesthetization with the injection of (100 mg/kg of Ketamine and 5 mg/kg Xylazine (Alfasan /Turkey); kidneys tissues were immediately removed after autopsy according to the procedure cited in Ref.[18]. Kidneys were cut into two parts; one half was fixed in (10% formalin) for histopathological examinations, while the other half was placed into liquid nitrogen (BIOBASE/ Meihua /China) at -20℃ for genetic assessment according to Asci *et al*.[19].

2.3. Histological Valuation

Kidney fixed in 10% formalin were embedded in paraffin, and tissue sections were cut at 5µm by Microtome (BIOBASE/ Meihua /China), mounted on slides after staining with Hematoxylin Eosin Y solutions (HE Stain Kit/TissuePro/USA) according to Ref.[18]. Sections were examined by a light microscope (Optika/Italy) and photographed by mounted Camera (Optica- Italy 4083-B5). Significant histopathological abnormalities were evaluated and recorded accordingly.

2.4. Genetic assessment of Kidneys DNA methylation pattern

Five methyl Cytosin levels were relatively quantified after the enzymatic hydrolysis of the genomic DNA with HpaIIand MspI restriction enzymes (1U / 1µg DNA/Thermo Scientific/France). DNA extractions were performed using the DNA isolation kit (Genomic Prep /Amersham Biosciences/UK) following the instructions of the manufacturer. Then extracted DNA was resuspended in Tris-EDTA, and the quantity of the extracted DNA checked by spectrophotometer (Schimadzo/Japan) as described in [20]. For this analysis, RAPD-PCR (Eppendorf /Germany) primer set (Ready-To-Go RAPD- PCR analysis kit/ Amersham Biosciences/ UK) used as instructed by the manufacturer company. Three samples of Kidney DNA (control, MTX 10mg, MTX 15mg) were subjected to PCR with a single primer concentration of 25 pmol in a reaction volume of 25 μl and a DNA marker of (pUC19 DNA MspI/HpaII Marker/ Thermo Scientific/ France). Each change observed in RAPD profiles was scored as described in [22].

Results

This study was conducted to observe the histological and epigenetic changes in the kidneys tissue due to methotrexate treatment for three months of (10 mg/m^2 BSA/ week) dose associated with the therapeutic doses of the juvenile idiopathic arthritis (JIA) but under low water intake circumstance. The histopathological examination of the kidneys of the study groups experimental animals showed the following results:-

-Macroscopic Histopathological Changes:

The excised kidneys of the ($\mathbf{G_{m1}}$) group of mice that received intraperitoneal injections of (10 mg/m^2 BSA/ week) treatment doses for three months durations with low water intake showed congestion and enlargement signs. While the kidneys of the ($\mathbf{G_{m2}}$) group of animals that received the same treatment with the same duration but with average water intake showed normal kidneys texture except for the last two weeks of the third month

where excised kidneys of the (G_{m2}) group showed congestion marks. On the other hand, the (G_c) group that received intraperitoneal injections of (0.1 ml) of normal saline showed normal shape and size of kidneys.

-Microscopic Histopathological Changes:

The examined kidney tissues of the (G_{m1}) group of mice showed marked aggregation of lymphocyte, congestion with fatty degeneration, and dilated tubules in the second month of treatment as declared in (Figure 1).

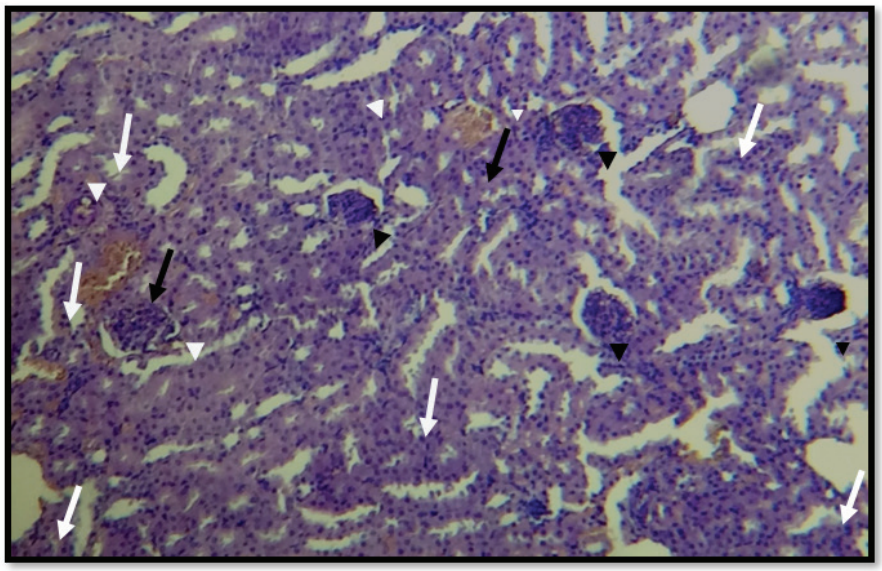

Figure 1:- Microscopical findings of the kidney tissues in the methotrexate treatment group (G_{m1}) for two months with low water intake showing marked aggregation of lymphocyte (black arrows head), congestion (black arrows), fatty degeneration (white arrows), dilated tubules (white arrows head) examined under a light microscope by different magnifications.

Whereas, the prolonged treatment for the third month of the (G_{m1}) group of mice with continued low water intake caused marked hemorrhage, cortical tubular basophilia, necroses and degeneration in the autopsied kidney tissues for both sexes of animals as manifested in (Figure 2).

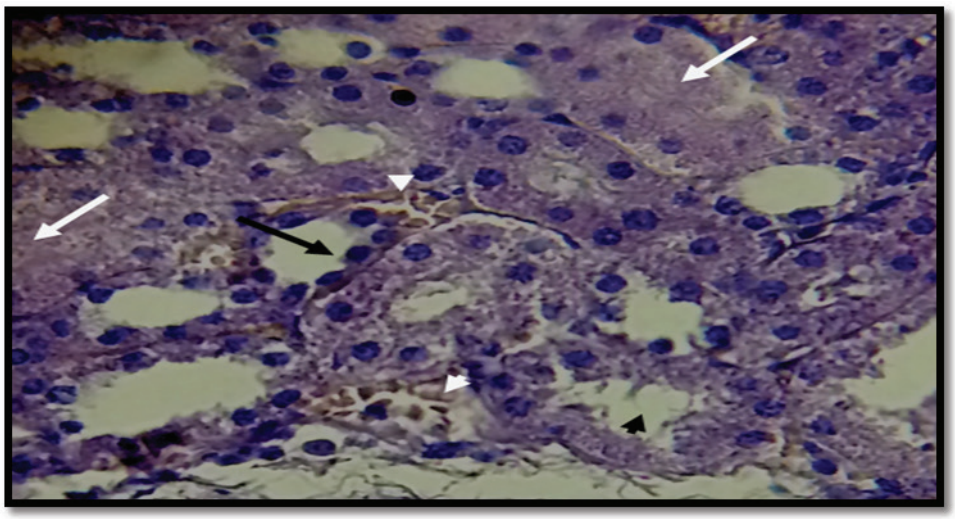

Figure 2:- Microscopical findings of the kidney tissues in the methotrexate treatment group (G_{m1}) for three months with low water intake showing marked hemorrhage (arrows head), cortical tubular basophilia (black arrow), necroses (white arrows)degeneration(black arrowhead) examined under a light microscope

by different magnifications.

Yet, the (G_{m2}) group of mice treated with the same methotrexate dose but with sufficient water content showed signs of cortical tubules basophilia with infiltration of lymphocytes only in the third month of methotrexate treatment. However, the epigenetic effect of methotrexate treatment on the genomic DNA methylation declared in (Figure 3).

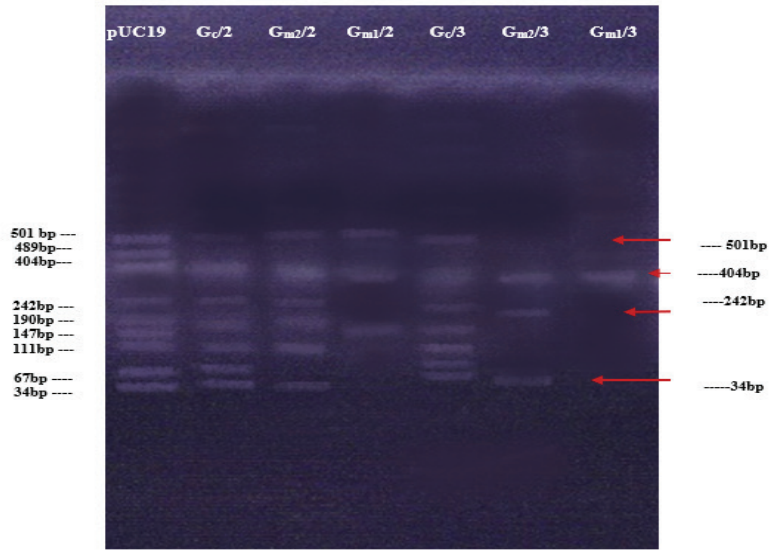

Figure 3:- Gel picture of RAPD-PCR products of Kidney tissues of treatment samples for two and three months of methotrexate associated with JIA doses on (1.7%) agarose gel electrophoresis stained with ethidium bromide. Gel visualized under UV-Transilluminator and pictured using a gel documentation system. pUC19 Lane is Marker DNA; Lane G_c is control group kidney DNA; Lane G_{m1} is a methotrexate treated group with low water content; Lane G_{m2} is a methotrexate treated group with sufficient water content. Numbers 2/3 means DNA extracts of two and three months of treatment.

The summary of RAPD-PCR products after the analysis of the banding pattern of genomic DNA methylation is outlined in (Table 2).

Table 2:- RAPD-PCR profile of DNA methylation pattern of kidney tissues treated with Methotrexate.

Banding profile of pUC19 DNA bp	Banding profile of Gc /2 DNA/bp	Banding profile of Gc/3 DNA/bp	Banding profile of Gm2/2 DNA/bp	Banding profile of Gm2/3 DNA/bp	Banding profile of Gm1/2 DNA/bp	Banding profile of Gm1/3 DNA/bp
501	+ve	+ve	+ve	-ve	+ve	-ve
404	+ve	+ve	+ve	+ve	+ve	+ve
242	+ve	+ve	+ve	+ve	+ve	-ve
190	+ve	+ve	+ve	-ve	-ve	-ve
111	+ve	+ve	+ve	-ve	-ve	-ve
67	+ve	+ve	-ve	-ve	-ve	-ve
34	+ve	+ve	+ve	+ve	-ve	-ve

*+ve refers to band presence; -ve refers to band absence in the agarose gel banding profile

Discussion

Juvenile idiopathic arthritis (JIA) is an autoimmune disease associated with functional disability and many complications in the child's life [16]. It is prevalent in children and teenagers, causing uncontrolled inflammations and irreversible damage to joints and other tissue [23]. In Iraq, there is a significant increase in the disease ratio between post-school and young children [24-26]. Thus, the improvement of the disease outcomes of children with JIA remains a critical challenge for the clinician, where most of them rely on methotrexate as their first-line of treatment agent[3&4]. Methotrexate is a standard treatment used in many countries for children who suffer from moderate to severe arthritis; it is primarily cleared through kidneys where renal excretion eliminates about 80% of the dose[25 & 26].

However, the elimination of the methotrexate dose depends on the urine alkalinization where methotrexate is a weak acid and tends to precipitation at urine pH below (6.0) leading to nephrotoxicity [11-13]. Accordingly, the present research intended to evluate the effect of methotrexate administration on kidney tissues of mice under insufficient oral hydration conditions. The equivalences of Dutta and Sengupta, 2016 [27] for men and mice ages were considered in the experimental animals to be in correspondence to the ages of young children in humans. Besides, treatment groups were divided into two groups according to the oral hydration circumstances in order to study the effect of methotrexate on children with low water intake, where most of the children might forget to drink sufficient water for at least six hours during the school day.

The kidneys of the treatment group with inadequate oral hydration (G_{m1}) appeared more affected in all treated animals than the other group (G_{m2}), where the kidneys showed marked cortical dilatation of tubules and necrosis. The same result obtained by Chelab and Majeed in 2009[26] where they had indicated that methotrexate crystals accumulated in the nephron tubules and the dilatation of nephric tubules of renal cortex occur due to thickening of basement membranes of tubules and precipitation of the methotrexate crystals in the nephron of the kidneys due to water dehydration. The same results were confirmed by Asci *et al*., 2017[19], where they established the etiology of MTX-induced nephrotoxicity.

Hence, genomic instability results in multiple molecular events believed to be a driving force in cancer etiology [22]. In this study, the RAPD-PCR technique with two methylation-sensitive restriction enzymes were used to analyze the genomic instability. Banding profile showed no change in the bands of the control group. While the (G_{m1}) group showed a significant difference in the banding pattern; where four bands of the molecular weights of (190bp,111bp,67bp, and 34bp) were missing from the profile after two months of treatment. Moreover, six bands were missing from the RAPD-PCR profile after three months of treatment with the molecular weight of (501bp, 242bp, 190bp, 111bp, 67bp, and 34bp) respectively.

Accordingly, the (G_{m2}) group showed changes in the DNA profile of the methylation pattern with one missing band of (67bp) molecular weight upon two months of treatment and three absent bands of molecular weights of (190bp, 111bp, and 67bp) at the end of the third month of methotrexate treatment respectively. These results, however, might indicate a probable role of methotrexate as a carcinogen and a teratogen that might affect the life quality of the treated child in the future. Those results agree with the results obtained by de Andres *et al*., in 2015[5] where they confirmed global DNA hypomethylation in patients with rheumatoid arthritis treated with methotrexate and the role of aberrations in the DNA methylation system as an essential tool in the initiation of certain human diseases.

Conclusions

The consequence of this study is related to the dose and route of administration of methotrexate in JIA issued to prevent treatment side effects as it was concluded that sufficient oral hydration could reduce histopathological side effects of methotrexate but not hypomethylation. Also, the nephrotoxicity of methotrexate necessitates caution when considering it by the physicians for the treatment of patients with kidney function impairment. Last, JIA treatment with methotrexate predetermines the warning of the child's parent about the outcomes and life quality after this drug's treatment since it is proven carcinogenic and probably teratogenic.

Conflict of Interest: The authors declare that there

is no conflict of interest.

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Ethical Clearance: No ethical issues.

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