

Protective effect of pentoxifylline in renal toxicity after methotrexate administration

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Abstract. – Introduction: Nephrotoxicity is an important side-effect of treatment with Methotrexate (MTX). Pentoxifylline (PTX) is an anti-inflammatory and anti-oxidant agent. We hypothesized that pentoxifylline may afford renal protection by downregulating TNF- α as well as by improving cellular anti-oxidant activity.

Materials and Methods: Forty five male Wistar rats were assigned to 3 groups of 15 animals each: Group 1: control group (0.9% saline). Group 2: MTX; injected with 20 mg/kg MTX intraperitoneally (i.p.). Group 3: MTX+PTX injected i.p. MTX (20 mg/kg) + PTX (50 mg/kg) i.p. PTX was administered since 3 days before MTX administration and continued for 6 days. After 6 days rats were anesthetized and serum sampled and renal tissue removed for biochemical and histological evaluation.

Results: Data showed that glutathione peroxidase (GPx), superoxide dismutase (SOD) activities were lower in PTX+MTX group comparing to MTX group significantly ($p < 0.05$). Renal tissue injury index and percent of TUNEL positive cells, renal tissue malondialdehyde (MDA) levels, serum BUN (Blood Urea Nitrogen), creatinine (Cr) and TNF- α levels were higher in MTX group comparing to MTX+PTX group significantly ($p < 0.05$).

Conclusions: In this study, the increased level of tissue MDA and serum TNF- α level together may be suggested that the underlying mechanism is related to direct toxicity of MTX rather than blockage in folate synthesis in kidneys. PTX administration also attenuated renal tissue injury and number of apoptic cells and suppressed the elevation of BUN and Cr levels. However, further studies are essential to elucidate the exact mechanisms of MTX-induced renal toxicity, and protection and the effect of PTX.

Key Words:

Methotrexate, Kidney, Pentoxifylline, Rat.

Introduction

Methotrexate (MTX), a folic acid antagonist, is widely used as a treatment for malignancies as well as in the treatment of various inflammatory and autoimmune disorders. MTX is classified as an antimetabolite drug, which means that it is capable of blocking the metabolism of cells. The efficacy of MTX is often limited by severe side-effects and toxic sequelae, such as intestinal injury hepatotoxicity and suppression of bone marrow. In fact, MTX depletes folate species and the lack of folate affects several biochemical pathways, including purine metabolism. These metabolic alterations are responsible for both the therapeutic and the toxic effects of MTX¹⁻³. Furthermore, MTX can cause increased serum creatinine levels, uremia and hematuria, while its administration in high doses has been reported to cause acute renal failure. Therefore, nephrotoxicity is an important side-effect of this drug. The mechanism of MTX-induced renal toxicity has been proposed as follows: the direct toxic effect of MTX⁴; inhibition of several enzymes relating to DNA synthesis⁵; enhancement of the production of reactive oxygen species (ROS)⁶. Endogenous sources of ROS include mitochondrial electron transport chain, cytochrome P450s and NADPH oxidase. Xanthine dehydrogenase/oxidase (XD/XO) is another source that has a close association with inflammation⁷. Thus, MTX may cause cellular injury through detrimental effects on DNA, proteins, lipids and other cellular structures^{8,9}. Severe MTX poisoning with acute hepatorenal dysfunction has been treated with plasma exchange and hemodialysis¹⁰. MTX-induced toxicity appears to be a consequence of the interaction of many factors: dosing schedule and length

of treatment, patients' risk factors, type of disease, presence of genetic and molecular apoptotic factors¹¹.

In addition, MTX inhibits cytosolic NADP-dependent dehydrogenase and NADP malic enzyme and causes a decrease in intracellular NADPH levels. NADPH is essential for glutathione reductase enzyme that sustains the levels of reduced glutathione (GSH), which is an important cytosolic antioxidant substance. Thus, the reduction in the levels of GSH due to MTX leads to a weakening of the effectivity of the antioxidant defense system protecting the cell against ROS¹².

Prevention and treatment of MTX-induced renal dysfunction are essential to prevent potentially life-threatening MTX-associated toxicities, especially myelosuppression, mucositis, and dermatitis. In addition to conventional treatment approaches, dialysis-based methods have been used to remove MTX with limited effectiveness. Regarding above mentioned problems, finding agents in which can prevent or decrease side effects of MTX is necessary.

Pentoxifylline (PTX) (3,7-dimethyl-1-(5-oxohexyl) xanthine) is a methylxanthine derivative that is used in patients with venous leg ulcers or peripheral vascular disease to improve blood flow¹³. It is a nonspecific phosphodiesterase inhibitor and down regulates several proinflammatory cytokines including TNF- α ¹⁴. The enhancement of blood flow and oxygenation of the tissues are the basic points of the therapeutic effects of PTX^{15,16}. PTX prevents extra release of superoxide and hydrogen-peroxide ions, and its demonstrated that PTX has anti-oxidant activity¹⁷. We thus hypothesized that pentoxifylline affords renal protection by downregulating TNF- α as well as by improving cellular antioxidant activity.

Materials and Methods

Animals

Male Wistar rats were obtained from Laboratory Animals Care Center of Tabriz University of Medical Sciences (Tabriz, Iran). Rats were weighing 230-270 g and they were approximately 7-8 weeks old. The experiments were performed according to the guidelines of our Ethics Committee. After an adaptation period of 3 days, rats (n=45) were assigned to 3 groups of 15 animals each:

Group 1: Control group (0.9% saline).

Group 2: MTX; injected with 20 mg/kg MTX intraperitoneally i.p.

Group 3: MTX+PTX; injected i.p. MTX (20 mg/kg) + PTX (50 mg/kg) i.p. PTX was administered since 3 days before MTX administration and continued for 6 days. At 6th day, rats were administered anesthetics with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketalar, Eczacibasi, Istanbul, Turkey) and were sacrificed and the renal tissue were quickly removed and were stored at -20°C until biochemical analysis including glutathione peroxidase (GPx), superoxide dismutase (SOD) and malondialdehyde (MDA). Blood also sampled and serum obtained for measuring blood urea nitrogen (BUN) and creatinine (Cr) and tumor necrosis factor-alpha (TNF- α).

Histopathological Analyzes

The specimens fixed in 10% formalin were embedded in paraffin. Sections of 4 μ m were prepared, stained with hematoxylin and eosin and then, examined, by a pathologist under a light microscope. The histopathologic scoring analysis was performed according to previously described methods, the assessment was expressed as the sum of the individual score grades from 0 (no findings), 1 (mild), 2 (moderate), to 3 (severe) for each of the following 4 parameters from kidney sections: tubular cell swelling, cellular vacuolization, pyknotic nuclei and medullary congestion¹⁷.

TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was used to assess the DNA fragmentation in paraffin-embedded tissue sections. The assay was conducted according to the manufacturer's instructions (Roche, Mannheim, Germany). Total cell population and TUNEL positive cells were counted. TUNEL positive cells were expressed as percentage of total cells¹⁸.

Measurement of BUN, Cr and TNF- α levels

The serum BUN and Cr levels were determined spectrophotometrically (Pars Azmoon, Tehran, Iran). Serum level of TNF- α was measured by an enzyme-linked immunosorbent assay (ELISA) using rat serum TNF- α immunoassay kit (Bender Med Systems, Vienna, Austria)¹⁹.

GPx Activity

One kidney of each animal was snap-frozen in liquid nitrogen and stored at -70°C until further preparation. In order to measure cytosolic enzyme activity, the kidney samples were homogenized in 1.15% KCl solution. GPx activity in kidney was measured using the method described by Paglia and Valentine²⁰. GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione is immediately converted to reduced form with a concomitant oxidation of NADPH to NADP^+ . The decrease in absorbance at 340 nm is measured (Ransod, Randox Laboratories Ltd., United Kingdom). Results obtained were expressed as GPx Units/mg protein²⁰.

SOD Activity

SOD activity in kidney tissue was determined by using xanthine and xanthine oxidase to generate superoxide radicals which then react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity was then measured by the degree of inhibition of this reaction (Ransod, Randox Laboratories Ltd., United Kingdom). Results obtained were expressed as SOD Unit/mg protein²¹.

MDA Level

Kidney MDA levels were studied using the thiobarbituric acid (TBA) abduction method as described previously. Briefly, 50 μl of sample was introduced into a tube containing 1 ml of distilled water. After addition of 1 ml of a solution containing 29 mmol/l TBA (Sigma Chemical Co., St Louis, MO, USA) in acetic acid (8.75 mmol/l, pH of the reaction mixture: 2.4-2.6) and mixing, the samples were placed in water bath and heated for 1 hour at $95-100^{\circ}\text{C}$. The samples were then cooled under running cold water. Twenty five μl of HCl (5 mol/l) was added and the reaction mixture was extracted for agitation with 3.5 ml of n-butanol (Sig-

ma Chemical Co.) for 5 minutes. After centrifugation, the butanol phase was separated and the fluorescence of the butanol extract was measured by spectrophotometry^{22,23}.

Statistical Analysis

Data were expressed as means \pm SD. Differences among various groups were tested for statistical significance using the one-way ANOVA test and Tukeys post test. A p value of less than 0.05 denoted the presence of a statistically significant difference.

Results

GPx and SOD Activity

Data on the enzyme activities of the control group, MTX treated group and MTX+ PTX group are shown in Table I. Renal tissue of rats showed significantly reduction of GPx and SOD activity after MTX treatment. GPx and SOD levels were higher in MTX+ PTX group comparing to MTX group significantly ($p < 0.05$).

Lipid Peroxidation Assay

The level of MDA, which is a major degradation product of lipid peroxidation, was significantly elevated in the renal tissue of rats treated with MTX ($p < 0.05$). However, this elevation was significantly suppressed when PTX treatment followed MTX administration ($p < 0.05$) (Table I).

Effects of PTX on Serum TNF- α , BUN and Cr Changes After MTX Treatment

For clarifying MTX administration effect on serum TNF- α , BUN and Cr, we measured these factors. MTX treatment increased TNF- α level significantly and elevated serum BUN and Cr levels. TNF- α , BUN and Cr levels were higher in

Table I. Renal tissue biochemical parameters (GPx, SOD, MDA).

	Control	MTX	MTX + PTX
GPx (U/mg protein)	3.73 \pm 0.41	1.86 \pm 0.45	2.61 \pm 0.47
SOD (U/mg protein)	2.70 \pm 0.26	1.28 \pm 0.58	1.92 \pm 0.26
MDA (nMol/ml)	0.83 \pm 0.38	2.46 \pm 0.55	1.74 \pm 0.44

The values are shown as a mean \pm SD for rats in each group and difference of ($p < 0.05$) considered significant. MTX: Methotrexate group, MTX+PTX: Methotrexate+Pentoxifylline group. GPx: glutathione peroxidase, SOD: superoxide dismutase and MDA: malondialdehyde.

MTX group comparing to MTX+PTX group significantly ($p<0.05$, Table II). TNF- α level was higher in MTX group comparing to saline group but there were no difference in BUN and Cr levels between MTX and MTX+PTX groups significantly ($p<0.05$).

Renal Tissue Injury

Semi-quantitative assessments of histological lesions produced significantly higher scores for MTX treated groups than the normal controls at day 6th. Renal tissue injury index was higher in MTX group comparing to MTX+PTX group significantly, and it was higher in MTX+PTX group comparing to control group significantly ($p<0.05$, Table III).

Apoptosis

The percent of TUNEL-positive cells increased in the renal tissue after MTX administration, and the percent of TUNEL-positive cells were lower in MTX+PTX group than MTX group significantly ($p<0.05$, Table III).

Discussion

Our study demonstrates that MTX administration causes oxidative tissue damage, as assessed by increased lipid peroxidation and decreased GPX and SOD activities in the kidney, while PTX treatment prevents the oxidative damage. Elevated serum level of the cytokine TNF-alpha and histological analyses demonstrated the severity of MTX induced systemic inflammatory response.

Antitumor drugs are being increasingly utilized as adjuvant therapy for patients at high risk for recurrent disease²⁴. Recent advances showed that oxygen radicals and hydrogen peroxides are linked with the development of several patholog-

ical processes associated with chemotherapy, including adverse effects of antitumor drugs^{25,26}.

The delayed therapeutic and toxic effect of MTX is due to its conversion to polyglutamated form which has a longer metabolic half life²⁷. MTX competitively and irreversibly inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis²⁸. DHFR catalyses the conversion of dihydrofolate to the active tetrahydrofolate. MTX has about 1000-fold greater affinity for DHFR compared to that of folate²⁹. Since folic acid is needed for the *de novo* synthesis of the nucleosides, by interacting with DHFR, MTX contributes to inhibition of nucleic acid synthesis. Specifically, MTX exhibits its cytotoxic action during the S-phase of the cell cycle^{30,31}.

The mechanisms of MTX-induced renal toxicity have not been exactly established yet. However, free radicals are expected to play a role in MTX induced renal toxicity. In our study we used MDA levels to show damage to the kidney caused by lipid peroxidation. Elevated observed MDA levels suggest that lipid peroxidation, mediated by oxygen free radicals, which is believed to be an important cause of destruction and damage to cell membranes, was an important contributing factor to the development of MTX-mediated tissue damage³². However, MTX-induced lipid peroxidation was prevented by PTX implicating an antioxidant effect of this molecule.

In normal condition, peroxidation injuries will promote anti-oxidation adaptation within human body. SOD and GPx are two different anti-oxidation enzymes major in the cytosol of living cells. We measured these two enzymes in kidney tissue of rats³³. Function of intracellular GPx is degradation of H₂O₂ and hydroperoxides of free fatty acids, whereas in plasma GPx catalyses degradation of H₂O₂ and hydroperoxides of phospholipids. In addition, GPx exert a protective effect on membrane phospholipids by inhibiting their

Table II. Clinical chemistry (kidney function tests and TNF- α) parameters of rats.

	Control	MTX	PTX + MTX
Cr (U/L)	0.49 \pm 0.06	0.73 \pm 0.07	0.56 \pm 0.07
BUN (U/L)	14.11 \pm 1.76	26.33 \pm 7.51	19.75 \pm 4.02
TNF- α (pg/ml)	30.22 \pm 4.54	73.11 \pm 5.60	55.25 \pm 9.40

The values are shown as a mean \pm SD for rats in each group and difference of ($p<0.05$) considered significant. MTX: Methotrexate group, MTX+PTX: Methotrexate+Pentoxifylline group. Cr: Creatinine, BUN: Blood urea nitrogen.

Table III. Renal tissue injury index.

	Control	MTX	MTX + LA
Renal tissue injury index	0.50 ± 0.052	2.20 ± 0.6	1.2 ± 0.6
% of TUNEL-positive cells	1.8 ± 0.3%	11.02 ± 2.4%	5.08 ± 1.3%

The values are shown as a mean ± SD for rats in each group and difference of ($p < 0.05$) considered significant.

peroxidation processes³⁴. SOD, an other anti-oxidant enzyme, can convert superoxide radicals to hydrogen peroxide and subsequently convert to water by GPx³⁵. In the present study, MTX administration decreased renal tissue GPX and SOD activity while PTX treatment attenuated this suppression.

The renal proximal tubule is both the segment of the nephron responsible for the active excretion of toxic chemicals, and an important target tissue for many of those same chemicals. Thus, it should not be surprising to find a convergence of these aspects of proximal tubule function and dysfunction³⁶. Acute renal failure (ARF) is the most common form of renal toxicity due to high-dose methotrexate (HDMTX) and is seen in 2-10% of treatment cycles^{37,38}. The most commonly described pathophysiology of ARF is the precipitation of methotrexate and its metabolites in the acidic environment of the urine. Other possible causes of ARF have been previously described³⁹. High-dose methotrexate-induced ARF is almost universally reversible and, given adequate recovery time, rarely will patients require discontinuation of HDMTX or dose reductions upon future cycles. Maintenance of adequate urine flow and urinary alkalization while receiving HDMTX is essential to prevent this adverse effect^{37,40}. Progressive renal dysfunction, defined as steady increase in baseline serum creatinine, with each subsequent cycle of HDMTX has not been reported in the literature⁴¹.

Kidney tissue, which presented severe glomerular congestion and degeneration, dilatation in Bowman's space, inflammatory cell infiltration in interstitium, and tubular degeneration in the MTX-treated group, showed mild glomerular and tubular degeneration, and mild inflammatory cell infiltration in the interstitium of the PTX+MTX-treated group. MTX administration increased TUNEL positive cells in renal tissue while PTX administration decreased number of TUNEL positive cells in MTX+PTX group significantly comparing to MTX group.

Beside histologic impairment, MTX administration impaired renal functional test like BUN and Cr. BUN and Cr levels in serum were increased after MTX administration while PTX administration decreased this elevation and there was no significant difference in BUN and Cr levels in control group and MTX+PTX group. Cetiner et al³ reported that MTX administration doesn't elevate BUN and Cr significantly, although there were tendency to increase. On the other hand, Kolli et al⁴¹ have indicated that MTX administration increases plasma BUN and Cr levels significantly in which is in accordance with our results.

Both clinical and experimental studies have shown that any noxious tissue event is perceived by macrophages and monocytes, which in turn secrete cytokines such as interleukin-1 (IL-1) and TNF- α ⁴². As evidenced in the present investigation, MTX administration resulted in increased serum TNF- α , indicating the role of this cytokine in drug-induced toxicity, while PTX depressed the TNF- α response.

In conclusions, it has been suggested that PTX may be a promising drug against MTX-induced renal damage and oxidative renal stress. Further studies are warranted to define the exact mechanism of the protecting effect of PTX on MTX-induced nephrotoxicity and the optimum dosage of this compound. In addition, these data indicate that the activities of GPX and SOD enzymes content in rat kidneys may play a role in the pathogenesis of MTX-damage. In our study, the increased level of tissue MDA and serum TNF- α levels together may be suggested that the underlying mechanism is related to direct toxicity of MTX rather than blockage in folate synthesis in rat kidneys. PTX administration also attenuated renal tissue injury and number of apoptic cells and suppressed elevation of BUN and Cr levels. However, further studies are essential to elucidate the exact mechanisms of MTX-induced renal toxicity, and protection and the effect of PTX.

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