Journal of Kidney
ISSN: 2472-1220

Li et al., J Kidney 2016, 2:3
DOI: 10.4172/2472-1220.1000130

Research Article Open Access

Effect of Administration Duration of Low Dose Methotrexate on Development of Acute Kidney Injury in Rats

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Received date: June 20, 2016; **Accepted date:** July 13, 2016; **Published date:** July 20, 2016

Abstract

Methotrexate (MTX) is currently utilized as a key drug in treatment of both malignant tumors and rheumatoid arthritis. However, MTX treatment is often associated with various side effects, such as pulmonary damage, hepatotoxicity and nephrotoxicity. Recent report also revealed that, even though total administered dosage of MTX was same, longer duration of MTX administration caused more severe adverse effect rather than short duration of its administration in clinical. Despite the importance of appropriate usage of MTX, the mechanism of kidney injury caused by the difference in duration of MTX administration remains still unknown. Here, we established animal models to determine the effect of administration duration on MTX-induced kidney injury and evaluated the significant factor and mechanism responsible for MTX caused-kidney injury. The dosage of MTX (25 mg/kg) were intraperitoneally injected by short-administration (25 mg/kg by 1 injection: Short-MTX) or long-administration (5 mg/kg by 5 injections: Long-MTX), respectively. In Long-MTX group, body weight, water intake, and urine volume were significantly decreased. Urea nitrogen and creatinine (CRE) in urine were obviously decreased in Long-MTX group, while blood urea nitrogen (BUN) and CRE in serum were increased in Long-MTX group. In addition, Long-MTX group showed the significant increase of both neutrophil gelatinase-associated lipocalin (N-gal) and kidney injury molecule 1 (Kim-1), kidney injury markers. Interestingly, renal MTX concentrations in Long-MTX group was higher than those in Short-MTX group. Moreover, 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), reliable oxidative stress markers, were significantly increased in Long-MTX group. Taken together, the present findings suggest that longer duration of MTX administration caused a higher MTX accumulation in kidney, thereby leading to kidney injury through an increase in oxidative stress.

Keywords: Methotrexate; Administration duration; Kidney injury; Oxidative stress

Abbreviations

AKI: Acute Kidney Injury; BUN: Blood Urea Nitrogen; CRE: Creatinine; 4-HNE: 4-Hydroxynonenal; KIM-1: Kidney Injury Molecule-1; MDA: Malondialdehyde; MTX: Methotrexate; N-GAL: Neutrophil Gelatinase-Associated Lipocalin

Introduction

Methotrexate (MTX), an antifolate, is a drug widely used in the treatment of patients with various diseases, such as, malignant tumors [1], rheumatoid arthritis (RA) [1,2], and ectopic pregnancy [3]. The clinical application of MTX has been increased since 1980s [4], and MTX is currently the most frequently disease-modifying drug both in adult RA and juvenile idiopathic arthritis [5,6]. Moreover, it is also widely used in malignant tumors, such as leukemia, lymphoma, choriocarcinoma, head and neck cancer, and osteogenic sarcoma [7].

Although MTX is currently utilized as a key drug in the treatment of both malignant tumors and RA, the efficacy of MTX is often limited by severe side-effects [8]. It is well-documented that MTX exhibits adverse effects including myelosuppression, mucositis, and kidney damage [9]. Of those side effects, kidney injury is one of the most frequent complications of MTX treatment. Because MTX is largely excreted into urine via kidney in its original form, MTX-induced renal dysfunction results in sustained and elevated plasma MTX concentrations, which in turn may lead to cause enhancement of other MTX toxicities [10]. Despite the importance of appropriate usage of MTX, the mechanism underlying MTX-induced kidney injury remains still unknown. In addition, previous studies have also shown that neither dosage nor plasma concentration may be a suitable index to predict MTX-induced kidney injury [11-13].

MTX has been used in a wide range of doses in clinical treatments from low dose of 20 mg/m^2 to high dose of 33,000 mg/m^2 (1,000 times higher than the low dose) [14]. It has been reported that high dose MTX could cause kidney injury by the precipitation of MTX [12]. Because the solubility of MTX in urine is directly influenced by pH, MTX is crystallized and precipitated in acid urine [12]. On the other hand, low dose MTX-induced kidney injury also have been frequently observed in clinical practice [13], which is unexplained by crystal-induced injury mechanism. Moreover, it has shown that the toxicity of MTX is associated with the infusion duration in clinical usage [15]. Recent studies also indicated that longer duration of MTX administration caused more severe adverse effect rather than short duration of its administration in patients [16]. However, the mechanism of kidney injury caused by the difference in duration of MTX administration has yet to be determined, and a suitable model to explore its mechanism is not yet available.

The purpose of this study was to establish a suitable animal model to study the mechanism involved in MTX-induced kidney injury and the effect of administration duration.

Materials and Methods

Drugs and chemicals

MTX was obtained from Pfizer Inc. (New York, USA). MTX standard chemical was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). N-gal antibody was purchased from R&D Systems (Minneapolis, USA), 4-HNE antibody was purchased from Abcam plc. (Cambridge, UK). All other chemicals used in this study were of the highest purity grade available.

Animal experiments

Male Wistar rats, each 10 weeks old (300 g), were obtained from Kyudo, Co, Ltd (Tosu, Japan). MTX administration method was based on clinical usage [17,18]. MTX (25 mg/kg) were intraperitoneally injected by short- or long-administration duration. Short administration duration control group (saline, n=6: Short-Con) and MTX group (25 mg/kg MTX saline solution, n=6: Short-M) were administered by 1 injection at day 1. Long administration duration control group (saline, n=6: Long-Con) and MTX group (5 mg/kg MTX solution, n=6: Long-MTX) were administered by 5 injections from day 1 to day 5, once a day. Analysis of biochemistry, such as urea nitrogen and creatinine (CRE), was performed by SRL Inc. (Tokyo, Japan). All procedures for animal experiments were approved by Kumamoto University ethical committee concerning animal experiments (B25-120R1), and animals were treated in accordance with the Guidelines of Kumamoto University for the care and use of laboratory animals.

Sample collection

Serum samples were collected on day 7 and centrifuged (3,000 g, 10 min). Urine samples were collected by metabolism cages every 24 hrs from 1 day before administration. Kidney tissue samples were collected on day 7. All samples were stored at -80°C until analysis.

Western blot analysis

The homogenized kidney sample was denatured with SDS buffer, separated by 10% Tris-Tricine SDS-PAGE, and transferred onto nitrocellulose membrane. The membrane was incubated with N-gal antibody (R&D Systems) for overnight at 4°C, and then incubated with anti-goat IgG antibody (Santa Cruz Biotechnology, Inc., Texas, USA) for 1 hr at room temperature. The membrane was incubated with detection reagent (ECL Advance Western Blotting Detection Kit; GE Healthcare UK Ltd, Buckinghamshire, England), and detected with LAS4000 (Fujifilm) and analyzed with Image J.

Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The kidney tissue (500 mg) was homogenized in 1 ml of PBS. ELISA for Kim-1 was performed by using Kim-1 ELISA kit (R&D Systems), according to the manufacturer's protocol.

Liquid chromatography/mass spectrometry/MS (LC/MS/MS) analysis

MTX concentration was measured by using API 3200™ LC/MS/MS system (AB SCIEX, Foster City, CA, USA) with triple quadruple mass spectrometer following positive ion mode: MTX, m/z 455.021; MTX d3, m/z 308.200. Source parameters were optimized to get highest analyte peak. The chromatographic separation was performed on 2.1 × 50 C18 column (3 µm particle size). The mobile phase consisting ammonium acetate buffer (1 nM ammonium acetate and 0.2% formic acid v/v) and acetonitrile (82:18 v/v).

Determination of MTX in kidney tissue

The kidney tissue (500 mg) was homogenized, centrifuged (15,000 g, 3 min), and the supernatant was collected. The perchloric acid (25 µl) was added into 100 µl supernatant. The samples were centrifuged, and the supernatant (10 µl) was injected into LC/MS/MS system.

Determination of MTX in urine

The Evolute™ ABN cartridge was activated with 1 ml of MeOH and 1 ml of 0.1% aqueous formic acid v/v, 0.1 ml of centrifuged (15,000 g, 10 min) and diluted (1:1 with 1% aqueous formic acid v/v) urine sample were loaded into the cartridge. The cartridge was the washed with aqueous 5% MeOH solution. Finally, the analyses were eluted with 300 µl of MeOH. The samples were evaporated the equate under vacuum at 45°C and re-dissolved in mobile phase. The samples (10 µl) was then injected into LC/MS/MS system.

Histological analysis

Paraffin-embedded 4 µm-thick sections were used for Hematoxylin-Eosin (H&E) staining and immunohistochemical analysis. For H&E staining, the slides were stained with hematoxylin for 15 min, and stained with eosin for 2 min.

Immunohistochemistry

Paraffin-embedded 4 µm-thick sections were used for immunohistochemistry. Deparaffinized sections were activated antigen with proteinase K (Dako). The slides were incubated with 4-HNE antibody (200 times dilution) overnight at 4°C, and then incubated with secondary anti-mouse IgG antibody (GE Healthcare UK Ltd., 200 times dilution) at room temperature for 1 hr. Reactivity was visualized with the DAB Liquid System (Dako Denmark A/S, Glostrup, Denmark).

Malondialdehyde (MDA) analysis

The kidney tissue (50 mg) was collected and homogenized in 250 µl RIPA Buffer. MDA analysis was performed by using MDA assay kit (Cayman Chemical., Michigan, USA), according to the manufacturer's protocol.

Statistical analysis

All data was expressed as mean ± SD. Difference between groups were tested for statistical significance using Student's t-test. P-values of <0.05 were considered statistically significant.

Results

MTX-induced kidney injury by short or long administration duration

We first sought to establish the animal model exhibiting kidney injury caused by MTX to determine whether the administration duration affects the MTX-induced kidney injury. The dosage of MTX (25 mg/kg) were intraperitoneally injected by Short-administration (25 mg/kg by 1 injection) or Long-administration (5 mg/kg by 5 injections), respectively. As shown in Figure 1a, body weight was significantly decreased in long administration duration of MTX (Long-MTX) group compared to short administration duration MTX (Short-MTX) group.

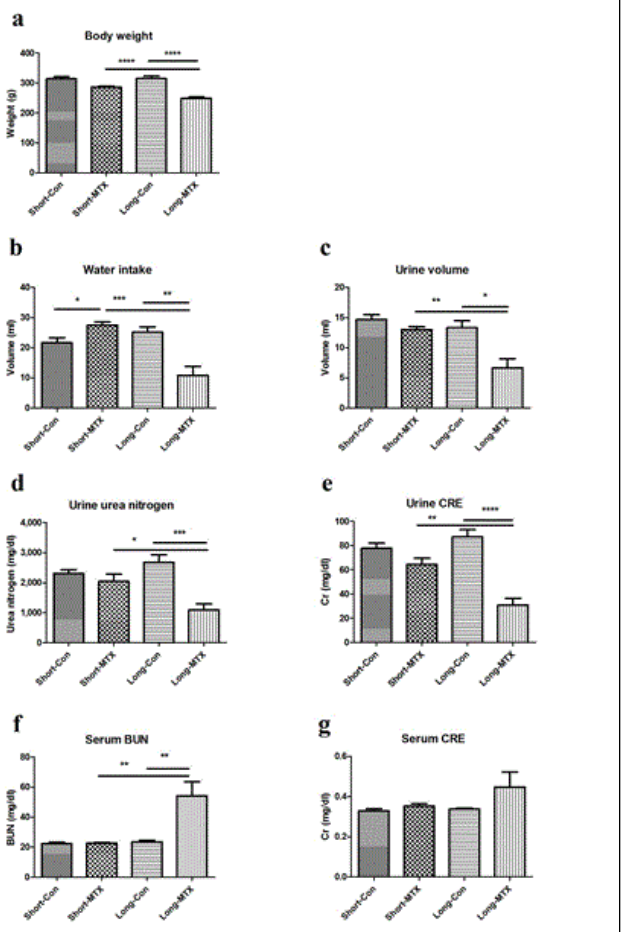


Figure 1: MTX-induced kidney injury by short or long administration duration. Body weight (a), water intake (b), and urine volume (c) were evaluated in the MTX-administrated rats by short-duration (Short-MTX, n=3) or long-duration (Long-MTX, n=6). Urine urea nitrogen (d), urine CRE (e) and serum BUN (f), serum CRE (g), were also determined in the MTX-administrated rats. Each bar represents the mean ± SD. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

In Long-MTX group, water intake and urine volume were also decreased markedly (Figures 1b and 1c), suggesting MTX-induced kidney injury. To further evaluate MTX-induced kidney injury, urea nitrogen and CRE were also measured in urine and serum samples. In urine, urea nitrogen and CRE were obviously decreased in Long-MTX group (Figures 1d and 1e). In contrast, serum BUN and CRE were increased in Long-MTX group (Figures 1f and 1g). Interestingly, no significant changes were observed in Short-MTX group (Figures 1d-1g). Those results suggested that longer duration of MTX administration caused more severe kidney injury rather than short duration of its administration in rat model.

Kidney tissue injury caused by long-MTX administration

By using established rat model, we attempted to determine the kidney tissue injury caused by long-MTX administration. As shown in Figure 2a, histological analysis showed that Long-MTX administration caused tubular and glomerular injury in cortex. Kidney hyperemia, tubular damage, and glomerulus atrophy were also observed in kidney tissue of Long-MTX group.

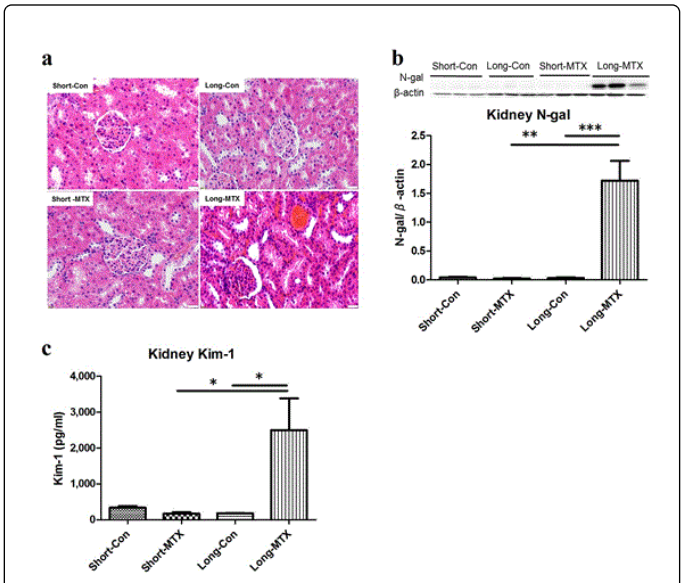


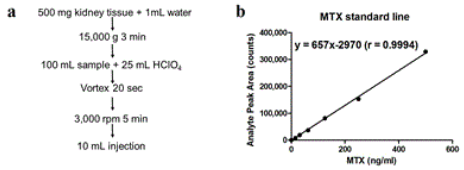
Figure 2: Kidney tissue damage caused by long-MTX administration. (a) H&E staining of kidney tissue containing tubules and glomerulus. Scale bars represent 20 μm. (b) Expression of N-gal protein in the kidney of MTX-administrated rats by Western blot and semiquantitative analysis. (c) Kidney Kim-1 protein expression in MTX-administrated rats by ELISA. *: p<0.05; **: p<0.01; ***: p<0.001.

As shown in Figure 2b, Western blot analysis clearly showed that N-gal protein expression was significantly increased in the kidney tissues of Long-MTX group compared to those of Short-MTX group. Consistent with the result shown in Figure 2b, Kim-1 protein expression was also markedly increased in the kidney tissues of Long-MTX group (Figure 2c), suggesting that longer duration of MTX administration caused more severe kidney tissue injury.

MTX concentration in urine and kidney

We next examined the mechanism involved in long-MTX administration-caused kidney injury by measuring MTX concentration. Because no obvious difference was observed between both groups in serum (Data not shown), we next focused on MTX

accumulation in kidney and built a simple method to evaluate MTX concentration in renal tissue (Figure 3a). The calibration curve was established with homogenized blank kidney sample and standard MTX chemical at the concentration between 15.625 ng/ml to 500 ng/ml (Figure 3b). As shown in Figure 3c, renal MTX concentrations in Long-MTX group was higher than those in Short-MTX group. As expected, in Long-MTX group, total amount of excrete MTX in urine was decreased (Figure 3c), suggesting that long-MTX administration-induced severe kidney injury was caused by the accumulation of MTX in renal tissue.



c

	Short-MTX (25mg/kg ×1)	Long-MTX (5mg/kg × 5)
Kidney MTX (ng/ g)	327.8 ± 168.0	436.7 ± 233.3
Total urine MTX (mg)	2.62 ± 1.18	0.97 ± 0.34

Figure 3: MTX concentration in and kidney and total MTX in urine. (a) Sample preparation method for measuring MTX concentration in kidney. (b) Standard line of renal MTX determination by LC-MS/MS. (c) Renal MTX concentration and total urine MTX in MTX-administrated rats.

Involvement of oxidative stress in kidney injury caused by Long-MTX administration

To further explore the mechanism of kidney injury caused by MTX accumulation in kidney, we evaluated 4-HNE, major end products of lipid peroxidation and known as an oxidative stress marker [19]. Immunohistochemical analysis showed that 4-HNE was increased in kidney tissue of Long-MTX group (Figure 4a).

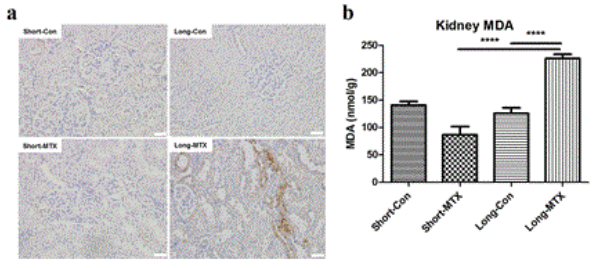


Figure 4: Involvement of oxidative stress in MTX-induced kidney injury. Immunohistochemical analysis for 4-HNE (a) and MDA analysis (b) in the kidney of MTX-administrated rats. Scale bars represent 20μm. ****: $p < 0.0001$.

Moreover, MDA, an another oxidative stress marker [20], was also significantly increased in the kidney tissue of Long-MTX group (Figure 4b), suggesting that the MTX accumulation in kidney by Long-MTX administration caused kidney injury through an increase in oxidative stress.

Discussion

In clinical practice, MTX is considered as a key drug in the treatment of both malignant tumors and RA. However, MTX treatment is often associated with various toxicities, such as kidney injury, which unexpectedly result in the interruption or discontinuation of medical treatment. Moreover, although it has been reported that the toxicity of MTX is associated with the infusion duration [15,16], the mechanism of kidney injury caused by the difference of MTX administration duration has yet to be determined. In the present study, we successfully established the animal model reflecting the clinical MTX-induced kidney injury caused by longer duration of MTX administration, and found that MTX accumulation in renal tissue by Long-MTX administration causes kidney injury through an increase in oxidative stress.

One of the interesting finding in this study is that, even though the administrated dosage of MTX was same (25 mg/kg), Long-MTX administration caused more severe kidney injury than Short-MTX administration. Goldie et al., reported the duration of MTX administration was important factor in MTX-induced toxicity [15]. Recent report also showed that shorter duration of MTX administration was more beneficial in view of reducing toxicity and enhancing central nervous system pharmacokinetics for lymphomas patients. In our experiments, the dosage of MTX (25 mg/kg) were intraperitoneally injected to the rats by Short-administration (25 mg/kg by 1 injection) or Long-administration (5 mg/kg by 5 injections), respectively. While the same dosage of MTX was administrated, longer duration of MTX administration caused more severe kidney injury than short duration of its administration in rat model (Figure 1). It is well-documented that neither dosage nor plasma concentration may be suitable index to predict MTX-induced kidney injury [11-13]. Previous study showed that, in high dose of MTX, toxicity was not related to plasma MTX area under the curve [21]. Meanwhile, MTX rapidly eliminated from the plasma at low dose, and plasma MTX concentration was also unrelated to toxic response, indicating MTX plasma levels may not be a reliable and not appropriate index for therapeutic drug monitoring [11]. As expected, no obvious correlation between serum MTX concentration and kidney tissue injury was observed (Data not shown). Interestingly, our further analysis focusing on MTX concentration in kidney tissue, suggested that MTX accumulation in kidney by Long-MTX administration. Since previous studies have shown that oxidative stress plays important roles in MTX-induced kidney injury [22], we also determined the oxidative markers, 4HNE and MDA, in kidney. The result shows longer administration duration of MTX caused kidney injury through increasing oxidative stress (Figure 3). Recent studies revealed that MTX underwent polyglutamation and transformed into polyglutamylated MTX (MTX-PGs) in the cell, and MTX-PGs showed severe cytotoxicity [23]. The MTX-PGs stayed at the cytoplasm for longer time until MTX-PGs changed back into MTX [23]. Because intracellular MTX-PGs concentration increases and sustained over time while administration [24], MTX in kidney cells may be more likely to transfer into MTX-PGs, while low dosage of MTX was administrated with longer duration. Therefore, long MTX

administration duration may cause more severe kidney injury due to MTX intracellular polyglutamation. Our results also showed increased oxidative stress in MTX-accumulated kidney (Figure 4). The above finding suggests that higher intracellular MTX-PGs concentration may trigger higher oxidative stress and severe kidney injury. Future studies will focus on determining the involvement of MTX-PGs accumulation in kidney tissue and further exploring the molecular mechanism underlying Long-MTX administration-induced kidney injury.

Attempts have been made to establish animal models showing MTX-induced kidney injury, as of this moment, the suitable model to study the effect of administration duration on MTX-induced kidney injury is not yet available. In this study, we successfully established the animal model with renal failure caused by longer duration of MTX administration. We observed the significant decrease of body weight, water intake, and urine volume in this model (Figures 1a-1c). As expected, urea nitrogen and CRE in urine were obviously decreased in Long-MTX group (Figures 1d and 1e), while BUN and CRE in serum were increased in Long-MTX group (Figures 1f and 1g). Additionally, in Long-MTX group, both N-gal and Kim-1, kidney injury markers, were also markedly increased (Figure 2). Previous studies have shown that N-gal is one of the most reliable markers in kidney after ischemic or nephrotoxic acute kidney injury (AKI) [25], and N-gal expression is elevated in renal tissue after kidney injury [26]. In addition, it is well-documented that Kim-1 is also a very useful kidney injury marker, and Kim-1 expression is rapidly increased after kidney injury [27]. Moreover, 4-HNE and MDA were significantly increased in Long-MTX group (Figure 4), indicating that, at least in significant parts, this model showed the clinical phenotype of MTX-induced kidney injury. Interestingly, our model showed that both N-gal and Kim-1 were significantly increased in kidney tissue by longer duration of MTX administration (Figure 2). Recent studies have shown that both N-gal and Kim-1 are recognized as sensitive and early diagnostic markers for kidney injury [25,26]. Thus, N-gal and Kim-1 levels in urine or serum may have potential to be a useful biomarker for kidney injury caused by Long-MTX administration. In clinical case report, it has been reported that serum CRE is increased during MTX-induced kidney injury [13]. However, in this model, urine CRE was obviously decreased in Long-MTX group without the significant increase of serum CRE. It may due to the deceased CRE synthesis in Long-MTX group. Because tetrahydrofolate (THF) is necessary for CRE synthesis [28], and MTX shows its therapeutic effect by inhibiting the synthesis of THF [29], MTX administration may lead to a decrease in CRE synthesis. It should be noted that folic acid was used with MTX in clinical usage, but not in animal experiment [9,13], indicating the difference between animal model and clinical case in CRE synthesis. Further investigation will be needed to determine the more detailed molecular mechanism and verify the usefulness of this model for studying MTX-induce kidney injury.

In conclusion, we provided first hand evidence that longer duration of MTX administration caused more severe kidney injury in rat model. MTX accumulation in renal tissue by Long-MTX administration caused kidney injury through an increase in oxidative stress. This finding may bring new insights into understanding of MTX-induced kidney injury, and may lead to clinical application for appropriate treatment with MTX.

Acknowledgements

This work was supported by Grant (JP25293040 and JP25670080) from the Japan society for the promotion of science KAKENHI. The first author was supported by State-Sponsored Scholarship Program (No.201306370035) from the China scholarship council.

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