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Evaluating the effect of methotrexate on the rate of renal fibrosis by elastography and fibrosis-related gene expression

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ABSTRACT

Methotrexate is mainly used to treat diseases such as rheumatoid arthritis (RA), but its potential for nephrotoxicity has always been a significant concern on the use of this medication. This study aimed to determine the rate of renal fibrosis using transient elastography and its relationship with cumulative dose and duration of drug use in patients with rheumatoid arthritis treated with methotrexate. TGFβ gene expression was also assessed for further evaluation. Patients with rheumatoid arthritis who received methotrexate for more than six months were included. Renal fibrosis was determined by measuring the stiffness of the kidney by elastography (FiberScan Device). RA patients were divided into two groups based on kidney stiffness measurement with and without renal fibrosis, and demographic, clinical, and biochemical parameters were compared to investigate the relationship between cumulative dose and duration of methotrexate treatment and renal fibrosis. Also, in this study, 50 controls (healthy people) and 50 cases (RA patients) were used to evaluate the expression of the TGFβ gene by real-time PCR method. The existence of kidney fibrosis was observed in 10 patients. There was no significant relationship between renal fibrosis and the cumulative dose (P = 0.21) and duration of methotrexate (P = 0.30). Multivariate regression analysis showed that the chances of developing renal fibrosis in patients increase with increasing serum ALT levels (P = 0.01). The results of the TGFβ gene expression showed that the expression of this gene in the group of RA patients with fibrosis was higher than the control group (healthy people) and the group of RA patients without fibrosis (P <0.01). These results showed that evaluation of renal fibrosis by elastography method is recommended for scanning RA patients while they are being treated with methotrexate, which is also confirmed by the results of the fibrosis-related-gene expression.

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Introduction

Rheumatoid arthritis (RA) is an inflammatory disease associated with joint and external manifestations that usually lead to disability due to the destruction of articular cartilage (1). The condition may be slow or very destructive (2). Various drugs used to treat this disease are consumed for nonspecific suppression of inflammatory or immunological phenomena to suppress or limit the disease associated with preventive and obstructive movement (3).

Methotrexate (MTX) is one of the most effective drugs commonly prescribed for treating RA and for the treatment of lupus, psoriatic arthritis, myositis, vasculitis, and some other rheumatic diseases (4). MTX is an immunosuppressive drug and is one of the drugs known as anti-rheumatic drugs that change the course of the disease, which in addition to eliminating the symptoms of the disease, also slows down the progress of the disease (5).

Nephrotoxicity and fibrosis are the main side effects of the long-term use of this drug (6). However, nephrotoxicity has been partially controlled with a reduced dose of the medicine and weekly administration (5 to 15 mg per week) instead of daily administration (7). However, due to this drug's prolonged and long-term use, especially in rheumatoid arthritis, cases of severe nephrotoxicity have been observed, and with neglect, it can lead to kidney

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failure (6). As a result, monitoring and diagnosis of methotrexate-induced kidney damage during treatment periods seems necessary (8).

Renal fibrosis is measured by various methods, including levels of biochemical kidney markers (such as serum creatinine (SCr) and urine), radiographic tests (ultrasound, kidney scan, and MRI), and histopathological tests by kidney biopsy (9). Kidney biopsy, which is used as the gold standard for assessing fibrosis, is invasive and painful and has side effects, so physicians and patients rarely accept it (10). On the other hand, laboratory tests are not accurate and reliable enough to diagnose kidney fibrosis.

Another way to diagnose renal fibrosis is to evaluate the expression of genes involved in fibrosis (11). One of the most important genes in the diagnosis of fibrosis is the Transforming growth factor-β (TGFβ) gene (12). TGFβ is produced as a pro-fibrotic cytokine by various kidney cells (11). Some studies have shown an important role for TGFβ in the early stages of fibrosis. Activation of smad2/3 (activated proteins in the fibrotic kidney) by TGFβ is involved in epithelial-mesenchymal transmission (EMT) and the spread of glomerular and interstitial fibrosis (13).

Elastography is a new imaging technique that has recently been recognized as an accurate method for diagnosing and evaluating the progression of renal fibrosis in chronic kidney disease (14). This technique quickly and non-invasively measures kidney stiffness. In studies to monitor kidney fibrosis by fibroscopy in patients with RA treated with MTX, different results have been reported from the association of kidney stiffness with the cumulative dose of methotrexate (15). This study aimed to evaluate the rate of kidney fibrosis in patients with rheumatoid arthritis treated with methotrexate and its relationship with the cumulative dose of the drug and the duration of drug use. We also evaluate TGFβ gene expression to better understand the importance of elastography in the diagnosis of renal fibrosis.

Materials and methods

Study design

The present study, which was designed as a descriptive-analytical study, was performed in a rheumatology clinic. Fifty patients with rheumatoid arthritis treated with methotrexate participated in the current study. Also, fifty healthy individuals were participated in evaluating the expression of the TGFβ gene.

Inclusion criteria included individuals who had RA (based on the 2010 Criteria for Rheumatology Association of America/European Union against Rheumatism) (16), were treated with methotrexate, were over 18 years of age, and had been treated for at least six months. Subjects were excluded from the study if they had signs and symptoms of diabetes, a history of chronic kidney diseases, AIDS, chronic renal failure, congestive heart failure, a history of alcohol consumption, and weight gain.

The sampling method was simple random. The samples were selected among the eligible patients by creating a sampling framework and giving a number to each patient using a table of random numbers.

Collection of demographic and clinical information

Demographic data, duration of illness, duration of MTX use, and cumulative dose of MTX were collected by the physician using a questionnaire from patients. Disease activity was measured according to the DAS 28 formula, which was calculated and recorded based on several joints with tenderness, some swollen joints, ESR (Erythrocyte Sedimentation Rate), and measuring pain by VAS (Visual Analog Scale) method. Also, the attending physician measured blood pressure (mmHg) with a digital sphygmomanometer in all participants.

Para-clinical studies

The rate of kidney fibrosis in the studied subjects was evaluated by elastography method and using FibroScan (EchoSens, Paris, France) by the gastroenterologist. The results of fibrosis were reported in kilopascals, and based on the study of Tsochatzis *et al*. (17), the cutoff value for the presence of fibrosis was considered to be 6kPa. The CAP (Controlled Attenuation Parameter) of FibroScan was used to evaluate tissue elasticity, and the results were reported as dB/m. Serum levels of ALT, AST, and ALP were measured in all participants. Ultrasound was also performed to assess renal obstructions in patients.

Expression of TGFβ gene

Five milliliters of peripheral blood prepared from patients were used to extract mRNA of TGFβ gene. RNeasy Kits (QIAGEN, Singapore) was used to extract RNA. The cDNA was synthesized by the relevant kit (Fermentas, Canada). Gene-specific primers were designed by Biosoft AlleleID7 software. The characteristics of primers are given in Table 1. The β-actin gene was used as intra control.

Table 1. The characteristics of primers for β-actin and TGFβ genes

Gene	Accession No.		Primer Sequences	Annealing Temp.	Product Size (bp)
β-actin	NM_002046.3	Forward	5'-ACACCAACTATTGCTTCAG-3'	60°C	159
		Reverse	5'-TGTCCAGGCTCCAAATG-3'		
TGFβ	NM_001130916	Forward	5'-GCACCGTCAAGGCTGAGAAC-3'	60°C	138
		Reverse	5'-TGGTGAAGACGCCAGTGGA-3'		

BioFACT Sybergreen kit was used to amplify the genes by PCR. Then, mRNA expression of related genes was measured by the RT-PCR technique. RT-PCR steps (Biosystems StepOne) include initial denaturation at 95°C for 15 minutes, then denaturation at 95°C for 15 seconds, annealing reaction at 60°C for 25 seconds and then extension step at 72°C for 15 seconds, then 95°C for 15 seconds and finally 60°C for 15 seconds. In order to quantify the amounts of gene expression, the formula $2^{-\Delta\Delta CT}$ was used.

Statistical analysis

Data analysis was performed using SPSS software version 22. Kolmograph-Smirnov test was used to evaluate the normality of quantitative data. Qualitative variables were described as frequency (percentage), quantitative variables with normal distribution were described as mean ±standard deviation, and quantitative variables with abnormal distribution were described as median (25-75 Percentiles). Independent t-test or, Mann-Whitney test (for non-parametric equivalent), and Chi-square test were used to investigate the relationship between quantitative and qualitative variables with kidney fibrosis. Then, modeling was performed using logistic regression to investigate the relationships between the variables to control possible confounders. A P-value less than 0.05 was considered as a significant level.

Results and discussion

Demographic, clinical, and biochemical characteristics

Demographic, clinical, and biochemical characteristics of all patients in the study are shown in Table 2. The mean age of patients was 52.68 ± 7.42, with a minimum of 32 years and a maximum of 69 years. Most of them (86%) were women. The mean duration of illness was 6.2 years (in the range of 2.1 to 9.5 years), and the median (25-75 percentile) of disease activity measured on the basis of DAS28 in patients was calculated 2.41 (1.79-3.22). The median (75-25 percentile) of kidney stiffness measured in all patients treated with MTX was 4.68kPa (3.61-5.48). The cumulative dose of MTX was less than 4000 mg in 43 patients and more than 4000 mg in 7 patients. Also, the mean duration of drug use in the studied patients was 54.14 ± 41.03 months.

Table 2. Demographic, clinical and biochemical characteristics in the studied patients

Variable	Patients (n=50)
Age (year)	52.68 ± 7.42
Gender (Female)	43 (86%)
BMI (Body Mass Index) (kg/m^2)	26.94 ± 3.37
ALT (IU/L)	24.47 (18.19-32.27)
AST (IU/L)	21.35 (17.16-26.12)
ALK (IU/L)	164.09 ± 41.23
DAS28	2.41 (1.79-3.22)
Duration of MTX consumption (month)	54.14 ± 41.03
Kidney stiffness (kPa)	4.68 (3.61-5.48)
Renal elasticity (dB/m)	235.18 ± 56.67
Renal obstructions (confirmed by ultrasound)	10 (20%)
Hypertension	18 (36%)

In the study of the relationship between the studied variables with renal fibrosis, the results showed that kidney fibrosis, equal to or greater than 6kPa with renal stiffness values measured in FibroScan, was observed in 10 out of 50 patients. In one of these 10 patients, significant fibrosis (FibroScan> 7.2kPa) and in two patients severe fibrosis (FibroScan> 9.6kPa) were observed.

When patients were divided into two groups based on kidney fibrosis, serum levels of AST (P = 0.01), ALT (P<0.01), and the presence of obstructions (confirmed by ultrasound) in patients with significant fibrosis were significantly higher than the other group. In contrast, there was no significant difference between the two groups in comparing the cumulative dose of MTX and the duration of MTX use (Table 3).

Table 3. Comparison of demographic, clinical, and biochemical characteristics between two groups of patients (based on kidney stiffness measurement)

Variables	FibroScan Score		P-value
	≥6 (n = 10)	<6 (n = 40)	
Age (year)	54.32 ± 7.34	51.04 ± 7.50	0.51
Gender (Female: Male)	8:2	35:5	0.47
BMI (kg/m^2)	28.21 ± 3.16	25.67 ± 3.58	0.18
Illness duration (year)	5.3 (1.9-9.7)	7.1 (2.3-9.3)	0.53
MTX accumulation dose (<4000 mg)	10 (100%)	35 (87.5%)	0.21
MTX accumulation dose (>4000 mg)	0 (0%)	5 (12.5%)	
Duration of MTX consumption (month)	43.66 ± 26.12	64.62 ± 55.94	0.30
Kidney stiffness (kPa)	6.83 (6.69-9.34)	2.53 (0.53-4.62)	<0.01
DAS28	2.38 (1.61-3.19)	2.44 (1.97-3.25)	0.85
ALT (IU/L)	28.12 (27.81-45.19)	22.66 (16.34-31.11)	<0.01
AST (IU/L)	27.72 (19.98-31.09)	19.61 (15.76-25.13)	0.01
ALK (IU/L)	178.41 ± 47.16	158.86 ± 53.51	0.28
Systolic blood pressure (mmHg)	132 (112-151)	115 (109-128)	0.05
Diastolic blood pressure (mmHg)	71 (70-79)	70 (69-80)	0.81
Hypertension	5 (50%)	13 (32.5%)	0.16
Renal obstructions (confirmed by ultrasound)	5 (50%)	5 (12.5%)	0.04
Renal elasticity (dB/m)	256.32 ± 61.99	214.04 ± 51.35	0.16

The logistic regression analysis results showed several variables that with increasing ALT, the chance of developing fibrosis in patients with RA receiving MTX treatment increases (OR = 1.07; 95% CI: 1.01 to 1.13; P = 0.01)(Table 4).

Evaluation of TGFβ gene expression

The results of the TGFβ gene expression showed that the expression of this gene in the group of patients with fibrosis was statistically higher than the control group and the group of patients without fibrosis (P <0.01) (Figure1).

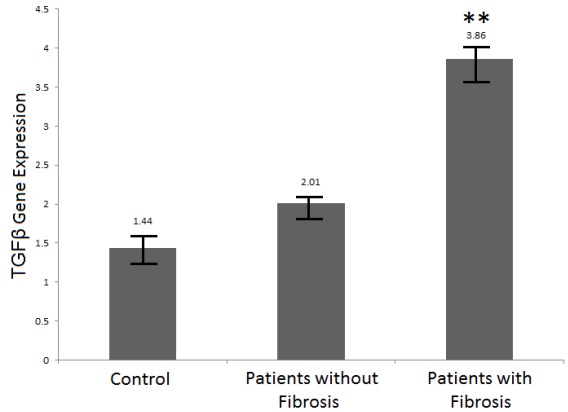

Figure 1. The expression of TGFβ gene in the control group (healthy people), RA patient without kidney fibrosis group, and RA patient with kidney fibrosis group; **: P<0.01

Table 4. Results of multivariate logistic regression analysis on risk factors associated with kidney fibrosis

Independent Variable	OR	95% Confidence Interval (CI)	P-value
Systolic Blood Pressure	1.03	0.99-1.06	0.12
Renal obstructions (confirmed by ultrasound)	2.64	0.46-15.01	0.27
AST	1.01	0.90-1.15	0.80
ALT	1.07	1.01-1.13	0.01

The effect of long-term use of MTX on renal fibrosis remains controversial (18, 19). In the present study, the rate of kidney stiffness in 50 RA patients treated with weekly MTX for an average of 54.14 ± 41.03 months with a minimum and maximum of 6 to 180 months was assessed by elastography technique. The main findings of this study indicate that increasing the duration of use and the cumulative dose of MTX does not affect the incidence of kidney fibrosis; however, the chances of developing renal fibrosis increase with increasing ALT, and there is a significant difference in serum ALT levels between patients with and without renal fibrosis.

Park *et al*. (20) examined 177 patients with RA treated with MTX for kidney fibrosis for more than three years. In this study, patients were divided into two groups receiving doses of less and more than 4000 mg in terms of the cumulative amount of the drug. No significant relationship was observed between the degrees of kidney stiffness in the two groups. Also, there was no significant relationship between the degree of kidney stiffness measured with Transient Electroscopy (TE) and the cumulative dose of the drug. Still, there was a significant correlation between kidney stiffness and AST values. In the study of Kumar *et al*. (15), which included 160 patients with RA treated with low-dose MTX for more than five years, no significant relationship was observed between the duration of use and the cumulative dose of renal fibrosis. Mansour-Ghanaei *et al*. (21) also examined the kidney of 101 patients with psoriasis and RA by TE treated with methotrexate for at least two years and did not find a significant relationship between the presence of renal fibrosis with cumulative dose and duration of methotrexate use. Unlike the previous two studies, obese and alcoholics were also examined in this study.

In the present study, long-term use of the medication was not associated with an increased risk of renal fibrosis in FibroScan findings, consistent with the above studies. In the present study, significant and severe fibrosis was observed in 3 patients. In a similar study, out of 160 patients with long-term RA treatment with MTX with a median cumulative dose of 4225 mg, only 12 of them reported significant fibrosis (21). Therefore, the occurrence of severe renal fibrosis measured in TE is not as common as in our study.

On the other hand, in several studies, significant kidney fibrosis has not been reported in patients consuming a wide range of MTX from 1125 to 7000 mg who underwent kidney biopsy (15, 22, 23). Therefore, the findings of this study are confirmed by other studies in which kidney fibrosis have been evaluated by needle biopsy (15). Based on these results, it appears that kidney fibrosis is not a significant concern in MTX treatment. TE can also be useful as a non-invasive tool for assessing kidney fibrosis. Our results also showed that molecular evaluation of fibrosis-related gene expression (TGFβ) confirms these results.

Renal fibrosis increases the function of specific cytokines such as TGFβ and chaperone proteins such as HSP72 (24). TGFβ is a cytokine with hypertrophic and proasclerotic properties. It is well established that high expression of TGFβ in renal fibrosis causes kidney cell hypertrophy and increased production and accumulation of extracellular matrix and interstitial fibroblast cells (25). Pathological conditions are closely related to the overexpression of TGFβ (26). The function of TGFβ in kidney disease has introduced it as a significant target with a therapeutic approach (27).

Several factors can explain the low prevalence of fibrosis. Folic acid supplementation appears to reduce the increase in transaminases (28). Using lower doses and weekly consumption instead of daily are other factors. Taking a weekly low-dose MTX may cause a lower degree of kidney damage, and there may be enough time for the kidney to heal and repair the damage (29). Excessive alcohol consumption, obesity, and diabetes are known to be risk factors for developing progressive kidney fibrosis in patients with RA receiving MTX. In this study, people with these conditions were excluded (30).

FibroScan is a reliable test for assessing renal fibrosis by measuring kidney stiffness with 60% sensitivity and 80% specificity in HCV, NASH, RA, and HBV patients (31). However, obesity and high ALT levels reduce the sensitivity of this method to detect fibrosis. ALT can interfere with TE measurements more than twice as normal (32, 33). In our study, ALT levels were not twice normal in any patients. As a result, kidney stiffness levels are not affected by ALT levels.

The results of our study showed that increased ALT is an independent risk factor for increased kidney stiffness measured by elastography and may indicate that this biological marker can help predict increased kidney stiffness and progression of kidney fibrosis.

Conclusions

The present study shows that significant and severe renal fibrosis is uncommon in patients treated with methotrexate. Increasing the duration of methotrexate and the cumulative dose of the drug does not increase its incidence. The incidence of renal fibrosis was not significantly related to the drug's cumulative amount and duration. Still, with increasing serum ALT levels

in these patients, the rate of kidney stiffness tended to grow, and the chances of fibrosis increased. Evaluation of renal fibrosis by elastography method is recommended for patients with rheumatoid arthritis treated with methotrexate, which is confirmed by the evaluation results of the fibrosis related-gene expression.

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Interest conflict

None.

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