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Rutin Attenuates Methotrexate-Induced Hepatic Oxidative Stress in Rats*

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Abstract: This study aimed to assess the antioxidant effects of rutin on methotrexate-induced hepatic oxidative stress. Wistar Albino rats (n=24) were separated into 4 groups: control group (C); methotrexate (M, 20 mg/kg, single dose) group; rutin (R, 50 mg/kg/day, for fifteen days) group; and methotrexate + rutin [MR, methotrexate (20 mg/kg, single dose) + rutin (50 mg/kg/day, for fifteen days)] group. Rats were sacrificed on the sixteenth day and liver tissues were removed. The liver glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPx), malondialdehyde (MDA), nicotinamide adenine dinucleotide phosphate (NADPH), total glutathione (tGSH), and vitamin C were determined colorimetrically. Malondialdehyde increased and vitamin C decreased in the M group compared to the others ($P<0.001$). Total glutathione reduced in both M and MR groups compared to C group ($P<0.05$). An important decrease in M group NADPH and GPx compared to C group ($P<0.001$) was observed. Nicotinamide adenine dinucleotide phosphate of R group were determined to be higher than M group ($P<0.001$). In this study, it might demonstrate that methotrexate caused oxidative stress in the liver and rutin had antioxidant effects on methotrexate-induced hepatic oxidative stress. Consequently, it can be suggested that rutin may be useful in attenuating the side effects of methotrexate on the liver.

Keywords: Liver, Methotrexate, Nicotinamide adenine dinucleotide phosphate, Rutin, Vitamin C.

Rutin Ratlarda Metotreksata Bağlı Hepatik Oksidatif Stresi Hafifletir

Öz: Bu çalışma, rutinin ratlarda metotreksata bağlı hepatik oksidatif stres üzerindeki potansiyel antioksidan etkilerini değerlendirmeyi amaçlamıştır. Wistar Albino ratlar (n=24) dört gruba ayrıldı: Kontrol grubu (K); metotreksat (M, 20 mg/kg, tek doz) grubu; rutin (R, 50 mg/kg/gün, 15 gün) grubu; ve metotreksat + rutin [MR, metotreksat (20 mg/kg, tek doz) + rutin (50 mg/kg/gün, 15 gün)] grubu. Ratlar onaltıncı gün sakrifiye edildi. Karaciğer dokuları alındı. Malondialdehit (MDA), vitamin C, total glutatyon (tGSH), nikotinamid adenin dinükleotid fosfat (NADPH) seviyeleri ve glutatyon peroksidaz (GPx) ve glukoz-6-fosfat dehidrojenaz (G6PD) aktiviteleri kolorimetrik olarak belirlendi. M grubunda diğer gruplara göre MDA düzeyleri artmış ve C vitamini düzeyleri azalmıştır ($P<0.001$). tGSH düzeyleri, hem M hem de MR gruplarında K grubuna göre azaldı ($P<0.05$). M grubu NADPH düzeylerinde ve GPx aktivitesinde K grubuna kıyasla önemli azalma ($P<0.001$) gözlendi. R grubunun NADPH düzeyleri M grubuna kıyasla daha yüksek belirlendi ($P<0.001$). Bu çalışma, metotreksatın karaciğerde oksidatif strese neden olduğunu ve rutinin de metotreksata bağlı hepatik oksidatif stres üzerine antioksidan etkisinin olduğunu göstermektedir. Sonuç olarak rutinin metotreksatın karaciğer üzerindeki yan etkilerini hafifletmede faydalı olabileceği düşünülmektedir.

Anahtar Kelimeler: Karaciğer, Metotreksat, Nikotinamid adenin dinükleotid fosfat, Rutin, Vitamin C.

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INTRODUCTION

Some drugs and chemical agents are known to have potential hepatotoxic effects in humans and animals (1). Drug-induced hepatotoxicity may end up cellular death, inflammation, and acute liver disorder (2,3). Methotrexate, 4 - amino, 10 - methyl analog of folate, is an antimetabolite, inhibits dihydrofolate reductase, which is involved in the formation of tetrahydrofolate from dihydrofolate and ultimately it inhibits DNA synthesis and cell proliferation (4). Researches have reported that methotrexate, which is widely used in cancer treatment, increases the risk of liver toxicity during or after cancer treatment (5-7). It has been reported that high-dose and continuous use of methotrexate has side effects, especially on the liver (6) and also leads to a significant reduction in body weight (3). In short, methotrexate is a drug used to treat different varietes of cancer and hepatotoxicity, a common complication with its use, limits the clinical use of methotrexate (8,9). According to the literature review, methotrexate has been reported to increase myeloperoxidase (9,10), nitric oxide (3,9,11,12) and malondialdehyde (MDA) (10-12) and decrease glutathione (GSH) (3,9,12), glutathione peroxidase (GPx) (3,12) and glutathione-s-transferase (3,4), superoxide dismutase (3,11), catalase (12,13) in liver tissue. Abnormal production of reactive oxygen species has been suspected in the methotrexate-induced hepatotoxicity pathophysiology (14). Thus, reducing oxidative stress may represent an impressive strategy to protect against methotrexate-induced hepatotoxicity (3). Natural agents are effective for the prevention and/or treatment of liver diseases such as liver fibrosis and cirrhosis, which are associated with prolonged oxidative stress and especially reactive oxygen types (15). Various antioxidant and anti-inflammatory agents are recommended for the prevention or treatment of methotrexate-induced liver toxicity (10).

Rutin (3, 3', 4', 5, 7 - pentahydroxyflavone - 3 - ramnoglycoside) is also termed rutosid, rutin trihydrate, quercetin - 3 – O - rutinoside, phytomin and sophorin, and a citrus flavonoid found in plants such as buckwheat, cranberry, berry, citrus fruit and fruit peels, tea, apple and passionflower (16-18). Rutin possesses anti-allergic, anti-angiogenic, anti-inflammatory, antioxidant, and antiviral features (17,19). Rutin has also hepatoprotective effects (18,20). Moreover, a wide variety of studies showed that rutin has beneficial effects against cyclophosphamide (17), mercury-chloride (21), iron (20), ethanol (16) and cadmium (22).

In the present study, rutin administration (50 mg/kg, orally, for fifteen days) was applied one week before and after the methotrexate administration (20 mg/kg, i.p.), and it was aimed to demonstrate the antioxidant effects of rutin on liver oxidative damage in male rats by changes in biochemical parameters (MDA, Vitamin C, tGSH, nikotinamid adenin dinükleotid fosfat (NADPH) levels and GPx and glucose-6-phosphate dehydrogenase (G6PD) activities). It was thought that the findings obtained from the study may contribute to scientific studies on the use of rutin against oxidative damage in the liver.

MATERIAL and METHODS

Animals and Experimental Design

Adult male Wistar Albino rats, acquired from the Experimental Research Application and Research Center, Hatay Mustafa Kemal University were used in this study. The rats were housed in polypropylene cages under laboratory humidity (44 ± 5%), standard temperature (24 ± 2 °C), and twelve hours light/dark cycle terms during the experiment. Food and water were provided *ad libitum* during the study. The Experimental Animals Ethics Committee approved the present study of Hatay Mustafa Kemal University (Ethical number: 2019/07-12). Experiments were performed according to international guidelines on the ethical use of rats.

In this study, rats (n=24) were randomly separated into four groups (six rats for each group). The experimental procedure was given in Table 1.

The animals were administered with rutin (rutin hydrate, ABCR, Germany, oral gavage) and distilled water by oral gavage (21). Methotrexate (Kocak Pharma, Turkey) and normal saline (0.09% NaCl) were administered intraperitoneally (9). On the sixteenth day of the study, the rats were anesthetized with ketamine and xylazine hydrochloride. Then, the rats were sacrificed, liver tissues were received and stored at -80 °C for biochemical analyses.

Table 1. Experimental design.
Tablo 1. Deneysel tasarım.

	Treatments-Duration		
Groups	Day 1-7	Day 8	Day 9-15
C Group	dw	dw + normal saline	dw
M Group	dw	20 mg/kg M	dw
R Group	50 mg/kg R	50 mg/kg R + normal saline	50 mg/kg R
MR Group	50 mg/kg R	20 mg/kg M + 50 mg/kg R	50 mg/kg R

C: control, M: methotrexate, R: rutin, MR: methotrexate + rutin, dw: distilled water.

Control (C) group obtained distilled water by oral gavage for fifteen days and injected with normal saline (0.09% NaCl) intraperitoneally (i.p.) on the eighth day of the study. Methotrexate (M) group, the animals were orally administered with distilled water for fifteen days and methotrexate was administered in single dose of 20 mg/kg i.p. on the eighth day of the study. Rutin group (R) received 50 mg/kg rutin orally for fifteen days and normal saline was administered i.p. on the eighth day of the study. Methotrexate + rutin (MR) group were orally administered with rutin during fifteen days of the experimental procedure. On the 8th day of the study, animals were injected with single dose of 20 mg/kg methotrexate i.p.

Preparation of Tissue Supernatants

Before analysis, the livers were homogenized in phosphate buffer solution (1:10 w/v, pH: 7.4) by ultrasonic homogenizer (Bandelin Electronic UW 2070, Germany) by using cooled tubes within ice. The homogenates were centrifuged at 5000 rpm, 30 minutes/+4 °C, and the supernatants were separated for further use.

Analysis

Protein levels were spectrophotometrically estimated by using Lowry's method (23). Tissue MDA assays were performed in compliance with the guidelines of Ohkawa et al. (24). Total GSH levels were measured in the liver tissue homogenates by using the commercially available kit (GSH-420^{TM} OxisResearch, USA). The method described by Haag (25) was used to measure the level of vitamin C. NADPH levels in the liver tissue homogenates was measured with a commercial NADP/NADPH Quantitation kit (MAK038, Sigma-Aldrich, USA). The levels of these parameters were represented as nmol/g tissue. Glutathione peroxidase (26) and G6PD (27) activities were estimated spectrophotometrically in compliance with the methods described by Beutler.

Statistical Analysis

Windows statistical package for the social sciences program (IBM SPSS 22 version, USA) was used to analyze for the obtained data. The multiple groups' comparison was assigned by variance analysis (one-way ANOVA), with a post hoc Duncan test. Differences were considered significant at $P<0.05$. All variables were represented as the mean ± standard error (SE).

RESULTS

Malondialdehyde was determined to be increased in the M group (50.12 ± 3.60 nmol/g tissue, $P<0.001$, Table 2) compared to other groups (C: 31.78 ± 1.17 nmol/g tissue; R: 27.55 ± 1.48 nmol/g tissue; MR: 32.47 ± 0.94 nmol/g tissue, Table 2). However, vitamin C levels decreased in the M group (138.23 ± 9.51 µg/g tissue, $P<0.01$, Table 2) compared to other groups (C: 193.29 ± 15.46 µg/g tissue; R: 201.52 ± 9.87 µg/g tissue; MR: 174.94 ± 5.06 µg/g tissue, Table 2). Total glutathione levels reduced both in M and MR groups (33.88 ± 2.27 µmol/g protein; 35.19 ± 0.60 µmol/g protein, respectively, Table 2) compared to C

group (42.46 ± 3.13 µmol/g protein, P<0.05, Table 2). A significant decrease in M group NADPH levels (201.69 ± 9.87 nmol/g tissue, P<0.001) compared to C group (250.95 ± 2.92 nmol/g tissue) was observed. Moreover, NADPH levels of R group (228.95 ± 1.78 nmol/g tissue, P<0.001) were found to be higher than M group (201.69 ± 9.87 nmol/g tissue) (Table 2). The antioxidant enzyme, GPx activity was decreased in M group (59.40 ± 6.75 U/g protein, P<0.01) compared to C (87.61 ± 8.04 U/g protein) (Table 2). In addition, it was found that the activities of GPx in R and MR groups (33.23 ± 6.02 U/g protein; 29.36 ± 2.93 U/g protein, P<0.01, respectively) were lower than C and M groups (Table 2). Meanwhile, G6PD activity increased in the M group (2.82 ± 0.25 U/g protein, Table 2) compared to the C group (2.55 ± 0.56 U/g protein, Table 2), but this increase was not significantly different (P>0.05).

Table 2. Effects of rutin and methotrexate MDA and some antioxidant parameters in rat liver.
Tablo 2. Rutin ve metotreksatın rat karaciğerinde MDA ve bazı antioksidan parametreler üzerine etkileri.

Groups	MDA (nmol/g tissue)	Vitamin C (µg/g tissue)	tGSH (µmol/g protein)	NADPH (nmol/g tissue)	GPx (U/g protein)	G6PD (U/g protein)
C	31.78±1.17^{b}	193.29±15.46^{a}	42.46±3.13^{a}	250.95±2.92^{a}	87.61±8.04^{a}	2.55±0.56
M	50.12±3.60^{a}	138.23±9.51^{b}	33.88±2.27^{b}	201.69±9.87^{c}	59.40±6.75^{b}	2.82±0.25
R	27.55±1.48^{b}	201.52±9.87^{a}	37.47±0.97^{ab}	228.95±1.78^{b}	33.23±6.02^{c}	2.35±0.17
MR	32.47±0.94^{b}	174.94±5.06^{a}	35.19±0.60^{b}	235.71±3.75^{ab}	29.36±2.93^{c}	2.96±0.28
P	<0.001	<0.01	<0.05	<0.001	<0.01	>0.05

M: methotrexate; R: rutin; tGSH: total glutathione; NADPH: nicotinamide adenine dinucleotide phosphate; GPx: glutathione peroxidase G6PD: glucose - 6 - phosphate dehydrogenase. Values were represented as mean ± SE. Different superscripts (a–c) in the same row indicate significant difference (P<0.001; P<0.01; P<0.05) among groups.

DISCUSSION and CONCLUSION

The mechanisms underlying methotrexate-induced hepatotoxicity are not fully understood, however, it has been proposed that both free radicals and inflammatory cytokines cause hepatotoxicity (8,12). Previous studies (12,15,28) presented that overproduction of free radicals, increased lipid peroxidation and depletion of antioxidant defense systems play a role in methotrexate-induced liver damage.

Lipid peroxidation induces damage to the cell membrane and alters the physiological function of the cell (29). Malondialdehyde, a metabolite of lipid peroxidation, is used as an oxidative stress indicator in cells (10,12). Previous studies (3,12,29) have reported that methotrexate (20 mg/kg, single dose, i.p.) leads to toxicity by triggering liver MDA production. Consistently with these studies (3,12,29), in our study liver MDA levels were found to increase following methotrexate (20 mg/kg, single dose i.p.) application. That increase might be related to the reactive oxygen species generating effects of methotrexate in the liver. Erdogan et al. (29) reported that post-methotrexate rutin administration caused a decrease MDA levels in liver, leading to a decrease in lipid peroxidation. Similarly, in the current study, it was found that pre and post-methotrexate rutin administration reduced MDA levels. According to these results, it can be said that rutin has a protective role in liver damage.

One of the low molecular weight antioxidants vitamin C is involved in the first line of antioxidant defense together with GSH and vitamin E, and plays an important role in cellular redox balance (4,30). Yamamoto et al. (31) reported an essential decrease in plasma vitamin C levels in chronic hepatitis patients. Olayinka et al. (4) reported that liver vitamin C decreased in male Wistar albino rats treated with 0.2 mg/kg methotrexate for seven days. Similarly, in this study, it was determined that methotrexate administered at a single dose of 20 mg/kg decreased liver vitamin C level. Shenbagam and Nalini (16) reported that liver vitamin C level did not change with rutin (100 mg/kg, orally) administration for 30 days in rats. In accordance with this study, it was determined that the rutin administered orally at a dose of 50 mg/kg for fifteen days did not have any effect on liver vitamin C. In our literature search, we could not find studies demonstrating the effects of rutin on vitamin C levels in rats treated with methotrexate. Nonetheless, Nègre-Salvayre et al. (32) reported that rutin increases the free radical scavenging capacity of

vitamins E and C. Shenbagam and Nalini (16) reported that, ethanol-induced decreased liver vitamin C levels were reversed by rutin supplementation along with ethanol in rats. Similarly, in this study, it was observed that rutin administration with methotrexate prevented the decrease of vitamin C caused by methotrexate in rats.

Nicotinamide adenine dinucleotide phosphate is used by glutathione reductase to maintain the reduced state of cellular GSH (12). The main enzymes that produce NADPH in cells are 6 - phosphogluconate dehydrogenase, NADP - malic enzyme, glucose - 6 - phosphate dehydrogenase, and NADP - isocitrate dehydrogenase (33). To our knowledge, there is not any study evaluating the effects of rutin and methotrexate on liver NADPH levels and G6PD activity in rats. In an *in vitro* study using the HeLa cell line, it has been suggested that methotrexate inhibited NAD(P) - dependent dehydrogenase and NADP malic enzymes and can reduce the presence of NADPH with an unknown mechanism in the cell (34). In this study, it was found that rutin and methotrexate decreased the level of liver NADPH and did not change the G6PD activity. It is thought that methotrexate and/or rutin may have NADPH inhibitory effects through NADPH producing enzymes (NADP - malic enzyme, NADP - isocitrate dehydrogenase, and 6 - phosphogluconate dehydrogenase) except G6PD. Higher levels of NADPH levels in methotrexate plus rutin compared to that in the methotrexate group, may indicate that the rutin possesses a protective effect on the liver in the presence of oxidative stress.

Glutathione is an essential cytosolic radical scavenger, and the levels have been reported to decrease through a lack of NADPH (10). Studies reported that methotrexate causes a decrease in liver GSH levels (3,4,12). Similar to previous studies, it was determined that methotrexate reduced tGSH levels like NADPH levels. Shenbagam and Nalini (16) reported that rutin did not change liver GSH levels in rats. In accordance with this study, it was found that the rutin did not change the level of liver tGSH. Caglayan et al. (21) reported that rutin administration with mercury-chloride decreased GSH levels compared to the control group. Similarly, it was determined that the use of rutin with methotrexate reduced the tGSH level compared to the control group in this study.

In accordance with the results of the current study, in previous studies (3,10,12,29), it has been reported that methotrexate causes a decrease in liver GPx activity. Although Shenbagam and Nalini (16) reported that rutin (100 mg/kg, thirty days) did not change liver GPx activity, in this study, it was found that rutin crucially reduced liver GPx activity. Caglayan et al. (21) demonstrated that rutin (50 mg/kg) administration with mercury-chloride reduced GPx activity compared to the control group. Similarly, in this study, it was determined that the use of rutin with methotrexate reduced GPx activity compared to the control group. Erdogan et al. (29) demonstrated that methotrexate (20 mg/kg, single dose) and rutin (100 mg/kg, for ten days, i.p.) increased GPx activity compared to methotrexate. In this study, it was found that the use of rutin, which was administered orally at a dose of 50 mg/kg for fifteen days, together with methotrexate (20 mg/kg, single dose) decreased GPx activity compared to the methotrexate group. This may be due to the different dose, administration duration, and routes of the rutin.

In this study, it was found that methotrexate-induced oxidative stress by increasing MDA levels and decreasing antioxidant (Vit C, tGSH, NADPH, GPx) levels in liver tissue. It has been observed that rutin reduced methotrexate-induced oxidative stress by decreasing the MDA levels and also by increasing vitamin C and NADPH levels.

Consequently, this study suggests that rutin administered pre- co- and post-methotrexate has antioxidant effects on methotrexate-induced oxidative hepatic damage. Moreover, it is predicted that rutin administered orally may be useful in reducing the side effects of chemotherapy, and it is thought that it can be used as a potential agent in clinical applications with methotrexate in chemotherapy. It is suggested to investigate the

exact mechanisms underlying the hepatoprotective potential of the rutin, in future studies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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