Rutin ameliorates methotrexate induced hepatic injury in rats[1]

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ABSTRACT

PURPOSE: To investigate the possible protective effect of rutin on methotrexate induced hepatotoxicity in rats.

METHODS: Twenty-two rats were divided into three experimental groups; Control-saline, Mtx, Mtx+Rutin. Hepatic tissue was taken for histological assessment and biochemical assays. Oxidative stress parameters malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were investigated. Liver markers aspartate aminotransferase (AST), alanine aminotransferase (ALT) were analyzed in serum.

RESULTS: Mtx+Rutin group showed lower histological injury compared to Mtx group, MDA and ALT levels were increased, while SOD and GSH-Px were decreased in Mtx group compared with Control-saline group. MDA and ALT levels were increased, while SOD and GSH-Px were decreased in Mtx group, compared with Mtx +Rutin group. Serum AST levels were similar among the groups.

CONCLUSION: Rutin may be a potential adjuvant drug to reduce the hepatic side effects observed during Mtx therapy for various clinical conditions.

Key words: Methotrexate. Rutin. Drug Effects. Liver. Rats.

Introduction

The use of chemotherapeutics is known to cause acute toxic effects in multiorgan systems. Methotrexate (Mtx), a folic acid analogue, is an effective cytotoxic agent and has been widely applied in chemotherapeutic-based treatments for malignancies. Methotrexate is also an effective immunosuppressive and anti-inflammatory agent and used for treatment of some chronic inflammatory disease including psoriasis, rheumatoid arthritis, Crohn's disease[1,2]. Side effects of Mtx limit its clinical uses. The most common adverse effects include hepatotoxicity, ulcerative stomatitis and decreased white blood cell count. Methotrexate has toxic side effect on liver particularly in high dose or long-term administration[3].

The exact mechanisms underlying Mtx hepatotoxicity are unclear. Mtx inhibits the cytosolic nicotinamide adenosine diphosphate (NADP)-dependent dehydrogenases. NADPH is the reduced form of NADP. Glutathione reductase, a cytosolic antioxidant, uses NADPH to maintain the reduced state of cellular glutathione and protects against reactive oxygen species (ROS). ROS damage the biomolecules such as lipids, proteins, DNA and ultimately lead to death of the cells[2-4]**.** In animals treated with Mtx, antioxidants were decreased and the levels of oxidants increased that contribute Mtx-induced oxidative stress[1,5,6]. It is suggested that Mtx associated hepatotoxicity is related to the accumulation of main Mtx metabolite, 7- hydroxymethotrexate as polyglutamate which inhibits the synthesis of tetrahydrofolate by dihydrofolate reductase in the hepatocyte. The decrease in tetrahydrafolate levels is suggested as the other mechanism for Mtx hepatotoxicity. Addition of folate in Mtx treatment reduces hepatotoxic side effects. However, it also reduces the efficacy of the Mtx treatment[7].

Flavonoids are natural polyphenols found ubiquitously in various fruits, leaves and seeds. Many flavonoids have antioxidant activity, free-radical scavenging capacity, anti-atherogenic, and anticancer activity. Quercetin and Rutin (quercetin-3-O-rutinoside) are the flavonoids most abundantly found in foods[8]. Rutin as a bioflavonoid compound (a glycoside derivative of quercetin) has anticancer, anti-inflammatory, anti-viral, antioxidant effects and has vasoactive properties. It is suggested that Rutin inhibits membrane lipid peroxidation by chelating metal ions, such as ferrous cations[9]. Rutin attenuated oxidative stress by improving antioxidant enzyme levels and inhibited lipid peroxidation in vitro as previously reported[10].

Rutin has high radical scavenging activity and antioxidant capacity that are potentially beneficial in preventing oxidative stress related diseases[10]. However to the best of our knowledge, there is no report about the effect of Rutin against Mtx induced hepatic oxidative stress and anti-inflammatory activity. The aim of this study is to investigate the possible protective effects of Rutin in Mtx induced hepatotoxicity in rats.

Methods

Animals and experimental design

The study was performed with the approval of the local animal ethics committee, (2015-01, 15/6). Twenty-two adult female Wistar albino rats ranging from 200 to 250 grams in weight were kept in temperature, and a 12-h light/dark cycle at 22± 2˚C in accordance with ethical guidelines. Water and food were provided ad libitum. Rats were randomly divided into three experimental groups; Group 1: Control-saline, Group 2: Mtx and Group 3: Mtx+Rutin groups. Group 1 was treated with 0.5 ml saline intraperitoneally (i.p) (n=6), Group 2 was treated with a single dose of 20 mg/kg Mtx (Methotrexate, Kocak Farma, Turkey) i.p at day 1 (n=8). Group 3 was treated with a single dose of 20 mg/kg Mtx i.p at day 1 followed by 100 mg/kg Rutin i.p (Rutin trihydrate, Santa Cruz, USA) for 10 consecutive days (n=8). At the end of the study, the rats were anesthetized by ketamin (20 mg/kg)-xylasine (5 mg/kg), then blood and liver tissue samples were obtained.

Histological analysis

Liver tissue specimens were fixed in formalin for light microscopic analyses. After fixation, the liver tissues were processed routinely for embedding in paraffin. Tissue sections of 4 μm were stained with Hematoxylin-Eosin (H&E) and Masson's Trichrome to evaluate the parenchymal and stromal structure of the liver tissues. The sections were examined under a light microscope. A modified scoring system was used to evaluate the severity of hepatic injury according the parameters with the extent of inflammatory cell infiltration, sinusoidal dilatation, congestion and hydropic degeneration (cytoplasmic vacuolization and swelling of hepatocytes). Liver injury parameters were scored as 0 (normal), 1 (mild), 2 (moderate), or 3 (severe) as described before[11]. Slide scores were obtained after examination of the complete microscopic field for each sample section.

Biochemical analysis

Liver tissue samples from each animal were immediately immersed in liquid nitrogen and kept at –80 prior to biochemical analyses. Frozen tissues were homogenized in phosphate buffer (pH 7.4) on an ice cube, by a homogenizer (Heidolph Silent Crusher M, Heidolph Instruments, Schwabach, Germany). The supernatant was stored at −80°C. The protein content of tissue homogenates was measured by the Lowry method.

In liver tissues, oxidative stress parameters such as malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were evaluated. MDA, a lipid peroxidation product, was measured by the thiobarbituric acid (TBA) reaction[12]. This method was used to obtain a spectrophotometric measurement (Biotek µQuant, Biotek Instruments, Winooski, WT, USA) of the color produced during the reaction of tothiobarbituric acid (TBA) with MDA and the absorbance was measured at 535 nm. The MDA levels were expressed as mmol/gr protein. SOD activity was assayed using the nitroblue tetrazolium (NBT) method. Nitroblue tetrazolium was reduced to blue formazan by O_2^-, which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The calculated SOD activity is expressed as U/gr protein. GSH-Px activity was measured using the method in which GSH-Px activity was coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was spectrophotometrically followed up at 340 nm at 37°C. The absorbance at 340 nm was recorded for 5 min. The activity was the slope of the lines as mmol of NADPH oxidized per minute. GSH-Px activity is expressed as U/gr protein.

For the analyses of serum hepatic injury markers such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), blood samples were taken from each animal, centrifuged at 3000 g for 10 minutes. Serum activities were measured with a spectrophotometric technique by the Olympus AU-2700 autoanalyzer (Olympus, Hamburg, Germany) using commercial kits and the results were expressed as U/L.

Statistical analysis

Histologic scores, tissue oxidative stress parameters and serum liver markers were analyzed to evaluate the effect of the Rutin on the Mtx induced hepatic injury in rats by using IBM SPSS Statistics 20.0 and Graphpad Prism 6.0. The differences among the groups were compared by Kruskal-Wallis test. The differences between two groups were compared using Mann Whitney U test. A p value < 0.05 was regarded as significant. All results were expressed as means +standard error (SEM).

Results

Histological analysis

The results of histological injury scores of the groups were summarized in Table 1. Dilatation and congestion in Mtx+Rutin group were increased significantly compared with the Control-saline group (p=0.0003, p=0.002 respectively). Inflammation, hepatocyte vacuolization, dilatation and congestion in Mtx group were increased significantly compared with Mtx+Rutin group (p=0.007, p=0.003, p=0.028, p=0.015 respectively). The Control-saline group showed a normal liver histology, radial arrangement and stroma (Figure 1A and 2A). In Mtx group, we observed intense hepatocyte vacuolization, sinusoidal dilatation, disruption in radial arrangement (Figure 1B), and intense congestion in the stromal areas (Figure 2B). In Mtx+Rutin group, we observed decrease in hepatocytes vacuolization, sinusoidal dilatation and partially disruption in radial arrangement (Figure 1C) and moderate congestion in the stromal areas (Figure 2C) as compared to Mtx group.

TABLE 1 - Histologic injury results of the liver tissue sections in the study groups.

Histologic Parameters	Groups		
	Control-Saline	Mtx	Mtx+Rutin
Inflammation	0.33±0.33[b]	1.75±0.25[a,c]	0.63±0.18[b]
Vacuolization	0±0[b]	2.00±0.27[a,c]	0.62±0.18[b]
Dilatation	0.17±0.17[b,c]	2.25±0.25[a,c]	1.37±0.18[a,b]
Congestion	0±0[b,c]	2.25±0.25[a,c]	1.25±0.63[a,b]

Mtx: Methotrexate
[a]: Compared with Control-saline group. p<0.05
[b]: Compared with Mtx group. p<0.05
[c]: Compared with Mtx+Rutin group. p<0.05

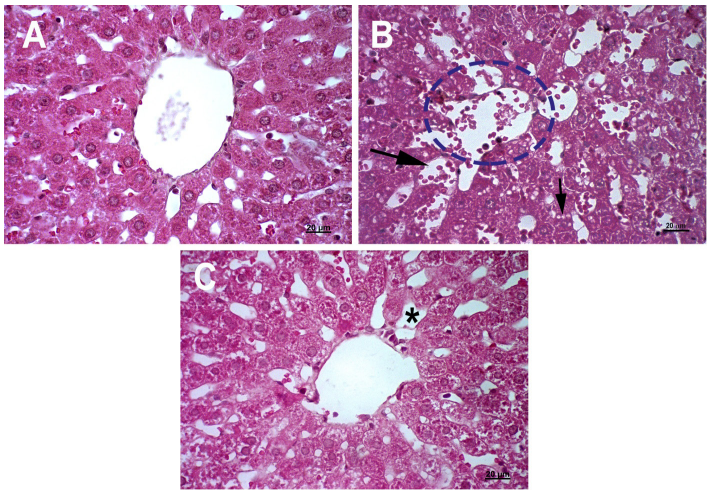

FIGURE 1 - Hematoxylen Eosin staining of the study groups. (**A**) Normal histological structure of liver in Control-saline group. (**B**) Hepatocytes vacuolization (thin arrow), sinusoidal dilatation (thick arrow) and disruption in radial arrangement around central vein (dashed circle) in Mtx group. (**C**) Sinusoidal dilatation (star) and partially disruption in radial arrangement around central vein in Mtx+Rutin group (H&E, Scale bar 20 µm).

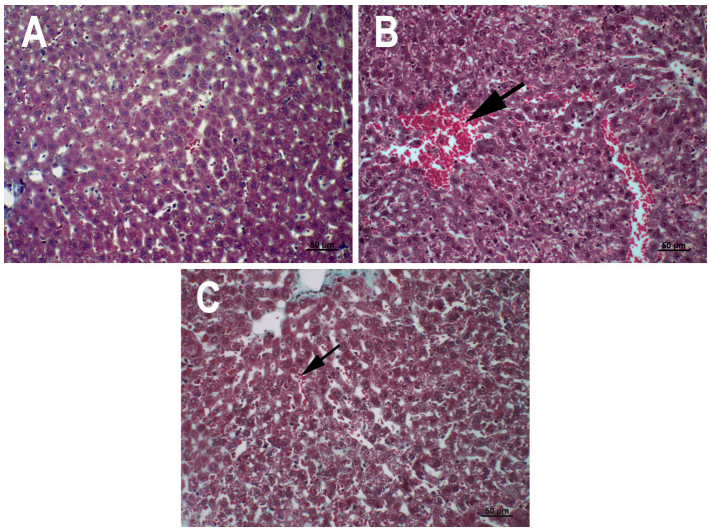

FIGURE 2 - Masson's Trichrome staining of the study groups. (**A**) Normal histological structure of liver in Control-saline group. (**B**) Intense congestion (thick arrow) in stromal areas in Mtx group. (**C**) Moderate congestion (thin arrow) in stromal areas in Mtx+Rutin group (Masson's Trichrome, Scale bar 50 µm).

Biochemical analysis

Liver tissue MDA, SOD, GSH-Px results are summarized in Table 2. MDA were increased significantly in Mtx group and Mtx+Rutin group compared with the Control-saline group (p=0.001, p=0.008 respectively). MDA level in Mtx+Rutin group was significantly lower than Mtx group (p=0.000). GSH-Px activity in Mtx group and Mtx+Rutin group were found significantly lower than Control-saline group (p=0.001, p=0.001 respectively). GSH-Px activity in Mtx group was significantly lower than Mtx+Rutin group (p=0.000). SOD activity in Mtx group was found significantly lower than Control-saline group (p=0.001) and Mtx+Rutin Group (p=0.007). SOD measurements were not significant between Mtx+Rutin and Control-saline groups (p=0.228).

TABLE 2 - Liver tissue malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) results in the study groups.

	Groups		
Oxidative Stress Parameters	Control-Saline	Mtx	Mtx+Rutin
MDA (mmol/g protein)	1.16±0.53[b,c]	2.41±0.19[a,c]	1.45±0.63[a,b]
SOD (U/g protein)	975.93±63.64[b]	671.58±23.40[a,c]	845.78±37.68[b]
GSH-Px (U/g protein)	224.63±9.94[b,c]	164.82±2.38[a,c]	189.40±2.07[a,b]

Mtx: Methotrexate
[a]: Compared with Control-saline group. p<0.05
[b]: Compared with Mtx group. p<0.05
[c]: Compared with Mtx+Rutin group. p<0.05

Serum AST and ALT results are presented in Table 3. ALT activity in Mtx group was significantly higher than Control-saline group (p=0.001) and Mtx+Rutin group (p=0.000). The difference between the ALT activity in Mtx+Rutin and Control-saline groups was not significant (p=0.228). The difference between the AST levels in all groups was not significant (p>0.05).

TABLE 3 - Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) results in the study groups.

	Groups		
Serum Liver Markers	Control-Saline	Mtx	Mtx+Rutin
AST	196.00±14.68[d]	248.00±17.72[d]	214.5±14.81[d]
ALT	79.67±8.20[b]	153.25±11.24[a,]	90.13±4.67[b]

Mtx: Methotrexate
a: Compared with Control-saline group. $p<0.05$
b: Compared with Mtx group. $p< 0.05$
c: Compared with Mtx+Rutin group. $p<0.05$
d: Compared with all 3 groups. $p>0.05$

Discussion

Chemotherapeutic agents such as Mtx are used in various cancer and inflammatory diseases. Hepatotoxicity is the most serious side effect in long term Mtx treatment. Approaches for reducing this complication are valuable for improving the life quality of the patients and success of the treatment[3,6]. We demonstrated Rutin, a flavonoid molecule with antioxidant capacity to have beneficial effects against Mtx induced hepatotoxicity.

In the present study Mtx treatment caused oxidative tissue damage and histological liver injury in rats in accordance with the literature[3]. Patients treated with Mtx show nonspecific liver histological changes such as fatty change, focal hepatocytes necrosis, portal tract inflammation, and fibrosis. Mtx increases the production of free radicals that are released by stimulated neutrophils, and thus accelerates cellular damage that can lead to, inflammation, necrosis and fibrosis[13]. We observed histologic changes in liver sections such as dense lymphocytic infiltration, vacuolization in hepatocytes, sinusoidal dilatation and congestion related to liver injury after Mtx treatment in our rat model. Rutin was able to improve the Mtx related histological changes in liver tissues. Our observations about the protective effects of are in agreement with in rats that protected against induced by observations about the protective effects of Rutin against drug induced hepatotoxicity are in agreement with two recent studies[14,15]. In one of them Nafees *et al*.[16] evaluated the beneficial effect of Rutin pretreatment against cyclophosphamide induced hepatotoxicity in a rat model. They evaluated inflammatory cytokines TNF-α and IL-6, transcription factor NFκB and p38-MAPK as well as oxidative enzymes. They suggested that Rutin alleviated ROS induced oxidative stress and concurrent inflammation by possibly interfering with the NFκB and MAPK pathway. They applied Rutin before chemotherapy in contrast to our study which we applied Rutin after cyclophosphamide treatment.

The other study investigated the beneficial effects of Rutin treatment against hepatotoxicity induced by another platinium compound, oxaliplatin in a mouse model[15]. The animals were treated with oxaliplatin on 6 different days where Rutin was administered everyday during a study period of 42 days. Rutin treatment showed beneficial effects against liver inflammation microscopically and decreased apoptosis as shown by decreased caspase-3 positive cells. Although there are differences in the design of above two studies compared to our study, all three studies reported similar findings about the beneficial effects of Rutin against drug induced hepatotoxicity.

Lipid peroxidation can be defined as the oxidative degradation of lipids containing carbon-carbon double bonds, especially fatty acids. Lipid peroxidation results in damaged cell membrane and altered the physiological function[16]. Malondialdehyde, a lipid peroxidation byproduct, is used as an indicator of oxidative stress in cells and tissues[17]. In our study, Mtx-depended increase in MDA levels confirmed the oxidative stress and explained the tissue damage as previously reported[6]. Rutin treatment following Mtx resulted a decrease in liver tissue MDA levels that indicates a decrease in lipid peroxidation. Rutin showed similar anti-oxidant effect in previous studies using cardiac and testicular tissue injury models[18].

The cell has enzymatic and non-enzymatic antioxidant systems to protect itself against oxidative damage. One of antioxidant enzyme in cytoplasm is GSH-Px. It degrades hydrogen peroxides and thus protects the cell against oxidative stress[10,19]. Superoxide dismutase converts superoxide radicals into hydrogen peroxide, and catalase, which converts hydrogen peroxide into water and oxygen gas[20]. Reduction of SOD and GSH-Px enzymes activity occur due to an increase of oxidant stress in the cell. According to previous studies with Mtx, decrease in SOD and GSH-Px-activities resulted with further increase in oxidative stress

in the tissue[2,19]. Our increased SOD and GSH-Px activity results correspond with a previous study about hepatoprotective effect of Rutin following carbon tetrachloride (CCl4) treatment[21]. In our study, tissue SOD and GSH-Px activity significantly decreased while MDA levels increased in Mtx group. Our results are in consonance with the previous studies related to Mtx toxicity on liver, kidney and testis[1,5,22]. In our study, Rutin treatment increased the tissue SOD and GSH-Px activity following Mtx treatment. Previous studies demonstrate that hepatoprotective effects of Rutin were associated with upregulation of antioxidant enzyme activities[14]. The improvement of the antioxidant enzyme activities that inhibit lipid peroxidation may explain the beneficial effects of Rutin against hepatotoxicity.

Increase of serum ALT activity in the Mtx group demonstrates the hepatotoxicity. The use of Rutin showed an important decrease in ALT enzyme activity compared with the Mtx group. But AST in our experiment was not changed significantly in all groups. Serum ALT is more specific for liver pathology, and AST is thought to be as a specific marker for long-term liver toxicity[23]. For that reason our study period may not be enough to observe the AST alteration. Rutin treatment was previously shown to be beneficial by decreasing serum ALT levels in alcohol induced hepatotoxicity in rats[24]. Assessment of transaminase levels (AST and ALT) did not reveal any significant alteration in mouse blood serum after the treatments, although there was a clear tendency of diminishing ALT in the treatment groups[15].

All the histological and biochemical results showed that Rutin treatment had preventive effects on Mtx induced hepatotoxicity in a rat model.

Conclusion

Rutin may be a potential adjuvant drug in Mtx chemotherapy to reduce the hepatic side effects.

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