**Introduction**

Methotrexate (MTX), is an inhibitor of folic acid synthesis. MTX have been used to treat psoriasis, psoriatic and rheumatoid arthritis, leukemia, sarcoidosis, vasculitis and inflammatory bowel disease [1].

Long-term clinical use of MTX is connected with a raised risk of liver injury and fibrosis [2]. About 3% of rheumatoid arthritis (RA) patients, on low dose MTX evolve critical liver fibrosis [3]. Additionally, patients with psoriasis have a much more significant risk for liver fibrosis, with reported incidences ranging from 8 to 23%, leading to recommendations for assessment of liver damage after every 1.5 g of total intake [4]. MTX-induced liver toxicity is an outcome of many factors including total applied dosage, hepatoxicity predisposing factors, underlying disease and presence of genetic and molecular apoptotic factors [5].

Leflunomide (N-[4-tri-urormethylphenyl]-5-methylisoxazol-4-carboxamide) was created as disease-modifying antirheumatic drug [6]. Leflunomide is a pro-drug that is metabolized to active A77 1726 (N- [4-tri-urormethylphenyl]-2-cyano-3-hydroxy-crotonic acid amide). A77 1726, is a potent inhibitor of nuclear factor kappa B (NF-KB) activation induced by tumor necrosis factor (TNF) and various inflammatory agents [7]. In addition, Leflunomide presents antioxidant activity. Leflunomide inhibits the release of reactive oxygen species (ROS) from leukocytes [8]. Ozturk et al. [9] showed that leflunomide had antioxidant properties in septic rats. Manna [10] demonstrated that Leflunomide reduced TNF related cellular responses and blocked TNF-induced caspase activation and decreased TNF-induced ROS generation and lipid peroxidation. Yao et al. [11] demonstrated that leflunomide diminished proinflammatory cytokines with the formation of malondialdehyde (MDA) and nitric oxide (NO) and raised antioxidant activity in CCl4-induced liver injury. Leflunomide is also commonly used in combination with MTX in certain autoimmune disorders. However, there is no experimental or clinical study investigating the effects of leflunomide in MTX toxicity.

In this study, we aimed to explore the role of leflunomide in the prevention of MTX induced hepatoxicity.

**Materials and methods**

All experiments were conducted with approval of the Animal Research Committee at Pamukkale University Medical Center, Denizli, Turkey (Date: 17.02.2009, Number: B.30.2.PAÜ.0.01.00.00.400-1/10). Wistar albino rats purchased from Pamukkale University Faculty of Medicine Research Laboratory. All experiments were conducted with the approval of the Animal Research. The experimental study included 39 male Wistar albino rats (185-254 g). Before experiments, animals were given free passage to food and water. The room was maintained on a 12 hours light-dark cycle and at a temperature of 24°C. Food was withdrawn 12 hours before the experiment.

MTX (Methotrexate; Onco-Tain Mayne Pharma Pty Ltd) was injected intraperitoneally. After one dose of MTX (in physiological saline, 20 mg/kg), either carboxymethylcellulose (Group 1, *n*=9) or leflunomide (Arava; Aventis Pharmaceuticals) 9 mg/kg/day (group 2, *n*=10) was given intragastrically for the sequential 5 days. Because of Leflunomide was insoluble in water; 1% sodium carboxymethylcellulose (CMC) was used as a vehicle. In this study, the model of the MTX-induced liver toxicity was applied according to Uraz et al. [12] and leflunomide was administered according to a previous report [11].In group 3, following a single dose, intraperitoneally saline injection leflunomide was administered 9 mg/kg/day intragastrically for the consecutive 5 days. In group 4 following a single dose intraperitoneally physiological saline injection,1% CMC was given intragastrically for the sequential 5 days. After 5 days, all the animals were sacrificed. After decapitation, intracardiac blood was collected and liver was removed. The liver tissue samples were put in 10% neutral buffered formaldehyde solution for histopathological examination or stored at -70 C for subsequent determination of activities of myeloperoxidase (MPO), superoxide dismutase (SOD) and glutathione (GSH) levels. Serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured using Abbott Architect C8000 autoanalyzer with commercial kits. The results are expressed as IU/L.

**Biochemical analysis**

**Glutathione (GSH) assays**

GSH assay was applied according to the previous report in the literature [13]. Tissue GSH levels are indicated as μmolGSH/ g tissue.

**Myeloperoxidase (MPO) activity**

MPO activity was measured according to the previous report in the literature [14]. Results were expressed as U/g tissue.

**Superoxide dismutase (SOD) activity**

Total liver SOD activity was determined according to the previous report in the literature [15]. SOD activity was expressed as U/mg protein.

**Histopathological preparation and analysis**

Microscopic scoring was evaluated by one experienced pathologist, who was unknowing of the treatment groups. The light microscopic investigation was done following stained hematoxylin and eosin (H&E). Histopathological examination was graded according to two different systems. First, semiquantitative oxidant liver injury was graded according to Demling and Sener et al [16, 17]. Second, MTX induced liver toxicity was graded according to the Roenigk classification ranging from grade 1 to grade 4 [18].

**Statistical analyses**

SPSS 10.0 package program was used for analyses. Methods including Kruskal Wallis Variance Analysis, Post-hoc comparisons, Mann Whitney U test, Bonferroni Correction were used for calculation. The statistical signification was set at *P*<0.05.

**Results**

Administration of one dose MTX 20 mg/kg induced liver toxicity characterized by high concentrations of serum ALT, and ALP levels which were 56.7+9.13 IU/L, 514+120 IU/L in group 1. Control group 4 had ALT and ALP levels 27+13 IU/L and 103+121 IU/L respectively (*P*=0.0001). Histopathologic score according to semiquantitative scale were significantly higher in MTX induced liver injury group 1 compared to control group 4. (5.1+2 vs 2.1+1.8 score *p*=0.03). Rising in Kuppfer cell activation is one of the criteria in semiquantitative scale. Increase in the number of activated Kupffer cells score of group 1 were detected significantly higher than that of group 4 (2.5+1.01 versus 0.9+1.1, *P*=0.01), (Figure 1a).Tissue GSH levels of the group 1 were measured significantly lower than that of the group 4 (9.6+1.4μmolGSH/ g tissue versus 13,01+1.8μmol GSH/ g tissue *P*=0.02). Results of biochemical tests including levels of ALT, ALP, GSH, SOD, and MPO are summarized in Table 1.

Group 2 was treated with both MTX an leflunomide in order to evaluate effects of leflunomide on MTX toxicity.Serum ALT levels of the group 2 were measured significantly lower than the levels of group 1 (40.68+13.6 IU/L versus 56.7+9.13 IU/L, *P*=0.0001). Serum ALP levels of the group 2 were measured significantly lower than that of the group 1 (169+234 IU/L versus 514+120 IU/L, *P*=0.0001). GSH levels of tissue in the group 2 were measured higher than that of group 1, but the difference was not significant (10.93±0.8 μmolGSH/ g versus 9.6±1.41 μmolGSH/ g, *p*>0.05). Tissue SOD levels of the group 2 were measured slightly higher than that of the group 1. (5.2±0.6 U/mg versus 4.7+0.2 U/mg, *p*>0.05). Although statistically non-significant tissue MPO levels in group 2 were measured lower than that of the group 1 (5.6±1.9 U/mg versus 7.8±3.2 U/mg, *p*>0.05).

Total histopathologic score according to semiquantitative scale in group 2 was lower than group 1 (1.1+0.7 versus 5.1+2 respectively, *p*=0.0001). Leflunomide significantly ameliorated Kuppfer cell activation. Rising in the number of activated Kupffer cells score was suppressed from 2.5+1.01 to 0.2+0.6, *p*<0.001 in group 1 and group 2 respectively (Figure 1b). Results of semiquantitative oxidant liver injury score of all groups are summarized in Figure 2.

According to Roenigk classification, 22.1% of rats were grade1 and 77.8% of rats were grade 2 in group 1. According to Roenigk classification, 100% of rats were grade1 in group 2. The difference between group 1 and 2 was statistically significant (*p*=0.001). This difference between two group Roenigk score a consequence of significantly different nuclear pleomorphism score which is one of the criteria in Roenigk classification. Rising in the number of activated Kupffer cells score was found 2.25+1.38 in the liver specimens of grade 2 according to Roenigk classification. Rising in the number of activated Kupffer cells score was found 0.84+1.04 in the liver specimens of grade 1 according to Roenigk classification. This difference was statistically significant (*p*<0.001). In this study fibrosis or cirrhosis were not detected in any of the liver specimens.

**Discussion**

The results of our study showed that leflunomide treatment ameliorated MTX induced liver toxicity. It has been suggested that combination therapy with MTX and leflunomide was safe in a previous report [19]. However, it is not known if MTX toxicity is less common in patients using leflunomide concomitantly for their autoimmune diseases.

MTX induced hepatotoxicity appears to be an outcome of the interaction of many factors: [9]. Cytosolic NADP-dependent dehydrogenase and NADP malic enzymes are suppressed by MTX. Diminished reserve of NADP caused by MTX decreases glutathione levels which eventually make hepatocytes tender to damage from reactive oxygen molecules like superoxide anions, hydroxyl radicals, hydrogen peroxide and hypochlorite radicals [20]. The new investigations about the mechanism of MTX related liver injury concentrate on apoptosis and apoptotic genes [9]. Caspase activity found to be directly associated with apoptosis [21].

A metabolite of leflunomide, A77 1726, is a powerful inhibitor of NF-KB activation [13]. Leflunomide presents antioxidant activity [14,15] and has a suppressive effect on TNF-induced caspase activation [16]. Although many treatment modalities such as ursodeoxycholic acid [12], N-acetylcysteine [22], grape seed extract [23] and melatonin in MTX induced liver toxicity treatment were experienced [24]. Beneficial effects of leflunomide have been demonstrated on experimental models of liver injury including CCl4 induced [11], biliary obstructed [25], T cell-mediated [26] or acetaminophen-induced liver injuries [27] in previous reports. In this study, we found that leflunomide significantly suppressed histopathologic damage and decreased serum levels of ALT, ALP in MTX induced liver toxicity. The liver GSH, MPO, and SOD levels were similar between leflunomide treated and untreated groups. These results suggest that the effect of leflunomide on MTX induced liver toxicity was independent of its antioxidant activity.

In this study leflunomide treatment significantly decreased the number of activated Kupffer cells in MTX induced liver toxicity. Little is known about the MTX induced liver toxicity and its relation to Kupffer cell activation in the literature. Hall et al. [28] demonstrated enlarged and activated Kupffer cells in an experimental model of MTX induced liver toxicity in rats. Our results suggested that MTX induced liver toxicity was strongly related to Kupffer cell activation. Beside, Kupffer cell activation was highly correlated with worse Roenigk histopathologic grade. Kupffer cells are the largest group of the mononuclear phagocytic system [29]. Many of the immunological critical roles of Kupffer cells are related their high activity of the NF-KB family of transcription factors [30]. Many inhibitors of NF-KB, such as caffeic acid [31], pyrrolidine dithiocarbamate [32] resveratrol [33], silymarin [34], thalidomide [35], spiriluna [36] andberberine [37] have shown antinecrotic, anticholestatic, antifibrotic and anticancer activities in the liver.[Bilasy SE](https://www.ncbi.nlm.nih.gov/pubmed/?term=Bilasy%20SE%5BAuthor%5D&cauthor=true&cauthor_uid=25560583) et al reported that leflunomide and MTX combination had more hepatotoxic effect in a rat model of RA [38]. Curtis et al. suggested that the combination of MTX and leflunomide was associated with more risk of hepatotoxicity than MTX monotherapy in human[39]. Our study is in disagreement with these reports. In our experiment, biochemical results and histopathologic analysis of liver suggested that leflunomide significantly ameliorates MTX induced liver toxicity.

One of the limitations of our study is lack of fibrosis in our MTX induced liver injury model in experimental groups. Therefore we were not able to test the effects of leflunomide in MTX induced liver fibrosis. As already known cumulative MTX usage may lead advanced liver fibrosis and even cirrhosis in patients and the potential effect of leflunomide on liver fibrosis should be investigated in similar experimental models. Lack of NF-KB measurement is also another limitation of our study. However, potent inhibitory effects of leflunomide on NF-KB is already known and was expected as one of the protective mechanism of it on MTX injury in this study. In conclusion, our study shows that leflunomide has protective properties in MTX triggered liver toxicity and the preventive effect of leflunomide on the MTX induced liver toxicity may be related to its effect on inhibition of Kuppfer cell activation. Further experimental studies are needed to explain exact mechanism of protective effects of leflunomide on MTX induced liver toxicity. Prospective clinical studies surging the prevalence of liver toxicity in long-term MTX and leflunomide usage in, as for example, RA patients might also give hints about the clinical reflection of our experimental study.

**Acknowlegment:** The authors declare no conflict of interest.

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**Table 1.** Biochemical results and tissue glutathione (GSH), superoxide dismutase (SOD), myeloperoxidase (MPO) levels of groups.

**Figure 1a.** Histopathological section of liver showing an increase in the number of activated Kupffer cells (square) which is one of the criteria in semiquantitative oxidant liver injury score (H&E stain x400).

**Figure 1b.** Histopathological section of liver from MTX group treated with leflunomide showing less increase in the number of activated Kupffer cells (square) which is one of the criteria in semiquantitative oxidant liver injury score (H&E stain x400).

**Figure 2.** Results of the semiquantitative oxidant liver injury score of all groups. Total score in MTX + leflunomide (Group 2) group was significantly lower than MTX + CMC (Group 1) (1.1+0.7 versus 5.1+2 respectively, *p*<0.01).