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Can leflunomide prevent methotrexate induced liver toxicity?

Leflunomid methotrexat'a bağlı karaciğer toksisitesini önleyebilir mi?

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Abstract

Purpose: Long-term clinical use of methotrexate is connected with a raised risk of liver injury and fibrosis. Leflunomide is a disease-modifying drug. Leflunomide has a powerful inhibitory effect on nuclear factor kappa B activation. Leflunomide also presents antioxidant activity. In this experimental study, we aimed to investigate the effects of leflunomide treatment on methotrexate -induced hepatotoxicity.

Materials and methods: Thirty-nine rats were divided into 4 groups. A single dose of 20mg/kg methotrexate was injected intraperitoneally for methotrexate-induced hepatotoxicity. After induction, leflunomide (10 mg/kg) was administered into the stomach for consecutive 5 days. Then, serum samples and homogenated liver tissues were collected for analyzed serum alanine aminotransferase, alkaline phosphatase, superoxide dismutase activity, myeloperoxidase activity, glutathione levels and assessment of histopathology.

Results: Leflunomide treatment significantly ameliorated total histopathologic score according to semiquantitative scale compared to the untreated group, (Pathological score 1.1+0.7 versus 5.1+2 respectively, p<0,01). Leflunomide treatment significantly ameliorated Kuppfer cell activation. (Elevation of the activated Kupffer cells score were 0.2+0.6 and 2.5+1.01 respectively, p = 0.001). The serum alanine aminotransferase, alkaline phosphatase levels were lower and glutathione levels, myeloperoxidase activity, and superoxide dismutase activity were similar between leflunomide treated and untreated methotrexate toxicity groups.

Conclusion: Leflunomide treatment ameliorated methotrexate induced liver toxicity in this experimental model.

Key words: Leflunomide, methotrexate, liver toxicity

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Özet

Amaç: Uzun dönem methotrexat kullanımı artmış karaciğer hasarı ve fibrozis riski ile ilişkilidir. Leflunomid hastalık modifiye edici bir ilaçtır. Leflunomid nükleer faktör kappa B aktivasyonunun güçlü bir inhibitörüdür. Aynı zamanda anti-oksidan aktivitesi de vardır. Bu deneysel çalışmada methotrexat'ın neden olduğu karaciğer toksisitesinde leflunomid tedavisinin etkinliği araştırılmıştır.

Gereç ve yöntem: 39 rat 4 gruba ayrılmıştır. Methotrexat'a bağlı karaciğer toksisitesi tek doz 20mg/kg methotrexat'ın periton içine injeksiyonu ile oluşturulmuştur. Ardından leflunomid 5 gün boyunca 10 mg/kg dozda verilmiştir. Ardından serum örnekleri ve homojenize karaciğer örnekleri toplanmıştır. Serum alanin aminotransferaz, alkalin fosfataz, superoxide dismutaz, myeloperoxidaz aktivitesi, glutatyon düzeyleri çalışılmış ve histopatolojik değerlendirme yapılmıştır.

Bulgular: Leflunomid tedavisi tedavi almayan gruba göre karaciğer semikantitatif skalaya bağlı histopatolojik değerlendirmede anlamlı düzelme sağlamıştır (Patolojik skor 1.1+0.7 ve 5.1+2, p<0,01). Leflunomid tedavisi Kuppfer hücre aktivasyonunu anlamlı derecede iyileştirmiştir. (Aktive Kuppfer hücre skorunda yükselme 0.2+0.6 ve 2.5+1.01, p = 0.001). Leflunomid tedavisi alan grupta Methotrexat toksisite grubuna göre serum alanin aminotransferaz, alkalin fosfataz düzeyleri düşük, glutatyon seviyesi, superoxide dismutaz ve myeloperoxidaz aktivitesi benzer bulunmuştur.

Sonuç: Bu deneysel modelde leflunomid tedavisi methotrexat'a bağlı karaciğer toksisitesini iyileştirmektedir.

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Anahtar sözcükler: Leflunomid, methotrexat, karaciğer toksisitesi

Kutluana U, Oruç N, Dinçer-Tekekoğlu S, Çallı-Demirkan N, Akdağ B, Yılmaz M, Yönetçi N, Özütemiz Ö. Leflunomid methotrexat'a bağlı karaciğer toksisitesini önleyebilir mi? *Pam Tıp Derg* 2018;11(3);321-327.

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 β (NF-Kβ) 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 unaware 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.

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

Groups	ALT (IU/L)	ALP (IU/L)	GSH (µmolGSH/ g tissue)	SOD (U/mg prot)	MPO (U/mg prot)
1. MTX+CMC	56.7 ± 9.13*	514±120*	9,6 ± 1.3†	4.7±0.2	7.8 ±SD 3.2
2.MTX+leflunomide	40.68± 13.6	169±234	10,9 ± 0.8	5.2 ±SD 0.60	5.65 ±SD 1.9
3. Leflunomide	33± 12	211±48.0	11,02 ± 1.4	5.1 ±SD 0.63	3.5± 1.3
4. CMC (Control)	27±13	103±121	13,01 ± 1.8	5.4±0.8	4.6± 0.9

MTX, methotrexate; ALT, alanine aminotransferase; CMC, sodium carboxymethylcellulose.
*p < 0.05. Group 1 compared to group 2, group 3 and 4.
†p< 0.05. Group 1 compared to group 4.

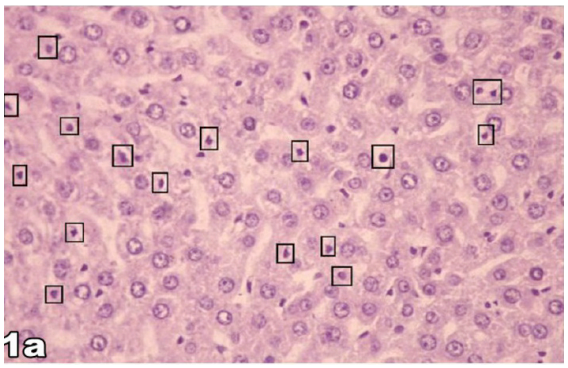

Figure 1a. Histopathological section of liver showing an increase in the number of activated Kupffer cells (square) which is one of the criteria in semiquantitative oxidant liver injury score (H&E stain x400).

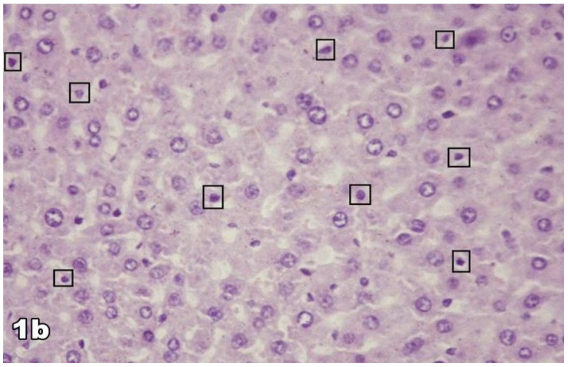

Figure 1b. Histopathological section of liver from MTX group treated with leflunomide showing less increase in the number of activated Kupffer cells (square) which is one of the criteria in semiquantitative oxidant liver injury score (H&E stain x400).

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 s cale 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.

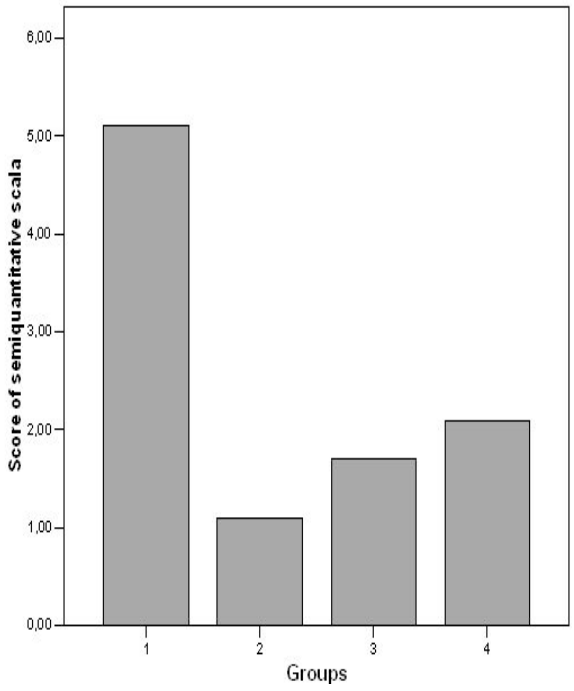

Figure 2. Results of the semiquantitative oxidant liver injury score of all groups. Total score in MTX + leflunomide (Group 2) group was significantly lower than MTX + CMC (Group 1) (1.1+0.7 versus 5.1+2 respectively, p<0.01).

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 combined 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 CCl_4 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] and berberine [37] have shown antinecrotic, anticholestatic, antifibrotic and anticancer activities in the liver. Bilasy SE et al [38] reported that leflunomide and MTX combination had more hepatotoxic effect in a rat model of RA. Curtis et al. [39] suggested that the combination of MTX and leflunomide was associated with more risk of hepatotoxicity than MTX monotherapy in human. 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.

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