

Original Article

Protective effect of infliximab on methotrexate-induced liver injury in rats: Unexpected drug interaction

ABSTRACT

Aims: Although methotrexate (mtx) is a widely used agent to treat cancer and inflammatory diseases, its hepatotoxic effect limits for clinical utility. We aimed to investigate whether infliximab (inf), an inhibitor of tumor necrosis factor-alpha (TNF- α) has a protective effect against mtx-induced hepatotoxicity.

Materials and Methods: For mtx group, the animals received an intraperitoneal single dose injection of mtx at a dose of 20 mg/kg. For inf group, the animals received an intraperitoneal single dose injection of inf at a dose of 7 mg/kg. For mtx + inf group, the single dose of inf at a dose of 7 mg/kg was given 72 h prior to mtx injection. After 72 h, a single dose of mtx 20 mg/kg was given. All rats were sacrificed 5 days after mtx injection.

Results: TNF- α and nitric oxide (NO) levels of mtx group was significantly higher than the control ($P < 0.001$), inf ($P < 0.001$) and mtx + inf ($P < 0.001$) groups. Total score of histological damage was higher in the mtx group when compared with the mtx + inf group. Arginase and carbamoyl phosphate synthetase 1 (CPS-1) of mtx group was suppressed in comparison with the control group and was markedly increased in mtx + inf group.

Conclusion: Inf may partially prevent mtx-induced hepatic damage in rats. However, the combined usage of mtx and inf increases arginase and CPS-1 enzyme activities and at the same time blocks TNF- α . This combination especially in cancer patients may lead to cancer cell invasion and metastasis.

KEY WORD: Arginase, carbamoyl phosphate synthetase 1, hepatotoxicity, infliximab, methotrexate

INTRODUCTION

Methotrexate (mtx), a folate antagonist, is commonly used in the treatment of many different types of cancer and inflammatory diseases such as rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus and dermatomyositis.^[1] However, due to its hepatotoxicity it has a narrow spectrum and a limited clinical usage. The mechanism of mtx-induced hepatotoxicity is not well-known. Previous studies have proposed some mechanisms such as that mtx decreases the defense system and increases lipid peroxidation and oxidative stress leading to an increase in reactive oxygen species (ROS).^[2] Additionally, an intense proinflammatory cytokine release due to excessive ROS formation has been reported during mtx treatment.^[3,4] Excessive ROS formation leads to neutrophil infiltration and proinflammatory cytokine release, which trigger apoptosis, cell damage and death.

Infliximab (inf) is a potent tumor necrosis factor-alpha (TNF- α) inhibitor that is used safely

in many inflammatory diseases such as rheumatoid arthritis, ankylosing spondylitis and Crohn disease.^[5] Inf has been proven experimentally to prevent liver tissue damage by suppressing the formation of TNF- α , other proinflammatory cytokines and nitric oxide (NO).^[6] On the other hand, several studies have reported the development of hepatotoxicity during inf treatment. Even though the mechanism of inf-induced hepatotoxicity has not been fully understood it has been reported to be a direct toxic effect of the drug on liver tissue, or more likely to be immunomediated or induced via Fc receptor-mediated interactions that lead to ROS formation, which is toxic to liver.^[7]

Arginase and carbamoyl phosphate synthetase 1 (CPS-1) enzymes are involved in urea cycle and are abundant in liver tissue. The inhibition of these enzymes increases arginine and NO levels and their overexpression decreases arginine and NO levels and increases ornithine level.^[8] Arginine, NO and ornithine are important substrates for growth or suppression of tumor cells.^[9,10]

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The aim of this study was to determine whether inf has a preventive or toxicity enhancing effect in mtx-induced liver toxicity and whether it has an effect on urea cycle enzymes such as arginase and CPS-1.

MATERIALS AND METHODS

Animals

Forty Wistar albino male rats, weighing 250-300 g (12-15 weeks old), were used in the current study. The rats were randomly divided into four groups: Control group ($n = 10$), mtx group ($n = 10$), inf group ($n = 10$) and mtx + inf group ($n = 10$). This research was performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, 1985), and approved by the local ethical committee (Approval numbers: 2012/16).

Experimental design

For the control group, only isotonic saline solution (an equal volume of mtx) was administered by intraperitoneal injection. In the mtx group, the animals received an intraperitoneal single dose injection of mtx (Emthexate-s, 50 mg ampoule) at a dose of 20 mg/kg and were sacrificed 5 days after mtx injection. Only one single dose of 7 mg/kg inf (Remicate©) was administered intraperitoneally to inf group. One single dose of 7 mg/kg inf was administered intraperitoneally to mtx + inf group. After 3 days, a single dose of 20 mg/kg mtx was administered intraperitoneally to mtx group. All animals were sacrificed 5 days after the mtx injection. All groups were sacrificed under anesthesia with ketamine hydrochloride (Ketalar, 50 mg/kg, intramuscularly; Parke-Davis Eczacibasi, Istanbul, Turkey). The liver tissues were then removed from the animals and immediately stored at -80°C until analysis.

Biochemical parameters

Blood samples (10 ml) were taken from all the rats and collected into tubes for evaluation of biochemical tests. The blood was centrifuged at 3,000 rpm for 10 minutes, after standing at room temperature for 15 minutes. The biochemical parameters, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were checked in the serum using commercial kits (ARCHITECT c16000, Abbott Laboratories, IL, USA).

Tissue homogenates

The samples were homogenized in phosphate-buffered saline (PBS) at pH 7.4 and centrifuged at 10,000 g for 20 minutes. Aliquots of the supernatant were put into tubes and frozen at 80°C .

Measurement of protein

For the tissue homogenate protein assay, a turbidimetric procedure was used, with application of benzethonium chloride as a protein denaturing agent. Proteins in the form of a fine suspension were quantitated turbidimetrically at 404 nm (ARCHITECT c16000, Abbott Laboratories, IL, USA).

Tissue TNF- α

The concentration of TNF- α was measured by using a commercially available rat TNF- α enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, Vienna, Austria).

Tissue interleukin-1 beta (IL-1 β)

The IL-1 β concentration was also measured using a commercially available rat IL-1 β ELISA kit (eBioscience).

Tissue NO

The concentrations of NO were measured using the colorimetric assay method, with a commercially available NO kit (Cayman Chemical Company, USA).

Statistical analyses

The results were reported as the means \pm standard deviation. Data analyses were performed using the statistical software SPSS for Windows (version 13.1; SPSS, USA). The biochemical parameter analyses were performed using the one-way ANOVA, followed by Bonferroni analyses. The Kruskal–Wallis test was used to compare the groups for histopathologic parameters. A Bonferroni-adjusted Mann–Whitney U test was used to compare the two groups. The results are given as the mean \pm SD. *P* values of less than 0.05 were regarded as statistically significant.

Immunohistological evaluation

For immunohistochemical staining, 3-4 μm thick sections of the liver tissues were cut off and allowed to stay in xylene for 20 minutes before the application of an alcohol series (50-100%), afterward allowed to stay for 10 minutes in an H_2O_2 solution. After being washed with PBS, these sections were heated in a citrate buffer solution at 800 W for 4-5 minutes, and allowed to stay in secondary blocker substance for 20 minutes. Each slide was allowed to stay for 75 minutes in different dilutions of the primary antibody (Anti-CPS-1 at 1 $\mu\text{g}/\text{mL}$ and anti-arginase at 1/250-/500), before being stained by anti-arginase and anti-CPS-1. A diaminobenzidine solution was used as an achromogen, Mayer's hematoxylin as a counterstain for 3-5 minutes, and PBS as a negative control. The preparations were photographed after being covered with the eligible covering materials. Consequently of the immunohistochemical staining, the preparations were divided into 4 categories according to the tissue percentage of immunopositive reaction areas: mild (+), moderate (++) , severe (+++) and very severe (++++) . The blocked tissues were cut into 4-5 μm thick sections before being stained with hematoxylin and eosin (H and E), and then the fields found to be suitable for histopathological assessment were photographed. The tissues were appraised in groups by two expert histologists.

RESULTS

Protective effect of Inf on liver enzyme and cytokines release

AST level of mtx group was significantly higher than the control ($P < 0.001$, $P = 0.026$), inf ($P < 0.001$, $P = 0.038$) and

mtx + inf ($P = 0.012$) groups. ALT level of mtx group was also significantly higher than control ($P = 0.026$) and inf ($P = 0.038$) groups. While TNF- α level of mtx group was higher than the control ($P < 0.001$, $P = 0.026$), inf ($P < 0.001$, $P = 0.038$) and mtx + inf ($P = 0.012$) groups, TNF- α level of mtx + inf was similar to the control and inf groups. While IL-1 β level was similar in mtx and mtx + inf groups, it was significantly higher than the control and inf groups. NO level of mtx group was significantly higher than the other three groups. NO level of mtx + inf group was significantly higher than the control and inf groups. All biochemical results are shown in Table 1.

Prevention of liver structure damage by Inf

The morphologic structures of the tissues in the control group were observed to have a normal histology [Figure 1A]. In inf group, hepatocytes had mild necrosis, granulocyte clusters and dilatations with cellular deformities. When compared to the control group, cell morphologic features were found to be similar to each other. The number and activation of Kupffer cell number in the inf group were similar with the control group [Figure 1B]. In mtx group, hepatocytes were observed to have cellular deformities with mild necrosis [Figure 1C]. Hepatocyte degenerations were observed to be near the portal region and intensively around sinusoids. Morphological changes were observed such as polygonal-shaped morphology with inward curling, and beside structures with dense eosinophilic cytoplasm there were pyknotic nucleated cells. These cells were observed to be in groups scattered around the central vein region and to contain acidophilic apoptotic cells with basophilic bodies. When analyzing the morphological characteristics of the cells, there were dense chromatin and irregularly shaped nuclei. Cells with this type of morphology were observed in areas close to the central vein in the wall of sinusoids with dilatations. Portal inflammation and steatosis formation with microvesicular structure associated with mtx-induced hepatotoxicity were observed to be moderate. Endothelial cell swelling associated with intracellular edema within the portal area were observed. There was an increase in intricate structures on the surface of hepatocytes, degenerations associated with mild cytoplasmic vacuolization and neutrophil infiltration. There was an increase in vascular congestion in the sinusoids. Beside sinusoidal dilatations, Kupffer cells that were found on the sinusoidal walls were swollen, protruding into the lumen and had lost its round squamous property. There was an increase in the amount of activated Kupffer cells. There were balloon degeneration and necrotic loss of some of the hepatocytes found in the

surroundings of central vein and in the area limited to portal region [Figure 1C].

In the mtx + inf group, there were lower cellular and tissue deformities than mtx group [Figure 1D]. Radial line up of the hepatic cords of this group was more prominent with less sinusoidal dilatations not completely recovered and long parallel array structures observed similar to the control group. Kupffer cells that were found on the sinusoidal walls of this group were observed to be more squamous and darker and basophilic than mtx group [Figure 1D]. Hepatocytes with radial line up had low apoptotic-shaped cells and low degenerations surrounding central vein. Nuclei of hepatocytes distanced from portal region and portal vein were stained euchromatic and were irregular in size, and their cytoplasmic membrane had normal shape with regular surroundings. Even though activated Kupffer cells were increased, cell activations were observed to be decreased.

In immunoperoxidase staining, while central vein and portal region surroundings of the control groups were less immune reactive, endothelial cells of the dilated regions were stained intensely positive. In mtx-applied groups, there were more cells surrounding central vein and portal region and their nuclei had negative immunoreactivity. In mtx + inf applied groups, immunoreactivity of both cell nuclei and cytoplasm was observed to be very intense [Figure 2]. While CPS-1 showed heterogeneous distribution in all the adult rat liver

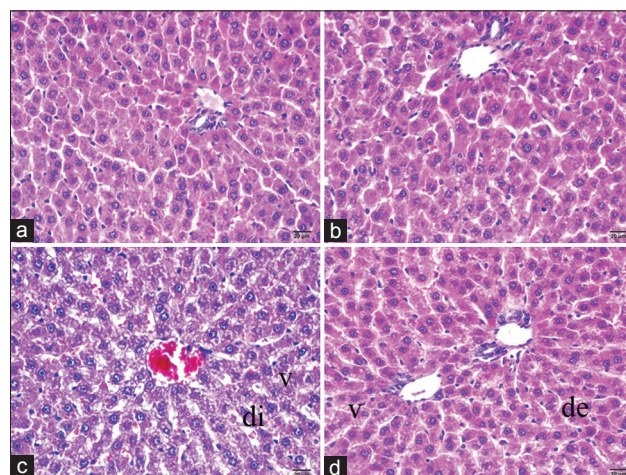


Figure 1: Histopathologic examination of liver tissue by light microscopy; A: control group, B: inf group, C: mtx group, di: dilatation, v: vacuolization, D: mtx+inf group, de: degenerate cell, H and E stain

Table 1: All the biochemical result of four groups

	Control	INF	MTX	MTX+INF	F value
AST IU/L	34.0±7.7	33.7±4.2	66.4±10.2 ^{a,*}	53.6±16.8 ^{*,*}	21.89 ^a
ALT IU/L	35.1±6.6	35.9±5.8	46.2±13.6 ^{a,#}	44.0±13.8	2.778
TNF- α pg/mg protein	310.2±54.9	285.1±39.6	449.1±95.1 ^{*,*x}	360.9±53.7	12.67 ^a
IL-1 β pg/mg protein	136.3±37.1	144.3±25.9	233.1±58.0 ^{*,*}	211.6±36.6 ^{*,*}	13.76 ^a
NO pg/mg protein	2.5±0.4	2.7±0.3	6.9±1.0 ^{*,*}	5.5±0.7 ^{*,*}	97.16 ^a

MTX=Methotrexate, INF=Infliximab, TNF- α =Tumor necrosis factor-alpha, IL-1 β =Interleukin-1 beta, NO=Nitric oxide. ^a $P < 0.001$ (P value for Anova), ^{*} $P < 0.001$, ^o $P = 0.026$ vs. control group, [#] $P < 0.001$, [#] $P = 0.038$ vs. INF group, [†] $P < 0.001$, ^x $P = 0.004$, ^β $P = 0.012$ vs. MTX+INF

parenchyma other than the narrow region of the surroundings of terminal hepatic venules, surroundings of the central vein region were found to be intensely immune reactive. Immunohistochemical methods revealed immunoreactivity for CPS-1 in both cytoplasm and mitochondrial matrix of the hepatocytes [Figure 3]. All histologic results are given in Table 2.

DISCUSSION

In our study, the levels of TNF- α , IL-1 β and NO of the liver tissue of mtx group were higher than both the control and mtx+inf group. Serum levels of ALT and AST of mtx group were high. Histopathological examination of the liver tissue in mtx given group showed significant cell damage. Mtx+inf group had lower damage than mtx group. While TNF- α and IL-1 β levels of mtx+inf group were suppressed more than mtx group, NO level in mtx was higher than the control group. TNF- α and IL-1 β levels of inf group were lower than the control group. Histopathologic properties of the liver tissue in inf group were similar to the control group. Interestingly, the immunohistochemical evaluation of mtx group revealed arginase and CPS-1 enzyme levels to be lower than in the control group. Only in inf group, they were found to be higher than in the control group. Additionally in mtx+inf group, arginase and CPS-1 enzyme levels were increased extensively. Mtx-increased TNF- α , IL-1 β and NO may cause liver toxicity. Inf ameliorated mtx-induced hepatotoxicity by reduced these cytokines and NO level.

Although TNF- α was discovered as a cytokine that could kill tumor cells, it can also lead to proliferation, invasion and metastasis of these cells.^[11] Thus, high levels of TNF- α may have harmful consequences for the organism. Excessive release of TNF- α , IL-1 β and other proinflammatory cytokines leads to acute and chronic liver injury.^[12] Mtx overdose leads

to oxidative stress that causes hepatotoxicity.^[13] Increased oxidative stress leads to release of more proinflammatory cytokines that leads to further tissue damage. In our study, while mtx group had more proinflammatory cytokines than the control group they were found to be suppressed in mtx+inf group. Inf treatment might prevent liver tissue damage by suppressing proinflammatory cytokines. On the other hand, even though TNF- α level is associated with anti-angiogenesis, their low levels are proangiogenic.^[14] In literature, there are case reports regarding patients treated with inf who developed cancer even though the release of TNF- α is suppressed.^[15] In our study, even if excessive suppressing of TNF- α in mtx + inf group was preventive against liver injury, the usage of this combination may be limited in patients with cancer due to elevated levels of arginase while TNF- α is suppressed. Perhaps this combination can increase growth and spread of tumor tissue or may lead to cancer of normal cell. Further studies are needed in this regard.

Normally, NO bioavailability may be critically regulated by arginase by competing with NOS for their extensive substrate L-arginine.^[16] Arginase, CPS-1 and NOS are immensely important for maintaining the delicate balance in the organism. Arginase increases over-expression of tumor cells, accelerates urea cycle and increases ornithine level.^[17,18] Likewise elevated CPS-1 enzyme level accelerates urea cycle and may increase ornithine level. The elevation of ornithine level may lead to the proliferation of cancer cells by decreasing apoptosis and cell death.^[19] At the same time, cancer cells release excessive amount of NOS. Increased NO levels lead to an increase in peroxynitrite radicals, a decrease in T cell-related apoptosis and a proliferation of tumor cells.^[20,21] In our study, while arginase level of mtx group was suppressed both arginase and mtx of mtx + inf group were higher than the control group. Additionally, arginase level of inf group was higher than both mtx and the control groups. NO level

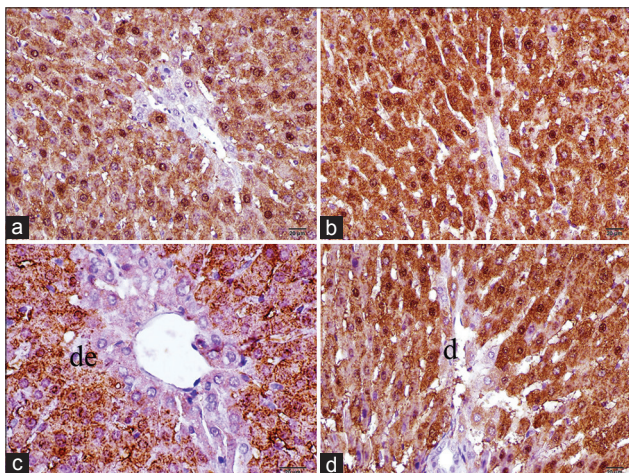


Figure 2: Histopathologic examination of liver tissue stained by immunoperoxidase method by light microscopy A: Control group, B: Inf group, C: mtx group, de: Degenerate cell, D: mtx+inf group, d: Dilatation, immunoperoxidase-stained anti-arginase antibody

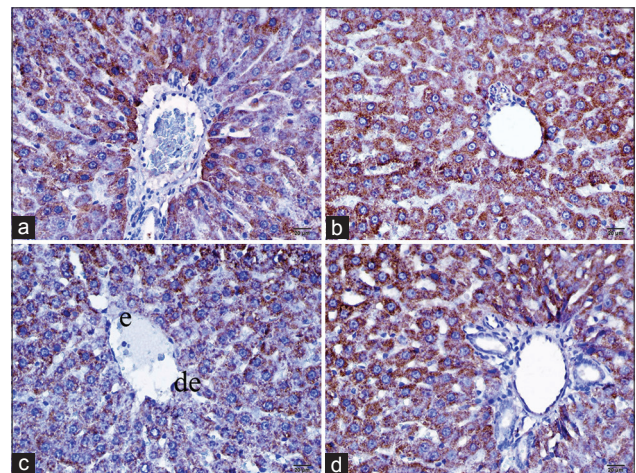


Figure 3: Histopathologic examination of liver tissue stained by immunoperoxidase method by light microscopy A: Control group, B: Inf group, C: mtx group, e: Edema, de: Degenerate cell, D: mtx+inf group, immunoperoxidase-stained anti-CPS-1 antibody

Table 2: Histopathologic examination of liver tissue

	Control	Inf	Mtx	Mtx+inf
Apoptotic body	1.0±0.5	1.2±0.4	3.3±0.5* ^β	2.0±0.5* ^α
Sinusoidal dilatation	0.0±0.0	0.8±0.4*	3.9±0.3* ^β	2.8±0.4* ^α
Hepatocyte degeneration	0.0±0.0	0.2±0.4	3.1±0.3* ^β	2.1±0.3* ^α
Neutrophil infiltration	0.0±0.0	0.7±0.5*	2.0±0.4* ^β	1.1±0.3* ^α
Arginase	2.2±0.8	3.0±0.9	1.9±0.7 ^α	3.3±0.9* ^α
CPS-1	2.5±0.7	3.6±0.5 ^α	1.8±0.6 ^β	3.5±0.7* ^α

CPS-1=Carbamoyl phosphate synthetase-1, Inf=Infliximab, mtx=Methotrexate. **P*<0.001, ^α*P*=0.002, ^β*P*=0.007, ^γ*P*=0.011 vs, control group, ^δ*P*<0.001, ^ε*P*=0.002, ^ζ*P*=0.012 vs. Inf group, ^η*P*<0.001, ^θ*P*=0.003 vs. Mtx group

of mtx+inf group was obviously unsuppressed. However, the full effect of inf on arginase enzyme is not known. In the current study, arginase level of inf group has been found to be similar to the control group. Adalimumab (ADA) is another TNF- α blocker that has been found to increase arginase level in ADA + ischemia reperfusion (I/R) model more than only I/R group. However, arginase level of the group given ADA was similar to the control group.^[8] Perhaps, TNF- α blockers may increase arginase level. Previously a study reported that inf does not affect arginase level.^[22] However our study showed that mtx + inf increased arginase level. In literature, there is only one study that has reported mtx to increase arginase level.^[23] Mtx + inf combination may excessively increase arginase level because of interaction. We suggest that if mtx and inf are administered at the same time, they may adversely affect patients with cancer.

On the other hand, accumulating evidence has shown that the amino acid, arginine is of importance in cancer. Arginine is important mediators in the defense against cancer cells. It influences T cell-mediated immunity, cytokine induction and macrophage-mediated tumor toxicity.^[24] In our study, elevated arginase levels accompanied by excessive arginine usage may lead to reduction in arginine levels. Reduced arginine levels may decrease defense against tumor cells. Combination of mtx and inf may diminish arginine level, thus they can cause reduced cell defense against cancer cells.

CONCLUSION

The combination of mtx and inf has shown a protective effect against mtx-induced liver injury. However, in rats given mtx and inf combination beside TNF- α blockage, arginase and CPS-1 levels were increased and NO level was decreased. According to these results, the combination of mtx and inf in patients with cancer may accelerate the growth and spread of tumor cells. Perhaps it may also lead to cancerous transformation of normal cells. Our study is a pilot study and thus further studies are needed in this regard.

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