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Research Article

# The Protective Effect of N-acetylcysteine Against Methotrexate-Induced Hepatotoxicity in Rat

**Tuba Demirci,[1] Semin Gedikli,[2] Nurinnisa Ozturk,[3] Nevra Aydemir Celep[2]**

[1]Department of Histology and Embryology, Ataturk University Faculty of Medicine, Erzurum, Turkey
[2]Department of Histology and Embryology, Ataturk University Faculty of Veterinary Medicine, Erzurum, Turkey
[3]Department of Biochemistry, Ataturk University Faculty of Medicine, Erzurum, Turkey

## Abstract

**Objectives:** In the present study, we investigated the probably curable effects of N-acetylcysteine (NAC) on Methotrexate (MTX)-induced hepatotoxicity as biochemically and histopathologically.

**Methods:** For this purpose 24 male rats were divided into four groups as; control, MTX, NAC+MTX and NAC groups. Control and MTX groups were given 0.09% NaCl solution for 7 days; and MTX group was administrated single dose MTX at fourth day, additionally. NAC+MTX and NAC groups were given NAC during 7 days; and NAC+MTX group was applied single dose MTX at fourth day. On the eighth day, rats were sacrificed, blood samples and livers were taken.

**Results:** According to biochemical analysis results, NAC caused a reduction in the MTX-induced increased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) enzymes. Histopathologically, cytoplasmic swelling-vacuolization, sinusoidal dilatation and congestion seen in the MTX group were decreased with NAC administration. Although abundant lipid droplets were found in the sections stained with Oil Red O of MTX, NAC usage decreased this situation to the minimum level. While NAC given with MTX decreased Bax immunopositivity, it increased Bcl-2 immunopositivity. Also, TUNEL-positive cells were less in NAC+MTX group according to MTX group.

**Conclusion:** Findings obtained in the current study suggest that NAC may be effective on the MTX-induced hepatotoxicity.

**Keywords:** Hepatotoxicity, histopathology, methotrexate, n-acetylcysteine, TUNEL

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Chemotherapeutic agents have acute toxic effects on many systems in the body.[1] Methotrexate (MTX), a folic acid antagonist, is an extremely effective cytotoxic agent that inhibits cell growth by disrupting cellular metabolism. With this feature, it's been commonly used as a chemotherapeutic drug for treatment of malignant disease.[2,3] Also, because of MTX has effective immunosuppressive and anti-inflammatory properties; it's been used in the treatment of many inflammatory diseases such as rheumatoid arthritis, psoriasis and Crohn disease.[4,5]

The most important complications of MTX treatment are that it causes the hepatotoxicity, ulcerative stomatitis and a decrease in the number of white blood cells; and this situation restricts the clinical usage of MTX.[3,6] Although the hepatotoxicity mechanism of MTX has not been known yet, experimental and clinical studies have supported the idea that MTX-induced hepatic damage could be a result of oxidative stress.[4,7] Under normal conditions, there are antioxidant defense mechanisms to neutralize the harmful effects of free radicals in the liver and these mechanisms

**Address for correspondence:** Semin Gedikli, MD. Erzurum Ataturk Universitesi Veteriner Fakultesi, Histoloji ve Embriyoloji Anabilim Dali, Erzurum, Turkey
**Phone:** +90 442 231 71 25 **E-mail:** semingedikli@gmail.com

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constitute the first step of the defense system for protecting the cells against the harmful effects of oxidative stress.[8] In a study on rabbits, it's been shown that the production of reactive oxygen species (ROS) increases and antioxidant defense system is suppressed in the environment due to the development of MTX-induced hepatotoxicity.[9]

N-acetylcysteine (NAC) is an antioxidant, commonly used in vivo and in vitro studies,[10–12] and a cytoprotective drug that it's effect against drug-induced hepatotoxicity is proven.[13] In the studies, it has been shown that NAC can protect the cells from oxidative stress by inhibiting the formation of hydrogen peroxide ($H_2O_2$).[14] NAC may also show its antioxidant potential on cells by suppressing of the expression of adhesion molecules, reducing cytokine and ROS production in cells, decreasing bacterial translocation and increasing nitric oxide production.[15–17]

In the literature survey, it's been seen that the effects of NAC on MTX-induced hepatotoxicity in rats were evaluated in terms of antioxidant effects, but, in MTX-induced hepatotoxicity in the liver, it's been encountered that no study investigating the presence of the apoptosis and the effect of the NAC on apoptosis. In this study, we aimed to detect the structural damage in MTX-induced hepatotoxicity in the rat liver, the presence of the apoptosis and the effect of the NAC on apoptosis by histochemical, immunohistochemical, biochemical and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays.

## Methods

### Experimental Animals

In our study, twenty-four adult male Sprague Dawley rats weighing 250-300 g were used. The rats were obtained from Atatürk University Medical Experimental Application and Research Center. Experimental practices were carried out in accordance with the ethical rules after the getting approval of Ataturk University Local Ethics Committee of Animal Experiments (24.05.2017/Meeting: 4, Decision No: 59).

During the applications, all experimental animals were put in metal cages and were kept alive by feeding with standard pellet rat chow and giving tap water, in rooms with optimal temperature (22±2 °C) and humidity (45-50%) under a 12/12 hour light/dark cycle. Rats were divided randomly into four groups as control group (n=6), MTX group (n=6), NAC+MTX group (n=6) and NAC group (n=6).

Control group was given only 0.09% NaCl solution 0.5 mL/day intraperitoneally (i.p.) during seven days without applying any drugs. The rats of MTX group were administered 0.09% NaCl solution 0.5 mL/day i.p. for seven days, and on the fourth day of the experiment MTX (Methotrexate® 10 mg/ml, Kocak Pharma) was applied as i.p in a single dose of 20 mg/kg.[18] NAC+MTX group was given 50 mg/kg/day oral NAC (NAC 600 © eff. Tb.R, Basel) via gavage every day during study, and MTX was applied as i.p in a single dose of 20 mg/kg only on the fourth day, as in MTX group. The rats of the NAC group received 50 mg/kg/day oral NAC via gavage for seven days starting from the first day.[19] At the eighth day of the study, the rats were sacrificed under the general anaesthesia supplied by applying intramuscular 10 mg/kg xylazine hydrochloride and 70 mg/kg ketamine hydrochloride. Blood samples taken from the animals were used for biochemical analyses and liver tissues were used for histopathological examinations.

### Biochemical Analysis

ALT, AST and GGT levels were determined spectrophotometrically by using Beckman Coulter (Mijdrecht, Netherlands) kits in the AU5800 Clinical Chemistry System (Beckman Coulter, Brea, CA, USA) in the blood obtained from the rats sacrificed at the end of the experiment. Methotrexate level was determined by immunoassay method using Siemens (Siemens Healthcare Diagnostic Inc. Germany) brand kit in the AU5800 Clinical Chemistry System (Beckman Coulter, Brea, CA, USA).

### Histochemical Procedure

At the end of the study, some part of the liver tissues from the rats sacrificed under anesthesia were put in 10% neutral buffered formaldehyde solution for histopathological evaluation. Following fixation, tissue samples were passed alcohol and xylol series and then embedded into paraffin. Paraffin blocks were cut 5 µm thickness with Leica RM2125RT microtome (*Leica Microsystems, Wetzlar, Germany*). Sections of all groups were stained with Crossman's modified Mallory triple staining method to enable evaluation of histopathological structure. Liver tissue sections of the control and experiment groups were examined under the light microscope (Nikon Eclipse i50, Tokyo, Japan) with camera attachment and were evaluated the severity of the hepatic injury by using a scoring system.[19] According to this scoring system; sinusoidal dilatation, inflammatory cell infiltration, congestion and hydropic degeneration (cytoplasmic vacuolization and/or swelling of hepatocyte) were evaluated. Each of these features was scored as 0 (normal), 1 (mild), 2 (moderate), or 3 (severe). The maximum score of the hepatic injury was 12.

Some parts of the liver tissues were made to Oil Red O (Biognost Kit, Croatia) staining for evaluation of lipid accumulation. Samples of the fresh liver tissues obtained from rats were taken between serum physiologic impregnated gauze in a petri dish. The tissues were frozen in a cryostat

cabinet (Leica CM1520, Germany) at temperatures of -20 ºC and following, sections were taken 10-12 µm thicknesses with a microtome in the cryostat. Frozen sections were stained according to manufacturer's instructions with Oil Red O kit. Slides were evaluated under the light microscope (Nikon Eclipse i50, Tokyo, Japan) and photographs of the sections were taken.

### Immunohistochemical Procedure

Sections of 4-5 µm thicknesses were obtained from the paraffin-embedded tissues and placed on positively charged slides. The liver sections were stained immunohistochemically using Bax primary antibody (dilution: 1/50, Abcam, Cambridge, UK) and Bcl-2 primary antibody (dilution: 1/50, Abcam, Cambridge, UK) with streptavidin-biotin-peroxidase method.

The sections were assessed under the light microscope (Nikon Eclipse i50, Tokyo, Japan) and photographed, and the intensity of the immunoreactivity observed was evaluated using the semi-quantitative method.[20]

### TUNEL Procedure

The presence of apoptosis in the liver sections belonging to the control and experiment groups was determined with TUNEL assay using the In Situ Cell Death Detection Kit (Indianapolis, IN,USA) according to manufacturer instructions. Apoptotic hepatocytes were stained as dark brown while normal ones were pale and purple. Slides were examined under light microscope (Nikon Eclipse i50, Tokyo, Japan) and their photomicrographs were taken. The sections were evaluated as none (-), mild 5-10 cells (+), moderate 11-20 cells (++), and severe 20 < number of cells (+++) according to the Tunel positivities.

### Statistical Analysis

Statistical analyses were performed with SPSS Version 21.0 statistical software package (SPSS Inc., Chicago, IL, United States). The differences among the groups were evaluated by using the Kruskal-Wallis test. The comparisons between every two groups were made using Mann-Whitney U test. P<0.05 value was considered significant.

## Results

### Biochemical Evaluation

The MTX group showed significantly higher levels of serum ALT, AST and GGT compared with the control group (p<0.05). In the NAC+MTX group, it was detected that NAC treatment caused a significant decrease the levels of serum ALT, AST and GGT (p<0.05). There was no significantly difference between the NAC and control groups according to the biochemical parameters (p>0.05) (Table 1).

### Histopathological Evaluation

The rat liver tissues stained with Crossman's modified Mallory triple staining were examined as histopathologically. In the control group, it was observed that hepatocytes, the portal area, sinusoids and sinusoidal cells had normal histological appearance (Fig. 1a). In the liver sections of MTX applied group, significant dilatation and congestion were viewed in sinusoids. There were cytoplasmic swelling and vacuolization in the hepatocytes, especially around the central vein. Again, in particular around the central vein, a prominent increase in connective tissue was remarkable. On the other hand, it was not detected mononuclear cellular infiltration (Fig. 1b). When the sections of the NAC+MTX group examined, sinusoidal dilation, that there was a significant improvement compared to the MTX group (Fig. 1c). The histological view of the sections of the NAC group were similar to those in the control group (Fig. 1d). The results of histopathological damage scoring, made to demonstrate occurred histopathologic damage in the liver, are shown in Table 2. When compared with the other groups, MTX group had the highest histopathological damage score and there were statically significant differences between the groups (p<0.05). The MTX-induced irregularities in the liver structure were healed by NAC treatment and the improved score of the NAC+MTX group had statistical significance (p<0.05).

**Table 1.** Biochemical parameters of damage in the rat livers of the control and the experimental groups

| | Groups | | | | p* |
|---|---|---|---|---|---|
| | Control Med (min-max) | MTX Med (min-max) | NAC+MTX Med (min-max) | NAC Med (min-max) | |
| AST | 62.5 (52-70) | 112.5 (95-120)[a] | 71 (60-78)[b, c] | 57.5 (45-70)[c] | <0.05 |
| ALT | 58 (40-68) | 107.5 (95-115)[a] | 65 (60-75)[b, c] | 57 (45-65)[c] | <0.05 |
| GGT | 47.5 (40-60) | 67.5 (60-80)[a] | 55 (45-65)[b, c] | 47.5 (42-57)[c] | <0.05 |

MTX: Methotrexate; NAC+MTX: N-acetylcysteine+Methotrexate; NAC: N-acetylcysteine; * Kruskal-Wallis test; [a]Significantly increased when compared with control group (p<0.05, Mann-Whitney U test); [b]Significantly decreased when compared with MTX group (p<0.05, Mann-Whitney U test); [c]No significant difference when compared with control group (p>0.05, Mann-Whitney U test).

In the examination of the Oil Red O stained liver cryostat sections, performed for the purpose of showing the lipid accumulation, while no significant reaction was observed in the hepatocytes and perisinusoidal cells in the sections belonging the control group (Fig. 1e), in the MTX-treated group the presence of dense Oil Red O positive lipid droplets in hepatocytes was remarkable (Fig. 1f). It was seen that positive lipid droplets in the NAC+MTX group were significantly reduced compared to the MTX group (Fig. 1g). Also, in the sections of the NAC group, an image where there was no lipid accumulation and close to the control group was observed (Fig. 1h).

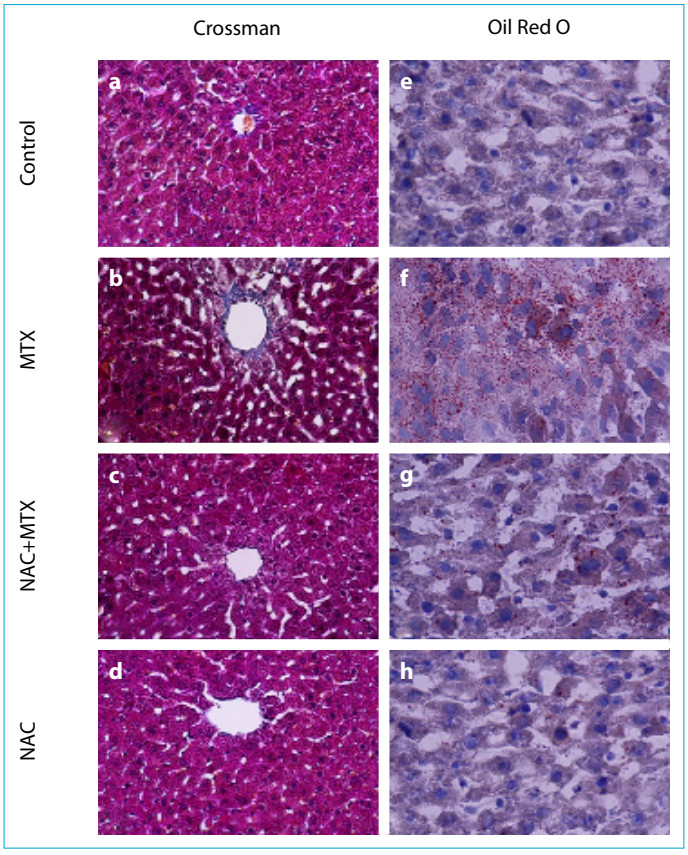


**Figure 1.** Photomicrographs of representative liver tissues stained Crossman's modified Mallory triple staining **(a-d)** and Oil Red O **(e-h)** are shown for the control groups **(a, e)** and experimental groups **(b-d and f-h)**. MTX: Methotrexate; NAC+MTX: N-acetylcysteine+Methotrexate; NAC: N-acetylcysteine Magnification: X20 (A-D), X40 **(e-h)**.

## Immunohistochemical Evaluation

Immunohistochemical staining was evaluated semi-quantitatively. Staining localizations for Bax and Bcl-2 were evaluated separately in liver sections of the all groups. The positive cells proportion score and intensity of staining score were recorded of each group and the immunoreactive score (IRS) was obtained by multiplying these two scores (Table 3).[20] The immunoreactive scores (IRS) of Bax and Bcl-2 for each group were shown in Table 4; also staining in the liver sections of all groups was shown in Figure 2.

When the liver tissue sections were evaluated according to Bax immunoreactivity, it was seen that IRS of MTX group was higher than control group and this increase was significantly ($p<0.05$). The IRS of NAC+MTX group was found as decreased compared with that of MTX group ($p<0.05$). IRS of NAC group was close to the control group ($p>0.05$) (Table 4, Fig. 2).

When the sections were evaluated in terms of Bcl-2 immunoreactivity, the lowest IRS is detected in the MTX group. While IRS of the sections in the control group was similar to that of the NAC group ($p>0.05$), it was higher than the IRS of the MTX and NAC+MTX groups. The difference between the control and MTX groups was statistically significant ($p<0.05$), however, there was not a significant difference between the control and NAC+MTX groups ($p>0.05$) (Table 4, Fig. 2).

## Results of TUNEL assay

In the TUNEL staining made to detect apoptosis in the liver

**Table 3.** The immunoreactive score (IRS) (20)

| Percentage of positive cells (0-4) | Intensity of staining (0-3) | IRS (0-12) |
|---|---|---|
| 0 = no positive cells | 0 = no color reaction | 0-1 = negative |
| 1 = <10% | 1 = mild | 2-3 = mild |
| 2 = 10-50% | 2 = moderate | 4-8 = moderate |
| 3 = 51-80% | 3 = intense | 9-12 = strongly positive |
| 4 = >80% | | |

IRS: Multiplication of percentage of positive cells and intensity of staining.

**Table 2.** Histopathological damage scores of the control and experimental groups

| | Groups | | | | p* |
|---|---|---|---|---|---|
| | Control Med (min-max) | MTX Med (min-max) | NAC+MTX Med (min-max) | NAC Med (min-max) | |
| Score | 1 (0-3) | 9 (7-11)[a] | 5.5 (4-6)[a, b] | 1 (0-2)[c] | <0.05 |

MTX: Methotrexate; NAC+MTX: N-acetylcysteine+Methotrexate; NAC: N-acetylcysteine; * Kruskal-Wallis test; [a]Significantly increased when compared with control group ($p<0.05$, Mann-Whitney U test); [b]Significantly decreased when compared with MTX group ($p<0.05$, Mann-Whitney U test); [c]No significant difference when compared with control group ($p>0.05$, Mann-Whitney U test).

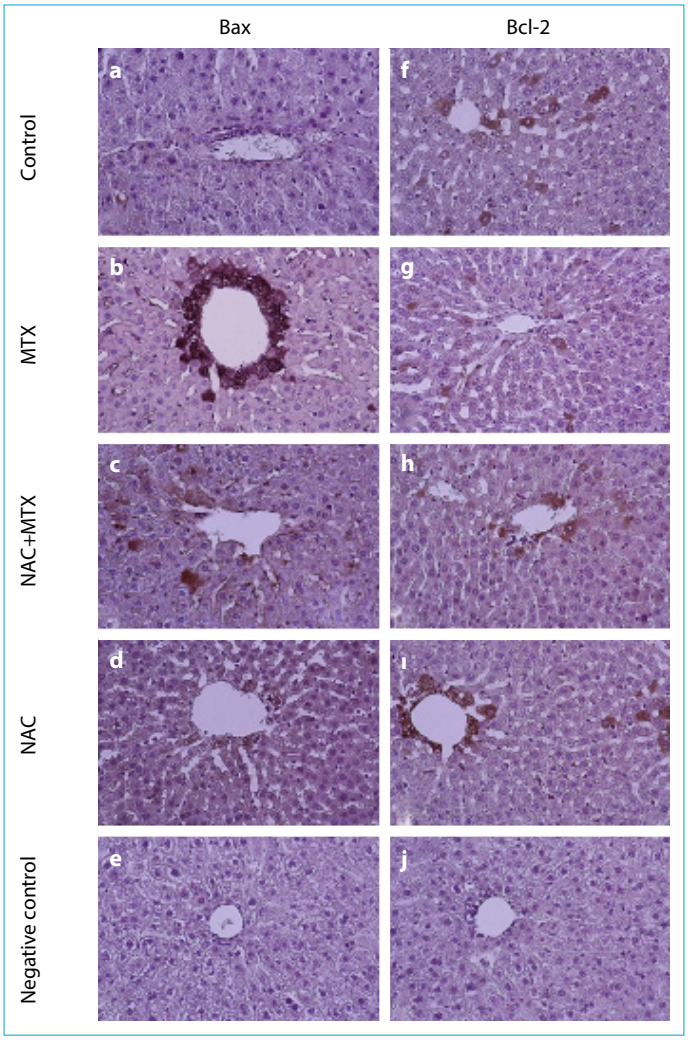


**Figure 2.** Photomicrographs of representative liver tissues showing expression of Bax **(a-e)** and Bcl-2 **(f-j)** in the control groups **(a, f)** and experimental groups **(b-e and g-j)**. MTX: Methotrexate; NAC+MTX: N-acetylcysteine+Methotrexate; NAC: N-acetylcysteine. Stain: Streptavidin-biotin-peroxidase method. Magnification: X20.

**Table 4.** Immunoreactivity scores of the control and experimental groups

| | Groups | | | | |
|---|---|---|---|---|---|
| | Control Med (min-max) | MTX Med (min-max) | NAC+MTX Med (min-max) | NAC Med (min-max) | p* |
| Bax IRS | 1 (0-1) | 7 (6-9)[a] | 3 (2-4)[a,d] | 1 (0-2)[e] | <0.05 |
| Bcl-2 IRS | 6 (4-8) | 1 (0-2)[b] | 4 (2-6)[c,e] | 7 (4-8)[e] | <0.05 |

MTX: Methotrexate; NAC+MTX: N-acetylcysteine+Methotrexate; NAC: N-acetylcysteine; * Kruskal-Wallis test; [a]Significantly increased when compared with control group ($p<0.05$, Mann-Whitney U test); [b]Significantly decreased when compared with control group ($p<0.05$, Mann-Whitney U test); [c]Significantly increased when compared with MTX group ($p<0.05$, Mann-Whitney U test); [d]Significantly decreased when compared with MTX group ($p<0.05$, Mann-Whitney U test); [e]No significant difference when compared with control group ($p>0.05$, Mann-Whitney U test).

sections of rats, it was observed that TUNEL positive cells were found intensively in the MTX group compared to the control group. It was noted that the concentration of apoptotic cells decreased prominently in the NAC+MTX group. And also, it was seen that TUNEL staining of the NAC group was very close to the control group (Fig. 3). The TUNEL findings were summarized in Table 5.

## Discussion

Methotrexate-like chemotherapeutic agents have been used broadly in the treatment of various cancer diseases and some inflammatory diseases. One of the serious adverse effects caused by use of MTX is hepatotoxicity. Approaches to reduce this complication are valuable in order to improve the quality of life of patients and to ensure that treatment is more successful.[6,7]

The underlying reason of MTX-induced hepatotoxicity has not been understood exactly[21,22] but it has been thought that drug shows this harmful effect by causing the increase of oxidative stress as a consequence of triggering ROS formation. Moreover, it has been detected that MTX-induced toxicity is associated with increase in lipid peroxidation in many organs such as liver, ileum and kidney.[2,5,23–25] Free radical-mediated lipid peroxidation is one of the most important destructive elements damaging cell membrane,

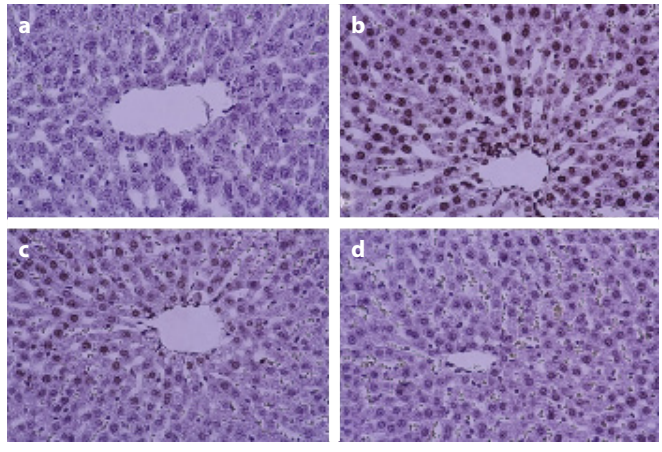


**Figure 3.** Representative photomicrographs of TUNEL-stained liver sections. **(a)** Control, **(b)** Methotrexate, **(c)** N-acetylcysteine+Methotrexate, **(d)** N-acetylcysteine groups. TUNEL-positive apoptotic cells are distinguished by their dark brown stained nuclei among the non-apoptotic cells with pale stained nuclei. Magnification: X20.

**Table 5.** TUNEL scores of the control and experimental groups

| | Groups | | | |
|---|---|---|---|---|
| | Control | MTX | NAC+MTX | NAC |
| TUNEL positive cell | - | +++ | + | - |

and it has been thought that it is a factor causing the development of MTX-induced tissue damage.[6]

While evaluating the biochemical effects of many drugs on the organism, cellular enzymes are being used. Even the minimal increases of these enzymes in plasma and serum are accepted as indicator of the cellular damage. Some of the cellular enzymes, commonly used to show liver damage, are aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) enzymes. From these enzymes, AST and ALT indicate hepatocellular damage and GGT mostly points to cholestasis. When the hepatocellular damage occurred, the levels of the AST, ALT and GGT in serum increase.[26] An increase in the blood level of ALT and AST found in the cytosol of hepatocytes under normal circumstances is indicative of toxicity, that is, cellular damage in the liver. Because, increased levels of ALT and AST in blood flow indicate that the functional integrity of the cell membrane is lost and cellular leakage occurred.[27] Studies have shown that MTX-induced hepatotoxicity increases serum ALT and AST levels and therefore impairs liver functions.[22,27,28] In addition to, it has been reported that an increase in GGT blood level occurs in liver, bile duct and pancreatic diseases.[29]

In this study, we also found that serum AST, ALT and GGT activities were significantly increased ($p<0.05$) in the methotrexate group compared to the control group. In the NAC + MTX group, we detected that NAC given for protection purpose decreased the levels of these enzymes significantly ($p<0.05$) compared to the MTX group. Antioxidant functions and mechanisms of NAC have been identified in human studies. NAC realizes this feature by inhibiting $H_2O_2$ formation and thus protecting the cells from oxidative stress. Because of antioxidant feature of NAC, it serves in reducing liver injury.[19] It has been detected that applied NAC in hepatotoxicity significantly attenuates serum AST and ALT levels and decreases proportion of damaged hepatocytes.[30]

The occurred histopathological damage in the liver by applied MTX was supported by the existing biochemical results. When the liver sections stained with Crosmann's modified Mallory triple stain of the control group were evaluated histopathologically, we observed that hepatocytes, portal area, sinusoids and sinusoidal cells have normal histological structure. In the sections of the MTX group, cytoplasmic swelling and vacuolization were present in hepatocytes, especially in the cells around the central vein. Significant dilatation and congestion in the sinusoids, as well as increased connective tissue around the central vein, were notable. In addition to, we didn't observe intense mononuclear cell infiltration in the sections. At the end of our researches, we have seen in the literature that there are some studies showing that MTX administration causes histopathological damage in the liver. For example, Patel et al.[27] have detected that steatosis and necrosis were found around the bile duct in the portal area in MTX-treated rats. Armağan et al. have shown that the presence of mononuclear cell infiltration, hepatocyte degeneration, sinusoidal dilation and congestion in the portal area.[18] It has also been shown that MTX administration increases the microscopic damage score in the liver tissue.[2] This data was consistent with our findings, so, while the histopathological damage score of the control group was 1, the score of the MTX group was 9, and this increase was statistically significant ($p<0.05$). In the NAC+MTX group there was a significant improvement compared to the MTX group; cytoplasmic swelling and vacuolization in hepatocytes, sinusoidal dilatation and congestion were less. The histopathological score of this group was decreased compared to the MTX group and it became 5.5, this decrease was also statistically significant ($p<0.05$). Light microscopic findings and histopathological score of the NAC group were in parallel with the control group. All these data demonstrate antioxidant properties of NAC against MTX-induced liver damage.

Recent studies have shown that MTX leads to leaky gut syndrome caused to the development and progression of fatty liver disease[31] by disturbing the intestinal epithelial barrier.[32] There are also studies presenting that MTX administration causes hepatic steatosis as a result of inhibition of fatty acid oxidation and triglyceride release in the liver.[33,34] Actually, it has been determined that MTX-induced hepatotoxicity has some risk factors associated with the development of non-alcoholic fatty liver disease (NAFLD), chronic hepatitis B or C, diabetes, and obesity.[35] Scale et al. have seen that fat accumulation in parenchymal cells, and they have determined the presence of fat drops is a response against hazardous effects of MTX. Scale et al.[36] have determined that fat accumulation in parenchymal cells, and they have expressed that the presence of fat drops is a response against hazardous effects of MTX. Because, ROS increases the oxidative stress by causing DNA damage,[37] and as a result, occurring liver damage leads to lipid accumulation of kinds of triglyceride in hepatocytes.[36] We also detected with Oil Red O stain that MTX administered at a dose of 20 mg/kg caused an increase in lipid accumulation in the rat liver. This situation probably was an event developing due to MTX-induced hepatotoxicity, and there were intense Oil Red O positive lipid droplets in the hepatocytes in the MTX group compared to the control group. Additionally, we observed that lipid accumulation in hepatocytes prominently decrease in NAC+MTX group according to MTX group, and in NAC group there was a view similar to that of the control group.

Toxins and cellular stressors playing a role as an inducer may stimulate apoptosis. Oxidative stress appears as a central element in the regulation of apoptotic pathways, activated by environmental stress factors.[38] In many studies, it has been shown that besides oxidative stress, inflammation also causes apoptosis in hepatocytes[39] and pro-apoptotic proteins were activated in MTX-induced hepatotoxicity.[40,41] Bax and Bcl-2, associated proteins with apoptosis, are the main components of mitochondrion-mediated apoptosis.[42,43] While Bax increases apoptosis of the cell, Bcl-2 inhibits apoptosis.[43] MTX administration leads to increase of Bax expression and decrease of Bcl-2 expression.[41, 44] We have detected that Bax expression increases in the MTX group compared to the control group, and also decreases in NAC+MTX group according to the MTX group. Bax expression in the NAC group had a similar view with the control group. When the sections evaluated in terms of Bcl-2 expression, we have noticed that there was a reduction in immunopositivity in the MTX group according to the control group, conversely there was an increase in the NAC+MTX group; and again in the NAC group, there was an expression close to the level in the control group.

Apoptotic cell death has been tried to detect by using TUNEL assay, the most common method to determine these cells. While there were fairly intense TUNEL positive cells in the sections of MTX-applied rats, the proportion of TUNEL positive cells was quite rare in the NAC+MTX group in comparison with the MTX group. Based on our research results, we can say that the protective effect of NAC against apoptotic cell death has been shown.[44]

## Conclusion

In conclusion, the current study demonstrates the potent protective effects of NAC against MTX-induced liver injury. The hepatoprotective effects of NAC depend on its ability of the enhancement of antioxidant defense system, and its ability to reduce pro-inflammatory and apoptosis signal pathway. However, to understand of molecular mechanism exactly, it needs to be performed further studies.

### Disclosures

**Ethics Committee Approval:** Ataturk University Local Ethics Committee of Animal Experiments (24.05.2017/Meeting: 4, Decision No: 59).

**Peer-review:** Externally peer-reviewed.

**Conflict of Interest:** None declared.

**Authorship Contributions:** Concept – T.D., S.G.; Design – T.D., S.G.; Supervision – T.D., S.G.; Materials – T.D., S.G., N.O., N.A.C.; Data collection &/or processing – T.D., S.G., N.O., N.A.C.; Analysis and/or interpretation – T.D., S.G., N.O.; Literature search – T.D., S.G., N.A.C.; Writing – T.D., S.G.; Critical review – T.D., S.G.

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