

ANTIOXIDANT EFFECT OF TRANS-3 HYDROXYCINNAMIC ACID AGAINST LIVER DAMAGE METHOTREXATE INDUCED IN RATS

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Abstract

The aim of this study was to determine the possible antioxidant effect of trans-3 hydroxycinnamic acid (HC) as an antioxidant agent against oxidative liver damage induced by MTX in rats. In the present study, the animals were divided into 4 groups of 6 rats in each group. The groups were designated as control, methotrexate (First day-single dose), HC (5 days-5 consecutive doses) and MTX + HC (First day- MTX single dose and then HC 5 days-5 consecutive dose). Rats were sacrificed on 5 day of experiment and the liver tissues were removed. Superoxide dismutase (SOD) activity, glutathione (GSH), and malondialdehyde (MDA) levels were evaluated in the liver tissue samples. MDA levels increased in the MTX group according to control group. But MDA levels decreased significantly in HC group. Also, SOD activity decreased in MTX group according to control group. However, this enzyme activity increased depending on HC group. On the other hand, GSH level in the rat's liver tissue were significantly reduced in MTX group compared to control. In conclusion, HC have an antioxidant effect against oxidative liver damage induced-MTX in rats.

Keywords: Methotrexate, oxidative stress, liver damage, trans-3 hydroxycinnamic acid.

INTRODUCTION

Methotrexate (MTX) is a folic acid analogue and an antimetabolite agent, which is commonly used in blood and solid organ malignancies, dermatological and rheumatic diseases and ectopic pregnancy termination (Smith *et al.*, 2008). It has been noted that the high dose of MTX used in the treatment of diseases such as acute leukemia or psoriasis is associated with hepatic toxicity that lead to massive hepatic fibrosis and cirrhosis (Hytioglou *et al.*, 2004). Moreover, it was reported that the common side effects of MTX are gastrointestinal, liver, kidney and bone marrow toxicities (Sener *et al.*, 2006).

Oxidative stress is one of the important mechanisms in liver damage (Prosser *et al.*, 2006). It can be described as the degradation of cellular oxidant/antioxidant balance in favor of oxidant (Sinclair *et al.*, 1990). It is known that lipid peroxidation, which is caused by oxidative stress-induced free oxygen radicals, plays a role in cancer, atherosclerotic heart diseases, diabetes mellitus, rheumatoid arthritis, cataracts, liver diseases and pathogenesis of toxic cell damages (Halliwell and Chirico, 1993; Droge, 2002). In many studies, it has been shown that there is a relationship between oxidative stress in liver diseases and liver damage associated with liver diseases and fibrosis (Shimiziu, 2001).

Cinnamic acid is α , β -unsaturated aromatic fatty acid which is common in plants and is used as a sweetener (Hoskins, 1984). Cinnamic acid and its

derivatives are naturally found in strawberries, plums, cinnamon, cloves and in plants used as spices (Beuchat and Golden, 1989). Its toxicity is low and it is approved to be used in food by the Food and Drug Administration (FDA) and the acceptable daily intake is 1.25 mg/kg (Hoskins, 1984; Beuchat and Golden, 1989; Kirk and Othmer, 1993). Cinnamic acid is a natural antimutagenic agent (Mitcher *et al.*, 1992; Liu *et al.*, 1995). However, there is no study showing the effect of trans 3 hydroxycinnamic acid compound on the oxidative stress produced by MTX.

Recently, the efficacy of various agents for the prevention or treatment of methotrexate-induced liver damage has been investigated by many scientists (Pinar *et al.*, 2018; Mehrzadi *et al.*, 2018). The study was planned to search the possible antioxidant effect of trans-3 hydroxycinnamic acid, a cinnamic acid derivative, against hepatotoxicity MTX-induced in rats.

MATERIALS AND METHODS

Ethical Approval: This experimental study was carried out at Atatürk University Experimental Animal Research Center, Atatürk University Faculty of Pharmacy Biochemistry Laboratory and Ağrı İbrahim Çeçen University Central Research Laboratory. The approval of Atatürk University Experimental Animals Local Ethics Committee was taken (2016-148).

Animals and Experimental Design: In this study, 24 male Wistar Albino rats weighing 200-220 grams were used. The rats were fed for up to 1 week with enough

amount of water and feed (standard rat food) at standard temperature (25 ± 1 °C) and light (12 h light, 12 h darkness), humidity ($55 \pm 5\%$). Rats were fasted 24 hours before the experiment.

The animals were divided into 4 groups. The groups were designated as control, methotrexate (MTX) (first day-single dose), trans-3 hydroxycinnamic acid (HC) (5 days-5 consecutive doses) and methotrexate+trans-3 hydroxycinnamic acid (MTX+HC). There was not given treatment to the control group. MTX in liquid form was administered at a dose of 20 mg/kg as intraperitoneal (IP) injection in a single dose on the first day of study to the MTX group. HC was given at dose of 100 mg/kg for only 5 days and as IP administration in HC group. A solution was prepared by dissolving 100 mg/kg of HC in 20% ethanol 1 cc of this solution was administered intraperitoneally at a dose of 100 mg/kg. A single dose of 20 mg/kg MTX was given intraperitoneally to MTX + HC group on the first day. Then 100 mg/kg HC was applied for 5 days. and HC was prepared as fresh every day. The rats were weighed on the first and last day of the experiment. Drugs were performed according to the weight of the rats. All groups' injections were initiated on the same day as IP and they all received injections for 5 days and were terminated on the same day. After the rats were sacrificed, liver tissue samples were taken for tissue MDA, SOD and CAT measurements.

Superoxide Dismutase (SOD) Enzyme Activity Assay:

Liver tissue samples were taken for tissue MDA, SOD and GSH measurements. These tissue samples were stored in a deep freeze at -80 °C to maintain enzyme activity until they were analyzed for MDA, SOD and GSH determinations. Liver tissues were treated in liquid nitrogen and grinded and weighed 100 mg and put in screw cap ependorf tubes. They were then homogenized by adding PBS buffer and centrifuging at 5000 rpm for 15 min. The homogenates were prepared and the supernatants obtained from the homogenates were separated for tissue MDA, SOD and GSH measurements.

SOD activity measurement was based on the method developed by Sun *et al* (Sun *et al.*, 1988). The principle of the assay is based on generation of $O_2^{\cdot-}$, which is released during xanthine oxidation and xanthine oxidase, to form the formazan by reducing nitroblue tetrazolium (NBT). As the SOD activity increases, the amount of $O_2^{\cdot-}$ entering the reaction with NBT decreases, consequently decreasing in formazan formation. Absorbance value of the formazan dye in the blue-violet range were read using a 96 well plate at 560 nm and the dilution coefficients were used to calculate the measurements using the standard graph made with the previously prepared SOD stock solution. The SOD activity of the samples was described as U/mg protein.

Total Glutathione (GSH) Level Measurement: GSH measurement is based on the method developed by Sedlak *et al* (Sedlak and Lindsay, 1968). The DTNB 5,5'-Dithiobis (2-nitrobenzoic acid) disulfide present in the measurement medium is a chromogen and is easily reduced by the sulfhydryl group-containing compounds. The resulting yellow color can be measured spectrophotometrically at 412 nm. Yellow color amounts were measured using a 96 well plate at 412 nm and the dilution coefficients were taken into consideration to calculate the measurements using the standard graph made with the previously prepared GSH stock solution. The GSH amounts of the samples were expressed as nmol/mg protein.

Malondialdehyde (MDA) Level Measurement: MDA amount was measured according to method developed by Ohkawa *et al* (Ohkawa *et al.*, 1979). The amount of MDA has shown the indication of lipid peroxidation. The amount of lipid peroxidation is measured according to the concentration of thiobarbituric acid reactive species. MDA forms a pink chromogen by reacting with thiobarbituric acid at $90-95$ °C. Standards prepared at different concentrations from the 100 mM stock standard solution were run on the same conditions as the samples. The amount of pink color was measured at Elisa Reader using a 96 well plate at 532 nm and standard graph were drawn with the obtained results. The slope constant obtained from this graph was applied to the samples and the amount of MDA was calculated. The MDA values of the samples were then expressed as nmol/mg protein.

Protein Assay: Protein concentrations were determined using commercial protein standards according to the Lowry method (Lowry *et al.*, 1951).

Statistical Analyses: For statistical analyzes and comparisons were made using IBM SPSS Statistic 19.0 software program. Results were given as Mean \pm Standard Deviation and P values below 0.05 were considered statistically significant. Analyses was performed according to One-Way ANOVA test and Duncan from Post Hoc.

RESULTS AND DISCUSSION

In our study, SOD activity and GSH level in liver tissue were higher in control group and MTX + HC group compared to MTX group ($P < 0,05$). In contrast, MDA levels in the control and the MTX + HC groups were significantly lower than the MTX group ($p < 0,05$). The liver damage in the MTX + HC group was significantly lower compared with the MTX group ($p < 0,05$) (Table 1).

Drugs with cytotoxic properties, such as MTX, effect not only cancer cells but also all healthy cells. Therefore, they have wide toxic effects (Reide and

Taylor, 2000). Toxic metabolites can cause damage of liver cells by many mechanisms. However, the two most important mechanisms are covalent attachment and oxidative stress. In a previous study, it was reported that MTX significantly altered the oxidant/antioxidant balance, GSH content, SOD and CAT activities decreased and MDA levels increased (Hemeida and Mohafez, 2008). In another study, it was found a reduction in GSH levels an increase in myeloperoxidase activity, as an indicator of inflammatory response, and apparent increase in MDA levels in blood, liver, kidney and small intestine tissues of rats administered intraperitoneal 20 mg/kg single dose of MTX (Jahovic *et al.*, 2004). Also, it was showed that there was an increase in SOD and catalase activities and a decrease in GSH levels in the rat small intestine and it was also suggested that oxidative stress plays an important role in the intestinal damage caused by MTX (Miyazono *et al.*, 2004). In a study conducted by Vardi *et al.* that the protective effects of apricot and beta-carotene on oxidative damage produced by MTX in the intestinal system in rats were investigated. It was also found that while MDA and MPO activity increased, SOD, catalase and GSH contents decreased in intestinal tissue of rats due to MTX administration (Vardi *et al.*, 2008). Also, hepatic oxidative damage in rats related to MTX was reduced by curcumin administration (Hemeida and Mohafez, 2008). Thus, it is suggested that antioxidant agents should be used to protect from MTX-toxicity (Khalifa *et al.*, 2017; Bu *et al.*, 2018; Pinar *et al.*, 2018). In our study, we investigated the possible antioxidant effects of HC, to reduce MTX-induced hepatotoxic effects.

Cinnamic acid is a compound found in nature. It is generally found in sweet compositions and in the structure of cinnamon oil as well as being a member of the plant family (Hoskins, 1984). In recent years, Cinnamic acid derivatives are highly attractive due to antiproliferative, antioxidant, antiangiogenic and antitumorogenic effects. Some cinnamic acid analogs have been suggested to act as tyrosine kinase inhibitors and inhibited tumor cell proliferation (Shiraishi *et al.*, 1989). Ekmekcioglu *et al.* examined the effect of cinnamic acid on cell proliferation and the differentiation markers of alkaline phosphatase, sucrose and

aminopeptidase N in human colon adenocarcinoma cells. In their study, it was demonstrated that cinnamic acid is an important antiproliferative agent and inhibits DNA synthesis in growing cells (Ekmekcioglu *et al.*, 1998). Soylemezoglu *et al.* have determined that trans-cinnamic acid derivatives are more sensitive and prone to isomerization than cis-structures. It is also important that the hypothesis that the cinnamic acid derivatives taken in the diet prevent the formation of nitrosamines, which are suspected of causing cancer, is also worth investigating (Söylemezoğlu, 2003). In the literature, no study has been conducted to determine whether can be prevented by trans-3 hydroxycinnamic acid against oxidative liver damage induced by MTX. In similar studies, it was reported that Hepatic oxidative stress increased due to MTX in rats. In many studies, it was conducted that this hepatotoxic effect could be reversed by N-acetylcysteine, melatonin, curcumin and grape kernel extract (Jahovic *et al.*, 2004; Miyazono *et al.*, 2004; Ciralik *et al.*, 2008; Cetin *et al.*, 2008). Two different studies have reported that oxidative stress plays an important role in the pathogenesis of MTX-induced toxicity in rat testis, and beta-carotene and caffeic acid have a protective effect on this oxidative stress (Armagan *et al.*, 2008; Vardi *et al.*, 2010). Caffeic acid is commonly used as an antioxidant in various oxidative stress models formed in animal models (Yildiz *et al.*, 2009). In our study like supporting previous studies' results, we also observed that SOD activity and GSH levels decreased due to MTX in liver tissue and these enzyme levels were significantly increased in MTX+HC group compared to MTX group (Table 1). MDA levels as a product of lipid peroxidation increased by the MTX treatment compared to the control group in the liver. In rats treated with MTX+HS, MDA levels were significantly reduced compared to the MTX group (Table 1). In conclusion, MTX causes oxidative damage in rat liver tissue by lowering antioxidant enzyme activities and increasing lipid peroxidation. The protective effect of HC has been demonstrated against tissue damage caused by MTX by lowering the lipid peroxidation level and increasing the levels of antioxidant enzymes. These results suggest that HC may be used as a protective supplement against liver damage due to chemotherapeutic drugs. However, more molecular and clinical studies are needed about this matter.

Table 1. Mean ± Standard Deviation (Mean ± SD) values of superoxide dismutase (SOD (U / mg protein) activity, glutathione (GSH) (nmol / mg protein) and malondialdehyde (MDA) (nmol / mg protein) levels of all groups were presented in the table.

	SOD (U/mg protein)	GSH (nmol/mg protein)	MDA (nmol/mg protein)
CONTROL (n=6)	35.18±5.49 ^c	3.89±0.58 ^c	1.79±0.27 ^a
MTX (n=6)	20.91±3.37 ^a	1.53±0.31 ^a	2.68±0.56 ^c
HC (n=6)	31.51±6.48 ^{b,c}	3.77±0.85 ^c	1.74±0.39 ^a
MTX+HC (n=6)	26.04±4.52 ^b	2.90±0.45 ^b	2.20±0.54 ^b

There is a statistically significant difference between the groups with different letters (p<0.05).

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