ORIGINAL RESEARCH

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Carvacrol prevents methotrexate-induced renal oxidative injury and renal damage in rats

Abstract

Purpose: The purpose of this study was to investigate the effect of carvacrol (CAR) on methotrexate (MTX)-induced renal damage in rats.

Methods: Twenty-four male rats were equally divided into three groups: group I, control treatment; group II, MTX-treated; and group III, MTX+CAR-treated. A single dose of CAR (73 mg/kg) was administered intraperitoneally to group III on the first day of the experiment and a single dose of MTX (20 mg/kg) was administered intraperitoneally to groups II and III on the second day of the experiment. Blood samples and kidney tissue were obtained from each animal on day 8 for the measurement of malondialdehyde (MDA), total oxidant status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI). Light microscopy was used for histopathological examination of kidney specimens.

Results: MDA, TOS and OSI levels were significantly greater in the group receiving MTX alone relative to the control animals, while the TAS level was significantly reduced in the MTX group compared with the control group. The administration of CAR was associated with significantly decreased MDA, TOS, and OSI levels and increased TAS levels relative to the rats treated with MTX alone. Animals treated with CAR exhibited decreased tubular degeneration and architectural impairment relative to animals treated with MTX alone; however, the difference in histological scores did not meet the threshold of statistical significance.

Conclusions: MTX treatment results in oxidative damage to the rat kidney; damage which is partially abrogated by the administration of CAR.

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The folic acid antagonist methotrexate (MTX) is used clinically to inhibit the synthesis of purines and pyrimidines [1]. MTX is commonly used at high doses to limit the growth of malignancies, but may also be used at low doses in inflammatory diseases, including psoriasis and rheumatoid arthritis, to inhibit the proliferation of inflammatory leukocytes [2-4]. MTX remains an essential component of modern clinical therapy for acute lymphoblastic leukemia and is the primary treatment for malignant gestational trophoblastic disease [5]. In addition to anti-proliferative activity, MTX has anti-inflammatory and immunomodulatory properties; however, cytotoxic effects and other side effects limit the efficacy of MTX as an anti-inflammatory. Significant side effects of MTX have been described in several organ systems. In particular, MTX has a detrimental effect on kidney function [6]; however, the mechanism of MTX-induced toxicity remains unclear. More than 90% of MTX is filtered by the kidneys [7] and MTX treatment is known to cause renal failure at high doses [8]. Renal impairment can result in altered metabolism of MTX, delayed drug excretion and, subsequently, systemic toxicity. The toxic effects of MTX are typically treated through hydration and alkalinization of the patient; however, these methods are insufficient to prevent all instances of MTX toxicity. Reactive oxygen species (ROS) have been implicated in the pathogenesis of MTX-induced renal damage [9, 10]. MTX produces free oxygen radicals, resulting in enhanced lipid peroxidation. These free radicals lead to functional impairment of the mitochondria [11]. Increased malondialdehyde (MDA) production and decreased glutathione levels in the blood, liver, and kidney are associated with MTX administration [12]. Enhanced neutrophil infiltration and oxidative stress are likely to contribute to MTX-induced renal damage [13].

We hypothesized that antioxidant compounds may inhibit MTX-mediated renal toxicity. Carvacrol (CAR) or cymophenol (2-methyl-5-isopropylphenol), is the predominant monoterpene phenol in many essential oils derived from plants in the family *Labiatae* including *Origanum*, *Satureja*, *Thymbra*, *Thymus*, and *Corydorthymus* species [14]. CAR is the active component producing the biological activities of *Origanum*. CAR has been widely consumed as a food or food additive for many years. Several reports have indicated that CAR exhibits anti-inflammatory [15], anti-microbial [16] and anti-viral activities [17]. Anti-carcinogenic and anti-tumorigenic activity [16, 18], and antioxidant properties [19-21] have also been attributed to CAR. A recent study conducted in rats demonstrated that CAR was not hepatotoxic at the applied dosage and, in fact, protected against ischemia/reperfusion-associated damage to the liver [22]. Another study concluded that CAR had signifi-

cant hepatoprotective and antioxidant effects in D-galactosamine-treated rats, a conclusion supported by and histologic and biochemical analysis of the liver and kidneys [23]. Application of CAR may be useful in the prevention of MTX-induced renal pathology.

No study has yet been published evaluating the protective effects of CAR in MTX-induced renal impairment; therefore, we aimed to investigate the effects of CAR treatment in a rat model of MTX-induced oxidative renal injury and to evaluate the associated morphologic changes using biochemical methods and histological examination.

Methods

Animals and Experimental Design

All experimental procedures were reviewed by the Dicle University Animal Ethical Committee and were carried out in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals prepared by the Dicle University Animal Ethical Committee. Male Wistar albino rats aged 3 months and with a mean body weight of 225 ± 30 g were provided by the animal laboratory at Dicle University. All animals were housed under standard conditions with an ambient temperature of $25 \pm 2^\circ\text{C}$ and 12/12 hours of light-dark cycle and standard animal cages and were treated in compliance with the National Institutes of Health guidelines. All experimental procedures were in compliance with the animal use regulations of Dicle University Experimental Research Center, Diyarbakir, Turkey.

The rats were randomly divided into three groups of eight animals each. Group I was the experimental control group and received no additional treatment other than standard care and housing. Group II animals received MTX treatment. Group III animals were treated with both MTX and CAR. On the first day of the experimental protocol, animals in the Group III group received intraperitoneal injections of CAR (73 mg/kg) according to the methods recommended by Canbek [22] *et al.* MTX (Medac, Wedel, Germany) was administered by intraperitoneal injection in a single 20 mg/kg dose to both the MTX treatment group and the MTX+CAR treatment group on second day of the experimental protocol. The total duration of the experiment was 8 days. On the eighth day, the experimental animals in all three groups animals were euthanized by decapitation.

Plant extract

The plant substance tested in this study, CAR (2-methyl-5-(1-methylethyl)-phenol), was isolated from steam distilled essen-

tial oils of *Origanum onites* L. collected in West Anatolia as described by Canbek [22] *et al.* Carvacrol-rich fractions were bulked to obtain CAR with 99% purity (gas chromatography and mass spectrometer analyses).

Surgical Procedures

After completion of the experimental and control treatments, all animals were anesthetized with an intramuscular injection of ketamine HCl (50 mg/kg) (Ketalar; Parke-Davis, Karachi, Pakistan) and peritoneal injection of xylazine (10 mg/kg) on the eight day and euthanized after the onset of surgical anesthesia. A total of 5 ml of blood was collected from the cardiac cavities of the rats immediately following euthanasia. The kidneys of all rats were then immediately excised, decapsulated, and divided longitudinally into two equivalent sections. One section was retained for biochemical analysis and the other section was fixed in 10% formalin for histopathological examination.

Biochemical Analysis

Blood samples were drawn into tubes without anticoagulant. Serum was separated by centrifugation at 4000 rpm at 4°C for 10 min and stored at -80°C for biochemical analyses. Tissues from both the control and experimental rats were excised, rinsed with ice-cold saline and immediately stored at -80°C for later biochemical analyses. Tissues from each rat were weighed and homogenized in 50 mM pH 7.0 phosphate buffered saline (PBS). Homogenized kidney tissues were then centrifuged at 10,000 rpm at 4°C, for 15 min to separate the supernatant for use in the biochemical analyses. MDA concentrations in serum and homogenized kidney tissue were determined using an assay kit (Northwest Life Science Specialties, Vancouver, Canada). This test is based on the reaction of MDA with thiobarbituric acid (TBA), which results in the production of MDA-TBA₂; a compound with measurable absorbance at 532 nm [24]. Total antioxidant capacity and total oxidant status of the tissues and serum were measured by the colorimetric methods described by Erel [25, 26]. TAS results are expressed as $\mu\text{mol Trolox Equivalence per gram of tissue}$ and TOS results are expressed as $\mu\text{mol H}_2\text{O}_2 \text{ Equivalence per gram of tissue}$. The OSI value was calculated according to the following formula: $\text{OSI (Arbitrary Unit)} = \text{TOS} / \text{TAS}$ [27].

Histopathological Assessment

Following excision for the euthanized animals, the kidneys were fixed in 10% formalin solution for 48 h. After routine histological tissue preparation, all specimens were embedded in

wax, sectioned in 4–5 μm thickness and stained with hematoxylin and eosin stain according to standard methods. For histopathological evaluation, all kidneys were examined and photographed using light microscopy (Zeiss, Axiphot, Germany) by a pathologist who was blinded to the study groups. Renal injury of the cortex and medulla, including tubular degeneration, tubular dilatation, tubular cell swelling and tubular architectural impairment, was scored semi-quantitatively using three slides from each kidney specimen at $\times 200$ to $\times 400$ magnification according to the following criteria [28]: normal, 0; involvement of $\leq 10\%$ of the cortex and medulla, 1; involvement of 11–20% of the cortex and medulla, 2; involvement of 21–30% of the cortex and medulla, 3; involvement of $> 30\%$ of the cortex and medulla, 4. Mononuclear inflammatory cells in the interstitium were scored as follows: no accumulation, 0; slight accumulation, 1; moderate accumulation, 2; extensive accumulation, 3.

Statistical Analysis

All data are expressed as mean $\pm$ standard deviation ($\pm\text{SD}$). Variation relative to a normal distribution was evaluated using the Kruskal–Wallis analysis of variance method, followed by the application of the non-parametric Mann–Whitney U-test with Bonferroni correction for binary comparisons to evaluate differences between the experimental groups. Pearson's chi-square test was used to compare kidney pathology grades between groups. *P* values < 0.05 were established as the threshold of statistical significance. All data were processed using the SPSS 18.0 for Windows (SPSS Inc., Chicago, USA) statistical software package.

Results

Serum Biochemistry Results

TOS ($p=0.001$), OSI ($p=0.001$) and MDA ($p=0.002$) levels were increased significantly in the MTX group relative to the control group; however, a significant decrease in TAS ($p=0.002$) was observed in MTX-treated rats in comparison with the control animals. CAR treatment was associated with significantly decreased TOS ($p=0.003$), OSI ($p=0.002$) and MDA ($p=0.005$) levels and increased TAS ($p=0.016$) levels in comparison with the animals that received MTX alone. The results of biochemical analyses of rat serum are shown in Table 1.

TABLE 1. Serum oxidant and antioxidant parameters by treatment group

Groups	TAS (mmol Trolox Equiv/L)	TOS (mmol H ₂ O ₂ Equiv/L)	OSI (Arbitrary Unit)	MDA (μ M)
CONTROL (1)	1.67 $\pm$ 0.08	11.40 $\pm$ 3.38	6.89 $\pm$ 2.30	2.47 $\pm$ 0.55
MTX (2)	1.34 $\pm$ 0.15	35.57 $\pm$ 26.45	26.60 $\pm$ 19.01	3.55 $\pm$ 0.34
MTX+CAR (3)	1.55 $\pm$ 0.05	12.28 $\pm$ 6.21	7.90 $\pm$ 3.94	2.37 $\pm$ 0.69
p value between groups				
1-2	0.002	0.001	0.001	0.002
2-3	0.016	0.003	0.002	0.005
1-3	0.005	0.834	0.529	0.636

TOS: Total oxidant status, TAS: Total antioxidant capacity, MTX: methotrexate, CAR: Carvacrol, MDA: malondialdehyde, data presented as mean $\pm$ SD

Kidney Biochemistry Results

In the MTX treatment group, MDA ($p=0.046$), TOS ($p=0.010$) and OSI ($p=0.010$) levels in the kidney tissue were increased significantly relative to the control group. The administration of CAR to the rats was associated with significantly decreased MDA ($p=0.016$), TOS ($p=0.006$) and OSI ($p=0.001$) levels in the homogenized kidney tissue in comparison with the animals treated with MTX alone. In addition, TAS levels were decreased in the MTX group relative to the control group ($p=0.012$). Cumulatively, the administration of CAR prior to MTX significantly suppressed the negative effects of MTX injury ($p=0.046$). The results of biochemical analysis of rat kidney tissues are shown in Table 2.

Kidney Histopathological Analysis

Extensive histopathological evaluation indicated that the renal tissues of the control group had normal tissue structure with no evidence of pathological changes (Figure 1A). Prominent degenerative changes with cell swelling (arrows), prominent nu-

clear loss (arrowheads), nuclear pyknosis of the tubular cells, infiltration of mononuclear inflammatory cells and moderate architectural impairment of the tubular structures were observed in the group treated with MTX alone (Figure 1B). The tubular structure changes, nuclear loss and cellular swelling were less severe in the tissues harvested from animals receiving CAR prior to MTX administration when compared with kidney tissues collected from animals treated with MTX alone (Figure 1C). CAR treatment prior to the administration of MTX resulted in observable protection from MTX-induced tubular degeneration and architectural impairment but the results were not statistical significance ($p=0.097$ and $p=0.091$, respectively).

Discussion

The main objectives of this study were to investigate the role of oxidative stress in MTX-induced renal toxicity and the effect of CAR administration in a rat model of MTX-induced renal toxicity. The results of this study demonstrate that prophylactic administration of CAR has protective effects on MTX-

TABLE 2. Renal tissue oxidant and antioxidant parameters by treatment group

Groups	TAS (μ mol Trolox Equiv/g tissue)	TOS (μ mol H ₂ O ₂ Equiv/g tissue)	OSI (Arbitrary Unit)	MDA (μ mol/ g tissue)
CONTROL (1)	3.74 $\pm$ 1.73	53.73 $\pm$ 23.82	22.21 $\pm$ 17.56	50.63 $\pm$ 15.57
MTX (2)	1.52 $\pm$ 0.66	83.10 $\pm$ 15.23	63.92 $\pm$ 30.56	67.09 $\pm$ 10.33
MTX+CAR (3)	2.48 $\pm$ 1.13	49.74 $\pm$ 23.36	21.53 $\pm$ 9.94	52.86 $\pm$ 16.94
p value between groups				
1-2	0.012	0.010	0.010	0.046
2-3	0.046	0.006	0.001	0.016
1-3	0.093	0.439	0.699	0.834

TOS: Total oxidant status, TAS: Total antioxidant capacity, MTX: methotrexate, CAR: Carvacrol, MDA: malondialdehyde
Data presented as mean $\pm$ SD

FIGURE 1. Control group, normal renal tubular structures (H&E x100) (A). MTX group, prominent mononuclear inflammatory cell infiltration (star), degenerative changes with cell swelling (small arrows), extensive nuclear loss (arrowheads); in the tubular cells, moderate tubular dilatation (large arrows) (H&E x100), and small cytoplasmic shape (H&E x200) (B). MTX+CAR group; improvement in the tubular structures, reduction of nuclear loss and minimal cellular swelling compared with MTX group (H&E x400) (C).

induced oxidative damage to kidney tissue in rats. The expression of TOS and MDA was increased significantly by MTX administration while TAS levels were significantly decreased in the renal tissues of MTX-treated rats relative to the control animals; therefore, decreased TAS and increased TOS and MDA levels appear to be characteristic of MTX-induced renal toxicity. Additionally, renal TOS and MDA levels were reduced and TAS levels elevated in MTX-induced renal injury by prophylactic administration of CAR.

Potentially life-threatening toxicity may occur following both high-dose and low-dose MTX therapy. Significant nephrotoxicity is associated with high doses of MTX, which may have detrimental effects on kidney function via two distinct mechanisms [2]. First, MTX precipitates in the renal tubules, directly inducing tubular injury. Second, MTX induces a transient decline in glomerular filtration rate following each dose. The risk of MTX-induced renal dysfunction in patients receiving MTX therapy can be reduced by hydration and by alkalization of the urine. Despite these measures, the risk of renal toxicity after MTX remains at approximately 2% in patients

receiving MTX therapy [2]. Experimental studies have demonstrated the increased production of oxygen free radicals following MTX treatment, and these free radicals may lead to mitochondrial functional impairment [11]. MTX-induced toxicity is characterized by activation of the inflammatory response and significantly increased production of pro-inflammatory cytokines [29]. MTX-induced renal damage is further exacerbated by subsequent neutrophil infiltration and oxidative stress contribute [13]. Another study has demonstrated that MTX administration results in increased MDA levels in the kidney and other tissues [12].

CAR is a natural compound extracted from many plants in the genera *Origanum* and *Thymus*. Numerous studies have demonstrated the bactericidal [30], fungicidal [31] and insecticidal [32] activity of CAR. CAR has shown anti-tumorigenic activity in hepatocellular carcinoma, both *in vitro* and *in vivo* [33, 34]. Additional anti-inflammatory and antitumor activities of CAR have been reported [16, 18-21]. Jayakumar *et al.* [33] showed that CAR pretreatment had protective effect against diethylnitrosamine-induced hepatocellular carcinoma

in rats. In the present study, CAR was administered one day before the MTX-treatment. Canbek *et al.* [22] demonstrated that CAR is not hepatotoxic at the applied dosage in rats and, in fact, protected the liver against injury caused by ischemia/reperfusion. In another study, [23] it was shown that CAR had significant hepatoprotective and antioxidant effects against D-galactosamine-induced injury in rats. Our findings demonstrate that CAR is useful in the prevention of MTX-induced renal pathology. Increased levels of TOS and MDA in the serum and kidney tissues of rats treated with MTX were significantly reduced by prophylactic CAR administration. Additionally, the level of antioxidant TAS was significantly elevated by CAR treatment. This study demonstrates, for the first time, that CAR has the ability to suppress oxidative damage caused by MTX-induced oxidative stress in rat serum and kidney tissues.

MDA is a major oxidative degradation product of membrane unsaturated fatty acid and an indicator of lipid peroxidation that has been shown to be highly biologically active [35]. Previous studies have demonstrated that MTX administration results in increased MDA levels in the blood, liver and kidneys [36]. In this study, MTX significantly increased MDA levels in the serum and renal tissue of rats. Our findings demonstrate that a single dose of MTX (20 mg/kg) is sufficient to produce oxidative stress in rats. Prophylactic CAR treatment significantly ameliorated MTX-induced lipid peroxidation in serum and kidney tissue. Increased lipid peroxidation levels may be modulated by oxidative stress induced by MTX directly or in the indirect effects of elevated ROS in MTX injury.

Conclusion

Our results demonstrate that oxidative stress plays an essential role in MTX-induced renal toxicity in rats. CAR may provide protection by reducing the production of oxidant products and enhancing the activity of the antioxidant system. The modulation of oxidative stress with CAR could be an effective means of reducing renal impairment caused by MTX therapy. Further research will be necessary to understand the mechanisms by which CAR is able to prevent renal damage.

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