Original Article
Protective effects of carvacrol against methotrexate-induced testicular toxicity in rats

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Abstract: To investigate the effect of carvacrol (CAR) on methotrexate (MTX)-induced testis damage in rats. Twenty-four male rats were equally divided into three groups: group I control treatment; group II MTX-treated; group III MTX + CAR-treated. A single dose of CAR was administered intraperitoneally to group III on the first day of the experiment and a single dose of MTX was administered intraperitoneally to groups II and III on the second day of the experiment. The total duration of the experiment was 8 days. Blood samples and testis tissue were obtained from each animal for the measurement of malondialdehyde (MDA), Total oxidant status (TOS), Total Antioxidant Status (TAS), and Oxidative stress index (OSI). Light microscopy was used to complete the histopathological examination of testis specimens from each animal. Analysis of serum and testis sampled revealed that MDA, TOS and OSI levels were significantly greater in the group receiving MTX alone relative to the control treated animals while the TAS level was significantly reduced in the MTX group when 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. All of these quantitative values demonstrate that CAR alleviates deleterious effects of MTX on testicular tissue. Use of antioxidants such as CAR may protect germ cells against oxidative stress and apoptosis when used in combination with MTX.

Keywords: Carvacrol, methotrexate, rats, testis

Introduction

Methotrexate (MTX) is commonly used as a therapeutic agent in the treatment of a wide variety of malignancies (such as, acute lymphoblastic leukemia, osteosarcoma) and autoimmune diseases as rheumatoid arthritis, psoriasis [1-5]. The mechanisms of MTX-induced toxicity have not been completely determined [3, 6]. Testicular toxicity of MTX has an important side effect which may cause subsequent infertility. Previous studies have shown disorganization in the seminiferous tubules of the testis, a decrease in sperm number, and sperm DNA damage following administration of MTX [4, 7].

Various hypotheses related to oxidative stress have been suggested [8, 9]. Oxidative stress is reported to play an important role in the pathogenesis of MTX-induced testicular damage [10]. Atrophy within the testicular seminiferous tubules and apoptosis in spermatocytes is associated with an increase in reactive oxygen species (ROS) [7].

Carvacrol (CAR), [2-Methyl-5-(1-methylethyl) phenol (Figure 1) the predominant monoterpenic phenol which occurs in many essential oils of the family Labiatae including Origanum, Thymus and Corydothymus species, was used for centuries as a source of taste in food [11]. Antibacterial, antifungal and insecticidal effects of carvacrol have been described on different organisms in previous studies [11-14]. Analgesic and antioxidant effects have also been reported [15, 16]. There is no study in the literature about protective effects of CAR in MTX-induced testicular toxicity. The goal of this study was to
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Figure 1. Chemical structure of CAR and MTX.

investigate the likely protective role of CAR on MTX-induced testicular toxicity in rats.

Materials and methods

Animals and experimental design

Experiments were performed 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. All of the experimental procedures were in accordance with the animal use regulations of Dicle University Experimental Research Center, Diyarbakir, Turkey.

The study was performed on 24 male rats (Albino Wistar type) ranging in weight from 195 to 245 g, which were provided by the animal laboratory at Dicle University. Albino rats were housed under standard conditions with an ambient temperature of 25 ± 2°C, 12/12 hours of light-dark cycle and standard animal cages. They were treated in accordance with the National Institutes of Health guidelines.

All rats were randomly separated into 3 groups of 8 rats each. Group 1 was the experimental control group and received no additional treatment other than standard care and housing. The rats in group 2 received MTX. Group 3 rats were treated with both MTX and CAR. On the first day of the experimental protocol, Group 3 received intraperitoneal injections of CAR (73 mg/kg) according to the methods recommended by Canbek et al [17]. On the second day of the experiment, group 2 and group 3 received single intraperitoneal injections of MTX at 20 mg/kg (Medac GmbH, Theaterstrasse 6, D-22880 Wedel/Germany). The total duration of the experiment was 8 days. On last day of the experiment, the experimental animals in all three groups animals were euthanized by decapitation.

Plant extract

The plant extract investigated in this study, CAR (2-methyl-5-(1-methylethyl) phenol), was isolated from steam distillation essential oil of Origanum onites L. collected from West Anatolia.

Surgical procedures

After completion of the experimental and control treatments, all animals were anesthetized with an intramuscular injection of ketamine HCL (50 mg/kg) and a peritoneal injection of xylazine (10 mg/kg) on the eighth day and euthanized after the surgical procedure. A total of 5 ml of blood was collected from the cardiac cavities of the rats shortly after euthanasia. After the collection of blood samples, the testes of all rats were immediately excised, decapsulated, and divided longitudinally into two equivalent sections. One section was preserved for biochemical analysis and the other section was fixed in 10% formalin for histopathological examination.

Biochemical analysis

Anticoagulant tubes were used to take blood samples. Serum was removed by centrifugation at 4000 rpm at 4°C for 10 min and stored at -80°C for biochemical analysis. Tissues from both the control and experimental rats were excised, rinsed with ice-cold saline and instantaneously stored at -80°C for later biochemical analysis. Tissues from the testis of each rat were weighed and homogenized in 50 mM pH 7.0 phosphate buffered saline (PBS). Homogenized
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Testis tissues were then centrifuged at 10,000 rpm at 4°C, for 15 min to separate the supernatant for use in the biochemical analysis. MDA concentrations in serum and homogenized testis tissue were determined using an assay kit (Northwest Life Science Specialties, LLC, Vancouver, Canada). TAS and TOS of the tissues and serum were measured by the colorimetric methods described by Erel [18]. TAS results are expressed as µmol Trolox Equivalence per gram of tissue and TOS results are expressed as µmol H_2O_2 Equivalence per gram of tissue. OSI value was calculated according to the following formula: OSI (Arbitrary Unit) = TOS/TAS [19].

Histopathological assessment

After excising the testicular tissue of the euthanized animals, the testes were fixed in 10% formalin solution for 48 hours. After routine histological tissue preparation, all specimens were embedded in wax and sectioned in 4-5 µm thickness, and stained with H&E according to standard methods. For histopathological evaluation, all testes were examined and photographed using light microscopy (Zeiss, Axioshot, Germany) by a pathologist who was blinded to the study groups.

Structural changes of testes from each animal were evaluated both qualitatively and quantitatively. Quantification was done as described by Padmanabhan et al [5]. In each testis, thirty seminiferous tubules were randomly examined and each tubule was scored based on the severity of the damage. Tubules showing no damage were scored as 0, mild damage as 1, moderate damage as 2 and extensive damage as 3. The respective scores obtained from each seminiferous tubule were added to get sum score and it was divided with thirty to get the final mean seminiferous tubule damage score of the rat.

Statistical analysis

For statistical analyses SPSS 18.0 (SPSS Inc, Ca, Ill, USA) software package program was used. Mean (± standard deviation), values of data were calculated. Compatibility of variables to normal distribution pattern was investigated using Kolmogorov-Smirnov tests. Data not complying with the normality of distribution were compared with Mann-Whitney U test for the difference between two different groups. Categorical values were compared with a chi-square test. P values < 0.05 were established as the threshold of statistical significance.

Results

Serum biochemistry results

While TOS, OSI and MDA levels in the MTX group were significantly higher than those of the control group and lower levels of TAS were

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>TAS (mmol Trolox Equiv./L)</th>
<th>TOS (mmol H_2O_2 Equiv./L)</th>
<th>OSI</th>
<th>MDA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (1)</td>
<td>1.67 ± 0.08</td>
<td>11.40 ± 3.38</td>
<td>6.89 ± 2.30</td>
<td>2.47 ± 0.55</td>
</tr>
<tr>
<td>MTX (2)</td>
<td>1.34 ± 0.15</td>
<td>35.57 ± 6.45</td>
<td>26.60 ± 19.01</td>
<td>3.55 ± 0.34</td>
</tr>
<tr>
<td>MTX + CAR (3)</td>
<td>1.55 ± 0.05</td>
<td>12.28 ± 6.21</td>
<td>7.90 ± 3.94</td>
<td>2.37 ± 0.69</td>
</tr>
</tbody>
</table>

P value 1-2   | 0.002                      | 0.001                       | 0.001        | 0.002         |
| P value 2-3  | 0.016                      | 0.003                       | 0.002        | 0.005         |

estimated relative to the control group. However, in the MTX + CAR group levels of TOS, OSI, and MDA were found to be significantly lower in the MTX administered group. TAS levels in the MTX + CAR group were higher than those in the MTX monotherapy group (Table 1).

**Testes biochemistry results**

In the MTX group levels of TOS, OSI, and MDA were higher than those of the control group and TAS levels were relatively lower. TOS, OSI, and MDA levels in the MTX + CAR group were relatively lower than those in the MTX monotherapy group. Also, TAS value estimated in the MTX + CAR group was significantly higher than the MTX monotherapy group (Table 2).

**Testes histopathological analysis**

Histological assessment of the testes was done on the basis of the seminiferous tubule damage score (Figure 2). A significant increase in the testicular damage was evident with once weekly treatment of MTX 20 mg/kg as compared to the control groups (p = 0.001). While mean testicular damage score was 1.36 ± 0.22 in MTX-treated group, it was 1.20 ± 0.17 in the post-treated with CAR group. It seems that CAR treatment reduces testicular damage caused by MTX, however this reduction was not statistically significant in our results (p = 0.097).

**Discussion**

In this study, single doses of MTX (20 mg/kg) was used to caused oxidative stress in testes of rats. After prophylactic use of CAR, decreases in serum, and testicular tissue MTX levels were detected. After administration of MTX, cellular lipid peroxidase activity increases leading to oxidative stress. However following administration of CAR, previously higher levels of lipid peroxidase activity and oxidative stress decreased markedly. Also, serum, and testicular TOS, OSI, and MDA levels in the MTX + CAR group of rats were only lower than those found in the MTX group. Serum, and testicular tissue TAS levels were detected to be higher in the MTX + CAR group. Our study demonstrated that administration of CAR before application of MTX markedly decreases apoptotic process in the seminiferous tubuli.

Previous studies have demonstrated that various doses of MTX accelerate the apoptotic process via oxidative stress, and may cause harmful effects on spermatogenesis leading to infertility [5, 7, 20]. Therefore, for patients under MTX therapy, protection of germinal cells is important. Oxidative stress develops as a result of an imbalance between reactive oxygen radicals and antioxidant system. Excess amounts of reactive oxygen radicals induce production of abnormal sperms and infertility [21].

CAR is a natural compound derived from Origumum and Thymus plants. In many studies, CAR has had fungicidal, insecticidal, and antimicrobial activity [11-13, 22]. Potent antimutagenic effects and antioxidant properties of CAR have been demonstrated from in vitro studies [23, 24]. In a separate study, important roles of...

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**Figure 2.** Photomicrographs of H&E stained histological slides of the testis after 1 weeks of experimental period, at both high (400×) and low (100×) magnification; control (A), MTX (B), CAR + MTX (C). (A) Normal seminiferous tubules, (B) An evident disorganization and vacuolization is seen in MTX group and (C) mild vacuolar changes are seen in CAR + MTX group. Vacuolizations are shown by black arrows.
CAR in the ischemia-reperfusion process were reported that CAR has had hepatoprotective effects in rats with damaged livers [17]. Additionally, our study demonstrated the prophylactic effects of CAR in the prevention of MTX-related testicular damage. MDA signifies the levels of unsaturated fatty acids and lipid peroxidation [25]. The process of oxygen-dependent lipid peroxidation speeds up because the sperm wall is quite rich in polyunsaturated fatty acids. These higher serum, and testicular tissue MDA levels in rats are in concordance with the outcomes of previous studies [10].

In conclusion, the results of our study demonstrate the important role of oxidative stress in the development of testicular damage related to MTX therapy. Use of antioxidants such as CAR may protect germ cells against oxidative stress and apoptosis when used in combination with antitumoral drugs. Consequently, CAR might prevent development of infertility caused by chemotherapeutic drugs. Finally, further experimental and clinical studies are needed to be able to fully understand the mechanism of action of CAR.

Disclosure of conflict of interest
None.

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