

Neuroprotective effects of carvacrol and pomegranate against methotrexate-induced toxicity in rats

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Abstract. – **BACKGROUND:** Studies related to the use of various antioxidant and anti-inflammatory drugs to decrease the toxic side effects related to MTX have been carried out. However, since no medicine providing full protection against the side effects of MTX has been discovered, the discovery of new agents is required.

AIM: The aim of this study was to determine whether methotrexate (MTX) causes oxidative stress and an inflammatory response in sciatic nerve, as well as whether carvacrol (CAR) and pomegranate (POM) have protective effects against the resulting oxidative stress and inflammatory response.

MATERIALS AND METHODS: 32 adult male Wistar albino rats were used in the study. The animals were divided into 4 groups: Group C: the rats were not given any medication; Group MTX: On study day 2, the rats were given a single dose of 20 mg/kg MTX, administered intraperitoneally; Group MTX+CAR: On study day one, the rats were administered a single dose of 73 mg/kg CAR intraperitoneally. On study day two, a single dose of 20 mg/kg MTX was administered intraperitoneally; Group MTX+POM: For seven days starting from the study day one, rats were given 225 mg/kg POM extract once a day through orogastric gavage. On study day two, a single dose of 20 mg/kg MTX was administered intraperitoneally. All animals were sacrificed on the day eight. TOS, TAS, MDA, TNF- α and IL-1 β levels were evaluated in the sciatic nerve tissue.

RESULTS: In comparison to the control group, a decrease in TAS levels and an increase in TOS, MDA, IL-1 β and TNF- α levels were detected in the MTX group. Compared to the MTX group, the MTX+CAR group had a significant increase in TAS level and significant decreases in TOS, MDA, IL-1 β and TNF- α levels. In comparison to the MTX group, the MTX+POM group had a significant decrease in MDA, IL-1 β and TNF- α levels. When the MTX+CAR and MTX+POM groups were compared, the TNF- α level measured was lower in the MTX+CAR group.

CONCLUSIONS: In this work, we have shown that MTX causes a significant oxidative stress and inflammatory response in rats' sciatic nerve tissue and that CAR had an antioxidant effect in this system. Furthermore, we have proven, for the first time, that both CAR and POM decreased the pro-inflammatory response.

Key Words:

Methotrexate-related neurotoxicity, Carvacrol, Pomegranate.

Introduction

Methotrexate (MTX), commonly used for the treatment of malignancies, has many systematic side effects. Long treatment durations or high doses used on patients may cause toxic effects. A folic acid antagonist, MTX disrupts the synthesis of DNA. It is used in high doses for the treatment of solid tumors and hematologic malignancies and in low doses in cases of rheumatoid arthritis and inflammation. MTX-related side effects include hypersensitivity pneumonia, central and peripheral nervous system toxicity, liver and gastrointestinal system dysfunctions, and hematologic failures¹⁻⁵. Though the mechanism of MTX neurotoxicity has not been fully revealed, the latest studies hold oxidative stress responsible as one possible mechanism⁵. Therefore, studies related to the use of various antioxidant and anti-inflammatory drugs to decrease the toxic side effects related to MTX have been carried out. However, since no medicine providing full protection against the side effects of MTX has been discovered, the discovery of new agents is required¹⁻⁵.

Anti-hypertensive, proliferative, protective, antioxidant, anti-inflammatory and anti-carcinogenic effects of pomegranate (POM), contained in various fruits and plants such as pomegranate, raspberry and strawberry, have been shown⁶⁻¹².

Carvacrol (CAR) is a natural substance obtained from thyme, which is commonly grown in our country. Antioxidant, anti-microbial, antispasmodic, anti-hepatotoxic and anti-carcinogenic properties of thyme have been shown in animal trials^{13,14}.

In the English literature, we did not find any studies investigating whether CAR and POM are protective against MTX-related neurotoxicity.

In this study, our aim was to look into whether MTX caused oxidative stress (total oxidant status, total antioxidant status and malondialdehyde) and inflammatory response (IL-1 β and TNF- α) in the sciatic nerve and, if so, whether CAR and POM had protective effects against oxidative stress and inflammatory response.

Materials and Methods

Animals

This research was conducted using a total of 32 adult male Wistar albino rats weighing 225 \pm 30 g. The animal protocols were approved by Dicle University Ethics Committee (Form date and number: 13.03.2012-2012/11). Animal care and experiments were performed according to the guidelines set out by the same Ethics Committee. At the beginning of the experiments and on the eighth day, the rats were weighed. The doses of compounds administered were calculated relative to the weights of the animals. Orogastric applications were performed with a gavage needle. No animals died during the experiment. The animals were sacrificed under general anesthesia.

Experimental Protocol

In this study, the animals were randomly assigned into four groups:

Group C: control group (n=8); not given any medication.

Group MTX: Methotrexate group (n=8); on the second day of the study, a single dose of 20 mg/kg methotrexate (Medac, GmbH, Wedel, Germany) was administered intraperitoneally as described by Jahovic et al¹⁵; no other medicine was administered. For seven days, the rats were fed their usual nutrition.

Group MTX+CAR: Methotrexate+carvacrol (n=8); on the first day of the study, the rats were administered a single dose of 73 mg/kg CAR intraperitoneally. The CAR was isolated from steam distilled essential oil of *Origanum onites* L. collected from West Anatolia as described by Canbek et al¹⁶. On the second day, a single dose of 20 mg/kg methotrexate was administered intraperitoneally.

Group MTX+POM

Methotrexate+pomegranate (n=8); for seven days starting from the first day of the study, 225 mg/kg pomegranate extract, which contains 40% ellagic acid, was administered to the rats through orogastric gavage once a day. On the second day, a single dose of 20 mg/kg methotrexate was administered intraperitoneally. The POM was obtained from GNC Herbal Plus[®] Standardized (Pittsburgh, PA, USA). The eighth day, after all the test subjects included in the study were anaesthetized using 50 mg/kg ketamine HCL and 10 mg/kg xylazine intraperitoneally, intra-cardiac blood was obtained from the rats. The rats were then sacrificed through perfusion with transcardiac isotonic saline and 10% formol, after which the sciatic nerve was extracted. Total antioxidant capacity (TAS), total oxidant capacity (TOS), malonyldialdehyde (MDA), interleukin-1 beta (IL-1 β) and Tumor necrosis factor alpha (TNF- α) were measured in the extracted sciatic nerve tissue samples.

Biochemical Analysis

The excised tissue samples were weighed, immediately stored at -70°C. Tissues minced, then homogenized in five volumes (w/v) of phosphate buffer. Assays were performed on the supernatant of the homogenate that is prepared at 14,000 rpm for 30 min at +4°C¹⁷.

TAS and TOS were measured by Erel's methods^{18,19}, TAS results were expressed as mmol Trolox equivalent/L and TOS results were expressed as μ mol H₂O₂ equivalent/L. MDA content were measured spectrophotometrically as described previously²⁰. TNF- α and IL-1 β (Bender MedSystems, Vienna, Austria) levels were determined using the enzyme-linked immunosorbent assay method.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA). Data were presented as mean \pm standard deviation for

Table I. Oxidant-antioxidant cytokines in sciatic nerve tissue.

	C	MTX	MTX + CAR	MTX + POM	p
TAS	3.32 ± 1.08	2.31 ± 0.56 ^a	3.21 ± 1.01 ^b	2.43 ± 0.85	0.037*
TOS	2.84 ± 6.35	5.38 ± 9.74 ^a	3.95 ± 7.39 ^b	4.36 ± 9.35	0.001*
MDA	2.99 ± 6.61	4.74 ± 1.01 ^a	2.86 ± 4.49 ^b	3.11 ± 6.86 ^b	0.004*

Abbreviations: C: Control group; MTX: methotrexate group; MTX + CAR: methotrexate + carvacrol group; MTX + POM: methotrexate + pomegranate group. TAS: total antioxidant status; TOS: total oxidant status; MDA: malonyldialdehyde. ^aDifferent from C group ($p < 0.05$); ^bDifferent from MTX group ($p < 0.005$). Values are given as mean ± standard deviation. *Statistically significant.

biochemical values. Groups were compared using the nonparametric Kruskal-Wallis test. Mann-Whitney U test was used for binary comparisons. p values of less than 0.05 were considered significant.

Results

Among the parameters evaluated in the sciatic nerve tissues extracted from the rats, a decrease in TAS levels was seen in the MTX group compared to the control group while TOS, MDA, IL-1 β and TNF- α levels were increased ($p < 0.05$) (Tables I, II). In comparison to the MTX group, in the MTX+CAR group, there were significant decreases in TOS, MDA, IL-1 β and TNF- α levels while TAS levels had increased ($p < 0.05$). In comparison to the MTX group, in the MTX-POM group, there were significant decreases in MDA, IL-1 β and TNF- α levels ($p < 0.05$); there was not a significant change in TAS and TOS levels ($p > 0.05$). Moreover, when the MTX + CAR and MTX+POM groups were compared, TNF- α level was observed to be lower in the MTX+CAR group ($p = 0.01$) and other parameters (TAS, TOS, MDA, IL-1 β) were similar ($p \geq 0.05$) (Tables I, II).

Discussion

Methotrexate has many reported side effects such as pancytopenia, teratogenicity, infertility, hepatotoxicity, nephrotoxicity and neurotoxicity¹⁻⁴. MTX neurotoxicity has three different clinical subtypes: acute, subacute and chronic. Five to ten days after the administration of medium dose systemic MTX, subacute toxicity typically appears. Generally, MTX-related subacute toxicity is mild and can be reversed. Sequels are rare. Medulla spinalis and cerebral changes are related to subacute and chronic toxicity^{21,9}. Methotrexate-related neurotoxicity is an important clinical problem in cancer patients²¹⁻²⁴.

Methotrexate affects the brain, spinal cord and peripheral nerves according to the administered dosage, administration method (oral, iv, intrathecal) and any co-administered neurotoxic agents²³⁻²⁶.

Although methotrexate-related neurotoxicity mechanisms have not been fully explained, methotrexate is known to have a direct effect on the central and peripheral nerves. Events such as an increase of oxidants and a decrease of antioxidants in blood and cerebrospinal fluid is what is known of the mechanism²⁷⁻²⁹. The oxidative stress resulting from some neoplastic and chemical toxic agents is a significant event blamed for neurotoxicity^{26,30}.

Table II. Proinflammatory cytokines in sciatic nerve tissue.

	C	MTX	MTX + CAR	MTX + POM	p
IL-1 β	1.52 ± 1.04	4.99 ± 1.86 ^a	1.87 ± 1.07 ^b	2.31 ± 2.08 ^b	0.008*
TNF- α	2.99 ± 2.04	4.32 ± 3.36 ^a	1.47 ± 3.36 ^b	2.62 ± 5.76 ^{b,c}	0.000*

Abbreviations: C: Control group; MTX: methotrexate group; MTX + CAR: methotrexate + carvacrol group; MTX + POM: methotrexate + pomegranate group. IL-1 β : interleukin-1 beta; TNF- α : Tumor necrosis factor alpha. ^aDifferent from C group ($p < 0.05$); ^bDifferent from MTX group ($p < 0.005$). Values are given as mean ± standard deviation. *Statistically significant.

Studies have shown that methotrexate causes oxidative stress in many tissues such as the sciatic nerve and medulla spinalis. Overproduction of oxidant substances damages the cell and causes neurotoxicity^{5,15,31,32}. Other studies have shown that methotrexate decreased the efficiency of antioxidant system^{26,27,33}. In our work, similar to the study of Uzar et al⁵, we have shown that methotrexate caused neurotoxicity in the sciatic nerve tissue by increasing oxidative stress. TAS is defined as the total of enzymatic and non-enzymatic antioxidants while TOS is defined as the total oxidants. TAS is the basic defense mechanism against oxidative distress in the body. MDA is a marker of oxidative stress^{34,35}. In our research, while MTX caused an increase in TOS, MDA, IL-1 β and TNF- α in the sciatic nerve of rats, it caused a decreased in total antioxidants. This suggests that MTX causes oxidative stress and inflammatory response in the sciatic nerve tissue. In this report, we looked into the effects of pomegranate and carvacrol, both of which have the potential to decrease MTX-related oxidative stress and inflammation in nerve tissue.

Pomegranate is the extract of pomegranate, which contains 40% ellagic. Ellagic acid is a polyphenol compound with antioxidant and anti-proliferative properties and which is contained in many fruits and plants (pomegranate, raspberry, pecan, strawberry, etc.) as an active substance⁶⁻¹². In the literature, the studies conducted on pomegranate and ellagic acid show their anti-atherogenic, anti-hypertensive, proliferative, protective, antioxidant, anti-inflammatory and anti-carcinogenic effects^{6,36-39}. Pomegranate has not yet been used to decrease or prevent MTX-caused damage.

Carvacrol (CAR) has been shown in various studies to have protective effects against hepatotoxicity in rat liver ischemia-reperfusion, and also to have antioxidant, anti-microbial, anti-spasmodic, anti-carcinogenic and anti-proliferative effects⁴⁰⁻⁴³. We looked into its protective effects in MTX-related neurotoxicity.

Superoxide radicals produced as a result of oxidative stress are converted to hydrogen peroxide (H₂O₂). H₂O₂ accesses tissues and organs remote from its point of origin and enters through the cell membrane. If there are transitional metals in the area where H₂O₂ has reached, hydroxy radicals, which are even more dangerous than superoxide radicals, are produced through the Fenton reaction. This results in an increase of oxidative stress. This increased oxidative stress causes neurotoxicity. In our study, we have shown that TAS

levels increased significantly in the MTX+CAR group compared to the MTX group and that there was not any statistically significant difference in the MTX+POM group compared to the MTX group. This may prove that CAR is a good antioxidant against MTX neurotoxicity. Additionally, in comparison to the MTX group, while the TOS level decreased significantly in the MTX+CAR group, the decrease in the MTX+POM group was not significant. This may prove that CAR is a better antioxidant than POM. Additionally, as a marker of oxidative stress, MDA levels were compared. The MTX group had significantly higher levels of MDA than both the MTX+CAR group and the MTX+POM group. These findings have shown that, in the case of MTX-related oxidative stress in nerve tissue, the antioxidant effect of CAR was prominent while POM's antioxidant effect was weaker.

TNF- α and IL-1 β are proinflammatory cytokines and are mediators related to cell death and inflammation⁴⁴. In studies, while small doses of methotrexate are used as anti-inflammatories, high doses increase pro-inflammatory cytokines, cause inflammation and eventually cause neurotoxicity⁴⁵. Çetiner et al⁴⁶ discovered that TNF- α levels increased in the methotrexate-treated group and caused inflammation in their study. Geurjeon et al⁴⁷ specified that in their studies with rats, TNF- α and IL-1 β did not decrease the neuron count but caused neuron growth, which is rather interesting. In our study, however, in both the MTX+CAR and MTX+POM groups, TNF- α and IL-1 β levels were found to be significantly lower compared to the MTX group. When comparing MTX+CAR and MTX+POM, MTX+CAR was observed to have a lower TNF- α level. This situation might mean that CAR is more efficient, but both CAR and POM extracts provide protection against the harmful effects of methotrexate by decreasing pro-inflammatory cytokines.

Conclusions

In this report, we have shown that MTX causes oxidative stress and an inflammatory response in sciatic nerve tissue through an increase in TOS, MDA, IL-1 β , and TNF- α levels and a decrease in TAS level. We have, for the first time to our knowledge, empirically shown that the antioxidant effect of CAR was effective against this oxidative stress and that both CAR and POM decreased the MTX-related pro-inflammatory response.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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