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HEPATORENAL PROTECTIVE EFFECTS OF WALNUT OIL AGAINST ANTICANCER DRUG METHOTREXATE IN EXPERIMENTALLY INDUCED LIVER AND KIDNEY TOXICITY IN RATS

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Abstract

The aim of this study was to investigate the hepatorenal protective effects of walnut oil (WO) against anticancer drug methotrexate (MTX)-induced kidney and liver toxicity.

In our study, 40 male Sprague Dawley rats weighing between 200–250 g were used. The rats were randomly divided into four groups; Group 1, control group (corn oil by gavage for 14 days and intraperitoneal (i.p.) physiological saline on the third day, $n = 10$), Group 2, WO group (2 ml/kg WO by gavage for 14 days and i.p. physiological saline on the third day, $n = 10$), Group 3, MTX group (corn oil by gavage for 14 days and 20 mg/kg MTX single dose i.p. on the third day, $n = 10$), Group 4, MTX + WO group (2 ml/kg WO by gavage for 14 days and 20 mg/kg MTX single dose i.p. on the third day, $n = 10$). At the end of the experiment, the rats were decapitated. Kidney and liver were preserved at $-86\,^{\circ}$C and biochemical measurements were performed.

Thiobarbituric acid reactive substances (TBARS) levels increased and superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx) and catalase (CAT) activities decreased in kidney and liver tissues in the methotrexate alone group compared to the control group. In the MTX + WO treated group, TBARS level decreased and GSH, CAT, SOD and GPx activities increased significantly compared to the MTX alone treated group.

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It was found that MTX caused oxidative damage in kidney and liver tissues and WO prevented this damage. Walnut oil is protective against MTX-induced kidney and liver toxicity.

Key words: kidney, liver, methotrexate, oxidative damage, walnut oil

Introduction. Methotrexate (MTX) is a folic acid antagonist acting by dihydrofolate reductase enzyme inhibition and is used as an antineoplastic agent in the treatment of haematological and solid tumours, autoimmune and inflammatory diseases such as inflammatory bowel diseases, rheumatoid arthritis, and gynaecological pathologies such as ectopic pregnancy and gestational trophoblastic diseases. Nevertheless, serious side effects limit the use of MTX. Side effects can be observed in various organs and tissues, including the gastrointestinal tract, liver, kidneys and nervous system. These side effects have been associated with oxidative stress and inflammatory processes [1].

Walnut oil obtained from walnut by hydraulic pressing technology contains approximately 5–6% saturated fatty acids and 47.14% unsaturated fatty acids. Approximately 80% of unsaturated fatty acids are linoleic acid (omega-6) and 20% are α-linolenic acid (omega-3) [2]. The fact that walnut contains both omega-3 and omega-6, which are polyunsaturated fatty acids, is the most important feature that makes walnut distinguishable among hard-shelled fruits. It has been stated that monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) reduce total plasma and LDL cholesterol concentration when consumed instead of saturated fatty acids. Omega-3 and Omega-6 are essential fatty acids that cannot be synthesised by the body and must be taken with food [3]. This feature has made the consumption of walnut indispensable. The preventive role of polyunsaturated fatty acids in cardiovascular diseases has been known for many years. It is known that these fatty acids have a positive effect on blood lipid levels in humans and animals [3]. Polyunsaturated fatty acids are reported to be anti-inflammatory, antihypertensive, they reduce blood lipid levels, prevent thrombosis and vascular occlusion in preventing cardiovascular diseases. Fan et al. [4] reported that daily regular consumption of walnut oil increased the activity of antioxidant enzymes such as superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT) and glutathione peroxidase (GSH-Px) and reduced oxidative stress of the body.

The aim of this study was to investigate the potential protective effects of WO against MTX-induced oxidative damage of kidney and liver. For this purpose, thiobarbituric acid reactive substance (TBARS) level as an oxidative stress marker and superoxide dismutase (SOD), reduced glutathione (GSH), glutathione peroxidase (GPx) and catalase (CAT) activities as antioxidant markers have been measured.

Materials and methods. ***Research and editorial ethics.*** This study was ethically approved by the decision of the Animal Experiments Ethics Committee of Medicine Faculty, dated 24.10.2020 and numbered 371, and the studies were conducted in the Experimental Research Centre Laboratories of Medicine Faculty.

Chemicals. Methotrexate (Methotrexate Koçak Pharma, Istanbul, Turkey; 500 mg/20 ml solution for injection) was purchased from a pharmacy and walnut oil was purchased from a market selling vegetable oil products (Aytorus Walnut oil, Juglans Regia, Cold Press, 100% Walnut oil, Antalya, Turkey; 5 L/10 L), all other chemicals were of analytical highest purity.

Experimental animals and study design. The rats used in the study were bred and raised in the Experimental Animal Research Centre. A total of 40 male Sprague Dawley rats weighing between 200–250 g were used. During the study, the rats were kept in polyprolene cages at an ambient temperature of 21 °C and a 12-hour light-dark cycle, and feed and water were given ad libitum.

Rats were randomly divided into four groups.

Group 1 (Control group, $n = 10$): Rats in the control group were given corn oil by gavage once a day for 14 days and a single dose of physiological saline was injected into the peritoneum on the third day.

Group 2 (WO group, $n = 10$): Rats in the WO group were given 2 ml/kg WO by gavage once a day for 14 days and a single dose of physiological saline was injected into the peritoneum on the third day.

Group 3 (MTX group, $n = 10$): Rats in the MTX group were given corn oil by gavage once daily for 14 days and a single dose of 20 mg/kg MTX was injected intraperitoneally on the third day.

Group 4 (MTX + WO group, $n = 10$): Rats in the MTX + WO group were given 2 ml/kg WO by gavage once daily for 14 days and a single dose of 20 mg/kg MTX was injected intraperitoneally on the third day.

On the fifteenth day of the study, rats under general anaesthesia with a mixture of 10% Ketamine (Ketasol; Richter Pharma Ag. Wels, Austria; 0.8–1.3 ml/kg) and 2% Xylazine (Basilazine; Bayet, Istanbul, Turkey; 2–5 mg/kg) were decapitated. Kidney and liver tissues were rapidly removed and cryopreserved at −86 °C for biochemical measurements.

Biochemical measurements. Tissues were homogenised in 0.2 M Tris-HCl buffer (pH: 7.4) under cold chain conditions to obtain a 1:10 (w/v) dilution of the whole homogenate. Direct homogenate was used for TBARS measurements. SOD, CAT and GPx activities and GSH levels were determined from the supernatants obtained by centrifugation of homogenates at 3220 rpm for 30 min (4 °C). TBARS levels, an index of lipid peroxidation, were measured using Yagi's method [5]. In this method, two molecules of thiobarbituric acid react with one molecule of TBARS to form a pink coloured product. The products were evaluated spectrophotometrically at 532 nm and the results were expressed as nmol/g tissue. SOD, CAT and GPx are members of the cellular enzymatic antioxidant defence system. SOD converts superoxide anion to hydrogen peroxide, CAT and GPx in mitochondria reduce hydrogen peroxide to water and form the first line of antioxidant defence which provides the strongest defence against free oxygen radicals. Spectrophotometric method was used to determine SOD, CAT and GPx

activities and the results were expressed as unit/mg tissue protein. The method of Sun et al. [6] was used to determine SOD activity. In this method, the superoxide radical formed during the conversion of xanthine to uric acid by xanthine oxidase reacts with nitrobluetetrazolium (NBT) in the medium to form purple coloured formazone. In the presence of SOD in the medium, the superoxide radical formed will be converted to hydrogen peroxide and NBT reduction and thus formazone formation will decrease. CAT activity was determined according to the method of Aebi [7], which is based on the principle that the destruction of hydrogen peroxide in the medium by the action of CAT is monitored as absorbance decrease at 240 nm wavelength. Absorbance difference per unit time was used as a measure of CAT activity. GPx activity was determined according to the method of Paglia and Valentine [8]. In this method, GPx, which reduces hydrogen peroxide to water, converts reduced glutathione to oxidised form. In the presence of glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH), oxidised glutathione is reduced back to reduced glutathione. The decrease in NADPH in the medium is followed by a decrease in absorbance at 340 nm. GSH levels were determined spectrophotometrically by measuring at 412 nm wavelength according to the method of Sedlak and Lindsay [9]. In this method, sulfhydryl groups in the structure of GSH react with 5,5′-dithiobis-2-nitrobenzoic acid to form a yellow-green coloured chromogen compound.

Statistical analyses. Statistical analyses were performed using IBM SPSS Statistics 22.0 for Windows (SPSS Inc., Chicago, Illinois, United States). The normal distribution of the data was analysed by Shapiro–Wilk test. One-way analysis of variance (ANOVA) test was used to compare variables between groups. The results were expressed as mean ± standard deviation (mean ± SD). $P < 0.05$ value was considered statistically significant.

Results. Kidney and liver tissue TBARS and GSH levels and SOD, GPx and CAT activities are given in Table 1 and Table 2.

Table 1

Changes in TBARS and GSH levels and SOD, GPx and CAT activities in kidney tissues of MTX and WO treated rats ($n = 10$)

KIDNEY	TBARS (nmol/g)	GSH (nmol/mg)	SOD (U/mg)	GPx (U/mg)	CAT (U/mg)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Control	103.77 ± 3.87^{a}	9.58 ± 0.68^{ab}	4.53 ± 0.23^{a}	0.99 ± 0.11^{a}	662.36 ± 49.70^{a}
WO	114.22 ± 2.54^{a}	11.49 ± 0.39^{b}	4.66 ± 0.30^{a}	1.04 ± 0.13^{a}	693.21 ± 13.49^{a}
MTX	132.69 ± 1.14^{b}	9.24 ± 0.41^{a}	3.58 ± 0.33^{b}	0.87 ± 0.12^{b}	509.12 ± 18.19^{b}
MTX + WO	119.32 ± 2.75^{a}	10.17 ± 0.18^{ab}	6.16 ± 0.57^{a}	1.15 ± 0.12^{a}	684.96 ± 20.30^{a}

TBARS: Thiobarbituric acid reactive substance, GSH: Reduced glutathione, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, CAT: Catalase, WO: Walnut oil, MTX: Methotrexate, MTX + WO: Methotrexate + Walnut oil, Mean ± SD: Mean ± Standard Deviation, ^{a,b}: Means with different superscripts in the same column are statistically significantly different ($p < 0.05$)

Table 2

Changes in TBARS and GSH levels and SOD, GPx and CAT activities in liver tissues of MTX and WO treated rats ($n = 10$)

KIDNEY	TBARS (nmol/g)	GSH (nmol/mg)	SOD (U/mg)	GPx (U/mg)	CAT (U/mg)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Control	59.51 ± 5.05^{a}	8.31 ± 0.59^{ad}	4.80 ± 0.11^{a}	1.42 ± 0.13^{a}	2220.79 ± 136.45^{a}
WO	58.17 ± 2.97^{a}	9.23 ± 0.64^{a}	5.31 ± 0.30^{ac}	1.50 ± 0.18^{a}	2661.71 ± 115.98^{a}
MTX	67.06 ± 4.07^{b}	6.30 ± 0.26^{bc}	3.64 ± 0.14^{b}	1.19 ± 0.10^{b}	1384.15 ± 77.69^{b}
MTX + WO	62.44 ± 6.06^{a}	6.96 ± 0.71^{cd}	6.05 ± 0.28^{c}	1.55 ± 0.14^{a}	1760.51 ± 136.55^{c}

TBARS: Thiobarbituric acid reactive substance, GSH: Reduced glutathione, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, CAT: Catalase, WO: Walnut oil, MTX: Methotrexate, MTX + WO: Methotrexate + Walnut oil, Mean ± SD: Mean ± Standard Deviation, ^{a,b,c,d}: Means with different superscripts in the same column are statistically significantly different ($p < 0.05$)

In this study, MTX alone treatment caused a statistically significant increase in TBARS levels, a statistically significant decrease in SOD, GPx and CAT activities, and a statistically significant decrease in GSH activity in kidney tissue compared to the control group. MTX + WO treatment group caused a statistically significant decrease in TBARS level, a statistically significant increase in SOD, GPx and CAT activities, and an increase in GSH activity that did not reach statistical significance compared to MTX alone treatment group (Table 1).

In the liver tissue, in the group administered MTX alone, TBARS levels increased, although not statistically significantly, and GSH, SOD, GPx and CAT activities decreased statistically significantly compared to the control group. In the group administered MTX+WO, compared to the group administered MTX alone, there was a statistically insignificant but significant decrease in TBARS levels and a statistically significant increase in GSH, SOD, GPx and CAT activities (Table 2).

Discussion. MTX, a cytotoxic antineoplastic agent, is a folate antimetabolite used in the treatment of many malignancies. It is also used in the treatment of rheumatoid arthritis and some other rheumatic and inflammatory diseases due to its immunosuppressive/modulatory effect. MTX causes serious side effects in many tissues and organs, especially in high proliferation tissues, requiring dose reduction or discontinuation. Hepatotoxicity, nephrotoxicity, pulmotoxicity, haematotoxicity, neurotoxicity, cardiotoxicity, gastrointestinal toxicity, gonadal toxicity and neocarcinogenic effect are the main side effects [10]. Side effects depend on dose, route of administration, frequency of administration and concomitant use of folinic acid.

In this study, 20 mg/kg single dose MTX administration caused an increase in TBARS levels and a decrease in SOD, GPx, GSH, CAT activities in kidney and liver tissues of rats compared to the control group. The results of our study were in accordance with the results of previous studies which determined the oxidative

damage caused by MTX [10,11]. In these studies, a single dose of 20 mg/kg MTX administration significantly increased malondialdehyde (MDA) levels and significantly decreased SOD, CAT and GPx activities in kidney, liver and testicular tissues of rats. In a study by Fikry et al. [11], it was found that oral administration of 14 mg/kg MTX 2 times at one week intervals caused a significant increase in MDA level and a significant decrease in GSH level and CAT activity in the heart tissue of rats. In another study, they found that benfotiamine administration resulted in significant biochemical and histological improvement in MTX-mediated nephrotoxicity [12]. Furthermore, Vardi et al. [13] demonstrated that increased oxidative stress due to MTX-mediated hepatotoxicity was significantly reversed by beta carotene administration. Hafez et al. [1] investigated the effects of etanercept, a TNF-α inhibitor, and aminoguanidine, an inducible nitric oxide synthase inhibitor (iNOS), on MTX-induced renal and hepatic damage separately and in combination and found that both agents had organ protective effects by suppressing oxidative stress pathways.

Vanderhoof et al. [14] showed that fish oil improved MTX-induced mucosal damage; Horie et al. [15] showed that DHA strongly inhibited small intestinal damage caused by oral MTX administration in mice; another study showing that MTX inhibits the side effects of MTX on bones revealed that fish oil significantly reduced inflammatory/oxidative processes on bone tissue at biochemical and histological levels [16]. Elbarbary et al. [17], who evaluated the side effects of methotrexate through the oxidant-antioxidant system, administered 1000 mg omega-3 fatty acids derived from fish oil together with MTX used in the treatment of acute lymphoblastic leukaemia patients and showed a significant improvement in SOD and GPx activities, serum total antioxidant capacity and MDA levels in this group. In another study, the effect of garlic juice extract on MTX-induced nephrotoxicity was investigated and it was revealed that garlic juice given before MTX administration increased renal functions and antioxidant enzyme levels in tissue [18]. There is a study showing that walnut oil has a protective effect in paracetamol-induced liver damage and experimental studies showing that it is protective against alcoholic liver disease [19]. However, a study investigating the effects of walnut oil against the side effects of methotrexate has not been conducted before. In this study, administration of 2 ml/kg WO caused a decrease in TBARS levels and a significant increase in CAT, SOD and GPx activities in kidney and liver tissues compared to MTX group. This study is important because it is the first experimental study in the literature showing that WO is protective against the toxic effects of MTX on kidney and liver tissues.

Conclusion. In conclusion, this study revealed that MTX causes oxidative damage in kidney and liver tissues and the potential of WO to prevent this damage. Therefore, WO may be useful in the prevention of nephrotoxicity and hepatotoxicity that may occur in patients receiving MTX treatment.

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