



Investigation of the potential beneficial effects of fish oil against methotrexate-induced brain injury

Kursat Kaya^{a,*}, Ali Gurel^b, Volkan Ipek^c

^aPamukkale University, Faculty of Medicine, Department of Medical Biochemistry, Denizli, Turkey

^bFırat University, Faculty of Medicine, Department of Internal Medicine, Department of Nephrology, Elazığ, Turkey

^cMehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Pathology, Burdur, Turkey

ARTICLE INFO

Keywords:

Methotrexate

Fish oil

Brain injury

Neurological damage

Oxidative stress

Received: Jun 29, 2021

Accepted: Nov 30, 2021

Available Online: Apr 29, 2022

DOI:

[10.5455/annalsmedres.2021.06.476](https://doi.org/10.5455/annalsmedres.2021.06.476)

Abstract

Aim: Methotrexate (MTX), a folic acid antagonist, is widely used in the treatment of many malignancies. However, various serious side effects caused by MTX limit its clinical use. Neurotoxicity is one of the most important side effects seen during MTX treatment. Oxidative damage is explained as one of the side-effect mechanisms caused by MTX. In addition to the many health benefits of fish oil (FO), its antioxidant properties have also been shown in various studies. In this study, it was aimed to investigate the potential beneficial effects of FO against oxidative brain damage caused by MTX.

Materials and Methods: Twenty-eight rats were randomly divided into 4 groups (n = 7). The first group is the control group. The second group was determined as the FO group and 1000 mg / kg / day FO was given by gavage to the rats in this group for two weeks. The third group was determined as MTX group and a single dose of 20 mg / kg MTX was injected intraperitoneally to the rats in this group on the third day. The fourth group was determined as MTX + FO group, and the rats in the group were given MTX and FO in similar doses together.

Results: The administration of MTX caused significant increase in TBARS levels compared to the control group, and significant decrease in SOD, GPx and CAT activities. However, the administration of FO with MTX caused statistically significant decrease in TBARS level and a significant increase in SOD and GPx activities compared to MTX group. On the other hand, there was no difference between the groups in terms of GSH levels. In addition, administration of MTX did not cause a histopathologically significant lesion in the brain tissue.

Conclusion: Our results support that the use of FO may benefit against neurological oxidative damage caused by MTX.



Copyright © 2022 The author(s) - Available online at www.annalsmedres.org. This is an Open Access article distributed under the terms of Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

Introduction

Folic acid is one of the B-complex vitamins that acts as a coenzyme in the metabolism of nucleic acids and amino acids, and in the reactions of transporting single-carbon units. Methotrexate (MTX) is a folic acid antagonist and inhibits cell proliferation by inhibiting de novo pyrimidine and purine synthesis [1]. MTX, which binds to dihydrofolate reductase with great affinity and blocks this enzyme, thus prevents the conversion of dihydrofolate to tetrahydrofolate and stops protein synthesis [2]. MTX is widely used in the treatment of many malignancies such as leukemia, various autoimmune and inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease, and some gynecological disorders such as gestational

trophoblastic diseases and ectopic pregnancy [1, 2].

However, various serious side effects caused by MTX limit its clinical use [5]. During MTX treatment, serious side effects can be observed in many organs and tissues, especially in the gastrointestinal system, liver, kidneys and nervous system tissues [6]. Methotrexate-related toxicity occurs due to the interaction of many factors such as dose, duration of treatment, risk factors of patients, type of disease, and genetic and molecular apoptotic factors [7]. Neurotoxicity, which is frequently seen during MTX treatment, can occur in acute, subacute and late forms after various administration methods [6, 7]. High-dose MTX has been associated with demyelination, white matter necrosis, loss of oligodendroglia, axonal swelling, microcystic encephalomalacia, and deep cerebral white matter atrophy in both intravenous and intrathecal administration [9]. Acute and chronic MTX neurotoxicity is thought to be associated

*Corresponding author:

Email address: kursatkaya023@gmail.com (Kursat Kaya)

with a reduction in hippocampal neurogenesis and specifically affects behaviors controlled by this brain region [10]. Neurological symptoms such as aphasia, weakness, sensory deficits, ataxia, and seizures are seen in acute MTX neurotoxicity. The incidence of acute MTX neurotoxicity varies between 3-10%, depending on the dose, route of administration, frequency of administration, and use of leucovorin [9].

Although not fully explained, oxidative stress and some inflammatory processes have been suggested to explain the toxicity caused by MTX. An imbalance among oxidants and antioxidant defense systems causes oxidative damage and, oxidative damage has been described as one of the most important causes of tissue damage caused by MTX [3]. The central nervous system is very sensitive to ROS attacks due to various reasons such as high oxygen intake, neuronal membrane lipids rich in polyunsaturated fatty acids, and having a moderate antioxidant defense system. Hence, conditions such as MTX application, where free radical formation and antioxidant defense capacity are exceeded, cause oxidative stress-mediated membrane disruption and cellular dysfunction [11]. In particular, peroxidation products of polyunsaturated fatty acids caused by oxidative substances interact with substances such as nucleic acids and proteins, thereby exacerbating oxidative damage [12]. It also accelerates oxidative processes by reducing the availability of NADPH used by glutathione reductase to maintain the reduced state of cell glutathione, an important cytosolic antioxidant that protects against reactive oxygen species [7].

Many studies have shown the beneficial effects of fish oil (FO) on human health due to its richness in n-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). In addition to the necessity of n-3 fatty acids for normal growth and development, important beneficial effects in both prevention and treatment of coronary artery diseases, hypertension, diabetes, inflammatory diseases, autoimmune diseases and cancer are stated [13]. Potential beneficial effects of n-3 fatty acids are associated with protecting many tissues against oxidative damage due to their location in the structure of biological membranes [14].

In the literature review conducted by us, there is no study investigating the effects of FO against MTX-induced oxidative damage. Therefore, this study was aimed to research the potential useful influences of FO against oxidative brain damage caused by MTX.

Materials and Methods

Chemicals

MTX (Koçak Farma, 500 mg / 20 ml) and fish oil [Solgar Omega-3 950 mg (soft gelatin capsule; each capsule containing 504 mg EPA and 378 mg DHA)] were purchased from the pharmacy. All other chemicals used were of analytical purity or the highest purity available and were purchased from Sigma-Aldrich.

Animals and Experiment Design

Ethics committee approval of the study was obtained from Adiyaman University Animal Experiments Local Ethics

Committee (2020 / 040-04.06.2020). In the study, 28 healthy male Sprague Dawley rats with a body weight of 250-300 grams were used. Sample size was determined by power analysis. Excessive body weight loss, inability to walk properly, severe reluctance to take food and water, and significantly reduced response to stimuli were determined as exclusion criteria. However, no animals were excluded from the study due to exclusion criteria throughout the study. Rats were obtained from Adiyaman University Experimental Animals Production, Application and Research Center. During the study, feed and water were given "ad libitum" to rats kept in polypropylene cages, at 21 ° C ambient temperature and 12-hour light-dark cycle.

A total of 28 rats were randomly divided into 4 groups (n = 7). The first group was determined as the control group and the rats in the control group were given corn oil [14] by gavage once a day for 2 weeks and one dose of physiological saline was injected intraperitoneally (i.p.) on the third day. The second group was named as FO group, and 1000 mg/kg of FO was given by gavage once a day for 2 weeks and one dose of physiological saline was injected i.p. on the third day. The third group was named as MTX group and rats were given corn oil by gavage once a day for 2 weeks and one dose of 20 mg/kg MTX was injected i.p. on the third day. The last group was named MTX + FO and rats in this group were given 1000 mg/kg of FO oil by gavage once a day for 2 weeks and one dose of 20 mg/kg MTX was injected i.p. on the third day. On the fifteenth day, rats were euthanized by the exsanguination method under general anesthesia with xylazine-ketamine mixture. The brain tissues were rapidly removed as a whole, cut in half from the sagittal plane on a cold glass. One of the brain hemispheres was stored for histopathological examinations. The other hemisphere was quickly frozen at -86 ° C for later biochemical measurements.

Biochemical Measurements]Biochemical Measurements Tissues were homogenized in 0.2 M Tris-HCl buffer (pH: 7.4) under cold chain conditions to obtain a 1:10 (w / v) dilution of the entire homogenate. Homogenate was used directly for thiobarbituric acid reactive substance (TBARS) measurements. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities and reduced glutathione (GSH) levels were determined from the supernatants obtained by centrifuging homogenates at 3220 rpm for 30 minutes (4 °C). TBARS levels, a lipid peroxidation marker, was measured using Yagi's method [15]. The products were evaluated spectrophotometrically by measuring at 532 nm and the results were shown as nmol / g tissue.

SOD, CAT and GPx are members of the cellular enzymatic antioxidant defense system. By conversion superoxide anion to hydrogen peroxide by SOD, CAT and GPx reduce hydrogen peroxide to water and by this way they form the first antioxidant defense line that makes the strongest defense against free oxygen radicals [16].

The spectrophotometric method was used to determine SOD, CAT, and GPx activities and the results were expressed as units / mg tissue protein. The method of Sun et al., was used to determine the SOD activity [17]. In this method, which is based on the inhibition of nitroblue tetrazolium (NBT) reduction caused by the superoxide radical

produced by the xanthine/xanthine oxidase system, the enzyme activity that inhibits NBT reduction by 50% is accepted as 1 SOD activity. CAT activity was measured using the method of Aebi et al. [18], based on the principle of following the degradation of hydrogen peroxide by CAT effect in the form of absorbance decrease at 240 nm wave length. The change in absorbance per unit time was evaluated as a measure of CAT activity. GPx activity was determined according to the method of Paglia and Valentine [19]. In this method, GPx, which reduces hydrogen peroxide to water, converts the reduced glutathione to its oxidized form. In the presence of glutathione reductase and NADPH in the environment, the oxidized glutathione is reduced back to reduced glutathione. NADPH reduction is followed as absorbance decrease at 340 nm. This decrease in absorbance is directly proportional to the GPx activity. GSH is one of the members of the second line antioxidant defense system. It cleans free radicals by giving them electrons [16]. GSH levels were determined spectrophotometrically by measuring at 412 nm wave length according to Sedlak and Lindsay's method [20]. Tissue GSH levels were expressed as nmol/mg tissue protein. The Lowry method was used to determine the amount of brain tissue protein [21].

Histopathological Evaluation

Brain tissues were taken after necropsy were fixed by immersion in 10% buffered formaldehyde. Tissues undergoing routine tissue follow-up procedures were embedded in paraffin and sections with a thickness of 5 microns were taken. Sections were stained with hematoxylin-eosin and evaluated under a light microscope. Histopathologically, in the telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon; evaluations were made in terms of the presence of degeneration, necrosis, chromatolysis, satellitosis, demyelination, myelin pallor, neuropil vacuolization, axonal swelling and spheroid formation, oligodendroglial swelling, intramyelin edema, macrophage infiltrations, gemistocytic astrocytes and glial activation and the groups were compared.

Statistical Analysis

All the statistical analyses of the obtained experimental data were performed using Statistical Package for the Social Sciences (SPSS) v.25 (IBM Corp., Armonk, NY, USA). Continuous variables were provided as mean \pm standard error. Shapiro Wilk test were used for determination of normal distribution. For independent groups comparisons, we used One Way Analysis of Variance (post hoc: Tukey method) when parametric test assumptions were provided. The Games-Howell test was used for the variables that did not show homogeneous variance. A p value of <0.05 was set as the limit for statistical significance.

Results

Biochemical Results

Brain tissue TBARS, GSH levels and SOD, GPx and CAT activities are shown in table 1. In our study, the application of MTX caused meaningful rise in TBARS levels compared to the control group, and meaningful reduce in

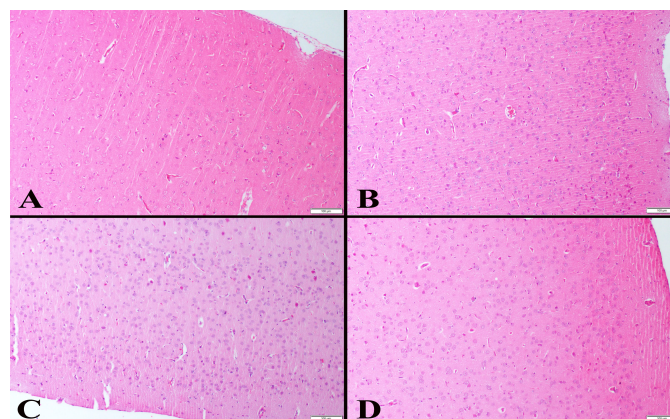


Figure 1. Histopathological views of the cerebral cortex in the control (A), FO (B), MTX (C) and MTX-FO (D) groups. H&E Bar: 100 μ m.

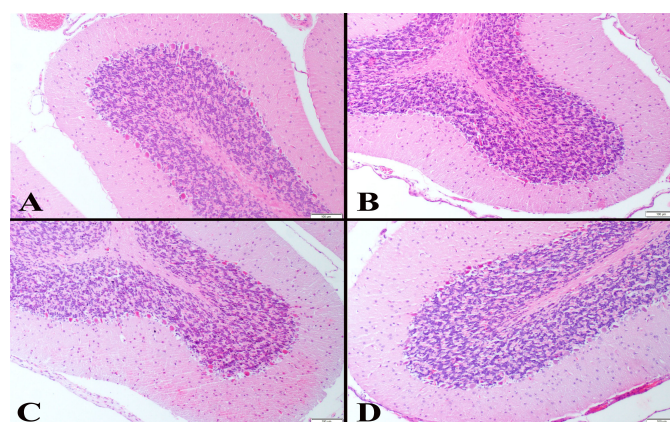


Figure 2. Histopathological views of the cerebellum in the control (A), FO (B), MTX (C) and MTX-FO (D) groups. H&E Bar: 100 μ m.

SOD, GPx and CAT activities. A decrease in GSH levels was observed, but this was not statistically significant. Application of FO with MTX caused statistically a meaningful reduce in TBARS level and a meaningful rise in SOD and GPx activities compared to MTX group. The increase in CAT activity was not statistically significant.

Histopathological Results

Slight morphological changes were observed in all groups due to the detection of brain tissues by the formalin immersion method. However, in the histopathological examinations of the mentioned areas of the brain and cerebellum sections, no significant difference was observed between the groups (Figure 1-3).

Discussion

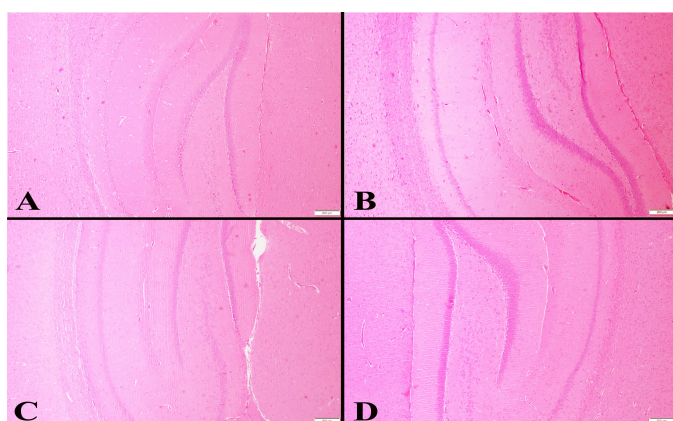
MTX, a cytotoxic chemotherapeutic agent, is an antifolate metabolite widely used in the treatment of many types of cancer. In addition to its use in malignancies, it is the main active ingredient preferred in the treatment of rheumatoid arthritis and other rheumatic diseases [17, 18].

MTX-polyglutamates, which are formed as a result of polyglutamation of MTX after it enters the cell, bind to

Table 1. Changes in TBARS, GSH levels and SOD, GPx and CAT activities in the brain tissues of MTX and FO applied rats (n = 7).

Brain	TBARS(nmol/g wet tissue)	GSH(nmol/mg tissue prot.)	SOD(U/mg tissue prot.)	GPx(U/mg tissue prot.)	CAT (U/mg tissue prot.)
(I) Control	32.37±0.95 ^a	8.04±0.20	13.53±0.29 ^a	0.89±0.03 ^a	12.26±0.67 ^a
(II) FO	30.75±0.86 ^a	7.91±0.36	14.11±0.48 ^a	0.89±0.03 ^a	11.16±0.51 ^a
(III) MTX	37.70±1.24 ^b	6.15±0.92	10.97±0.50 ^b	0.67±0.02 ^b	9.68±0.48 ^b
(IV) MTX+FO	32.90±1.13 ^a	8.52±0.23	14.74±0.63 ^a	0.93±0.05 ^a	10.48±0.18 ^{a, b}
<i>p</i> values					
I-II	0.703	0.991	0.838	1.000	0.409
I-III	0.008	0.281	0.006	0.005	0.006
I-IV	0.984	0.458	0.328	0.925	0.079
II-III	0.001	0.352	0.001	0.005	0.046
II-IV	0.488	0.537	0.804	0.920	0.771
III-IV	0.019	0.151	<0.001	0.001	0.668

The means with different superscripts in the same column are statistically significantly different. Mean ± SEM, NS: not significant

**Figure 3.** Histopathological views of the hippocampus region in the control (A), FO (B), MTX (C) and MTX+FO [D] groups. H&E Bar: 200 µm.

dihydrofolate reductase enzyme with high affinity, rendering this enzyme dysfunctional. Inhibition of dihydrofolate reductase enzyme stops protein synthesis by stopping the synthesis of thymidine and purines, which need tetrahydrofolate for their synthesis [2]. Besides its widespread use, MTX causes serious side effects in many tissues and organs especially tissues with high proliferation that require dose limitation [4, 18]. Many previous studies have demonstrated hepatotoxic, nephrotoxic, pulmonary, gastrointestinal, hematopoietic, carcinogenic, cardiac, testicular and neurotoxic side effects of MTX [17, 19–21].

Although its incidence varies depending on the dose, route of application, frequency of administration and the use of folic acid; neurotoxicity is among the most important side effects that occur with MTX use [9]. How MTX-induced central nervous system damage develops has often been explained by various mechanisms related to impaired folate balance and adenosine accumulation [3, 22]. However, oxidative stress is one of the most important causal factors in tissue damage caused by MTX use [3]. MTX causes a decrease in glutathione level by decreasing the level of NADPH and a decrease in S-adenosyl

methionine (SAM) level by decreasing the level of 5-methyltetrahydrofolate. The decrease of these antioxidant compounds results in the increase of reactive oxygen species and lipid peroxidation [23, 24]. In addition, the brain is one of the organs most susceptible to oxidative damage. The reason for this is that the ability to resist oxidative stress is limited together with the large oxidative capacity brought about by consuming 20% of all the oxygen used in the body [28].

In our study, administration of a single dose of 20 mg/kg MTX reasoned a meaningful rise in TBARS levels and a meaningful reduce in SOD, GPx and CAT activities in rat brain tissue compared to the control group. GSH levels also decreased, but without statistical significance. The conclusions of our work are in accordance with the results of prior works that revealed oxidative damage caused by the effect of MTX in both brain tissue and various other tissues. Pınar et al., showed that a single dose of 20 mg/kg MTX administration significantly increased MDA levels in rat liver and kidney tissues [26] and rat testis tissue [23] and significantly decreased SOD, CAT and GPx activities. In the study of Fikry et al. [29], it was reported that oral MTX administration of 14 mg/kg 2 times a week caused a significant increase in rat heart tissue MDA level and a significant decrease in GSH level and CAT activity. In the study of Vardi et al. [11], administration of a single dose of 20 mg/kg MTX significantly increased MDA level in rat cerebellum tissue and significantly decreased GSH level and SOD and CAT activities. In the study of Kushwaha et al. [30], oral MTX intake of 2 mg per day for 28 days significantly increased MDA levels and decreased GSH levels in mouse brain tissues.

In many past studies, the potential beneficial effects of various substances such as chlorogenic acid [11], caffeic acid [27], alpha-lipoic acid [23], tempol [26] and gallic acid [31] have been investigated in order to prevent the side effects associated with MTX use. However, there are very few studies investigating the effects of FO against MTX-induced damage. Vanderhoof et al. [32], demonstrated that FO ameliorates MTX-induced mucosal damage. Horie et al. [33], reported that DHA strongly in-

hibited small intestine damage caused by oral MTX administration in mice. Nadhanan et al. [34], demonstrated that FO significantly prevented the side effects of MTX on bones. However, in the literature review conducted by us, it was seen that there was only one study evaluating the effects of FO against damage caused by MTX in terms of oxidative parameters. In this study, Elbarbary et al [12]. showed that 1000 mg FO given in addition to MTX used in the treatment of acute lymphoblastic leukemia patients provided a significant improvement in SOD and GPx activities, serum total antioxidant capacity and MDA levels.

There is no study has been encountered in the literature investigating the effects of FO against MTX-induced brain damage. Our study is the first research conducted in this context. In our study, administration of 1000 mg oral FO caused a meaningful reduce in TBARS levels and a meaningful rise in SOD and GPx activities compared to the MTX group. The increase in CAT level was not statistically significant. Our results are in agreement with the results of a limited number of similar previous studies.

This study, the effects of MTX and FO on the brain were also evaluated histopathologically. Several previous studies have shown that MTX administration causes the Purkinje cells to lose their shape and shrink and the density of Nissl bodies to decrease [11], suppression of cell proliferation in the dorsal hippocampus [35] and the formation of abundant pycnotic neuroepithelial cells along the telencephalic wall in fetal brain tissue [4]. In our study, it was observed that a single intraperitoneal administration of MTX at a dose of 20 mg/kg on the 3rd day of the study did not cause a histopathologically significant lesion in the brain. This can be explained by the fact that the level of MTX-mediated lipid peroxidation is such that it does not cause morphologically significant cell damage in the brain tissue. As a matter of fact, two scenarios can be mentioned for the decrease of antioxidant enzymes in the case of oxidative stress. The first is defense-related enzyme depletion, in which the body may become unable to produce any more enzymes against damage. The other scenario is cyclic enzyme oscillations that occur when oxidative attack and defense are at equal levels. In the first stage of the attack on lipids, cells produce large amounts of antioxidant enzymes, and enzyme levels appear to increase at this stage. However, when enzymes capture these radicals, enzyme production may stop for a while due to the cyclic state [36]. The increase in TBARS biochemically detected in our study and the decrease in antioxidant enzyme levels indicate an active defense state in the brain that includes the aforementioned mechanisms. Thus, a significant histopathological damage occurrence in the defensive brain may have been prevented. However, depending on the application of higher doses of MTX, different administration routes and duration, it is possible that a damage may occur not only at the biochemical level, but also at the morphological level.

Conclusion

In conclusion, this research demonstrated that MTX causes oxidative damage in brain tissue and FO has the potential to attenuate this damage. Therefore, we suggest that FO may be beneficial in alleviating the neurological

oxidative damage that may occur in people receiving MTX therapy.

References

- Hirako A, Furukawa S, Takeuchi T, Sugiyama A. Effect of methotrexate exposure at late gestation on development of telencephalon in rat fetal brain. *J Vet Med Sci.* 2016;78(2):213–20.
- Howard SC, McCormick J, Pui C, et al. Preventing and managing toxicities of high-dose methotrexate. *Oncologist* 2016;21(12):1471–82.
- Hafez HM, Ibrahim MA, Ibrahim SA, et al. Potential protective effect of etanercept and aminoguanidine in methotrexate-induced hepatotoxicity and nephrotoxicity in rats. *Eur J Pharmacol* 2015;768:1–12.
- Sun J, Sugiyama A, Inoue S, et al. Effect of methotrexate on neuroepithelium in the rat fetal brain. *J Vet Med Sci* 2014;76(3):347–54.
- Campbell JM, Bateman E, Peters MDJ, et al. Fluoropyrimidine and platinum toxicity pharmacogenetics: An umbrella review of systematic reviews and meta-analyses. *Pharmacogenomics* 2016;17(4):435–51.
- Bernsen EC, Hagleitner MM, Kouwenberg TW, et al. Pharmacogenomics as a tool to limit acute and long-term adverse effects of chemotherapeutics: An update in pediatric oncology. *Front Pharmacol* 2020;11:1–19.
- Şener G, Ekşiöğlü-Demiralp E, Çetiner M, et al. β -glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immunomodulatory effects. *Eur J Pharmacol* 2006;170–8.
- Leite RA, Vosgrau JS, Cortez Neto L, et al. Brainstem auditory pathway of children with acute lymphoid leukemia on chemotherapy with methotrexate. *Arq Neuropsiquiatr* 2020;78(2):63–9.
- Rollins N, Winick N, Bash R, et al. Acute methotrexate neurotoxicity: Findings on diffusion-weighted imaging and correlation with clinical outcome. *Am J Neuroradiol* 2004;25(10):1688–95.
- Yang M, Kim JS, Kim J, et al. Acute treatment with methotrexate induces hippocampal dysfunction in a mouse model of breast cancer. *Brain Res Bull* 2012;89(1–2):50–6.
- Vardi N, Parlakpinar H, Ates B. Beneficial effects of chlorogenic acid on methotrexate-induced cerebellar Purkinje cell damage in rats. *J Chem Neuroanat* 2012;43(1):43–7.
- Elbarbary NS, Ismail EAR, Farahat RK, et al. ω -3 fatty acids as an adjuvant therapy ameliorates methotrexate-induced hepatotoxicity in children and adolescents with acute lymphoblastic leukemia: A randomized placebo-controlled study. *Nutrition* 2016;32(1):41–7.
- Koppelman T, Pollak Y, Mogilner J, et al. Reversal of severe methotrexate-induced intestinal damage using enteral n-3 fatty acids. *Br J Nutr* 2013;109(1):89–98.
- Kamisli S, Ciftci O, Cetin A, et al. Fish oil protects the peripheral and central nervous systems against cisplatin-induced neurotoxicity. *Nutr Neurosci* 2014;17(3):116–26.
- Yagi K. Simple assay for the level of total lipid peroxides in serum or plasma. *Methods Mol Biol* 1998;108:101–6.
- Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria J Med* 2018;54(4):287–93.
- Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. *Clin Chem* 1988;34(3):497–500.
- Aebi H. Catalase. *Methods Enzym Anal* 1974;673–84.
- Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967;70(1):158–69.
- Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* 1968;25(C):192–205.
- Lowry OH, Rosebrough NJ, Farr AL, et al. The folin by oliver. *Readings* 1951;193(1):265–75.
- Wang W, Zhou H, Liu L. Side effects of methotrexate therapy for rheumatoid arthritis: A systematic review. *Eur J Med Chem* 2018;158:502–16.
- Pınar N, Çakırca G, Özgür T, et al. The protective effects of alpha lipoic acid on methotrexate induced testis injury in rats. *Biomed Pharmacother.* 2018;97:1486–92.

24. Abdel-Daim MM, Khalifa HA, Abushouk AI, et al. Diosmin attenuates methotrexate-induced hepatic, renal, and cardiac injury: A biochemical and histopathological study in mice. *Oxid Med Cell Longev*. 2017;2017.
25. Bhojwani D, Sabin ND, Pei D, et al. Methotrexate-induced neurotoxicity and leukoencephalopathy in childhood acute lymphoblastic leukemia. *J Clin Oncol* 2014;32(9):949-59.
26. Pinar N, Kaplan M, Özgür T, et al. Ameliorating effects of tempol on methotrexate-induced liver injury in rats. *Biomed Pharmacother* 2018;102:758-64.
27. Uzar E, Koyuncuoglu HR, Uz E, et al. The activities of antioxidant enzymes and the level of malondialdehyde in cerebellum of rats subjected to methotrexate: Protective effect of caffeic acid phenethyl ester. *Mol Cell Biochem* 2006;291(1-2):63-8.
28. Kumar GP, Khanum F. Neuroprotective potential of phytochemicals. *Pharmacognosy Reviews* 2012;6:81-90.
29. Fikry EM, Hassan WA, Gad AM. Bone marrow and adipose mesenchymal stem cells attenuate cardiac fibrosis induced by methotrexate in rats. *J Biochem Mol Toxicol* 2017;31:11.
30. Kushwaha S, Tripathi DN, Vikram A, et al. Evaluation of multi-organ DNA damage by comet assay from 28 days repeated dose oral toxicity test in mice: A practical approach for test integration in regulatory toxicity testing. *Regul Toxicol Pharmacol* 2010;58(1):145-54.
31. Asci H, Ozmen O, Ellidag HY, et al. The impact of gallic acid on the methotrexate-induced kidney damage in rats. *J Food Drug Anal* 2017;25(4):890-7.
32. Vanderhoof JA, Blackwood DJ, Mohammadpour H, et al. Effect of dietary menhaden oil on normal growth and development and on ameliorating mucosal injury in rats. *Am J Clin Nutr* 1991;54(2):346-50.
33. Horie T, Nakamaru M, Masubuchi Y. Docosahexaenoic acid exhibits a potent protection of small intestine from methotrexate-induced damage in mice. *Life Sci* 1998;62(15):1333-8.
34. Raghu Nadhanan R, Fan CM, Su YW, et al. Fish oil in comparison to folic acid for protection against adverse effects of methotrexate chemotherapy on bone. *J Orthop Res* 2014;32(4):587-96.
35. Elens I, Dekeyster E, Moons L, et al. Methotrexate affects cerebrospinal fluid folate and tau levels and induces late cognitive deficits in mice. *Neuroscience* 2019;404:62-70.
36. Porokhovnik LN, Passekov VP, Gorbachevskaya NL, et al. Active ribosomal genes, translational homeostasis and oxidative stress in the pathogenesis of schizophrenia and autism. *Psychiatr Genet* 2015;25(2):79-87.