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Research Article
Potential Therapeutic Effect of Vitamin C on Methotrexate-Induced Damage in the Cerebral Cortex

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Abstract

Background and Objective: Methotrexate is an anti-metabolic medication used to treat cancer. It causes oxidative stress in nerve tissue and has neurotoxic effects. A strong antioxidant and effective free radical scavenger is vitamin C. The current research aims to investigate the potential protective impact of vitamin C and the toxic consequences of methotrexate. **Material and Methods:** Thirty-six rats were used in this research and group one (Group 1) got no treatment at all. For 4 weeks, (Group 2) underwent a single intraperitoneal injection of methotrexate at a dose of 20 mg/kg once a week and (Group 3) got methotrexate at the same dosage as Group 2 and vitamin C (20 mg/kg) intragastrically every other day for four weeks. Rats were killed after the experiment and brain hemispheres were removed and prepared for light microscopic analysis. The cerebral hemispheres were ready for biochemical analysis to determine the brain tissue's concentrations of MDA, CAT, GSH and SOD. Data analysis was conducted using SPSS software version 20. **Results:** In the methotrexate (2)-treated group, there were histological alterations manifested as a reduction in granular layer thickness. Purkinje cells exhibit a reduction in number, a shrinking of the cell bodies and a loss of monolaminar organization. Reduced cellularity was seen in the molecular layer. These cellular alterations are lessened and the thickness of the granular and molecular cell layers is restored following vitamin C treatment. When compared to the MTX+Vitamin C group, vitamin C greatly attenuates the biochemical and histological alterations caused by MTX. **Conclusion:** Results concluded that although methotrexate is a toxic medication that damages the brain cortex, its toxicity is reduced when vitamin C is taken with it.

Key words: Methotrexate, vitamin C, antioxidant, cerebral cortex, neurotoxicity, oxidative stress

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

One of the B-complex vitamins, folic acid functions as a coenzyme in the transport of single-carbon units and the metabolism of amino acids and nucleic acids. As a folic acid antagonist, methotrexate (MTX) prevents the synthesis of purines and pyrimidines from scratch, thereby limiting the growth of cells[1]. Protein synthesis is stopped and dihydrofolate is not converted to tetrahydrofolate by MTX, an enzyme that binds to dihydrofolate reductase with considerable affinity and inhibits this enzyme. Many autoimmune and inflammatory diseases, including inflammatory bowel disease and rheumatoid arthritis, as well as some gynecological conditions, including gestational trophoblastic diseases and ectopic pregnancy are treated with MTX on a broad basis[2]. However, MTX's numerous harmful side effects restrict its practical application[3]. Serious side effects from MTX treatment have been reported in a variety of organs and tissues, most notably the liver, kidneys, gastrointestinal tract and nervous system tissues[4]. Numerous factors, including dosage, length of treatment, illness kind and molecular and genetic apoptotic factors, interact to cause methotrexate-related toxicity[5]. The MTX treatment frequently results in neurotoxicity, which can manifest in acute, subacute and late forms following different modes of administration. Both intravenous and intrathecal injections of high-dose MTX have been linked to demyelination, loss of oligodendroglia, white matter necrosis, axonal swelling and deep cerebral white matter atrophy[6]. Acute MTX neurotoxicity is characterized by neurological symptoms such as aphasia, paralysis, sensory impairments, ataxia and seizures. Depending on leucovorin use, dosage, delivery method and frequency of administration, the incidence of acute MTX neurotoxicity ranges from 3 to 10%[6].

Due to several factors, including high oxygen consumption, lipids in neural membranes that are rich in polyunsaturated fatty acids and a modest antioxidant defense mechanism, the central nervous system is particularly vulnerable to ROS attacks. Because of this, circumstances like the use of MTX, which raises levels of free radical production and antioxidant defense, result in oxidative stress-mediated membrane disruption and cellular malfunction[7].

Ascorbate or vitamin C, is an antioxidant in the aqueous phase found in biological fluids. Tissue damage and oxidative stress are brought on by vitamin C deficiency. Additionally, it offers a significant antioxidant defense against the damage caused by hypochlorous acid in atherosclerosis[8]. Antioxidant that binds to free radicals and shields biological macromolecules like proteins, lipids and DNA from damage. Hypochlorous acid, hydroxyl and reactive oxygen species are all easily scavenged by vitamin C. It is well known to be a significant electron source that can provide electrons to radicals or oxidants[9]. Known for its ability to scavenge free radicals and other reactive oxygen, vitamin C is a water-soluble antioxidant created during normal metabolism, by active immune cells and through exposure to toxins. Additionally, because of their free radical activity, it prevents the production of cytotoxic Low-Density Lipoprotein (LDL) when exposed to reactive oxygen species. *In vitro*, vitamin C inhibits the expression of pro-inflammatory cytokines in adult whole blood cells, including Interleukin (IL)-6 and tumor necrosis factor alpha[10,11]. The study aimed to investigate if vitamin C could protect adult albino rats' cerebral cortex histology from the harmful effects of methotrexate.

MATERIALS AND METHODS

Study area: This work was conducted in an animal house at, College of Pharmacy, Prince Sattam Bin Abdulaziz University's Al-Kharj. The study was carried out from April, 2023 to September, 2024.

Study design: About 36 mature male albino rats weighing between 200 and 220 g were bought from PSA University's Pharmacy Faculty. All of the rats were housed at 24°C with 60% humidity. They were fed regular food and given free access to water. The rats were housed for one week before the start of the investigation.

Ethical consideration: The experiment was approved on 17 October, 2023, by the PSA University Ethical Committee (SCBR-168/2023).

Methods: Methotrexate is offered as an injectable with 25 mg/mL in a 2 mL vial. The source of it was Mylan Pharmaceutical Company. After being diluted in 3 mL of saline, 1 mL of MTX contains 25 mg of MTX. Vitamin C was purchased from Hikma Pharmaceuticals Company and was supplied as 500 mg capsules of C Retard Ascorbic Acid. Each capsule contained 50 mg of vitamin C and 10 mL of distilled water were used to dissolve it. For 10 days, all the rats were divided equally into three groups, each consisting of twelve rats. The rats in the control group were not given any medication.

MTX group: On the fifth day of the trial, rats received a single intraperitoneal injection of MTX (20 mg/kg b.wt.)[12]. The MTX+Vitamin C group: Rats received MTX single intraperitoneal injection on day five, the same as Group 2, 1 hr after receiving 250 mg/kg b.wt., of vitamin C via oro-gastric tube from the first to the tenth day[13]. By the tenth day's closing, intraperitoneal injections of ketamine hydrochloride had rendered all of the rat's unconscious. After the cerebrum was extracted from the skull, the blood stain was carefully removed by rinsing it in cold saline water. Biochemical indicators were analyzed on a single specimen.

To facilitate histological examinations, the remaining portion was placed in 10% formalin. In the Scientific Research Centre, Faculty of Pharmacy, PSA University, tissue specimens were frozen in an ultracold potassium phosphate buffer and minced at 20°C in preparation for biochemical analysis.

The combination was centrifuged for 30 min and the resulting supernatant was used to measure the level of malondialdehyde (MDA). Analysis were conducted to determine the tissue antioxidant enzyme activity of GSH (reduced glutathione), CAT (catalase) and SOD (superoxide dismutase). The brain hemisphere samples were placed in a 10% formalin solution and handled for light microscopic examination, which included alcohol dehydration, xylene clearing, paraffin fixation and sectioning at 4-5 μm thickness for subsequent histopathological and immunohistochemical analysis. Hematoxylin and Eosin (H&E) staining for standard histological examination. Following that, several sections underwent polysaccharide detection staining. The Nissl granules were identified using a toluidine blue stain.

Statistical analysis: By analyzing the collected data with (SPSS) version 20 (Chicago, SPSS Inc., Illinois, USA). When the $p<0.05$, it was considered non-significant; if the $p>0.05$, it was considered very significant. Each data point was presented as mean SD.

RESULTS

The MDA readings in the MTX-treated group were significantly higher than those in the control group. In the third group, the administration of vitamin C along with MTX dramatically lowers the MDA level. Conversely, the second group's SOD, CAT and GSH levels were considerably lower than those of the control group. The SOD, CAT and GSH levels in the third group were substantially greater than those in the second group, although they were still significantly different from those in the control group (Table 1).

To highlight the results, the histopathological changes in the cerebral cortex tissue after exposure to MTX were examined. The control cerebrums' parasagittal slices stained with Hematoxylin and Eosin (H&E) revealed almost identical normal neuronal arrangement, cortical morphology and intact pia matter attachment. Six layers-three outer and three inner cortical layers-make up the cerebral cortex. There were tiny cortical neuronal cells seen in the outer cortex. Large cortical neurons with vesicular nuclei and a border of basophilic cytoplasm were visible in the inner cortex. These cortical neurons exhibited intact blood arteries with tiny perivascular gaps, encased in a normal homogeneous acidophilic neuropil. The nuclei of neuroglial cells were clearly defined and either bright or darkly pigmented. A dense network of glial cell processes, axons and dendrites that covers most of the voids created by the cell bodies in CNS tissue is known as the neuropil. The nuclei seen in the cell bodies of neurons are typically big, spherical, euchromatic and visible. Every neuron is closely connected to a network of glial cells, which are much smaller and more abundant than neurons and provide various forms of support for the neurons (Fig. 1, 2a-b).

Examining sections of a normal brain stained with toluidine blue stain revealed darkly stained cytoplasm including Nissl's granules and vesicular nuclei in normal neural cell bodies; normal neurons also had darkly stained cell membranes and normal darkly stained glial cells (Fig. 2c-d).

Comparing the second MTX-treated group to the control group, stained with Hematoxylin and Eosin (H&E) pathological alterations were noted, including vacuolated and condensed neurons with narrow diameters, expanding neuronal cell bodies, deeply stained pyknotic nuclei of glial cells and a sponge-like vacuolated neuropil of the cerebral cortex. Examining slices of the MTX-treated group stained with toluidine blue revealed a decrease in the staining of toluidine blue in the cytoplasm of neurons, glial cells and neural cell bodies (Fig. 3a-d).

Table 1: Biochemical analysis of groups under study

Parameter	Control group	MTX group	MTX+Vitamin C group
SOD (μ/g)	4.47±0.47	2.11±0.41	2.62±0.27
MDA (nmol/g)	12.29±1.30	29.33±1.87	19.08±2.81
GSH (nmol/g)	2.52±0.28	1.03±0.15	1.83±0.19
CAT (μ/g)	1.87±0.21	0.72±0.15	0.99±0.12

SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione, CAT: Catalase and ±: Mean±Standard Deviation (SD), showing the average value and the variability or dispersion of data around the mean

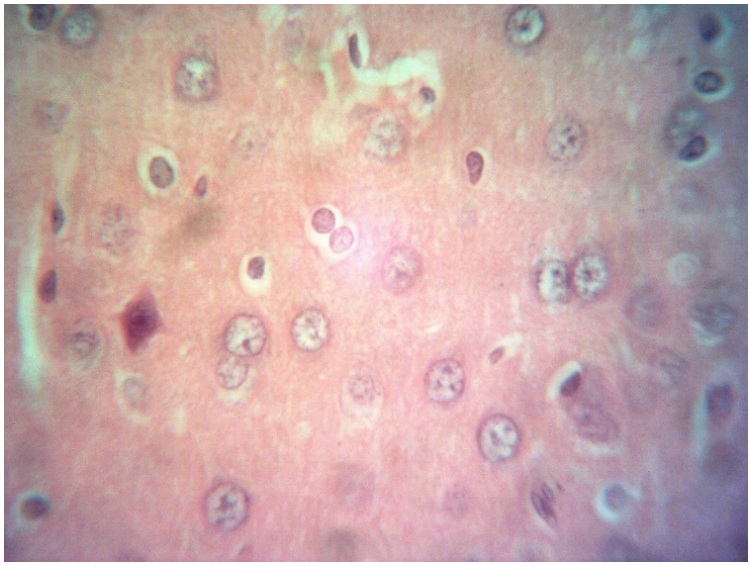

Fig.1: Control group's brain's Hematoxylin and Eosin (H&E) images display typical glial and neuronal cells in addition to a normal dense network of background
×1000

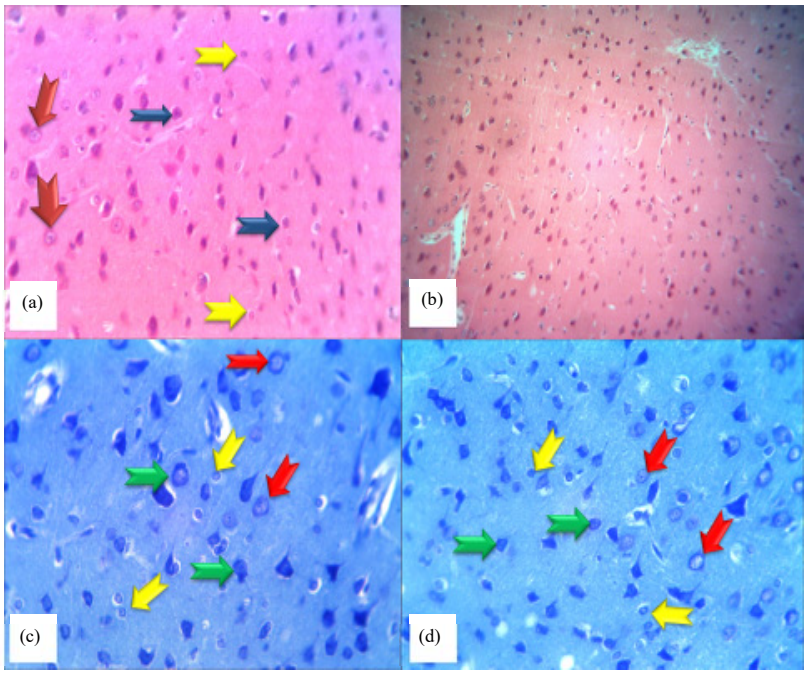

Fig. 2(a-d): Different images of the brain of the control group (a) Brain H&E from the control group displayed normal neurons (red arrows), glial cells (yellow arrows) and neuronal cell bodies (blue arrows), (b) Lower magnification H&E of the control group's brain demonstrating healthy neurons, glial cells and neuronal cell bodies, (c) Toluidine blue stain demonstrates the darkly stained glial cells (yellow arrows), normal neurons with darkly stained cell membranes (red arrows) and normal neuronal cell bodies (green arrows) and (d) Toluidine blue stain demonstrates the darkly stained glial cells (yellow arrows), normal neurons with darkly stained cell membranes (red arrows) and normal neuronal cell bodies (green arrows)
(a) ×400, (b) ×200, (c) ×400 and (d) ×400

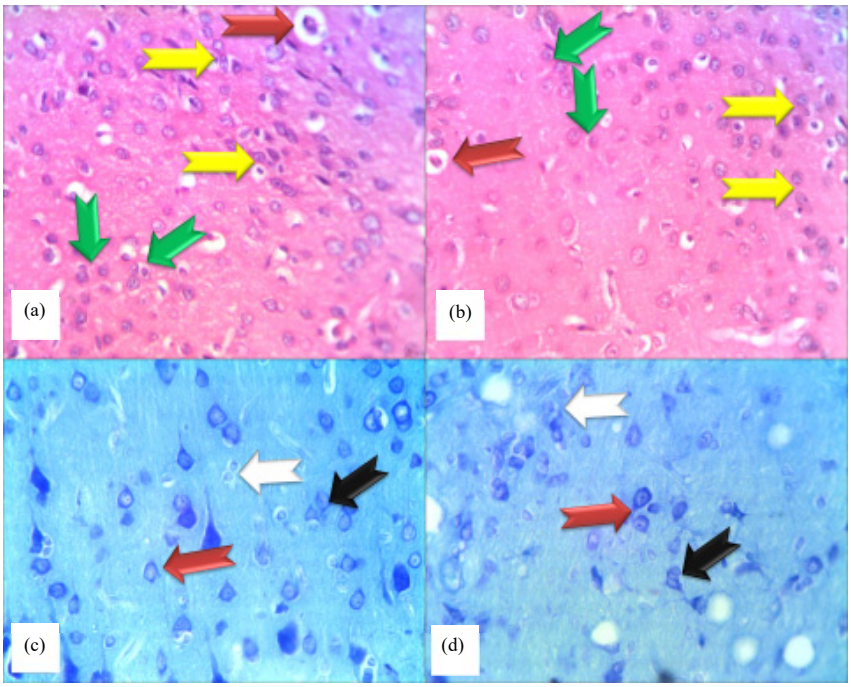

Fig. 3(a-d): Different images of the brain of MXT group, (a) An illustration of the brain after MXT treatment, stained with H&E, displaying highly stained pyknotic nuclei of glial cells (green arrows), condensed neurons with narrow diameters (yellow arrows) and vacuolated and expanding neuronal cell bodies (red arrow), (b) H&E stain on brain rats given MXT treatment reveals vacuolated and growing neuronal cell bodies (red arrow). Green arrows indicate the barely stained cytoplasm of neural cell bodies; yellow arrows indicate the weakly stained neurons and glial cells, (c) Comparing the MXT group to the control group, a photomicrograph of the cerebral cortex showed neuronal cell bodies with weakly stained neurons (black arrow), faintly stained glial cells (white arrow) and slightly stained cytoplasm (red arrow) and (d) Comparing the MXT group to the control group, a photomicrograph of the cerebral cortex showed neuronal cell bodies with weakly stained neurons (black arrow), faintly stained glial cells (white arrow) and slightly stained cytoplasm (red arrow)
×400

The third group (MTX+Vitamin C) stained with Hematoxylin and Eosin (H&E) showed considerable degree of protection after AFA treatment, as no cellular pathological changes were detected (Fig. 4a-b). Additionally when the third group stained with toluidine blue it exhibited a marked increase in intensity of toluidine blue stain in comparison to the second group (Fig. 4c-d).

DISCUSSION

In this study, the biochemical examination of the group treated with MXT revealed a significant elevation in MDA level compared to the control group. When compared to the control group, the antioxidant enzyme activity levels of SOD, CAT and GSH were considerably lower in the MTX-treated group. This suggests that MTX plays a role in oxidative stress induction and the impairment of biochemical analysis in brain tissue. On the other hand, MTX caused damage to the typical histological features of brain tissue in our study.

A popular chemotherapy drug; MTX has detrimental effects on several organs, including the central nervous system[14]. When the body produces more oxidants than it can use antioxidants to protect against them, oxidative stress results. This imbalance may cause lipids, proteins and DNA in cells to be damaged by oxidation. Experimental studies have demonstrated that oxidative stress is the main factor causing organ damage. The MTX has the potential to enhance oxidative stress in the brain and other tissues. Because white matter has a high concentration of polyunsaturated fatty acids and low quantities of antioxidants, it is especially vulnerable to oxidative damage[15].

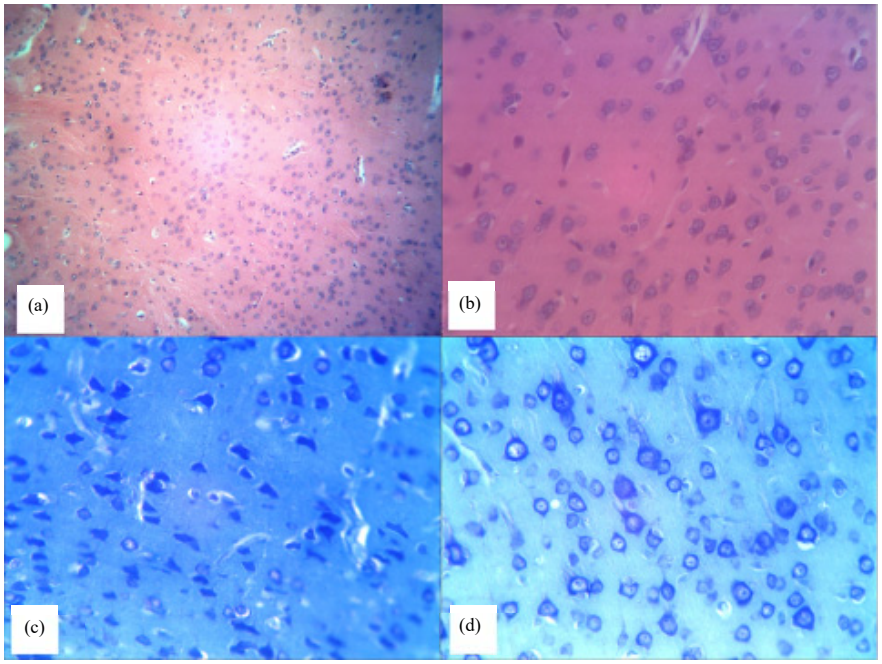

Fig. 4(a-d): Different images of the brain of MXT and vitamin C group, (a) H&E (×200), (b) H&E (×400) and (c-d) Toluidine blue stain (×400)

This causes MTX-induced lipid peroxidation to grow, which in turn causes the cerebral cortex's membrane permeability, neurotransmitter-receptor interaction and cell death. Research has demonstrated that in conditions of oxidative stress, the rat cerebral cortex's antioxidant activity can be reduced by ROS produced by MTX[16]. Ahmed *et al.*[17] and Sritawan *et al.*[18] have shown that the treatment of MTX causes the brain's levels of antioxidant enzymes to decrease and MDA levels to rise. The results of this study showed that rats given MTX had higher levels of oxidative stress, as shown by lower SOD, CAT and GSH levels. Another study of Behairy *et al.*[19] that looked into whether giving spirulina platensis (SP) and/or thymoquinone (TQ) could lessen the neurotoxic effects of methotrexate (MTX) produced similar results. The potential advantages of several compounds, including tempol, gallic acid, alpha-lipoic acid, caffeic acid and chlorogenic acid, have been examined in numerous previous studies to reduce the negative effects of MTX use[20]. By impairing mitochondrial processes and releasing an excessive amount of reactive oxygen species, MTX causes neurotoxicity and may cause cell death. Oral MTX delivery in the form of a single high dosage or a protracted low dose may cause MTX neurotoxicity[21].

Vitamin C has been linked to the maturation and growth of glial and neuronal cells. It can lessen oxidative damage by lowering lipid peroxidation and neuronal loss[22]. When compared to the MTX group, vitamin C administration in this study significantly reduced the MDA level and significantly increased the levels of antioxidant enzymes, SOD and GSH. These findings were corroborated by other authors who claimed that vitamin C administration improved the antioxidant system and reduced the oxidative stress brought on by MTX[23]. This was clarified by demonstrating how vitamin C could enhance the antitumor effects of low doses of methotrexate. Vitamin C reduces cellular damage by interacting with superoxide and hydroxyl radicals.

CONCLUSION

The study's findings demonstrated MTX's adverse effects on the cerebral cortex. The cerebral cortex is adversely affected by methotrexate. Vitamin C lessens the negative effects of MTX. According to these findings, vitamin C supplementation avoided neurotoxicity and may improve the anti-cancer, rheumatoid arthritis and psoriasis medication's selectivity in patients who needed MTX for therapy. Based on the findings of this work, it is recommended that more research be done to see whether vitamin C added to MXT can be utilized as an adjuvant treatment for neurological impairments that damage the brain.

SIGNIFICANCE STATEMENT

By generating oxygen reactive species and lowering the levels of antioxidant enzymes, methotrexate damages neurological tissue and crosses the blood-brain barrier. This oxidative stress results in apoptosis, tissue destruction and neurotoxicity. Vitamin C is present in nervous tissue at a higher level than other organs. It has an important role in the appropriate functions of the nervous system and it participates in the antioxidant defense of the brain. It plays an essential role in neuronal maturation and neurotransmission. Therefore, this work was planned to assess the role of vitamin C in ameliorating the harmful impacts of methotrexate on the cerebral cortex of adult male albino rats. Results suggested that vitamin C may be employed as a possible pharmacological treatment for cancer patients exposed to MTX based on the findings of the current study.

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