Research Article

NEURO PROTECTIVE EFFECTS OF WHEAT GERM OIL ON THE BRAIN INFLAMMATION INDUCED BY METHOTREXATE IN MICE

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ABSTRACT

The aim of this study is to evaluate the neurotoxicity of methotrexate (MTX) in mice, as well as the neuroprotective effects of wheat germ oil (WGO). One group of mice received corn oil orally for 10 days and a single dose of methotrexate (20 mg/kg) intraperitoneally on the 7th day. Another group was given both wheat germ oil orally for 10 days and methotrexate single dose (20 mg/kg) intraperitoneally on the 7th day. Our results suggest that the administration of MTX produced neurochemical and neurotoxic alterations in the brain causing severe damage including peripheral neuropathy and encephalopathy. It also led to brain tissue-damage by reactive oxygen species (ROS) generation, caused alteration in mitochondrial permeability and tissue injury as a response to the oxidative stress. WGO has neuroprotective action through scavenging the oxygen free radicals, decreasing lipid peroxidation and ameliorating the histopathological changes induced by MTX. WGO is recommended to patients undergoing MTX treatment.

Keywords: Methotrexate, Wheat germ oil, Neurotoxicity, Antioxidant effect, Mice

INTRODUCTION

Methotrexate (MTX) is a drug commonly used for the treatment of many forms of cancer by the way of inhibiting cancer cells from multiplying¹. It was introduced into malignant disease treatment more than 50 years ago² however, its therapeutic effect is restricted by its low Blood brain barrier (BBB) permeability and poor brain targeting³. It is considered as a basic chemotherapy drug for the treatment of brain tumors, especially leptomeningeal metastatic cancer and primary central nervous system lymphoma (PCNSL)⁴. MTX is also used to reduce inflammation in both psoriasis and rheumatoid arthritis¹. It can enter the cells via two mechanisms active transport and passive diffusion, but active transport is the most dominant way⁵, by the reduced folate carrier⁶. It inhibits dihydrofolate reductase enzyme that reduces dihydrofolate to tetrahydrofolate (the active form of folate). Tetrahydrofolate is critical for many metabolic pathways, e.g. DNA/RNA synthesis, amino acid synthesis, and DNA/protein methylation⁷. MTX also inhibits aminoimidazole carboxamide ribonucleotide transferase and thymidine synthase enzymes in folate metabolism^{6,8-11}. Down-regulation of these enzymes impairs neurogenesis including cell proliferation, survival, and differentiation^{6,12,13}. MTX is often associated with neurotoxicity and severe morbidity resulting from neurological problems that damage the brain and spinal cord causing impairment of higher cognitive functions, seizures, cerebraledema, leuko encephalopathy and secondary malignancies¹⁴⁻¹⁸. Shao *et al.* also demonstrated the severe cytotoxicity on astrocytes after treatment with MTX¹⁹.

Several studies suggest that ROS are implicated in the etiology of several diseases such as inflammatory diseases, diabetes, ischemia-reperfusion injury²⁰, neurodegenerative disorders,

cancer, aging and cardiovascular diseases,²¹⁻²³. The brain is more prone to oxidative stress (OS) compared to other organs of the body due to its high rate of oxygen consumption^{24, 25}. MTX induces oxidative stress by inducing lipid peroxidation and altering the antioxidant defenses in the nervous system²⁶.

Moreover,²⁷ implicated that MTX increases the protein carbonyl level, increases the activity of GPx, SOD, catalase as well as the lipid peroxidation while decreasing the protein thiol and GSH levels in all the regions of the brain, which indicates free radical formation due to MTX treatment.

It should be noted, that some drugs including MTX may cause severe side effects, as seen above, therefore substances that are naturally present in the human diet should be considered as therapeutic agents with strong impact.

Wheat germ oil (WGO) is one of these natural substances extracted from the germ of the wheat kernel. This oil is a rich source of vitamins such as vitamin E (tocopherols), A, D and B6, in addition to many fatty acids including linoleic acid and α -linolenic acid²⁸. These fatty acids ameliorate brain development and cognitive functions in normal and diseased conditions²⁹ increase endurance, lower cholesterol levels, and ameliorate muscular dystrophies and other neuromuscular disorders³⁰. The vitamin E content of WGO is a potent antioxidant agent that has been nominated to protect the brain from oxidative stress by directly scavenging toxic radicals³¹, ameliorating the brain impairment process³² and protecting DNA and cell membranes against oxidative damage. Moreover, WGO contains β -carotenes, which also have an antioxidant effect^{33,34} declared that WGO not only prevents the autoxidation of unsaturated fatty acids but also leads to the generation of DNA protective properties.

The main objective of our study is to declare the role of WGO as a natural therapeutic supplement in ameliorating the neurotoxic effects of MTX administration.

MATERIALS AND METHODS

Experimental Materials

Methotrexate (MYLAN- France) (50 mg/2 mL) ampoule for injection. Wheat germ oil (99 % purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Kits for biochemical analysis were bought from the Bio-diagnostic Company (Giza, Egypt).

Experimental Animals

Male C57BL/6 mice, 5–6 weeks age and 22–25 grams in weight were purchased from the National Research Center (NRC, Dokki, Giza). Mice were randomly assigned to polyacrylic cages (eight mice per cage) and acclimatized for seven days at a temperature of 22°C– 25°C and humidity-controlled conditions in 12/12 h light/dark cycle and access *ad libitum* to fresh tap water and rodent food. The experimental protocols and animal procedures were approved by the Cairo University, Faculty of Science, Institutional Animal Care and Use Committee (IACUC) (Egypt) (CUFS/F/21/15). All the experimental procedures were performed according to the international guidelines Guide for Care and Use of Laboratory Animals.

Experimental Design

A total of 32 mice were divided into 4 groups as follows: control group, MTX group, WGO group and WGO+MTX group

The control group: mice received oral administration of corn oil (0.5 mg/ kg) for 10 days and a single dose of saline intra peritoneal on day seven of the experiment.

The WGO group: mice received oral administration of wheat germ oil WGO (1400 mg/kg) for 10 days. The dose and mode of injection of WGO in our work were managed according to³⁵.

The MTX group: mice received oral administration of corn oil (0.5 mg/ kg) for 10 days and a single dose of methotrexate MTX (20 mg/kg) intraperitoneally on the 7th day of the experiment. The dose and mode of injection of MTX in the present study were managed according to^{36,37}.

The WGO + MTX group: mice received oral administration of WGO for 10 days and a single dose of MTX (20 mg/kg) intraperitoneally on the 7th day of the experiment.

Preparation of Specimens

At the end of the experimental period, mice were sacrificed by decapitation, brain was collected from all experimental groups and stored at -80°C, small slices of brain tissues were homogenized in ice-cold methanol 70% then the homogenate was centrifuged at 3000 r.p.m at 4°C for 15 min and the supernatant was subjected to biochemical analyses.

Histopathological Investigations

Other portions of the brain collected from all experimental groups were fixed in 10% buffered formal saline. The specimens were

washed, dehydrated by alcohol, cleared using xylene, embedded in paraffin and sectioned at five μ m thickness³⁸. These sections were stained with hematoxylin and eosin and examined with a light microscope for histopathological examination.

Biochemical Analysis Assayed

Brain tissues were taken at the end of the experiment to determine cell energy content of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP). They were evaluated by high-performance liquid chromatography HPLC according to³⁹ and the results were expressed as μ g/g tissue. The antioxidant levels of malondialdehyde (MDA), which is a marker of oxidative stress and indicator for lipid peroxidation was estimated by HPLC according to⁴⁰ and the results were expressed in nmol/g tissue. Reduced glutathione (GSH) and oxidized glutathione (GSSG) content were determined by HPLC, according to⁴¹ with the results expressed in μ mol/g tissue. The level of nitric oxide (NO) was determined by HPLC, as described by⁴² and the results were expressed in μ mol/g tissue. The level of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was determined by HPLC, according to⁴³ and the results were expressed as pg/g tissue and determination of monoamines as nor adrenaline (NE), dopamine (DA) and serotonin (5-HT) were evaluated in brain tissue, according to⁴⁴ and the results were expressed in μ g/g tissue.

Data and Statistical Analysis

Data analysis was accomplished using Graph Pad software (Version 5.01, San Diego, USA). The data were expressed as means $\pm$ SEM standard error of eight mice in each group. Unpaired Student's t-test was used to compare the treated group with the control and the MTX groups. Differences were considered significant at *P < 0.05, **P < 0.01 as compared to the control group, #P < 0.05; ##P < 0.01 as compared to the MTX group.

RESULTS

Histological and Histopathological Investigation of Cerebrum

Microscopic examination of the cerebral cortex of the control group and the wheat germ oil group (WGO 1400 mg/kg) were similar and showed no histological changes. They revealed normal architecture of neurons with distinct cell borders and large dense nuclei in addition to neuroglial cells and the neuropil (dense feltwork of cytoplasmic processes of nerve cells and neuroglial cells) (Figures 1 and 2).

On the other hand, the group that received a single dose of methotrexate (MTX 20 mg/kg) showed cerebral cortical infarction with edema, neuropil vacuolization, neurons degeneration with dark pyknotic nuclei, shrunken cytoplasm and blood vessels congestion (Figures 3 and 4). Examination of specimens attained from mice treated with WGO for 10 days concomitantly with a single dose of MTX (20 mg/kg) showed improvement in nerve tissues (Figures 5 and 6). The neuropil appeared less vacuolated and most of the nerve cells retained their normal architecture as a control group with normal appearance of the blood vessel. However, (Figures 5 and 6) showed necrosis of some neurons, the nerve cells appeared shrunken with darkly stained nuclei.

Figure 1: Photomicrograph of the cerebral cortex of mice in the control group showing the general histological structure of neurons with large nuclei (N) and normal healthy neuroglia (G), the surrounding dense felt work neuropil is also normal (arrow). (H & E X 400)

Figure 2: Photomicrograph of the cerebral cortex of mice treated with WGO showing no histopathological changes, normal neurons (N), neuroglia (G) and blood capillaries (C). (H & E X 400)

Figure 3 and 4: Photomicrographs of the cerebral cortex of mice treated with MTX showing congestion of blood capillaries (thick arrows), cerebral cortical infarction with edema, neuropil vacuolization (thin arrows), neurons degeneration with dark pyknotic nuclei and shrunken cytoplasm (head arrows). (H & E X 400)

Figure 5 and 6: Photomicrographs of the cerebral cortex of mice treated with MTX+WGO showing no histopathological changes except for mild necrosis of some neurons (arrow in Figure 5), some neurons appear dark red in color and are slightly shrunken (thin arrow Figure 6). Normal neurons (N), neuroglia (G), blood capillaries (C) and neuropil. (H & E X 400)

Figure 7: Effect of wheat germ oil on ATP, ADP and AMP level in brain of mice treated with MTX. Treated groups are compared with control and MTX groups by using Unpaired Student's *t*-test values are means $\pm$ SE. * $P < 0.05$ and ** $P < 0.01$ compared to control group and # $P < 0.05$ and ## $P < 0.01$ compared to MTX group

Figure 8: Effect of wheat germ oil on MDA, GSSG, NO and GSH concentration in brain of mice treated with MTX. Treated groups are compared with control and MTX groups by using Unpaired Student's t-test values are means $\pm$ SE. *P < 0.05 and **P < 0.01 compared to control group and #P < 0.05 and ## P < 0.01 compared to MTX group

Figure 9: Effect of wheat germ oil on 8HDG, NE, DA and 5HT concentration in brain of mice treated with MTX. Treated groups are compared with control and MTX groups by using Unpaired Student's t-test values are means $\pm$ SE. *P < 0.05 and **P < 0.01 compared to control group and #P < 0.05 and ## P < 0.01 compared to MTX group

These histological and histopathological results are consistent with biochemical analysis.

Biochemical Analysis

The results in (Figure 7) show that the level of ATP decreased significantly ($p < 0.01$) in the brain of mice that received MTX compared with the control group, while ADP and AMP levels showed significant increase ($p < 0.01$) compared to control group. Treatment with wheat germ oil in WGO + MTX group increased the level of ATP ($p < 0.05$) compared to the MTX group and decreased the level of ADP and AMP significantly ($p < 0.01$) compared to the MTX group.

Intra peritoneal administration of MTX increased MDA, GSSG and NO level in the brain of mice and decreased the concentration of GSH compared with the control group and the variance was statistically significant ($p < 0.01$). Wheat germ oil reduced oxidative stress by decreasing the level of MDA ($p < 0.01$) in the brain of mice and highly decreasing GSSG and NO level ($p < 0.05$) compared to the MTX group (Figure 8).

Biochemical analysis of 8-hydroxy-2-deoxyguanosine, a biomarker of global DNA oxidation, showed significant increase ($p < 0.01$) in the brain of the MTX group compared with the control group. Treatment with WGO in WGO + MTX group reduced the harmful effect of MTX and the difference appeared statistically significant ($p < 0.05$) compared to the MTX group (Figure 9).

Moreover (Figure 9) shows the destructive effect of a single dose of MTX administration on brain monoamines. Neurotransmitters norepinephrine and dopamine decreased significantly ($p < 0.05$), as well as serotonin ($p < 0.01$) compared to the control group. In contrast, wheat germ oil reduced the MTX harmful effect, and the level of DA, NE and 5HT were increased in WGO + MTX group compared to the MTX group.

DISCUSSION

Methotrexate is chemically known as 4-amino-4-deoxy-N¹⁰-methyl pteroylglutamic acid⁴⁵. Methotrexate (MTX) is used as an anti-inflammatory drug for the treatment of systemic lupus erythematosus, psoriasis, and inflammatory bowel disease; it also reduces inflammation in rheumatoid arthritis and damage to joints^{46, 47}. MTX is one of the most commonly antineoplastic drugs used for the treatment of osteosarcoma acute leukemia, lymphomas, breast and head cancer⁴⁸⁻⁵⁰.

However, MTX has adverse effects extensively described in recent years, including neurotoxicity^{48, 51} ataxia, tremor, somnolence, headaches and cognitive and psychiatric disturbances⁵². Its molecular structure is very close to that of folic acid; therefore, it was considered as an analogue and an antifolate drug. It enters the cells via active transport⁵ and inhibits the activation of folic acid in the body and thereby decreases the synthesis of both purines and pyrimidines⁵³ which in turn inhibit DNA synthesis, proteins, and RNA synthesis. In addition to depleting folate stores, methotrexate also induces oxidative stress^{54, 55} and excitotoxicity⁵⁶.

In the present study, the authors examined the histological changes in the cerebral cortex after administration of MTX and the improvement effect of wheat germ oil on these variations. The intraperitoneal administration of MTX induced the degeneration of most of granular and pyramidal neurons and the neuroglia, this result is consistent with^{57, 58} who reported that methotrexate suppresses the neurogenesis and induces microglial

inflammation. Sieklucka *et al.* studied the damage of white matter in the brain of albino swiss mice after a single intraperitoneal injection of MTX⁵⁹. To the best of the authors' knowledge, the distraction of glial cell homeostasis is associated with nervous system diseases related to myelin dysfunction including catatonia⁶⁰ and multiple sclerosis⁶¹.

Our microscopic examination revealed some dark neurons with condensed cytoplasm which reflects a phase of apoptosis. These small dark neurons are almost due to significant abnormalities in the capillary wall and subsequently structural elements of the blood-brain barrier⁶². The presence of some distorted and shrunken cells after administration of a single dose of MTX in our study is a result of damage in the biosynthesis of nucleic acids, certain enzymes and various neurotransmitters⁶³. As reported before, MTX inhibits the synthesis of tetrahydrofolate which is critical for DNA/RNA synthesis, amino acid synthesis and DNA/protein methylation.

Concerning the cerebral cortical infarction with edema and the vacuolation in the surrounding neuropil, this study has confirmed that these histological abnormalities were detected in MTX group in mice, this vacuolation could be attributed to the shrinkage of cells and withdrawal of their processes leaving peri-cellular spaces consistent with neuronal necrosis⁶⁴. Scott *et al.* declared that the cytoplasmic vacuoles corresponded with swollen mitochondria while the neuropil vacuoles denoted the swollen neuronal processes and presynaptic nerve endings⁶⁵.

The biochemical results in our study are consistent with the histological alterations. Moreover, they showed a decrease in the level of ATP in the brain of mice that received MTX compared with the control group. A new study in Proceedings of the National Academy of Sciences USA indicates that two-thirds of the brain's energy is used to help neurons send signals and the remaining third is used for cell-health maintenance, most of this energy is attained from ATP molecules through mitochondrial oxidative phosphorylation. Thus, we conclude that the reduction in ATP production is due to the harmful effects of MTX on the mitochondria, this result was previously recorded by⁶⁶ who established that MTX treatment caused swelling in the mitochondria of rats and significantly decreased adenosine triphosphate (ATP).

The MTX group showed increase in the MDA (end product of lipid peroxidation), GSSG and NO (pro-inflammatory mediators), levels in the brain of mice compared with the control group, this observation indicated that MTX could increase lipid peroxidation and produce brain tissue-injury by the generation of reactive oxygen species (ROS)²⁷. Moreover, GSH which is an indicator of ROS overproduction was decreased after administration of MTX. GSH reacts with the free radicals and can protect cells from hydroxyl radical, singlet oxygen, and superoxide radical damage⁶⁷.

These findings revealed that MTX induced oxidative stress in the nervous system causing brain injury. This result was also observed in previous literature which declared that (ROS) generation altered neurotransmission⁶⁸ and/or induced structural brain alterations¹³ while also causing mitochondrial damage which in turn causes rapid degeneration of the cells⁶⁹ and plays an important role in pathogenesis of neurodegenerative disorders, cancer and aging²¹⁻²³. In addition,⁵⁷ revealed that methotrexate induced changes in neuroinflammatory markers and cellular proliferation, along with structural changes in the corpus callosum in the brain.

The oxidized nucleoside, 8-hydroxy-2'-deoxyguanosine (8HDG), one of the oxidative DNA damage products, has been measured in this work after MTX administration showed significant increase in the brain compared to control group. This result was in agreement with⁷⁰ who found that the methotrexate treatment leads to the accumulation of (8-OHdG) in cells. The significant increase in 8HDG indicated the severe damage of DNA; this damage is due to MTX inhibiting effect on the synthesis of tetrahydrofolate which is critical for DNA synthesis as mentioned above and the harmful effect of reactive oxygen species.

On the other hand, MTX administration reduced the concentration of brain neurotransmitters (norepinephrine, dopamine, and serotonin) this result is confirmed by⁷¹, he reported that methotrexate decreases main brain amines in different brain areas. Various types of neurotoxicity are due to interference in the supply of neurotransmitters after MTX administration⁷². Moreover,^{73,74} believed that methotrexate reduces the bioamine synthesis leading to an imbalance in some neurotransmitter systems.

Wheat germ oil (WGO), which is extracted from the germ of the wheat kernel, has an excellent nutritional value, contains a high percentage of fatty acids, the highest tocopherol content of all vegetable oils, and the highest content of α -tocopherol (vitamin E) which is considered the potent natural antioxidant⁷⁵. The present study has reported that WGO has the capability to protect against MTX neurotoxicity, examination of specimens of mice treated with MTX concomitantly with Wheat germ oil (WGO) showed improvement in the nerve tissues, the neuropil appeared less vacuolated, most of nerve cells retained their normal architecture and scarcely the affected cells are observed. In addition, WGO attenuates the harmful neurotoxic effects of MTX by increasing the level of ATP, GSH and monoamines and decreasing the level of MDA, GSSG, NO and 8-hydroxy-2'-deoxyguanosine (8HDG). Thus, WGO can protect against neurotoxicity induced by a variety of oxygen radical generators³⁰.

Wheat germ oil as a rich source of vitamin E acts as an anti-inflammatory and an inhibitor of lipid peroxidation^{30,76,77}. Furthermore, WGO inhibits oxidation processes in body tissues and protects cells against the effects of free radicals by directly scavenging toxic radicals³¹. Wheat germ oil is widely used in the treatment of diseases involving oxidative damage⁷⁸. Moreover, it creates DNA protective properties³⁴. Thus, a dose of wheat germ oil may protect the brain tissue through its cytoprotective properties.

CONCLUSION

In conclusion, our results suggest that the administration of MTX produces neurochemical and neurotoxic alterations in the brain causing severe damage including peripheral neuropathy and encephalopathy. We can explain these effects by many concepts. MTX affects the structure of nucleic acids, inhibits the activity of dihydrofolate reductase enzyme, and decreases the levels of various neurotransmitters. It also leads to brain tissue-damage by ROS generation, causes alteration in mitochondrial permeability, results in tissue injury and loss of the myelin sheath as a response to the oxidative stress. WGO has neuroprotective actions through scavenging the oxygen free radicals, decreasing lipid peroxidation and eliminating the histopathological changes induced by MTX, thus WGO is recommended to patients using MTX. Some degenerative cells still observed in this study after WGO treatment require further experimental study to adjust the dose and the exposure frequency.

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