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Protective benefits of quercetin and lipoic acid on methotrexate-induced oxidative stress in rat spleen

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ABSTRACT

The study aims to examine the effect of Methotrexate (MTX) treatment on rat spleen and investigate the antioxidant properties of quercetin (Que) and lipoic acid (LA) on MTX. Rats were divided into groups as Controls, MTX-given group, MTX+Que-given group, and MTX+LA-given group. At the end of the experiment, total protein, glutathione (GSH), lipid peroxidation (LPO), nitric oxide (NO), and total sialic acid (SA) levels, also superoxide dismutase (SOD), and catalase activities were determined in spleen homogenates. MTX increased oxidant parameters and both Que and LA effectively decreased LPO, SA, and NO levels. Administration of MTX increased GSH levels and decreased SOD activity, besides Que normalized GSH level and SOD activity, LA decreased GSH activity. Que and LA may be effective in recovering MTX-induced toxicity by decreasing oxidative stress and can be useful in normalizing the damage situation in rat spleen.

Keywords: methotrexate, oxidative stress, quercetin, lipoic acid, spleen

INTRODUCTION

Methotrexate (MTX) (4-amino-N10-methyl-pteroylglutamic acid), a weak bicarboxylic organic acid, acts as an anticancer agent and immunosuppressant[1,2].

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It was developed based on its structural similarity to folic acid and its capacity to inhibit folate-dependent enzymes[3]. After its initial use in the treatment of acute leukemia, MTX has also been used to treat various types of cancer, as well as autoimmune and inflammatory diseases[1,3].

The primary pharmacological mechanism of MTX is to competitively inhibit dihydrofolate reductase, an intracellular enzyme that reduces folic acid to tetrahydrofolate cofactors[4]. MTX has 100x greater affinity to dihydrofolate reductase than folic acid, which effects the conversion of folic acid to tetrahydrofolate which is required for DNA synthesis[5]. Inhibition of purine and pyrimidine thymidylate synthesis by MTX leads to deoxynucleotide pool imbalance, decreased folate, purine, and thymine deficiency in actively proliferating cells such as tumor cells, leading to blockage of DNA and RNA synthesis, and eventually cell death[4,5].

MTX has a toxic effect on the body since either monoglutamate, the natural form of the drug, or polyglutamate are inhibitors of several enzymes. The polyglutamated forms are stored in the cells and kept in the body for weeks, resulting in a decrease in folate levels and both drug forms are not specific to malignant cells[3,5]. The toxicity caused by MTX may vary depending on the dose and duration of use according to the disease and patient profile[6]. These side effects may be life-threatening such as a decrease in the immune system, dysfunction of blood cells, various liver complications and organ involvement. The exact way in which MTX causes toxicity in the body is not completely known. However, it is mainly linked to the generation of oxidative stress in organs such as the kidney, liver, and heart[7]. This is caused by an increase in reactive oxygen species (ROS), which leads to high levels of neutrophil infiltration and release of pro-inflammatory cytokines[8].

Quercetin (Que) is a potent antioxidant belonging to the flavonol subclass of flavonoids. It is not synthesized in the body but is taken from various vegetables and fruits through diet and the best-known source of Que is onion[9,10]. Que is known to have anti-oxidative effects as well as anti-inflammatory, antidiabetic, anti-proliferative, anti-viral, and anti-carcinogenic properties[11-13]. By possessing hydroxyl groups within its structure, Que has lipophilic properties, and it demonstrates an exceptional ability to cross the blood-brain barrier easily and therefore it has been reported to protect from neurodegenerative diseases[10]. Que shows a strong antioxidant property and can scavenge many ROS due to the presence of the phenolic hydroxyl group and the double bonds in its structure[9,14]. These hydroxyl groups in its structure act as active hydrogen donors and reduce free radicals. Thus, Que makes them stable and prevents

unsaturated fatty acid oxidation[14,15]. Que also protects the organism from oxidative damage by taking an active role in maintaining the oxidant/antioxidant balance[9,14].

Lipoic acid (LA, 1,2-dithiolane-3-pentanoic acid) is a natural dithiol compound synthesized enzymatically in mitochondria[16,17]. LA is a crucial cofactor for the mitochondrial enzymes, therefore it is critical in mitochondrial energy metabolism[16,18]. Unlike other antioxidants, LA is soluble in both water and fat, so it can act anywhere in the body[19]. In addition, its small size and high lipophilicity allow it to easily cross biological membranes[6].

The spleen is the largest of the lymphoid tissue organs. It is the center for the destruction of red blood cells and platelets. It also filters out old blood cells that are coated with antibodies or incorrectly produced. It plays an active role in the immune system by filtering out foreign bodies such as bacteria as well as abnormal erythrocytes. It is also the organ where red blood cells are stored[20,21].

There are limited studies focused on the oxidative effects of MTX on spleen tissue. Thus, we aimed to examine the protective benefits of Que and LA on MTX-induced oxidative stress in the spleen tissue of rats.

METHODOLOGY

Experimental groups

This study was approved by the Marmara University Animal Experiments Local Ethics Committee (MÜHDEK) (Protocol No: 01.2023mar). In the study, Wistar albino rats (2 months old, 200-250 gr.), taken from Istanbul University Aziz Sancar Experimental Medicine Research Institute, were used. The animals were housed in cages at room temperature with a maximum of 4 rats. They were fed ad libitum and consumed standard rat feed and tap water. MTX (50 mg/5 ml) was obtained from Koçak Farma Pharmaceuticals and Chemicals Industry Inc., Türkiye. Lipoic acid (catalog number: 1077-28-7) and quercetin (catalog number: 117-39-5) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Experimental groups were created as follows:

Control group (n=6): 0.1 cc/100 g saline i.p. was administered to each rat for 5 days.

MTX-given group (n=6): Given 20 mg/kg MTX i.p. on day 1st, followed by 0.1 cc/100 g saline i.p. for 5 days.

MTX + Que- given group (n=6): Given 20mg/kg MTX i.p. on day 1st, followed by 50mg/kg Que i.p. dissolved in 0.1 cc/100 g saline for 5 days.

MTX + LA-given group (n=6): Given 20 mg/kg MTX i.p. on day 1st, followed by 50 mg/kg LA i.p. dissolved in 0.1 cc/100 g saline for 5 days.

Administration dose, timing, and experimental protocol were determined based on previous studies[22,23]. On the 6th day, anesthesia of the animals was performed by intraperitoneal injection of sodium pentothal (50 mg/kg). After euthanasia by taking cardiac blood, spleen tissues were taken and homogenized with saline solution (0.9% g NaCl). Total protein[24], lipid peroxidation (LPO)[25], nitric oxide (NO)[26], total sialic acid (SA)[27], glutathione (GSH)[28] levels, and superoxide dismutase (SOD)[29], and catalase (CAT)[30] activities were determined in 10% (w/v) homogenates prepared from spleen tissue.

Statistical analysis

Graph-Pad Prism 9.0 (GraphPad Software, San Diego, CA, USA) software was used for statistical analysis. The one-way ANOVA method and Tukey's test were used for the comparison of more than two group averages and the interpretation of the differences between them. The t-test was used for the comparison of two groups. $P<0.05$ was accepted as significant.

RESULTS and DISCUSSION

The study focused on examining the effects of Que and LA on MTX-induced oxidative stress on rat spleen tissue. Since the spleen plays an important role in regulating blood cells in the body, when it is damaged, the immune system is also affected. Despite its widespread use in the treatment of cancer, autoimmune, and inflammatory diseases, MTX is known to have toxic effects, but studies on its effect on the spleen tissue are limited. Due to mice, one of the frequently preferred experimental animals, synthesize folic acid in their intestines[31], they were not suitable for the present study.

Oxidant parameters; LPO, NO, and SA levels results are shown in Figure 1.

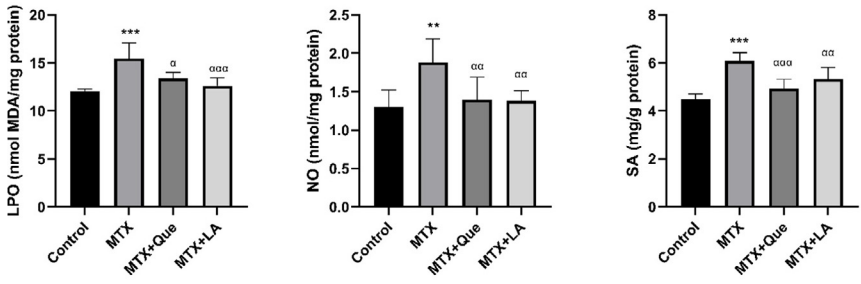

Figure 1. Levels of LPO, NO, and SA in spleen tissue. Values are given as mean±standard deviation, Control: Control group; MTX: Methotrexate-given group; MTX-Que: Methotrexate and Quercetin-given group; MTX-LA: Methotrexate and Lipoic acid-given group; LPO: Lipid Peroxidation; MDA: Malondialdehyde; NO: Nitric Oxide; SA: Sialic Acid. **p<0,01, ***p<0.001 significantly different from Control group; $^{\alpha}$p<0.05, $^{\alpha\alpha}$p<0.01, $^{\alpha\alpha\alpha}$p<0.001 significantly different from MTX group.

LPO levels of the spleen tissues increased significantly in the MTX group compared to the controls (p<0.001) and also decreased significantly in the MTX+ Que and MTX+LA groups compared to the MTX-given group (p<0.05, p<0.001, respectively). Splenic NO level increased significantly in the MTX-given group compared to the controls (p<0.01), while significant decreases were observed in the MTX+Que and MTX+LA groups compared to the MTX-given group (p<0.001, p<0.01, respectively). A significant increase in the SA level of the spleen tissue was detected in the MTX group compared to the controls (p<0.001), while significant decreases were observed in the MTX+ Que and MTX+LA groups compared to the MTX-given group (p<0.001, p<0.01, respectively).

Antioxidant parameters; GSH level, SOD, and CAT activities results are shown in Figure 2.

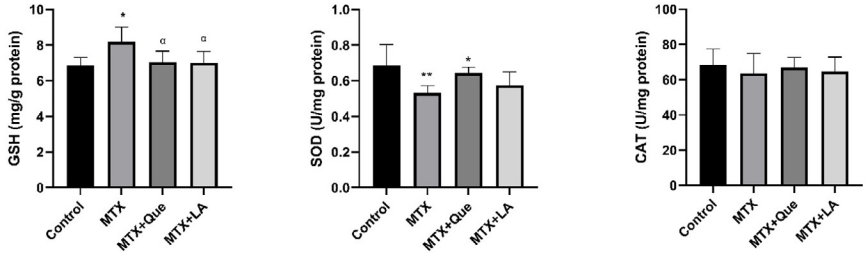

Figure 2. Level of GSH, activities of CAT and SOD in spleen tissue. Values are given as mean±standard deviation, Control: Control group; MTX: Methotrexate-given group; MTX-Que: Methotrexate and Quercetin-given group; MTX-LA: Methotrexate and Lipoic acid-given group; GSH: Glutathione; CAT: Catalase; SOD: Superoxide Dismutase, *p<0,05, **p<0,01 significantly different from Control group; [a]p<0.05 significantly different from MTX group.

GSH level of the spleen tissue increased significantly in the MTX-given group compared to the controls (p<0.05) and decreased significantly in the MTX+Que and MTX+LA groups compared to the MTX-given group (p<0.05). SOD activity decreased significantly in the MTX group compared to the controls (p<0.01). Administration of Que significantly increased SOD activity compared to the MTX-given group (p<0.05). Although there was a slight increase in the MTX-LA group, the result was insignificant.

No significant change was observed in the CAT activity of the groups.

Although MTX is preferred in the treatment of various cancers and autoimmune diseases, serious side effects up to organ involvement may be observed in patients due to long-term and high dose use[32]. In addition, while MTX-polyglutamate forms accumulate in intracellular stores with MTX use, folate stores decrease. These MTX-polyglutamate can act for weeks even if MTX administration is stopped, which increases the toxic effect of the drug[32,33]. The main reason for the side effects of MTX is that it can harm healthy cells as well as cancerous ones. MTX can also cause oxidative damage in the surrounding tissues and organs because the antioxidant system is unable to eliminate the reactive species that increase during treatment. The oxidative stress may cause involvement or loss of function in various organs such as the spleen.

Que reacts directly with radicals and neutralizes them. It also shows antioxidant properties by inhibiting LPO by breaking down lipid peroxyl radicals and chelating metals[15]. In many studies on animals and cells, it has been documented that Que induces GSH synthesis, increases CAT and SOD activities, and decreases MDA content[9,14,34]. Likewise, external supplementation with LA acts as a potent antioxidant and can reduce oxidative stress both in vitro and in-vivo[35].

It has been reported that LA protects DNA, membrane lipids, and intracellular proteins from oxidative damage[33]. In addition to its antioxidant capacity, LA has been proven to participate in the regeneration of oxidized forms of other endogenous antioxidant agents such as vitamin E, C, and GSH, which are depleted especially during oxidative stress[17,35].

In our study, significant increases were found in oxidant parameters consisting of LPO, NO, and SA in the spleen tissue of MTX-given rats compared to the controls. Besides, administration of Que and/or LA to the MTX-given group was effective in reducing and/or normalizing these parameters. In studies conducted with serum and different tissues of animals treated with MTX, it has been proven that the MDA level, which is an indicator of LPO, is increased[33,36]. In parallel with the results of our study, Soliman et al.[7] reported that the administration of MTX increased LPO levels in the spleen tissue. Also, in previous studies with different tissues, decreased MDA levels were found with the administration of Que[15] and LA in MTX-given groups[17].

NO is one of the nitrogen-derived reactive species in the organism. Previous studies showed that MTX administration increased NO levels in rat spinal cord[37], serum[36], liver[38,39], heart[40], and kidney[41]. Excessively produced NO in the cell can react with superoxide radicals and cause nitration of proteins and the formation of peroxynitrite radicals. In addition, the production of NO in the organism leads to rapid depletion of GSH, which is an antioxidant in the cell[38]. In our study, MTX increased NO levels in spleen tissue. Besides, we found that the NO level in spleen tissue was significantly reduced by the antioxidant effects of Que and LA compared to the MTX-given group. Thus, we can suggest that Que and LA may be effective in preventing NO-induced oxidative damage in spleen tissue.

SA, found at the terminal end of many glycoconjugates in all biological membranes, is an acetylated derivative of neuronic acid[40]. SA is also a marker of oxidative stress and a more accurate indicator of inflammation since it shows little variability between individuals[42]. In our study, a significant increase was observed in the SA level of the MTX group compared to the controls. Consistent with the literature, SA levels decreased significantly with the treatment of Que and LA compared to the MTX group[43]. Increased SA levels following MTX treatment may be a response of spleen tissue to protect itself against oxidative damage.

In previous studies, GSH levels were examined in various tissues of animals treated with MTX, and decreases were found compared to the control group. Besides, treatment with antioxidants, such as LA and Que, leads to an increase in GSH level[6,7,15]. However, in our study, GSH levels increased significantly in the spleen tissue of MTX-given rats. We suggest that this increase is due to

the activation of the antioxidant defense mechanism in the spleen, which is an important part of the immune system and develops an immune response to radical formation. Besides this, we found that GSH levels in the MTX+Que and MTX+LA groups decreased significantly compared to the MTX group and approached the control levels. Based on our experimental design, we believe that starting antioxidant treatment following the MTX administration on the first day and continuing the treatment for five days resulted in mild MTX-induced oxidative damage in the spleen. Additionally, the use of Que and LA may help protection of spleen tissue against oxidative damage by increasing GSH levels.

Considering the animal trials in which MTX treatment was applied, a decrease in SOD activity was found[17,39], and a significant elevation was observed with the administration of Que[34,43] and LA[6,35]. Following these studies, we found a decrease in SOD activity in the spleen tissue of the MTX group. The inhibition of cellular NADPH by as a result of MTX-induced oxidative stress may cause a decrease in SOD activity. Although a significant increase was observed with Que treatment, LA did not change SOD activity. In some previous studies CAT activity, an antioxidant enzyme in various tissues, decreased with MTX administration, while an increase was observed after Que and LA treatment[35,44-46]. In the present study, no difference was found in the CAT activity of the groups; this may be a result of the animals developing an immune response to a single dose of 20 mg/kg MTX administration and tolerating the oxidative damage caused by MTX. In the literature, SOD and CAT activities have been found to decrease with MTX administration, but there are also studies with different results. In the study conducted by Armağan et al. increased levels of liver SOD and CAT activities were detected and the administration of LA decreased SOD and CAT activities slightly compared to the controls[47]. Besides, in kidney tissue, SOD activity level was observed to decrease compared to the control group, while CAT activity level was observed to increase compared to the controls, but these results were insignificant. Uzar et al. administered MTX to rats at a dose of 20 mg/kg and found that cerebellum SOD and CAT activities were significantly increased in the MTX-treated group compared to the control group[37]. Additionally, Devrim et al. applied MTX to 60 mg/m^2 body surface area to rats for 7 weeks and detected a non-significant increase in SOD and CAT activities in kidney tissue compared to the control group[48]. However, Soylu Karapınar et al. measured a significant decrease in CAT activity of the rat ovarian tissue after MTX administration compared to the controls, but after LA treatment there was a slight increase in the MTX+LA group compared to the MTX-given group[49].

The current study showed that MTX-induced oxidative stress causes tissue toxicity and damages spleen tissue. Que and LA were able to restore the oxidative state by fixing the damage. Que and LA protected the antioxidant system by regulating GSH levels and SOD enzymes, both of which were useful in decreasing oxidant parameters.

STATEMENT OF ETHICS

Ethics approval is not required in this study, as no human and experimental animal samples are involved.

CONFLICT OF INTEREST STATEMENT

Declared none.

AUTHOR CONTRIBUTIONS

Concept: A.A., Ş.O., Design: Ş.O., Data Collection and Processing: A.A., Ş.O., Analysis or Interpretation: A.A., Ş.O., Literature Search: A.A., Ş.O.

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