ORIGINAL RESEARCH

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Comparison of the Protective Effect of Alpha Lipoic Acid and Quercetin in Methotrexate-Induced Lung Damage

Metotreksat ile Oluşturulan Akciğer Hasarında Alfa Lipoik Asit ve Kuersetinin Koruyucu Etkilerinin Karşılaştırılması

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Abstract

Objective: The aim of this experiment is to investigate and compare the effects of alpha lipoic acid (ALA) and quercetin (QUE) on methotrexate (MTX)-induced lung injury in rats.

Method: Wistar Albino rats were distributed into control, MTX, MTX+ALA and MTX+QUE groups with each consisting of 6 rats. Except control group, MTX administrated to rats as a single dose (20 mg/kg) intraperitoneally (i.p.) on the first day. Saline (0.1 cc/100 gr/day, i.p.) was injected to rats in control and MTX groups for 5 days. In MTX+ALA and MTX+QUE groups, rats had injections of ALA (50 mg/kg/day, i.p.) and QUE (50 mg/kg/day, i.p.) for 5 days. After sacrification on day 6, lung tissues were excised out for histopathologic and biochemical investigation.

Results: MTX group showed massive hemorrhage with edema in the interstitium, significant inflammatory cell infiltration, and severe alveolar destruction and vascular congestion. Additionally, significant increases in oxidative stress markers as malondialdehyde and sialic acid and significant decreases in antioxidants as glutathione, superoxide dismutase and catalase were detected at the tissue level in MTX group ($p<0.0001$, $p<0.0001$, $p<0.0001$, $p=0.03$ and $p<0.0001$, respectively). Both ALA and QUE treatment led to a prominent improvement in morphologic damage. Moreover, ALA and QUE resulted in the reversal of the alterations seen in the tissue oxidative damage markers and antioxidant activities as well. We could not reveal a significant difference between MTX+ALA and MTX+QUE group in terms of morphologic damage and biochemical markers of oxidative injury ($p>0.05$).

Öz

Amaç: Bu çalışmanın amacı sıçanlarda metotreksat (MTX) ile oluşturulan akciğer hasarında alfa lipoik asit (ALA) ve kuersetinin (QUE) koruyucu etkilerinin araştırılması ve karşılaştırılmasıdır.

Yöntem: Wistar Albino her biri 6 adet sıçandan oluşan kontrol, MTX, MTX+ALA ve MTX+QUE gruplarına ayrıldı. Kontrol grubu hariç tüm gruplarda deneyin ilk gününde sıçanlara tek doz MTX (20 mg/kg) intraperitoneal (i.p.) olarak verildi. Kontrol ve MTX gruplarındaki sıçanlara 5 gün serum fizyolojik (0,1 cc/100 gr/gün, i.p.) verildi. MTX+ALA ve MTX+QUE gruplarındaki sıçanlara 5 gün ALA (50 mg/kg/gün, i.p.) ve QUE (50 mg/kg/gün, i.p.) enjeksiyonu yapıldı. Deneyin 6. gününde sıçanlar sakrifiye edilerek akciğer dokuları histopatolojik ve biyokimyasal inceleme için çıkartıldı.

Bulgular: MTX grubunda interstisyumda yoğun hemorajiyle birlikte ödem, belirgin enflamatuvar hücre infiltrasyonu ve ciddi alveolar yıkımı ve vasküler konjesyon görüldü. Bu bulgulara ek olarak, MTX grubunda malondialdehit ve sialik asit gibi oksidatif stres belirteçlerinde önemli bir artış ve glutatyon, süperoksit dismutaz ve katalaz gibi antioksidanlarda önemli bir azalış olduğu doku düzeyinde saptandı (sırasıyla $p<0,0001$, $p<0,0001$, $p<0,0001$, $p=0,03$ ve $p<0,0001$). Hem ALA hem de QUE tedavisinin morfolojik hasar üzerinde belirgin bir iyileşmeye neden olduğu tespit edildi. Ayrıca ALA ve QUE tedavisi MTX'in yol açtığı doku oksidatif hasar belirteçlerinde ve antioksidan aktivitedeki olumsuz değişimleri tersine çevrilmesine neden olduğu tespit edildi. Morfolojik hasar ve oksidatif hasarın biyokimyasal belirteçleri açısından MTX+ALA ve MTX+QUE grupları arasında anlamlı bir fark saptanmadı ($p>0,05$).

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Abstract

Conclusion: Our study showed the similar protective effect of ALA and QUE in MTX induced lung damage. Further studies are warranted to verify the results of our outcome.

Keywords: Alpha lipoic acid, lung, methotrexate, quercetin

Öz

Sonuç: Çalışmamız MTX ile oluşturulan akciğer hasarı üzerinde ALA ve QUE'nin benzer koruyucu etkilerinin olduğunu gösterdi. Sonuçlarımızı doğrulamak için daha ileri çalışmalara ihtiyaç duyulmaktadır.

Anahtar kelimeler: Akciğer, alfa lipoik asit, kuersetin, metotreksat

Introduction

Methotrexate (MTX) is a folic acid analog that frequently preferred in the treatment of both systemic and inflammatory diseases like psoriasis, rheumatoid arthritis and malignancies. The basic mechanism of its action occurs through the inhibition of folate metabolism and suppression of the synthesis of inflammatory cytokines such as like tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β), and thereby leading to the downregulation of inflammatory reactions (1,2). Pulmonary toxicity has been regarded as the main adverse effect of the long-term treatment with MTX or its overdose. In a dose-dependent fashion, MTX could cause pneumonitis, interstitial lung disease, and lung fibrosis and the development of these adverse effects usually ends up with early discontinuation of the therapy (3). Oxidative stress caused by overproduction of reactive oxygen species (ROS) is regarded as the underlying mechanism behind MTX-induced lung toxicity (4).

Recently there has been an on-going effort to tackle MTX-induced pulmonary toxicity and many natural and synthetic agents with a proposed antioxidant action have been used in the treatment of this dismal complication. Quercetin (QUE), its name originating from quercetum (oak forest), is a natural product which acts as a pigment giving color to fruits and vegetables (5). It was shown that QUE has a role in prevention of many chronic diseases like diabetes mellitus (DM), obesity and cardiovascular diseases via its antioxidant effects (5, 6). Several investigators revealed that QUE supplementation in rats treated with MTX caused a beneficial effect against the development of lung, liver and renal toxicities (7-9). Furthermore, alpha lipoic acid (ALA) is a commonly used herbal medicine and it is a synthetic form of lipoic acid produced by plants and animals (10). ALA has a crucial role in energy metabolism and regeneration of cellular antioxidant stores, therefore inducing an antioxidant action in the body (11). Clinically, ALA supplementation in diabetic patients regulates the blood sugar and improves DM-related complications (12). In a study by Arpag et al. (13), combination of ALA with MTX was found to reduce the MTX-related tissue damage in rats. In MTX-related pulmonary toxicity, although the organ protective effects of the above-given herbals, ALA and QUE, have been shown in several studies, the evidence has still remained weak. In our study, we aimed to study the effect of ALA and QUE in pulmonary toxicity related to MTX experimentally and compare the effects of these two agents.

Materials and Methods

Chemicals

Quercetin (Q4951, catalog number:117-39-5) and ALA (T1395, catalog number: 1077-28-7) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Methotrexate (Metotrexate, 50 mg/5 mL) was obtained from Koçak Farma İlaç ve Kimya Sanayi A.Ş., Turkey.

Animals

Twenty-four adult male Wistar Albino rats (2 months old, 200-250 gr) were housed under the standard laboratory conditions. They received standard rat pellet and water ad libitum. Throughout the experiment, "the Guide for the Care and Use of Laboratory Animals" process guidelines were followed. The experimental procedure was approved by Marmara University Animal Care and Use Committee (protocol number: 50.2022.mar and date: 11.10.2022).

Experimental Design

Rats were distributed into four group: In control group (C, n=6), rats were injected with saline (0.1 cc/100 gr/day) intraperitoneally (i.p.) for 5 days. In MTX group (n=6), rats were injected with a single dose of MTX (20 mg/kg, i.p.) on the first day and saline (0.1 cc/100 g/day, i.p.) for 5 days. In MTX+ALA group (n=6), rats were injected with a single dose of MTX (20 mg/kg, i.p.) on the first day and ALA (dissolved in saline, 50 mg/kg/day, i.p.) for 5 days. In MTX+QUE group (n=6), rats were injected with a single dose of MTX (20 mg/kg, i.p.) on the first day and QUE (dissolved in saline, 50 mg/kg/day i.p.) for 5 days. On the sixth day of the experiment, all rats were sacrificed under general anesthesia with sodium pentothal (50 mg/kg, i.p.) and lung tissues were excised out for histopathological and biochemical investigation.

Histopathologic Analysis

For light microscopic analysis, fixation of lung tissue samples was done with 10% formaldehyde. After routine tissue processing, they were embedded in paraffin blocks. For detection of the morphological alterations, hematoxylin and eosin staining was applied to approximately 5 µm thick paraffin section. All stained section was investigated by a microscope (Olympus CX41, Tokyo, Japan) and photographed with a camera (Kameram Dijital Mikroskopi, Türkiye). Histopathologic scoring for each sample was done at least 5 microscopic areas and four criteria were evaluated including (1) congestion, (2) interstitial edema, (3) inflammatory cell infiltration and (4) alveolar degeneration. Each criterion was semiquantitatively scored on a scale ranging from 0-3 (0: Absent, 1: Mild, 2: Moderate and 3: Severe) (14).

Biochemical Analysis

To assess the biochemical parameters, lung tissue homogenates (10% w/v) were made using saline solution and kept in the freezer at -20 °C. Malondialdehyde (MDA), glutathione (GSH) and sialic acid (SA) levels and superoxide dismutase (SOD), catalase (CAT) activities were determined via following previously described methods (15).

Statistical Analysis

All data was analyzed by GraphPad Prism 8.42 (GraphPad Software, San Diego, CA, USA). Following the normal distribution of data was identified by Shapiro-Wilk test, the One-Way Analysis of Variance (ANOVA) test and Tukey's multiple comparison tests were done. The results were given as mean ± standard deviation and a value of $p<0.05$ was accepted as significant.

Results

Histopathologic Results

Microscopic investigation revealed normal lung architecture in control group. In MTX group, massive hemorrhage with edema in the interstitium, significant inflammatory cell infiltration, and severe alveolar destruction and vascular congestion were observed. In addition to a quite regular alveolar structure in most areas of the tissue, a reduction in hemorrhage with edema in the interstitium, a decrease in inflammatory cell infiltration and mild vascular congestion were seen in MTX+ALA group. In MTX+QUE group, a prominent amelioration in alveolar structure, regression in hemorrhage with edema in the interstitium, reduced inflammatory cell inflammation and a moderate vascular congestion were present (Figure 1).

Compared to control group, exposure to MTX resulted in a significantly higher histopathologic score ($p<0.0001$). In comparison, the histopathologic score was found to be lower in MTX+ALA and MTX+QUE groups when compared to MTX group ($p<0.0001$, for both). Additionally, there was no significant difference in the histopathologic score between MTX+ALA and MTX+QUE groups ($p>0.05$, Table 1).

Biochemical Results

The MDA and SA levels in MTX group were found to be significantly higher in comparison with control group ($p<0.0001$, for both). However, MDA level significantly decreased in both MTX+ALA and MTX+QUE groups when compared with MTX group ($p=0.006$ and $p=0.02$, respectively). Moreover, SA level significantly decreased in both MTX+ALA and MTX+QUE groups in comparison with MTX group ($p=0.001$, for both). Contrasted with the control, the GSH level, SOD and CAT activities significantly reduced in MTX group ($p<0.0001$, $p=0.03$ and $p<0.0001$, respectively). The level of GSH significantly increased in

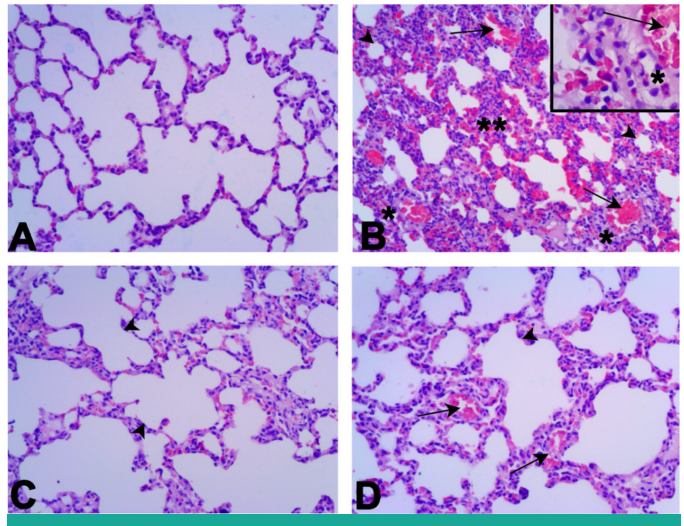

Figure 1. Representative light photomicrographs of lung tissues in experimental groups. In control group (**A**), normal lung morphology were observed. In methotrexate (MTX) group (**B**), Massive hemorrhage with edema in the interstitium (**), inflammatory cell inflammation (*), severe vascular congestion (arrows) and alveolar destruction (arrows) were seen. In MTX + alpha lipoic acid (ALA) group (**C**), Quite regular alveolar structure (arrow heads) were observed in most areas of the tissue. In MTX+quercetin (QUE) group (**D**), Quite regular alveolar structure (arrow heads) in many areas of the tissue and moderate vascular congestion (arrows) were seen. Original magnification x 100 and x200 (inset), Hematoxylin and eosin staining

both MTX+ALA and MTX+QUE groups compared to MTX group (p=0.0005 and p=0.005, respectively). Also, CAT activity was significantly increased in both MTX+ALA and MTX+QUE groups compared to MTX group (p=0.009 and p=0.007 respectively). There was no a significant difference in SOD activity in both MTX+ALA and MTX+QUE groups compared to MTX group (p>0.05, Table 2).

Discussion

Our results showed that both ALA and QUE have the antioxidant potential to suppress the generation of ROS in the lung tissue of the animals treated with MTX. The benefit of these agents was also verified at the morphologic level and administration of both ALA and QUE with MTX lessened the inflammation in the lung tissue and preserved the alveolar structure in the similar fashion.

In our study, MTX treatment resulted in the generation of ROS and lung damage that was in accordance with the current literature data. As a folate antagonist and S-phase active agent, MTX inhibits the activity of dihydrofolate reductase enzyme and synthesis of purine and pyrimidine bases necessary for production of nucleic acids in rapidly dividing malignant cells (16). It was shown that most of the patients treated with MTX for malignancy and inflammatory conditions like rheumatoid arthritis have pulmonary symptoms such as dyspnea, cough, pneumonitis and interstitial lung disease (3,4,17). In patients with MTX-associated pneumonitis, IL-8 and chemotactic factors secreted by the airway epithelium increase (18). Toxic doses of MTX promotes a more profound lung inflammation by increasing the secretion of IL-1 and TNF-α, MDA and myeloperoxidase (MPO) which were shown to be the most crucial players of chronic inflammation and pneumonitis (2,19).

To tackle the pulmonary toxicity, anti-inflammatory and antioxidant agents have been increasingly tested in many experimental studies. Kurt et al. (20) revealed that infliximab, TNF-α inhibitor, is protective against MTX overdose-associated lung damage by decreasing endothelin-1 release and oxidative stress. In an experimental study by Kaymak et al. (21), Vitamin B12 pretreatment was shown to lessen oxidative damage and inflammation in MTX-induced lung toxicity. Moreover, immunohistochemically, expressions of TNF-α, alpha-smooth muscle actin (α-SMA), laminin, and the number of apoptotic cells were found to decrease significantly with vitamin B12 in the lung tissue samples. The protective

Table 1. Comparison of histopathologic score in experimental groups

Parameters	C (n=6)	MTX (n=6)	MTX+ALA (n=6)	MTX+QUE (n=6)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Histopathologic score	1.08±0.37	10.17±1.16	4.91±0.91	5.91±0.66
p-values		<0.0001*	<0.0001*; <0.0001$^{\alpha}$	<0.0001*; <0.0001$^{\alpha}$

Tukey's multiple comparison test. Value of p<0.05 was accepted as significant. *represents comparisons with control group; $^{\alpha}$ represents comparisons with MTX group. SD: Standard deviation, C: Control, MTX: Methotrexate, ALA: Alpha lipoic acid, QUE: Quercetin

Table 2. Comparison of biochemical markers in experimental groups

Parameters	C (n=6)	MTX (n=6)	MTX+ALA (n=6)	MTX+QUE (n=6)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
MDA (nmol/mg protein)	39.92±6.79	65.49±11.34	49.09±6.72	51.94±3.04
p-values		<0.0001*	0.006$^{\alpha}$	0.02$^{\alpha}$
SA (ng/g protein)	12.75±1.73	21.31±2.31	16.85±1.22	17.10±1.31
p-values		<0.0001*	0.002*; 0.001$^{\alpha}$	0.001*; 0.001$^{\alpha}$
GSH (mg/g protein)	4.14±0.29	2.59±0.38	3.52±0.31	3.32±0.31
p-values		<0.0001*	0.02*; 0.0005$^{\alpha}$	0.001*; 0.005$^{\alpha}$
SOD (U/mg protein)	1.26±0.10	1.08±0.08	1.63±0.14	1.13±0.07
p-values		0.03*		
CAT (kU/mg protein)	5.52±0.42	4.31±0.35	5.03±0.24	5.05±0.32
p-values		<0.0001*	0.009$^{\alpha}$	0.007$^{\alpha}$

Tukey's multiple comparison test. Value of p<0.05 was accepted as significant. *represents comparisons with control group; $^{\alpha}$represents comparisons with MTX group. SD: Standard deviation, C: Control, MTX: Methotrexate, ALA: Alpha lipoic acid, QUE: Quercetin, MDA: Malondialdehyde, GSH: Glutathione, SA: Sialic acid, SOD: Superoxide dismutase, CAT: Catalase

roles of N-acetylcysteine and erythropoietin in MTX-related lung damage were also demonstrated in the literature (22).

The balance between ROS and antioxidants determines the sensitivity and resistance of the organism to oxidative stress. Endogenous and/or exogenous factors that change this balance cause cell injury. Antioxidant defense systems protect the cell against the harmful effects of ROS. These molecules are divided into two groups as enzymatic, such as SOD and CAT activity, and nonenzymatic, such as GSH, and are involved in antioxidant defense mechanisms at different levels in the cell (23).

As a flavonoid, QUE is extensively found in plants in nature, including berries, grapes, apples, brassica vegetables, tomatoes and onions as well as in many seeds, nuts, and leaves (5). Flavonoids are natural products and have several therapeutic effects such as antioxidant, antitumor, and anti-inflammation properties. QUE is an active flavonoid and has the ability to scavenge free radicals (24). Owing to these properties, it has been remained as a popular herbal agent (25). The impact of QUE in MTX-induced organ damage was studied in several studies. Aydin (26) demonstrated the hepatoprotective effect of QUE in MTX-related hepatotoxicity experimentally. In a study by David et al. (9), numerous inflammatory cell infiltration, congested septal capillaries and abnormally large alveoli in lungs were detected with MTX (0.125 mg/kg) and increasing the dosage of MTX to 0.250 mg/kg resulted in more severe damage. Co-administration of QUE with MTX inhibited the most of the pathologic alterations in the lung tissue. In our study, QUE treatment increased the antioxidant capacity of the lung tissue exposed to MTX by increasing the tissue levels of GSH, CAT and SOD. Moreover, MDA, a product of lipid peroxidation, decreased with QUE treatment. The benefit of QUE treatment was also evident in histopathologic evaluation with amelioration of alveolar structure and regression in hemorrhage, edema, inflammatory cell inflammation and vascular congestion after MTX treatment.

Biochemically, ALA, as a cofactor, is fat and water soluble and involves in the conversion of glucose into energy. The antioxidant capacity of ALA has been shown in many *in vitro* and *in vivo* studies as it acts like a metal chelator, free radical scavenger, regenerator of antioxidant defense and repairer of already injured tissues by ROS (27). Its anti-inflammatory and antioxidant action was shown in many studies (12). The antioxidant effect of ALA on kidney, liver and peripheral nerves, and kidney was shown experimentally (28-30). Guais et al. (31) gathered attention on the fact that when cellular cofactors like lipoic acid and hydroxy citrate were combined with chemotherapeutic agents in cancer models, it resulted in a better basic treatment protocol efficacy. The role of ALA in MTX-induced lung damage was questioned in only one study performed by Arpag et al. (13). The investigators treated animals with ALA (60 mg/kg) for 5 days after a single dose of MTX and found that ALA treatment resulted in significant decreases in the production of MDA, MPO, IL-1 and TNF-α. Also, in histologic evaluation, ALA reduced chronic inflammation and damage. In our study, MTX and ALA were applied in similar doses as those given in literature. In accordance with the above mentioned study, our study also proved the antioxidant effect of ALA in MTX induced pulmonary toxicity. We could not reveal a superiority of QUE on ALA or vice versa in terms of antioxidant capacity or tissue protection.

Study Limitations

Our study has a few apparent limitations. First, to test the protective roles of ALA and QUE, we mainly focused on the tissue markers of oxidative stress. Incorporating immunohistochemical tests of tissue cytokines like IL-6 and TNF-α and apoptosis would certainly increase the power of the study. Second, while demonstrated the protective effect of ALA and QUE individually against MTX-induced lung damage, it would be better to test the combination of two agents in a separate group to understand whether they exert a synergistic effect when used in combination.

Conclusion

Our study verified the fact that MTX causes lung damage through augmentation of oxidative stress in the tissue. Moreover, both ALA and QUE exert a protective role in lung injury by lessening the oxidative stress related to methotrexate exposure. We could not detect any difference in the magnitude of the tissue protection between ALA and QUE against MTX-induced lung damage.

Ethics

Ethics Committee Approval: The experimental procedure was approved by Marmara University Animal Care and Use Committee (protocol number: 50.2022.mar and date: 11.10.2022).

Informed Consent: N/A.

Peer-review: Internally and externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: E.A., A.M., Ş.Ç., Ş.O., Concept: E.A., Ş.O., Design: E.A., Ş.O., Data Collection or Processing: E.A., A.M., Ş.Ç., Ş.O., Analysis or Interpretation: E.A., A.M., Ş.Ç., Ş.O., Literature Search: E.A., A.M., Ş.Ç., Ş.O., Writing: E.A., A.M., Ş.Ç., Ş.O.

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