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Original Article

Therapeutic Properties of Alpha Lipoic Acid and Quercetin on Methotrexate-Induced Oxidative Stress in Rat Liver

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Abstract

Introduction: Methotrexate (MTX), is a widely used drug however it can cause tissue toxicity; but the concurrent use of antioxidants may help reduce this damage. This study investigates the therapeutic properties of alpha-lipoic acid (α-LA) and Quercetin (Que) on MTX-induced oxidative stress in the rat liver.

Methods: The experimental groups were designed as: C: Control, MT: MTX-given group (20 mg/kg, i.p. single dose), MT+α-LA: MTX+α-LA given group (20 mg/kg, i.p. for 5 days), and MT+Q: MTX+Que given group (20 mg/kg, i.p. for 5 days). Liver tissues were analyzed using biochemical, histopathological, and electrophoretic mobility shift assays.

Results: There was a significant increase in lipid peroxidation (LPO) and nitric oxide levels (NO) and a decrease in glutathione (GSH) levels, superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase activities in the MT group compared to the control. MTX caused a significant increase in alkaline phosphatase (ALP) and sialic acid (SA) levels and decreased boron level and tissue factor activity. Biochemical results related to hepatotoxicity were also correlated with the histological examinations. Administration of α-LA and Que regulated LPO, GSH, ALP, SA, and boron levels, as well as SOD and CAT activities. Besides, Que was found to be more effective at restoring these values to normal levels.

Conclusions: The results suggest that α-LA and, in particular, Que may help alleviate MTX-induced liver damage.

Keywords: Alpha lipoic acid, methotrexate, oxidative stress, rat; quercetin;

1. Introduction

Methotrexate (MTX), a folate antimetabolite, is widely utilized in the treatment of various immune-mediated and neoplastic disorders (1). It is clinically employed to manage chronic inflammatory diseases, such as rheumatoid arthritis and psoriasis, and the treatment of certain malignancies, including osteosarcoma (2). The primary mechanism of MTX in oncology is the inhibition of dihydrofolate reductase, which blocks the intracellular production of reduced tetrahydrofolate (3). This suppression limits the synthesis of thymidylate and certain amino acids, thereby inhibiting DNA synthesis and cell proliferation. Additionally, MTX inhibits thymidylate synthase, an enzyme essential for the formation of deoxythymidine monophosphate (dTMP). The disruption of these pathways by MTX impairs the synthesis of DNA, RNA, and ATP, ultimately halting cellular replication (4).

Folate deficiency induced by MTX impacts mitochondrial function because mitochondria require folate for various processes and results in cellular dysfunction that increases the generation of reactive oxygen species (ROS) due to impaired antioxidant defense mechanisms and mitochondrial dysfunction. Besides, MTX inhibits the deamination pathway of adenosine and adenosine monophosphate. Thus, increased extracellular adenosine levels result from low-dose MTX's anti-inflammatory and immunosuppressant effects (3,4). Moreover, MTX causes various toxicological side effects, such as teratogenicity, infertility, and neurotoxicity by forming ROS that damage cell components. Depletion of folate, which causes disturbances in the metabolism of purine and pyrimidine bases, is thought to cause liver damage (5).

Alpha lipoic acid (α-LA), or thioctic acid, is produced in the mitochondria in all prokaryotic and eukaryotic cells. It is mainly found in the heart, kidney, and liver in animal tissues; it is also found in tomatoes, spinach, and broccoli in plants, thus, it can be used as a food supplement. Dihydrolipoic acid, the reduced form of α-LA, repairs functional parts of the cell, such as proteins and lipids, protects DNA from damage in oxidative reactions, and acts as a cofactor for various enzymes in the antioxidant defense system and as a chelating agent for heavy metals (6). Alpha-LA is indicated for the amelioration of complications of several neurological disorders, including diabetic neuropathy and Alzheimer's disease (7).

Quercetin (Que), the most abundant flavonoid in the Mediterranean diet, is found in many seeds, vegetables, and fruits. Que prevents cell death and oxidative damage by scavenging oxygen radicals and chelating metal ions to protect against lipid peroxidation. In addition to its therapeutic activities, Que stimulates mitochondrial biogenesis and inhibits lipid peroxidation (8).

The liver is an organ that performs various tasks in maintaining life. It has many vital functions, such as detoxification, defence against infections, control of energy sources, and production of some proteins. Since MTX is primarily metabolized in the liver by enzymes like dihydrofolate reductase and glutamate hydrolase, the metabolic products of MTX can be toxic to hepatocytes, leading to liver injury. While several studies have examined the effects of antioxidants in conjunction with MTX therapy, there is a relative lack of comparative studies evaluating the differences in their protective effects. Therefore, this study examines the potential protective and therapeutic role of α-LA and Que on MTX-induced liver damage in rats.

2. Methods

2.1. Drugs and Chemicals

MTX (50 mg/5 mL) was obtained from Koçak Farma Pharmaceutical and Chemical Co. (Istanbul, Turkey), and α-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). All other chemicals and solvents used were of best available analytical grades.

2.2. Animals and Ethics Statement

Approval of the study was granted by the Marmara University Animal Care and Use Committee (71.2015.mar). Adult male Wistar albino rats (200-250 g) were purchased from Aziz Sancar Experimental Medicine Research Institute, Istanbul University, Istanbul, Turkiye.

This study used four animal groups, six rats for each group as follows: i) C: the control group, received only physiological saline, ii) intraperitoneal (i.p.) MT: injected MTX (20 mg/kg body weight, i.p. single dose), iii) MT+ α-LA: injected α-LA (20 mg/kg body weight, i.p. for five days) after MTX (20 mg/kg body weight i.p. injection), and iv) MT+Q: injected Que (20 mg/kg body weight i.p. for five days) after MTX (20 mg/kg body weight i.p. injection). On day 6th, rats were sacrificed under anesthesia, liver tissues were resected and divided into two parts – the left half of the liver tissues were taken for histopathological analysis, and the right half of the tissues were taken and stored at – 20°C until the day of the experiment for biochemical analyses.

2.3. Biochemical Analysis

Liver homogenates [10% (w/v)] were prepared with physiological saline (0.9% g NaCl) solution for the biochemical analysis. Total protein level and the parameters investigate oxidative stress; lipid peroxidation (LPO) and nitric oxide (NO), antioxidant parameters; glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and structural and functional parameters; alkaline phosphatase (ALP), sialic acid (SA), boron and tissue factor (TF) were measured spectrophotometrically, also electrophoretic mobility shift assays were done in the liver according to the methods described previously (9).

2.4. Histopathologic Analysis

For light microscopic investigation, liver tissue samples taken from the experimental animals were fixed in 10% neutral buffered formaldehyde at room temperature. The tissues were dehydrated by ascending alcohol series (70%, 90%, 96%, and 100%), cleared in toluene, kept in paraffin in an oven at 60°C, and embedded in paraffin blocks at room temperature. Following the staining of the approximately 5 µm thick paraffin section with hematoxylin and eosin (H&E), they were examined and photographed with a microscope (ZEISS-Axio Scope A, Göttingen, Germany) attached camera (ZEISS Axiocam 105 color, Göttingen, Germany). For histopathological analysis, each section was evaluated by the following criteria: i) vacuolization of hepatocytes and pyknotic nucleus, ii) vascular congestion and dilatation of sinusoids, and iii) activation of the Kupffer cells and inflammatory cell infiltration. Each criterion was scored using the semiquantitative scale as follows: 0 (none), 1 (mild), 2 (moderate), and 3 (severe).

2.5. Statistical Analysis

GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) program was used for statistical analysis. The one-way ANOVA method and Tukey test were used to compare the mean of more than two groups and to interpret the differences between subgroups in the variables with a difference. P value <0.05 was considered significant.

3. Results

3.1. Effects of MTX, α-LA, and Que administration on hepatic oxidative stress markers

The effects of MTX, α-LA, and Que on hepatic ROS generation were investigated by evaluating the levels of LPO and NO (Table 1). Administration of MTX significantly elevated LPO and NO levels than group C ($p<0.01$). Both α-LA and Que reduced LPO levels compared to the ($p<0.01$). Administration of α-LA decreased the NO level significantly compared to the MT group ($p<0.05$). Although a slight decrease was observed in NO level after Que administration to the MT group, the result was insignificant ($p>0.05$).

Table 1. Comparison of LPO and NO values in liver tissues between the experimental groups.

Experimental Groups (n=6)	LPO (nmoL MDA/mg protein)	NO (nmoL MDA/mg protein)
C	5.94±0.57	15.34±2.04
MT	10.31±0.56**	21.34±1.8**
MT+α	7.17±1.48$^{\alpha\alpha}$	19.05±1.18$^{\alpha}$
MT+Q	6.3±1.76$^{\alpha\alpha}$	19.72±0.68

n: number of animals. Values are given as mean ± standard deviation. LPO: Lipid peroxidation, NO: Nitric oxide, C: Control group, MT; Methotrexate group, MT+α-LA: Alpha lipoic acid given methotrexate group, MT+Q: Quercetin given methotrexate group. **$p<0.01$ compared to the group C, $^{\alpha}p<0.05$, $^{\alpha\alpha}p<0.01$ compared to the MT group.

3.2. Effects of MTX, α-LA, and Que on hepatic antioxidant markers

As shown in Table 2, the GSH level and activities of SOD, CAT, and GST were decreased significantly in the MT group than the group C ($p<0.05$). GSH level and, SOD and CAT activities increased significantly in the MT+α-LA group compared to the MT group ($p<0.05$). Besides, Que was also effective in elevating hepatic GSH levels and SOD and CAT activities compared to the MT group ($p<0.05$) (Table 2). Neither α-LA nor Que changed GST activity ($p>0.05$).

Table 2. Comparison of GSH, SOD, CAT, and GST values in liver tissues between the experimental groups.

Experimental Groups (n=6)	GSH (mg/g protein)	SOD (U/mg protein)	CAT (U/mg protein)	GST (U/g protein)
C	4.03±0.16	1.14±0.39	92.62±2.97	94.42±7.7
MT	2.06±0.65**	0.63±0.16*	77.06±6.53**	78.38±8.31*
MT+α	3.04±0.41α	1.10±0.23αα	84.76±3.14α	80.88±6.38
MT+Q	3.16±0.64α	1.11±0.25αα	85.88±3.82α	85.86±4.53

n: number of animals. Values are given as mean ± standard deviation. GSH: Glutathione, SOD: Superoxide dismutase, CAT: Catalase, GST: Glutathione-S-transferase, C: Control group, MT; Methotrexate group, MT+α-LA: Alpha lipoic acid given methotrexate group, MT+Q: Quercetin given methotrexate group. *p<0.05, **p<0.01 compared to the group C, αp<0.05 compared to the MT group.

3.3. Effects of MTX, α-LA, and Que on Hepatic Structural and Functional Markers

The effect of MTX, α-LA, and Que administration on ALP, SA, and boron values and TF activities in liver tissue is shown in Table 3. MTX caused an increase in ALP and SA levels compared to the group C ($p<0.05$). Que decreased ALP and SA levels significantly compared to the MT group ($p<0.05$); however, the decrease with α-LA administration was insignificant. Hepatic boron levels reduced significantly in the MT group, and Que elevated the results significantly ($p<0.05$). Prolonging the time in TF activity means that the activity decreases. TF activity of the MT group reduced significantly than group C ($p<0.05$).

Table 3. Comparison of ALP, SA, Boron and TF values in liver tissues between the experimental groups.

Experimental Groups (n=6)	ALP (U/mg protein)	SA (mg/g protein)	Boron (ppm)	TF activity (second)
C	261.5±16.84	6.06±0.42	2.79±0.48	74.83±7.46
MT	300.8±19.73*	7.23±0.43**	1.32±0.74**	85.83±3.06*
MT+α	281.2±11.75	6.28±0.9	2.2±0.56	78.33±10.07
MT+Q	273±8.36α	6.04±1.11α	2.4±0.62α	85.0±5.72

n: number of animals. Values are given as mean ± standard deviation. ALP: Alkaline phosphatase, SA: Sialic acid, TF: Tissue factor, C: Control group, MT; Methotrexate group, MT+α-LA: Alpha lipoic acid given methotrexate group, MT+Q: Quercetin given methotrexate group. *p<0.05, **p<0.01 compared to the group C, αp<0.05 compared to the MT group.

3.4. SDS-polyacrylamide Gel Electrophoresis Results

The liver samples analyzed through SDS-PAGE showed protein bands at the same position for all samples, with molecular weights ranging from 21 to 136 kD. When assessed through the Image J program, some unsignificant differences in the densities of the protein bands were detected in the electrophoretic examination of the groups. The MT group showed an increase in the band density of the protein with a molecular weight of 117, 110, 84, 78, 38, and 24 kD than those of the group C, and a decrease was observed in these bands in the MT+α-LA and MT+Q groups compared to the MT group (Fig. 1).

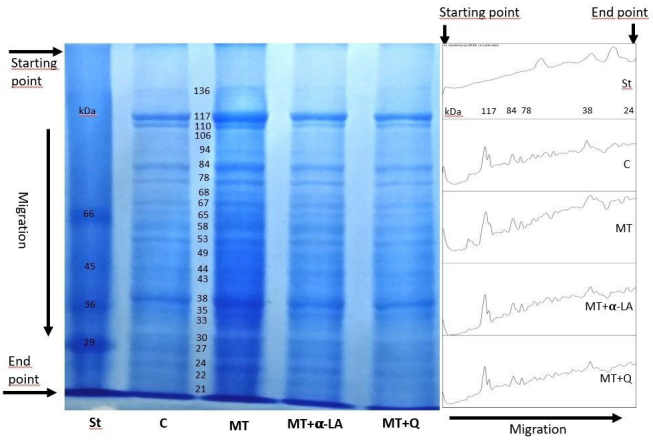

Figure 1. SDS-PAGE patterns of liver tissue protein of the groups. St: Standard protein mixture (bovine albumin:66 kD; egg albumin: 45 kD; glyceraldehyde-3-phosphate: rabbit muscle, 36 kD and bovine carbonic anhydrase: 29 kD); MW: Molecular weight: C: Control group, MT; Methotrexate group, MT+α-LA: Alpha lipoic acid given methotrexate group, MT+Q: Quercetin given methotrexate group.

3.5. Histological Results

A normal morphology of liver parenchyma with regular hepatocytes and sinusoids was seen in the group C (Fig. 2A). In the MT group, severe degeneration of hepatocytes with cytoplasmic vacuolization and pyknotic nucleus, sinusoidal dilatation and congestion, inflammatory cell infiltration, and increased activated Kupffer cells were observed (Fig. 2B). On the other hand, all

these histological alterations were decreased in the liver parenchyma of the MT+α-LA and MT+Q groups compared to the MT group (Figure 2C, D). The histopathologic score of the MT group was significantly higher than the C group ($p<0.01$). On the other hand, the histopathological scores of the MT+α-LA and MT+Q groups were significantly lower compared to the MT group ($p<0.01$). There was no statistically significant difference between the histopathological score of the MT+α-LA group and the MT+Q group (Fig. 2E).

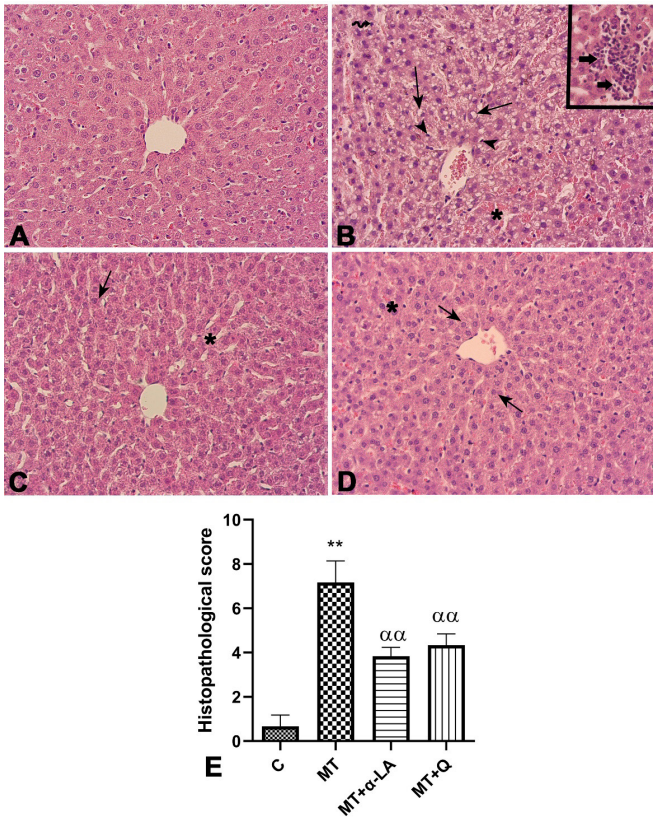

Figure 2. Representative light micrographs (A-D) and the graph of the histopathologic score (E) of the liver in the experimental groups. A: Normal liver parenchyma with hepatocytes and sinusoids was seen in the control group. B: Degenerated hepatocytes with cytoplasmic vacuolization (arrows), pyknotic nucleus (broken arrow), marked sinusoidal dilatation and congestion (asterisk), and Kupffer cells (arrowheads), and inflammatory cells (thick arrow-inset) were seen in the MT group. C: Besides hepatocytes with normal morphology (arrows) in many areas, degenerated hepatocytes and mild sinusoidal dilatation (asterisk) were observed in the MT+α-LA group. D: Besides hepatocytes with normal morphology (arrows) in many areas, degenerated hepatocytes and mild sinusoidal congestion (asterisk) were observed in the MT+Q group. Original magnification: x200, H&E stain. E: **$p<0.01$ compared to the C group, αα$p<0.001$ compared to the MT group. C: Control group, MT; Methotrexate group, MT+α-LA: Alpha lipoic acid given methotrexate group, MT+Q: Quercetin given methotrexate group

4. Discussion

Chemotherapeutic drugs show their anticancer effects by disrupting tumor cells' structures or metabolic pathways. However, they cannot be used selectively against cancer cells and can cause potentially serious side effects at therapeutic doses. The group of antimetabolites, including the frequently used MTX, is also among the treatments that cause serious side effects (2,10). MTX can cause a wide variety of toxicological effects, biochemical dysfunctions, and severe changes in enzyme levels and cellular structures-functions. It is known that MTX is converted to 7-hydroxymethotrexate in the liver, its extracellular metabolite, and stored as its polyglutamated form. However, long-term use of MTX can cause the accumulation of MTX polyglutamates, which MTX hepatotoxicity (2).

According to peer-reviewed studies conducted to understand the MTX-related liver toxicity, it is thought to develop due to oxidative stress (11). Excessive free radicals resulting from oxidative stress overwhelm the body's defence system and disrupt metabolism, and the reason behind the damage is the loss of antioxidant molecules. This situation can result in increased production of reactive substances, such as MDA (a reactive substance of LPO), and NO (a gas-formed free radical in the cell), resulting in hepatotoxicity and tissue damage. Mitochondrial dysfunction and concomitant ROS generation have been proven to mediate autophagic cell death (5).

MTX also can reduce the antioxidant levels, which in turn lowers the effectiveness of the body's natural defense system against ROS. GSH plays a crucial role in maintaining cell integrity, while SOD, CAT, and GST are antioxidants that are effective in detoxification. These antioxidants are essential for protecting the cell against oxidative stress. According to previous studies (5,12,13), MTX administration causes elevated MDA and NO levels. Besides, it was determined that GSH levels and antioxidant enzymes, such as SOD and CAT decreased significantly following MTX intoxication

(14). In a related study (15), GST and CAT activities in the MTX-induced damage group were found to be decreased. It was concluded that MTX administration triggers ROS production, thereby increasing oxidants and reducing antioxidants (16). Consistent with previous findings, our study showed that LPO levels (an indicator of ROS generation) increased significantly in the MTX-treated group compared to Group C; conversely, GSH levels, as well as CAT, GST, and SOD activities, were significantly reduced. According to these results, MTX-induced damage appears to occur through two primary mechanisms: direct cellular toxicity and increased ROS production. Furthermore, MTX inhibits several antioxidant enzymes, thereby elevating LPO levels and exacerbating oxidative stress.

ALP is a vital enzyme in the calcification process; its expression is linked to inflammation, and abnormal levels of ALP may indicate tissue damage (17). SA is a part of the cell structure and plays a crucial role in functioning biological systems. Also, SA determines the inflammatory state and tissue destruction (18). Boron is an important trace element for the body because it regulates biological processes and metabolism. It affects brain functions and skeletal health and regulates the immune system (19). TF is one of the regulators of thrombosis, an initiator of the coagulation cascade in the body. In case of organ damage, TF quickly stops bleeding to prevent excessive blood loss and ensures hemostasis (9). To our knowledge, this is the first study that attempts to investigate both α-LA and Que treatment on MTX-induced tissue damage on boron level and TF activity of the liver tissue in rats. Exposing MTX caused an increase in ALP and SA levels and decreased boron levels and TF activity in the liver, which is a sign of structural and functional changes in the liver tissue caused by MTX administration. Elevated liver ALP levels may be a sign of disrupted normal functions, and the increase in liver SA levels may be a response to tissue self-protection against damage caused by MTX administration. Also, this increase may be due to SA's protective effect in reducing damage by creating a negative charge on cell membranes. Decreased boron levels are also another sign of damage in liver tissue. Boron, which functions as an antioxidant in the organism, may have decreased due to the reduced activities of the other antioxidant enzymes in the tissue. Prolonged TF activity may indicate that the body is delayed in repairing the injury. The electrophoretic examination shows that MTX caused a boost in the band density of the proteins with a molecular weight of 117, 110, 84, 78, 38, and 24 kD compared to the control. The structural and functional proteins in the liver maintain the proper and regular functioning of the tissue. Any disruption or change in the arrangement of these proteins may cause dysfunction or insufficiency of the liver.

Our altered biochemical results related to hepatotoxicity were also correlated with the histological examinations. Both α-LA and Que efficiently neutralized the structural and functional changes resulting from the MTX application; Que, in particular, brought the parameters to a controlled level. Our results suggest that both α-LA and Que treatment on MTX had positive effects on the liver, especially since Que was more effective in neutralizing damage and using substances with antioxidant properties to prevent tissue damage caused by MTX. Alpha LA helps to form cellular antioxidants by making them reusable by regenerating (20). According to previous studies, α-LA treatment to MTX administration elevates SOD activity and GSH levels and reduces MDA levels, and also reverses the decreased antioxidant parameters (21,22). Besides, α-LA has beneficial effects on fatty liver disease patients by reducing ALP levels were reported (23). The antioxidant capacity of α-LA in the inflammatory process by reducing SA levels in plasma was also pointed out (24). In our study, we found that α-LA administration to MT significantly decreased MDA and NO levels, and decreased GSH levels, SOD and CAT activities, compared to the MT group.

The protective effects of Que have been documented in various models of chemically-induced hepatotoxicity (25). For instance, Que administration was shown to normalize elevated MDA and depleted GSH levels in ethanol-induced injury (26). Similarly, Similarly, in MTX-administered rats, Que acted as a therapeutic agent

by reversing MTX-induced lipid peroxidation and restoring SOD activity (27). While most studies, such as those involving lipopolysaccharide/D-galactosamine-induced injury, report an increase in GSH levels following Que treatment (28), contrasting results have been observed in other models. In chlorpyrifos-induced testicular damage, for example, Que was found to decrease previously elevated antioxidant enzymes and ALP levels (29). This reduction in ALP levels suggests that Que possesses membrane-stabilizing activity (30). Consistent with these findings, our research demonstrated that Que administration to MTX-treated rats significantly reduced MDA, ALP, and SA levels while elevating GSH content and the activities of SOD and CAT.

The experimental study demonstrated that MTX induced oxidative stress in rat liver tissue, leading to increased generation of ROS and a significant reduction in antioxidant levels. Both α-LA and Que were effective in reducing oxidative stress and protecting tissue against damage, and thus, they may be promising drugs for preventing MTX-induced liver damage.

5. Conclusion

We experimentally demonstrated that oxidative damage caused by MTX administration could be biochemically reversed by α-LA and Que administration in liver tissue. Que was more effective in normalizing the investigated biochemical and functional parameters, although both α-LA and Que are hepatoprotective agents for oxidative stress. The present study, however, is limited by the absence of parameters in the blood, as the sample amount was insufficient for determining parameters.

Conflict of interest

The authors declare that there are no conflicts of interest associated with this research.

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Ethics approval

Ethical approval for this study was obtained from the Marmara University Animal Care and Use Committee (71.2015.mar)

Author Contributions

Conception/Design of Study – Ş.S, E.A., S.A., Ş.O..; Data Acquisition– Ş.S, Ş.O. ; Data Analysis/Interpretation – Ş.S, E.A., Ş.O.; Drafting Manuscript – Ş.S, E.A., S.A., Ş.O; Critical Revision of Manuscript – E.A., S.A., Ş.O.; Final Approval and Accountability – Ş.S, E.A., S.A., Ş.O.

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