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The Effect of Alpha-Lipoic Acid against Methotrexate on Testicular Damage in Rats

Metotreksat'a Karşı Alfa-Lipoik Asit'in Sıçanlarda Testis Hasarına Etkisi

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Abstract

Aim: The toxic effects of methotrexate, a chemotherapeutic, on the testicles is an important side effect. Methotrexate impairs spermatogenesis and fertility and causes oligospermia. In this study, we aimed to minimize the testicular toxicity, those being the side effects of methotrexate, by using the probable protective effects of α-lipoic acid, a potent antioxidant.

Material and Method: Twenty-eight male Sprague Dawley rats that we employed in this research were separated into three groups as control (0.09% PS) (n=8), methotrexate (20 mg/kg) (n= 10), and methotrexate (20 mg/kg) + α-lipoic acid (100 mg/kg) (n= 10). We performed a histochemical analysis on the testicular tissue of rats using hematoxylin-eosin and Masson's trichrome. We performed an immunohistochemical analysis using inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) primer ab.

Results: The histochemical evaluation revealed a significant decrease in the methotrexate-induced testicular toxicity in the α-lipoic acid-treated groups. On the other hand, TNF-α and iNOS immunostaining results were also observed to support these results.

Conclusion: The treatment use of α-lipoic acid succeeded in protecting against methotrexate-induced testicular damage through an α-lipoic acid-mediated antioxidant and anti-inflammatory mechanisms. α-lipoic acid can be used in combination with methotrexate as a protector against side effects during anticancer therapy. In the present study, it was shown that α-lipoic acid can be used in combination with methotrexate as a protector against side effects during anticancer treatment.

Keywords: Alpha-lipoic acid, anti-inflammatory, methotrexate, testicular damage, oxidative stress

Öz

Amaç: Bir kemoterapötik ajan olan metotreksatın, testisler üzerindeki toksik etkisi önemli bir yan etkidir. Metotreksat, oligospermiye neden olup spermatogenezi ve fertiliteyi bozar. Bu çalışmada, güçlü bir antioksidan olan α-lipoik asidin olası koruyucu etkilerini kullanarak metotreksat'ın yan etkisi olan testiküler toksisiteyi en aza indirmeyi amaçladık.

Gereç ve Yöntem: Araştırmada kullandığımız 28 adet erkek Sprague Dawley cinsi ratlar kontrol (0.09% SF) (n= 8), metotreksat (20 mg/kg) (n= 10) ve metotreksat (20 mg/kg) + α-lipoik asit (100 mg/kg) (n= 10) olmak üzere üç gruba ayrıldı. Ratların testis dokusunda Hematoksilen-Eozin ve Masson Trikrom boyama yöntemlerini kullanarak histokimyasal analizler yapıldı. İndüklenebilir nitrik oksit sentaz (iNOS) ve tümör nekrozis faktörü-alfa (TNF-α) primer antikoru kullanılarak immünohistokimyasal analizler yapıldı.

Bulgular: Histokimyasal değerlendirmede, α-lipoik asitle tedavi edilen gruplarda metotreksat'ın neden olduğu testiküler toksisitede önemli bir azalma olduğu ortaya çıkarıldı. Öte yandan TNF-α ve iNOS immünboyama değerlendirme sonuçlarının da bu sonuçları desteklediği gözlendi.

Sonuç: Tedavide α-lipoik asidin kullanımı, α-lipoik asit aracılı antioksidan ve antiinflamatuvar mekanizmalar yoluyla metotreksat'ın neden olduğu testis hasarına karşı koruma sağladı. α-lipoik asit, kanser tedavisi sırasında yan etkilere karşı koruyucu olarak metotreksat ile birlikte kullanılabilir. Mevcut çalışmada, kanser tedavisi sırasında, α-lipoik asid ve metotreksat'ın kombine kullanımı yan etkilere karşı koruyucu bir ajan şeklinde kullanılabileceği gösterilmiştir.

Anahtar Kelimeler: Alfa-lipoik asit, antiinflamatuvar, metotreksat, testis hasarı, oksidatif stres

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INTRODUCTION

The methotrexate (MTX) drug, folic acid analogue from the point of structure, is a potent cytotoxic substance and has been extensively used against many malignancies as a chemotherapeutic, but the side effects of MTX limit its clinical uses.[1] It is known that the use of chemotherapeutics causes acute toxic effects in several organs, and one of them is the testes. People can become infertile when they receive cancer treatment for other organs not involved in reproduction.[2] This is not an acceptable outcome. Thus, this side effect of MTX should be reduced or destroyed. In our study, we aimed to minimize or eliminate the side effects of MTX.

The complete mechanisms underlying MTX testicular toxicity are unclear. It is known that MTX inhibits cytosolic forms of nicotinamide adenosine diphosphate (NADP)-dependent dehydrogenase enzymes.[3] NADPH is the oxidatively reduced form of NADP. Being an antioxidant enzyme that resides in cytoplasm, glutathione reductase, utilizes NADPH to conserve level of the reduced form of cellular glutathione and keeps safe against the effects of reactive oxygen species (ROS). If their level is not kept under control, damages biomolecules like lipids, proteins, and DNA and eventually leads to cell death.[4-6] In the animals treated by MTX, antioxidant levels reduced and oxidant levels enhanced, a situation that contributes to MTX-induced oxidative stress.[7,8] MTX is used in low doses as anti-inflammatories in diseases such as anti-rheumatic diseases. However, it has an inflammatory effect when used at high doses as a chemotherapeutic to treat various malignancies, including lymphoma, head and neck carcinoma, osteosarcoma, breast carcinoma and acute lymphoblastic leukemia due to the toxic effect of the tissue.[9-11] As a result, MTX induces oxidative stress and inflammation in tissues. Inducible NO synthase (iNOS) is a predominant parameter of ROS-mediated tissue damage due to produce NO which is free oxygen radical.[12] iNOS is also known as an inflammatory cytokine-like tumor necrosis factor-α (TNF-α), meaning it increases inflammatory conditions.[13]

α-Lipoic acid (ALA) is found in some foods and is synthesized de novo in the body. It is a natural antioxidant, which is why it is so important in terms of easy retrieval. Lipoic acid, which prevents free radical damage, is unique among antioxidants in terms of its ability to dissolve in oil and water.[14] Its reduced form, Dihydrolipoic acid, is more biologically active. Many studies have mentioned that ALA has antioxidant and anti-inflammatory effects, and it creates these properties with different mechanisms.[15] The antioxidant effects of ALA are due to increase iNOS expression. It provides the formation of NO which is one of the free oxygen radical. ALA also shows this effect by increasing antioxidant enzymes, namely glutathione reductase, the restorating of the reduced / oxidized glutathione ratio, and lowering the NADP level. ALA's anti-inflammatory effects are attributable to repression of inflammatory activities from IL-6, TNF-α and NF-kB and elevation of anti-inflammatory proteins, like nuclear erythroid 2- related factor (Nrf2).[16]

In this experiment, a rat model was conceived to evaluate whether ALA had any protective effects opposed to ROS-mediated damage caused via MTX-induced testicular injury. For that purpose, immunohistochemical receptor activity and histopathological changes were evaluated in MTX-administered animals with and without ALA treatment. We believe that the outcomes of this research will play a significant role in showing that it is probable to reduce testicular toxicity, which is one of the most crucial side-effects that restrict the effective usage of a chemotherapeutic drug for instance MTX, with antioxidant and anti-inflammatory agents such as ALA.

MATERIAL AND METHOD

Chemicals

Hematoxylin (HX86017674), eosin (HX378237), ethanol 96% (1009712500), ksilen (1086612500), entellan (HX87112361), and hydrochloric acid (143007) were obtained from Merck. iNOS antibody (ab15323) and TNF-α (ab66579) were obtained Abcam. Hydrogen peroxide (H2O2) (ThermoFisher Scientific, FSH40069), acetic acid (Fluka, 52220) were used. Phosphate buffered saline (PBS999), SensiTek HRP Anti-Polyvalent (SHP125) and DAB chromogen (ADK125) were obtained from ScyTek. Picric acid (P6744), biebrich scarlet-acid solution (HT151), fosfotungistik acid solution (HT152), fosfomolibdik acid solution (HT153), aniline blue (HT154) solution were obtained from Sigma-Aldrich.

Experimental Animals

Twenty-eight male-gendered Sprague-Dawley stock rats were employed in the experiment. The rats were obtained from the Experimental Animals Research Laboratory Production Unit of the School of Medicine, Süleyman Demirel University. The rats were maintained in regulated conditions of temperature (22±2°C) and humidity (50±10%) on a 12 h light/dark cycle during the experiment. All rats had ad libitum access to food and water (Standard Issue Rat Chow of Animal Food Institution). This study was approved by the Local Ethical Committee on Animal Research of Süleyman Demirel University, Isparta and was performed according to ethical rules (Protocol number: 15.09.2022 06/70).

Experiment Protocols

Animals were split into three groups at random. Group number 1 (C) was the control group consisting of eight animals (n=8). In this group, for ten days starting from the first day, animals have taken intraperitoneally (i.p.) administered physiologic saline (PS) (0.09% NaCl) in approximately the equal volume same as the drugs given to the other groups on the similar day. Group number 2 (MTX) was the methotrexate group consisting of ten animals (n=10). In this group, on the fourth day of the study, MTX (Methotrexate® available in 50 mg/ml, Koçak Farma, injectable solution) was administered in a single dose of 20 mg/kg i.p.[17] PS was given i.p. on days when the drug was not administered. Group number 3 (MTX

+ ALA) was the methotrexate + α-lipoic acid group consist of ten animals (n=10). In this group, MTX was administered only on the fourth day of the study, same as in Group number 2. ALA (Thioctacid® 600T available in 600 milligrams in 24 milliliters per ampoule, MEDA Pharma, injectable solution) was administered in a dose of 100 mg/kg i.p.[18] for ten days starting on the first day.

Sample Collection and Preparation

At the conclusion of the experiment (the eleventh day), rats were anesthetized under a mixture of xylazine hydrochloride (10 mg/kg) + ketamine hydrochloride (90 mg/kg) and then euthanized. Subsequently, the bilateral testes were isolated, the fat was separated, and the testes were weighed and subsequently fixed in 10% neutral formalin for 24 h for histochemical and immunohistochemical investigations. After fixation, the testes were dehydrated stepwise with an ethanol series, at least 1 h for each step. Then, testicular tissues were embedded in paraffin, sectioned at 3-4 μm thickness, mounted on microscope slides, and dried overnight at room temperature.

Histochemical Analysis

The next preparations were deparaffinized and dehydrated. After deparaffinization and dehydration, the testis sections were stained with routine hematoxylin and eosin (H-E) stain for a general histochemical evaluation and for an examination of cellular toxicity. Sections were stained with Masson's trichrome to better observe the interstitial area. The prepared slides were examined and imaged with a camera-equipped light microscope (DM500, Leica, Germany). Testicular specimens were evaluated for typical histopathological features associated with MTX-induced testicular toxicity (spermatogenetic and interstitial degeneration), and these parameters were scored as 0-3 damage score (0=no, 1=mild, 2=moderate, 3=severe).

iNOS and TNF-α Immunoreactivity Analysis

To detect iNOS and TNF-α immunoreactivity in the testes, we have done the following immunohistochemistry procedure. Paraffin-embedded testicular tissues were cut into thin (3-4 μm) sections with a sliding microtome (SM2000R, Leica, Germany) and sections mounted on poly-l-lysine coated glass slides. The next preparations were deparaffinized and dehydrated. Then, the testis sections were first immersed in 3% hydrogen peroxide (ready-to-use, Thermo Scientific) to incapacitate intrinsic peroxidase activity and then in super-block (ready-to-use, ScyTek) to prevent non-specific antibody attachment. They were later incubated overnight at 4°C with the rabbit polyclonal to iNOS antibody (Abcam, Cambridge, USA) diluted 1:50 in antibody diluent and the rabbit polyclonal to TNF-α antibody (Abcam, Cambridge, USA) diluted 1:100 in antibody diluent. After washing in phosphate-buffered saline (PBS), the spot of the immunoreaction was visualized through incubating the sections sequentially with a biotinylated goat anti-polyvalent antibody, horseradish peroxidase-conjugated streptavidin (ready-to-use ScyTek Laboratories, Logan, UT), and 3,3′-diaminobenzidine solution (5.6% ml, ScyTek Laboratories, Logan, UT). Biotinylated goat anti-polyvalent antibody (ready-to-use, ScyTek Laboratories, Logan, UT) was used instead of the anti-iNOS and anti-TNF-α antibody for a negative control. After washing with the PBS, sections were lightly counterstained with hematoxylin solution. Next, the sections were passed through a graded series of ethanol, cleared by xylene, and covered with entellan. These specimens were evaluated according to the staining intensity and scored as 0-3. The sections were then examined and imaged with a camera-equipped light microscope (DM500, Leica, Germany).

Statistical Analysis

The statistical analysis was accomplished by using a Windows® compatible SPSS® 16.0 program. Results were examined as histological measurements. For these histopathological findings, the Kruskal-Wallis's test was the nonparametric test, and the Mann–Whitney U test was used for comparisons of measurements among the two groups. The level of significance was taken as $p < 0.05$. The values were conveyed as mean±standard deviation.

RESULTS

Effect of ALA Treatment on Testicular Histochemistry

As a result of a histochemical investigations of testicular tissue, a usual histological structure was found in the control group. Effects of MTX treatment and the intervention of ALA on the cellular alterations were investigated by a histological evaluation of the testes. The spermatogenetic degeneration (loss of spermatozoa in the lumen and vacuolization of germinal epithelium), interstitial degeneration (interstitial fibrosis, interstitial congestion, and interstitial mononuclear cell infiltration) were considered in the assessment of the testicular toxicity. Significant regression of these parameters was identified in the ALA treatment group.

Histopathological analysis of the testes was done based on spermatogenetic degeneration and the interstitial degeneration score (**Figure 1, 2**). A significant rise in the testicular damage was marked with the one with treatment of MTX 20 mg/kg compared to the control group ($p < 0.05$). While the mean spermatogenetic damage score was 2.80±0.42, interstitial damage was 2.70±0.67 in the MTX-treated group, they were respectively 0.44±0.72 and 0.55±0.72 in the ALA-treated group. It seems that ALA treatment significantly reduced MTX-induced testicular damage (**Table 1**).

Table 1. Scoring of histopathological changes in groups of testicular tissue

	Control	MTX	ALA-treated
Spermatogenetic degeneration	0.25±0.46	2.80±0.42[a]	0.44±0.72
Interstisial degeneration	0.12±0.35	2.70±0.67[a]	0.55±0.72

Values are expressed in mean±SD, [a]Significantly changed when compared with the control group ($p < 0.05$)

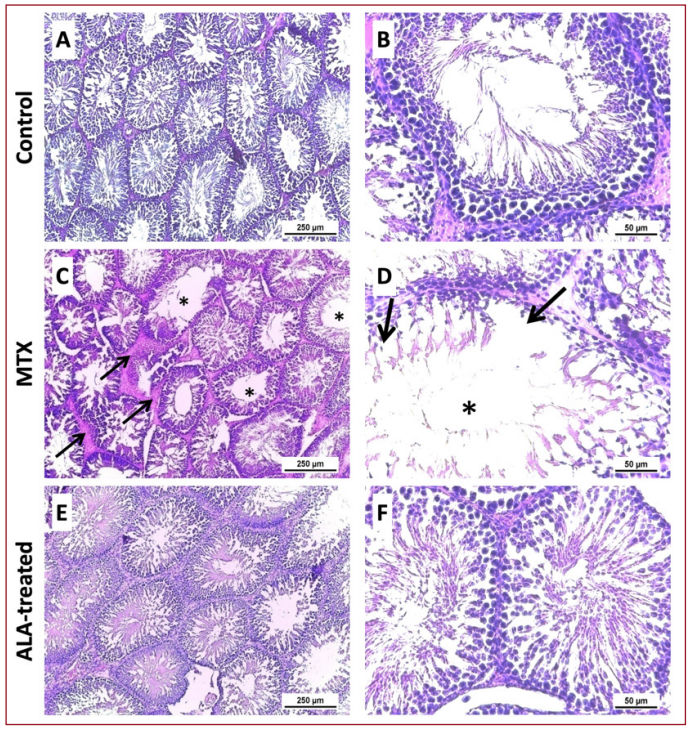

Figure 1: Effect of methotrexate (MTX), alpha lipoic acid (ALA) on testicular tissue. Histopathological sections from control group (A, B) and ALA-treated group (E, F) showed near-normal testis histology. Whereas, sections from the MTX group (C, D) showed extensive testicular damage, such as loosing of spermatozoa in the lumen (black asterix), vacuolization of germinal epithelium (thin arrow), interstitial fibrosis (thick arrow). H–E staining, scale bar 250 µm (×100 magnification), and scale bar 50 µm (×400 magnification).

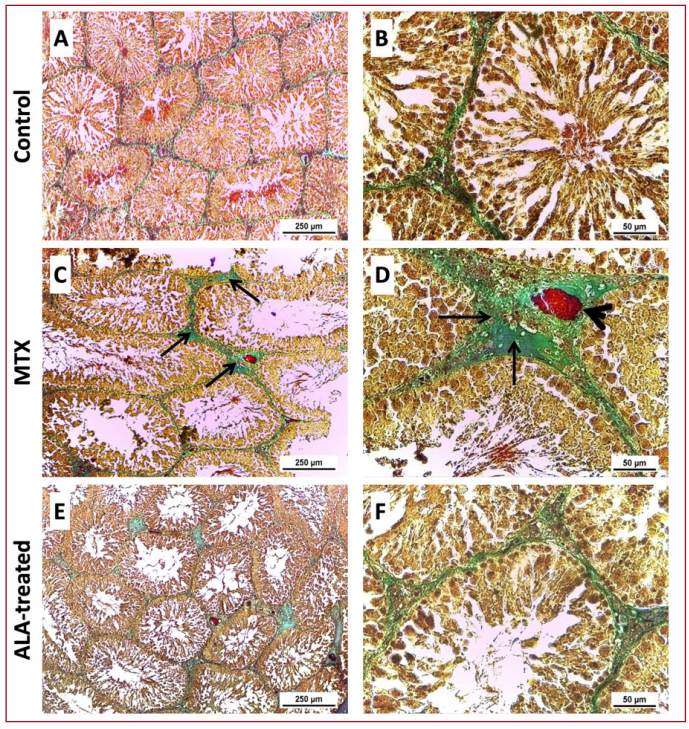

Figure 2: Effect of Methotrexate (MTX), alpha lipoic acid (ALA) on testicular tissue. Histopathological sections are stained with a stain that shows connective tissue well. Control group (A, B) and ALA-treated group (E, F) showed near-normal testis histology. Whereas sections of the MTX group (C, D) showed extensive testicular damage, such as interstitial fibrosis (arrow), interstitial congestion (arrowhead). Masson thricrome staining, scale bar 250 µm (×100 magnification), and scale bar 50 µm (×400 magnification).

Effect of ALA Treatment on iNOS and TNF-α Immunoreactivity

Strong iNOS and TNF-α staining were noted in the testes of the rats that took MTX treatment as compared to the control group ($p < 0.05$). The ALA treatment led to a significant reduction in iNOS and TNF-α positive receptors compared to the MTX group (**Figure 3**, **Table 2**).

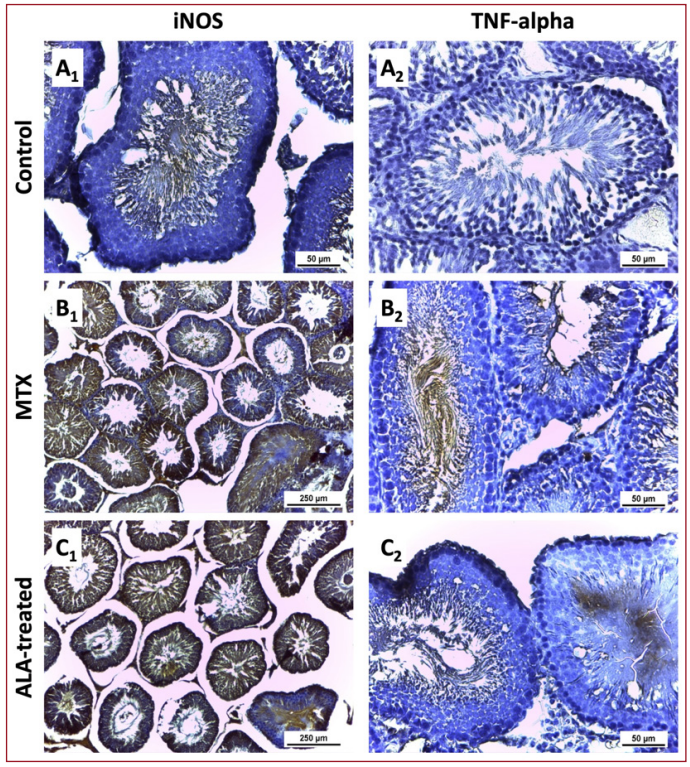

Figure 3: Effect of methotrexate (MTX), alpha lipoic acid (ALA) on testicular tissue. Immunohistological sections from control group (A_1, A_2) showed less staining intensity and ALA-treated (C_1, C_2) group showed moderate staining intensity for both iNOS and TNF-α. On the contrary, sections of the MTX group (B_1, B_2) showed strong staining intensity. iNOS and TNF-α immunostaining, scale bar 250 µm (×100 magnification), and scale bar 50 µm (×400 magnification).

Table 2. iNOS and TNF-α immunoreactivity grades in groups of testicular tissue

	Control	MTX	ALA-treated
iNOS immunoreactivity	(+)	(+++)[a]	(++)
TNF-α immunoreactivity	(-/+)	(+++)[a]	(+)

Values are expressed in mean±SD, [a]Significantly changed when compared with the control group ($p < 0.05$)

DISCUSSION

MTX, an antagonist of folate, is broadly utilized in the therapy of psoriasis, malignancies and rheumatoid arthritis.[19] Toxicities of MTX in different organs in the gastrointestinal, urinary and lung systems have already been reported.[20-22] Additionally, the testicular toxicity of MTX is a significant adverse effect and may lead to infertility. MTX impairs spermatogenesis and fertility and causes oligospermia.[2,7] While some sources mention that this damage returns after the use of cytotoxic drugs, some sources recommend

sperm-freezing before treatment against possible irreversible damage.[23] Various theories have been proposed for the machineries behind MTX toxicity, involving the interference of oxidative stress,[24] inflammation, and apoptosis.[25] In this study, we aimed to inspect whether there will be a difference in the iNOS immunoreactivity of the MTX-administrated animal group and also whether application of ALA could forestall these effects. In this study, MTX clearly caused alteration in the immunohistochemical findings confirmed by the distorted histological image of testes specimens such as spermatogenetic and interstitial degeneration, which was in accord with previous studies.[26-28] Mechanisms that are participating in MTX-induced testicular damage were studied, and our findings demonstrated MTX-induced oxidative stress, marked with increased iNOS, which was in concurrence with previous studies.[20] Additionally, recent studies have shown that MTX leads to iNOS expression and thus increases NO level which causes oxidative stress.[29,30] In another study, it is mentioned that 20 mg/kg single dose MTX administration increases NO and TNF-α expressions in the liver.[31] This imbalance between oxidant/antioxidant and inflammatory signaling in other organs may also be the cause of MTX-induced testicular toxicity. Here, we showed that ALA, with well-documented powerful antioxidant properties,[32] confers protection against MTX-induced testicular damage. MTX has been reported to induce iNOS in the small intestine,[33] the brain,[34] and liver and kidney (20). Here, we observed that MTX can also be caused ROS-mediated testicular damage, shown through an increase in iNOS immunoreactivity. Additionally, in this study, we showed that MTX elevated the expression of TNF-α as did the expression of iNOS. It has been reported that methotrexate increases TNF-α levels in rat brains and the findings are associated with cognitive and behavioral disorders.[35] Earlier researches have stated that MTX increased the levels of TNF-α in kidney and liver tissues.[36-38] To the very best of our understanding, no validation of MTX stimulation of iNOS and TNF-α expression in testis tissue was proposed so far.

The ALA-treated group that received 100 mg/kg ALA for 10 days and 20 mg/kg MTX administered in a single dose on the fourth day showed improved spermatogenesis and also testis histology when compared with MTX alone. In this research, we also showed that ALA ameliorates MTX-induced testis toxicity, enhancing the histology of the testis when compared with MTX alone. Although ALA, also known as thioctic acid, is an endogenous compound, it can also be obtained exogenously from some plant foods. It has complementary roles in damaged tissue associated with a strong antioxidant and anti-inflammatory effect.[39,40] The processes participating included reversing MTX-induced testicular damage, as marked with decreased iNOS and TNF-α immunoreactivity. In addition, ALA reversed MTX-induced inflammatory hints because it notably reduced the level of TNF-α, which is consistent with the earlier stated antioxidant/anti-inflammatory effects of ALA.[20] Other studies have also shown that ALA has anti-inflammatory effect by decreasing the level of TNF-α.[41,42] Furthermore, ALA reversed MTX-induced ROS-mediated damage, inflammation, and disruption in the testis as evidenced by decreasing iNOS and TNF-α immunoreactivity and histopathological improvement compared with MTX alone, which is in agreement with prior studies.[20] Here, we showed that the ALA-treated decline in NO levels was attributable to downregulation of iNOS expressions in the testis. We recently reviewed the interaction among apoptotic, inflammatory, nitrosative and oxidative pathways[43] and are thus cognizant of difficult it is to ascertain whether the association among these pathways is a reason or a result of one another. Mitochondria is the primary organelle that produces ROS and is the main target of ROS-mediated damage[44] This is supported by the fact that Kolli et al. mention MTX-induced intestinal damage, including oxidative stress and mitochondrial dysfunction.[45] The improvement effect of ALA on mitochondrial performance is mentioned in the literature and it can be said that it minimizes an MTX-induced damage by this mechanism. ALA acts as an enzymatic cofactor that can regulate mitochondrial biogenesis. The antioxidant capacity of ALA is associated with two thiol groups that can be oxidized or reduced, and its effect on improving mitochondrial performance may be explained by this.[46] In conclusion, we postulate that MTX is initiating the testis damage through the stimulation of oxidative stress and an inflammatory process. ALA grants protection from MTX-induced toxicity through blocking these mechanisms.

In this experiment, we studied the molecular machinery behind protective effects of ALA against MTX-induced toxicity. iNOS is in charge for the generation of NO and is considered to be the primary stage originator of toxicity beneath the oxidative stress.[47] Increased levels of iNOS were detected in MTX-received rats. To the best of our understanding, it is the first time such a finding has been reported in MTX-induced testicular damage. ALA caused a noteworthy decline in iNOS and TNF-α levels, countering the MTX effect. The mechanism which ALA down-regulated iNOS is unclear, but this may be owing to the reduction in TNF-α establishment or because of its direct scavenging activity on NO through its carboxyl group. We also examined the expressions of TNF-α, a proinflammatory cytokine, in reply to MTX exposure, and we noticed an important increase in its expression on testicular tissue. TNF-α is found in the testis at seminiferous tubules and is highly up-regulated in pathophysiological conditions.[48,49] ALA might prevent reactive intermediates participating in testicular damage by reducing TNF-α formation.[50]

CONCLUSION

Treatment use of ALA has been successful in preserving from MTX-induced testicular damage through ALA-mediated anti-inflammatory and antioxidant processes. In the present study, it was shown that ALA can be used in combination with MTX as a protector against side effects during anticancer treatment.

ETHICAL DECLARATIONS

Ethics Committee Approval: This study was approved by the Local Ethical Committee on Animal Research of Süleyman Demirel University, Isparta and was performed according to ethical rules (Protocol number: 15.09.2022 06/70).

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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REFERENCES

1. Alarcoan GS, Tracy IC, Blackburn WD et al. Methotrexate in rheumatoid arthritis. Toxic effects as the major factor in limiting long-term treatment. Arthritis Rheum 1989;32(6):671-6.
2. Boekelheide K. Mechanisms of Toxic Damage to Spermatogenesis. J Natl Cancer Inst Monogr 2005 ;2005(34):6-8.
3. Safaei F, Mehrzadi S, Khadem Haghighian H et al. Protective effects of gallic acid against methotrexate-induced toxicity in rats. Acta Chir Belg 2018;118(3):152-60.
4. Miyazono Y, Gao F, Horie T. Oxidative stress contributes to methotrexate-induced small intestinal toxicity in rats. Scand J Gastroenterol 2004;39(11):1119-27.
5. Sener G, Eksioglu-Demiralp E, Cetiner M et al. Beta-glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immunomodulatory effects. Eur J Pharmacol 2006;542(1-3):170-8.
6. Vardi N, Parlakpinar H, Cetin A, Erdogan A, Cetin Ozturk I. Protective effect of beta-carotene on methotrexate-induced oxidative liver damage. Toxicol Pathol 2010;38(4):592-7.
7. Daggulli M, Dede O, Utangac MM, et al. Protective effects of carvacrol against methotrexate-induced testicular toxicity in rats. Int J Clin Exp Med 2014;7(12):5511-6.
8. Jahovic N, Cevik H, Sehirli AO et al. Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. J Pineal Res 2003;34(4):282-7.
9. Asci H, Ozmen O, Ellidag HY et al. The impact of gallic acid on the methotrexate-induced kidney damage in rats. J Food Drug Anal 2017;25(4):890-7.
10. Selimoglu Sen H, Sen V, Bozkurt M et al. Carvacrol and pomegranate extract in treating methotrexate-induced lung oxidative injury in rats. Med Sci Monit 2014;20:1983-90.
11. Widemann BC, Balis FM, Kempf-Bielack B et al. High-dose methotrexate-induced nephrotoxicity in patients with osteosarcoma. Cancer 2004;100(10):2222-32.
12. Aktan F. iNOS-mediated nitric oxide production and its regulation. Life Sci 2004;75(6):639-53.
13. de Castro MRT, Ferreira APO, Busanello GL et al. Previous physical exercise alters the hepatic profile of oxidative-inflammatory status and limits the secondary brain damage induced by severe traumatic brain injury in rats. J Physiol 2017;595(17):6023-44.
14. Bilska A, Wlodek L. Lipoic acid - the drug of the future? Pharmacol Rep 2005;57(5):570-7.
15. Anthony RM, MacLeay JM, Gross KL. Alpha-Lipoic Acid as a Nutritive Supplement for Humans and Animals: An Overview of Its Use in Dog Food. Animals 2021;11(5):1454.
16. Tibullo D, Li Volti G, Giallongo C et al. Biochemical and clinical relevance of alpha lipoic acid: antioxidant and anti-inflammatory activity, molecular pathways and therapeutic potential. Inflamm Res 2017;66(11):947-59.
17. Asvadi I, Hajipour B, Asvadi A et al. Protective effect of pentoxyfilline in renal toxicity after methotrexate administration. Eur Rev Med Pharmacol Sci 2011;15(9):1003-9.
18. Ozbal S, Ergur BU, Erbil G et al. The effects of alpha-lipoic acid against testicular ischemia-reperfusion injury in Rats. ScientificWorldJournal 2012;2012:489248.
19. Al Maruf A, O'Brien PJ, Naserzadeh P et al. Methotrexate induced mitochondrial injury and cytochrome c release in rat liver hepatocytes. Drug Chem Toxicol 2018;41(1):51-61.
20. Armagan I, Bayram D, Candan IA et al. Effects of pentoxifylline and alpha lipoic acid on methotrexate-induced damage in liver and kidney of rats. Environ Toxicol Pharmacol 2015;39(3):1122-31.
21. Conway R, Low C, Coughlan RJ et al. Methotrexate use and risk of lung disease in psoriasis, psoriatic arthritis, and inflammatory bowel disease: systematic literature review and meta-analysis of randomised controlled trials. BMJ 2015;350:h1269.
22. Yamamoto A, Itoh T, Nasu R et al. Sodium alginate inhibits methotrexate-induced gastrointestinal mucositis in rats. Biol Pharm Bull 2013;36(10):1528-34.
23. Semet M, Paci M, Saïas-Magnan J et al. The impact of drugs on male fertility: a review. Andrology 2017;5(4):640-63.
24. Yulug E, Turedi S, Alver A et al. Effects of resveratrol on methotrexate-induced testicular damage in rats. ScientificWorldJournal 2013;2013:489659.
25. Ali N, Rashid S, Nafees S et al. Protective effect of Chlorogenic acid against methotrexate induced oxidative stress, inflammation and apoptosis in rat liver: An experimental approach. Chem Biol Interact 2017;272:80-91.
26. Morgan AM, Ibrahim MA, Noshy PA. Reproductive toxicity provoked by titanium dioxide nanoparticles and the ameliorative role of Tiron in adult male rats. Biochem Biophys Res Commun 2017;486(2):595-600.
27. Shah NA, Khan MR. Increase of glutathione, testosterone and antioxidant effects of Jurenia dolomiaea on CCl4 induced testicular toxicity in rat. BMC Complement Altern Med 2017;17(1):206.
28. Singh A, Arvinda S, Singh S et al. IN0523 (Urs-12-ene-3alpha,24beta-diol) a plant based derivative of boswellic acid protect Cisplatin induced urogenital toxicity. Toxicol Appl Pharmacol 2017;318:8-15.
29. Dabrowska M, Uram L, Zielinski Z et al. Oxidative stress and inhibition of nitric oxide generation underlie methotrexate-induced senescence in human colon cancer cells. Mech Ageing Dev 2018;170:22-9.
30. El-Sheikh AA, Morsy MA, Abdalla AM, et al. Mechanisms of thymoquinone hepatorenal protection in methotrexate-induced toxicity in rats. Mediators Inflamm 2015;2015:859383.
31. Najafi N, Mehri S, Ghasemzadeh Rahbardar M, et al. Effects of alpha lipoic acid on metabolic syndrome: A comprehensive review. Phyther Res 2022;36(6):2300-23.
32. Lebda M, Gad S, Gaafar H. Effects of lipoic Acid on acrylamide induced testicular damage. Mater Sociomed 2014;26(3):208-12.
33. Leitao RF, Brito GA, Oria RB et al. Role of inducible nitric oxide synthase pathway on methotrexate-induced intestinal mucositis in rodents. BMC Gastroenterol 2011;11:90.
34. Yang M, Kim JS, Kim J et al. Acute treatment with methotrexate induces hippocampal dysfunction in a mouse model of breast cancer. Brain Res Bull 2012;89(1-2):50-6.
35. Gupta P, Makkar TK, Goel L, Pahuja M. Role of inflammation and oxidative stress in chemotherapy-induced neurotoxicity. Immunol Res 2022;70(6):725-41.

36. Ibrahim MA, El-Sheikh AA, Khalaf HM et al. Protective effect of peroxisome proliferator activator receptor (PPAR)-alpha and -gamma ligands against methotrexate-induced nephrotoxicity. Immunopharmacol Immunotoxicol 2014;36(2):130-7.
37. Mukherjee S, Ghosh S, Choudhury S et al. Pomegranate reverses methotrexate-induced oxidative stress and apoptosis in hepatocytes by modulating Nrf2-NF-kappaB pathways. J Nutr Biochem 2013;24(12):2040-50.
38. Hafez HM, Ibrahim MA, Ibrahim SA et al. Potential protective effect of etanercept and aminoguanidine in methotrexate-induced hepatotoxicity and nephrotoxicity in rats. Eur J Pharmacol 2015;768:1-12.
39. Barletta MA, Marino G, Spagnolo B et al. Coenzyme Q10 + alpha lipoic acid for chronic COVID syndrome. Clin Exp Med 2022;DOI: 10.1007/s10238-022-00871-8.
40. Heidari R, Ahmadi A, Mohammadi H et al. Mitochondrial dysfunction and oxidative stress are involved in the mechanism of methotrexate-induced renal injury and electrolytes imbalance. Biomed Pharmacother 2018;107:834-40.
41. Moura FA, de Andrade KQ, dos Santos JC et al. Lipoic Acid: its antioxidant and anti-inflammatory role and clinical applications. Curr Top Med Chem 2015;15(5):458-83.
42. Barut EN, Engin S, Saygın İ et al. Alpha-lipoic acid: A promising adjuvant for nonsteroidal anti-inflammatory drugs therapy with improved efficacy and gastroprotection. Drug Dev Res 2021;82(6):844-51.
43. Taye A, El-Sheikh AA. Lectin-like oxidized low-density lipoprotein receptor 1 pathways. Eur J Clin Invest 2013;43(7):740-5.
44. Zhang T, Zhang D, Zhang Z, et al. Alpha-lipoic acid activates AMPK to protect against oxidative stress and apoptosis in rats with diabetic peripheral neuropathy. Hormones (Athens). 2022;10.1007/s42000-022-00413-7.
45. Kolli V, Natarajan K, Isaac B et al. Mitochondrial dysfunction and respiratory chain defects in a rodent model of methotrexate-induced enteritis. Hum Exp Toxicol 2014;33(10):1051-65.
46. dos Santos SM, Romeiro CFR, Rodrigues CA et al. Mitochondrial Dysfunction and Alpha-Lipoic Acid: Beneficial or Harmful in Alzheimer's Disease? Oxid Med Cell Longev 2019;2019:1-14.
47. Amin A, Abraham C, Hamza AA et al. A standardized extract of Ginkgo biloba neutralizes cisplatin-mediated reproductive toxicity in rats. J Biomed Biotechnol 2012;2012:362049.
48. El-Sheikh AA, Morsy MA, Al-Taher AY. Multi-drug resistance protein (Mrp) 3 may be involved in resveratrol protection against methotrexate-induced testicular damage. Life Sci 2014;119(1-2):40-6.
49. Akhigbe R, Ajayi A. Testicular toxicity following chronic codeine administration is via oxidative DNA damage and up-regulation of NO/TNF-α and caspase 3 activities. Yenugu S, editor. PLoS One 2020;15(3):e0224052
50. Huk-Kolega H, Ciejka E, Skibska B et al. Influence of lipoic acid on the level of TNF-alpha in spleen homogenates. Pol Merkur Lekarski 2014;36(216):379-81.