RESEARCH ARTICLE

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Melatonin-lycopene combination improves methotrexate-induced liver toxicity in rats via anti-inflammatory effect

Ratlarda, melatonin-likopen kombinasyonu antiinflamatuvar etki ile metotraksat ilişkili toksisiteyi iyileştirebilir

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ABSTRACT

Introduction: This study aimed to investigate the effect of melatonin (MLT)-lycopene (LYC) combination on methotrexate-induced hepatotoxicity (MIT).

Materials and Methods: Twenty-seven Sprague-Dawley adult male rats were divided into 3 equal groups: Group I= control group, fed with oral corn oil; Group II, a single dose of methotrexate (MTX) was injected into the rats and they were fed with oral corn oil; Group III, rats were treated with a melatonin-lycopene combination for 5 days after a single dose of MTX intraperitoneal. Serum aspartate and alanine aminotransferase activities were measured as hepatic function tests. The supernatant fraction of the liver tissue homogenate was used to measure the levels of total antioxidant capacity (TAC) and total oxidant status (TOS) in order to assess the presence of oxidative stress, tumour necrosis factor-alpha, and interleukin 1beta, which in turn indicate the presence of inflammation. Five sections from each rat liver tissue were prepared for histopathological examination and stained with hemotoxil-eosin. Then the specimens were examined under a light microscope and was evaluated semi-quantitatively.

Results: This study showed that serum aspartate aminotransferase and tissue proinflammatory cytokines such as tumour necrosis factor-alpha and interleukin 1beta increased with MTX use and decreased significantly when using a combination of MLT-LYC, but oxidative stress parameters such as TOS and OSI did not change in MIT.

Conclusion: The LYC-MLT combination improved the acute toxic effects of MTX on the liver. It improved both liver function and the histopathology level. However, these effects seem to be associated with the anti-inflammatory effect than the reduction of oxidative stress.

Keywords: *Methotrexate-induced hepatotoxicity, melatonin, lycopene, oxidative stress, inflammation*

ÖZ

Giriş: Bu çalışmada melatonin-likopen (MLT-LYC) kombine tedavisinin metotreksatla (MTX) indüklenmiş hepatotoksisite üzerinde etkisini araştırmak amaçlanmıştır.

Materyal ve Metod: 27 Sprague-Dawley cinsi erkek rat 3 eşit gruba ayrıldı; Grup I sadece oral mısır yağıyla beslendi, Grup II ratlara tek doz MTX enjekte edildi ve oral mısır yağıyla beslendi, Grup III ise ratlar tek doz intraperitoneal MTX uygulamasından sonra 5 gün boyunca MLT-LYC kombinasyonu ile tedavi edildi ve oral mısır yağıyla beslendi. Genel anestezi altında dekapitasyonla ötenaziyi müteakiben kan örnekleri ve karaciğer dokusu alındı. Serum aspartat aminotransferaz ve alanin aminotransferaz aktivitelerinin ölçümü ile karaciğer fonksiyonları değerlendirildi. Karaciğer doku homojenatının supernatant fraksiyonu kullanılarak total antioksidan kapasite (TAC), total oksidan seviye (TOS), tümör nekroz faktörü-alfa (TNF-α) ve interlökin-1beta (IL-1β) seviyeleri ölçüldü. Histopatolojik inceleme için her bir rat dokusundan beş kesit hazırlandı ve hemotoksilen-eozin ile boyandı. Daha sonra kesitler ışık mikroskobunda incelendi ve semi-kantitatif olarak değerlendirildi.

Bulgular: Biyokimyasal analizde MTX kullanımı ile aspartat aminotransferaz, TNF-α ve IL-1β seviyelerinin arttığı, MLT-LYC tedavisinin kullanılması ile bu parametrelerin seviyelerinin azaldığı, TOS ve OSI gibi oksidatif stres parametrelerinin seviyesinin değişmediği gözlendi.

Sonuç: LYC-MLT kombinasyonu MTX'in karaciğer üzerindeki akut toksik etkilerini hem biyokimyasal olarak hem de histopatolojik olarak düzeltmektedir. Fakat bu etkiler oksidatif stres azalmasından ziyade antiinflamatuvar etki ile ilişkili gibi görülmektedir.

Anahtar Kelimeler: *Metotreksat ilişkili hepatotoksisite, melatonin, likopen, oksidatif stres, inflamasyon*

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INTRODUCTION

Methotrexate (MTX), a folat analogue agent with immunosuppressive and antineoplastic effects, is used to treat various malignancies and autoimmune diseases (1,2). High doses of MTX have the antineoplastic effect and the low doses have the immunosuppressive effect (3). The toxic effects of MTX such as impairment of cell proliferation rate are seen mainly on target cells such as cancer cells. At time, the cytotoxic effects of MTX may be life-threatening. Side effects may be seen in many organs and tissues of the body. The liver is one of the organs faced with the most serious side effects of MTX. MTX cannot be used in high doses for hepatotoxicity-almost any dose is risky. These side effects limit the use of MTX in some cases such as chronic liver disease, cirrhosis, and alcoholism (1,2,4-6). Although a few mechanisms have been suggested (7,8), the cause of methotrexate-induced hepatotoxicity (MIT) is not fully understood. MIT may be due to increased production of free radicals and proinflammatory cytokines (9,10). MTX administration increases oxidative stress and significantly reduces the levels of antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase in the liver and other tissues and organs (6,11). A few antioxidants have been shown to reduce these oxidant effects (11). For example, the cytoprotective effects of ascorbat, amifostine, and N-acetylsistein against MIT in rats have been demonstrated (6). Hadi et al. indicated that the effect of inflammation may have a role in the pathogenesis of MIT and metformin might ameliorate this side effect (12).

Lycopene (LYC) is a bright red carotene and carotenoid pigment found in red fruits and vegetables such as tomato and watermelon. It has powerful antioxidant, anti-inflammatory, and anti-cancer properties (13-16). The antioxidant effects of LYC are the most studied and the best known effects (17). These effects may be associated with anti-inflammatory effects, especially on adipose tissue (18). The most obvious protective effects of LYC are seen in cases of ischemic heart disease and cancers (19). Melatonin (N-acetyl-5-methoxytryptamine melatonin, MLT) is found in animals, plants, fungi, and bacteria. In humans, it is one of the most important products of the pineal gland. MLT is a very powerful free radical recipient and a potent antioxidant (20). The antioxidant effects of MLT are occurred either direct or through metabolites. These effects manifest as direct free radical scavenging, increased efficiency of mitochondrial oxidative phosphorylation, antioxidant enzymes stimulation, decreased free radical generation, and increased efficiency of other antioxidants (21,22). Moreover, the anti-inflammatory effects of MLT are well known (23). In a study, it is similar between LYC and LYC + Mel treated groups in levels of TNF-α and IL-1 β, in renal tissue (24). In the same study, the levels nitric oxide was similar with LYC and combination of LYC + MLT. Ceruloplasmine levels significantly reduced with the only LYC and combinations of LYC + MLT. In another study, in MTX applied rats according to controls, has been shown antioxidant and anti-inflammatory effects were obtained with LYC (25).

The combination of MLT-LYC, namely, powerful antioxidant and anti-inflammatory effects, may lead to serious improvements in MIT. However, in the literature, there are studies investigating the joint effect. Therefore, this study aimed to investigate the effect of the MLT-LYC combination on MIT.

MATERIALS and METHODS

Chemicals

Methotrexate, melatonin, and lycopene (Redivivo lycopene 10%; CWS/S-TG, Basel, Switzerland) were purchased from a chemist's shop. The ELISA kits and chemicals used for biochemical analysis were purchased from medical stores.

Animals

In this study, twenty-seven Sprague-Dawley adult male rats (weighing 200-275 g) obtained from Dollvet Animal Laboratory (Sanliurfa, Turkey) were used. To acclimate the rats to standard laboratory conditions (12-h light:12-h dark)) in a room with controlled temperature (24 ± 3°C) one week prior to the experimental study, the animals were kept and fed a standard commercial pellet diet ad libitum with free access to water. The study was approved by the Dollvet Animal Care and Use Committee. Experimental procedures were based on the Guide for the Care and Use of Laboratory Animals.

Experimental Protocol

After 7 days of acclimatization, the rats were divided into 3 groups having 9 rats each and were fed according to the following protocols:

Group I (9 rats): control group; rats in this group were fed with oral corn oil only.

Group II (9 rats): A single dose of 20 mg/kg MTX intraperitoneal was injected into the rats and they were fed with oral corn oil (26).

Group III (9 rats): The rats were treated with a combination of lycopene (10 mg/kg) and melatonin (25 mg/kg, intraperitoneal) for 5 days after a single dose of MTX (20 mg/kg, intraperitoneal) (26,27).

Under general anaesthesia (intraperitoneal xylazine 8 mg/kg, intraperitoneal ketamine 75 mg/kg), all rats were euthanized by decapitation. Blood samples were collected and serum samples were separated by centrifugation at 3000 rpm for 10 min at room temperature.

The liver was removed and divided into two parts for histopathological examination and biochemical analysis. One part of tissue samples was fixed in 10% buffered formalin solution at room temperature for histopathological examination and the other part was stored at -80°C for biochemical analysis.

Histopathological Assessment of Hepatic Tissue

Liver tissues were cut into small sections and fixed in 10% formaldehyde solution. The sections (5 µm) were stained with haematoxylin-eosin (HE) for evaluating liver structure. Five pathological sections were prepared from the liver tissue of each rat. Each stained section was evaluated under a light microscope (Olympus Bx51 microscope with 200x magnification) by a histologist blinded to the treatment group. For semi-quantitative evaluation, the following scoring system used by Akbulut et al. was applied (6).

1. Sinusoidal dilatation
2. Inflammatory cell infiltration
3. Congestion
4. Hydropic degeneration (cytoplasmic vacuolization /swelling of hepatocyte)

0= normal, 1= mild, 2= moderate, and 3= severe, and the total score is calculated by adding all scores.

For example: the maximum score of 12 indicates the severest hepatic injury.

Biochemical Analysis

Measurements of liver function tests

As hepatic function tests, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activities were measured using commercially available kits in an auto analyser (Aeroset® Abbott Laboratories, Chicago, IL).

Tissue Sampling And Measurement of Oxidative Stress Biomarkers

Tissue sampling and homogenization

Before biochemical assays, hepatic tissues were weighed, broken down into very small pieces, and placed in glass tubes. Then, 1 mL of 140 mM KCl solution per gram of tissue was added to each tube and all tissues were homogenized in a motor-driven homogenizer. The homogenate was centrifuged at 2.800 × g for 10 min at 4°C. The supernatant fraction of the homogenate was used for measurement of total antioxidant capacity (TAC), total oxidant status (TOS), TNF-α, and IL-1β levels.

Measurement of Total Antioxidant Capacity

TAC of the supernatant fractions was determined using a novel automated measurement method developed by Erel (28). In this method, the hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. Sequentially produced radicals such as brown-coloured dianisidinyl radical cation, generated by the hydroxyl radical, are potent radicals as well. Using this method, antioxidative effect of the sample against potent-free radical reactions, which are initiated by the produced hydroxyl radicals, is measured. The results are expressed as nmol Trolox Equiv/L.

Measurement of Total Oxidant Status

TOS of supernatant fractions was determined using a novel automated measurement method, developed by Erel (29). Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to the ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are present abundantly in the reaction medium. The ferric ion forms a coloured complex with xylenol orange in an acidic medium. The colour intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed as nmol H_2O_2 Equiv/L.

Oxidative Stress İndex

The percentage ratio of the TOS level to the TAC level has been accepted as the oxidative stress index (OSI) (30). The OSI value was calculated according to the following formula: OSI= (TOS/TAC) x 100. This formula can be applied after synchronizing the TAC and TOS units.

Measurement of TNF-α and IL-1β Levels

In the supernatant of hepatic tissue, TNF-α and IL-1β concentrations were measured using commercial ELISA kits (TNF-α RayBiotech, Inc., Diaclone, Cayman Chemical; IL-1β, Diaclone, Besancon, France).

Statistical Analysis

All data were analyzed using the Statistical Package for the Social Sciences 11.5 for Windows (SPSS Inc., Chicago, USA) statistical software package and data were presented as mean ± standard deviation (± SD). The Kruskal-Wallis analysis of variance method and the non-parametric Mann-Whitney U test in that order were used to evaluate the differences in pathological scores between the experimental groups. P values < 0.05 were considered statistically significant.

RESULTS

Histopathological Results

Group II (MTX), the group treated with only MTX. Compared with the control group (Group I), in the liver tissue of this group, sinusoidal dilatation and hydropic degeneration were increased significantly, but inflammatory cell infiltration was similar (Figure 1, Figure 2A,B,C,D and Table 1).

Group III (MTX-LYC-MLT), the group treated with both MTX and the combination of MLT-LYC. Compared with group II, in this group, the histopathological parameters were improved significantly with the exception of cell infiltration (Figure 3 and Table 1).

Biochemical Results

Serum liver function, tissue pro-inflammatory cytokines, and oxidative and antioxidant parameters among groups are summarized in Table 2.

Serum ALT activity was similar between Groups I and II and Groups II and III ($p > 0.05$), but AST activity increased significantly in Group II compared to Group I, and decreased in Group III compared to Group II ($p < 0.05$).

In the liver tissue, IL-1β and TNF-α increased significantly in Group II compared to Group I and decreased in Group III compared to Group II ($p < 0.05$). TOS and OSI were similar between Groups II and I; Groups II and III ($p > 0.05$). TAC was similar between

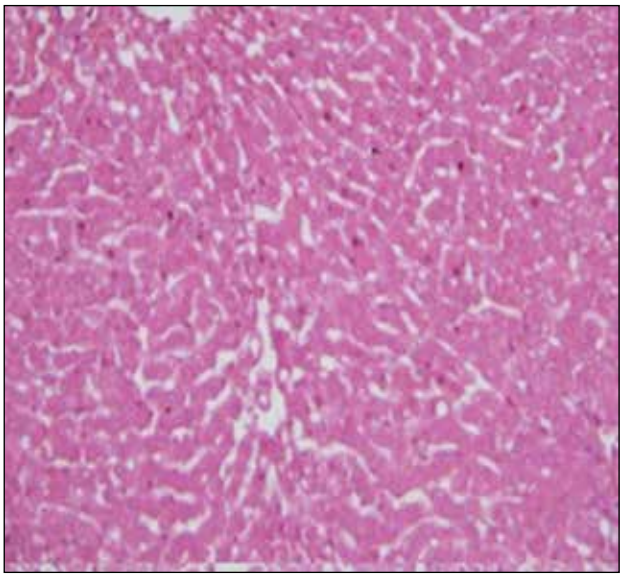

Figure 1. *Normal histological appearence of HE stained control liver tissues.*

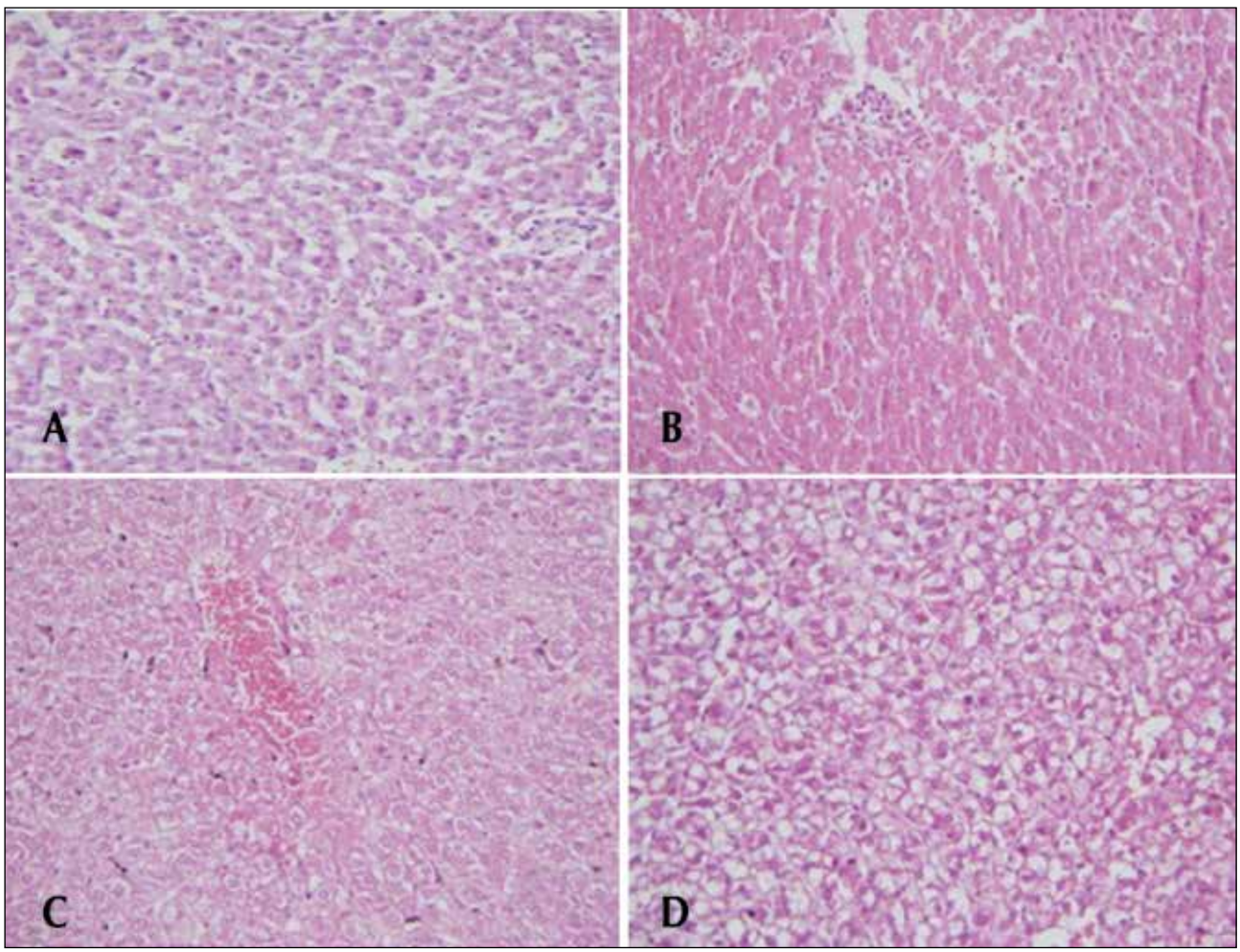

Figure 2. *Histopathological effects of high-dose methotrexate on the liver structure* ***(A)*** *sinusoidal dilatation* ***(B)*** *inflammatory cell infiltration* ***(C)*** *congestion* ***(D)*** *hydropic degeneration.*

Table 1. Histopathologic examination

Histopathologic parameters	Groups		
	Control	MTX	MTX-MLT-LYC
Sinusoidal dilatation	1.00 (0.0-1.0)	2.00 (1.0-3.0)[a]*	0.00 (0.0-1.0)[b]*
Inflammatory cell infiltration	0.00 (0.0-0.0)	0.00 (0.0-1.0)	0.00 (0.0-0.0)
Congestion	0.00 (0.0-1.0)	1.00 (0.0-2.0)[a]*	0.00 (0.0-1.0)[b]*
Hydropic degeneration	0.00 (0.0-1.0)	1.00 (0.0-3.0)[a]*	1.00 (0.0-1.0) [b]*

Data were expressed as median (min-max).
[a] Significance methotrexate compared with control.
[b] Significance methotrexate-melatonin-lycopene compared with methotexate.
** p< 0.05.*

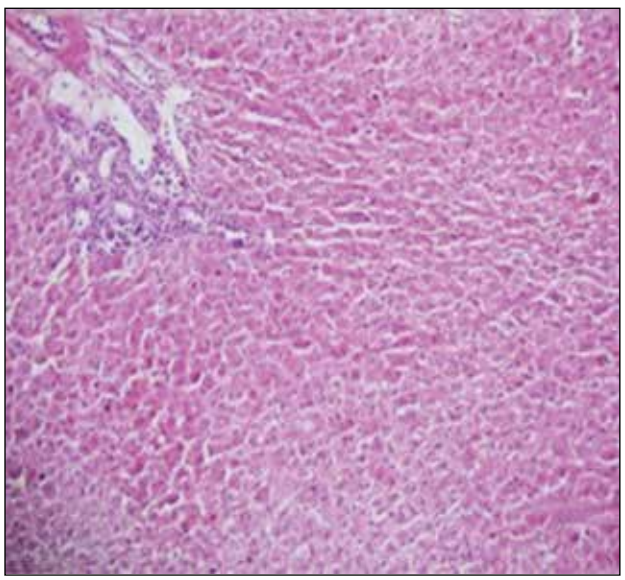

***Figure 3.** After application Melatonin-lycopen, significant improvement in sunosoidal dilatation.*

Groups II and I (p< 0.05), but considerably higher in Group III compared to Group II (p< 0.05).

DISCUSSION

In this study, we showed that serum AST activity and tissue pro-inflammatory mediators such as TNF-α and IL-1β increased with MTX use and decreased significantly with the use of the MLT-LYC combination, but oxidative stress parameters such as TOS and OSI did not change in MIT. This study is the first study in the literature to examine the effect of the combination of MLT-LYC on MIT.

The acute toxic side effects of anti-cancer drugs on multiple tissue and organ systems are known. These side effects are often seen in quickly renewed cells such as gastrointestinal tract, mucosal membranes, bone marrow, and hair follicles (31). The liver is among the organs most affected by anti-cancer drugs. This is because the liver metabolizes many medications, including MTX, and comprises cells with rapid regeneration capability (32). MTX-induced liver damage may occur following the administration of a high single dose or chronic administration of MTX (33,34). The cause of the MTX-induced hepatotoxicity is not fully understood. A few theories ascribe it to the accumulation of free radicals (33-36). Oxidative stress increase and significantly reduces levels of antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase with MTX administration in liver (33-36). The cytoprotective effects of

Table 2. Histopathologic examination

Histopathologic parameters	Groups		
	Control n= 9	MTX n= 9	MTX-MLT-LYC n= 9
TNF-α, pg/mL	**1862 ± 136**	**2354 ± 221[a]***	**2022 ± 259[b]***
Interleucin-1β, pg/mL	**32.10 ± 5.16**	**51.63 ± 6.79[a]***	**39.58 ± 6.01[b]***
TAC, nmoL Trolox Eqv./mg protein	0.28 ± 0.02	**0.28 ± 0.03[a]***	**0.35 ± 0.06[b]***
TOS, nmoL H_2O_2 Eqv./mg protein	14.42 ± 1.07	15.61 ± 2.01	15.19 ± 1.78
OSI, Arbitrary Unite	5.11 ± 0.47	5.44 ± 1.21	5.41 ± 0.69
Aspartate aminotransferase , U/L	**66.00 ± 8.16**	**173.00 ± 41.71[a]***	**47.80 ± 10.23[b]***
Alanine aminotransferase , U/L	36.20 ± 7.30	50.50 ± 8.38	43.00 ± 10.48

Data were expressed as mean ± standard deviation.
[a] Significance methotrexate compared with control.
[b] Significance methotrexate-melatonin-lycopene compared with methotrexate.
** p< 0.05.*

ascorbat, beta glucan, ursodeoxycholic acid, beta carotene, amifostine, resveratrol, and N-acetylsistein against MTX-induced hepatotoxicity have been demonstrated (6,33-36). Melatonin is a potent antioxidant because of its free radical scavenging property (37). Jahovich et al. showed that MLT was prevented MTX-induced hepatotoxicity in rats (26). MLT has also been found to be effective against hepatotoxicity induced by cyclosporine A, carbon tetrachloride, lipopolysaccharide, and thioacetamide (38-41). LYC has powerful antioxidant, anti-inflammatory, and anti-cancer properties (13-16). We opined that the combination of two agents with powerful antioxidant, anti-inflammatory, and anti-cancer properties would be more effective against MIT, and we intend to use the MLT-LYC combination against MIT. To that end, we first created liver damage using methotrexate. We used ALT and AST enzyme activities, as well as histopathological imaging to confirm the damage. ALT activities were similar between Groups I and II, but the AST activity in Group II was higher. In cases with acute injury, AST can also be caused by more sensitive enzymes. Again, we used histopathological imaging and pro-inflammatory cytokines such as TNF-α and IL-1β to show inflammation of hepatic tissue.

Both histopathological images and increased levels of the two proinflammatory cytokines in Group II samples showed that MTX caused liver inflammation. This study showed histopathologically and biochemically that inflammation was healed in Group III samples. This is evidence of the remedial effect of the MLT-LYC combination on MIT. However, it was not possible to determine the healing mechanism of hepatotoxicity with the application of the MIT-LYC combination. However, some predictions can be made. As an indicator of oxidative status, we measured TAC, TOS, and OSI, which indicate the total oxidative status in an organism. TAC, TOS, and OSI were not changed with MTX. TAC increased, but TOS and OSI remained unchanged in the liver tissue when using the MLT-LYC combination. The improvement in response to oxidative stress parameters in previous studies, we have seen to be MLT-LYC ineffective on the total oxidative balance in hepatic tissue. Therefore, the increase in TAC in Group III is consistent with the literature. The main healing effect of MLT-LYC on MTX-induced hepatotoxicity might not be limited to decreased oxidative stress and might include other mechanisms. Indeed, there is evidence of the presence of anti-inflammatory effects in the literature. In particular, there are significant effects on IL-1β and IL-1β induced matrix metalloproteinase enzymes (36,42-44). Similarly, in our study, the levels of IL-1β and TNF-α decreased significantly. Therefore, the MLT-LYC combination might have suppressed inflammation by inhibiting IL-1β and TNF-α.

LYC, a carotenoid, has powerful antioxidant, anti-inflammatory and anti-cancer properties (45). It has been shown to be protective against carbon tetrachloride-, acetaminophen (APAP)-, amiodarone-, cyclosporine A-induced hepatotoxicity (27,46,47). However, its effect on MTX-induced hepatotoxicity was not known. In this study, we examined the antioxidant and anti-inflammatory effects of the combination of LYC-MLT. Anti-inflammatory effects were observed, but the oxidative status remained unchanged. These results demonstrate that the antioxidant effect of MLT and LYC may be caused by the anti-inflammatory effect.

As a result, the LYC-MLT combination improves the acute effects of MIT. It improves both liver function and the histopathology level. However, these effects seem to be associated with the anti-inflammatory effect than the reduction of oxidative stress. To clarify this issue, further studies are needed.

CONFLICT of INTEREST

None declared.

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