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Protective Effect of Linoleic Acid on Liver Toxicity Induced By Methotrexate

Efecto Protector del Ácido Linoleico sobre la Toxicidad Hepática Inducida por Metotrexato

Ayse Gül Kabakcı[1] & Memduha Gülhal Bozkır[1]

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SUMMARY: We aimed to investigate the protective effect of linoleic acid on liver toxicity induced by methotrexate. The study was carried out in partnership with the Department of Anatomy and Department of Medical Pharmacology of Çukurova University Faculty of Medicine, using the laboratory facilities of the Department of Medical Pharmacology. Human hepatocyte cell line (CRL-11233) cells obtained from the American Type Culture Collection Organization (ATCC) were used. Expressions of apoptotic pathway markers, apoptosis inducing factor (AIF), BAX, BCL 2, GADD 153, 78-kDa glucose-regulated protein (GRP78), and CASPASE-3 were evaluated. All analyzes were examined in four groups (Group 1; control, Group 2; linoleic acid given, Group 3; methotrexate given and Group 4; linoleic acid and methotrexate given). The mean ± standard error values of the obtained results as nanogram / milliliter (ng / ml) are in Group I, Group II, Group III and Group IV, respectively; AIF values, 0.4150 ± 0.1208, 0.3633 ± 0.2389, 1.792 ± 0.3611 and 1.077 ± 0.1646, BAX values, 0.900 ± 0.1864, 1.002 ± 0.2098, 8.352 ± 1.467 and 4.295 ± 1.522, BCL 2 values, 13.93 ± 1.198, 13.92 ± 1.739, 2.938 ± 1.059 and 9.250 ± 1.492, GADD 153, 0.7333 ± 0.1751, 0.7067 ± 0.2115, 1.650 ± 0.2950 and 1.237 ± 0.1805, GRP78, 0.4767 ± 0.1804, 0.5233 ± 0.1590, 2.183 ± 0.2639 and 1.112 ± 0.2693, CASPASE-3 values , 1.127 ± 0.2033, 0.8317 ± 0.3392, 13.50 ± 1.871 and 8.183 ± 1.030. It was determined that linoleic acid has a protective effect on methotrexate-induced liver toxicity.

KEY WORDS; Anatomy; Hepatocyte; Liver; Linoleic acid; Methotrexate.

INTRODUCTION

The liver is the target organ for drug toxicity as it is responsible for the metabolism of many foreign substances due to its location in the gastrointestinal tract. The incidence of drug-induced liver injury in general populations is about 14-19 per 100,000 people. The reported incidence and severity of drug-induced liver injury varies among drugs, suggesting that drug properties have a role in drug-induced liver injury risk determination. Conversely, drugs with drug-induced liver injury potential cause liver injury only in a small portion of patients indicating that host factors play a major role in drug-induced liver injury development (Sgro, *et al*., 2002; Björnsson *et al*., 2013). Hepatotoxicity has a considerable impact on health because many of the hepatic reactions induced by pharmaceutical preparations can be very severe. Drug-induced organ toxicity is a frequently encountered obstacle in the field of medical practice that limits the use of numerous pharmacologically valuable drugs. Drugs are an important cause of liver injury. More than 900 drugs, toxins, and herbs have been reported to cause liver injury, and drugs account for 20-40 % of all instances of fulminant hepatic failure. Approximately 75 % of the idiosyncratic drug reactions result in liver transplantation or death (Lewis, 2000). Antituberculosis drugs, methotrexate, niacin, vitamin A, antiandrogens can be given as examples of drugs that increase the risk of hepatotoxicity in chronic liver disease. Methotrexate (MTX)-induced organ toxicity is unfortunately the rate-limiting factor for its clinical application (Helal & Said, 2020). The clinical use of MTX is significantly limited due to the associated various organ toxicities, including kidney, liver, lung, bone marrow, and gastrointestinal toxicities (Andrade *et al*., 2007). Methotrexate is a folic acid antagonist with anti-inflammatory and immunosuppressive effects (Salim *et al*., 2006). It is used in the treatment of ALL, the treatment of meningeal carcinomatosis, the prophylaxis and treatment of meningeal leukemia and lymphoma, the combination therapy

[1] Cukurova University, Faculty of Medicine Department of Anatomy, Balcalı-Adana, Turkey.

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of non-hodging lymphomas, the adjuvant therapy of osteosarcoma, the treatment of rheumatoid arthritis, resistant psoriasis, and also in the treatment of breast, head-neck, ovary and bladder cancer (Chen *et al*., 2009). In addition, MTX has been the most commonly used immunosuppressive agent after prednisolone in the treatment of various skin diseases by dermatologists for more than fifty years. It is cheap, has a reducing effect on steroid dose, is well known about its toxicity and side effects, and the availability of efficacy data has increased its use in dermatology (Bangert & Costner, 2007). MTX side effects that occur during treatment are quite common. Generally, these side effects resolve after the end of treatment or dose reduction. Approximately 30 % of the patients who receive MTX treatment are discontinued due to drug toxicity (van Ede *et al*., 2001). For this reason, it comes to the conclusion that it should be used together with antioxidants to avoid MTX toxicity. In the literature, melatonin, nicotinamide, methionine, vitamin E and n-acetylcysteine, alpha lipoic acid, lipoic acid, vitamin C, melatonin, coconut, folic acid, antioxidant agents, anti-inflammatory and vasodilator agents have been tried to protect tissues from MTX damage. Also, studies on molsidomine, inulin, coconut, improved metformin, misoprostol,vitamin E, Indole-3-Carbinol, balanites aegyptiaca extract, melatonin and ursodeoxycholic acid, sitagliptin, silymarin, turmeric and naringin to prevent hepatotoxicity caused by MTX are available (Kirbas *et al*., 2015; Cline & Jorizzo, 2017; Arpag *et al*., 2018; Famurewaa *et al*., 2018; Samdanci *et al*., 2019; Kalantaria *et al*., 2019). Also, studies on molsidomine, inulin, coconut, improved metformin, misoprostol, vitamin E, Indole-3-Carbinol, balanites aegyptiaca extract, melatonin and ursodeoxycholic acid, sitagliptin, silymarin, turmeric and naringin to prevent hepatotoxicity caused by MTX are available. We think that linoleic acid (LA) can be an alternative prophylactic option.

The hypothesis of our study is that linoleic acid prevents hepatotoxicity caused by MTX. LA has anticarcinogenic effects on human metabolism, enhancing the immune system, lowering cholesterol, lowering the risk of arteriosclerosis, promoting development and growth, reducing fat accumulation in the body, protecting against diabetes, enhancing muscle growth, eliminating free radicals, antibacterial and antioxidative effects. The aim of our study is to investigate the protective effect of LA in MTX-induced liver damage. Thus, we aim to prevent prolongation of the treatment process by interrupting the treatment or reducing the dose that should be used due to hepatotoxicity caused by MTX. While there are many studies in the literature using experimental animals to prevent the side effects of MTX, this study is first examining the effects of LA to prevent the side effects of MTX and on human metabolism. We think that it will contribute to the literature on this subject.

MATERIAL AND METHOD

Experimental Design: In this study, human hepatocyte (CRL-11233) cells obtained from the American Type Culture Collection Organization (ATCC) were used. All experimental procedures were approved by "Non-Invasive Clinical Research Ethics Committee of Çukurova University Faculty of Medicine for our study (Decision No: 104/9). All the test procedures were performed after ethics committee approval according to the Helsinki Declaration of Principles and and the measures were done in Cukurova University Faculty of Medicine, Department of Medical Pharmacology. Cell lines were randomly divided into four groups (6 cell lines per group) as follows;

Group I; Healthy control group. No substance was given to this group.
Group II; Only MTX in liquid form has been given to this group.
Group III; Only LA has been given to this group.
Group IV; MTX + LA was given to this group.

Steps followed in the experimental process;
1.Cell culture medium was prepared to support the growth of cells.
2. The cell media prepared was incubated under appropriate conditions (in an oven with 37 ° C and 5 % CO2), and the proliferation of the cells was ensured.
3. Proliferated cells were frozen at -20 ° C in an Eppendorf tube.
4. Cells frozen by homogenization were lysed.
5. For the ELISA test standardization of the homogenized cells, protein measurements were made by the Bradford method.
6. AIF, BAX, BCL-2, GADD153, GRP78 and CASPASE-3 apoptotic mediators were examined by ELISA test.

Cell culture medium was prepared using the ingredients (10 % Fetal Bovine Serum (FBS) (Hyclone), 1 % L-Glutamine (Hyclone), 1 % Penicillinstreptomycin (Hyclone), Dulbecco's modification of Eagle's medium (DMEM) (GIBCO)) to support the growth of cells. Cells were incubated in sterile flasks in a temperature 37 ° C and 5 % CO2 oven (Core EN400) in the prepared cell medium. Thus, the proliferation of the cells was achieved. After the incubation step, the experimental groups were frozen in an Eppendorf tube at -20 C.

Tissue Homogenization. In order to homogenize the tissues, in an eppendorf tube per 1 gram; 3 ml RIPA (Radio-Immunoprecipitation Assay) buffer, 30 microliters (ml) phenylmethanesulfonylfluoride (PMSF), 30 ml sodium

vanadate and 30 ml protease inhibitor was added and homogenates were obtained from the tissues by being crushed on ice with an ultrasonic disrupter. The homogenates were centrifuged at 10,000 rpm (RPM) for 10 minutes (min) (Core NF200) and the supernatants separated from the upper part were taken, and the lower precipitates, ie pellets, were discarded.

Cell Culture. Human hepatocyte cell line (CRL-11233) cells (ATCC, USA) were grown in medium containing 10 % FBS (Hyclone, USA), trypsin-EDTA. Culture was carried out under sterile conditions at 37 ° C, in oven containing 5 % CO 2. Cells were grown in a single layer of 1x105 live cells in 1 ml in 75cm2 standard cell culture plates. The medium prepared cells were incubated at 37 ° C and 5 % CO2 in an oven (Core EN400) for 48 hours by treating the cells with MTX, LA and MTX + LA.

Protein Quantification. Protein quantification of homogenized cells was made by the Bradford method. Using bovine serum albumin (1mg / ml), a standard was prepared at concentrations of 1, 2, 3, 5, 7, 8, 10 (mg / ml) and 10 ml of each sample was taken and completed to 100 ml with distilled water. After adding 1ml of Bradford solution on the standard and samples and mixed with vortex, the amount of absorbance was measured manually at a wavelength of 595 nanometers in the spectrophotometer. Protein quantification was performed in mg / ml according to the standard curve drawn in the Prism program (GraphPad Prism 8.1.2. CA, USA). Protein quantification was carried out for standardization of ELISA experiments.

ELISA (Enzyme Linked Immunosorbent Assay) Test. Expressions of apoptotic pathway mediators AIF, BAX, BCL-2, GADD153, GRP78 and CASPASE-3 were analyzed by ELISA test (Awareness Technology Inc., ChroMate Elisa Reader, US). As a result of protein quantification, 25 ml of each standard and samples were added to the ELISA plate and 200 ml (working reactant = 50A solution: 1 B solution) was added to the plate and the plate was shaken in a shaker for 3 seconds, and then it was incubated at 37 degrees for 30 minutes and read on the spectrophotometer at 562 nm.

Statistical analysis. Relaxation responses of tissues were expressed as a percentage of contractions. It is shown with standard errors. GraphPad Prism 8.1.2 for drawing graphs and for statistical analysis. (CA, USA) program was used. One way (ANOVA) and post-hoc test (Bonferroni method) were used for statistical comparisons. The results were evaluated at a 95 % confidence interval.

RESULTS

When the expression levels of AIF, BAX, BCL2, GADD153, GRP78 and CASPASE 3 were examined, it was found that LA had a protective effect on MTX-induced hepatotoxicity (Table I).

AIF; Group I (Control); 0.4150 ± 0.1208 ng / ml, only in Group II with LA application; 0.3633 ± 0.2389 ng / ml, only in Group III with MTX application; In Group IV where 1.792 ± 0.3611 ng / ml and LA + MTX was applied; It was found to be 1.077 ± 0.1646 ng / ml. AIF expression when compared to the control group; It increased in the group administered MTX, increased in the group administered MTX and LA, and decreased in the group given only LA (Fig. 1).

Table I. Effects of Methotrexate (MTX) and Linoleic Acid (LA) on human liver hepatocyte cells.

	Group I Mean ± SD (min-max)	Group II Mean ± SD (min-max)	Group III Mean ± SD (min-max)	Group IV Mean ± SD (min-max)
AIF	0.4150±0.1208 (0.3200-0.6500)	0.3633±0.2389 (0.1000-0.7000)	1.792±0.3611 (1.250-2.200)	1.077±0.1646 (0.8800-1.300)
BAX	0.900±0.1864 (0.6500-1.120)	1.002±0.2098 (0.7500-1.300)	8.352±1.467 (5.900-10.42)	4.295±1.522 (2.500-6.120)
BCL2	13.93±1.198 (12.80-16.10)	13.92±1.739 (11.80-16.50)	2.938±1.059 (1.770-4.200)	9.250±1.492 (7.700-11.60)
GADD153	0.7333±0.1751 (0.5500-0.9900)	0.7067±0.2115 (0.3500-0.9500)	1.650±0.2950 (1.300-2.100)	1.237±0.1805 (0.9900-1.500)
GRP78	0.4767±0.1804 (0.3300-0.7500)	0.5233±0.1590 (0.3300-0.7300)	2.183±0.2639 (1.800-2.600)	1.112±0.2693 (0.7500-1.500)
CASPASE 3	1.127±0.2033 (0.9500-1.500)	0.8317±0.3392 (0.3800-1.300)	13.50±1.871 (11.00-16.00)	8.183±1.030 (6.800-9.500)
BAX/BCL2	0,065	0,072	2,843	0,464

(n=6, ANOVA, Post hoc: Bonferroni). SD; Standard Deviation, Min; Minimum, Max; Maximum.

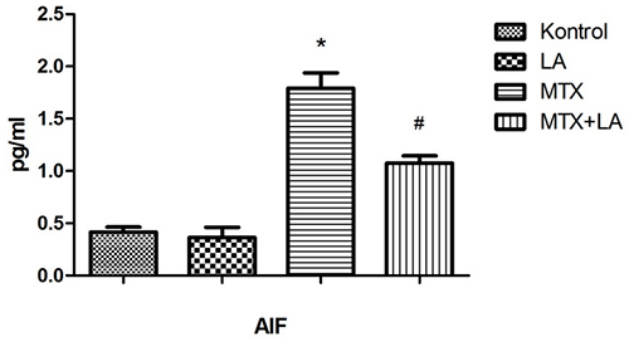

Fig. 1.Distribution of AIF apoptotic marker among groups.

BAX; Group I (Control); 0.900 ± 0.1864 ng / ml, only in Group II with LA application; 1.002 ± 0.2098 ng / ml, only in Group III with MTX application; In Group IV where 8.352 ± 1.467 ng / ml and LA + MTX was applied; It was found to be 4.295 ± 1.522 ng / ml. When BAX expression is compared to the control group; It increased in Group II, III, IV. However, the highest increase was seen only in Group III, where MTX was applied (Fig. 2).

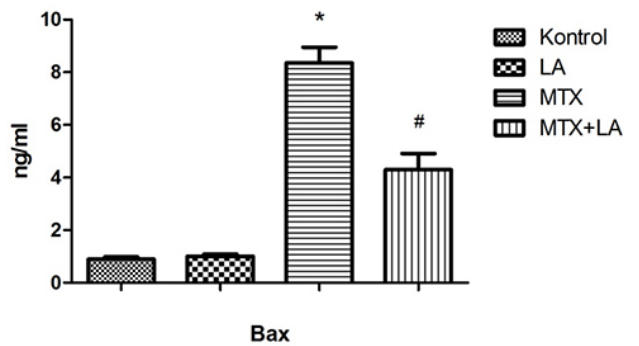

Fig. 2.Distribution of BAX apoptotic marker among groups.

BCL 2; Group I (Control); 13.93 ± 1.198 ng / ml, only in Group II with LA application; 13.92 ± 1.739 ng / ml, only in Group III with MTX application; In Group IV where 2.938 ± 1.059 ng / ml and LA + MTX was applied; It was found to be 9,250 ± 1,492 ng / ml. BCL-2 expression when compared to the control group; It decreased in the MTX group. It increased in the group given MTX and LA compared to the group given MTX and in the group given only LA (Fig. 3).

GADD 153; Group I (Control); 0.7333 ± 0.1751 ng / ml, only in Group II with LA application; 0.7067 ± 0.2115 ng / ml, only in Group III with MTX application; In Group IV where 1.650 ± 0.2950 ng / ml and LA + MTX was applied; It was found to be 1.237 ± 0.1805 ng / ml. When the expression of GADD153 is compared to the control group; It increased in the MTX group, but decreased in the LA applied group. When the LA + MTX group was compared with the MTX group, a decrease was observed in the LA + MTX group (Fig. 4).

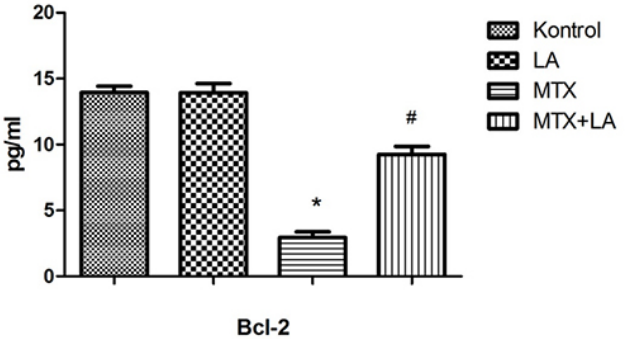

Fig. 3.Distribution of Bcl-2 apoptotic marker among groups.

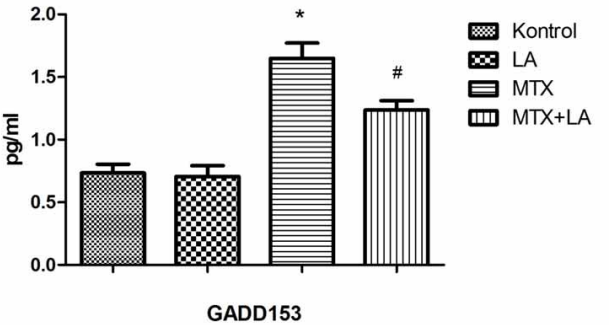

Fig. 4. Distribution of GADD153 apoptotic marker among groups.

GRP 78; Group I (Control); 0.4767 ± 0.1804 ng / ml, only in Group II with LA application; 0.5233 ± 0.1590 ng / ml, only in Group III with MTX application; In Group IV where 2.183 ± 0.2639 ng / ml and LA + MTX was applied; It was found to be 1.112 ± 0.2693 ng / ml. When GRP78 expression is compared to the control group; An increase was observed in the groups given MTX, LA and LA + MTX. When the LA + MTX group was compared with the MTX group, a decrease was observed in the LA + MTX group (Fig. 5).

CASPASE 3; Group I (Control); 1.1270 ± 0.2033 ng / ml, only in Group II with LA application; 0.8317 ± 0.3392 ng / ml, only in Group III with MTX application; In Group IV where 13.50 ± 1.871 ng / ml and LA + MTX was applied; It was found to be 8.183 ± 1.030 ng / ml. When caspase 3 expression is compared to the control group; there was a decrease in the group receiving LA, and an increase in the groups given MTX and LA + MTX. When the LA + MTX group was compared with the MTX group, a decrease was observed in the LA + MTX group (Fig. 6).

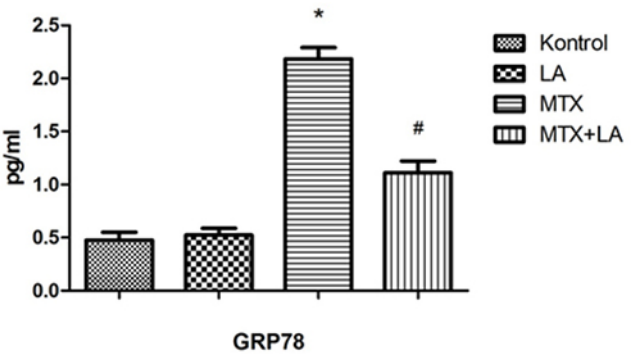

Fig. 5.Distribution of GRP78 apoptotic marker among groups.

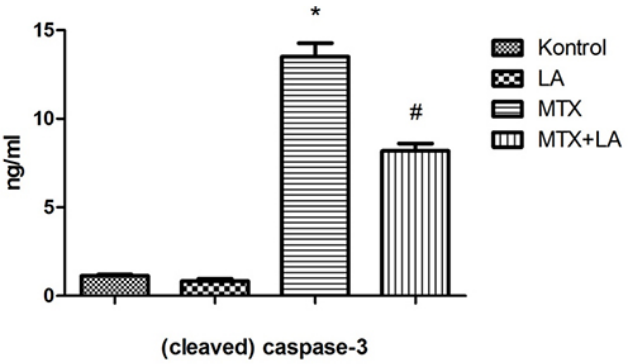

Fig. 6.Distribution of CASPASE 3 apoptotic marker among groups.

DISCUSSION

Drugs were held responsible for more than 50 % of liver disease. Antituberculosis drugs, methotrexate, niacin, vitamin A, antiandrogens can be given as examples of drugs that increase the risk of hepatotoxicity in chronic liver disease. MTX, an antineoplastic drug, belongs to the group of antimetabolites. It acts as a folic acid antimetabolite. It reversibly inhibits the dihydrofolate reductase enzyme. It prevents the conversion of dihydrofolate to tetrahydrofolate so it acts as a folate antagonist. While it is used as an antineoplastic in high doses, it is used as an immunosuppressive and anti-inflammatory in low doses (Martin *et al*., 2009). There are many studies in the literature about pure squamous cell cancer of the urinary tract (Griffiths *et al*., 2019), in cancer types (Wippel *et al*., 2019), romatoid artrit (Boone *et al*., 2019), in dermatology (Thi *et al*., 2019), where methotrexate is used. MTX creates side effects, especially nephrotoxicity and hepatotoxicity, against these wide indications for use. These side effects are thought to be the result of oxidative damage caused by reactive oxygen species. In addition, other toxic effects of MTX are neurotoxicity, pulmonary fibrosis, testicular toxicity, pancreatic toxicity and intestinal mucositis (Mercantepe *et al*., 2018). There are studies in the literature to prevent hepatotoxicity and nephrotoxicity, which are the most important side effects caused by MTX (Yang *et al*., 2018). Recent studies have shown that the development of MTX-mediated toxicity is associated with free oxygen radicals and hydrogen peroxide. There are studies in the literature on molsidomine, inulin, coconut, enhanced metformin, misoprostol, vitamin E, Indole-3-Carbinol, balanites aegyptiaca extract, melatonin and ursodeoxycholic acid, sitagliptin, silymarin, turmeric and naringin to prevent hepatotoxicity caused by MTX (Montasser *et al*., 2017; Abo-Haded *et al*., 2017). In this study, the effect of LA, which has anticarcinogenic, antimutagenic, anti-inflammatory and fat mass reducing effect, to prevent hepatoxicity caused by MTX was investigated. LA has skin barrier, immune, cardiovascular, neurobiological, reproductive, thermoregulatory and digestive functions (Guyenet & Carlson, 2015). However, although there are many studies on experimental animals (Draycott *et al*., 2019), the number of studies examining the effects of LA on human metabolism is very few. There are studies that LA reduces body fat percentage (Venø *et al*., 2018), slows down the development of atherosclerosis, lowers cholesterol level (Wannamethee *et al*., 2018), reduces the risk of diabetes, strengthens immunity, accelerates body metabolism (Andersson-Hall *et al*., 2018), neuroprotective activity (Mann *et al*., 2018), anti-inflammatory activity (Matin *et al*., 2018) and anticarcinogen activity (den Hartigh, 2019).

In the study where we examined the protective effect of LA against hepatotoxicity induced by MTX, apoptotic markers were examined and evaluated. Apoptotic markers; The changes in the values of AIF, BAX, BCL2, GADD153, GRP78, CASPASE 3 among the groups, respectively, in the control group; 0.4150±0.1208 ng/ml, 0.900±0.1864 ng/ml, 13.93±1.198 ng/ml, 0.7333±0.1751 ng/ml, 0.4767±0.1804 ng/ml, 1.127±0.2033 ng/ml, in the LA group; 0.3633±0.2389 ng/ml, 1.002±0.2098 ng/ml, 13.92±1.739 ng/ml, 0.7067±0.2115 ng/ml, 0.5233±0.1590 ng/ml, 0.8317±0.3392 ng/ml, in the MTX group; In the group receiving 1.792±0.3611 ng/ml, 8.352±1.467 ng/ml, 2.938±1.059 ng/ml, 1.650±0.2950 ng/ml, 2.183±0.2639 ng /ml, 13.50±1.871 ng/ml, in the group receiving LA +MTX; It was found to be 1.077±0.1646 ng/ml, 4.295±1.522 ng/ml, 9.250±1.492 ng/ml, 1.237±0.1805 ng/ml, 1.112±0.2693 ng/ml, 8.183±1.030 ng/ml.

In their study on mice, Ge *et al*., examined the protective effect of tempol against acute hepatotoxicity caused by acetaminophen and found that it reduced pro-apoptotic protein expressions CASPASE 3, BAX and

increased anti-apoptotic BCL2 (Ge *et al*., 2019). In another study examining the preventive effect of Nigella Sativa oil in mice against apoptosis and hepatotoxicity caused by the galactose-induced aging process, they found that the level of BAX protein increased in the group treated with D-galactose and no change was observed in the level of BCL2 protein. Therefore, the ratio of BAX / BCL2 increased significantly and decreased from 1.34 ± 0.15 to 0.75 ± 0.19 in the group given black seed oil (0.1ml / kg) compared to the group treated with D-galactose ($P < 0.001$). CASPASE 3 level increased in the group given D-Galactose compared to the control group and decreased (from 116 ± 4 to 63 ± 0.5) in mice exposed to black seed oil (0.1ml / kg) (Shahroudi *et al*., 2017). Yang *et al*. (2020) investigated the protective effect of the polysaccharide D-Isofluoridocide obtained from Laurencia undulata on alcohol-induced hepatotoxicity in HepG2 cells, and found that decrease in BAX, BCL2 and CASPASE 3 proteins in the group given D-Isofluoridocide. Zhang *et al*. (2020) investigated the protective effect of aspirin on acute liver injury due to paraquat in rats, and found that BAX, AIF and CASPASE 3 decreased, BCL2 value increased after aspirin treatment. In a study by Ramachandran *et al*. (2011) examined the effect of acetaminophen hepatotoxicity on mitochondrial oxidative stress, DNA and liver damage, they found an increase in AIF and BAX values. In another study conducted by Ramachandran *et al*. (2015) they examined the effect of hepatitis c virus on acetaminophen-induced liver damage in mice, and stated that increase in AIF and GADD153 values compared to the control group and a decrease in GRP78 in mice with hepatitis c with liver damage. Similarly, Kouam *et al*. (2017) investigated the protective effect of Khaya grandifoliola (Meliaceae) used in Cameroon traditional medicine to prevent acetaminophen-induced hepatotoxicity. They found a decrease in BAX and AIF values in the group treated with Khaya grandifoliola (Kouam *et al*., 2017). In another study conducted on mice to prevent acetaminophen-induced hepatotoxicity, the protective effect of Folium Microcos was examined, and increase in CASPASE3, BAX values and decrease in BCL2 were observed in the acetaminophen given group compared to the acetaminophen + Folium Microcos group (Wu *et al*., 2017). In another study examining the effect of vitamin E and Metallothionein in fish to prevent the toxicological effect of cadmium on the liver, increase in CASPASE 3, AIF, BAX values and decrease in GRP 78 value were found in the group given saline compared to the group given vitamins and Metallothionein (Duan *et al*., 2018). Hamed *et al*. (2016) examined the protective effect of strawberries against hepatotoxicity due to carbon tetrachloride and found that BAX, CASPASE 3 value decreased and BCL2 value increased in the group receiving strawberries. In the study conducted by Orazizadeh *et al*. (2020) who examined the effect of glycyrrhizin acid on BAX and BCL2 expression in hepatotoxicity caused by Titanium dioxide nanoparticles in rats, they found that increase in BAX expression and decrease in BCL2 expression.

In a study conducted with a lung cancer cell line examining the effect of linoleic acid on the expression of apoptotic genes in lung cancer, decrease in BAX level and increase in BCL2 level were found as a result of 72-hour LA treatment (Stowikowski *et al*., 2020). In another study examining the effect of LA supplementation on in vitro maturation, embryo development and apoptotic related gene expression in sheep, it was reported that increase in the mRNA expression of the BAX (BCL2, associated X) gene in the group given LA compared to the control group (Amini *et al*., 2016). A study mouse cell line HEP2G with liver cancer demonstrated the antiproliferative effect of LA by inducing apoptosis mediated by upregulation of BAX and downregulation of BCL2 (Mondal *et al*., 2016). In a study in which HepG2 and Hep3B cell lines were used to investigate the effects of LA on cell viability and cell proliferation ability, it was observed that in the HepG2 cell line, there was increase in BAX and CASPASE 3 level and decrease in BCL 2 level in the group given LA compared to the control group (Lu *et al*., 2015). In a study evaluating visceral adipose tissue in mice without thymus gland, mice given the stearic acid diet were found to have significantly less belly fat compared to mice given LA, lower BCL2 levels and higher levels of BAX and CASPASE 3 (Shen *et al*., 2014). Similarly, in a study involving the monitoring of dorsal adipose fat ratio in pigs, it was found that there was decrease in BCL 2 level and increase in BAX level in the group given LA (Qi *et al*., 2014).

In another study examining the protective effect of berberine against MTX-induced nephrotoxicity, the anti-apoptotic properties of berberine were demonstrated by suppressing BAX and CASPASE-3 and increasing BCL2 expression when compared to the MTX-administered group (Hassanein *et al*., 2019). In the study examining the protective effects of Moringa oleifera leaf extract against oxidative stress and apoptosis in the liver and kidney due to MTX in mice, the BAX value was found to be higher in the MTX group compared to the group receiving Moringa + MTX, while the BCL2 value was found to be lower (Soliman *et al*., 2020a). Similarly, in another study conducted to prevent damage to the spleen due to MTX in mice, Moringa oleifera leaf extract had higher BAX and CASPASE 3 values in the MTX group than the Moringa + MTX group (Soliman *et al*., 2020b). In the study of Samdanci *et al*. (2019) they examined the protective effect of molsidomine against MTX-induced hepatotoxicity in rats, they found that the BCL 2 ratio was higher in the group given only MTX compared to

the molsidomine + MTX group. In a study by Abo-Haded *et al*. (2017) examined the protective effect of sitagliptin to prevent MTX-induced hepatotoxicity in mice, found that increase in the immuno-expression of the pro-apoptotic protein BAX, CASPASE 3 levels and decrease in anti-apoptotic BCL2 level in the MTX-treated group. In the study of Rong *et al*. (2018), they examined that MTX improved spinal cord injury and found that CASPASE 3, GRP 78 apoptotic factors increased in spinal cord injury, but the values decreased as MTX was administered. Our study findings have resulted in support of the literature. In our study, it was found that MTX biochemically significantly increased liver apoptotic markers BAX, GADD153, GRP78 and CASPASE 3 protein levels compared to the control group. However, in the group which LA + MTX was used, it was observed that decrease in these protein values compared to the group using MTX. This result of our study also supports the positive effect of LA on hepatocyte apoptosis. At the BCL2 protein level, it was found that the apoptotic effect of MTX on hepatocyte cells decreased. But BCL2 protein level increased in the LA + MTX group. MTX-associated toxicities have a multifactorial history, meaning they occur through a combination of genetic and environmental factors. The pharmacological metabolism of MTX includes many transporters and enzymes that can affect the efficacy and toxicity of MTX. We think that small differences between studies are also caused by these reasons. In addition, we think that factors such as cell line usage, clinical applications, use of experimental animals, ethnic origin, difference and amount of active ingredients will affect the study results.

As a result, this study demonstrated that LA has a significant protective effect on MTX-induced hepatotoxicity in humans. Unlike the literature, this study, which was carried out with healthy human hepatocyte cell line for the first time, revealed that LA can be used in MTX treatment and prophylaxis.

CONCLUSION

Path markers AIF, BAX, BCL2, GADD153, GRP78 and CASPASE3 were examined in the study. AIF expression increased in the MTX given group and the MTX + LA given group, and decreased only in the group given LA. When BAX expression is compared to the control group; It increased in Group II, III, IV. However, it was determined that the highest increase was only in the MTX group. BCL2 expression when compared to the control group; It decreased in the MTX group. It was found to be increased in the group given MTX and LA compared to the group given MTX and in the group given only LA. When the expression of GADD153 is compared to the control group; It was found that it increased in the MTX group, decreased in the LA group and the LA + MTX group when the MTX group were compared. When GRP78 expression is compared to the control group; It was found that increase in the MTX, LA and LA + MTX groups, and a decrease in the LA + MTX group when the LA + MTX group and the MTX group were compared. CASPASE 3 expression when compared to the control group; It was observed that there was a decrease in the group receiving LA, and an increase in the groups given MTX and LA + MTX. When the LA + MTX group was compared with the MTX group, decrease was observed in the LA + MTX group. When the expression levels of markers were examined, it was concluded that hepatotoxicity was induced in the MTX given groups. When the expression levels of these markers were examined, it was concluded that LA had a protective effect on MTX-induced hepatotoxicity. It has been demonstrated that LA supplementation can be used to prevent hepatotoxicity in patients who need to use MTX, thus contributing to the prolongation of hospital stay in patients using MTX. It is thought to contribute to the interruption or termination of MTX treatment. We recommend that these studies be continued with different supplements aimed at preventing the hepatotoxic side effect of MTX in different tissues and organs at the same or different markers.

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KABAKCI, A. G. & BOZKıR, M. G. Efecto protector del ácido linoleico sobre la toxicidad hepática inducida por metotrexato. *Int. J. Morphol., 41(1)*:237-245, 2023.

RESUMEN: Nuestro objetivo fue investigar el efecto protector del ácido linoleico sobre la toxicidad hepática inducida por metotrexato. El estudio se llevó a cabo en colaboración con el Departamento de Anatomía y el Departamento de Farmacología Médica de la Facultad de Medicina de la Universidad de Çukurova, utilizando las instalaciones del laboratorio del Departamento de Farmacología Médica. Se usaron células de la línea celular de hepatocitos humanos (CRL-11233) obtenidas de la American Type Culture Collection Organisation (ATCC). Se evaluaron las expresiones de marcadores de vías apoptóticas, factor inductor de apoptosis (AIF), BAX, BCL 2, GADD 153, proteína regulada por glucosa de 78 kDa (GRP78) y CASPASE-3. Todos los análisis se examinaron en cuatro grupos (Grupo 1; control, Grupo 2; se administró ácido linoleico, Grupo 3; se administró metotrexato y Grupo 4; se administró ácido linoleico y metotrexato). Los valores medios ± error estándar de los resultados obtenidos como

nanogramo/mililitro (ng/ml) se encuentran en el Grupo I, Grupo II, Grupo III y Grupo IV, respectivamente; Valores de AIF, 0,4150 ± 0,1208, 0,3633 ± 0,2389, 1,792 ± 0,3611 y 1,077 ± 0,1646, valores de Bax, 0,900 ± 0,1864, 1,002 ± 0,2098, 8,352 ± 1,467 y 4,295 ± 1,522, BCL 2 valores, 13,93 ± 1,199. 2,938 ± 1,059 y 9,250 ± 1,492, GADD 153, 0,7333 ± 0,1751, 0,7067 ± 0,2115, 1,650 ± 0,2950 y 1,237 ± 0,1805, Grp78, 0,4767 ± 0,1804, 0,5233 ± 0,1590, 2,183, ± 1,263. 1,127 ± 0,2033, 0,8317 ± 0,3392, 13,50 ± 1,871 y 8,183 ± 1,030. Se determinó que el ácido linoleico tiene un efecto protector sobre la toxicidad hepática inducida por metotrexato.

PALABRAS CLAVE: Anatomía; Hepatocito; Hígado; Ácido linoleico; Metotrexato.

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Corresponding author:
Ayse Gül Kabakcı (Dr)
Cukurova University
Faculty of Medicine
Department of Anatomy
Balcalı-Adana
TURKEY

E-mail: akabakci@cu.edu.tr

Ayse Gül Kabakci: Orcid 0000-0001-7144-8759
Memduha Gülhal Bozkır: Orcid 0000-0003-4164-4227