Effect of Methotrexate and Linoleic Acid on BAX/BCL2 Ratio in Human Hepatocyte Cell

ABSTRACT

Aims: To investigate the effect of methotrexate and linoleic acid on BAX/BCL 2 ratio in human hepatocyte cell.
Study design; Original Research Article.
Place and Duration of Study: 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.
Methodology: 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 BAX, BCL2 and BAX/BCL2 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).
Results: 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; 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 and BAX/BCL2 values 0.065, 0.072, 2.843 and 0.464.
Conclusion: While BAX/BCL2 level increased in the group given methotrexate, it decreased in the group given linoleic acid and methotrexate.

Keywords: BAX/BCL2, hepatocyte, liver, linoleic acid, methotrexate.

1. 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 [1]. 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. 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.The clinical use of MTX is significantly limited due to the associated various organ toxicities, including kidney, liver, lung, bone marrow, and gastrointestinal toxicities [1-2]. Methotrexate is a folic acid antagonist with anti-inflammatory and immunosuppressive effects [3]. 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 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 [4-6]. 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 [7]. 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 [8]. 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 [9-17]. 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 [14,17-26]. We think that linoleic acid (LA) can be able to reduce the BAX/BCL2 ratio and prevent apoptosis. The hypothesis of our study is that linoleic acid prevents hepatotoxicity and MTX caused hepatotoxicity. 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 [27]. The aim of our study is to investigate the effect of methotrexate and linoleic acid on BAX/BCL 2 ratio in human hepatocyte cell.

2. MATERIAL AND METHODS

2.1 Experimental Design

In this study, human hepatocyte (CRL-11233) cells obtained from the American Type Culture Collection Organization (ATCC) were used. All experimental procedures have been approved by “Non-Invasive Clinical Research Ethics Committee of Çukurova University Faculty of Medicine for our study (Decision No: 104/9). This study is derived from the Anatomy Doctor of Philosophy program thesis supported by Çukurova University Research Fund (project no: TDK-2018-10773). 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.

2.2 Experimental process;

BAX, BCL-2, apoptotic mediators were examined by ELISA test.

2.2.1 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 µl of each standard and samples were added to the ELISA plate and 200 µl (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.

2.3 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.

3. RESULTS

When the expression levels of BAX, BCL2 were examined, it was found that LA had a protective effect on MTX-induced hepatotoxicity (Table 1).

Table 1. 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)**
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)
BAX/BCL2	0,065	0,072	2,843	0,464

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

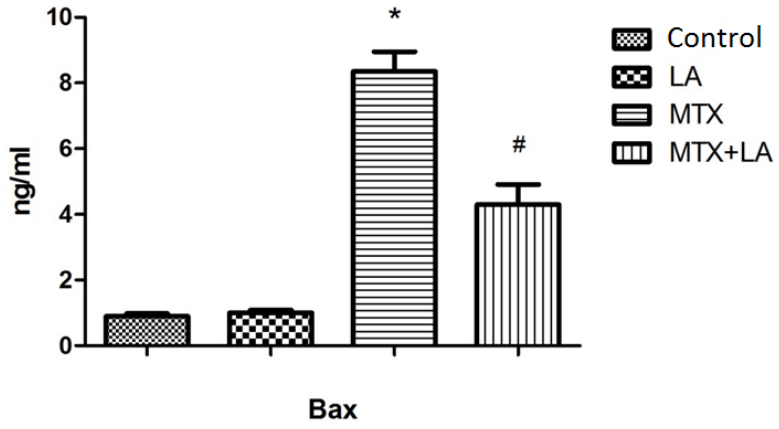

Figure 1.Distribution of BAX apoptotic marker among groups

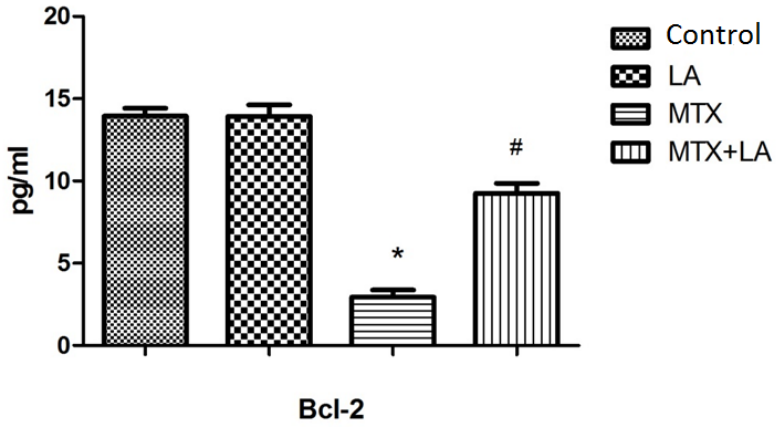

Figure 2. Distribution of Bcl-2 apoptotic marker among groups

In the study where we examined the effect of LA and MTX on hepatociyte cell. The changes in the values of BAX, BCL2 between the groups, respectively, in the control group; 0.900±0.1864 ng/ml, 13.93±1.198 ng/ml, in the LA group; 1.002±0.2098 ng/ml, 13.92±1.739 ng/ml, in the MTX group; 8.352±1.467 ng/ml, 2.938±1.059 ng/ml, in the group receiving LA +MTX; 4.295±1.522 ng/ml, 9.250±1.492 ng/ml were found (Figure 1, Figure 2). 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 levels of markers were examined, it was concluded that hepatotoxicity was induced in the MTX given groups.

4. DISCUSSION

Drugs were held responsible for more than 50% of liver disease. MTX, an antineoplastic drug, belongs to the group of antimetabolites. It acts as a folic acid antimetabolite [4]. There are many studies in the literature about pure squamous cell cancer of the urinary tract [28], in cancer types [29], rheumatoid arthritis [30] in dermatology [31] 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 [32-34]. There are studies in the literature to prevent hepatotoxicity and nephrotoxicity, which are the most important side effects caused by MTX [16,33,34]. 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 [35]. However, although there are many studies on experimental animals the number of studies examining the effects of LA on human metabolism is very few.

In the study where we examined the protective effect of LA against hepatotoxicity induced by MTX, apoptotic markers were examined and evaluated. Apoptotic markers; 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. Also, 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. While the BAX/BCL2 ratio was similar between the LA group and the control group, a significant increase was observed in the MTX group. In the MTX+LA group, the BAX/BCL2 ratio decreased compared to the MTX-administered group.

BAX, BCL2 and the BAX/BCL2 ratio are apoptotic markers that are frequently evaluated in the literature. 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 BAX and increased anti-apoptotic BCL2 [36]. 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) [37]. Yang et al. 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 and BCL2 proteins in the group given D-Isofluoridocide [33]. Zhang et al. investigated the protective effect of aspirin on acute liver injury due to paraquat in rats, and found that BAX value decreased, BCL2 value increased after aspirin treatment [38]. In a study by Ramachandran et al., examined the effect of acetaminophen hepatotoxicity on mitochondrial oxidative stress, DNA and liver damage, they found an increase in BAX values [39]. Similarly, Kouam et al., investigated the protective effect of Khaya grandifoliola (Meliaceae) used in Cameroon traditional medicine to prevent acetaminophen-induced hepatotoxicity. They found a decrease in BAX value in the group treated with Khaya grandifoliola [40]. In another study conducted on mice to prevent acetaminophen-induced hepatotoxicity, the protective effect of Folium Microcos was examined, and increase BAX value and decrease in BCL2 were observed in the acetaminophen given group compared to the acetaminophen + Folium Microcos group [41]. 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 BAX value was found in the group given saline compared to the group given vitamins and Metallothionein [42]. Hamed et al. examined the protective effect of strawberries against hepatotoxicity due to carbon tetrachloride and found that BAX value decreased and BCL2 value increased in the group receiving strawberries [43]. In the study conducted by Orazizadeh et al., 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 [44].

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 [45]. 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 [46]. 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 [47]. 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 level and decrease in BCL 2 level in the group given LA compared to the control group [48]. 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 level of BAX [49]. 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 [50].

In the another 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 [51]. In the study of Samdanci et al., 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 [17]. In the study of Kalantari et al., investigating the effect of inulin to prevent MTX-induced hepatotoxicity in mice, they stated that MTX administration caused significant liver damage in all mice and found decrease in BCL2 value compared to the control group [52]. In a study by Abo-Haded et al., 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 level and decrease in anti-apoptotic BCL2 level in the MTX-treated group [23]. Tabatabaei et al., investigated the neuroprotective effects of CU and LLLT on the BAX/BCL2 expression ratio in PC12 Cells induced by 6-OHDA. They found the combination of LLLT and CU has a neuroprotective effect on PC12 cells against 6-OHDA-induced neurotoxicity due to an increase cell viability and decrease an increase in the Bax/Bcl2 ratio which shows cell susceptibility to apoptosis [53]. In another study, the expression of apoptotic genes including BAX and BCL2 was evaluated in B16-F10 melanoma cancer and L929 cells by real-time PCR assay. As results shows in study, the expression of all genes and the BAX/BCL2 ratio were significantly changed after CAP treatment in B16-F10 tumor cells in comparison to untreated controls (BAX ($P = 0.028$), BCL2 and CASP3 ($P = 0.014$), and BAX/BCL2 ($P < 0.0001$)). CUR significantly changed the mRNA expression of BCL2 in B16-F10 tumor cells in comparison to untreated control cells (BCL2 ($P = 0.039$)). However, the BAX gene did not significantly increase in the CUR-treated cells, and BAX/BCL2 ratio was significantly increased in B16-F10 tumor cells ($P < 0.0001$). The expression of BAX ($P = 0.034$), BCL2 ($P = 0.042$), and BAX/BCL2 ratio ($P < 0.0001$) was found significantly altered after combination therapy in B16-F10 cells in comparison with untreated cells. CAP and CUR treatments were found had no significant effects on the expression of apoptotic genes in L929 normal cells [54].

Our study findings have resulted in support of the literature. In our study, it was found that MTX biochemically significantly increased liver apoptotic marker BAX protein level compared to the control group. However, in the group which LA + MTX was used, it was observed that decrease in these protein value 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.

5. CONCLUSION

- 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.
- 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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COMPETING INTERESTS

We wish to confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

AUTHORS' CONTRIBUTIONS

Idea, design, collection of resources by: AGK, MK, EŞ, MGB, application by: AGK, MK, EŞ, MGB, analysis and interpretation of results and literature by: AGK, MK, EŞ, MGB written and reviewed by: AGK and MGB.

CONSENT

In this study, human hepatocyte (CRL-11233) cells obtained from the American Type Culture Collection Organization (ATCC) were used.

ETHICAL APPROVAL

Approval was obtained from Çukurova University Faculty of Medicine Clinical Ethics Committee (approval number: 104/9). All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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