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Determination of Oxidative Damage Caused by Methotrexate and 5-Fluorouracil in Liver, Heart and Kidney Tissue in Rats

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Abstract

The 5-Fluorourosil (5-FU) is a pyrimidine analog used as a chemotherapy drug in many types of cancer. Methotrexate (MTX) is used as a therapeutic drug and chemotherapeutic agent in many diseases. However, in addition to its therapeutic properties, it causes damage to organs such as liver and kidney. In this study, 2-month-old male Sprague Dawley rats were divided into Group 1: Control, Group 2: MTX, Group 3: 5-FU groups with 5 animals in each group to determine the oxidative damage in liver, kidney and heart tissues. One day after the administration, the rats were anesthetized, the anterior abdominal wall was opened with an incision after cervical dislocation and liver, kidney and heart tissues were removed. Malondialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) levels were determined in these tissues. Liver GSH levels were significantly lower ($p<0.001$) and MDA and NO levels were significantly higher ($p<0.05$) in MTX and 5-FU groups compared to the control group. Similarly, renal GSH levels were significantly lower ($p<0.01$) in MTX and 5-FU groups compared to the control group, while MDA levels were significantly higher ($p<0.01$) in both groups and NO levels were significantly higher ($p<0.05$) only in MTX group. In heart tissue, GSH levels were significantly lower ($p<0.01$), MDA was significantly higher ($p<0.05$) and NO was significantly higher ($p<0.05$) in MTX and 5-FU groups compared to the control group. In conclusion, it is thought that MTX and 5-FU may cause oxidative damage in kidney, liver and heart tissue and may cause changes in oxidative damage markers and antioxidant levels.

Keywords: *Malondialdahyde, methotrexate, nitric oxide, oxidative damage, reduced glutathione*

1. Introduction

The 5-Fluorouracil (5-FU) is known as a pyrimidine analog, uracil antimetabolite and used as a chemotherapeutic agent. Although its mechanism of action is not fully known, it inhibits DNA and RNA synthesis in living organisms. While most of 5-FU is metabolized in the liver, the remaining part is excreted as urea. 5-FU is reported to be a therapeutic agent in many cancer types such as colorectal, gastrointestinal system, lung and liver cancer.[1] 5-FU has been reported to increase oxidative stress in the liver, resulting in both structural and functional damage to liver cells.[2] Methotrexate (MTX) is an anti-folic acid substance and has been reported to be cytotoxic.[3] Although it was first used to treat malignancies, it is now used in the treatment of many diseases such as skin diseases, rheumatoid arthritis and gynecological complaints. In addition to this feature, it has been reported to have serious side effects such as hepatotoxicity and nephrotoxicity.[4] MTX and 5-FU are anti-cancer drugs that have been widely used in the past.[5] Metabolites of 5-FU inhibit pyrimidine de novo synthesis by negatively affecting the enzyme thymidylate synthase. MTX further potentiates this.[7] Free radicals are high-energy atoms or molecules carrying one or more unpaired electrons in the outer orbital. Free radicals damage lipids, proteins and DNA at high concentrations.[8] As a result, cell death and DNA fragmentation have been reported to occur.[9] It has been reported that nitric oxide (NO) is a free radical of the reactive nitrogen species (RNS) group and is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS).[10] Reduced glutathione (GSH), an important antioxidant found in all living cells, has been reported to be a tripeptide consisting of glycine, glutamic acid and cysteine.[11] GSH regulates cell signaling by reducing the sulfhydryl group in proteins and protecting them against oxidation. It also ensures the passage of amino acids through the membrane.[9] It has been reported that the determination of malondialdehyde (MDA) level is most preferred in the determination of lipid peroxidation.[12] As a result of the increase in MDA levels due to lipid peroxidation, cell membrane integrity is disrupted.[13] In this study, it was aimed to investigate the oxidative damage caused by MTX and 5-FU in liver, heart and kidney tissues in rats.

2. Materıal and Methods

2.1. Study groups

This study was initiated after the approval of the ethics committee of Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK) dated 23.10.2020 and coded 2020/140. In the study, 15 2-month-old male Sprague Dawley rats from Kafkas University Experimental Animal Housing, Application and Research Unit were used. During the 15-day housing period, rats were given feed and drinking water ad libitum. Necessary care and cleaning of the animals were performed regularly. No restriction or deprivation was applied. Animals were kept in a 12-hour day a 12-hour dark period. Groups:

Group I (Control Group, n=5): Intraperitoneal 0.05% sali-

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ne was administered. No special treatment was applied during the experiment and the usual nutritional conditions were provided.

Group II (MTX Group, n=5): A single dose of 200 mg/kg intraperitoneal MTX dissolved in saline was administered for the first 6 days and 200 mg/kg intraperitoneal MTX dissolved in saline was administered on the 7th day.

Group III (5-FU, n=5): Saline for the first 6 days and 200 mg/kg single dose of intraperitoneal 5-FU dissolved in saline on day 7.

One day after the treatments, the animals were euthanized with ether. The anterior abdominal wall was incised and liver, kidney and heart tissues were removed. The tissues were washed with saline and homogenized. The homogenates were centrifuged at 1500 rpm for 15 min and the supernatants were stored at -20 ^{0}C until the measurements were performed.

2.2. Biochemical analysis

GSH, MDA ve NO levels were measured by chemical method in the Kaskas University Veterinary Faculty Biochemistry Laboratory.

GSH analysis was performed according to the Beutler method. [13] The analysis method is as follows.

From the tubes to be marked as blind, standard and test, 200 µL of EDTA blood was taken into the test tube, 200 µL of the standard solution was taken into the standard tube, 1.8 ml of distilled water (for hemolysis of the tissue) and 3 ml of precipitating solution were added. To the tube marked as blind, 800 µL of distilled water was pipetted with 1.2 ml of precipitating solution. The tubes were mixed and kept in ice water for 5 minutes and centrifuged at 3000 rpm for 10'. The blind tube was kept intact and 2 ml each of supernatant was taken into new tubes marked as standard and test. Add 8 ml of phosphate solution to each tube and mix. 1 ml of DTNB was added and the optical density of the standard and test was read against the blind at 412 nm wavelength.

Tissue GSH concentration (mg/dl) = (Optical Density of Assay / Optical Density of Standard) x Concentration of Standard (20 mg/dl).

MDA analysis was performed according to the Yoshioka method. [14] The analysis method is as follows:

Glass test tubes to be marked as test and blind were taken and 0.5 ml of plasma was pipetted into the test tube. 3 ml of 20% TCAA was added to the blind tube and 2.5 ml to the test tube. Then 1 ml of TBA was added to both tubes and the tubes were incubated in a 90°C water bath for 30 minutes, cooled, pipetted with 4 ml of n-butanol and centrifuged at 3000 rpm for 10 minutes. The n-butanol layer was then transferred to another tube and the absorbance of the assay against the blind at 535 nm was read on a spectrophotometer. The results were obtained from the calibration curve.

NO levels were determined by the Miranda method.[15] The analysis method is as follows:

For nitrate analysis, 100 µL of sample was pipetted into the microplate wells. 100 µL of VaCl3 was added to all wells. Immediately afterwards, 100 µL of Griess reagent was pipetted. After incubating for 30 minutes at 37 ºC in an oven, absorbances were read at 540 nm wavelength. For nitrite analysis, 100 µL of sample was pipetted into the microplate wells. Immediately afterwards, 100 µL of Griess reagent was pipetted. After incubating for 30 minutes at 37 ºC in an oven, absorbances were read at 540 nm wavelength. Nitric oxide concentration was found by summing the nitrate and nitrite concentrations based on the calibration curve. Nitric Oxide (µM) = Nitrate (µM) + Nitrite (µM)

2.3. Statistical Analyses

SPSS 16 Windows package program was used for statistical analysis of GSH, MDA and NO levels in liver, kidney and heart tissues of rats. One way ANOVA and Duncan test for multiple comparisons were used to compare the values.

3. Results

The GSH level (Figure 1) in the liver tissue of rats was significantly lower ($p<0.001$) in the MTX and 5-FU groups compared to the control group, and the GSH level in the kidney and heart tissue was significantly lower ($p<0.01$) in the MTX and 5-FU groups.

MDA levels (Figure 2) of MTX and 5-FU groups were significantly higher in liver tissue ($p<0.05$), significantly higher in kidney tissue ($p<0.01$) and significantly higher in heart tissue ($p<0.05$) compared to the control group.

NO levels (Figure 3) in MTX and 5-FU groups were significantly higher in liver tissue ($p<0.05$), significantly higher in kidney tissue only in MTX group ($p<0.05$) and significantly higher in heart tissue only in 5-FU group ($p<0.05$) compared to the control group.

4. Discussion

Although 5-FU is reported to be a chemotherapeutic drug as a pyrimidine analog, it inhibits DNA synthesis and impairs RNA function in living organisms.[1] The metabolic product of 5-FU is 5-fluoro-2-deoxyuridine monophosphate, an irreversible inhibitor of the enzyme thymidylate synthase, which is required for thymine synthesis. 5-FU limits the production of deoxythymidine monophosphate (dTMP), which is crucial for DNA replication and repair in the organism. dTMP deficiency leads to cellular toxicity. However, it is converted to dihydrouracil in the liver. This in turn is broken down into α-fluoro-β-alanine, ammonia, urea and carbon dioxide, leading to nephrotoxicity.[17] It can be used in combination with other chemotherapeutic drugs for tumors of the colon, stomach, breast, pancreas and brain. However, due to the exposure of healthy cells to the drug, it causes side effects such as low white blood cell count, low platelet count, anemia, nausea, vomiting, diarrhea, leukopenia and hand-foot syndrome.[1,18] These effects of 5-FU increase oxidative stress parameters in the organism.[2] Intraperitoneal administration of 5-FU has been reported to cause renal toxicity. It was concluded that this toxicity was due to the production of free radicals that cause an increase in oxidative stress.[19]

Several studies have reported that MDA and NO levels increased and GSH levels decreased in liver, kidney and

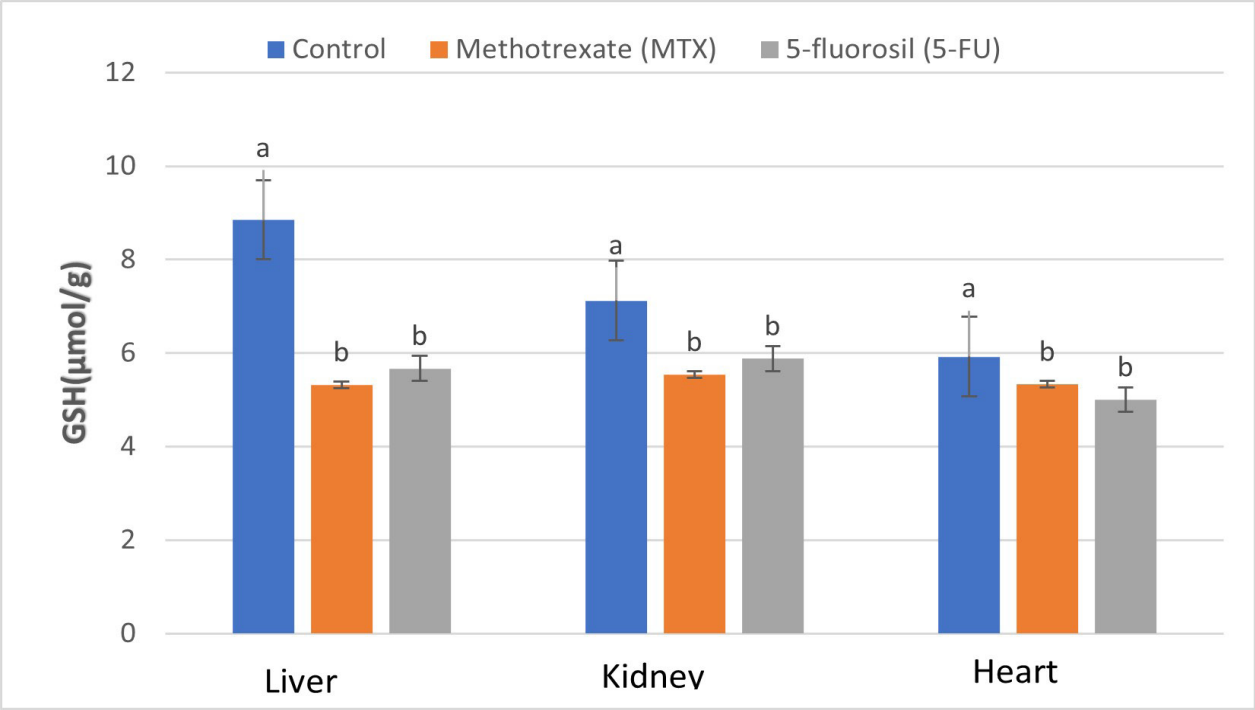

Figure 1. Changes in GSH levels in liver, kidney and heart tissues (a,b: the difference between groups with different letters is significant).

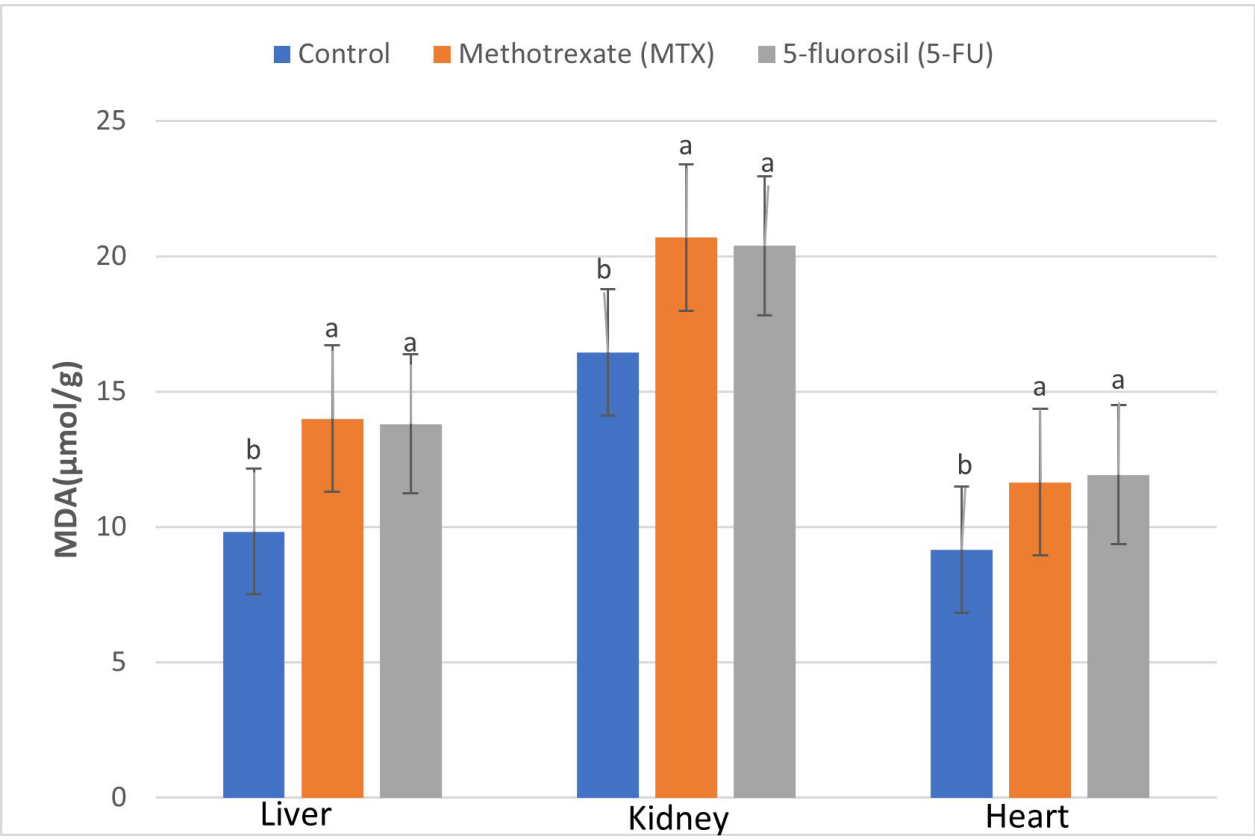

Figure 2. Changes in MDA levels in liver, kidney and heart tissue (a,b: the difference between groups with different letters is significant).

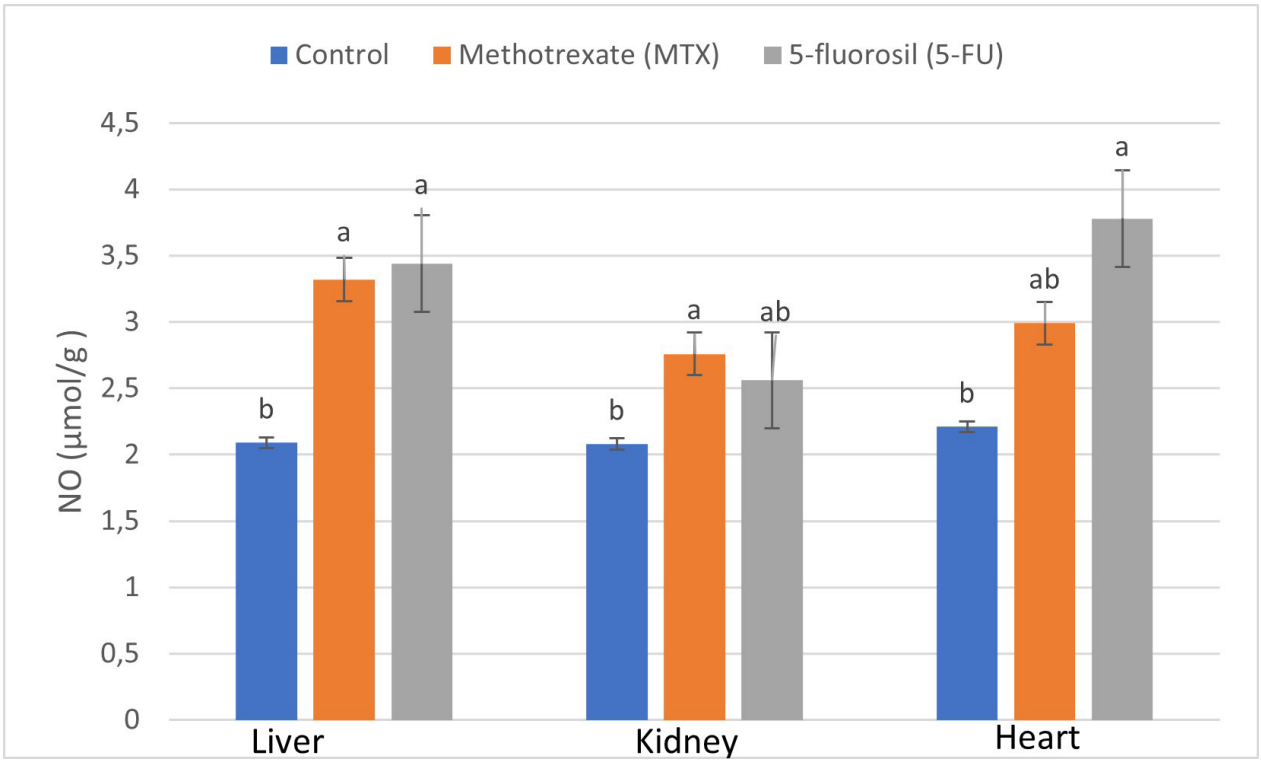

Figure 3. Changes in NO in liver, kidney and heart tissue.

heart tissues of 5-FU-treated animals.[20-26]

The mechanism of 5-FU can be explained based on the synthesis of anabolic reactions that produce active metabolites such as fluoro-deoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). FdUMP binds to thymidylate synthase (TS) and causes inhibition of DNA synthesis and its effect is irreversible. FdUTP and FUTP bind to DNA and RNA respectively and disrupt their production of genetic material and cause cell death. 5-FU increases the production of ROS in mitochondria through the p53 signaling pathway. 5-FU increases the formation of ROS by increasing the release of cytochrome c from mitochondria. 5-FU affects the whole system in the organism. This means that healthy tissue cells are also affected by 5-FU toxicity. Vital organs such as the liver and kidneys are constantly exposed to the metabolites of 5-FU and are adversely affected. Complaints such as hepaptitis, steatosis, steatohepatitis, nephropathy and chest pain have been reported in patients treated with 5-FU. It is emphasized that the reason for these pathological findings is the triggering of oxidative stress and inflammation as a result of the chematheropathic administration of 5-FU.[27] 5-FU, which is frequently used as a chemotherapeutic agent, is highly toxic to the vascular entothelium using endothelial NO synthase. It can cause coronary disorders by utilizing the Protein Kinase C pathway.[28] The mechanism of 5-FU injury in liver tissue is still unclear and needs to be further investigated and studied.[29]

In this study, GSH levels were found to be low and MDA and NO levels were found to be high in liver, kidney and heart tissues of 5-FU-treated rats in accordance with the other studies mentioned above.[20-26]

MTX is a folic acid antagonist and its structure is similar to propylglutamic acid. It is widely used in the treatment of hematologic disorders and various autoimmune system diseases. It has also been reported to be used as a chemotherapeutic agent in all kinds of cancer diseases.[30] MTX binds to dihydrofolate reductase and decreases thymidylate, purine synthesis and cell poliferation. It also suppresses other folate-dependent enzymes and produces anti-inflammatory effect by suppressing the proliferation of lymphocyte cells with adenosine accumulation, adhesion of these cells as a result of free radical formation and neutrophil chemotaxis.[31]

Although MTX has a wide range of uses, it shows side effects such as nephrotoxicity and hepatoxicity.[32,33] Oxidative damage caused by ROS (reactive oxygen species) is held responsible for these side effects. MTX is highly excreted through the urinary system. Therefore, high dose administration of MTX causes acute renal failure. As a result, nephrotoxicity has been reported to be one of the most important side effects of MTX.[33,34]

MTX causes oxidative damage in many tissues, especially kidney and liver tissues. In some studies, it was reported that MDA and NO levels increased and GSH levels decreased in liver, kidney and heart tissues of MTX-treated rats.[35-43]

Consistent with other studies, liver, kidney and heart GSH levels were found to be lower in MTX-treated animals compared to the control group.[39-43] consistent with the studies, liver, kidney and heart MDA levels were found to be higher in the liver, kidney and heart compared to the control group.[36,39-43] consistent with the studies, liver, kidney and heart NO levels were found to be higher in the liver, kidney and heart compared to the control group.[36,39,41]

In this study, MDA and NO levels of liver, kidney and heart tissues of MTX-treated animals were found to be high, while GSH levels were found to be low, in accor-

dance with the studies mentioned above.[35-43]

5. Conclusion

In conclusion, it was concluded that oxidative damage occurred in the liver, kidney and heart tissues of mice administered MTX and 5-FU with increased levels of MDA, the end product of lipid peroxidation, and the antioxidant system was insufficient to prevent this damage.

Notes

This study was previously presented as a poster.

Presentation title: Determınation O1of Oxidative Damage Caused by Methotrexate And 5-Fluorouracil in Liver, Heart And Kidney Tissue in Rats

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There is no financial support for this study.

Conflict of Interest

There is no conflict of interest between the authors.

Ethics Statement or Informed Consent

This study was initiated after the ethics committee approval of Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK) dated 23.10.2020 and coded 2020/140.

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