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The Effect of Dimethyl Fumarate on Tissues in Methanol Poisoning

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ABSTRACT

Objective: This study aimed to investigate the effects of an antioxidant, dimethyl fumarate (DMF), on methanol (MeOH)-induced liver, kidney, testicular, and ocular toxicity in Wistar rats.

Methods: Six experimental groups were established: a control group; a group receiving (DMF; 100 mg/kg); a group receiving methotrexate [(MTX); 0.3 mg/kg/day]; and three groups receiving MTX followed by MeOH (3 g/kg, oral, 20% solution) with either 30 mg/kg or 100 mg/kg DMF, or no DMF. MTX was administered for seven days prior to MeOH, and DMF was administered two hours before MeOH, on the eighth day. Animals were sacrificed eight hours after MeOH administration.

Results: Minimal changes were seen in the urine test, such as a high protein score and increased pH value in the MTX + MeOH group, and a significant level of bilirubin excretion in the MTX + MeOH + DMF100 group. Only the liver weight/body weight X100 index was lower in the DMF group. There were no histopathological changes in liver, kidney, testicular, and eye tissues.

Conclusion: DMF was found to be safe for the liver, kidney, testicular, and ocular tissues. However, it was not possible to achieve the MeOH toxicity model in the specified doses and routes in the rats, contrary to the related literature. Future studies are needed to develop a reliable model for MeOH toxicity in rats.

Keywords: Alcohol, animal experimentation, antioxidant, ocular toxicity, methotrexate, toxicology

INTRODUCTION

Methanol (MeOH) is used in a wide range of consumer products, such as model car fuel, aviation fuel, fragrances, gas line antifreeze, and copy machine fluid. Unfortunately, MeOH is sometimes illegally substituted for ethanol due to its lower cost and easier availability (1). Inhalation of carburetor cleaner is another source of MeOH poisoning. Notably, in India, Türkiye, and Tunisia, a large proportion of MeOH poisoning cases result from the absorption of fragrances and colognes (1). Accidental or intentional ingestion of MeOH-containing substances, is an important public health problem as its toxicity may cause severe morbidity and mortality (1-3). The enzymatic activity of alcohol dehydrogenase on MeOH first produces formaldehyde, which is then converted to formic acid by aldehyde dehydrogenase. This process decreases nicotinamide adenine dinucleotide (NAD+) concentrations. When the conversion of NAD+ to NADH diminishes, anaerobic respiration is promoted. These metabolic changes can lead to hypoglycemia and lactic acidosis (4). Acidosis leads to oxidative stress, production of reactive oxygen species, lipid peroxidation, cell damage, and mitochondrial dysfunction in many organs, especially in the optic nerve (1,3). Formic acid itself is also considered responsible for ocular toxicity (1,2). Meanwhile, several antioxidants have been reported as effective medications against tissue damage of MeOH poisoning (2,3,5).

Dimethyl fumarate (DMF) is an immunomodulatory, anti-inflammatory, and antioxidant, pharmaceutical agent indicated for the therapeutic management of inflammatory pathologies, specifically psoriasis and multiple sclerosis (6,7). Evidence suggests that DMF facilitates a cascade of processes, leading to the final expression of a variety of antioxidant and detoxifying genes. Furthermore, systemic administration of this agent for psoriasis treatment reduced levels of several cytokines, including interleukin

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(IL)-6, IL-17, IL-22, granulocyte-macrophage colony-stimulating factor, transforming growth factor-alpha, and interferon-gamma, while conversely increasing IL-10 levels. Similarly, the anti-inflammatory mechanism of multiple sclerosis treatment on glial cells involves inhibiting the release of various pro-inflammatory molecules, such as nitric oxide, IL-1 beta, IL-6, and tumor necrosis factor (TNF)-alpha (7). The findings of Sangineto et al. (6) demonstrated that DMF protected mice from ethanol-induced liver damage, steatosis, and inflammation by specifically reducing hepatic triglyceride and alanine aminotransferase levels, downregulating the hepatic expression of inflammatory cytokines (TNF-α, IL-1β, CXCL11), and decreasing the presence of neutrophils and macrophages in the liver tissue of ethanol-fed mice. A recent review provided several examples of the benefits of DMF in ocular diseases, such as age-related macular degeneration, uveitis, cystoid macular edema, light-induced photoreceptor loss in mice, and corneal transplantation in rats (7).

Nevertheless, research has not yet examined the effects of DMF on MeOH toxicity at the organ level, especially considering the significant side effects on the retina. Therefore, this study aims to investigate the efficacy of DMF as an antioxidant and anti-inflammatory agent in MeOH poisoning.

METHODS

Since no human embryos, fetuses, tissues derived from them, or other human cells and tissues were used in any part of this study, obtaining a patient consent form is not required. Animal experiments were conducted at the Animal Center of Aydin Adnan Menderes University, and all animal protocols were approved by the Animal Ethics Committee for Experiments at Aydin Adnan Menderes University (decision no: 64583101/2024/32, date: 21.03.2024) before the experiments were conducted. The study used 54 male Wistar albino rats that were 10-12 weeks old and were kept in a room with a temperature of 22±2 degrees celsius, 40-50% humidity, and a 12-hour light-dark cycle. Because rats are resistant to MeOH toxicity, methotrexate was given to establish the animal model (2,3). Each group consisted of 9 animals; the groups were as follows:

Control: The control group was administered intraperitoneal saline solution for 7 days, followed by 1 mL of oral saline solution on the 8th day.

DMF100: The group receiving a single high dose of DMF by gavage in the study was intended to demonstrate the safety of the treatment agent at this dose and route.

Methotrexate (MTX): The group administered methotrexate (0.3 mg/kg/day) by gavage for 7 days.

MTX + MeOH: The group administered methotrexate (0.3 mg/kg/day) by gavage for 7 days, followed by methyl alcohol (3 g/kg, 20%) by gavage on the 8th day.

MTX + MeOH + DMF30: The group was administered methotrexate (0.3 mg/kg/day) by gavage for 7 days, then DMF (30 mg/kg) by gavage on the 8th day, and finally oral methyl alcohol (3 g/kg, 20%) administered 2 hours later.

MTX + MeOH + DMF100: The group was administered methotrexate (0.3 mg/kg/day) by gavage for 7 days, followed by DMF (100 mg/kg) by gavage on the 8th day. Subsequently, oral methyl alcohol (3 g/kg, 20%) was given 2 hours later.

The animals were euthanized 8 hours after MeOH intoxication (2,8), using a combination of 50 mg/kg ketamine and 5 mg/kg xylazine anesthesia. Liver, kidney, testicular, and eye samples were placed in the 10% formalin solution.

Clinical Follow-up

All animals' body weights were assessed to adjust the drug doses. Liver, kidney and testicular wet weights have been adjusted according to body weight: [(sum of liver weights)/body weight] × 100; [(sum of kidney weights)/body weight] × 100, and [(sum of testicular weights)/body weight] × 100. To identify organ changes, such as edema, vasoconstriction and necrosis, in cases of acute organ damage where body weight remained unchanged, organ weights were compared to body weight. Blood glucose level was measured with a glucometer (Clever Chek, Istanbul, Türkiye) from the tail blood sample.

The urine samples were collected just prior to the sacrifice, to perform the strip test. Using a monitor (Hua 90M portable urine monitor, China), numeric data was obtained to show kidney and liver functions for urinary density, pH alteration, presence of leukocytes, nitrite, urobilinogen, calcium, blood ketone, bilirubin, creatinine, and glucose (9).

Pathological Evaluation

In the pathological examination, tissues were fixed in 10% neutral buffered formalin and subjected to routine tissue processing. Following this procedure, 4 µm thick sections were prepared from the tissue samples embedded in paraffin blocks using a rotary microtome. These sections were stained with hematoxylin and eosin (HE) and evaluated under a light microscope (BX51, Olympus, Tokyo, Japan) at 10x, 20x, and 40x magnifications. For findings that were present, semi-quantitative scoring was applied based on severity: mild (1), moderate (2), or severe (3).

For the liver: portal inflammation, confluent necrosis, spotty necrosis, piecemeal necrosis, and fibrosis were evaluated as either present or absent.

In the kidney tissues, interstitial inflammation, glomerular sclerosis, tubular necrosis, and vascular changes were evaluated as present/absent (0).

In the testicular tissues, necrosis and spermatogenesis arrest, as present/absent (1/0), were evaluated.

In the ocular tissues, damage findings in the cornea, retina, and optic nerve, including edema, inflammation, hemorrhage, increased vascularity, and necrosis, were evaluated as present or absent, with 0 indicating absence.

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Statistical Analysis

The conformity of quantitative variables to normal distribution was examined by the Kolmogorov-Smirnov test. One-Way Analysis of Variance or Kruskal-Wallis test were used, according to the distribution structure of the variables. Pathologic examination results were analyzed using chi-square tests. Descriptive statistics were given as mean ± standard error, median (25^{th}-75^{th} percentiles), or frequency (%). For all statistical analyses, $p<0.05$ was accepted as the significance level.

RESULTS

Clinical findings

None of the rats died during the study. The presence of leukocytes, nitrite, urobilinogen, protein, calcium, blood, ketone, creatinine, and glucose in the urine was not significantly different among the groups. The MTX + MeOH group had a urine protein score of 3.33, which was higher than the control group's 2.75. However, no significant differences were observed among the other groups: DMF (2.13), MTX (2), MTX + MeOH + DMF30 (2.25), and MTX + MeOH + DMF100 (2.88).

Bilirubin excretion score of MTX + MeOH + DMF100 group was 0.78, significantly higher than the control group score, which was 0 (p=0.014). The MTX group score was 0.44 and the MTX + MeOH + DMF30 group score was 0.25; both scores showed a tendency to increase.

Urine density was the same in all groups. The urine pH values in the DMF treatment groups (30 mg/kg and 100 mg/kg) were found to be similar to the control group, respectively, with measurements of 6.06±0.42, 6.89±0.16, and 6.88±0.25. Only DMF-taken group's urine pH was 6.44±0.11. In the MTX + MeOH group, the pH value increased to 7.31±0.25, but there was no statistically significant difference when compared to the control group. Blood sugar was not different among the groups, although it tended to be lower with MeOH. The animals' kidney to body weight and testicular weight to body weight ratios were insignificant (Table 1).

A significant reduction in the liver weight/body weight ratio was observed when compared to the Control group (3.53±0.11) in the DMF100 group (3.17±0.06, p=0.010), the MTX + MeOH + DMF30 group (3.14±0.06, p=0.035), and the MTX + MeOH + DMF100 group (3.01±0.06, p=0.014). (Table 1).

Pathological Evaluation

Pathological evaluations were performed on the liver (inflammation, necrosis, fibrosis); kidney (inflammation, sclerosis, necrosis, vascular changes); testes (necrosis, spermatogenesis arrest); and eyes (edema, inflammation, hemorrhage, increased vascularity, necrosis in cornea, retina, optic nerve) using HE staining. No abnormalities were detected in any group across these tissues. (Figure 1).

DISCUSSION

In an effort to replicate and extend previous findings, we applied DMF within our experimental model, using the same setup and strictly adhering to published techniques. Regrettably, we could not demonstrate that this route and dose of administration, with HE staining, produced MeOH toxicity, or that DMF attenuated systemic inflammation in a rat model of MeOH toxicity. MeOH had minimal effects on the systems in our rats.

In a previous study, male Wistar rats exposed to 3 g/kg of a 50% w/v solution of MeOH showed the highest increase in liver lipid peroxide products, 24 hours later (5). Similarly, Nugrahanti et al. (8) induced liver toxicity in 2-3 month-old male Wistar rats by administering 3.5 and 7 g/kg of a 20% w/v MeOH solution and sacrificing them 8 hours later, without MTX pretreatment. This dosage resulted in an abnormal liver condition. In contrast, Sahin et al. (2) examined retinal and optic nerve damage, administering 3 g/kg of a 20% w/v MeOH solution intraperitoneally after 7 days of MTX (0.3 mg/kg/d) treatment, and sacrificing the rats 8 hours post-MeOH. They noted that rats are less susceptible to MeOH poisoning than humans due to higher liver folic acid and rapid formic acid metabolism, the latter of which can be compromised by nitrous oxide or MTX. MTX treatment impairs folate-dependent

Table 1. Biochemical findings of all experimental groups

	Urine pH	Urine density	Blood sugar	Liver weight /BW X100	Kidney weight /BW X100	Testicular weight /BW X100
Control	6.5 (6.5-7.0)	1030 (1030-1037.5)	198 (168.5-219.5)	3.53±0.11	0.83 (0.83-0.89)	0.87 (0.81-0.88)
DMF100	6.5 (6.13-6.5)	1030 (1030-1037.5)	201.5 (160-228.75)	3.17±0.06*	0.91 (0.83-0.96)	0.89 (0.85-0.98)
MTX	7 (5.75-7.25)	1030 (1030-1030)	189 (168.5-240)	3.51±0.09	0.89 (0.85-0.96)	0.89 (0.88-0.93)
MTX+MeOH	7 (6.75-8.0)	1030 (1030-1030)	172 (156-189)	3.37±0.10	0.90 (0.84-0.99)	0.97 (0.92-1.01)
MTX+MeOH+ DMF30	5.75 (5.0-7.38)	1030 (1030-1055)	174 (155.5-191.75)	3.14±0.06**	0.88 (0.83-0.97)	0.92 (0.84-0.97)
MTX+MeOH+ DMF100	7 (6.5-7.25)	1030 (1030-1035)	166 (161-177)	3.01±0.06***	0.87 (0.81-0.94)	0.93 (0.89-0.97)
p-value	0.122	0.428	0.252	<0.001	0.586	0.058

*: DMF100 (p=0.010), **: MTX + MeOH + DMF30 (p=0.035) and ***: MTX + MeOH + DMF100 (p=0.014) are statistically different from the Control group.
DMF100: Dimethyl fumarate (100 mg/kg), MTX: Methotrexate (0.3 mg/kg/7 days), MeOH: Methyl alcohol (3 g/kg, 20%), DMF30: Dimethyl fumarate (30 mg/kg), DMF100: Dimethyl fumarate (100 mg/kg)

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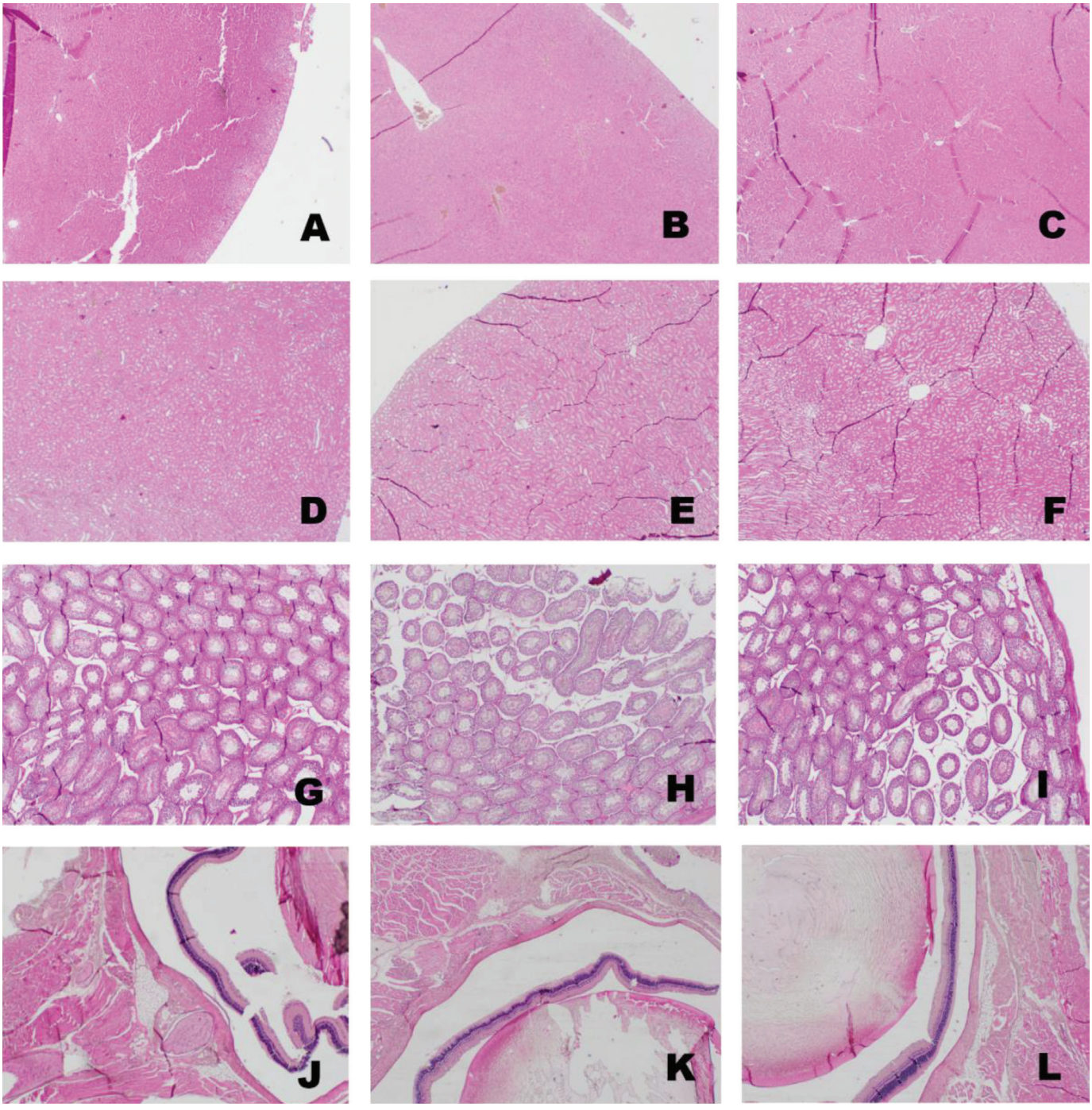

Figure 1. Pathological appearance of liver (A, B, C), kidney (D, E, F), testis (G, H, I) and eye (J, K, L) tissues. A, D, G, J are the Control groups; B, E, H, K are the 100 mg/kg Dimethyl Fumarate groups; C, F, I, L are Methotrexate+ Methanol groups

formate metabolism and facilitates MeOH poisoning in rats (3). Human livers contain approximately half the tetrahydrofolate levels found in rat livers. Furthermore, the activity of the enzyme 10-formyl tetrahydrofolate dehydrogenase is lower in human livers than in rat livers. Consequently, rats metabolize formate twice as quickly as humans. This rapid metabolism prevents the accumulation of formic acid in rats, protecting them from acidosis, ocular damage, and other toxic effects observed in human MeOH poisoning (1). Yazgan et al. (10) and Gursul et al. (3) also reported that they successfully used this MTX/MeOH model for liver injury. Therefore, to mitigate the risk of sudden death or gastric hemorrhage associated with a 3.5 or 7 g/kg MeOH dose alone, we pretreated rats with MTX for 7 days and applied 3 mg/kg of a 20% w/v MeOH solution, strictly adhering to established protocols. We have also used 10-12 week old male Wistar rats according to previous literature. These previous reports have evaluated the MeOH exposure samples through electron microscopy (5) and by measuring the leakage of alanine aminotransferase and aspartate

aminotransferase into the blood (3,5). Additionally, they have assessed malondialdehyde and total (anti)oxidant status (3) and conducted docking affinity studies for alcohol dehydrogenase (2). Unlike previous studies, we employed urine tests and pathological examinations to evaluate toxicity in the liver, kidneys, testicles, and eyes.

Silva et al. (9) demonstrated that a urine test strip, used in semiquantitative biochemical urinalysis, is a straightforward and safe technique for evaluating urinary and systemic function in ischemia and reperfusion injury in the kidney and in streptozotocin diabetes models. Similar to their technique, we placed the animals on the bench and waited for spontaneous urination. They reported an average urine pH of 8.5 in their mouse model. Conversely, our experiments with normal rats showed a significantly lower urine pH, averaging 6.88. The application of MTX + MeOH to rats increased the urine pH to 7.31 (a 6.25% increase). This group also showed a high protein score in urine 3.33 (a 21% increase over the control value), indicating an early sign of kidney stress; however, prominent kidney pathology was not detected using HE staining following the administered MeOH dose. Our urine analyses also supported these normal histological findings, which showed creatinine within normal levels and no hematuria in the urine samples.

Bilirubin is typically absent from urine. However, some animals showed bilirubin excretion in urine following treatment with MTX and MTX + MeOH + DMF30, while the MTX + MeOH + DMF100 group exhibited a significant increase. This effect was not observed in the control or DMF groups, suggesting that it is related to MTX. As MTX can indirectly affect bilirubin levels due to its potential impact on the liver (11), this side effect may be more pronounced when used in combination with MeOH or DMF. Nugrahanti et al. (8) reported that liver specimens exposed to only, 3.5 and 7 g/kg of MeOH exhibited inflammatory cells, necrosis, cell degeneration, and fibrosis. This was similar to findings in a previous study, where 3 g/kg of a 20% w/v MeOH solution, administered with MTX for 7 days, resulted in severe pyknosis, hemorrhage, hydropic degeneration, and mononuclear cell infiltration in the hepatocytes of the MeOH + MTX group (3). However, we did not observe any of these signs in HE staining of our liver samples.

In our study, testicular tissue was not damaged by this route and dose in the MeOH + MTX group, as evaluated through histopathological examination of HE staining.

Hypoglycemia and lactic acidosis are known consequences of MeOH poisoning (4). However, it has been reported that inducing the severe acidosis seen in human MeOH poisoning is not possible in rodents (1). This limitation might explain why our study did not show severe urine and blood parameter changes. While blood glucose levels slightly decreased, this difference was not statistically significant compared to the control group.

Sahin et al. (2) showed that MeOH + MTX exposure caused oxidative stress in rat retinas and optic nerves, leading to retinal ganglion cell loss. This damage was prevented by the antioxidant caffeic acid phenethyl ester. Although we used the same MeOH + MTX protocol, we did not observe, using HE staining, edema, inflammation, hemorrhage, increased vascularity, or necrosis in the cornea, retina, or optic nerve.

In this study, DMF was determined to be safe for the liver, kidney, testicular, and ocular tissues, based on the parameters inspected. DMF has been shown to be effective against ethanol-induced hepatic injury, steatosis, and inflammation in mice (6). Furthermore, its beneficial effects have been proposed in many experimental eye models (7). Similarly, we would like to evaluate its potential, especially in ocular tissue in MeOH toxicity. Unfortunately, we could not prove our hypothesis. At least we could prove its safety in this dose and route on four tissues. First, an additional replicable MeOH model should be established.

Study Limitations

A key limitation of this study is that the model did not produce expected toxicity symptoms in urine and tissue samples. Furthermore, only HE staining was used to evaluate the four tissue samples, unlike previous research that employed electron microscopy for more detailed damage assessment.

CONCLUSION

DMF was found to be safe for the liver, kidney, testicular, and ocular tissues. The administered dose and route of MeOH, in conjunction with MTX, did not result in toxicity in rats, as has been observed in previous reports. Our study provides foundational techniques and ideas for future research.

Ethics

Ethics Committee Approval: Animal Ethics Committee for Experiments at Aydin Adnan Menderes University (decision no: 64583101/2024/32, date: 21.03.2024).

Informed Consent: Since no human embryos, fetuses, tissues derived from them, or other human cells and tissues were used in any part of this study, obtaining a patient consent form is not required.

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Footnotes

Author Contributions: Surgical and Medical Practices - A.A.U., B.D.; Concept - A.I.A.U., I.M., B.D.; Design - A.I.A.U., A.A.U., B.D.; Data Collection and/or Processing - A.A.U., I.M., B.D.; Analysis and/or Interpretation - A.I.A.U., I.M., B.D.; Literature Search - A.I.A.U., A.A.U., B.D., Writing - A.I.A.U., B.D.

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

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