Research Paper

Efficacy of Ethanol and Intravenous Lipid Emulsion on Methanol Toxicity in Rats: Biochemical and Histopathological Insights

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ABSTRACT

Background: This study evaluated the therapeutic efficacy and interactions of intravenous lipid emulsion (ILE) and ethanol in methanol toxicity, focusing on biochemical parameters, organ function, and histopathology.

Methods: Sixty-four male rats were assigned to seven groups: control, methanol, methanol + ethanol, methanol + ILE, methanol + ethanol + ILE, ethanol, and ILE. Methanol (4 g/kg) was administered orally, followed by ethanol (1 g/kg) an hour later. ILE (1.5 ml/kg) was given within 30 minutes of methanol ingestion, with two additional doses every six hours. After five days, blood and organ samples were analyzed.

Results: Ethanol significantly reduced ALT, AST, and ALP, while LDH was lowest in the methanol + ethanol + ILE group. Ethanol improved kid`ney function ($p<0.001$), whereas ILE alone increased creatinine. No significant differences were observed in 8-OHdG ($p=0.572$). Histopathology showed ethanol reduced liver congestion ($p=0.0067$), lung inflammation ($p=0.0186$), and congestion ($p=0.0067$). The combined therapy showed no significant advantage over ethanol alone.

Conclusion: Ethanol remains the most effective treatment, while ILE's role appears complex and timing-dependent, warranting further investigation.

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Introduction

Alcohol consumption is recognized globally as one of the five major mortality factors. The production and consumption of unrecorded alcoholic beverages is of significant concern both in Türkiye and worldwide [1-3]. Alcoholic beverages likely to contain methanol (M) adversely affect nearly every body system, reducing the effectiveness of treatment for alcohol-related diseases. Additionally, alcohol consumption has been linked to various cancers, the production of reactive oxygen and nitrogen species, alterations in folate metabolism, DNA methylation, and nutritional deficiencies. Once M enters the body, it undergoes enzymatic reactions in the liver, leading to the formation of toxic metabolites such as formaldehyde and formic acid. Due to the time required for these metabolites to form (12–24 hours), patients often delay seeking medical attention. In treatment, ethanol (E) competes with M for alcohol dehydrogenase, preventing the conversion of M into formaldehyde [4,5].

Acute M poisoning is life-threatening, with risks of death, severe acidosis, visual impairment, and neurological disorders [6,7]. Treatment typically involves administering E or fomepizole, which have a high affinity for alcohol dehydrogenase, blocking the formation of toxic metabolites. In the brain, M poisoning can cause necrosis and hemorrhaging in the putamen, as well as lesions in the white matter. Permanent blindness occurs in 25–33% of survivors [8,9]. While the primary use of intravenous lipid emulsion (ILE) is as parenteral nutrition for children, adults, and newborns, it also acts as a carrier for drugs such as amphotericin B and propofol [10]. Recently, ILE has been recognized as an antidote for treating systemic toxicity caused by lipophilic toxins, including local anesthetics. Treatment protocols have been established due to ILE's ability to absorb these toxins [10,11].

In Turkey, many individuals suffer disability or death each year due to M poisoning. This study aimed to examine the effects of E and/or ILE, used in the treatment of acute M intoxication, on tissue and organ damage. It was assessed by analyzing pathological and biochemical changes in the body fluids, tissues, and organs of rats.

Materials and Methods

Animal experiments

This experimental study was conducted on male rats at the Research Center for Animal Husbandry, Balikesir University, in accordance with the ARRIVE guidelines and the UK Animals (Scientific Procedures) Act, 1986, and associated guidelines. The study was approved by the Ethics Committee of Balikesir University (Reference number: 2023/01).

A total of 64 male Wistar Albino rats, aged 7 to 8 weeks and weighing 250–300 g, were used in this study to avoid any influence from gender-related variations. The rats were divided into seven groups, ensuring similar body weights among them. Throughout the experiments, they were housed individually in cages under controlled conditions, with a temperature of 21±2 °C, humidity of 50±10%, and a 12-hour artificial light-dark cycle. The rats had ad libitum access to a standard diet provided by Korkutelim Food Company (Antalya, Türkiye), consisting of commercial rat food with the following composition: 88.0% dry matter, 23.5% crude protein, 3.3% ether extract, 6.1% crude fiber, 5.3% ash, and 2800 kcal/kg metabolizable energy, along with filtered tap water.

Anesthetic agents were not administered to prevent any interference with biochemical results, and all necessary measures were taken to minimize animal discomfort. Since the study aimed to investigate the effects of E, M, and ILE without considering sex differences, only male rats were used to ensure that sex did not affect the research outcomes.

Selected Reagents and Chemicals

Saline Solution (0.9% isotonic sodium chloride) was obtained from Polifleks, Türkiye.

M, (Methyl Alcohol, CH3OH, 99.9% purity) was obtained from Merck, Germany.

E, (Ethyl Alcohol, CH3CH2OH, Purity: 99.8 was obtained from Sigma Aldrich, Germany.

ILE, marketed as Clinoleic by Baxter in Belgium, was used in its 20% emulsion form. The product, Clinoleic 20% Emulsion, comes in 500 ml packages and contains 80% olive oil, 20% soybean oil, along with egg phospholipids, glycerol, sodium oleate, and sodium hydroxide.

Experimental Design and Group Allocation

In the study, 64 male rats were distributed across seven groups, with eight rats each in groups one, six, and seven, and 10 rats in the remaining groups.

Group 1: Saline Solution (Control) – Rats received oral saline once daily at a dose of 1.5 ml/kg.

Group 2: M – Rats were administered oral M once daily at a dose of 4 g/kg, diluted to 50%.

Group 3: M+E – Rats received oral M (4 g/kg, diluted to 50%), followed by oral E via gavage (1 g/kg, diluted to 50%) one hour later.

Group 4: M+ILE – Rats were given oral M (4 g/kg, diluted to 50%), followed by ILE at a dose of 1.5 ml/kg. The ILE was administered via IV, with the first dose within 30 minutes after M ingestion and subsequent doses every 6 hours, for a total of three doses.

Group 5: ME+E+ILE – Rats were administered oral M (4 g/kg, diluted to 50%), followed by oral E (1 g/kg, diluted to 50%) one hour later. Additionally, intravenous lipid emulsion (ILE) was given at a dose of 1.5 ml/kg, with the first dose within 30 minutes of M ingestion and subsequent doses every 6 hours, for a total of three doses.

Group 6: E – Rats received oral E via gavage once daily at a dose of 1 g/kg, diluted to 50%.

Group 7: ILE – Rats were given intravenous lipid emulsion (ILE) at a dose of 1.5 ml/kg via IV, administered within 30 minutes and repeated every 6 hours for a total of three doses within a single day.

In this study, groups 1 (Control), 6 (E), and 7 (ILE) initially included eight male rats each. However, in compliance with the principle set by the Local Ethics Committee for Experimental Animals to minimize the use of animals, the number of rats in these three groups was reduced.

Collection of specimens

In the experimental study, male rats were exposed to various chemicals. Saline was exclusively administered to the control group, while E, M, and ILE were administered to the remaining groups.

Throughout the 5-day observation period, notable incidents occurred during the chemical applications. In the control group, the eighth animal presented a cystic structure in the right kidney, necessitating the inclusion of the left kidney in the study. In group 3 (M and E), the death of the 4th and 10th animals on the fourth day prevented blood sampling. However, organ specimens were obtained immediately after death (1-2 hours). Furthermore, the third animal in Group 5 (ME+E+ILE) was euthanised on day 4 due to deteriorating health condition, which facilitated the collection of blood and organ specimens.

Following the completion of chemical administrations, male rats were observed for 5 days during the experiment, and surviving animals were euthanized on the fifth day. Cervical dislocation was performed without anesthesia to avoid altering biochemical measurement values. Subsequently, the brain, eye, optic nerve, liver, lung, kidney, heart, and testis organs were collected for pathological examinations. Among the tissue specimens collected, brain, eye, optic nerve, and testes were reserved for future studies, while liver, lung, kidney, heart, and biopsy analyses were conducted.

After the animals were euthanized, blood specimens were immediately collected using a glass funnel for biochemical analysis. The glass funnels were washed and rinsed with saline and dried for each animal. Blood collected in gel-separated tubes was kept at room temperature for about 30 minutes for clotting.

For biochemical analysis, each collected blood specimen was individually numbered for each of the seven groups and placed into blood centrifuge tubes. These were numbered from one to eight for groups 1, 6, and 7, and from one to ten for the other groups. After the study concluded, the collected blood tubes were transported to the biochemistry laboratory for analysis. The specimens were centrifuged at 2000 rpm at 4°C for 15 minutes and stored at -40°C until analysis. ALT, AST, and ALP levels were measured using a Beckman Coulter AU680 autoanalyzer (USA) after centrifugation.

For pathological examinations, collected tissue specimens were preserved in containers with 10% buffered formalin for fixation. Following appropriate sampling, tissue tracing was conducted. Liver, lung, heart, and kidney specimens were embedded in paraffin, and 4-micron-thick sections were stained with hematoxylin and eosin. Histopathological changes in the tissues were evaluated and recorded using light microscopy.

Biochemical analyses

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and total protein levels were assessed to evaluate liver function. In contrast, urea and creatinine levels were measured to assess kidney function. All analyses were performed using the Beckman Coulter AU680 autoanalyzer (USA). To assess DNA damage, serum 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels were measured with an ELISA kit (Elabscience, E-EL-0028; USA), following the manufacturer's instructions, using a Thermo Scientific Varioskan Flash ELISA reader (USA).

Pathological and histopathological examinations

In the experimental study, tissue specimens from the liver, lungs, heart, and kidneys were carefully collected

from male rats. Each specimen was individually numbered and placed in containers filled with 10% buffered formalin for fixation. After approximately 3.5 hours, the samples were transferred to the pathology laboratory for further processing. At the pathology laboratory, tissue tracking and sampling were performed. The specimens were then embedded in paraffin, and 4-micron-thick sections were prepared (Leica, RM 2125, Germany) and stained with hematoxylin and eosin (Merck Millipore, MA, USA). Histopathological alterations were examined and documented using a light microscope. (Olympus Corporation, CX41, Japan). Selected relevant images were identified, marked, and numbered for inclusion in the study.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics, Version 22.0 (SPSS Inc., Chicago, USA). Results were expressed as mean ± standard deviation. A one-way ANOVA test was conducted to compare numerical variables between groups. Post-hoc tests, either Bonferroni or Games-Howell (for ALP), were used to identify significant differences between groups for biochemical parameters. A p-value of <0.05 was considered statistically significant.

Results

Biochemical Findings

The biochemical parameter levels in rat sera are presented in Table 1. Concerning liver enzymes, the M+E group displayed the lowest levels of ALT, AST, and ALP, while LDH levels were lowest in the M+E+ILE group.

The M+E group showed significantly lower AST levels compared to the E and ILE groups ($p=0.008$ and $p=0.026$, respectively), and ALT levels were significantly lower in the M+E and E groups compared to the M-only group ($p=0.032$ and $p=0.004$, respectively). LDH levels were significantly lower in the M+ILE and M+E+ILE groups compared to the M-only group ($p=0.022$ and $p<0.001$, respectively). Additionally, there was a significant increase in ALP levels in the M+ILE group compared to the M+E group ($p=0.023$). The TP levels were lowest in the group where E was administered to rats with M toxicity compared to the other groups. There were significantly lower TP levels in the M, M+E, and M+E+ILE groups compared to the control group ($p=0.031$, $p<0.001$, and $p=0.016$, respectively). Only ILE administration significantly increased total protein levels compared to ME administration alone ($p=0.009$).

Regarding kidney function tests, the group that received both M and E exhibited the lowest urea and creatinine levels, which were significantly different from the control and M groups ($p<0.001$, $p=0.003$, $p<0.001$, and $p<0.001$, respectively). However, M+E+ILE application significantly increased urea and creatinine levels ($p=0.022$ and $p=0.008$, respectively) compared to the M+E group, while M+ILE application only increased creatinine levels ($p=0.022$). There were no significant differences between the groups in terms of 8-OHdG, a DNA damage product ($p=0.572$).

In this study, E administration significantly reduced AST, ALT, ALP, LDH, and TP levels in rats with M intoxication. These improvements in biochemical parameters indicate that E effectively mitigates liver damage caused by M toxicity (Table 1; Figures 5–6). However, given the small sample size and the E dosage used, these findings should be interpreted with caution. Further studies are necessary to explore the impact of ethanol on M toxicity in greater detail.

Histopathological Findings

Intergroup comparisons revealed significant differences across multiple organ systems outlined in Table 2.

n comparing methanol+intravenous lipid emulsion vs methanol+ethanol groups, liver congestion showed significant differences ($p=0.006738$); lung inflammation ($p=0.01857$) and lung congestion ($p=0.006738$) were significantly improved in the methanol+ethanol group. In comparing control vs intravenous lipid emulsion groups, significant differences were observed in lung fibrosis ($p=0.03389$). Kidney inflammation showed notable variations ($p=0.03389$). In comparing methanol vs methanol + ethanol groups, a significant difference was found only in liver hydropic degeneration ($p=0.01111$). Other parameters showed no significant differences ($p>0.05$). In comparing methanol + ethanol + intravenous lipid emulsion vs methanol + ethanol groups, no significant differences were observed in any parameters ($p>0.05$).

Microscopic examination of lung tissues (Figures 1, 2, and 3) showed severe inflammation in the lung parenchyma (Figure 3). In contrast, microscopic examination of liver tissues (Figures 4, 5, and 6) showed mild inflammation in the liver parenchyma (Figure 6).

Discussion

In many cultures, the consumption of alcoholic beverages is deeply embedded in social activities and often associated with recreational enjoyment.

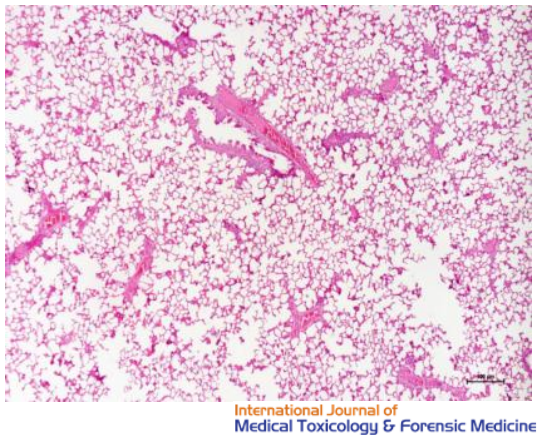

Figure 1. Control group lung parenchyma tissue (Hematoxylin Eosin), x100.

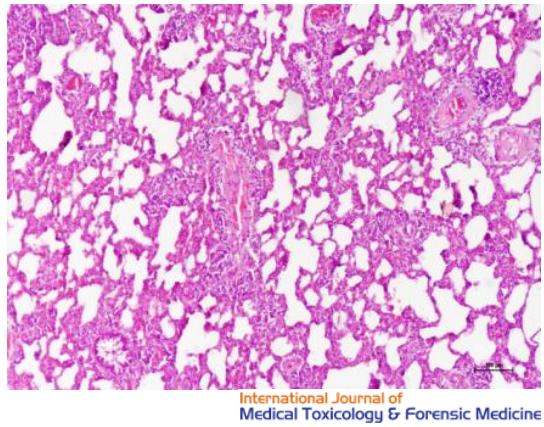

Figure 2. Mild inflammation of the lung parenchyma (Hematoxylin Eosin), x100.

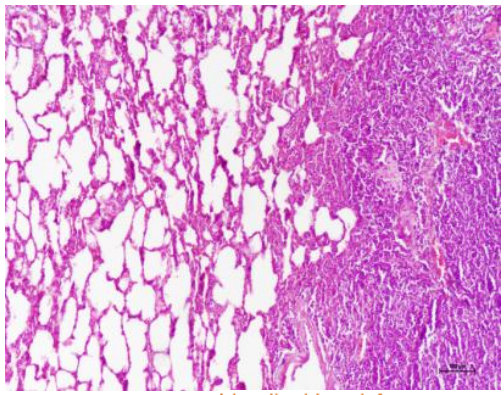

Figure 3. Severe inflammation in lung parenchyma (Hematoxylin Eosin), x100.

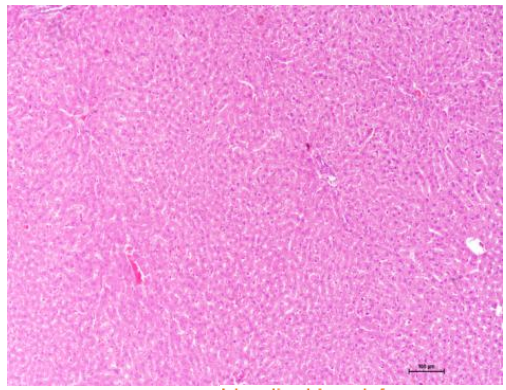

Figure 4. Control group liver parenchyma tissue (Hematoxylin Eosin), x100.

However, excessive alcohol consumption poses significant public health concerns, contributing not only to chronic liver disease but also to cardiovascular disorders, various cancers, and neuropsychiatric conditions. Therefore, the development of effective therapeutic strategies for the management of acute methanol intoxication is of critical importance. To the best of our knowledge, this study represents the first investigation into the effects of ILE in the treatment of acute M intoxication.

M poisoning, often resulting from the unintentional consumption of pure M or mixtures of E and M, is a global public health issue with high morbidity and mortality rates [12–16]. This problem is particularly pronounced in developing countries [12, 16–18]. Both E and fomepizole (4-methylpyrazole) are well-established antidotes for M poisoning. In this study, E demonstrated a protective effect by improving biochemical parameters and mitigating histopathological damage to the liver and kidneys.

A study by Aslan et al. analyzed 56 samples of illicit alcohol known as *Boğma Rakı* and found M in 75% of the samples. This underscores the widespread presence of M in illicit alcoholic beverages and served as the rationale for this investigation into how E and ILE can modulate the effects of M [19]. Similarly, Gökçe et al. reported that illegally produced alcoholic beverages containing trans-anethole exhibited greater hepatotoxicity compared to commercial products [20].

A study by Çelik et al. at the Ankara Branch of the Council of Forensic Medicine documented 74 deaths caused by M and E poisoning between 2001 and 2011, accounting for 0.69% of all forensic autopsy cases [21]. M poisoning was responsible for 47.3% (n=35) of these deaths, with the majority of cases involving males (90.5%). These findings highlight the significant contribution of M poisoning to mortality rates, emphasizing the importance of this study.

Statistical analysis revealed significant differences between the Methanol plus ILE group and the Methanol plus Ethanol group in the parameters of liver congestion, lung inflammation, and lung congestion. These findings highlight the protective effects of ethanol in reducing methanol-induced damage to these organs. No statistically significant differences were observed for the parameters of cardiac congestion, liver

Table 1. The levels of biochemical parameters in rats.

Parameters	Control	M	M+E	M+ILE	M+E+ILE	E	ILE	F/p values
AST (IU/L)	200,75 ±33,78	200,50 ±25,43	152,88 ±27,88	197,70 ±46,93	178,80±39,59	226,75 ±40,62	219,25±52,13	3,298/ 0,008
ALT (IU/L)	72,88± 8,39	84,80± 11,97	53,75± 28,70	82,20± 31,72	77,00 ±18,65	66,13± 8,90	79,00 ±11,24	2,556/ 0,030
LDH (IU/L)	1380,38±317,41	1518,70±277,23	1151,00±341,44	1072,80±285,40	904,10±89,76	1420,13±331,66	1328,50±336,84	5,498/ 0,000
ALP (IU/L)	139,38 ±18,29	134,20 ±20,83	116,25 ±34,16	183,70 ±69,49	150,10±25,44	128,75 ±25,60	167,63±35,71	3,478/ 0,023
TP (g/L)	67,22± 4,50	61,15± 4,38	55,54± 4,77	63,51± 4,85	59,24 ±5,87	66,00± 4,84	69,56 ±2,97	8,954/ 0,000
Urea (mg/dL)	54,38± 6,57	51,00± 5,73	39,25± 6,86	45,70± 6,04	49,30 ±5,58	46,63± 6,76	45,38 ±5,40	5,129/ 0,000
Creatinine (mg/	0,515± 0,043	0,476± 0,054	0,375± 0,043	0,444± 0,041	0,436 ±0,031	0,420± 0,039	0,469 ±0,036	9,251/ 0,000
8-OHdG (ng/m	10,44± 3,38	12,13± 4,02	10,46± 2,14	10,52± 2,94	10,03 ±2,57	10,28± 2,47	9,27± 2,20	0,803/ 0,572

M:methanol, E:ethanol, ILE:intravenous lipid emulsion, AST:aspartate aminotransferase, ALT:alanine aminotransferase, LDH:lactate dehydrogenase, ALP:alcaline phpsphatase, TP:total protein, 8-OHdG:8-Hydroxy 2 Deoxyguanosine

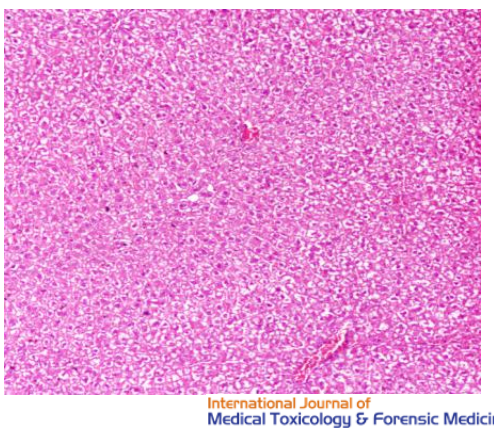

Figure 5. Hydropic degeneration of liver parenchyma (Hematoxylin Eosin), x100.

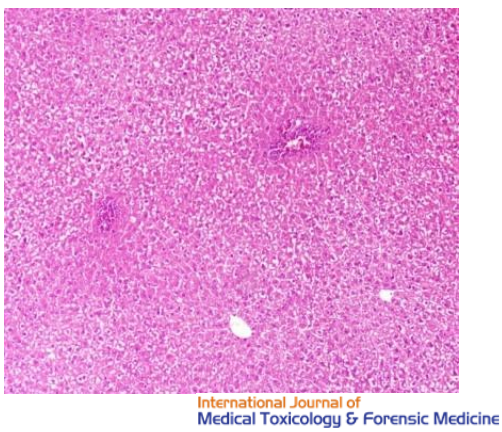

Figure 6. Mild inflammation of liver parenchyma (Hematoxylin Eosin), x100.

hydropic degeneration, liver inflammation, liver fibrosis, or kidney inflammation across the groups studied. When comparing the Control group to the ILE group, significant differences were identified for lung fibrosis and kidney inflammation ($p < 0.05$). These findings suggest that ILE administration may have negatively impacted these parameters, potentially highlighting an adverse effect of ILE on pulmonary and renal tissues under normal conditions. No significant differences were observed in any parameters ($p > 0.05$) when comparing groups treated with ethanol alone after methanol toxicity to those treated with both ethanol and ILE post-methanol toxicity. This suggests that the addition of ILE did not provide further benefits in mitigating methanol-induced damage when ethanol was already administered. Significant differences were observed when comparing the group that received ILE alone to the group that received ethanol post-methanol toxicity. Specifically, liver congestion ($p = 0.0067$), lung inflammation ($p = 0.0186$), and lung congestion ($p = 0.0067$) showed significant improvements in the Methanol plus Ethanol group. These findings further support the superior efficacy of ethanol over ILE in alleviating methanol-induced tissue damage in these parameters.

Previous studies have identified several factors predictive of mortality in M intoxication cases. Lee et al. demonstrated that Glasgow Coma Scale (GCS)

Table 2. Pathological examination results of organs.

		Liver congestion	Lung inflammation	Lung congestion	Lung fibrosis	Heart congestion	Liver hydropic degeneration	Liver inflammation	Kidney congestion
Control	absent	7	5	4	8	6	7	8	8
	Slightly positive	1	3	4	0	2	1	0	0
	moderate positive	0	0	0	0	0	0	0	0
	Serious positive	0	0	0	0	0	0	0	0
E	absent	4	3	1	7	6	7	3	6
	Slightly positive	4	3	7	1	2	1	5	2
	moderate positive	0	2	0	0	0	0	0	0
	Serious positive	0	0	0	0	0	0	0	0
	absent	2	6	2	8	8	8	8	7
	Slightly positive	6	2	6	0	0	0	0	1
	moderate positive	0	0	0	0	0	0	0	0
	Serious positive	0	0	0	0	0	0	0	0
M	absent	1	0	0	6	10	3	8	3
	Slightly positive	9	5	9	4	0	7	2	7
	moderate positive	0	5	1	0	0	0	0	0
	Serious positive	0	0	0	0	0	0	0	0
M+ILE	absent	4	7	3	9	10	8	10	7
	Slightly positive	6	3	7	1	0	2	0	3
	moderate positive	0	0	0	0	0	0	0	0
	Serious positive	0	0	0	0	0	0	0	0
M+E	absent	2	3	0	10	10	10	10	7
	Slightly positive	8	5	8	0	0	0	0	3
	moderate positive	0	2	2	0	0	0	0	0
	Serious positive	0	0	0	0	0	0	0	0
M+E+ILE	absent	0	8	4	10	10	9	10	8
	Slightly positive	10	2	6	0	0	1	0	2
	moderate positive	0	0	0	0	0	0	0	0
	Serious positive	0	0	0	0	0	0	0	0
p	0.001		0.007	0.042	0.032	0.046	0.01	0.001	0.0001

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M:methanol, E:ethanol, ILE:intravenous lipid emulsion

scores, hypothermia, and serum creatinine levels are critical predictors of mortality [22], while Cook et al. suggested that elevated blood creatinine levels may indicate the severity of M poisoning [23]. Animal studies have further revealed that M intoxication causes both liver and kidney damage, with significant increases in serum creatinine levels [24]. Treatment approaches for M poisoning often include bicarbonate, E, and hemodialysis [25]. For instance, Babuş et al. reported that 83.9% of patients were treated with dialysis and 58.1% with ethanol [26]. Furthermore, blood creatinine levels have been identified as a predictor of hemodialysis duration in M poisoning cases [27].

ILE has gained recognition for its ability to enhance recovery rates and improve outcomes in various lipophilic drug toxicities. Although the precise mechanisms underlying its antidotal effects remain unclear, several pharmacokinetic and pharmacodynamic pathways have been proposed, including its potential to improve myocardial performance [28]. In this study, ILE administration reduced mild hepatic congestion from 90% in M-exposed animals to 40% in animals treated with M and ILE. Similarly, mild hepatic hydropic degeneration decreased from 70% in M-exposed animals to 20% in those treated with M and ILE. These findings align with studies reporting favorable outcomes of ILE use in toxicity models, such as verapamil poisoning in rabbits [29], as well as successful human treatments for propranolol and nebivolol overdose [30].

Despite its benefits, ILE administration may have adverse effects, particularly on lung function. In critically ill patients, ILE has been associated with increased pulmonary arterial pressure, venous mixing, and reduced oxygen partial pressure, although these changes typically resolve upon discontinuation [31]. In this study, ILE administration after M exposure positively influenced renal function tests and total protein levels. However, a significant increase in ALP levels was observed, suggesting potential bile duct damage following methanol and ILE administration. The dose and duration of ILE treatment, as well as factors affecting intoxication rates, may have

contributed to these findings. A preprint has previously been published [32].

Limitations

The limitation of this study is that while it was planned to use 70 rats in seven groups, eight rats were used in 3 groups (control, E, and ILE groups), and 64 rats had to be used in 7 groups, depending on the decision of Balikesir University Animal Experiments Ethics Committee. The results were based on the ethics committee's rule

Conclusion

In conclusion, this study highlights the protective effects of E and ILE in mitigating M-induced organ damage. While ethanol effectively improved liver biochemical parameters, ILE demonstrated positive effects on renal function and reduced hepatic damage—however, potential adverse effects, such as bile duct damage, warrant further investigation. Given the limited studies on this topic, these findings provide valuable experimental data and underscore the need for additional research to optimize treatment strategies for M intoxication.

Conflicts of Interest

The authors report there are no competing interests to declare.

Clinical Implications

These results underscore the importance of carefully considering the sequence and timing of antidotal treatments for methanol toxicity. Ethanol remains a highly effective treatment, and while ILE shows potential, its benefits are contingent on the timing and context of administration. The findings emphasize the need for evidence-based protocols that balance the therapeutic interactions of multiple antidotes.

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Ethics Statement

This study was conducted in accordance with Guidelines for the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes (2011/28141/TR) and approved by the Ethics Committee of Balıkesir University (Reference number: 2023/01).

Written informed consent was not necessary because no patient data were included in the manuscript.

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