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Effects of intravenous lipid emulsions on irisin, MMP9, NF-κB, TNF-α in rat kidneys with bupivacaine toxicity: An immunohistochemical study

Efectos de emulsiones lipídicas intravenosas sobre irisin, MMP-9, NF-κB, TNF-α en riñones de ratas con toxicidad por bupivacaína: estudio inmunohistoquímico

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ABSTRACT

This study aims to investigate the effects of bupivacaine-induced toxicity on renal tissue and the potential protective role of intravenous lipid emulsion. 28 adult male Wistar Albino rats were randomly assigned to four groups as follows: Control, local anesthesia, intravenous lipid emulsion, and local anesthesia + intravenous lipid emulsion. group local anesthesia received a 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ bupivacaine infusion, group intravenous lipid emulsion received a 1.5 mL bolus followed by 0.25 $\mu g \cdot kg^{-1} \cdot min^{-1}$ intravenous lipid emulsion infusion, and group local anesthesia + intravenous lipid emulsion received bupivacaine with intravenous lipid emulsion intervention upon toxicity signs. All animals were closely monitored throughout the study. In group local anesthesia, bupivacaine induced significant renal alterations histopathologically, and increased irisin, matrix metalloproteinase-9 (MMP-9), nuclear factor-kappa B (NF-κB), and tumor necrosis factor-α (TNF-α) immunoreactivity compared to control (P<0.05). Histopathology revealed marked edema, congestion, tubular degeneration, inflammatory cell infiltration, glomerular degeneration, and tubular cell shedding (P<0.001). Conversely, rats in the local anesthesia + intravenous lipid emulsion group showed decreased renal tissue edema and congestion, attenuated tubular degeneration, and reduced infiltration, glomerular degeneration, and tubular cell shedding, and reduced immunoreactivity of irisin, MMP-9, NF-κB, and TNF-α (P<0.001), indicating a nephroprotective effect of intravenous lipid emulsion. These findings suggest that irisin, MMP-9, NF-κB, and TNF-α serve as reliable biomarkers of bupivacaine-induced nephrotoxicity. Intravenous lipid emulsion administration mitigates these biochemical and histopathological changes, highlighting its potential as a protective agent against local anesthetic-induced renal damage. The study underscores the importance of monitoring these biomarkers and provides evidence for the therapeutic benefits of intravenous lipid emulsion in the management of bupivacaine toxicity.

Key words: Intravenous lipid emulsion; irisin; MMP-9; NF-κB; TNF-α

RESUMEN

Este estudio investigó los efectos de la toxicidad inducida por bupivacaína en el tejido renal y el posible papel protector de la emulsión lipídica intravenosa. Veintiocho ratas Wistar Albino macho adultas fueron asignadas aleatoriamente a cuatro grupos: Control, anestesia local, emulsión lipídica intravenosa y anestesia local + emulsión lipídica intravenosa. El grupo anestesia local recibió una infusión de bupivacaína de 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$, el grupo emulsión lipídica intravenosa recibió un bolo de 1,5 mL seguido de una infusión de emulsión lipídica intravenosa de 0,25 $\mu g \cdot kg^{-1} \cdot min^{-1}$, y el grupo anestesia local + emulsión lipídica intravenosa recibió bupivacaína con intervención de emulsión lipídica intravenosa al observar signos de toxicidad. Todos los animales fueron monitoreados de cerca durante el estudio. En el grupo anestesia local, la bupivacaína indujo alteraciones renales significativas, incluyendo un aumento en la inmunorreactividad de irisina, metaloproteinasa de matriz-9, factor nuclear kappa B y factor de necrosis tumoral-α en comparación con los controles (P<0,05). La histopatología reveló edema, congestión, degeneración tubular, infiltración, degeneración glomerular y desprendimiento de células tubulares significativos (P<0,001). Por el contrario, las ratas del grupo anestesia local + emulsión lipídica intravenosa mostraron disminución del edema y congestión renal, atenuación de la degeneración tubular y reducción de la infiltración, degeneración glomerular y desprendimiento celular tubular (P<0,001), indicando un efecto nefroprotector de emulsión lipídica intravenosa. Estos hallazgos sugieren que irisina, factor nuclear kappa B, metaloproteinasa de matriz-9 y factor de necrosis tumoral-α son biomarcadores fiables de la nefrotoxicidad inducida por bupivacaína. La administración de emulsión lipídica intravenosa atenúa estos cambios bioquímicos e histopatológicos, destacando su potencial como agente protector frente al daño renal inducido por anestésicos locales. El estudio subraya la importancia de monitorear estos biomarcadores y proporciona evidencia de los beneficios terapéuticos de emulsión lipídica intravenosa en el manejo de la toxicidad por bupivacaína.

Palabras clave: Emulsión lipídica intravenosa; irisina; MMP-9; NF-κB; TNF-α

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INTRODUCTION

Local anesthetics (LA) are drugs that temporarily inhibit some or all of the sensory, motor, or autonomic functions [1] and are widely used in local/regional anesthesia and postoperative pain management. Although adverse side effects are rare, the increased prevalence of their use has led to a higher incidence of local anesthetic systemic toxicity (LAST), most frequently triggered by inadvertent intravascular injection or the use of excessively high doses during application [2].

Local anesthetic systemic toxicity can impair perfusion and function of vital organs such as the central nervous system, kidneys, and cardiovascular system [3], potentially resulting in organ failure [4]. High-dose LA induces molecular and cellular changes in renal cells, including inflammation and apoptosis, contributing to renal injury [5].

Various biomarkers and molecular analyses have been explored to clarify these mechanisms, including the myokine irisin [5] and matrix metalloproteinase-9 (MMP-9) [6] tumor necrosis factor alpha (TNF-α) and nuclear factor kappa B (NF-κB) are key mediators of kidney damage [7, 8]; TNF-α initiates inflammation and activates NF-κB [9, 10], which sustains inflammation via upregulation of MMP-9 and other genes [11]. Irisin may modulate these processes by balancing TNF-α/NF-κB pathways [10, 12].

In severe instances of LAST, insufficient or delayed intervention may lead to renal and systemic complications, ultimately progressing to cardiac arrest and even death [2, 13]. The American Society of Regional Anesthesia emphasizes early airway management and prompt intralipid emulsion (ILE) therapy in LAST to prevent the harmful cycle of hypoxia and acidosis in advanced toxicity [14].

Evidence supports ILE use in bupivacaine-induced LAST [15, 16], though its effects on renal tissue remain underexplored. This study aims to characterize bupivacaine-induced renal toxicity and evaluate the potential protective effects of ILE on these alterations.

MATERIALS AND METHODS

This study was approved by the Animal Studies Ethics Committee of Adıyaman University (ADIYAMAN-HADYEK: 06.06.2024–2023/014), and all procedures followed NIH guidelines for laboratory animal care. Twenty-eight adult male Wistar-Albino rats (300–350 g) were used, provided with standard diet and water ad libitum, and maintained under controlled temperature (22–25°C), humidity (50–55%), and a 12-hour light/dark cycle.

Sample size calculation, based on previously published myocardial bupivacaine data [17], indicated that seven rats per group were sufficient to detect an effect size of 1.55 with 80% statistical power and 5% type I error rate. This sample size was therefore considered adequate to ensure the statistical validity and reliability of the research findings.

Rats were anesthetized under Veterinary supervision with intramuscular Xylazine hydrochloride (Rompun, Bayer Turkish Pharmaceutical Co. Ltd., 20 mg·kg^{-1}) and Ketamine hydrochloride (Ketalar, Eczacıbaşı, Istanbul, Türkiye, 50 mg·kg^{-1}). Subjects were randomly assigned to four groups (n = 7 each):

- **Control (Group C):** Rats in this group did not receive any treatment [18].
- **Local Anesthesia (Group LA):** In this group, rats were provided with intravenous access via the tail vein. Following anesthesia and cardiac monitoring, a continuous bupivacaine infusion (Marcaine 0.5%, AstraZeneca Ltd, Istanbul, Türkiye) was administered at 3 μg·kg^{-1}·min^{-1} to induce experimental LAST and to monitor the onset of cardiac toxicity symptoms. The infusion was discontinued immediately upon the appearance of arrhythmia or bradycardia [19].
- **Intravenous Lipid Emulsion (Group ILE):** Rats received intravenous access through the tail vein, and after anesthesia and cardiac monitoring, a bolus of 1.5 mL·kg^{-1} ILE (Intralipid® 20%, soybean oil–based intravenous lipid emulsion, Fresenius Kabi AB, Uppsala, Sweden) was given, followed by a continuous infusion at 0.25 μg·kg^{-1}·min^{-1} for 15 min.
- **Local Anesthesia + Intravenous Lipid Emulsion (Group LA + ILE):** In this group, following anesthesia and cardiac monitoring, rats first received a continuous intravenous bupivacaine infusion via the tail vein (Marcaine® 0.5%, AstraZeneca Ltd, Istanbul, Türkiye) until arrhythmia or bradycardia was observed. Immediately after the bupivacaine infusion was stopped, a bolus of 1.5 mL·kg^{-1} ILE (Intralipid® 20%, Fresenius Kabi AB, Uppsala, Sweden) was administered via the same intravenous route, followed by a continuous infusion at 0.25 μg·kg^{-1}·min^{-1} for 15 min. Oxygen support was provided throughout, and the infusion was terminated once hemodynamic stability was achieved.

Kidneys were harvested from all animals under deep anesthesia: in the LA group immediately after the onset of cardiac toxicity (bradycardia), in the ILE and LA + ILE groups after completion of the ILE infusion, and in the control group at the end of the experimental procedures. Euthanasia was performed immediately after tissue collection, and kidneys were placed in freshly prepared, phosphate-buffered 10% formalin for histological analysis.

Histological analyses

Kidney tissue samples were fixed in 10% formaldehyde for further analysis. After fixation, tissue samples underwent routine processing, then were paraffin-embedded and blocked. 5 μm thickness sections were obtained from these blocks using a microtome (ThermoScientific, HM325, USA). For the morphological evaluation, the sections were stained with Hematoxylin & Eosin (H&E).

Morphological alterations were scored semi-quantitatively for parameters including edema, congestion, tubular degeneration, inflammatory cell infiltration, glomerular degeneration, and tubular cell detachment using a light microscope (Nikon ECLIPSE E200, Japan) and photographed. The semi-quantitative scoring system was defined as follows: 0 = no damage, 1 = ≤10%, 2 = 10–25%, 3 = 25–50%, 4 = 50–75%, and 5 = > 75% histopathological damage. This scoring system was adapted and modified from previously published rat kidney histopathology studies [20].

Immunohistochemical evaluation

Immunohistochemical staining was carried out as previously reported using the avidin-biotin-peroxidase complex method [21, 22]. 5 µm thickness sections were deparaffinized. Primary antibodies were diluted 1:200 using a commercial kit (ThermoScientific™ TP-015-HA) to detect the following proteins: Irisin (Rabbit Polyclonal, H-067–17, PhoenixPharmaceuticals Inc., California, USA), MMP-9 (Rabbit Polyclonal, BS-4593R, Bioss Inc., Massachusetts, USA), TNF-α (Rabbit Monoclonal IgG, ab220210, Abcam, Cambridge, UK), and NF-κB (Rabbit Monoclonal IgG, ab32536, Abcam, Cambridge, UK).

After application of the 3-Amino-9-Ethylcarbazole chromogen (AEC), the sections were counterstained with Mayer's hematoxylin. Then the immunostained tissues were examined under a light microscope and photographed. A histoscore was calculated based on the extent (0.1: < 25%, 0.4: 26–50%, 0.6: 51–75%, 0.9: 76–100%) and intensity (0: none, +0.5: very weak, +1: weak, +2: moderate, +3: strong) of immunoreactivity. The histoscore was determined using the following formula: Histoscore = extent × intensity [23].

Statistical analysis

Statistical analyses were performed using SPSS version 22. Continuous data were expressed as median and range (minimum–maximum). For comparisons among more than two groups, the Kruskal-Wallis test was applied. Pairwise comparisons following the Kruskal-Wallis test were performed using Dunn's post hoc test. Dependent variables were analyzed with Two-Related-Samples Tests, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The experiment started with 28 subjects and ended with the same number.

Histopathological Findings

Histopathological analysis presented that kidney tissues from the control and ILE groups exhibited normal architecture, including glomeruli, cortical and medullary tubules, and peritubular regions, with no statistically significant differences between these groups (*P*=0.802). Compared to the control group, in the LA group demonstrated marked renal injury, including edema, congestion, tubular degeneration, inflammatory cell infiltration, glomerular degeneration, and tubular cell detachment (*P*<0.001).

Notably, these alterations were significantly attenuated in the LA + ILE group, with reductions in edema, congestion, tubular degeneration, and inflammatory changes (*P*<0.001) (TABLE 1 and FIG. 1).

Local anesthetics are widely applied in clinical practice; however, use of LA, especially at high concentrations, may induce detrimental effects on renal tissue. These effects can result from direct cytotoxic mechanisms or indirect physiological disruptions. Lee *et al.* [5] demonstrated that lidocaine, bupivacaine, and tetracaine induced apoptosis in a concentration-dependent manner, leading to kidney damage by impairing both the structural and functional integrity of renal cells.

Additionally, in a study by Chen and Zhuang [24] neurotoxicity was induced in mice through bupivacaine application, which led to an excessive inflammatory response that could potentially kidney tissue injury. In the present study, histopathological changes consistent with these reports were observed, indicating that bupivacaine induces renal damage. Furthermore, administration of ILE mitigated bupivacaine-induced renal injury. Reductions in edema, tubular degeneration, and inflammatory cell infiltration in the LA + ILE group reveal that ILE may counteract both the direct cytotoxic and indirect inflammatory effects of high-dose bupivacaine [14, 16].

These results highlight the critical importance of timely intervention in cases of LAST. Rapid administration of therapeutic measures, such as intravenous lipid emulsions, is essential to prevent both systemic and organ-specific toxic effects. Delayed intervention can allow the accumulation of the anesthetic in the circulation and target organs, exacerbating cardiotoxicity, neurotoxicity, and renal injury. Therefore, early recognition and prompt treatment are crucial to minimize adverse outcomes and improve overall safety in experimental and clinical settings.

Immunohistochemical findings

Immunohistochemical analysis demonstrated the expression of irisin (FIGS. 2. 1a to 1d), MMP-9 (FIGS. 2. 2a to 2d), NF-κB (FIGS. 2. 3a to 3d), TNF-α (FIGS. 2. 4a to 4d) in kidney tissues under light microscopy. Immunoreactivity was similar between the control (FIG. 2. 1a, 2a, 3a, 4a) and ILE groups (FIG. 2. 1d, 2d, 3d, 4d), with no significant differences observed (*P*>0.05, FIG. 3). In contrast, the LA group (FIG. 2. 1b, 2b, 3b, 4b) showed markedly

TABLE I
Semiquantitative scoring of kidney tissues of rats from all groups

Groups	Edema Median (min-max)	Congestion Median (min-max)	Tubular degeneration Median (min-max)	Infiltration Median (min-max)	Glomerular degeneration Median (min-max)	Tubular cell shedding Median (min-max)
Control	0.00 (0.00–1.00)	0	0	0.00 (0.00–1.00)	0.00 (0.00–1.00)	0
LA	4.00 (3.00–5.00)[a]	4.00 (3.00–5.00)[a]	2.00 (2.00–3.00)[a]	2.00 (2.00–4.00)[a]	3.00 (2.00–3.00)[a]	3.00 (2.00–4.00)[a]
LA + ILE	2.00 (2.00–3.00)[b]	1.00 (1.00–2.00)[b]	2.00 (1.00–2.00)[b]	1.00 (1.00–2.00)[b]	1.00 (0.00–1.00)[b]	1.00 (0.00–1.00)[b]
ILE	0.00 (0.00–1.00)	0	0.00 (0.00–1.00)	0	0	0

Values are summarized as median (min–max). [a] Significantly different from the control group, and [b] Significantly different from the LA group (*P*<0.01)

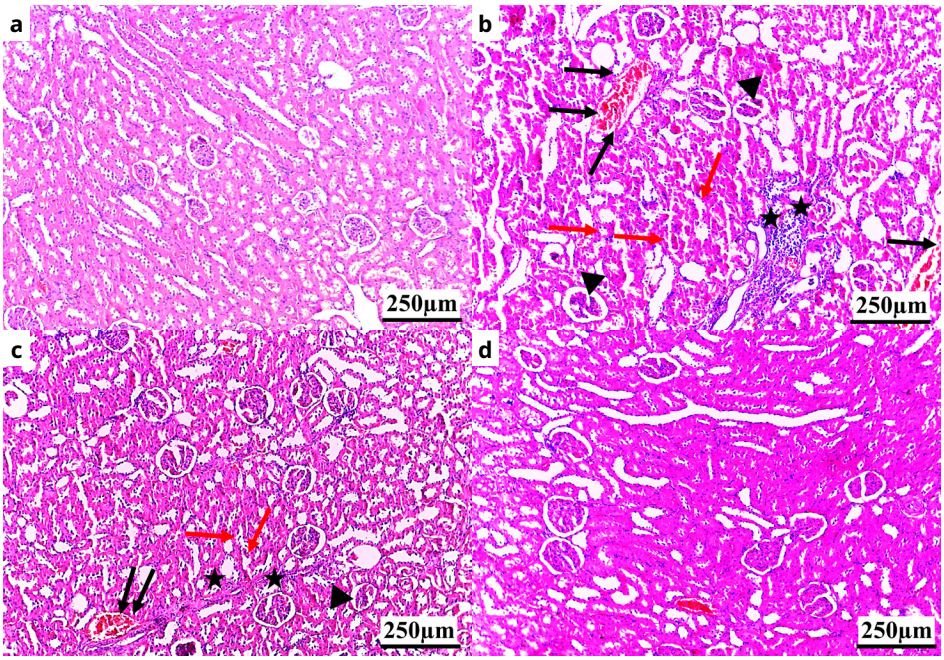

FIGURE 1. H&E staining of control (a), LA (b), LA+ILE (c), and ILE (d) groups. Normal morphology in kidneys of control and ILE; in the LA group, glomerular degeneration (arrowhead), tubular degeneration (red arrow), inflammatory infiltration (asterisk), and vascular congestion (black arrow), reduced pathology in LA+ILE. Scale bar: 250 µm, magnification 100×

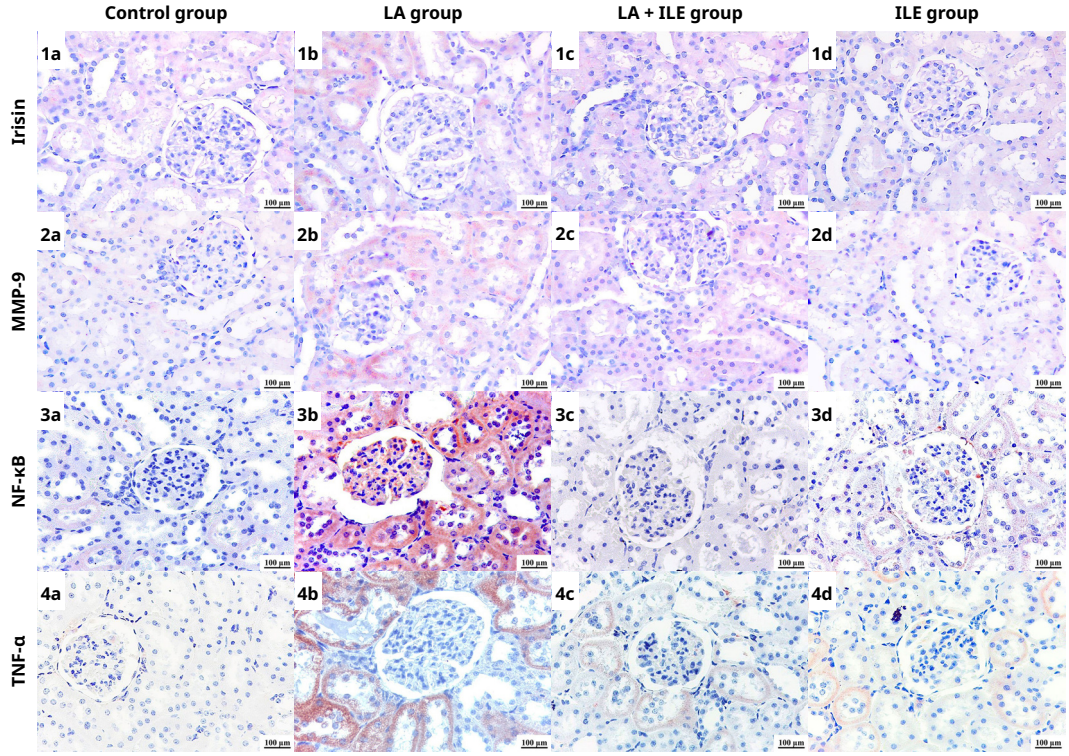

FIGURE 2. Immunohistochemical staining of irisin (1a–1d), MMP-9 (2a–2d), NF-κB (3a–3d), and TNF-α (4a–4d) in kidney tissues showing similar expression in control and ILE, increased in LA, and reduced in LA + ILE (Immunohistochemical staining, AEC chromogen, Mayer's Hematoxylin, Scale bar: 100 µm, 400× magnification)

elevated immunoreactivities for all markers compared to the control group (P<0.05, FIG. 3). Administration of ILE in the LA + ILE group (FIG. 2. 1c, 2c, 3c, 4c) significantly attenuated these increases compared with the LA group, suggesting a protective effect against bupivacaine-induced renal alterations (P<0.05, FIG. 3).

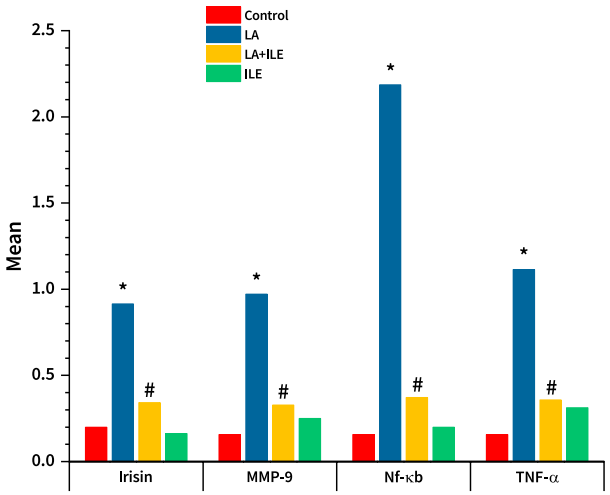

FIGURE 3. Irisin, MMP-9, NF-κB, TNF-α immunostaining histoscore levels of all groups. * Significant difference compared with the control group (P<0.05). # Significant difference compared with the LA group (P<0.05)

Irisin a proteolytic fragment of Fndc5 [25], is expressed in multiple tissues, including the liver, heart, and kidneys [26, 27], and exhibits anti-inflammatory, anti-apoptotic [10], antioxidant [28], and anti-fibrotic effects [29]. Recent studies have shown that local anesthetics can induce renal cell apoptosis, serving as a marker for kidney damage [5].

Peng *et al.* [30] and Liu *et al.* [31] reported that irisin reduced kidney histopathological alterations and fibrosis in mice and acted as a myokine protecting against ischemia-reperfusion injury, respectively. In the present study, immunoreactivity of irisin increased in the LA group, indicating a potential protective response to bupivacaine-induced toxicity, whereas the decrease in the LA + ILE group suggests mitigation of kidney damage following ILE infusion.

The matrix metalloproteinase-9 is an endopeptidase that is responsible for the degradation of the extracellular matrix. Its ability to activate cytokines [32] and its pivotal role in various physiological and pathological processes have been well-documented [33, 34]. While some studies demonstrate that MMP-9 may help protect kidney function [35, 36], most studies highlight its harmful effects.

Wang *et al.* [37] observed that mice deficient in MMP-9 had a lower likelihood of developing morphological damage and displayed milder renal interstitial fibrotic lesions in an obstructive nephropathy model. Another study reported that reperfusion injury after acute myocardial ischemia was associated with elevated levels of inflammatory cytokines and MMP-9 in both the myocardium and renal cortex, initiating signaling pathways implicated in the progression of renal cortical fibrosis [6]. MMP-9 has also been implicated in the fibrosis of the lungs, liver, myocardial infarction, and chronic kidney disease [38].

In this study, the increased MMP-9 immunoreactivity in the LA group indicates kidney damage caused by bupivacaine, while the reduction in MMP-9 immunoreactivity following ILE administration suggests that ILE protects against bupivacaine-induced kidney injury.

The TNF-α is a pro-inflammatory cytokine that activates intracellular signaling pathways, including the transcription factor NF-κB, and contributes significantly to inflammatory processes implicated in renal injury [9, 10]. Although bupivacaine toxicity has been associated with neurotoxicity via TNF-α and NF-κB activation [24], immunohistochemical evidence regarding their role in kidney damage is limited. In this study, increased NF-κB and TNF-α immunoreactivity in the LA group reveals that local anesthetic-induced inflammation contributes to renal damage.

These findings emphasize the importance of the TNF-α and NF-κB pathways in the pathogenesis of kidney injury. Agents that inhibit NF-κB activation or TNF-α production have been reported to offer protective effects in various experimental models. Studies have shown that compounds such as pioglitazone [39], sevoflurane [40], calycosin [9], neuropeptide Y [41], puerarin [42], chlorogenic acid [43], and bicyclol [44] present anti-inflammatory, antifibrotic, antioxidant, and immunomodulatory properties by significantly reducing TNF-α and NF-κB expression. Targeting these pathways may therefore represent a feasible strategy for preventing or alleviating kidney damage associated with local anesthetic toxicity.

In the present study, administration of ILE reduced the immunoreactivity of both NF-κB and TNF-α, indicating a protective effect against bupivacaine-induced kidney injury. While some local anesthetics at clinical doses, such as articaine [45], procaine [46], lidocaine [47], and ropivacaine [48], can reduce NF-κB and TNF-α expression and alleviate inflammation, the toxic dose of bupivacaine used in this study is much higher than clinical levels, which likely overwhelms the regulatory mechanisms and leads to increased expression of these inflammatory markers, resulting in nephrotoxicity. TNF-α activation consequently promotes NF-κB signaling, which induces the synthesis of enzymes such as matrix metalloproteinases, including MMP-9, thereby enhancing tissue injury and inflammation [11].

In another study, ischemia-reperfusion injury in rabbit kidneys were shown to activate NF-κB [49], and exogenous TNF-α application in proximal tubule cells increased inflammatory mediators, including MMP-9, causing the exacerbation of renal damage [50]. In line with these reports, increased immunoreactivity of TNF-α, NF-κB, and MMP-9 in the LA group led to kidney injury, whereas ILE administration reduced these effects, implying a protective role.

The NF-κB and TNF-α also play crucial roles in promoting both inflammation and apoptosis during kidney injury. Irisin has been reported to exert protective effects by inhibiting these processes [10, 12]. In present study, increased immunoreactivity of NF-κB, irisin, and TNF-α in the LA group may reflect a compensatory protective response of irisin against bupivacaine toxicity. In contrast, the LA + ILE group exhibited reduced immunoreactivity

of these biomarkers, indicating that ILE infusion improves bupivacaine-induced renal damage.

Lipid emulsion therapy is an established treatment for LAST. It facilitates the binding and removal of lipophilic local anesthetics from critical organs, including the kidneys [51]. Previous studies reported that bupivacaine-induced toxicity causes significant bradycardia in rats [19], and ILE effectively treats early cardiovascular depression before cardiac arrest [51].

Administration of ILE during the initial stages of LAST, such as arrhythmias and bradycardia, helps maintain hemodynamic stability [51, 52]. Experimental evidence also demonstrates that ILE accelerates bupivacaine clearance and reduces its concentrations in critical tissues, including the heart, brain, and kidneys [53]. In the present study, ILE reduced TNF-α, NF-κB, and MMP-9 immunoreactivity, supporting its protective and therapeutic potential against bupivacaine-induced kidney injury.

High plasma concentrations of bupivacaine have been shown to induce necrotic cell death and inflammatory responses, resulting in severe tubular necrosis, medullary congestion, and hemorrhage in rat kidneys [5]. The findings of this study are consistent with these observations and demonstrate that ILE can attenuate such effects. Bupivacaine and other local anesthetics are metabolized by hepatic microsomal enzymes, and both the parent compounds and their metabolites are eliminated via the kidneys. The immunohistochemical markers used in this study, including irisin, MMP-9, NF-κB, and TNF-α, reflect renal tissue damage caused by these agents.

A limitation of this study is that ILE's renal effects were assessed over a short duration. Therefore, long-term outcomes and potential side effects may have been overlooked. Additionally, results obtained from animal models may not fully translate to humans and should be interpreted cautiously in clinical contexts. Further studies are warranted to evaluate the long-term efficacy and safety of ILE in preventing local anesthetic-induced kidney injury.

CONCLUSION

The study suggests that irisin, MMP-9, NF-κB, and TNF-α are significant biomarkers of LA-induced renal injury. The decrease in these parameters following ILE administration reflects its nephroprotective effect. Furthermore, ILE appears to modulate bupivacaine-induced renal toxicity and ameliorate histopathological changes, including inflammation, glomerular degeneration, tubular damage, and congestion.

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Conflict of interests

The authors have no conflict of interest to declare concerning the authorship or publication of this article**.**

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