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Original article

Linezolid mitigates tissue injury in experimental model of pediatric testicular torsion: TLR-4/MAPK/NF-κB involvement

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Background: Testicular torsion is a urological emergency that requires prompt surgery to prevent orchiectomy. Pharmacological interventions may slow the progression of damage and reduce reperfusion injury after surgical correction.

Purpose: This study evaluated the protective effects of linezolid against testicular torsion-detorsion (T/D) injury in rats by focusing on the mechanisms involving the Toll-like receptor 4 (TLR-4) pathway.

Methods: Eighty-four male Wistar rats were allocated into 8 groups; of them, one was subjected to a sham operation and another was subjected to 4-hour ischemia via 720° of torsion followed by 24-hour reperfusion. Linezolid (3–100 mg/kg) was assessed for its effects on T/D injury using histopathological evaluation, oxidative stress markers (malondialdehyde [MDA], superoxide dismutase [SOD]), and inflammatory biomarker tumor necrosis factor-alpha (TNF-α). Mechanistic investigations have focused on TLR-4 the mitogen-activated protein kinase (MAPK)/nuclear factor kappa B (NF-κB) pathway. Molecular docking and in silico analyses were conducted to predict interactions with key inflammatory proteins.

Results: Linezolid 25, 50, and 100 mg/kg significantly reduced the histopathological damage, with 50 mg/kg being the most effective dosage. Within the 6–50 mg/kg range, linezolid reduced MDA, increased SOD, decreased TNF-α, and suppressed TLR-4/NF-κB pathway activity, with maximal reductions in MDA, TNF-α, NF-κB, and TLR-4 of 64%, 77%, 56%, and 53%, respectively, and an enhancement in SOD of 47%. In silico docking predicted strong binding interactions with TLR-4 pathway proteins, including p38 MAPK and JNK, with affinities of -7.4 to -8.3 kcal/mol.

Conclusion: Linezolid protects against testicular torsion by reducing oxidative stress and inflammation via modulating the TLR-4/NF-κB pathway, suggesting its therapeutic potential and need for further study.

Key words: Linezolid, Spermatic cord torsion, Reperfusion injury, Toll-like receptor 4, Mitogen-activated protein kinases

Key message

Question: What pharmacological strategies can limit ischemia-reperfusion injury in pediatric patients with testicular torsion?

Finding: In a rat model of testicular torsion, linezolid reduced oxidative stress, inflammation, and tissue injury via the Toll-like receptor 4/mitogen-activated protein kinase/nuclear factor kappa beta pathway.

Meaning: Linezolid may offer a pharmacological approach to attenuate testicular damage in pediatric patients with testicular torsion, warranting further clinical investigation.

Introduction

Testicular torsion is a urological emergency affecting 3.8 in 100 000 males under 18 annually in the United States.[1)] The twisting of the spermatic cord impairs blood flow to the testicle, causing ischemia and subsequent oxygen and nutrient deprivation. This leads to energy depletion, electrolyte imbalance, and mitochondrial dysfunction, resulting in progressive tissue damage.[2)] Without timely intervention, irreversible damage may occur, potentially

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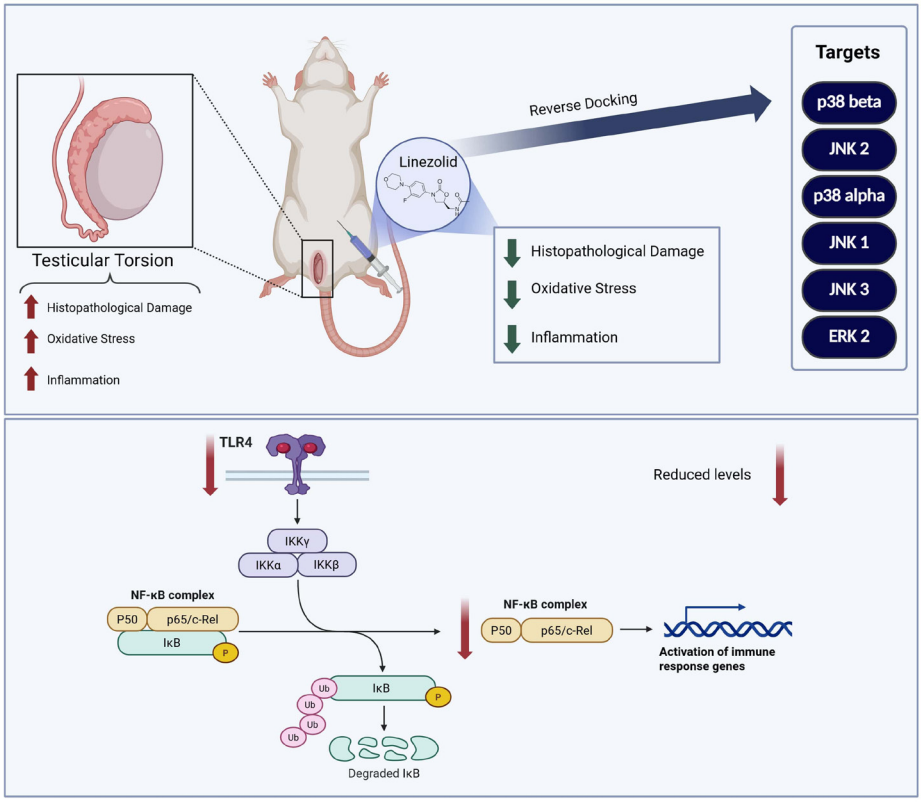

Graphical abstract. TLR-4, Toll-like receptor 4; NF-κB, nuclear factor kappa B; IKK, Inhibitor-κB kinase; Ub, Ubiquitin; IkB, Inhibitor-κB.

necessitating orchiectomy.[1] Despite extensive research, there is currently no approved pharmacological option to protect against testicular torsion and mitigate damage. The rat model of testicular torsion-detorsion (T/D) provides a clinically relevant paradigm that closely mimics the pathophysiology of human cases, making it suitable for exploring potential interventions.[3]

Ischemia triggers complex signaling cascades, with inflammatory pathways playing a critical role. Energy depletion and mitochondrial dysfunction activate inflammatory responses within affected cells. Additionally, the release of damage-associated molecular patterns recruits both local and systemic immune cells, intensifying the inflammatory response.[4] Given these mechanisms, agents with anti-inflammatory properties present promising therapeutic potential for mitigating ischemia-related damage in testicular torsion. The central role of mitogen-activated protein kinases (MAPKs) in inflammation further highlights them as a plausible target for anti-inflammatory therapies.[5] Elevated MAPK levels in testicular T/D injury[6] and the efficacy of MAPK-modulating drugs in testicular torsion[7-9] reinforce this pathway as a key therapeutic focus. Additionally, Toll-like receptor 4 (TLR-4) modulation, as a key upstream regulator of the MAPK pathway, has emerged as a potential therapeutic target in inflammatory conditions.[10]

Linezolid, an oxazolidinone antibiotic, has exhibited notable anti-inflammatory properties in both infectious and non-infectious conditions,[11-15] making it a potential therapeutic candidate for testicular torsion. While its primary mechanism involves inhibiting bacterial protein synthesis by targeting 23S ribosomal RNA, evidence suggests its effects extend to mammalian cells.[12-14,16,17] The structural similarity between mitochondrial and bacterial ribosomes may partially explain its influence on mitochondrial function and related anti-inflammatory actions. Additionally, studies have implicated pathways such as MAPK in mediating these effects.[16] Consistent with MAPK modulation, oxazolidinones have also been reported to influence the TLR-4 pathway.[18,19]

Given the complex and not fully understood mechanism of linezolid's anti-inflammatory action, we employed molecular docking techniques to predict potential pathways involved in its protective effects against testicular torsion. We hypothesized that linezolid exerts protection against T/D injury by modulating the TLR-4 pathway. Using a rat model of testicular T/D, we evaluated the efficacy of linezolid by assessing histopathological changes, measuring pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), evaluating oxidative stress markers (malondialdehyde [MDA] and superoxide dismutase [SOD]), and quantifying nuclear factor kappa-light-chain-enhan-

cer of activated B cells (NF-κB) and TLR-4 levels as key components of the hypothesized pathway.

Methods

1. Computational approach

1) Ligand preparation

Linezolid was selected as the ligand for this study. Its structure was obtained from the PubChem database (CID: 441401) in the 3-dimensional (3D) Signed Distance Fields format and converted to the Mol2 format using OpenBabel. The Mol2 structure was minimized using Chem3D to enhance docking accuracy and the final Mol2 format was used for further analysis.

2) Target protein prediction

The SwissTargetPrediction tool (https://www.swisstargetprediction.ch/) was utilized to predict potential protein targets of linezolid. The software uses chemical similarity-based methods and bioactivity data to predict potential targets in the human proteome. The input ligand, linezolid, was screened, and 100 proteins with the highest probability of binding were identified. The list of the identified proteins was downloaded and used for further docking and protein-protein interaction studies.

3) Protein-protein interaction network

The STRING database (https://string-db.org/) was used to explore the protein-protein interaction network of the 100 identified targets. The list of 100 proteins was submitted, and the enrichment analysis was conducted, generating information on cellular components, biological processes, molecular functions, Kyoto Encyclopedia of Genes and Genomes pathways, Reactome pathways (RCTM), tissue expression profiles, and WikiPathways.

4) Molecular docking

ReverseDock (https://www.reversedock.org/) was employed to dock linezolid with the 100 proteins identified from SwissTargetPrediction. The docking was performed using AutoDock Vina,[20] with web server default parameters. The 3D protein structures were retrieved from the AlphaFold by providing the proteins UniProt IDs. Linezolid-protein interaction representations were created using Discovery Studio Visualizer (Ver.17.2) (https://www.3dsbiovia.com/products/collaborative-science/biovia-discovery-studio) and PyMol version 1. Level (https://pymol.org).

2. *In vivo* experiment

1) Animals

We acquired 84 seven-week-old male Wistar rats, each weighing 200±15 g, from the Department of Pharmacology at Tehran University of Medical Sciences. To minimize potential environmental confounding factors, the rats were housed in groups of six, with each cage containing animals from different experimental groups. The housing facility maintained controlled conditions, including a temperature of 22°C±2°C, relative humidity of 45%–55%, and a 12-hour light/dark cycle. Rats had unrestricted access to food and water throughout the study. Prior to the experiments, a one-week acclimation period was provided. All animal handling and experimental procedures adhered to EU Directive 2010/63/EU for animal experiments and received approval from the institutional ethics committee (IR.TUMS.VCR.REC. No.03-2-101-73303). We also followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Each rat served as an individual experimental unit in all experiments, and exclusion criteria included abnormally small testes or other genital abnormalities.

2) Chemicals

The chemicals used in this study included: Linezolid (PZ0014, Sigma-Aldrich, USA), dimethyl sulfoxide (DMSO) (102952, Merck, Germany), ketamine hydrochloride (10% w/v solution, Alfasan Pharmaceuticals, The Netherlands), and xylazine hydrochloride (2% w/v solution, Bremer Pharma, Germany). Due to the limited aqueous solubility, linezolid was prepared in a 5% v/v DMSO/physiological saline vehicle. Treatments were administered at doses of 3, 6, 12, 25, 50, and 100 mg/kg, with a final administration volume of 8-mL/kg body weight. Linezolid dosages were determined by extrapolating the clinical dose (1,200 mg/day) to preclinical rat models using a 6-fold scaling factor, approximating 100 mg/kg, consistent with prior studies demonstrating anti-inflammatory effects within the 5–100 mg/kg range.[11-15]

3) Testicular torsion

Anesthesia was induced via intraperitoneal injection of a ketamine-xylazine cocktail (60/8 mg/kg) prior to surgical intervention. A 2-cm vertical midline incision was made on the right scrotum to expose and release the right testis from surrounding tissues. Testicular torsion was induced by rotating the right testis 720° counterclockwise and securing the tunica albuginea to the scrotal wall with a 5/0 nylon suture, avoiding injury to the testicular parenchyma. After a 4-hour ischemic period, detorsion was performed by removing the suture and rotating

the testis 720° clockwise to restore its original position, followed by suturing of the scrotal skin for a 24-hour reperfusion period. Successful detorsion was confirmed by observing smooth testicular movement through the spermatic cord. During the procedure, rats were placed on a warm pad, and moist sterile gauze was applied to the testis. Due to the limited duration of ketamine anesthesia (approximately 45 minutes), additional ketamine (20 mg/kg) was administered as needed. After detorsion, 6 mL/kg of physiological saline was given to each rat to compensate for fluid loss during surgery.

4) Experimental groups

AS shown in Fig. 1, the investigation was conducted in 2 phases. In the first phase, 48 rats were randomly assigned to 8 experimental groups (n=6 per group) using computer-based block randomization: (1) Sham-operated, which involved a scrotal incision without testicular torsion, followed by closure after 4 hours; (2) T/D group, which underwent the T/D procedure with vehicle administration 2 hours after ischemia; (3–8) T/D + linezolid groups, where rats underwent the T/D procedure and received intraperitoneal linezolid administration at varying doses (3, 6, 12, 25, 50, or 100 mg/kg) 2 hours after ischemia.[21] Histopathological evaluations using Cosentino grade served as the primary outcome measure to assess dose efficacy.

The second phase involved 36 rats divided into 6 groups using computer-based block randomization (n=6 per group): (1) Sham-operated and (2) T/D groups, identical to those in phase one; (3–6) T/D + linezolid groups, where linezolid was administered at doses of 6, 12, 25, or 50 mg/kg, 2 hours after ischemia. Secondary outcome measures

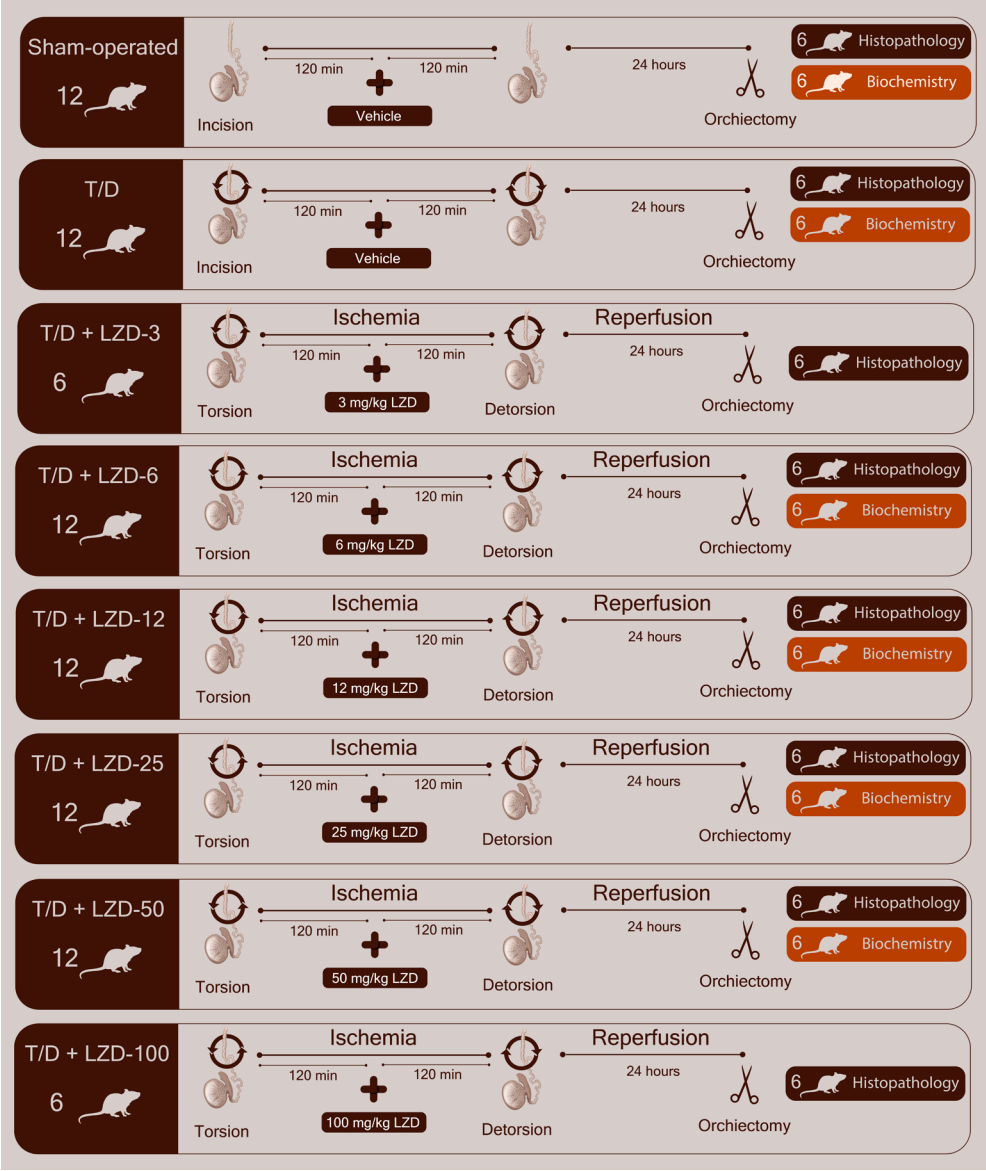

Fig. 1. Experimental design and timeline for testicular torsion-detorsion (T/D) induction, treatments, and sample collection. LZD, linezolid.

included biochemical parameters and changes in testicular weight. Sample sizes were determined based on previous research indicating n=6 as adequate for histopathological and biochemical analyses.[21]

5) Biochemistry

Following a 24-hour reperfusion period, animals were euthanized using a high-dose ketamine/xylazine cocktail (200/20 mg/kg). Ipsilateral orchiectomy was then performed. Testicular tissue protein content was quantified using the bicinchoninic acid assay (Nadford, Navand Salamat, Iran), and all subsequent measurements were normalized to tissue protein content and expressed per milligram of tissue protein. Levels of proinflammatory cytokine TNF-α (Karmania Pars Gene, Iran), oxidative stress markers (MDA [Teb Pazhouhan Razi, Iran] and SOD [Nasdox, Navand Salamat, Iran]), and inflammatory mediators (NF-κB-p65 [E-EL-R0674, Elabscience, USA] and TLR-4 [MBS705488, MyBioSource, USA]) were quantified using enzyme-linked immunosorbent assay kits, following the manufacturer's protocols.

6) Histopathology

Twenty-four hours after detorsion, rats were euthanized using a high-dose ketamine/xylazine cocktail (200/20 mg/kg), followed by ipsilateral orchiectomy. The excised testes were fixed in 10% phosphate-buffered formalin and subsequently dehydrated in 70% ethanol. Sections of 5-μm thickness were obtained from the superior, medial, and inferior regions of the testis. The tissue sections underwent deparaffinization and were stained with hematoxylin and eosin. Histopathological evaluation was conducted under ×400 magnification by an experienced pathologist who was blinded to the experimental groups. Testicular damage was quantified using the Cosentino scoring system, as previously described.[21] Morphometric analysis of seminiferous tubules was performed using a light microscope with a calibrated micrometer, and tubular area measurements were conducted using the Sketchandcalc online tool (https://www.sketchandcalc.com/). The percentage of the tubule covered with cells was determined by dividing the area covered by cells in the outer layer by the total tubular area. For each rat, the area and cell coverage percentages were calculated by averaging measurements taken from the three roundest tubules in each of the superior, medial, and inferior regions, resulting in an average based on nine tubules per rat. Seminiferous tubule areas were expressed relative to the mean value of the sham group for comparison.

7) Testis weight

Twenty-four hours after detorsion, bilateral orchiectomy was performed. The weights of both ipsilateral and contralateral testes parenchyma were measured using a digital scale with an accuracy of 0.01 mg. The ipsilateral-to-contralateral testicular weight ratio was then calculated.

8) Data analysis

Statistical analyses and data visualization were performed using GraphPad PRISM 10 (GraphPad Software Inc., USA). Data are presented as mean±standard deviation (SD) for parametric data and as median±interquartile range for nonparametric data. The Shapiro-Wilk test was used to assess normality, while Levene test evaluated the equality of variances. Parametric data with equal SDs, including levels of MDA, SOD, TLR-4, NF-κB, seminiferous tubule area, cell coverage percentage, and testicular weight ratio, were analyzed using 1-way analysis of variance (ANOVA). For data with unequal SDs (TNF-α), Welch ANOVA was applied, followed by Dunnett post hoc test for comparisons with the T/D group. Nonparametric histopathological scores were assessed using the Kruskal-Wallis test with Dunn post hoc test for pairwise comparisons. Statistical significance was defined as $P<0.05$, and all analyses were conducted by a blinded investigator (AB).

Results

1. Docking

The list of 100 targets predicted using the SwissTarget Prediction tool, along with their binding affinities and enrichment analysis results, is available in the supplementary data. Based on enrichment analysis findings, six key proteins, predominantly associated with the pathophysiology of testicular torsion and ischemia-reperfusion injury as indicated by previous research, were identified for further investigation. These targets include:

- MAP kinase ERK2 (UniProt ID: P28482)
- c-Jun N-terminal kinase 1 (JNK1) (UniProt ID: P45983)
- c-Jun N-terminal kinase 2 (JNK2) (UniProt ID: P45984)
- c-Jun N-terminal kinase 3 (JNK3) (UniProt ID: P53779)
- MAP kinase p38 alpha (p38α) (UniProt ID: Q15759)
- MAP kinase p38 beta (p38β) (UniProt ID: Q16539)

The binding affinities for these targets ranged from -7.4 to -8.3 kcal/mol, within an acceptable range. The protein with the highest affinity interactions was further examined, as detailed in Table 1 and Fig. 2.

2. Histopathological evaluations and testes weight change

In the first phase of the study, protective effects of linezolid across a range of doses were evaluated using the Cosentino score as the primary outcome measure. As anticipated, testicular torsion caused substantial tissue

Table 1. Molecular docking of mitogen-activated protein kinases

Protein	UniProt ID	Binding affinity (kcal/mol)	Interactions
MAP kinase p38 beta	Q15759	-8.3	4 Hydrogen Bonds with Thr 106, Met 109, Asp 112, and Asn 115 5 different π interactions with Val 38, Lys 53, Leu 75, Ile 84, and Ileu 167, and 8 van der Waals interactions
c-Jun N-terminal kinase 2	P45984	-8.1	
MAP kinase p38 alpha	Q16539	-8.1	
c-Jun N-terminal kinase 1	P45983	-7.9	
c-Jun N-terminal kinase 3	P53779	-7.8	
MAP kinase ERK2	P28482	-7.4	

MAP, mitogen-activated protein.

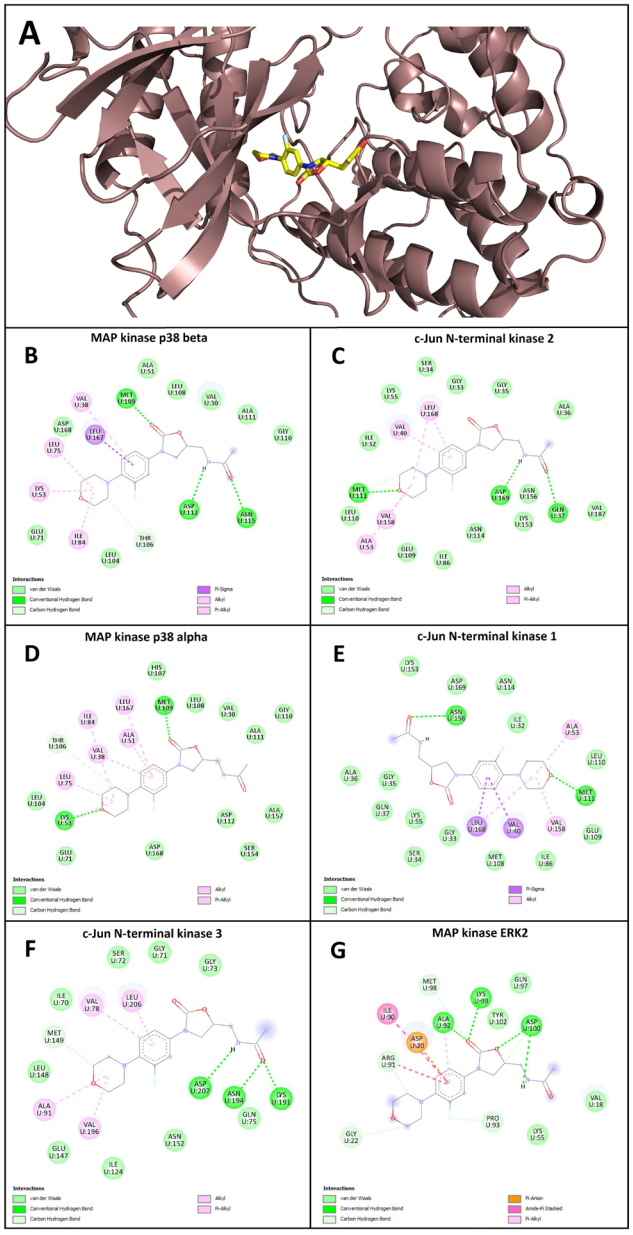

Fig. 2. Linezolid-protein interactions. (A) Three-dimensional representation of linezolid and MAP kinase p38 beta interactions. Two-dimensional representation of linezolid and MAP kinase p38 beta (B), c-Jun N-terminal kinase 2 (C), MAP kinase p38 alpha (D), c-Jun N-terminal kinase 1 (E), c-Jun N-terminal kinase 3 (F), and MAP kinase ERK2 (G). MAP, mitogen-activated protein.

damage with extensive cell necrosis, evidenced by a mean score of 3.8 compared to 1.0 in the sham group (P< 0.0001). Linezolid demonstrated protective effects at doses of 25, 50, and 100 mg/kg, with the 50 mg/kg showing the greatest efficacy (mean score=2.0, P<0.01). In addition to histopathological scoring, morphometric analysis of seminiferous tubules was conducted by measuring tubular area and the percentage of area covered by cell layers. Testicular torsion induced significant tubule shrinkage (P< 0.05), reducing the seminiferous tubule area from 48,761 to 34,367 μm², without a significant change in cell coverage percentage (from 74.4% to 68.9%, P=0.11). Linezolid treatment at 50 and 100 mg/kg increased cell coverage to levels surpassing those of the sham group (76.8% and 77.4%, respectively), although it did not reverse tubule shrinkage (P=0.83 and P=0.86, respectively). Regarding the weight ratio of ipsilateral to contralateral testes, testicular torsion significantly decreased this ratio; however, linezolid at 50 (97.0% vs. 85.1% in the vehicle group, P=0.013) and 100 mg/kg (95.8%, P=0.019) effectively restored it (Fig. 3).

3. Antioxidative and anti-inflammatory effects of linezolid

Testicular T/D induced significant oxidative stress, as reflected by elevated MDA levels (3.46 μmol/mg vs. 1.07 μmol/mg, P<0.0001), and a reduction in the antioxidant activity of SOD (391 U/mg vs. 673 U/mg, P<0.0001). Linezolid treatment mitigated these oxidative changes by restoring SOD activity and reducing MDA levels. Although all tested doses of linezolid lowered MDA levels, only the 50 mg/kg dose significantly restored SOD activity from 391 to 578 U/mg (P=0.005). Regarding inflammation, testicular T/D led to a marked increase in TNF-α levels, rising from 18.2 to 55.1 pg/mg (P<0.0001). Linezolid at 6 mg/kg demonstrated a decreasing trend in TNF-α levels, though it did not achieve statistical significance (40.1 pg/mg, P=0.13). However, higher doses of 12, 25, and 50 mg/kg significantly reduced TNF-α levels, with the 50 mg/kg exhibiting the greatest effect, lowering TNF-α levels from 55.1 to 12.6 pg/mg (P= 0.0002), even below the sham group level, highlighting its potential anti-inflammatory efficacy (Fig. 4).

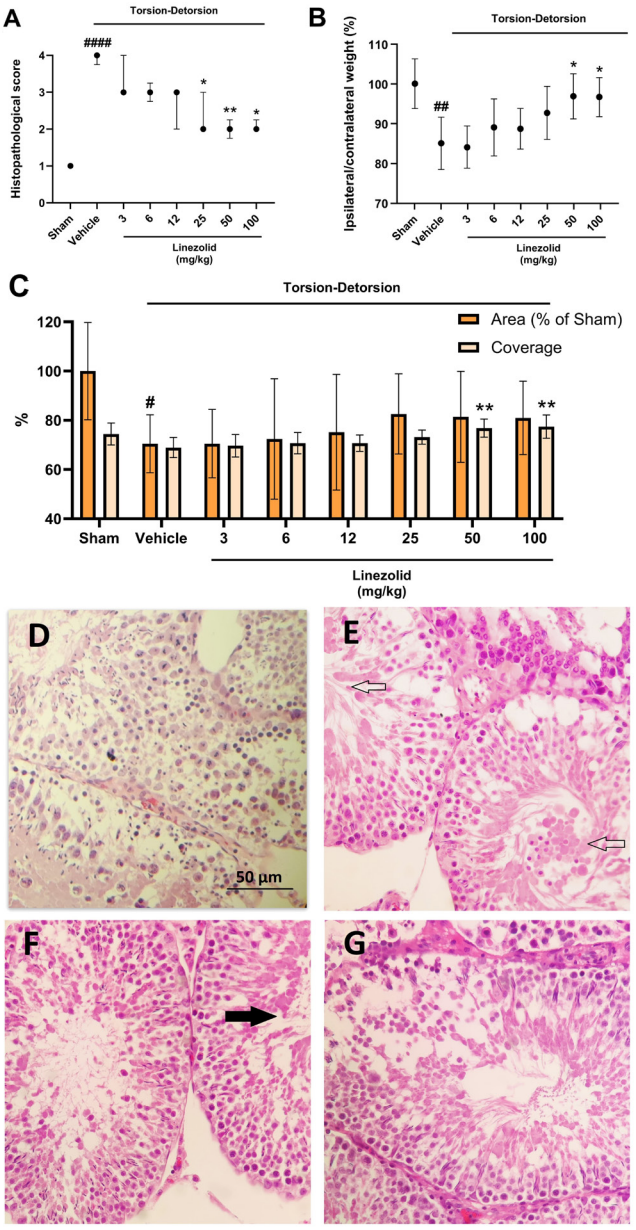

Fig. 3. Histopathologic and morphometric evaluations. (A) Histopathologic scores of the different groups based on the Cosentino scoring system. (B) Ipsilateral/contralateral testis weight ratio (%). (C) Seminiferous tubule area (orange bars, % of sham group) and the percentage of the tubule covered with cells (light apricot bars). Panels D-G show representative hematoxylin and eosin–stained sections of testes from experimental groups under 400× magnification. (D) Normal seminiferous structure observed in the Sham group. (E) Extensive necrosis observed in the torsion-detorsion (T/D) group and that treated with linezolid 3 mg/kg (open arrows). (F) Sloughed germ cells with shrunken, pyknotic nuclei as seen in the groups treated with linezolid 6 and 12 mg/kg (solid arrow). (G) Closely packed seminiferous tubules observed in the groups treated with linezolid 25, 50, and 100 mg/kg. The rats in the eight groups were subjected to a sham operation or testicular T/D and received either vehicle or linezolid (3–100 mg/kg). Data are expressed as median±interquartile range (A) or mean±standard deviation (B and C). N=6 rats for each measurement in each group. #, ##, ###, and #### indicate P<0.05, 0.01, 0.001, and 0.0001, respectively, compared to the Sham group, while * and ** denote P<0.05 and 0.01 compared to the T/D+vehicle group. The statistical analysis was performed using the Kruskal-Wallis test followed by Dunn *post hoc* test (A) or 1-way analysis of variance followed by Dunnett multiple comparisons post hoc test (B and C).

4. TLR-4 signaling pathway

Similar to TNF-α, NF-κB levels increased significantly during testicular T/D, rising from 76.6 to 357.1 pg/mg (P< 0.0001). TLR-4, an upstream regulator of NF-κB, also exhibited a marked increase from 0.13 to 0.62 ng/mg during T/D (P<0.0001). Linezolid treatment at doses of 25 and 50 mg/kg effectively reduced both TLR-4 and NF-κB levels, with the 50 mg/kg demonstrating the greatest efficacy, lowering TLR-4 and NF-κB levels by 53% (0.29 ng/mg, P=0.0009) and 56% (155.6 pg/mg, P<0.0001), respectively. Additionally, the 12 mg/kg dose significantly decreased NF-κB levels (211.3 pg/mg, P<0.01) but did not produce a statistically significant reduction in TLR-4 levels (0.44 ng/mg, P=0.099) (Fig. 5).

Discussion

To the best of our knowledge, this study is the first to demonstrate the protective effects of linezolid against ischemia-reperfusion injury in a rat model of testicular T/D. Recognizing inflammation as a key factor in testicular T/D, our findings reveal the anti-inflammatory properties of linezolid, independent of its effects in infectious conditions. This aligns with prior research on the anti-inflammatory actions of linezolid in both *in vivo* and *in vitro* models. Linezolid attenuated T/D injury by modulating the TLR-4 and MAPK pathways, reducing oxidative stress and inflammatory biomarkers. Although the clinically equivalent dosage for rats is approximately 100 mg/kg, we observed promising protective effects at a lower dose of 50 mg/kg, thereby minimizing concerns regarding toxicity.

Although the anti-inflammatory effects of linezolid in infectious diseases have been widely reported and are often attributed to inhibition of prokaryotic metabolism, previous research has demonstrated its anti-inflammatory actions across various conditions independent of infectious pathogens. For instance, treatment with linezolid in a carrageenan-induced paw edema model in rats reduced the edema rate, whereas other antibiotics failed to produce similar effects.[11] In human peripheral blood mononuclear cells activated with lipopolysaccharide (LPS), linezolid treatment reduced interleukin-6 (IL-6) expression.[13] An in vitro study using phorbol-12-myristate-13-acetate-stimulated neutrophils revealed decreased IL-8 secretion following linezolid treatment.[15] Furthermore, cytokine expression studies in LPS-treated human whole blood cells showed decreased levels of IL-1β, IL-6, TNF-α, and IL-8 mRNA following linezolid administration.[14] In a study examining neutrophils, linezolid did not impact methicillin-resistant Staphylococcus aureus (MRSA) viability in the

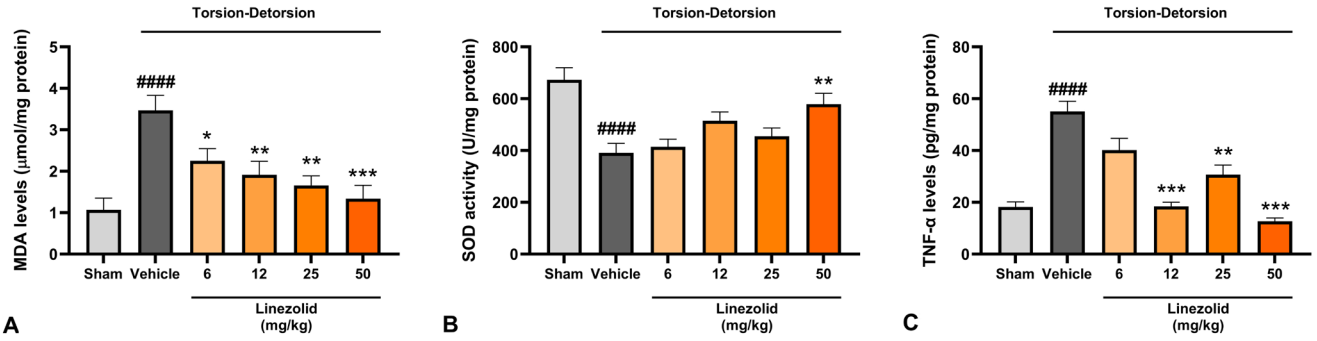

Fig. 4. Oxidative and inflammatory biomarker levels in testicular tissue. (A) Malondialdehyde (MDA). (B) Superoxide dismutase (SOD). (C) Tumor necrosis factor-alpha (TNF-α). The rats were divided into 6 groups that were subjected to a sham operation or testicular torsion-detorsion (T/D) and received vehicle or linezolid (6, 12, 25, or 50 mg/kg). Data are expressed as mean±standard deviation. N=6 rats for each measurement in each group. #, ##, ###, and #### indicate *P*<0.05, 0.01, 0.001, and 0.0001, respectively, compared to the Sham group; *, **, and *** denote *P*<0.05, 0.01, and 0.001 compared to the T/D + vehicle group. Statistical analysis was performed using the ordinary 1-way (A and B) or Welch (C) analysis of variance followed by Dunnett multiple comparisons *post hoc* test.

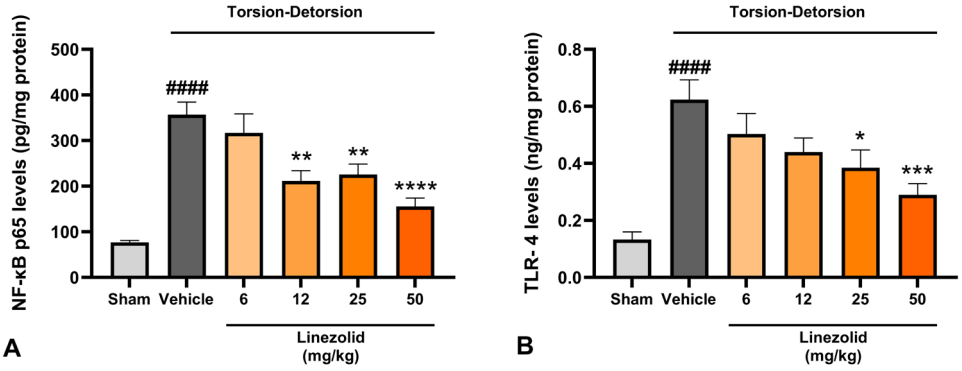

Fig. 5. Toll-like receptor 4 (TLR-4) pathway evaluation. (A) NF-κB-p65 levels in testicular tissue. (B) TLR-4 levels in testicular tissue. The rats were divided into six groups and subjected to a sham operation or testicular torsion-detorsion (T/D) and received vehicle or linezolid (6, 12, 25, or 50 mg/kg). Data are expressed as mean±standard deviation. N=6 rats for each measurement in each group. #, ##, ###, and #### indicate *P*<0.05, 0.01, 0.001, and 0.0001, respectively, compared to the Sham group, while *, **, and *** denote *P*<0.05, 0.01, and 0.001 compared to the T/D+vehicle group. The statistical analysis was performed using 1-way analysis of variance followed by Dunnett multiple comparisons *post hoc* test. NF-κB, nuclear factor kappa B.

presence of healthy neutrophils but reduced viability in impaired neutrophils treated with C5a, and it also inhibited IL-8-induced neutrophil transmigration.[12] Conversely, some studies reported increased IL-1β expression after linezolid incubation in immune cells under physiological conditions in the absence of bacteria or endotoxins,[22] as well as reactive oxygen species (ROS)-independent activation of the NOD-like receptor family pyrin domain-containing 3 (NLRP-3) inflammasome by linezolid.[23] While these findings may appear contradictory, they are consistent with the mechanisms underlying linezolid's actions.

Linezolid binds to the bacterial 23S ribosomal RNA of the 50S subunit, thereby preventing the formation of a functional 70S initiation complex and inhibiting bacterial protein synthesis by disrupting translation. Due to structural similarities between mitochondrial and bacterial ribosomes, linezolid administration can lead to mitochondrial dysfunction, which may explain its adverse effects during prolonged clinical use, such as thrombocytopenia, optic neuropathy, peripheral neuropathy, and lactic acidosis.[24] Additionally, linezolid has been shown to inhibit mitochondrial complex IV, impairing oxidative phosphorylation.[25] This impairment leads to oxidative and nitrosative stress, resulting in increased production of ROS.[26] While elevated ROS levels can activate the NLRP-3 inflammasome and enhance IL-1β maturation, linezolid-induced mitochondrial dysfunction can also activate the NLRP-3 pathway in a ROS-independent manner through cardiolipin-mediated mechanisms.[23] These oxidative and proinflammatory effects are typically observed under physiological conditions. However, in the context of a pathological inflammatory state, linezolid exhibits an opposing, protective role.

During the ischemic phase of T/D, oxygen and glucose deprivation inhibits cellular respiration at mitochondrial complex IV. So, the additional inhibition of complex IV by linezolid may not significantly exacerbate the existing

mitochondrial dysfunction. Also, linezolid's anti-inflammatory effects may outweigh its pro-oxidant actions. In some studies, linezolid treatment has been associated with reduced neutrophil infiltration in inflamed tissues.[27] Given that neutrophils contribute significantly to ROS production during reperfusion, reduction in neutrophil infiltration could help minimize oxidative damage during the reperfusion phase, thus indirectly protecting the testicular tissue in the T/D model.[28] Also, in a T/D model, this limited exposure could mean that any mitochondrial dysfunction induced by linezolid does not have enough time to significantly affect testicular cells or exacerbate I/R injury. It's possible that linezolid's effect on mitochondria only becomes problematic at higher doses or with prolonged treatment, so in a short-term or moderate-dose context, the effects might not be substantial enough to worsen T/D injury.

Overactivity of tissue-specific and circulating immune cells is a well-documented phenomenon during ischemia-reperfusion, including in testicular T/D. Mitigating this immune overactivity can provide protective effects against tissue damage. In fact, various mitochondrial inhibitors have demonstrated protective effects against ischemia.[29-31] Linezolid has been shown to confer protection in experimental autoimmune encephalomyelitis through the inhibition of T helper-17 cell effector function.[32] Additionally, linezolid has been found to dampen neutrophil-mediated inflammation in MRSA-induced pneumonia, thereby protecting the lungs from associated damage.[27] These observations are consistent with our findings of protection against testicular T/D, characterized by a reduction in TNF-α levels. Interestingly, linezolid treatment of human peripheral blood mononuclear cells stimulated with LPS did not reduce TNF-α levels,[13] suggesting that the observed TNF-α reduction in our study likely results from indirect modulation via anti-inflammatory mechanisms rather than direct suppression of TNF-α production.

Despite the oxidative effects of linezolid, our study demonstrated protective effects against testicular T/D, reflected by reduced MDA levels and increased SOD activity. Oxidative stress during ischemia-reperfusion occurs in three phases: oxygen and glucose deprivation inhibit cellular respiration at mitochondrial complex IV; intracellular ATP depletion activates xanthine oxidase, resulting in ROS production; and rapid blood flow restoration causes a mismatch in mitochondrial complexes, leading to additional ROS generation.[33] Linezolid's inhibition of complex IV suggests that it does not exacerbate oxidative stress, as this complex is already inhibited. However, during the reperfusion phase, slowing electron transport may help reduce ROS production, a phenomenon supported by the protective role of carbon monoxide—known to inhibit complex IV—against ischemia-reperfusion injury.[34]

Although linezolid is primarily recognized as an inhibitor of the formation of a functional 70S initiation complex, evidence suggests it may also target other proteins. For instance, Enterococcus faecium cultured with subinhibitory concentrations of linezolid exhibited altered protein expression patterns, with some proteins showing decreased levels and others showing increased levels—a phenomenon not solely attributable to inhibition of protein synthesis.[35] Furthermore, linezolid reduced MRSA supernatant-induced MUC5AC overexpression in human airway epithelial cells, accompanied by a decrease in ERK phosphorylation.[16] Additionally, 2,3,4-Triaryl-1,2,4-oxadiazol-5, a synthetic oxazolidinone structurally similar to linezolid, demonstrated potent inhibitory effects on p38 MAPK.[36] Similarly, 2-pentadecyl-2-oxazoline inhibited LPS-induced microglia activation by interfering with TLR-4 signaling,[18] while 4-oxo-4-(2-oxo-oxazolidin-3-yl)-but-2-enoic acid ethyl ester, another oxazolidinone, inhibited TLR-4 homodimerization.[19] These findings prompted us to focus on the TLR-4 pathway, which acts through MAPKs, as potential targets for linezolid's mechanism of action. Consistent with previous studies and molecular docking results, our data indicate decreased TLR-4 levels and a reduction in NF-κB-p65 as a downstream mediator.

Testicular torsion induces an elevation in p38, ERK, and JNK levels.[6] Modulating p38 and JNK activity by Acetyl-11-keto-β-boswellic acid has been shown to prevent testicular T/D injury in rats.[7] Dysregulation of p38 is also implicated in male infertility, highlighting it as a potentially effective therapeutic target.[37] Activation of peroxisome proliferator-activated receptors protects the testes from ischemia-reperfusion injury by reducing ERK phosphorylation.[8] Additionally, exposure to acrylamide inhibits testosterone production in mice testes and Leydig cells through ERK1/2 phosphorylation activation.[38] SP600125, a JNK inhibitor, alleviates oxidative DNA damage, germ cell apoptosis, and mitochondrial dysfunction during testicular torsion.[9] IL-1β has been shown to induce JNK phosphorylation, leading to neutrophil recruitment to the testes through increased E-selectin expression.[39] Collectively, these findings underscore the critical role of MAPK activation in testicular T/D, suggesting that modulating this pathway could yield protective effects. Consistent with these observations, our docking results indicate that MAPKs are probable targets for linezolid's mechanism of action, which is supported by decreased NF-κB levels.

The present study uncovered several key findings with potential implications for future research. Protective anti-inflammatory effects of linezolid observed at subinhibitory concentrations, along with the lower doses used in

our study compared to equivalent therapeutic doses, minimize concerns regarding safety in clinical settings. Furthermore, adverse effects associated with linezolid treatment typically arise during prolonged administration, whereas pharmacological intervention for testicular torsion is inherently acute.[24] Given that testicular T/D serves as a model for ischemia-reperfusion injury, linezolid's protective effects may extend to other ischemia-reperfusion conditions, such as myocardial infarction, stroke, and renal or hepatic ischemia. Additionally, structural modifications of linezolid could potentially yield more potent and selective oxazolidinones with reduced side effects.

Despite the novel and promising findings, our study faces several limitations. First, while the testicular T/D rat model closely replicates the pathophysiology of human testicular torsion, further clinical trials are necessary to validate the efficacy and safety of linezolid in human subjects. Second, although our data demonstrating decreased levels of TNF-α and NF-κB align with our docking results, further assessment of MAPKs activity would strengthen our hypothesis. Third, the use of a four-hour ischemic period caused extensive damage, potentially obscuring the protective effects of lower doses of linezolid under milder ischemic conditions. Some studies have suggested using shorter ischemic periods to better evaluate therapeutic interventions.[40] Finally, while administering linezolid during the ischemic phase aligns with typical clinical scenarios, where the median delay from diagnosis to surgery is approximately 1.8 hours,[41] evaluating administration during different phases of T/D injury could more thoroughly elucidate its therapeutic role.

In conclusion, this study uniquely demonstrates the protective effects of linezolid against testicular T/D injury, primarily through its anti-inflammatory properties independent of infectious states. Linezolid's modulation of the TLR-4 and MAPK pathways, alongside reduced oxidative stress and inflammatory biomarkers, underscores its therapeutic potential. Effective at doses significantly below conventional clinical levels, it mitigates concerns about toxicity. Despite recognized mitochondrial dysfunction-related proinflammatory effects in physiological settings, linezolid's targeted inhibition of immune overactivity during ischemia-reperfusion highlights its potential for acute therapeutic applications. Future clinical trials and mechanistic studies are warranted to further elucidate its efficacy, safety, and dosing strategies in ischemic contexts.

Footnotes

Conflicts of interest: No potential conflict of interest relevant to this article was reported.

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