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PDE5 Inhibitor Tadalafil Attenuates Liver Sinusoid Endothelial Cell Dysfunction in Rat Hepatic Ischemia-Reperfusion Injury by Reducing Platelet-Secreted NO and Suppressing LSEC Pyroptosis

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Background: Hepatic ischemia-reperfusion (I/R) injury, an inexorable event after liver surgery, is associated with circulating platelet activation and aggregation. Phosphodiesterase 5 inhibitor (PDE5i) is a promising option for treating organ I/R injury. Therefore, this study investigated the underlying mechanism of PDE5i in hepatic I/R injury.

Methods: Rats received tadalafil pre-treatment (a PDE5i; 5 mg/kg/day) and underwent hepatic I/R modeling. The impact of tadalafil against liver injury was assessed by measuring the nitric oxide (NO) content as well as the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), followed by histological analysis and TdT-mediated dUTP nick end labeling (TUNEL) assay. The liver sinusoid endothelial cells (LSECs) isolated from normal rats were pre-treated with tadalafil (100 nM) and co-cultured with platelets obtained from hepatic I/R rats. Additionally, cell viability, lactate dehydrogenase (LDH) activity, and degree of pyroptosis were evaluated using Western blot analysis in *in vitro* experimental settings.

Results: Tadalafil protected rats against hepatic I/R by reducing serum levels of AST and ALT and attenuating liver tissue damage, cell apoptosis, and NO content ($p < 0.01$). Tadalafil inhibited the secretion of NO by platelets in hepatic I/R-induced rats ($p < 0.001$). Furthermore, it enhanced cellular viability and decreased LDH activity and pyroptosis of LSECs in platelets derived from hepatic I/R rats ($p < 0.01$). Additionally, tadalafil suppressed LSEC pyroptosis in hepatic I/R rat-derived platelets by down-regulating the expression levels of nucleotide-binding oligomerization domain (NOD)-like receptor thermal protein domain associated protein 3 (NLRP3), apoptosis-associated speck-like protein (ASC), C-caspase 1, N-terminal fragment of gasdermin D (GSDMD-N), interleukin (IL)-1β, and IL-18. Furthermore, tadalafil treatment decreased phosphorylation levels of vasodilator-stimulated phosphoprotein (VASP) and nuclear factor kappa-B (NF-κB) inhibitor alpha (IKB-α) ($p < 0.001$).

Conclusion: Tadalafil attenuates LSEC dysfunction in rat hepatic I/R injury by reducing the levels of platelet-secreted NO and suppressing LSEC pyroptosis. These observations indicate the dual role of tadalafil in modulating platelet function and directly protecting LSECs, offering novel insights into the underlying mechanistic pathway.

Keywords: hepatic ischemia-reperfusion injury; phosphodiesterase 5 inhibitor; platelet; nitric oxide; liver sinusoid endothelial cells

Introduction

Hepatic ischemia-reperfusion (I/R) injury is an inevitable condition after liver surgery, such as liver transplantation and hepatectomy [1]. I/R injury is a two-stage phenomenon, during which temporary occlusion of the vasculature results in the hypoxic damage of cells and this damage is exacerbated upon restoration of blood flow [2]. Despite a variety of approaches to prevent and treat hepatic I/R injury, its associated adverse outcomes, including graft dysfunction and transplant liver failure, remain major challenges in clinical practice [3,4]. Therefore, the investigation of safe and effective agents to alleviate liver damage after I/R is a prominent focus of current research.

Recently, there has been increasing interest in the role of platelet activation during I/R-induced organ injury [5–7]. Platelets, as cell fragments released from megakaryocytes, can be activated under inflammatory conditions and implicated in endothelial dysfunction and atherosclerosis [8,9]. Hepatic I/R injury is an innate immunity-driven persistent inflammatory response, involving the interaction among hepatocytes, liver sinusoid endothelial cells (LSECs), Kupffer cells, macrophages, infiltrating neutrophils, and platelets [10,11]. Among these cell types, LSECs form the vascular wall of the liver sinusoid and play a pivotal role in hepatic homeostasis by regulating vascular tone, inflammation, toxicant clearance, and thrombosis [12]. Previous research has suggested an intimate association between damaged LSECs following hepatic I/R injury with platelet activation and platelet-neutrophil aggregation within vascular beds [13].

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Additionally, it has been shown that extravasated platelet aggregation in the space of Disse, initiated by LSECs damage due to hepatic I/R or immunosuppressive treatment, and the subsequent activation of platelets may primarily contribute to liver damage in liver transplantation. Therefore, endothelial protective therapy or antiplatelet treatment may be useful in treating hepatic I/R following extravasated platelet aggregation [14]. Hence, it indicates that targeting platelets holds promise for preventing and treating hepatic I/R injury.

Phosphodiesterases (PDEs) have been recognized for their role in modulating platelet function by catalyzing the hydrolysis of cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) [15,16]. Among these, the isoform PDE5 is expressed in platelets and specifically binds to cGMP. This interaction has been found to activate protein kinase G (PKG)-mediated intracellular downstream targets, thus dampening platelet activity [17]. Phosphodiesterase 5 inhibitors (PDE5is), including tadalafil and sildenafil, are commonly used for treating conditions like erectile dysfunction, lower urinary tract symptoms, and pulmonary artery hypertension [18]. Several studies have demonstrated the protective effect of PDE5is on myocardial, intestinal, and acute retinal I/R injuries [19–21]. Although less attention has been paid to the impact of PDE5is on hepatic I/R injury, there is evidence that tadalafil can alleviate endothelial damage by reducing nitric oxide (NO)-mediated nitrosative stress [22].

Therefore, this study aims to elucidate the effect and underlying mechanism of PDE5i tadalafil in hepatic I/R injury, focusing on LSEC dysfunction, to provide a theoretical basis for the clinical application of PDE5i for treating hepatic I/R injury.

Materials and Methods

Animals

A specific pathogen-free environment was set to house 50 seven-week-old male Wistar rats (weighing 230–270 g) sourced from Hangzhou Medical College, China. The rats were provided with food and water *ad libitum*. This study received approval from the Ethics Committee of Taizhou Enze Medical Center (group) for Experimental Animals Welfare (No. TEY-2021159), and the experiments involving animals were conducted following the guidelines of the China Council on Animal Care and Use.

Preparation of Tadalafil

Tadalafil (purity >99%, CAS: 171596-29-5, chemical structure: $C_{22}H_{19}N_3O_4$; Cat. No.: M3473) was purchased from Abmole (Houston, TX, USA) and was dissolved in dimethyl sulfoxide (DMSO; GC203005, Servicebio, Wuhan, China). Subsequently, tadalafil was diluted in ethanol (1:100, PHR1070, Sigma-Aldrich, St. Louis, MO, USA) with 28 mL of drinking water.

Rat Modeling and Drug Administration

Following one week of acclimation, rats were randomly categorized into the Sham, I/R, I/R + PDE5i, and PDE5i groups, each comprising 10 rats. Rats in the I/R and I/R + PDE5i groups underwent hepatic I/R injury modeling [23]. For this purpose, rats were anesthetized with 1.5–3% isoflurane (PHR2874, Sigma-Aldrich, St. Louis, MO, USA) and ventilated employing a rodent ventilator (Frequency: 60 beats/min; Tidal volume: 3 mL/kg; Inspiratory to expiratory ratio: 1:1.5; ALC-V8S, Shanghai Alcott Biotech, Shanghai, China). After this, a midline laparotomy was conducted to access the abdominal cavity. The portal vein and hepatic artery supplying blood to the left and middle lobes were isolated and clamped with an atraumatic microvascular clamp to induce 70% hepatic ischemia. Post 30 minutes occlusion, the microvascular clamp was removed followed by a 2-hour reperfusion. Rats in the Sham and PDE5i groups received the same surgical procedures but without vascular clamping. Before modeling, rats in the I/R + PDE5i and PDE5i groups received 5 mg/kg tadalafil daily for 15 days [24], while those in the Sham and I/R groups received drinking water (vehicle) in a similar manner. After reperfusion, all rats were euthanized under deep anesthesia (150 mg/kg of 3% pentobarbital sodium (P3761, Sigma-Aldrich, St. Louis, MO, USA)), and blood and liver samples were collected and stored at –80 °C for subsequent analysis.

Biochemical Assay

The blood samples were centrifuged at 1000 ×g for 10 minutes, and the resultant serum samples were collected in fresh tubes. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were assessed using specific kits (SP12839/SP30121, Wuhan Saipei Biotechnology Co., Ltd, Wuhan, China). Absorbance at 450 nm was recorded utilizing a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland).

Histological Analysis

The fresh liver tissues were fixed using 4% paraformaldehyde (PFD) (P0099, Beyotime, Shanghai, China), embedded in paraffin, and subsequently sectioned into 5 μm thick slices. In the next step, the tissue sections underwent dewaxing and rehydration. These sections were then covered with hematoxylin (C0107, Beyotime, Shanghai, China) for 10 minutes, followed by staining with eosin (C0109, Beyotime, Shanghai, China) at room temperature (RT) for 4 minutes. Finally, they were observed using a light microscope (×100 magnification, AXio Lab.A1, ZEISS, Oberkochen, Germany), and the hepatic tissue injury was determined in four randomly selected fields and analyzed using a histopathological scoring system [25].

TdT-Mediated dUTP Nick End Labeling (TUNEL) Assay

Cellular apoptosis rate in liver tissues was assessed using diaminobenzidine (DAB) (SA-HRP) TUNEL Cell Apoptosis Detection Kit (G1507-50T, Servicebio, Wuhan, China). Briefly, liver sections were treated with 50 µL of equilibration buffer for 10 minutes, followed by the addition of Biotin-dUTP Labeling Mix (5 µL) along with Recombinant TdT enzyme (1 µL). Subsequently, Streptavidin-HRP reaction solution was added and incubated for 30 minutes. After washing with PBS, 50–100 µL of DAB color-developing working solution was added. The reaction was terminated by cleaning with pure water after positive reaction was detected. In the next step, the tissue sections were stained with hematoxylin solution for 3–5 minutes. Finally, images of TUNEL-positive cell nuclei indicating brownish-yellow fluorescence in random fields (×400 magnification) were captured utilizing a confocal microscope (FV3000, Olympus, Tokyo, Japan).

Isolation of LSECs

To isolate LSECs, the fresh liver tissue obtained from normal rats was digested with 0.05% collagenase (A004194, Sangon Biotech, Shanghai, China) and subsequently treated with anti-CD31 antibody (MA1-80069, Thermo Fisher, Waltham, MA, USA) at 37 °C for one hour. After this, immunomagnetic sorting was conducted. Then, the collected cell suspension was assigned to gradient centrifugation and centrifugal elutriation, as previously described [26]. Each rat liver yielded approximately 1×10^8 LSECs with a viability of >95%. The cells were then cultured in a specialized complete medium (CM-R040, Procell, Wuhan, China) at 37 °C with 5% CO_2. Meanwhile, the cells were tested negative for mycoplasma contamination.

Fluorescence-Activated Cell Sorting

According to reference [27], LSECs were suspended in flow solution (PBS buffer containing 0.5% fetal bovine serum + 0.1% sodium azide), followed by staining with VEGF receptor 3 (VEGFR3, 1:100, AF743, R&D system, Minneapolis, MN, USA) on ice for 30 minutes. Following centrifugation at 1200 rpm for 5 minutes, LSECs were washed three times. After this, they underwent staining with Fitc-labeled donkey anti-goat fluorescent secondary antibody (1:200, F0107, R&D system, Minneapolis, MN, USA) and eFluro660 directly labeled CD34 antibody (1:100, 50-0341-82, Thermo Fisher, Waltham, MA, USA) on ice for 30 minutes. Following centrifugation at 1200 rpm for 5 minutes and washing three times, LSECs were examined using a flow cytometer (FC500, Beckman, Brea, CA, USA).

Platelet Pre-Treatment and Co-Culture

Platelets were isolated from the whole blood of either normal rats or rat models using density gradient centrifugation. The platelets were subsequently co-cultured with LSECs at a ratio of 40:1, followed by incubation at 37 °C for 24 hours [28]. Before co-culture, 1×10^8 platelets derived from rat model were pre-treated with 100 nM tadalafil in 1 mL of HEPES-buffered Tyrode's solution (PB180340, Procell, Wuhan, China) for 4 hours [29].

Measurement of NO Level

The NO levels in rat liver tissues or platelets were determined utilizing a Micro NO Content Assay Kit (BC1475, Solarbio, Beijing, China). Briefly, 0.2 g fresh tissues were homogenized in 1 mL of extraction solution at 4 °C followed by centrifugation at 12,000 ×g for 15 minutes. The resultant supernatant or platelet-cultured medium was mixed with a working solution from the kit. After incubation, absorbance at 550 nm was recorded using a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland).

Cell Viability Assay

After co-culture, LSECs at the logarithmic growth phase were seeded into 96-well plates (5×10^3 cells/well) and incubated for 24 hours. The LSECs were reacted with 10 µL of methylthiazolyldiphenyl-tetrazolium bromide solution (ST316, Beyotime, Shanghai, China) for four hours at 37 °C. Following formazan dissolution with 100 µL DMSO, the absorbance (450 nm) was assessed using a microplate spectrophotometer (Multiskan FC, Thermo Fisher, Waltham, MA, USA). Furthermore, cell viability was calculated as follows: Cell viability (%) = $(A_{experimental} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$.

Lactate Dehydrogenase (LDH) Activity

After co-culture, the LDH activity of LSECs was determined by quantifying LDH release using the LDH Activity Assay Kit (E-BC-K046-M, Elabscience, Wuhan, China). Briefly, 1×10^6 cells were homogenized in PBS, followed by centrifugation. Subsequently, the resultant supernatant was sequentially treated with Substrate Buffer, Coenzyme I, Chromogenic Agent, and Alkali Reagent, following the manufacturer's instructions. Finally, the absorbance was determined at a wavelength of 450 nm employing a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland).

Hoechst 33342/Propidium Iodide (PI) Double Staining Assay

LSECs apoptosis rate was examined using Hoechst 33342/PI double staining. The co-cultured cells (1×10^5) were resuspended in 1 mL culture medium, and then 5 µL of Hoechst 33342 (C0031, Solarbio, Beijing, China) and 5 µL of PI (G1021, Servicebio, Wuhan, China) were added. After a 30 minutes incubation (4 °C), the cells were washed with PBS, and PI-positive cells were observed under a fluorescent microscope at ×200 magnification.

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Western Blot

Total protein was extracted from LSECs using radioimmunoprecipitation assay (RIPA) Lysis Buffer (89901, Thermo Fisher, Waltham, MA, USA) supplemented with protease inhibitors (278447, Thermo Fisher, Waltham, MA, USA). They were subsequently quantified utilizing a Bicinchoninic Acid assay (BCA) Protein Assay Kit (23225, Thermo Fisher, Waltham, MA, USA). After this, 20 µg/group of protein extract was resolved through 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (36124ES10, Yeasen, Wuhan, China). The membranes were blocked with Fast Blocking Buffer (36122ES60, Yeasen, Wuhan, China) for 10 minutes at room temperature. The membranes were incubated overnight at 4 °C with primary antibodies (Abcam, Cambridge, UK). The following day, they underwent incubation with secondary antibodies at room temperature for two hours (Thermo Fisher, Waltham, MA, USA). The antibodies used in Western blot analysis were as follows: vasodilator-stimulated phosphoprotein (VASP; 1:1000, ab267471, 47 kDa), phosphorylated (p)-VASP (1:500, ab194747, 40 kDa), nuclear factor kappa-B (NF-κB) inhibitor alpha (IKB-α; 1:10,000, ab32518, 35 kDa), p-IKB-α (1:1000, ab133462, 40 kDa), nucleotide-binding oligomerization domain (NOD)-like receptor thermal protein domain associated protein 3 (NLRP3, 1:1000, ab263899, 35 kDa), apoptosis-associated speck-like protein (ASC, ab180799, 22 kDa), C-caspase 1 (1:1000, ab286125, 17 kDa), N-terminal fragment of gasdermin D (GSDMD-N; 1:1000, ab219800, 53 kDa), interleukin-1β (IL-1β; 1:1000, ab254360, 30 kDa), interleukin-18 (IL-18; 1:1000, ab191860, 22 kDa), loading control GAPDH (1:10,000, ab181602, 36 kDa), and horseradish peroxidase-conjugated Goat anti-Rabbit IgG (1:10,000, 31460, Thermo Fisher, Waltham, MA, USA). Finally, the immunoblots were visualized using an EZ-ECL Chemiluminescent Detection Kit (20-500-120, Biological Industries, Beit Haemek, Israel), and protein bands were analyzed through Image Quant LAS 4000 system (GE Healthcare, Uppsala, Sweden).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). The data obtained from three independent experiments were expressed as the mean ± standard deviation. Moreover, multi-group comparisons were performed using one-way analysis of variance, followed by post hoc test (Tukey). Statistical significance was defined as a p-value < 0.05.

Results

Tadalafil Enhanced Liver Function and Alleviated Liver Injury in Rats after Hepatic I/R

To explore the attenuation of hepatic I/R injury through PDE5 inhibition, the levels of liver function indicators (AST and ALT) were examined in hepatic I/R-induced rats with or without pre-administration of tadalafil (a PDE5i). Serum AST and ALT levels were increased in the I/R group relative to the Sham group (Fig. 1A,B, $p < 0.001$), indicating the impairment of liver function. However, a similar trend was observed in the I/R + PDE5i group compared to the PDE5i group (Fig. 1A,B, $p < 0.001$). Pre-administration of tadalafil (5 mg/kg/day) was found to reduce serum AST and ALT levels in model rats (Fig. 1A,B, $p < 0.001$). Furthermore, hepatic I/R induced tissue damage and cell apoptosis in the rat liver, as determined by histological analysis and TUNEL assay (Fig. 1C–E). However, tadalafil treatment alleviated tissue damage and reduced cell apoptosis in the liver of model rats (Fig. 1C–E).

Tadalafil Reduced NO Content in the Liver of Rats after Hepatic I/R

We observed that hepatic I/R elevated NO levels in rat liver (Fig. 1F, $p < 0.001$). However, the level of NO was reduced in model rats pre-treated with tadalafil (Fig. 1F, $p < 0.001$).

Impact of Tadalafil on LSECs Function and NO Secretion by Platelets in Hepatic I/R-Induced Rats

The expression levels of VEGFR3 and CD34 in the LSEC were assessed using fluorescence-activated cell sorting. We observed that nearly all (99.71%) LSECs were $VEGFR3^{+}CD34^{-}$ (Fig. 2A), implying the high purity of LSECs isolated from the liver. Moreover, our findings demonstrated that model rats showed an increase in platelet-secreted NO levels relative to rats in the sham group (Fig. 2B, $p < 0.001$). However, platelets isolated from the whole blood of hepatic I/R-induced rats pre-administered with tadalafil exhibited an alleviation in NO levels (Fig. 2B, $p < 0.001$).

Tadalafil Facilitated Survival yet Decreased Pyroptotic Cell Death of LSECs in the Presence of Hepatic I/R Rat-Derived Platelets

We investigated the impact of PDE5i on LSEC function during hepatic I/R by regulating platelets-mediated secretion of NO. As shown in Fig. 2C,D, co-culture with hepatic I/R rat-derived platelets reduced viability and enhanced LDH activity of LSECs ($p < 0.001$). In contrast, normal rat platelets did not affect LSEC viability and LDH activity. LSECs showed increased viability and decreased LDH activity in the presence of hepatic I/R rat-derived platelets with pre-treatment of tadalafil (100 nM) (Fig. 2C,D, $p < 0.01$). Furthermore, we observed that platelets derived from hepatic I/R rats increased the PI-positive cells count (Fig. 2E,F, $p < 0.001$), whereas normal rat platelets did not affect their number. Moreover, the promoting effect of hepatic I/R rat-derived platelets on PI-positive cells was reversed by tadalafil pre-treatment (Fig. 2E,F, $p < 0.001$). However, it has been reported that NLRP3-mediated pyroptosis is closely associated with en-

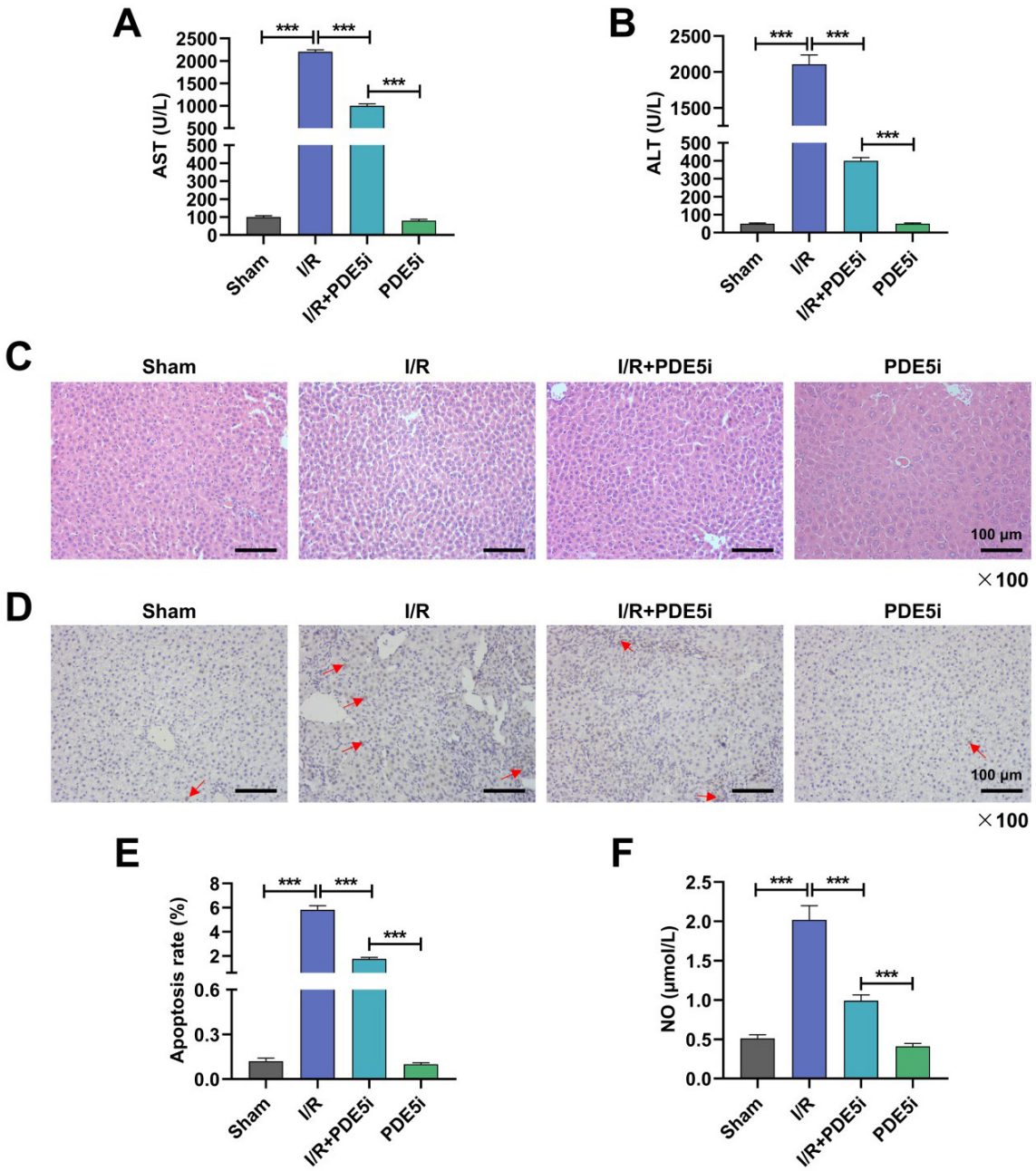

Fig. 1. Effects of tadalafil on liver function and nitric oxide (NO) levels in rats after hepatic ischemia-reperfusion (I/R). (A,B) Serum AST and ALT levels in rats after pre-administration of PDE5i tadalafil (5 mg/kg) or/and hepatic I/R (specific kits). (C) Liver tissues of rats after tadalafil pre-administration of tadalafil or/and hepatic I/R (histological analyses) (magnification: ×100, scale bar = 100 μm). (D,E) Cell apoptosis in liver tissues of rats after pre-administration of tadalafil (5 mg/kg) or/and hepatic I/R (TUNEL) (arrow: apoptotic cells) (magnification: ×100, scale bar = 100 μm). (F) The levels of NO in the liver tissues of rats pre-administered with tadalafil (5 mg/kg) or/and hepatic I/R (specific kit). Data are expressed as mean ± standard deviation. $^{***}p < 0.001$. n = 3. Abbreviation: AST, aspartate aminotransferase; ALT, alanine aminotransferase; PDE5i, phosphodiesterase 5 inhibitor; I/R, ischemia-reperfusion; TUNEL, TdT-mediated dUTP nick end labeling assay.

dothelial dysfunction in I/R injury [30]. In LSECs, the levels of pyroptosis-related proteins, including NLRP3, ASC, C-caspase 1, GSDMD-N, IL-1β, and IL-18, were significantly increased after co-culture with platelets obtained from hepatic I/R rat (Fig. 3A–G, $p < 0.001$), whereas remained unaffected after co-culture with platelets derived from normal rat. After hepatic I/R rat-derived platelets treated with pre-treatment of tadalafil (100 nM) and co-culture with LSECs, the expression levels of NLRP3, ASC, C-caspase 1, GSDMD-N, IL-1β and IL-18 were decreased in LSECs (Fig. 3A–G, $p < 0.001$).

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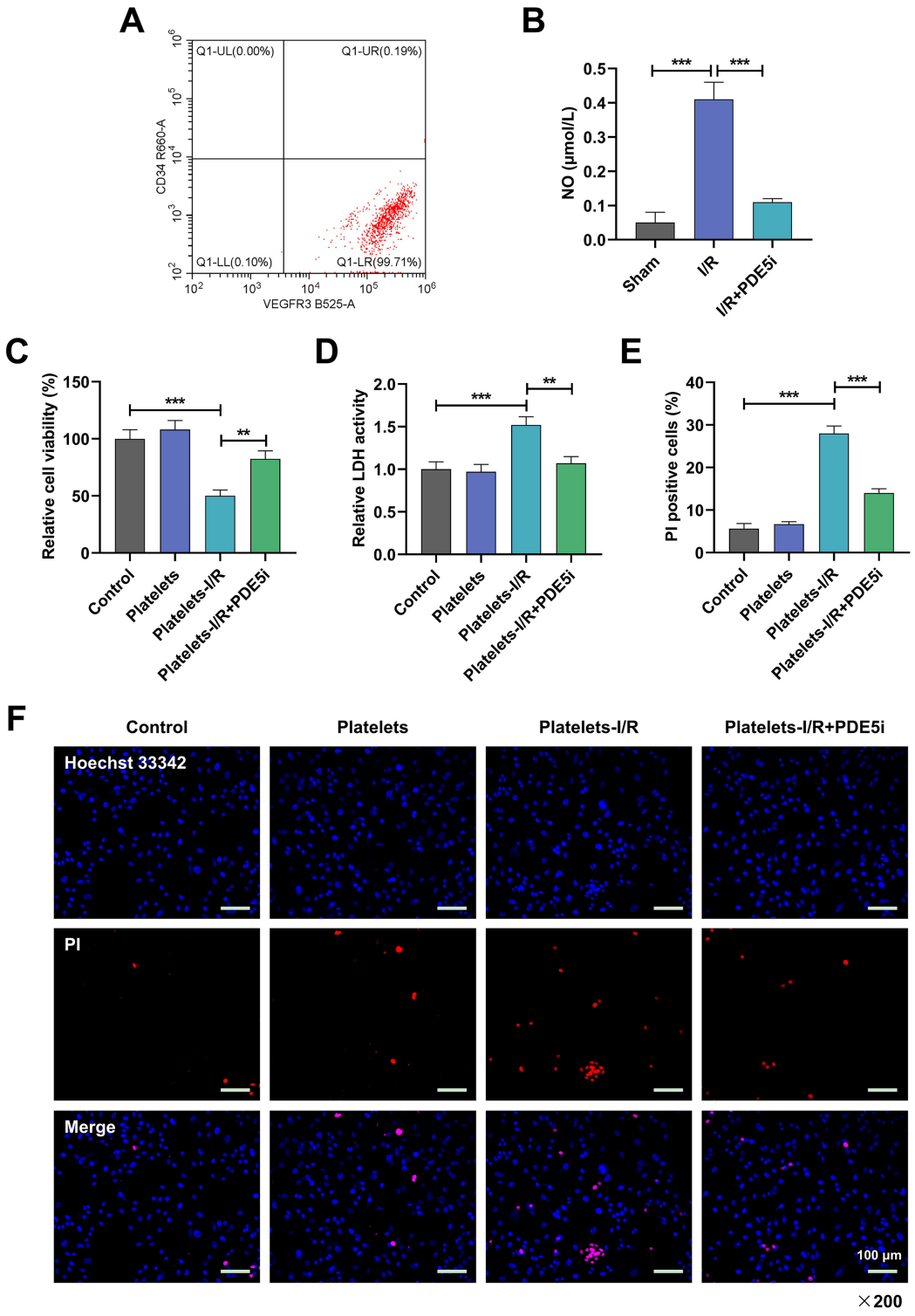

Fig. 2. Impact of tadalafil on LSECs function and nitric oxide (NO) secretion by platelets in hepatic ischemia-reperfusion (I/R)-induced rats. (A) VEGFR3 and CD34 expressions in the LSEC were determined using fluorescence-activated cell sorting. (B) Platelets were isolated from the whole blood of hepatic I/R-induced rats with or without pre-administration of tadalafil (5 mg/kg), and the level of platelet-secreted NO in the medium was examined (specific kit). (C) Viability of LSECs after hepatic I/R rat-derived platelets were pre-treated with or without tadalafil (100 nM) and co-cultured with LSECs (methylthiazolyldiphenyl-tetrazolium bromide assay). (D) LDH activity of LSECs after hepatic I/R rat-derived platelets were pre-treated with or without tadalafil (100 nM) and co-cultured with LSECs (LDH activity assay kit). (E,F) The percentage of PI-positive cells was determined using Hoechst 33342 (blue)/PI (red) double staining (magnification: ×200, scale bar = 100 μm). Data are expressed as the mean ± standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3. Abbreviation: LDH, lactate dehydrogenase; PI, propidium iodide; LSECs, liver sinusoid endothelial cells.

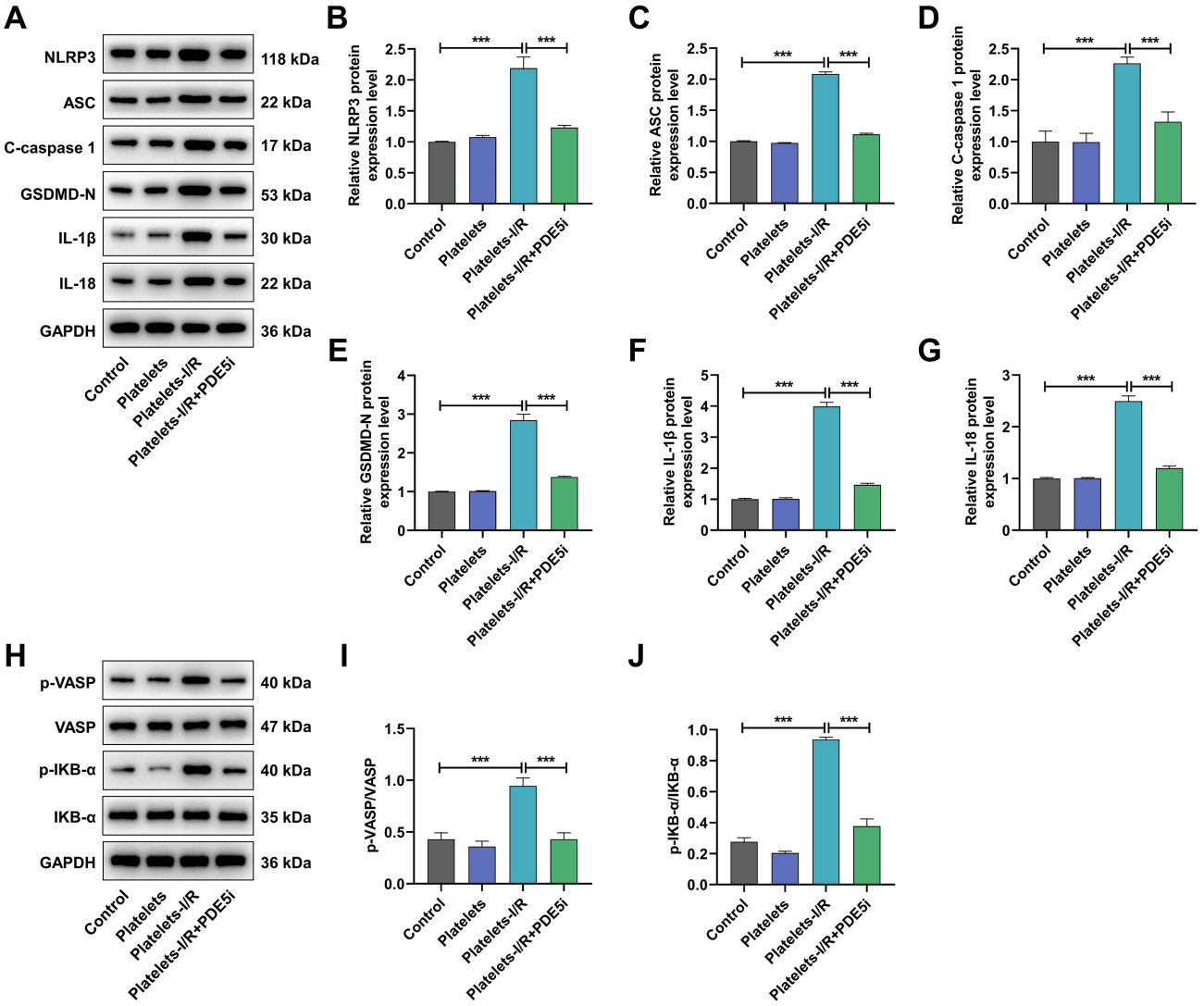

Fig. 3. The attenuating impact of tadalafil on LSEC pyroptosis through regulation of platelet-secreted nitric oxide (NO) in hepatic ischemia-reperfusion (I/R)-induced rats. (A–J) The expression levels of NLRP3, ASC, C-caspase 1×, GSDMD-N, IL-1β, IL-18, p-VASP/VASP, and p-IKB-α/IKB-α in LSECs after co-culture with hepatic I/R rat-derived platelets with or without pre-treatment of tadalafil (100 nM) (Western blot, GAPDH as a loading control). Data are expressed as the mean ± standard deviation. $^{***}p < 0.001$. n = 3. Abbreviation: NLRP3, nucleotide-binding oligomerization domain (NOD)-like receptor thermal protein domain associated protein 3; ASC, apoptosis-associated speck-like protein; GSDMD-N, N-terminal fragment of gasdermin D; IL-1β, interleukin-1β; IL-18, interleukin-18; p-VASP, phosphorylated vasodilator-stimulated phosphoprotein; p-IKB-α, phosphorylated nuclear factor kappa-B inhibitor alpha.

Tadalafil Suppressed VASP/NF-κB Pathway in LSECs Co-Cultured with Platelets from Hepatic I/R Rats

Our finding revealed that p-VASP/VASP and p-IKB-α/IKB-α levels were upregulated in LSECs by hepatic I/R rat-derived platelets (Fig. 3H–J, $p < 0.001$), whereas remained unaffected by normal rat platelets. Moreover, the promoting effects of platelets derived from hepatic I/R rat on phosphorylation levels of VASP and IKB-α were counteracted by tadalafil treatment (Fig. 3H–J, $p < 0.001$).

Discussion

In this study, we observed that tadalafil enhanced liver function and attenuated liver injury in rats after hepatic I/R, possibly through the interaction between platelets and liver sinusoid endothelium. Furthermore, evidence from *in vitro* experimental findings indicated that tadalafil alleviated LSEC dysfunction by reducing platelet-secreted NO and suppressing LSEC pyroptosis.

Histopathologically, liver I/R injuries are characterized by oxidative stress, inflammation, and apoptosis [31]. Additionally, serum ALT and AST are widely used as bio-

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chemical indicators of liver function [32]. We found that the levels of ALT, AST and NO were increased, tissue damage and cell apoptosis were induced in hepatic I/R. However, these results were reduced by tadalafil. These findings are consistent with those reported by Bektas *et al*. [22]. However, how tadalafil alleviates liver I/R injury needs further investigation.

During the development of hepatic I/R injury, neutrophils have been reported to function as intrinsic immune effectors that induce platelet activation, leading to immunothrombosis and exacerbating local and distant organ damage after liver inflammatory stress [13]. Tadalafil, as a PDE5i, can reduce platelet activity and promote vasodilation by activating cGMP [33]. Therefore, it is reasonable to surmise that tadalafil may against hepatic I/R by modulating platelet functions.

NO is a soluble gas synthesized by three NO synthase (NOS) enzymes: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) [34]. In cardiovascular diseases, NO is widely recognized as a strong endothelium-derived relaxing factor that protects vascular endothelium [35]. However, the role of NO in hepatic I/R injury is complicated and inconclusive, as it depends on its production sources and downstream pathways [36]. Available evidence suggests that platelets can produce NO independently [37]. However, the role of platelet-derived NO in hepatic I/R injury is poorly understood. A previous study indicated increased levels of eNOS and iNOS in liver tissues of hepatic I/R rats, which can produce excessive NO, leading to the production of peroxynitrite through nitrosative stress, thereby inducing cell apoptosis and tissue damage [38].

Additionally, it has been shown that the PDE5i sildenafil reduced iNOS expression in inflammation-induced colorectal cancer models, and tadalafil has been indicated to inhibit iNOS expression in hepatic I/R-induced rat liver tissues [22,39]. Intriguingly, we found upregulated NO in both liver tissues of model rats and the medium obtained from platelets of these rats, whereas the effect was reversed by tadalafil. These findings indicate that tadalafil alleviates hepatic I/R injury by reducing platelet-secreted NO.

Additionally, platelet adhesion in the sinusoid endothelium, which limits sinusoidal blood flow, is a crucial mechanism underlying hepatic I/R injury and is associated with disease severity [40,41]. Recently, studies have indicated that tadalafil mitigates endothelial dysfunction [42–44]. Furthermore, NLRP3-mediated pyroptosis has been frequently implicated in organ I/R injury, exacerbating inflammatory response by releasing inflammatory factors such as IL-1β and IL-18 [45]. Accumulation of the NLRP3 inflammasome can cleave caspase 1, triggering the program of pyroptosis by cleaving GSDMD [46]. In this study, increased expressions of NLRP3, ASC, C-caspase 1, GSDMD-N, IL-1β, and IL-18 were found in LSECs after co-culture with platelets obtained from hepatic I/R rats, suggesting that platelets facilitate LSEC pyroptosis during the hepatic I/R injury. Nevertheless, the promoting effect of hepatic I/R rat-derived platelets on the above-mentioned pyroptosis-related proteins was counteracted by tadalafil, indicating that tadalafil opposes hepatic I/R by protecting the sinusoid endothelium.

Moreover, in fatty liver disease, it has been demonstrated that the activation of NLRP3 inflammasome in hepatocytes and Kupffer cells is regulated by VASP phosphorylation and NF-κB inhibition [47]. It has been shown that VASP-deficient mice exhibited enhanced activity of NF-κB in Kupffer cells [48]. NF-κB, a nuclear transcription factor, is crucial in regulating cell pyroptosis by activating the NLRP3 inflammasome [49]. Notably, we found that VASP phosphorylation and IKB-α phosphorylation were increased in LSECs after co-culture with platelets derived from hepatic I/R rat, which was prevented by tadalafil. These observations indicate that NF-κB is involved in the protective effect of tadalafil on LSECs against hepatic I/R. However, the regulatory mechanism of VASP/NF-κB/NLRP33 on LSECs against hepatic I/R injury needs further investigation. Furthermore, regarding cigarette smoke-induced vascular injury, upregulation of iNOS has been unveiled to promote aortic endothelial pyroptosis by activating NLRP3 inflammasome via soluble guanylate cyclase (sGC)/cGMP/PKG/TACE/TNF-α axis [50]. Therefore, we proposed a hypothesis that tadalafil might suppress the platelets-derived secretion of NO through the iNOS-mediated pathway, thereby suppressing LSEC pyroptosis during hepatic I/R injury. However, this speculation needs further investigation.

There are some limitations in our study. Firstly, we did not investigate varying concentrations of tadalafil to determine the optimal dosage. Secondly, we did not thoroughly analyze the NO/VASP/NF-κB/NLRP3 correlation between platelets and LSEC. Thirdly, we did not design randomized controlled trials to validate our findings.

Conclusion

This study provides new evidence that the PDE5i tadalafil attenuates LSEC dysfunction in rat hepatic I/R injury. Furthermore, it reveals that tadalafil protects LSECs against hepatic I/R injury by inhibiting platelet-secreted NO and suppressing LSEC pyroptosis. These findings offer novel insights into the mechanism of tadalafil in hepatic I/R injury and support its use as a potential therapeutic option for treating hepatic I/R injury after liver surgery.

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding authors on reasonable request.

Author Contributions

Substantial contributions to conception and design: SDL. Data acquisition, data analysis and interpretation: JL, TC, BFW, BCC. Drafting the article or critically revising it for important intellectual content: SDL. Contributions to editorial changes in the manuscript: all authors. Final approval of the version to be published: all authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: all authors.

Ethics Approval and Consent to Participate

All animal experiments in this study had been approved by the Ethics Committee of Taizhou Enze Medical Center (group) for Experimental Animals Welfare (No. TEY-2021159), and experimental procedures were conducted based on the guidelines of the China Council on Animal Care and Use.

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Conflict of Interest

The authors declare no conflict of interest.

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