Article

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Hyperbaric Lidocaine Aggravates Diabetic Neuropathic Pain by Targeting p38 MAPK/ERK and PINK1/Parkin-Mediated Mitophagy Signaling Pathway

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Background: Diabetic neuropathic pain (DNP) is a complication of diabetes mellitus (DM). Hyperbaric lidocaine (HL), a local anesthetics drug, has neurotoxicity. The present study aims to study the effect and molecular mechanisms of HL on spinal nerve injury in DNP.

Methods: The DNP rat model was established through a high-fat-glucose diet in combination with Streptozotocin (STZ) administration. SB203580 and PD98059 were utilized to inhibit p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase (ERK). The mechanical paw withdrawal threshold (PWT) and the thermal paw withdrawal latency (PWL) were tested to evaluate rats' mechanical allodynia and thermal hyperalgesia. Hematoxylin-eosin (H&E) and terminal deoxynucleotidyltransferase-mediated dUTP nick-end Labeling (TUNEL) staining were performed to evaluate the pathological changes and neuron apoptosis in spinal cord tissues of L4-5. Western blotting analysis and reverse transcription-polymerase chain reaction (RT-qPCR) assay were used to measure the levels of proteins and mRNAs, respectively.

Results: PWT and PWL were decreased in DNP rats with serious spinal nerve injury. HL administration downregulated the PWT and PWL and aggravated spinal nerve injury in DNP rats, but isobaric lidocaine had no effects on these changes. Meanwhile, p38 MAPK/ERK signaling and PTEN-induced kinase 1 (PINK1)-mediated mitophagy were activated in DNP, which was enhanced by HL but not isobaric lidocaine. Blocking p38 MAPK/ERK signaling could effectively attenuate HL-induced spinal nerve injury and inhibit mitophagy.

Conclusion: In summary, HL can aggravate spinal cord tissue damage in DNP rats by inducing PINK1-mediated mitophagy via activating p38 MAPK/ERK signaling. Our data provide a novel insight that supports the potential role of p38 MAPK/ERK signaling in acting as a therapeutic target for HL-induced neurotoxicity.

Keywords: diabetic neuropathic pain; lidocaine; hyperbaric factor; p38 MAPK/ERK signaling; mitophagy

Introduction

Long-term diabetes mellitus (DM) will result in multiple organs bearing an undue burden, thus inducing the occurrence and development of multiple disorders, including diabetic retinopathy, diabetic nephropathy, and cardiocerebral vascular disease [1,2]. Moreover, neuropathy is also one of the common complications of DM. There are approximately 425 million patients with DM globally, and an estimated 6% to 51% have also suffered from neuropathy [1,3]. Neuropathic pain is a chronic pain symptom caused by nerve injury and characterized by hyperalgesia, spontaneous pain, and tactile allodynia [4]. Diabetic neuropathic pain (DNP) is a complication of DM and results from diabetic neuropathy. It was reported that nearly 30% of DM patients suffer from DNP, leading to a severe decline in the quality of life of the patients [5].

It is inevitable for patients with DM to undergo various types of surgery under local anesthesia. Previous studies revealed that patients with DNP have a higher sensitivity to neurotoxicity caused by peripheral nerve blocking than healthy controls [6,7]. Yu *et al*. [8] reported that administration of 0.5% ropivacaine did not influence healthy rats but could slow the motor nerve conduction velocity and extend the time of sensory and motor nerve blocking, leading to subsequent sciatic nerve injury in DM model rats. Notably, the above effects of ropivacaine on the DM rats were enhanced by dexmedetomidine. Therefore, the inappropriate usage of anesthetic drugs during the operation might result in nerve injury in patients with DM.

Lidocaine has been utilized as a local anesthetic drug during operation all the time. As an amino amide anesthetic, lidocaine could block the voltage-dependent sodium channels and thus interrupt the impulse initiation and transmission in axons [9]. In addition, lidocaine has also been

utilized for analgesia in clinical, including visceral pain, dysrhythmias, hyperalgesia, centrally mediated pain, postherpetic neuralgia, postoperative pain, and most importantly, neuropathic pain [9]. However, although lidocaine is an appealing local anesthetic drug, a high risk of transient neurological symptoms limits its application. Clinically, the standard concentration of lidocaine is 1%–2%. During spinal anesthesia, local anesthetic drugs in combination with glucose are often formulated into hyperbaric local anesthetic drugs for operation [10,11]. Our previous study indicated that the administration of hyperbaric factor or isobaric factor in DNP results in a different influence on nerve injury in spinal cord tissues. Hyperbaric lidocaine (HL) increased the loss of neurons in the spinal cord tissues of DNP rats, but isobaric lidocaine (IL) did not affect the survival of neurons. We also proved that HL may induce nerve injury by activating the p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway [12].

Accumulating evidence has demonstrated that p38 MAPK/extracellular signal-regulated kinase (ERK) signaling plays a crucial role in neuropathic pain. Blocking the p38 MAPK/ERK signaling pathway alleviates neuropathic pain [13]. Li *et al*. [14] reported that curcumin could effectively improve the mechanical and thermal pain hypersensitivity and sciatic nerve fiber injury in diabetes peripheral neuropathy, and the activity of p38 MAPK/ERK signaling and PI3K/AKT signaling were inhibited in that process. In the current study, we further probed the downstream target of p38 MAPK/ERK signaling and excavated the regulatory mechanism of lidocaine in inducing spinal nerve injury in DNP model rats. Our research may propose a novel idea for improving spinal nerve injury in DNP rats.

Materials and Methods

Reagents

The high-fat, high-glucose, and normal diets were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Streptozotocin (STZ; diluted in 0.01 M sodium citrate, pH4.5) was from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany), and lidocaine was obtained from Xuzhou Laien Pharmaceutical Co., Ltd. (Xuzhou, China). P38 MAPK inhibitor SB203580 and ERK inhibitor PD98059 were purchased from MedChemExpress (Shanghai, China). Hematoxylin-eosin (H&E) staining kit (G1120), terminal deoxynucleotidyltransferase-mediated dUTP nick-end Labeling (TUNEL) staining kit (T2196), RIPA lysis buffer (R0010), and BCA kit (PC0020) were obtained from Solarbio (Beijing, China). The primary antibodies, including anti-p38 (ab308333), anti-ERK (ab32537), anti-phosphorylated p38 (ab4822), anti-phosphorylated ERK (ab201015), anti-LC3-II/LC3-I (ab48394), anti-p62/sequestosome 1 (SQSTM1) (ab109012), anti-PTEN-induced kinase 1 (PINK1) (ab216144), anti-Parkin (ab77924), anti-GAPDH (ab8245), goat anti-Mouse Immunoglobulin G (IgG) H&L (Horseradish Peroxidase (HRP)) (ab205719) and goat anti-Rabbit IgG H&L (HRP) (ab205718), were obtained from Abcam (Cambridge, UK).

Laboratory Animals

A total of 48 healthy Sprague-Dawley rats (gender, male; weight, 220–300 g; and age, 2–3 months) were utilized in the current study. All rats were provided by the Nanchang University of Traditional Chinese Medicine (Nanchang, China) and were fed in a stable environment with 25 ± 5 °C temperature and 60 ± 5% relative humidity. The lighting was set to be bright for 12 hours and dark for 12 hours. All animal experiments were carried out in accordance with the rules of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital, Jiangxi Medical College, Nanchang University (No. CDYFY-IACUC-202206QR002).

DNP Rat Model

Adaptive feeding for one week, the rats were utilized to establish the DNP rat model through a high-fat-glucose diet (21% fat, 0.15% cholesterol, 20% glucose) in combination with STZ administration. The rats used to establish the DNP model were accepted with the high-fat and high-glucose diet for four weeks and then were fasted with free water overnight. Afterward, the rats were accepted with a single intraperitoneal injection of 50 mg/kg of STZ dissolved in 0.1 N citrate-buffered saline (pH 4.5). Three days after STZ treatment, the rats with a fasting blood glucose level higher than 16.65 mmol/L were recognized as DM rats. The DNP rat model was further ensured through the pain behavioral tests [12].

Experimental Groups and Drug Administration

All rats were randomly divided into 6 groups (n = 8 for each group): (i) Health group: rats were fed with normal diet and intraperitoneally injected with citrate-buffered saline; (ii) DNP group; (iii) DNP + HL group: at 7 weeks after STZ injection, rats were injected intrathecally with 10 µL of 2% lidocaine with 10% glucose for three consecutive days, once a day; (iv) DNP + IL group: at 7 weeks after STZ administration, rats were injected intrathecally with 10 µL of 2% lidocaine for three consecutive days, once a day; (v) DNP + HL + SB203580 group: rats were given with the hyperbaric solution of lidocaine and glucose, and injected intrathecally with 10 µg/10 µL of SB203580 for five days; (vi) DNP + IL + PD98059 group: rats were given with the hyperbaric solution of lidocaine and glucose, and injected intrathecally with 10 µg/10 µL of PD98059 for five days. Pain behavioral studies were performed five days after lidocaine administration, and spinal cord tissues were collected for the following studies [12].

Pain Behavioral Studies

(i) Mechanical pain assessment: Rats were allowed to adapt to the test environment for 30 min before testing, and then the rat's hind paw was stimulated by von Frey filament with a linearly increasing pressure; the measurement interval was 30 s. The maximum intensities of the von Frey filament were set as 15 g. The 50th percentile paw response threshold across the rats was regarded as the paw withdrawal threshold (PWT). All testing was performed blindly. (ii) Thermal hyperalgesia tests: The thermal paw withdrawal latency (PWL) of rats was measured in a square and transparent glass box to verify the presence of thermal hyperalgesia [15]. Rats were allowed to adapt to the test environment for 30 min before testing, and then the left hind foot of the rats was stimulated with a radiant thermal stimulus through the glass floor. When stimulation was given, the timer was on. The elapsed time from the start of the heat stimulus to the rats lifting their feet was recorded as PWL. If the rat did not lift their feet, the PWL of the rat was recorded as 30 seconds. Throughout all experiments, the intensity of the heat stimulus was held at a constant level, and all testing was performed blindly.

H&E Staining and TUNEL Staining

At 5 days after the last lidocaine treatment, the rats were euthanized with 150 mg/kg sodium pentobarbital. and then the spinal cord from L4 to L5 was removed, fixed with 4% paraformaldehyde, and embedded in paraffin. Afterward, paraffin-embedded tissues were cut into slices 4-μm thick and stained with H&E and TUNEL according to the corresponding kits. Five random fields of the stained slices were photographed under a microscope. Regarding H&E, the pathological changes of spinal cord tissues were evaluated according to previous studies [16,17]. As referring to TUNEL, the slices were incubated with 4′,6-diamidino-2-phenylindole (DAPI, Beyotime, Shanghai, China) for 5 min and then observed using a fluorescence microscopy (M205 FA, Leica, Wetzlar, Germany). The TUNEL-positive cells were counted using the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). Subsequently, the apoptotic rate of neurons was calculated as (number of TUNEL-positive cells/total number of nuclei) × 100%.

Western Blotting Analysis

The tissues were ultrasonically homogenized using radioimmunoprecipitation assay (RIPA) lysis buffer at a low temperature and centrifuged at 1400 g for 15 min at 4 ℃ to collect supernatants. Subsequently, the protein concentrations in supernatants were measured using a bicinchoninic acid (BCA) kit, and 25 μg equal quality of total proteins for each group was separated by 12% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Next, after total proteins were transferred onto the nitrocellulose transfer membrane, the membrane was blocked with 5% non-fat milk for one hour at room temperature and was then maintained with primary antibodies like anti-p38, anti-ERK, anti-phosphorylated p38, anti-phosphorylated ERK, anti-LC3-II/LC3-I, anti-p62/SQSTM1, anti-PINK1 or anti-Parkin at 4 ℃ overnight. All primary antibodies were diluted as 1:1000 for usage. GAPDH served as an internal reference. Subsequently, the membrane was incubated with the HRP-conjugated anti-mouse or rabbit IgG antibody for one hour at room temperature. Secondary antibodies were diluted to 1:2000. Finally, protein bands were determined using an ECL kit and quantified using Image-Pro Plus 6.0 software.

RT-qPCR Assay

Similarly, rats' lumbar spinal cord tissues (L4-5) were collected for subsequent detection. The levels of p62/*SQSTM1*, BCL2 interacting protein 3 (*BNIP3*), *PINK1*, and Parkin mRNAs were analyzed using the RT-qPCR method [18]. The primers used in current study as follows: p62/SQSTM1: 5′-GGACCCACAGGGCTGAAGGAAG-3′ (sense) and 5′-CTGGTGAGCCAGCCGCCTTCAT-3′ (antisense); BNIP3: 5′-CGTGTGTGTGGAAAGCTGTG-3′ (sense) and 5′-GTGCA GGCCACAAAAGACAG-3′ (antisense); PINK1: 5′-CATGGCTTTGGATGGAGAGT-3′ (sense) and 5′-TGGGAGTTTGCTCTTCAAGG-3′ (antisense); Parkin: 5′-CTTGACTCGAGTGGACCTCA-3′ (sense) and 5′-GGTTTAGCTGCTGGACCTCT-3′ (antisense); GAPDH: 5′-TCCTGCACCACCAACTGCTTAG-3′ (sense) and 5′-GATGACCTTGCCCACAGCCTTG-3′ (antisense). GAPDH was used as an internal control. The levels of p62/*SQSTM1*, *BNIP3*, *PINK1*, and Parkin mRNAs were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All experimental studies were repeated three times. All results were analyzed using SPSS 19.0 statistical software (IBM SPSS, Armonk, NJ, USA) and presented as mean ± SD. Shapiro-Wilk test was used to determine whether the data fit the normal distribution, and the results indicated that the data of each group met the normal distribution characteristics. Therefore, one-way analysis of variance (ANOVA) was used to analyze the differences between multiple groups. If the results of one-way ANOVA were significant, the SNK-q test was used to compare the differences between groups. $p < 0.05$ was recognized as statistically significant.

Results

Validation of Diabetic Model Rats

As shown in **Supplementary Fig. 1**, the blood glucose of rats increased sharply after intraperitoneal injection of STZ combined with high-fat food and remained high for 7 weeks afterward. Blood glucose levels remained normal in the control group (**Supplementary Fig. 1A**). The weight of diabetic rats decreased gradually along the disease time

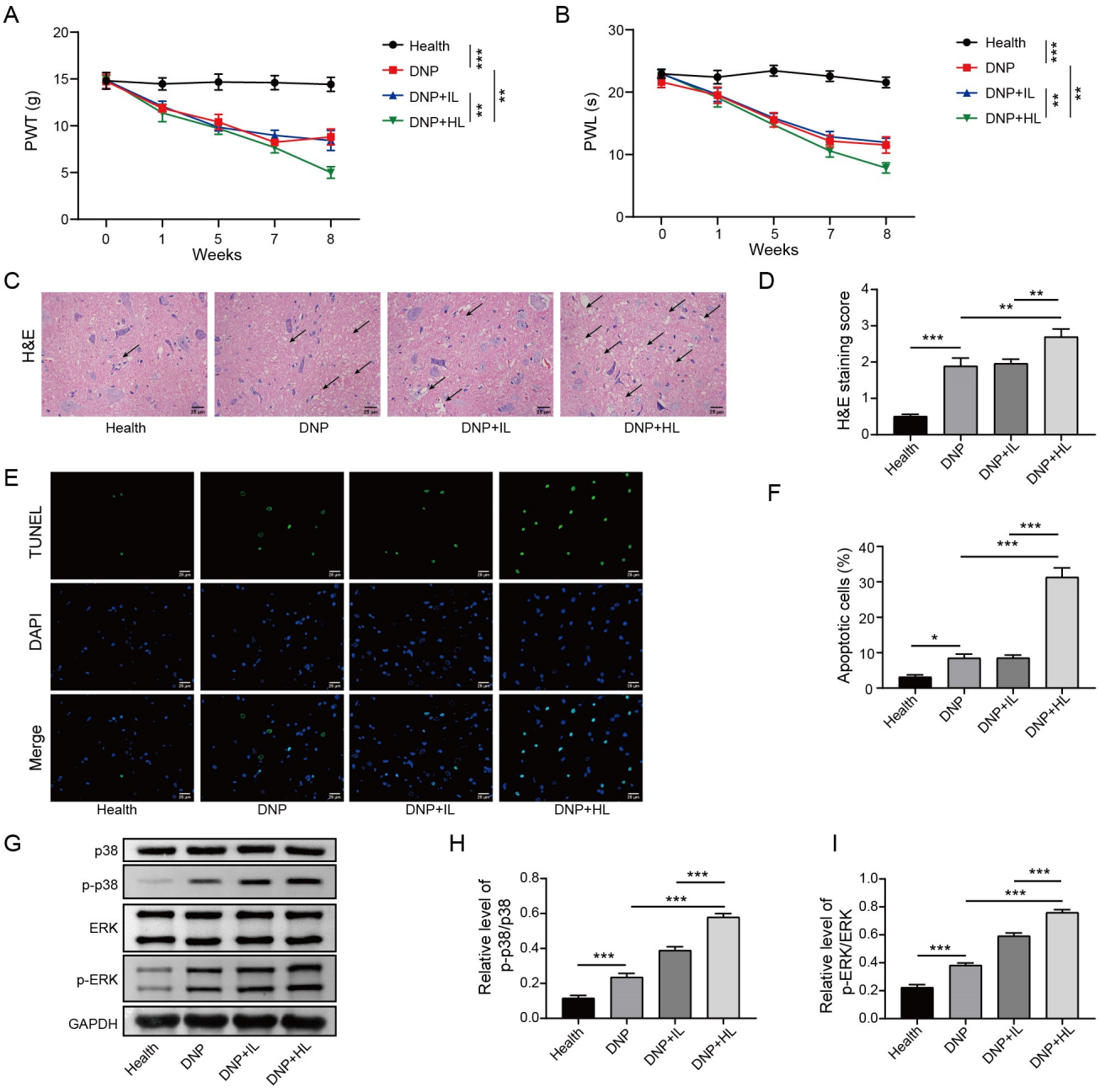

Fig. 1. Hyperbaric lidocaine aggravated spinal nerve injury in diabetic neuropathic pain (DNP) rats. Before Streptozotocin (STZ) injection, at 1, 5, and 7 weeks after Streptozotocin (STZ) injection, and at 5 days after Hyperbaric lidocaine (HL) injection, the paw withdrawal threshold (PWT) (A) and paw withdrawal latency (PWL) (B) were measured to evaluate the mechanical and thermal pain hypersensitivity, respectively (n = 6). At 5 days after HL or IL administration, the spinal cord tissues from L4 to L5 of rats were collected for Hematoxylin-eosin (H&E) staining (C,D) and terminal deoxynucleotidyltransferase-mediated dUTP nick-end Labeling (TUNEL) staining (E,F) to examine the pathological changes and neuron apoptosis in the tissues, respectively (n = 3). Moreover, the protein levels of p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase (ERK) and their phosphorylation were determined utilizing a western blotting assay (G–I) (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale = 25 µm.

course. The weight of rats in the control group increases over time. This caused a significant weight difference between the two groups (**Supplementary Fig. 1B**). Thus, we infered that STZ treatment effectively mimicked DM in rats and that all rats were modeled successfully.

Hyperbaric Lidocaine Deteriorated the Pathological Damages in DNP Rats

The behavioral testing results demonstrated that the values of PWT and PWL were no different among DNP, DNP + IL, and NDP + HL groups but lower than that in

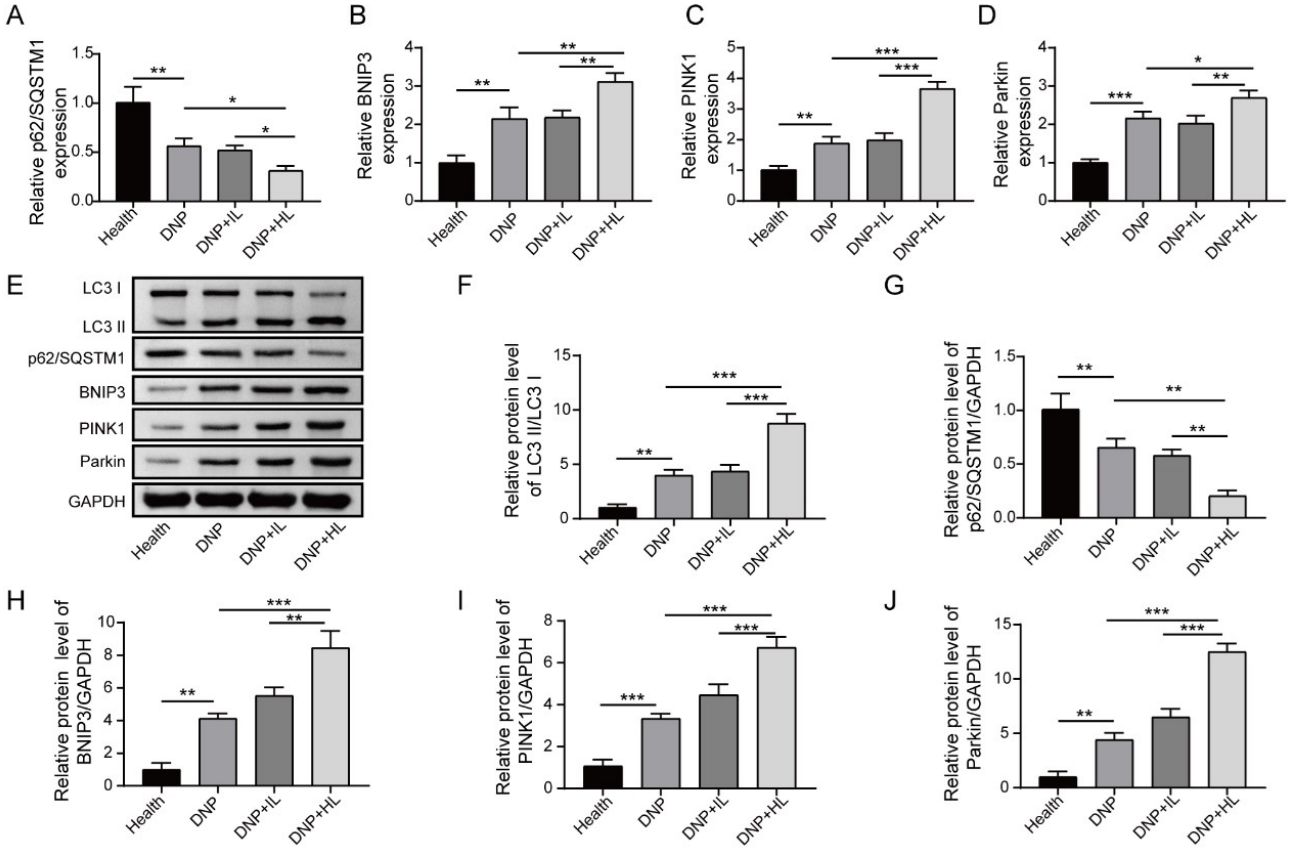

Fig. 2. Hyperbaric lidocaine enhanced mitophagy in DNP rats. At 5 days after HL or IL administration, the spinal cord tissues from L4 to L5 were collected for the examination of the levels of p62/*SQSTM1* mRNA (A), BCL2 interacting protein 3 (*BNIP3*) (B), PTEN-induced kinase 1 (*PINK1*) mRNA (C) and Parkin mRNA using RT-qPCR (D) (n = 6), and the protein levels of LC3-II/LC3-I, p62/SQSTM1, BNIP3, PINK1, and Parkin (E–J) using western blotting assay (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

the Health group before IL and HL administration, which revealed the presence of DM-resulted allodynia. 7 weeks after STZ injection, model rats were administered HL or IL. Then, the values of PWT and PWL of the model rats were further decreased after HL administration, but IL did not influence their values (Fig. 1A,B). In the spinal cord tissues of DNP rats, nerve fibers were damaged, neurons were partly lost, and inflammatory cells were infiltrated. IL treatment did not influence the pathological damage in the spinal cord tissues of DNP rats, while HL administration significantly aggravated the damages (Fig. 1C,D). Furthermore, the number of apoptotic cells was higher in the spinal cord tissues of DNP rats than in the Health group. Similar to the results of H&E staining, IL administration did not influence cell apoptosis in DNP rats, while HL administration markedly enhanced cell apoptosis in DNP rats (Fig. 1E,F). Proof by facts, HL administration, enhances the neuropathic pain and pathological damage in DNP rats. A previous study indicated that the p38 MAPKK/ERK signaling pathway is activated in STZ-induced DM rats and may be involved in DM-induced neuropathic pain [19]. Activated p38 MAPK/ERK signaling pathway in DNP rats also was found in our study, and HL administration could further enhance the phosphorylation of p38 MAPK and ERK (Fig. 1G–I). Thus, the p38 MAPK/ERK signaling pathway may be crucial in HL-induced neuropathic pain and pathological damage in DNP rats.

PINK1-Medicated Mitophagy was Enhanced in Hyperbaric Lidocaine-Treated DNP Rats

Mitophagy is reported to be activated in neuropathic pain and positively associated with pain-related behaviors in neuropathic pain model rats [20]. Lei *et al.* [21] indicated that the activation of the p38 MAPK/ERK signaling pathway suppresses TNF-α-induced apoptosis in microglial BV2 cells by activating BINP3-mediated mitophagy. To probe the role of mitophagy in HL-induced neuropathic pain in DNP rats and the association between mitophagy and p38 MAPK/ERK signaling, we determined the expression of mitophagy-related proteins in gene and protein levels. The mRNA level of autophagic substrate p62/*SQSTM1* in DNP rats was lower than that in healthy rats, and HL administration further downregulated the mRNA level (Fig. 2A). Oppositely, the mRNA level of mitophagy receptor *BNIP3* in DNP rats was higher than that in healthy rats, and HL administration enhanced the expression of *BNIP3*

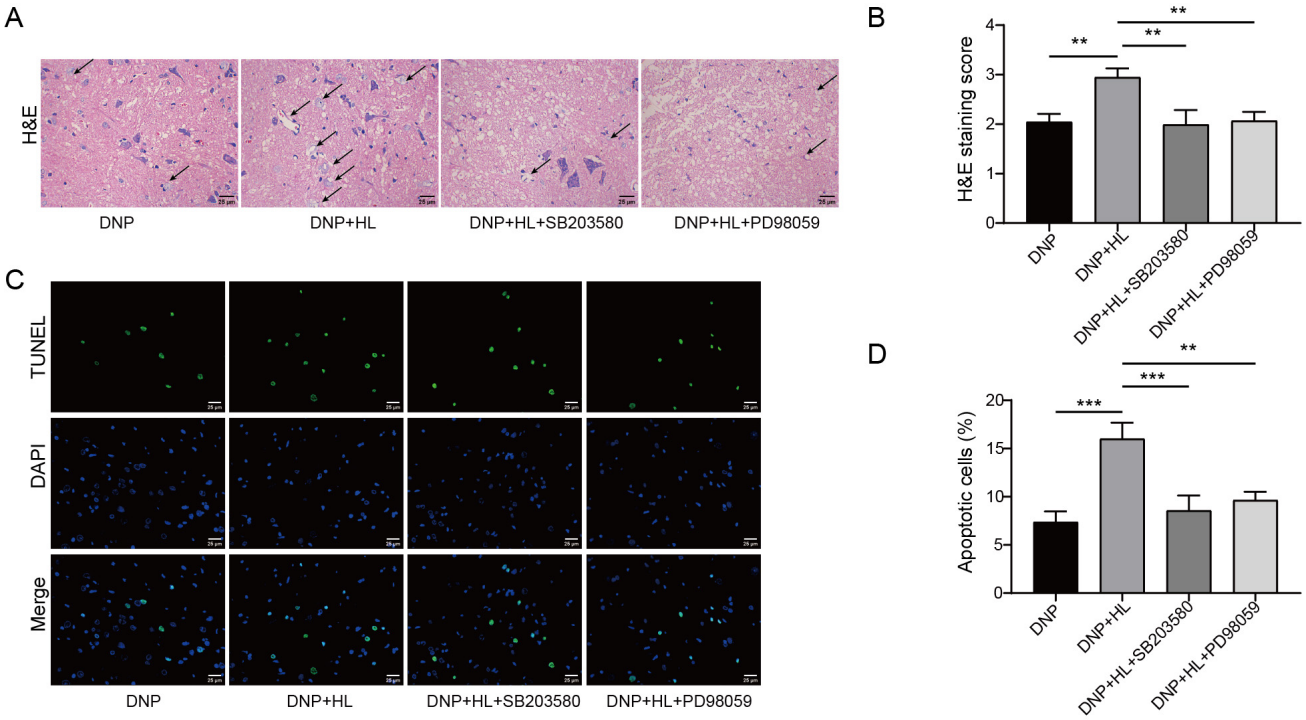

Fig. 3. Inhibition of p38 MAPK/ERK signaling improved hyperbaric lidocaine-induced spinal nerve injury in DNP rats. To probe whether HL aggravates spinal nerve injury in DNP rats, the DNP rats administrated with HL were injected with SB203580 or PD98059. At 5 days after HL administration, spinal cord tissues were collected for H&E staining (A,B) and TUNEL staining (C,D) to measure the pathological changes and neuron apoptosis, respectively (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$. Scale = 25μm. The black arrow for pointing out the representative pathological damage in the spinal card tissues.

mRNA in DNP rats (Fig. 2B). Furthermore, the mRNA levels of mitophagy mediators *PINK1* and Parkin in DNP rats were higher than those in healthy rats, and HL administration significantly upregulated their levels (Fig. 2C,D). The above results of qRT-PCR were also supported by a western blotting assay, which revealed that the protein level of p62/SQSTM1 declined, but the levels of BNIP3, PINK1, and Parkin proteins were upregulated in DNP rats when contrasted to healthy rats. In DNP rats, HL administration markedly suppressed p62/SQSTM1 expression and facilitated BNIP3, PINK1, and Parkin expression (Fig. 2E,G–J). The western blotting assay also demonstrated that the LC3-II/LC3-I level in DNP rats was higher than that in healthy rats, which was strengthened by HL administration (Fig. 2E,F). In summary, HL administration aggravated the activation of mitophagy in DNP rats.

Inhibition of p38 MAPK/ERK Signaling Attenuated Hyperbaric Lidocaine-Induced Damages in Spinal Cord Tissues

To delve into the role and regulatory mechanism of p38 MAPK/ERK signaling in HL-induced neuropathic pain in DNP rats, we blocked this signaling using SB203580 (inhibitor of p38 MAPK) and PD98059 (inhibitor of ERK). The pathological damages, such as inflammatory cell infiltration and neuron loss, in the spinal cord tissues of DNP rats caused by HL administration were attenuated by the blocking of the p38 MAPK/ERK signaling pathway (Fig. 3A,B). The enhancement effect of HL on neuron apoptosis in the spinal cord tissues of DNP rats was effectively recused by p38 MAPK/ERK signaling pathway blocking (Fig. 3C,D). Overall, blocking the p38 MAPK/ERK signaling pathway effectively ameliorated the damage to spinal cord tissues caused by HL administration.

Blocking of the p38 MAPK/ERK Signaling Pathway Suppressed Hyperbaric Lidocaine-Induced Mitophagy

After p38 MAPK/ERK signaling was blocked, the level of p62/*SQSTM1* mRNA (Fig. 4A) was upregulated. In contrast, the levels of *BNIP3* mRNA (Fig. 4B), *PINK1* mRNA (Fig. 4C), and Parkin mRNA (Fig. 4D) were downregulated in the spinal cord tissues of HL-administrated DNP rats. The results of the western blotting assay were consistent with qRT-PCR. As shown in Fig. 4E–J, the level of p62/SQSTM1 protein was lower, while the levels of LC3-II/LC3-I, BNIP3, PINK1, and Parkin were higher in the spinal cord tissues of HL-administrated DNP rats than that in DNP rats without HL or IL treatment. Nevertheless, the expression of the above proteins in the HL-treated DNP

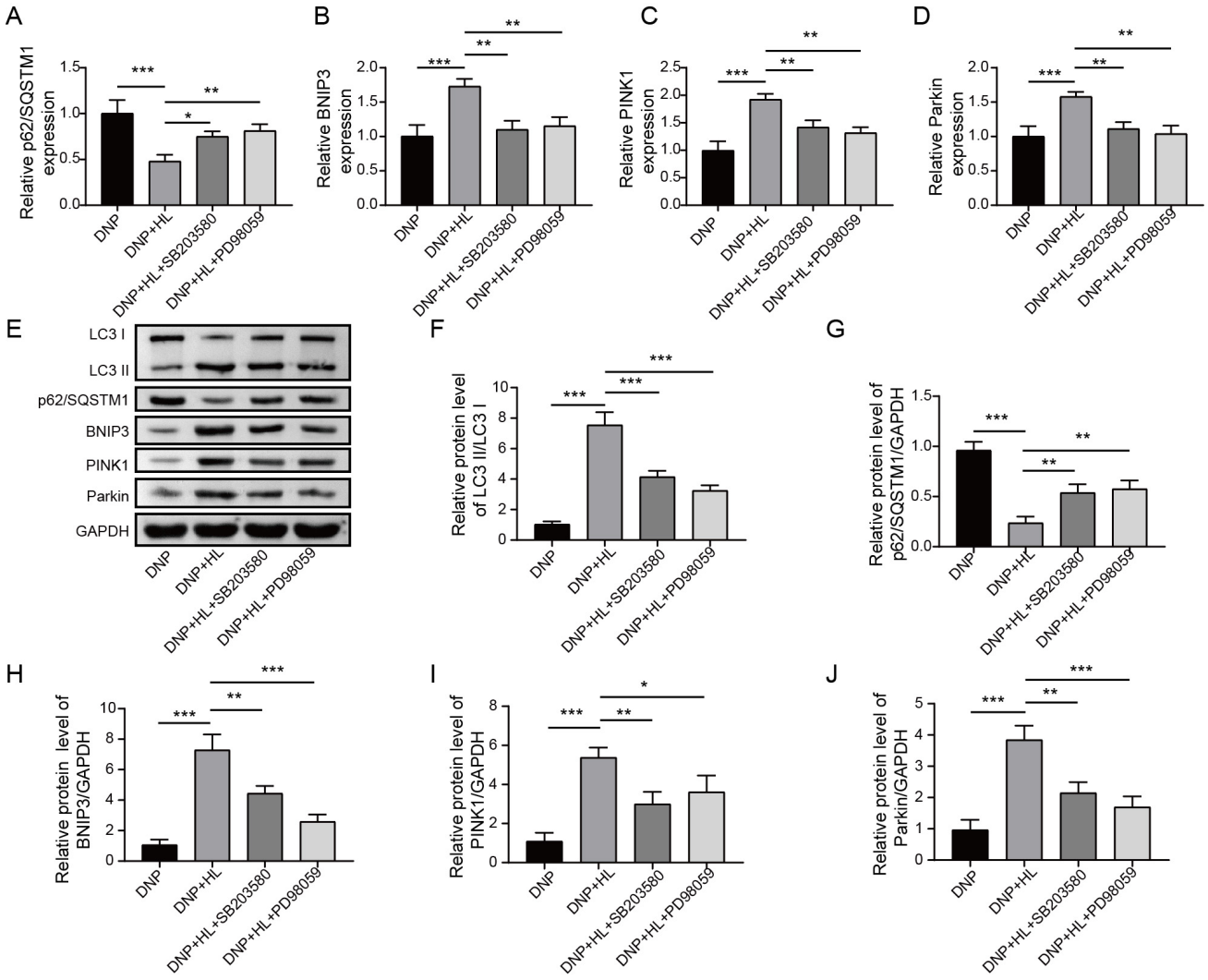

Fig. 4. Inhibition of p38 MAPK/ERK signaling limited hyperbaric lidocaine-induced mitophagy in DNP rats. The DNP rats administrated with HL were injected with SB203580 or PD98059. At 5 days after HL administration, spinal cord tissues were collected. Then, the levels of p62/*SQSTM1* mRNA (A), *BNIP3* mRNA (B), *PINK1* mRNA (C), and Parkin mRNA (D) were measured by RT-qPCR (n = 6). The levels of LC3-II/LC3-I, p62/SQSTM1, BNIP3, PINK1, and Parkin proteins were determined utilizing western blotting assay (E–J) (n = 3). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

rats was partly recused by p38 MAPK/ERK signaling pathway blocking. In summary, in the HL-administrated DNP rats, p38 MAPKK/ERK signaling blocking was accompanied by the inactivation of mitophagy.

Discussion

Spinal anesthesia is a generally anesthetic means during various types of operation, and the selection of anesthetic drugs is essential for it. Clinically, the hyperbaric solution formulated with 2% lidocaine and glucose is commonly used for spinal anesthesia [22,23]. However, some clinical reports and experimental studies showed the potential of lidocaine to induce nerve injury and even transient spinal neurologic disorders [24]. The studies of Takenami *et al.* [25] suggested that the different concentrations ranging from 0% to 20% of lidocaine could result in varying degrees of spinal cord tissue injury and neurotoxicity in rats. To probe whether the hyperbaric factor of lidocaine affects the survival and normal function of spinal nerves during the operation of DNP patients, we established a DNP rat model, which was administrated with HL or IL. To ensure the DNP rat model was set successfully, we tested the pain-related behaviors of rats, and the data demonstrated that mechanical and thermal pain hypersensitivity was induced after STZ administration, as reflected in the decreased PWT and PWL. Persistent allodynia and hyperalgesia are the important clinical features of DM patients with neuropathic pain, revealing that we established the DNP rat model successfully. Notably, the results showed that hyperbaric lidocaine could deepen the mechanical allodynia and heat hyperalgesia of DNP rats. Still, isobaric lidocaine did not influence

the pain-related behaviors of DNP rats. Previous studies authenticated that 5% or 10% lidocaine with 10% glucose has neurotoxicity and results in spinal nerve injury in rats [26–28]. The hyperbaric solution of 2% lidocaine and 10% glucose was also proved to induce spinal nerve injury in DNP rats in the current study. Consistently, isobaric lidocaine had no effects on the STZ-caused spinal cord tissue injury and loss of spinal nerves in rats. This study evaluated the influence of HL on mechanical and thermal pain hypersensitivity in DNP rats and then probed its regulatory mechanism.

MAPKs are crucial biomolecules involved in various cellular events and thus play an essential role in multiple pathogenesis. Accumulating evidence discovered that MAPK signaling is activated in the spinal cord tissues of several neuropathic pain animal models, like chronic constriction injury and trigeminal neuralgia, and the activity of MAPK signaling is decreased following drug treatment [29,30]. Accordingly, we conjectured that activated MAPK signaling is associated with HL-induced mechanical allodynia and heat hyperalgesia in DNP rats. ERK, p38, and c-Jun NH2-terminal kinases are the key components of MAPK signaling [30]. Our previous studies demonstrated that HL increased the phosphorylation of p38 [12], but we did not detect the expression of p38 and ERK and the phosphorylation level of ERK. In the current study, we further measured the activity of MAPKs in the spinal cord tissues of the DNP rats with a decreased PWT and PWL. The results demonstrated that the phosphorylation levels of p38 and ERK were significantly increased in the spinal cord tissues of DNP rats. HL administration facilitated the phosphorylation of p38 and ERK. Furthermore, the current study verified the improvement effect of p38 MAPK inhibitor and ERK inhibitor on the HL-induced spinal nerve injury in DNP rats and indicated that mitophagy may be involved in the HL-induced spinal nerve injury.

Maintenance of cell survival and function is inseparable from normal mitophagy, which is regulated by a series of proteins, including PINK1, Parkin, and BNIP3 [31]. During the damage-induced mitophagy, the PINK1 located on the mitochondrial outer membrane activates Parkin. Then, activated Parkin facilitates voltage-dependent anion channel ubiquitination, which is necessary to maintain the LC3-interacting region containing p62/SQSTM1 (autophagic substrate) [31]. In addition, BNIP3 is also an important receptor of mitophagy in mammalian systems. When mitophagy occurs, BNIP3 is recruited to abnormal mitochondria and bound with LC3 II to clean the damaged mitochondria [32]. Yi *et al.*'s [33] study demonstrated that PINK1-mediated mitophagy is activated in neuropathic pain model rats and associated with spinal nerve injury. The PINK1/Parkin pathway is an important amplification mechanism that improves the efficiency of mitosis. Mutations in this pathway are involved in the pathogenesis of neurodegenerative diseases [34]. Consistent with the previous study, our study's findings indicated that mitophagy was activated in DNP rats, as reflected in increased LC3-II/LC3-I, BIMP3, PINK1, and Parkin and decreased p62/SQSTM1, strengthened by HL administration.

SB 203580 is the most commonly studied selective p38α, p38β MAPK inhibitor [35]. The study showed that SB 203580 can inhibit the catalytic activity of p38 MAPK by binding ATP-binding bags. SB 203580 does not affect activation from upstream p38 MAPK (i.e., does not affect phosphorylation of p38 MAPK) [36]. PD98059 is a potent and selective MEK inhibitor. PD98059 binds to the inactive form of MEK, thereby preventing upstream kinases from activating MEK1 and MEK2 [37]. Meanwhile, PD98059 is an inhibitor of ERK1/2 signaling. PD98059 also inhibited autophagy induced by Mycobacterium bovis Bacillus Calmette-Guerin (BCG) [38,39]. Previous studies suggested that the activation of PINK1/Parkin is affected by ERK signaling [40]. Interestingly, there was crosstalk between AKT, EGFR, and p38; p38 decreased AKT activation and EGFR expression, while a chemical inhibitor of p38 (SB203580) and P38-specific siRNA increased AKT activation and EGFR expression [41]. Moreover, in pathologic conditions, PINK1 may play a role in the repair process. The deficiency of PINK1 in astrocytes may lead to mitochondrial defects, which in turn regulate the signaling pathway of proliferation by activating p38 and AKT; Epidermal growth factor receptor expression, which can lead to abnormal tissue repair in the injured brain and increase the risk of Parkinson's disease [36]. Hwang *et al.* [41] found that the p38 specific inhibitor (SB203580) alleviated the aggravating effects of Parkin loss on ethanol-induced ROS production, mitochondrial autophagy, mitochondrial dysfunction, and cell death *in vitro* and *in vivo*, and alleviated ethanol-induced dopaminergic neuron damage. In this study, activated mitophagy was partly suppressed by p38 MAPK and ERK inhibitors in the HL-administrated DNP rats, indicating that PINK1-mediated mitophagy may be a downstream signaling of p38/MAPK/ERK.

However, there are still some limitations in our current research. Whether the connection between HL and p38/MAPK/ERK signaling cascade is direct or indirect remains unclear; the precise downstream effectors of HL in DNP rats would also be an essential issue in our follow-up study.

Conclusion

In conclusion, our data suggest that the hyperbaric factor of lidocaine can cause spinal nerve injury in DNP rats by inducing PINK1-mediated mitophagy via activating p38 MAPK/ERK signaling. These results provide novel experimental evidence that p38 MAPK/ERK signaling may be a therapeutic target for the prevention of HL-induced neurotoxicity and neuropathic pain.

Availability of Data and Materials

Data involved in the present work are available from the corresponding author upon request.

Author Contributions

XLZ and FMY designed the research study. XLZ and FMY performed the research. LC, CLF, SJX and SBC provided help and advice on experiments. LC, CLF, SJX and SBC analyzed the data. All authors were involved in drafting and critical revision of the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments were carried out in accordance with the rules of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital, Jiangxi Medical College, Nanchang University (No. CDYFY-IACUC-202206QR002).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202436184.92.

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