Neuroprotective effect of Indobufen against pyroptosis following cerebral ischemia-reperfusion injury both in vivo and in vitro

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Research article

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Abstract

Background: Indobufen is a new generation of antiplatelet agents and has been shown to have antithrombotic effects in animal models. However, the efficacy of Indobufen on cerebral ischemia/reperfusion (I/R) injury and its mechanisms remain to be investigated.

Methods: In this study, the efficacy of Indobufen with both pre- (5days) and post- (15days) treatment on rats suffering middle cerebral artery occlusion/reperfusion (MCAO/R, 2h of ischemia and 24h/15days of reperfusion) was investigated. Furthermore, human umbilical vein endothelial cells (HUVECs) were cultured and underwent oxygen glucose deprivation/reoxygenation (OGD/R) injury for in vitro studies. Relationship between Indobufen and pyroptosis associated NF- κ B/Caspase-1/GSDMD pathway was preliminarily discussed.

Results: The pharmacodynamic tests revealed that Indobufen ameliorated I/R injury by decreasing the platelet aggregation, infarct size, brain edema and neurologic impairment in rats and rescuing cell apoptosis/pyroptosis in HUVECs. The underlying mechanisms were probably related to pyroptosis suppression by platelet inhibition induced regulation of the NF- κ B/Caspase-1/GSDMD pathway.

Conclusion: Overall, these studies indicates that Indobufen exerts protective and therapeutic effects against I/R injury by pyroptosis suppression via downregulating NF- κ B/Caspase-1/GSDMD pathway.

1. Background

Stroke is characterized by high morbidity and disability rate, and is also one of the deadliest diseases globally [1]. Ischemic stroke occupy the vast majority of stroke caused by blockage of blood circulation to the brain, resulting in pathophysiologic events like excitatory amino acids toxicity, ion imbalances and oxidative stress, leading to accumulation of hypoxia and toxic substances such as inflammatory cytokines, as well as the death of brain cells, and may induce subsequent neurobehavioral deficits in stroke survivors. However, current treatment strategies for ischemic stroke are far from optimal [2], which are limited by narrow treatment window and risk of bleeding [3, 4]. Therefore, the prevention and treatment of stroke has important clinical value.

There are three classic forms of brain cell death in stroke: necrosis, apoptosis and pyroptosis, among which pyroptosis, also known as caspase-1-dependent cell death, has been the focus of research in recent years [5]. Caspase-1 is activated by the inflammasome NLRP3 after receiving the signal of NF- κ B nuclear translocation, followed by the transformation of inflammatory cytokines IL-1 β and IL-18 into active forms, which play important roles in the maintenance and development of inflammatory responses [6]. In addition, Caspase-1 also specifically cleaves Gasdermin D protein (GSDMD) into carboxy-terminal Gasdermin-c (GSDMD-C) and amino-terminal Gasdermin-n (GSDMD-NT), where GSDMD-NT is associated with cell membrane rupture [7]. Recent studies have shown that hemoglobin induced platelet activation is mediated by ROS-driven proteasome activity and inflammasome activation [8]. In addition, platelets license NLRP3 to activate inflammasomes in innate immune cells and shape IL-1 β at the same time,

whose count is associated with IL-1 β in plasma [9]. These results suggest that targeted oxidative stress and platelet activation inhibition may promote the down-regulation of pyroptosis in cerebral ischemia and become a new therapeutic strategy for cerebral ischemia.

Indobufen (lab no. K3920, Figure 1), with a chemical structure of 2-[p-(1-oxo-2-isoindoline) phenyl] butyrate, is a new generation of antiplatelet aggregation drugs that reversely inhibits platelets, cyclooxygenase and reduces the production of thromboxane A₂ (TXA₂) [10]. We previously reported that Indobufen could inhibit blood coagulation process of APTT, PT, TT, platelet factor 3 (PF3), clotting factor I, II, V, IX and X content to reduce thrombosis [11], so we speculated that Indobufen may also have therapeutic and preventive effects against ischemia/reperfusion (I/R) injury. Its potential mechanisms may be closely related to inhibition of inflammation/apoptosis/pyroptosis induced by its platelet inhibition and antithrombotic effects, but research about efficacy of Indobufen in treatment/prevention of cerebral ischemia has not been reported. In this study, we investigated by prophylactic and therapeutic administration the effects of Indobufen on I/R injury as well as its anti-apoptosis/apoptosis efficacy in rat brains and HUVEC cells, conjecturing that the molecular mechanisms involved signal regulation of NF- κ B/GSDMD/NLRP3 both in vivo and in vitro. This study may provide us new insights into the effects of Indobufen on I/R injury and suggest Indobufen as a potential strategy for ischemic stroke.

2. Methods

• Animals and Treatments

Male SPF Sprague–Dawley (SD) rats (250–300g) were purchased from Qinglongshan Animal Farm of Nanjing, China (Production License No. SCXX (su) 2018-0019; SCXK (zhe) 2019-0002). All animals were housed under a 12 hour light-dark cycle at temperature of 22-26°C in a room of 40-70% humidity. Rats were fasted for 12 hours before the surgery. Rats were randomly divided into 4 groups: (1) sham operation group; (2) MCAO/R model group; (3) Indobufen (20 mg/kg) group; (4) Aspirin (10 mg/kg) group. The in vivo trial was divided into 5 day prophylactic administration (5-day tests) and 15 day therapeutic administration (15-day tests). In the 5-day prophylactic administration experiment, oral administration was given for 5 days, and surgery was performed 30min after the 5th-day administration. In the 15-day treatment administration experiment, the drugs were administered 3 hours after the operation, once a day for 15 days. All rats were kept at a relative humidity of 55 $\pm$ 5% (at 25 $\pm$ 2 °C) in compliance with institutional guidelines of China Pharmaceutical University and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All experiments were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University (SYXK (Su) 2016-0011).

• Drug administration

Indobufen (Huadong medicine Co., LTD) and Aspirin (Shanghai yuanye Bio-Technology Co., LTD) were both dissolved in 0.5% sodium carboxymethyl cellulose (CMC-Na). Drugs were administered

intragastrically with a volume of 0.5mL/100g body weight. Sham and model group were given the same volume of 0.5% CMC-Na.

- **In vivo MCAO/R establishment**

MCAO/R injury was established as previously described after anesthetization with 3% isoflurane [12] with 2h of ischemia followed by reperfusion. After the operation, the rats with successful surgery according to the behavioral scoring were retained. All efforts were made to minimize animal suffering and reduce the number of animals used. Five rats died from anesthesia and ten died from intracranial hemorrhage, which were excluded in the analysis.

- **Neurological defect scoring, cylinder test and postural reflex test**

One days following MCAO/R, the neurological function of the rats was scored from 0 to 4 according to Bederson's method [13]. For the limb-use asymmetry test and postural reflex test, the rats were tested as previous reported [3]. In 5-day tests: neurological scoring was conducted at 24 h after reperfusion. In 15-day tests: cylinder test and posture reflex test were carried out 30 min after drug administration as shown in Figure 2B.

- **Measurement of infarct size**

In the 5-day tests, rats were sacrificed by cervical dislocation 24h after the MCAO surgery and in the 15-day tests, rats were sacrificed by cervical dislocation 30min after the last drug-administration. 2,3,5-Triphenyltetrazolium chloride (TTC, Sangon Biotech CO., LTD) staining were used to measure brain infarct size [14]. The percentage of infarction was calculated as following: Infarct rate (%) = (left hemisphere area – right white brain infarction area)/left hemisphere area × 100%.

- **Evaluation of brain edema**

Brain sections were weighed right after TTC staining to obtain wet weight. Then the brain was placed in an oven at 110 °C for 24 h to dry to constant weight to obtain dry weight, and the brain water content was calculated: Brain water content (%) = (1 – dry weight/wet weight) × 100% [15].

- **Morris water maze (MWM)**

In the 15-day tests, Morris Water Maze evaluation was conducted as mentioned before [16, 17]. The escape latency and swimming path were recorded along the first five days and percentage of time spent in the quadrant IV and number of crossing platform was measured in probe trial of the fifth day of MWM.

2.8 Assay of Intracellular Total ROS Levels

By measuring the fluorescence intensity of 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA), ROS production in the ischemic penumbra of rats in the 5-day administration group was evaluated by ROS kit (Nanjing Jiancheng Bioengineering Institute). At 24h after MCAO/R, 0.1g ischemic penumbral zone was

taken from rats in each group. Single-cell suspension was prepared by pancreatin digestion method and treated with 10 μ M DCFH-DA PBS for 45 min. The excess dye was removed with PBS. DCF fluorescence was determined using multifunctional microplate reader (POLARstar Omega, USA) with 485-nm excitation and 525-nm emission filters [18].

2.9. Primary cell culture of HUVECs

Primary cultures of Human umbilical vein endothelial (HUVEC) were purchased from ScienCell Research Laboratories, Inc (Cat. #8000). Endothelial cell medium (ECM, ScienCell Research Laboratories, Inc, Cat. #1001) containing 5% heat-inactivated fetal bovine serum (FBS) with 100 U/mL penicillin and 100 μ g/mL streptomycin was used and cells was placed in a humidified atmosphere with 5% CO₂ at 37 °C. The medium was changed every 2 days.

2.10. Oxygen-glucose deprivation followed by recovery (OGD/R)

HUVEC cells were randomly divided into groups below: (1) control; (2) OGD/R; (3) Indobufen (200 μ M) (4) Aspirin (100 μ M);. All groups were incubated for 24 h with medium containing different drugs. Then HUVEC cells were subjected to hypoxic conditions (95% N₂, 5% CO₂) with no-glucose DMEM for 4 h, followed by reoxygenation for 2 h [19].

2.11. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

Cell viability was determined by MTT assay (Wanleibio, Shenyang, China; LOT#16B109). The optical density (OD) value at 570nm was measured to calculate the cell viability as follows: Cell viability (%) = (OD value in the administration group-OD value in the solvent control group)/ (OD value in the blank group-OD value in the solvent control group) $\times$ 100%.

2.12. ELISA assay

The concentrations of 6-keto-PGF_{1 α} and TXB₂ in the brain tissue homogenates and culture cell supernatants were assessed by ELISA (Nanjing Jiancheng Bioengineering Institute) according to manufacturer's instructions. The levels were normalized to cell protein concentrations.

2.13. Real-time PCR

Changes in mRNA levels of IL-18, IL-1 β and NLRP3 were detected in brain tissues in the 5-day pretreatment groups, and real-time PCR was performed as described previously [20]. Quantitative PCR (Mastercycler ep realplex, Eppendorf) was performed in the presence of SYBR Green I. Expression of IL-18, IL-1 β and NLRP3 was normalized to β -actin analyzed by 2^{- $\Delta\Delta$ Ct}. The primers (Sangon Biotech Co., LTD., Shanghai, China) were as follows:

Rat IL-18-F: ACCGCAGTAATACGGAGCAT; R: GTCTGGGATTCGTTGGCTGT

Rat IL-1 β -F: AGGCTGACAGACCCCAAAG; R: CTCCACGGGCAAGACATAGG

Rat NLRP3-F: GTGGAGATCCTAGGTTTCTCTG; R: CAGGATCTCATTCTCTTGGATC

Rat- β -actin-F: AGACCTTCAACACCCCAG; R: CACGATTTCCCTCTCAGC

2.14. Immunofluorescence (IF) assay

At 24 h after MCAO/R in the 5-day pretreatment groups, the rats were anesthetized, 4% paraformaldehyde was perfused through the heart, and brain tissue was quickly removed and fixed in 4% paraformaldehyde. Brain samples were performed on fixed frozen ultrathin sections (Leica CM3050s, Germany) as previously described [21]. As for in vitro experiments, HUVEC cells were fixed with 4% paraformaldehyde for 20 min after OGD/R treatment. Thereafter, brain sections or culture cells were incubated with rabbit antibody NF- κ B p65 (1:200, Cell Signaling Technology), rabbit antibody NLRP3 (1:100, proteintech, Wuhan, China; CAT#17168-1-AP) or rabbit antibody Caspase-1 (1:100, proteintech, Wuhan, China; CAT#22915-1-AP) at 4 °C overnight. After being rinsed with 0.3% Triton-X PBS (PBST), cells were incubated with goat anti-rabbit IgG/Cy3 antibody (1:200; Bioss, Beijing, China; CAT#bs-0295G-Cy3) or goat anti-rabbit IgG (H+L) (1:200, proteintech, Wuhan, China; CAT#SA00013-4) in dark for 2h, and then stained with 4',6-diamidino-2-phenylindole (DAPI, Beyotime Institute of Biotechnology, Shanghai) for 30 min. Images were captured with a fluorescence microscope (Nikon Ts2R, Japan).

2.15. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and GSDMD co-staining

The pyroptosis cells emerged from rat MCAO/R or HUVECs OGD/R were detected by TUNEL staining and immunofluorescence assay. The brain slides or HUVEC cells were incubated with GSDMD rabbit antibody (1:100, abbexa, Houston, TX, USA CAT#abx136074) overnight at 4 °C. Subsequently, CoraLite594-conjugated goat anti-rabbit IgG (H+L) (1:200, proteintech, Wuhan, China; CAT#SA00013-4) was incubated for 2 h. TUNEL staining (Keygenbiotech, Nanjing, China; CAT#KGA7073) were processed followed with DAPI staining for 30 min and photograph by a fluorescence microscope (Nikon Ts2R, Japan).

2.16. Western blot analysis

Equivalent protein samples (50 μ g) of brain tissues or HUVEC cells were separated by 10% SDS/PAGE gel electrophoresis. Following primary antibodies were incubated: rabbit antibody NF- κ B p65 (1:1000, Cell Signaling Technology), rabbit antibody Caspase-1 p20 (1:500, Wanleibio, Shenyang, China), mouse antibody β -actin (1:1000, proteintech, Wuhan), and rabbit antibody Histone H3 (1:1000, proteintech, Wuhan, China) and membranes were visualized via chemiluminescence.

2.17. Statistical analysis

The data in this study were expressed as mean $\pm$ SD. Kruskal-Wallis test was used for behavioral tests, the number of crosses in the probe test in quadrant IV of MWM. The remaining data were analyzed by one-way ANOVA followed by Bonferroni test post hoc test for equal variance data and Dunnett 3 post hoc test for unequal variance by IBM SPSS 25.0 software. Differences were considered significant when p-

values were smaller than 0.05. Photoshop 2020, Image J and GraphPad Prism Version 8.0 were used for statistical analysis.

3. Results

- **Pre-treatment with Indobufen alleviates cerebral ischemic injury in MCAO/R rats**

MCAO was conducted to mimic human cerebral ischemic injury in rats [22]. The diagram in Fig. 2 showed the timeline of in vivo studies. In 5-day tests, 24 h after MCAO/R, TTC staining showed that infarcted white zone in MCAO/R model group was apparent (Fig. 2C) and held $32.73 \pm 1.83\%$ (Fig. 2D) of the brain, which marked the successful establishment of MCAO. Quantification showed that Aspirin (10 mg/kg, $20.20 \pm 2.55\%$) exhibited a significant reduction in the infarct size ($P < 0.01$, Fig. 2D), with Indobufen (20 mg/kg, $15.03 \pm 2.20\%$) exhibiting better effects on infarction alleviation ($P < 0.01$, Fig. 2C). Similarly, Indobufen (20 mg/kg, $79.31 \pm 0.64\%$) and Aspirin (10 mg/kg, $79.38 \pm 1.12\%$) significantly reduced the water content compared with model group ($P < 0.05$, Fig. 2E). Indobufen (20 mg/kg, 1.75 ± 0.71) and Aspirin (10 mg/kg, 1.625 ± 0.74) showed an apparent decrease on the neurological score ($P < 0.05$, Fig. 2F). TXB_2 and 6-keto-PGF 1α levels in the model group both increased versus sham group ($P \leq 0.01$, Fig. 2G). Indobufen group showed obvious reduction in TXB_2 content compared with MCAO/R group ($P \leq 0.01$, Fig. 2G) while Aspirin exhibited no apparent difference. The 6-keto-PGF 1α levels in both the Indobufen and Aspirin groups had no significant difference versus the MCAO/R group.

- **Post-treatment with Indobufen promotes exercising, learning and memory ability recovery in MCAO/R rats**

Therapeutic effects of 15-day post-treatment of Indobufen with the rat MCAO/R model was also investigated. As in Fig. 2B, TTC staining were conducted and representative photographs was shown in Fig. 3A. Indobufen (20 mg/kg) and Aspirin (10 mg/kg) group showed a marked decrease in infarct size ($P < 0.01$, Fig. 3B).

Representative swimming tracks in MWM of rats on the 2nd day on visible platform were presented in Fig. 3C. Other results of MWM were in Fig. 3D-F. The MCAO/R group had an apparently longer escape latency than the sham group (Day10, Day11, Day13: $P < 0.01$; Day12, Day14: $P < 0.01$, Fig. 3D). Compared with model group, escape latency of Indobufen and Aspirin groups were significantly reduced in the visible platform trial and escape latency of Indobufen group was significantly reduced in the invisible trial (Fig. 3C). In the probe trial, model group had fewer numbers of platform crossings ($P < 0.01$) compared with sham group, while Indobufen group increased the crossing numbers ($P < 0.01$, Fig. 3E). Time spent in the target quadrant also showed an increase in Indobufen group ($P < 0.01$, Fig. 3F).

In both the cylinder test and posture reflex test, Indobufen groups showed a significant difference (Cylinder test: $P < 0.01$; Posture reflex test: Day 2, 10,15: $P < 0.01$, Day5: $P < 0.05$) compared with model group, and particularly showed better effects than Aspirin in cylinder test (Day10 and 15, $P < 0.01$), which suggested that Indobufen (20 mg/kg) had an obvious effect on behavioral improvement (Fig. 3G-H).

3.3. Indobufen reduces cerebral pyroptosis and regulates ROS/NF-κB/Caspase-1/GSDMD expressions in vivo

To evaluate the protective effects of Indobufen on cerebral cell survival especially pyroptosis against I/R injury, 24 h after MCAO/R, ROS as well as pyroptosis related components Caspase-1, NLRP3 and NF-κB-p65 was measured through IF in the brains of rats in 5-day tests. GSDMD, a hallmark of pyroptosis, was stained along with TUNEL assay to distinguish cerebral pyroptosis cells (Fig. 4-5). Fig. 4A-B showed the ipsilateral cortex with Caspase-1 or NLRP3 costaining with DAPI. It was obvious that MCAO/R group showed more apparent Caspase-1/NLRP3 expression compared with sham group, while Aspirin and Indobufen groups exhibited similar expression as sham group. Fig. 5A, B showed the ipsilateral cortex with NF-κB costaining with DAPI or GSDMD costaining with TUNEL and DAPI. It was also apparent to see that MCAO/R group showed more nuclear import of NF-κB compared with sham group, while Aspirin and Indobufen groups exhibited similar level of nuclear import as sham group. Results from Fig. 5B showed that GSDMD-positive TUNEL cells were markedly reduced in both the pretreated groups, indicating the protective effect of Indobufen against cerebral cell pyroptosis.

During stroke associated pyroptosis, Caspase-1 plays a central role, which, activated by inflammasome NLRP3 following the signal from NF-κB nuclear transportation, cleaves the inactive pro-IL-18 and pro-IL-1β into mature inflammatory cytokines IL-18 and IL-1β, mediating pyroptosis and also promoting GSDMD-NT expression, leading to cell membrane rupture [23]. At the same time, ROS was proven to be able to induce inflammation and subsequently aggravate pyroptosis after acute ischemic stroke [8]. To explore whether Indobufen inhibits cerebral pyroptosis through ROS/NF-κB/Caspase-1/GSDMD signal, ROS detection via fluorescence intensity of DCFH-DA, western blot and PCR were used to examine the expressions of ROS, Caspase-1, IL-18, IL-1β, NF-κB-p65 at 24 h after MCAO/R. The results revealed that ROS, IL-18, IL-1β, NLRP3 mRNA, Caspase-1 protein expression and NF-κB-p65 nuclear import were all significantly enhanced in model group versus sham group ($P < 0.01$, Fig. 4C-F, 5C-F), indicating that pyroptosis was markedly activated after I/R injury, while Indobufen (20 mg/kg) exhibited an reverse effect on pyroptosis activation ($P < 0.01$, Fig. 4C-E, 5C-F), which was similar to Aspirin (10mg/kg), identical to the results of IF.

3.4 Indobufen protects OGD/R induced cell injury in HUVEC cells

HUVEC cells were used to determine the effect of Indobufen on cell viability using the MTT assay. As shown in (Fig. 6A-B), compared with the control group, the HUVEC cells subjected to OGD for 4h and reperfusion for 2h exhibited a decrease in cell viability, and this effect was potently reversed by pretreatment of Indobufen (200μM) and Aspirin (100μM). Moreover, consistent with the in vivo results, Indobufen showed similar effects on TXA2/PGI2 balance. (Fig. 6C).

3.5. Indobufen reduces pyroptosis and regulates NF-κB/Caspase-1/GSDMD expressions in vitro

To investigate whether Indobufen also exerts protective efficacy on pyroptosis via the NF-κB/Caspase-1/GSDMD signaling pathway in vitro, the pyroptosis-associated cellular proteins, including Caspase-1,

NLRP3, NF- κ B and GSDMD-NT in HUVEC cells were examined with IF and western blot. The results showed that OGD/R exposure enhanced the Caspase-1, NLRP3, GSDMD protein expressions, and induced NF- κ B nuclear import. IF results showed that Indobufen (200 μ M) exhibited a reverse effect on Caspase-1/NLRP3/GSDMD activation (Fig. 7A-D). Typical bands of protein expression also revealed that Indobufen had reverse influence against the Caspase-1 up-regulation and NF- κ B nuclear transportation (Caspase-1: $P < 0.05$; NF- κ B $P < 0.01$, Fig. 7E-I), which was in accordance with the results in the in vivo experiments.

4. Discussion

In this research, we first examined the neuroprotective effects of Indobufen against cerebral I/R injury and proved its possible mechanism of reducing pyroptosis by inhibiting NF- κ B/Caspase-1/GSDMD signal pathway.

Our previous studies showed that Indobufen had antiplatelet, antithrombotic and anticoagulant effects on rats [11]. Therefore, we speculated that Indobufen may be able to treat cerebral ischemia reperfusion injury. In MCAO/R induced I/R injury, exacerbate damage from numerous inflammatory responses occurs, leading to cell injury and eventually programmed cell death, in which pyroptosis depends on inflammatory responses and is a hot topic recently [24]. Recent studies have shown that platelet activation is mediated by ROS-driven inflammasome activation [8]. In addition, NLRP3 are licensed by platelets to activate innate inflammasomes in immune cells, and platelet amount is positively correlated with IL-1 β in plasma [9]. It suggests that targeting oxidative stress and platelet activation inhibition may promote the inhibition of pyroptosis in cerebral ischemia to reduce cell death and promote functional recover. In vivo pharmacodynamic tests with pretreatment or post-treatment were managed to assess the efficacy of Indobufen on I/R injury. Aspirin was used as the positive drug for its common use in the clinic [25, 26]. In the pre-treatment test, Indobufen significantly alleviated I/R injury by reducing infarct area and water edema 24 hours after MCAO/R treatment, and Indobufen could adjust the balance of TXA2 and PGI2 expression after I/R injury both in vivo and in vitro, preliminarily proving its anti-thrombotic ability. In post-treatment tests, the assessment of motor function, learning and memory ability 24 hours to 15 days after reperfusion showed that the I/R injury was significantly reduced in rats treated with Indobufen, and Indobufen significantly improved spatial cognition and motor function in rats over a longer period of time. In vitro, we simulated I/R injury with HUVEC cells by OGD/R, and Indobufen pretreatment reduced OGD/R-induced cytotoxicity and cellular morphological changes, and increased cell viability. The reduction of I/R injury and the improvement of motor, memory and learning abilities are all closely related to the reduction of brain cell injury and death by Indobufen.

Recently, more and more studies have shown that cell pyroptosis, also known as inflammatory death, can be caused by cerebral ischemia-reperfusion injury [27]. A hallmark of pyroptosis is GSDMD-NT-dependent membrane rupture due to Caspase-1 activation, which, unlike apoptosis, allows the release of intracellular pathogens such as pro-inflammatory mediators (e.g., IL-1 β and IL-18) [28]. Inflammation and oxidative stress are strongly connected with NF- κ B activation [29]. NF- κ B is normally found in cytoplasm linked to

regulatory proteins I κ Bs. After ischemic stroke, I κ B is phosphorylated and degraded, causing NF- κ B p65 translocation from the cytoplasm to the nucleus, promoting the transcription of a number of genes, such as IL-1 β [30]. Moreover, latest research provided new evidence that ROS is related to platelet and inflammatory pyroptosis activation [31] and is able to regulate NF- κ B as a second messenger [32, 33]. Caspase-1 can be stimulated by the typical signal of NF- κ B-p65 entry into the nucleus and is activated by canonical inflammasomes such as NLRP3, which promotes brain tissue damage [8]. Therefore, in order to determine the role of Indobufen against cerebral ischemia and cell pyroptosis, IF, PCR, western blot method were conducted 24 hours after MCAO/R in rats/HUVECs to detect the Indobufen in vivo and in vitro NF- κ B/Caspase-1/GSDMD gene and protein expression in composition of pyroptosis signaling pathways, and using TUNEL co-staining with GSDMD-NT to measure the apoptosis/pyroptosis levels in the 5-day pretreatment rats. The results showed that Indobufen significantly reduced the ROS level and mRNA expression levels of IL-1 β , IL-18 and NLRP3 in the rat brain, reducing the entry of NF- κ B into the nucleus, and apparently inhibited the expression levels of Caspase-1 and NLRP3, thus lowering the number of pyroptosis cells, exhibiting certain anti-apoptosis/pyroptosis effects. Consistent with the results in vivo, Indobufen had a strong anti-pyroptosis effect on OGD/R-induced cell death by potentially decreasing the pyroptosis signal of NF- κ B/Caspase-1/GSDMD in HUVEC cell culture. In addition, TUNEL+ GSDMD-NT co-staining also confirmed this anti-pyroptosis effect in vitro. However, since this experiment was only a preliminary study of the relationship between Indobufen and pyroptosis related pathway, further studies are needed on whether Indobufen directly inhibits pyroptosis signals and whether it further activates the downstream signals.

5. Conclusion

Collectively, we observed that pre-treatment or post-treatment of Indobufen exerts anti-pyroptosis effects against cerebral I/R injury and is beneficial to long term memory, learning and cognitive recovery. The underlying mechanisms are probably related to its potential of down-regulating pyroptosis related NF- κ B/Caspase-1/GSDMD signaling pathway. This study suggests that aside from anti-thrombotic and anti-coagulant effects, Indobufen may have neuroprotective efficacy in cerebral ischemic injury and is ideal as a new candidate drug for ischemic stroke in clinical trials.

Abbreviations

I/R: cerebral ischemia/reperfusion; MCAO/R: middle cerebral artery occlusion/reperfusion; HUVECs: human umbilical vein endothelial cells; OGD/R: oxygen glucose deprivation/reoxygenation; TXA2: thromboxane A2; GSDMD: gasdermin D; CMC-Na: sodium carboxymethyl cellulose; TTC: 2,3,5-Triphenyltetrazolium chloride; MWM: Morris Water Maze.

Declarations

Ethics declarations and consent to participate: All animal protocols were approved by the local-research ethics review board of the Animal Ethics Committee of China Pharmaceutical University Health Science

Center.

Consent for publication: Not applicable.

Availability of data and materials: The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests: The authors declare no conflict of interest.

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Figures

Figure 1

Chemical structures of Indobufen

Figure 2

Effects of Indobufen pretreatment on changes in infarct size, water content, neurological deficits and TXA₂/PGI₂ release after ischemic stroke injury. (A) Study of the protective effects of Indobufen administration in MCAO/R-induced pyroptosis in vivo. (B) Study of the therapeutic effects of Indobufen administration in MCAO/R-induced pyroptosis in vivo. (C) Representative images of cerebral infarct area after cerebral ischemia 24h after MCAO/R; (D) Quantification of cerebral infarct area (ANOVA followed by Bonferroni post hoc test); (E) Quantification of cerebral edema; (F) Neurological scoring statistics (Kruskal-Wallis test); (G). Effects of Indobufen on TXB₂ and 6-keto-PGF_{1α} in brain homogenate (ANOVA followed by Bonferroni post hoc test). Data are shown as mean $\pm$ SD, n=8. *P < 0.05, **P < 0.01 vs. MCAO/R; #P < 0.05, ##P < 0.01 vs. Sham.

Figure 3

Post-treatment of Indobufen promotes learning, memory and motor ability of MCAO/R rats. (A) Representative images of cerebral infarct area after cerebral ischemia 15 days after MCAO/R; (B) Quantification of cerebral infarct area (ANOVA followed by Bonferroni post hoc test); (C) Representative swimming tracks of MWM. (D) Escape latency to find the visible/non-visible platform (ANOVA followed by Bonferroni post hoc test). (E) Number of crosses in the probe test in quadrant IV (Kruskal-Wallis test).

(F) Time spent in quadrant IV (ANOVA followed by Bonferroni post hoc test). (G-H) Postural reflex test and cylinder test (Kruskal-Wallis test). Data are shown as mean $\pm$ SD, n = 8. *P < 0.05, **P < 0.01 vs. Model; #P < .05, ##P < 0.01 vs. Sham.

Figure 4

In vivo Indobufen reduces cerebral pyroptosis and regulates Caspase-1/IL-18/IL-1β/NLRP3 expressions. (A, B) Representative images showing Caspase-1 or NLRP3-positive cells in ipsilateral cortex; green-positive staining; blue-DAPI counterstaining of nuclei ($\times 200$ magnification, scale bar=50 μ m). (C) ROS levels in the cerebral cortex (% of the sham group). (D) mRNA expression and quantification of key components in NF- κ B/Caspase-1/GSDMD signaling pathway: IL-18, IL-1β, NLRP3; (E, F) Western blot analysis of Caspase-1 expression in the cortex (ANOVA followed by Bonferroni post hoc test). Data are shown as mean $\pm$ SD, n = 3. *P < 0.05, **P < 0.01 vs. Model; #P < 0.05, ##P < .01 vs. Sham.

Figure 5

In vivo Indobufen reduces cerebral pyroptosis and regulates NF-κB/GSDMD expressions. (A) Representative images showing NF-κB-positive cells in ipsilateral cortex, red-positive staining; blue-DAPI counterstaining of nuclei ($\times 200$ magnification, scale bar = $50\mu\text{m}$). (B) Representative images showing GSDMD/TUNEL-positive cells in ipsilateral cortex, green-TUNEL-positive staining; red-TUNEL-positive staining; blue-DAPI counterstaining of nuclei ($\times 200$ magnification, scale bar = $50\mu\text{m}$). (C, D) Western blot analysis of the ratio of NF-κB-p65 expression in the cortex (ANOVA followed by Bonferroni post hoc test). Data are shown as mean $\pm$ SD, $n=3$. * $P < 0.05$, ** $P < 0.01$ vs. MCAO/R; # $P < 0.05$, ## $P < 0.01$ vs. Sham.

Figure 6

Effects of drugs pretreatment on OGD/R-induced cytotoxicity in cultured HUVEC cells. (A) Microscope images showing respective cell morphology of HUVEC cells caused by OGD 4 h and Reperfusion 2 h ($\times 400$ magnification, scale bar=50μm); (B) Cell viability (ANOVA followed by Bonferroni post hoc test); (C) Effects of Indobufen on TXB₂ and 6-keto-PGF_{1α} (ANOVA followed by Bonferroni post hoc test). Data are shown as mean $\pm$ SD, n=8. *P < 0.05, **P < 0.01 vs. OGD/R; #P < 0.05, ##P < 0.01 vs. Control

Figure 7

In vitro Indobufen reduces HUVEC pyroptosis and regulates NF-κB/Caspase-1/GSDMD expressions after OGD/R. (A-D) The representative images of (A) Caspase-1, (B) NLRP3, (C) NF-κB, and (D) TUNEL/GSDMD in HUVEC cells were detected by IF staining (×200 magnification, scale bar=50μm). (E-I) Western blot analysis of the ratio of Caspase-1 and NF-κB-p65 expression in HUVEC cells. Data are shown as mean ± SD, n=3. *P < 0.05, **P < 0.01 vs. OGD/R; #P < 0.05, ##P < 0.01 vs. Control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [NC3RsARRIVEChecklist.pdf](#)