

# Elevated intracranial pressure induces IL-1 $\beta$ and IL-18 overproduction via activation of the NLRP3 inflammasome in microglia of ischemic adult rats

HONGGUANG DING<sup>1</sup>, YA LI<sup>1,2</sup>, MIAOYUN WEN<sup>1</sup>, XINQIANG LIU<sup>1</sup>, YONGLI HAN<sup>1</sup> and HONGKE ZENG<sup>1</sup>

<sup>1</sup>Department of Emergency and Critical Care Medicine, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong 510080; <sup>2</sup>School of Medicine, South China University of Technology, Guangzhou, Guangdong 510006, P.R. China

Received June 20, 2020; Accepted October 23, 2020

DOI: 10.3892/ijmm.2020.4779

**Abstract.** Elevated intracranial pressure (ICP) is one of the most common complications following an ischemic stroke, and has implications for the clinical and neurological outcomes. The aim of the present study was to examine whether elevated ICP may increase IL-1 $\beta$  and IL-18 secretion by activating the NOD-like receptor protein 3 (NLRP3) inflammasome in microglia of ischemic adult rats. Sprague-Dawley rats that underwent middle cerebral artery occlusion were used for assessment of ICP. Reactive oxygen species (ROS) production was detected, and western blotting and immunofluorescence staining were used to determine the expression levels of Caspase-1, gasdermin D-N domains (GSDMD-N), IL-1 $\beta$  and IL-18 in microglial cells. ICP levels were significantly increased, which was accompanied by ROS overproduction, in the brain tissue following ischemia-reperfusion (IR) injury in rats. Treatment with 10% hypertonic saline by intravenous injection significantly reduced the ICP and ROS levels of the rats. Furthermore, high pressure (20 mmHg) combined with oxygen-glucose deprivation (OGD) treatment resulted in increased ROS production in BV-2 microglial cells compared with those subjected to OGD treatment alone *in vitro*. Elevated pressure upregulated the expression of Caspase-1, GSDMD-N, IL-18 and IL-1 $\beta$  in IR-treated or OGD-treated microglia both

*in vivo* and *in vitro*. More importantly, Caspase-1, GSDMD-N, IL-18 and IL-1 $\beta$  expression in microglia was significantly downregulated when elevated pressure was reduced or removed. These results suggested that elevated ICP-induced IL-1 $\beta$  and IL-18 overproduction via activation of the NLRP3 inflammasome by ischemia-activated microglia may augment neuroinflammation.

## Introduction

Ischemic stroke is one of the most common causes of death worldwide (1,2). Cerebral edema and elevated intracranial pressure (ICP) are common complications following ischemic stroke (3,4). Elevated ICP is a key factor affecting the clinical and neurological outcomes of stroke (5-7); however, the underlying mechanisms remain unclear.

Inflammation in the central nervous system is an important cause of secondary brain injury that occurs following cerebral ischemia (8). The microglial cells are the resident immunocytes in the brain and are activated within the first few hours following cerebral infarction and release a large number of inflammatory cytokines (9-12). Recent studies have reported that intracellular NOD-like receptors, including NOD-like receptor protein 3 (NLRP3), are widely expressed in microglia (13-16). NLRP3 inflammasome serves a key role in initiating and amplifying the inflammation in the central nervous system (17-19). To exert its functions, the activation of the NLRP3 inflammasome is first required. As several studies have reported, the NLRP3 inflammasome can be triggered by reactive oxygen species (ROS) (20-23). The expression of IL-1 $\beta$  and IL-18 can be upregulated following activation of the NLRP3 inflammasome, which promotes inflammation in the central nervous system and eventually leads to the aggravation of brain injuries following ischemic stroke (24,25). Whether ICP can mediate NLRP3 inflammasome activation in ischemic microglia remains to be clarified.

In the present study, it was hypothesized that elevated ICP may aggravate nerve injury that occurs following cerebral ischemia. The possible underlying mechanism was determined to involve elevated ICP, which in-turn increases IL-1 $\beta$  and IL-18 secretion via activation of the NLRP3 inflammasome.

**Correspondence to:** Professor Hongke Zeng, Department of Emergency and Critical Care Medicine, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, 106 Zhongshan Er Road, Guangzhou, Guangdong 510080, P.R. China  
E-mail: zenghongke@vip.163.com

**Abbreviations:** ICP, intracranial pressure; ROS, reactive oxygen species; MCAO, middle cerebral artery occlusion; GSDMD-N, gasdermin D-N domains; IR, ischemia-reperfusion; OGD, oxygen-glucose deprivation; NLRP3, NOD-like receptor protein 3

**Key words:** ischemic stroke, intracranial pressure, microglia, NLRP3 inflammasome

## Materials and methods

**Animals and treatment.** Male adult Sprague-Dawley rats aged 3–4 months weighing 220–250 g were provided by the Institute of Laboratory Animal Science of Jinan University (Guangzhou, China). The rats were fed standard chow and water, and housed under standard experimental conditions (temperature, 20–25°C; humidity, 50–70%) with a 12-h light/dark cycle for a week. As few animals as possible were used in the experiments. A total of 160 rats were randomly divided into four groups ( $n=40$  rats per group): i) Sham-operated group (sham group); ii) cerebral ischemia-reperfusion (IR) group (IR group); iii) cerebral IR + normal saline group (NS group); and iv) cerebral IR + 10% hypertonic saline group (HS group). Rats in the IR, NS and HS groups were subjected to middle cerebral artery occlusion (MCAO). Rats in the sham group were subjected to all the procedures without occlusion. The tail vein was cannulated for intravenous infusion of 10% HS or normal saline. After IR, the rats in the NS group and HS group were continuously administered NS (0.3 ml/h) and 10% HS (0.3 ml/h) by intravenous injection, respectively. All animals were observed closely for 24 h.

**Rat model of cerebral ischemia.** Before the surgical procedure, all rats were fasted with access to water overnight. Cerebral ischemia was induced by right-sided MCAO as described previously (26). The rats were anesthetized with pentobarbital sodium (30 mg/kg intraperitoneal injection) followed by a midline incision. The right common carotid artery, internal carotid artery and external carotid artery were carefully exposed. A head-end spherical nylon suture was inserted from the external carotid artery into the middle cerebral artery until resistance was felt. The suture remained in place for 2 h, after which it was withdrawn to allow reperfusion. The health and behavior of the rats were monitored every 2 h after surgery. The rats ( $n=23$ ) who could not walk spontaneously and had a depressed level of consciousness, were excluded from the study. The rats were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneal injection) and euthanized with 0.9% sodium chloride intravenous perfusion and arterial exsanguination. The Research Ethics Committee of Guangdong Provincial People's Hospital and Guangdong Academy of Medical Sciences approved all animal procedure protocols [approval no. GDREC2012106A(R1); Guangzhou, China].

**Measurement of ICP.** The ICP was measured 0, 2, 4, 8, 12, 16, 20 and 24 h after surgery ( $n=8$  for each group). To evaluate the ICP, a midline incision over the vertex was performed following anesthesia, and then a hole caudal to the coronal suture was drilled, 4 mm from the midline. The dura was punctured and a microsensor for ICP was inserted intracranially (3). An ICP monitor (Integra CAMO2; Integra LifeSciences) was used to measure the ICP.

**Measurement of ROS levels in brain tissue.** The ROS levels in brain tissues were evaluated using a ROS ELISA kit (cat. no. DG21175D-96; DG Biotech) 24 h after IR. Briefly, samples and standards (50  $\mu$ l/well) were added to the plate wells coated with HRP-conjugated antibodies, which were used to capture ROS. The plates were incubated for 1 h

at 37°C. After washing completely, substrate A (50  $\mu$ l/well) and substrate B (50  $\mu$ l/well) were added to incubate the plate in the dark for 15 min at 37°C. Then, the stop buffer was added, and the optical density was measured spectrophotometrically at a wavelength of 450 nm. The concentrations of ROS in the samples were then determined by comparing the optical density of the samples to the standard curve.

**BV-2 microglial cell cultures and treatment.** BV-2 microglial cells (cat. no. 7-1502) were purchased from CHI Scientific, Inc., and were cultured and treated as described in our previous study (27). Briefly, the cells were cultured on six well plates at a density of  $1.5 \times 10^6$  cells/well with DMEM high glucose (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The microglial cells were randomly divided into three groups: i) Control group; ii) oxygen-glucose deprivation (OGD) + 20 mmHg group; and iii) OGD group. The cells in the OGD group were cultured for 2 h with glucose-free medium in an airtight hypoxia chamber with 0.1% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C, and then the cells were switched to DMEM high glucose in an incubator with 5% CO<sub>2</sub>/95% air for 24 h. The cells in the OGD + 20 mmHg group were exposed to a higher atmospheric pressure (20 mmHg) for 24 h with a high-pressure installation when the oxygen-glucose supply was reinstated. The cells in the control group were cultured in the DMEM high glucose containing 10% FBS.

**ROS measurement in microglia.** The ROS production in the BV-2 microglial cells was evaluated using a ROS assay kit (cat. no. BB-4705-2; BestBio), according to the manufacturer's protocol. Briefly, DCFH-DA was diluted with DMEM high glucose without FBS (1:1,500). The coverslips with adherent BV-2 microglial cells were cultured in DMEM high glucose supplemented with 10% FBS. Following treatment, the medium was changed to diluted DCFH-DA (2 ml/well). Then, the plates were incubated for 20 min at 37°C and 5% CO<sub>2</sub>. The coverslips were washed with DMEM high glucose without FBS. Finally, the coverslips were mounted using a fluorescent mounting medium and visualized under a fluorescence microscope (Olympus DP73 Microscope; Olympus Corporation).

**Western blotting analysis.** Total proteins from the peri-infarcted cerebral cortex and BV-2 microglial cells ( $n=4$  per group) were extracted using a Total Protein Extraction kit (cat. no. BB-3101-100T; BestBio) as described previously (26). Protein concentration was determined using a Pierce™ BCA Protein assay kit (cat. no. 23227; Pierce; Thermo Fisher Scientific, Inc.). Equal quantities of protein from each sample (40  $\mu$ g per lane) were separated via 10% SDS-PAGE, and then transferred to PVDF membranes, which were blocked with 5% non-fat milk for 1 h at room temperature. Subsequently, the following primary antibodies were added to incubate the membranes overnight at 4°C: Caspase-1 (1:1,000; cat. no. 24232S; Cell Signaling Technology, Inc.), IL-1 $\beta$  (1:1,000; cat. no. 12703S; Cell Signaling Technology, Inc.), IL-18 (1:1,000; cat. no. ab207323; Abcam) and gasdermin D-N domains (GSDMD-N; 1:1,000; cat. no. 36425S; Cell Signaling Technology, Inc.). The membranes were washed the following day, and the HRP-conjugated goat anti-rabbit antibody (1:2,000; cat. no. 7074S; Cell Signaling Technology, Inc.) was

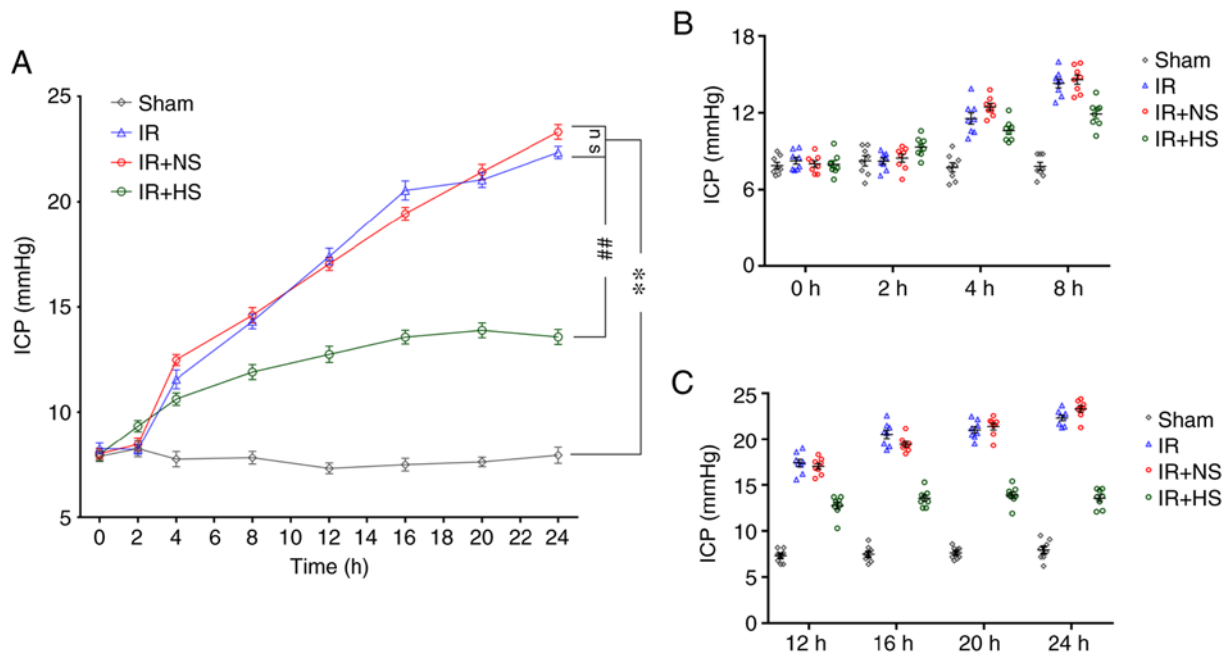


Figure 1. ICP levels 0-24 h after IR *in vivo*. (A) ICP levels in the IR group and the IR + NS group were significantly higher compared with the sham group. There was no significant difference between the IR group and the IR + NS group. ICP levels in the IR + HS group were significantly lower than the IR group. (B) The exact data points of ICP 0-8 h after IR are presented as a dot plot. (C) The exact data points of ICP 12-24 h after IR are presented as a dot plot. n=8 per group. \*\*P<0.01 vs. sham group; ##P<0.01 vs. IR group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group; ns, non-significant.

added and the membrane was incubated for 2 h at 4°C. The immunoblots were visualized using a chemiluminescence kit (Bioworld Technology, Inc.), and detected using an imaging densitometer (ImageQuant™ LAS 500; Cytiva). The relative density was semi-quantified using FluorChem 8900 software (version 4.0.1; ProteinSimple).  $\beta$ -actin was used as the loading control.

**Double immunofluorescence labeling.** After 24 h of reperfusion, the rats were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneal injection) and transcardially perfused with saline and 4% paraformaldehyde sequentially. The brains were harvested and post-fixed in 4% paraformaldehyde for 24 h at 4°C. These tissue samples were then dehydrated in a graded series of sucrose solutions, embedded in optimal cutting temperature compound and cut into 10- $\mu$ m thick sections. *In vitro*, the coverslips with adherent BV-2 microglial cells were fixed with 4% paraformaldehyde for 20 min at room temperature 24 h after treatment.

The sections/coverslips were blocked in 5% normal donkey serum (cat. no. ab7475; Abcam) for 0.5 h at room temperature. Subsequently, they were incubated with the following primary antibodies overnight at 4°C: Caspase-1 (1:100; cat. no. 24232S; Cell Signaling Technology, Inc.), IL-1 $\beta$  (1:100; cat. no. 12703S; Cell Signaling Technology, Inc.), IL-18 (1:100; cat. no. ab207323; Abcam), and Iba1 (1:100; cat. no. ab15690; Abcam). The sections/coverslips were washed the following day, and the secondary antibodies, Alexa Fluor® 549 goat anti-rabbit IgG (H+L) (1:100; cat. no. ATRJN1301; Invitrogen; Thermo Fisher Scientific, Inc.) and Alexa Fluor® 488 Goat anti-mouse IgG (1:100; Invitrogen; cat. no. ATRMR2301; Thermo Fisher Scientific, Inc.) were added to the sections and

incubated for 1 h at room temperature. Finally, the sections were mounted using a fluorescent mounting medium with DAPI (Sigma-Aldrich; Merck KGaA) and visualized using a fluorescence microscope.

**Statistical analysis.** Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc.). All values are expressed as the mean  $\pm$  standard error of the mean. Repeated measures ANOVA was used to analyze the repeated measurement data. A one-way ANOVA was used to analyze the data of three or four-group univariate-factor measurements. Following ANOVA, multiple comparisons were performed using Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**ICP levels following MCAO.** The ICP levels in the IR group and the IR + NS group were significantly higher than the sham group (P<0.01). There were no significant differences between the IR group and the IR + NS group (P>0.05). The ICP levels in the IR + HS group were significantly lower compared with the IR group (P<0.01; Fig. 1A-C; Table I).

**Elevated ICP promotes ROS overproduction.** The ROS levels in the IR group and the IR + NS group were significantly higher compared with the sham group (P<0.01). There was no significant difference between the IR group and the IR + NS group (P>0.05). The ROS levels in the IR + HS group were significantly lower compared with the IR group when ICP levels were reduced by HS (P<0.01; Fig. 2A). *In vitro*, increased ROS immunofluorescence was observed in the

Table I. ICP levels 0, 2, 4, 8, 12, 16, 20 and 24 h after ischemia-reperfusion *in vivo*.

Time Group, h	Sham, mmHg	IR, mmHg	IR + NS, mmHg	IR + HS, mmHg
0	7.89±0.72	8.26±0.74 <sup>a</sup>	8.01±0.69 <sup>a</sup>	7.98±0.82 <sup>b</sup>
2	8.25±1.10	8.23±0.68 <sup>a</sup>	8.46±0.89 <sup>a</sup>	9.34±0.77 <sup>b</sup>
4	7.75±1.02	11.56±1.26 <sup>a</sup>	12.48±0.75 <sup>a</sup>	10.63±0.82 <sup>b</sup>
8	7.83±0.86	14.31±1.01 <sup>a</sup>	14.60±1.03 <sup>a</sup>	11.91±1.02 <sup>b</sup>
12	7.31±0.72	17.39±1.12 <sup>a</sup>	17.04±0.87 <sup>a</sup>	12.75±1.10 <sup>b</sup>
16	7.49±0.85	20.55±1.30 <sup>a</sup>	19.44±0.84 <sup>a</sup>	13.56±0.93 <sup>b</sup>
20	7.63±0.65	21.05±1.00 <sup>a</sup>	21.43±1.03 <sup>a</sup>	13.89±1.00 <sup>b</sup>
24	7.94±1.09	22.35±0.84 <sup>a</sup>	23.33±0.98 <sup>a</sup>	13.58±1.00 <sup>b</sup>

n=8 per group. <sup>a</sup>P<0.01 vs. sham group; <sup>b</sup>P<0.01 vs. IR group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group.

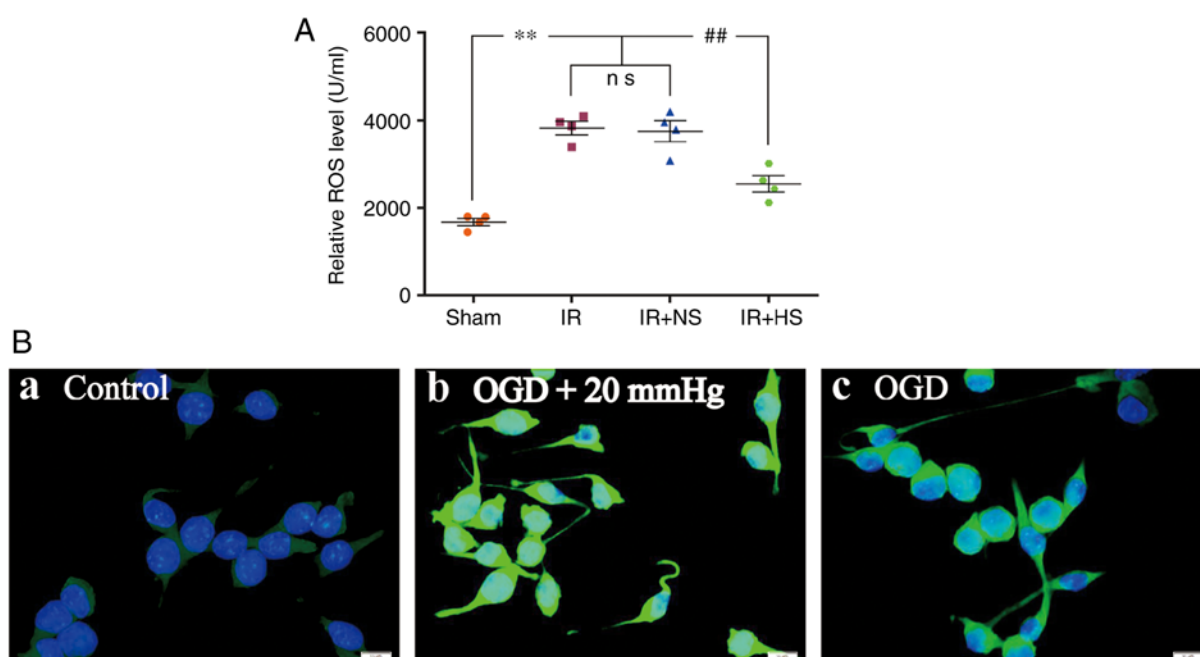


Figure 2. Elevated ICP promotes ROS overproduction in ischemic microglia both *in vivo* and *in vitro*. (A) ROS levels in the IR group and the IR + NS group were significantly higher compared with the sham group. There was no significant difference between the IR group and the IR + NS group. ROS levels in the IR + HS group were significantly lower compared with the IR group when ICP levels were reduced by HS. (B) Immunofluorescence images show the production of ROS (green) in the (B-a) control, (B-b) OGD + 20 mmHg and (B-c) OGD groups. Enhanced ROS immunofluorescence was observed in the BV-2 microglial cells of the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, ROS fluorescence was notably reduced in the OGD group without high-pressure treatment. Scale bar, 10 μm. n=4 per group. \*\*P<0.01 vs. sham group; ##P<0.01 vs. IR group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group; OGD, oxygen-glucose deprivation; ns, non-significant; ROS, reactive oxygen species.

OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, ROS fluorescence was notably reduced in the OGD group without high-pressure treatment (Fig. 2B).

**Elevated ICP promotes Caspase-1 activation.** *In vivo*, the protein expression levels of Caspase-1 were significantly increased in the IR group and the IR + NS group compared with the sham group (P<0.01). There was no significant difference between the IR group and the IR + NS group (P>0.05). The levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS

(P<0.01; Fig. 3A and B). Double immunofluorescence staining was used to examine Caspase-1 expression in the microglia of the peri-infarcted brain tissue. Increased Caspase-1 immunofluorescence was observed in the IR group and the IR + NS group compared with the sham group. When ICP levels were reduced by HS, Caspase-1 fluorescence was noticeably attenuated (Fig. 3C).

*In vitro*, the protein expression levels of Caspase-1 were significantly increased in the OGD + 20 mmHg group compared with the control group (P<0.01). Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure

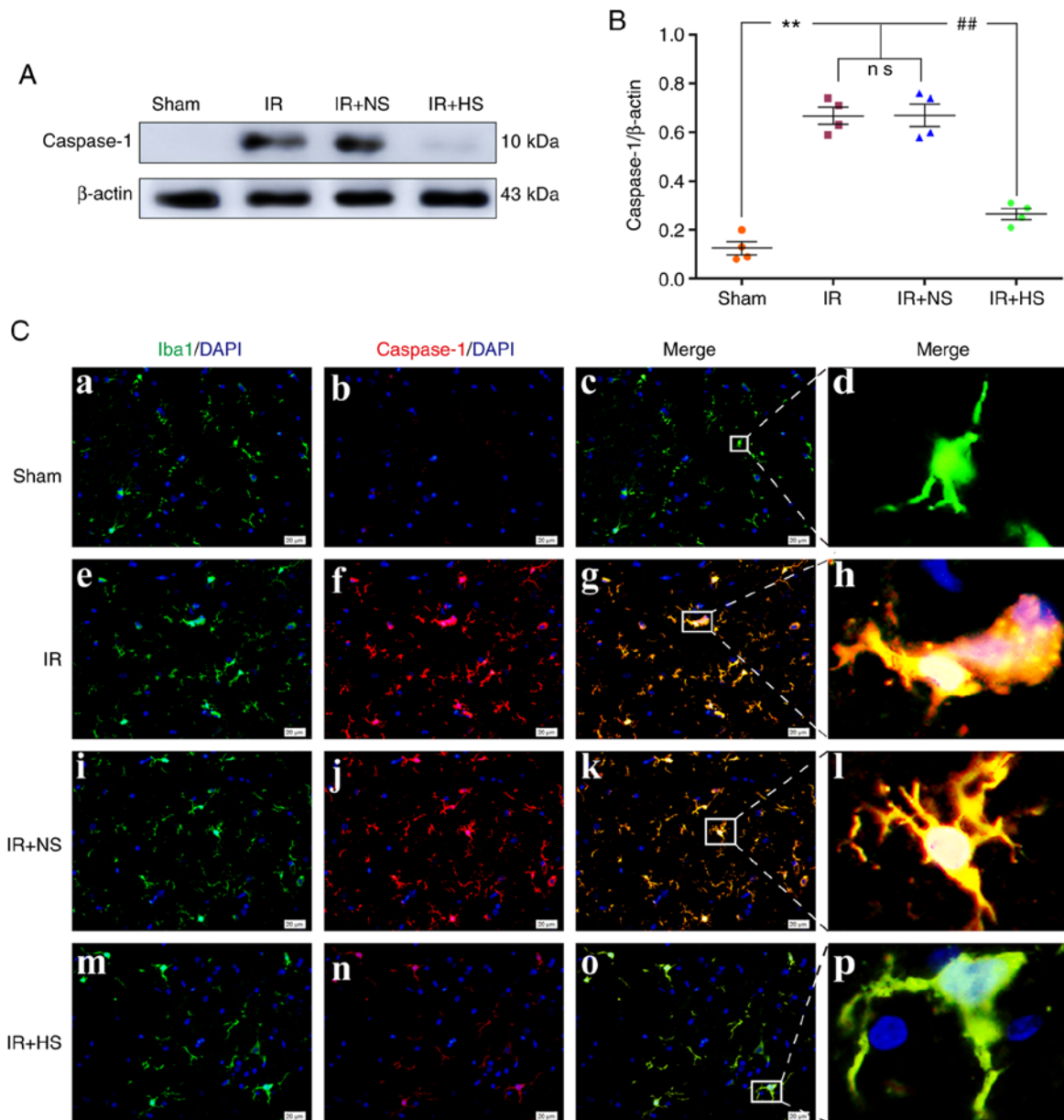


Figure 3. Elevated ICP promotes Caspase-1 expression in microglia following IR *in vivo*. (A) Immunoreactive bands of Caspase-1 (10 kDa) and  $\beta$ -actin (43 kDa). (B) Protein expression levels of Caspase-1 were significantly increased in the IR group and the IR + NS group compared with the sham group. There was no significant difference between the IR group and the IR + NS group. The levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS. (C) Immunofluorescence images showing the expression of (C-a, C-e, C-i and C-m) Iba1+ microglia (green), (C-b, C-f, C-j and C-n) Caspase-1 (red), (C-c, C-g, C-k and C-o) the co-localization of Caspase-1 and microglia, and (C-d, C-h, C-l and C-p) high amplification images of the microglial cells in peri-ischemic cortex. Increased Caspase-1 immunofluorescence was observed in the IR group and the IR + NS group compared with the sham group. When ICP levels were reduced by HS, Caspase-1 fluorescence was notably attenuated. Scale bar, 20  $\mu$ m. n=4 per group. \*\*P<0.01 vs. sham group; ##P<0.01 vs. IR group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group; ns, non-significant.

treatment (P<0.01; Fig. 4A and B). Double immunofluorescence staining was used to examine Caspase-1 expression in the BV-2 microglial cells. Increased Caspase-1 immunofluorescence was observed in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the fluorescence was notably reduced in the OGD group without high-pressure treatment (Fig. 4C).

**Elevated ICP increases IL-1 $\beta$  expression.** The protein expression levels of IL-1 $\beta$  *in vivo* were significantly increased in the IR group and the IR + NS group compared with the

sham group (P<0.01). There were no significant differences between the IR and the IR + NS group (P>0.05). The IL-1 $\beta$  expression levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS (P<0.01; Fig. 5A and B). Double immunofluorescence staining was used to examine IL-1 $\beta$  expression in the microglia of the peri-infarcted brain tissue. Increased IL-1 $\beta$  immunofluorescence was observed in the IR group and the IR + NS group compared with the sham group. When ICP levels were reduced by HS, IL-1 $\beta$  fluorescence was notably attenuated (Fig. 5C).



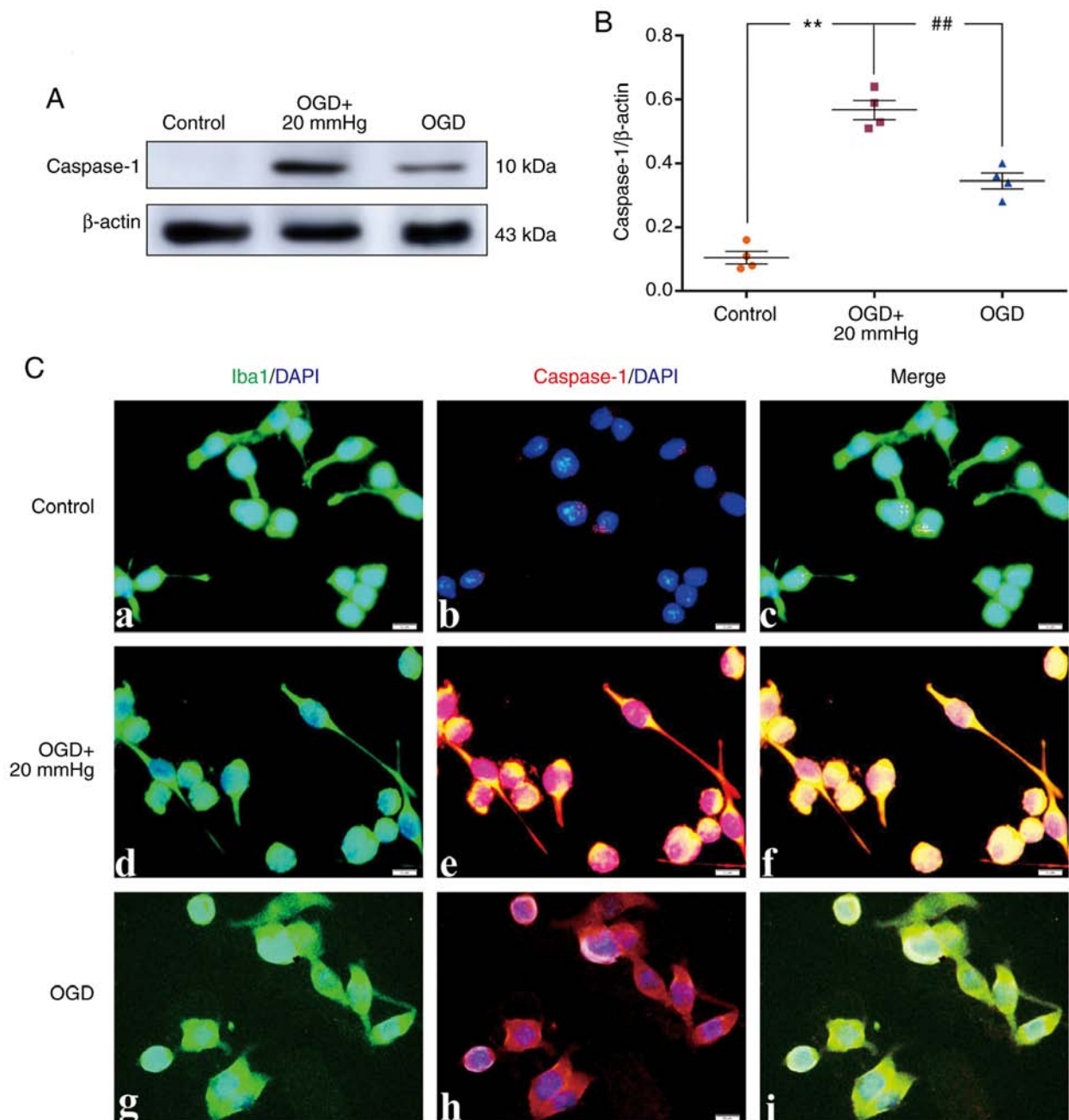


Figure 4. Elevated pressure promotes Caspase-1 expression in BV-2 microglial cells following OGD *in vitro*. (A) Immunoreactive bands of Caspase-1 (10 kDa) and  $\beta$ -actin (43 kDa). (B) Protein expression levels of Caspase-1 were significantly increased in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment. (C) Immunofluorescence images showing the expression of (C-a, C-d and C-g) Iba1+ microglia (green), (C-b, C-e and C-h) Caspase-1 (red), and (C-c, C-f and C-i) the co-localization of Caspase-1 and microglia. Increased Caspase-1 immunofluorescence was observed in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the fluorescence was notably reduced in the OGD group without high-pressure treatment. Scale bar, 10  $\mu$ m. n=4 per group. \*\*P<0.01 vs. control group; ##P<0.01 vs. OGD + 20 mmHg group. OGD, oxygen-glucose deprivation.

*In vitro*, the protein expression levels of IL-1 $\beta$  were significantly increased in the OGD + 20 mmHg group compared with the control group (P<0.01). Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment (P<0.01; Fig. 6A and B). Double immunofluorescence staining was used to examine IL-1 $\beta$  expression in the BV-2 microglial cells. Enhanced IL-1 $\beta$  immunofluorescence was observed in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg

group, the fluorescence was notably reduced in the OGD group without high-pressure treatment (Fig. 6C).

**Elevated ICP increases IL-18 expression.** The protein expression levels of IL-18 *in vivo* were significantly increased in the IR group and the IR + NS group compared with the sham group (P<0.01). There were no significant differences between the IR group and the IR + NS group (P>0.05). IL-18 expression levels in the IR + HS group were significantly lower when ICP levels were reduced by HS compared with the IR group

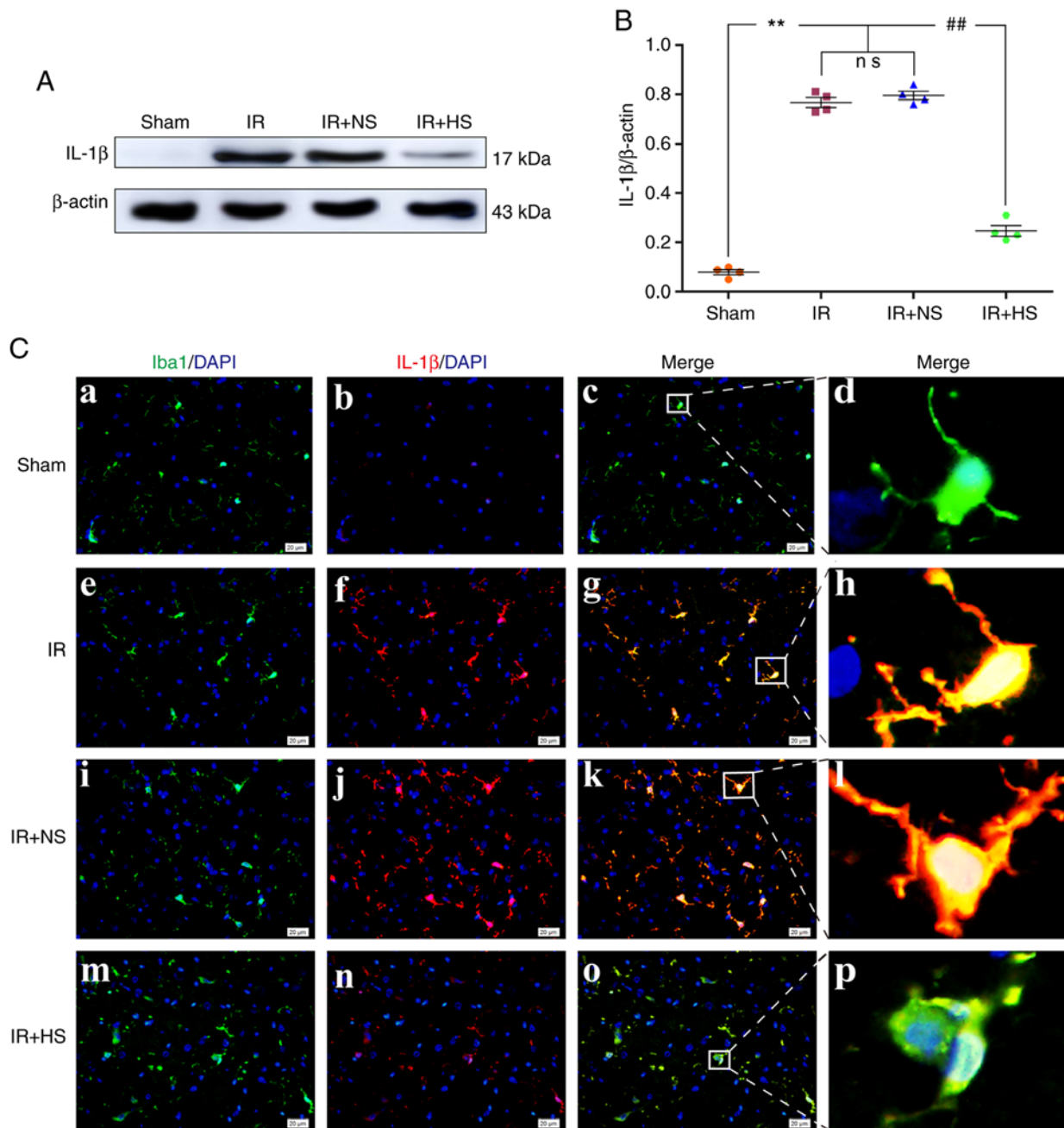


Figure 5. Elevated ICP increases IL-1 $\beta$  expression in the microglia following IR *in vivo*. (A) Immunoreactive bands of IL-1 $\beta$  (17 kDa) and  $\beta$ -actin (43 kDa). (B) Protein expression levels of IL-1 $\beta$  were significantly increased in the IR and the IR + NS groups compared with the sham group. There were no significant differences between the IR and the IR + NS group. The levels in the IR + HS group were significantly lower than in the IR group when ICP levels were reduced by HS. (C) Immunofluorescence images showing the expression of (C-a, C-e, C-i and C-m) Iba1+ microglia (green), (C-b, C-f, C-j and C-n) IL-1 $\beta$  (red), (C-c, C-g, C-k and C-o) the co-localization of IL-1 $\beta$  and microglia, and (C-d, C-h, C-l and C-p) high amplification images of the microglial cells in peri-ischemic cortex. Enhanced IL-1 $\beta$  immunofluorescence was observed in the IR and the IR + NS groups compared with the sham group. In the IR + HS group, IL-1 $\beta$  fluorescence was notably decreased. Scale bar, 20  $\mu$ m. n=4 per group. \*\*P<0.01 vs. sham group; ##P<0.01 vs. IR group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group; ns, non-significant.

(P<0.01; Fig. 7A and B). Double immunofluorescence staining was used to examine IL-18 expression in the microglia of the peri-infarcted brain tissue. Enhanced IL-18 immunofluorescence was observed in the IR group and the IR + NS group compared with the sham group. When ICP levels were reduced by HS, IL-18 fluorescence was noticeably attenuated (Fig. 7C).

*In vitro*, the protein expression levels of IL-18 were significantly increased in the OGD + 20 mmHg group compared with the control group (P<0.01). Compared with

the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment (P<0.01; Fig. 8A and B). Double immunofluorescence staining was used to examine IL-18 expression in the BV-2 microglial cells. Enhanced IL-18 immunofluorescence was observed in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, fluorescence was notably reduced in the OGD group without high-pressure treatment (Fig. 8C).

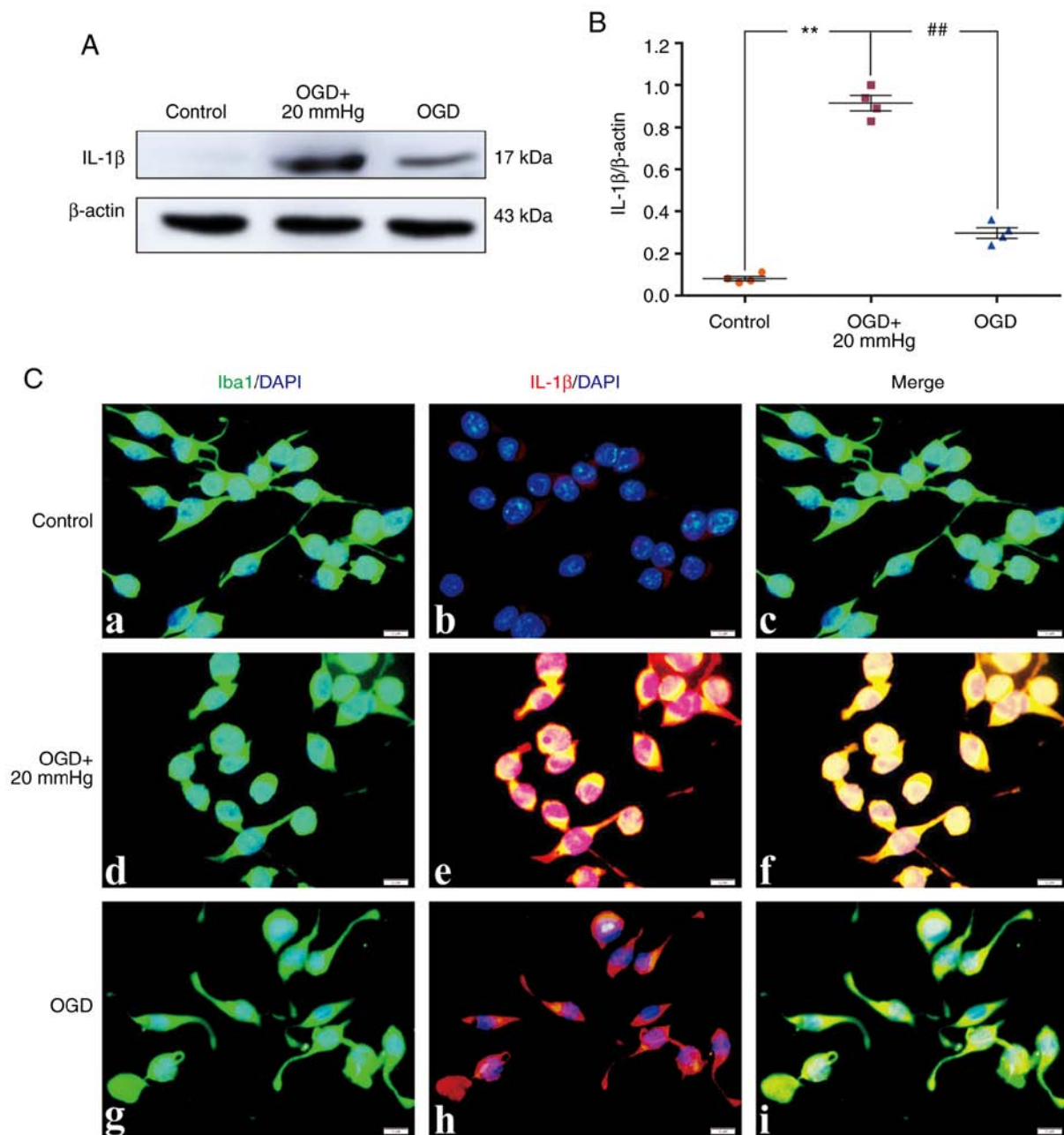


Figure 6. Elevated pressure increases IL-1 $\beta$  expression in BV-2 microglial cells following OGD *in vitro*. (A) Immunoreactive bands of IL-1 $\beta$  (17 kDa) and  $\beta$ -actin (43 kDa). (B) Protein expression levels of IL-1 $\beta$  were significantly increased in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment. (C) Immunofluorescence images showing the expression of (C-a, C-d and C-g) Iba1+ microglia (green), (C-b, C-e and C-h) IL-1 $\beta$  (red), and (C-c, C-f and C-i) the co-localization of IL-1 $\beta$  and microglia. Enhanced IL-1 $\beta$  immunofluorescence was observed in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the fluorescence was notably reduced in the OGD group without high-pressure treatment. Scale bar, 10  $\mu$ m. n=4 per group. \*\*P<0.01 vs. control group; ##P<0.01 vs. OGD + 20 mmHg group. OGD, oxygen-glucose deprivation.

**Elevated ICP increases GSDMD-N expression.** The protein expression levels of GSDMD-N *in vivo* were significantly increased in the IR group and the IR + NS group compared with the sham group (P<0.01). There were no significant differences between the IR and the IR + NS group (P>0.05). The levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS (P<0.01; Fig. 9A and B).

*In vitro*, the protein expression levels of GSDMD-N were significantly increased in the OGD + 20 mmHg group compared with the control group (P<0.01). Compared with

the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment (P<0.01; Fig. 9C and D).

## Discussion

In the present study, it was shown that elevated ICP promoted NLRP3 inflammasome activation in microglia of ischemic adult rats. This was evident based on the increased expression levels of Caspase-1, GSDMD-N, IL-18 and IL-1 $\beta$  in the ischemia-activated microglial cells.



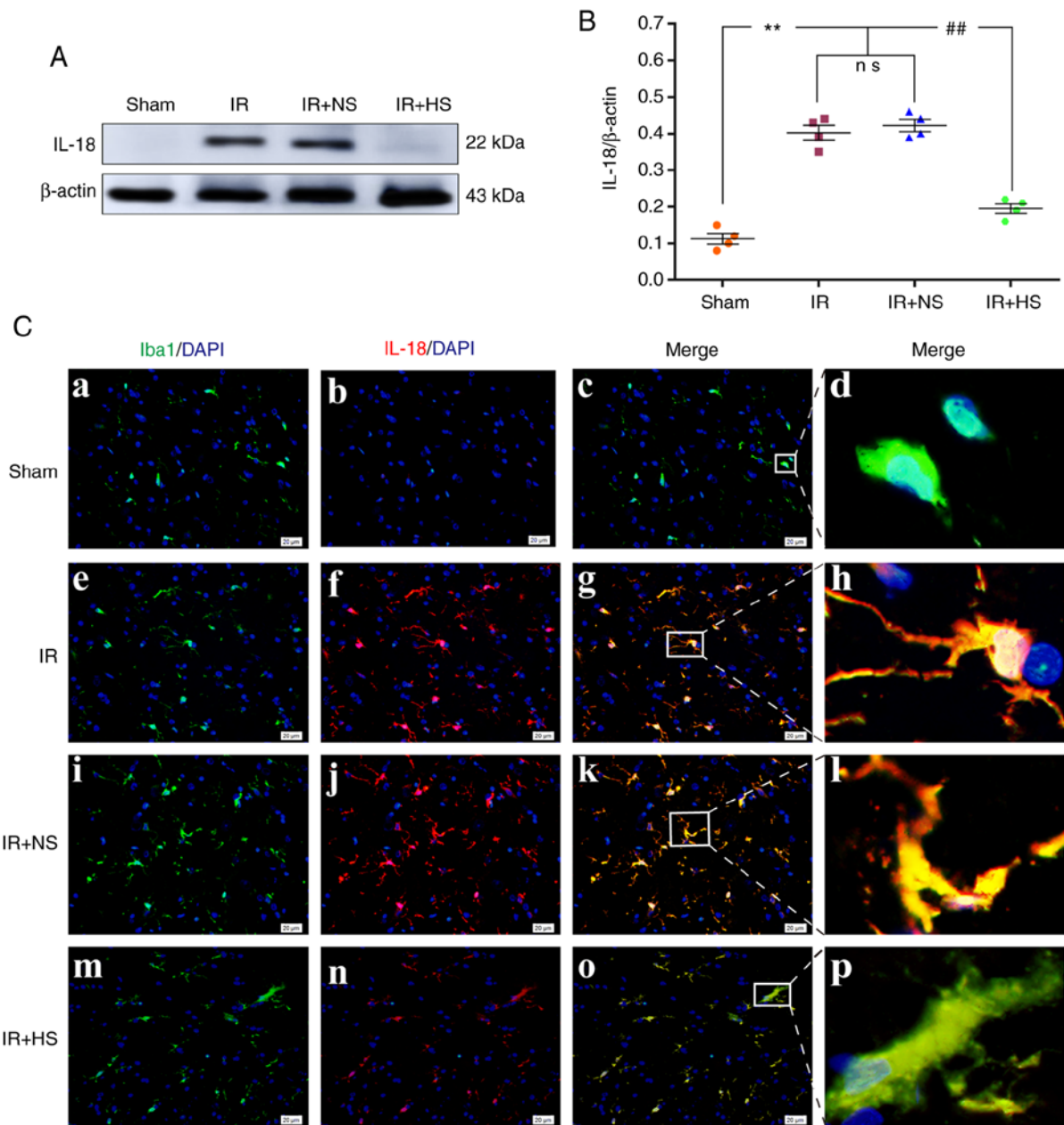


Figure 7. Elevated ICP increases IL-18 expression in microglia following IR *in vivo*. (A) Immunoreactive bands of IL-18 (22 kDa) and  $\beta$ -actin (43 kDa). (B) Protein expression levels of IL-18 were significantly increased in the IR group and the IR + NS group compared with the sham group. There was no significant difference between the IR group and the IR + NS group. The levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS. (C) Immunofluorescence images showing the expression of (C-a, C-e, C-i and C-m) Iba1+ microglia (green), (C-b, C-f, C-j and C-n) IL-18 (red), (C-c, C-g, C-k and C-o) the co-localization of IL-18 and microglia, and (C-d, C-h, C-l and C-p) high amplification images of the microglial cells in peri-ischemic cortex. Increased IL-18 immunofluorescence was observed in the IR group and the IR + NS group compared with the sham group. In the IR + HS group, IL-18 fluorescence was notably reduced. Scale bar, 20  $\mu$ m. n=4 per group. \*\*P<0.01 vs. sham group; ##P<0.01 vs. IR group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group; ns, non-significant.

ICP management is an essential means of preventing secondary injury in the central nervous system following ischemic stroke (5-7). Osmotherapy has been used as a foundation for managing elevated ICP levels induced by cerebral edema (5,6). Commonly used osmotic agents include HS and mannitol (28,29). In the present study, to determine the effects of HS on ICP following ischemic stroke, the ICP of the rats were examined 0, 2, 4, 8, 12, 16, 20 and 24 h after MCAO. The ICP levels were significantly increased following cerebral IR. Following 10% HS treatment by intravenous injection, the ICP of the rats after MCAO was significantly reduced. This was

consistent with the effects of HS on traumatic brain injury (30) and subarachnoid hemorrhage (31).

The NLRP3 inflammasome can be triggered by ROS (20-23). It has been reported that ROS levels are increased during high altitude exposure in lowlanders, which induced passive hypobaric hypoxia; optic nerve sheath diameter (ONSD), which is an indirect measurement of ICP, is concurrently increased. However, regression analysis did not infer a causal relationship between oxidative stress biomarkers and changes in ONSD (32). In the present study, the ROS levels of the brain tissue were increased when ICP was increased

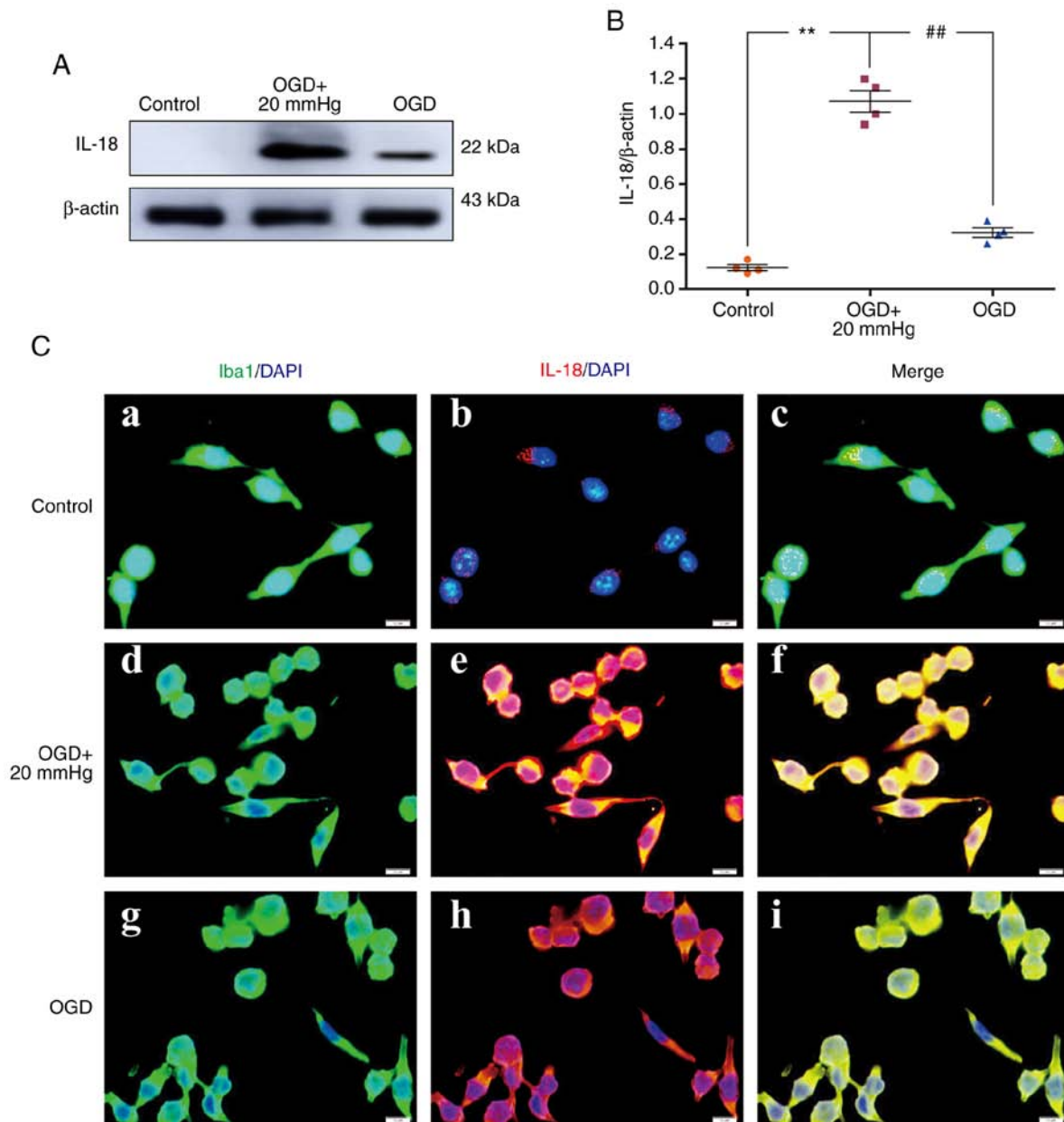


Figure 8. Elevated pressure increases IL-18 expression in BV-2 microglial cells following OGD *in vitro*. (A) Immunoreactive bands of IL-18 (22 kDa) and  $\beta$ -actin (43 kDa). (B) Protein expression levels of IL-18 were significantly increased in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment. (C) Immunofluorescence images showing the expression of (C-a, C-d and C-g) Iba1+ microglia (green), (C-b, C-e and C-h) IL-18 (red), and (C-c, C-f and C-i) the co-localization of IL-18 and microglia. Enhanced IL-18 immunofluorescence was observed in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the fluorescence was notably reduced in the OGD group without high-pressure treatment. Scale bar, 10  $\mu$ m. n=4 per group. \*\*P<0.01 vs. control group; ##P<0.01 vs. OGD + 20 mmHg group. OGD, oxygen-glucose deprivation.

following cerebral IR. Additionally, ROS overproduction was inhibited by reduced ICP. Furthermore, high pressure (20 mmHg) combined with OGD treatment increased ROS production in the BV-2 microglial cells compared with those subjected to OGD treatment alone *in vitro*. These results suggested that elevated ICP can enhance oxidative stress. To determine whether elevated ICP activated the NLRP3 inflammasome via ROS overproduction in ischemia-activated microglia, expression of Caspase-1, GSDMD-N, IL-18 and IL-1 $\beta$  in the microglia were determined both *in vivo* and *in vitro*. It was shown that elevated pressure upregulated the expression of Caspase-1, GSDMD-N, IL-18 and IL-1 $\beta$  in

IR- or OGD-treated microglia both *in vivo* and *in vitro*. More importantly, Caspase-1, GSDMD-N, IL-18 and IL-1 $\beta$  expression in microglia was significantly downregulated when the elevated pressure was reduced or removed. These results suggested that elevated ICP increased NLRP3 inflammasome activation in ischemia-activated microglial cells via induction of ROS overproduction.

However, there are limitations to the present study. Firstly, it has been reported that activated microglia secrete pro-inflammatory cytokines, including TNF- $\alpha$ , IL-18 and IL-1 $\beta$  (33-35). However, the lack of investigation of other cytokines (such as TNF- $\alpha$ ) in the present study, is a limitation

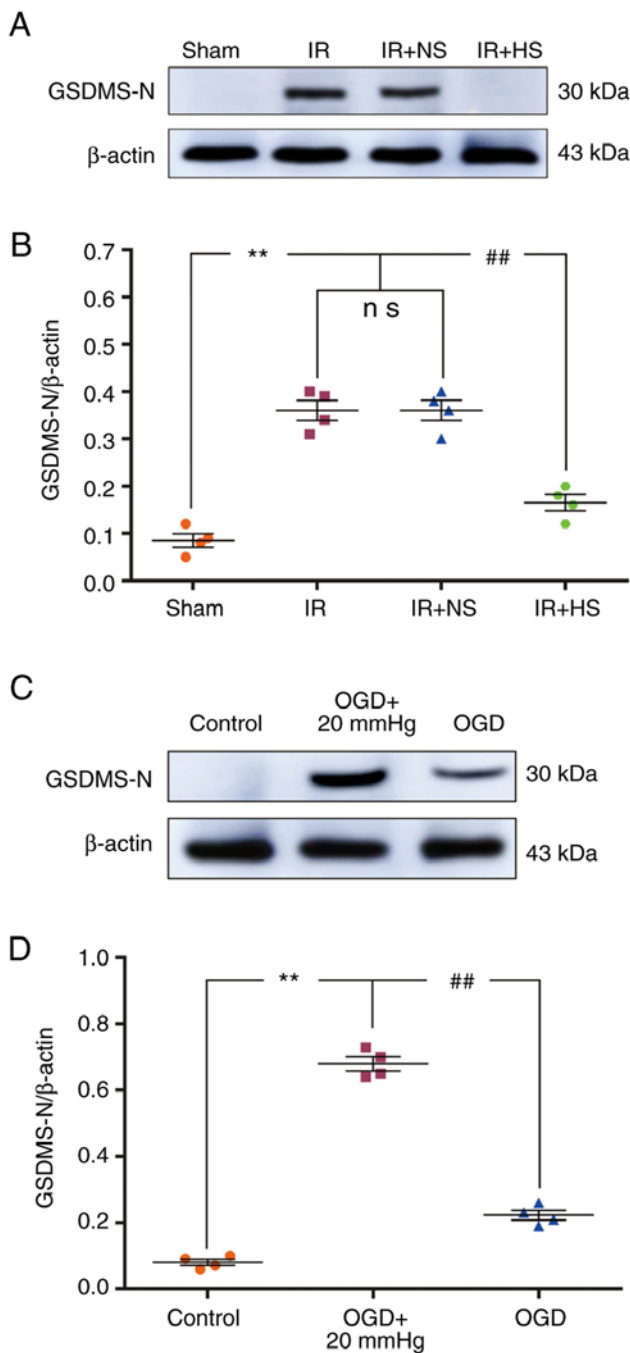


Figure 9. Elevated ICP increases GSDMD-N expression in ischemic microglia both *in vivo* and *in vitro*. (A and C) Immunoreactive bands of GSDMD-N (30 kDa) and  $\beta$ -actin (43 kDa). (B) Protein expression levels of GSDMD-N were significantly increased in the IR group and the IR + NS group compared with the sham group. There was no significant difference between the IR group and the IR + NS group. The levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS. (D) *In vitro*, the protein expression levels of GSDMD-N were significantly increased in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment.  $n=4$  per group. \*\* $P<0.01$  vs. sham group or control group; ## $P<0.01$  vs. IR group or OGD + 20 mmHg group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group; OGD, oxygen-glucose deprivation; ns, non-significant; GSDMD-N, gasdermin D-N domains.

and an area for future research. Secondly, the present study did not determine the upstream mechanism by which elevated

ICP promotes ROS overproduction in ischemic stroke. It has been found that ischemia damages mitochondria and induces ROS production (36,37). Mitophagy can eliminate damaged mitochondria, reduce ROS production and then alleviate NLRP3 inflammasome activation (38). Elevated ICP may promote ROS overproduction by inhibiting mitophagy. It has been reported that the NLRP3 inflammasome is expressed in astrocytes (39). However, the lack of determination of NLRP3 inflammasome expression in astrocytes in the present study is another limitation of the present study. Finally, all animals were observed closely for 24 h after IR in the present study. However, the lack of determination of factors after various time points is another limitation.

In summary, elevated ICP was found to upregulate the expression of Caspase-1, GSDMD-N, IL-18 and IL-1 $\beta$  in ischemic microglia, which was significantly downregulated when ICP was reduced. Thus, elevated ICP-induced Caspase-1, GSDMD-N, IL-18 and IL-1 $\beta$  overproduction in the microglia may be a potential target for mitigating neuroinflammation following an ischemic stroke.

### Acknowledgements

The authors would like to thank Mr Zhengkang Ding and Miss Zixi Yang (Guangdong Provincial People's Hospital, Guangzhou, China) for the technical support.

### Funding

The present work was supported by the National Natural Science Foundation for Young Scientists of China (grant no. 82002074), the Medical Scientific Research Foundation of Guangdong Province (grant nos. A2019135 and A2017284), the Scientific research project of Guangdong Traditional Chinese Medicine Bureau (grant no. 20201045), and the Science and Technology Program of Guangzhou (grant no. 202002030338).

### Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

HZ conceived the project and designed the experiments. HD carried out the assessment of ICP and ROS evaluation. YL established the rat model of MCAO. MW carried out BV-2 microglial cell cultures and treatment. XL and YH performed western blotting and immunofluorescence staining. HD conducted the statistical analysis. HD and YL wrote the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The Research Ethics Committee of Guangdong Provincial People's Hospital and Guangdong Academy of Medical Sciences approved all animal procedure protocols [approval no. GDREC2012106A(R1); Guangzhou, China].



## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

- Phipps MS and Cronin CA: Management of acute ischemic stroke. *BMJ* 368: l6983, 2020.
- Khandelwal P, Yavagal DR and Sacco RL: Acute ischemic stroke intervention. *J Am Coll Cardiol* 67: 2631-2644, 2016.
- Blaha M, Schwab J, Vajnerova O, Bednar M, Vajner L and Michal T: Intracranial pressure and experimental model of diffuse brain injury in rats. *J Korean Neurosurg Soc* 47: 7-10, 2010.
- Caltagirone C, Cisari C, Schievano C, Di Paola R, Cordaro M, Bruschetta G, Esposito E and Cuzzocrea S: Co-ultramicrosized palmitoylethanolamide/luteolin in the treatment of cerebral ischemia: From rodent to man. *Transl Stroke Res* 7: 54-69, 2016.
- Robinson JD: Management of refractory intracranial pressure. *Crit Care Nurs Clin North Am* 28: 67-75, 2016.
- Shah A, Almenawer S and Hawryluk G: Timing of decompressive craniectomy for ischemic stroke and traumatic brain injury: A review. *Front Neurol* 10: 11, 2019.
- Wei CC, Zhang ST, Tan G, Zhang SH and Liu M: Impact of anemia on in-hospital complications after ischemic stroke. *Eur J Neurol* 25: 768-774, 2018.
- Jin R, Yang G and Li G: Inflammatory mechanisms in ischemic stroke: Role of inflammatory cells. *J Leukoc Biol* 87: 779-789, 2010.
- Zhao SC, Ma LS, Chu ZH, Xu H, Wu WQ and Liu F: Regulation of microglial activation in stroke. *Acta Pharmacol Sin* 38: 445-458, 2017.
- Wang S, Zhang H and Xu Y: Crosstalk between microglia and T cells contributes to brain damage and recovery after ischemic stroke. *Neurol Res* 38: 495-503, 2016.
- Zhao TZ, Ding Q, Hu J, He SM, Shi F and Ma LT: GPER expressed on microglia mediates the anti-inflammatory effect of estradiol in ischemic stroke. *Brain Behav* 6: e449, 2016.
- Ma Y, Wang J, Wang Y and Yang GY: The biphasic function of microglia in ischemic stroke. *Prog Neurobiol* 157: 247-272, 2017.
- Goldmann T, Tay TL and Prinz M: Love and death: Microglia, NLRP3 and the Alzheimer's brain. *Cell Res* 23: 595-596, 2013.
- Cho MH, Cho K, Kang HJ, Jeon EY, Kim HS, Kwon HJ, Kim HM, Kim DH and Yoon SY: Autophagy in microglia degrades extracellular  $\beta$ -amyloid fibrils and regulates the NLRP3 inflammasome. *Autophagy* 10: 1761-1775, 2014.
- Houtman J, Freitag K, Gimber N, Schmoranzler J, Heppner FL and Jendrach M: Beclin1-driven autophagy modulates the inflammatory response of microglia via NLRP3. *Embo J* 38: e99430, 2019.
- Panicker N, Sarkar S, Harischandra DS, Neal M, Kam TI, Jin H, Saminathan H, Langley M, Charli A, Samidurai M, *et al*: Fyn kinase regulates misfolded  $\alpha$ -synuclein uptake and NLRP3 inflammasome activation in microglia. *J Exp Med* 216: 1411-1430, 2019.
- Liu HD, Li W, Chen ZR, Hu YC, Zhang DD, Shen W, Zhou ML, Zhu L and Hang CH: Expression of the NLRP3 inflammasome in cerebral cortex after traumatic brain injury in a rat model. *Neurochem Res* 38: 2072-2083, 2013.
- Zhang N, Zhang X, Liu X, Wang H, Xue J, Yu J, Kang N and Wang X: Chrysophanol inhibits NALP3 inflammasome activation and ameliorates cerebral ischemia/reperfusion in mice. *Mediators Inflamm* 2014: 370530, 2014.
- Gustin A, Kirchmeyer M, Koncina E, Felten P, Losciuto S, Heurtaux T, Tardivel A, Heuschling P and Dostert C: NLRP3 inflammasome is expressed and functional in mouse brain microglia but not in astrocytes. *PLoS One* 10: e130624, 2015.
- Xu X, Zhang L, Ye X, Hao Q, Zhang T, Cui G and Yu M: Nrf2/ARE pathway inhibits ROS-induced NLRP3 inflammasome activation in BV2 cells after cerebral ischemia reperfusion. *Inflamm Res* 67: 57-65, 2018.
- Wang H, Zhong D, Chen H, Jin J, Liu Q and Li G: NLRP3 inflammasome activates interleukin-23/interleukin-17 axis during ischemia-reperfusion injury in cerebral ischemia in mice. *Life Sci* 227: 101-113, 2019.
- Kelley N, Jeltima D, Duan Y and He Y: The NLRP3 inflammasome: An overview of mechanisms of activation and regulation. *Int J Mol Sci* 20: 3328, 2019.
- Harijith A, Ebenezer DL and Natarajan V: Reactive oxygen species at the crossroads of inflammasome and inflammation. *Front Physiol* 5: 352, 2014.
- Fann DY, Lee SY, Manzanero S, Chunduri P, Sobey CG and Arumugam TV: Pathogenesis of acute stroke and the role of inflammasomes. *Ageing Res Rev* 12: 941-966, 2013.
- Yang F, Wang Z, Wei X, Han H, Meng X, Zhang Y, Shi W, Li F, Xin T, Pang Q and Yi F: NLRP3 deficiency ameliorates neurovascular damage in experimental ischemic stroke. *J Cereb Blood Flow Metab* 34: 660-667, 2014.
- Huang LQ, Zhu GF, Deng YY, Jiang WQ, Fang M, Chen CB, Cao W, Wen MY, Han YL and Zeng HK: Hypertonic saline alleviates cerebral edema by inhibiting microglia-derived TNF- $\alpha$  and IL-1 $\beta$ -induced Na-K-Cl Cotransporter up-regulation. *J Neuroinflammation* 11: 102, 2014.
- Ding HG, Deng YY, Yang RQ, Wang QS, Jiang WQ, Han YL, Huang LQ, Wen MY, Zhong WH, Li XS, *et al*: Hypercapnia induces IL-1 $\beta$  overproduction via activation of NLRP3 inflammasome: Implication in cognitive impairment in hypoxemic adult rats. *J Neuroinflammation* 15: 4, 2018.
- Changa AR, Czeisler BM and Lord AS: Management of elevated intracranial pressure: A review. *Curr Neurol Neurosci Rep* 19: 99, 2019.
- Fernando SM, Tran A, Cheng W, Rochwerf B, Taljaard M, Kyeremanteng K, English SW, Sekhon MS, Griesdale D, Dowlatshahi D, *et al*: Diagnosis of elevated intracranial pressure in critically ill adults: Systematic review and meta-analysis. *BMJ* 366: 14225, 2019.
- Wu AG, Samadani U, Slusher TM, Zhang L and Kiragu AW: 23.4% hypertonic saline and intracranial pressure in severe traumatic brain injury among children: A 10-year retrospective analysis. *Pediatr Crit Care Med* 20: 466-473, 2019.
- Pasarikovski CR, Alotaibi NM, Al-Mufti F and Macdonald RL: Hypertonic saline for increased intracranial pressure after aneurysmal subarachnoid hemorrhage: A systematic review. *World Neurosurg* 105: 1-6, 2017.
- Strapazzon G, Malacrida S, Vezzoli A, Dal Cappello T, Falla M, Lochner P, Moretti S, Procter E, Brugger H and Mrakic-Sposta S: Oxidative stress response to acute hypobaric hypoxia and its association with indirect measurement of increased intracranial pressure: A field study. *Sci Rep* 6: 32426, 2016.
- Abdullah Z, Rakkar K, Bath PM and Bayraktutan U: Inhibition of TNF- $\alpha$  protects in vitro brain barrier from ischaemic damage. *Mol Cell Neurosci* 69: 65-79, 2015.
- Wong R, Lénárt N, Hill L, Toms L, Coutts G, Martinecz B, Császár E, Nyíri G, Papaemmanouil A, Waisman A, *et al*: Interleukin-1 mediates ischaemic brain injury via distinct actions on endothelial cells and cholinergic neurons. *Brain Behav Immun* 76: 126-138, 2019.
- Kho DT, Johnson R, Robilliard L, du Mez E, McIntosh J, O'Carroll SJ, Angel CE and Graham ES: ECIS technology reveals that monocytes isolated by CD14+ve selection mediate greater loss of BBB integrity than untouched monocytes, which occurs to a greater extent with IL-1 $\beta$  activated endothelium in comparison to TNF $\alpha$ . *PLoS One* 12: e180267, 2017.
- Liang JM, Xu HY, Zhang XJ, Li X, Zhang HB and Ge PF: Role of mitochondrial function in the protective effects of ischaemic preconditioning on ischaemia/reperfusion cerebral damage. *J Int Med Res* 41: 618-627, 2013.
- Li H, Feng J, Zhang Y, Feng J, Wang Q, Zhao S, Meng P and Li J: Mst1 deletion attenuates renal ischaemia-reperfusion injury: The role of microtubule cytoskeleton dynamics, mitochondrial fission and the GSK3 $\beta$ -p53 signalling pathway. *Redox Biol* 20: 261-274, 2019.
- Deretic V and Levine B: Autophagy balances inflammation in innate immunity. *Autophagy* 14: 243-251, 2018.
- Heneka MT, McManus RM and Latz E: Inflammasome signalling in brain function and neurodegenerative disease. *Nat Rev Neurosci* 19: 610-621, 2018.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.