

Expression of interferon regulatory factor 4 and inflammation in secondary injury of intracerebral haemorrhage

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Abstract

The aim of this study was to investigate the expression of interferon regulatory factor 4 (IRF4) and the inflammatory response in secondary injury of intracerebral haemorrhage (ICH). Twelve SD rats were randomly divided into a sham group and an ICH group, with 6 rats in each group. A rat model of ICH was established by injecting collagenase type IV into the right striatum of rats. The expression of IRF4 was measured by western blot and immunohistochemistry 48 h after ICH. In addition, 15 μ m of hemin-induced PC12 cell injury was used to simulate an *in vitro* ICH model. IRF4 expression was detected by immunofluorescence (IF). Moreover, the inflammatory cytokines (IL-1 β and IL-6) were measured by ELISA. The behavioural score of ICH rats was the lowest at 48 h after operation. The expression of IRF4 was significantly higher in the striatal tissue of ICH rats compared with the sham group ($p < 0.05$). Meanwhile, IF results showed that hemin induced the upregulation of IRF4 expression in rat pheochromocytoma cells PC12. In addition, IL-1 β and IL-6 levels were significantly increased in the serum of ICH rats and in the supernatant of hemin-induced PC12 cells ($p < 0.01$). The inflammation in ICH is related to the increase of IRF4. It provides a theoretical basis for the clinical treatment of ICH.

Key words: intracerebral haemorrhage (ICH), secondary injury, interferon regulatory factor 4 (IRF4), inflammation, treatment.

Introduction

Intracerebral haemorrhage (ICH) refers to bleeding caused by non-traumatic vascular rupture within the brain. It can trigger blood to enter the brain tissue, leading to brain injury and ultimately disability or death [8]. After haemorrhage, the perihematomal brain tissue is compressed and ischaemic, and the specific mechanisms of this process include the activation of excitatory amino acids and their receptors, persistent neuronal depolarization and the release of inflammatory cytokines, and the activa-

tion of protein kinases [11,26]. Among cerebrovascular diseases, ICH is a common event, accounting for approximately 10-15% of cases. ICH is associated with a 1-month mortality rate of about 40%, which increases with age [13,24]. In addition to primary brain injury caused by direct mechanical injury from haemorrhage, ICH can lead to secondary brain injury, resulting in neurological deterioration [14,15]. Perihematomal oedema occurs within a few hours after the onset of ICH, leading to secondary injury as shown by blood-brain barrier dysfunction – mac-

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rophage and neutrophil infiltration and haematoma expansion occur, and ultimately neuronal death [4,12]. Secondary injury after ICH is complex. Inflammatory cytokines are important players in secondary injury after ICH, and an increase in pro-inflammatory cytokines may exacerbate tissue damage [7,30]. Therefore, it is of great clinical significance to deeply study the cellular and molecular mechanisms of haemorrhagic brain injury.

Interferon regulatory factors (IRFs) are critical transcription factors for the differentiation and maturation of T cells, B cells, and plasma cells [1]. IRFs not only regulate interferons to participate in immune regulation, but also play an important role in regulating cell differentiation, proliferation, and apoptosis [9,17,18]. As a crucial member of the IRF family, IRF4 can perform regulation in the transcription of a variety of genes and has a wide range of biological roles [19,20]. For example, the involvement of IRF4 in autoimmune diseases (such as systemic lupus erythematosus, multiple sclerosis, and inflammatory bowel disease) is achieved by regulating the development and function of T cells, B cells, and dendritic cells [16,28]. The regulation of IRF4 in pressure overload-triggered cardiac hypertrophy is available by activating the transcription of cardiac CREB [10]. Also, after cerebral ischaemia-reperfusion injury, an increase of IRF4 expression in neural cells and its involvement in inflammation are confirmed in the literature [5,6]. However, there are no literature reports on the role of IRF4 in secondary injury of ICH. Therefore, we examined the expression of IRF4 in ICH by *in vivo* rat ICH model and *in vitro* cellular model to investigate the role of IRF4 and inflammation in ICH. This study is clinically meaningful and provides a theoretical basis for the development of new targeted drugs for the prevention and treatment of ICH.

Material and methods

Animals

A total of 12 adult male SD rats (weight: 280-320 g, age: 9-11 weeks) were obtained from the Laboratory Animal Science Centre of Hebei Medical University. The experimental animals were kept under identical conditions (room temperature 25°C, 12-hour light-dark cycle) and were allowed free access to food and water. The experimental protocol conformed to the guidelines of Hebei Medical University and the "Guide for the Care and Use of Laboratory Ani-

mals" (National Institutes of Health Publication No. 80-23). Additionally, our trials were conducted with the approval of the Animal Care and Use Committee of Hebei Medical University. Each rat was assigned a random identification number.

Induction of intracerebral haemorrhage

The ICH rat model was induced by collagenase type IV [29]. After fasting for a night, the rats were given pentobarbital sodium (40 mg/kg, intraperitoneal injection) for anaesthesia. On completion of anaesthesia, the rats were fixed on a stereotaxic device (Neurostar, model: SD252). The scalp was incised (2.4 mm posterior to and 3.5 mm right lateral to the bregma) at a depth of 6 mm. Subsequently, 1 µl of bacterial collagenase (0.35 U, type IV, Sigma Aldrich) dissolved in 0.9% sterile saline was injected into the right globus pallidus using a 5 µl Hamilton syringe. The injection lasted for 5 min, and the needle was retained for 5 min. The bone hole was closed with bone wax, and the scalp wound was sutured. The final step was to place each rat in an incubator for rehabilitation. In the sham operation group (sham group, $n = 6$), the same site was injected with 1 µl of 0.9% sterile saline without collagenase.

Sampling

On completion of anaesthesia using 0.4% pentobarbital sodium (40 mg/kg, intraperitoneal injection), the thoracic cavity of each rat was opened to expose the heart. The needle was inserted into the left ventricle with precooled 0.9% normal saline, and the right auricle was cut open to completely exclude the blood from the body. Afterwards, the brain was removed and the fur on the dorsal side of the brain was longitudinally cut along the centre. The skull was then dissected to completely remove the brain. The final step was to dissect and obtain the perihematomal striatum.

Cell culture

Well-differentiated rat pheochromocytoma cells (PC12) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in 1640 complete medium (Gibco, USA) supplemented with 10% foetal bovine serum (FBS, Gibco) with culture conditions of 37°C and a humidified atmosphere of 5 CO₂. Finally, the cells were exposed to 150 µM hemin for 4 h [22].

In the experiment, the cells were separated into the control group and the hemin group.

Forelimb placing test

The trunk of each rat was grasped to suspend its forelimbs freely, and the rat was gently shaken up and down to relax its muscles. Then whiskers of the rat were touched with the edge of the table corner. The healthy rats quickly placed their ipsilateral forelimb above the table corner, but this action was impaired in the contralateral forelimb of ICH rats. Each forelimb of each rat was tested 10 times, and the percentage of the number of times that the forelimb was correctly placed was recorded.

Western blot

Lysis buffer for extraction of total protein from 40 mg of striatal tissue was utilized. Then, on completion of quantification by BCA method, conventional western blotting for determination of IRF4 and β -actin expression was carried out with rabbit anti-IRF4 antibody (1 : 20,000; Abcam, UK) and anti- β -actin antibody (1 : 300,000; AbClon, Korea). Image J software was employed to obtain the greyscale values of protein bands. β -actin was an internal reference. Relative expression of the protein was represented as the greyscale value ratio of the target band to the internal reference band.

Immunohistochemistry (IHC)

Rat striatum tissue was collected and fixed in 4% paraformaldehyde. After embedding the sections (5 μ m), the sections were soaked in 3% H₂O₂ at room temperature for 30 min. The sections were then blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 20 min and incubated with primary anti-IRF4 antibody (1 : 300; Abcam, UK) at 4°C overnight. Subsequently, the sections were rinsed 3 times in PBS and incubated with secondary antibodies. Finally, immunohistochemical reactions were detected with a DAB substrate kit (Boster, China).

Immunofluorescent staining (IF)

PC12 cells grew on coverslips for 24 h and were then divided into groups for different treatment. The slips covered with treated cells were fixed by 4% paraformaldehyde, followed by supplement of

1% Triton X-100 for permeabilization and 5% serum for blocking. Subsequently, overnight incubation of the slips and primary antibody (IRF4, 1 : 300) at 4°C was carried out. The next day, they were rewarmed at room temperature for 15 min and incubated with fluorescent secondary antibody (KPL 072-03-15-06) for 1.5 h at room temperature. The coverslips were finally mounted utilizing DAPI mounting medium (Cell Signalling #8961) and observed with a fluorescence microscope (OLYMPUS, Japan) in the dark.

ELISA

The levels of IL-1 β and IL-6 in the serum and medium of different groups were measured according to the ELISA kit (ABclonal RK00020) instructions, with background values as controls. Measurement of optical density (450 nm) was calibrated with a wavelength of 570 nm. Based on the calculated values of the constructed standard curve, each sample was tested in triplicate.

Statistics analysis

Each experiment was conducted at least in triplicate, and the experimental data were statistically analysed utilizing STATISTICA 6.0. The outcomes were expressed in the form of mean \pm standard error of mean (SEM). *T*-test and one-way analysis of variance were performed to compare the differences between 2 groups and among multiple groups, respectively. *P* < 0.05 was used as the criterion of a significant difference.

Results and discussion

Behavioural scores of rats with intracerebral haemorrhage

We performed behavioural tests in rats with successful modelling using the forelimb placing test. In comparison with the sham group, the rats showed severe neurobehavioral disorders at 6, 12, 24, 48, and 72 h after ICH induction, with the most severe at 48 h after surgery (Fig. 1). Therefore, the rats at 48 h after surgery were finally selected as ICH models.

IRF4 expression was up-regulated in rats with intracerebral haemorrhage

Some studies have suggested that IRF4 is a protein unique to the immune system [6] and is mainly involved in innate and adaptive immune responses.

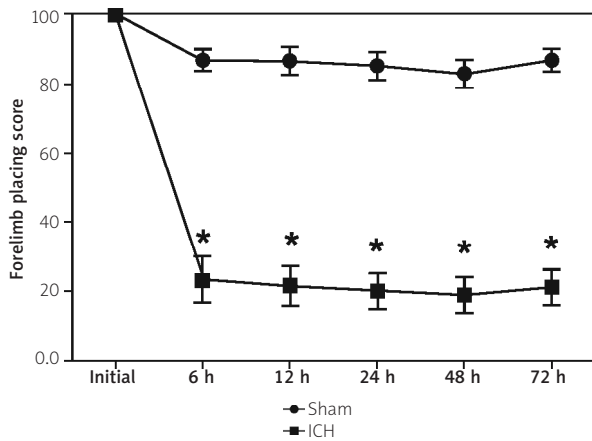


Fig. 1. Forelimb placing test assessment of the behavioural changes of rats at 6, 12, 24, 48, and 72 hours after haemorrhage by forelimb placing test. Values are mean ± SEM, $n = 6$. * $p < 0.05$ vs. sham group.

However, its involvement in the development and progression of haematological malignancies and in maintaining tumour cell survival is also supported in the literature [20,23]. In addition to the above functions, IRF4 as a transcription factor still has many functions to be studied [2]. For example, regulation of cardiac hypertrophy by IRF4 and expression of IRF4 in humans and mice have been revealed [21]. IRF4 can also act on adipocytes; it participates in fat catabolism by

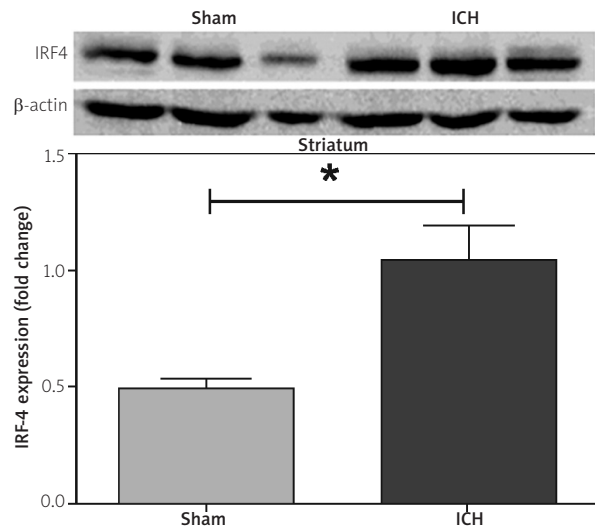


Fig. 2. IRF4 expression in rats with intracerebral haemorrhage. Measurement of IRF4 expression in rats with intracerebral haemorrhage utilized western blot with β-actin as an internal reference. Values are mean ± SEM, $n = 6$. * $p < 0.05$ vs. sham group.

regulating triglyceride lipase and hormone-sensitive lipase [27]. In a model of cerebral ischaemia-reperfusion injury, IRF4, with a protective effect on neural cells, can be induced in neurons [3]. Additionally, IRF4 can regulate the activation of glial cells thereby affecting the prognosis of ischaemic stroke. However,

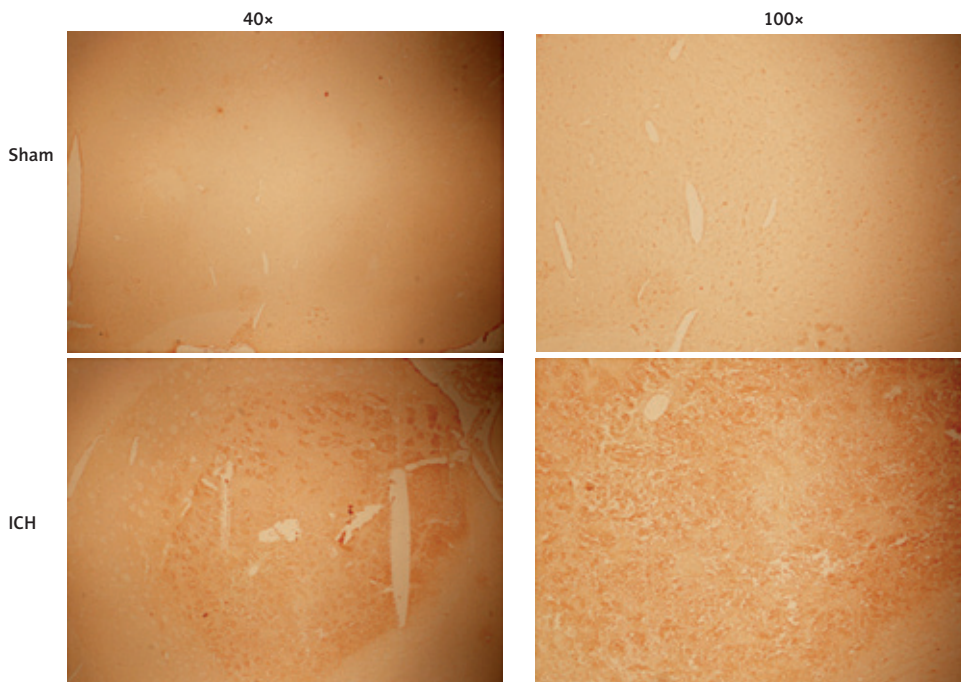


Fig. 3. IRF4 expression in perihematomal striatum of rats with intracerebral haemorrhage.

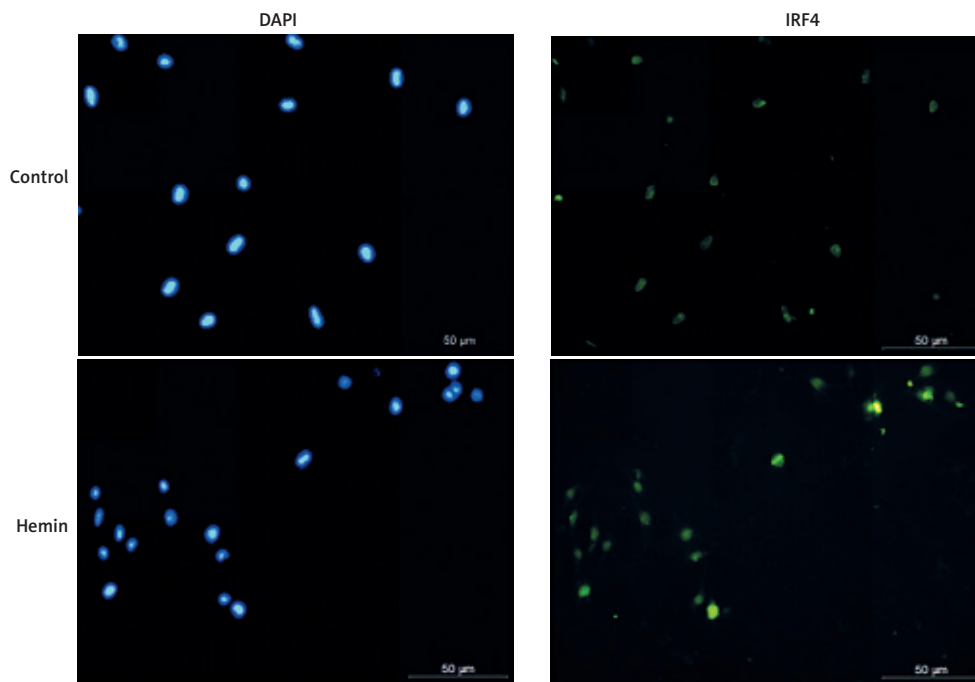


Fig. 4. IRF4 content in PC12 cells treated with hemin.

for acute ischaemic stroke lacking effective treatment options, investigation of IRF4 is conducive to elucidate the molecular mechanism of the development of this disease, thus providing a potential effective therapeutic target. Related studies regarding IRF4 in acute ischaemic stroke are also a good reference for exploring other similar diseases.

In this study, western blot was carried out to determine IRF4 expression in the striatum around the haematoma site in the sham and ICH groups. From the results of Figure 2, the protein expression of IRF4 in the ICH group was significantly increased compared with the sham group ($p < 0.05$), and the IHC results (Fig. 3) were consistent with the western blot, validating that IRF4 expression in the striatum was higher in ICH rats.

IRF4 content in PC12 cells treated with hemin

The increase of IRF4 expression was subject to further confirmation by *in vitro* experiment. Therefore, with PC12 cells treated with hemin as an *in vitro* model of ICH, measurement of IRF4 expression in the model was carried out. By fluorescence staining (Fig. 4), detection of IRF4 content in PC12 cells treated with 150 μM hemin for 4 h was conducted. According to the fluorescence intensity, in compar-

ison with the control group, a significant increase of IRF4 expression in PC12 cells was confirmed.

Concentration of inflammatory factors in serum and PC-12 cells of intracerebral haemorrhage rats

It has been found that IRF4 functions in inhibiting inflammation and reducing injury after renal ischaemia-reperfusion, thus preventing the progression of acute renal failure [25]. However, IRF4 expression still has an unknown relationship with inflammation in ICH. Therefore, ELISA was adopted for the examination of the expression of inflammatory factors in ICH rat serum and hemin-treated PC-12 cells. In comparison with the sham group and control group, the expression of IL-1 β and IL-6 in both ICH rat serum and hemin-treated PC-12 cells was increased (Fig. 5).

Conclusions

Based on the above results, our results showed that the inflammation in ICH is related to the increase of IRF4. However, the specific signalling pathway of this regulation requires further exploration. This study provides a basis for investigating the mechanism of IRF4 and inflammation in the future, and for clinically developing a new targeted drug

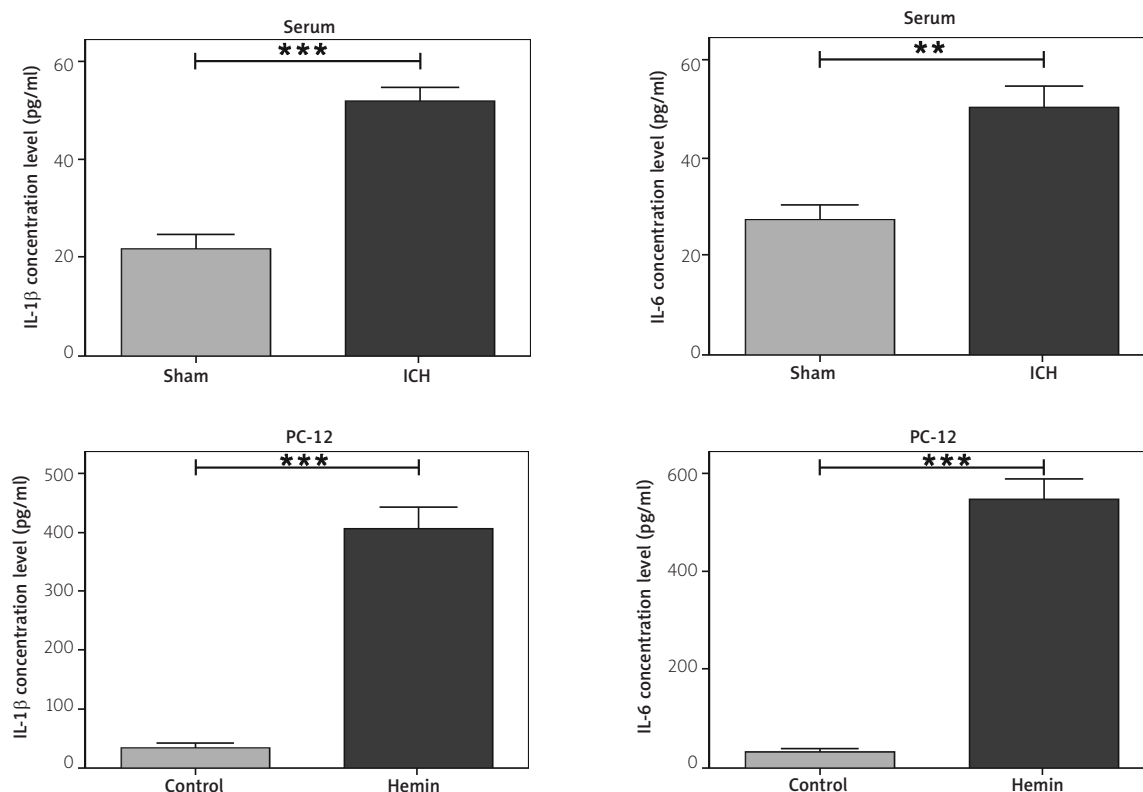


Fig. 5. Concentration of inflammatory factors in serum and PC-12 cells of ICH rats. The concentrations of IL-1β and IL-6 in the serum of the sham and ICH groups and in the PC12 cells of hemin groups were measured by ELISA. Values are mean ± SEM, n = 6. **0.01 < p < 0.05 vs. sham group; ***p < 0.001 vs. control group.

to effectively prevent and treat haemorrhagic brain injury.

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Disclosure

The authors report no conflict of interest.

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