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PF4 induces inflammatory response through NF-κB signal pathway in rats with intracerebral haemorrhage

Na Hu[1,2,3], Ran An[2,3], Kun Yu[2,3], Yanzhong Chang[1,4], Weijuan Gao[2,3]

[1]Hebei Normal University Biology Postdoctoral Research Station, College of Life Science, Hebei Normal University, China, [2]Hebei University of Chinese Medicine, China, [3]Hebei Key Laboratory of Chinese Medicine Research on Cardio-Cerebrovascular Disease, China, [4]College of Life Sciences, Hebei Normal University, China

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Abstract

Intracerebral haemorrhage (ICH) is a lethal cerebrovascular disorder with a high mortality and morbidity. Although it is a major public health problem, there is no effective treatment for ICH. After ICH, the primary and secondary mechanisms are mentioned when discussing brain injury. The transcription factor, nuclear factor-kappa B (NF-κB), is an important regulator of inflammatory responses. The role of platelet factor 4 (PF4) in ICH is unclear. To study the effect of PF4 on inflammatory response of rats in ICH, a rat model of striatum ICH was established by injecting autologous blood from the autogenous femoral artery into the right striatum of rats. Forty-eight hours after ICH, the expression of PF4, NF-κB (P-P65) and inflammatory changes in rats were determined with WB and ELISA. Heme was used to induce PC12 cell damage, simulate the ICH model in vitro, and detect PF4, P-P65 and striatal inflammatory changes. Short hairpin RNA (shRNA-PF4) was used to knock-down the expression of PF4 in PC12 cells to detect changes in inflammatory factors. The results showed that 48 hours after surgery, the behavioural score of cerebral haemorrhage was the lowest. The expression of PF4 and P-P65 in the striatum of the ICH group was significantly higher compared with the sham surgery group. The expression of interleukin (IL)-6 and IL-1β in the ICH group was also greatly improved. After inhibiting NF-κB expression, PF4 expression was decreased. In short, ICH enhances the expression of PF4, which induces an inflammatory response in rats with cerebral haemorrhage through the NF-κB signalling pathway. Reducing the expression of PF4 can attenuate the inflammatory response.

Key words: *cerebral haemorrhage, PF4, NF-κB, inflammation.*

Introduction

Nerve function due to intracerebral haemorrhage (ICH) is difficult to repair [3]. In addition to direct mechanical damage and the mass effect that leads to primary brain injury, secondary brain injury (SBI) is considered a major cause of adverse outcomes [11]. SBI is induced by oxidative stress, the activation of TRP channels and excitatory toxicity caused by iron overload and calcium-induced accumulation of reactive oxygen species (ROS). The coagulation cascade (especially thrombin), haemoglobin breakdown products (such as hemin or iron), changes in cerebral blood flow, inflammation, and complement reactions all play a role in SBI [19]. Hemin plays an important role in the cytotoxicity in pheochromocytoma (PC12, a rat pheochromocytoma cell line derived from adrenal medulla) and neuroblastoma (SH-SY5Y, a human neuroblastoma cell line cloned from a human bone marrow biopsy-derived line called SK-N-SH) cell lines [7].

Intracerebral haemorrhage, also known as a haemorrhagic stroke, refers to bleeding caused by rupture of primary cerebral parenchymal blood vessels. It is a common cerebrovascular disease [14]. Pathological

Communicating authors:
Yanzhong Chang, College of Life Sciences, Hebei Normal University, China, e-mail: Hebei_Normal@163.com; Weijuan Gao, Department of Biochemistry, Basic Medicine College, Hebei University of Chinese Medicine; Hebei Key Laboratory of Chinese Medicine Research on Cardio-Cerebrovascular Disease, China, e-mail: hbydhuna1983@126.com

changes in brain injury hours after ICH are hematoma compression and destruction of surrounding brain tissue [17]. Peripheral tissue oedema caused by hematoma can induce a progressive inflammatory response [15,24]. Platelet factor 4 (PF4, also known as CXCL4) is a factor produced by platelets α granules synthesized basic polypeptide tetramer protein [2]. PF4 is a low molecular weight protein involved in the hematopoietic process, vascular inhibition, organ fibre formation, mitosis, tumour growth, and metastasis [8]. The transcription factor kappa-light chain enhancers that activate B cells (NF-κB) were first discovered in 1986 as a regulator of B-cell specific transcription factor. NF-κB is a key nuclear transcription factor that has been considered a key regulator of inflammatory response since its discovery [18]. NF-κB is expressed in many tissues and cells throughout the body, and NF can be expressed in neurons, astrocytes and microglia in neurons in the nervous system. NF-κB activation can cause activation and expression of a range of pro-inflammatory factors. During ICH, these activated inflammatory factors play an important role in apoptosis caused by inflammatory response [4,20,21]. In this study, using rats with cerebral haemorrhage as the experimental model and PC12 cells as the object of study, this study explored whether PF4 regulates the expression of inflammatory factors in cerebral haemorrhage through NF-κB-mediated signal transduction pathways.

Material and methods

Adult male Sprague Dawley rats (9 weeks old) purchased from the experimental animal science centre of the Hebei Medical University were used in this experiment. The animals used in this experiment were raised in the SPF feeding room of the animal centre (room temperature 25°C, 12-hour light-dark cycle) and allowed free access to food and water. The experimental protocol conforms to the ethical principles and standards of laboratory animal welfare and has been approved by the Laboratory Animal Welfare Ethics Committee of Hebei Medical University.

Preparation and administration of the intracerebral haemorrhage rat model

The ICH model in this experiment uses a rat model that injects autologous arterial blood into the brain. After anaesthesia, the rats were fixed in the supine position, disinfected with 75% alcohol, incised in the inguinal skin to expose the femoral artery, and 100 µl of arterial blood was drawn with a microsyringe. After stopping the bleeding with a cotton swab, rats drawn with femoral artery blood were fixed on a brain stereotaxer. The skin was cut on the top of the rat's head, the surface of the rat's skull was wiped with 75% alcohol, the anterior fontanelle was exposed, the skull was gently drilled (3.5 mm on the right and 0.5 mm in front of the rat's anterior fontanelle), the microsyringe was inserted slowly (5.0 mm in depth), stayed for 5 minutes, continued to be inserted 0.5 mm, stayed for 10 minutes, Blood from a microsyringe was injected into brain tissue at a rate of 10 µl/min for 50 µl.

After another 10 min, the remaining 50 µl of blood was injected into the rat brain tissue at the same rate. After the injection was completed for 20 minutes, the syringe was slowly withdrawn, the brain wound was sutured, and after the rat regained consciousness, the experimental rat was sent back to the rat cage and raised alone.

Brain tissue sampling

The brain tissue was taken before the rat fasting for 12 h, weighed during the experiment, 0.4% pentobarbital sodium of 1 ml/100 g was used for intraperitoneal injection anaesthesia, after anaesthesia, the rat chest cavity was opened to expose the heart, the right atrial ear was cut open, the pre-chilled 0.9% normal saline from the tip of the heart was injected into the left ventricle, so that the blood in the blood vessels was discharged. The dorsal hairs on the rat's brain were then shaved off, and the skin and skull were removed longitudinally along the centre of the rat, and the overall brain tissue was taken out to obtain the striatum around the hematoma.

Cell

Rat pheochromocytoma cell PC12 (high differentiation) was purchased from the Chinese Academy of Sciences Cell Bank (TCR8) under the condition of 1640 medium, 37°C, 5% CO_2 containing 10% foetal bovine serum. Then incubated in different concentrations of heme for 4 h, the experiment was divided into the normal group, the Hemin treatment group, and the Hemin PDTC group.

Adenovirus shRNA was used to knock down PF4 expression in PC-12 cells

PC-12 cells were randomly divided into Sh-NC groups (i.e., control groups) and shRNA-PF4 groups. After 48 h of cell culture, the constructed carriers Sh-NC (5 µg/ml) and shRNA-PF4 (5 µg/ml) were transfected into PC-12 cells according to the transfection reagent instructions. After transfection of PC12 cells for 72 h, the expression of PF4 protein in PC-12 cells was detected by Western-blot and immunofluorescence techniques.

Forelimb placement experiment

Before the experiment, the rat torso was held, its forelimbs were let hang freely, and slowly the rat was

moved up and down to relax its muscles and reduce resistance activity. Healthy rats can quickly place the ipsilateral forelimbs on the corner of the table after touching the whiskers. In rats with cerebral haemorrhage, depending on the degree of injury, this effect has different degrees of damage. Each experimental rat tested the forelimbs 10 times on each side, recording the percentage of correct placement of the ipsilateral forelimbs behind the tentacles to the corner of the table.

Real-time PCR to detect IRF4 mRNA expression around the hematoma

The total striatal RNA around the hematoma was extracted according to the instructions in the RNA extraction kit (RNeasy MiNi QIAcube Kit, QIAGEN). According to the reverse transcription kit [stock number K1691, Thermo Fisher Scientific (China Co., Ltd.)], the total RNA of 1 μg of reverse transcription is cDNA. Real-time PCR was performed to detect interleukin (IL)-6, IL-1β mRNA, β-actin (β-actin) relative expression levels as an internal reference. IL-6, forward: 5'-CTGCAAGAGACTTCCATCCAG-3', reverse: 5'-AGTGGTATAGACAGGTCTGTTGG-3'; IL-1β forward: 5'-ATAAGCCCACTCTACACCT-3', reverse: 5'-ATTGGCCCTGAAAGGAGAGA-3'; GAPDH, forward: 5'-ACCCAGAAGACTGTGGATGG-3', reverse: 5'-TTCAGCTCAGGGATGACCTT-3'. The results were calculated using the $2^{-\Delta\Delta Ct}$ (Ct is the number of cycles).

Immunohistochemical imprinting (Western-blot)

Whole-cell proteins were extracted with RIPA Buffer lysate containing 1% phosphatase inhibitors and protease inhibitors. Total protein concentration was determined using BCA with a separation gel concentration of 12% and a concentrated gel concentration of 4%; loading volume of 15 μl/well, concentrated gel 80 V, 30 min, separation gel 120V, electrophoresis for 1 h; 200 mA transfer for 2 h (PVDF membrane); 5% milk closed at room temperature for 2 h; primary antibody: PF4 (1 : 1000, Abcam), P-P65 (1 : 2000, Abcam), β-actin (1 : 50000, Abcam), incubated overnight at 4°C; secondary antibody: 1 : 10000 incubation at room temperature for 1 h, chemiluminescence method with ECL luminescence solution to scan PVDF film for imaging.

ELISA detects levels of inflammatory factors

According to the manufacturer's instructions, the levels of IL-1β, IL-6 cytokines released into different groups of animal serum and culture medium were measured using the ELISA kit (ABclonal RK00020). The background values were analysed as controls. The microplate reader detected absorbance at a wavelength of 450 nm (using 570 nm for the calibration wavelength). The standard curve was constructed based on the measurements of the standard, three wells were repeated for each sample and the experiment was repeated three times independently.

Statistical analysis

All experiments were repeated independently at least 3 times, with experimental data expressed as mean ± standard errors, and the statistical analysis was made using GraphPad Prism5.0 software. For intergroup comparisons, paired *T*-tests or one-way ANOVA was used, and *p*-values less than 0.05 were considered statistically significant.

Results

Behavioural score in rats with cerebral haemorrhage

The forelimb placement experiment was used to detect the neural behaviour of rats, and the modelling was successful. Compared with the sham surgery group, rats in the 12-hour, 24-hour, 48-hour, and 72-hour post-ICH group exhibited severe neurobehavioral disorders, with the lowest scores and more severe behavioural disorders at 48 hours after bleeding (Fig. 1).

Expression of PF4 and p-p65 in striatum around oedema in rats with intracerebral haemorrhage

We detected the expression of PF4 in the striatum around the oedema by immunohistochemistry, and the expression of PF4 and P-P65 by Western blotting.

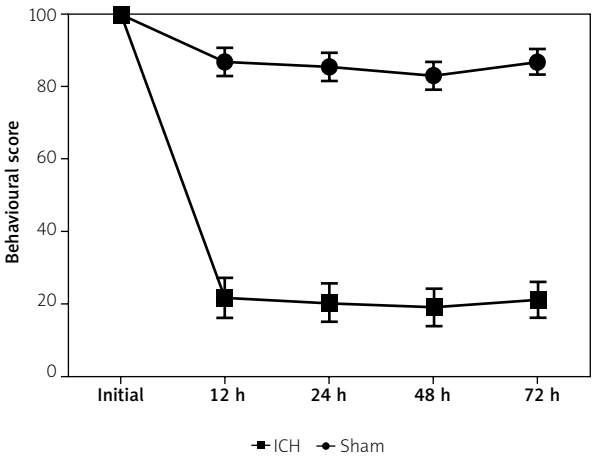


Fig. 1. Neurobehavioral score testing in rats with cerebral haemorrhage.

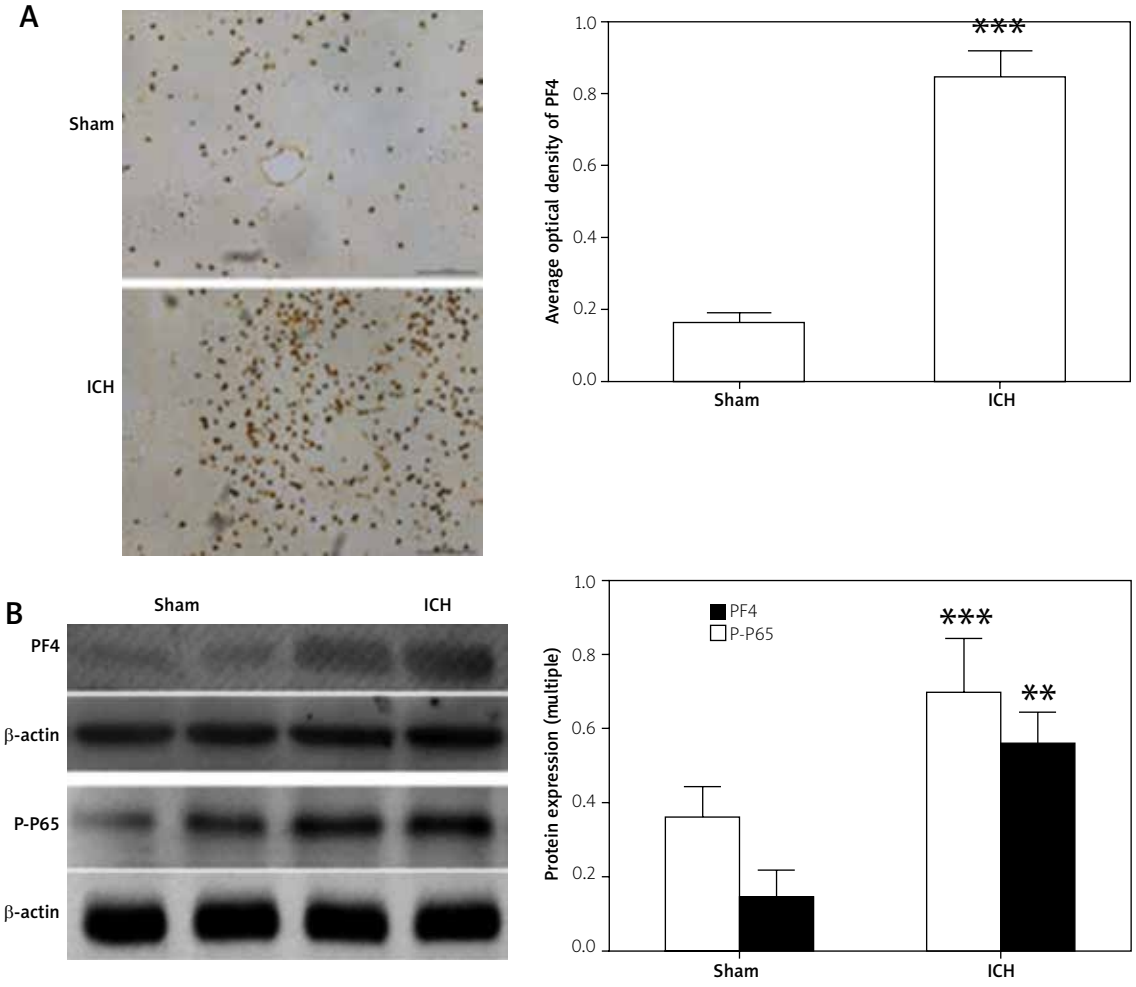


Fig. 2. Expression of PF4 and P-P65 in the striatum surrounding hematomas in rats with cerebral haemorrhage. **A**) The expression of PF4 was observed by immunohistochemical staining; **B**) The expression of PF4 and P-P65 in intracerebral haemorrhage was detected by Western blot. Take β-actin as the internal reference; $n = 3$. **$0.01 < p < 0.05$, ***$p < 0.01$.

The results showed that the expression of PF4 and P-P65 in ICH group were significantly higher than that in the Sham group ($p < 0.05$) (Fig. 2).

Expression and content of inflammatory factor IL-6 and IL-1β in striatum and serum of rats with intracerebral haemorrhage

To determine the level of inflammation in ICH rats, we measured the expression levels of IL-6mRNA and IL-1βmRNA in the striatum around the hematoma with RT-qPCR, and the ELISA method detected the expression of IL-6 and IL-1β in the serum of ICH rats. The results showed that there was a statistically significant difference in the expression of IL-6 and IL-1β in the striatum and serum around the hematoma of ICH rats compared with the Sham group (Fig. 3).

Expression of PF4 and P-P65 in PC12 cells after treatment with heme and silencing of PF4

Later, in *in vitro* experiments, we transfected PC12 cells with Sh-RNA-PF4 adenovirus for 72 h, or 100 μM Hemin treated PC-12 cells for 4 h and detected the expression of PF4 proteins with cell immunofluorescence staining. According to the fluorescence intensity and statistical results, the expression of PF4 in PC12 cells after Hemin treatment increased with statistical

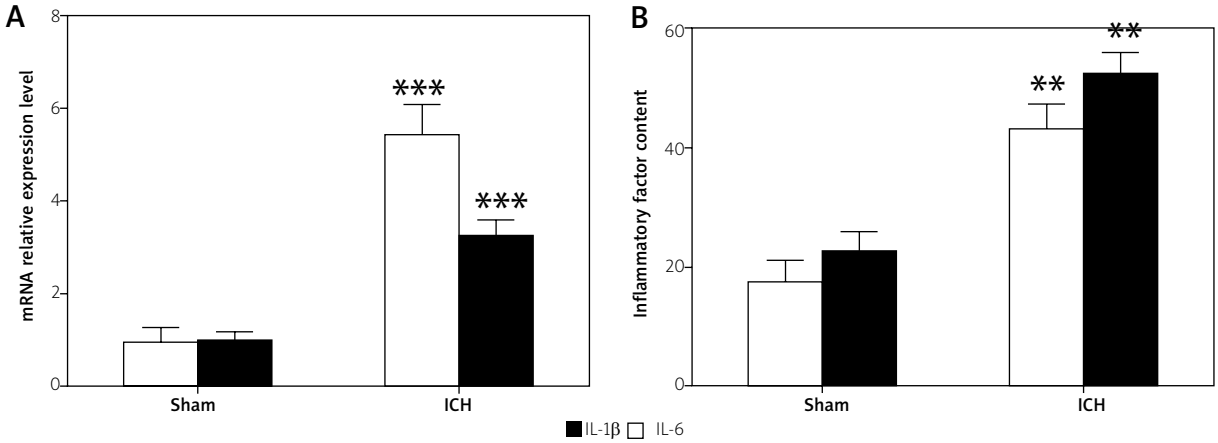


Fig. 3. Expression of IL-6, IL-1β in intracerebral haemorrhage (ICH) rats. **A**) RT-qPCR detects the expression of IL-6mRNA and IL-1βmRNA of the striatum around the hematoma; **B**) The concentration of IL-6 and IL-1β in serum of ICH rats was detected by ELISA. **0.01 < p < 0.05, *** p < 0.01.

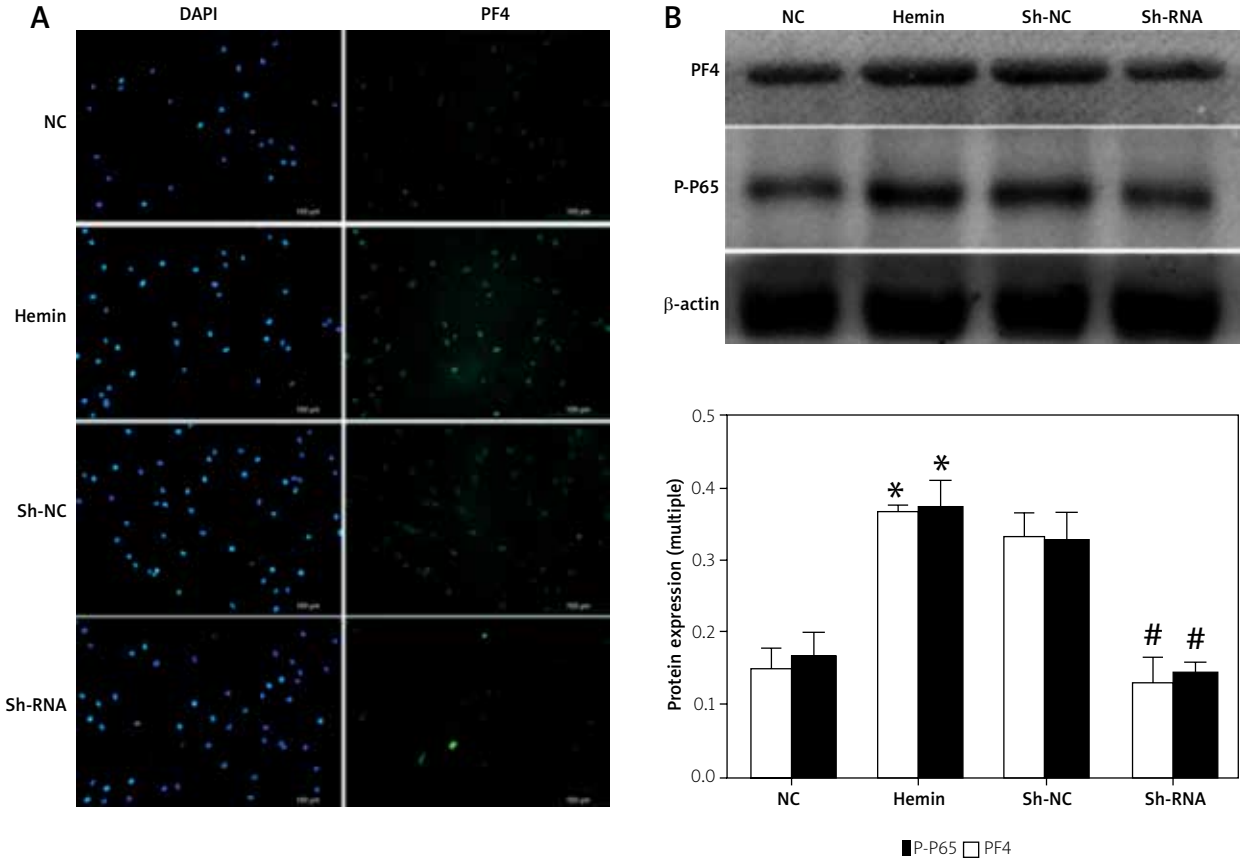


Fig. 4. Contents of PF4 and P-P65 in PC12 cells in different treatment groups. **A**) Fluorescence staining observed PF4 expression in the NC group, Hemin group, Sh-NC group, and Sh-RNA group; **B**) Western blotting detected the expression of PF4 and P65 in different treatment groups. DAPI: nucleus; n = 3. *p < 0.05 vs. NC, #p < 0.05 vs. Hemin.

difference ($p < 0.05$) compared with untreated cells, and the expression of PF4 in PC-12 cells was reduced by short hairpin RNA (shRNA), which was statistically different from that of the Hemin treatment group. Later, PF4 and P-P65 were detected with Western-blot and the same results were obtained (Fig. 4).

Content of the intracellular inflammatory factors IL-6 and IL-1β in PC12 cells after Hemin treatment and PF4 knockdown

To determine the effect of reducing PF4 on cellular inflammation levels, we used ELISA to detect the expression of inflammatory factors in PC-12 cells, and the results showed that the expression of IL-1β and IL-6 in Hemin-treated PC-12 cells was elevated compared to the control group, with statistical differences. After silencing PF4, the expression of IL-6 and IL-1β in PC-12 cells decreased, and there was a statistical difference compared with the Hemin treatment group ($p < 0.05$) (Fig. 5).

Expression of PF4 and P65 after treatment of inflammatory cells with NF-κB (P-P65) inhibitors

To further verify that PF4 is regulated by the NF-κB pathway to validate the response, we treated PC12 cells that had been treated with Hemin using the NF-κB inhibitor PDTC, and found that the expression of PF4 and P-P65 decreased after treatment with the inhibitor (Fig. 6).

Discussion

The incidence and mortality of ICH is high, and to date, no drug or surgical therapy has significantly reduced morbidity or mortality after ICH [13]. Intracerebral haemorrhage usually causes brain damage within the first few hours, which is the result of a mass effect of hematoma formation. Haemoglobin, heme, and iron released after lysis of erythrocytes in advanced cerebral haemorrhage cause direct toxicity and inflammation, and exacerbate neurological deficits [6,23]. After ICH, the activity of NF-κB in the brain tissue surrounding the hematoma increases significantly, and the expression of cytokines downstream of NF-κB such as IL-6 and IL-1β increases significantly, and these inflammatory factors play an important role in secondary brain injury after ICH [9]. Normally, NF-κB exists and is localized in cytoplasm in the form of a trimer polymerized with IKB or in the dimer form of polymerization with a precur-

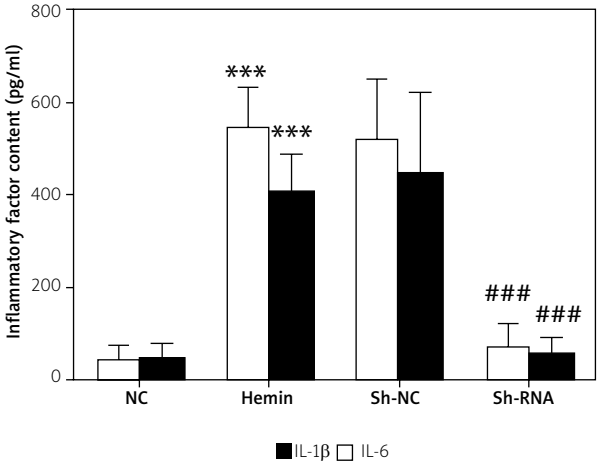
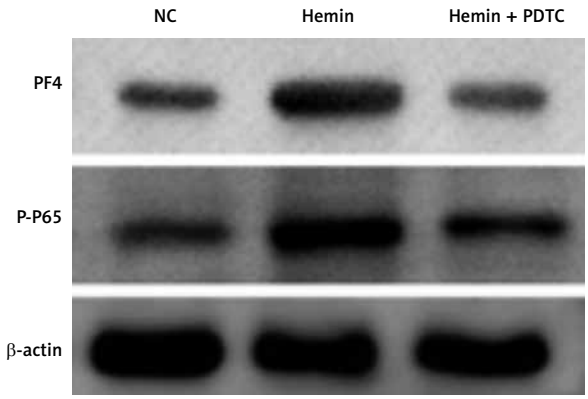


Fig. 5. ELISA method detects the content of IL-6 and IL-1β in PC12 cells. ***$p < 0.01$ vs. NC, ###$p < 0.01$ vs. Hemin.



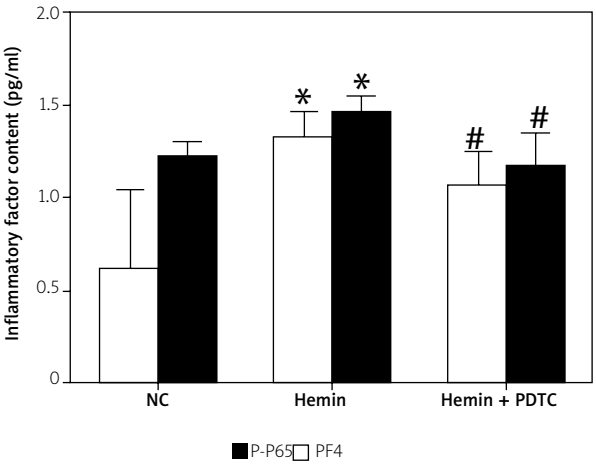


Fig. 6. The content of PF4 and P-P65 after treatment of PC12 cells by inhibitors. ***$p < 0.01$ vs. NC, ###$p < 0.01$ vs. Hemin.

sor protein, which interacts with the RHD structure of NF-κB, obscuring the NLS sequence (nuclear localization sequence) of the NF-κB transcription factor. When ICH occurs, a series of factors can cause the IKB protein to phosphorylate, the effect of proteolytic enzymes is weakened, NF-κB can be exposed to its nuclear localization sequence, rapidly entering the nucleus from the cytoplasm, and binding to the specific sequence on the DNA in the nucleus, thereby initiating or enhancing the transcription of the associated gene [22].

PF4 is a chemokine protein that activates platelet release, and its main physiological function is to promote coagulation function [1] but its cell surface specific receptors have not been studied, and other biological effects leading to PF4 and their mechanisms are not well understood. The PF4 gene is a member of the polygenic family (the small inducible gene (SIG) family, whose protein amino acid sequences are homologous and all have similar genomic exon structures, suggesting that they may have evolved from common ancestral genes [5]. At present, the effect of PF4 on the inflammatory response in the hematoma area of cerebral haemorrhage has not been reported in the literature. This experiment only preliminarily revealed part of the complex effect of PF4 on brain tissue damage in cerebral haemorrhage, that is, PF4 can promote the expression of inflammatory factors through the NF-κB pathway in ICH.

Chlorinated metheme (Hemin) is one of the breakdown products of haemoglobin in the red blood cell lysate and a leading cause of neuronal death [24]. Hemin stimulates PC12 cells and can lead to massive deaths through the NF-κB pathway, mainly manifested by apoptosis and inflammatory response. In ICH models, hemin has been widely used to induce PC-12 cytotoxic effects [10]. A variety of drugs have been reported to reduce damage to PC-12 cells by reducing oxidative stress, inflammation, and inhibition of apoptosis [12,16].

Based on the above findings, we explored the expression and inflammatory effects of PF4 in cerebral haemorrhage. First, we used rat autologous blood to make a rat model of cerebral haemorrhage, then performed behavioural scoring, and finally selected rats 48 h after surgery as a model of cerebral haemorrhage. After that, the expression of PF4 and NF-κB (P-P65) in the striatum around the oedema site of rats in the sham surgery group and the ICH group was detected, and the expression of PF4 and P-P65 in the ICH group was significantly higher than that in the sham surgery group. To further clarify this phenomenon, we examined the expression of PF4 and P65 in cells using an *in vitro* model of Hemin-treated PC12 cells simulating ICH and obtained the same results. Whether the expression of PF4 in ICH is also associated with inflammation is unclear. Therefore, we also detected the expression of the inflammatory factors IL-6 and IL-1β in ICH rats, and found that the expression of IL-6 and IL-1β was increased significantly in the bleeding rat model. Based on these results, we believe that the inflammatory response in ICH is associated with an increase in PF4, and then silence PF4 by RNA (short hairpin RNA, shRNA)-PF4 to reduce its expression in heme-treated PC12 cells, further exploring its effect on the inflammatory response. We found that after heme treatment of PC12 cells inhibiting PF4, P-P65 was also downregulated, slowing down the inflammatory response, and P-P65 and PF4 expression decreased after treating PC12 cells with NF-κB inhibitors. In order to further explore the mechanism of action of PF4 and inflammatory response in cerebral haemorrhage, this study suggests that PF4 may upregulate the expression of inflammatory factors in cerebral haemorrhage through the signalling pathway of NF-κB.

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Disclosure

The authors report no conflict of interest.

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