

The effects of deferoxamine on inhibition for microglia activation and protection of secondary nerve injury after intracerebral hemorrhage in rats

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Abstracts: To investigate the effects of the iron chelator deferoxamine (DFA) on inhibition of microglia activation and protection of secondary nerve injury after intracerebral hemorrhage (ICH) in rats. The rats were randomly divided into sham operation group, ICH group and DFA treatment group. The collagenase was used to prepare ICH model of basal ganglia in rats and 1h after the beginning of the operation, the intraperitoneal injection with DFA was arranged every 12 h and for a total of 7d. The changes of Iron ion concentration were measured at perihematoma at different time points after the medicine was given. OX42 immunohistochemical staining observed microglia change at perihematoma. ELISA method determined the changes of IL-1 β and TNF- α content of brain tissue. Neurological deficit scores and Nissl staining were used to observe the situation of neurological function and neuronal loss of rats after DFA treatment. 1 d After the start of ICH, the concentration of iron in perihematoma was significantly higher than that of animals in sham-operated group and could sustain for 28 d. At the same time, the quantities of local microglial cells were significantly increased. After applying DFA, the concentration of iron ions in the brain tissue around the hematoma was significantly reduced, so did the number of microglial cells and activation of neurotoxic cytokines (IL-1 β and TNF- α content) secreted by microglial cells was significantly reduced. At the same time, the loss of neurons in the tissue around of the hematoma was significantly reduced and neurological deficit scores were significantly reduced. Iron ions which were sustainedly released by hematoma after ICH can activate the local microglia and cause secondary brain injury. DFA curbed excessive activation of microglia and reduced neuronal death of ICH by means of clearing away iron ions of brain tissue surrounding the hematoma, thus improving secondary neurological dysfunction.

Keywords: cerebral hemorrhage, iron, deferoxamine, secondary nerve injury, microglia.

INTRODUCTION

Intracerebral hemorrhage (ICH) is a type of emergency of severe central nervous system with high morbidity and mortality. In the acute phase, if the patient could survive, intracerebral hemorrhagic site and its surrounding brain tissue would occur series of reactions, which could cause secondary damage of local neurons and severe sequelae such as neurological dysfunction (Qureshi *et al.*, 2001). Therefore, the prevention and treatment for secondary nerve damage after ICH can reduce subsequent neurological dysfunction, improve the quality of life of patients, which has very important clinical significance. After ICH, with the decomposition of hemoglobin, the concentration of the local iron ions continues to rise, which becomes one of the important factors of secondary brain injury of ICH (Wagner *et al.*, 2003; Wu and Zhang, 2008). Some studies suggest that their iron ions accumulating around the hematoma may continuously activate microglia in local brain tissues, these abnormally activated microglia could cause series of secondary brain injury by means of neurotoxic (Karwacki *et al.*, 2006; Hua *et al.*, 2006). Thus, effective removal of local iron ions may inhibit the abnormal activation of microglial cells and reduce neuronal death.

In this study, collagenase in rat with basal ganglia ICH model was used to observe changes of concentration of iron ions in the brain tissues around the hematoma after ICH and activation of microglia and the iron chelator deferoxamine (DFA) was further used to reduce concentration of iron ions of hematoma around brain tissue and inhibiting microglial activation and observe neurotoxic cytokines IL-1 β and changes of TNF- α content secreted by microglia cells and the loss of local neurons for the sake to investigate the activation effect of iron on microglia and the inhibiting effect of DFA on the activation of microglial and protective effect for secondary brain damage after ICH, which provided theoretical basis for the clinical treatment for ICH.

MATERIALS AND METHODS

Laboratory instruments and reagents

Rat brain stereotaxic apparatus was Japanese Narishige products and optical microscope was Japanese Olympus products. The collagenase of IV type was purchased from Sigma and rat IL-1 β and TNF- α ELISA kit were purchased from R & D. Mouse anti-OX42 monoclonal antibody was purchased from Chemicon and immunized mice of SABC kit was purchased from Beijing Zhongshan Biotechnology Co.,

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Ltd., and other reagents were analytical grade products.

Animals and grouping

Clean male Sprague-Dawley (SD) rats (body weight 250~270g) were purchased from the Experimental Animal Center of Xi'an Jiaotong University. The animals were randomly divided into sham group, the experimental group (ICH group) and DFA treatment group. They were used to do nerve dysfunction score, iron ion concentration, morphology observation and ELISA measurement.

Preparation for rat cerebral hemorrhage model

Referring to Brown *et al.* (1995) method, after being arranged intraperitoneal injection of anesthesia with 10% chloral hydrate (400mg/kg) experimental animals were fixed on the rat brain stereotaxic apparatus. Then their skin on the top of calvarium was cut along the longitudinal center line for 2cm, positioned 0.4mm in front of the anterior fontanelle, and 2.6mm left the center line. Miniature hand drill was used to drill a hole with diameter of 1 mm, IV collagenase solution of 1 μ L was sucked by micro syringe, and needle depth was 5.2mm and the solution was injected into the caudate putamen at the speed of 0.2 μ L/min, slowly back the needle was removed 10 min later. Then the bone wax was used to block drilling and the wound was sutured. Next, they were put in the insulation with temperature of 37°C. After the animals revived, they were respectively caged and feeded and routinely given water. The sham group received the same amount of sterile saline injected caudate putamen.

Administration

Intraperitoneal injection for DFA (100mg/kg) was arranged 1h after the surgery and once every 12h and the cumulative injection was arranged for 7 d. Sham group and experimental control group was injected with the same amount of saline.

Neurological deficit scores

By referring to the comprehensive score method of neural dysfunction after experimental strokes in rats reported by Reglodi, *et al.*, the grading was arranged at 2 h, 6 h, 1 d, 2 d, 3 d, 5 d, 7 d after ICH for the sake of grading the neural dysfunction after ICH injury of rats. Reglodi *et al.* (2003). The main tests included posture, gait, balance function, muscle tension and muscle symmetry, spontaneous exercise. Total scores of all items were the final scores of neural dysfunction for each animal at this time point.

Detection for the concentration of iron ions

Iron ion concentration in brain tissue was measured after administration of 1, 3, 5, 7, 14, 28 d. After the animals were under narcosis with 10% chloral hydrate anesthesia, they were implemented left ventricular cannula and then the blood was washed with normal saline, then the brains were removed, and coronal brain slices of about 4 mm thick were cut at the front and back of the needle tract and

then the slices were divided into two slices along with the middle line and were weighed. The measurement for the concentration of iron ions in brain tissues was arranged according to the method of Wu *et al.* (2003) and had been improved. The cut brain tissue was put into 1ml of concentrated hydrochloric acid of 8.5mol/L to be sufficiently homogenized, and then was placed in water bath of 90°C and was hydrolyzed for 1h; after the solution was cooling, 2ml of trichloroacetic acid of 20% was added, after the procedure of sufficient vibration, it was placed on the ice for 20 min to precipitate the protein; the solution was centrifuged for 10 min at the speed of 400 r/min and the supernatant was sucked out for standby application; 0.5ml of concentrated hydrochloric acid and 20% trichloroacetic acid were added in precipitation and then the solution was thoroughly washed and the supernatant was collected by centrifugation; the supernatant solution of two times was mixed and adjusted to pH 3.1 with 1mol/L of sodium citrate, and the volume was set to 15ml. 1ml of reducing agent was put into 1ml of sample solution and then the mixture was put at room temperature for 5 min after. Then 0.8 ml of acetic acid and 0.2ml of Philo triazine amine color-developing agent were added, absorbance was measured at a wavelength of 562nm, the concentration of iron ions of brain tissue was calculated according to a standard curve.

IL-1 β and TNF- α concentrations were measured

ELISA method was used to measure IL-1 β and TNF- α content around perihematoma after administration 1, 3, 7, 14d. After the animals were treated with 10% chloral hydrate anesthesia and the brains were removed. Cut the brain tissue with 2mm wide around the hematoma and weighed in the needle in the front and rear track with 4 mm coronal slices. PBS was added with 1ml/100 mg of brain tissue, homogenized in 12000g centrifugation under ice-cold for 10min and got the supernatant. ELISA operations performed according to kit instructions, the standard curve was made to calculate the IL-1 β TNF- α concentrations.

Fixing the organization, implementing the paraffin sectioning and staining

After the rats were treated with 10% chloral hydrate intraperitoneal injection of anesthesia, left ventricular cannula, 300ml of normal saline was rapidly infused to rush blood, and then 4% para formaldehyde perfused with fixation. Decapitated and set in 4% formaldehyde to fix and overnight after 4°C. Embedded in paraffin, do coronal slices and track the needle centered. Slice thickness is 10 μ m, mounted on glass slides treated chrome vanadium gelatin, dried and stored at room temperature. It was used for HE, Nissl staining and immunohistochemical OX42 staining and analysis.

OX42 immunohistochemistry staining

Paraffin sections were dewaxed to water, incubated with 0.3% H₂O₂ at room temperature for 10 min to inactivate

endogenous per oxidase and normal goat serum closed at room temperature with 1h; dropped anti-mouse OX42 monoclonal antibody (1: 200) and incubated at 4°C overnight; Dropped biotinylated goat anti-mouse secondary antibody (1: 200) and incubated at room temperature for 2h; dropped ABC complex, incubated at room temperature for 1h; DAB was colored, fully washed, conventional dehydration was mounted, optical microscopy and photographed. At high magnification, took three horizons at the outside of the striatal hemorrhage and counted the number of positive cells.

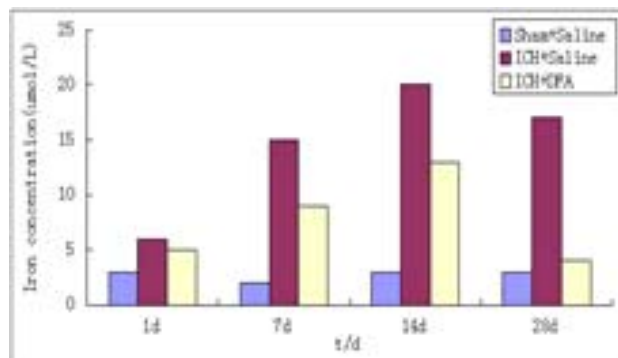


Fig. 1: Effect of DFA on iron content in the perihematoma brain tissues. Compared with Sham group, * $P < 0.05$; Compared with ICH group, # $P < 0.05$.

STATISTICAL ANALYSIS

All data were expressed as mean \pm standard. Statistical analysis use SPSS10.0 software for statistical analysis. Univariate analysis of variance is used to compare different treatment. Tukey's post-hoc was used to analyze the differences among groups and $P < 0.05$ was considered statistically significant.

RESULTS

DFA reduced the concentration of iron in brain tissue of ICH, significantly

The concentration of iron ions in brain tissue in the sham-operated group of animals was maintained at physiological levels at each detection time. Compared with sham group, the concentration of iron ions in brain tissue significantly increased 3 d after ICH, the peak got to the highest after ICH for 14d and it decreased slightly after ICH for 28d; after applying of DFA, iron concentration was significantly lower than the control group after administration of 5d and after stopping treatment of DFA for 7 d and 14 d, it remained lower than the control group, significantly (fig. 1).

Score changes of neurological deficit after DFA treatment

Neurological scores in Sham-operated group of animals at each time were significantly lower than that of blood group and treatment group, stabilized at a low level. After ICH injury, animal neurological score was significantly

higher than the sham group and reached a peak after hemorrhagic 2d and then decreased, significantly. A slow decline appeared after bleeding injury within 2~5d and stabilized at the level of significantly higher than the sham group after 6d. There was no significant difference between DFA treatment and the control group in neurological deficit scores after 1d treatment, but the score is significantly lower than hemorrhage group after 2d (fig. 2).

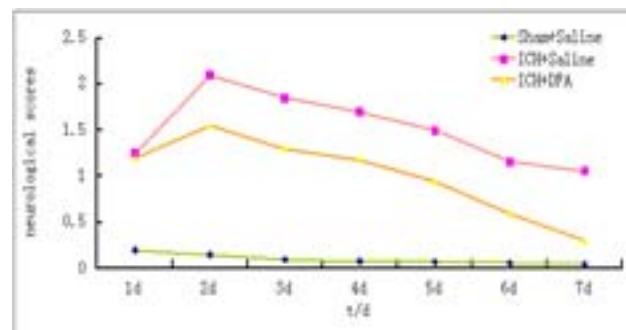


Fig. 2: Effect of DFA on neurological deficiency scores after ICH. Compared with Sham group, * $P < 0.05$; Compared with ICH group, # $P < 0.05$.

DFA reduced perihematoma neuronal loss

In order to observe the protective effects of DFA on neurons stimulate injury after ICH injury, Nissl staining was used to observe perihematoma neuronal loss (fig. 3). The results showed that neurons behave in sham group of animals had normal morphology (fig. 3A). After ICH 3 d, the performance of the neural cellular body was swelling, and part of the nerve cell was lost (fig. 3C); after ICH 7 d, these injury neurons showed nuclear condensation, and cell body shrinkage was in deformation (fig. 3D); after ICH 14d, these neurons lost almost exhausted, only a small amount of residual shrinkage with deformation neurons were existed (fig. 3E). After DFA treatment for 3 d, the neuronal edema decreased compared with ICH group (fig. 3F); neuronal survival was significantly higher than ICH group at 7d and 14d and the survival of neurons generally showed normal morphology (fig. 3G ~ I).

DFA curb excessive activation within Perihematoma microglia

In order to observe the impact of DFA on microglial activation after ICH, OX42 immunohistochemistry was used to coloring. We observed changes in the brain tissue around the hematoma microglia (fig. 4). There were only a few OX42-positive cells in caudate putamen of sham-operated animals (fig. 4B). The number of microglial cells significantly at hematoma surrounding brain tissue increased after ICH for 3d, reached a peak after ICH for 7d and there was still more visible microglial cell around the hematomas after ICH for 14d (fig. 4C~E). After applying DFA, compared with ICH group, the number of microglial cells decreased significantly on 7d and 14d (fig. 4A, F, G, H).

Changes of IL-1 β and TNF- α content at perihematoma

Compared with the sham group, IL-1 β was significantly increased after ICH for 1d and it continuously had high expression to ICH for 3d. It gradually declined after ICH for 7d and 14d, but it was still significantly higher than the sham group, and TNF- α showed similar expression variation. After DFA treatment, expression levels of these two factors began to decline significantly after ICH for 3 d, approaching the level of expression of the sham group (fig. 5) to 14 d.

DISCUSSION

Main ICH's pathological changes included cerebral edema and neuronal loss, which could be divided into two stages: acute injury and secondary injury. Secondary injury often occurred after ICH for 3~5 d and sustainable to ICH for 2~4 weeks. Secondary injury would further promote neuronal loss around perihematoma, increasing neurological dysfunction and later became one of the key causes of epilepsy, Alzheimer's disease, Parkinson's disease and other degenerative diseases of the secondary of central nervous system to ICH (Qureshi *et al.*, 2001). Thus, an effective treatment was research of secondary injury after ICH, one of the important goals of treatment of the disease.

The reasons of secondary brain edema and neuronal loss after ICH were currently under study. As microglia of immune cells within the central nervous system, may play an important role. Under physiological conditions, microglia was in a resting or dormant and had a smaller number. In traumatic brain injury, cerebral ischemia, cerebral hemorrhage, central nervous system infections and other pathological conditions, microglia could be activated, proliferated, expressed various signaling molecules and cytokines locally (Kowiański *et al.*, 2003; Zhang *et al.*, 2004). Activated microglia could participate pathological process of brain damage in three ways: increasing MHCII antigen and complement receptor of CR3 and other immune molecules, eliciting an immune response mediated by T cells; playing phagocytosis effect of macrophages; producing neurotoxic molecules and neurotrophic factors and involving in brain damage and repair (Zhang *et al.*, 2004). Kowiański *et al.* (2003) found that, ICH could induce microglial get to the state of rapid activation transition from the resting state, and significantly increased CR3 complement receptor. The expression of MHCII antigen and labeled molecules of mononuclear phagocyte antigen: OX42, OX6, ED1 and neuron death and injury around the hematoma are associated with activation of microglia. In addition, microglia activation after ICH was significantly more intense and persistent than cerebral ischemia (Zhang *et al.*, 1997). Some studies found brain tissue around the hematoma remains a large number of activated microglia

after the ICH for 7~14d. On one hand, the excessive activation of microglia can direct contact with nerve cells, exerting toxicity function of phagocytic cells in the brain; on the other hand, by releasing a series of nerve and secrete toxic molecules, such as oxygen free radicals, IL-1 β , TNF- α , NO, lead to peripheral neuronal degeneration and death, causing secondary brain injury occurred (Chen *et al.*, 2005).

Currently, the causes of persistent activation of microglia after ICH were not very clear. It was worth noting that the red blood cells within the hematoma began to dissolve after ICH, releasing large amounts of hemoglobin. Hemoglobin and its degradation products may play an important role in secondary brain injury. Iron is the main degradation product of hemoglobin, iron gathered continuously within the hematoma and surrounding brain tissue after ICH, leading to iron overload (Wagner *et al.*, 2003; Wu *et al.*, 2003). Abnormally elevated iron ions can act on glial cells around and neurons, inducing lipid peroxidation and free radical formed, further causing oxidative damage of local neurons (Wan *et al.*, 2009; Pérez de la Ossa *et al.*, 2010; Qiu *et al.*, 2009). Vitro experiments showed that treat with FeCl₂ for 30 min, then incubated for 24h, cultured microglia appears shape changes of activation, and the number of OX6 positive cells increased significantly, suggesting that iron had a direct role in the activation of microglia (Su and Tong, 2010; Saleppico *et al.*, 1996).

This study shows that concentration of iron in the brain tissue around the hematoma continues to rise after ICH, with sustained activation of microglia and a continues rise to expression levels on neuronal toxicity cytokine, accompanied by the death of a large number of local neuronal and serious neurological dysfunction. This is consistent with previous reports (Karwacki *et al.*, 2006; Wagner *et al.*, 2003; Kowiański *et al.*, 2003).

As chelating agents of iron, DFA is widely used to remove excess iron ions *in vivo* in clinical, and experiments show that DFA can quickly go through the blood-brain barrier and get into the brain tissue. Therefore, it can be used to remove iron overload in the brain (Jiang *et al.*, 2006). The study shows that application of DFA can reduce iron overload hematoma surrounding brain tissue, with the significant reduction in the number of local activation of microglia and reducing release of the local microglial neurotoxicity associated with cytokines IL-1 β and TNF- α , reducing the loss of neurons in the brain tissue around the hematoma, and performance of nerve dysfunction in rats is improved. Further proving that the local release of iron ions after ICH can sustained active microglia, which causes secondary neuronal damage, and remove the local iron ions inhibit the activation of microglia and reduce secondary neuronal injury after ICH (Wu *et al.*, 2011).

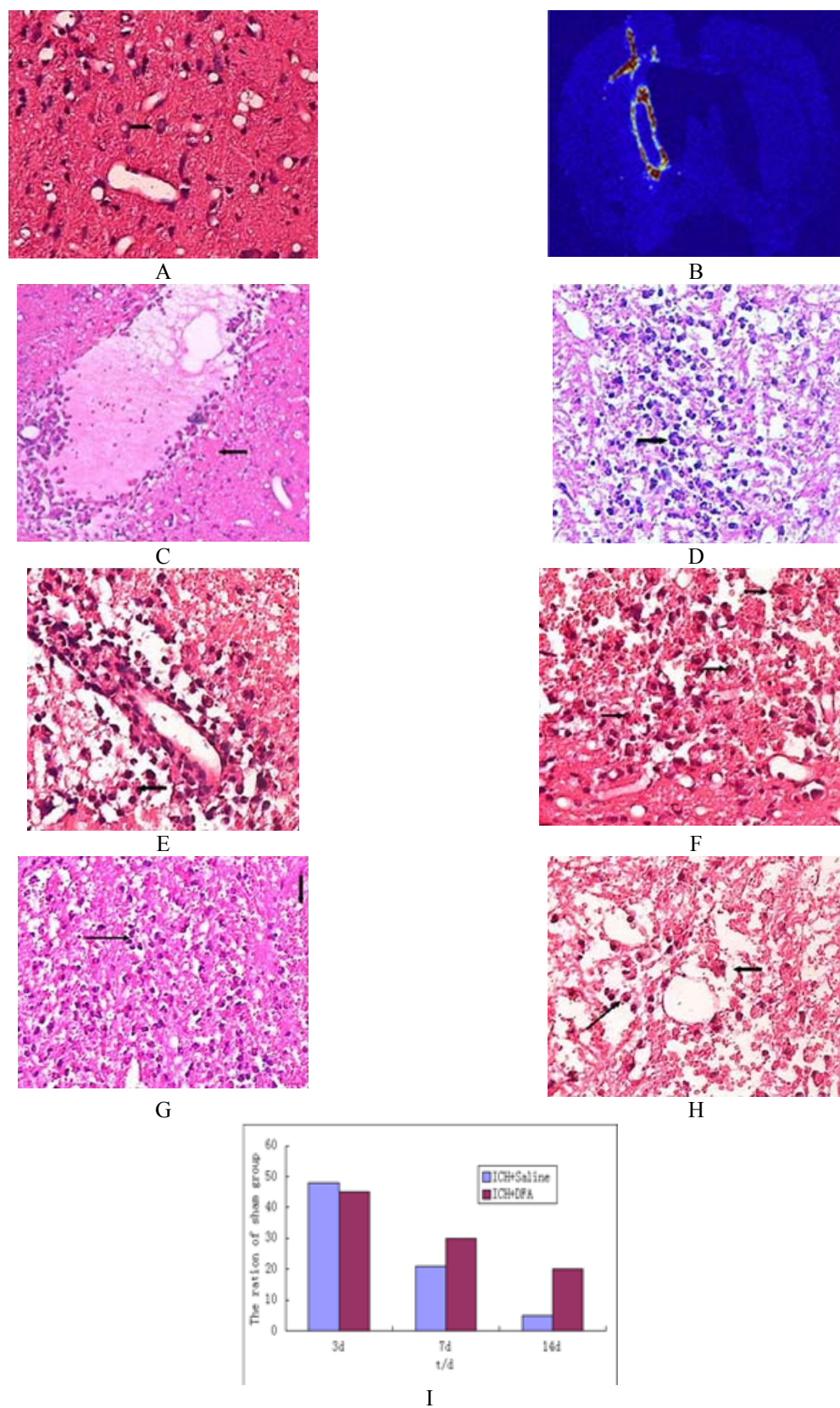


Fig. 3: Effect of DFA on neuronal loss in the perihematoma brain tissues (Original magnification: $\times 200$). Compared with Sham group, $*P < 0.05$; Compared with ICH group, $\# P < 0.05$.

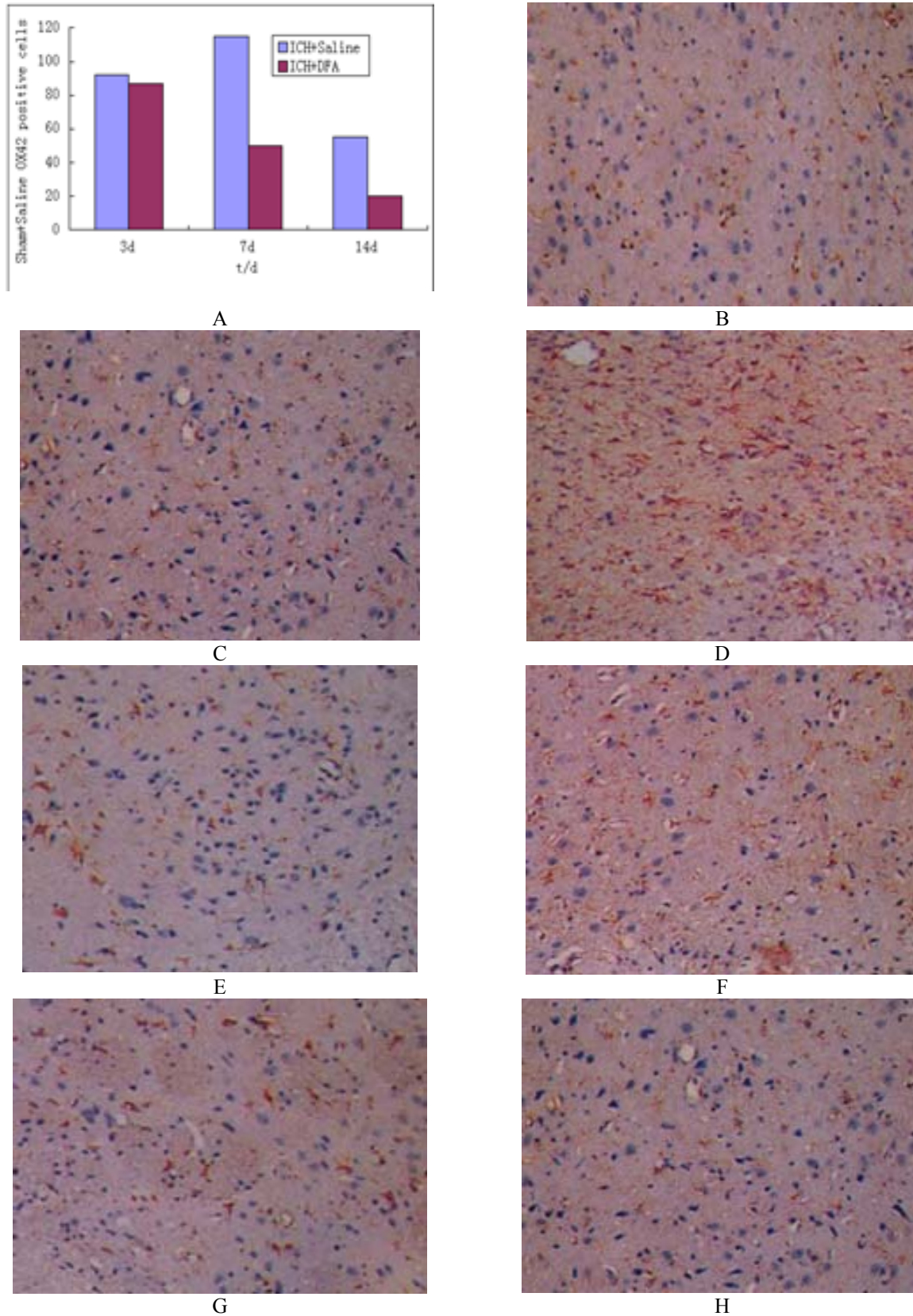


Fig. 4: (A) Effect of DFA on microglia in the perihematoma brain tissues compared with ICH group, * $P < 0.05$; (B-H) OX42 Immunohistochemical staining.

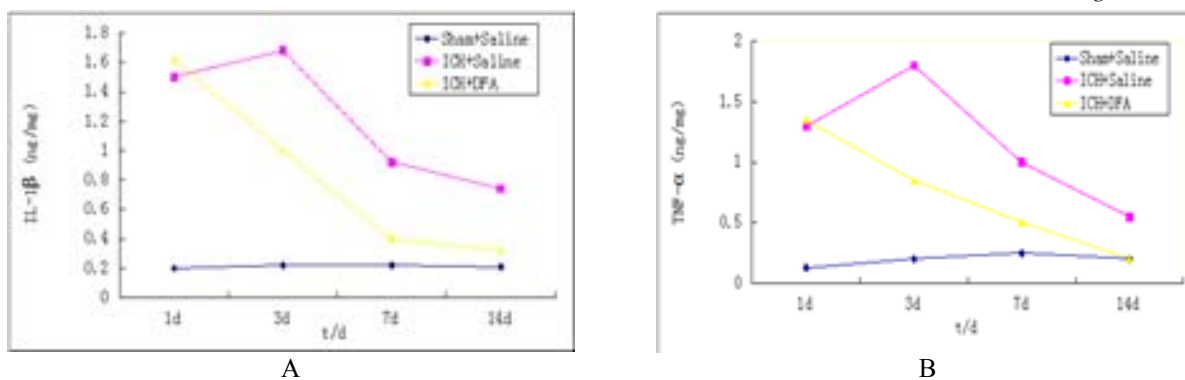


Fig. 5: Effects of DFA on IL-1 β and TNF- α levels of perihematoma brain tissue after ICH. Compared with Sham group, *P<0.05; Compared with ICH group, # P<0.05.

In conclusion, the study finds that releasing iron ions around the hematoma after ICH can activate microglia, causing secondary brain damage and considerable loss of neurons, which may be an important reason for the high disabling and sequelae of neurological dysfunction of ICH, and the specific mechanism needs further study. Application of iron ion scavenger can effectively inhibit excessive activation of microglia and reduce neurotoxic microglial cells secrete cytokines, reducing neuronal loss, and it will become a new way of clinical treatment of ICH.

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