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# Effects and mechanism of neuregulin on oxidative stress injury and Nrf2-are signaling pathway in rat intracerebral hemorrhage

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***Abstract:*** **To investigate the effect of neuregulin (NRG-1β) on neurological function and cerebral edema in rats with intracerebral hemorrhage and to explore the mechanism of neuregulin in intracerebral hemorrhage by detecting the expression of Nrf2-ARE, an endogenous antioxidant pathway. Method: Ninety adult male SD rats were randomly divided into control group, ICH group, ICH+vehicle group and ICH+NRG-1β group (2ug/Kg), and the rat model of intracerebral hemorrhage was established by stereotaxic caudate nucleus injected with type Ⅳ collagenase. After 24 hours of operation, the neurological dysfunction and the brain water content were measured and the expression of Nrf2 and HO-1 protein was detected by immunohistochemistry in every group. Results: Compared with the ICH group, the neurological score and the degree of cerebral edema were significantly reduced in the NRG-1β group after intracerebral ICH. The expression of Nrf2 and HO-1 protein in NRG-1β treatment group was significantly higher than that in ICH group. Conclusion: Neuregulin can significantly reduce the secondary damage of ICH, and its mechanism may be through the activation of Nrf2-ARE antioxidant pathway inducing the expression of downstream antioxidant enzymes, detoxification enzymes to protect nerve cells.**

***Keywords:*** Neuregulin; Intracerebral Hemorrhage; Oxidative stress

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## 1. Introduction

Intracerebral hemorrhage (ICH) is a common disease with high morbidity and high mortality [1]. In the world, there are about 200 million new patients with intracerebral hemorrhage every year, which expected to double the incidence by 2050, but so far, it is still lack of effective treatment in clinical practice [2]. The early stage of intracerebral hemorrhage injury is due to the rapid accumulation of blood in the brain cells lead to normal anatomical structure damage and hematoma mass effect, and secondary injury has a variety of complex pathological mechanisms. Compared with simple hematoma compression, intracerebral hemorrhage secondary injury is the main cause of neurological dysfunction and prognosis after intracerebral hemorrhage [3], so the treatment of secondary injuryof intracerebral hemorrhage has become the main way to treat intracerebral hemorrhage in recent years. Studies have shown that excessive oxidative stress is the beginning of multiple reactions in secondary injury of intracerebral hemorrhage [4], therefore, the study of the key factors affecting oxidative stress injury can be used as a therapeutic target to reduce secondary injury of intracerebral hemorrhage.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the family of transcription factors of basic leucine zipper structure. It is the regulating center of antioxidant stress in the body and can be transferred from cytoplasm to the nucleus and bind to the ARE under oxidative stress, upregulating the expression of heme-oxygenase (HO-1), quinone oxidoreductase NQO1, glutathione-s-transferase (GST) and other antioxidant enzymes [4]. Studies have shown that Nrf2-ARE pathway activation after intracerebral hemorrhage can play an effective protective effect.

NRG-1β is a member of the epidermal growth factor family with the activity of lanine kinase. By binding to epidermal growth factor receptor, it can activate multiple downstream signaling pathways and play a variety of regulation effect of neuronal growth, survival and function. In addition, a large number of studies have shown that NRG-1β has the protection of the blood-brain barrier, inhibition of inflammation, anti-oxidative stress, reducing neuronal apoptosis and other neuroprotective effects in the cerebral ischemic injury [5,6]. However, neuregulin on the protective

 

function of nerve cells and the mechanism is rarely reported, the experiment is to find the neuroprotective effect and the neuroprotective mechanism by neuromodulin intervention in the rat model of cerebral hemorrhage and observing the extent of cerebral edema, neurological function scores and the changes of expression of Nrf2-ARE pathway.

## 2. Materials and Methods

### *2.1. Experimental animals*

In this study, 80 healthy male SD rats weighing 250-300 g were used, and the experimental animals were purchased from Qingdao Paite Fude Colonial Cooperative. Sub-cage in the animal room, the standard particles feeding, room temperature being 22 ± 2℃, to adapt to the environment for 3 days, free to eat drinking water. All procedures are in line with the National Institutes of Health's Laboratory Animal Care and Use Guidelines.

### *2.2 Experimental design*

80 SD rats were randomly divided into two groups: control group (n=20); ICH group (n=20); ICH + vehicle group (n=20) and ICH+NRG-1β group (n=20). In the ICH + NRG-1β group, the recombinant human NRG-1β was dissolved and diluted to 1.5% solution with 0.1 mol/L PBS, and the tail vein was injected into NRG-1β (1μg/kg) after 30minutes of ICH. At an appropriate time, the rats were rejected into an equal volume of 0.1mol/L PBS in the ICH + vehicle group.

### *2.3. Intracerebral hemorrhage model*

Preoperative preparation: first of all, the rats had no food for 12h and no water for 4h before the surgery. Modeling process: Step one: anesthesia (400mg/kg) was injected into abdomen with 10% chloral hydrate solution. Step two:The experimental rats were fixed on the brain stereotaxic device, adjusting the stereoscopic positioner to make anterior fontanel and posterior keep the same plane, disinfecting head skin preparation, cutting 1 cm surgical incision along the sagittal skin in the middle of the skull, bluntly separating left and right skin, removing the periosteum with a cotton swab to fully expose the anterior fontanel and the posterior fontanel, in the left caudate nucleus surface, drilling a 1mm diameter hole with a dental drill and in the sense of breakthrough immediately stopping to avoid injuring intracranial tissue. Step three: The microinjector was used to extract the configured collagenase solution (0.2U/1ul) and fixed to the stereo locator. Then the needle is into the left caudate nucleus surface fixation point where 2ul collagenase were slowly injected into the caudal nucleus with the needle depth of 5.5mm (ICH group, ICH + vehicle group, ICH+NRG-1β group) and control group with micro-injector into the same amount of sterilized saline, leaving the needle for 5 minutes to prevent the solution from flowing back, slowly withdrawing the micro-injector, closing drilling holes with bone wax, suturing incision with iodophor disinfection. These rats were returned to the cage after surgery.

### *2.4. Neurological score*

After 24h of model, the neurological severity score (mNSS) was evaluated according to the internationally recognized modified neurological severity score (mNSS). The exercise tests included muscle strength assessment and abnormal exercise respectively. The sensory test included deep feeling the feeling of test. The higher the score, the more severe neurological dysfunction in rats, whereas the other is relatively mild. The maximum score is 18 points and the test is done by the same person who does not know the grouping.

### *2.5. Determination of brain water content*

After 24hours of model, 5 rats in each group were randomly chosen and taken out their brains, making the coronal section of the anterior and posterior 4mm injected into the cerebral hemisphere as slice, quickly removing the brain tissue samples and placing them in the labeled glass bottle to take wet weight, and then the vial on the drying oven at 100℃ for 72h, and then again weighing dry weight. Finally, the Blliot formula is used to calculate: brain water content = (wet weight -dry weight) / wet weight x 100%.

### *2.6. Statistical methods*

The experimental data were analyzed by SPSS 17.0 software. The data were expressed as mean ± standard deviation (X ± S), comparison of multiple groups using single factor analysis of variance. The two groups were compared between the t test and $P<0.05$ for the difference that was statistically significant.

## 3. Experimental Results

### *3.1. Effects of neuregulin-1β on neurological function after intracerebral hemorrhage in rats*

The test is done by the same researcher who does not know the grouping. The scores of neurological function scores in each group after intracerebral hemorrhage showed that compared with control group, the neurological dysfunction in ICH group was significantly higher than that in control group (*$P<0.05$), ICH + NRG-1β group was lower than ICH group and ICH +vehicle (#$P<0.05$) and here was no significant difference between ICH + vehicle group and ICH group (▲$P>0.05$). These results indicate that neuregulin -1β has the function of protecting nerve cells and reducing the damage of nerve cells after intracerebral hemorrhage.

### *3.2. Brain tissue water content*

The results of this study showed that the brain water content in the ICH group was significantly increased

(*P <0.01) compared with the control group, and compared with ICH group and ICH + vehicle group, the intervention of NRG-1β could significantly decrease the brain water content after intracerebral hemorrhage (#P<0.05). The brain water content of ICH group and ICH + vehicle group have no significant difference(▲p>0.05).

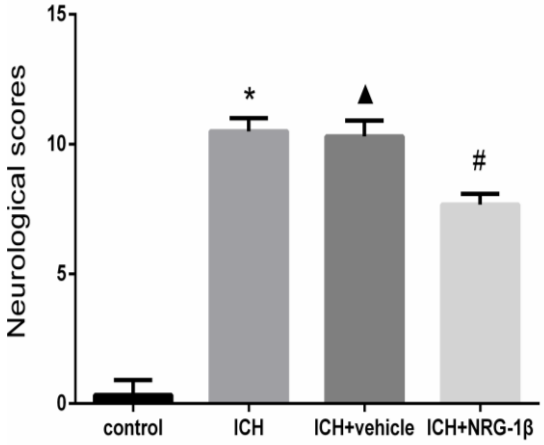


**Figure 1. Neurological score for each group. Data are expressed as mean ± standard deviation. *P <0.05 compared with the control group; # p <0.05 compared with the ICH group and ICH+vehiclegroup; ▲p> 0.05 compared with ICH group.**

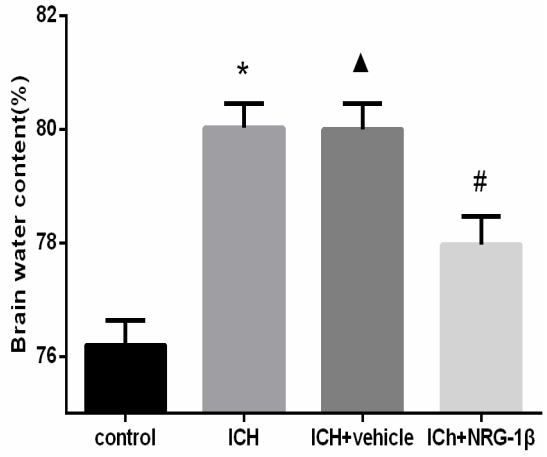


**Figure 2. Percentage of brain water content in each group. As shown in the figure, brain water content of ICH group compared to the control group significantly is increased and compared with ICH group and control group, the neuregulin -1β treatment can significantly reduce brain water content after intervention. Data are expressed as mean ± standard deviation. *P<0.05, compared with the control group; #P<0.05 compared with the ICH group and ICH+vehicle group; ▲P>0.05, compared with the ICH group.**

### *3.3. The change of the expression of Nrf2 and HO-1 protein detected by immunohistochemistry*

In order to study the effect of neuregulin -1β on the activation of Nrf2 signaling pathway after intracerebral hemorrhage, the expression of Nrf2 and HO-1 in cerebral hemorrhage was detected by immunohistochemistry. The results showed that the control group Nrf2 was mainly expressed in the cytoplasm, and no significant expression was observed in the nucleus (Nrf2 was not activated and nuclear transfer occurred). Control group had little expression of HO-1 in the cytoplasm and a large number of positive cells of Nrf2 and HO-1 were observed in ICH group and ICH + vehicle group. Compared with ICH group and ICH + vehicle group, the number of Nrf2 and HO-1 positive cells were further increased in ICH + NRG-1β group, the difference was statistically significant (#P<0.05), which indicated that neuregulin -1β can make the activation of Nrf2 signal path after intracerebral hemorrhage.

## 4. Discussions

The results of this study show that the Nrf2-ARE pathway is activated in the rat model of cerebral hemorrhage, and the expression of Nrf2 pathway can be further up-regulated by NRG-1β, regulating a variety of antioxidant enzymes and detoxification gene transcription and playing the role of antioxidant stress. At the same time, the intervention of neuregulin can reduce neurological dysfunction and the degree of cerebral edema. These results suggest that neuregulin can effectively relieve secondary injury of intracerebral hemorrhage. This neuroprotective action may be associated with the activation of the antioxidant effect of the Nrf2-ARE pathway.

After intracerebral hemorrhage, red blood cells' decomposition products such as iron ions, heme, thrombin, etc. can promote the body to produce a large number of free radicals, inducing oxidative stress. More and more studies have shown that oxidative stress is involved in multiple pathophysiological processes such as inflammatory response, endoplasmic reticulum stress, apoptosis, necrosis and autophagy after intracerebral hemorrhage, playing an important role of secondary injury in intracerebral hemorrhage. Recent studies have shown that the release of reactive oxygen species in oxidative stress, resulting in adjacent brain tissue protein, DNA, lipid oxidative damage, and can cause distant parts of brain tissue microcirculation [7], leading to cell ischemia and hypoxia, blood Brain barrier damage, triggering extensive nerve cell damage [8,9].

NRG-1β is a member of the epidermal growth factor family with the activity of lanine kinase. By binding to epidermal growth factor receptor (ERB), it can activate multiple downstream signaling pathways and play a variety of roles of regulation of neuronal growth, survival and function [10]. In addition, in vivo and vitro experiments show that NRG in the central

 

nervous system is an anti-inflammatory substance, can inhibit neuronal apoptosis, glial reaction and inflammatory response because of cerebral ischemia-reperfusion injury, inducing induction of neurotrophic factor expression with neuroprotection effect [11]. Human brain microvascular endothelial cells and H2O2 were cultured for 18 hours in vitro. The cell death rate was 44% in the control group and 33% in the NRG-1β group. The addition of NRG-1β and AG825 (ErbB2 inhibitor) increased it to 42 %, Thus confirming that the NRG-1β / ErbB pathway is resistant to oxidative stress injury [12]. Recent studies have shown that NRG-1β can significantly reduce the microglia surrounding the lesion, neutrophil activation and invasion, thereby reducing the activation of microglia and neutrophils that release large amounts of nitric oxide, hydrogen peroxide and super oxygen free radicals, aggravating secondary brain injury [13]. NRG-1β antioxidant activity has been confirmed by a large number of in vivo and in vivo experiments, however, the potential signal transduction mechanism is not very clear. Therefore, this paper through the establishment of rat model of cerebral hemorrhage studies the NRG-1β on cerebral hemorrhage protection and potential signal transduction mechanism.

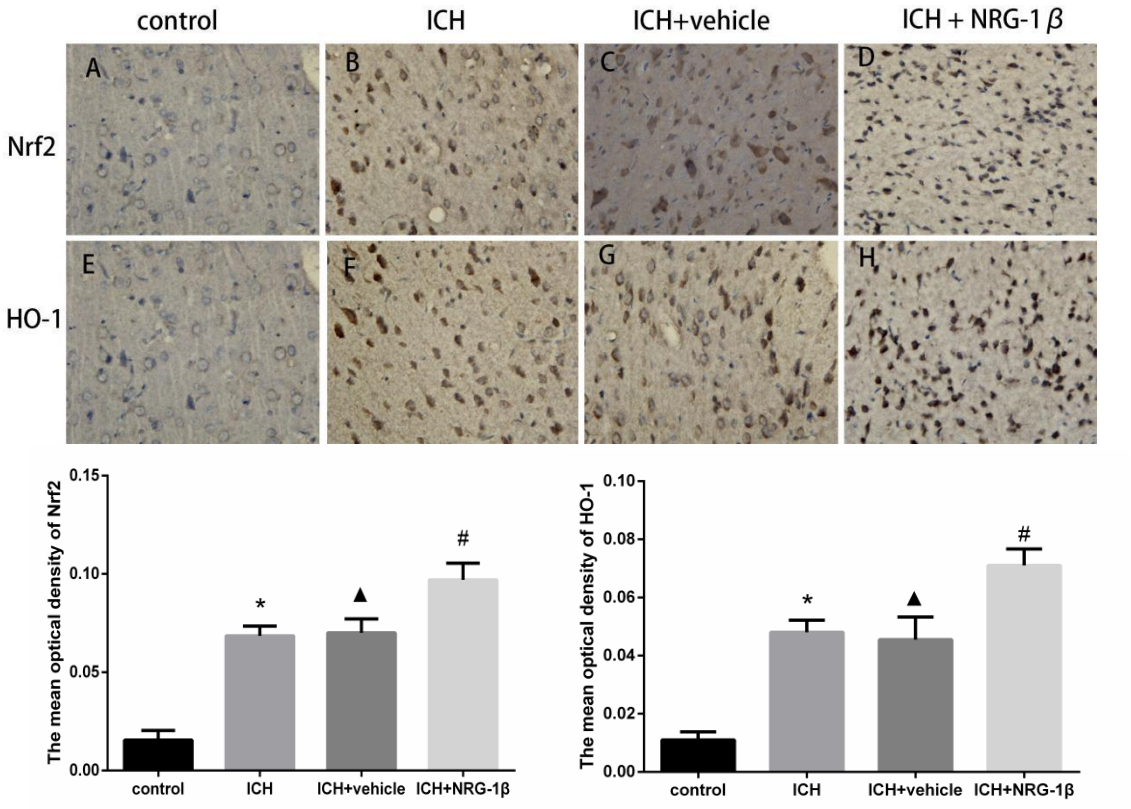


**Figure 3. Results of nef2 and HO-1 immunohistochemical staining. As shown in the figure, few Nrf2 and HO-1 positive cells were observed in the control group, and many Nrf2 and HO-1 positive cells were observed in the ICH group and the ICH + vehicle group, and the therapeutic intervention of the neuregulin -1β can significantly increase the number of Nrf2, HO-1 positive cells after intracerebral hemorrhage, indicating that the administration of neuregulin -1β can activate the Nrf2 signaling pathway after intracerebral hemorrhage. Data are expressed as mean ± standard deviation. *P<0.05, compared with control group; #P<0.05, compared with ICH group and ICH + vehicle group; ▲P>0.05, compared with ICH group.**

There is growing evidence that Nrf2 plays an important protective role in a variety of neurological disorders as the core of the endogenous antioxidant system. Under pathological conditions, Nrf2 is transferred into the nucleus and bound to the ARE, activating a series of cytoprotective enzymes to protect cells from oxidative stress injury [14]. Recent studies have shown that hematoma volume increases, leukocyte infiltration increases, reactive oxygen production and DNA damage are aggravated in the mouse cerebral hemorrhage model lack of Nrf2 knockout. The above damage of the Nrf2 gene expression group was significantly less [15], indicating that Nrf2 antioxidant pathway has an important protective effect.in intracerebral hemorrhage. NRG-1β has been shown to upregulate the expression of SOD, glutathione, catalase, glutathione reductase and other antioxidant enzymes in previous studies. However, the

 

molecular mechanism of NRG-1β is not clear, suggesting that NRG-1β / ERB can activate downstream PI3Kpathway expression [16]. The current study suggests that mitogen-activated protein kinase (MAPKs)[17], protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3K) [18] and other protein kinase pathway involved in the Nrf2-ARE activation process. Therefore, we assume that NRG-1β can activate Nrf2 pathway in intracerebral hemorrhage and upregulate the expression of various antioxidant enzymes to reduce oxidative damage. In the results of this study, we found that the tissue around ICH group hematoma where Nrf2, HO-1 protein content increased significantly, indicating that the Nrf2-ARE pathway was activated after ICH and exhibited antioxidant protection. Compared with ICH group and ICH + vehicle group, the expression of Nrf2 and HO-1 protein was further upregulated by NRG-1β for 24 h, and the neurological score and brain edema were significantly decreased, which indicated that NRG-1β can activate the Nrf2 pathway, upregulate the expression of downstream antioxidant enzymes, and play an anti-oxidative effect to relieve secondary injury of intracerebral hemorrhage.

The blood components into the brain because of intracerebral hemorrhage can induce oxidative stress and inflammatory response. HO-1 can decompose hemoglobin into bilirubin, biliverdin, CO and Fe2 + in time, promoting the chelating of Fe2 + to ferritin and reducing the free radical reaction induced by excess Fe2 +. Bilirubin and biliverdin have the role of scavenging oxygen free radicals and reducing lipid peroxidation. CO can activate cGMP pathway, and can be combined with hemoglobin, inhibiting hemoglobin on NO removal [19]. Thus, antioxidant free radicals can work, reducing the peripheral blood cell apoptosis, inflammatory cell infiltration [20], making the activation of oxidative factors and promoting cells' synthesis of catalase and glutathione to play an antioxidant effect [21]. Thus, in this study, NRG-1β upregulates the expression of HO-1 by activating the Nrf2 pathway to reduce oxidative stress and secondary injury of intracerebral hemorrhage. However, the mechanism by which NRG-1β is activated for the Nrf2 -ARE pathway is not well understood. For example, inositol phospholipase-3 kinase (PI3K) and extracellular signal-regulated kinase (ERK) can activate the Nrf2 pathway in some external stimuli [22,23]. On the other hand, it has been reported that NRG-1β can induce PI3K and ERK [16, 24] under certain conditions, which may be involved in NRG-1β and Nrf2 pathway activation mechanisms that are needed in further studies and investigations.

In summary, the results of this study show that NRG-1β can reduce neurological dysfunction, cerebral edema, significantly alleviate the secondary injury of intracerebral hemorrhage. The mechanism may be related to the activation of the Nrf2 pathway upregulating a variety of antioxidant enzymes that play a neuroprotective effect. However, there are some limitations in our research. Firstly, NRG-1β treatment was performed only once, and we did not know whether the multiple treatments were still effective or had other unknowable effects. In addition, NRG-1β may reduce ICH secondary injury through other protective mechanisms. Finally, this study did not validate the protective effect of NRG-1β in mice lack of Nrf2, so a comprehensive study was expected.

## 5. Conclusions

Neonectin-1β has a recovery effect on neurological dysfunction after intracerebral hemorrhage in rats.Intracerebral hemorrhage can activate the Nrf2 signaling pathway, promoting the expression of downstream HO-1 protein Neonectin-1β can activate the Nrf2 signaling pathway after intracerebral hemorrhage in rats, and further upregulate the expression of downstream antioxidant enzyme HO-1.

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