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Expression changes and significance of neurite outgrowth inhibitor A (*Nogo-A*), glial fibrillary acidic protein and insulin-like growth factor-1 in rat brain tissues after craniocerebral injury.

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Abstract

The aim of this study was to study the expression changes of neurite outgrowth inhibitor A (*Nogo-A*), Glial Fibrillary Acidic Protein (GFAP), and insulin-like growth factor 1 (IGF-1) in rat brain tissues after craniocerebral injury, and to investigate the neural repair mechanism of experimental craniocerebral injury in rats. The control group was only cut the top scalp and removed the skull, while the model group was performed moderate brain contusion. The expression levels of *Nogo-A*, GFAP and IGF-1 in the peripheral lesion tissues were detected by immunohistochemical assay. The immunohistochemical results showed that *Nogo-A*, GFAP and IGF-1 were expressed in the control group at various time points, while the change amplitudes were small. The expressions of *Nogo-A*, GFAP and IGF-1 in the model group at each time point were higher than the sham-surgery group (P<0.05), GFAP reached the peak at 3 d, while *Nogo-A* and IGF-1 reached the peaks at 7 d; after that, they all gradually decreased and approached normal levels at 28 d. The expressions of *Nogo-A*, GFAP and IGF-1 in rat brain tissues after craniocerebral injury were increased; through reducing the inhibitory factors of axonal growth and the formation of glial scar barrier, as well as promoting the expressions of the related neurotrophic factors, the prognosis of craniocerebral injury could be improved.

Keywords: Craniocerebral injury, *Nogo-A*, GFAP, IGF-1.

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Introduction

Craniocerebral injury (CCI) is a common severe neurosurgical emergency characterized by high mortality and high morbidity [1]. With the continuous improvements of economy levels, the incidences of traffic accidents and industrial accidents, as well as the resulted casualty number, are increased year by year, Jiang et al. [2] statistically analyzed the CCI database and found that the mortality in patients with severe CCI was 27.23% in 2014, and the mortality and severe disability rate was >53.17%, similar to those globally [3]. Although the levels of pre-hospital care and CCI diagnosis and treatment are continuously improved, there is no significant improvement in the mortality and disability rate of CCI patients in recent years [4]. Currently, scientific community has achieved the consensus that mature brain structures and functions are malleable; therefore, promoting nerve repair and regeneration after CCI has become one way to treat nerve injury. Recent studies had found the post-CCI neurological disorders were related to the regeneration and repair disorders of the central nervous system [5,6]. Neurite outgrowth inhibitor A (*Nogo-A*) protein is widely distributed inside the cell bodies and projections of oligodendrocytes, as well as in the nuclei of neurons, and is the strongest inhibitory factor, among the myelins of central nervous system, against the growth of neuronal neuritis [7]; glial fibrillary acidic protein (GFAP) is a mature astrocyte-specific protein, and related with the proliferation of glial scars [8]; insulin-like growth factor 1 (IGF-1) is a neurotrophic factor, and could promote the differentiation, proliferation, repair, and regeneration of nerve cells [9]. In order to study the impacts of CCI on the expressions of *Nogo-A*, GFAP and IGF-1 in rat brain tissues, and to explore the neural repair-related mechanisms in experimental CCI rats, we prepared the free-fall induced CCI animal model, then detected the protein expression levels of Nogo A, GFAP and GAP-43 after injury to study the possible mechanisms of these nerve regulators and to provide theoretical basis for clinical treatment.

Materials and Methods

Animal and grouping

50 healthy male SD rats (body weight 250 ± 20 g) (provided by the Laboratory Animal Center, Zhejiang Academy of Medical Sciences) were bred with standard diet and water in quiet environment. Room temperature 23-25°C and constant humidity (about 70%) were kept from one week before the

experiment until the end of the experiment. The rats were randomly divided into the control group and the model group using random number table method (n=25), and divided into five sub-groups (D1, D3, D7, D14 and D28, n=5); the rats in each subgroup were killed at the designed post-CCI time points, respectively. The control group was only cut the top scalp and removed the skull; the model group was performed moderate brain contusion on the right top using the improved Feeney free fall CCI device to prepare the CCI model. During the procedures of surgery and reanimation, animals' body temperature was maintained around 37°C. The rats with consciousness loss after reanimation, neurological deficits and death were excluded, and the rats from the same batch were then selected to receive the same treatment. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First People's Hospital of Wenling.

Preparation of rat CCI model

After anesthetized with intraperitoneal injection of 10% chloral hydrate (3 mg/kg), the rat was placed in the prone position and fixed, cut 2.5 cm scalp along the sagittal line, revealed the right parietal bone and closely pressed for hemostasis; set the cross point, 2.5 mm right to the midline and 1.5 mm posterior to the coronal crack as the center, used a skull drill to carefully drill one 5 mm skull window; kept the dura intact, padded an appropriate amount of foam under the rat head, fixed the guide rod vertically, and placed the polyethylene striking head against the bone window; used 40 g hammer falling from 20 cm height to hit the polyethylene striking head to cause moderate CCI in rat cerebral hemisphere; the signs of brief limb twitching and apnea after the strike indicated the successful experimental injury.

Specimen collection

The rats of each group were decapitated at the corresponding time points; anesthetized with 4% chloral hydrate, then quickly opened the chest and pericardium, and exposed the heart; cut a small open on the left ventricle, inserted the perfusion needle along the left ventricular towards the right atrium, fixed the needle; cut open the right atrial appendage; slowly bolus-perfused 250 ml of 0.1 mol/L PBS, until the effluent from the right atrial appendage became clear; changed to 250 ml of 0.1 mol/L PBS-prepared 4% paraformaldehyde to continue cardiac perfusion; decapitated the rat immediately after the perfusion completion; sampled the brain tissues at the hippocampal and cortical border zone; after fixation, washing, ethanol dehydration, hyalinization and paraffin-embedding, performed 4 μm serial slicing for future use.

Immunohistochemistry

The paraffin sections were grilled, dewaxed, hydrated, and performed antigen retrieval and closed with goat serum; incubated with rabbit anti-mouse Noga-A polyclonal antibody (abcam Co., Cambridge, UK) 1:200, goat anti-mouse polyclonal GFAP antibody (abcam Co., Cambridge, UK) 1:200, and rabbit anti-mouse IFG-1 polyclonal antibody (SANTA CRUZ Co., CA, USA) 1:200 at 4°C overnight, rinsed with PBS 5 min × 3; added FITC-conjugated mouse anti-goat IgG (Beijing Bioss Co., Beijing, China) and FITC-conjugated mouse anti-rabbit IgG (Beijing Bioss Co., Beijing, China) and incubated at room temperature in darkness for 1 h, rinsed with PBS 5 min × 3. The sections were then performed DAB staining, dehydrated, and mounted for examination.

Data collection

Five slices were sampled from the same site of each group, and then each slice was microscopically magnified by 200 times and sampled five non-overlapping high-power fields for photography to count the number of positive cells and calculate the average.

Statistical analysis

SPSS17.0 software was used for statistical analysis, the measurement data were expressed as $\bar{x} \pm s$, the inter- and intragroup comparison used ANOVA.

Results

Expression of Nogo-A

The positive immunohistochemical products were mainly distributed in oligodendrocytes. In the control group, *Nogo-A* protein was expressed at various time points, while the change amplitude was small. Compared with the control group, the expression of *Nogo-A* protein in the brain tissues of the model group at each time point was higher than the control group ($P<0.05$); the number of positive cells reached the peak on D7, then gradually decreased and approached normal level on D28 (Table 1).

Table 1. *Comparison of Nogo-A expression in rats at each time point after CCI ($x \pm s$).*

Group	1 d	3 d	7 d	14 d	28 d
Control	19.04 ± 7.51	19.08 ± 7.39	19.00 ± 7.45	18.96 ± 7.49	19.12 ± 7.48
Model	27.44 ± 8.27	35.84 ± 9.74	42.12 ± 9.37*	34.64 ± 8.72	28.96 ± 8.46
P value	0.131192	0.0154586	0.0025506	0.0158182	0.0872018

*The number of *Nogo-A* positive cells reached the peak on D7 in the model group compared with the control group.

Expression of GFAP

The positive immunohistochemical products were mainly distributed in the astrocytes. In the control group, GFAP protein was expressed at various time points, while the change amplitude was small. Compared with the control group, the

expression of GFAP protein in the brain tissues of the model group at each time point was higher than the control group (P<0.05); the number of positive cells reached the peak on D3, then gradually decreased and approached normal level on D28 (Table 2).

Table 2. *Comparison of GFAP expression in rats at each time point after CCI (x ± s).*

Group	1 d	3 d	7 d	14 d	28 d
Control	28.68 ± 4.13	29.12 ± 4.58	29.36 ± 4.67	28.88 ± 4.33	28.84 ± 3.99
Model	35.28 ± 5.68	45.36 ± 7.59*	39.48 ± 6.53	37.84 ± 6.35	34.92 ± 5.96
P value	0.068782	0.003455	0.022538	0.031286	0.094617

*The number of GFAP positive cells reached the peak on D3 in the model group compared with the control group.

Expression of IGF-1

The positive immunohistochemical products were mainly distributed in nerve cells. In the control group, IGF-1 protein was expressed at various time points, while the change amplitude was small. Compared with the control group, the expression of IGF-1 protein in the brain tissues of the model group at each time point was higher than the control group (P<0.05); the number of positive cells reached the peak on D7, then gradually decreased and showed no significant difference than the control group on D28 (Table 3).

Table 3. *Comparison of IGF-1 expression in rats at each time point after CCI (x ± s).*

Group	1 d	3 d	7 d	14 d	28 d
Control	2.04 ± 0.09	2.08 ± 0.07	1.96 ± 0.11	2.04 ± 0.09	2.00 ± 0.13
Model	20.08 ± 0.72	31.24 ± 1.53	39.52 ± 1.67*	4.08 ± 0.56	1.96 ± 0.15
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.664236

*The number of IGF-1 positive cells reached the peak on D7 in the model group compared with the control group.

Discussion

Post-CCI neurological disorders are related to the regeneration disorders after the central nervous system is injured; currently, the main reasons are considered as: there are ingredients inside microenvironments which could inhibit the growth of neuritis, together with the space obstruction effects of glial scars and the deficiency of neurotrophic factors, promoting the repair and regeneration of nerves after CCI has become an important way to improve the prognosis. Savio and Scawab [10] transplanted the spinal cord dorsal root ganglion cells of adult rats into the spinal gray matter, and found that it could extend the axons; while when they were transplanted into the white matter, the axons could not regenerate; therefore, it was found that the spinal white matter could produce axonal growth inhibitors, and one inhibitory protein was successfully separated from the differentiated oligodendrocytes and named Nogo protein, which meant "No Go for Neuron". *Nogo-A* protein belongs to sphingomyelin associated protein, and is one neurite growth inhibitory factor encoded by Nogo gene. It had been identified that it could inhibit the regeneration of neuronal synapse after the central nervous system was injured [11]. Huber et al. [12] used confocal and immuno-electron microscopy and revealed that *Nogo-A* protein was mainly expressed in the cell bodies and projections of oligodendrocytes, and located adjacently to axons and on myelin membranes, which was an convenience condition for it to play its axon inhibition effects. This study observed on D1 after CCI, the expression of *Nogo-A* protein was significantly increased, which was then continuously increased and reached the peak on D7, and that, it would gradually decrease and approach normal level on D28. The model group had the same time-dependent changes, but the expression level at each time point was significantly higher than the control group, indicating that after the central nervous system was injured, the dissolved oligodendrocytes could rapidly release *Nogo-A* protein; meanwhile, because the blood brain barrier was damaged and the permeability was increased, *Nogo-A* protein would bind with its specific receptor complex, then regulated the microfilament protein in the growth cone via the second messenger, so the growth cone would degenerate and inhibit the axonal regeneration [13]; when the damaged oligodendrocytes gradually repaired themselves, the release and shedding of *Nogo-A* would be reduced, and its expression would also be gradually decreased.

The proliferation of glial scars is the main morphological manifestation during the central nervous system repairs injury, which fills the tissue defect of the injured nerves and restores its structural integrity; however, regarding the nerve regeneration, the compact structures formed by the proliferation of glial scars could spatially hinder the nerve regeneration and functional reconstruction, hinder the migration and regeneration of the newborn neurons, thus it would become one of the important factors affecting the nerve regeneration and functional reconstruction. Glial cells in the central nervous system are mostly the astrocytes, which have the heterogeneous characteristics, while the significance of this heterogenicity is not clearly understood [14]. The astrocytes are the main component composing the glial scars, as the intermediate filament and cytoskeletal protein of the astrocytes [15], GFAP is essential for maintaining the morphological and structural stabilities of the astrocytes, and could decide the response extent of the astrocytes towards nerve injury, so monitoring the expression level of GFAP could reflect the proliferation extent of glial scars. In this study, we found that the expression level of GFAP in the control group was low, and the change was not significant among different time points; while after injured, the model group exhibited significantly increased GFAP positive cells than the control group, which reached the peak on D3, then gradually decreased and approached normal level on D28, indicating that after CCI, the repair process characterized by the proliferation of the astrocytes would immediately appear and reach the peak on D3, consistent with the foreign researches [16]. CCI could

cause inflammations; meanwhile, the astrocytes would be activated as the neural immune cells; and this increased activity would speed the synthesis and secretion of inflammatory cytokines. Therefore, by inhibiting the lipid peroxidation, clearing the oxidative free radicals, and inhibiting the neurologic inflammatory factors, the astrocyte-related brain injury responses could be inhibited, thereby reducing the formation of glial scars and promoting the neurological recovery.

IGF-1 is a cytokine with in vitro and in vivo biological activities, and widely presents in the normal brain tissues. The recent study had found that IGF-1 was very important in regulating the nerve growth [17], and it could participate in the brain development, promote the axonal growth, maintain and regulate the expressions of nerve functions. IGF-1 would be activated in CCI, thus exhibiting important protection and restoration towards the injured nerve cells, and promoting the nerve regeneration [9]; therefore, it could help the damaged nerve cells to restore their functions and reduce the brain injury. In this study, we found that after injured, the IGF-1 positive cells in the model group were significantly increased than the control group, gradually increased with the injury time extending, reached the peak on D7 and decreased gradually, consistent with the trend of *Nogo-A*, indicating that after CCI, the expression of IGF-1 would be increased, thus playing the roles of protecting neurons, and this might be related with the roles of reducing the regeneration inhibition of neuronal synapses by *Nogo-A*. Carlson et al. [18] observed in the rat model with cortical impact injury that the IGF-1 expression in the hippocampus region was significantly increased, and the proliferation of the astrocytes was significantly activated, on D7 after trauma, a large number of immature neurons were seen proliferated around the injured areas; so, it was considered that IGF-1 could promote the nerve synthesis after CCI. Madathil et al. [19] found through the rat model with controlled cortical injury that the IGF-1 expression was significantly increased in the injured areas including the hippocampus, and accompanied by a significant proliferation of the astrocytes. Meanwhile, the GFAP expression was also significantly increased. IGF-1 could reduce the degeneration of hippocampal neurons in the acute phase (<3 d) of CCI through the Akt phosphorylation, and peaked on D10 after injury. While certain study also showed that as a specific neurotrophic factor, IGF-1 could not only be conducive to the growth, repair and regeneration of nerve cells, but also prevent the apoptosis and death, and help the function recovery after the nerve cells were injured. IGF-1 is an important factor synthesized by the myelin, and has certain effects in multiple sclerosis and other demyelinating diseases. Through PCR quantitative analysis, Chesik et al. [20] found that after using the progesterone to open the blood brain barrier, the levels of IGF-1 and its receptors in the astrocytes and oligodendrocytes could be upregulated, and the sites were consistent with those in which *Nogo-A* was expressed and played its functions, suggesting that IGF-1 might antagonize *Nogo-A*'s roles of inhibiting the axonal growth, thus demonstrating the effects of promoting the neuronal repair. Baykara et al. [21] discovered from the 7-day-old pedo-rat model with brain contusion that the progesterone treatment could significantly improve the IGF-1 level in plasma and reduce the corticosterone level, thus reducing the post-CCI anxiety status; all these indicated that IGF-1 had neuroprotective effects.

In summary, the expressions of *Nogo-A*, GFAP, and IGF-1 in rat brain tissues after CCI were increased, which could reduce the inhibitory factors against the axonal growth and reduce the formation of glial scar barriers, thus promoting the expressions of the related neurotrophic factors and improving the post-CCI prognosis. But the expression changes of *Nogo-A*, GFAP, and IGF-1 with time courses and their mutual relationships still needed further studies.

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