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OPN (OSTEOPONTIN) REGULATES NEUROINFLAMMATION IN RATS WITH HYPERGLYCEMIC CEREBRAL HEMORRHAGE

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Abstract: Objective: To investigate the intervention effect of OPN (osteopontin) on the expression of nuclear factor-KB (Fume-xappab, NF-KB) and interleukin-1 (IL-1) after hyperglycemic cerebral hemorrhage in rats. Methods: The hyperglycemia cerebral hemorrhage model was made by autologous blood injection. The neurological function behavior score was performed by Garcia method in each group. The changes of brain water content were measured by wet weight method. Results: The expression of NF-KB and IL-1 protein was significantly increased after hyperglycemia cerebral hemorrhage, the expression of NF-KB and IL-1 protein was significantly decreased after osteopontin intervention, and the brain water content was significantly decreased, which was consistent with the cerebral nerve dysfunction score. Conclusion: Osteopontin may inhibit the expression of IL-1 through the NF-KB pathway, reduce the inflammatory damage of the brain tissue around the hematoma after hyperglycemia cerebral hemorrhage, and play a protective role on the brain tissue.
Keywords: Hyperglycemia cerebral hemorrhage; Osteopontin; Nuclear factor -KB; Interleukin 1 beta

1 INTRODUCTION

Hyperglycemic intracerebral hemorrhage has a high mortality and disability rate. About 50% of patients die within 6 months, and about 80% of survivors cannot fully recover. At present, the mechanism of secondary injury caused by hyperglycemic intracerebral hemorrhage is not clear [1]. Research shows that inflammation is an important factor in secondary injury after hyperglycemic intracerebral hemorrhage. The research of molecular biology shows that nuclear factor kappaB (NF kB) causes the expression of various inflammatory factors by initiating and enhancing the activation of KB site, which is closely related to inflammation [2] . Osteopontin, as an endogenous cholesterol inhibitor, can improve microcirculation and reduce brain edema in hemorrhagic brain injury [3]. Whether osteopontin plays an anti-inflammatory role through NF kB pathway after hyperglycemic intracerebral hemorrhage remains to be further studied. Therefore, this experiment further explored the mechanism of secondary inflammatory damage caused by hyperglycemic intracerebral hemorrhage and the intervention effect of osteopontin by establishing a hyperglycemic intracerebral hemorrhage model.

2 MATERIALS AND METHODS

2.1 Laboratory Animals

66 healthy male SD rats with a body mass of 250~300g, SPF grade, were purchased from Beijing Huafukang Biotechnology Co., Ltd., and ate and drank freely. The rats were randomly divided into sham operation group (Sham), hyperglycemic intracerebral hemorrhage model group (ICH), osteopontin intervention group (ICH+SIM), 20 rats in each group. Osteopontin Zhejiang Nanyang Pharmaceutical Co., Ltd., batch number: H20073719.

2.2 Modeling of Hyperglycemic Cerebral Hemorrhage

The experimental hyperglycemic cerebral hemorrhage model was established by Fredrik method. After the rats were anesthetized successfully by intraperitoneal injection of 10% chloral hydrate, they were fixed on the brain stereotaxic apparatus, cut the skin, exposed the front fontanelle, and positioned the tip of the microinjector 0.2 mm in front of the front halogen, and 3.0 mm on the right side of the midline. Use a dental drill to drill a hole with a diameter of about 3mm here. Use a micro sampler to inject a needle along the drill hole, with a depth of 6.0mm, into the basal ganglia area of the caudate nucleus of the rat, inject 50 μ L of autologous femoral artery blood, complete the injection in 5min, keep the needle for 15min, then slowly withdraw the needle, seal the small hole with sterile bone wax, and suture it. In the sham operation group, the model was made in the same steps, and normal saline was injected. In the intervention group, osteopontin 3mg · kg^{-1} · d^{-1} was administered by gavage [4] after complete recovery.

2.3 Neurologic Dysfunction Scores and Cerebral Water Content Measurements

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After hyperglycemic intracerebral hemorrhage in rats, the activity of the contralateral limb of the lesion was disadvantageous. In this experiment, the Garcia method was used to conduct the neurobehavioral score (NDS). The lower the score, the more serious the neurological dysfunction. Double blind method was used for evaluation. Determination of brain tissue water content (BWC): kill the animals, take out the brain, weigh the brain tissue with an analytical balance, place it in an incubator for drying, take out the dry weight, and calculate the brain water content: (wet weight dry weight)/wet weight × 100% [5].

2.4 Immunohistochemical Observation of Protein Expression of NF kB and IL-1 β

After anesthesia, the rats in each group were perfused with 4% paraformaldehyde phosphate buffer solution, then decapitated and brain was taken, embedded in paraffin, and sliced for immunohistochemical analysis. NF kB primary antibody and interleukin 1B (IL-1 β) primary anti polyclonal antibody are provided by Wuhan Bode Biological Reagent Co., Ltd; SP kit and 3,3 'diaminobenzidine (DAB) are provided by Fujian Maxin Company; DAB coloration, hematoxylin re staining of nuclei, distilled water washing, dehydration, transparency, and film. The positive staining was brown granules in cytoplasm or nucleus. In the negative control, PBS was used to replace the primary antibody, and the other steps were the same as those of [6].

2.5 Protein Expression Levels of NF kB and IL-1 β Detected by Western Blot

The rat brain was quickly removed after decapitation, and the supernatant was taken after homogenization centrifugation. The protein content was measured by BCA method. Take 30 μ L of protein sample after treatment and electrophoresis it on 12% SDS-PAGE gel and transfer it to PVDF membrane. With standard protein maker as reference, 5% skimmed milk powder is closed. The corresponding bands are added with NF-KB antibody (1:1000) and IL-1 β antibody (1:400) respectively and incubated overnight at 4 °C. ECL color display, scan with fluorescent CCD camera [7].

2.6 Immunohistochemical detection of OPN

The distribution and level of OPN expression on the wall of basilar artery were detected by routine immunohistochemical technique: (1) After paraffin sections were dewaxed and hydrated, PBS solution was used to wash them three times (3min/time); (2) Citric acid buffer solution was used for high temperature and high pressure repair. 50 μ L peroxide blocking solution was added to each slice at room temperature and incubated for 10 minutes; (3) Rinse the PBS solution for three times (3min/time), add 50 μ L of normal non immune animal serum to each slice after removing the PBS solution, incubate at room temperature for 10min, remove the serum, add a drop of 1:100 diluted RatAnti OPN and RatAnti MMP9 to each slice at room temperature, and incubate for 60min; (4) Flush with PBS solution for 3 times, 3-5 min each time; (5) Remove PBS solution, add 50 μ L biotin labeled second antibody to each slice at room temperature, and incubate for 10 minutes; (6) Flush with PBS solution for 3 times, 3min each time; Remove PBS solution, add 50 μ L streptomycin avidin G peroxidase solution to each slice at room temperature, and incubate for 10 minutes; (7) Rinse with PBS solution for 3 times (3min/time), remove PBS solution, add 50 μ L fresh DAB solution to each slice, and observe under microscope for 3-10min; (8) Rinse with tap water, re dye with hematoxylin, and return to blue after washing with PBS solution; (9) Dehydrated, transparent, and sealed. Judgment standard: positive staining is brown or brown yellow. Observe the staining of the intima, media and adventitia of each vessel wall under the microscope (400 times), and indicate (-) (+) (+++) for non staining, mild or local, full layer severe or strong staining respectively, and (++) for those between the latter two [8].

2.7 Image Analysis and Statistical Processing

Enlarge the stained section 100 times under the microscope, randomly select 5 non overlapping visual fields, count the number of positive cells and measure their gray value under the computer by the image analyzer, and the measured data is expressed by $x \pm s$. Using the SAS6.12 statistical software, the hypothesis test uses the square error analysis and the inter group t-test. P<0.05 is statistically significant.

3 RESULTS

3.1 Analysis of Neurological Dysfunction Scores and Brain Tissue Water Content

The experimental rats woke up about 3 hours after the operation, and the rats in each group were scored by Garcia method at 5 hours after the operation. Both the model group and osteopontin intervention group of rats with hyperglycemic intracerebral hemorrhage model after operation showed severe neurological dysfunction, unstable standing, limb weakness, tail chasing, etc. BWC significantly increased, indicating the formation of brain edema. After the intervention of osteopontin, the neurological dysfunction of rats was significantly reduced, and the brain water content was significantly reduced, with a statistically significant difference (P<0.05, Table 1).

Table 1 Neurological Dysfunction Score of Rats and Water Content of Brain Tissue around Hematoma after Operation (n=10, $\overline{x} \pm s$)

Groups	BWC	NDS
Sham	78.25±0.55	17.22±0.26
ICH	82.15±0.45a	9.32±2.45a
ICH+SIM	79.29±0.39ab	11.79±0.52ab

Note: [a]P<0.05 compared with sham group; [b]P<0.05 compared with ICH group

3.2 Immunohistochemical Detection

In the sham operation group, abundant nerve cells can be seen in the basal ganglia of brain tissue. The cell bodies are round and orderly arranged, with dense structure, clear nucleoli and no edema (Figure 1). In the model group, the number of nerve cells in the basal ganglia area was significantly reduced, and the arrangement was disordered and sparse. The positive cells in the basal ganglia area were brown yellow. Compared with the model group, the positive expression of NF - in the intervention group was significantly reduced (P<0.05, Table 2).

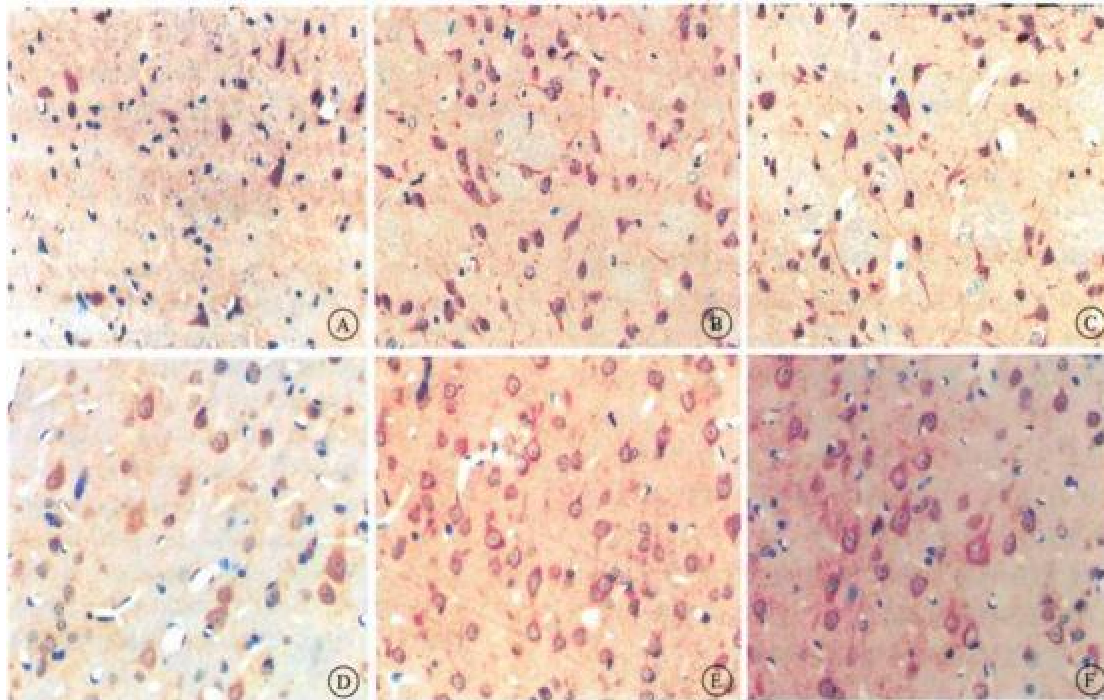

Figure 1 Protein Expression of IL-1 β and NF kB around Hematoma, Immunohistochemical Color-X400

Table 2 Changes in the Number of IL-1 β and NF kB Immunoreactive Cells (n=20, $\overline{x} \pm s$)

Groups	IL-1β	NF-kB
Sham	8.45±0.53	10.26±1.35
ICH	25.05±2.16a	30.65±1.15a
ICH+SIM	16.55±1.82ab	21.25±2.36ab

Note: [a]P<0.05 compared with sham group; [b]P<0.05 compared with ICH group

3.3 Protein Expression of NF-KB and IL-1 β Detected by Western Blot

According to the band sensitivity analysis of NF-KB electrophoresis, compared with the sham operation group, the expression of NF kB gene in the model group began to increase after hyperglycemic intracerebral hemorrhage (P<0.05), and after osteopontin intervention, the expression of NF kB gene was significantly lower than that in the model group (P<0.05). The protein expression of IL-1 β was consistent with that of NF kB.

4 DISCUSSION

OPN belongs to SIBLING glycoprotein and was first found in osteoblasts in 1986. Also known as bone sialoprotein I (BSP1), early T lymphocyte activation 1 (Eta1) 1, this protein has high homology in different species

and is highly conservative. The OPN gene encoding of different species is slightly different. The OPN gene encoding human is located on chromosome 4 q13, which is a single copy gene with a length of about 5.4~8.2 kb. The coding sequence includes 7 exons and 6 introns [9]. The OPN gene encoding rat was located in the rice region 15,16 of chromosome 5. The human OPN protein is a single chain polypeptide, which contains about 300 amino acids. There are 16 amino acid residues to form a signal peptide, which is mixed with sialic acid and sulfate during translation. OPN is rich in glutamic acid residues, aspartic acid residues and glutamic acid residues [10]. The regulatory mechanism of OPN expression with molecular weight of 44-75KD is unknown. There are multiple response elements in the promoter region of OPN gene, including transforming growth factor - β (TGF - β), high glucose, glucosamine, etc; There are vitamin D response element, glucocorticoid response element, activator protein-1 (AP-1) binding site and AP-1 binding site. Therefore, OPN can be regulated by many factors, such as PTH, vitamin D3, calcium, phosphorus, etc., which can regulate the expression of OPN, thus playing a corresponding biological role. It has been confirmed that TGF - β 1 and EGF can increase OPN gene transcription, thus increasing OPN expression; IL-l can also up regulate the expression of OPN mRNA and protein in a dose-dependent manner, and IL-1 receptor antagonist can inhibit this expression. Therefore, there are many ways to regulate OPN. Through corresponding intervention, the biological function of OPN can be affected.

The main pathological change of nerve tissue after hyperglycemic intracerebral hemorrhage is that the hematoma in the brain compresses the peripheral nerve tissue to form a space occupying effect, resulting in localized or extensive brain tissue edema and inflammatory reaction. Research shows that inflammatory reaction is one of the main reasons for secondary damage of nerve tissue after hyperglycemic intracerebral hemorrhage. Pang Chunyan et al. [11] showed that the pro-inflammatory cytokine IL-1 β participates in the inflammatory response caused by brain injury through various ways, mediates neuronal necrosis and loss, and is an important link in the formation of a series of pathophysiological changes and secondary inflammatory injury. The results of this study showed that compared with the sham operation group, the model group had severe neurological dysfunction, significantly increased water content in brain tissue, high levels of inflammatory factor IL-1 β positive cells in brain tissue around the hemorrhage, brown cytoplasm, and edema fissure expression between cells. It is suggested that hyperglycemic intracerebral hemorrhage may cause secondary inflammatory damage, and the proinflammatory factor IL-1 β plays an important role in inflammatory response. It is important to study the expression and regulation mechanism of IL-1 β for formulating prevention and treatment strategies for spontaneous hyperglycemic intracerebral hemorrhage. Some studies believe that NF kB, as an upstream regulatory factor of many inflammatory cells, plays an important role in inflammatory response and may participate in the pathophysiological process of inflammatory response. When activated, it enters the nucleus and binds to DNA to promote the formation of inflammatory cascades [12] through transcriptional regulation of inflammatory factors such as TNF - α, IL-1 β, etc. We speculate that NF kB may be involved in regulating the expression of inflammatory factor IL-1 β after hyperglycemic intracerebral hemorrhage in rats. Therefore, this study established a rat model of hyperglycemic intracerebral hemorrhage to observe the expression of NF kB and IL-1 β [13] . Immunohistochemical results showed that, compared with the sham operation group, the model group had high levels of NF-KB and IL-1 β expression, and both were positively correlated. Immunoblotting results showed that the expression levels of both were consistent. It is suggested that activation of NF kB signaling pathway may be an important reason for IL-1 β - mediated inflammatory reaction around brain tissue after hyperglycemic intracerebral hemorrhage. So inhibiting this signal pathway can inhibit the expression of IL-1 β, and reduce the inflammatory reaction in the surrounding brain tissue after hyperglycemic intracerebral hemorrhage. Statins can not only play a role in reducing blood lipids, but also play a role in the prevention and treatment of cardiovascular and cerebrovascular diseases through non lipid regulating effects, improving the function of vascular endothelial cells, anti-inflammatory and other ways. There is evidence that statins can significantly reduce the volume of cerebral infarction during cerebral ischemia-reperfusion, effectively reduce the expression of inflammatory factors mediated by NF kB pathway, and inhibit the occurrence of inflammatory reaction. So the anti-inflammatory effect of statin osteopontin after hyperglycemic intracerebral hemorrhage may also play a protective role in brain tissue through the above pathways. In order to confirm this hypothesis, this experiment used osteopontin for intervention treatment. The results showed that after the intervention of osteopontin, the score of neurological impairment was significantly reduced, the water content of brain tissue was reduced, the expression and amount of NF kB were significantly lower than that of the model group, and the expression of IL-1 β was consistent with that of NF kB. It is suggested that osteopontin may reduce the expression of IL-1 β inflammatory factors by inhibiting the activity of NF kB, thereby alleviating inflammatory damage.

Recently, researchers found that OPN can reduce the production of matrix metalloproteinase-9 (MMP-9) by inhibiting the activity of nuclear factor kB, which can alleviate the early brain injury after intracerebral hemorrhage. However, the relationship between the expression, distribution and level of OPN in the vascular wall and cerebral vasospasm after intracerebral hemorrhage has not been reported [14]. In this study, a rat model of intracerebral hemorrhage (ICH) was successfully established by secondary blood injection into the cistern magnum. The behavioral changes of rats were identified through the observation of rats; The morphological changes of the basilar artery were observed by measuring the inner diameter and wall thickness of the basilar artery; The pathological changes of the basilar artery were confirmed by observing the cells and structures of the adventitia, media and intima of the basilar artery; The above behavioral changes of rats, morphological changes of rat basilar artery, and pathological changes of rat basilar artery all confirmed that cerebral vasospasm occurred in the basilar artery after intracerebral hemorrhage in rats, and the degree of cerebral vasospasm was judged by the diameter. We found that after intracerebral

hemorrhage, cerebral vasospasm began to develop at 72 hours and reached its maximum at 6 days. Immunohistochemical results showed that the expression of OPN on the basilar artery wall was up-regulated after intracerebral hemorrhage, and increased sharply at 72h, which was consistent with the occurrence of cerebral vasospasm [15]. OPN can transform and proliferate the phenotype of vascular smooth muscle and myofibroblast, endothelial proliferation, collagen deposition, fibrosis and other factors lead to vascular remodeling, which is similar to the pathological changes of cerebral vasospasm after intracerebral hemorrhage. These results suggest that OPN may play an important role in the pathogenesis of cerebral vasospasm after intracerebral hemorrhage, and may become a marker to predict the occurrence of cerebral vasospasm. The role of OPN in cerebral vasospasm after intracerebral hemorrhage needs further experimental demonstration.
In conclusion, osteopontin may inhibit the inflammatory reaction mediated by IL-1 β through the NF-KB pathway, improve the neurological damage, thereby reducing the secondary neurological damage after hyperglycemic intracerebral hemorrhage, and play a protective role in brain tissue. Its mechanism may provide new ideas for the prevention and treatment of secondary damage caused by hyperglycemic intracerebral hemorrhage.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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