RELATIONSHIP BETWEEN EXPRESSION CASPASE-3 AND APOPTOSIS IN THE HIPPOCAMPUS OF RATS AFTER SUBARACHNOID HEMORRHAGE

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ABSTRACT

Introduction: In this experiment, the expression of Caspase-3 and apoptosis of hippocampal neurons was detected, and the relationship between Caspase-3 and apoptosis of neurons after subarachnoid hemorrhage (SAH) was discussed.

Materials and methods: 48 male SD rats were randomly divided into two groups, 6 rats in the blank group, and 42 rats in the experimental groups. The experimental groups were then randomly divided into 1h, 3h, 6h, 12h, 24h, 48h, 72h, and Each subgroup of 6.

Results: The results of HE staining showed that there were more red blood cells in the subarachnoid space. Subarachnoid hemorrhage groups 1h(16.50±0.55), 3h(15.80±0.41), 6h(15.33±0.52), 12h(14.50±0.55), 24h(13.67±0.52), 48h(14.10±0.41), 72h(15.00±0.63) had lower neurological score and had statistically significant compared with the control group (17.83±0.41) (P<0.05). The neurological behavior scores of rats in the SAH group showed a trend of first decrease, and the 24h was the lowest, the difference was statistically significant (P<0.05). TUNEL results showed that the apoptosis of SAH cells increased at first and then decreased. 1H began to appear in the cell apoptosis, 1H and 3H apoptosis cells were less, 6h increased, and the apoptosis reached the peak at 24. The apoptosis of 48h and 72h decreased gradually, and the difference was statistically significant (P<0.05). Caspase-3 protein expression: 3h, 6h, 12h, 24h, 48h, 72hSAH, 1H group were significantly higher than the control group, the difference was statistically significant (P<0.01), 1H group SAH began to rise, 6h rose rapidly, 24 hours to reach the peak, 72h, 48h began to decline, the difference was statistically significant (P<0.05). There was positive correlation between Caspase-3 and neuronal apoptosis (r=0.730, P<0.05).

Conclusions: Caspase-3 plays an important role in the pathological process of early brain injury after SAH.

Keywords: Subarachnoid hemorrhage, Early brain injury, Caspase-3, Apoptosis.

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Introduction

Subarachnoid hemorrhage (SAH) is a common intensive cerebrovascular disease in neurosurgery. As an aging population, changes in diet, the number of patients with subarachnoid hemorrhage will continue to increase, therefore, the prevention and treatment of subarachnoid hemorrhage is in an urgent need. Recent studies have shown that early brain injury (EBI) is the leading cause of death in patients with SAH, but its pathogenesis is not fully understood[1].

EBI means a direct injury of whole brain tissue and all the involved pathology and physiological processes after SAH within 72h[2], experimental studies have highlighted the importance of this period, and speculated several pathophysiological mechanisms[3,4,5] including changes in brain physiology, inflammatory factor, microcirculation dysfunction and neural apoptosis. Some scholars have suggested that apoptosis may be involved in early brain injury after SAH, and early brain injury may lead to later neurological deficit[6]. Our previous study demonstrated that the endoplasmic reticulum-specific factor Caspase-12 played an important role in post-SAH EBI, but the role of Caspase-3 in early brain injury after SAH and its relationship with apoptosis were unknown. This experiment detected the expression of Caspase-3 and the apoptosis of hippocampal neurons to further explore the role of Caspase-3 in EBI after SAH and provide a theoretical basis for the time win-
dow of clinical treatment of brain injury after SAH, thereby to further guide clinical treatment.

Materials and methods

Animals and groups

48 adult SD rats (male), weighing 250g ~ 350g, were purchased from Xinjiang Urumqi Autonomous Region Center for Disease Control and Prevention (Certificate of Conformity: SYXK (new), 20030003). Deeded under drying, ventilating condition, room temperature and humidity were maintained at 25°C and 40% to 50%. Animals were randomly divided into eight groups: blank group, 1h after SAH group, 3h after SAH group, 6h after SAH group, 12h after SAH group, 24h after SAH group, 48h after SAH group, 72h after SAH group, each group 6 rats respectively.

Main reagents and equipment

Caspase-3 antibody (Abcam company); β-actin antibody, goat anti-mouse secondary antibody, goat anti-rabbit secondary antibody (Beijing Zhongshan); SDS-PAGE and other protein extraction reagent (Soledad treasure); proteinase K (Merck); TUNEL kit (Roche). rat brain Stereotactic, skull drilling, thermostatic dissecting table, surgical instruments (Huaibei Zhenghua).

Animal model

Early brain injury model was established by injecting SAH blood into prechiasmatic pool: Rats were intraperitoneal anesthesia by 10% chloral hydrate (0.35mL / 100g), after 10min they were fixed in a stereotactic apparatus and keeping the skull at the top level, forehead were routinely disinfected and towel, the surface of the skin was incisioned, bluntly dissected the muscle and periosteum, drilled a 2mm hole which was 5mm in front of the coronal suture, and 3mm away from the midline. Meninges were carefully pierced by 1ml syringe, cerebrospinal fluid were then seen outflowing, a homemade pipe was pushed along the binaural imaginary midpoint line about 10mm further.

In the control group the skull was just drilled; in the SAH group after drilling, 0.3ml autologous arterial blood was injected slowly through the fixed puncture tube in 10s into the subarachnoid space. Closed the hole by paraffin after pulled out the pipe, then sutured the scalp, and kept the head down for 30 minutes. rats were put to die in 1h, 3h, 6h, 12h, 24h, 48h, 72h after the modelings were successed, then separated and extracted the hippocampus, one part was stored with 4% paraformaldehyde, and the others were frozen in a -80 °C refrigerator.

HE staining model

The 4% paraformaldehyde fixed hippocampal tissue was paraffin-embedded and sliced, HE stained, and DAB colored. Light microscope was used to detect the presence of red blood cells in the subarachnoid (× 100).

Neuroethology scoring

By using Garcia scoring:

• Activities: 3 point means normal, 2 points means slightly affected, and seriously affected was 1 point, no activity means 0 point.
• Evaluation the symmetry when the rats limbs were suspended; symmetry was 3 points, asymmetric is 2 points, 1 point means hemiplegia.
• caught the tail of the rats and observing the forelimb activities during walking: symmetrical is 3 points, a slight asymmetry is 2 points, 1 point is obviously asymmetrical, 0 points means one forelimb can’t move.
• proprioception: bilateral equal is 3 points, 2 points means moving sluggishly when stimulated one side, and no activites when stimulated means 1 point.
• the rats were placed on wire cages to observe their ability to climbing and attaching: climbing slightly and catching tightly is 3 points, 2 points means one side does not work, and can’t climbing or tend to circling is 1 point.
• observe the reaction when brushing tentacle of each sides: symmetrical is 3 points, 2 points means asymmetrical, 1 point means one side doesn’t react. Scoring ranges from 4 to 18 points, 4 points is the worst, 18 points is normal, and rats were scored in 1h, 3h, 6h, 12h, 24h, 48h, 72h after SAH respectively, calculate the average value at different time points by double-blind method.

TUNEL was used to detect apoptosis

• Remove hippocampal tissue fixed in 4% paraformaldehyde, and then embedded in paraffin, serially sectioned (5μm).
• after dewaxing, and hydration the tissue was digested 15min with proteinase K, 37 °C incubated (each 50uL).
• soaked in DEPC water for 60mins, vibration washed and dipped with TUNEL mixture (A: B = 1: 15, each 32μL, 60min, 37 °C incubated).
• vibration washed again, dropping POD solution (each 32μL, 30min, 37 °C incubator).
• DAB color, hematoxylin colored, water back to blue gradient ethanol dehydration, xylene, sealed with neutral gum.
• observe the apoptosis with light microscope (× 400).

**Western-Blot was used to detect the expression of Caspase-3**

Specific steps see (7,8) Removed frozen hippocampal tissue from the -80 °C refrigerator, extracted protein, and quantified protein by BCA. Take total 50ug protein sample to do 10% SDS-PAGE electrophoresis (80v, about 150min), using semi-dry film rotating device to transfer membrane: Caspase-3 (23V,42min), β-actin (23V,43min), and 5% nonfat milk were used to block for 1h,and add anti-Caspase-3 (1: 1000) and then incubated overnight at 4 °C, washed with TBS buffer 6 × 5min, added horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG antibody 1: 20,000 ) incubated at room temperature for2h. washed with TBS buffer 6x5min and then colored by ECL. The experiment was repeated 6 times with reference to the β-action.

**Statistical analysis**

Statistical analysis were performed by using SPSS 17.0 software for data analysising, comparing between groups by using ANOVA (One-way ANOVA), when doing correlation analysis, Person test was used if variance is homogeneity and Spearman test was used if it is not . P <0.05 was considered statistically significant.

**Results**

Morphology: results of observing by the naked eye:in subarachnoid hemorrhage group more blood were on the brain surface, surrounding the circle of Willis compared with the blank group. Results of observing HE staining under a light microscope, a large number of red blood cells were seen in subarachnoid (Figure 1).

**Rats neurobehavioral scoring**

Neurobehavioral scores in control group and subarachnoid hemorrhage group were (17.83 ± 0.41) points, 1h (16.50 ± 0.55) points, 3h (15.80 ± 0.41) points, 6h (15.33 ± 0.52) points, 12h (14.50 ± 0.55) points, 24h (13.67 ± 0.52) points, 48h (14.10 ± 0.41) points, 72h (15.00 ± 0.63) points respectively. rats neurobehavioral scoring was reduced and then elevated later, and in the 24h group the scoring was the lowest, compared with the blank group (17.83 ± 0.41) differences were statistically significant (P <0.05).

**Figure 1:** pictures of rat brain tissue and HE staining (x 100): A, B are the pictures of blank group and the subarachnoid hemorrhage group respectively; C, D are the HE staining of control group and the subarachnoid hemorrhage group respectively.

**Apoptosis**

TUNEL results showed that the blank group showed a small amount of brown neurons which means positive response, a large number of positive cells were observed in subarachnoid hemorrhage groups, and the neurons were smaller, nuclear membrane was shrinkage, pyknosis and colored deeply.

**Figure 2:** TUNEL staining in each groups (DAB chromogenic, x 400), A: control group B: SAH group 1h C: SAH group 3h D: SAH group 6h E: SAH group 12h F: SAH group 24h G: SAH group 48h H: SAH group 72h, the positive cells rates of subarachnoid hemorrhage groups was significantly increased compared with the blank group.

Positive cells in the control group and SAH group were (6.17 ± 0.98)% , 1h (11.67 ± 1.03)% , 3h (19.17 ± 1.17)% , 6h (25.50 ± 1.05)% , 12h (33.67 ± 1.63)% , 24h (41.50 ± 2.62)% , 48h (34.50 ± 1.38)% , 72h (33.33 ± 1.37)% respectively. Apoptosis in SAH groups trend to increase at the first and then decreased: apoptosis began from 1h, cells apoptotic...
at 1h, 3h groups were less, and increased from the 6h, and reached the peak at the 24h, and decreased from 48h compared with the blank group, differences were statistically significant (P <0.05) (Figure 2).

Changes of Caspase-3 protein expression

Expression of Caspase-3 protein of 1h, 3h, 6h, 12h, 24h, 48h, 72h SAH group were significantly higher than the control group respectively, the differences were statistically significant (P <0.01), the expression in SAH group began to rise from 1h, and rised rapidly in 6h group, 24h reached the peak, and 48h began to decline, the different time points had statistically significant differences (P <0.05) (Figure 3).

Correlation analysis of rat Caspase-3 expression and apoptosis

Positive correlation existed between expression of neuronal Caspase-3 and apoptosis (r = 0.730, P <0.05).

Discussion

Subarachnoid hemorrhage (SAH) is a acute cerebeal apoplexy with highly rate of mortality and disability. EBI means a direct injury and all the pathology and physiological processes involved after SAH within 72h, some scholars have suggested that apoptosis may be involved in early brain injury after SAH, and early brain injury may lead to neurological defect. Apoptosis is a common mechanism for the developing of EBI, studies have been reported, Nau et detected neurons apoptosis after SAH in the cerebral cortex and hippocampus. Apoptosis, programmed cell death, is main manifestation of early brain damage after SAH. Pathways of cell apoptosis are: cell death receptor mediated apoptosis pathway, apoptosis pathway mediated by mitochondria, endoplasmic reticulum mediated apoptosis pathway.

The endoplasmic reticulum is an important place for synthesising of cell protein and the storage of calcium, is an important organ in cells which regulates protein synthesising, folding and gathering. Caspase family is the hydrolases which finish the final apoptosis under endoplasmic reticulum stress, in the process Caspase-12 is located in the endoplasmic reticulum membrane, is a key molecule mediating endoplasmic reticulum stress-induced apoptosis, usually exists in an inactive zymogen form. Endoplasmic reticulum stress injury induce the activation of Caspase-12, activated Caspase-12 cuts and activates Caspase-9. Activated Caspase-9 induces the Caspase-3 effect, eventually leading to cell apoptosis.

Our early study has confirmed the positive correlation between the expression of Caspase-12 and the number of cells apoptotic. Persistent and excessive endoplasmic reticulum stress can provoke a large expression of CHOP, by regulating downstream proapoptotic Bax / Bak and Caspase-3 and anti-apoptotic gene Bcl-2, etc which leading cell apoptosis. Caspase-3 is the most important effector of apoptosis, a key role is to collect a variety of apoptotic stimuli transducting singal, and has been widely used as a indicator of apoptosis. In this study we detected EBI through behavioral function scoring, detecting expression of Caspase-3 and measuring apoptosis of neurons, and further explored the role of Caspase-3 in EBI after subarachnoid hemorrhage by correlation analysis.

In this study, Garcia method was used to score the rats neurological behavior, and activities of rat subarachnoid hemorrhage group were found unnatural, limb movement was asymmetrical during suspension, climbing ability was impaired and so on. And neurological function was severely damaged, all the results are consisten with Yang Peng, Tianweidong et al. After SAH rats neurological behavior scoring reduced at first and then tend to increase, the lowest was at the 24h, and then gradually increased, indicating that the rats brain injury gradually increased in a short time after SAH, and most serious at the 24h, then the brain Injury eased. By using TUNEL assay to detect neuronal apoptosis in rat hippocampus showed, apoptosis began from 1h, cell apoptotic were less at 1h, and 3h, and began to increase from 6h, reached the peak at 24h and decreased at 48h and 72h, differences were statistically significant (P <0.05) compared with the blank groups. Detecting expression of Caspase-3 protein by Western-Blot showed: Caspase-3 after SAH 1h,
3h, 6h, 12h, 24h, 48h, 72h were significantly higher than blank group, the differences were statistically significant (P < 0.01), SAH group began to rise from 1h, and rised rapidly at 6h, reached the peak at 24 h, and decreased from 48h, differences at different time points were statistically significant (P < 0.05). The expression of Caspase-3 and neuronal apoptosis were positive correlated (r = 0.730, P < 0.05). This experiment showed rats after SAH appeared to apoptosis, and apoptosis was positively correlated with the expression of Caspase-3, further confirmed the endoplasmic reticulum stress induced apoptosis in rat hippocampus neurons, illustrating the Caspase-3 plays an important role in the pathogenesis of early brain injury after SAH.

In this study, the hippocampus was treated by TUNEL and staining, the result showed more positive cells in subarachnoid hemorrhage group than the blank group, which was consistent with Zhang Xiang San et al.(19-20). Park S put forward that neurological function was closely related with neuronal apoptosis(19-20). The study found that neuronal apoptosis in subarachnoid hemorrhage groups and control group were trend to opposite correlated with the neurobehavioral scoring, neuronal apoptosis may be negative correlated with neurobehavioral scoring, and the extent should be further explored.

References


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