Neuroglobin Expression on Rat Brain Tissue with Subarachnoid Hemorrhage

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ABSTRACT
This paper sets to analyse the mortality and disability rates induced by subarachnoid hemorrhage (SAH). It adopts the prechiasmatic cisterna injection method to construct the models and extracts the brain tissue proteins for the research. The results show that, before and after the SAH, the neuroglobin protein expressions are significantly different, while the other subgroups do not have statistically-significant differences to the control group. It is the conclusion of this paper that the rat's post-SAH neuroglobin and gene expression on the temporal lobe cortex have time-relevant change patterns, and the cell type and expression location of the neuroglobin expression are identified.

Key Words: Neuroglobin, Subarachnoid Hemorrhage, Nerve Function
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Introduction
Subarachnoid hemorrhage (SAH) means rupture of the diseased vessels and bleeding into the subarachnoid space. It is a severe and common disease with high mortality and disability rates. About 12.4% of the patients die before they get to the hospital, and an additional 40% to 50% patients die within 30 days of the attack. Among those survivors, about 10% to 20% suffer from severe nerve dysfunction. The SAH-induced brain damages not only bring emotional sufferings to the patients and families, but also heavy economic burden to the society and the families. In recent years, though with advanced technologies intracavitary artery of aneurysm, diagnosis, perioperative management, and operation skills, the SAH-induced mortality and disability rates remain high.

Pathological process after SAH and precise control mechanism are not fully understood. Currently, the changes taking place after SAH include: elevation of intracranial pressure, decrease of cerebral blood flow, decrease of cerebral perfusion pressure, destruction of blood brain barrier, brain swelling, cerebral edema, severe cerebral vasospasm, and self regulating dysfunction. The direct damages led by these changes are referred to as early brain injury (EBI) (Nicholls et al., 2000). In this phase, the intracranial changes activate many molecular activations, including inflammatory mediators, apoptosis mediators and endogenous protective factors (Hop et al., 1997). The post-SAH intracranial pathological process may activate many endogenous protective factors to protect the brain (Teasdale GM et al., 2005). Fully tapping the potential of these endogenous protective factors, especially the protective factors in neuron expressions, may be the new treatment direction to reduce the SAH-induced mortality and disability (Gupta et al., 2007).

Neuroglobin was first discovered in 2000 by German scientists in the rat's nervous system and it features the extensive expressions in the
nervous system (Weber et al., 2001). Since the discovery of neuroglobin, the academia has conducted a great number of research on its structure, molecular mechanism and protection function with a surge of research (Pesce et al., 2002). It is discovered that it is a single sub-unit protein in blood red, and has 21% sequencing resemblance to that of vertebrate myoglobin, and 25% sequencing resemblance to that of hemoglobin (Burmeister et al., 2000). However, different from vertebrate myoglobin and hemoglobin, the histidine on both ends of the neuroglobin and in the bag structure formed by hemoglobin can directly bind with the iron ion in the hemoglobin. This is caused by the specific structure (Cahill et al., 2006). As of now, the biological function is not fully understood, but it is detected that its function may be relevant to the below: 1. it stores oxygen, and in hypoxia, promotes oxygen transfer to mitochondria or directly interferes with the oxygen transfer to mitochondria for neuroglobin has a similar protein structure to that of hemoglobin and myoglobin, and it can bind with oxygen. 2. it performs oxygen sensing function and scavenges active oxygen, NO group and other active substances. Neuroglobin may be used as an oxygen sensor to regulate intracellular signal transduction pathway according to the change of oxygen concentration. It scavenges active oxygen, groups and other active substances since it 3. regulates the signal pathway and 4. protects mitochondrial function, and through inhibition of separation from protein and promotion of complex release, thereby reducing cytochrome c.

This paper selects 84 adult male rates of 280 grams to 320 grams in good health conditions supplied by professional health care institutions. Use random digital table method to separate the rates into a control group (12) and SAH group (72). The SAH group is then divided into 6 subgroups when the animal models are created upon 3 hours, 6 hours, 12 hours, 24 hours, 48 hours and 72 hours. Each subgroup has 12 rats, among which 6 are used for preparation of fresh brain tissue tested with PCR. And the other 6 rats formaldehyde fixated for immunohistochemical staining and immunofluorescence double staining.

Methods

Neuroglobin rat model

This paper adopts the prechiasmatic cisterna injection method to construct the models. We conduct intraperitoneal injection of chloral hydrate to sedate the rate, put it in the prone position, fix the head of the rat with a stereotactic instrument and maintain its horizontal position. After the preparation of the skin and the routine disinfection, cut the skin, muscle and periosteum, and drill the skull with a dental drill along the 7.5mm median line of the anterior fontanelle. Then use a tube with a round head with a side hole, place the tube in at an angle, pierce the skull with the tube along the midline, and stop at a distance of about 2-3mm from the valerer. The gap between the bone hole and the catheter is closed with bone wax to prevent the cerebrospinal fluid from revealing. Convert the rats to supine position. Then dissect the right femoral artery under microscope, and inject autologous arterial blood 0.3ml slowly into the subarachnoid space through the tube. Pull out the tube, immediately close the bone hole with bone wax and sew the scalp. Set the rat head low for about half an hour and then place it back in cage. On the control group, no treatment is performed.

Brain tissue protein extraction

(1) Obtain the frozen brain tissue, separate 0.1 g of temporal lobe brain tissue, dissolve at room temperature, and pre-cool the glass grinder on ice.

(2) Put the brain tissue in the pre-cooled glass grinder, then grind the brain tissue with a grinder.

(3) In the RIPA solution, according to the ratio of 100-200 microlysate per 20 mg tissue, add to the lysis solution to suspend the brain tissue. Add 1 ml of RIP to each 250 mg brain tissue.

(4) Prepare the uniform on ice until it is smooth.

(5) Transfer the uniform to the pre-cooled Eppendorf tube.

(6) Centrifuge at 4°C, 12000 rpm for 15 minutes, be careful to keep the supernatant and remove sediment.

(7) Transfer the supernatant to another centrifuge tube.

Experiment methods

(1) Clean the glass plate: use one hand to hold tight the glass plate, and the other hand to dip into detergent powder for gently scrubbing. When both sides are washed, rinse with tap water and then steamed water, stand the plates in a basket to dry.

(2) Separate the glue, concentration glue and sample: according to the molecular protein weight measured at 17kDa in this experiment, choose SDS-PAGE glue at concentration rate of 12%.
(3) Align the glass plates and clip them tightly. Then place them vertically on the rack ready for glue filling. (two glass plates should be aligned to avoid leakage during operation).

(4) Prepare the glue according to the formula. After adding ammonium persulfate to the glue, share the mixture immediately, and glue is ready to be applied. When applying the glue, it can be released onto the glass by a glue gun, and stop when the glue surface is raised to the height of the middle line of the green belt. Then add a layer of water on the glue so that the coagulation is faster after liquid seal. The glue must flow along the glass plate so that there will be no bubbles in the glue. When adding water, seal it very slowly, otherwise the glue will be deformed.

(5) When there is a refracting line between the water seal and the glue, it indicates that the glue has been coagulated. Wait for another 3 minutes for the glue to fully solidify, pour the water seal and dry it with absorbent paper.

(6) Prepare 4% concentrated glue according to the formula. After adding ammonium persulfate to the glue, shake the mixture immediately, and the glue is ready to be applied. Fill the remaining gap with the concentrated glue and then insert a comb into the glue. When applying the glue, make sure glue flow down the glass plate so that no bubbles will be created. Keep the comb horizontally when inserting the comb. Since the glue will shrink when it is solidified, the volume of the sample with sampled hole will be reduced, so it is necessary to apply glue regularly on two sides during the solidification. When the concentrated gel is solidified, use both hands to hold sides of the comb, and pull it up vertically slowly.

(7) Add enough electrophoretic fluid to prepare the sample. The electrophoresis solution should be at least over the level of the smaller glass plate. Sample with a micro injector and suck out the sample without causing bubbles. Insert the syringe needle into the sample hole and add the sample slowly (if the sample is added too fast, it may overflow through the sample hole. If any bubble is created, the sample may also overflow. When processing with the next sample, change the gun head to avoid cross contamination).

Results and discussion

Quantitative analysis of animal experiment
This experiment design uses 84 rats to obtain brain tissues. 10 rates fail in the process of building the neuroglobin models, with 1 death after intraperitoneal injection of chloral hydrate, and other 8 deaths in the process of prechiasmatic cisterna injection perhaps due to fast injection. In addition, after establishment of the neuroglobin models, there are 3 death before the testing points. The accidental deaths that result in failure of obtaining animal samples are all excluded from the experiment and supplemented randomly so that data on 84 rats are collected.

Neuroglobin test of rat brain tissue
The neuroglobin protein stripe occurs at around the position of 17kDa and has dynamic changes as in Fig 1. In the control group, the protein expression of neuroglobin is relatively low. After SAH, the protein level starts to raise at 3h, and peaks at 24h. Then it is on a gradual decline but remains higher than normal 72 hours after SAH. Compared to the control group, 24 hours after SAH, the difference in protein expression of neuroglobin is statistically significant, and the others sub-groups and control group do not have significant statistical differences (see Figure 2).

![Figure 1. Changes in the position of 17kDa in the brain erythrocyte egg](image1)

![Figure 2. Changes in the cortex of the facial cortex of rats after analysis](image2)
temporal lobe cortex from the 24h SAH group and control group for comparison. The control group has fewer strong positive cells from the immune reaction of the neuroglobin with weaker positive reaction, while the 24h SAH group has increased numbers of positive cells of neuroglobin protein in the same areas at varied levels. The staining is also significant with strong positive (See Figure 3 and Figure 4).

![Figure 3. Brain cell response in group A](image1)

![Figure 4. Brain cell response in group B](image2)

Besides the temporal lobe cortex, immunoreactive cells are observed in the hippocampus, cerebellum, and brain stem as in Fig 5. Typical neurons that have positive neuroglobin reaction are observed in the slicing, and mainly distributed in the cytoplasm as in Fig 6. After statistical analysis, the differences in immunoreactive cell rates of the latter group has statistical significance.

![Figure 5. Hippocampal brain cell response](image3)

![Figure 6. Cerebellar cell response](image4)

**Conclusion and forecast**

In this experiment, we, for the first time, conduct analysis on the expression and distribution of neuroglobin in the SAH model rats and have below findings: this experiment adopts the the prechiasmatic cisterna injection method to simulate SAH in the rats and observe the time-relevant change patterns in the neuroglobin and gene expressions in temporal lobe cortex, and observes the cell type and expression position within the cell of neuroglobin expression. The content of neuroglobin in normal brain tissues is relatively low, but after SAH, it has time-expression amount relation. Within the time-expression amount relation, the expression amount is on the rise for the 1st hour after SAH, and peaks within three hours. It then gradually declines but remains at a higher-than-normal level at 72nd hour after SAH. The level of Ngb m RNA is low in normal brain tissue, but is on a rapid rise for the first three hours, and peaks at the 6th
hour before the decline. The Ngb is mostly expressed in the cerebral cortex, and also observed in the hippocampus and cerebellum.

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References