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Basic Investigation

Effects of Batroxobin on Spatial Learning and Memory Disorder of Rats with Temporal Ischemia and the Expression of HSP32 and HSP70

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The effect of Batroxobin on spatial memory disorder of left temporal ischemic rats and the expression of HSP32 and HSP70 were investigated with Morri`s water maze and immunohistochemistry methods. The results showed that the mean reaction time and distance of temporal ischemic rats in searching a goal were significantly longer than those of the sham-operated rats and at the same time HSP32 and HSP70 expression of left temporal ischemic region in rats was significantly increased as compared with the sham-operated rats. However, the mean reaction time and distance of the Batroxobin-treated rats were shorter and they used normal strategies more often and earlier than those of ischemic rats. The number of HSP32 and HSP70 immune reactive cells of Batroxobin-treated rats was also less than that of the ischemic group. In conclusion, Batroxobin can improve spatial memory disorder of temporal ischemic rats; and the down-regulation of the expression of HSP32 and HSP70 is probably related to the attenuation of ischemic injury.

Snake venom is a well-known traditional Chinese medicine used to treat some diseases, including ischemic cerebrovascular diseases. Batroxobin is a thrombin-like enzyme isolated from snake venom that enables to remove specifically fibrinogen. Our previous studies found that Batroxobin is an excellent drug for treatment of acute ischemic cerebrovascular diseases[1]. Batroxobin could

also attenuate cerebral edema in ischemic gerbils and inhibit c-fos gene expression of rat brains during ischemia and reperfusion[2-4]. Our previous study also found that Batroxobin could improve the learning and memory disorder of rats with left temporal ischemia[5]. But the mechanism remains unclear.

Heat shock protein (HSPs) are a group of cellular proteins known to emerge after various noxious stimuli. HSP 70 has been found to be preferentially synthesized after cerebral ischemia in gerbils and rats. Induction of HSP32 and HSP70 occurred more intensely in the areas less vulnerable to global ischemia, suggesting a protective effect of HSP32 and HSP70, which was considered to be an early marker for neuronal injury [6]. However, the relationship between HSPs and selective tissue vulnerability as well as delayed neuronal death remain uncertain. The association of memory disorder induced by temporal ischemia and HSPs expression as well as the effect of Batroxobin are unclear.

A recently developed spatial memory task is the Morri's water maze (MWM). We had used MWM to investigate the effect of Batroxobin on learning and memory disorder of temporal ischemic rats. We had found in the behavioral experiment that the mean reaction time and distance of temporal ischemic rats in searching a goal were significantly longer than those of the sham-operated rats[7]. However, the mean reaction time and mean distance of the Batroxobin-treated rats were shorter, and the Batroxobin-treated rats used normal strategies more often and earlier than those of ischemic rats. Morris water maze has been proven very useful for characterizing the neurochemical basis of spatial learning and memory in rodents[8]. In this study, we used the Morri's water maze to investigate the effect of Batroxobin on the expression of HSP32 and HSP70 in the temporal ischemic rats with learning and memory disorders.

Materials and Methods

Animal and group: Thirty-six male Wistar rats weighing 180~220g were randomly divided into the following three groups, the ischemia group (n=12), the Batroxobin-treated group (n=12) and the sham-operated group (n=12). Four days after operation, a five-day behavioral training was carried out during 10:00 am ~ 5:00 pm. The rats were free for food and water during the whole time of experiment. After the behavioral experiment was finished, the rats were killed, and HSP32 and HSP70 expression of brain tissue was studied with immunohistochemistry method.

Animal model: Selective left temporal ischemia of rat model was induced by the photochemical method[5]. Each rat was anaesthetized with 10% chloral hydrate (0.3g/kg, i.p.). The rats were placed in a stereotaxic frame and the rectal temperature was kept at 36.5℃±0.7℃ with a heating lamp. The left temporal scalp incision was performed to expose the skull surface. For skull illumination, standard equipment was used. A light guide fiber cool light was positioned on the left temporal skull surface. Rose bengal dye (Sigma Chemical Ltd; 20mg/ml in sterile 0.9% NaCl) was injected slowly via a lateral tail vein, and the skull was illuminated for 20 minutes. The light was then interrupted, the scalp wound closed with sutures, and the animal allowed to

recover. The Batroxobin-treated rats received an injection of Batroxobin (8BU/kg, i.p. Tobishi Pharmaceutical Co. Ltd) 30 min before illumination. The sham-operated control animals were prepared with the same procedures except illumination.

Morri's water maze behavioral experiment[7]: The behavioral experiment equipment consists of Morri's water maze and Morri's Maze Experimental Assistant System (MMEAS). MWM is a large circular pool (d=94mm, h=55cm) filled with cool water rendered opaque with milk powder. Submerged just below the water surface somewhere within the pool is a platform (s=20cm^2) onto which the rat can climb to emerge from the cool water and escape from the necessity of swimming. MMEAS consists of image collect card, video camera and automated computer tracking system. The experimental data were collected and analyzed automatically.

The behavioral experiment was performed four days after operation and continued for five days (including 1 day of pretraining and 4 days of training trials). Each rat accepted a total training of 24 times and 6 trials per day at a 15-min interval for each training trial. The experiment data included response time (s), distance (cm) and strategies. The mean values of the three trials in response time and distance were considered as one unit respectively, and the total eight unit values were obtained by a four-day training trials. F and χ^2 test were analyzed with SPSS software package.

Immunohistochemistry: After the behavioral experiment, six rats from each group were anaesthetized with 10% chloral hydrate (0.3g/kg, ip), brains were fixed by transcardiac perfusion with 4% paraformaldehyde 40 ml, then the brains were quickly removed and kept in the fixation of 4% paraformaldehyde at 4℃. They were subsequently cryostat sectioned (slice thickness, 30 μm) and stained for HSP32 and HSP70 by use of monoclone antibody (Santa Cruz Ltd) and SP kit[9]. Five slices of the ischemic brain tissue from each rat were observed under microscope, and the number of HSP32 and HSP70 immune positive cells were counted.

Results

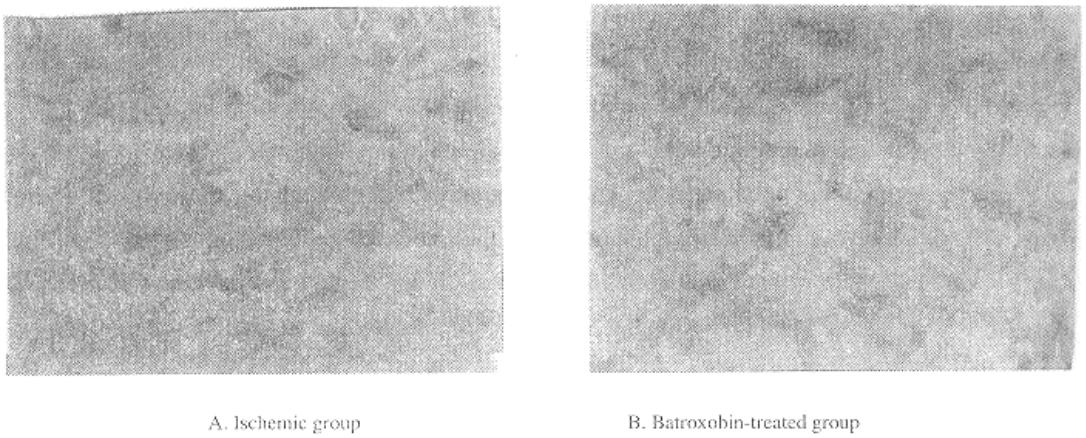
The number of HSP32 and HSP70 immune positive cells of the left temporal ischemic rats was significantly increased as compared with that of the sham-operated group. However, the number of HSP32 and HSP70 immune positive cells of the Batroxobin-treated rats was less than that of the ischemic group (see the following Table and Fig.).

Table. The number of HSP32 and HSP70 immune positive cells

Group	n	HSP32	HSP70 ($\bar{x}\pm s$)
Ischemic	6	254.3±31.0	55.5±6.6*
Batroxobin-treated	6	46.3±12.8	25.8±4.9*#
Sham-operated	6	2.8±2.3	0-3

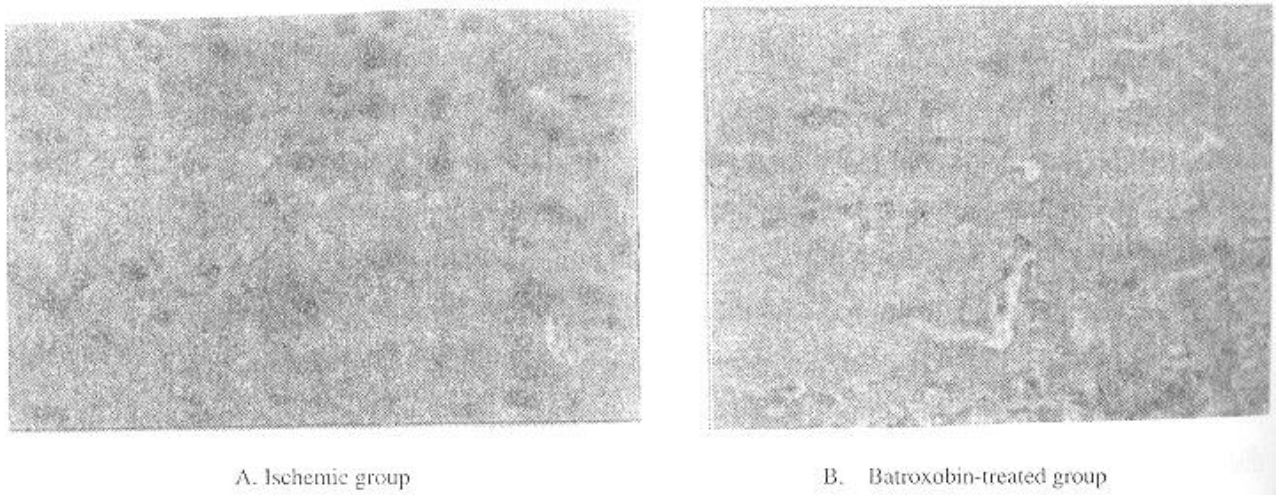
* $P<0.01$, compared with the sham-operated group

\# $P<0.01$, compared with the ischemic group



A. Ischemic group　　B. Batroxobin-treated group

Fig 1. HSP70 immune positive cells of the cerebral cortex during ischemia (20×10)



A. Ischemic group　　B. Batroxobin-treated group

Fig 2. HSP32 immune positive cells of the cerebral cortex during ischemia (20×10)

Discussion

In the present Morri's water maze behavioral experiment and immunohistochemistry study, we found that the spatial learning and memory ability was disordered in the left temporal ischemic rats; and the HSP70 expression of the ischemic temporal cortex was increased. This result is in accordance with our previous study that the HSP70 expression was increased during anoxia of hippocampus neurons[4]; and the present result further suggests that there may be a relation among left temporal ischemia, spatial memory disorders and HSPs expression.

HSPs are also known to be induced in a variety of conditions, including brain injury, status epilepsy, cerebral ischemia, and hyperthermia. HSPs are selectively expressed in neurons subjected to sublethal stress; and lethal ischemic damage could be ameliorated by preceding hyperthermia, brief ischemia, or oxidative stress; which could induce HSPs. Our previous study also found that the HSP70 was expressed in the cultured hippocampus neurons during anoxia[9]. HSP32 and HSP70 are putative

protective proteins induced after ischemia, although the biological significance of this protein in brain remains uncertain[10]. HSPs are considered to control the conformation, stabilization, and transport of normal and partially denatured proteins. HSP70 is essential for the restoration of normal ribosome assembly, promotion of new ribosomal synthesis, and acceleration of the recovery of nucleolar morphology after heat shock by ATP-dependent mechanisms[6].

Our MWM study has found that the mean reaction time and distance of the Batroxobin-treated rats are shorter and they use normal strategies more often and earlier than those of the ischemic rats[7]. The result is in accordance with our previous study that Batroxobin may ameliorate the dysfunction of learning and memory in temporal ischemic rats measured with step-down methods[5]. The MWM result further indicates that the Batroxobin may also ameliorate the dysfunction of spatial learning and memory in temporal ischemic rats measured with Morri' s water maze. It has been demonstrated that batroxobin can reduce the morbidity and mortality of ischemic stroke in gerbils and rats, ameliorate cerebral edema, and attenuate the dysfunctions of arginine vasopression, excitatory amino acids, nitric oxide and down-regulation of endothelin-1 gene expression[1-4,11]. Based on these mechanisms, it is reasonably proposed that Batroxobin may be of down-regulation of HSP32 and HSP70 expression in ischemic brain tissue due to ameliorating ischemic brain injury, and may therefore improve the spatial learning and memory disorder of the left temporal ischemic rats.

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