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The effects of MCA-MAO on cAMP pathway in rats with cerebral hemorrhage

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Abstract. – OBJECTIVE: To explore the effects of MCA-MAO on the cAMP pathway in rats with cerebral hemorrhage.

MATERIALS AND METHODS: Forty SD male rats were randomly divided into four groups: the sham operation group (n=10), the model group (n=10), the negative control group (n=10) and the experimental group (n=10). To prepare rat models for cerebral hemorrhage, autogenous femoral arterial blood was injected into the caudate nucleus. In the case of rats in sham operation group, normal saline was injected into the caudate nucleus. Rats in the negative control group received a proper amount of saline via an injection into the abdominal cavity. Rats in the experimental group were injected with 500 μL/kg MCA-MAO into the abdominal cavity. Five rats from each group were executed after 1 to 3 days, the water contents of gray and white matters were detected using far infrared moisture analyzer, the MAO activity was measured by the histochemical method. The cAMP level was measured by radio-immunity method and the protein kinase A (PKA) level was measured by Western blot. cAMP response element binding (CREB) mRNA expression level was detected by RT-PCR.

RESULTS: Water content, MAO activity, cAMP, PKA, and CREB mRNA expression levels in the model, and the negative control groups were significantly higher than those of the sham operation and the experimental groups, the differences were statistically significant (*p*<0.05).

CONCLUSIONS: MAO may mediate the pathophysiological process of hemorrhage via cAMP signaling pathway.

Key Words:
MCA-MAO, Cerebral hemorrhage model of rat, CAMP signaling pathway.

Introduction

Cerebral hemorrhage is a cerebral vascular disease characterized by high morbidity and high mortality rates as well as high disability rate that can be a serious threat to patient's life and impose a heavy burden on the society[1]. The blood-brain barrier destruction, oxygen free radicals generation, calcium overload, inflammatory reaction, and apoptosis or necrosis of cerebral neurons are involved in this condition[2]. A recent study[3] revealed that in this condition the monoamine oxidase (MAO) and alkaline phosphatase (AKP) enzyme activities in cerebral capillaries and veins were elevated. Also, the permeability of blood brain barrier was increased which suggested the probability that changes in enzyme activity might be involved in the molecular transport and blood-brain barrier function after a cerebral hemorrhage. Monoamine neurotransmitters including norepinephrine, epinephrine, 5-hydroxytryptamine and dopamine play a very important role in the pathophysiological process after a cerebral hemorrhage. This study focuses on the effects of MCA-MAO after cerebral hemorrhage and its mediation mechanism via a cAMP signal pathway. The cAMP signal pathway is a major pathway playing a significant "bridge" role in the body[4].

Materials and methods

Animals, reagents and instruments

Animals: Forty, 6 weeks old, male SPF level SD rats weighting (250±10) g were used in this

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study. Animals were provided by Beijing Vital River Co., Ltd. (Beijing, China) (production license: SCXK, Beijing, 2006-0009). Rats were fed under 12 to 12-hour light-dark cycle and at the temperature of 20°C to 25°C.

Main Reagents: MCA-MAO (Serotec, Sigma-Aldrich, St. Louis, MO, USA), PKA kit, Western Blot assay kit and cAMP kit were purchased from Wuhan Boster-Bio Engineering Co., Ltd. (Wuhan, Hubei, China), RT-PCR reagent: Trizol-reagent (Invitrogen, Carlsbad, CA, USA), reverse transcriptase, PCR reagent (Promega, Madison, WI, USA), agarose (Sigma), PCR premier (synthesized by Beijing Aoke Biological Technology Co. Ltd., Beijing, China).

Main Instruments: Far infrared moisture analyzer (Denver, CO, USA), CM1850 cryostat microtome (Leica, Solms, Germany), 303-1 type electric thermostatic multipurpose box (provided by Huyue Scientific Experiment Instrument Factory, Beijing, China), image-forming system Nikon Eclipse E600 microscope, Nikon U-III multi-point sensor system camera system, Miaspro image analysis system (Denver, MA, USA), full-automatic γ free counter FJ-2008 was purchased from Xi'an Nuclear Instrument Factory (Xi'an, China), Cold CCD gel imaging system was purchased from USA General Healthcare Company (Marlborough, MA, USA); RT-PCR instrument: high speed freezing centrifuge (Sigma-Aldrich, St. Louis, MO, USA), ultraviolet spectrophotometer (Unicam), PCR instrument (MJ, Edgewood, Canada), image analysis system (Marlborough, MA, USA).

Rat cerebral hemorrhage model: Rats were injected with 3.5 mL/kg 10% chloral hydrate in the abdominal cavity. After anesthesia, head and thigh skins were prepared and disinfected by iodophors. Consequently, the skin on the right thigh region was cut open with a 15 mm incision and arteria femoralis was exposed and fixed onto the stereotaxic apparatus. The stereotaxic apparatus was adjusted to keep rats' anterior and posterior fontanelle at the same horizontal level. The skin on the head region was cut open with a 15 mm incision and periosteum was denudated with 3% aquae hydrogen dioxide. Anterior and posterior fontanelle were exposed, and a single 1 mm hole was drilled using a 3.5 mm bit at the right side of the midline (endocranium was not involved). 50 µl non-coagulated blood was drawn out by a micro injector and local compression hemostasis was made while the micro injector was fixed onto the stereotaxic apparatus. A needle was inserted vertically into the hole (up to 5.5 mm) and the injection was performed slowly at a rate of 2 µl/min. After 20 min, the needle was withdrawn, and the hole was closed by bone wax and the incision was sutured and skin was infected. According to Longa method, neurological function defect score of 1 to 4 points after 2 h were considered to be successfully established models.

Grouping method and observation index: Rats were randomly divided into four groups: the sham operation group (n=10), the model group (n=10), the negative control group (n=10) and the experimental group (n=10). Autogenous femoral arterial blood was injected into caudate nucleus and cerebral hemorrhage rat models were constructed. In the sham operation group, rats were injected with normal saline into the caudate nucleus. In the negative control group, a proper amount of saline was injected into the abdominal cavity. In the experimental group, 500 µL/kg MCA-MAO were injected into the abdominal cavity. After one to three days, five rats from each group were executed and the water contents of gray and white matters were detected by far infrared moisture analyzer. MAO activity was measured by the histochemical method, the cAMP level was evaluated by the radio-immunity method and the protein kinase A (PKA) level was measured by Western blot. The expression level of CREB (cAMP response element binding) mRNA was measured by RT-PCR. To measure the average percentage of moisture, a 0.02 g sample from gray and white matters were cut from the frontal part and were examined using the far infrared moisture analyzer (parameter: temperature of 180°C for 5 min and slope of 0.05%).

Glenner staining and MAO activity: Skin was cut into 10 µm pieces and incubated in MTT incubation solution at 37 C for 30 to 60 min. Samples were then rinsed and fixed in 10% formalin for 24 h, rinsed again and mounted with water soluble mounting medium. Under 25X objective lens, 3 randomly selected fields were selected and images from those fields were imported into Miaspro image analysis system (parameter: µm, scale of 0.818, average optical density of 0.69, average gray of 51.9, field area of 175103.55 to 175284.10).

cAMP radioimmunoassay (RIA) kit: We used bovine serum albumin (BSA), acetylation reagent, ^{125}I-cAMP, cAMP standard solution, cAMP anti-blood serum, goat anti-rabbit IgG serum, normal rabbit serum, and acetic acid buffer. We followed the instructions provided by cAMP RIA kit while related parameters, standard curve and

sample concentration were figured out from the pre-programmed γ counter.

PKA level determination: Protein was purified and samples were prepared. Separation gel (12%) and 5% spacer gel were prepared pro rata The polymerized gel was transferred to the electrophoresis tank and samples were loaded and performed electrophoresis at proper voltage, current and power. Till the front of bromophenol blue reached the end of the gel, suspended electrophoresis. Stained with Coomassie brilliant blue and protein to be tested was determined. After the transfer to the membrane, Ponceau staining, distilled water rinse, PKA primary antibody was added and closed at room temperature for 2 h, rinsed by TBST buffer solution. A secondary antibody was then added and incubated at room temperature for 2 hrs, followed by rinsing with TBST buffer solution. Finally, the membrane was soaked into PBS solution, until color was developed and pictures were taken by cold CCD gel imaging system.

RT-PCR: CREB and internal reference β-action gene primers were determined according to references. The reaction system used was: 25 μL in all with 12.5 μL of PCR MIX, 3 μL of RT sample, 7.5 μL of RNA free enzyme and 1 μL of each target gene primers. The Loop parameters used were: reverse transcription at 50 C for 30 min, then at 94°C for 2 min; PCR cycle: denaturation at 94°C for 30 s, annealing at 57 C for 30 s, and extension at 72°C for l min, for a total 27 cycles. Reactions were continued for 8 min at 72°C in the last cycle. PCR products (8 μL) were transferred to agarose gel (1.2%) for electrophoresis, and the size of amplified fragments was determined. The optical density of the bands was determined using FluorChem image analysis system and OD values were recorded.

Statistical Analysis

SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical. Measurement data were expressed as mean±standard deviation and the group comparison was made by adopting the analysis of variance. Count data were expressed as a percentage (%) and the group comparison was made by adopting the X^2 test. Correlation analysis was made by Pearson test. $p<0.05$ was considered to be statistically significant.

Results

Comparison of water content in gray and white matters: At day 1 and day 2, the water content of the model group and the negative control group were significantly higher than those in the sham operation group and the experimental group. Differences were statistically significant ($p<0.05$). At day 3, the differences were more noticeable and the differences were statistically significant ($p<0.05$) (Table I).

Comparison of MAO activity, cAMP content, PKA content and CREB mRNA expression level: At day 1 and day 3, MAO activity, cAMP, PKA, and CREB mRNA expression levels in the model group and the negative control group were significantly higher than those in the sham operation group and the experimental group. Differences were statistically significant ($p<0.05$) (Table II and Figure 1).

Discussion

Monoamine oxidase (MAO) is usually found bound to the outer membrane of mitochondria and is involved in monoamines metabolism. The release and aggregation of excessive monoamine caused by cerebral hemorrhage can aggravate the brain injury[5]. Prior works[6] showed that norepinephrine, epinephrine, 5-hydroxytryptamine and dopamine can cause vascular spasm, cerebral microcirculation disturban-

Table I. Comparison of water content in gray and white matters (%).

	Water content of gray matter				Water content of white matter			
Group	**1d**	**3d**	***t***	***p***	**1d**	**3d**	***t***	***p***
Sham operation group	69.2±7.2	68.4±7.3	0.364	0.127	65.6±6.9	65.3±6.7	0.967	0.463
Model group	82.4±6.9	86.5±6.6	2.865	0.041	76.4±6.3	79.8±6.4	2.765	0.041
Negative control group	83.2±8.2	85.7±8.4	2.769	0.042	77.3±7.5	80.3±7.8	2.698	0.042
Experimental group	71.6±5.9	69.3±5.8	3.362	0.039	67.8±5.4	64.6±5.5	3.421	0.039
F	4.625	4.968			4.567	4.932		
P	0.037	0.035			0.037	0.035		

Table II. Comparison of MAO activity, cAMP, PKA and CREB expression levels.

Group	MAO activity (unit area of optical density)		cAMP (pmol/ml)		PKA (x10^6)		CREB mRNA	
	1d	3d	1d	3d	1d	3d	1d	3d
Sham operation group	0.49±0.06	0.48±0.05	386.7±78.3	378.5±80.2	12.6±3.5	12.5±3.3	1.3±0.5	1.4±0.3
Model group	0.94±0.03	0.98±0.05	854.2±82.7	886.9±76.6	24.7±4.2	26.9±4.3	2.6±0.8	2.8±0.6
Negative control group	0.97±0.05	0.98±0.07	863.3±85.2	893.5±82.4	25.3±4.4	28.2±4.5	2.8±0.7	2.9±0.5
Experimental group	0.55±0.04	0.52±0.03	397.4±80.5	356.4±67.8	13.9±4.7	10.3±3.8	1.7±0.4	1.2±0.5
F	5.346	5.632	5.541	5.879	5.768	6.201	6.302	6.634
p	0.027	0.024	0.025	0.023	0.023	0.019	0.015	0.012

ce and increased permeability of blood brain barrier. Also, it can aggravate cerebral ischemia, hypoxia and cerebral edema. Norepinephrine and 5-hydroxytryptamine bind to specific cell membrane receptors and activate Ca^{2+} channel, increase the internal Ca^{2+} flow and aggravate edema. Dopamine can generate a large number of free radicals via the metabolic process of MAO-B and oxidize the cell membrane as well as organelle membrane system and, finally, result in the degeneration and necrosis of dopaminergic neurons.

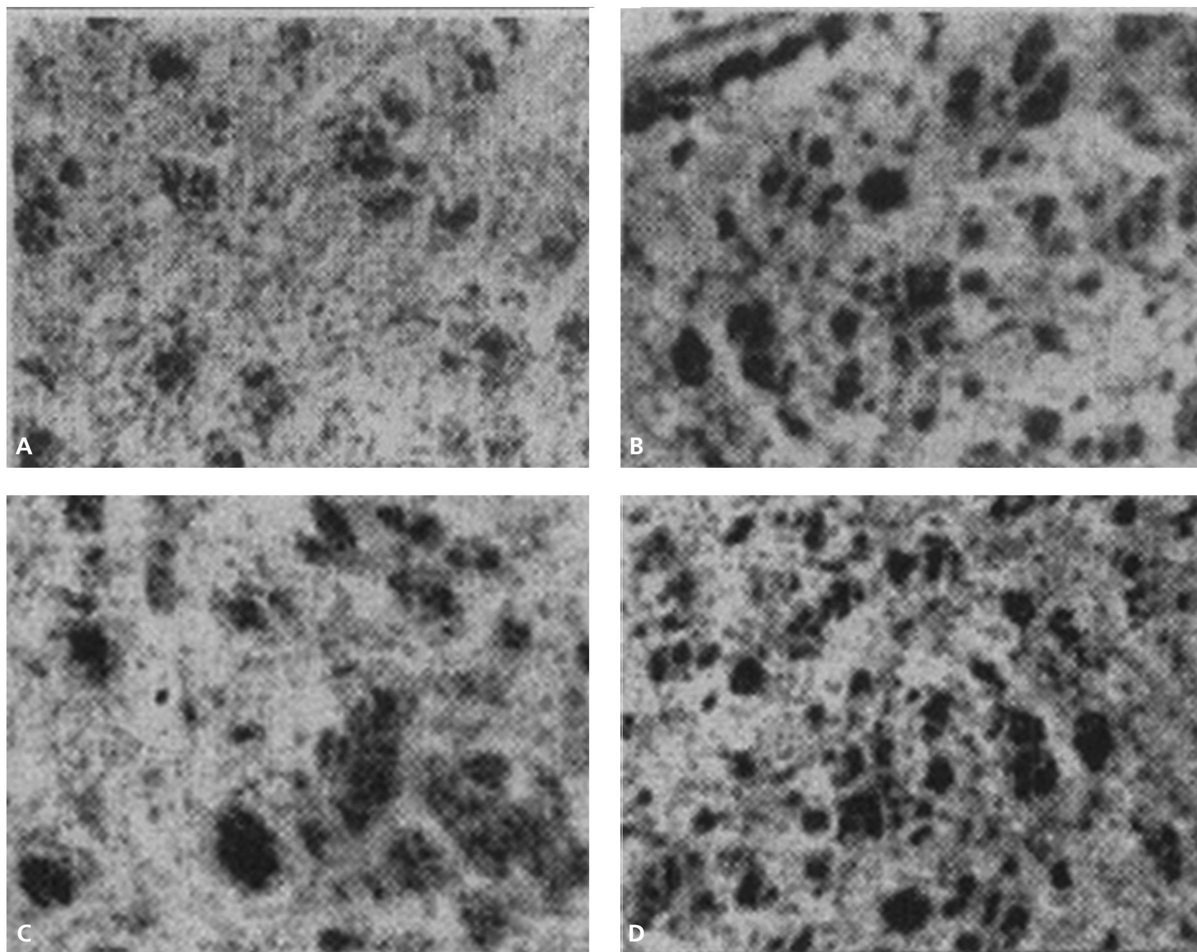

Figure 1. Glenner staining method for MAO activity 200× (***A,*** sham operation group; ***B,*** model group; ***C***, negative control group; ***D,*** experimental group; staining of group b and c were significantly stronger than a and d).

Prior studies confirmed that an increase in MAO activity was closely correlated with Alzheimer disease, Pick disease, Parkinson disease, senile schizophrenia and depression. It was also revealed that MAO inhibitors were quite effective in the treatment of these conditions. In the early period of acute cerebral ischemia, MAO inhibitors could effectively reduce neuron damages and stroke symptoms[8].

Adenylate cyclases are capable of integrating signals that act directly from G protein-coupled receptors through stimulation of the G-protein alpha and beta/gamma subunits or indirectly via intracellular signaling by protein kinases A (PKA), Stimulation through G-protein is the major mechanism, by which adenylate cyclase is activated and cAMP levels are elevated. PKA is a significant target of cAMP. PKA phosphorylates and stimulates cAMP responsive element binding protein (CREB).

CREB is a gene regulatory protein and a target protein activated by cAMP-dependent protein kinase. Activated PKA could enter the nucleus and phosphorylate CREB. Phosphorylated CREB can attach to target gene regulatory sequence, and enhance the expression of the target genes[9]. Since the monoamine neurotransmitter receptors are mostly G protein-coupled receptors, there are reasons to believe that the cAMP signaling pathway is playing a "bridge" role in the pathophysiological process of MAO mediation of cerebral hemorrhage[10].

Results obtained from our study showed that the water content, MAO activity, cAMP, PKA, and CREB mRNA levels in the model and the negative control groups were significantly higher than those in the sham operation and the experimental groups. These findings suggested that the edema secondary to cerebral hemorrhage would become more obvious by time. Our results were consistent with the clinical symptoms. We showed that MCS-MAO could significantly inhibit the MAO activity, reduce cAMP as well as PKA levels, decrease CREB expression levels and thereby greatly improve the edema. There were no significant differences in above indexes between the experimental group and the sham operation group, which indicated that MCA-MAO could significantly improve cerebral hemorrhage.

Conclusions

MAO may mediate the pathophysiological process of hemorrhage via cAMP signaling pathway. An intervention at the MAO function channel could be a potential target for cerebral hemorrhage treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests

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