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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, autologous femoral arterial blood was injected into the caudate nucleus. In the case of rats in the sham operation group, normal saline was injected into the caudate nucleus. Rats in the negative control group received a proportional amount of saline via an injection into the abdominal cavity. Rats in the experimental group were injected with 500 μL/kg MCA-MAO to the abdominal cavity. Five rats from each group were executed after 1 to 3 days, the brain tissue was collected, gray and white matters were separated using far infrared moisture analyzer, the MAO activity was measured by the histochemical method. The cAMP level was measured by radioimmunity method and 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 experimental groups, the differences were statistically significant ($p<0.05$).

CONCLUSIONS: MCA-MAO may mediate the pathological 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 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 Fa (Xi'an, China), Cold CCD gel imaging s m was purchased from USA General Health Company (Marlborough, MA, USA); RT-P instrument: high speed freezing centrifuge (Sigma-Aldrich, St. Louis, MO, US raviolet spectrophotometer (Unicam), ument (MJ, Edgewood, Canada), ima analysi ystem (Marlborough, MA, USA).

Rat cerebral hemorrhage l: Rat injected with 3.5 mL/kg % ch ydrate in the abdominal cavity. r anesthe d and thigh skins were pre d and disinfect io-dophors. Conseque kin on the right high region was cut with mm incision and arteria femoralis was expose fixed onto the stereotaxic a ratus. The stere apparatus was adjust o keep rats' anterior and posterior fontanell the sa horizontal level. The skin on the regio was cut open with a 15 mm incision teum w enudated with 3% aquae hydr ioxide terior and posterior f le were nd a single 1 mm hole s d using mm bit at the right side of the dline (endo anium was not involved). 50 µl coagulated blood was drawn out by a icro inj local compression hemostasis ade while the micro injector was fixed onto the taxic apparatus. A needle was inserted vertically into the hole (up to 5.5 mm injection was performed slowly at a of 2 µ min. After 20 min, the needle was w rawn, and the hole was closed by bone wax the incision was sutured and skin was infecte ording to Longa method, neurological functio score of 1 to 4 points after 2 h consid be successfully established m els.

Grouping method an bservati index: R were randomly divide o four ups: the sham operation group (n=1 m l group 10), the negative cont group and t xperimental group (n). Autoge m arterial blood was in d into caudate s and cerebral hem t models were constructed. In the sh oper roup, rats were injected with norm saline caudate nucleus. In the n ve control grou per amount of saline s injected into the ab minal cavity. In the ex imental group, 500 µL/kg MCA-MAO were i ed into the abdominal cavity. After one to th days, five rats om each group were exec nd the wa contents of gray and white mat ted by far infrared moisture analyze activity was measured by the hi-chemical method, the cAMP level was evaluated by radio-immunity method and the protein kinase (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 gen mers. The Loop parameters used were: reverse n-scription at 50 C for 30 min, then at 94°C for 2 PCR cycle: denaturation at 94°C for 30 s, anneal at 57 C for 30 s, and extension at 72°C for l min, f a total 27 cycles. Reactions were cont r 8 mi at 72°C in the last cycle. PCR pro were transferred to agarose gel (1.2%) electro oresis, and the size of amplified fragn was de ined. The optical density of the ba s dete using FluorChem image alysis and OD values were recorded.

Statistical Anal

SPSS 19.0 stati al so (SPSS Inc., Chicago, IL, USA) was used for sta Measurement data were expressed as mean±standard and the group comparison was made adopti the analysis of variance. Count data expressed as a percentage (%) and the group parison was made by adopting the X^2 test. C n analysis was made by Pearson test. $p<0.05$ was ered to be statistically significant.

Results

Comparison of ter c n gray white matters: At day nd day 2, te ntent of the model gr and the negati rol group were signifi her than thos n the sham operation up a experimental group. Differences re statis significant ($p<0.05$). At da the differenc e more noticeable and differences were s tistically significant (p 05) (Table I).

mparison of MAO activity, cAMP content, P content and CR B mRNA expression level: A l and day MAO activity, cAMP, PKA, and expression levels in the model group a egative control group were significantly higher than those in the sham operation and the experimental group. Differences re tically significant ($p<0.05$) (Table II nd 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-

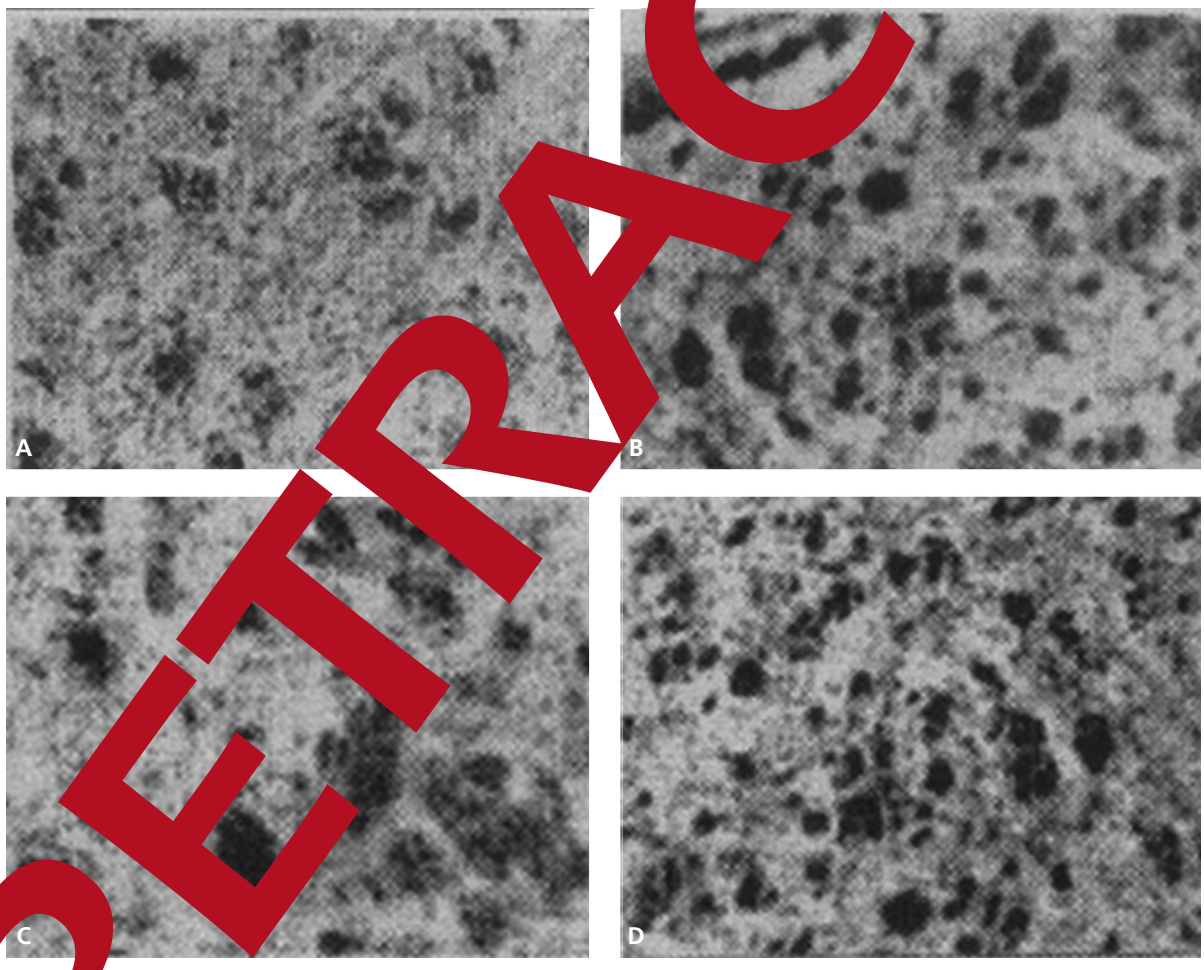
Table I. C arison o ter content in gray and white matters (%).

	Water content of gray matter				Water content of white matter			
Group	1d	3d	*t*	*p*	1d	3d	*t*	*p*
ation grou	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 up	82.4±6.9	86.5±6.6	2.865	0.041	76.4±6.3	79.8±6.4	2.765	0.041
Negati 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
Exp	71.6±5.9	69.3±5.8	3.362	0.039	67.8±5.4	64.6±5.5	3.421	0.039
	4.625	4.968			4.567	4.932		
	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 (×10⁶)		B 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	1.3±0.5	1.
Model group	0.94±0.03	0.98±0.05	854.2±82.7	886.9±76.6	24.7±4.2	26 .3	2.6±0.8	2.8±
Negative control group	0.97±0.05	0.98±0.07	863.3±85.2	893.5±82.4	25.3±4.4	±4.5	2. 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	3.8	0.4	1. ±0.5
F	5.346	5.632	5.541	5.879	5.768		.302	4
p	0.027	0.024	0.025	0.023	0.0	0.0	0.015	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 aggrava Dopamine can enerate a large numbe free via the metabolic process of MAO-B nd oxidiz ll membrane as well as organ membrane syste finally, result in the deg ation and necrosis of opaminergic neurons.



staining method for MAO activity 200× (***A,*** sham operation group; ***B,*** model group; ative control group; ***D,*** experimental group; staining of group b and c were significantly stronger tha 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 be that the cAMP signaling pathway is playing a dge" role in the pathophysiological process of M mediation of cerebral hemorrhage[10].

Results obtained from our study showed tha the water content, MAO activity, cA KA, and CREB mRNA levels in the mode gative control groups were significantl gher th those in the sham operation and the eriment ups. These findings suggested that t ma se to cerebral hemorrhage wo beco re obvious by time. Our results wer nsistent wi linical symptoms. We showe MCS-MAO co ni-ficantly inhibit the vity, reduce cAMP as well as PKA level crea B expression levels and thereby greatly improve th a. There were no significant ferences in abo xes betwe-en the exp ental group and the sham operation group, wh indicate hat MCA-MAO could signi-ficantly ove ce al hemorrhage.

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MAO ay mediate e pathophysiological process of hem ge via cAMP signaling pathway. An in-vention AO function channel could be a p al target for cerebral hemorrhage treatment.

Conflict of Interests

The Authors declare that they have no conf f interests

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