

Original Article

Nasal administration of muscone promotes cAMP-PKA-CREB signaling in rats with traumatic brain injury

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Abstract: There is a lack of data on the effects of muscone and on the best administration approach for the management of traumatic brain injury (TBI). The goal of this study was to observe the effect of muscone intranasal administration on cAMP-PKA-CREB signaling in TBI rats, and to explore the mechanism of muscone on protecting the brains. Sprague-Dawley rats were randomized into the sham operation group, TBI group, the muscone group (nasal administration), and the control group (intragastric administration) (50/group). The TBI model was established through controlled cortical impact. In the muscone group, muscone (1.8 mg/kg) was administered by nasal circulation perfusion for 7 days, 30 minutes/time, 2 times/day. Brain water content was detected before muscone intervention (T1) and on the third (T2), fifth (T3), and seventh (T4) days after intervention. Immunohistochemistry was used to detect the expression of PKA and CREB in brain tissues. Compared with the sham group, the brain water content of the TBI group was higher ($P<0.05$) and the expression of PKA-CREB were lower on T2-T4 ($P<0.05$). Compared with the TBI group, the brain water content was lower ($P<0.05$) and the PKA-CREB levels were higher in both muscone groups ($P<0.05$), especially in the muscone group with intranasal administration. Muscone can reduce cerebral edema and activate the PKA-CREB signal pathway, providing some effective brain protection. Intranasal administration of muscone could be a new way for brain protection in the treatment of TBI. Nasal administration could be more efficient than gastric administration. Clinical trials could be implemented to examine this way of administration.

Keywords: Muscone, traumatic brain injury, intranasal administration, PKA, CREB

Introduction

Traumatic brain injury (TBI) is a condition associated with high morbidity and mortality [1-4]. The neuronal damage mechanisms of TBI include primary and secondary brain injuries. Primary brain injury is the direct mechanical injury on the central nervous system, and it is mostly irreversible. On the other hand, secondary brain injury occurs from minutes to a few days after injury, and is mainly due to variations in brain microenvironment induced by cerebral ischemia and hypoxia resulting from microcirculation disorders and hemodynamic changes after brain injury, and manifesting as traumatic penumbra neuronal apoptosis [5-7]. Secondary brain injury is a potentially reversible process, and proper management is the key to improve patients' prognosis.

The protein kinase A-cAMP (cyclic adenosine monophosphate) response element binding protein (PKA-CREB) signal transduction pathway plays a key role in the central nervous system, promoting the survival, regeneration, and differentiation of nerve cells. The PKA-CREB signal transduction pathway is closely associated with synaptic plasticity and learning and memory abilities [8, 9]. Patients with TBI often suffer from advanced cognitive dysfunction, with varying degrees of decreased abilities of learning and memory, related to the degeneration and necrosis of the neurons in the hippocampus, directly or indirectly caused by TBI and post-TBI intracranial hypoperfusion (cerebral ischemia and hypoxia) [10, 11]. In addition, the cAMP-PKA-CREB signal transduction pathway has been found to play important roles in cerebral ischemia, and acute cerebral ischemia can

activate this endogenous protective mechanism [12, 13].

Muscone (3-methyl-cyclopentadecanone, C₁₆H₃₀O) is the main active ingredient of the traditional Chinese medicine musk, which shows protective effects against many kinds of brain injuries, reducing the damage to brain neurons, and accelerating the recovery of brain functions [14, 15]. Changes in permeability of the blood-brain barrier caused by ischemia and hypoxia, as well as protein leakage were reduced when rat models of cerebral ischemia injury were fed muscone [16, 17]. Additionally, Jogani V et al. pointed that drugs could be delivered by nasal administration through cerebrospinal fluid and capillaries in the lungs, which allowed rapid drug entry into the brain [18].

Nevertheless, there is a lack of data on the effects of muscone in TBI and on the best administration way for the management of TBI. Therefore, the hypothesis of this study was that muscone could play a neuroprotective role on TBI through regulating the PKA-CREB signaling pathway. This study aimed to observe the effects of muscone intranasal administration on cAMP-PKA-CREB signaling in TBI rat models, and to explore the mechanism of muscone on protecting the brains.

Methods

Animals

A total of 200 healthy male Sprague-Dawley (SD) rats, weighing 300-350 grams, were provided by the Animal Experimental Center of Zhejiang Chinese Medical University (Hangzhou, China). The rats were fed in a specific pathogen-free laboratory. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Zhejiang Chinese Medical University (No.2015-0078).

TBI rat model

Before model establishment, the rats were acclimatized for at least 3 days. The rats were randomized to the muscone, control, sham, or TBI groups (n=50/group). The TBI rat model was established as previously described [19]. Briefly, the rats were fasted for 12 hours before the operation and anesthetized using 2% pentobarbital (50 mg/kg, i.p.) (Sinopharm, Beijing,

China). After incising the scalp in the midline, stripping the periosteum, removing the right parietal, and exposing the endocranium, the striker head of an electromagnetic brain damage impactor was vertically fixed on the endocranium. The striker head of 5 mm diameter was used to mimic TBI, with an impacting depth of 2 mm and velocity of 4 m/s. The striker was removed and the scalp incision was sutured immediately. After the spontaneous breathing recovered, the rats were fed before further analysis. All the procedures were aseptically performed. The rats in the sham group did not receive striker. The rats in the muscone group were treated with muscone (1.8 mg/kg diluted with phosphate buffer containing 1% Tween-80; administered by nasal circulation perfusion; Hongsheng Tang Pharmaceutical Co., Ltd., Jinan, China) for 7 days, twice daily. The rats in the control group were subjected to intragastric perfusion of muscone for 7 days, once daily, at the dose of 2 mg/kg. The rats in TBI and sham groups received an intragastric administration of an equal volume of phosphate buffer, with 1% DMSO.

Muscone (99.3% purity; batch number 20120-609), was purchased from Jinan Hongsheng Tang Pharmaceutical Co., Ltd. (Jinan, China). The 1 g/L muscone emulsion was obtained by dilution with normal saline containing 1% Tween-80.

Brain water content

Ten rats in each group were decapitated on the day before muscone intervention (T1) and on the third (T2), fifth (T3), and seventh (T4) days after intervention. The brain tissues of the injured rats were taken out immediately, weighed, and baked at 110°C for 24 hours until a constant weight was obtained. The water content of the brain tissue (%) = (wet weight - dry weight)/wet weight ×100%.

Radioimmunoassay

Radioimmunoassay was used to detect the cAMP content in the hippocampus, according to the instructions of the cAMP kit (Beijing Huanyataike Biomedical Technology Co., Ltd., China).

Immunohistochemistry (IHC)

The brain tissues were obtained from the injured cortex in each group after the interven-

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Table 1. Water content of brain tissues in TBI rats

Groups	T1	T2	T3	T4
Sham (n=10)	78.8±1.0*	78.5±1.0*	78.7±1.0*	78.6±1.0*
TBI (n=10)	85.5±0.6	87.6±0.7	88.1±0.5	86.3±0.2
Muscone (n=10)	81.4±0.4*#	80.8±0.5*#	79.2±0.4*#	78.9±0.7
Control (n=10)	83.5±0.5*	82.6±0.8*	81.1±0.6*	79.9±0.8

*P<0.05 vs. the TBI group, #P<0.05 vs. the control group.

Table 2. cAMP content in the hippocampus of TBI rats

Groups (n=10)	cAMP (nmol/g)
Sham	0.9234±0.0351*
TBI	0.3653±0.0468
Muscone	0.6036±0.0265*#
Control	0.4785±0.0421*

*P<0.05 vs. the TBI group, #P<0.05 vs. the control group.

tion. The expression levels of PKA and CREB were detected by IHC. The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. The brain tissues were fixed with formalin and frozen sections of 15 µm were harvested and stored at -20°C. Then, 3% H₂O₂ was used to quench endogenous peroxidase and tissue sections were blocked with 5% BSA for 30 min at room temperature. Primary antibodies against PKA (1:100, Boster Biological Engineering Co., Ltd., Wuhan, China) or CREB (1:100, Boster Biological Engineering Co., Ltd.) were incubated at 4°C overnight and the corresponding secondary antibody was incubated at 37°C for 30 minutes at room temperature. The SABC working solution of horseradish peroxidase-labeled streptomycin was added and incubated at 37°C for 30 minutes. Finally, the DAB reagent was added and incubated for 20 minutes at room temperature. The slides were dehydrated, sealed, and observed under a light microscope (Olympus, Tokyo, Japan). The CM-LAS color pathological image analysis system (Beihang University, China) was used to calculate the expression of PKA and CREB, as reflected by the average optical density (OD) values.

Statistical analysis

SPSS 17.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The data are expressed as mean ± standard deviation. Multiple samples were compared by single-factor analysis of variance (ANOVA) with the post hoc q test. Two-

sided *P*-values <0.05 were considered statistically significant.

Results

Muscone lowered the water content of brain tissues in TBI rats

Food and water intake, as well as the activity of the rats were signifi-

cantly decreased after TBI model establishment, with less lustrous furs. In contrast, the rats with nasal administration or intragastric perfusion were more active than the rats in the TBI group. Compared with the rats in the intragastric perfusion control group, the food and water intake were significantly higher and the furs were more lustrous in the muscone group. Compared with the sham group, the brain water content of the TBI group was significantly higher at the time points after TBI (T1-T4) (all *P*<0.05). Compared with the TBI group, the brain water content was significantly lower at T1-T4 in both muscone-treated groups, including the muscone group and intragastric perfusion control group (all *P*<0.05), especially in the muscone group (**Table 1**). These results suggest that muscone could alleviate brain water content induced by TBI.

Muscone improved the cAMP content in the hippocampus of TBI rats

The content of cAMP in the hippocampus of the different groups was measured by radioimmunoassay. The results show that the cAMP content in the TBI group was significantly lower than in the sham group (*P*<0.05). Meanwhile, the cAMP content was significantly lower in the muscone and intragastric perfusion control groups (*P*<0.05). The cAMP content was higher in the muscone group than in the intragastric perfusion control group (**Table 2**). These findings suggest that the cAMP content may be associated with muscone-mediated brain protection.

Muscone increased the expression of PKA-CREB in TBI rats

Next, the expression levels of PKA and CREB were detected by IHC in the injured brain tissues. The positive reaction of PKA was found in the cytoplasm, while that of CREB was found in the cytoplasm and dendrites (**Figure 1**). The images and the OD values show that the expres-

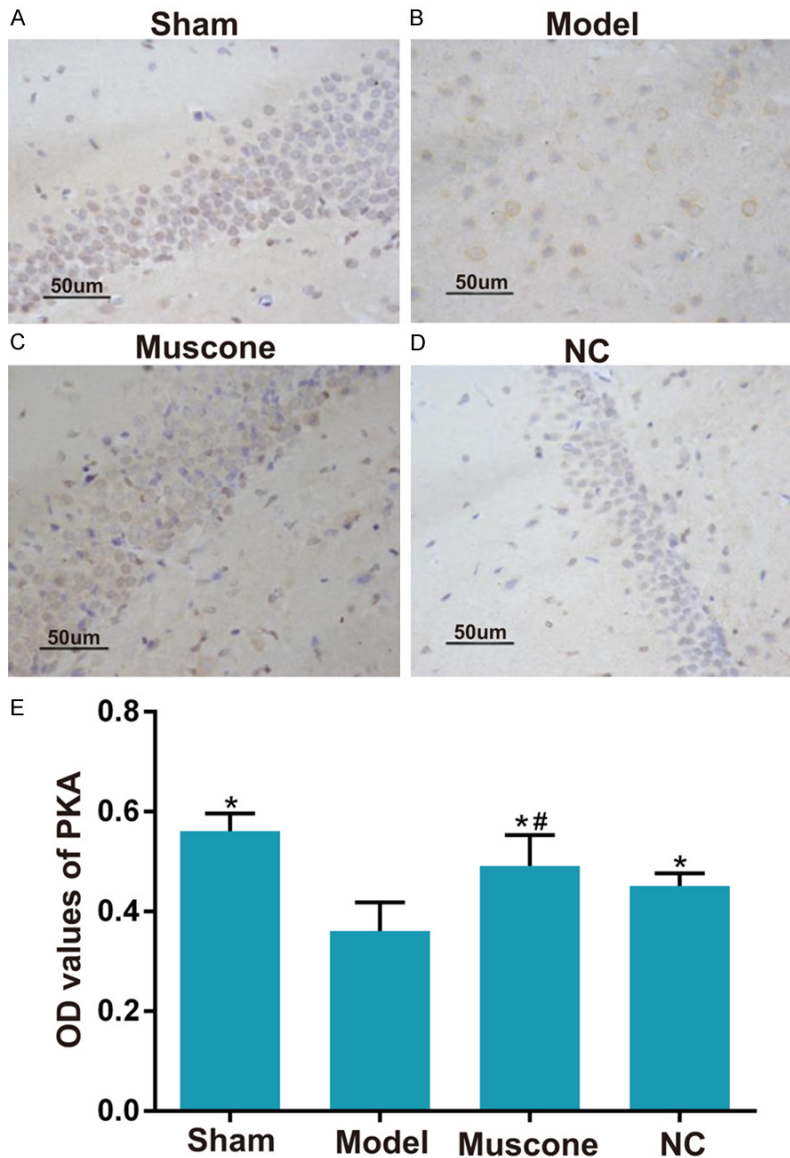


Figure 1. Muscone promoted the expression of PKA in rats with traumatic brain injury. A-D. Immunohistochemistry of PKA in injured brain tissues. E. Optical density values of PKA detected by immunohistochemistry. * $P < 0.05$ vs. the TBI group, # $P < 0.05$ vs. the intragastric muscone control group.

sion levels of PKA were significantly lower in the TBI group compared with the sham group ($P < 0.05$). On the other hand, expression levels of PKA in the muscone and intragastric perfusion control groups were significantly increased (all $P < 0.05$), especially in the muscone group (Figure 1). In addition, the impact of muscone on the expression levels of CREB was similar to that of PKA (all $P < 0.05$) (Figure 2).

Discussion

There is a lack of data on the effects of muscone and on the best administration way for the

management of TBI. This study aimed to observe the effect of muscone intranasal administration on cAMP-PKA-CREB signaling in TBI rats, and to explore the mechanism of muscone on protecting the brains. The results suggest that muscone can reduce cerebral edema and activate PKA-CREB signal pathway, providing some effective brain protection. Intranasal administration of muscone could be a new way for brain protection in the treatment of TBI. In traditional Chinese medicine, the nasal administration of muscone could be more efficient than gastric administration. Clinical trials could be implemented to examine this way of administration.

The treatment strategies for TBI are limited since no drug has been shown to have a definite clinical efficacy so far [3, 4, 20, 21]. Studies showed that muscone can reduce the volume of cerebral infarction in rat models of cerebral ischemia-reperfusion injury and alleviate the damage of nerve cells involved in cerebral ischemia-reperfusion injury [22-24]. Therefore, muscone could have some effects against TBI.

Previous studies showed that acute cerebral ischemia activates the endogenous protection mechanism of the PKA-CREB pathway, which stimulates transcription of related downstream genes, such as NGF and BDNF, playing important roles in promoting the survival and repair of nerve cells [8, 9, 12, 13, 25]. Cheng et al. [26] showed that focal cerebral ischemia stimulated production of dentate gyrus neurons in adult rats, which was associated with the activation of the transcription factor CREB and the transcription promotion of downstream BDNF. Nagakura et al. [27] found that the escape

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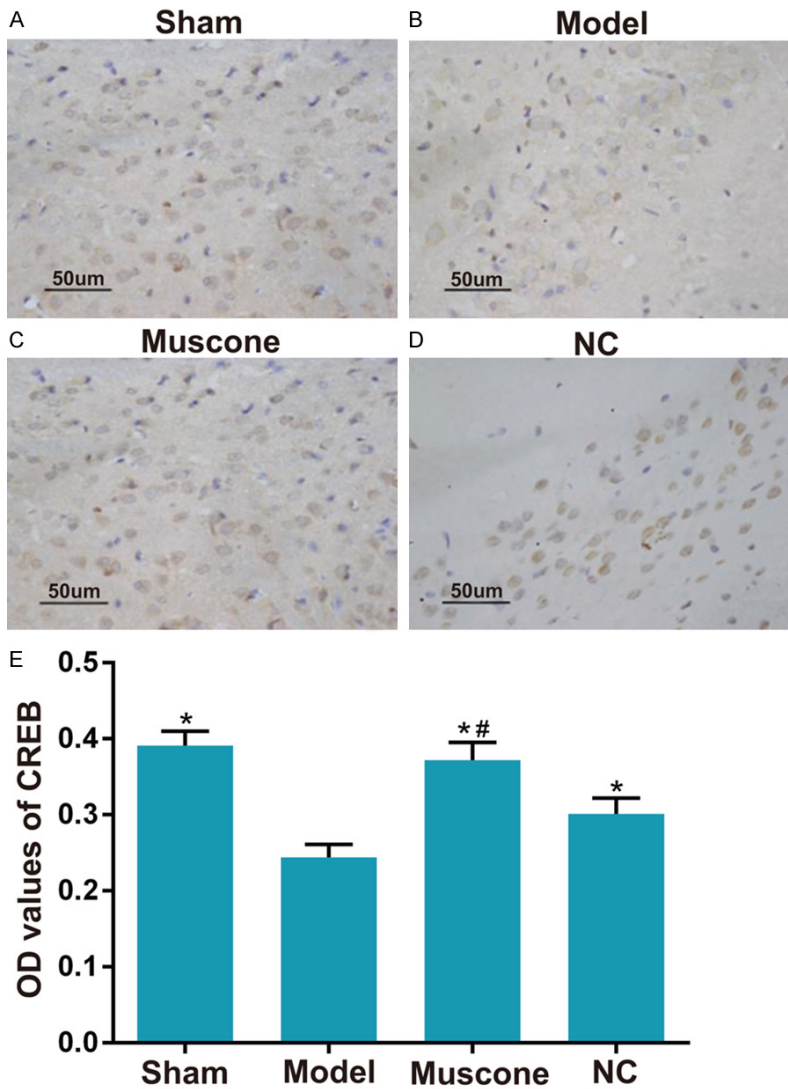


Figure 2. Muscone promoted the expression of CREB in rats with traumatic brain injury. A-D. Immunohistochemistry of CREB in injured brain tissues. E. Optical density values of CREB detected by immunohistochemistry. * $P < 0.05$ vs. the TBI group, # $P < 0.05$ vs. the intragastric muscone control group.

latency of rats with vascular dementia was prolonged after training in water maze, compared with normal rats. In addition, the levels of PKA-Ca and pCREB were decreased in the nucleus of the corresponding hippocampus. Therefore, activation of the PKA-CREB signaling pathway may be an important intrinsic neuroprotective mechanism against ischemic and hypoxic brain injury.

Cerebral ischemia and hypoxia after TBI is the main cause of neuronal damage in patients with second brain injury, and the pathogenesis of TBI is similar to cerebral ischemia-reperfusion injury [28, 29]. The present study shows

that muscone probably played neuroprotective roles through the PKA-CREB signaling pathway in rat with TBI. The TBI rats were more active and cerebral water content was decreased when treated with muscone. In addition, cAMP, PKA, and CREB were significantly higher in brain lesions when the rats were treated with muscone. Nevertheless, other pathways are involved in TBI and in the effects of muscone [14, 17, 23], and additional studies are necessary to determine the exact beneficial mechanisms of muscone in TBI.

TBI rats treated with muscone through nasal-brain way showed more intake of food and drink, more decrease of brain water content, compared with rats treated with muscone through intragastric perfusion. The exact reasons for those observations remain to be determined, but it can be hypothesized that nasal administration provides a more direct route for diffusion in the cerebrospinal fluid, compared with intragastric administration, which depends upon intestinal absorption and dilution in the blood.

One possible explanation for this may be nasal administration, as it was considered as a rapid and highly effective drug delivery method. Nasal administration preparations, which are absorbed by the nasal mucosa, could exert local or systemic therapeutic effects [30]. Future pharmacokinetics studies are necessary to address this issue.

Conclusion

In conclusion, the present study provides evidence that PKA-CREB was part of the mechanism of muscone for reducing neuronal damage after TBI. These results suggest a new way

of muscone treatment for brain protection. The nose-brain delivery route seems to provide more medicine to the brain, improving its effectiveness. It is worth further clinical study in the future.

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Disclosure of conflict of interest

None.

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