Occlusion of Middle Cerebral Artery Induces Apoptosis of Cerebellar Cortex Neural Cells via Caspase-3 in Rats

Orta Serebral Arterin Tikanmasının Ratlarda Kaspaz-3 Yolu ile Apopitozu İndüklemesi

ABSTRACT

AIM: Occlusion of the middle cerebral artery in rats may cause secondary injury that is not associated with middle cerebral artery feeding zone. This entity has been investigated very rarely.

MATERIAL and METHODS: HE staining method observed the changes of cerebellar cortex after MCAO operation. Electron Microscopy and TUNEL methods observed the apoptosis of neural cells of cerebellar cortex after MCAO in rats. Immunohistochemical analyses method observed the caspase-3 in neural cells of cerebellar cortex.

RESULTS: The results of HE staining indicated that no ischemia-necrosis changes of cerebellar cortex tissue were observed after MCAO operation by HE staining. Further experiments by Electron Microscopy and TUNEL assay revealed that the apoptosis of neural cells of cerebellar cortex were induced after MCAO in rats. Furthermore, immunohistochemical analyses showed that caspase-3 played an important role on MCAO-induced apoptosis of neural cells of cerebellar cortex.

CONCLUSION: These data showed for the first time that the role of caspase-3 in the mechanism of secondary injury of separated infarction in cerebellar cortex after middle cerebral artery occlusion in rats and it might give a new treatment strategy for individuals with human ischemic stroke.

KEYWORDS: Cerebral infarction, Stroke, Cerebellar cortex neural cells, Apoptosis, Caspase-3

INTRODUCTION

Stroke is a major health burden with high rates of incidence, morbidity, mortality and relapse (15). There are approximately 795,000 new or recurrent strokes per year and a stroke patient appears every 40 seconds averagely in the United States. Preliminary data from 2006 indicate that stroke accounted for about 1 of every 18 deaths and stroke had become the third leading underlying cause of death in the U.S., behind heart disease and cancer, and the leading cause of long-term disability (21). There are two main stroke categories of etiologic importance: ischemic stroke, accounting for about 83% of cases, and hemorrhagic stroke. Ischemic strokes are attributable to arterial thrombosis (20%), embolism (25%),...
small-vessel disease (25%), and cryptogenic causes (30%).
As ischemic stroke is the cause of significant morbidity and
mortality among the people, it is urgent to clarify the
molecular mechanism of ischemic stroke.

Apoptosis and necrosis are suggested to be the two poles of a
continuum of cellular death after ischemic stroke (11). Within
minutes of a focal ischemic stroke occurring, the core of brain
tissue exposed to the most dramatic blood flow reduction is fatally
injured and subsequently undergoes necrotic cell death. In contrast
to necrosis, apoptosis occurs in the ischemic penumbra or periinfarct zone, where collateral blood flow reduces the degree of hypoxia after several hours or days (20). In the apoptotic cells, the chromatin is condensed and the DNA becomes fragmented forming vesicles known as “apoptotic bodies” (7,19). Apoptosis induction might be achieved in several ways. For example, apoptosis was medicated by promoting the expression of pro-apoptotic factors, or reducing the expression of anti-apoptotic factors only in the tumor cells (1). In ischemic stroke, apoptosis can be initiated by two general pathways. One is the intrinsic pathway which originates from mitochondrial release of cytochrome c and associated stimulation of caspase-3; the other is the extrinsic pathway which originates from the activation of cell surface death receptors, resulting in the stimulation of caspase-8 (4). It is obvious that caspase family is important in the stroke-induced apoptosis of neuronal cells.

Caspases are cysteine proteases that are homologous to the
tematode ced-3 gene product. The first identified caspase, caspase-1, was actually interleukin-1β converting enzyme in vertebrates (14,23). Thus far, 14 members of the caspase family have been identified, 11 of which are present in humans (9,23). Caspase-3 is one of caspase family members and can be activated in animal models of ischemic stroke (2). In apoptotic neuronal cells, caspase-3 is the main executioner that it can be activated through both extrinsic and intrinsic signaling pathway (16). These findings suggest that caspase-3 might be a key mediator of apoptosis in ischemic stroke.

In this study, we focus on the secondary injury of separated infarction in cerebellar cortex after MCAO in rats. We found that the apoptosis of neural cells of cerebellar cortex were induced after MCAO. Further experiments indicated that it was caspase-3 that involved in and played an important role in MCAO-induced apoptosis of neural cells of cerebellar cortex. Our data for the first time showed the mechanism of secondary injury of separated infarction in cerebellar cortex after MCAO in rats and it might be useful for introducing a new treatment for individuals with human ischemic stroke.

**MATERIAL and METHODS**

**Animals handling and groups**

All procedures were performed in accordance with current
guidelines for Animal Experimentation at the Institutional
Animal Care and Use Committee of China Medical University.
Adult male Wistar rats (n=70, body weight 200-250 g, Animal
Center of China Medical University, Shenyang, China) were
housed in groups of 3 to 5 and maintained under a natural
12/12-hour light/dark cycle, mechanically ventilated, relative
humidity between 50-60% and environmental temperature
between 23-27°C. Rats were fed a regular diet and given tap
water ad libitum. The surgery was performed after at least one
week and rats were fasted for 12 hours before surgery. Wistar
rats were randomly assigned to two groups: sham group (n =
35) and cerebral ischemia with MCAO group (n = 35). Five rats
each of group were sacrificed after surgery at five time-points: 1
day, 3 days, 5days, 7days, and 10days, respectively, which
were used for experimental verification of the secondary
injury of separated infarction in cerebellar cortex after MCAO.

**Ischemic Stroke Model**

The ischemic stroke model was built by MCAO method as
previously described (10). Wistar rats were anesthetized with
10 % chloral hydrate (4 ml / kg) ip and placed supine on a table.
The right CCA was exposed through a midline neck incision, and,
using glass rods, was carefully dissected free from surrounding
nerves and fascia from its point of bifurcation to the base of the skull and the occipital artery branches of the right ICA and ECA were isolated, respectively. A 5-0 silk suture was tied loosely around the mobilized ECA stump. Before a
notch in the distal end of the ECA was made, two microartery
clamps were clipped on the right CCA and the ICA. A 2-cm
length of No 0.2 to 0.4 monofilament nylon suture was
inserted through the distal end of the ECA into the ICA, and
then advanced approximately 8 to 10 mm intracranially from
the CCA point of bifurcation, with distance varying according
to the animal’s body weight. Once the nylon suture had
entered the circle of Willis, it effectively occluded the MCA. The
silk suture around the right ECA stump was tightened around
the intraluminal nylon suture to prevent bleeding. After the
intraluminal suture was placed, the neck incision was closed
with a silk suture. Anesthesia and vascular dissection were
performed only in sham operation group.

Neurological examinations were performed 2, 4, and 8 hours
after the onset of occlusion and then daily until sacrifice. The
neurological findings were scored on a six-point scale: Score 0,
no neurological deficit; Score 1, a mild focal neurologic deficit
(failure to extend left forepaw fully); Score 2, a moderate focal
infarction (falling to the left); Score 4, no walk spontaneously
with a depressed level of consciousness; and Score 5, dead.
MCAO rats with neurological deficit scores 1-3 were used as
an ischemic stroke model for the further experiments.

**HE staining and immunohistochemical analyses**

Wistar rats were anesthetized with 10% chloral hydrate (4
ml / kg) ip and sacrificed at each time-point after operation
with MCAO and sham. Then the left cerebellum were rapidly
removed and postfixed for 24 hrs in Formalin. After postfixed
tissues were embedded in paraffin wax, 6-μm-thick serial
coronal sections were obtained and mounted on poly-
L-lysine-coated glass slices. For assay of the histological
change in MCAO and sham group, the paraffin-embedded
left cerebellum sections were stained with hematoxylin-eosin (HE) according to standard protocol. For assay of the expressions of caspase-3 in cerebellum after MCAO operation, immunohistochemistry was performed. Briefly, paraffin-embedded sections were deparaffinized with xylene and hydrated through graded alcohols. Epitope unmasking was retrieved by heating sections in 10 mL citrate buffer (pH 6.0) in a microwave oven for 15 min, and sections were then cooled in de-ionized water. Endogenous peroxidase activity was quenched by incubation of the sections for 10 min with 3% hydrogen peroxide in PBS. Subsequently, sections were blocked with 10% normal sera for 30 min and immunoincubated with the primary antibody against caspase-3 (Santa Cruz Biotechnology, 1:200 dilutions) for 16 hrs at 4°C. After rinsing in PBS, all sections were incubated with a biotinylated goat anti-rabbit secondary antibody (Boshide Biotechnology Company, Wuhan, China) diluted 1:200 for 30 min at room temperature. The ABC kit (Boshide Biotechnology Company, Wuhan, China) was used to localize the primary antibody and the dianaminobenzidine (DAB) kit (Boshide Biotechnology Company, Wuhan, China) was used to visualize the catalyzed peroxidase-reaction product. Three slices of each sample were observed and examined under the non-overlapping eight regions by microscope (400 times). Images were collected using software by computer image analysis system and a method of average positive stained area percentage was used to measure the immunohistochemical results.

**Electron Microscopy**

Wistar rats were anesthetized with 10% chloral hydrate (4 ml / kg) ip and sacrificed at each time-point. Then the left cerebellum were rapidly removed and cerebellar cortex was cut into 1 mm x 1 mm x 1 mm pieces and placed in a freshly prepared fixative buffer (2.5% gluteraldehyde) at 4°C for 2 hrs. After being fixed in 1% osmium tetroxide (pH 7.3-7.4) at 4°C for 2 hrs, the tissue was exposed twice to an increased concentration of Acetone (70%, 80%, 90% and 99.5%) at 15°C for 2 hrs, the tissue was exposed twice to an increased concentration of Acetone (70%, 80%, 90% and 99.5%) at 15-min intervals for partial dehydration. Tissue was embedded with epoxy resin and ultrathin sections (1 μm) were cut and transferred to 300-mesh nickel grids. After being stained with tannic acid, saturated uranyl acetate in 50% alcohol and 0.01% lead citrate, sections were then cooled in de-ionized water. Endogenous peroxidase activity was quenched by incubation of the sections for 10 min with 3% hydrogen peroxide in PBS. Slides were placed in equilibration buffer and then placed in working-strength TdT enzyme, followed by working-strength stop/wash buffer. After two drops of antidigoxigenin-peroxidase were applied to the slides, DAB was used to detect peroxidase and then washed with de-ionized water. Three slices of each sample were observed and examined by microscope (400 times). TUNEL-positive cells were counted under the non-overlapping eight regions of each sample by an observer blind to the design of these experiments.

**Statistical analysis**

Data are presented as mean ± SD. Statistical analysis was performed using SPSS version 13.0 software. Difference between the groups were assessed by one-way ANOVA(22). Significance was inferred at P<0.05.

**RESULTS**

**Effect of MCAO on histopathologic changes of cerebellar cortical tissue**

The method of MCAO-induced ischemic stroke has been widely utilized as an animal model of human stroke. MCAO-induced ischemic stroke has been widespread researched in recent years. Most studies focused on the changes of ischemic region, yet the secondary injury of separated infarction after MCAO, such as cerebellar cortex, was seldom attentive. In this study, we mainly explored the histopathologic changes of cerebellar cortex after MCAO-stimulated. The left cerebellums of Wistar rats (one of each group) were rapidly removed and the paraffin-embedded sections were made and stained with hematoxylin-eosin (HE). Figure 1B-F showed the histopathologic changes of cerebellar cortical tissue after MCAO operation at 1 day, 3 days, 5days, 7days, and 10days, respectively. No significant histopathologic changes were observed in MCAO group (Figure 1B-F) compared with sham group (Figure 1A). These results indicated that there were no ischemia-necrosis changes of cerebellar cortical tissue after MCAO operation.

**Effect of MCAO on ultrastructure of neural cells of cerebellar cortex**

Next, we explore the morphological changes of neural cells of cerebellar cortex after MCAO-stimulated by electron microscopy. As shown in Figure 2A, there were no apoptotic neural cells appeared on the sham group. Also, no apoptotic neural cells of cerebellar cortex were detected in MCAO operation 1 day group (Figure 2B). Remarkable, cell shrinkage and pyknosis appeared first in MCAO operation 3 days group by electron microscopy (Figure 2C). The cells were smaller in size, the cytoplasm was dense and the organelles were more tightly packed, and the chromatin was condensed, the most characteristic feature of the early process of apoptosis (Figure 2C). At MCAO operation 5 days group, the morphologic hallmarks of the late stages of apoptosis appeared in neural cells of cerebellar cortex besides the characteristic feature of the medium stages of apoptosis (Figure 2D, E). As shown in Figure 2E, apoptotic bodies were formed as the nucleus...
Figure 1: Effect of MCAO on histopathologic changes of cerebellar cortical tissue. Wistar rats (one of each group) were sacrificed at each time-point after operation with MCAO and sham. Then the left cerebellums were rapidly removed and the paraffin-embedded sections were made and stained with hematoxylin-eosin (HE). The histopathologic changes of cerebellar cortical tissue after MCAO and sham operation were detected by microscope. Three slices of each sample were observed and examined under the non-overlapping eight regions. Images were collected using software by computer image analysis system (original x 400). No significant histopathologic changes were observed in sham and MCAO group. A) sham group; B) 1 day after MCAO operation; C) 3 days after MCAO operation; D) 5 days after MCAO operation; E) 7 days after MCAO operation; F) 10 days after MCAO operation.

Figure 2: Effect of MCAO on ultrastructure of neural cells of cerebellar cortex. Wistar rats (one of each group) were sacrificed at each time-point after operation with MCAO and sham. Then the left cerebellum were rapidly removed and cerebellar cortex was cut into 1 mm × 1 mm × 1 mm pieces and embedded with epoxy resin. Ultrathin sections (1 μm) were cut and transferred to 300-mesh nickel grids. The samples were examined under transmission electron microscope after stained. Three ultrathin sections of each sample were observed and examined under the non-overlapping eight regions. The images of morphological changes of neural cells of cerebellar cortex after MCAO-stimulated were collected using software by computer image analysis system (original x 800). Apoptotic neural cells were not detected in the sham group (A) and MCAO operation 1 day group (B). Early process of apoptosis (small cell size, dense cytoplasm pyknosis are seen in the MCAO operation 3 days group (C). Characteristic features of the medium stage of apoptosis (D) and late stages of apoptosis (E) are shown in the MCAO operation 5 days group. Changes of myelinated fiber (F) and the hyperplasia of glial cells were found in MCAO operation 5 days and MCAO operation 10 days group, respectively (G,H).

rapidly completes its shrinkage and separates into individual fragments. With the time of MCAO operation extended, more and more neural cells of cerebellar cortex became into apoptotic and represented the morphologic hallmarks of the medium and late stages of apoptosis.
Occlusion of the Middle Cerebral Artery

To further confirm the effect of MCAO on the apoptosis of neural cells of cerebellar cortex, TUNEL assay was utilized to confirm the pro-apoptotic effect of MCAO operation. TUNEL-positive neural cells of cerebellar cortex were observed as early as 3 days after MCAO. Appearance of TUNEL-positive cells was observed progressively after MCAO (Figure 3). Next, TUNEL-positive cells through the non-overlapping eight regions of each sample were counted by an observer blind to the design of these experiments. As shown in Figure 4, the numbers of TUNEL-positive cells were significantly increased with the time of MCAO operation extended comparing with sham control group. For example, there were only few of neural cells became into apoptotic at each time-point of sham control group. At MCAO operation 1 day group, a mean 3.6 ± 0.1 % (± SD) of the neural cells of cerebellar cortex became apoptotic. Apoptosis was observed in a mean 29.6 ± 0.6 % (± SD) and 83.7 ± 2.0 % (± SD) of the neural cells of cerebellar cortex at MCAO operation 3 days and 7 days group, respectively (Fig. 4). These data indicated that the apoptosis of neural cells of cerebellar cortex were effectively induced after MCAO in rats.

**Effect of MCAO on the apoptosis of neural cells of cerebellar cortex**

To further confirm the effect of MCAO on the apoptosis of neural cells of cerebellar cortex, TUNEL assay was utilized to confirm the pro-apoptotic effect of MCAO operation. TUNEL-positive neural cells of cerebellar cortex were observed as early as 3 days after MCAO operation (Figure 3). Appearance of TUNEL-positive cells was observed progressively after MCAO (Figure 3). Next, TUNEL-positive cells through the non-overlapping eight regions of each sample were counted by an observer blind to the design of these experiments. As shown in Figure 4, the numbers of TUNEL-positive cells were significantly increased with the time of MCAO operation extended comparing with sham control group. For example, there were only few of neural cells became into apoptotic at each time-point of sham control group. At MCAO operation 1 day group, a mean 3.6 ± 0.1 % (± SD) of the neural cells of cerebellar cortex became apoptotic. Apoptosis was observed in a mean 29.6 ± 0.6 % (± SD) and 83.7 ± 2.0 % (± SD) of the neural cells of cerebellar cortex at MCAO operation 3 days and 7 days group, respectively (Fig. 4). These data indicated that the apoptosis of neural cells of cerebellar cortex were effectively induced after MCAO in rats.

**Activation of caspase-3 is responsible for MCAO-induced apoptosis of neural cells of cerebellar cortex**

Furthermore, we explored the mechanism of MCAO-induced apoptosis of neural cells of cerebellar cortex. It has been...
MCAO operation (Figure 5C, brown). With the time of MCAO operation extended, more and more caspase-3-positive cells were detected although there were no positive neural cells were found in MCAO operation 1 day group (Figure 5 B-F). A method of average positive stained area percentage was used to measure the immunohistochemical results. The percentage of caspase-3-positive cells was significantly increased at MCAO operation 3 days group (29.4± 0.9%) compared to sham control group (1.6 ± 0.1%) (Figure 6); the peak percentage was observed at MCAO operation 7 days group with a mean percentage of 35.0 ± 0.8 % (± SD) (Figure 6). These findings were consistent with the apoptotic results described above (Figure 3, 4) and suggested that caspase-3 might play a major role in MCAO-induced apoptosis of neural cells of cerebellar cortex.

**DISCUSSION**

In ischemic lesions of the brain, attention has been focused on the pathophysiological and clinical changes of the site of ischemia, but not the secondary injury which attracted interest of researchers only recently. Some evidences suggested that brain damage by cerebral infarction was not only confined to local infarction but also leaded to exofocal postischemic neuronal death (EPND) (3,8,12,13,24). In 1989, Kataoka et al. revealed degenerated synaptic terminals in the contralateral hemisphere after MCAO (8). Zhao et al reported that the decreased number of neurons and the proliferation of astrocytes on the left side of the substantia nigra in the left MCAO rat model (24). Also, the secondary changes of microglia and astrocyte activated were observed in the regions of hypothalamus, substantia nigra pars reticulata,
Caspase-3 was up-regulated in the human brain tissue after reperfusion (6). Importantly, Rami et al. also observed this occurred up to nine hours after brief MCAO and that the rat (18). Fink and coworker observed that caspase activation mRNA was elevated after transient focal brain ischemia in after MCAO. Schmidt-Kastner et al. indicated that caspase-3 apoptosis in animal models of the focal site of ischemic stroke had been identified that caspase-3 was a key mediator of cortex induced by MCAO in rats. Also, previous study of the apoptosis of neural cells of cerebellar cortex was induced after MCAO probably with the interruption of the nerve pathways. Neuronal damage is caused not only by a direct injury on neural cells, but by the axonal degeneration. The cerebellar cortex is the origin of cortical pathway and its projection fibers can be conducted to the contralateral cerebellar hemisphere to impulse the excitability of granule cells. Contralateral cerebellar dysfunction can be induced when the cortical pathway was damaged. In our study, the MCAO-induced apoptosis of neural cells of cerebellar cortex might be related to the interruption of the cortical pathway instead of direct ischemic-necrosis lesions.

Finally, we explored the mechanism of MCAO-induced apoptosis of neural cells of cerebellar cortex. As shown in Figure 5, it was caspase-3 that involved in the MCAO-induced apoptosis of neural cells of cerebellar cortex. The caspase-3 positive cells were increased by the time course of MCAO operation (Figure 6). These data were consistent with the TUNEL assay results indicating that caspase-3 played an important role in the apoptosis of neural cells of cerebellar cortex induced by MCAO in rats. Also, previous study had been identified that caspase-3 was a key mediator of apoptosis in animal models of the focal site of ischemic stroke after MCAO. Schmidt-Kastner et al. indicated that caspase-3 mRNA was elevated after transient focal brain ischemia in rat (18). Fink and coworker observed that caspase activation occurred up to nine hours after brief MCAO and that the ischemic damage could be reduced by caspase inhibitors after reperfusion (6). Importantly, Rami et al. also observed that caspase-3 was up-regulated in the human brain tissue after cerebral ischemia (17). Interestingly, caspase-3 and its cleavage products were detected in mice cerebellar granule TUNEL-positive cells during early reperfusion after two hours MCAO. These results including our finding suggested that caspase-3 played a key role in the apoptosis of neural cells in both focal cerebral ischemia damage and secondary injury of separated infarction after MCA occlusion.

In summary, our data revealed that the apoptosis of neural cells of cerebellar cortex were induced after MCAO. It was the first report showed that the importance of caspase-3 in the nerve injury of the remote area of brain infarction in rats. These findings might give a new clue to the treatment of individual with human ischemic stroke.

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THE ABBREVIATIONS
CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; EM, Electron Microscopy; EPND, exofocal postischemic neuronal death; HE staining, hematoxylin-eosin staining; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; PBS, phosphate-buffered saline; TUNEL assay, Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling assay

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