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Effects of Cervical-Lymphatic Blockade on Brain Edema and Infarction Volume in Cerebral Ischemic Rats

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Abstract

To observe the effects of cervical-lymphatic blockade (CLB) on brain edema and infarction volume of ischemic (MCAO) rat, we examined changes in cerebral water content, Ca^{2+} and glutamate concentrations, cerebral infarction volume and mRNA expression levels of N-methyl-D-aspartame receptor 1 (NMDA receptor 1) in the ischemic (left) hemisphere. The present results demonstrated that all the above indices in rats with middle cerebral artery occlusion plus cervical lymphatic blockade (MCAO+CLB) were markedly higher than those with only middle cerebral artery occlusion (MCAO) at different time points. These results indicate that CLB can aggravate cerebral ischemia by increasing brain edema and infarction volume.

Key Words: middle cerebral artery occlusion, cervical-lymphatic blockade, Ca^{2+}, glutamate, NMDA receptor

Introduction

Many studies have demonstrated that the central nervous system (CNS) is not a lymphatic lining of endothelial cell but is involved in lymph drainage. Lymphatic drainage is involved not only in circulating cerebrospinal fluid (CSF) (21, 22, 39), but also in the absorption of macromolecules in the CSF and the interstitial space of the brain (30). Thus, it is important for the brain in homeostasis of the internal environment, regulating intracranial pressure and maintaining normal physiological functions (14, 34). Obstruction of lymphatic drainage pathway can cause retention of macromolecular material, such as plasma protein, and hence to brain edema and changes in brain morphology and function, known as "lymphostatic encephalopathy" (10, 21). A number of pathological conditions, such as inflammation of the head and cervical areas, lymphatic lesions and brain diseases, may also cause obstruction of the pathway, thereby aggravating cerebral ischemia. However, the reasons are still unclear. Middle cerebral artery occlusion with cervical lymphatic blockade (MCAO+CLB) was induced in rat to elucidate possible mechanisms.

Zakharov *et al*. (41) and Silver *et al*. (36) found that cervical lymphatic vessels played an important role in the outflow of CSF. Kiwic *et al*. (26) believed that CSF outflow to the lymphatic system could influence CNS immunology, hydrocephalus and olfactory sensation. However, disorders of the CSF system, such as hydrocephalus and intracranial hypertension, might relate directly or indirectly to a lymphatic CSF absorption deficit (23). Many studies demonstrated that brain microcirculation disorders, caused by intracranial hypertension, hydrocephalus and CSF absorption deficit, could increase Ca^{2+} and glutamate content and expression levels of NMDA receptor 1 in brain tissue and impair brain function (17, 27, 37, 40).

In the healthy brain, high-affinity glutamate transporters actively clear glutamate released into the synaptic cleft and, thereby, prevent excitotoxicity (13, 20). However, in hypoxic-ischemic conditions, pre-synaptic neurons release excessive glutamate, promoting the rapid accumulation of glutamate in the

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synaptic cleft. In addition, decrease of reuptake and massive glutamate overflow from dead cells could also increase glutamate concentration in the synaptic cleft (24, 31). The excessive glutamate continuously activated postsynaptic NMDA receptors, depolarized the cell, induced a massive cellular influx of Ca^{2+} and then activated a series of cytotoxicity-related enzymes, resulting in ongoing neuronal death (7, 15, 32).

The aim of this study is to clarify why CLB would aggravate brain edema and infarct volume by observing the dynamic change of Ca^{2+} and glutamate levels and the expression of NMDA receptor 1 in ischemic brain tissue before and after CLB. These findings will be used to further illustrate the CLB mechanisms aggravating cerebral ischemia.

Materials and Methods

Animal experiments were approved by the local review committee and were conducted in accordance with the guidelines for animal research established by the Shandong University regulations on laboratory animal care. Every effort was made to minimize animal suffering and to reduce the number of animals used. Male Wistar rats (obtained from the Experimental Animals Center of Shandong University, P. R. China, No. 20021024) weighing 250-300 g were fasted for 24 h before being they were used in the experiments. These rats were anesthetized with chloral hydrate (400 mg/kg) administered intraperitoneally. Femoral artery catheterization was performed for monitoring arterial pressure and blood gas concentrations. A rectal probe was used for temperature monitoring, and rectal temperature was maintained at 37.0 ± 0.5°C using a heating pad and heating lamp during the period of operation. These parameters were measured before and after MCAO and cervical lymph node removal.

Experimental Models

Permanent focal cerebral ischemia was induced by a suture (Prolen, Anahuac, Coapa Mexico City, Mexico) occlusion technique, according to Longa *et al*. (33). After making a midline incision in the neck, the left external carotid artery (ECA) was carefully exposed and dissected, and a 0.25-mm diameter suture inserted from the ECA into the left internal carotid artery (ICA) to occlude the origin of the left middle cerebral artery. The ECA and suture were ligated together, and then the wound was closed. Sham-operated rats underwent identical surgery, except that the suture was not inserted into the ICA. MCAO+CLB rats first underwent a 15-min permanent MCAO and were then subject to CLB, according to the method of Casley-Smith and Foldi (6). The cervical-lymph nodes, including bilateral submandibular superficial and deep nodes were identified, isolated, deligated and removed under a dissecting microscope. The afferent and efferent lymphatics were also deligated. However, the MCAO+Sham CLB rats underwent identical surgery to the MCAO+CLB rats, except that cervical-lymph nodes and lymphatics were not isolated, deligated and removed.

Experimental Protocol

All rats were divided into five groups randomly: Normal, Sham-operated, MCAO, MCAO+Sham-CLB and MCAO+CLB groups. Each group was assigned to seven time points: 3, 6, 12, 24, 48, 72 and 96 h. Cerebral water content, infarct volume, Ca^{2+} and glutamate concentrations and expression levels of NMDA receptor 1 in cerebral tissue were assessed at the different time points in all rats.

Determination of Edema Formation

There were eight rats in this measurement at each time point. Measuring regional water content assessed brain edema, according to the Elliott formula (9). After the rats were sarcrificed, the brains were rapidly removed. Fresh tissue samples were immediately weighed on pre-weighed aluminum foils to yield the wet weight. The fresh weight of each specimen was noted. Then, the tissue was dried in an electronic oven with a temperature of 105°C for 24 hours to achieve the constant dried tissue. The dried tissue was then weighed on the balance again. Water content was expressed as percentage, calculated as [wet weight-dry weight]/wet weight × 100%.

Measurement of Total Ca^{2+} in Brain Tissue

Ca^{2+} content in brain tissue was measured by an atomic absorption technique (8). The above-mentioned dry brain tissue was sufficiently digested with 5 ml of mixed acids (nitric acid/perchloric, 3:1, v/v) and then treated to reach an achromatic color on a homoiothermy plate. The tissue was cooled to room temperature and the volume was adjusted to 10 ml using deionized water. Finally, the total Ca^{2+} content in brain tissue was directly measured using an atomic absorption spectrophotometer (unit: μmol/g · dry wt).

Quantification of Cerebral Infarction Volumes

There were six rats per measurement at each time point. After the rats were sacrificed, the brains were immediately removed, briefly cooled in ice-cold saline and successively sliced into 2-mm coronal sections using a brain matrix (Harvard Bioscience

Inc. Holliston, MA, USA). The brain sections were incubated in phosphate-buffered saline containing 2% 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) at 37°C for 30 min, subsequently immersed in 10% phosphate-buffered formalin and examined after 1 week to measure the infarction size, as described by Bederson *et al*. (2). The infarction area of each brain slice was determined with an image analyzer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Infarction volume was calculated by summing the infarct areas measured in the component brain slices. Total infarction volume was expressed as a percentage of total left hemispheric volume using computerized imaging software (NIH Image).

Glutamate Content Detecting

There were ten rats per measurement at each time point. The left cerebral cortex of rat at each time point was isolated and washed with ice-cold phosphate buffered saline (PBS, pH 7.4) to remove residual blood. The cerebral cortex homogenate was prepared using a manual-type homogenizer in 0.1× phosphate-buffered saline and was subsequently centrifuged at 18,000 x g for 30 min at 4°C. The cerebral cortex homogenate supernatants were kept at -20°C before use. When the frozen supernatants in PBS were used, they were thawed in the cold room at 4°C and then glutamate concentration was determined using a glutamate kit (Sigma, St. Louis, MO, USA).

Detection of NMDA Receptor 1 mRNA by Northern Blot

Northern blot was performed using 20 μg of total RNA from rat cerebral tissue at different time points. The NMDA receptor-1 cDNA fragment was labeled with [α-^{32}P]dCTP by Priming, a gene labeling system from Promega, and used for hybridization probe. The blots were autoradiographed at -80°C for up to 6 days. In addition, primers for isolation of NMDA receptor 1 gene were as follows: 5'-ATGAGCACCATGCACCTGCTGAC-3', 5'-TCAGCTCTCCCTATGACGGGAAC-3'. Quantity One Gel Analysis Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure and calculate light intensity area of each electrophoresis band and the corresponding GAPDH band, and to compare average values (ratios showed the relative content of each sample mRNA).

Statistical Analysis

Data are expressed as mean ± S.D. One-way ANOVA (analysis of variance) was used for comparing different groups in each measurement. Differences were considered significant at $P < 0.05$.

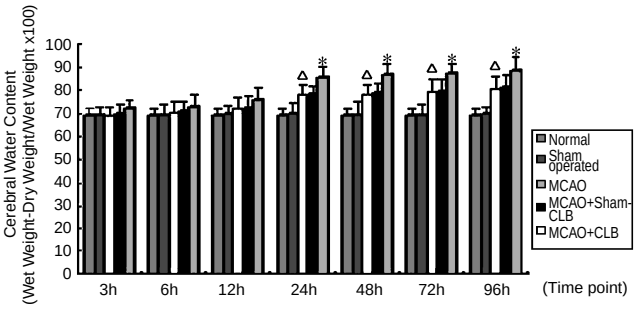

Fig. 1. Cerebral water content of the ischemic hemisphere. The y-axis for cerebral water content is the relative percentage ratio of (wet weight-dry weight) to wet weight. Each bar represented the mean ± SD of data from eight rats per group. The histograms, from the extreme left, are as follows: normal, sham-operated, MCAO, MCAO+Sham-CLB and MCAO+CLB groups. The x-axis indicated different time points.

Results

Physiological Parameters

Baseline pH in blood was 7.36 ± 0.03, P_{O_2} 102.94 ± 6.39 mmHg, P_{CO_2} 34.6 ±3.31 mmHg, with no significant changes among the groups during the experiments. Normothermia (37°C) was maintained in all rats.

Cerebral Water Content

There was no obvious change in cerebral water content of the left hemisphere between normal and sham-operated groups (in Fig. 1). In MCAO group, cerebral water content increased by 10.14%, 11.59%, 13.04% and 14.49% at 24, 48, 72 and 96 h, respectively, compared with sham-operated group ($P < 0.05$). In MCAO+CLB group, cerebral water content increased by 10.52%, 10.38%, 10.25% and 10.14% at 24, 48, 72 and 96 h, respectively, compared with MCAO+Sham-CLB group ($P < 0.05$).

Ca^{2+} Content

The mean values and standard deviations of Ca^{2+} content of different groups is presented in Table 1. Ca^{2+} content in cerebral tissue showed no obvious change in the normal and sham-operated groups, but was significantly increased at 24 h and lasted to 96 h in other three groups. In MCAO group, Ca^{2+} content increased by 83.34%, 91.67%, 113.08% and 142.11% at 24, 48, 72 and 96 h, respectively compared with sham-operated group ($P < 0.05$ at 24 and 48 h, $P < 0.01$ at 72 and 96 h). In MCAO+CLB group, Ca^{2+} content increased by 42.22%, 38.19%, 34.27% and 32.68% at 24, 48, 72 and 96 h, respectively, compared with MCAO+Sham-CLB group ($P < 0.05$).

Table 1. Effects of middle cerebral artery occlusion (MCAO) with or without cervical lymphatic blockage (CLB) on calcium concentrations (mean ± SD, μmol/g.dry wt) in rat brain tissues at different time intervals.

Group	3 h	6 h	12 h	24 h	48 h	72 h	96 h
Normal	10.70 ± 1.36	–	–	–	–	–	–
Sham-operated	11.27 ± 1.23	11.15 ± 1.37	11.21 ± 1.31	11.35 ± 1.42	11.29 ± 1.40	11.35 ± 1.38	11.24 ± 1.26
MCAO	11.36 ± 1.39	15.52 ± 2.21	17.19 ± 2.54	20.81 ± 2.68*	21.64 ± 2.72*	25.32 ± 2.63**	27.21 ± 2.59**
MCAO+Sham-CLB	11.41 ± 1.26	16.32 ± 1.87	17.96 ± 2.19	21.01 ± 2.56	2.39 ± 2.57	25.76 ± 2.23	28.12 ± 2.43
MCAO+CLB	12.02 ± 1.97	18.25 ± 2.01	22.17 ± 2.14	29.88 ± 2.05$^{\Delta}$	30.94 ± 2.18$^{\Delta}$	34.59 ± 2.34$^{\Delta}$	37.31 ± 2.41$^{\Delta}$

MCAO: middle cerebral artery occlusion, MCAO+Sham-CLB: middle cerebral artery occlusion with sham-cervical lymphatic blockage, MCAO+CLB: middle cerebral artery occlusion with cervical lymphatic blockage. *P <0.05, **P <0.01 *vs.* sham-operated; $^{\Delta}P$ < 0.05 *vs.* MCAO+Sham-CLB.

Table 2. Changes of infarction volume in brain tissues of ischemic rats before and after CLB (mean ± SD).

Time	MCAO		MCAO+Sham-CLB		MCAO+CLB	
	(mm^3)	(%)	(mm^3)	(%)	(mm^3)	(%)
3 h	151.18 ±15.01	17.53 ±3.21	155.16 ±18.85	17.96 ±3.93	155.62 ±20.27	18.01 ±4.21
6 h	182.10 ±16.52	20.87 ±3.47	185.80 ±17.87	21.27 ±3.74	215.82 ±20.73	24.51 ±4.30
12 h	228.48 ±22.87	25.88 ±4.69	233.10 ±26.15	26.38 ±5.37	268.10 ±25.35	30.16 ±5.21
24 h	303.21 ±17.29	33.84 ±3.92	306.51 ±20.62	34.31 ±4.28	338.55 ±24.74	37.77 ±5.09
48 h	300.14 ±15.86	33.57 ±3.62	315.30 ±20.05	35.26 ±4.17	346.23 ±20.82	38.60 ±4.32
72 h	321.01 ±14.12	35.88 ±3.11	330.02 ±18.07	36.74 ±3.78	330.24 ±20.81*	46.45 ±4.10*
96 h	330.86 ±16.13	36.94 ±3.68	332.34 ±19.74	37.10 ±4.11	428.81 ±18.53*	47.52 ±3.87*

MCAO: middle cerebral artery occlusion, MCAO+sham-CLB: middle cerebral artery occlusion with sham-cervical lymphatic blockage, MCAO+CLB: middle cerebral artery occlusion with cervical lymphatic blockage. *P < 0.05 *vs.* MCAO+sham-CLB.

Table 3. Effects of middle cerebral artery occlusion (MCAO) with or without cervical lymphatic blockage (CLB) on glutamate concentration (mean ± SD, ng/mg protein) in rat brain tissues at different time intervals.

Group	3 h	6 h	12 h	24 h	48 h	72 h	96 h
Normal	0.55 ± 0.15	–	–	–	–	–	–
Sham-operated	0.51 ± 0.11	0.57 ± 0.19	0.56 ± 0.16	0.56 ± 0.24	0.55 ± 0.19	0.54 ± 0.20	0.56 ± 0.21
MCAO	1.37 ± 0.27**	0.51 ± 0.13	0.63 ± 0.16	0.61 ± 0.21	0.57 ± 0.18	0.68 ± 0.22	0.67 ± 0.27
MCAO+Sham-CLB	1.39 ± 0.22	0.55 ± 0.16	0.66 ± 0.20	0.64 ± 0.19	0.60 ± 0.11	0.69 ± 0.25	0.71 ± 0.25
MCAO+CLB	1.21 ± 0.19	2.01 ± 0.31$^{\Delta\Delta\Delta}$	0.71 ± 0.18	0.56 ± 0.20	0.56 ± 0.21	0.71 ± 0.23	0.74 ± 0.24

MCAO: middle cerebral artery occlusion, MCAO+Sham-CLB: middle cerebral artery occlusion with sham-cervical lymphatic blockage, MCAO+CLB: middle cerebral artery occlusion with cervical lymphatic blockage. **P < 0.01 *vs.* sham-operated; $^{\Delta\Delta\Delta}P$ < 0.001 *vs.* MCAO+Sham-CLB.

Infarction Volume

Cerebral infarction, measured by TTC staining, significantly increased in the MCAO, MCAO+Sham-CLB and MCAO+CLB animals. The mean volumes of infarction as a percentage of total left hemispheric volume, measured at 3, 6, 12, 24, 48, 72 and 96 h, respectively, are presented in Table 2. The infarct volume at different time points significantly increased in the MCAO group compared with those in the sham-operated group. Furthermore, CLB could obviously promote this increasing trend in infarct size of MCAO+Sham-CLB rats (P < 0.05 at 72 and 96 h).

Glutamate Concentrations in Cerebral Tissue Homogenates

Table 3 shows that glutamate concentrations in

Table 4. Effects of middle cerebral artery occlusion (MCAO) with or without cervical lymphatic blockage (CLB) on relative levels of mRNA expression of NMDA receptor 1 in rat brain tissues (NMDA receptor 1/ GAPDH light intensity).

Time	MCAO	MCAO+Sham-CLB	MCAO+CLB
Sham	1.00 ± 0.01	–	–
3 h	3.01 ± 0.04**	3.04 ± 0.03	2.69 ± 0.04
6 h	1.81 ± 0.02	1.83 ± 0.02	$3.46 \pm 0.04^{\Delta\Delta}$
12 h	1.07 ± 0.02	1.13 ± 0.03	1.47 ± 0.03
24 h	1.45 ± 0.01	1.41 ± 0.01	1.38 ± 0.01
48 h	1.67 ± 0.03	1.73 ± 0.02	1.69 ± 0.02
72 h	1.93 ± 0.01	2.01 ± 0.01	2.19 ± 0.02
96 h	2.22 ± 0.04*	2.34 ± 0.04	2.75 ± 0.03

MCAO: middle cerebral artery occlusion, MCAO+Sham-CLB: middle cerebral artery occlusion with sham-cervical lymphatic blockage, MCAO+CLB: middle cerebral artery occlusion with cervical lymphatic blockage. *$P < 0.05$, **$P < 0.01$ *vs.* sham-operated; $^{\Delta\Delta}P < 0.01$ *vs*. MCAO+Sham-CLB at 6 h.

tissue homogenates were not significantly changed in normal and sham-operated groups, whereas the concentration varied greatly at different time points in the MCAO, MCAO+Sham-CLB and MCAO+CLB groups. For instance, the peak concentrations of glutamate occurred at 3 h in the MCAO (1.37 ±0.27 ng/mg protein, $P < 0.01$ versus sham-operated) and MCAO+Sham-CLB (1.39 ±0.22 ng/mg protein, $P < 0.001$ versus MCAO+Sham-CLB) groups, but at 6 h in the MCAO+CLB (2.01 ± 0.31 ng/mg protein) group. Moreover, glutamate concentrations rapidly decreased after 3 h (MCAO and MCAO+Sham-CLB groups) or after 6 h (MCAO+CLB group), but then returned to a normal level at the 24th and lasted to the 96th h in the MCAO, MCAO+Sham-CLB and MCAO+CLB groups.

Detection of NMDA Receptor 1 mRNA by Northern Blot

Fig. 2 and Table 4 show the mRNA expression levels of NMDA receptor 1 of cerebral tissue in different groups: mRNA expression level for NMDA receptor 1 was very low in both the normal and sham-operated rats, but significantly increased, reaching maximum levels at 3 h in the MCAO ($P < 0.01$ versus sham-operated) and MCAO+Sham-CLB groups or at 6 h in the MCAO+CLB group ($P < 0.01$ versus MCAO+Sham-CLB), respectively, and then rapidly returned to normal levels, increased again at the 24th and lasted to the 96th h in the MCAO ($P < 0.05$ versus sham-operated at 96 h), MCAO+Sham-CLB and MCAO+CLB rats.

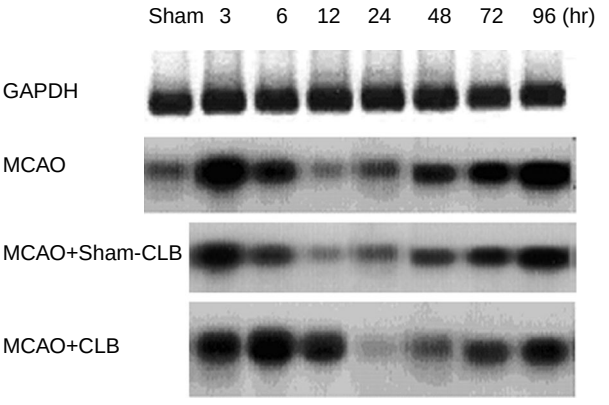

Fig. 2. RNA gel-blot analysis of NMDA receptor-1 in cerebral tissue. The ethidium bromide-stained GAPDH band in the agarose gel is shown as a loading control.

Discussion

This study has shown that the administration of CLB after MCAO can significantly increase Ca^{2+} and glutamate content, and up-regulate the expression level of NMDA receptor 1 mRNA in ischemic cerebral tissue to aggravate rat brain injury, such as increasing cerebral water content and infarction volume. It mimics the clinical situation seen, namely the aggravation of ischemic stroke in patients after suffering from cervical lymph-node removal of carcinoma of larynx, tonsil resection, various kinds of inflammations in head and cervical part and lymphatic lesion (12, 18).

Our experiment results revealed that CLB can markedly increase cerebral water content to aggravate cerebral edema of ischemic rats. Some studies (3, 28) demonstrated that after MCAO both cerebral water content and Ca^{2+} concentration in ischemic animal brain tissue significantly increased, and Ca^{2+} concentration was highly, positively correlated with cerebral water content. Hypoxia-ischemia induced by MCAO was found to be able to promote excessive release of glutamate in ischemic rat brain tissue, continuously activate NMDA receptor 1 and then result in a massive cellular influx of Ca^{2+} (1). Elevated levels of intracellular Ca^{2+} can further enhance glutamate release

from pre-synaptic stores and then induce more accumulation of Ca^{2+} in ischemic brain tissue to form the chain metabolism of arachidonic acid to aggravate vasogenic edema (5). Our results also demonstrated that both MCAO and administration of CLB after MCAO could significantly increase cerebral water content and Ca^{2+} concentration in ischemic brain tissue, especially in the administration of CLB after MCAO. Meanwhile, glutamate content and expression level of NMDA receptor-1 mRNA were also markedly enhanced. Thus, we believed that CLB increasing cerebral water content of ischemic rat is associated with CLB aggravated hypoxia-ischemia, and hence significantly increased glutamate and Ca^{2+} content and over-activated NMDA receptor 1. A possible mechanism is as follows: after CLB, the increased aggravation of hypoxia-ischemia may further enhance Ca^{2+} and glutamate concentrations in ischemic brain tissue, gradually worsening the swelling and necrosis of cerebral blood-capillary endothelium, and significantly increasing blood-brain barrier (BBB) permeability. Subsequently, plasma components, such as plasma proteins, water and Na^{+} were elevated in the interspaces of brain tissue to form vasogenic edema. Meanwhile, the over-accumulated Ca^{2+} may speed up excessive Ca^{2+} inflowing into the cerebral blood-capillary endothelium, worsen the swelling and necrosis of endothelium and further aggravate vasogenic edema.

Sun *et al.* (38) and Foldi (11) found that, although surgical blockade of the cervical-lymphatics would not block all CSF drainage, simple CLB could also markedly increase protein content and colloid osmotic pressure in the brain interstitial fluid, accelerate some micro-molecular materials, such as H_2O and Na^{+}, to penetrate into the brain tissue interspace and aggravate interstitial brain edema and form the lymph-stagnation brain edema. Our previous study also demonstrated that, after administrating simple CLB to rats, the meninges tightened, and the brain swelled overtly. The adventitia of the brain arteries and the perivascular Virchow-Robin space dilated irregularly. In some cerebral cortical neurons, both the nucleus and cytoplasm condensed, chromatin gathered near the nuclear membrane. Some other neurons appeared to be degeneration and necrosis. There was intracellular edema of the astrocytes. The above alterations could be seen in the third layer of cerebral cortical of parietal lobus mainly.

The results of our experiment showed that the more cerebral water content in ischemic brain tissue was, the severer the brain edema would become. We thought it was because CLB further increased water pressure in the interstitial space, resulting in the increase of intracranial pressure and the compression of blood vessels.

Thus, we suggested that the significant increase in cerebral water content in ischemic brain tissue was an artificial result of CLB aggravating vasogenic edema and interstitial brain edema, thereby promoting lymph-stagnation brain edema.

After MCAO over-released glutamate induced by ischemia could continuously activate NMDA receptor-1 promote the influx of excessive extracellular Ca^{2+} into cells, causing calcium overload to speed up the ongoing neuronal death (4, 15). In addition, calcium overload could activate protein kinase C to stimulate blood platelet producing the maximum-release response to aggravate ischemic infarction (29). A previous study also demonstrates that calcium overload could induce penumbra to develop into infarction by aggravating the cell apoptosis in ischemic penumbra (25). In this study, we find that CLB can markedly increase brain infarction volume. Therefore, we thought that it was because CLB aggravated calcium overload.

Shimada *et al.* (35) discovered that, in cats, a massive release of glutamate in the ischemic region could cause significant brain injury when cerebral blood flow decreased to 36% of normal value. Hossmann (19) observed that the rapid increase in extracellular glutamate content could increase depolarized and decrease repolarized cells in ischemic penumbra to expand infarction volume. Hakim (16) believed that cerebral ischemia and post-ischemic secondary injury could result in penumbra being in a state of dynamic change: either a functional restoration by improving cerebral blood flow or an expansion of necrosis from the ischemic core to peripheral regions by failing to obtain adequate blood supply.

In this study, increased infarction volume was due to obstruction of the brain lymph drainage pathway caused by CLB's increasing intracranial hypertension and brain edema, worsening cerebrospinal fluid and blood circulation, aggravating hypoxia-ischemia to reduce the blood flow of the penumbra region and inducing excessive release of glutamate and Ca^{2+}. A possible mechanism is as follows: After CLB, the increasing aggravation of hypoxia-ischemia could further enhance glutamate concentrations in ischemic penumbra, over-stimulating NMDA receptors to induce the influx of extracellular excessive Ca^{2+} into cell to form calcium overload. In addition, calcium overload could also speed up release of glutamate in ischemic penumbra to worsen calcium overload. These results aggravated developmental excitotoxic or secondary injury, such as rapid depolarization, post-ischemic inflammatory reaction and apoptosis, which promoted penumbra to develop into infarction.

In conclusion, this study has proved that CLB could promote the development of permanent focal ischemia due to increasing cerebral water content, Ca^{2+} and glutamate concentrations, cerebral infarction volume and mRNA expression level in brain tissue.

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