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Excitatory amino acid changes in the brains of rhesus monkeys following selective cerebral deep hypothermia and blood flow occlusion☆

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Abstract
Selective cerebral deep hypothermia and blood flow occlusion can enhance brain tolerance to ischemia and hypoxia and reduce cardiopulmonary complications in monkeys. Excitotoxicity induced by the release of a large amount of excitatory amino acids after cerebral ischemia is the major mechanism underlying ischemic brain injury and nerve cell death. In the present study, we used selective cerebral deep hypothermia and blood flow occlusion to block the bilateral common carotid arteries and/or bilateral vertebral arteries in rhesus monkey, followed by reperfusion using Ringer's solution at 4°C. Microdialysis and transmission electron microscope results showed that selective cerebral deep hypothermia and blood flow occlusion inhibited the release of glutamic acid into the extracellular fluid in the brain frontal lobe and relieved pathological injury in terms of the ultrastructure of brain tissues after severe cerebral ischemia. These findings indicate that cerebral deep hypothermia and blood flow occlusion can inhibit cytotoxic effects and attenuate ischemic/hypoxic brain injury through decreasing the release of excitatory amino acids, such as glutamic acid.

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neural regeneration; brain injury; selective deep hypothermia; microdialysis; rhesus monkey; glutamic acid; excitatory amino acids; brain protection; high performance liquid chromatogram; ultrastructure; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights
(1) Selective deep hypothermia resuscitation after severe ischemia in monkey brain stimulated release of excitatory neurotransmitters such as glutamic acid and inhibited cytotoxicity, but did not damage cell ultrastructure in brain tissues.
(2) Selective deep hypothermia enhanced brain tolerance to ischemia and hypoxia.

Abbreviation
Glu, glutamic acid

INTRODUCTION

Olney *et al* [1] and Choi[2] proposed for the first time that excitotoxicity may serve as a common pathway leading to neuronal death. Huang *et al* [3] cultured primary nerve cells and found that hypothermia (32°C) after hypoxia can reduce glutamic acid (Glu) release and maintain low levels of extracellular Glu. Microdialysis results showed that hypothermia after cerebral ischemia reduced Glu release in the infarction region and delayed Glu

distribution to the ischemic penumbra[4]. Selective cerebral deep hypothermia and blood flow occlusion can selectively and greatly reduce brain temperature and maximize the period of “safe” cerebral ischemia/hypoxia while also preventing the complications induced by general deep hypothermia[5]. However, the influence of this technique on neurotransmitter metabolism in the extracellular fluid of brain tissues remains poorly understood.

In the present study, we used microdialysis to harvest the extracellular fluid of brain tissues and detected Glu changes prior to and following selective cerebral deep hypothermia and blood flow occlusion using high performance liquid chromatography-ultraviolet detection. Moreover, we observed the ultrastructure of brain tissues using transmission electron microscope to investigate the cerebroprotective mechanism of cerebral deep hypothermia and blood flow occlusion.

RESULTS

Quantitative analysis of experimental animals

A total of seven rhesus monkeys were randomly assigned to four-vessel (bilateral common carotid artery and bilateral vertebral artery) occlusion (n = 4), and two-vessel (bilateral common carotid artery) occlusion (n = 3) groups. All seven monkeys were included in the final analysis.

Glu content changes in the frontal lobe prior to and following cerebral deep hypothermia and blood flow occlusion

In the four-vessel occlusion group, Glu was immediately significantly elevated following occlusion of the bilateral common carotid arteries and the bilateral vertebral arteries at room temperature (P < 0.05), but was significantly reduced following hypothermic perfusion, to levels even lower than those prior to ischemia (P < 0.05). The Glu level remained unchanged during perfusion, and was elevated early after rewarming reperfusion following blood flow restoration (P < 0.05), becoming gradually greater than the levels prior to perfusion (P < 0.05; Table 1).

In the two-vessel occlusion group, Glu was significantly elevated following occlusion of the bilateral common carotid arteries at the common temperature (P < 0.05), but was significantly reduced following hypothermic perfusion compared with that at the common temperature or in the four-vessel occlusion group (P < 0.05). The Glu level remained unchanged after rewarming reperfusion following blood flow restoration compared with that after hypothermic perfusion (Table 1).

Table 1 Glutamic acid content (μM) in monkey brain before and after ischemic hypothermic perfusion

Time	Four-vessel occlusion group (n = 4)	Two-vessel occlusion group (n = 3)
Prior to ischemia	2.05±0.64^{bfg}	2.12±0.46^{bfg}
10 minutes after occlusion (common temperature)	2.97±1.36^{acde}	3.11±0.84^{acde}
30 minutes after occlusion (early after hypothermic perfusion)	1.49±0.48^{bfg}	2.11±0.38^{bf}
50 minutes after occlusion (during hypothermic perfusion)	1.66±0.66^{bfg}	2.35±0.71^{bg}
70 minutes after occlusion (end of hypothermic perfusion)	1.77±0.68^{bfg}	1.99±0.46^{bf}
Early rewarming after blood flow restoration	3.44±0.12^{acde}	2.36±0.35
Late rewarming after blood flow restoration	3.78±0.37^{acde}	2.08±0.43

^{a}P < 0.05, *vs*. prior to ischemia; ^{b}P < 0.05, *vs*. 10 minutes after occlusion; ^{c}P < 0.05, *vs*. 30 minutes after occlusion; ^{d}P < 0.05, *vs*. 50 minutes after occlusion; ^{e}P < 0.05, *vs*. 70 minutes after occlusion; ^{f}P < 0.05, *vs*. early rewarming after blood flow restoration; ^{g}P < 0.05, *vs*. late rewarming after blood flow restoration. Data are expressed as mean ± SD and analyzed by one-way analysis of variance.

Ultrastructure of brain tissues following cerebral deep hypothermia and blood flow occlusion

In the two-vessel occlusion group, transmission electron microscopic observation of brain tissues, including the frontal, temporal, parietal and occipital cortex, hippocampus, basal ganglia, cerebellum, and brain stem, showed that the cell membrane and nuclear membrane were intact and the cytoplasm was normal (Figures 1A, B). In addition, mitochondria in the cytoplasm and the endoplasmic reticulum were normal (Figures 1C, D). In the four-vessel occlusion group, nerve cells were swollen (Figure 1E), and this was accompanied by karyopyknosis (Figure 1F) in addition to organelle pathological changes, such as mitochondrial swelling, cristae lysis (Figures 1G, H), and expansion of the endoplasmic reticulum with the shedding of particles (Figure 1I).

DISCUSSION

Traditional deep hypothermia can induce proarrhythmia, coagulation disorders and pulmonary nonfunction. Moreover, clinical application is limited by safe time limits. Ohta *et al* [6] reported that selective cerebral deep hypothermia and blood flow occlusion with cerebral bloodless circulation was successfully restored in dogs.

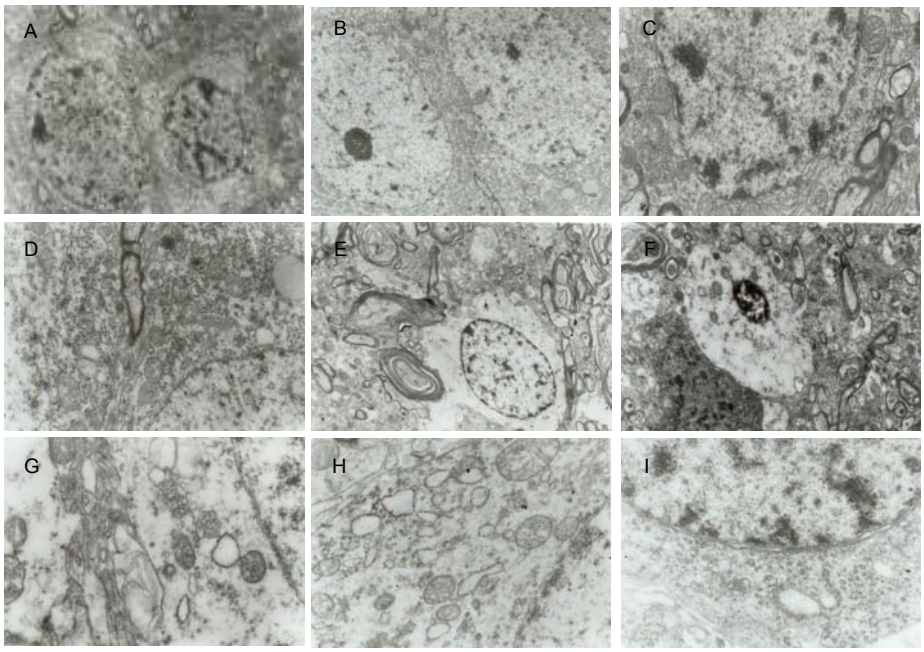

Figure 1 Ultrastructure of brain tissues with ischemia/reperfusion injury (uranyl acetate-lead citrate staining, transmission electron microscope).

In the two-vessel occlusion group, the nuclear membranes were intact and the cytoplasm was normal in the occipital lobe (A, × 3 300) and hippocampus (B, × 2 600); mitochondrial swelling or pyknosis was not observed in the occipital lobe (C, × 10 000); the endoplasmic reticulum was normal in the hippocampus (D, × 10 000).

In the four-vessel occlusion group, nerve cells were swollen in the parietal lobe (E, × 2 600), and this was accompanied by karyopyknosis (F, × 3 300); mitochondrial swelling and cristae lysis were observed in the hypothalamus (G, × 16 000) and hippocampus (H, × 16 000); expansion of the endoplasmic reticulum and shedding of particles were observed in the basal ganglia (I, × 20 000).

Liang *et al* [5, 7] established a stable and reliable monkey model of selective cerebral deep hypothermia and blood flow occlusion, and conducted deep hypothermic perfusion for 60 minutes following ischemia at 20°C for 10 minutes, and then successfully resuscitated the monkeys. These results indicate that selective cerebral deep hypothermia and blood flow occlusion in monkeys can enhance brain tolerance to ischemia and hypoxia, while not damaging organs of the body. However, the metabolism of excitatory amino acids and other transmitters in brain tissues following cerebral deep hypothermia and blood flow occlusion remains poorly understood. Glu is the major excitatory amino acid transmitter in the nervous system of mammals. It functions together with Glu receptors at postsynaptic membranes to increase receptor sensitivity, inducing neurogenic edema induced by a large amount of Na^+, Cl^-, and H_2O inflow, followed by calcium release, resulting in calcium ion overload in cells and neuronal death or other cascade reactions[8-10]. Moreover, a large number of free radicals are produced in these processes, damaging cell membrane structure, cytoskeleton and mitochondrial function[11-13]. Therefore, Glu release after cerebral ischemia/hypoxia is an important step in the initiation of nerve cell death. The results from the present study indicate that Glu content is rapidly increased after blood flow occlusion in the four-vessel occlusion group, and selective cerebral deep hypothermic perfusion significantly reduced Glu content. In addition, Glu levels were significantly elevated at room temperature after ischemia in the two-vessel occlusion group, but significantly decreased following hypothermic perfusion at room temperature compared with the four-vessel occlusion group. These results indicated that selective cerebral deep hypothermia can inhibit excessive Glu release following blood flow occlusion to reduce Glu-mediated Ca^{2+} inflow and inhibit Ca^{2+} inflow-mediated neuronal death and other cascade reactions, consistent with the results of previous results[14-15]. Following hypothermic circulatory arrest, a Glu receptor antagonist was shown to significantly inhibit neuronal apoptosis[16]. Currently, sub-hypothermia treatment has been used clinically to treat severe cerebral trauma and ischemic brain injury. Although Glu reduction cannot fully contribute to the cerebroprotective effects of hypothermia[17], and the increase in Glu levels

is not the only factor underlying nerve cell death after ischemia, a high concentration of extracellular Glu can induce overexcitation of N-methyl-D-aspartate receptors and increase the levels of free Ca^{2+} in ischemic nerve cells; these processes contribute substantially to the pathological process of nerve cell death resulting from Glu-induced excitotoxicity.

After the blood flow was restored, the brain was subjected to reperfusion rewarming, and the Glu level was immediately increased in the four-vessel occlusion group, rapidly exceeding the levels prior to perfusion. This may be associated with several factors. During rewarming, the sympathetico-adrenomedullary system is excited, brain oxygen consumption is increased and the brain oxygen supply is imbalanced, resulting in dysfunction of sodium-potassium pumps and calcium pumps, cell membrane long-lasting depolarization, and release of Glu. Moreover, injured gliocytes and insufficient ATP induce reuptake disorder, and high K^+ in brain tissue extracellular spaces results in a reverse of the amino acid transport system, such that amino acids enter the extracellular fluid[18-20]. Compared with hypothermic perfusion, Glu was not increased following reperfusion rewarming after blood flow restoration in the two-vessel occlusion group. During rewarming reperfusion, the brain oxygen supply is unbalanced and oxygen consumption is increased, resulting in increased anaerobic metabolism, production of free radicals, and an amino acid metabolism disorder in the brain, which enhances excitotoxicity. Reperfusion rewarming following blood flow restoration after selective cerebral deep hypothermia and blood flow occlusion (occlusion of bilateral common carotid artery) did not significantly change Glu content compared with hypothermic perfusion, indicating that it may be safer to block the bilateral common carotid arteries during selective cerebral deep hypothermia and blood flow occlusion.

Transmission electron microscopic observations showed no abnormality in the cell ultrastructure of brain tissues in the two-vessel occlusion group, but nerve cell edema, demyelination in myelinated nerve fibers, mitochondrial swelling, endoplasmic reticulum expansion, and particle shedding were all seen in the four-vessel occlusion group, consistent with the changes seen in the microdialysis curves for excitatory amino acids in the extracellular fluid[21].

After blocking the bilateral common carotid arteries, resuscitation of selective cerebral deep hypothermia was performed to inhibit cytotoxic effects through inhibiting the release of excitatory neurotransmitters such as Glu. This method does not damage the ultrastructure of brain cells and may improve brain tolerance to ischemia/hypoxia. However, further studies are needed to investigate the influence of selective cerebral deep hypothermia on neurotransmitter metabolism and ultrastructure at different temperatures.

MATERIALS AND METHODS

Design

A randomized, controlled, animal study.

Time and setting

A total of seven healthy adult rhesus monkeys, of both genders, weighing 9.2 ± 2.6 kg, were purchased from the Experimental Center of Kunming Institute of Zoology (license No. SYXK (Dian) 2005-0004). The experiments were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[22].

Methods

Establishment of selective cerebral deep hypothermia and blood flow occlusion model

After anesthesia, mean arterial blood pressure was monitored by intubation of the proximal right inguinal artery during hypothermic perfusion, while intubation of the proximal left inguinal vein was used for transfusion of systemic circulation blood flow after rewarming. The right internal carotid artery and both internal carotid veins were clamped by an aneurysm blocking clamp and connected to a circulation pump (blood pump, DKP-22, NIKKISO, Japan). An ultrafilter (F-60, FRESENIUS, Germany) and water bank (SARNS TCM, ANN ARBOR MICHINA, USA) were used to build local extracorporeal circulation. Central venous pressure was monitored by intubation of the proximal right internal jugular vein. A pin-shaped brain temperature sensor was inserted into the right frontal lobe and connected to a brain temperature monitoring system (TH-5; Department of Medical Engineering, The University of Virginia, VA, USA). General heparinization was conducted prior to temperature reduction by intravenous injection of 50 IU/kg heparin.

The bilateral external jugular veins, the left common carotid artery and the internal jugular vein were clamped in the two-vessel occlusion group, and the bilateral vertebral arteries were also clamped in the four-vessel

occlusion group. After ischemia for 10 minutes at common temperature, Ringer's solution (Shanghai Baite Medical Product Co., Ltd., Shanghai, China) at 4°C was infused *via* the right internal carotid artery, at 10 mL/kg per minute. In addition, perfusion solution was refluxed through the right internal jugular vein, and systemic circulation blood was refluxed through the right inguinal vein. Redundant water was removed through the ultrafilter (F-60, Fresenius, Germany) and the blood was rewarmed to 38°C and transfused into the left inguinal vein. After the brain temperature was reduced to ≤18°C, the perfusion rate was reduced or interrupted, at 2–10 mL/kg per minute to maintain the brain temperature at ≤18°C. Hypothermic perfusion was terminated after 60 minutes, and the right internal carotid artery and vein were unclamped to restore the brain temperature to 36°C[5, 7]. The monkeys exhibited stable vital signs after surgery and recovered from surgery within 24 hours. They could move and take food normally, and their visual acuity was normal.

Microdialysis sample collection and processing

A microdialysis probe (dialysis-membrane effective length 4 mm, molecular weight cutoff 20 ku) was implanted into the right frontal lobe (the probe was implanted into the right frontal lobe and brain tissues from different sites were harvested for pathological detection). The right frontal lobe was slowly microdialysed using a micro- injection pump at 2.5 μL/min 90 minutes prior to blood flow blocking, and dialyzate was collected at 60 minutes. Extracellular fluid was collected prior to ischemia, after ischemia for 10 minutes, after hypothermic perfusion for 60 minutes and after rewarming for 40 minutes, with one tube (100 μL) every 20 minutes for seven tubes in total. The tubes were stored at –70°C until amino acid analysis was performed.

Derivatization reactions

Dialyzate (50 μL) was harvested, mixed with drying agent (absolute alcohol: triethylamine: water = 2:2:1) for 3 minutes, dried in a 60–100 Mt vacuum, mixed with 100 μL of freshly prepared derivatized reagents (alcohol: water: triethylamine:phenyl isothiocyanate=7:1:1:1), and shaken for 5 minutes for derivation in a dark box for 10 minutes. The products were dried in vacuum for 15 minutes, mixed with 100 μL of dilution solution (Na_2HPO_4 710 mg/L, 10% H_3PO_4, pH 7.40, followed by acetonitrile to a final content of 5%), and filtered using a syringe filter (0.45 μm). Filtrate (20 μL) was harvested and sampled.

Detection of Glu content in the brain

The Glu chromatographic peak from various samples was detected by chromatography (Waters 510 pump, MA, USA), and the retention time of unknown amino acid peaks was qualified and quantified according to the retention time of standard amino acid peaks. The chromatographic peak area of four amino acid samples was calculated using high performance liquid chromatography-ultraviolet detection[23] with the formula: C = R1/R2 × D × N; R1, peak area of detected samples; R2, peak area of amino acid standard sample; D, concentration of amino acid standard sample (μM); N, dilution times; C, concentration of detected sample (μM).

Ultrastructural observation of brain tissues

Bilateral brain tissues of monkeys were cut into tissue blocks of 1 mm^3, fixed with glutaral (3%)-osmium tetroxide (10 g/L), dehydrated with 30–100% acetone, embedded with epoxy resin, coronally sectioned using LKV-V ultramicrotome (50 nm thick), stained with uranyl acetate-lead citrate, and observed under a JEM-100CX transmission electron microscope (Tokyo, Japan) to detect the structure of the cell membrane, nuclear membrane, cytoplasm, mitochondria and endoplasmic reticulum.

Statistical analysis

Data were analyzed using SPSS 12.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Intergroup differences in Glu content at different time points were compared using one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

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Ethical approval: This study received permission from the Animal Ethics Committee of Beijing Tiantan Hospital, Capital Medical University, China.

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