JECT. 2002;34:185–189
The Journal of The American Society of Extra-Corporeal Technology

The Assessment of Brain Injury with Measurement of Calcium Fluorescent Intensity of a Vital Brain Slice

Yulong Guan, MD; Peiqing Dong, MD; Caihong Wan, MD; Jing Yang, MD; Meiling He

Beijing Anzhen Hospital, Beijing Capital Medical University, Beijing Heart, Lung, and Blood Vessel Medical Institute, Beijing, People's Republic of China

Abstract: Retrograde cerebral perfusion (RCP) is one of cerebral protection methods during deep hypothermic circulatory arrest (DHCA). However, the mechanism is unclear. In this study with laser confocal scanning microscope (LCSM), calcium fluorescent intensity of vital brain slice was compared between RCP and DHCA group. Sixteen swine, weighing from 19 ~ 20 kg and supplied by Beijing College of Agriculture, were used for the experiment. After 90 min of DHCA or RCP through the superior vena cava, the animals were rewarmed for 120 min. Through tentorium of the cerebellum and enucleation of the eyeball, vital brain slices (cerebellula and retina) were obtained and fluorescein labeled in artificial cerebrospinal fluid containing 5 μmol/L Fluo-3/AM (fluoresence probe). The calcium fluorescent intensity was examined by LCSM. The results indicated that calcium fluorescent intensity of vital brain slice was lower in the RCP group (cerebellula, 9.16 ± 3.98; retina, 21.48 ± 14.27) than that in the DHCA group (cerebellula, 31.97 ± 20.59; retina, 44.07 ± 21.01) ($p < .05$). More moderate and severe eosinophilic degeneration was found in the DHCA group than in the RCP group ($p < .05$) through morphological examination. The statistical analysis also indicated the calcium fluorescent intensity of the vital brain slice was correlated with the level of moderate and severe eosinophilic degeneration of thee neuron ($r = 0.86$, $p < .05$). So "calcium overload" contributes to the injury of neuron after DHCA. RCP is able to attenuate "calcium overload," which has the effect of cerebral protection. **Keywords:** calcium overload, laser confocal scanning microscope, *JECT. 2002;34:185–189*

As a means of cerebral protection, retrograde cerebral perfusion (RCP) through the superior vena cava has been enthusiastically adopted during aortic arch operations. Ueda, Dong, and Lin et al. (1–3) have reported successful clinical use of RCP; however, a greater understanding of the effect of RCP as it relates to cerebral protection remains unknown. It is believed that intracellular calcium overload is a significant factor in cerebral ischemia–reperfusion injury according to recently reported studies (4,5). Direct evidence of calcium overload has not been determined. Zhou et al.(6), Zhao et al. (7), and Yu et al. (8) reported that the laser confocal scanning microscope (LCSM) was used to demonstrate calcium overload. The present study is designed to explore the mechanism of cerebral injury by measuring the calcium fluorescent intensity of a vital brain slice.

MATERIALS AND METHODS

Animals

The study was carried out with 16 healthy young swine, weighing from 19 ~ 20 kg, supplied by Beijing College of Agriculture. Animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (National Institutes of Health, Publications 85–23; revised 1985).

Anesthesia and Surgical Procedure

Anesthesia was induced with a peritoneal injection of 3% pentobarbital sodium (1.5 ~ 2.0 mL/kg) and intravenous pancuronium (0.1 mg/kg) was used as a muscle relaxant. The anesthesia was maintained with intravenous fentanyl (10 μg/kg). After tracheotomy, the animals were maintained on positive pressure ventilation with 100% oxygen. Through a median sternotomy, conventional cardiopulmonary bypass (CPB) was established. The extracorporeal circuit of CPB was described in detail in a previous study (9). The animals were heparinized (300–400 units/kg IV) to maintain an activated clotting time above 480 s. The ascending aorta was cannulated with 16-F arterial cannula, two separated venous cannulas (22 F), and vena caval tapes were applied with clamping of the azygos vein and the hemiazygos vein. A bubble oxygenator was primed with 500 mL-hydroxyethyl starch, 400 mL sodium chloride, furosemide (1 mg/kg), heparin (100 units/kg), and potassium chloride (1 mEq/kg). CPB was carried out at a flow rate of 80 ~100 mL/kg to maintain perfusion

Address correspondence to Yulan Guan, M.D. Extracorporeal Circulation Department, Beijing Heart, Lung, and Blood Vessel Medical Institute, Beijing Anzhen Hospital, Beijing, P.O. Box 100029, PRC, E-mail: guanyulong@yahoo.com.
Received April 2002; accepted July 2002.

pressure at 60 ~ 70 mm Hg. During the cooling phase, ice packs were placed around the animals' heads to enhance cerebral hypothermia. The discrepancy between water temperature and blood temperature was below 10°C. The velocity of cooling and rewarming was about 0.33°C/min. pH-stat management during the cooling phase, which maintained the pH at 7.40 ± 0.05 with an arterial CO_2 tension of 35–40 mmHg, and alpha-stat management in the rewarming phase was utilized. Cardiac arrest was induced by cold crystalloid cardioplegia through the aortic root when the aorta was clamped at an esophageal temperature of 30°C. CPB was discontinued when the animals were cooled to attain a nasopharyngeal temperature of 18°C and rectal temperature of 20°C. In the RCP group, RCP was established by perfusing blood through the superior vena cava. The retrograde flow rate was regulated to maintain a perfusion pressure of 25 mm Hg at 5.5 ~ 9.5 mL/kg per min. In the DHCA group, perfusion was discontinued after applying a cross clamp. Ninety minutes later, CPB was then reestablished, and the animals were rewarmed for 120 min to attain nasopharyngeal temperature of 37°C and rectal temperature of 35°C. Cerebellar hemisphere tissue (300 ~ 500 mg) was obtained after craniotomy through the tentorium of the cerebellum. The retina was also procured after enucleation of the eyeball and dissection.

Preparation of Vital Brain Slice and Measurement of Calcium Fluorescent Intensity

The cerebellar tissue and retina were incubated in a 4°C artificial cerebrospinal fluid (NaCl 124 mmol/L, KCl 1.8 mmol/L, $MgSO_4$ 1.8 mmol/L, $CaCl_2$ 1.6 mmol/L, NaH_2PO_4 1.25 mmol/L, D-glucose 10 mmol/L, $NaHCO_3$ 26 mmol/L, pH 7.4) that was aerated with 95% oxygen and 5% carbon dioxide for 5 min. The cerebellar tissue was then sectioned coronally into 400 ± 10 μm brain slices. After incubation for 30 min at room temperature, the vital brain slice (400 ± 10 μm cerebellar brain slice and retina) was fluorescein labeled in artificial cerebrospinal fluid containing 5 μmol/L Fluo-3/AM (Molecular probes Eugene, OR) lucifugally at room temperature for 45 min. The tissue was rinsed three times and incubated in artificial cerebrospinal fluid without 5 μmol/L Fluo-3/AM for 30 min again just before examination. The examination of calcium fluorescent intensity was made with LCSM (Leica TCS-NT, Leica Microsystems, Wetzlar, Germany). The cerebellar brain slice was observed with 5× microscope and the retinal slice with 20× microscope. The wavelength of the excitation laser is 480 nm and that of the emission fluorescent light is 530 nm. Photosection scanning was used. The format is 512 × 512. The gain of photoelectric multiplier tube is 884 in the cerebellar slice and 779 in the retinal slice. The laserpower is 58. The zoom is 2. The pinhole is 3.09. The laser confocal scanning imaging system (Leica Q550IW, Leica Microsystems, Wetzlar, Germany) was used for quantitative analysis.

The morphologic studies of cerebellar tissue and retina were made with a light microscope and an electron microscope. According to the description of "ischemic cell change" in *Greenfield's Neuropathology* (10), the major change of neuron is degeneration. The descriptions of the different grades of degeneration are: 1) *microvacuolation*—the cell may be normal in size or only slightly shrunken, the nucleus is also only slightly shrunken, and the perikaryon is vacuolated; 2) *mild eosinophilic degeneration*—the neuron is shrunken, stained darkly with cresyl violet, its cytoplasm becoming markedly eosinophilic; 3) *moderate eosinophilic degeneration*—all cytoplasm and nucleolei are eosinophilic; and 4) *severe eosinophilic degeneration*—appearance of incrustations that stain darkly in most preparations, even shrunken pyknotic and fragmented nucleus without recognizable cytoplasm.

Statistical Analysis

The data were expressed as mean ± standard deviation. Comparison between two groups was done by a set of *t* tests with SPSS 7.0 software (SPSS Inc, Chicago, IL). Correlation analysis was used to test the relationship between calcium fluorescent intensity of vital brain slice and histopathologic results. A p value less than .05 was considered significant.

RESULTS

Blood Gas and Hematocrit Data

During different periods, there was no difference in pH and hematocrit between the two groups ($p > .05$)

Analysis of Calcium Fluorescent Intensity of Vital Brain Slice

Significantly lower calcium fluorescent intensity of the vital brain slice was detected in the RCP group than that in the DHCA group (Figure 1 A–D) ($p < .05$, Table 1

Histopathologic Results

The histopathologic findings varied in different regions and in the neurons, showed ischemic changes. According to the description of "ischemic cell change" in *Greenfield's Neuropathology*, the pyramidal cells were observed with a 40× microscope, and quantitative analysis was made (the proportion of pathologic neurons to whole number of neurons) (Table 2). More moderate and severe eosinophilic degeneration was found in the DHCA group than in the RCP group ($p < .05$).

Ultrastructural Results

Electron microscope examination of the cerebellum showed the normal perikaryon reticula, mild swelling of rough-surfaced endoplasmic reticula and mitochondria in

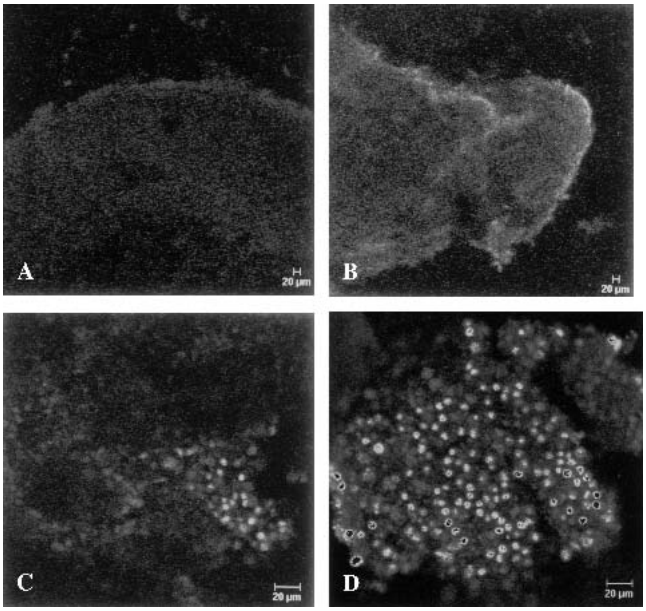

Figure 1. Photogragh of calcium fluorescent intensity of the vital brain slice. (A) cerebellular photogragh of RCP; (B) cerebellular photogragh of DHCA; (C) retinal photogragh of RCP; (D) retinal photogragh of DHCA.

the RCP as a group as compared with distinct swelling and degeneration of rough-surfaced endoplasmic reticula, distinct swollen mitochondria during which the mitochondrial sheath shortened, lessened, and disappeared in the DHCA group.

Analysis indicated that a correlation existed between calcium fluorescent intensity of the vital brain slice and moderate to severe neuronal eosinophilic degeneration ($r = 0.86$, $p < .05$).

DISCUSSION

The brain is the most important organ susceptible to ischemia and hypoxia. Ischemia produces an immediate cellular oxygen debt given that the electron transport chain of mitochondria can no longer oxidize reducing equivalents to generate ATP required for endergonic reaction and the maintenance of transmembrane ion potentials. It was demonstrated that cerebral tissue ATP decreased to 20% baseline at the end of 60 min of DHCA (11). During cerebral ischemia, because of an increase in release and a decrease in reuptake, the concentration of glutamate increased (12). Glutamate-induced Na^+ influx into neurons occurs during deplorization. The intra/extracellular balance of C^- is disturbed, and the ion flows down its electrochemical gradient into the neuron. Excessive activation of Na^+–Ca^{2+} exchange also resulted in an increase of calcium influx (13). At the same time, the glutamate release, in turn, activates kainate/quisqualate (K/Q) and N-methyl-D-aspartate (NMDA) cell surface receptors, resulting in an influx of Na^+ and calcium ions. Because of depletion of high-energy phosphate reserves, transmembrane ion pumping fails, leading to an accumulation of intracellular calcium ion and calcium overload. Calcium overload activates numerous intracellular reactions—including the activation of several lipases, proteases, and endonucleases—all of which attack the structural integrity of the cell (12). Excessive calcium also activates phospholipase C, contributes to the formation of oxygen free radicals, and leads to an uncoupling of oxidative phospholation with mitochondria (14). However, the direct evidence of calcium overload has not been determined because of technical limitations. In this study, LCSM was used to detect the calcium fluorescent intensity of the vital brain slice, and it was found that calcium fluorescent intensity of the vital brain slice correlated with cerebral injury, which demonstrated that calcium overload contributed to the pathogenesis of cerebral injury after DHCA.

Table 1. Calcium fluorescent intensity of the vital brain slice.

	Cerebellula	Retina
Retrograde cerebral perfusion	9.16 ± 3.98	21.48 ± 14.27
Deep hypothermic circulation arrest	31.97 ± 20.59	44.07 ± 21.01
p value	0.009	0.024

Comparison between retrograde cerebral perfusion and deep hypothermic circulation arrest.

After the first clinical application of continuous RCP reported by Ueda et al. (1), this technique was highly evaluated and adopted in some hospitals; however, the effectiveness of RCP was questioned. There were clinical studies indicating that RCP was capable of prolonging the safety duration of circulation arrest (15), and there was a case report of a 135 min endurance of RCP without postoperative neurological complications (16). In our previous study (17), technetium 99-labeled perfusion agent [ethyl cysteinate dimer (^{99m}Tc-ECD)] was injected into blood flowing retrograde through the superior vena cava. Cerebral perfusion images showed homogeneous perfusion of cerebrum, cerebellum, and medulla oblongata 3 min after the start of RCP. Moreover, in a recent study (9), retinal microvascular perfusions were observed by using fundus fluorescein angiography (FFA) and color Doppler sonography before CPB and retrograde cerebral perfusion during DHCA. FFA showed initial filling of the fundus venae in 2.5 min and complete filling in 4.5 min, with partial filling of the arteriae. Arteriae completely filled in 8 min, and all of the arteriae and venae filled from 15–17 min. Color Doppler sonograghy showed that the flow signals could be detected in all of the fundus vessels during RCP. Because the retinal vessels stem from cerebral circulation, the study demonstrated that RCP is able to perfuse the cerebral tissue. Pagano and associates (18) reported three cases in which a technetium 99-labeled perfusion agent

Table 2. Quantitative analysis of morphological measurement (%).

	Microvacuolation	Mild Eosinophilic Degeneration	Moderate Eosinophilic Degeneration	Severe Eosinophilic Degeneration
Retrograde cerebral perfusion	10.50 ± 20.15	19.42 ± 27.13	3.30 ± 12.18	0 ± 0
Deep hypothermic circulation arrest	2.42 ± 7.57	20.60 ± 25.08	30.63 ± 26.05	31.82 ± 18.35
p value	0.31	0.93	0.02	0.001

Comparison between retrograde cerebral perfusion and deep hypothermic circulation arrest.

[d,l-hexamethyl propylene amine oxime (^{99m}Tc-HMPAO)] was used during RCP to obtain a perfusion image with a portable gamma camera in the operating theater. Time activity curves showed homogeneous perfusion of both cerebral hemispheres in all patients during RCP.

Another focus on the RCP is the mechanism of cerebral protection of RCP. Observation during our previous clinical studies (2) showed a significant difference existed in the oxygen contents of perfusion blood and that of returned blood at 5, 10, 15, 20, and 25 min of RCP. When CPB was reestablished, the blood concentration of MDA in RCP is significantly lower than that in DHCA. The spectrum of electron spin resonance (ESR) indicated that Q/S did not increase significantly in RCP, as compared with that in DHCA, which demonstrated that no excessive free radials were produced. Moreover, the content of blood lactate in RCP was also lower than that in DHCA. Studies in animals (17) showed that a significant difference existed in the content of the blood lactate of jugular veins 120 min following RCP and DHCA. The activity of Na^+-K^+-ATP maintained in RCP, as compared with decreasing DHCA, which indicated that RCP could provide blood to the brain and mitochondria to continue the generation of ATP for maintaining the cellular function. Saitou et al. (19) reported that by mapping the brain tissue blood flow with colored microspheres, during RCP regional cerebral blood flow (mL/min/100g) with a driving pressure of 25 mm Hg were 12.1 ± 9.4, 7.0 ± 5.6, 4.4 ± 2.8, and 2.2 ± 1.4 in the frontal cortex, anterior, mid, and posterior cerebrum, respectively. Therefore, all of these experiments demonstrated that RCP maintained aerobic glycolysis and reduced acidosis.

The etiology of cerebral injury during DHCA may possibly be caused by the generally applied quick cooling that may result in the heterogeneity of brain temperature. It was found that the RCP group had a significantly lower mean epidural temperature than the DHCA group (20). Usui et al. (11) found cerebral tissue temperature increased during DHCA (20.4°C ± 1.4°C /22.7°C ± 0.7°C), in contrast to no increase of the cerebral tissue temperature during RCP. Sharma et al. (21) reviewed the midterm results of neurological and intelligence testing in infants operated upon using deep hypothermic CPB. It was found it is very important to ensure uniform and adequate cerebral cooling. In the present study, we chose to maintain the velocity of cooling and rewarming about 0.33°C/min. During the cooling phase, ice packs were placed around the head to enhance cerebral hypothermia.

Because of the continuous washings out the cerebral vessels during RCP, the possibility of particulate and air embolism could be reduced. An elegant study was performed by Yerlioglu et al. (22), during which neurological outcomes of animals subjected to embolization with polystyrene microsphere (250–750 μm in diameter) were studied followed by either antegrade or retrograde cerebral perfusion. The animals receiving antegrade perfusion after embolization had very poor neurological outcomes; whereas, the animals subjected to RCP with superior vena cava pressure less than 40 mmHg had favorable neurological outcomes. When looking at brain histology, RCP had a lower number of microspheres trapped in capillaries. Using selective antegrade cerebral perfusion (SCP) in 60 patients with atherosclerotic arch aneurysm, Ohmi and coworkers reported a higher prevalence of postoperative stroke in patients with occlusive arterial disease, especially in emergency cases in which the status of cerebral circulation was unknown (23). *Svensson* recommended that RCP should be used in patients when a lot of potential embolic material is expected (24).

LCSM can monitor the dynamic changes of free calcium in neurons and the concentration of calcium can be indicated quantitatively. It had been widely used in cellular calcium overload in vitro (7,8). Compared with other measurements for intracellular calcium, this technology is the more preferred method. LCSM is first used in this study to measure the calcium fluorescent intensity of the vital brain slice directly. It demonstrated that the calcium fluorescent intensity of the vital brain slice in the RCP group was significantly lower than that in the DHCA group. Moreover, morphological examination showed more severe cerebral injury in the DHCA group, which indicated RCP could attenuate calcium overload and had the effect of cerebral protection.

Because this study was carried out in animals, the results cannot be extrapolated in clinical study completely. Further research is needed to demonstrate the etiology and prevent neurological injury of patients.

In conclusion, calcium overload contributes to the cerebral injury after DHCA. RCP is able to attenuate neuronal calcium overload to protect the brain.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation, code 39770733; number C03030304.

REFERENCES

1. Ueda Y, Miki S, Kusuhara K, et al. Surgical treatment of aneurysm of dissection involving the ascending aorta and aortic arch, utilizing circulatory arrest and retrograde cerebral perfusion. J Cardiovasc Surg (Torino). 1990;31:553–8.
2. Dong PQ, Sun YQ, Yang J, et al. Extracorporeal. circulation for great vessels surgery: A review of 131 cases. JECT. 1995;27:216–21.
3. Lin PJ, Chang CH, Chang JP, et al. Surgical treatment of acute type A aortic dissection with an intraluminal sutureless graft. J Formosa Med Assoc. 1994;93:681–5.
4. Kristian T, Gido G, Kuroda S, et al. Calcium metabolism of focal and penumbral tissues in rats subjected to transient middle cerebral artery occlusion. Exp Brain Res. 1998;120:503–9.
5. Kuroda S, Siesjo BK. Reperfusion damage following focal ischemia: Pathophysiology and therapeutic windows. Clin Neurosci. 1997;4: 199–212.
6. Zhou F, Xiang Z, Feng WX, et al. Neuronal free Ca(2$^+$) and BBB permeability and ultrastructure in head injury with secondary insult. J Clin Neurosci. 2001;8:561–3.
7. Zhao P, Huang YL, Cheng JS. Taurine antagonizes calcium overload induced by glutamate or chemical hypoxia in cultured rat hippocampal neurons. Neurosci Lett. 1999;268:25–8.
8. Yu ZF, Bruce-Keller AJ, Goodman Y, et al. Uric acid protects neurons against excitotoxic and metabolic insults in cell culture and against focal ischemic brain injury in vivo. J Neurosci Res. 1998;53: 613–25.
9. Dong PQ, Guan YL, Yang J, et al. Fundus microvascular flow monitoring during retrograde cerebral perfusion: An experimental study. Ann Thorac Surg. 2000;70:1478–82.
10. Duchen LW. General pathology of neurons and neuroglia. In: Greenfield's Neuropathology. 4th ed. Adams JH., ed. London: Edward Arnold, 1984:1–11.
11. Usui A, Oohara K, Liu T, et al. Comparative experimental study between retrograde cerebral perfusion and circulatory arrest. J Thorac Cardiovasc Surg. 1994;107:1228–36.
12. Berger R, Garnier Y. Perinatal brain injury. J Perinat Med. 2000;28: 261–85.
13. Eigel BN, Hadley RW. Antisense inhibition of Na^+/Ca^{2+} exchange during anoxia/reoxygenation in ventricular myocytes. Am J Physiol Heart Circ Physiol. 2001;281:H2184–90.
14. Vannucci RC. Experimental biology of cerebral hypoxia-ischemia: Relation to perinatal brain damage. Pediatr Res. 1990;27:317–26.
15. Aomi S, Hashimoto A, Imamaki M, et al. Recent advances of surgical treatment of aortic arch aneurysm using retrograde cerebral perfusion. Kyobu Geka. 1993;46(Suppl)S682–5.
16. Sasaguri S, Yamamoto S, Hosoda Y. What is the safe time limit for retrograde cerebral perfusion with hypothermic circulatory arrest in aortic surgery? J Cardiovasc Surg (Torino). 1996;37:441–4.
17. Dong PQ. The cerebral protection in great vessels surgery. In: The Cardiovasc Anesthesia and Cardiopulmonary Bypass. Hu XO, eds. Beijing: The People's Medical Publishing House. 1997;489–98.
18. Pagano D, Boivin CM, Foroqui MH, et al. Retrograde perfusion through the superior vena cava perfuses the brain in human beings. J Thorac Cardiovasc Surg. 1996;111:270–2.
19. Saitou H, Wantanabe T, Zhang JW, et al. Regional tissue blood flow and pH in the brain during deep hypothermic retrograde brain perfusion. J Surg Res. 1997;72:135–40.
20. Juvonen T, Zhang N, Wolfe D, et al. Retrograde cerebral perfusion enhances cerebral protection during prolonged hypothermic circulatory arrest: A study in a chronic porcine model Ann Thorac Surg. 1998;66:38–50.
21. Sharma R, Choudhary SK, Mohan MR, et al. Neurological evaluation and intelligence testing in the child with operated congenital heart disease. Ann Thorac Surg. 2000;70:575–81.
22. Yerlioglu ME, Wolfe D, Mezrow CK, et al. The effect of retrograde cerebral perfusion after particulate embolization to the brain. J Thorac Cardiovasc Surg. 1995;110:1470–85.
23. Ohmi M, Tabayashi K, Hata M, et al. Brain damage after aortic arch repair using selective cerebral perfusion. Ann Thorac Surg. 1998;66: 1250–3.
24. Svensson LG, Nadolny EM, Penney DL, et al. Prospective randomized neurocognitive and S-100 study of hypothermic circulatory arrest, retrograde brain perfusion, and antegrade brain perfusion for aortic arch operations. Ann Thorac Surg. 2001;71:1905–12.