Hyperbaric Oxygen Reduces Neuronal Death and Improves Neurological Outcome After Canine Cardiac Arrest

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Background and Purpose—Studies suggest that hyperbaric oxygen (HBO) is neuroprotective after experimental cerebral ischemia, but the mechanism is unknown. This study tested the hypotheses that postischemic HBO affords clinical and histopathological neuroprotection after experimental cardiac arrest and resuscitation (A/R) and that this neuroprotection results from improved cerebral oxygen metabolism after A/R.

Methods—Anesthetized adult female beagles underwent A/R and randomization to HBO (2.7-atm absolute [ATA] for 60 minutes, 1 hour after A/R) or control (Po_2=80 to 100 mm Hg; 1 ATA). Animals underwent neurological deficit scoring (NDS) 23 hours after A/R. After euthanasia at 24 hours, neuronal death (necrotic and apoptotic) in representative animals was determined stereologically in hippocampus and cerebral neocortex. In experiment 2, arterial and sagittal sinus oxygenation and cerebral blood flow (CBF) were measured. Cerebral oxygen extraction ratio (ER_c), oxygen delivery (Do_2c), and metabolic rate for oxygen ($CMRo_2$) were calculated (baseline and 2, 30, 60, 120, 180, 240, 300, and 360 minutes after restoration of spontaneous circulation).

Results—NDS improved after A/R in HBO animals (HBO, 35±14; controls, 54±15; P=0.028). Histopathological examination revealed significantly fewer dying neurons in HBO animals; the magnitude of neuronal injury correlated well with NDS. HBO corrected elevations in ER_c (peak, 60±14% for controls, 26±4% for HBO) but did not increase Do_2c or $CMRo_2$, which decreased ≈50% after A/R in both groups.

Conclusions—HBO inhibits neuronal death and improves neurological outcome after A/R; the mechanism of HBO neuroprotection is not due to stimulation of oxidative cerebral energy metabolism. (***Stroke***. 2003;34:1311-1316.)

Key Words: cerebral ischemia ■ hyperbaric oxygenation ■ neurological deficits ■ oxygen ■ dogs

Recent evidence suggests that neurological outcome after cardiac arrest and resuscitation (A/R) may be greatly affected by the concentration of oxygen inhaled immediately after resuscitation; even short periods of postresuscitative hyperoxia resulted in increased oxidative neuronal injury and worsened neurological outcome.[1] Paradoxically, there is increasing evidence that postischemic hyperbaric oxygen (HBO), despite greatly elevating tissue oxygen, can afford neuroprotection after experimental complete global ischemia (CGI) induced by vascular occlusion.[2–5] No studies to date, however, have correlated clinical neurological injury with pathological evidence of neuronal death. Additionally, HBO has not previously been shown effective after experimental cardiac arrest–induced (rather than vascular occlusive) CGI.

Postischemic hypoperfusion causes a mismatch between cerebral oxygen delivery and demands.[6] This mismatch causes the brain to increase the percent of oxygen removed from the cerebral arterial circulation. Increased cerebral oxygen extraction (ER_c) is manifested by decreased oxygenation in the brain's venous outflow. Extraction during this phase can approach its theoretical critical value, the point at which no more oxygen can be extracted.[6] Oxygen demands beyond this point would cause decreased oxygen consumption ($CMRo_2$), which is directly indicative of ischemia. Critical postischemic values of extraction have been widely felt to be an indirect indicator of ongoing delivery-dependent ischemia during this period, when $CMRo_2$ is not directly measured.[6,7]

If diminished cerebral blood flow (CBF) (and thus oxygen delivery) during postischemic hypoperfusion causes ongoing cerebral ischemia, improving oxygen delivery should be neuroprotective. HBO has the potential to increase postischemic oxygen delivery, theoretically overcoming postresuscitative delivery-dependent cerebral ischemia. This study was designed to determine whether HBO would correct ongoing ischemia during postischemic hypoperfusion, thereby affording neuroprotection. The following 3 hypotheses were tested: (1) HBO delivered 1 hour after A/R reduces functional neurological deficits caused by CGI; (2) differences in neurological deficits

after A/R correspond to neocortical and hippocampal neuronal death, and neuronal death correlates closely with clinical neurological outcome; and (3) HBO delivered 1 hour after A/R improves oxygen delivery and cerebral oxygen consumption during postischemic hypoperfusion.

Materials and Methods

Animal Model of A/R

All animal experiments were approved by the George Washington University IACUC. Single-source female beagles (9 to 12 kg) were studied using our laboratory's well-established model of A/R.[1,8] A peripheral intravenous line was established, and anesthesia was induced with pentobarbital (12.5 mg/kg) and maintained with α-chloralose (75 mg/kg) during surgery and preparation. Animals were intubated and mechanically ventilated. Core body temperature was continuously monitored. Left femoral arterial and venous cutdowns were placed for drug delivery, central venous and blood pressure monitoring, and arterial blood gas sampling.

Animals in experiment 2 (cerebral oxygen measurements) had a small burr hole placed in the midline of the skull 2.5 cm anterior to the sagittal crest; a sagittal sinus catheter was inserted for venous sampling and intracranial pressure measurement. A left ventricular pigtail catheter was placed for injection of microspheres; a right femoral arterial catheter was placed for microsphere sampling.

Left lateral thoracotomy was performed on all animals. Ventricular fibrillation was initiated with a 250-Hz square-wave DC pulse to the right ventricle. Animals were left in cardiac arrest for 10 minutes. Epinephrine (0.02 mg/kg) and sodium bicarbonate (1 mEq/kg) were then administered, and open-chest CPR was performed for 3 minutes. Animals were defibrillated by 5-J DC epicardial countershock.

All animals were randomized to HBO therapy or standard intensive care. HBO was performed in an animal research chamber (WSF Industries) equipped with a hyperbaric ventilator (Sechrist 500A). HBO was initiated 60 minutes after resuscitation and consisted of a 25-minute descent to 2.7 atm, which was maintained for 60 minutes, followed by a 25-minute ascent. In the group randomized to standard intensive care, inspired oxygen was adjusted to maintain PaO_2 of 80 to 100 mm Hg at 1 atm absolute.

Experiment 1

Experiment 1 determined behavioral or histopathological outcomes. All animals (n=20) received intensive care for 23 hours. A morphine drip (0.1 mg · kg^{-1} · h^{-1}, with additional boluses as needed) provided analgesia during intensive care. Pancuronium (0.1 mg/kg) was used intermittently, only as needed to facilitate ventilation and only after verification that the animal was adequately sedated, feeling no pain. Ventilation was adjusted to maintain $PaCO_2$ of 30 to 35 and PaO_2 of 80 to 100 mm ,Hg. At 20 hours, anesthesia was lightened, and animals were weaned from controlled ventilation. At 23 hours morphine was reversed with naloxone (0.4 mg/kg), and neurological deficit scoring (NDS) was measured.

The NDS used was validated in other laboratories[9] and in ours.[1,8] The NDS is a multisystem test of 18 parameters in 5 categories (level of consciousness, respiration, cranial nerve, motor and sensory, and behavior), yielding a score between 0% and 100% (0=normal; 100=brain dead). Two investigators blinded to treatment scored each animal. After measurement of NDS, animals were reanesthetized with pentobarbital (12.5 mg/kg) and α-chloralose (75 mg/kg), intubated, and mechanically ventilated.

The sample size was chosen to allow a power of >80% to identify a difference in NDS of 10. Using 10 paired samples with significance set at $P<0.05$, a mean difference of 10, and an estimated SD of the differences of 10, we estimate the power of the study to be 0.81. The results of the NDS were analyzed by Wilcoxon signed-rank test, a nonparametric method. Physiological parameters were compared at baseline and at intervals over the course of the experiment by analysis of variance (ANOVA).

Histopathology

Tissue Preparation

At the end of the experimental period, after reinstitution of deep anesthesia, 9 dogs (4 HBO, 5 control) were perfused according to a protocol originally described for macaque monkeys[10,11] and adapted for use with canines.[12] Briefly, the chest was opened, the heart was exposed, and 1.5 mL of 0.1% sodium nitrite was injected into the left ventricle. The descending aorta was clamped, and the animals were perfused transcardially with cold 1% paraformaldehyde in phosphate buffer for 1 minute, followed by cold 4% paraformaldehyde for 10 minutes. The brains were then removed from the skull, cut into 3- to 5-mm-thick coronal blocks, postfixed for 6 to 10 hours in 4% paraformaldehyde at 4°C, and cryoprotected in a series of graded sucrose solutions (12% to 30%) in a phosphate-buffered saline.

Quantitative Analysis

Materials included in quantitative analyses were taken from the ventral hippocampus and from prefrontal area 8a. One hemisphere per animal was sectioned in 4-mm-thick blocks in the coronal plane. One block containing area 8a and 1 block containing the hippocampus were selected for quantitative analyses, carefully matching the rostrocaudal levels of these blocks across animals using a stereotaxic atlas of the dog brain.[13] Using a random starting point, a 1 in 10 series of 40-μm-thick sections was stained with cresyl violet and used for estimating neuronal densities.

These sections were sampled with a systematic random scheme that prevented counting bias toward any particular region of the tissue or toward including larger neurons preferentially. Quantitative analyses were performed on a computer-assisted image analysis system consisting of a Zeiss Axiophot photomicroscope equipped with a Zeiss MSP65 computer-controlled motorized stage, a Zeiss ZVS-47E video camera, a Macintosh G3 microcomputer (Apple Computers), and NeuroZoom, a custom-designed morphology and stereology software. Accurate maps of area 8a and the hippocampus showing the spatial distribution of neurons exhibiting characteristics of necrotic or apoptotic alterations were produced from these representative serial sections. The coordinates of each labeled element were recorded in each microscopic field, typically a fraction of a cortical layer visualized on the computer display at a magnification of ×20, relative to an origin on the section, and the map was automatically assembled. After outlining the boundaries of layers III and V of area 8a or the pyramidal layer of the CA1 field at low magnification (×5) on the computer graphic display in each section separately, the NeuroZoom software placed within each laminar boundary a set of optical disector frames (60×60 μm), in a systematic-random fashion, corresponding to a predetermined percentage of the sampled area that was kept constant throughout the study (2% in all layers of interest). Necrotic and apoptotic cells were then counted in optical disectors 12 μm in depth, according to stereologic principles. All analyses were performed with a 1.4 N.A. 100x Plan-NeoFluar Zeiss objective with a 1.4 N.A. auxiliary condenser lens to achieve optimal optical sectioning during disector analysis. Under these optical conditions, it was also possible to verify the histopathological features of the counted neurons. Subsequently, these estimates were converted into densities. In these materials, neurons showing clear accumulation of dense, globular materials in the cytoplasm with evidence of nuclear fragmentation were considered apoptotic, whereas necrotic neurons were defined as cells showing shrunken perikarya and darkly stained nuclei of reduced size.[14] Comparison of dying neurons between experimental groups was done with a 2-sample t test; $P<0.05$ was considered significant.

Experiment 2: Oxygen Metabolism and Blood Flow

Temporal Pattern of Cerebral Oxygen Extraction

Experiment 2 consisted of 2 parts. Part A determined the temporal pattern of cerebral oxygen extraction and the effects of HBO on extraction (n=5 per group). Arterial and sagittal sinus blood oxygenation were measured simultaneously at baseline and at 2, 30, 60,
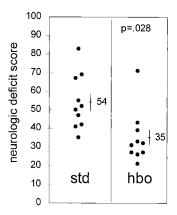
Figure 1. Comparison of NDS 24 hours after resuscitation for animals receiving either HBO or standard intensive care. NDS scores were significantly different at P=0.028.

120, 180, 240, 300, and 360 minutes after resuscitation (Instrumentation Laboratories Model 1302 Blood Gas Analyzer). The hyperbaric chamber used was designed so that arterial and sagittal sinus blood samples could be drawn even while the animal was under pressure. Cerebral oxygen extraction was calculated as follows: $ER_c = (C_{arterial} - C_{sagittal\ sinus})/C_{arterial}$, where C is oxygen content of blood. Comparisons were made using ANOVA with posthoc t test analysis.

Cerebral Oxygen Delivery and Utilization

Part B determined cerebral oxygen delivery (Do_2c) and utilization—ie, $CMRo_2$ (n=4 per group). In addition to arterial and sagittal sinus oxygenation, CBF was measured by the colored microsphere technique at baseline and at 30, 150, and 300 minutes after A/R. Colored microspheres (NuFLOW, Triton Technology) injected into the left ventricle of the heart disperse and lodge throughout the microcirculation in a flow-dependent density, which can be measured fluorocytometrically after death.[15] End-organ blood flow was referenced to the density in an arterial blood sample withdrawn at a known rate at the time of microsphere injection. Cerebral oxygen extraction, delivery, and $CMRo_2$ were calculated: $Do_2c = C_{arterial} \times CBF$ and $CMRo_2 = CBF \times (C_{arterial} - C_{sagittal\ sinus})$.

The total mass of tissue analyzed for regional CBF using microspheres was used to convert milliliters per minute per 100 g to millimeters per minute. The tissue sample averaged 80 ± 5 g. Comparisons were made using ANOVA with posthoc t test analysis.

In experiment 2, all animals were euthanized with sodium pentobarbital. In part B, the brain was immersion fixed in formaldehyde, sectioned, and sent to an independent laboratory (Interactive Medical Technologies) for tissue digestion and counting of microspheres. Analysis was performed by researchers blinded to the treatment.

Results

There were no significant differences in blood pressure, heart rate, core body temperature, pH, Pco_2 or intracranial pressure between groups at baseline or during recovery; Po_2 differed only during HBO. These physiological parameters closely matched those described in greater detail in previous presentations of this model.[8] All animals were successfully resuscitated.

Neurological Outcome

Animals treated with HBO had significantly better neurological outcomes than controls (Figure 1) (NDS: 35 ± 14 for HBO; 54 ± 15 for controls; P=0.028). Seven of 10 HBO animals had better NDS than any control dog. Control animals were poorly responsive, often demonstrating stereotypical "running movements" while lying on their sides. In contrast, most HBO animals appeared aware of surroundings,
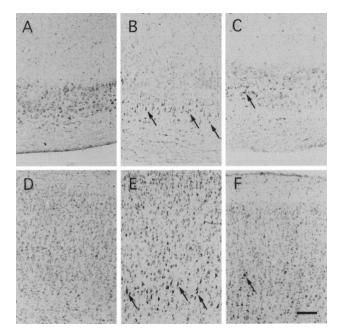

Figure 2. Ischemic changes in hippocampus (A through C) and neocortex (D through F) 24 hours after reperfusion without (B, E) or with (C, F) HBO vs nonischemic control (A, D). Note the preserved morphology of neurons in the control animal, whereas severe changes are seen in the standard 24-hour–reperfused animal (arrows, B, E). Animal treated with HBO (arrows, C, F) shows milder changes than the untreated animal (Nissl stain). Scale bar=100 μm.

were responsive to external stimuli, and attempted to stand. Several walked ataxically.

Histopathology

Neuronal densities in 9 animals were measured in hippocampus CA1 and 2 layers of cerebral cortex (II through III and V through VI). Total neuronal densities averaged from 40 to 60 000 neurons per 1 mm^3 in the regions examined (Figure 2). The density of dying neurons (changes consistent with necrosis or apoptosis) was then measured. Computer-generated maps of the distribution of ischemic changes in hippocampus and frontal cortex were prepared (Figure 3). The percentage of dying neurons was calculated. Differences in histopathological outcomes between groups were even more pronounced than clinical neurological outcome. HBO animals had significantly less neuronal injury in each region examined compared with controls (Figure 4a); the relevance of quantitative measurement of neuronal injury is highlighted by the close correlation of percent dying neurons in each animal with that animal's clinical NDS (Figure 4b). Linear regression of this correlation was significant (R^2=0.71, P=0.005).

Experiment 2: Oxygen Metabolism

The results of experiment 2A are shown in Figure 5. This figure shows cerebral oxygen extraction and sagittal sinus cerebral venous Po_2 from which it is calculated as a function of time after A/R. The ER_c falls precipitously from baseline after A/R but rapidly increases in controls to peak at $60 \pm 14\%$ at 240 minutes. HBO suppresses this response, with extrac-
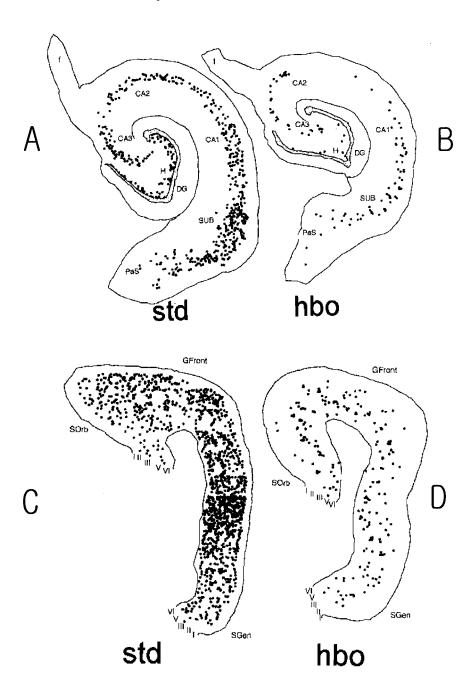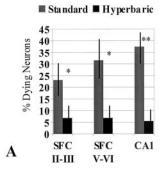
Figure 3. Computer-generated maps of distribution of ischemic changes in the hippocampus (A, B) and frontal cortex (C, D) of 24-hour–reperfused dogs with HBO treatment (B, D) or standard (std) intensive care without HBO (A, E). Neo-cortical layers are indicated by roman numerals. DG indicates dentate gyrus; f, fimbira; H, hilus; SUB, subiculum; PaS, parasubiculum, Gfront, gyrus frontalis; Sorb, sulcus orbitalis; and Sgen, sulcus genualis.

tion dropping to 26±4% during treatment and returning to a peak similar to that of controls after treatment.

The time points selected for CBF measurements in experiment 2B were based on the time course defined in experi-ment 2A. The results of experiment 2B are shown in Figure 6, with ER_c, Do_2c, $CMRo_2$, and CBF shown as a function of time after A/R. Thirty minutes after A/R, CBF was similar to baseline but dropped 50% by 150 minutes and was beginning
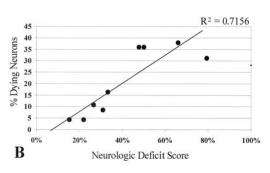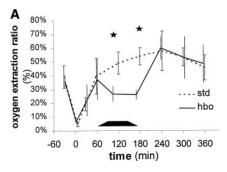

Figure 4. Quantification of neuronal cell death in superior frontal cortex (SFC) lay-ers II through III and V through VI, as well as in hippocampus CA1, for animals receiving HBO or standard intensive care. A, Values represent mean±SD. Cell death between groups was significantly different at $P<0.02$ (*) or $P<0.001$ (**). B, Cell death observed at 24 hours after A/R correlated well with NDS scores obtained at 23 hours after A/R ($R^2=0.7156$).
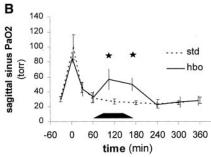
Figure 5. Cerebral oxygen extraction (a) and measured sagittal sinus P_{O_2} (b) after A/R in untreated and HBO-treated animals. Horizontal black bars indicate the onset and duration of HBO in the treatment group. *$P<0.05$.

to recover at 300 minutes. There were no differences in CBF between treatment groups ($P=0.42$). Oxygen delivery was 4.66 mL/min at baseline and then decreased with decreases in CBF in untreated animals. HBO increased oxygen content by 20% but did not significantly alter oxygen delivery. Baseline CMR_{O_2} was 1.58 mL/min. After A/R, there was a 50% reduction in CMR_{O_2} and in CBF in both groups. CMR_{O_2} was not significantly changed between HBO (0.82 mL/min) and controls (1.25 mL/min) ($P=0.20$).

Discussion

HBO has been shown to be effective in preventing neurological injuries in animal models of both focal ischemia[16–21] and CGI.[3–5] Previous CGI studies demonstrated improvement using several postischemic HBO treatments; ours establishes effectiveness with only 1 postischemic exposure. Moreover, the present study demonstrates for the first time that HBO both improves neurological function and reduces neuronal cell death. Neuroprotection does not, however, result from increased cerebral oxygen delivery or oxygen consumption after A/R. Most important, this is the first study to demon-

strate HBO neuroprotection after experimental cardiac arrest–induced CGI. Because of substantial physiological differences between cardiac arrest and vessel occlusion–induced CGI, efficacy in a model such as ours is imperative if clinical trials are to be considered at any point.

Because HBO can theoretically increase oxygen delivery to ischemic tissue, we hypothesized that treatments 1 hour after A/R were correcting ongoing ischemia, therefore stimulating aerobic cerebral energy metabolism. In focal ischemia, there is some evidence that HBO reduces infarct volume by increasing oxygen supply to the ischemic periphery.[18] Our data confirm that extraction, which can be used as a surrogate marker of ischemia, peaks during the treatment interval and is corrected by HBO. However, our data show no increase in cerebral oxygen delivery during treatment. A 20% increase in arterial oxygen content in our animals is offset by a 20% decrease in CBF during treatment. Moreover, the normalization of extraction with HBO was not associated with an increase in cerebral oxygen consumption, as it would be if HBO corrected ongoing ischemia. In fact, the normalization in extraction may be a result of the trend toward lower CMR_{O_2} during HBO treatment. These data are
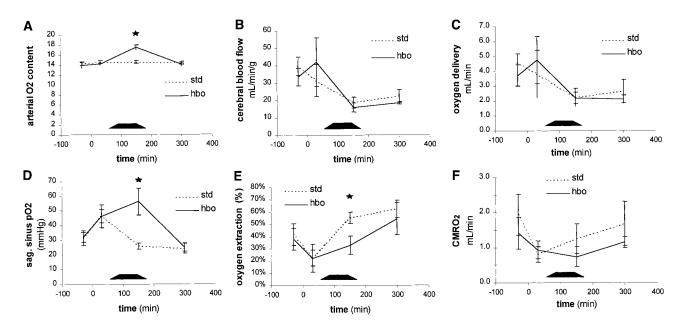

Figure 6. Cerebral oxygen metabolism after A/R in untreated and HBO-treated animals. Increased arterial oxygen content (a) during HBO is modest but significant ($P<0.05$). CBF (b) is initially preserved but then decreases after A/R. Oxygen delivery (c) is not significantly increased in HBO-treated animals. Measured sagittal sinus P_{O_2} (d) and cerebral oxygen extraction (e) show a transient decrease in extraction immediately after A/R, which then increase to critical levels in untreated animals. HBO-treated animals have lower extraction during treatment ($P<0.05$). Cerebral metabolic rate for oxygen (f) decreases after A/R, but there are no differences between treated and untreated animals. Horizontal black bars indicate onset and duration of HBO in the treatment group.

consistent with previous studies that suggest that there is no ongoing ischemia during postischemic hypoperfusion.[22] After CGI, therefore, energy metabolism may not be limited by oxygen delivery but rather by the activity of aerobic metabolic enzymes. Observations indicating that mitochondrial metabolism is impaired for many hours after transient CGI support this hypothesis.[23]

Several limitations of this work should be noted. Neurological outcome and histopathology were measured at only 1 time point. It is possible that clinical deficits and neuronal losses may only be delayed by HBO and that injury may mature with time. Further studies should extend these preliminary investigations to at least 72 hours to fully define neuronal injury and protection. It is also important to note that our estimates of neuronal injury are based on simple histopathology. Further studies should explore this question immunohistochemically, examining specific markers for neuronal death. Finally, we tested only 1 treatment of 1 dose of HBO. Further studies of optimal timing and HBO dosage are needed before clinical trials could be considered.[24]

Considering the lack of support for a direct metabolic effect of HBO treatment in our model, other mechanisms of neuroprotection must be considered. After focal cerebral ischemia, evidence is mounting that HBO protects vulnerable neurons through prevention of leukocyte infiltration into ischemic tissue.[17,25] However, although inflammation is known to play an important role in secondary injury after stroke,[26] the role of inflammatory damage after global ischemia is much less understood. As such, the importance of HBO-mediated prevention of white blood cell migration into the brain after A/R remains unclear.

A few studies have provided evidence that HBO alters cerebral gene expression in ways that could be neuroprotective. Multiple preischemic exposures to HBO significantly elevated brain levels of Bcl-2, an anti-apoptotic protein, and Mn-superoxide dismutase, an enzyme that detoxifies reactive oxygen species.[27] In addition to upregulating antioxidant gene expression, HBO downregulates the expression of cyclooxygenase-2,[21] a potential source of toxic reactive oxygen species that has been implicated in postischemic oxidative stress.[28,29] The combined effects of HBO on antioxidant and pro-oxidant enzyme activities could therefore provide a plausible explanation for its inhibitory effects on neuronal cell death and neurological impairment after cerebral ischemia.

Acknowledgments

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