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Effects of Permissive Hypercapnia on Transient Global Cerebral Ischemia–Reperfusion Injury in Rats

Qiang Zhou, M.D.,* Bo Cao, Ph.D.,† Li Niu, M.D.,‡ Xiaoguang Cui, M.D.,§ Hongwei Yu, M.D.,‖ Jinfeng Liu, M.D.,§ Haibo Li, M.D.,‡ Wenzhi Li, M.D.§

ABSTRACT

Background: Permissive hypercapnia is a widely practiced protective ventilatory strategy that has significant protective effects on several models of *in vitro* and *in vivo* neuronal injury. However, conclusive effects of permissive hypercapnia on cerebral ischemia are still unknown.

Methods: One hundred sixty male Wistar rats were divided into five groups: S group (control), ischemia–reperfusion (I/R) group, P1 group, P2 group, and P3 group. I/R was induced by bilateral occlusion of the common carotid arteries, combined with controlled hypotension for 15 min. In groups P1, P2, and P3, the rats inhaled carbon dioxide for 2 h during reperfusion to keep $Paco_2$ within the ranges of 60–80 mmHg, 80–100 mmHg, and 100–120 mmHg, respectively. After 24 and 72 h, neurologic deficit scores, ultrastructural changes, apoptotic neurons, and brain wet-to-dry weight ratios were observed. Caspase-3 and aquaporin-4 protein expression and caspase-3 activity were analyzed.

Results: Compared with groups I/R and P3, groups P1 and P2 had better neurologic deficit scores and fewer ultrastructural histopathologic changes. I/R-induced cerebral apoptosis was also significantly reduced. The neuroprotective effect was significantly increased in the P2 group compared with the P1 group. There was a significant increase of brain water content and of aquaporin-4 levels in the P3 group.

Conclusions: Mild to moderate hypercapnia ($Paco_2$ 60–100 mmHg) is neuroprotective after transient global cerebral I/R injury. Such a protection might be associated with apoptosis-regulating proteins. In contrast, severe hypercapnia ($Paco_2$ 100–120 mmHg) increased brain injury, which may be caused by increased brain edema.

What We Already Know about This Topic

- Hypercapnia is often avoided after global cerebral ischemia because it may increase intracranial pressure
- In immature animals, however, hypercapnia is neuroprotective after ischemia

What This Article Tells Us That Is New

- In normal adult rats, neurologic outcome and histologic brain injury from global cerebral ischemia were improved by moderate (pCO_2 60–100 mmHg) permissive hypercapnia
- Greater degrees of hypercapnia, however, resulted in more brain edema

TRANSIENT global cerebral ischemia–reperfusion (I/R) injury is a major complication during the perioperative period of cardiac arrest and resuscitation. Prophylactic pharmacologic interventions for neuroprotection against cerebral I/R injuries could be of great benefit to patients undergoing these surgeries. However, to date, there are no safe and efficacious agents available to protect the brain from I/R injury.

Permissive hypercapnia is a widely practiced ventilatory strategy to improve patient outcome, in which tidal volume and alveolar ventilation are reduced to decrease the potential for ventilator-induced lung injury. Acute hypercapnia increases intracranial pressure (ICP), primarily through cerebral vasodilatation and increases in cerebral blood volume, which, clinically, is the most important effect of hypercapnia on the central nervous system. It is usually considered that increasing ICP can reduce cerebral perfusion, induce cerebral ischemia as a result of vascular steal, or perhaps increase perfusion with propensity to hemorrhage or reperfusion injury in previously underperfused vascular beds. However, counterintuitively, permissive hypercapnia protects against hypoxic ischemic brain injury in immature animal models.[1–3] Recent studies have shown that the underlying neuroprotective mechanisms include reductions of excitatory amino acids levels, augmentation of oxygen delivery, and a reduction

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* Attending Doctor, ‡ Associate Professor, § Professor, Department of Anesthesiology, The Second Clinical Hospital, Harbin Medical University. † Associate Professor, ‖ Lecturer, Department of Histology and Embryology, Harbin Medical University.

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Drs. Zhou and Cao contributed equally to this work.

Address correspondence to Dr. Li: Department of Anesthesiology, The Second Clinical Hospital, Harbin Medical University, Harbin, China. wenzhili9@126.com.

of cerebral metabolism. In addition, a growing body of clinical and laboratory data suggests that permissive hypercapnia may be cytoprotective against I/R injury in the lung, heart, and intestine.[4–7] Although previous studies have shown some putative neuroprotective effects, conclusive effects of permissive hypercapnia on both functional and histologic outcomes after transient global cerebral I/R injury are unknown.

In the current study, we hypothesized that permissive hypercapnia would attenuate histologic injury and improve functional neurologic outcome after transient global cerebral I/R in rats. Therefore, we examined the effects of permissive hypercapnia on neurologic behavioral assessments, ultrastructural histopathologic changes, neuronal apoptosis, and cerebral edema.

Materials and Methods

Animal Groups

This study was approved by the Harbin Medical University Animal Care and Use Committee, Harbin, China. A total of 160 male Wistar rats, aged between 8 and 9 weeks and weighing between 250 and 340 g, were randomly allocated to one of five experimental groups (32 animals in each group) using the SPSS 11.5 (SPSS Inc., Chicago, IL):

1. S group (the sham-operated group): these rats underwent the same anesthesia and surgical procedures, except that the bilateral common carotid arteries were not occluded as in the experimental groups.
2. I/R group: under the same anesthetic conditions and surgical procedures as group I, these rats were subjected to transient global cerebral ischemia for 15 min, followed by reperfusion and inhalation of 30% O_2 for 2 h.
3. P1 group ($PaCO_2$ 60–80 mmHg + I/R): these rats received the same ischemia–reperfusion and the same fraction of inspired oxygen as described for the I/R group, but in contrast to the I/R group, carbon dioxide was inhaled for 2 h after reperfusion to maintain the $PaCO_2$ between 60 and 80 mmHg.
4. P2 group ($PaCO_2$ 80–100 mmHg + I/R): these rats received the same ischemia–reperfusion and the same fraction of inspired oxygen as described for the I/R group, but in contrast to the I/R group, carbon dioxide was inhaled for 2 h after reperfusion to maintain the $PaCO_2$ between 80 and 100 mmHg.
5. P3 group ($PaCO_2$ 100–120 mmHg + I/R): these rats received the same ischemia–reperfusion and the same fraction of inspired oxygen as described for the I/R group, but in contrast to the I/R group, carbon dioxide was inhaled for 2 h after reperfusion to maintain the $PaCO_2$ between 100–120 mmHg.

Surgical Preparations and Transient Global Cerebral Ischemia

Surgery. All animals were fasted overnight before the experiments but were allowed free access to water. Body weights were measured before experiments. Each rat was anesthetized with chloral hydrate (360 mg/kg, intraperitoneally), and all surgical incisions were infiltrated with 0.25% bupivacaine. If the depth of anesthesia was considered inadequate, additional chloral hydrate (100 mg/kg, intraperitoneally) was administered. A sterile cutdown was performed in the left groin. The left femoral artery was cannulated with a 24-gauge Teflon cannula (Becton Dickinson, Sparks, MD) to monitor mean arterial pressure (MAP) and to collect arterial blood samples. MAP was measured using an MP150 Workstation and analyzed using AcqKnowledge software (BIOPAC Systems Inc., Santa Barbara, CA) according to the manufacturer's specifications. Arterial blood gases were examined using a Chiron Diagnostics model 248 blood gases/pH analyzer (Bayer Diagnostics, Norwood, MA). The left femoral vein was cannulated with a 22-gauge Teflon cannula to enable a continuous infusion of fentanyl and 0.9% saline as maintenance fluid. By means of a ventral neck incision, both common carotid arteries were identified, carefully separated from the vagus nerves, and encircled with silk sutures for later clamping. Temperature probes were inserted into each animal's rectum and the left-side temporalis muscle. Rectal and pericranial temperatures were kept constant at 37.5° ± 0.2°C using a servocontrolled overhead heating lamp and a thermostatically regulated warming blanket throughout the period of surgery and recovery. Needle electrodes were placed in the scalp for continuous recording of the frontoparietal electroencephalogram.

Global Cerebral Ischemia–Reperfusion. Fifteen minutes of transient global cerebral ischemia was achieved using bilateral carotid artery occlusion and hypotension. The experimental model was based on that previously reported, with several modifications.[8–10] In brief, the bilateral common carotid arteries were occluded with aneurysm clips. Fifteen minutes of global cerebral ischemia was achieved by the simultaneous reduction of mean arterial blood pressure to 35 mmHg using the hypotension technique.[9,11] Electroencephalogram was monitored to ensure isoelectricity within 10 s after the induction of ischemia. After 15 min of ischemia, the aneurysm clips were removed, and hypotension was terminated to allow reperfusion of the brain. The neck incision was closed with sutures. Rats were placed in a Plexiglass box (Hotson Acrylic Product Manufacturing Co. Ltd., Shenzhen, China) that was relatively airtight. The end-tidal carbon dioxide concentration and the inspiratory oxygen concentration were monitored continuously using a gas monitor (DATEX Instrumentarium, Helsinki, Finland). After 2 h of reperfusion, temperature probes, needle electrodes, and catheters were removed, incisions were closed, and the animals were extubated and placed in another Plexiglass box, and oxygen was insufflated into the box until the animals were awake. Free access to food and water was then allowed.

The physiologic variables were recorded at six time points: before hemorrhagic hypotension (baseline), after 10 min of cerebral ischemia (ischemia), and every 30 min after the start of reperfusion (reperfusion 30, 60, 90, and 120 min).

Intracranial Pressure

ICP was recorded from the cisterna magna as described.[12] In brief, after positioning the rat in the stereotactic frame, a 24-gauge needle, attached using short, noncompliant tubing to a pressure transducer, was inserted into the cisterna magna. ICP was measured using the MP150 Workstation and analyzed using AcqKnowledge software (BIOPAC Systems Inc.) according to the manufacturer's specifications.

Assessments of Neurologic Behavior

Neurologic Deficit. A neurologic evaluation was performed 24 and 72 h after the onset of reperfusion or 24 and 72 h after sham operation by the same investigator, who was not aware of the group assignment, using a neurologic deficit score (NDS) as described previously.[10,8,13–15] Scoring included consciousness, breathing, smell, vision and hearing, reflexes, motor function, overall activity, orientation, and presence of seizures. The NDS could range from 0 to 100, an NDS of 0 reflects normal brain function and an NDS of 100 reflects brain death.

Postmortem Brain Sampling and Storage

At the completion of the neurologic evaluation, rats were anesthetized with chloral hydrate (400 mg/kg, intraperitoneally). Sixteen animals from each group were killed by transcardiac perfusion with 200 ml heparinized saline followed by 200 ml freshly prepared ice-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). The rats were decapitated; their brains were removed carefully and postfixed for 24 h in the same fixative. The brains were then prepared for transmission electron microscopy and for the TdT-mediated dUTP nick end-labeling (TUNEL) assay. Twelve animals from each group were killed by transcardiac perfusion with 200 ml heparinized saline and then decapitated. The brains were quickly removed and inspected to confirm the absence of subarachnoid hemorrhage. Hippocampi and cortices were dissected, and hippocampi were snap-frozen in liquid nitrogen and stored at −80°C for the determination of caspase-3 activity and for Western blot analysis. The remaining brain tissues were used for the determination of brain wet-to-dry (W/D) weight ratios. Three hours after the onset of reperfusion, the brain tissues from four animals of each group were collected to determine the W/D ratios.

Ultrastructural Changes and Histopathologic Changes

Observation by Electron Microscopy. Bilateral hippocampal and cortical tissues were removed from the brains fixed with 4% paraformaldehyde and then incubated in 30 g/l (3%) glutaraldehyde for 2 h and subsequently postfixed in 1% osmium tetroxide (in 0.1 M phosphate buffer, pH 7.4) for 2 h at 4°C. Subsequently, the samples were dehydrated in a graded ethanol series with acetone, permeated and embedded in epoxide resin. Semi-thin sections of approximately 75 nm were prepared, stained with uranyl acetate and lead citrate, and then observed with an H-300 transmission electron microscope (Hitachi Electronic Instruments, Tokyo, Japan).

Neuronal Apoptosis

TUNEL Assay. Apoptosis was assessed using a TUNEL assay. This was performed with an In Situ Apoptosis Detection Kit (Roche Diagnostics, Indianapolis, IN), according to a previously described methodology.[16] In brief, the formaldehyde-fixed brains were stored in fresh buffer containing 20% sucrose, then frozen at −20°C in 2-methylbutane, and sectioned with a cryostat to a thickness of 20 μm. The sections were acetone fixed for 10 min at room temperature, air dried, and then kept at −80°C until processed. Slides were dewaxed and rehydrated in a graded series of ethanol and incubated with 20 μg/ml proteinase K (Sigma, St. Louis, MO). Fifty microliter of TUNEL mixture was then added to the samples and incubated in a humidified chamber for 60 min at 37°C in the dark. The samples were rinsed, and visualization was achieved with 0.025% 3,3′-diaminobenzidine. The sections were counterstained with hematoxylin, hyalinized with dimethylbenzene, and sealed with neutral gum for observation under an Eclipse E-600 optical microscope (Nikon, Kawasaki, Japan). TUNEL-positive cells were counted in the CA1 regions of the dorsal hippocampus and in the cortices using light microscopy (three different random high-power fields per sector, ×400 magnification) by an investigator who was blinded to group assignment.

Measurement of Caspase-3 Activity. Caspase-3 activity was measured using a commercial caspase-3 assay kit (PharMingen, San Diego, CA). Fifty milligrams of the hippocampal CA1 tissue was homogenized in lysis buffer at 4°C. The lysates were centrifuged, and the protein content of supernatants was quantified. Exactly 2 μg of the protein was loaded in each well of a 96-well plate. Caspase-3 activity was determined using an Fmax fluorescence microtiter plate reader (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

The Levels of Procaspase-3 and of the Active Form of Caspase-3. Western blot analysis was used to evaluate the levels of procaspase-3 and the active form of caspase-3. Rat hippocampus samples were pulverized under liquid nitrogen using a porcelain mortar and pestle. The pulverized brain tissues were incubated in lysis buffer and protease inhibitor cocktail (Sigma) for 1 h at 4°C. After a 10-min centrifugation at 10,000*g*, the supernatant was collected, and the protein content was quantified. Thirty micrograms of protein was loaded per lane, separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electroblotted onto polyvinylidene fluoride membranes for 2 h at a constant voltage of 10 V using semidry apparatus. The membrane was incubated with a polyclonal rabbit anti-rat caspase-3 antibody (Lab Vision, Fremont, CA) overnight at 4°C and then incubated with an anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). The blot was stripped and reprobed with a mouse GAPDH monoclonal antibody (Santa Cruz Biotechnology,

Table 1. Physiological Variables in the Experimental Groups

		Time					
				Reperfusion			
	Group	Baseline	Ischemia	30 min	60 min	90 min	120 min
MAP (mmHg)	S	115 ± 11	113 ± 10	116 ± 7	114 ± 7	119 ± 6	112 ± 5
	I/R	116 ± 9	35 ± 1*	111 ± 8	115 ± 6	117 ± 13	116 ± 7
	P1	115 ± 7	35 ± 1*	117 ± 10†	123 ± 9†	131 ± 7†	133 ± 11†
	P2	116 ± 6	35 ± 1*	118 ± 11†	127 ± 7†	134 ± 6†	131 ± 10†
	P3	114 ± 12	35 ± 1*	118 ± 6†	129 ± 11†	133 ± 9†	132 ± 8†
Pa_{O_2} (mmHg)	S	124 ± 9		121 ± 7	123 ± 6	131 ± 11	127 ± 8
	I/R	123 ± 13		159 ± 12	168 ± 11	167 ± 10	168 ± 7
	P1	123 ± 7		172 ± 6†	179 ± 11†	188 ± 8†	186 ± 10†
	P2	125 ± 10		207 ± 12†	212 ± 9†	217 ± 5†	221 ± 8†
	P3	121 ± 9		232 ± 9†	246 ± 11†	249 ± 13†	247 ± 5†
Pa_{CO_2} (mmHg)	S	32 ± 6		40 ± 9	38 ± 6	35 ± 7	36 ± 6
	I/R	34 ± 5		37 ± 7	35 ± 7	36 ± 4	33 ± 5
	P1	32 ± 6		63 ± 6†	73 ± 5†	72 ± 7†	70 ± 5†
	P2	36 ± 3		85 ± 7†	94 ± 6†	93 ± 8†	93 ± 5†
	P3	33 ± 4		106 ± 5†	109 ± 7†	114 ± 9†	111 ± 4†
Arterial pH	S	7.41 ± 0.04		7.38 ± 0.06	7.37 ± 0.03	7.38 ± 0.04	7.38 ± 0.03
	I/R	7.39 ± 0.03		7.37 ± 0.04	7.40 ± 0.02	7.38 ± 0.03	7.41 ± 0.05
	P1	7.40 ± 0.03		7.21 ± 0.07†	7.18 ± 0.05†	7.16 ± 0.07†	7.19 ± 0.04†
	P2	7.37 ± 0.06		7.13 ± 0.09†	7.15 ± 0.06†	7.11 ± 0.08†	7.12 ± 0.11†
	P3	7.40 ± 0.04		7.05 ± 0.10†	7.02 ± 0.07†	7.07 ± 0.05†	7.04 ± 0.08†

Data are presented as mean ± SD.

* Controlled parameter. † $P < 0.05$ *versus* ischemia–reperfusion (I/R) group.

MAP = mean arterial pressure; Pa_{O_2} = arterial oxygen tension; Pa_{CO_2} = arterial carbon dioxide tension.

Santa Cruz, CA) to assess loading in each lane. The signals were detected with an enhanced chemiluminescence kit (Pierce) and exposed on x-ray film. After the film was scanned with a GS-700 imaging densitometer (Bio-Rad, Hercules, CA), a quantitative analysis was performed using Multi-Analyst software (Bio-Rad).

Cerebral Edema

W/D Ratio. Brains were weighed, maintained at 70°C for 7 days, and reweighed to calculate the W/D ratio.

The Level of Aquaporin-4. The protein level of aquaporin-4 (AQP4) was determined by Western blot analysis using a rabbit monoclonal antibody against rat AQP4 (Santa Cruz), as described in the previous section.

Statistical Analysis

Statistical analyses were performed using SPSS 11.5. Continuous variables were presented as mean ± SD. The physiologic, arterial blood gas, ICP variables, TUNEL-positive cell counts, caspase-3 activity, W/D ratio and protein level of procaspase-3, the active form of caspase-3, and AQP4 were analyzed using repeated-measures ANOVA for multiple *t* tests. NDS was expressed as median ± twenty-fifth/seventy-fifth percentiles, and groups were compared using the Kruskal-Wallis test. If the result was significant, ranks of arranged NDS were analyzed by the Student–Newman–Keuls test for the multiple comparisons. *P* values of less than 0.05 were considered statistically significant.

Results

One animal from the I/R group, one from the P2 group, and two from the P3 group were excluded from this study because of the development of subarachnoid hemorrhage or because of technical experimental problems. Two animals from the I/R group and one from the P3 group died before histologic analysis and were also excluded from the study.

Physiologic Variables during Ischemia–Reperfusion

Body weight, pericranial, and rectal temperatures were not significantly different among the five experimental groups (data not shown). The MAP and arterial blood gas parameters are presented in table 1. At baseline, there were no among-group differences for MAP, Pa_{O_2}, Pa_{CO_2}, and pH. As intended, Pa_{CO_2} and pH differed during reperfusion because of different fraction of inspired oxygen in the P1, P2, and P3 groups compared with the I/R group. Significantly, MAP and Pa_{O_2} were higher in the P1, P2, and P3 groups compared with the I/R group, although MAP values were similar for the P1, P2, and P3 groups.

The ICP of all the groups is shown in figure 1. At baseline, there were no among-group differences. As predicted, ICP was lower in the I/R, P1, P2, and P3 groups compared with the S group during ischemia. Significantly, ICP was higher in the P1, P2, and P3 groups compared with the I/R group during carbon dioxide inhalation. Three hours after the onset of reperfusion, there were no statistically significant differences among the five experimental groups.

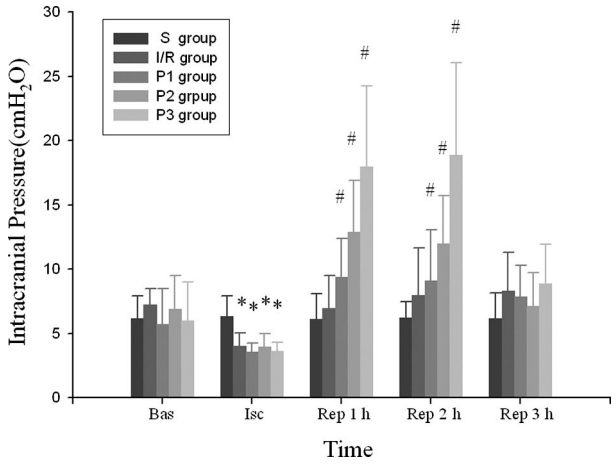

Fig. 1. Intracranial pressure measures following baseline (Bas), ischemia (Isc), 1 h (Rep 1 h), 2 h (Rep 2 h), and 3 h (Rep 3 h) after reperfusion. *$P < 0.05$ *versus* Isc S group; #$P < 0.05$ *versus* Rep 1 h and Rep 2 h S group. I/R = ischemia–reperfusion.

Neurologic Deficit Scores

The NDSs are shown in figure 2. Twenty-four and 72 h after reperfusion, the P1 and P2 groups demonstrated a better neurologic outcome compared with the I/R and P3 groups ($P < 0.01$), but there were no differences between the I/R and P3 groups.

Ultrastructural Changes

The ultrastructural changes are shown in figure 3. There are no obvious pathologic changes in the S group. In the I/R group, nuclear membranes lost definition, and the number of nuclear pore complexes was reduced. The chromatin was significantly aggregated toward the nuclear membrane. The mitochondrial cristae were broken down and sparsely distributed with vacuolar degeneration. The number of Nissl bodies was reduced. In the P1 group, the nuclear membranes were clear, and the granule-like aggregation of chromatin could be seen. In the P2 group, the nuclear structures were normal, the nuclear pore complexes were clear, the chromatin was distributed evenly, the mitochondrial structures were complete, and the number of Nissl bodies was copious. In the P3 group, chromosomes had partially degraded and disappeared. The electron density of the cytoplasm was reduced, and the number of lipofuscin particles of different sizes was increased.

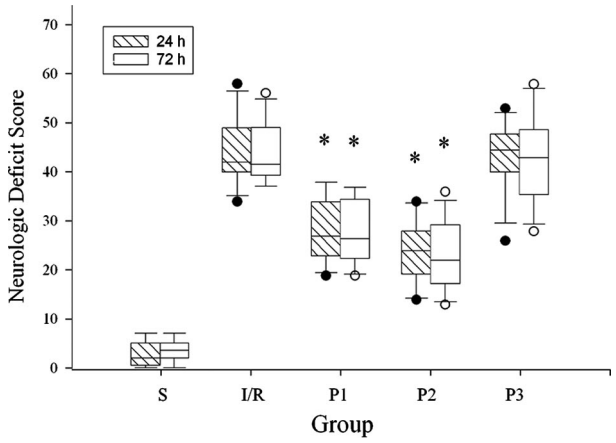

Fig. 2. Neurologic deficit scores after 24 and 72 h of reperfusion after bilateral carotid artery occlusion. Data are presented as medians ± twenty-fifth/twenty-fifth percentiles. *$P < 0.05$ *versus* I/R and P3 groups. I/R = ischemia–reperfusion.

Neuronal Apoptosis

Figures 4 and 5 illustrate the effect of hypercapnia on neuronal apoptosis. Figure 4A shows representative TUNEL-positive apoptotic cells in the CA1 region of the hippocampus and in the cortices. TUNEL-positive cell counts are shown in figure 4B. These data show that the P1 and P2 groups exhibited significantly fewer TUNEL-positive cells within the CA1 region of the hippocampus and in the cortices compared with the I/R and P3 groups, whereas the P2 group had fewer TUNEL-positive cells compared with those in the P1 group.

The levels of caspase-3 enzymatic activity, procaspase-3 (p32), and the active form of caspase-3 (p20) are presented in figure 5. As expected, the levels of caspase-3 activity, p32, and p20 caspase-3 were markedly increased in the I/R group compared with the S group. The levels of caspase-3 activity, p32, and p20 caspase-3 were significantly decreased in the P1 and P2 groups compared with their levels in the I/R group, whereas the P2 group had lower levels compared with those of the P1 group. However, there was no difference between the P3 and I/R groups in terms of the activities of caspase-3, p32, and p20 caspase-3.

Cerebral Edema

As shown in figure 6, the water content in the brain and the levels of AQP4 protein were significantly higher in the P3 group compared with those in the I/R group. The levels of brain water content and AQP4 protein were slightly decreased in the P1 and P2 groups compared with the I/R group, but there were no statistically significant differences among the P1, P2, and I/R groups.

Discussion

This study showed that after I/R, rats with $PaCO_2$ 60–100 mmHg (P1 and P2 groups) had an improved neurologic outcome and that fewer ultrastructural histopathologic changes occurred. Furthermore, I/R-induced cerebral apoptosis is obviously reduced, as shown by decreased TUNEL-positive cell counts and decreased caspase-3 activation. More importantly, the neuroprotective effect was significantly increased in rats with $PaCO_2$ 80–100 mmHg compared with rats with $PaCO_2$ 60–80 mmHg. There was a significant increase of NDSs, ultrastructural histopathologic injury, brain water content, and AQP4 levels in rats with $PaCO_2$ 100–120 mmHg. These results indicate that severe hypercapnia-induced brain injury may be mediated by an increase in brain edema.

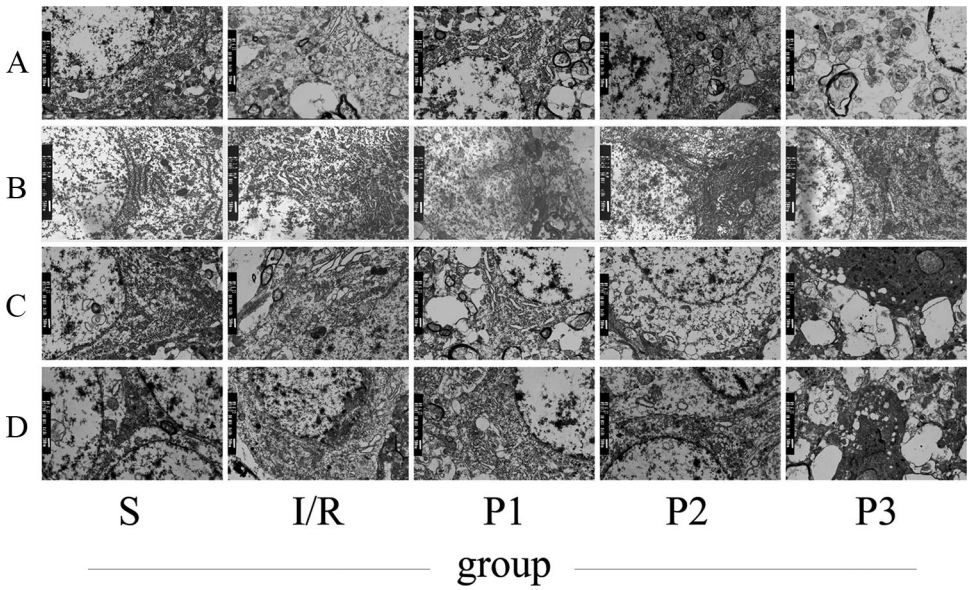

Fig. 3. Hippocampal and cortical ultrastructural histopathologic changes after 24 and 72 h of reperfusion (×5000). (*A*) Cortices after 24-h reperfusion; (*B*) hippocampus after 24 h reperfusion; (*C*) cortices after 72-h reperfusion; and (*D*) hippocampus after 72-h reperfusion. I/R = ischemia–reperfusion.

The specific effects of hypercapnia on the brain have not been well studied because the orthodox view considers that increased $PaCO_2$ produces an adverse effect on intracranial pathology. Thus, the original objective of our experimental design was to study hypercapnia-induced brain edema formation in rodent models of transient global cerebral ischemia–

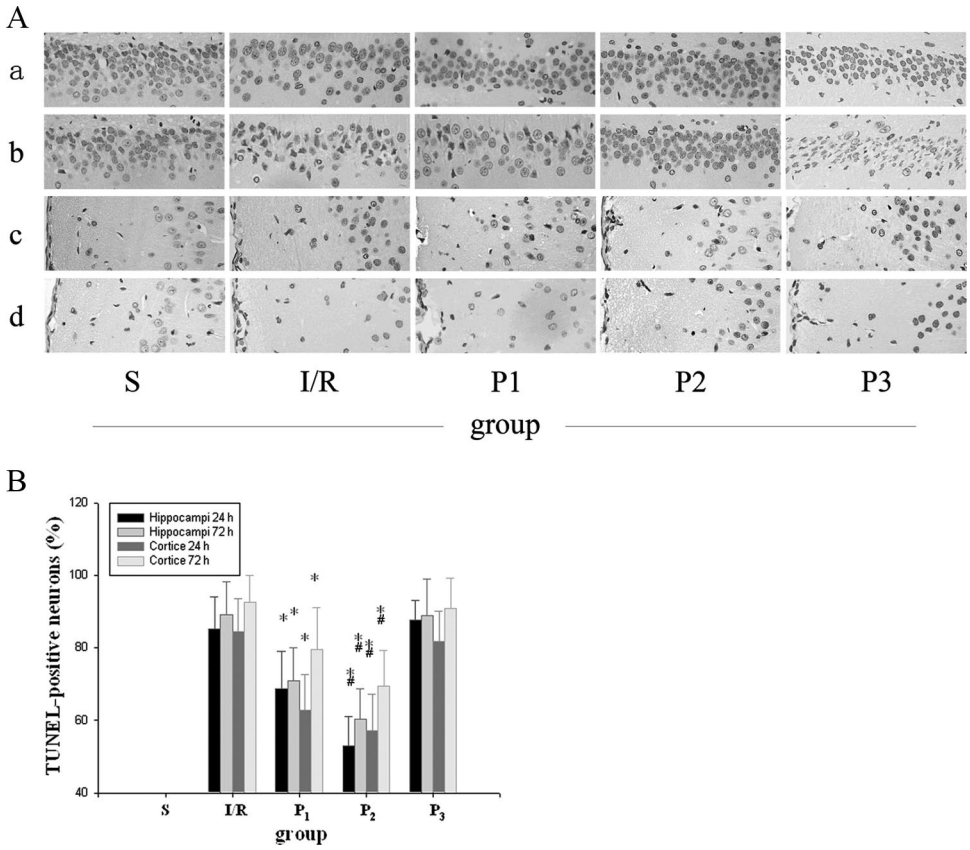

Fig. 4. (*A*) Immunohistochemical images of TdT-mediated dUTP nick end-labeling (TUNEL; ×400). (*a*) hippocampus after 24-h reperfusion; (*b*) hippocampus after 72-h reperfusion; (*c*) cortices after 24-h reperfusion; and (*d*) cortices after 72-h reperfusion. (*B*) TUNEL-positive cell counts in hippocampus and cortices after 24 and 72 h of reperfusion. Data are presented as means ± SD, $^{*}P < 0.05$ *versus* ischemia–reperfusion (I/R) and P3 groups; $^{\#}P < 0.05$ *versus* P1 group.

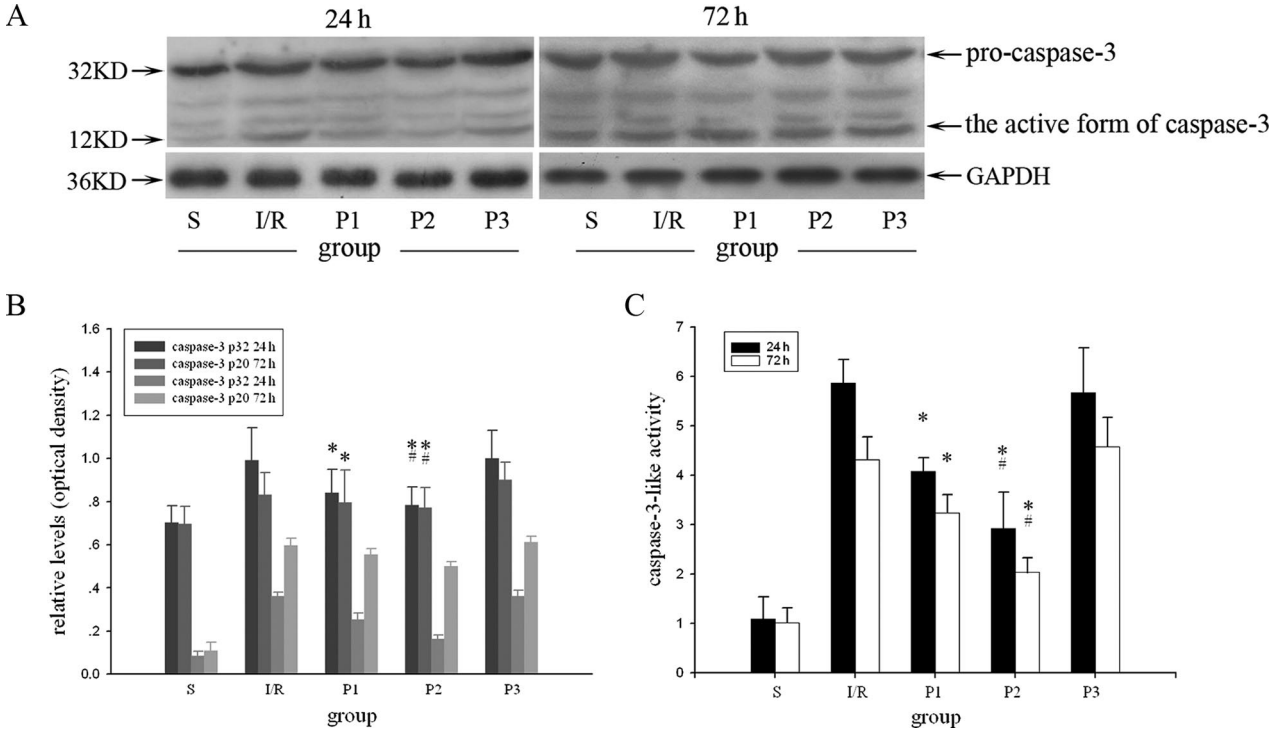

Fig. 5. Western blot analyses of caspase-3 (*A*, *B*) and measurements of caspase-3-like protease activity (*C*) after 24 and 72 h of reperfusion. Data are presented as means ± SD. (*B*) $^{*}P < 0.05$ *versus* caspase-3 p32 I/R and P3 groups; $^{\#}P < 0.05$ *versus* caspase-3 p32 P1 group. (*C*) $^{*}P < 0.05$ *versus* ischemia–reperfusion (I/R) and P3 groups; $^{\#}P < 0.05$ *versus* P1 group. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; KD = kilodalton.

reperfusion. Interestingly, in the preliminary studies, mild and moderate increases in carbon dioxide levels significantly attenuated brain edema formation compared with severe increases (data not shown). We also observed hypercapnia-induced histopathologic changes. Surprisingly, histologic improvements were observed in rats with $Paco_2$ 60–100 mmHg. Together, these results suggested that underlying mechanisms, other than the increase of ICP through increased cerebral blood volume, play a major role in the neuroprotective effect produced by mild and moderate hypercapnia. To elucidate the possible mechanisms, this study was performed to investigate the potential effects of permissive hypercapnia on neurologic behavioral assessments, ultrastructural histopathologic changes, neuronal apoptosis, and cerebral edema in an animal model of transient global cerebral ischemia.

Previous studies have demonstrated that hypercapnia seems to provide additional benefits.[17] For example, in I/R, xanthine oxidase intensifies the production of superoxide, and this is attenuated by the presence of hypercapnic acidosis.[18,19] Similarly, in an isolated blood-perfused heart model, hypercapnia improves coronary blood flow and functional recovery.[6] Furthermore, small tidal volume ventilation (associated with increased $Paco_2$ and hypercapnic acidosis) improves the outcome of patients with acute respiratory distress syndrome as a result of decreased mechanical stretch of the diseased pulmonary tissues.[20,21] In experimental brain studies, hypercapnia increases cerebral blood flow and decreases cerebrovascular resistance through vasodilatation of the precapillary cerebral arterioles, whereas hypocapnia does the opposite.[22,23] In a recent study in swine, hypercapnia and hypocapnia were shown to influence brain oxygen tension during hemorrhagic shock,[24] whereas hyperventilation and the resulting hypocapnia decreased cerebral oxygen pressure a further 56%. Hypercapnia has been used clinically to improve cerebral perfusion during carotid endarterectomy[25,26] and for emergency treatment of retinal artery occlusion.[27] These observations, taken together, suggest that permissive hypercapnia could increase brain oxygen supply and subsequently reduce I/R-induced brain injury after global cerebral I/R.

In this model of global cerebral I/R, the pyramidal cell layer of the hippocampus and the Purkinje-cell layer of the cerebellum are selectively vulnerable to ischemia.[28] The selective vulnerability of these neurons may be related to the high concentration of excitatory neurotransmitters (*i.e.*, glutamate) in these regions.[29] Excessive interstitial glutamate in the hippocampus has been found during transient global cerebral ischemia, and it may worsen the ischemic injury by facilitating calcium entry into the neuron.[30] A previous report by Vannucci *et al.*[1] showed that hypercapnia can reduce ambient levels of excitatory amino acids such as glutamate. In the current study, we found that rats with $Paco_2$ 60–100 mmHg had an improvement in ultrastructural histopathologic outcome. In contrast, the neurons in rats subjected to $Paco_2$ 100–120 mmHg were morphologically damaged 24 and 72 h after reperfusion. These results support the hypothesis that permissive hypercapnia in rats would attenuate histologic injury after transient global cerebral I/R.

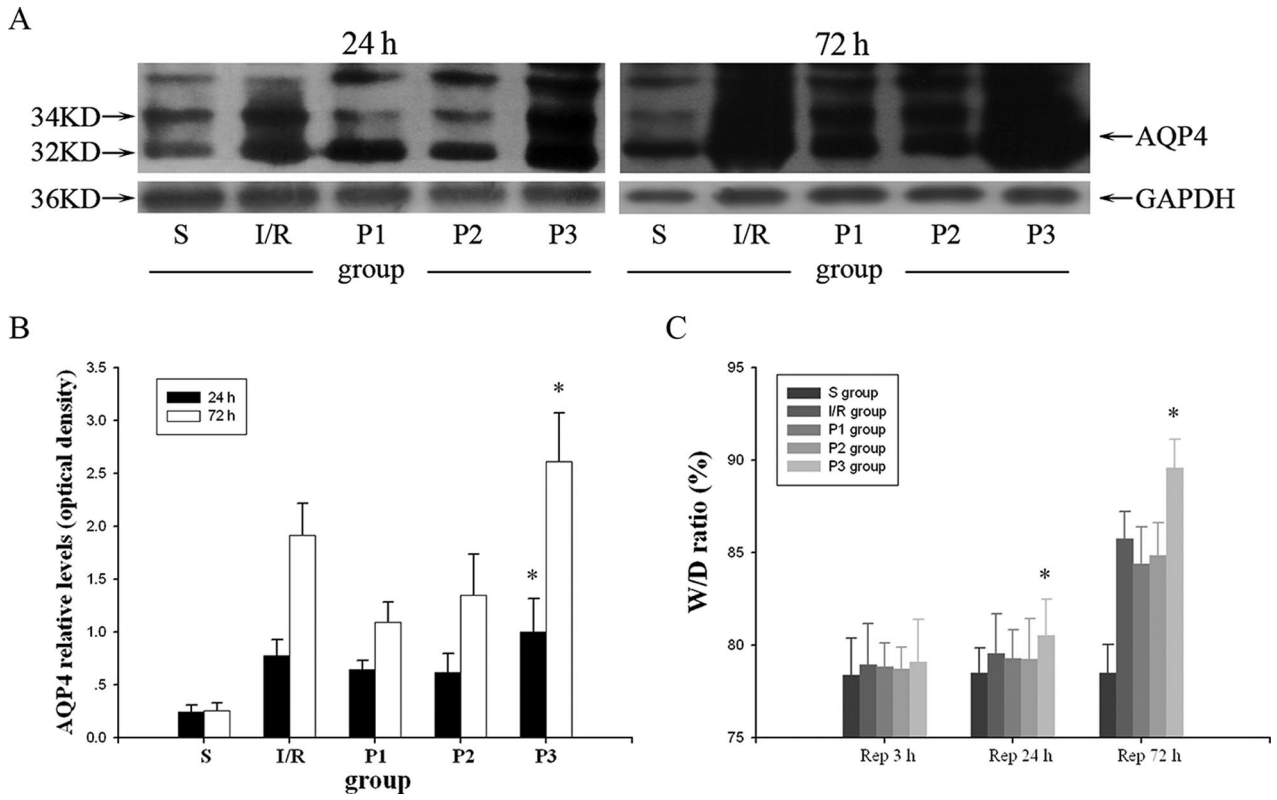

Fig. 6. Western blot analyses of AQP4 (*A*, *B*) and measurements of brain wet-to-dry (W/D) weight ratio after 24 and 72 h of reperfusion (*C*). Data are presented as mean ± SD. (*B*) **P* < 0.05 *versus* ischemia–reperfusion (I/R), P1, and P2 groups. (*C*) **P* < 0.01 *versus* I/R, P1, and P2 groups. AQP4 = aquaporin-4; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; KD = kilodalton.

The evaluation of the functional outcome, in addition to histologic changes, is increasingly acknowledged in the experimental cerebral I/R model because improvement of the functional outcome is a primary goal of cerebroprotective therapies. Several studies have shown that the peak of CA1 damage appears on the third day after ischemia in both rats and mice.[31,32] In our experiment, the NDS was significantly reduced in rats with Pa_{CO_2} 60–100 mmHg 24 and 72 h after reperfusion. These results support the hypothesis that permissive hypercapnia in rats attenuates functional injury after transient global cerebral I/R.

In the current study, relevant physiologic variables were monitored and controlled with one exception. Plasma glucose levels were not monitored because of concerns over blood volume after repeated blood gas analysis; a hypovolemic state may have influenced the overall outcome. Some physiologic differences were present among groups. Despite being statistically significant, these differences, except MAP, were unlikely to have influenced outcome. Blood pressure during reperfusion is an important determinant of neurologic outcome. An increase of MAP may improve neurologic function and CBF in subacute ischemic stroke[33] or may alleviate the degree of neurologic dysfunction during acute ischemic stroke.[34] In our experiment, the animals that were administered carbon dioxide had an obviously higher MAP after reperfusion. As expected, we found that mild and moderate hypercapnia had a neuroprotective effect in rats with cerebral I/R injury. Meanwhile, the various degrees of hypercapnia, despite higher ICP levels, did not increase the levels of brain water content 3 h after the onset of reperfusion. This result suggests that permissive hypercapnia does not cause cerebral edema during early reperfusion. Furthermore, severe hypercapnia resulted in higher brain water content and AQP4 levels 24 and 72 h after the onset of reperfusion. It is well known that AQP4 is the major water channel in the brain, and AQP4 has been proposed to play an important role in the pathophysiology of brain edema.[35–39]

An important mechanism by which neuronal apoptosis occurs is *via* the activation of caspases. Caspase-3 has been termed the "common executioner" for various cell death pathways and is initially present as a proenzyme (32 kd precursor protein) that is subsequently transformed into an active heterodimeric complex through a cascade of proteolytic events.[40,41] The active form of caspase-3 is composed of two subunits of 20 kd (p20) and 11 kd (p11), which are derived from proteolytic processing of the 32-kd precursor during apoptosis.[42–44] In the current study, mild and moderate hypercapnia reduces the number of TUNEL-positive neurons and favorably modulates apoptosis-regulating proteins. These findings provide evidence that permissive hypercapnia attenuates caspase-3 activation and promotes the survival of neurons after global cerebral I/R. In addition, our results indicate that Pa_{CO_2} 100 mmHg may be the upper limit of the hypercapnic neuroprotective range.

The current study suggests a promising therapeutic use for permissive hypercapnia in cerebral ischemia. However, the precise mechanism by which hypercapnia reduced the cerebral damage after global cerebral I/R is unknown. Much work remains to be done before the diverse effects of hypercapnia and acidosis are fully understood.

In conclusion, our study demonstrates that mild and moderate increases in the levels of carbon dioxide ($PaCO_2$ 60–100 mmHg) are neuroprotective in transient global cerebral I/R injury and that this protection is probably associated with influencing apoptosis-regulating proteins after cerebral I/R. In contrast, animals exposed to severe hypercapnia ($PaCO_2$ 100–120 mmHg) are more brain damaged compared with mild and moderate hypercapnia. This may be attributed to increased brain edema.

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