Focal cerebral ischemic tolerance and change in blood-brain barrier permeability after repetitive pure oxygen exposure preconditioning in a rodent model

*Xi Wang, MD, PhD,¹ Kai Kang, MD,^{2,3} Shiquan Wang, MD,² Jianhua Yao, MD,² and Xijing Zhang, MD, PhD^{2,4}

¹Institute of Neurosciences, Fourth Military Medical University; ²Department of Anesthesiology and ⁴ICU Center, Xijing Hospital and Fourth Military Medical University; and ³Hospital of PLA 451, Xi'an, China

OBJECTIVE The goal of this study was to demonstrate that repetitive pure oxygen exposure preconditioning (O₂PC) for 8 hours per day for 3 or 7 days, a practicable preconditioning for clinical use, is able to induce cerebral ischemic tolerance (IT) and further clarify the accompanying changes in the blood-brain barrier (BBB) that may be involved.

METHODS A total of 68 adult male Sprague-Dawley rats and eight 1-day-old rat pups were used in this study. The adult rats were exposed to pure O₂ (38 rats) 8 hours a day for 3 or 7 days or to room air (in an identical setup) for 8 hours a day for 7 days as controls (30 rats). Arterial O₂ tension (PaO₂) was measured in 6 rats exposed to O₂ and 3 controls. Focal cerebral ischemia was elicited by middle cerebral artery occlusion (MCAO) in 37 rats, of which 21 had been exposed to pure O₂ for 3 or 7 days and 16 to room air for 7 days as controls. Neurological behavior was scored with the Garcia score in 15 MCAO rats, of which 10 had been exposed to pure O₂ for 3 or 7 days and 5 to room air for 7 days as controls, and cerebral infarct volumes were assessed with TTC (2,3,5-triphenyltetrazolium chloride) staining in 10 rats (5 from each group) after 7 days of exposure. Formamide-extraction method was used to detect leakage of Evans blue (EB) dye in 7 rats exposed to pure O₂ for 7 days and 7 exposed to room air for 7 days. Fluorescence microscopy was used to analyze the leaked EB in the nonischemic areas of 4 rats exposed to pure O₂ for 7 days and 4 exposed to room air for 7 days before MCAO and the brain of the rats that had not been subjected to MCAO. Astrocyte changes associated with O₂PC were evaluated by means of fluorescence microscopy and electron microscopy in 14 rats that were exposed to the same O₂ or control conditions as the MCAO rats but without MCAO. Astrocytes were also obtained from 8 rat pups and cultured; levels of AQP4 and VEGF were detected by Western blot and ELISA in cells with and without O₂ treatment.

RESULTS A significant increase in PaO₂ was seen after O₂PC. The neurological score was significantly increased in the O₂PC groups (10.6 ± 0.6 in the 3-day O₂PC group, $p < 0.05$; 12 ± 0.84 in the 7-day O₂PC group, $p < 0.05$) compared with the control group (7 ± 0.55). The ratio of cerebral infarct volume to contralateral cerebral hemisphere volume was significantly lower in the O₂PC group than in the control group (0.204 ± 0.03 vs 0.48 ± 0.05, $p < 0.05$). The amount of leaked EB in the ischemic cerebral hemisphere was also lower in the O₂-treated rats than in controls (7.53 ± 1.4 vs 11.79 ± 3.3 µg EB/g brain weight, $p < 0.05$). However, fluorescence microscopy showed significantly greater BBB permeability in the nonischemic areas in the O₂PC group than in controls ($p < 0.05$). More red fluorescence could be observed in the nonischemic areas in both the ipsilateral and contralateral sides of the ischemic brain in the O₂PC animals than in the nonischemic areas in the corresponding sides of the controls. Further investigation of the effect of the O₂PC itself on the BBB of rats that were not subjected to MCAO showed that there was no EB leakage in the brain parenchyma in the rats exposed to room air, but some red fluorescence patches were noticed in the normal brain from the rats in the O₂PC group. Astrocytes, including those from areas around the BBB, were activated in the O₂PC group. Levels of both aquaporin 4 (AQP4) and vascular endothelial growth factor (VEGF) were significantly increased in cultured astrocytes after O₂PC.

CONCLUSIONS These findings suggest that O₂PC is able to induce IT, which makes it a strong candidate for clinical use. Moreover, O₂PC can also promote BBB opening, which may contribute to the induction of IT as well as representing a possi-

ABBREVIATIONS AQP4 = aquaporin 4; BBB = blood-brain barrier; BSA = bovine serum albumin; EB = Evans blue; FBS = fetal bovine serum; GFAP = glial fibrillary acidic protein; HBA = hyperbaric air; HBO = hyperbaric oxygen; IP = intraperitoneally; IT = ischemic tolerance; MCAO = middle cerebral artery occlusion; O₂PC = pure oxygen exposure preconditioning; PaO₂ = partial pressure of O₂, arterial; PC = preconditioning; TTC = 2,3,5-triphenyltetrazolium chloride; VEGF = vascular endothelial growth factor.

SUBMITTED September 24, 2014. **ACCEPTED** July 16, 2015.

INCLUDE WHEN CITING Published online January 29, 2016; DOI: 10.3171/2015.7.JNS142220.

* Drs. X. Wang and Kang contributed equally to this work.

ble strategy for promoting drug transportation into the CNS. Activated astrocytes are likely to be involved in these processes through astrocyte-derived factors, such as AQP4 and VEGF.

<http://thejns.org/doi/abs/10.3171/2015.7.JNS142220>

KEY WORDS repetitive pure oxygen exposure; preconditioning; ischemic tolerance; blood-brain barrier; astrocytes; vascular disorders

As pointed out by Durukan and Tatlisumak⁷ in their review article, Nietzsche's famous maxim—"What doesn't kill me makes me stronger"—aptly describes preconditioning and ischemic tolerance: surviving a sublethal noxious stimulus may result in the ability to survive an otherwise lethal subsequent ischemic insult. This phenomenon, referred to as preconditioning (PC) and ischemic tolerance (IT),^{7,14} holds substantial promise for identifying novel neuroprotective strategies for stroke, neurodegenerative diseases, and other various neurological disorders. IT is actually an endogenous protective mechanism. PC can involve different stimuli that trigger endogenous protective or regenerative mechanisms, thereby providing protection from subsequent ischemic injury.⁶ Different types of PC have been used to induce cerebral IT, including the use of endotoxin,⁷ hypoxia,^{1,13} hypothermia,¹⁹ hyperthermia,⁵ inhalation of anesthetics,³⁴ electroacupuncture,²⁹ 3-nitropropionic acid,²³ tumor necrosis factor- α ,¹⁷ and hyperbaric oxygen (HBO),^{25,26,30} since the first description of ischemic PC in the brain.¹⁴ However, the side effects or toxicity of these types of PC are problematic for translation into clinical applications, and a more practicable form of PC is needed. In HBO preconditioning, Wada et al.²⁶ found that pretreatment with HBO induced IT in the gerbil hippocampus; however, pretreatment with hyperbaric air (HBA) did not. They noted that oxygen was thought to induce IT through the generation of free radicals as opposed to hyperbaric effect. We think that treatment with pure oxygen (O₂) inhalation is simpler and more feasible than treatment with HBO. So, we directly investigated the role of pure O₂ inhalation at atmospheric pressure in inducing IT in our previous study. It was demonstrated that a single period of 24 hours, but not 6 or 12 hours, of pure O₂ inhalation could induce IT in focal cerebral ischemic/reperfusion rats.³² Therefore, we hypothesized that pure O₂ inhalation may be a strong candidate for clinical use as a powerful PC method before subsequent ischemic brain injury. In our previous study, we demonstrated the efficacy of 24 hours of pure O₂ inhalation for PC, but it is necessary to investigate shorter O₂ exposure periods that would be more feasible for clinical use. We have demonstrated that one possible mechanism by which pure O₂ inhalation induces IT is via induction of O₂ free radicals,³² but many more mechanisms remain to be investigated.

The blood-brain barrier (BBB) is a dynamic barrier between the bloodstream and the central nervous system that consists of microvascular endothelial cells, pericytes, and the basal lamina, as well as astrocyte endfeet. In contrast to the peripheral capillary vessels, the BBB has low and selective permeability because of its special structure. BBB permeability is regulated via different mechanisms on different cell types to match the brain activities, either for adjustment of circulating elements or for facilitation of local repair.

Although it plays a crucial role in the pathogenesis of ischemic brain injury, little attention has been paid so far to BBB in various types of PC. Cevik et al.,³ however, did report that BBB permeability to EB dye significantly increased in animals in an HBO treatment group compared with those in an HBA group, suggesting that the increased partial pressure of O₂ (rather than the hyperbaric condition itself) has an impact on BBB integrity. Therefore, O₂ may impact BBB permeability.

Astrocytes are glial cells whose endfeet form a lacework of fine lamellae closely apposed to the outer surface of the BBB endothelium and basement membrane of the BBB.² Astrocyte metabolic inhibition by fluorocitrate has been shown to abolish ischemic PC protection, confirming the critical role of astrocytes in ischemic PC protection.¹² Many astrocyte factors are able to induce specific BBB features. The membrane protein aquaporin 4 (AQP4) is expressed by astrocytes, particularly by perivascular astrocyte endfeet, ependymal glial cells, and the glia limitans, and a close relationship between AQP4 and BBB has been demonstrated.¹⁸ Transgenic mice lacking AQP4 have provided compelling evidence for involvement of AQP4 in cerebral water balance, astrocyte migration, and maturation of the BBB.^{18,24} AQP4 plays a crucial role in cerebral ischemia/reperfusion injury, and AQP4 knockout has been shown to aggravate ischemia/reperfusion injury in mice.³¹ Vascular endothelial growth factor (VEGF) is a secreted mitogen associated with angiogenesis and is also a potent vascular permeability factor. Administration of VEGF can increase BBB permeability.^{22,33} Several cell types express VEGF, including neurons, astrocytes, microglia, and choroid plexus epithelial cells.¹⁵ In ischemic stroke, VEGF expression is increased not only in the area around the infarct but also in remote cortical regions.¹⁵ VEGF is also able to induce IT. Neuroprotection by hypoxia preconditioning required the increase of VEGF and the activation of VEGF receptor.²⁸ A significant reduction of the infarct volume was induced with VEGF treatment in stroke rats.⁹

We investigated the use of 8 hours per day of pure O₂ inhalation, a short-duration PC per cycle, for 3 days or for 1 week to verify our hypothesis that repetitive, short-duration pure O₂ exposure PC is capable of inducing IT. We speculated that the permeability of the BBB is influenced by repetitive pure O₂ inhalation PC and activated astrocytes may be involved in these events through expression of AQP4 and VEGF, which are closely associated with BBB permeability and the induction of IT.

Methods

Study Animals

A total of 76 Sprague-Dawley rats were used for this study (68 adult males weighing 180–250 g and eight 1-day-

old pups). The animals were provided by the Laboratory Animal Center of the Fourth Military Medical University. All experiments were performed in compliance with the Ethics Committee for Animal Experimentation and carried out according to the Guidelines for Animal Experimentation of the Fourth Military Medical University.

Repetitive O₂ Exposure PC and IT

A total of 37 adult rats were used in this part of the experiment to study the effect of repetitive O₂ exposure PC (O₂PC) on IT. All of the animals were subjected to middle cerebral artery occlusion (MCAO).

Repetitive Pure O₂ Exposure PC

The procedure for pure O₂ exposure was described in detail in our previous report.³² In brief, adult male rats ($n = 21$) were placed in a pure O₂ chamber for 8 hours per day for 3 days ($n = 5$) or for 1 week ($n = 16$). An O₂ analyzer (Brüel & Kjær) was used to monitor the O₂ concentration in the container. After each preconditioning treatment, the rats were returned to their home cages. Sixteen male rats were placed in an identical chamber with room air for 8 hours per day for 7 days as controls.

Focal Cerebral Ischemia

Focal cerebral ischemia was elicited by MCAO. The surgery for MCAO was carried out under isoflurane anesthesia, as previously described.³² Twenty-four hours after the last exposure to pure O₂ or room air, MCAO was induced. Briefly, the right common carotid artery and the right external carotid artery were exposed and ligated. A blunt tip of 3-0 nylon monofilament suture was inserted into the internal carotid artery, 17–18 mm distal to the carotid bifurcation, until a mild resistance was felt. Thereby, the origins of the anterior cerebral and middle cerebral arteries were occluded. Reperfusion was accomplished by withdrawing the suture after 2 hours of ischemia. The rats were returned to their cages with free access to food and water after recovering.

Neurological Score

Twenty-four hours after reperfusion, 15 animals (5 from the 7-day O₂PC group, 5 from the 3-day O₂PC group, and 5 controls) were assessed neurologically according to the method of Garcia et al.¹¹ The Garcia neurological score is based on a sequence of 6 different physiological criteria: spontaneous activity, symmetry in the movement (4 limbs), forepaw outstretching, climbing, body proprioception, and response to vibrissae touch. The overall score for a healthy rat with no neurological deficit is 18 points.

Infarct Volume Assessment

TTC (2,3,5-triphenyltetrazolium chloride) staining was used to assess the infarct volume as previously described.³² After undergoing the neurological assessment described above, 10 rats (5 from the MCAO with one week of O₂PC group and 5 from the control MCAO without O₂PC group) were anesthetized with 4% isoflurane in O₂ and decapitated. The brains were rapidly removed and cooled in iced saline for 10 minutes. Six 2-mm-thick coronal sections were

stained with 2% TTC to evaluate the infarct volume. Unstained areas were defined as infarct, and the red-stained areas as noninfarct. The unstained area volume was calculated by subtracting the noninfarct area in the ischemic hemisphere from that of the contralateral hemisphere in each slice to avoid the effect of edema in the ischemic area, multiplying the result by slice thickness (2 mm), and then calculating the sum for 6 slices. The result was shown as the ratio of the volume of the unstained area to the volume of the contralateral cerebral hemisphere to avoid the effect of possible edema in the nonischemic area.

Evaluation of BBB Permeability

BBB permeability was evaluated by using Evans blue (EB, Sigma). EB is a diazo blue dye of 960 D. It binds to plasma albumins and becomes fluorescent after it is injected intravenously. It is not able to cross the intact BBB after binding to proteins and only leaks into the brain parenchyma through areas in which the BBB is absent, immature, or disrupted; thus, it is widely used to assess BBB integrity. A solution of 2% EB in normal saline was injected intravenously (4 ml/kg, through a tail vein) into 22 adult male rats (11 that had been exposed to 7-day O₂PC and 11 controls) 24 hours after MCAO and reperfusion. Twenty minutes later, the rats were anesthetized deeply with 1% sodium pentobarbital and transcardially perfused with 300 ml of normal saline to flush away the blood and EB in blood vessels. Then the brains were removed. Two methods were used to detect the leakage of EB in brain parenchyma. To detect the leaked EB in nonischemic brain areas, 40- μ m-thick coronal sections were cut with a cryostat microtome and mounted on slides for observation under the fluorescence microscope (BX51, Olympus). To detect leakage in the ischemic hemispheres, the hemispheres were removed and weighed before being cut into small pieces and then immersed in a formamide solution at 50°C for 72 hours for EB extraction. The solution containing the small pieces of brain was then centrifuged at 2000 rpm (340g) for 10 minutes to collect the supernatant. The EB content of the supernatant was detected using a spectrophotometer (wavelength 630 nm). The EB leakage was expressed as micrograms of EB per gram of brain weight.

Effect of O₂PC on Astrocytes in Normal Brain and in Culture

A total of 31 adult male rats and eight 1-day-old pups were used in studying the effect of O₂PC on astrocytes. None of these animals were subjected to MCAO.

Measurement of PaO₂

For determination of the partial pressure of oxygen (PaO₂, arterial O₂ tension), 9 adult rats were anesthetized with chloral hydrate (300 mg/kg, IP) and arterial blood was sampled from the femoral artery immediately after the end of exposure to O₂ ($n = 6$) or room air ($n = 3$). Arterial blood gases were measured with the OMNI modular system (AVL List GmbH Medizintechnik).

Evaluation of BBB Permeability

Briefly, 8 rats were exposed to O₂PC ($n = 4$) or normal

room air ($n = 4$) for 8 hours a day for 7 days as described under *Repetitive O₂ Exposure PC and IT*, and then, immediately after the last session, they were injected intravenously with 2% EB as described above (under *Evaluation of BBB Permeability* in the section on *Repetitive O₂ Exposure PC and IT*). Twenty minutes later, the rats were anesthetized deeply with 1% sodium pentobarbital and transcardially perfused with 300 ml of normal saline. The brains were removed and 40- μ m-thick coronal sections were cut with a cryostat microtome and mounted on slides for observation under the fluorescence microscope.

Ultrastructure Study Using Transmission Electron Microscopy

For ultrastructural study, 6 adult rats (3 that had been treated with O₂PC for 8 hours a day for 7 days and 3 controls exposed to room air under identical conditions, as described above under *Repetitive O₂ Exposure PC and IT*) were perfused transcardially with 150 ml of normal saline, followed by 500 ml of ice-cold mixture of 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The brain cortices were dissected out and postfixed by immersion in the same fixative for 2 hours at 4°C followed with 0.5% osmium tetroxide in 0.1 M phosphate buffer for 1 hour. Fixed samples were dehydrated in a graded ethanol series and then in propylene oxide and finally were embedded in embedding medium (Epon 812). Ultrathin sections were cut with an ultramicrotome (Leica EM UC6) and mounted on mesh grids and then counterstained with uranyl acetate and lead citrate. Stained ultrathin sections were observed under a JEM-1230 electron microscope (JEOL Ltd.).

Astrocyte Primary Culture

Astrocyte cultures were prepared as described previously.¹⁶ Briefly, cerebral cortices were harvested from eight 1-day-old pups and mechanically dissociated after the pups were decapitated. The cell suspension was diluted in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were seeded in 75-cm² flasks coated with poly-L-ornithine hydrobromide at a density of 2×10^6 cells/flask in a humidified tissue culture incubator with 5% CO₂ and maintained at 37°C. Ten days after seeding, microglia and oligodendrocytes were removed by orbital shaking of the culture flasks at 260 rpm for 24 hours at 37°C. A pure astrocyte layer was left adhering to the bottom.

The purified astrocytes were then subcultured. Some of the astrocytes were cultured in a dish and divided into 2 groups: astrocytes in the control group were incubated in air for 6 hours; astrocytes in the treatment group were incubated in 100% O₂ for 6 hours. Then, the supernatant and cell lysate were collected individually ($n = 3$ samples per group) to detect VEGF by means of enzyme-linked immunosorbent assay (ELISA). Some of the cells were cultured on poly-L-ornithine hydrobromide-coated coverslips to be stained with AQP4 antibody using immunofluorescence methodology ($n = 4$ samples per group).

Immunofluorescence Staining

For immunofluorescence staining of brain sections, 8

adult male rats (4 that had been pretreated with O₂ for 8 hours a day for 7 days, and 4 controls exposed to room air under identical conditions) were deeply anesthetized with pentobarbital (60 mg/kg) intraperitoneally (IP) and perfused transcardially with 4% paraformaldehyde. After perfusion, the animals' brains were removed and immersed in 20% sucrose at 4°C overnight. Forty-micron-thick coronal sections were cut with a cryostat microtome and mounted on gelatin-coated slides for staining.

The brain sections were incubated with rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1:3000, DAKO) at 4°C overnight after incubation in 1% bovine serum albumin (BSA), followed by incubation with goat anti-rabbit IgG antibody (Alexa Fluor 488, 1:400, Molecular Probes) for 2 hours at room temperature. Primary and secondary antibodies were diluted in phosphate-buffered saline containing 1% BSA.

For characteristic identification of cultured astrocytes and detection of AQP4 expression, cultured cells were seeded on poly-L-ornithine hydrobromide-coated coverslips and fixed with 4% paraformaldehyde for 20 minutes at 4°C. Fixed cells were incubated with rabbit anti-GFAP or rabbit anti-AQP4 (1:500, Millipore) antibodies at 4°C overnight after incubation in 1% BSA, and then incubated with goat anti-rabbit Immunoglobulin G (IgG) antibody (Alexa Fluor 488) for 2 hours at room temperature. Hoechst 33342 was used to stain the nuclei. The stained brain sections and cultured cells were observed and photographed under a fluorescence microscope (BX51, Olympus).

For quantification of GFAP and AQP4 expression, the GFAP-positive areas in the cortex and the fluorescence density of AQP4-positive cells were analyzed by using Image-Pro Plus 5.0 software (Media Cybernetics).

Western Blot

Western blot analysis was performed as previously described to further detect the expression of AQP4 in cultured astrocytes.²⁷ Briefly, cultured astrocytes were harvested in radioimmunoprecipitation assay (RIPA) buffer containing 2% protease inhibitor (Boehringer Mannheim). Cell lysates were loaded onto 10% polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were incubated with rabbit anti-AQP4 (1:1000) primary antibody at 4°C overnight and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:3000, Jackson ImmunoResearch) successively and developed with an ECL (enhanced chemiluminescence) kit (Millipore). The bands were then stripped from the membranes with stripping buffer, and the membranes were incubated with rabbit anti- β -actin (1:1000, Sigma) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody successively and then developed with an ECL kit again to observe the β -actin bands. The intensity of immunoblotting bands was analyzed using Image-Pro Plus 5.0 software. Band intensities of AQP4 were normalized to those of corresponding β -actin.

Enzyme-Linked Immunosorbent Assay

The supernatant and the cell lysate of cultured astrocytes were collected after exposure to pure O₂ or air to detect VEGF. Quantitation of VEGF was performed us-

ing a VEGF ELISA kit (R&D Systems) according to the manufacturer's instructions.

Statistical Analysis

All results are shown as mean $\pm$ SEM. All analyses were performed with statistical software SPSS (version 13.00). The differences between means were evaluated by 1-way ANOVA (Garcia score) or independent-samples t-test (all results except Garcia scores) and considered statistically significant at $p < 0.05$.

Results

Repetitive O₂ Exposure and IT

Analysis of PaO₂, Neurological Behavior, and Infarct Volume

At the end of the exposure period, the PaO₂ of animals exposed to pure O₂ (419.27 ± 27.94 mm Hg, $n = 6$) was much higher than that of controls exposed to room air (80.5 ± 5.67 mm Hg, $n = 3$; $p < 0.0001$; Fig. 1A).

Twenty-four hours after focal cerebral ischemia/reperfusion, neurological behavior was assessed according to the method of Garcia et al.¹¹ The post-MCAO Garcia score was 7 ± 0.55 in the control group ($n = 5$) and significantly higher in both of the O₂PC groups (10.6 ± 0.6 in the 3-day O₂PC group, $p < 0.05$; 12 ± 0.84 in 7-day O₂PC group, $p < 0.05$; $n = 5$ in each group) than in the control group (Fig. 1B). Additional studies were subsequently conducted using the 7-day O₂PC protocol.

The brains of the rats were removed and stained with 2% TTC to evaluate the infarct volume 24 hours after the focal cerebral ischemia/reperfusion. The results showed that the ratio of the infarct volume in the MCAO rat brain was significantly lower in the O₂PC group (0.204 ± 0.03 , $n = 5$) than in the control group (0.48 ± 0.05 , $n = 5$; $p < 0.05$; Fig. 1C and D).

Effect of O₂PC on the BBB Permeability After MCAO

The integrity of the BBB was assessed with EB extravasation. EB extravasation was detected in the ischemic area by means of the formamide-extraction method.⁸ There was significantly less EB extravasation in the O₂PC group (7.53 ± 1.4 μ g EB/g brain weight, $n = 7$) than in the control group (11.79 ± 3.3 μ g EB/g brain weight, $n = 7$; $p < 0.05$; Fig. 2A).

In the nonischemic areas, EB leakage was assessed by means of fluorescence microscopy. Leaked EB in the brain can excite red fluorescence. Red fluorescence should be absent from the normal brain parenchyma, except in the pineal body and choroid plexus, which lack BBB. However, red fluorescence patches of different sizes were observed in the parenchyma under the fluorescence microscope in all MCAO rats. Light EB leakage was observed in the nonischemic areas in all MCAO rats in the control group (Fig. 2B), but there was more EB leakage in the O₂PC group than in the control group in both ipsilateral and contralateral nonischemic areas ($n = 4$ in each

FIG. 1. PaO₂, Garcia score, and the infarct volume of MCAO rats. **A:** Arterial blood gas analysis showing the PaO₂ in the control group (CON, $n = 3$) and rats exposed to pure O₂ for 8 hours (O₂PC group, $n = 6$). **B:** Neurological assessment ($n = 5$ in each group) showing the increased Garcia score in the O₂PC group (10.6 ± 0.6 in the 3-day O₂PC group; 12 ± 0.84 in 7-day O₂PC group) compared with that in the control group (7 ± 0.55). **C:** Representative images of TTC staining showing the infarct brain sections in the control group and the 7-day O₂PC group. **D:** Bar graph showing the decreased ratio of the infarct volume relative to the contralateral hemisphere volume in the O₂PC group compared with that in the control group ($n = 5$ in each group). Error bars indicate SEMs. d = day. * $p < 0.05$, ** $p < 0.0001$ versus controls. Figure is available in color online only.

FIG. 2. Blood-brain barrier permeability of MCAO rats evaluated using EB. **A:** Content of leaked EB in the ischemic cerebral hemisphere of the MCAO rats detected by using formamide-extraction method. The amount of leaked EB was decreased in the O₂PC group compared with the control group ($n = 7$ in each group). **B:** Representative photomicrographs showing the leaked EB patches in sections from the nonischemic areas in the ipsilateral and the contralateral cerebral hemispheres in the control group and O₂PC group. **C:** Bar graph showing the increased area of the leaked EB patches in the nonischemic area of the ipsilateral cerebral hemisphere of the MCAO rats in the O₂PC group compared with that in the control group ($n = 4$ in each group). **D:** Bar graph showing the increased area of the leaked EB patches in the nonischemic area of the contralateral cerebral hemisphere of the MCAO rats in the O₂PC group compared with that in the control group ($n = 4$ in each group). * $p < 0.05$ versus controls. Figure is available in color online only.

group, $p < 0.05$; Fig. 2B–D), which suggested higher BBB permeability in nonischemic areas in the O₂PC group than in the control group.

Effect of O₂PC on the Normal Rat Brain and Cultured Astrocytes

To investigate the mechanism of IT induction, the effect of O₂PC on BBB integrity was studied in animals that had not been subjected to MCAO. Meanwhile, the astrocytes, one of the important players in the constitution of the special structure of BBB, were focused on in this study in both their structure and secretion function.

Effect of O₂PC on the BBB Integrity of the Normal Brain

Fluorescence microscopy showed no red fluorescent patches in the cerebral parenchyma of the controls; in contrast, some small red fluorescent patches appeared in some areas in the O₂PC group ($p < 0.0001$, Fig. 3A and B). The results indicated that O₂PC induced BBB opening.

Effect of O₂PC on GFAP-Positive Astrocytes

To investigate whether the astrocytes were involved in

the induction of BBB opening by O₂PC, the activation of astrocytes in the brain after O₂PC was observed. Astrocyte reactions are characterized by hypertrophy and hyperplasia of astrocytes. GFAP was used here as the astrocyte marker to observe the astrocyte reactions. In the control group, GFAP-positive cells were widespread in the brain with small cell bodies and short and thin processes (Fig. 4A). The mean GFAP-positive area per unit ($10^5 \mu\text{m}^2$) in the control group was $2370.81 \pm 427.276 \mu\text{m}^2$. In contrast, the GFAP-positive cell area was significantly greater in the O₂PC group ($6407.67 \pm 624.6 \mu\text{m}^2$, $p < 0.0001$, Fig. 4A and B), which suggested that the astrocytes in the O₂PC group were activated. The cell bodies of GFAP-positive cells were bigger and the processes were longer and thicker. The numbers of GFAP-positive cells in the 2 groups were not significantly different (Fig. 4C).

Astrocyte Ultrastructure Changes Following O₂PC

The ultrastructure of the astrocytes was also observed. Polyribosomes in the cytoplasm of astrocytes were seen infrequently in the control group (Fig. 4D), but there were more polyribosomes, especially membrane-bound polyri-

FIG. 3. BBB permeability of the normal rat brain after O₂PC. **A:** Representative photomicrographs showing the increase in leaked EB patches in the brain in the O₂PC group compared with that in the control group ($n = 4$ in each group). **B:** Bar graph showing the area of the leaked EB patches in the O₂PC group compared with that in the control group. ** $p < 0.0001$ versus controls. Figure is available in color online only.

bosomes, in the cytoplasm of astrocytes in the O₂PC group ($p < 0.05$, Fig. 4D and E). The increased presence of membrane-bound polyribosomes indicated that the astrocytes in the O₂PC group were in an active condition of synthesizing proteins, especially secretory proteins.

Effect of O₂PC on AQP4 Expression in Cultured Astrocytes

Astrocytes were isolated from the rat brain cortices and cultured *in vitro* to further study their synthesizing and secreting functions. GFAP was used to determine the purity

of the cultures. The result showed that more than 98% of the cultured cells were astrocytes (Fig. 5A).

AQP4 is expressed by astrocytes, and its expression has been demonstrated to be closely related with BBB permeability¹⁸ and ischemia/reperfusion injury.³¹ After staining with anti-AQP4 antibody, the fluorescence density of AQP4-positive cells was compared between the control and O₂PC groups. The fluorescence density was obviously stronger in the O₂PC group than in the control group ($p < 0.0001$, Fig. 5B and C), indicating much more AQP4

FIG. 4. Activated astrocytes after O₂PC in normal rat brain. **A:** Representative photomicrographs showing the GFAP-positive astrocytes in the rat cortices from the control and O₂PC groups as demonstrated by immunofluorescent staining. **B:** Bar graph showing the increased GFAP-positive cell area in sections from the O₂PC group compared with that in sections from the control group ($n = 4$ in each group). **C:** Bar graph showing the GFAP-positive cell numbers in the control and O₂PC groups. There was no significant difference between the 2 groups ($n = 4$ in each group). **D:** Representative transmission electron micrographs showing the polyribosomes and the membrane-bound polyribosomes (dark dots) in the cytoplasm of astrocytes in sections from the control and O₂PC groups. The right 2 images are magnifications of the frames in the left 2 images, respectively. **E:** Bar graph showing more polyribosomes in sections from the O₂PC group than in those from the control group ($n = 3$ in each group). * $p < 0.05$; ** $p < 0.0001$ versus controls. Figure is available in color online only.

FIG. 5. Changes of astrocyte-derived AQP4 and VEGF after incubation in pure O₂ for 6 hours. **A:** Cultured astrocytes identified by GFAP staining with > 98% purity. **B:** Representative images of immunofluorescent staining showing enhanced AQP4 expression in cultured astrocytes that were incubated in pure O₂ for 6 hours compared with those that were incubated in room air (control condition). **C:** Bar graph showing the ratio of AQP4-positive cell fluorescence density in the O₂-treated cells relative to the controls. **D:** Representative Western blot and bar graph showing the increased AQP4 protein in the O₂-treated cells compared with that in the controls. **E:** Bar graph showing the ratio of VEGF in the astrocyte lysate in the O₂-treated cells relative to that of the controls as demonstrated by ELISA. **F:** Bar graph showing the ratio of VEGF in the supernatant of cultured astrocytes treated with O₂ relative to that of controls as demonstrated by ELISA. **p* < 0.05 versus controls; ***p* < 0.0001. Figure is available in color online only.

protein was synthesized in the O₂PC group. Western blot results further showed the increased expression of AQP4 protein in the O₂PC group compared with the control group (*p* < 0.05, Fig. 5D).

Effect of O₂PC on VEGF Expression in Cultured Astrocytes

VEGF is a secreted mitogen associated with angiogenesis; it is also a potent vascular permeability factor and IT inducer.^{9,22,23} In the present study, the secreted VEGF in the supernatant of the cultured astrocytes and the expression of VEGF in the cell lysate were detected individually with ELISA methods. In the control group, low levels of VEGF were found in the supernatant and cell lysate. After O₂PC, levels of VEGF in both supernatant and cell lysis were increased compared with those in the control group in each group (*p* < 0.05, Fig. 5D and E), indicating that O₂PC enhanced the synthesis and secretion of VEGF from astrocytes.

Discussion

This study shows that repetitive pure oxygen exposure of 8 hours per day for 3 or 7 days is able to induce IT. We refer to this as repetitive pure oxygen exposure preconditioning (O₂PC) for purposes of this paper. In the current study, we found that in comparison with controls exposed to room air in an equivalent setting, rats in the O₂PC group had improved neurological behavior, decreased infarct

volume, and reduced EB leakage in the ischemic hemisphere after MCAO and reperfusion. These results suggest that O₂PC is a strong candidate for clinical use as a powerful PC for subsequent ischemic brain injury.

The results of this study also show that O₂PC induces hyperpermeability of the BBB in the nonischemic areas and the normal rat brain. It has been reported that BBB integrity in the ischemic area is severely disrupted following brain ischemia/reperfusion.²¹ BBB plays a crucial role in the pathogenesis of ischemic injury, but researchers seldom paid attention to it in PC studies. The BBB is capable of responding to various local physiological or pathological changes by changing its permeability. In most cases, interruption of BBB integrity is considered harmful because of its role in the maintenance of CNS homeostasis. But in some cases, increased BBB permeability is needed for therapeutic purposes. For example, in some brain diseases, it would be desirable for a drug to cross the BBB and reach the brain parenchyma via the blood circulation; drug transport to the brain is hampered by this almost impermeable and highly selective barrier, but researchers in different fields are working on developing methods of increasing BBB permeability, and it is likely that a multidisciplinary approach will be most fruitful.⁴ BBB hyperpermeability might also contribute to the resorption of the widespread edema in the later time points after cerebral ischemia.²⁰ BBB opening is actually a 2-edged sword, sometimes harmful and sometimes useful depending on

the brain situation. Since O₂PC is able to induce slight BBB opening in the normal brain, it is reasonable to use it as an effective therapeutic way to help medication cross the BBB to the brain parenchyma in some brain diseases.

BBB permeability increased in the nonischemic areas and the normal rat brain after O₂PC in this study. Is it good or bad for the induction of IT and the recovery of the brain from ischemia/reperfusion injury? It is believed that preconditioning induces 2 phases of IT: early and delayed IT. The development of early IT involves rapid changes in activity and posttranslational modifications of existing proteins, whereas delayed IT requires gene induction and de novo protein synthesis.⁷ It is possible that O₂PC induces IT by facilitating the communication between the CNS and blood via the appropriately opening BBB, which would make it possible to use proteins or other factors existing within or outside of the CNS to induce gene expression and de novo protein synthesis. It is also possible that O₂PC played a role in the recovery of the brain from cerebral ischemic/reperfusion injury by promoting the removal of deleterious substances produced after ischemia/reperfusion, by allowing neuroprotective agents to cross the more permeable BBB to reach the brain parenchyma, and by facilitating resorption of the widespread edema in the late time points after the cerebral ischemia and/or by other yet-unknown mechanisms. It was recently shown that the enhanced BBB permeability associated with ischemia/reperfusion injury facilitated a significant increase in penetration of ginkgolide B, a potential neuroprotective agent, through the BBB in an ischemia/reperfusion injury rat model compared with what was observed in normal rats.¹⁰ Further investigation is needed to elucidate the role of BBB opening in the O₂PC model.

In contrast to the composition of the peripheral capillary, extracellular base membrane, pericytes, and astrocytes are all integral parts of the BBB in addition to endothelial cells. However, much more attention has still been paid to the endothelial cells than to other components in BBB studies. It was reported recently that reactive astrocytes mediate disruption of BBB integrity through the angiogenic factor VEGF as an important astrocyte-derived inducer of BBB disruption to drive vascular permeability and CNS damage in acute inflammatory lesions.¹ In this study, we focused on astrocyte changes in order to investigate part of the mechanism of O₂PC. Astrocytes were found to be activated after O₂PC, with an increase in GFAP expression, polyribosomes, and membrane-bound polyribosomes. Many factors can be synthesized and secreted by activated astrocytes. AQP4 and VEGF, 2 important factors closely related to BBB permeability and the induction of IT, were found to be significantly increased following O₂PC. These results suggest that astrocytes may be involved in the induction of BBB opening and IT by O₂PC, and AQP4 and VEGF from the astrocytes may contribute to this process.

Conclusions

In summary, we have shown that O₂PC is able to induce IT in this study. O₂PC is simple and feasible and represents a promising strategy as a powerful PC for the mitigation

of subsequent ischemic brain injury. We also found that O₂PC increases the permeability of BBB in the nonischemic area after MCAO/reperfusion and in the normal rats, which may contribute to the induction of IT and/or be used to promote drug transportation from blood to CNS. It is further proved that astrocytes can be activated and be caused to increase the expression of AQP4 and VEGF, 2 important factors closely related to BBB opening and the induction of IT. We speculate that astrocytes are involved in the induction of IT and BBB opening through synthesizing and secreting some related factors, such as AQP4 and VEGF, during O₂PC. Meanwhile, much more investigation still needs to be done to definitively determine the role of BBB opening in the O₂PC model.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81171156 to Xi Wang and 81272135 to Xijing Zhang).

References

- Bernaudo M, Nedelec AS, Divoux D, MacKenzie ET, Petit E, Schumann-Bard P: Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. *J Cereb Blood Flow Metab* 22:393–403, 2002
- Cardoso FL, Brites D, Brito MA: Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches. *Brain Res Brain Res Rev* 64:328–363, 2010
- Cevik NG, Orhan N, Yilmaz CU, Arican N, Ahishali B, Kucuk M, et al: The effects of hyperbaric air and hyperbaric oxygen on blood-brain barrier integrity in rats. *Brain Res* 1531:113–121, 2013
- Chen Y, Liu L: Modern methods for delivery of drugs across the blood-brain barrier. *Adv Drug Deliv Rev* 64:640–665, 2012
- Chopp M, Chen H, Ho KL, Dereski MO, Brown E, Hetzel FW, et al: Transient hyperthermia protects against subsequent forebrain ischemic cell damage in the rat. *Neurology* 39:1396–1398, 1989
- Dirnagl U, Becker K, Meisel A: Preconditioning and tolerance against cerebral ischaemia: from experimental strategies to clinical use. *Lancet Neurol* 8:398–412, 2009
- Durukan A, Tatlisumak T: Preconditioning-induced ischemic tolerance: a window into endogenous gearing for cerebroprotection. *Exp Transl Stroke Med* 2:2, 2010
- Durward QJ, Del Maestro RF, Amacher AL, Farrar JK: The influence of systemic arterial pressure and intracranial pressure on the development of cerebral vasogenic edema. *J Neurosurg* 59:803–809, 1983
- Eicker SO, Hoppe M, Etminan N, Macht S, Perrin J, Steiger HJ, et al: The impact of experimental preconditioning using vascular endothelial growth factor in stroke and subarachnoid hemorrhage. *Stroke Res Treat* 2013:948783, 2013
- Fang W, Deng Y, Li Y, Shang E, Fang F, Lv P, et al: Blood brain barrier permeability and therapeutic time window of Ginkgolide B in ischemia-reperfusion injury. *Eur J Pharm Sci* 39:8–14, 2010
- Garcia JH, Wagner S, Liu KF, Hu XJ: Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. *Stroke* 26:627–635, 1995
- Gesuele R, Orsini F, Zanier ER, Albani D, Deli MA, Bazzoni G, et al: Glial cells drive preconditioning-induced blood-brain barrier protection. *Stroke* 42:1445–1453, 2011

13. Gidday JM, Fitzgibbons JC, Shah AR, Park TS: Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. **Neurosci Lett** **168**:221–224, 1994
14. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Ninobe M, et al: 'Ischemic tolerance' phenomenon found in the brain. **Brain Res** **528**:21–24, 1990
15. Ma Y, Zechariah A, Qu Y, Hermann DM: Effects of vascular endothelial growth factor in ischemic stroke. **J Neurosci Res** **90**:1873–1882, 2012
16. McCarthy KD, de Vellis J: Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. **J Cell Biol** **85**:890–902, 1980
17. Nawashiro H, Tasaki K, Ruetzler CA, Hallenbeck JM: TNF- α pretreatment induces protective effects against focal cerebral ischemia in mice. **J Cereb Blood Flow Metab** **17**:483–490, 1997
18. Nico B, Ribatti D: Morphofunctional aspects of the blood-brain barrier. **Curr Drug Metab** **13**:50–60, 2012
19. Nishio S, Chen ZF, Yunoki M, Toyoda T, Anzivino M, Lee KS: Hypothermia-induced ischemic tolerance. **Ann N Y Acad Sci** **890**:26–41, 1999
20. Pillai DR, Dittmar MS, Baldaranov D, Heidemann RM, Henning EC, Schuierer G, et al: Cerebral ischemia-reperfusion injury in rats—a 3 T MRI study on biphasic blood-brain barrier opening and the dynamics of edema formation. **J Cereb Blood Flow Metab** **29**:1846–1855, 2009
21. Schell RM, Cole DJ, Schultz RL, Osborne TN: Temporary cerebral ischemia. Effects of pentastarch or albumin on reperfusion injury. **Anesthesiology** **77**:86–92, 1992
22. Shi L, Zeng M, Fu BM: Temporal effects of vascular endothelial growth factor and 3,5-cyclic monophosphate on blood-brain barrier solute permeability in vivo. **J Neurosci Res** **92**:1678–1689, 2014
23. Sugino T, Nozaki K, Takagi Y, Hashimoto N: 3-Nitropropionic acid induces ischemic tolerance in gerbil hippocampus in vivo. **Neurosci Lett** **259**:9–12, 1999
24. Verkman AS, Binder DK, Bloch O, Auguste K, Papadopoulos MC: Three distinct roles of aquaporin-4 in brain function revealed by knockout mice. **Biochim Biophys Acta** **1758**:1085–1093, 2006
25. Wada K, Ito M, Miyazawa T, Katoh H, Nawashiro H, Shima K, et al: Repeated hyperbaric oxygen induces ischemic tolerance in gerbil hippocampus. **Brain Res** **740**:15–20, 1996
26. Wada K, Miyazawa T, Nomura N, Tsuzuki N, Nawashiro H, Shima K: Preferential conditions for and possible mechanisms of induction of ischemic tolerance by repeated hyperbaric oxygenation in gerbil hippocampus. **Neurosurgery** **49**:160–167, 2001
27. Wang X, Wang BR, Duan XL, Zhang P, Ding YQ, Jia Y, et al: Strong expression of interleukin-1 receptor type I in the rat carotid body. **J Histochem Cytochem** **50**:1677–1684, 2002
28. Wick A, Wick W, Waltenberger J, Weller M, Dichgans J, Schulz JB: Neuroprotection by hypoxic preconditioning requires sequential activation of vascular endothelial growth factor receptor and Akt. **J Neurosci** **22**:6401–6407, 2002
29. Xiong L, Lu Z, Hou L, Zheng H, Zhu Z, Wang Q, et al: Pretreatment with repeated electroacupuncture attenuates transient focal cerebral ischemic injury in rats. **Chin Med J (Engl)** **116**:108–111, 2003
30. Xiong L, Zhu Z, Dong H, Hu W, Hou L, Chen S: Hyperbaric oxygen preconditioning induces neuroprotection against ischemia in transient not permanent middle cerebral artery occlusion rat model. **Chin Med J (Engl)** **113**:836–839, 2000
31. Zeng XN, Xie LL, Liang R, Sun XL, Fan Y, Hu G: AQP4 knockout aggravates ischemia/reperfusion injury in mice. **CNS Neurosci Ther** **18**:388–394, 2012
32. Zhang X, Xiong L, Hu W, Zheng Y, Zhu Z, Liu Y, et al: Preconditioning with prolonged oxygen exposure induces ischemic tolerance in the brain via oxygen free radical formation. **Can J Anaesth** **51**:258–263, 2004
33. Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, et al: VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. **J Clin Invest** **106**:829–838, 2000
34. Zhao P, Peng L, Li L, Xu X, Zuo Z: Isoflurane preconditioning improves long-term neurologic outcome after hypoxic-ischemic brain injury in neonatal rats. **Anesthesiology** **107**:963–970, 2007

Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Zhang, X Wang. Acquisition of data: X Wang, Kang, S Wang, Yao. Analysis and interpretation of data: Zhang, X Wang. Drafting the article: Zhang, X Wang. Critically revising the article: Zhang, X Wang. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Zhang. Statistical analysis: Zhang, X Wang. Administrative/technical/material support: Zhang, X Wang. Study supervision: Zhang, X Wang.

Correspondence

Xijing Zhang, Department of Anesthesiology, ICU Center, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China. email: zhangxj918@163.com.