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Effects of Hyperbaric Oxygenation on Experimental Acute Cerebral Ischemia

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Kawamura S, Yasui N, Shirasawa M, Fukasawa H. Effects of hyperbaric oxygenation on experimental acute cerebral ischemia. J Hyperbaric Med 1990; 5(2):111–123.—Effects of hyperbaric oxygenation (HBO) on cerebral ischemia were investigated in rats. A 3-0 nylon suture was introduced through the extracranial internal carotid artery to occlude the left middle cerebral artery. Nontreated controls had a percent infarct volume of 27.9 ± 5.5%. The Lt:Rt hemispheric volume ratio was 109.3 ± 2.8%. Animals treated between 2.5 and 3.5 h after the occlusion had a percent infarct volume of 18.1 ± 9.7% ($P < 0.01$). The Lt:Rt hemispheric volume ratio was 104.5 ± 2.8% ($P < 0.001$). At least until 4 h after the occlusion, HBO reduced ischemic neuronal injury and brain edema in rats treated between 2.5 and 3.5 h after the ischemic insult. The rat model described here provides a simple and relatively noninvasive method of producing constant focal brain ischemia.

hyperbaric oxygen therapy, focal cerebral ischemia, cerebral edema, animal model, rats, middle cerebral artery

Introduction

Hyperbaric oxygenation (HBO) has been said to have favorable effects on cerebral ischemia and cerebral edema. If that is true, HBO would be beneficial for stroke patients as well as for animal models that have cerebral ischemia. However, previous investigations (1, 2) on patients with cerebrovascular diseases indicated that the effects of HBO are temporary. It must be considered, then, that further studies about pathophysiologic changes caused by HBO, using a reliable animal model, are further needed to establish HBO therapy.

Our purpose was to investigate the effects of HBO on acute cerebral ischemia. To do this, an improved rat model of reproducible focal cerebral ischemia (3, 4) was employed, and neurologic and neuropathologic findings, following unilateral middle cerebral artery occlusion, were quantitatively assessed. Discussion is focused on the methodology of operative techniques and on the pathophysiology of HBO effects on cerebral ischemia.

Materials and Methods

General Preparation

Adult male Sprague-Dawley rats, weighing 250–330 g, were housed at 24° ± 2°C, at 50 ± 10% humidity. They lived in a 12-h light/dark cycle, with free

access to standard laboratory food and water. Anesthesia was induced with 4% halothane and maintained with 1.2% halothane. Atropine-sulfate (0.25 mg per rat, i.p.) was given as premedication to prevent airway obstructions caused by mucus formation. The animals were allowed to breathe spontaneously with a nitrous oxide:oxygen mixture (2.5:1) delivered via a humidifier and through a face mask. Rectal temperature was maintained at 37.5° ± 0.1°C by a thermostatically regulated heating pad. Temperature and humidity during all procedures, including HBO, were the same as those in the rat house mentioned above. A PE-50 catheter was inserted into the tail artery for continuous monitoring of mean arterial blood pressure (MABP), as well as for repeated sampling of blood for serial measurements of arterial oxygen (Pa_{O_2}) and carbon dioxide tensions (Pa_{CO_2}), pH, hematocrit (Ht), and blood glucose. Gas analysis was performed using an ABL330 (Radiometer, Copenhagen, Denmark).

Operative Technique

The animal, under anesthesia, was placed in the supine position. A midline 1-cm skin incision was made in the neck. Under an operating microscope, the external carotid artery (ECA) was exposed on the left side; both the digastric and the sternomastoid muscles were retracted laterally, and the omohyoid muscle was retracted medially using small hooks attached to rubber string. The superior thyroid and occipital arteries, both of which are branches of the ECA, were dissected, electrocoagulated with a bipolar forceps, and cut. The ECA was then isolated. Next, the stylohyoid muscle was separated from the hyoid bone, and the internal carotid artery (ICA) was carefully separated from the adjacent nerves (including the sympathetic and the vagus nerves). Further dissection revealed the glossopharyngeal nerve, which was carefully dissected and positioned superiorly. The only branch of the extracranial ICA, the pterygopalatine artery (PPA), was then isolated. The origin of the PPA was occluded with a microvascular clip, so that the intraluminal suture (*see* below) was never introduced erroneously into the PPA.

Next, the ECA was ligated with a 6-0 silk suture, and cut. The mobilized ECA stump (approximately 3 mm in length) was gently pulled downward. A 6-0 silk suture was tied loosely around the ECA stump, and a microvascular clip was placed temporarily at the origin of the ECA stump. The ECA stump was punctured with a 27-gauge needle. A 20-mm, 3-0 monofilament nylon suture, with its tip tapered and rounded with fine sandpaper, was then introduced into the ECA stump. The silk suture around the ECA stump was tightened to prevent bleeding, and the microvascular clip was removed. Thereafter, the nylon suture was gently advanced into the ICA. The black nylon suture was easily identified through the semitranslucent wall of the ICA. A 17.5–18.0-mm nylon suture was advanced from the bifurcation of the common carotid artery (CCA) to the ICA. Faint resistance was then felt, and a slight curving of the nylon suture was observed, indicating that the tip of the suture had attached to the wall of the proximal segment of the anterior cerebral artery (ACA).

Consequently, the origin of the middle cerebral artery (MCA) was occluded by the intraluminal suture, and the blood flow from the ICA, ACA, and posterior cerebral artery (PCA) to the MCA was blocked at the Willis ring. Finally, the skin incision was closed. The operative procedure was performed in 15–20 min. The catheter in the tail artery was filled with 20–30 μl heparin (1000 IU/ml), and heat-sealed. The nitrous oxide:oxygen mixture was switched to room air 20 min after the MCA occlusion, and rats were returned to individual cages. All animals were killed 4 h after the onset of the MCA occlusion.

Animal Groups Studied and the HBO Schedule

Thirty rats were randomly assigned to 1 of 3 groups. In 1 group of 10 rats, HBO treatment was performed between 30 min and 1.5 h after the MCA occlusion (early treated group). Ten animals in a second group were treated between 2.5 and 3.5 h after the onset of ischemia (late treated group). The last group of 10 animals served as a control group.

All noncontrol animals were placed in a hyperbaric chamber (KHO-100, Kawasaki-Engineering, Japan) which was pressurized to 2 atmospheres absolute (atm abs) for 30 min, with 100% oxygen delivered via a humidifier. Compression was accomplished at 0.1 atm abs $\cdot$ min^{-1}, and decompression was accomplished at 0.05 atm abs $\cdot$ min^{-1}. The oxygen inflow and outflow rates of the chamber were about 2 m$^3 \cdot$ h^{-1} during HBO. Control animals were not treated with HBO but placed in normobaric room air.

Neurologic Examination

After cessation of the anesthesia, animals were able to move within 10 min and recovered completely from the anesthesia within 30 min. Rats were observed as often as possible until they were killed, and all behavioral changes were noted. As a rule, the neurologic status was evaluated 2 h after the onset of the MCA occlusion and 20–30 min before killing. The neurologic findings were scored on a four-point scale (5): a score of 0 indicates no neurologic deficit; a score of 1 (forelimb flexion) indicates a mild neurologic deficit; a score of 2 (decreased resistance to lateral push and forelimb flexion without circling) indicates a moderate neurologic deficit; and a score of 3 (same behavior as the score of 2, with a circling toward the paretic side) indicates a severe neurologic deficit.

Neuropathology

After the final neurologic examinations, animals were anesthetized with pentobarbital sodium (50 mg $\cdot$ kg^{-1}, i.p.). The spontaneously respirating rats were placed in the supine position on a heating pad. Rectal temperature was kept at 37.5° ± 0.1°C. Heparin (1000 IU $\cdot$ kg^{-1}) was administered into the femoral vein. The tail artery was again used for the continuous monitoring of MABP and for arterial blood sampling. The thorax was opened to expose the heart through a midline incision. A cannula was inserted into the ascending

aorta via the left ventricle and fixed with a 3-0 silk suture around the aorta. The inferior vena cava was clamped with a vascular clip, and the right atrium was incised. Perfusion fixation was performed under a pressure of 100–120 mmHg, and all perfusates as well as syringes used were warmed to 37.5°C in a heat-regulating bath until just before use. The animal was first perfused with 20 ml of heparinized saline and then with 150–200 ml of 10% buffered formalin (pH 7.0) over a period of 6–8 min, until the perfusate from the right atrium was bloodless.

Before the brain was removed from the skull, the skull base was carefully removed using a minirongeur under an operating microscope. This was done to observe the position of the intraluminal suture. The suture was recognized under the ophthalmic division of the left trigeminal nerve after the removal of the presphenoid bone and the underlying dura. The brains were stored in 15% buffered formalin solution for later sectioning.

Quantification of Ischemic Brain Damage

The forebrain was embedded in paraffin wax and sectioned at multiple levels. Sections 5-μm thick were stained with hematoxylin-eosin (HE) and Mallory azan, and examined by conventional light microscopy (40× and 200×). On conventional HE-stained sections, degenerating neurons were darkly stained and had mildly atrophied cell bodies and triangular shrunken nuclei. These cells were distinguishable from dark neuron artifacts. Around the degenerating neurons there were numerous microvacuolations, indicating brain edema.

Area measurements were done at 8 coronal levels. The coronal levels were chosen at 1.5-mm intervals, and the first level was 2.5 mm posterior to the frontal tip. Areas of ischemic neuronal injury or infarction were plotted on tracings from projections of the coronal sections, and areas of ischemic damage were measured using an image analyzer (Texture-Analyzing-System, Leitz, FRG). Measurements were performed separately for the striatum, the pallium, and each hemisphere. The volume of ischemic damage was then calculated using a computer program by means of numeric integration of infarct areas from the eight chosen sections, according to the distances between them. At the same time, the volume of each hemisphere between the eight levels was also calculated. The amount of ischemic damage was expressed both as a percent of the total cerebral volume (% infarct volume) and in absolute terms (mm^3). The volume of the left hemisphere was divided by that of the right, and the Lt:Rt hemispheric volume ratio (%) was then obtained. This ratio was considered an indicator of brain swelling or brain edema (6).

In the preliminary study, India-ink perfusion was done to observe the circulation defect associated with the MCA occlusion. Rats ($n = 2$) were perfused with India-ink solution at a pressure of 100–120 mmHg 4 h after the

onset of the MCA occlusion. The pallor or hypoperfusion area was located in the occluded MCA territory (Fig. 1).

Statistical Analysis

Mean values, standard deviations (SD), and standard errors of the mean (SEM) were calculated for all data. For a comparison of the control and other groups of HBO-treated animals, the unpaired Student's t test was used. A P value of <0.05 was considered significant.

Results

Physiologic Variables

Physiologic data for each group of spontaneously breathing animals during and after the surgery are presented in Table 1. In both control and experimental rats, MABP and the other variables remained stable. Several animals developed mild hypercapnia and respiratory acidosis under anesthesia; all other values were within their normal ranges. Physiologic variables just before killing also showed no significant difference between the control and the other groups (Table 2).

Operative Mortality

One rat was excluded from this study. This rat died just after cessation of the anesthesia (20 min after the MCA occlusion). An autopsy found no cause for death other than the MCA occlusion.

Neurologic Status

In no rat was a seizure observed after surgery. All control animals had a uniformly severe neurologic deficit (score 3) throughout the period of

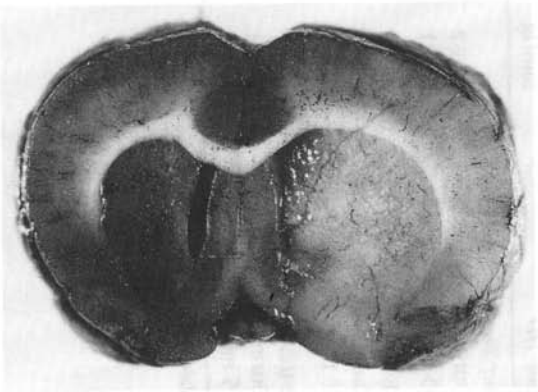


FIG. 1—Coronal section in the India-ink perfusion study, showing a hypoperfusion area in the left MCA territory.

Table 1: Physiologic Variables in Spontaneous Respirating Rats[a]

Group *(n)*	MABP, mmHg	Pa_{O_2}, mmHg	Pa_{CO_2}, mmHg	pH	Glucose, mg/dl	Ht, %	Body Weight, g
Nontreated controls (10)							
During occlusion	84±2	117±4	50±2	7.36±0.01	145±5	41±1	270±6
15 min After occlusion	85±1	104±2	53±2	7.35±0.01			
Early treated group (10)							
During occlusion	82±2	115±3	51±1	7.36±0.01	144±4	40±1	275±7
15 min After occlusion	86±4	108±2	52±2	7.36±0.01			
Late-treated group (10)							
During occlusion	82±4	116±3	50±1	7.36±0.01	146±4	41±0	274±5
15 min After occlusion	84±4	106±4	52±2	7.35±0.01			

[a]Values are mean ± SEM.

Table 2: Physiologic Variables Immediately Before Neuropathologic Study[a]

Group *(n)*	MABP, mmHg	Pa_{O_2}, mmHg	Pa_{CO_2}, mmHg	pH	Ht, %
Nontreated controls (10)	98±3	116±2	48±2	7.34±0.02	43±1
Early treated group (10)	104±5	116±4	46±2	7.36±0.01	42±1
Late-treated group (10)	95±4	117±4	48±1	7.35±0.01	43±1

[a]Values are mean ± SEM.

observation. Rats in the early treated group also revealed a neurologic score of 3, both 2 h after the occlusion and just before they were killed. However, 2 rats in this group did not show circling during HBO, and their neurologic status just after HBO was a score of 2. Thereafter, these 2 rats showed circling (score 3) within a few minutes. In the late-treated group, all animals had a neurologic deficit score of 3 until HBO treatment. The neurologic deficit of 9 rats in this group did not change after HBO. The other rat did not show circling during HBO and had a neurologic score of 1 after treatment. This rat was anesthetized 10 min later for the neuropathologic study.

Neuropathology

In all rats, the tip position of the suture was confirmed in the ACA. Intracranial hemorrhage due to vessel perforation was not seen in this study. Perfusion fixation seemed to be adequate in all animals. All brains had a slightly soft zone distal to the occluded MCA.

There was a remarkably consistent pattern of ischemic brain damage. A typical example is shown in Fig. 2. The lesions appeared as areas of early infarction within the territory of the occluded MCA; that is in the dorsolateral frontoparietal cortex, the caudoputamen, the globus pallidus, and the internal capsule. The boundaries between areas of ischemic damage and the adjacent normal parts of the brain could be sharply delineated. Infarct areas in the territory of the lenticulostriate arteries were particularly detectable. The infarct volume (mean ± SD) was 32 ± 6 mm^3 in the striatum and 118 ± 29 mm^3 in the pallium, with a total of 150 ± 33 mm^3. The percent infarct volume was 27.9 ± 5.5%. The Lt:Rt hemispheric volume ratio was 109.3 ± 2.8%. In rats from the early treated group, the infarct volume was 32 ± 6 mm^3 in the striatum and 113 ± 44 mm^3 in the pallium, with a total of 146 ± 46 mm^3. The percent infarct volume was 25.9 ± 8.2%. The Lt:Rt hemispheric volume ratio was 106.8 ± 4.5%. All these values were the same for the control and early treated groups. However, if 1 rat with a 116% Lt:Rt hemispheric volume ratio was excluded from the early treated group, the Lt:Rt ratio of the other 9 was 105.8 ± 3.5% ($P < 0.02$ vs. control).

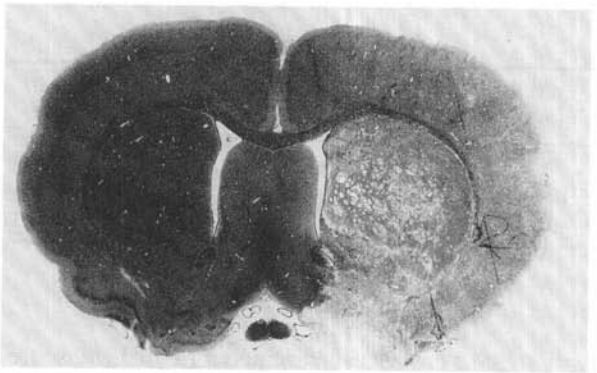
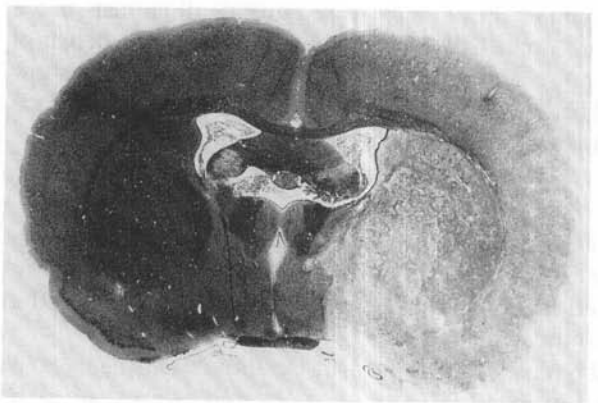


FIG. 2—A representative example of ischemic brain damage in a nontreated control. HE stain.



Conversely, late-treated animals had significantly less damage than the controls (Figs. 3 and 4). The infarct volume was 26 ± 7 mm^3 in the striatum ($P < 0.05$) and 73 ± 47 mm^3 in the pallium ($P < 0.01$), for a total of 98 ± 52 mm^3 ($P < 0.01$). The percent infarct volume was 18.1 ± 9.7% ($P < 0.01$). Decrease of the ischemic neuronal damage was remarkable in the frontoparietal cortex and in the medial areas of the striatum. Brain edema was also less in the late-treated group than in the control group. The Lt:Rt hemispheric volume ratio was 104.5 ± 2.8% ($P < 0.001$).

Discussion

Methodology in Operative Techniques

The intraluminal suture technique produced constant focal brain ischemia in rats, without the necessity of using hypotension, hypoxia, and hypovolemia

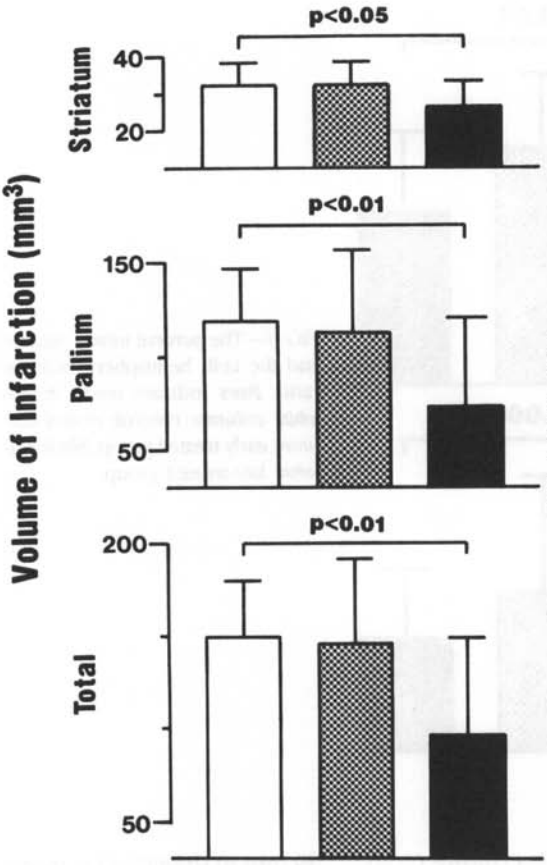


FIG. 3—Infarct volume (mm^3) in the striatum (*top*), in the pallium (*middle*), and the total (*bottom*). *Bars* indicate mean ± SD; *white column*: control; *dotted column*: early treated group; *black column*: late-treated group.

(3, 4). This technique does not necessitate a craniectomy, and therefore provides a more simple and relatively noninvasive model than others in which the MCA becomes occluded under direct visual control when craniectomy is performed (7, 8). Moreover, the intracranial physiology can be preserved; for example, intracranial pressure during the experiments was not affected by the operative procedures.

Previously, a 4-0 nylon suture (0.15–0.199 mm in diameter, *U.S. Pharmacopeia* size) was used for the intraluminal suture technique. The suture of Longa et al. (4) was made with its tip rounded by heating near a flame. Koizumi et al. (3) infused resin into the aorta under a pressure of 100–120 mmHg, and measured the vessel diameter on the resin cast. The internal diameter of both the intracranial ICA proximal to its bifurcation and the proximal ACA was approximately 0.3 and 0.2 mm, respectively. Therefore, they used a 4–0 nylon suture covered with a silicon rubber cylinder approximately 5 mm in length and 0.2–0.3 mm in diameter after the tip of the suture was rounded by heating.

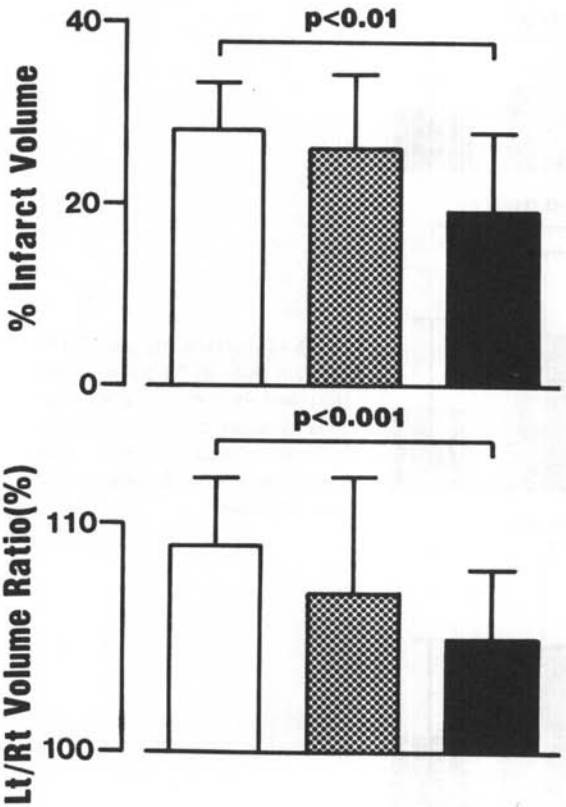


FIG. 4—The percent infarct volume and the Lt:Rt hemispheric volume ratio. *Bars* indicate mean ± SD; *white column*: control; *dotted column*: early treated group; *black column*: late-treated group.

Thus it is presumed that the 3–0 suture (0.20–0.249 mm in diameter) is more suitable to occlude the MCA than the 4-0 suture. Additionally, considering the diameter decrease from the ICA to the ACA, a tapering suture for insertion into the ACA must be considered reasonable as an embolic source. Accordingly, a simple 3-0 nylon suture, with its tip tapered and rounded, was employed in this study. The surgical procedures of Longa et al. (4) included further dissection of the ECA, along with the terminal lingual and maxillary arteries, and ligation of the PPA (the deepest structure in this preparation) with a suture. Koizumi et al. (3) ligated the CCA as well as the ECA, and introduced the suture through the CCA distal to the ligation. Therefore, the physiologic blood stream into the MCA could not be obtained when reperfusion was performed.

If reperfusion had been used in the model described in our study, it could have been easily carried out by pulling the suture. Following these considerations, the rat model in this study could be considered more simple and less invasive. This study indicates that the MCA occlusion in the control animals resulted in a uniformly severe neurologic deficit (score 3) throughout the observation period. The neuropathologic pattern was also remarkably

constant: the percent infarct volume was 27.9 ± 5.5% (mean ± SD), and the Lt:Rt hemispheric volume ratio was 109.3 ± 2.8% (n = 10). Longa et al. (4) measured infarct areas taken from a single section of each brain at the level of the optic chiasm in the MCA-occluded rats that were killed 48 h after the occlusion. The area of neuronal injury was then divided by the total area of the coronal section of the entire brain to obtain the percent infarct area. Their results showed that the percent infarct area was 37.6 ± 5.5% (n = 5) (4). Thus the rat models in this study provided a reliable method to constantly produce neurologic deficits, the amount of ischemic neuronal damage, and brain edema, without craniectomy. The mortality rate (1 rat out of 31) was considerably lower.

Pathophysiology in HBO Effects on Cerebral Ischemia

It is thought that HBO increases tissue oxygen supply, resulting in an improved oxidative metabolism. Brain ischemia may be improved by this treatment, whereas vasoconstriction due to an increase of arterial oxygen tension induces a decrease of the cerebral blood flow (CBF) (9–11). The CBF decrease as well as vasoconstriction can induce a decrease in cerebral blood volume (CBV), resulting in a decrease of intracranial pressure (ICP). The beneficial effects for brain edema may be related to the dual effects of the available oxygen increase and the CBV decrease. Therefore, both the oxygen supply increase (in spite of the CBF decrease) and the ICP decrease are expected to improve the brain ischemia.

This study clearly demonstrates that HBO therapy decreased ischemic neuronal injury and brain edema in the MCA-occluded rats that were treated between 2.5 and 3.5 h after the ischemic insult. The decrease in infarct volume was remarkable in the frontoparietal cortex and in the medial striatum. These areas may be perfused by the collateral circulation from the ACA and PCA, suggesting that HBO can improve the damage of ischemic penumbra (12). All the animals had an infarct in the internal capsule. This is why all late-treated rats, except one, showed no improvement on their neurologic score (motor disturbance), in spite of the decrease of the infarct size in the caudoputamen and the globus pallidus, as well as in the pallium.

The early treated animals had no significant improvement in either the neurologic or neuropathologic findings. The reason for this is uncertain, but similar results have been observed elsewhere (13). Shiokawa et al. (13) studied the effects of HBO on acute cerebral ischemia in rats that received bilateral CCA occlusion. Their findings indicate that animals treated 3 h after ischemia had longer survival times and less of an increase in ischemic cerebral metabolite (lactate) than nontreated controls ($P < 0.05$). Conversely, animals treated 1 h after ischemia failed to show significant improvement in either the survival time or brain metabolism. The threshold for cellular damage may explain the mechanism. This threshold should be determined according to the severity and duration of ischemia (14, 15). The results of Jones et al. (14)

suggest an infarction threshold that rises over the period of a few hours to a plateau, at a CBF value of about 17–18 $ml \cdot 100\ g^{-1} \cdot min^{-1}$, below which normal tissue structure is irreversibly damaged.

We believe that in the early treated animals the ischemic neuronal injury could not be reduced because several hours delay after therapy is enough to produce irreversible brain damage. However, 9 out of the 10 early treated animals in this study showed a noticeable decrease in brain edema. This suggests the persistent effects of HBO, whereas 2 of these 9 animals had a neurologic score of 2 until only a few min after HBO. However, this study provides no conclusion about whether the effects of HBO are temporary or persistent because the observation time after the ischemic insult was only 4 h. Further studies about whether the favorable effects observed in this study persist are needed.

Conclusion

Hyperbaric oxygen therapy, at least 4 h after an ischemic insult, reduced ischemic neuronal injury and improved brain edema following MCA occlusion in rats that were treated between 2.5 and 3.5 h after the ischemic insult. Favorable effects of HBO treatment are expected in the ischemic penumbra.

The rat model of focal cerebral ischemia described here is simple and relatively noninvasive. It also provides a reliably constant source for neuronal injury. Thus, this model is suitable for the pathophysiologic investigation of ischemic stroke and for the testing of potential therapies.

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