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Preconditioning with Hyperbaric Oxygen and Hyperoxia Induces Tolerance against Spinal Cord Ischemia in Rabbits

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Background: **The aim of this study was to determine if the ischemic tolerance could be induced in the spinal cord by pretreatment with hyperbaric oxygen (HBO) and what components of HBO (hyperoxia, hyperbaricity, and combination of these two) were critical in the induction of tolerance against ischemic injury.**

Methods: **In experiment 1, 21 rabbits were randomly assigned to one of three groups (n = 7 each): animals in the control group received no HBO before spinal cord ischemia; animals in the HBO-1 and HBO-2 groups received HBO (2.5 atmosphere absolute [ATA], 100% O_2) pretreatment 1 h/day for 3 and 5 days before ischemia, respectively. In experiment 2, 48 rabbits were randomly assigned to one of four groups (n = 12 each): the control group received no HBO (21% O_2, 1 ATA, 1 h/day, 5 days) before spinal cord ischemia; the HB group received 1-h treatment in 21% O_2 at 2.5 ATA each day for 5 days; the HO group received 1-h treatment in 100% oxygen at 1 ATA each day for 5 days; and the HBO group received HBO (2.5 ATA, 100% O_2) treatment 1 h/day for 5 days. Twenty-four hours after the last treatment, spinal cord ischemia was induced by an infrarenal aorta clamping for 20 min. Forty-eight hours after reperfusion, hind-limb motor function and histopathology of the spinal cord were examined in a blinded fashion.**

Results: **In experiment 1, the neurologic outcome in the HBO-2 group was better than that of the control group ($P = 0.004$). The number of normal neurons in the anterior spinal cord in the HBO-2 group was more than that of the control group ($P = 0.021$). In experiment 2, the neurologic and histopathologic outcomes in the HBO group were better than that of the control group ($P < 0.01$). The histopathologic outcome in the HO group was better than that in the control group ($P < 0.05$).**

Conclusions: **Serial exposure to high oxygen tension induced ischemic tolerance in spinal cord of rabbits. Simple hyperbaricity (2.5 ATA, 21% O_2) did not induce ischemic tolerance.**

PARAPLEGIA is the most serious complication after thoracoabdominal aneurysm repair.[1] Hypothermia, cerebrospinal fluid drainage, *N*-methyl-D-aspartate receptor antagonists, calcium channel blockers, and free radical scavengers have been suggested to protect against ischemic spinal cord injury,[2–6] but the improvement in outcome is marginal. Therefore, a novel therapy to protect against spinal cord ischemic injury requires further study.

In 1990, Kitagawa *et al.*[7] found that neuronal cells in the gerbil hippocampus developed resistance to ischemic neuronal damage after a brief period of reversible ischemia. This phenomenon, which has been designated as "ischemic tolerance", has drawn attention in the hope that understanding the mechanism will lead to new therapy for ischemic neuronal damage. A similar phenomenon is also demonstrable in the spinal cord.[8] Unfortunately, the idea of exposure to brief periods of ischemia before an anticipated cerebral or spinal cord ischemic event does not appear to be clinically relevant. Recently, a number of substances have proven effective in inducing ischemic tolerance in the brain. These substances include endotoxin,[9] cytokines,[10,11] potassium chloride,[12] and neurotoxin 3-nitro-propionic acid (a selective inhibitor of mitochondrial succinic dehydrogenase).[13] However, the possibility of clinical application of these substances is yet to be elucidated because of their toxicity or side effects.

Oxygen free radicals, which are generated by hyperbaric oxygen (HBO),[14] are also known to induce ischemic tolerance in gerbil hippocampal neurons.[15] Wada *et al.*[16] demonstrated that repeated administration of HBO induced tolerance against subsequent lethal ischemia in gerbil CA1 hippocampal neurons. The similar results were further reported by Prass *et al.*[17] and our group[18] in focal cerebral ischemia both in mice and rats. Because HBO is proven to protect against cerebral ischemic injury[19] and is applicable when treating carbon monoxide poisoning, air embolism, or decompression sickness, HBO is easily applied clinically as a possible way of preconditioning.

The current study was undertaken to determine if ischemic tolerance could be induced in the spinal cord by repeated administration of HBO and, if so, what components of HBO (hyperbaricity or hyperoxia) were critical in the induction of tolerance against ischemic injury.

Materials and Methods

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation and was conducted according to the Guidelines for Animal Experimentation of our institutes. The animals were studied at Xijing Hospital of the Fourth Military Medical University (Xi'an, China).

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Animals and Surgical Preparation

Sixty-nine male New Zealand White rabbits (weight, 2.1–2.3 kg) were used in this study. After an overnight fast with unrestricted access to water, the rabbits were anesthetized with 4% halothane in an oxygen–room air mixture. After induction, rabbits were maintained with 1.5% halothane delivered by mask while spontaneously breathing. An ear vein catheter was inserted, and lactated Ringer solution (4 ml · kg^{-1} · h^{-1}) was administered intravenously. In all animals, a 24-gauge catheter was inserted into the ear artery to measure proximal blood pressure, an another catheter was inserted into the left femoral artery to measure distal blood pressure. After all cannulae were placed, 400 units of heparin was injected intravenously. Blood pressure was monitored continuously using a calibrated pressure transducer connected to an invasive pressure monitor (Spacelabs Medical, Inc., Redmond, WA). Heart rate was calculated by counting blood pressure wave form on the monitor. Rectal temperature was maintained at 38.5 ± 0.5°C by a heating blanket and overhead lamps during the experiment. Arterial blood was sampled for determination of arterial oxygen tension (Pa_{O_2}), arterial carbon dioxide tension (Pa_{CO_2}), *p*H, and plasma glucose. Arterial blood gases were measured using the OMNI Modular System (AVL List GmbH Medizintechnik, Kleiststraβe, Graz, Austria).

Spinal Cord Ischemia

The induction of spinal cord ischemia was performed as previously described by Johnson *et al.*[20] Briefly, the abdominal aorta was exposed at the level of the left renal artery through a 3- to 4-cm medial incision. Small-diameter plastic tubing was placed around the aorta just distal to the left renal artery. The ends of the tubing were threaded through a small plastic button and then through a plastic tube of large diameter, forming a snare ligature. Aortic occlusion was performed by pulling and clamping the small tube around the aorta. After the occlusion, distal blood pressure decreased immediately, and the pulsatility disappeared. Ischemia lasted 20 min. At the end of the ischemic period, the tubing was released to restore the flow through the aorta, and the abdominal wall was closed with wound clips. The local infiltration around the wound with 0.25% bupivacaine hydrochloride was applied for postoperative analgesia. Halothane was discontinued. The infusion of lactated Ringer solution was continued until the animals began to drink. An antibiotic (40,000 IU gentamicin) was administered intramuscularly immediately after operation. The animals were then returned to their home cages and observed for 2 days. Bladder contents were expressed manually as required.

Experimental Protocol

This study consisted of two experiments. Experiment 1 was designed to determine if HBO with variable exposure times could induce ischemic tolerance in the spinal cord. Experiment 2 was undertaken to elucidate whether hyperoxia (high oxygen tension) or hyperbaricity induced ischemic tolerance in the spinal cord.

Experiment 1. A total of 21 male New Zealand White rabbits were randomly assigned to one of three groups: the control group (n = 7), the HBO-1 group (n = 7), or the HBO-2 group (n = 7). Rabbits in the HBO-1 group received 1 h of HBO at 2.5 atmosphere absolute (ATA) in 100% oxygen each day for 3 days using an animal hyperbaric chamber. The animals in the HBO-2 group received identical HBO for 5 days. The animals in the control group were placed in a chamber (21% O_2), which was not pressurized for sham treatments, on the same schedule as for the HBO pretreatment groups for 5 days. Twenty-four hours after the last treatment, animals were subjected to spinal cord ischemia for 20 min.

Experiment 2. A total of 48 male New Zealand White rabbits were randomly assigned to one of four groups (n = 12 each): the control group, HB group, HO group, or HBO group. Rabbits in the control group received 1-h treatment in 21% O_2 at 1 ATA each day for 5 days. Rabbits in the HB group received 1-h treatment in 21% O_2 at 2.5 ATA each day for 5 days. Rabbits in the HO group were exposed to 100% O_2 at 1 ATA 1 h each day for 5 days. Rabbits in the HBO group were exposed to HBO in 100% O_2 at 2.5 ATA 1 h each day for 5 days. All animals received their treatments in the animal hyperbaric chamber. Twenty-four hours after the last pretreatment, animals were subjected to spinal cord ischemia for 20 min.

Neurologic and Histopathologic Evaluation

At 4, 8, 12, 24, and 48 h after reperfusion, the rabbits were neurologically assessed by an observer who was unaware of the grouping, using the modified Tarlov criteria[21] : 0, no voluntary hind-limb function; 1, movement of joints perceptible; 2, active movement but unable to stand; 3, able to stand but unable to walk; 4, complete normal hind-limb motor function.

After completion of the evaluation of hind-limb motor function at 48 h after reperfusion, the animals were reanesthetized. Transcardiac perfusion and fixation were performed with 1,000 ml heparinized saline followed by 500 ml buffered formalin, 10%. The lumbar spinal cord was removed and refrigerated in 10% phosphate-buffered formalin for 48 h. After dehydration in graded concentrations of ethanol and butanol, the spinal cord was embedded in paraffin. Coronal sections of the spinal cord (L5 level) were cut at a thickness of 6 μm and stained with hematoxylin and eosin. Neuronal injury was evaluated at a magnification of 400× by an observer who was unaware of the grouping. Ischemic neurons were

Table 1. Physiological Variables

	MAP				Arterial Blood Gases			
	Proximal mmHg	Distal mmHg	HR	T (°C)	pH	Pa_{O_2} mmHg	Pa_{CO_2} mmHg	Glucose mM
Preischemia								
Control	74 ± 9	73 ± 7	223 ± 29	38.1 ± 0.3	7.34 ± 0.07	217 ± 50	40 ± 7	5.0 ± 2.1
HBO-1	79 ± 9	80 ± 11	232 ± 26	38.4 ± 0.4	7.35 ± 0.02	207 ± 32	36 ± 5	4.1 ± 0.4
HBO-2	81 ± 17	78 ± 16	250 ± 20	38.4 ± 0.5	7.33 ± 0.05	200 ± 44	39 ± 5	4.0 ± 1.4
Ischemia 10 min								
Control	74 ± 5	10 ± 1.3	216 ± 15	37.8 ± 0.3	7.36 ± 0.08	264 ± 70	36 ± 3	6.4 ± 1.8
HBO-1	71 ± 12	10 ± 2.2	217 ± 26	38.0 ± 0.5	7.35 ± 0.03	242 ± 51	32 ± 6	7.1 ± 1.9
HBO-2	80 ± 17	10 ± 2.3	229 ± 29	37.9 ± 0.4	7.34 ± 0.04	223 ± 34	37 ± 2	5.9 ± 1.4
Reperfusion 10 min								
Control	70 ± 5	78 ± 7	227 ± 25	38.0 ± 0.3	7.38 ± 0.04	314 ± 36	31 ± 2	7.2 ± 1.9
HBO-1	77 ± 15	74 ± 13	231 ± 12	38.0 ± 0.5	7.36 ± 0.04	291 ± 69	29 ± 5	5.4 ± 1.2
HBO-2	78 ± 9	76 ± 11	237 ± 12	38.2 ± 0.2	7.33 ± 0.04	295 ± 34	34 ± 2	6.6 ± 1.5

Data are mean ± SD.

MAP = mean arterial pressure; HR = heart rate; T = rectal temperature.

identified by cytoplasmic eosinophila with loss of Nissl substance and by the presence of pyknotic homogenous nuclei. In each slice, normal neurons in the anterior spinal cord (anterior to a line drawn through the central canal perpendicular to the vertebral axis) were counted in two sections for each animal and then averaged.

Statistical Analysis

Changes in mean arterial pressure, heart rate, rectal temperature, arterial *p*H, Pa_{O_2}, Pa_{CO_2}, and blood glucose concentration were compared using analysis of variance. The scores of hind-limb motor function and the number of normal neurons in the anterior spinal cord were analyzed using a nonparametric method (Kruskal–Wallis test) followed by the Mann–Whitney U test with Bonferroni correction. The correlation of hind-limb motor function scores and the number of normal neurons in the anterior spinal cord were analyzed using Spearman rank correlation. A P value < 0.05 was considered statistically significant.

Results

Experiment 1

Physiologic Variables. Physiologic variables are shown in table 1. The hemodynamics, rectal temperature, arterial *p*H, Pa_{O_2}, Pa_{CO_2}, and plasma glucose were similar in all groups, regardless of the treatment patterns.

Neurologic Outcome. All animals survived until the final neurologic assessment at 48 h after reperfusion. The neurologic outcome in the HBO-2 group was better than that of the control group ($P = 0.004$; fig. 1). Four animals in the HBO-2 group and two in the HBO-1 group showed completely normal motor function (Tarlov score = 4) at 48 h after reperfusion. No animals in the control group showed normal motor function (neurologic score < 3 in all animals).

Histopathologic Outcome. The normal neurons in the anterior spinal cord of the HBO-2 group were more than that of the control group ($P = 0.021$). There was no difference in the number of normal neurons in the anterior spinal cord between the control and HBO-1 groups (fig. 2).

Correlation of Neurologic and Histopathologic Outcomes. There was a significant correlation between the final neurologic status at 48 h after reperfusion and the number of normal neurons in the anterior spinal cord ($r = 0.80$; $P < 0.001$).

Experiment 2

Physiologic Variables. The hemodynamics, rectal temperature, arterial *p*H, Pa_{O_2}, and Pa_{CO_2} were similar in all groups, regardless of the treatment pattern.

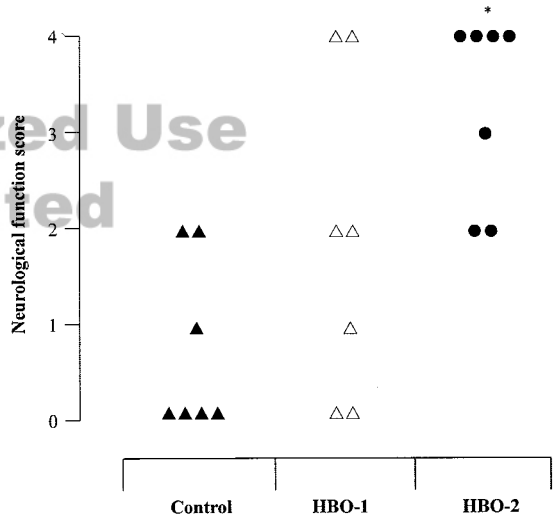

Fig. 1. Experiment 1: neurologic outcome 48 h after reperfusion (*$P < 0.05$ *vs.* control). HBO-1 = group that received 1 h of hyperbaric oxygen at 2.5 ATA in 100% O_2 each day for 3 days; HBO-2 = group that received 1 h of hyperbaric oxygen at 2.5 ATA in 100% O_2 each day for 5 days.

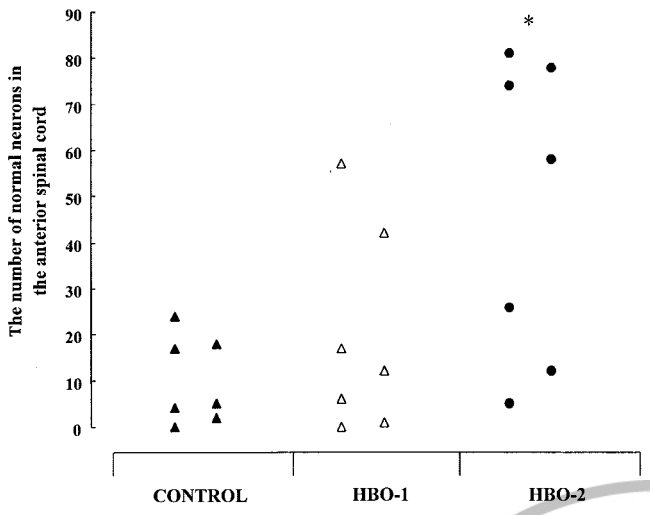

Fig. 2. Experiment 1: histopathologic outcome 48 h after reperfusion (*$P < 0.05$ *vs.* control). HBO-1 = group that received 1 h of hyperbaric oxygen at 2.5 ATA in 100% O_2 each day for 3 days; HBO-2 = group that received 1 h of hyperbaric oxygen at 2.5 ATA in 100% O_2 each day for 5 days.

Neurologic Outcome. All animals survived until the final neurologic assessment at 48 h after reperfusion. The neurologic outcome in the HBO group was better than that of the control group ($P = 0.0013$; fig. 3). There was no significant difference in neurologic outcome among the HO, HB, and control groups.

Histopathologic Outcome. The normal neurons in the anterior spinal cord of the HO and HBO groups were more than that of the control group ($P = 0.0431$ and 0.0045, respectively). However, no difference was found in the number of normal neurons at the anterior spinal cord between the HO and HBO groups. There was no difference in the number of normal neurons in the anterior spinal cord between the control and HB groups (fig. 4).

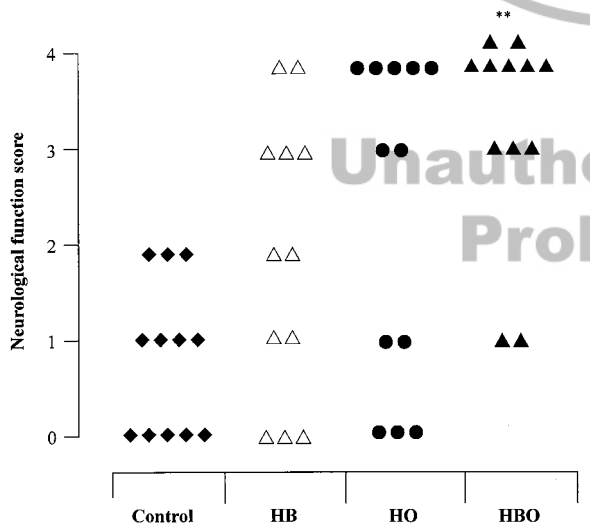

Fig. 3. Experiment 2: neurologic outcome 48 h after reperfusion ($P < 0.01$ *vs.* control). HB = group that received 1-h treatment in 21% O_2 at 2.5 ATA each day for 5 days; HO = group that was exposed to 100% O_2 at 1 ATA 1 h each day for 5 days; HBO = group that was exposed to hyperbaric oxygen in 100% O_2 at 2.5 ATA 1 h each day for 5 days.**

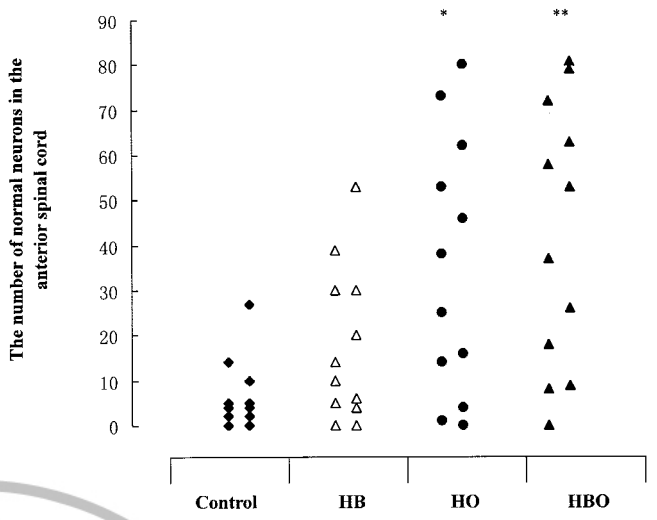

Fig. 4. Experiment 2: histopathologic outcome 48 h after reperfusion (*$P < 0.05$ *vs.* control; **$P < 0.01$ *vs.* control). HB = group that received 1-h treatment in 21% O_2 at 2.5 ATA each day for 5 days; HO = group that was exposed to 100% O_2 at 1 ATA 1 h each day for 5 days; HBO = group that was exposed to hyperbaric oxygen in 100% O_2 at 2.5 ATA 1 h each day for 5 days.

Correlation of Neurologic and Histopathologic Outcome. There was a significant correlation between the final neurologic status at 48 h after reperfusion and the number of normal neurons in the anterior spinal cord ($r = 0.809$; $P < 0.001$).

Discussion

The current study demonstrated that repeated exposure to HBO before ischemia induces ischemic tolerance against subsequent lethal ischemia in spinal cord. HO (1 ATA, 100% O_2) also induces ischemic tolerance, but simple hyperbaricity (2.5 ATA, 21% O_2) did not.

Spinal cord injury after a successful surgical operation on the thoracic aorta is an unpredictable but disastrous complication in human beings. The reported incidences of postoperative paraplegia vary from 0.9 to 40%.[1] The causes of acute spinal cord dysfunction is believed to be the result of spinal cord ischemia from hypoperfusion during aortic cross-clamping. Excitatory amino acids, accumulation of intracellular calcium, and free radicals are all suggested to play important roles in neuronal injury after ischemia.[22–24] However, ameliorative measures, including hypothermia, *N*-methyl-D-aspartate receptor antagonists, calcium channel blockers, and free radical scavengers[2–6] are still inadequate.

Accumulated evidence from recent studies provides possible new insights into neuroprotective measures: short ischemic episodes to the brain or spinal cord induce protection against a subsequent lethal ischemic injury. Unfortunately, inducing a brief period of suble-

thal ischemia before an anticipated cerebral or spinal cord ischemic event is hardly practical clinically. Various pharmacologic and chemical preconditionings, including cytokines,[10] endotoxin,[11] and the neurotoxin 3-nitropropionic acid,[12] have been shown to induce ischemic tolerance in the brain. However, the clinical application of these substances is still questionable because of their side effects and toxicity.

Production of free oxygen radicals, either by drugs or by exposure to HBO, is capable of inducing ischemic tolerance in the brain.[15–18] However, there have been no data concerning the optimal preconditioning, especially in the spinal cord. Furthermore, it is undefined what component of HBO pretreatment, namely, hyperbaricity or hyperoxia, contributes to induce ischemic tolerance. In the current study we observed a significantly better neurologic outcome in rabbits that received HBO preconditioning (for 5 days) than in the control group. Histopathologic examination also confirmed the ability of HBO preconditioning to curb neuronal necrosis in the anterior horn motor neurons after 5 consecutive days of HBO exposure (HBO-2 group). Chromatolysis of Nissl substance, swelling and vacuolization of perikarya, and karyolysis frequently seen in the control group were significantly less the HBO-2 group.

To elucidate whether hyperoxia (high oxygen tension) or hyperbaricity itself was critical in the induction of ischemic tolerance by HBO, we designed experiment 2. Animals in both the HBO (100% O_2, 2.5 ATA, 1 h/day, 5 days) and HO (100% O_2, 1 ATA, 1 h/day, 5 days) groups had a better histopathologic outcome compared with the control group. The neurologic outcome in the HBO group was significantly better than that of the control group. The results indicate that pretreatment with high oxygen tension, but not with simple hyperbaricity, played a key role in inducing ischemic tolerance against spinal cord ischemia. In experiment 2, the neurologic outcome in the HO group failed to demonstrate a better result than that of control group, indicating that the neurologic scoring is inherently less sensitive than histopathology.

In this study, we only investigated the HO and HBO preconditioning effect at a single interval (24 h) between the final pretreatment and subsequent ischemic insult. The determination of the optimal interval between preconditioning treatment and ischemia as well as the optimal "dose" of preconditioning merits further investigation. As to the mechanism for induction of ischemic tolerance with HO and HBO, the study exploring the roles of oxygen free radicals formation is now under way in our laboratory.

The postoperative analgesia for the animals in current study was fulfilled by infiltration of incision sites with bupivacaine, a long-acting local anesthetic. The animals with normal neurologic function after surgery moved around in their cages and ate normally. Some paraplegic animals lost their appetite. No obvious weight loss was found for the animals. All of the rabbits received proper care after surgery.

In conclusion, the current study is the first to clearly demonstrate that serial exposure to HBO induced ischemic tolerance in the spinal cord of rabbits. Hyperoxia (1 ATA, 100% O_2) also induced ischemic tolerance, but simple hyperbaricity (2.5 ATA, 21% O_2) did not.

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