Hyperbaric oxygenation therapy enhances the protective effect of moderate hypothermia against forebrain ischemia in the gerbil hippocampus.

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Wada K, Nishi D, Kitamura T, Ono K, Takahara T, Shirotani T, Shimizu A. Hyperbaric oxygenation therapy enhances the protective effect of moderate hypothermia against forebrain ischemia in the gerbil hippocampus. Undersea Hyperb Med 2006; 33(6):399-405. Moderate hypothermia may have a beneficial effect on the neurological outcome. However, ischemic deterioration such as brain swelling during rewarming has been reported as a notable complication after successful therapeutic cerebral hypothermia. In this study, we investigated the effects of hyperbaric oxygenation during rewarming. Forebrain ischemia was produced in 24 gerbils and sham ischemia in 8 animals. Then ischemia-treated animals were divided into 3 groups, whole-body moderate hypothermia (31°C for 60 min) and hyperbaric oxygenation (HBO_2) (2- atmosphere absolute for 60 min using 100% oxygen) during rewarming group (n = 8), moderate hypothermia without HBO_2 group (n = 8), and sham treatment without hypothermia and without HBO_2 group (n = 8). Both the hypothermia group (77.9 ± 48.1 neurons per mm, mean ± SD) and hypothermia + HBO_2 group (127.6 ± 29.7 neurons per mm,) showed significant preservation of CA1 pyramidal neurons in the hippocampus compared to that in the sham treatment group (6.4 ± 2.7) ($p < 0.01$). Furthermore, the hypothermia + HBO_2 group showed significantly greater preservation of CA1 pyramidal neurons than the hypothermia group ($p < 0.05$). These results suggest that HBO_2 during rewarming preserves the protective effect of hypothermia against ischemic neuronal damage.

INTRODUCTION

Various studies have demonstrated that intra-ischemic and/or post-ischemic hypothermia has a protective effect in patients with cerebral ischemia (1). To date, several techniques and methods of inducing and maintaining hypothermia have been investigated and developed to prevent complications of hypothermia (2). However, few methods of adjuvant therapy have been studied except for the speed of rewarming, although ischemic deterioration during rewarming such as brain swelling caused by mismatching of oxygen consumption and supply has been reported as a notable complication after successful therapeutic cerebral hypothermia (3).

Hyperbaric oxygen therapy (HBO_2) has been used in humans for the treatment of stroke (4), CO poisoning (5), gas gangrene, air embolism and decompression sickness. Preservation of blood-brain barrier function (6,7) and reduction of brain swelling (8) through oxygen delivery to the ischemic periphery (9) is considered a possible mechanism for the beneficial effect of HBO_2. Therefore, HBO_2 during rewarming after successful hypothermia

may protect against ischemic deterioration. In this study, we investigated the effects of hyperbaric oxygenation during rewarming on ischemia using a delayed neuronal death model in the gerbil hippocampus.

MATERIALS AND METHODS

Animals

A total of 32 male Mongolian gerbils, weighing 60-80 g, were used. The animals were allowed free access to food and water prior to and following treatment. The animals underwent 5-min forebrain ischemia induction or sham-surgery (n = 8). Ischemia-treated animals were then divided into 3 groups: whole-body moderate hypothermia (32°C for 60 min) and hyperbaric oxygenation (HBO_2) (2- atmosphere absolute for 60 min using 100% oxygen) during rewarming group (n = 8), moderate hypothermia without HBO_2 group (n = 8), and sham treatment without hypothermia and without HBO_2 group (n = 8). Seven days after ischemia or sham treatment, histological examination was conducted.

Brain ischemia

The surgical procedure for induction of forebrain ischemia has been described (10). Briefly, anesthesia was induced with 2% halothane and maintained with 1% halothane in a mixture of 30% oxygen and 70% nitrous oxide through a face mask. A midline cervical incision was made, and both common carotid arteries were gently exposed, then occluded with a small vascular clip, when reflex, but not spontaneous, movement was exhibited. Carotid flow was restored by releasing the clip following 5-min of occlusion. The rectal temperature was monitored and maintained close to 37.5 °C during the procedure using a feedback-controlled heating pad. A needle-type thermocouple probe was inserted in the skin on the right side of the head to monitor and maintain temperature close to 37.0°C during the procedure using a feedback-controlled heating lamp. The electroencephalogram (EEG) was also monitored during each ischemic insult, and animals that failed to exhibit severe depression of EEG activity during ischemia were excluded from the study. The animals in the sham-operated group underwent the same surgical procedure but without common carotid occlusion.

Hypothermia

Immediately after 5-min ischemia, hypothermia was induced in two groups. The gerbils were placed on an ice pack and the core temperature was maintained at 31 °C for one-hour. During hypothermia, anesthesia was induced with 1% halothane in a mixture of 30% oxygen and 70% nitrous oxide through a face mask. Each animal was then gradually rewarmed to 36 °C using a heating lamp for 10-min .

HBO_2 administration

For HBO_2 treatment, pure oxygen was supplied continuously at a pressure of 2-ATA for 1 hour. Before compression, the chamber was flushed for 1-min with pure oxygen. Compression was performed at 1 kg/cm^2/min, and decompression was carried out at 0.2 kg/cm^2/min. There was no seizure observed in any animal during the procedure. The animals in the sham-HBO_2 group were placed in the chamber, which was not pressurized for sham-treatment.

Histology

Seven days after the ischemic insult, animals were anesthetized with pentobarbital (50 mg/kg i.p.) and their brains were briefly perfused transcardially with heparinized saline, followed by perfusion-fixation with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min. The brains were removed 1-h later, immersed in the same fixative for 2 days, then

embedded in paraffin. Paraffin sections 5-μm thick were prepared at the level of the dorsal hippocampus, stained with hematoxylin and eosin, and examined by light microscopy. The neuronal density of the hippocampal CA1 subfield, i.e., the number of intact pyramidal cells per 1-mm length of CA1, was determined in a blind fashion (K.W.) according the method of Kirino et al (11).

Statistical analysis

Statistical comparisons were made by one-way ANOVA and the post-hoc Fisher test. Values of $p < 0.05$ were considered significant, and results are expressed as means ± SD.

RESULTS

Histology

The results of histological examinations of CA1 are summarized in Figure 1 and Table 1 (see page 3)**.** Sham operation alone did not cause neuronal death, and the neuronal density of sham-operated animals was 176.9 ± 16.4 (mean ± SD) per 1-mm length of the CA1 pyramidal cell layer. Gerbils that received sham treatment after 5-min ischemic insult exhibited extensive neuronal damage and a CA1 neuronal density of 6.4 ± 2.7/mm (3.6% of normal). The hypothermia group (77.9 ± 48.1; 44.0% of normal) and the hypothermia + HBO_2 group (127.6 ± 29.7; 72.1% of normal) showed preservation of CA1 pyramidal neurons in the hippocampus compared with the sham group ($p < 0.01$). Furthermore, the hypothermia + HBO_2 group showed significantly greater preservation of CA1 pyramidal neurons than the hypothermia group ($p < 0.05$).

DISCUSSION

This study clearly demonstrated that 2-ATA hyperbaric oxygenation (HBO_2) during rewarming promotes the protective effect of hypothermia against ischemic neuronal damage in the gerbil hippocampus. Experimental studies suggest that hypothermia is protective in cerebral ischemia or brain injury (12). However, a recent clinical study showed that hypothermia after acute brain injury had no effect (13). One reason for this discrepancy is that the rewarming conditions, such as rewarming speed, after successful hypothermia treatment have a critical impact (14). For this reason, slow, controlled rewarming, which is defined as temperature increase of 0.1°C to 0.2°C over 2 to 4 hours, is recommended after hypothermia treatment (15,16). However, despite slow rewarming, acute brain swelling may occur (3). The mechanism of such swelling appears to involve the uncoupling of cerebral circulation and metabolism, leading to an increase in extracellular glutamate and lactate. This causes brain swelling which leads to intracranial pressure elevation and irreversible neuronal cell damage (14).

Reduction of brain swelling and increased intracranial pressure is considered a possible mechanism of the beneficial effect of HBO_2 (17,18). Sukoff et al. reported that CSF pressure was reduced after HBO_2 in dogs with experimentally induced cerebral edema and compression (19,20). HBO_2 may cause vasoconstriction in nonischemic areas (21,22), shunting flow to ischemic regions and preventing functional deterioration of ischemic microcirculation. Atochin et al. reported that modulation of constitutive nitric oxide synthase (cNOS)-derived NO by HBO_2 is responsible for vasoconstriction responses (23). cNOS is reported to decrease in the ischemic region (24). Therefore, vasoconstriction caused by HBO_2 may occur only in the non-ischemic area, shunting flow to ischemic regions. Post-ischemic hypoperfusion causes a mismatch between cerebral oxygen delivery and demand. Neuroprotection by HBO_2 after ischemia is thought to be mediated by improved oxygen

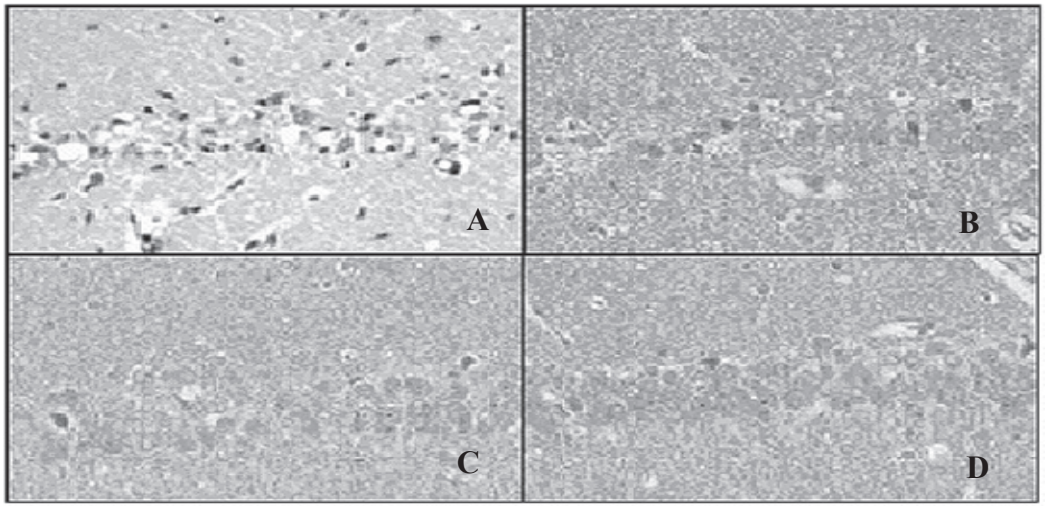
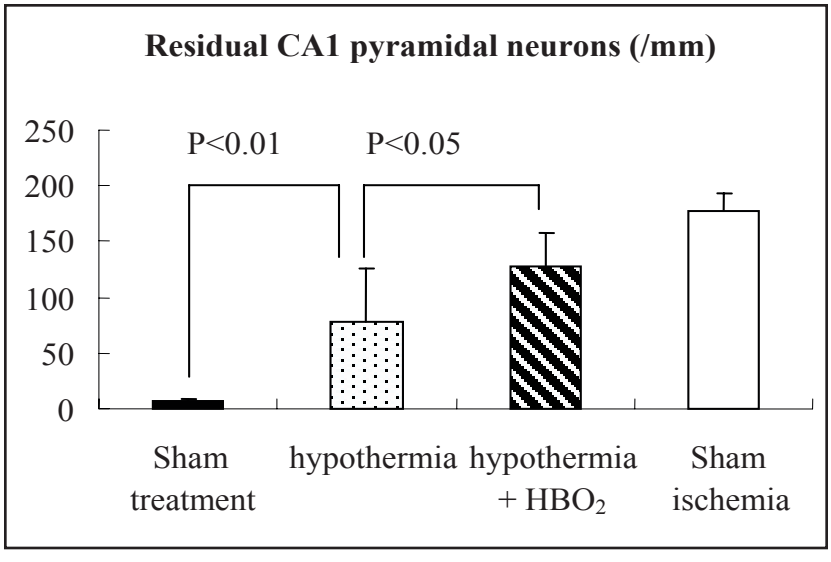


Fig. 1. Hematoxylin and eosin staining of surviving hippocampal CA1 neurons after 5-min of forebrain ischemia shows in this figure. Sham operation alone did not cause neuronal death (D). Gerbils that received ischemic insult for 5-min followed by sham treatment showed extensive neuronal damage (A). In animals that underwent hypothermia (B) or hypothermia + HBO (C), the CA1 cells were preserved. However, in animals that underwent hypothermia + HBO (C) preservation was better than that achieved by hypothermia alone (B).

Table 1. Residual CA1 pyramidal neurons in hippocampus at 7 days after ischemia

Group	Sham treatment	hypothermia	hypothermia + HBO_2	Sham ischemia
N	8	8	8	8
Residual CA1 pyramidal neurons (means ± SD)	6.4 ± 2.7	77.9 ± 48.1	127.6 ± 29.7	176.9 ± 16.4



Both hypothermia and hypothermia + HBO_2 groups were significantly preserved CA1 pyramidal neurons in hippocampus compared to sham treatment group ($p < 0.01$). Furthermore, hypothermia + HBO_2 group was significantly more preserved CA1 pyramidal neurons than hypothermia group ($p < 0.05$).

supply to the ischemic periphery. Furthermore, Ostrowski et al. have reported that HBO_2 can induce neuroprotection by preserving blood-brain barrier function (18). Improvement of the microcirculation and oxygen delivery in ischemic regions by HBO_2 during rewarming after hypothermia may reduce brain swelling through preservation of the blood-brain barrier function, thus preventing an increase of intracranial pressure.

Another possible mechanism of the protective effects by HBO_2 is the inhibition of apoptosis. In this study, we used the brief forebrain ischemia model described by Kirino (11). Even brief global cerebral ischemia causes irreversible damage to hippocampal CA1 neurons in rodents. Selective vulnerability of CA1 neurons results in delayed neuronal death (DND). This DND differs from necrosis because CA1 neurons survive at 24 hours after ischemic injury (11). The mechanisms of DND are still unclear. Some studies have suggested that delayed neuronal death differs from typical apoptosis, given the inhibition of protein synthesis as well as RNA synthesis associated with delayed neuronal death, or based on morphological findings (25). However, it is well known that apoptosis-regulating molecules such as Bcl-associated X (Bax) and Bcl-2 influence CA-1 neuronal survival after global ischemia (26). Therefore, it is conceivable that apoptotic cell death plays a role in delayed neuronal death. Hypothermia has been reported to rescue hippocampal CA1 through attenuating down-regulation of the AMPA receptor GluR2 subunit (27), and diminishing apoptosis (28). HBO_2 may also reduce apoptotic cells by reducing the expression of COX-2 (29), the expression of Nogo-A, Ng-R, or Rho A (30), expression of hypoxia-inducible factor-1alpha (18), and the expression of ICAM-1 (31) and inhibition of polymorphonuclear neutrophils (32). It is thus conceivable that HBO_2 administration reduces the apoptotic cascade process and/or inflammatory process, influencing the neuroprotective effect of hypothermia to CA1 neurons otherwise "destined to die". Indeed, protection by hyperbaric oxygenation against DND has been demonstrated in the gerbil hippocampus (33). Clinical benefits for human ischemic neuronal disease is controversial. Ryuniak have been reported that a 1-time treatment with HBO_2 at 2.5 ATA does not appear to be beneficial and may be harmful in patients with acute ischemic stroke (34). Despite multiple treatments with HBO_2 at 1.5 ATA have been reported benefit (35). We have been reported that pretreatment with 2-ATA HBO_2 once every other day for 5 sessions induced ischemic tolerance in experimental ischemia, but those with 2-ATA HBO_2 for one session or with 3-ATA HBO_2 once daily for 10 sessions did not. These results may indicate that preferential HBO_2 conditions for induction of ischemic tolerance by hyperbaric oxygenation exist (26). Therefore, the discrepancy of these two clinical trials caused by therapeutic pressure or/and number of treatments.

Further study is needed to precisely determine the mechanisms of the protective effect of HBO_2 during rewarming after successful brain hypothermia. Clinical application of HBO_2 will require determination of the ideal pressure and timing during rewarming, but this study suggests that HBO_2 during rewarming after successful hypothermia may be useful as adjuvant therapy for stroke patients.

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REFERENCES

1. Olsen TS, Weber UJ, Kammersgaard LP: Therapeutic hypothermia for acute stroke. *Lancet Neurol* 2003; 2: 410-416.
2. McIntyre LA, Fergusson DA, Hebert PC, Moher D,

Hutchison JS: Prolonged therapeutic hypothermia after traumatic brain injury in adults: a systematic review. *JAMA* 2003; 289: 2992-2999.
3. Iida K, Kurisu K, Arita K, Ohtani M: Hyperemia prior to acute brain swelling during rewarming of patients who have been treated with moderate hypothermia for severe head injuries. *J Neurosurg* 2003; 98: 793-799.
4. Nighoghossian N, Trouillas P, Adeleine P, Salord F: Hyperbaric oxygen in the treatment of acute ischemic stroke. A double-blind pilot study*: Stroke* 1995; 26: 1369-1372.
5. Weaver LK, Hopkins RO, Chan KJ, Churchill S, Elliott CG, Clemmer TP, Orme JF Jr, Thomas FO, Morris AH: Hyperbaric oxygen for acute carbon monoxide poisoning. *N Engl J Med* 2002; 347: 1057-1067.
6. Veltkamp R, Siebing DA, Heiland S et al. Hyperbaric oxygen induces rapid protection against focal cerebral ischemia. *Brain Res* 2005; 1037: 134-138.
7. Veltkamp R, Siebing DA, Heiland S et al. Hyperbaric oxygen reduces blood-brain barrier damage and edema after transient focal cerebral ischemia. *Stroke* 2005; 36:1679-83.
8. Schabitz WR, Schade H, Heiland S et al. Neuroprotection by hyperbaric oxygenation after experimental focal cerebral ischemia monitored by MRI. *Stroke* 2004; 35: 1175-1179.
9. Sunami K, Takeda Y, Hashimoto M, Hirakawa M: Hyperbaric oxygen reduces infarct volume in rats by increasing oxygen supply to the ischemic periphery. *Crit Care Med* 2000; 28: 2831-2836.
10. Wada K, Ito M, Miyazawa T, Katoh H, Nawashiro H, Shima K, Chigasaki H: Repeated hyperbaric oxygen induces ischemic tolerance in gerbil hippocampus. *Brain Res* 1996; 740: 15-20.
11. Kirino T: Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res* 1982; 239: 57-69.
12. Chatzipanteli K, Alonso OF, Kraydieh S, Dietrich WD: Importance of posttraumatic hypothermia and hyperthermia on the inflammatory response after fluid percussion brain injury: biochemical and immunocytochemical studies. *J Cereb Blood Flow Metab* 2000; 20: 531-542.
13. Clifton GL, Miller ER, Choi SC et al. Lack of effect of induction of hypothermia after acute brain injury. *N Engl J Med* 2001; 344: 556-563.
14. Nakamura T, Miyamoto O, Sumitani K, Negi T, Itano T, Nagao S: Do rapid systemic changes of brain temperature have an influence on the brain? *Acta Neurochir (Wien***)** 2003; 145: 301-307.
15. Steiner T, Friede T, Aschoff A, Schellinger PD, Schwab S, Hacke W: Effect and feasibility of controlled rewarming after moderate hypothermia in stroke patients with malignant infarction of the middle cerebral artery. *Stroke* 2001; 32: 2833-2835.
16. Suehiro E, Ueda Y, Wei EP, Kontos HA, Povlishock JT: Posttraumatic hypothermia followed by slow rewarming protects the cerebral microcirculation. *J Neurotrauma* 2003; 20: 381-390.
17. Miller JD, Ledingham IM: Reduction of increased intracranial pressure. Comparison between hyperbaric oxygen and hyperventilation. *Arch Neurol* 1971; 24: 210-216.
18. Ostrowski RP, Colohan AR, Zhang JH.: Mechanisms of hyperbaric oxygen-induced neuroprotection in a rat model of subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 2005; 25: 554-571.
19. Sukoff MH, Hollin SA, Espinosa OE, Jacobson JH 2nd: The protective effect of hyperbaric oxygenation in experimental cerebral edema: *J Neurosurg* 1968; 29: 236-241.
20. Sukoff MH: Effects of hyperbaric oxygenation. *J Neurosurg* 2001; 95: 544-546.
21. Miller JD, Fitch W, Ledingham IM, Jennett WB: The effect of hyperbaric oxygen on experimentally increased intracranial pressure. *J Neurosurg* 1970; 33: 287-296.
22. Miller JD, Ledingham IM, Jennett WB: Effects of hyperbaric oxygen on intracranial pressure and cerebral blood flow in experimental cerebral oedema. *J Neurol Neurosurg Psychiatry* 1970; 33: 745-755.
23. Atochin DN, Demchenko IT, Astern J, Boso AE, Piantadosi CA, Huang PL: Contributions of endothelial and neuronal nitric oxide synthases to cerebrovascular responses to hyperoxia. *J Cereb Blood Flow Metab* 2003; 23: 1219-1226.
24. Nakashima MN, Yamashita K, Kataoka Y, Yamashita YS, Niwa M: Time course of nitric oxide synthase activity in neuronal, glial, and endothelial cells of rat striatum following focal cerebral ischemia. *Cell Mol Neurobiol* 1995; 15: 341-349.
25. Kirino T, Tsujita Y, Tamura A: Induced tolerance to ischemia in gerbil hippocampal neurons. *J Cereb Blood Flow Metab* 1991; 11: 299-307.
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* 2001; 49:160-6.
27. Colbourne F, Grooms SY, Zukin RS, Buchan AM, Bennett MV: Hypothermia rescues hippocampal CA1 neurons and attenuates down-regulation of the AMPA receptor GluR2 subunit after forebrain ischemia. *Proc Natl Acad Sci U S A* 2003; 100:2906-2910.
28. Zhu C, Wang X, Cheng X et al. Post-ischemic hypothermia-induced tissue protection and diminished apoptosis after neonatal cerebral hypoxia-ischemia. *Brain Res* 2004; 996: 67-75.
29. Yin D, Zhou C, Kusaka I et al. Inhibition of

apoptosis by hyperbaric oxygen in a rat focal cerebral ischemic model. *J Cereb Blood Flow Metab* 2003; 23: 855-864.

30. Zhou C, Li Y, Nanda A, Zhang JH: HBO_2 suppresses Nogo-A, Ng-R, or RhoA expression in the cerebral cortex after global ischemia. *Biochem Biophys Res Commun* 2003; 309: 368-376.
31. Buras JA, Stahl GL, Svoboda KK, Reenstra WR: Hyperbaric oxygen downregulates ICAM-1 expression induced by hypoxia and hypoglycemia: the role of NOS. *Am J Physiol Cell Physiol* 2000; 278: C292-302.
32. Miljkovic-Lolic M, Silbergleit R, Fiskum G, Rosenthal RE: Neuroprotective effects of hyperbaric oxygen treatment in experimental focal cerebral ischemia are associated with reduced brain leukocyte myeloperoxidase activity. *Brain Res* 2003; 971: 90-94.
33. Konda A, Baba S, Iwaki T, Harai H, Koga H, Kimura T, Takamatsu J Hyperbaric oxygenation prevents delayed neuronal death following transient ischaemia in the gerbil hippocampus.*Neuropathol Appl Neurobiol*. 1996; 22: 350-60.
34. Rusyniak DE, Kirk MA, May JD, Kao LW, Brizendine EJ, Welch JL, Cordell, WH, Alonso, RJ. Hyperbaric oxygen therapy in acute ischemic stroke: results of the Hyperbaric Oxygen in Acute Ischemic Stroke Trial Pilot Study. *Stroke*. 2003 34:571-4