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 (HBO₂) (2-atmosphere absolute for 60 min using 100% oxygen) during rewarming group (n = 8), moderate hypothermia without HBO₂ group (n = 8), and sham treatment without hypothermia and without HBO₂ group (n = 8). Both the hypothermia group (77.9 ± 48.1 neurons per mm, mean ± SD) and hypothermia + HBO₂ 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 + HBO₂ group showed significantly greater preservation of CA1 pyramidal neurons than the hypothermia group (p < 0.05). These results suggest that HBO₂ 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 (HBO₂) 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 HBO₂. Therefore, HBO₂ 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 (HBO2) (2-atmosphere absolute for 60 min using 100% oxygen) during rewarming group (n = 8), moderate hypothermia without HBO2 group (n = 8), and sham treatment without hypothermia and without HBO2 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.

**HBO2 administration**

For HBO2 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/cm²/min, and decompression was carried out at 0.2 kg/cm²/min. There was no seizure observed in any animal during the procedure. The animals in the sham-HBO2 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 + HBO2 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 + HBO2 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 (HBO2) 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 HBO2 (17,18). Sukoff et al. reported that CSF pressure was reduced after HBO2 in dogs with experimentally induced cerebral edema and compression (19,20). HBO2 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 HBO2 is responsible for vasoconstriction responses (23). cNOS is reported to decrease in the ischemic region (24). Therefore, vasoconstriction caused by HBO2 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 HBO2 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

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham treatment</th>
<th>hypothermia</th>
<th>hypothermia + HBO₂</th>
<th>Sham ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Residual CA1 pyramidal neurons (mean ± SD)</td>
<td>6.4 ± 2.7</td>
<td>77.9 ± 48.1</td>
<td>127.6 ± 29.7</td>
<td>176.9 ± 16.4</td>
</tr>
</tbody>
</table>

Both hypothermia and hypothermia + HBO₂ groups were significantly preserved CA1 pyramidal neurons in hippocampus compared to sham treatment group (p < 0.01). Furthermore, hypothermia + HBO₂ 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 HBO2 can induce neuroprotection by preserving blood-brain barrier function (18). Improvement of the microcirculation and oxygen delivery in ischemic regions by HBO2 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 HBO2 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). HBO2 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 HBO2 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 HBO2 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 HBO2 at 1.5 ATA have been reported benefit (35). We have been reported that pretreatment with 2-ATA HBO2 once every other day for 5 sessions induced ischemic tolerance in experimental ischemia, but those with 2-ATA HBO2 for one session or with 3-ATA HBO2 once daily for 10 sessions did not. These results may indicate that preferential HBO2 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 HBO2 during rewarming after successful brain hypothermia. Clinical application of HBO2 will require determination of the ideal pressure and timing during rewarming, but this study suggests that HBO2 during rewarming after successful hypothermia may be useful as adjuvant therapy for stroke patients.

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