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Research report

Hyperbaric oxygenation prevented brain injury induced by hypoxia–ischemia in a neonatal rat model

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Abstract

The occurrence of hypoxia–ischemia (HI) during early fetal or neonatal stages of an individual leads to the damaging of immature neurons resulting in behavioral and psychological dysfunctions, such as motor or learning disabilities, cerebral palsy, epilepsy or even death. No effective treatment is currently available and this study is the first to use hyperbaric oxygen (HBO) as a treatment for neonatal HI. Herein, we sought out to determine if HBO is able to offer neuroprotectivity against an HI insult. Seven-day-old rat pups were subjected to unilateral carotid artery ligation followed by 2.5 h of hypoxia (8% O_2 at 37 °C). HBO treatment was administered by placing pups in a chamber (3 ATA for 1 h) 1 h after hypoxia exposure. Brain injury was assessed based on ipsilateral hemispheric weight divided by contralateral hemispheric weight, light microscopy, and EM. Sensorimotor functional tests were administered at 5 weeks after hypoxia exposure. After HI, the ipsilateral hemisphere was 52.65 and 57.64% ($P<0.001$) of the contralateral hemisphere at 2 and 6 weeks, respectively. In HBO treated groups, the ipsilateral hemisphere was 77.77 and 84.19% ($P<0.001$) at 2 and 6 weeks. There was much less atrophy and apoptosis in HBO treated animals under light or electron microscopy. Sensorimotor function was also improved by HBO at 5 weeks after hypoxia exposure (Chi-square, $P<0.050$). The results suggest that HBO is able to attenuate the effects of HI on the neonatal brain by reducing the progression of neuronal injury and increasing sensorimotor function.

Theme: Development and regeneration

Topic: Neuronal death

Keywords: Hyperbaric oxygen; Hypoxia–ischemia; Neonatal rat

1. Introduction

Hypoxia is one of the major pathological factors that induces neuronal cell injury, neurodegeneration, and cell death by causing a dysbalanced intracellular Ca^{2+} homeostasis followed by a cascade of potentially hazardous cellular challenges such as excitatory amino acid excitotoxicity, glycopenia, and acidosis [14,29,31]. It often acts in combination with ischemia due to a serious impairment in blood supply of brain tissue caused either by local or general circulatory failure. With ischemia occurring alongside hypoxia (termed hypoxia–ischemia, HI), the severity of the outcome of the hypoxic event increases and the chance of survival of the affected neurons decreases [29]. HI-induced brain dysfunction and neuronal death occur in two phases: an immediate loss of neuronal function (primary damage) and a delayed loss of function that could occur hours or days later (secondary damage) [31].

If an HI insult occurs during a critical cellular or tissue differentiation process, that episode might have a serious impact on brain maturation. For this reason alone the perinatal age is of great importance, but bear in mind that the process of delivery and the sudden adaptation to postnatal life are in their own right stressful and demanding events for the metabolic homeostasis of the newborn organism. Add on the event of an HI episode to this

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already stressful time and the results can be catastrophic at best or fatal at worst. The damage can be seen in two areas: anatomical or psychological. Anatomical consequences on the developing neurons vary from cell death to a hampered differentiation of cellular extremities, i.e. dendrites and axons. Even if the immature neurons are able to survive the HI insult, these abnormalities that have stricken the neurons may lead to behavioral and psychological dysfunctions, such as motor or learning disabilities, cerebral palsy, or epilepsy [18].

Various drugs [5,40] and hypothermia [9] have been used as a treatment with some degree of success, but nothing has emerged as an effective clinical treatment. The purpose of this study was to determine the effects of hyperbaric oxygen (HBO) on the neonatal rat brain after an HI insult and to determine if HBO can act as a suitable neuroprotective treatment against the events that follow an HI attack. HBO has been used as a treatment for stroke, CO poisoning, air embolism or decompression sickness, and wound healing in adults [16,25,41]. In newborns and children, HBO has been a successful treatment of radiation induced bone and soft tissue complications, cyanotic congenital heart disease, as well as CO poisoning [3,35]. With past studies mainly dealing with cerebral ischemia in adult models of different animals, this current study is the first to study HBO as a treatment in a neonatal hypoxic–ischemic rat model.

2. Material and methods

2.1. Hypoxia-ischemia model

The Animal and Ethics Review Committee at the University of Mississippi Medical Center evaluated and approved the protocol used in this study. The model used in this study is based on the Rice [33] modification of the Levine [24] preparation in the adult rat. Pups were housed with the dam under a 12:12 h light dark cycle, with food and water available ad libitum throughout the study. Unsexed 7-day-old (day 0, day of birth) Sprague–Dawley (Harlan) rats were anesthetized by inhalation with isoflurane (0.1%) in oxygen. The rats were kept at a temperature of 37 °C as the right common carotid artery of each pup was exposed and ligated with 5–0 surgical sutures. The duration of the anesthesia did not exceed 20 min and the pups were allowed to recover with their dams for 2 h. They were then placed in a jar perfused with a humidified gas mixture (8% oxygen balanced nitrogen) for 2.5 h. Both the jar and the gas mixture were kept at 37 °C. The pups were returned to their dams after the hypoxic exposure.

2.2. Experimental groups and HBO treatment

The pups were divided into the following three groups: (1) control ($n=75$); (2) HI ($n=76$); and (3) HI+HBO ($n=78$). Each group was composed of pups from each litter to obtain parity within the groups. The brains were removed and analyzed at 24, 48 and 72 h, and 1, 2 and 6 weeks. The pups that underwent HBO treatment were allowed to recover from hypoxic exposure for 1 h before being placed in the HBO chamber (Bethlehem Steel) with their dams (to control body temperature). The control pups and HI pups were kept under a heating lamp to maintain their temperature. The pups were kept in the chamber (3 ATA) for 1 h and returned to their cages after treatment. Animals were weaned at postnatal day 21 and separated by gender at postnatal day 42.

2.3. Brain weight

The pups were sacrificed under deep pentobarbital anesthesia (60 mg/kg, IP). After removal of the brain, the cerebellum and brain stem were removed from the forebrain. The hemispheres were separated by a midline incision and then weighed on a high precision balance (sensitivity ±0.001 g). The cerebellum was also weighed. Brain damage was expressed as the percent reduction of the ipsilateral (right) hemisphere compared to the contralateral (left) hemisphere.

2.4. Function test

The postural reflex test [10] was used to evaluate functional recovery in the pups 5 weeks after injury. The examiner was blinded to the experimental protocols. The pups were held by the tail 50 cm above the table. Normal rats extend both forelimbs toward the table (Score 0). Pups with brain damage flex the forelimb contralateral to the damaged hemisphere (Score 1). Thereafter, the pups were put onto the table, and a lateral pressure was applied behind the shoulder of the pup until the forelimbs slid. This was repeated several times, and a reduced resistance to lateral force toward the contralateral side was considered abnormal (Score 2).

2.5. Histology

Brains were fixed in 3.7% formaldehyde/PBS and kept at 4 °C. Two-mm thick coronal sections were cut from the brain with the most frontal cut surface being 2 mm from the frontal pole of the intact hemisphere. The sections were then dehydrated in 70% ethanol, embedded in paraffin wax, and sectioned. Five-micrometer sections were stained with hematoxylin and eosin and then examined with light microscopy. Pictures were taken with a digital camera (Canon) at a magnification of 24×.

2.6. Electron microscopy

For EM, the brain was removed and placed in a solution

of 2% glutaraldehyde for 4 days. Coronal sections were cut through the hippocampus and frontotemporal cortex and placed in 2% glutaraldehyde. Samples measuring 4 mm in height and 1 mm in thickness were post-fixed with osmium tetroxide, dehydrated in a graded series of acetone, embedded in epon-araldite epoxy resin, sectioned at 60 Å, and examined with a LEO 906 (Leo, Thoenwood, NY) transmission electron microscope (TEM). Each section was scanned at low magnification for location and identification of neurons. Using increased magnification, neurons were evaluated for the presence of apoptosis or necrosis.

2.7. Statistical analysis

The data was represented as the means±standard error (S.E.). Statistical differences were compared by using a one-way ANOVA and then a Tukey test if a significant difference was found; a P value <0.050 was considered to be statistically significant. Chi-square analysis was used for the test based on the scoring system.

3. Results

3.1. Mortality

The mortality of pups subjected to HI was variable between different litters with an overall mortality of 13%. The pups either died during the surgery or in the hypoxic chamber and no pup died after hypoxic exposure in the chamber.

3.2. Brain weight and morphology

The brain was divided into three regions (contralateral hemisphere, ipsilateral hemisphere, and cerebellum) to determine the degree of damage based on weight (Fig. 1). Total brain weight (Fig. 1A) was determined by adding the three regions. The degree of brain damage was assessed by dividing the ipsilateral hemispheric weight by the contralateral hemispheric weight (Fig. 1E) and expressed as a percentage. Based on the evaluation of brain damage by weight, HBO reduced brain damage by 26.8% at 1 week, 47.71% at 2 weeks and 46.10% at 6 weeks.

Over a period of 6 weeks following a HI-induced brain injury, the neonatal brain undergoes significant growth retardation. After HBO treatment, the progression of brain damage appears to be lessened. A side view (Fig. 2) of the ipsilateral hemisphere at 6 weeks shows that a HI insult leads to a significant loss of tissue, but after HBO treatment the loss of tissue is not as obvious. The loss of tissue in the HI+HBO seems to not be localized to a certain area, but rather dispersed over the entire hemisphere.

3.3. Body weight

Body weight (Table 1) was measured for each animal throughout the course of the study. A significant difference was only found between the HI and control groups and the HI+HBO and control groups at 2 weeks.

3.4. Histology

Coronal sections of striatum (Fig. 3) level show the progression of hypoxic–ischemic brain damage from slight neuronal loss to a marked degeneration of the ipsilateral hemisphere. Extensive cerebral cortical atrophy and damage can be seen on the ipsilateral hemisphere of the hypoxic–ischemic animals. A remarkable difference in the extension of injury was found among the animals that were treated with HBO. The damage is mostly confined to moderate neuronal loss with the ipsilateral hemispheres being reduced only slightly compared to the ipsilateral hemispheres of the hypoxic–ischemic animals. Furthermore, the hemisphere appears to be intact with the only abnormality being the slight reduction in size.

3.5. Electron microscopy

Sections of the dorsal dentate gyrus were examined with EM (Fig. 4) at 24 and 72 h after hypoxia exposure. Some neurons exhibited prominent characteristics of apoptotic bodies, such as: rounding up of cell bodies, cytoplasmic and nuclear compactions, and chromatin clumping and fragmentation into multiple spherical bodies. Some neurons also showed signs of necrosis, which is characteristic of numerous irregular chromatin clumps, a loss of cytoplasmic integrity, or swelling of the cytoplasm. Necrosis and early stages of apoptosis can be seen in the HI sections at 24 h. At 72 h, there is a dramatic loss in tissue and most of the cells demonstrate some characteristic of apoptosis. After HBO treatment, there is no sign of apoptosis at 24 and 72 h, but at 72 h there still was a dramatic loss of tissue and some neurons are necrotic. Control samples did not display any characteristics of apoptosis or necrosis.

3.6. Function test

The Postural Reflex Test shows that an HI insult affects the sensorimotor function of the animal. Score 0 represents normal sensorimotor function, whereas Score 1 and Score 2 represent a deficiency in sensorimotor function. All of the control animals scored a Score 0. 44.44% of the HI animals scored a Score 0, 55.56% a Score 1 and 55.56% a Score 2. 65.38% of the HI+HBO scored a Score 0, 34.62% a Score 1 and 15.38% a Score 2. A Chi-squared test ($P<0.050$) showed that there was a significant difference in the distribution between the groups.

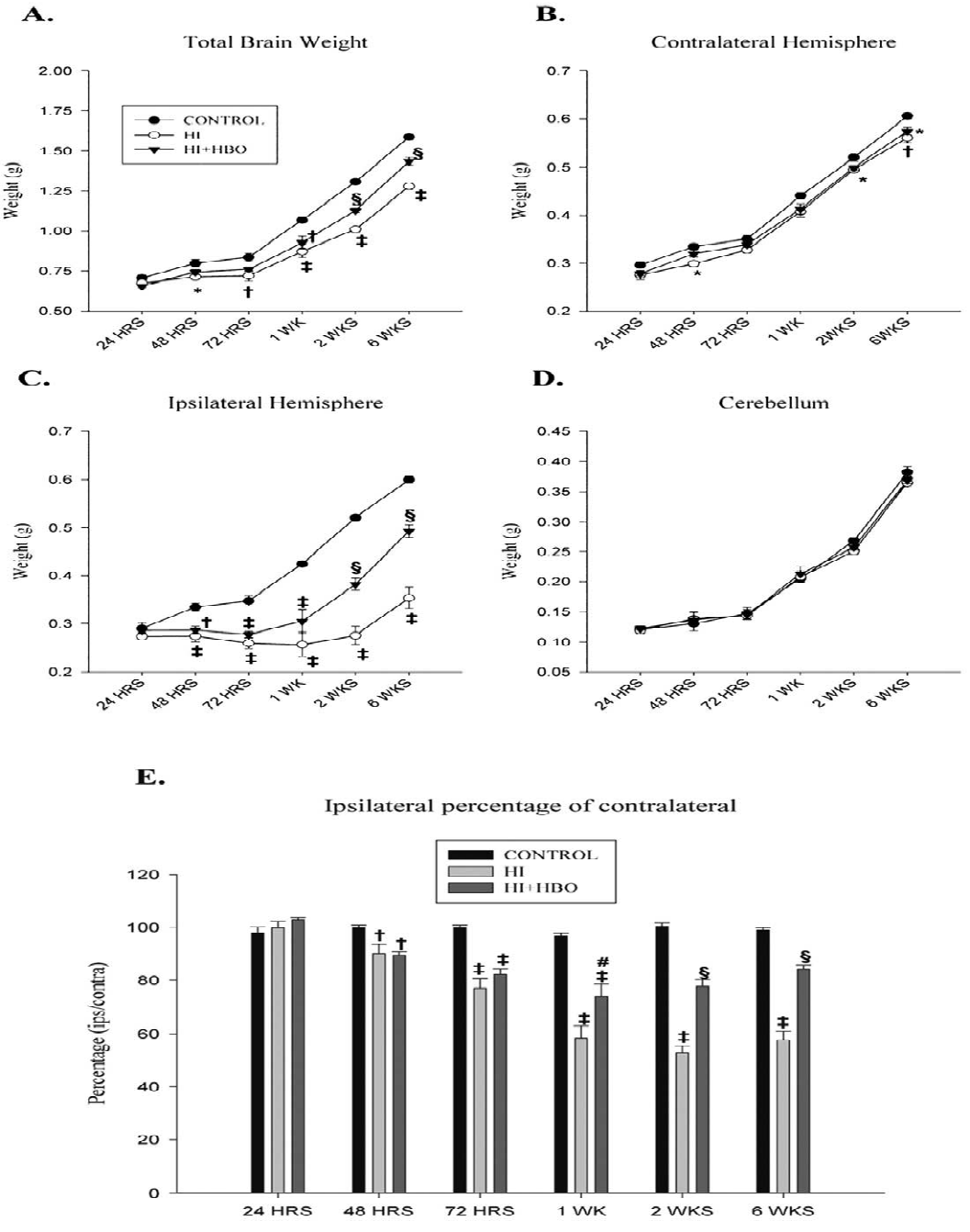

Fig. 1. Hypoxia-ischemic (HI) induced brain injury assessed by brain weight. (A) Total brain weight. (B) Contralateral hemispheric weight. (C) Ipsilateral hemispheric weight. (D) Cerebellum weight. (E) Ipsilateral hemisphere percentage of contralateral hemisphere. (*$P<0.050$, †$P<0.010$, ‡$P<0.001$ compared to control, §$P<0.001$ compared to control and HI, #$P<0.010$ compared with HI by ANOVA). HBO stands for hyperbaric oxygen. Error bars denote S.E. Symbols are defined in panels (A and E).

4. Discussion and conclusions

In the present study, we report that HBO is able to reduce the progression of brain damage induced by HI in the neonatal brain. We show that HBO is able to improve brain recovery in terms of weight and volume, which leads to a recovery of sensorimotor function. Furthermore, HBO offers a long-lasting neuroprotective effect against the tissue loss that accompanies HI, possibly by intervening in the apoptotic pathway. The neuroprotective qualities of HBO have been studied in adult stroke models for some time now, but this is the first study to demonstrate these qualities in a neonatal model.

In 1963, Jacobson and Lawson found that HBO treatment of 2 ATA after permanent occlusion of the middle cerebral artery (MCA) in dogs had no benefit on the

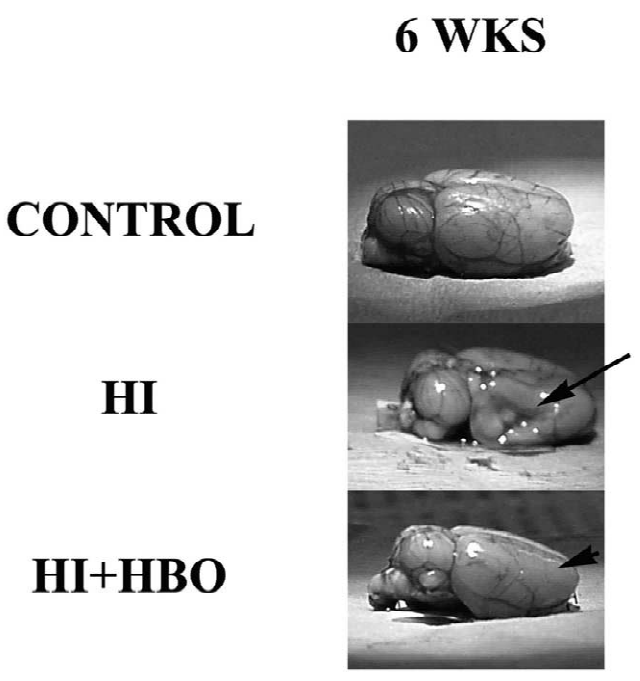

Fig. 2. Side view of control, HI and HI+HBO brains at 6 weeks. An empty space (arrow) can be seen in the ipsilateral hemisphere after HI. After HBO treatment, the ipsilateral hemisphere (arrowhead) does not have this empty space.

outcome of the animals [22]. Since that time, many studies have followed and some have shown promising results [11,23,36,39], whereas others have shown no benefit at all [26,34,39]. A detailed review of these experimental studies suggests that the methods used, the concepts studied, and the differences in the experimental design might account for this discrepancy [39].

Despite the critical clinical and socio–economic consequences of perinatal brain damage, no effective clinical therapeutic strategy has yet to be developed to prevent its causes. With the better understanding of the mechanisms that underlie neuronal cell death, several diverse possibilities have presented themselves for pharmacological intervention [6]. Past studies have focused on the administration of oxygen free radical scavengers [13], NO inhibitors [19], glutamate antagonists [1], calcium antagonists [7], potassium channel agonists [38], growth factors [2,28], and anticytokines [30]. Still, other studies have focused on hypothermia as a neuroprotective agent [9,17]. Many of these studies have shown to be effective in the laboratory, but nothing has been able to make the jump over into the clinic as an effective treatment.

We used a 7-day postnatal rat model [33] in our study because at this stage of development the rat's brain is histologically similar to that of a 32–34-week gestation human fetus or newborn infant. This model has proved useful in many studies and is used in the United States and abroad [37]. This model yields a reproducible pattern of hemispheric injury ipsilateral, but not contralateral, to the ligated carotid artery and furthermore, it allows for an assessment of mechanisms of brain injury and testing of neuroprotective agents and strategies [20]. Animals that experienced HI showed retardation in brain growth, especially to the hemisphere ipsilateral to the ligated artery. Similar to other studies, we showed that severe tissue loss and atrophy accompany HI and lead to this reduction in brain development [10,21]. In contrast, animals that were treated with HBO experienced less brain damage and overall HBO was able to reduce brain damage by up to 47%. Our data shows that at the light microscopy and EM levels neurons are being spared after treatment with HBO. Neuronal cell death in this model of hypoxia–ischemia is known to occur in at least three different forms (apoptosis, necrosis, or some hybrid of the two). These different forms can coexist in the same region of the brain or in different regions or may even coexist in the same cell [27]. Although severe ischemic insults may be dominated by necrotic mechanisms, apoptosis may represent a target for stroke treatment because it is likely involved in a different mechanism than necrosis and may occur in areas of milder injury for days after the initial insult [32]. Based on our findings that apoptosis is not present in HBO-treated samples 24 and 72 h after hypoxia exposure, HBO may offer a protective effect against the progression of injury by way of preventing apoptosis. More in depth studies are certainly needed to confirm if anti-apoptosis is one of the mechanisms for the therapeutic effect of HBO and if in fact it is a viable treatment target given that the presence of necrotic cells and tissue loss at 72 h furthers adds to the possibility that necrosis and apoptosis occur via different mechanisms. One of the reasons that we did not perform experiment examining the long-term effect of HBO on apoptosis was according to Northington et al. [27] that HI-induced apoptosis in the striatum and neocortex is complete by 6 days following the initial injury. In this

Table 1
Body weight

	24 h (g)	48 h (g)	72 h (g)	1 week (g)	2 weeks (g)	6 weeks (g)
Control	17.65±0.99	22.47±1.27	22.01±1.19	29.93±1.86	52.24±1.83	184.13±8.48
HI	14.81±0.88	19.82±0.62	20.41±0.88	26.64±2.85	39.70±1.39*	174.39±7.30
HI+HBO	15.30±0.63	19.27±1.88	20.42±0.99	31.62±0.53	43.66±1.15*	174.85±5.70

Values are mean±S.E. *$P<0.001$ compared to control (ANOVA).

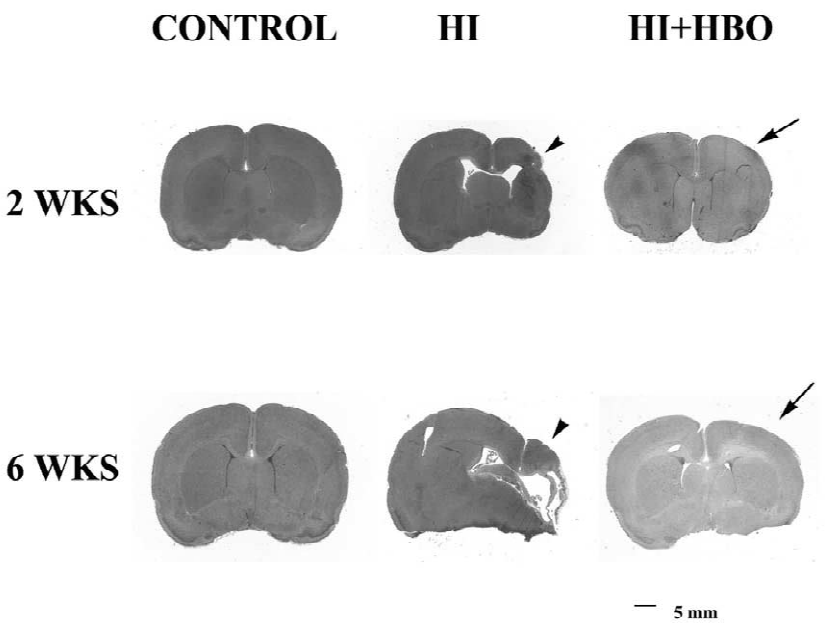

Fig. 3. Histology of coronal sections. Coronal sections at the level of the striatum from pups exposed to hypoxia–ischemia and treated with HBO at 2 and 6 weeks. Extensive cerebral and cortical atrophy and damage to the striatum on the ipsilateral side (arrowheads). Less atrophy can be seen in the HBO-treated animals (arrows). Bar, 5 mm. Hematoxylin-eosin.

study, we did not see any signs of necrosis or apoptosis in samples taken 1 week after the HI insult. However, it is definitely a possibility that there could be delayed apoptosis at some time following 1 week after the insult. This is something that should be explored, but it will take a more in depth study that is designed to look at apoptosis.

In agreement with Bona et al. [10], we found that animals that had experienced HI scored worse in the postural reflex test than control animals. In contrast, animals that were treated with HBO scored similar to control animals. The findings that HBO is able to prevent, in most cases, the sensorimotor deficits caused by HI is in agreement with a previous study [4] that demonstrated the same protective aspects of HBO as evaluated by a motor deficit test in a MCA occlusion adult model. Other authors [11,23,39] using a MCA occlusion model, found that HBO is able reduce behavioral deficits, infarction volumes, and edema which all lead to an improved outcome. With the onset of the biochemical events that follow an HI insult, the tissue of the developing brain is severely damaged. This damage can render certain areas of the brain nonfunctional, resulting in a number of neurological handicaps. The treatment of HBO appears to preserve viable tissue by: (1) improving tissue oxygen delivery, especially to areas of diminished flow; (2) enhancing neuronal viability by increasing the amount of dissolved oxygen in the blood without significantly changing blood viscosity; (3) increasing arterial oxygen pressure and content; (4) reducing edema; (5) reducing brain vascular permeability, and enhances blood–brain barrier integrity; (6) restoring ion pump function; (7) improving postischemic cerebral metabolism; and (8) allowing time for collateral circulation to develop [25]. The prevention of HI-induced damage by HBO allows the brain tissue to continue development.

There has been some controversy surrounding the use of HBO treatment. Some studies [8,12,15] have shown that exposure to oxygen of sufficient pressure and duration can result in CNS toxicity, mainly by the generation of oxygen free radicals, which in turn can lead to cell death. These studies have used pressures in excess of 4 ATA and have subjected the animals to repeated exposures of oxygen. Whereas, these studies have provided a good look at the limitations of high doses of oxygen and repeated exposures, they have also cast a dark shadow on the effectiveness of HBO treatment. At lower pressures and exposure times, HBO has been shown to improve outcome considerably without detrimental side affects [11,23,39]. So, it appears that oxygen in like any other treatment agent, in that too much can cause negative affects. But, we believe that the proper administration of HBO can lead to an affective treatment, which will benefit the lives of countless individuals.

In summary, the results of the present study suggest that HBO is able to attenuate the effects of HI on the neonatal brain by reducing the progression of neuronal injury and increasing sensorimotor function. Since HBO has been used to treat humans in the past with a certain degree of success and since it is currently being used as an effective treatment in infants with various disorders, it may provide to be an effective strategy for the prevention of numerous neurological handicaps that plague many children. However, concerns regarding the toxicity of oxygen that can occur when HBO is administered at high doses or over long periods of time needs to be addressed along with the

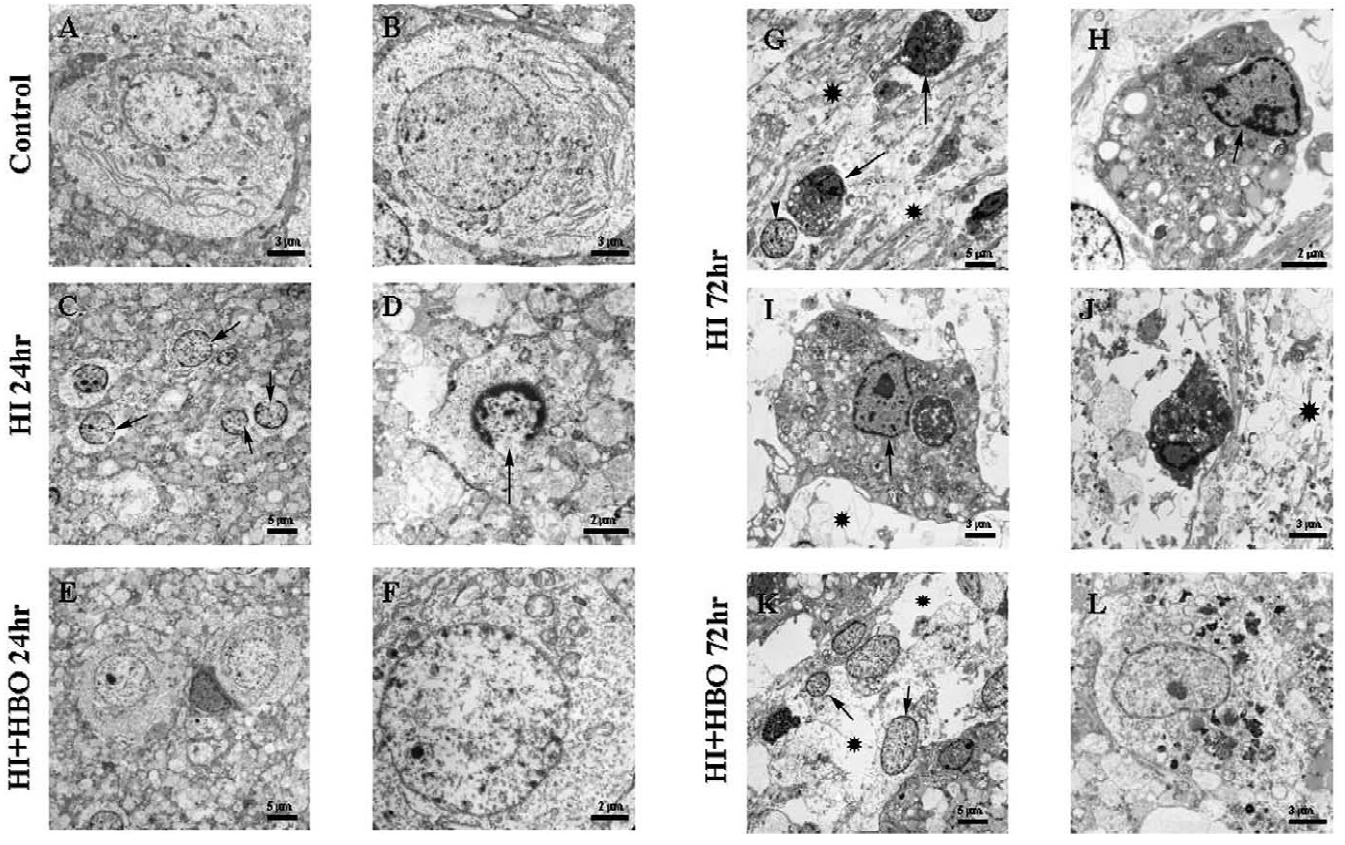

Fig. 4. EM of neonatal rat brain. The dorsal dentate gyrus 24 and 72 h after hypoxia–ischemia and HBO treatment. (A and B), nonischemic dorsal dentate gyrus (Bar, 3 μm). (C), Low magnification of dorsal dentate gyrus 24 h after hypoxia–ischemia. Cells can be seen undergoing early stages of apoptosis or necosis (arrows) (Bar, 5 μm). (D), High magnification of a cell that shows a breakdown in the nuclear membrane (arrow) (Bar, 2 μm). (E), Low magnification of dorsal dentate gyrus 24 h after treatment with HBO (Bar, 5 μm). (F), High magnification a cell that shows no sign of apoptosis 24 h after treatment with HBO (Bar, 2 μm). (G), Low magnification of dorsal dentate gyrus 72 h after hypoxia–ischemia. Apoptotic (arrows) and necrotic (arrowhead) cells can be seen among the cellular debris (stars) that is apparent at 72 h after hypoxia–ischemia (Bar, 5 μm). (H–J), High magnification of apoptotic cells showing degeneration of the nuclear membrane (arrows) surrounded by cellular debris (star). [Bar, 2 μm in (H), Bar, 3 μm in (I–J)] (K), Low magnification of dentate gyrus 72 h after treatment with HBO. No signs of apoptosis, but some cells are necrotic (arrows). There is still some debris (stars) surrounding the cells, but it is less than that seen in the hypoxia–ischemia samples (Bar, 5 μm). (L), High magnification of cell 72 h after treatment with HBO. Cell shows no signs of apoptosis (Bar, 3 μm).

establishment of a standard treatment plan before HBO can be used in the treating of newborns.

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