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Research Report

Hyperbaric oxygen therapy improves spatial learning and memory in a rat model of chronic traumatic brain injury

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ABSTRACT

In the present experiment we use a rat model of traumatic brain injury to evaluate the ability of low-pressure hyperbaric oxygen therapy (HBOT) to improve behavioral and neurobiological outcomes. The study employed an adaptation of the focal cortical contusion model. 64 Male Long–Evans rats received unilateral cortical contusion and were tested in the Morris Water Task (MWT) 31–33 days post injury. Rats were divided into three groups: an untreated control group ($N=22$), an HBOT treatment group ($N=19$) and a sham-treated normobaric air group ($N=23$). The HBOT group received 80 bid, 7 days/week 1.5 ATA/90-min HBOTs and the sham-treated normobaric air group the identical schedule of air treatments using a sham hyperbaric pressurization. All rats were subsequently retested in the MWT. After testing all rats were euthanized. Blood vessel density was measured bilaterally in hippocampus using a diaminobenzadine stain and was correlated with MWT performance. HBOT caused an increase in vascular density in the injured hippocampus ($p<0.001$) and an associated improvement in spatial learning ($p<0.001$) compared to the control groups. The increased vascular density and improved MWT in the HBOT group were highly correlated ($p<0.001$). In conclusion, a 40-day series of 80 low-pressure HBOTs caused an increase in contused hippocampus vascular density and an associated improvement in cognitive function. These findings reaffirm the clinical experience of HBOT-treated patients with chronic traumatic brain injury.

1. Introduction

Traumatic brain injury (TBI) is a disorder of major public health significance. Each year in the United States alone there are 100 new cases/100,000 population and 52,000 deaths (NIH Consensus, 1999). Most patients survive and add to an increasing prevalence of chronic TBI, estimated at 2.5–6.5 million individuals in 1998 (NIH Consensus, 1999). The impact of TBI

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on each new case is enormous. Direct and indirect costs have been estimated at $56 billion/year in 1995 (Thurman, 2001). All of these figures are felt to be gross underestimates due to reporting bias against (Alexander, 1995; Kraus and Nourjah, 1988) and significant underdiagnosis (NIH Consensus, 1999; Frankowski et al., 1985) of mild TBI. Unfortunately, there is no cure for chronic TBI and only a few studies suggest effectiveness under limited conditions (NIH Consensus, 1999).

HBOT is the use of greater than atmospheric pressure oxygen as a pharmacologic treatment of basic disease processes/states and their diseases (Harch and Neubauer, 1999). HBOT has a variety of physiological effects depending on the chronicity of the underlying pathology. When delivered repetitively in chronic shallow perfusion gradient wounds (Marx and Johnson, 1988) HBOT repairs wounds through stimulated fibroblast replication (Hehenberger et al., 1997), collagen synthesis (Ishii et al., 1999), and ultimately angiogenesis (Marx et al., 1990). The mechanisms are postulated to be a combination of upregulation of key growth hormone receptors (Bonomo et al., 1998) and hormones (Sheikh et al., 2000), improved wound responsiveness to growth hormones (Wu et al., 1995), and DNA signaling (Siddiqui et al., 1997; Ishii et al., 1999). No one has investigated these processes in chronic brain injury.

In the early 1990s we found that a lower pressure/dose of HBOT than traditionally employed for diving cases could be used to successfully treat delayed, recalcitrant, and chronic cases of decompression illness (Harch, 1996). The durable clinical improvements (cognitive, social, work/school, emotional, and neurological) were mirrored by improvements in SPECT brain imaging (Harch, 1996). We and others subsequently applied this low-pressure HBOT protocol to additional chronic cerebral disorder cases, including chronic traumatic brain injury, with similar clinical and SPECT improvements (Neubauer et al., 1992, 1994, 2004; Harch et al., 1996a, 2004; Neubauer and James, 1998; Harch and Neubauer, 1999, 2004; Golden et al., 2002). The present study is an attempt to develop an animal model of aspects of the human case series, to experimentally examine the efficacy of HBOT in promoting recovery after chronic brain injury, and to provide information about brain vascular changes that may underlie the SPECT brain blood flow improvement seen in human patients. To those ends we adapted the open focal cortical brain contusion model of Feeney et al. (1981). Previously we used 12 rats in a single control group blinded pilot trial and found that low-pressure HBOT after unilateral cortical contusion improved spatial learning (cognition) and simultaneously increased vascular density in the affected hippocampus (Harch et al., 1996b). Since the study was a small pilot trial we did not control for the effects of altitude (rats were injured in Albuquerque at ~5600 ft and treated in New Orleans at ~14 ft below sea level). The goal of the present study was to replicate the pilot findings with a greater number of rats and two control groups: hyperbaric air control group and no treatment altitude control group. We now report a replication of the pilot findings with greater statistical power and stronger experimental design. To our knowledge this is the first demonstration of noninvasive improvement of chronic brain injury in an animal model.

2. Results

One of the hyperbaric oxygen rats became anorexic and adipsic and died during the treatment phase of the experiment in New Orleans. Thus, all of this rat's data are omitted and our results included the 64 rats that completed all phases of the experiment.

2.1. *Forelimb placing*

Mean differences of percentage correct forelimb placing before and after treatment were nearly identical for the three groups. The means and standard errors were 0.014±0.044 (TBI+HBOT), −0.037±0.054 (TBI+Air), and 0.011±0.048 (TBI+Altitude). Comparison of forelimb placing between groups using one-way ANOVA showed no significant change after HBOT, air treatment, or staying at altitude ($F<1$, $p>0.7$).

2.2. *Morris water task*

2.2.1. *Before treatment*

The rats of all groups before treatment showed improved performance in the Morris Water Task across days and within a session during training in the first room. This is particularly clear during the trials of the 10th day of training. The rats of all three groups took approximately 15 s on average to find the hidden platform on the first trial but were finding it in less than 10 s by the final trials. A repeated measures ANOVA showed that this improvement in time to find the platform over trials on the last day of training was statistically significant ($F(7,427)=5.6$, $p<0.001$). There was no overall difference among the three groups on this measure ($F<1$) and the groups' improvements over trials did not differ ($F<1$).

When the rats were transferred to the second room a similar pattern of performance was observed. There was a statistically significant improvement in time over trials to find the platform ($F(6,360)=4.3$, $p<0.001$), but no difference among the groups ($F(2,60)=1.01$, $p=0.37$), and the improvement shown by all groups over trials did not differ ($F<1$) between groups. We compared the average performance at the end of training in the first room to average performance in the second room using a repeated measures ANOVA on mean time to find the platform across the first 7 trials. The rats took significantly longer to find the platform in the new room ($F(1,61)=7.3$, $p=0.009$), but there was no overall significant difference between groups ($F<1$), and the differences among the three groups in the effect of room change were not significant ($F<1$). During the no-platform probe test in the second room the percentage of swim path in the correct quadrant by all three groups was significantly greater than would be predicted by chance (25%). This was evaluated using three two-tailed, one-group t-tests (altitude group: $t(21)=3.65$, $p=0.001$; air group: $t(22)=7.38$, $p<0.001$; hyperbaric group: $t(18)=2.41$, $p<0.03$).

2.2.2. *After treatment*

Because there was considerable variability in swim performance before treatment we used the performance of the rats during the last day of training in the first room pretreatment as a covariate in a repeated measures ANOVA of the treatment

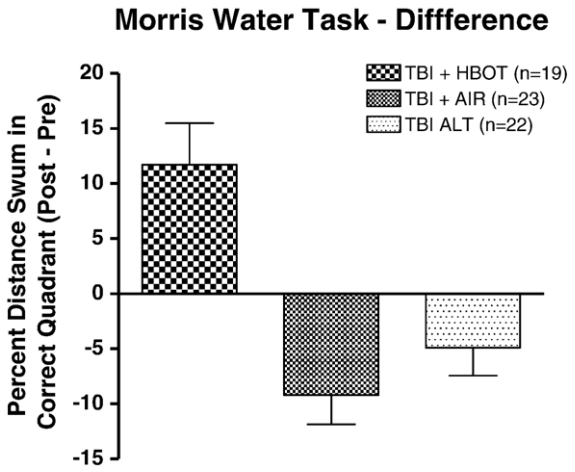

Fig. 1 – Morris Water Task Probe Trial results. Percent increase or decrease in swim distance in the correct quadrant (post-HBOT minus pre-HBOT) vs. group. HBOT group demonstrated significant increase compared to either control group, $p<0.001$.

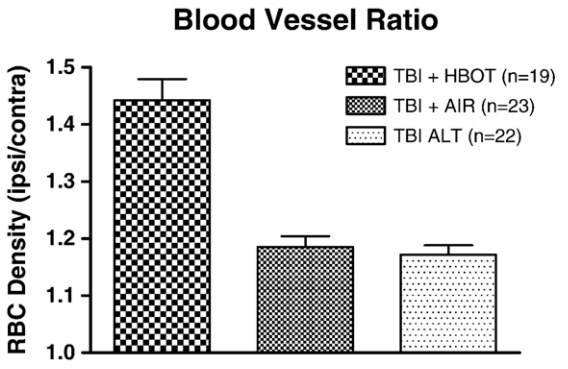

Fig. 3 – Cerebrovascular density in hippocampi. Ratio of ipsilateral to contralateral density vs. treatment group. Ratio (and blood vessel density) is greatest in the HBOT group, $p<0.001$.

effects. We analyzed time to find the platform in the first room for the trials of days 1, 2, and 10 after treatment. The covariate ANOVA on day 1 performance revealed that the groups significantly improved across trials ($F(7,427)=16.14$, $p<0.001$) but that the improvement was significantly different across trials for the treatment group ($F(14,427)=2.04$, $p<0.015$). This significant interaction effect between treatment and trials is due to the trend for the hyperbaric oxygen-treated group to take less time to find the platform on trials 3–6 than the other two groups, but similar times on the other trials. The overall difference in time to find the platform between groups was not statistically significant ($F(2,60)=1.25$, $p>0.6$). On day 2 of post-treatment testing there was a significant improvement in time to find the platform across trials ($F(7,427)=11.69$, $p<0.001$), but neither the interaction effect nor the main effect of treatment was statistically significant (respectively, $F(14,427)=1.54$, $p>0.09$; $F(2,60)=2.71$, $p>0.07$). This pattern of performance was seen at the last day of post-treatment testing as well, with a significant improvement across trials ($F(7,427)=5.71$, $p<0.001$), but not a significant interaction effect ($F<1$) nor a significant main effect of treatment ($F<1$).

When the rats were transferred to a new pool room (room 3) after treatment, they showed an increase in time to find the platform from the last day of room 1 testing after treatment ($F(1,61)=8.73$, $p=0.004$). The interaction between this increase and the treatments was not statistically significant ($F(2,61)=1.69$, $p>0.19$) nor was the main effect of treatment ($F<1$). The ANOVA with pretreatment day 10 performance as a covariate revealed that there was a statistically significant improvement in time to find the platform across trials in the new room ($F(6,366)=6.24$, $p<0.001$). This improvement was not different among the treatment groups ($F(12,366)=1.61$, $p=0.088$) nor was there a statistically significant overall effect of treatment on time to find the platform ($F(2,60)=1.57$, $p=0.216$).

During the no-platform probe trial a difference among the treatment effects emerged in their searching in the correct location. Again, because of the variability in pretreatment no-platform probe performance, we used percentage swim path in the correct quadrant in room 2 as a covariate in an ANOVA on swim path in the correct quadrant in room 3. There was a statistically significant effect of treatment on percent of swim distance in the correct quadrant ($F(2,60)=9.4$, $p<0.001$). This was due to the clear increase in percent distance in the correct quadrant by the hyperbaric oxygen-treated rats and decreases by the two other treatment groups (Fig. 1).

2.3. *Histology*

2.3.1. *Contusion*

The contusions produced a cystic cavity in the neocortex only in the right hemisphere (Fig. 2). The medial and lateral tissue boundaries of the cavity extended as far medial as the corpus

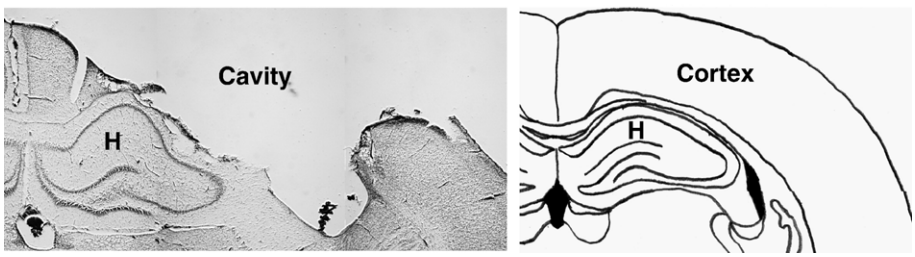

Fig. 2 – Coronal section through site of cortical impact (left) at time of sacrifice. Cavity is the area of cortical and underlying white matter necrosis caused by focal weight drop. "H" is the underlying hippocampus. On the right is a drawing of the same coronal section pre-injury.

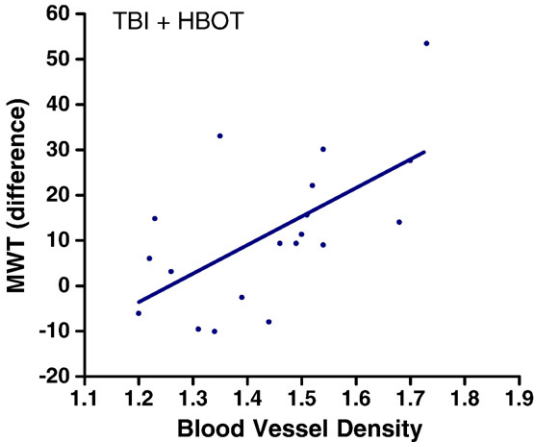

Fig. 4 – Behavioral (spatial learning)/vascular density correlation for HBOT group. Morris Water Task Probe Trial data from Fig. 1 vs. cerebrovascular density data from Fig. 3 for HBOT group. Morris Water Task and vascular density were highly correlated, $r=0.62$, $p<0.001$.

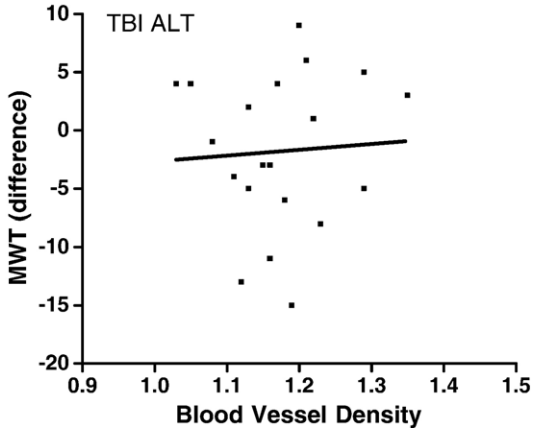

Fig. 6 – Behavioral (spatial learning)/vascular density correlation for altitude group. Morris Water Task Probe Trial data from Fig. 1 vs. cerebrovascular density data from Fig. 3 for altitude group. Morris Water Task and vascular density were not correlated, $r=0.060$, $p>0.8$.

callosum and at its widest point was typically 4.5–5.5 mm in lateral extent. TBI-induced contusion cavity size variance did not differ between groups. A one-way ANOVA on injury size revealed no statistically significant difference ($F<1$).

2.3.2. *Cerebrovascular density*

The DAB stain in the hippocampus consistently revealed greater blood vessel density ipsilateral compared to contralateral to the contusion in all three groups. A one-way ANOVA on the ratio of vessel density ipsilateral to contralateral revealed a statistically significant effect of treatment ($F(2,61)=36.69$, $p<0.001$). This effect was due to the higher ratio (1.44) shown by the hyperbaric oxygen-treated group compared to the ratios (1.19 and 1.17) of the two other treatment groups which were very similar to each other (Fig. 3).

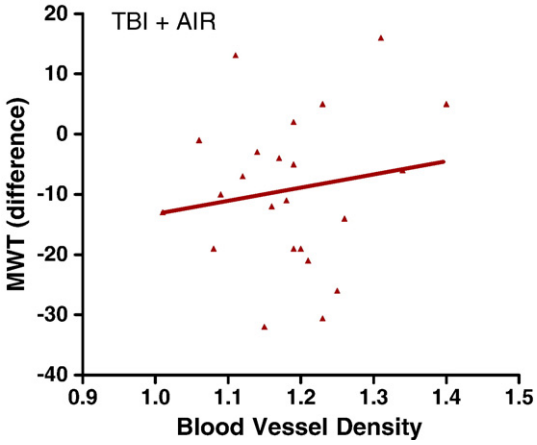

Fig. 5 – Behavioral (spatial learning)/vascular density correlation for air treatment group. Morris Water Task Probe Trial data from Fig. 1 vs. cerebrovascular density data from Fig. 3 for air treatment group. Morris Water Task and vascular density were not correlated, $r=0.15$, $p>0.4$.

2.3.3. *Behavioral correlations*

As a way of measuring if either the extent of injury or hippocampal cerebrovascular density predicted the major positive behavioral outcome, we calculated two correlations. The first was between our estimates of contusion-induced injury and the difference in percentage of swim path in the correct quadrant before and after treatment. We found that this correlation was not statistically significant ($r=0.07$, $p=0.59$). The second was between the ratio of ipsilateral to contralateral hippocampal vessel density (cerebrovascular density) and the difference in percentage of swim path in the correct quadrant before and after treatment. We found that the relationship between higher vessel density and better behavioural performance *after treatment* (Fig. 4) was statistically significant ($r=0.62$, $p<0.001$) for the hyperbaric oxygen group only. The same relationships in the air treatment group, Fig. 5 ($r=0.15$, $p>0.4$), and altitude group, Fig. 6 ($r=0.060$, $p>0.8$) were nearly identical to each other and non-significant. All three groups are represented in Fig. 7.

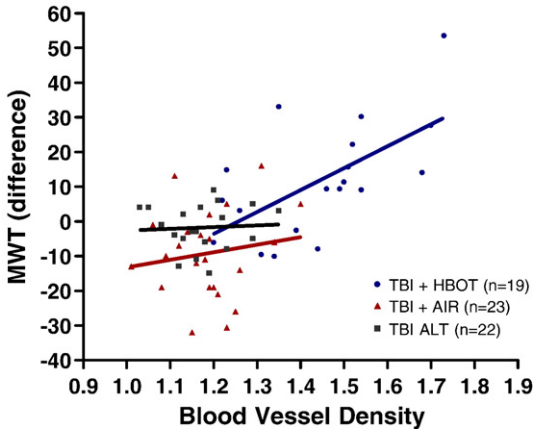

Fig. 7 – Composite graph of behavioral (spatial learning)/vascular density for all three groups.

3. Discussion

In the present study, we measured the effects of markedly delayed low-pressure hyperbaric oxygen therapy (HBOT) on several outcome measures following traumatic brain injury in rats. Our neocortical contusion procedure, the focal cortical weight-drop method (Feeney et al., 1981) produced extensive unilateral damage that involved sensorimotor cortex and to a lesser extent hippocampus (Weisend and Feeney, 1994; Golarai et al., 2001). As previously described, this form of traumatic brain injury produces long-lasting cognitive changes measured in the Morris Water Task (Sutherland et al., 1993). HBOT enhanced spatial performance in the Morris Water Task and increased blood vessel density in the hippocampus. Furthermore, the extent of improvement in cognition was associated with greater hippocampal blood vessel density increase. Thus, the present results replicate and extend the findings of an earlier pilot study (Harch et al., 1996b) on the effects of low-pressure HBOT on hippocampal vascular density and cognition in chronic traumatic brain injured rats. Significantly, our results offer experimental support for the conclusions about the efficacy of HBOT in chronic human TBI patients (Neubauer et al., 1994; Harch et al., 1996a, Harch and Neubauer, 1999, Golden et al., 2002) in which similar low-pressure HBOT demonstrated clinical, cognitive, and SPECT brain blood flow improvements. The HBOT-associated SPECT brain imaging findings in human patients consisted of a relative increased brain blood flow to low flow areas of brain and a relative decreased flow to higher flow areas. This corresponds to a shift from a diffuse heterogeneous pattern to a more homogeneous pattern of blood flow. The relative increase in brain blood flow to the areas of lower flow is consistent with our present results of increased vascular density in the damaged hippocampus.

The injury from the focal cortical weight-drop method consists of a cortical cavity that extends to the underlying white matter and a microscopic injury to the underlying structures, including the hippocampus (Weisend and Feeney, 1994; Golarai et al., 2001). Earlier work has shown a functional deficit in spatial learning and memory in this contusion model (Sutherland et al., 1993). The cortical cavitation is complete by day 15 (Feeney et al., 1981). The hippocampal injury is manifest by a maximal rate of neuronal loss and deafferentation in the first week post injury that subsequently slows over at least a 27-week period (Golarai et al., 2001). The cell loss is accompanied by gliosis and abnormal sprouting of granule cell axons (Golarai et al., 2001). Damage to hippocampal vessels has not been studied or reported. The deafferentation and neuronal loss of the contusion injury would be expected to produce some degree of inflammation and microvascular hippocampal damage due to focal edema from tissue injury. A resultant shallow perfusion gradient wound similar to the Marx model (Marx and Johnson, 1988) above would be likely, but this is not established.

HBOT involves the delivery of greater than atmospheric pressure oxygen in an enclosed chamber to treat basic pathophysiologic processes/states and their diseases (Harch and Neubauer, 1999). In chronic non-central nervous system (CNS) wounds characterized by ischemia/hypoxia the intermittent exposure to greater than atmospheric pressures of oxygen is thought to cause a steepening of the oxygen gradient from the center of the wound to the more normally vascularized periphery (Marx and Johnson, 1988). The steepened gradient is felt to cause DNA signaling (Ishii et al., 1999; Siddiqui et al., 1997), upregulation of key proteins involved in the wound repair process (Bonomo et al., 1998; Sheikh et al., 2000), and angiogenesis (Marx et al., 1990). Angiogenesis may be explained in part by HBOT-generated increases in VEGF (Sheikh et al., 2000). Our study is the first to investigate possible HBOT-induced angiogenesis in chronic brain injury.

In our study, we chose a diaminobenzadine (DAB) blood stain (Sherman and Paull, 1985) to measure vascular density (Rieder et al., 1995) as an indirect measure of angiogenesis. We were looking for vascular changes in the injured hippocampus to correlate with improvements in function on the Morris Water Task. DAB stains blood, allowing for an index of blood volume. Increases in blood volume can be caused by angiogenesis, increased metabolism/recruitment of existing unused vessels, recruitment of unused vessels alone, a change in vascular tone (dilation), or a combination of one or more of these factors. We feel that angiogenesis is the most likely explanation given the well-described presence of angiogenesis in other HBOT acute and chronic wound models (Marx et al., 1990; Ketchum et al., 1970; Manson et al., 1980). We suggest that this is the most parsimonious explanation for the change in DAB staining we observed in the hippocampus. As a cautionary note, without actual vessel counting, however, it is impossible to conclusively say with the present model, stain, and data that HBOT induced angiogenesis. Hence, our study strongly suggests, but does not reaffirm, the reported HBOT-induced angiogenesis reported in other chronic wound models.

We also chose to use ratios of blood vessel density instead of absolute numbers to control for shrinkage and other artifacts of the tissue harvest, preservation, preparation, and histology procedures. By taking a ratio of ipsilateral DAB hippocampal stain to ipsilateral hippocampal surface area much of the anatomic distortions should be eliminated. The important findings shown by the ratio data are that HBOT induced increases in blood volume (vessel density) in the injured hippocampus responsible for the deficit in spatial learning and that the increased blood volume/vascular density was highly correlated with an improvement in hippocampal relevant spatial learning and memory. Given the coupling of “blood flow and metabolism” and “metabolism and function” in normal brain (Reivich, 1974) and chronically injured brain (Raichle et al., 1976) it is apparent that metabolism, blood flow, and hence function as a whole were increased in the hippocampus.

The results of our study show an improvement in spatial learning and simultaneous increase in vascular density in the contused hippocampus and no effect on forelimb placing (data not reported). The absence of improvement in forelimb placing is not surprising. Forelimb placing is lost secondary to massive loss (cavity formation) of the sensorimotor cortex that subserves this function. Cavity size did not decrease in the HBOT group compared to the controls, hence no restoration of function was expected or found. Spatial learning is due to the hippocampal injury and is tested by the probe trial of the Morris Water Task. The improvement of the HBOT rats on the probe trial implies incomplete injury, partial reversibility of the injury, and a

capacity to respond to HBOT. In other words, there is partial retention of the components of the hippocampus responsible for the probe trial of the MWT and HBOT either revives these components, regenerates lost elements of the hippocampus responsible for the probe trial, prevents further loss of tissue, or a combination of the three choices. Unfortunately, the limited focus of this study does not permit a conclusion on this point. The improved spatial learning function and concomitantly increased vascular density in our study could be due to a variety of mechanisms or combination of mechanisms. Since neuronal loss continues for at least 27 weeks in the neocortical contusion model (Golarai et al., 2001), HBOT intervention may halt apoptosis as it has been shown to do in acute brain injury models (Kondo et al., 1996; Calvert et al., 2003; Rosenthal et al., 2003; Yin et al., 2003). Neocortical contusion-induced axon damage would be expected to produce excitatory amino acid elaboration (Hovda et al., 1995), and tissue inflammation, and its associated edema (Schoettle et al., 1990; Zhuang et al., 1993). Microscopic edema would cause vascular compression, stasis, and thrombosis of vessels leading to microscopic areas of wounding (Kimura et al., 1996). Microscopic shallow perfusion gradients would be established similar to both the microscopic and macroscopic injury in the Marx model (Marx and Johnson, 1988) above and in brain decompression sickness (Polkinghorne et al., 1988; Palmer et al., 1992; Staff et al., 1995, 1996; Harch, 1996; Macleod et al., 1996; Shields et al., 1997; Houston et al., 1998). HBOT could then act in a similar fashion to induce angiogenesis. This would be consistent with the increased vascular density demonstrated in this study.

Alternatively or in addition, microscopic ischemia would lead to a metabolic/protein synthesis insult to neurons, termed the "ischemic" freeze (Hossman, 1993) that may also explain apoptosis and the continued tissue loss over 27 weeks in this model (Golarai et al., 2001). HBOT could be overriding the ischemic freeze in this neocortical contusion model. The improved function that we have demonstrated could derive from improved metabolism in the hippocampus which would generate increased demand for blood flow.

HBOT-induced axon sprouting may also contribute to improved spatial learning. HBOT has caused axon sprouting in models of spinal cord injury and peripheral nerve (Gelderd et al., 1980; Haampaniemi et al., 1998). The underlying mechanism could be stimulation of growth hormones or production of new growth hormones as in non-CNS wound models. Researchers have also demonstrated anti-inflammatory/immunosuppressive effects of HBOT (Ginaldi et al., 1991, Weisz et al., 1997; Saito et al., 1991; Inamoto et al., 1991). HBOT may be slowing or halting the gliotic process demonstrated in this model. Improved synaptogenesis, synaptic remodeling, and dendritic changes are also possible. All of these processes normally occur as adaptive responses to brain injury (Gall et al., 1980; Jorgensen et al., 1997; Jones and Schallert, 1992). HBOT may upregulate these processes as it has been shown to do with cell surface growth hormone receptors (Bonomo et al., 1998). HBOT may be increasing neuronal replication or stimulating replication and/or differentiation of neuronal stem cells. Dividing hippocampal cells have been found in close association with angiogenesis (Palmer et al., 2000). If HBOT-induced angiogenesis explains the increased vascular density in our study, neurogenesis is also possible. Alternatively, HBOT could be stimulating release of bone marrow stem cells into the circulation which could home to the injured hippocampus. Such release into the circulation has been demonstrated in normal people, non-central nervous system radiation injured patients undergoing HBOT, and animals (Thom et al., 2006). Unfortunately, there are no direct data for any of the above speculative mechanisms in the present study.

The importance of this study is twofold. First, to our knowledge, this is the only demonstration of noninvasive improvement of chronic brain injury in an animal model. Second, and more importantly, this study reaffirmed our experience and the experience of others with HBOT in chronic human TBI patients (Neubauer et al., 1994; Harch et al., 1996a; Harch and Neubauer, 1999; Golden et al., 2002) which demonstrated clinical, cognitive, and SPECT brain blood flow improvements. We have demonstrated that the similar effect generated in humans with chronic traumatic brain injury, i.e., clinical, cognitive, and SPECT brain blood flow improvement, is underpinned by highly correlated/coupled vascular changes and cognitive improvements in an animal model of chronic traumatic brain injury. Importantly, the improved vascular density in our animal model is consistent with the pattern of HBOT-induced increased flow to areas of lower flow seen on SPECT brain imaging in hyperbaric oxygen-treated patients with chronic TBI. Given the similarity of this pattern and clinical response in the multiple other chronic neurological conditions treated with HBOT (Neubauer et al., 1992; Harch, 1996; Neubauer and James, 1998; Harch and Neubauer, 1999, 2004; Golden et al., 2002; Harch et al., 2004; Neubauer et al., 2004; Stoller, 2005; Vila et al., 2005; Rossignol and Rossignol, 2006) and the similarity of pathology in chronic brain injury irrespective of etiology (Cormio et al., 1997), the results of our study may be applicable to other non-traumatic forms of brain injury.

In conclusion, we have adapted a well-known acute animal model of focal traumatic brain injury to use in a chronic setting, subjected the rats to a human protocol of low-pressure HBOT, and caused an improvement in cognition and an increased vascular density in the hippocampus. Most importantly, the improvement in spatial learning and increased vascular density was strongly linked: HBOT induced a functional/cognitive improvement and simultaneously increased vascular density of the injured hippocampus. To our knowledge, this is the first demonstration of noninvasive improvement of chronic brain injury in an animal model.

4. Experimental procedure

This experiment was approved by the Animal Care and Use Committees of the Baromedical Research Institute of New Orleans, Louisiana State University School of Medicine, New Orleans, and University of New Mexico.

4.1. *Subjects*

Sixty-five male, Long–Evans hooded rats (supplied by Harlan Laboratories), each weighing between 300 and 360 g on the day of surgery, served in this three-phase experiment (see Fig. 8 for time-line). For the first and third phases of the experiment

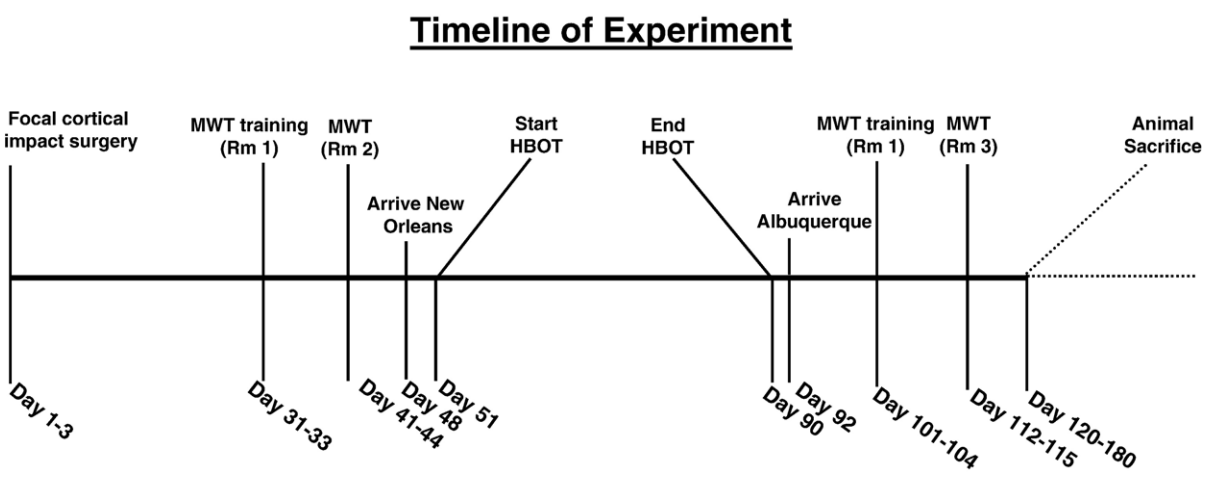

Fig. 8 – Time-line of experiment from cortical impact injury to animal sacrifice.

they were housed in the Department of Psychology, University of New Mexico. The rats were weighed every day for the 5 days before surgery and the 5 days after surgery, then weighed once a week. During the hyperbaric treatment phase (second phase) of the experiment, 43 rats were shipped to and housed in the vivarium at the Baromedical Research Institute (New Orleans, LA). 22 Rats remained at the New Mexico facility (Altitude control group). At both facilities the rats were housed individually in standard, hanging wire-mesh cages, maintained on a 12:12-h light:dark cycle, and were given free access to water and laboratory chow, except 12 h before surgery.

4.2. *Surgical procedure*

Surgery was performed under aseptic conditions and in accordance with NIH guidelines. Following induction (5.0% halothane in O_2 at 1.5 l/min) of general anesthesia and maintenance (1.5% to 3.0% halothane in O_2 at 1.5 to 2.5 l/min), the rats were placed in a stereotaxic frame, the scalp and periosteum were resected, and the right temporal muscle was blunt dissected away from the lateral aspect of the skull. A circular craniotomy, 5 to 7 mm in diameter, was performed over the right sensorimotor cortex, exposing the dura.

All rats received cortical trauma using the focal cortical weight-drop impact method (Feeney et al., 1981). Briefly, after completion of the craniotomy described above, the footplate of the contusion device is stereotaxically positioned over the right sensorimotor cortex, centered 2.5 to 3.0 mm posterior, and 2.5 to 3.0 mm lateral to bregma. Next, the guide tube was lowered 3.0 mm below the point at which the footplate rests on the exposed dura. This limited the depression of cortical tissue under the footplate to 3.0 mm. The device is constructed to prevent bouncing of the weight, thus allowing only a single compression producing a controlled cortical contusion. When the footplate was in position, a 20-g weight was dropped 20 cm through the guide tube, producing a 400-g/cm^2 impact to the brain underlying the footplate. The footplate was removed within 4 s after impact. Immediately following the removal of the footplate, the bone flap was replaced, the cranium was sealed with sterile bone wax, and the scalp was sutured. Animals were carefully monitored by observation during the procedure for adequacy of anesthesia. During recovery they were regularly administered parenteral narcotics to minimize pain.

4.3. *Hyperbaric treatment*

HBOT was delivered in a Vickers monoplace chamber with 100% oxygen commencing 51 days following cortical contusion and 3 days after air transport arrival from New Mexico. All rats receiving hyperbaric oxygen treatment ($N=20$) were placed in duplex cages, one rat to each side of the duplex. Six duplex cages were juxtaposed side to side on a tray with a second identical tray of six cages on top to form a double deck of cages. The double deck of cages was slid into the hyperbaric chamber on a gurney. Pressure within the chamber was increased at a constant rate from sea level (1.00 ATA—atmospheres absolute) to 1.50 atmospheres (16.5 FSW—feet of seawater) over 8 to 10 min. Oxygen flow rate was 200 l/min. The animals remained in the chamber at 1.50 ATA for approximately 70 min. At the 80-min mark the chamber was depressurized over 8–10 min to surface pressure. Total treatment time was 90 min. Treatments were administered twice in 24 h with a minimum 4-h break between treatments. The remaining rats (hyperbaric controls, $N=23$) were treated identically except that compressed air was administered in a sham pressure profile. The chamber was pressurized with air at the same compression rate as the oxygen group to a pressure of 1.13 ATA or 4 FSW for 2–12 min (average 7 min) and then allowed to drift back to surface pressure over the next few minutes. The chamber remained at surface pressure for the remainder of the dive such that the total dive time was equal to the oxygen group, 90 min. Flow rate was identical to the oxygen group. Animals in both groups were carefully observed throughout the dives for behavioral changes or signs of distress.

4.4. *Forelimb placing*

The placing reflex in the forelimb contralateral to the cortical contusion was measured in all rats. The response was elicited by touching the vibrissae and dorsal aspect of the paw on the

edge of a flat, horizontal surface while the rats was held with snout and both paws facing the surface. A successful response was scored if the rat lifted and placed the palmar surface of the paw on the surface. Each rat received 2 days of 10 placing trials in one session prior to hyperbaric oxygen 43 days following surgery and 2 days of 10 more placing trials in a second session 19 days after completion of treatment. The effect of treatment was assessed by comparing with one-way ANOVA the mean difference in each group of the percent of successful placing responses out of 20 trials after the treatments minus the percent of 20 trials before treatment for each rat.

4.5. *Morris water task*

A circular pool (1.5-m diameter) was filled to within 8 cm of the top of the pool wall with room temperature water (20–22 °C). A clear Plexiglas platform was placed in one quadrant 1.5 cm beneath the surface of the water and remained in that quadrant for all eight trials on a training day. Powdered skim milk was added to the water to hide the platform. Large, salient visible cues were placed on the walls of the pool room. The rat was placed into the water at the pool wall at one of the four cardinal compass points. Each start location was used once in every 4 trials according to a computer-generated random sequence. If the rats did not find the platform after 60 s they were lifted from the water by hand and placed on the platform for 10 s. Similarly, if they found the platform before the 60-s limit, they were allowed to stay on the platform for 10 s.

Beginning 31–33 days after surgery, but before hyperbaric treatment, rats received 8 trials a day for 10 consecutive days in one room. On day 11, rats received 8 trials in a single session in a second pool room containing different visible wall cues. One week following hyperbaric treatment, rats were tested for 10 consecutive days in the first room with the identical procedures as before hyperbaric treatment. On the 11th day, rats were tested in a third room, with a different set of visible wall cues, for 8 trials. The ninth trial conducted on the 11th day in rooms 2 and 3 were 20-s probe trials during which the platform was removed. This probe trial assessed the spatial accuracy of the rat's search pattern, and hence, spatial learning and memory. Search accuracy was assessed by calculating the percent of swim distance in the correct quadrant. This performance before and after hyperbaric oxygen in treated and untreated rats was compared within and between groups. In addition, pre-HBOT/post-HBOT differences were calculated and compared between rats that received HBOT and rats that did not receive HBOT.

4.6. *Histology*

All rats were euthanized in random triads 30–90 days after the last HBOT and 8-68 days after room 3 Morris Water Task testing using sodium pentobarbital (100 mg/kg, intraperitoneal). Immediately after cessation of breathing, the animals were submerged in an ice bath for 1–2 h. The brains were then extracted from the skull, immersed in a phosphate-buffered 4% paraformaldehyde/20% sucrose fixative for 48–72 h, frozen with CO_2 gas, and cut into 60-μm-thick coronal sections using a cryostat microtome. Every fifth section from the olfactory bulb to the posterior cerebellum was mounted on a glass slide and stained using Sherman's red blood cell staining technique (Sherman and Paull, 1985). Red blood cells are rendered dark brown and quantified using a computerized densitometry program (Rieder et al., 1995).

The extent of the brain injury was measured using the same sections. In every section the areas of left and right hemispheres were measured in standardized units using NIH Image. The difference in the sum of the areas of hemisphere contralateral and ipsilateral to the contusion injury was taken as the estimate of the size of the cerebral injury.

Measurements of hippocampal cerebrovascular density were taken ipsilateral and contralateral to the injured hemisphere from each section containing neocortical contusion injury. Thus the measures were from approximately the anterior half of each hippocampus, including measures of the CA1, CA3, and dentate gyrus subfields. Two measures were taken: total hippocampal area in each section and the total area within the hippocampus stained with DAB. A vessel ratio was calculated (vessel area/total area) for each hemisphere and a second ratio was determined of ipsilateral vessel ratio/contralateral vessel ratio. This number was termed the cerebrovascular density of the ipsilateral hippocampus. A mean cerebrovascular density was calculated for each of the three groups and compared using one-way ANOVA.

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