

Research report

Neuroprotective effects of hyperbaric oxygen treatment in experimental focal cerebral ischemia are associated with reduced brain leukocyte myeloperoxidase activity

Milena Miljkovic-Lolic^a, Robert Silbergleit^{b,*}, Gary Fiskum^c, Robert E. Rosenthal^{c,d}

^aDepartment of Medicine, St. Joseph Medical Center, Towson, MD, USA

^bDepartment of Emergency Medicine, University of Michigan, TC B1354/0303, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0303, USA

^cDepartment of Anesthesiology, University of Maryland, Baltimore, MD, USA

^dProgram in Trauma, Department of Surgery, University of Maryland, Baltimore, MD, USA

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Abstract

Objective: Hyperbaric oxygen (HBO) reduces cerebral infarct size after middle cerebral artery occlusion (MCAO) in rats through an unknown mechanism. In other forms of injury, cellular protection with HBO is associated with diminished infiltration of polymorphonuclear neutrophils (PMN). We hypothesized that HBO given prior to or after MCAO reduces PMN infiltration into the brain, and that decreased PMN infiltration is associated with improved functional and anatomic outcome. **Methods:** Forty rats underwent MCAO and were randomized to pretreatment with HBO (3 ATA) immediately prior to ($n=13$), or posttreatment immediately after surgery ($n=12$), or to control (air 1 ATA) ($n=15$). Five rats underwent sham surgery. Neurologic outcome was measured at 24 h in all animals. Brain myeloperoxidase (MPO) activity ($n=22$) and infarct volume ($n=23$) were determined. **Results:** MPO activity was significantly higher in controls (mean 0.28, 95% C.I. 0.17–0.38) than in the HBO pretreatment group (0.12, 0.08–0.16), HBO posttreatment group (0.16, 0.13–0.19), and the sham group (0.02, –0.02 to 0.05). HBO treated animals also had better neurologic outcomes (pretreatment 1.5, 0.9–2.1, posttreatment 2.6, 2.0–3.2) and smaller infarcts (pretreatment 27%, 18–37%, posttreatment 28%, 19–37%) than controls (neurologic outcome 3.7, 3.1–4.4, infarct volume 39%, 30–48%). Neurologic outcomes correlate better with MPO activity ($R^2=0.75$) than with infarct volume ($R^2=0.25$). **Conclusion:** These data confirm the neuroprotective effects of HBO in cerebral ischemia and suggest that the mechanism of this action may involve inhibition of PMN infiltration in the injured brain.

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1. Introduction

Treatment with hyperbaric oxygen has been shown to be neuroprotective in several animal models of cerebral ischemia [19,21,22,30], but the mechanism of this therapeutic response is unknown. One possibility involves attenuation of inflammatory secondary brain injury by inhibition of polymorphonuclear neutrophils (PMN) infil-

tration. Activation of PMN by surface molecules on the endothelial cells adjacent to ischemic neurons and supporting cells triggers infiltration of these inflammatory cells into the area of infarction and penumbra. The presence of PMN in injured areas after focal cerebral ischemia has been documented in several models by histology and assays for tissue myeloperoxidase (MPO) [4,5,8,13,28]. PMN may contribute to secondary brain injury by reducing microvascular blood flow, initiating thrombosis, and releasing oxygen free radicals [15,18,24]. The endothelial surface molecules responsible for PMN activation and adherence involve highly specific receptor ligand interac-

*Corresponding author. Tel.: +1-734-936-6284; fax: +1-734-936-9414.

E-mail address: robie@umich.edu (R. Silbergleit).

tions. These molecules include beta-2 integrins (sharing the common beta chain, CD-18) on the leukocyte and ICAM-1 on the endothelium. Administration of antibodies against either ICAM-1 [45] or CD11b/18 [11] prior to or immediately after focal cerebral ischemia in rats has been shown to reduce infarction.

Infiltration of the brain with activated PMN and increased expression of beta-2 integrins have also been implicated in experimental neurotoxicity with carbon monoxide (CO) [35], another form of brain injury thought amenable to treatment with hyperbaric oxygen (HBO). Interestingly, HBO (3 ATA) has also been shown to interfere with the function of beta-2 integrins and prevent adherence of activated leukocytes to damaged endothelium after CO poisoning in rats [34], reduces beta-2 integrin function in human neutrophils in vivo [20,37], and has been shown to reduce ICAM-1 expression in ischemic endothelial cell culture [9].

We hypothesized that HBO would reduce the PMN infiltration after focal cerebral ischemia. Furthermore we hypothesized that if HBO suppresses ICAM-1 or beta-2 integrins that the treatment would be equally effective when given either prior to or immediately after cerebral vascular occlusion.

2. Materials and methods

The Institutional Animal Care and Use Committee of the George Washington University Medical Center approved this study. Focal cerebral ischemia was produced by permanent distal middle cerebral artery occlusion (MCAO) and temporary occlusion of both common carotid arteries (CCA) for 60 min in the manner described by Chen [10] with modifications [26,27].

Adult male Sprague–Dawley rats weighing 300–350 g (Hilltop Lab) were anesthetized with one dose of intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg) and were maintained with one supplemental dose if needed. Core body temperature was monitored rectally and normothermia was maintained with a homeothermic pad. The right femoral artery was catheterized for measurement of blood pressure, arterial blood gases, and serum glucose. Both common carotid arteries were isolated through the middle neck incision. A 3 mm burr hole was drilled at the junction of the zygomatic arch and squamous bone via a 2 cm incision made midway between the right eye and ear. The dura was opened and the middle cerebral artery (MCA) was exposed, elevated above the cortical surface, cauterized with a microbipolar coagulator, and then cut. Both CCA were occluded with atraumatic arterial clamps immediately after MCAO. The craniotomy was closed in layers. After 60 min of carotid occlusion, the arterial clamps and femoral artery catheter were removed and the incisions were closed.

Forty animals underwent MCAO and were randomly assigned to one of three groups. Animals in the pretreatment group ($n=13$) were treated with HBO immediately prior to surgery. Animals in the posttreatment group ($n=12$) were treated with HBO immediately after surgery. HBO treatments were delivered at a dose of three atmospheres absolute (ATA). Control animals ($n=15$) received no treatment, but were exposed to air at ambient room pressure (1 ATA). All animals were kept for 60 min in a small animal hyperbaric chamber. Another five rats underwent sham surgery.

Neurologic outcome was measured 24 h after MCAO in all animals by an examiner blinded to treatment. Focal neurologic deficit was assessed using previously validated instruments [6,30,41] and was scored on a 6-point scale on which 0 indicates no neurologic deficit and 6 severe deficit. In brief, the neurologic score measures elements of forelimb motor function, suspended thorax twisting, resistance to lateral movement, spontaneous ambulation and circling behavior. After scoring, animals were euthanized by *trans*-cardiac cerebral perfusion with saline.

To determine PMN infiltration, brain myeloperoxidase (MPO) was assayed in 22 animals in the manner of Barone [4]. Following saline perfusion, the brain was rapidly removed and immediately frozen in liquid nitrogen. The frozen tissue sample was weighed, homogenized and washed twice in 5 mM phosphate buffer, pH 6.0 at 4 °C, centrifuged at 30 000×*g* for 30 min. The pellet was suspended in 0.5% hexadecyltrimethylammonium bromide in 50 mM/l potassium phosphate buffer, pH 6.0 at 25 °C, and subjected to three freeze–thaw cycles with sonication between the cycles. Samples were then centrifuged at 12 000×*g* for 15 min at 4 °C. MPO activity was measured spectrophotometrically at 460 nm. One unit of MPO activity was defined as that degrading 1 μM of peroxide per min at 25 °C. MPO activity was normalized on the basis of grams of tissue wet-weight.

Infarct volume was determined by staining with 2,3,5-triphenyltetrazolium hydrochloride (TTC) [7] in the remaining 23 animals. Following saline perfusion, animals were perfused with 50 ml of 2% TTC. The brains were then removed, immersed in 2% TTC at 37 °C for 30 min, and fixed in 10% phosphate-buffered formalin. Brains were sectioned into five 1-mm thick coronal slices between 4 and 9 mm from the anterior surface. Sections were digitally imaged and analyzed by planimetry. Infarct volume is presented as a percent of the volume of the contralateral hemisphere.

Results are expressed as means ± standard deviations and 95% confidence intervals. Hypothesis testing for group comparisons was performed using *t*-tests for parametric, and Kruskal Wallis test for nonparametric data. For all comparisons, significance was set at 0.05. Correlation between neurological outcome and both MPO activity and infarct volume was analyzed using nonparametric analysis with Spearman's rho reported.

3. Results

Baseline physiologic parameters including weight, temperatures, MABP, blood gases, and glucose were the same in all groups and were consistent with those previously found in this model [27]. Three animals died during surgery, one in each of the MCAO groups, and are excluded from the analysis. There were no deaths, seizures, or other signs of oxygen toxicity associated with HBO treatment.

MPO activity (Fig. 1A) was significantly higher in the normobaric (control) ischemic brains (mean $0.28 \pm$ standard deviation 0.10 , 95% C.I. 0.17 – 0.38) than in the brains of animals treated with HBO prior to ischemia (0.12 ± 0.04 , 0.08 – 0.16 , $P=0.01$) or after ischemia (0.16 ± 0.03 , 0.13 – 0.19 , $P=0.04$). There was no significant difference in

MPO activity between the two HBO treatment groups ($P=0.11$). MPO was significantly lower in sham animals (0.02 ± 0.03 , -0.02 – 0.05 , $P<0.01$) than in all three ischemic groups.

Neurologic outcome is shown in Fig. 1B. Animals treated with HBO prior to ischemia had better neurologic outcome scores (1.5 ± 1.0 , 0.9 – 2.1) than those treated with HBO after ischemia (2.6 ± 1.0 , 2.0 – 3.2 , $P=0.02$). Both HBO treated groups had significantly better neurologic outcome scores than did control animals (3.7 ± 1.2 , 3.1 – 4.4 , $P \leq 0.01$). Sham animals showed no deficits on the neurologic outcome scores, which was significantly better than all the ischemic groups (0.0 ± 0.0 , $P<0.01$).

Infarct volumes (Fig. 1C) were also smaller in HBO treated animals ($28 \pm 9.4\%$, 22 – 33% , $P=0.02$) than in control animals ($39 \pm 11\%$, 30 – 48%). There was no difference in infarct volumes between the two HBO treatment groups ($P=0.91$).

Neurologic outcome scores correlated with both MPO activity (Spearman's $\rho=0.86$) and infarct volumes (Spearman's $\rho=0.53$).

4. Discussion

HBO has been shown to reduce secondary injury in several models including both focal brain ischemia [3,32,38,42,44] and global brain ischemia [25,31,33]. The mechanisms of this protective effect are unknown, but several possibilities have been advanced. These include such diverse ideas as improved oxygen supply to penumbral areas [38], down regulation of COX-2 [44], reduced striatal dopamine release [42], restoration of striatal metabolites [3], and alteration of vascular permeability and blood flow [31]. Another possible mechanism is reduction of inflammatory mediated secondary brain injury resulting from the activation and infiltration of PMN, and this has been demonstrated in a single previous study of transient focal cerebral ischemia [2]. Neutrophil inflammatory responses in focal brain ischemia are well documented [16] and inhibition of those responses by antibodies to beta-2 integrins have been demonstrated to reduce neuronal injury [29,43] in animal models. Human trials of these therapies [1] have failed because of complications of antibody administration [17], but alternative methods of attenuating beta-2 integrin would still hold promise. There is evidence from several model systems other than cerebral ischemia that HBO has the potential to reduce neutrophil mediated inflammatory response in this manner. Thom has shown that HBO interferes with the function of beta-2 integrins and prevent adherence of activated leukocytes both in vitro [36] and in an in vivo model of CO poisoning in rats [34]. Buras has shown that HBO down regulates ICAM-1 expression in endothelial cell culture exposed to hypoxia and hypoglycemia simulating ischemia and reperfusion [9].

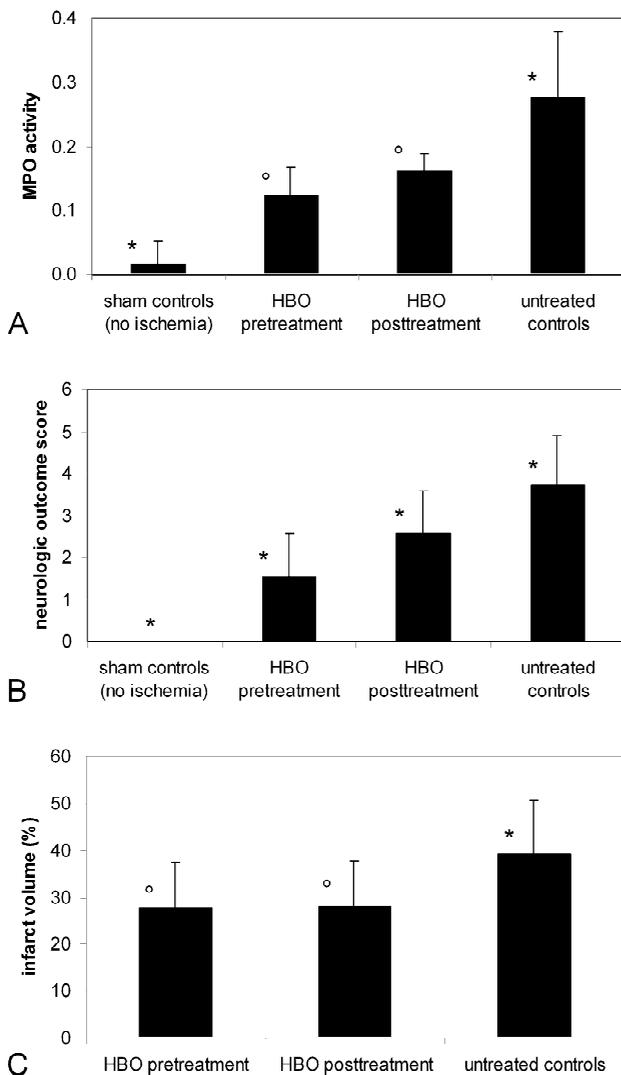


Fig. 1. Mean MPO activity (A), neurologic outcome score (B), and infarct volume (C) for each treatment group. Error bars show standard deviation; *, indicates $P<0.05$ vs. all other groups, °, indicates $P<0.05$ compared to untreated controls and sham.

HBO also reduced neutrophil infiltration and lipid peroxidation in a rat model of multisystem organ failure [12]. Finally, there is evidence that HBO reduces beta-2 integrin function in neutrophils in both healthy humans [20] and in humans after hepatic surgery [37].

Our data confirm that HBO is also associated with a decrease in leukocyte infiltration (as demonstrated by MPO assay) in a rat model of focal cerebral ischemia by permanent MCAO. Although these data cannot prove causality, pretreatment with HBO was at least as effective as treatment delivered after ischemia. This finding is consistent with the mechanism of anti-beta-2 integrin antibodies and other traditional neuroprotective pharmaceuticals and less suggestive of mechanisms such as enhanced oxygen supply to the penumbra, or restoration of metabolites.

Preconditioning with HBO has been shown in other models to induce ischemic tolerance [14,39], and it is conceivable that a similar mechanism was involved in our model. Elements of our data, however, suggest that the protection described here differs from that afforded by preconditioning. First, preconditioning has been shown to require longer exposures to HBO than those used in our model [23,40]. Second, our observation of similar protection from treatments given immediately before and immediately after the onset of ischemia cannot be explained by preconditioning alone.

Several limitations of this study should be noted. First, beta-2 integrin function was not directly measured in this study, so we cannot be certain that the suppression of leukocyte infiltration by HBO was mediated by down regulation of these molecules, although this seems likely based on prior work. Second, it is possible that HBO reduces infarct size by an unrelated mechanism and that decreased inflammation is not the cause but the result of a smaller infarction providing less nidus for leukocyte infiltration. Neurologic outcome, however, is more closely associated with MPO activity than with infarct volume in our data. This is indirect evidence that inflammation is causally related to neurologic outcome, rather than a side effect. Finally, the treatment times used in this model are selected to help define the potential mechanism of neuroprotection from HBO, but are not clinically realistic. The actual window of opportunity for effective treatment with HBO after brain ischemia remains poorly defined, but has been shown in some studies to be as late as 6 h post reperfusion [3,44].

One-hour treatment with HBO at 3 ATA immediately prior to or after the onset of experimental stroke reduces PMN infiltration in the injured brain, reduces infarct volume, and improves neurologic outcome. These data suggest that inflammation is involved in secondary brain injury after focal cerebral ischemia, and are consistent with a mechanism of action for HBO that involves inhibition of neutrophil infiltration.

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