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Effects of Normobaric Oxygen on the Progression of Focal Cerebral Ischemia in Rats

Elga Esposito, Emiri T. Mandeville, Kazuhide Hayakawa, Aneesh B. Singhal, and Eng H. Lo
Departments of Neurology and Radiology, Massachusetts General Hospital, Harvard Medical School, Boston, USA

Abstract

Normobaric oxygen (NBO) reduces infarction at 24–48 hrs in experimental models of focal cerebral ischemia. However, to be clinically relevant, longer term safety and efficacy must be explored. Here, we assessed the effects of NBO on glial activation, neurovascular recovery, and behavioral outcomes at 2 weeks after transient focal ischemia in rats. 100 min transient focal ischemia was induced by intraluminal occlusion of the middle cerebral artery in adult male Sprague-Dawley rats. Animals were randomized into sham, controls or 85 NBO started 15 minutes after ischemic onset. Infarct volumes and behavioral outcomes were blindly quantified. Immunohistochemistry was used to examine the effects of NBO on glial activation and neurovascular responses. After 2 weeks of reperfusion the infarct volume was markedly reduced in animals subjected to NBO. They also had better outcomes in forelimb placement test and in body-swing test and weight loss reduction. After 14 days, NBO decreased expression of Iba1, a marker of activated microglia, and GFAP, a marker of activated astrocytes. NBO treatment had no detectable effect on angiogenesis. These results suggest that protective effects of NBO may persist for up to 2 weeks post-stroke.

Keywords

normobaric oxygen therapy; stroke; neuroprotection

Introduction

Many experimental studies have demonstrated that NBO is able to reduce infarct volume and neurological deficits in rodents subjected to ischemic stroke (Kim, et al., 2005, Singhal, et al., 2002, Singhal, et al., 2002). Furthermore, NBO did not appear to worsen postischemic brain hemorrhage, edema, or blood-brain barrier damage, and it may not increase matrix metalloproteinase levels or various other markers of oxidative stress (Velthkamp, et al., 2006, Liu, et al., 2009). In addition, a recent study demonstrated that NBO did not worsen outcomes in a rat model of intracerebral hemorrhage (ICH), suggesting that treatments could potentially be started even before a definitive diagnosis to distinguish ischemic versus hemorrhagic stroke (Fujiwara, et al., 2011). Based on these preclinical animal studies, NBO

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Correspondence: Elga Esposito, Phone: (+49) 174 7747895, Address: Otfried-Müller-Strasse 27. 72076 Tübingen, Germany, elga.esposito@gmail.com or Aneesh B. Singhal, Address: 15 Parkman Street. Boston, MA 02114-3117, USA, Phone: (+1) 855-644-6387, asinghal@partners.org.

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treatment was extended to humans in a few early proof-of-concept clinical trials. The initial findings suggested that high-flow oxygen therapy was associated with a transient improvement of clinical deficits in patients with acute ischemic stroke (Singhal, et al., 2005, Singhal, et al., 2007, Wu, et al., 2012). Hence, NBO therapy might be a potentially viable approach for acute stroke (Singhal, 2007).

Nevertheless, the majority of experimental studies so far have shown a neuroprotective effect of NBO only during the acute phase, mostly after 24 or 48 hours of reperfusion. So the effect of NBO for longer periods of recovery post-stroke remains to be elucidated. Since some potential risks are associated with oxygen therapy in stroke, as increased of free radicals (Liu, et al., 2006, Singhal, et al., 2002) and vasoconstriction (Shin, et al., 2007), it may be important to assess efficacy and safety of NBO treatment for longer periods post-stroke. In this study, we examined the effect of NBO in a rat model of middle cerebral artery occlusion (MCAO) with 2 weeks of reperfusion.

Methods

Transient Middle Cerebral Artery Occlusion Model (tMCAO)

All experiments were performed under institutionally approved protocol in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were anesthetized with isoflurane (1.5%) in 30%/70% oxygen/nitrous oxide. A catheter in the tail artery was used for measuring blood pressure, pH, PaO₂, and PaCO₂. Transient focal ischemia was induced by suture occlusion of the middle cerebral artery (MCA), as previously described (Longa, et al., 1989). Adequate ischemia was confirmed by continuous laser doppler flowmetry (LDF) (Perimed, North Royalton, OH, U.S.A.) (Pignataro, et al., 2011). For placement of the LDF probe, a burr hole 2 mm to 3 mm in diameter was created in the right parietal bone (2 mm posterior and 6 mm lateral to bregma). After 100-minutes MCA occlusion, the monofilament suture was gently withdrawn in order to restore blood flow, and LDF values were recorded for 80 minutes after reperfusion. Rats that did not demonstrate a significant reduction to less than 30% baseline LDF values during MCAO, or rapid restoration of the LDF signal during reperfusion, were excluded. Rats were randomized into sham, control or 85 NBO groups. All animals were under anesthesia for 3 hours total. The control group received 30% O₂ during the 180 minutes. The sham-operated group received the same anesthesia procedure without inserting the filament into the MCA. The 85 NBO group received 100% O₂ for 85 minutes, from 15 minutes after MCAO until the end of the occlusion, and 30% O₂ during the 80 minute reperfusion period (Figure 1A). Arterial blood gases, mean blood pressure, and temperature were recorded. All procedures and measurements were performed in a blinded and randomized fashion.

Evaluation of Infarct Volume

Rats were euthanized 2 weeks after ischemia. The animals underwent transcardial perfusion with 60mL of phosphate-buffered saline (PBS) under deep (5%) isoflurane anesthesia. Brains were quickly removed and frozen, and coronal sections of 20 μ m thickness were prepared from frozen rat brains. Infarction volumes were quantified on Nissl-stained sections using the "indirect" morphometric method (Lin, et al., 1993) with Image J software. Total hemispheric, cortical, and subcortical lesion areas from each axial slice were calculated and integrated to yield infarction volumes.

Immunohistochemistry

Immunohistochemistry was performed as described before (Hayakawa, et al., 2010). After staining with primary antibodies anti-Iba1 (1:400, Santa Cruz for microglia activation), anti

GFAP (1:500, Invitrogen for astrocyte activation), Goat Anti-type IV collagen (1:500, Jackson ImmunoResearch Lab for vascular remodelling), and fluorescent-tagged secondary antibody, nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Immunostaining was analyzed with a fluorescence microscope (Nikon ECLIPSE Ti-S) interfaced with a digital charge-coupled device camera and an image analysis system.

Neurological Tests

All rats were assessed blindly at 2 weeks using three tests: forelimb placement test, body swing test, and a 5-point neuroscore scale. For the forelimb-placing test, the animals were held close to a tabletop and scored for the ability to place the forelimb on the tabletop in response to whisker, visual, tactile, or proprioceptive stimulation. For the body swing test, the animals were held approximately one inch from the base of their tails and elevated to an inch above a surface of a table. A swing was recorded whenever the rat moved its head out of the vertical axis to either side by more than 10° from vertical and then returned to the vertical position. Thirty total swings were counted per animal. For the forelimb placing test, 0=normal, and 8=maximally impaired. For the body swing the number of body swing to the ipsilateral side were recorded as a measure of recovery (Menniti, et al., 2009). Neuroscores were graded as 0=no apparent deficit; 1=slight deficit; 2=circling; 3=heavy circling or no movement at all; or 4=death, (Fujiwara, et al., 2011, Schabitz, et al., 2004).

Statistical Analysis

Values are expressed as mean±S.D. Infarct volumes were assessed with one-way ANOVA followed by post-hoc Fisher-protected least significant difference tests. Neurological outcomes (forelimb placement test, body swing test, neuroscore) were analyzed using the nonparametric Kruskal-Wallis test. Correlations between infarcts and neurological outcomes were assessed using Pearson's correlation coefficients. P values of <0.05 were considered statistically significant.

Results

Effect of NBO on Infarct Volume

Physiological parameters remained within normal range in all rats (Table 1). Before NBO treatment, arterial pO₂ levels were approximately 130mmHg in all rats. As expected, NBO rapidly and markedly elevated arterial pO₂ levels to around 400mmHg in 85 NBO rats. The arterial pO₂ levels decreased again to approximately 130mmHg during the reperfusion period. After MCA occlusion, cerebral perfusion dropped below 30% of pre-ischemic baselines and remained stable throughout the 100 minute duration of occlusion. After reperfusion, cerebral perfusion returned to 100% in all animals. NBO did not appear to affect perfusion levels. Nissl staining revealed well-defined infarcts in control animals subjected to 100 minutes of MCAO followed by 2 weeks of reperfusion (128.50±49.64 mm³). Infarct volumes were markedly reduced in animals subjected to 85 NBO (65.86±46.14mm³) compared to controls (Figure 1B–C).

Effect of NBO on Neurologic Impairment

NBO treatment appeared to reduce the weight loss incurred during stroke recovery (+9 g weight gain in NBO animals versus -29.2g weight loss in controls, Table 2). NBO treatment had a positive effect on neurological outcomes at 2 wks post-stroke (Figure 2). NBO-treated rats had significantly better outcomes in forelimb placement test (2.40±2.3) versus controls (5.29±3.3), and in the body-swing test (18.6±1.1) versus controls (25.14±4.2), although no effects were detected on neurological scores (1.33±1.2 85 NBO versus 1.4±0.5 control group). These effects may be related to the effects of NBO on infarction since infarct size

was correlated with outcome variables such as the forelimb placing test, the body swing test and weight loss (Figure 3).

Effect of NBO on Glial Activation and Vascular Remodeling

Activation of microglia and astrocytes was assessed by immunostaining for Iba1 and GFAP respectively. By 2 wks post-MCAO, all ischemic brains showed prominent glial reactivity in peri-infarct zones. However, the degree of immunostaining signal intensity for both Iba1-positive microglia and GFAP-positive astrocytes appeared to be lower in brains from rats treated with NBO compared to untreated rats (Figure 4). In addition to gliosis, peri-infarct areas were also assessed for vascular remodeling in terms of Type IV collagen positive microvessel staining. But no detectable increase in microvessel lengths were noted at 2 wks after ischemia compared with sham-operated brains, and NBO did not appear to alter these responses (Figure 5).

Discussion

NBO has been increasingly explored as a potential therapy for acute ischemic stroke (Calvert, et al., 2007, Singhal, et al., 2002). NBO is considered a good approach because it may have some clinical advantages. It is inexpensive, non-invasive, easily and readily applied to acute stroke patients. However, there always remains the worry that oxygen may potentially have detrimental effects due to the generation of reactive oxygen species and vasoconstriction (Chan, 2001, Omae, et al., 1998). The majority of experimental studies to date mostly focus on acute 24 or 48 hr outcomes. Hence, the purpose of this study was to examine whether NBO can be effective in the longer term. Our results here demonstrate that NBO significantly reduced tissue infarction and improved performance on some neurological tests at 2 wks after a 100 min period of transient focal cerebral ischemia in rats. And consistent with these tissue and functional outcomes, NBO also appeared to suppress microglia and astrocyte activation in remodeling cortex.

At the present time, the only FDA-approved therapy for acute ischemic stroke is reperfusion with either tPA or mechanical devices. In this regard, NBO may be especially useful. Previous studies have suggested that NBO may attenuates diffusion-weighted brain MRI abnormalities, and correspondingly extends the reperfusion time window (Veltkamp, et al., 2006). Furthermore, NBO was safe insofar as it did not worsen outcomes in a rat model of intracerebral hemorrhage (Fujiwara, et al., 2011). Therefore, at least theoretically, NBO might even be used as a combination therapy that can be started in the ambulance or hyperacute phases in order to widen the time window for tPA or mechanical devices.

Existing data suggest that NBO reduces 24–48 hr infarction in animal models of focal cerebral ischemia. But for clinical translation, it is essential to find out whether NBO's effects can persist for longer periods of time. In this study, the neuroprotective effects of NBO was found to persist even up to 2 wks later, and importantly, NBO's positive actions were also detected for some neurological tests. However, some caveats remain. First, although this study examined subacute time points at 2 wks, for human patients, stroke recovery should occur over months. Whether the benefits of NBO can still be detected up to months later warrants further examination. Second, the effects on various markers of gliosis and inflammation must be interpreted cautiously. Emerging data now suggest that gliosis and inflammation can be biphasic in nature, with both damaging as well as beneficial effects after stroke and brain injury (Iadecola and Anrather, 2011, Lo, 2008, Rolls, et al., 2009, Silver and Miller, 2004). Finally, it must be acknowledged that there are no perfect and validated neurological tests for stroke recovery in rodents, and furthermore, how these tests correlate to human function remains unclear. In this study, NBO appeared to improve

performance in some but not all tests that were used. Ultimately, the true efficacy of NBO against stroke must be tested in a well-controlled clinical trial.

In this study, NBO-induced infarct reduction was correlated with improvement in some neurological tests. But what might be some of the cellular mechanisms involved? Recovery after stroke involves a complex series of interactions between all cell types in the neurovascular unit. To further examine this effect of NBO on stroke recovery, we also assessed the various cellular responses in peri-infarct areas. After brain injury, microglia are known to rapidly shift their phenotype, including changes in morphology, proliferation, expression of various cytokines and immunomodulatory surface antigens (Weinstein, et al., 2010). Similarly, astrocytes may also play a key role, with reactive astrocytes being able to play a multifaceted role after ischemia, with potential to both enhance and impair neuronal survival and regeneration (Barreto, et al., 2011, Lo and Rosenberg, 2009). Finally, it is well known that the ischemic penumbra is an area of intense restoration and active angiogenesis. Many growth factors, such as VEGF and hypoxia-inducible factor-1, are described as inducing angiogenesis and endothelial cell proliferation (Liman and Endres, 2012). This may already start as early as 12–24 h after stroke and persist for up to several weeks after ischemia. In our model system, NBO was noted to affect some but not all of these processes. NBO-treated rats did not appear to alter their angiogenic response – microvessel density was the same in controls and NBO-treated brains. But a significant effect was detected for glial response. Treatment with NBO decreased Iba1 expression, a marker of activated microglia, and GFAP, a marker of activated astrocytes. It is likely that the ameliorated glial response is linked to the reduction of infarction. The present study provides translational assurance that beneficial effects of NBO may persist up to 2 wks. But whether the mechanisms by which NBO affects glia remains to be determined.

Taken together, our experimental results here suggest that NBO can be safely administered in acute focal cerebral ischemia, reducing infarction, improving recovery after 2 weeks and suppressing glial reactions in recovering brain. Further studies may be warranted to investigate the therapeutic applications of NBO for clinical stroke.

Acknowledgments

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Abbreviations

NBO	Normobaric oxygen
ICH	intracerebral hemorrhage
MCAO	middle cerebral artery occlusion
LDF	laser doppler flowmetry
PBS	phosphate-buffered saline
DAPI	4,6-diamidino-2-phenylindole
GFAP	glial fibrillary acidic protein
tPA	tissue plasminogen activator
FDA	Food and Drug Administration
VEGF	Vascular Endothelial Growth Factor

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Highlights

- We examine the effect of NBO in a rat model of MCAO with 2 weeks of reperfusion.
- NBO reduces infarction, improving recovery after 2 weeks and suppressing glial reactions in recovering brain.
- NBO can be safely administered in acute focal cerebral ischemia.

Figure 1. Quantification of ischemic volume after 2 weeks

(A) The control group received 30% O₂ during the 180 minutes. The 85 NBO group received 100% O₂ for 85 minutes, from 15 minutes after MCAO until the end of the occlusion, and 30% O₂ during the 80 minute reperfusion period. (B) Representative Nissl staining of brain sections showing ischemic infarctions at 2 wks. (C) In animals subjected to 85 NBO, when compared to the control group, the infarct volume was significantly reduced. *P<0.05. Each column represents the mean $\pm$ SD.

Figure 2. Neurological outcomes were quantified at 2 weeks

Forelimb placement test and body swing test had a significant positive effect after 85 NBO treatment when compared with the control group. *P<0.05. All data represented as mean $\pm$ SD. Neuroscore outcomes were not different.

Figure 3. Correlation analysis

Correlation analysis between forelimb placement test, body swing test and weight loss with ischemic volume. Forelimb placement test, body swing test and weight loss were correlated with infarct volumes at 2 wks post-stroke. * $P < 0.05$.

Figure 4. Effect of NBO on microglia and astrocytes activation

Immunohistochemistry was performed using primary antibodies anti-Iba1, a marker of activated microglia, and anti GFAP, a marker of activated astrocytes. Activation of microglia and astrocytes was observed in peri-infarct zones at 2 wks in sham, control and 85 NBO groups. The expression of Iba1 and GFAP at 2 wks post-ischemia was increased compared to sham rats. After 85 NBO treatment microglia and astrocytes levels decreased again.

Figure 5. Effect of NBO on vascular remodeling

Immunostaining for type IV collagen was used to assess microvessel lengths in peri-infarct cortical areas. Sham, control and 85 NBO groups were analyzed after 2 wks reperfusion. NBO did not appear to affect average microvessel lengths.

Table 1**Physiological Parameters**

	Control	85min NBO
MABP (mmHg)		
before MCAO	103±5.0	104±3.8
1h after MCAO	103±9.1	109±11.3
2.5 h after MCAO	97±8.3	100±11.2
pH		
before MCAO	7.48±0.023	7.41±0.049
1h after MCAO	7.45±0.048	7.41±0.027
2.5 h after MCAO	7.37±0.021	7.37±0.029
pO₂		
before MCAO	131.7±31.42	133.1±20.86
1h after MCAO	109.0±30.48	420.3±96.04
2.5 h after MCAO	114.5±32.75	137.6±36.18
pCO₂		
before MCAO	38.9±5.12	43.9±6.92
1h after MCAO	37.0±5.72	43.8±4.77
2.5 h after MCAO	43.9±4.21	42.1±4.17

MABP: Mean arterial blood pressure

Values are mean ± SD.

Table 2

Physiological Parameters*

	Control	85min NBO
body weight (g)		
day 0	372.8±49.9	335±16.6
day 14	343.6±77.3	344±47.7
gain/loss of weight	-29.2	9
rectal temperature (°C)		
pre-ICH	37.2±0.1	37.1±0.2
1hour	37.0±0.1	37.1±0.1
2hours	37.0±0.1	37.1±0.2

Values are mean±SD