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Neuroscience Letters 425 (2007) 141–145
Neuroscience Letters
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Delayed hyperbaric oxygenation is more effective than early prolonged normobaric hyperoxia in experimental focal cerebral ischemia

Christopher Beynon [a], Li Sun [a], Hugo H. Marti [b], Sabine Heiland [c], Roland Veltkamp [a,*]

[a] *Department of Neurology, University of Heidelberg, Germany*
[b] *Department of Physiology, University of Heidelberg, Germany*
[c] *Department of Neuroradiology, University of Heidelberg, Germany*

Received 26 May 2007; received in revised form 12 July 2007; accepted 13 July 2007

Abstract

Hyperbaric (HBO) and normobaric (NBO) oxygen therapy have been shown to be neuroprotective in focal cerebral ischemia. In previous comparative studies, NBO appeared to be less effective than HBO. However, the experimental protocols did not account for important advantages of NBO in the clinical setting such as earlier initiation and prolonged administration. Therefore, we compared the effects of early prolonged NBO to delayed HBO on infarct size and functional outcome. We also examined whether combining NBO and HBO is of additional benefit. Wistar rats underwent filament-induced middle cerebral artery occlusion (MCAO) for 150 min. Animals breathed either air, 100% O_2 at ambient pressure (NBO; initiated 30 min after MCAO) 100% O_2 at 3 atm absolute (HBO; initiated 90 min after MCAO), or a sequence of NBO and HBO. Infarct volumes and neurological outcome (Garcia score) were examined 7 d after MCAO. HBO (174 ± 65 mm^3) significantly reduced mean infarct volume by 31% compared to air (251 ± 59 mm^3) and by 23% compared to NBO treated animals (225 ± 63 mm^3). In contrast, NBO failed to decrease infarct volume significantly. Treatment with NBO + HBO (185 ± 101 mm^3) added no additional benefit to HBO alone. Neurological deficit was significantly smaller in HBO treated animals (Garcia score: 13.3 ± 1.2) than in animals treated with air (12.1 ± 1.4), but did not differ significantly from NBO (12.4 ± 0.9) and NBO + HBO (12.8 ± 1.1). In conclusion, HBO is a more effective therapy than NBO in transient experimental ischemia even when accounting for delayed treatment-onset of HBO. The combination of NBO and HBO results in no additional benefit.

Keywords: Hypoxia; Neuroprotection; Stroke; Experimental ischemia

Improving tissue oxygenation in ischemic stroke has been considered a promising therapeutic strategy for many years. The majority of experimental studies have been performed with hyperbaric oxygen treatment (HBO). Despite positive results in experimental focal cerebral ischemia [14,24–28,30], the effectiveness of HBO in the treatment of acute ischemic stroke in patients remains controversial [4,16]. Disappointing results of clinical pilot studies [1,17,19], potential side effects of HBO [3,5,16], and limited availability of HBO chambers are major limitations. Recent experimental studies suggest that normobaric hyperoxia (NBO) has substantial neuroprotective effects in focal cerebral ischemia [6,11,12,21–23]. NBO has several advantages compared to HBO [21]. Importantly, NBO therapy could be initiated earlier after stroke-onset by emergency medical personnel with only minimal risk.

The efficacy of NBO and HBO has been compared directly only in a limited number of studies in focal cerebral ischemia. In most experiments, HBO showed a significantly stronger neuroprotective effect than NBO. The interval between onset of ischemia and initiation of oxygen therapy is an important factor determining its effectiveness [14,30]. In the previous studies, however, NBO was administered for the same duration and initiated after the same interval after ischemia-onset as HBO [25–28], although this experimental design does not reflect the time-advantage that can be expected for initiation of NBO in the clinical setting. Another interesting approach may be to com-

* Corresponding author at: Department of Neurology, Ruprecht-Karls-University Heidelberg, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany. Tel.: +49 6221 568211; fax: +49 6221 564952.
E-mail address: roland_veltkamp@med.uni-heidelberg.de (R. Veltkamp).

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doi:10.1016/j.neulet.2007.07.009

bine the effects of both oxygen modalities sequentially, but this aspect has received only little attention so far [27]. The main purpose of the present study was to compare the effects of early initiated NBO to delayed HBO treatment on infarct size and neurological outcome in rats after transient focal cerebral ischemia. We hypothesized that initiating NBO earlier than HBO could compensate for inferiority of NBO in direct comparison of both therapies. We also addressed the question whether the combination of both therapies is superior to administration of NBO or HBO alone.

All experiments were performed on male Wistar rats ($n = 72$) weighing 300–350 g (Charles River, Germany) and approved by the local and regional governmental animal care authorities. Using a face mask, anaesthesia was induced with 4% halothane in O_2 and continued with 0.8–1.2 % halothane in a 70/30 mixture of nitrous oxide/oxygen under spontaneous respiration. During surgery, temperature was continuously monitored with a rectal probe and maintained at 37 °C with a thermostatically controlled heating pad. The femoral artery and vein were cannulated with PE-50 polyethylene tubing for continuous monitoring of arterial blood pressure and heart rate, to provide samples for blood gas measurements and to inject the MR–contrast agent. Focal cerebral ischemia was induced using the reversible filament occlusion model as introduced by Longa et al. [13] with some modifications as previously described [26]. After placement of the silicone coated filament and closure of the neck, rats were placed into a MRI scanner (Bruker Biospec, 2.35 T). Perfusion-weighted imaging (PWI) was performed to ensure hypoperfusion in the territory of the occluded MCA in all animals (see below). After PWI, rats were allowed to wake up. All animals were subjected to ischemia of 150 min duration. Treatment was performed according to one of four treatment protocols to which animals were randomly assigned (Fig. 1). Animals breathed either air, 100% O_2 at ambient pressure (NBO), 100% O_2 at 3 atm absolute (ata; HBO), or a sequence of NBO and HBO (NBO + HBO group). In the NBO group, NBO was initiated 30 min after MCAO and was continued for 120 min during ischemia plus 180 min during reperfusion. In the HBO group, HBO was begun 90 min after ischemia-onset and performed for 60 min. After reperfusion, these animals received no further therapy and breathed room air. In the NBO + HBO combination group, rats received NBO 30 min after onset of ischemia for a period of 60 min followed by HBO for 60 min plus 180 min of NBO during reperfusion. In all groups, a PWI MRI was performed just after removal of the filament to verify successful reperfusion in all animals. Animals were examined in a 2.35 T MRI scanner (Biospec 24/40, BRUKER Medizintechnik Ettlingen, Germany) with a previously described configuration and protocol [10]. For perfusion-weighted imaging (PWI), we used a gradient-echo echo-planar imaging (GE-EPI) sequence (repetition time = 1 s, echo-time = 15 ms, 20 repetitions with a time resolution of 1 s/image data set) for monitoring the bolus passage of 1 mmol/kg of a paramagnetic contrast agent (Omniscan, Nycomed Amersham, Oslo, Norway). For analysis of PWI during ischemia and after reperfusion, the relative cerebral blood volume (rCBV) and the relative mean transit time were calculated in two predefined regions of interest in the parietal cortex and the striatum in both hemispheres from the signal-time-curve determined from the PWI data set as previously described [9].

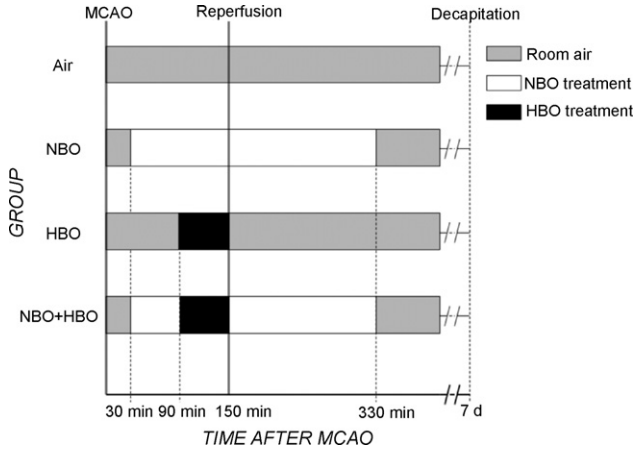

Fig. 1. Schematic overview of the experimental protocols.

Seven days after ischemia, rats were deeply anesthetized and transcardially perfused with 100 mL of heparinized saline. Brains were rapidly removed, frozen in isopentane and stored at −80 °C. For determination of infarct size, 20 μm coronal sections were cut at 400 μm intervals and stained with the high-contrast silver infarct method as previously described [29]. The public domain Scion image program was used for analysis of infarct size. No correction for focal edema was necessary at 7 d after ischemia.

Neurological deficit was graded 7 d after ischemia using a scale ranging from 3 to 18 introduced by Garcia et al. [7] for filament-induced MCAO in rats. On this scale, lower scores represent greater deficits. Scores were assessed by an observer blinded to experimental groups.

All values are expressed as mean ± standard deviation (S.D.). For comparison of physiological values, infarct volumes and MRI data, ANOVA was used followed by post hoc Fisher's protected least significant difference test. Between-group differences of behavioral scores were analysed with the Mann–Whitney U-test. All analyses were performed using SPSS analysis software. A p-value < 0.05 was considered statistically significant.

Physiological parameters before MCAO and 5 min after treatment were not significantly different between the air group and the different oxygen treatment groups except for arterial pO_2 (Table 1). Arterial pO_2 could not be measured during HBO treatment in the chamber. Perfusion deficit on MRI after MCAO in cortex and striatum did not differ among groups. Cortical rCBV (ischemic/nonischemic) was 0.57 ± 0.13 in the air, 0.54 ± 0.14 in the NBO, 0.60 ± 0.17 in the HBO group, and 0.62 ± 0.21 in the NBO + HBO combination group. Thus, all groups underwent ischemia of the same severity. Similarly, reperfusion after filament removal did not differ significantly among groups (data not shown).

Mortality did not differ significantly among the experimental groups during the 7 d observation period. In each of the air, NBO, and NBO + HBO combination treated groups three rats died, whereas only two rats died in the HBO group. Postmortem

Table 1
Physiological Parameters

	Air	NBO	HBO	NBO + HBO
Baseline				
pO_2 (mmHg)	136 ± 13.2	131.9 ± 13.5	136.9 ± 11.7	134.7 ± 8.9
pCO_2 (mmHg)	41.9 ± 4.6	41.8 ± 3.6	41.4 ± 3.4	43.4 ± 4.7
pH	7.45 ± 0.04	7.44 ± 0.03	7.45 ± 0.03	7.43 ± 0.04
Temperature (°C)	37.4 ± 0.3	37.2 ± 0.2	37.3 ± 0.2	37.3 ± 0.3
MAP (mmHg)	87.6 ± 7.9	88.7 ± 6.5	89.3 ± 9.4	86.4 ± 8.2
HF (bpm)	435 ± 46	433 ± 35	442 ± 49	429 ± 41
Reperfusion				
pO_2 (mmHg)	121.2 ± 19.8	385 ± 25.7*	570.3 ± 36.6*'**	546 ± 49.3*'**
pCO_2 (mmHg)	43.2 ± 4.2	44.7 ± 5.2	40.5 ± 5.8	41.6 ± 5.1
pH	7.39 ± 0.03	7.41 ± 0.03	7.42 ± 0.04	7.40 ± 0.03
Temperature (°C)	37.7 ± 0.3	37.6 ± 0.3	37.6 ± 0.4	37.5 ± 0.4

Except for arterial pO_2 after reperfusion, there were no significant differences in physiological parameters between groups (*$p < 0.05$ compared to air; **$p < 0.05$ compared to NBO; ANOVA and post hoc Fisher's protected least significant difference test).

examination of the animals which all died within the first 48 h after ischemia revealed massive brain edema with signs of uncal herniation as the most likely cause of death. These animals were not included in further data analysis.

Mean total infarct volumes were significantly smaller in HBO treated animals ($174 \pm 65\,mm^3$) than in animals treated with air ($251 \pm 59\,mm^3$) or NBO ($225 \pm 63\,mm^3$). Thus, HBO induced a 31% reduction of mean infarct volume compared to air despite a delay of treatment initiation (Fig. 2). In contrast, mean total infarct volume of NBO treated animals was not significantly smaller than of animals treated with room air. Treatment with NBO + HBO ($185 \pm 101\,mm^3$) resulted in a 26% reduction of mean infarct volume compared to air. Comparison of infarcted cortical and subcortical regions showed a significant reduction of mean cortical infarct volume in the HBO group compared to air ($79 \pm 49\,mm^3$ versus$132 \pm 37\,mm^3$) and to NBO ($117 \pm 54\,mm^3$). Mean cortical infarct volume of HBO + NBO treated animals ($83 \pm 58\,mm^3$) was significantly lower than of animals treated with air. There were no significant differences observed between mean subcortical infarct volumes among the groups, although a trend towards reduction was noticed between mean subcortical infarct volumes of HBO and air ($94 \pm 33\,mm^3$ versus $119 \pm 41\,mm^3$; $p = 0.08$). Mean subcortical infarct volumes were $108 \pm 34\,mm^3$ for NBO and $101 \pm 52\,mm^3$ for NBO + HBO.

Behavioral deficits evaluated 7 d after MCAO appeared to depend on the treatment strategy, being most severe in the air group (12.1 ± 1.4), followed by the NBO (12.4 ± 0.9), the NBO + HBO (12.8 ± 1.1), and finally the HBO group (13.3 ± 1.2) (Fig. 3). At a statistical significance level, though, only the HBO group showed a better outcome compared to air treatment. Absolute mean scores were rather high because the deficits measured by the Garcia scale had already improved 7 d after MCAO.

The findings in the present study may be of relevance for the translation of oxygen therapy from the experimental into the clinical setting. Consistent with previous studies from our and other groups [14,24–26,28], intraischemic HBO reduced histological infarct size and improved neurological outcome in rats subjected to transient focal cerebral ischemia. In contrast

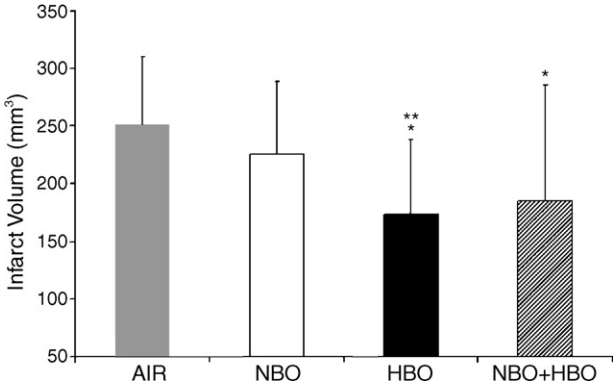

Fig. 2. Infarct volumes on silver stained sections 7 d after MCAO. HBO decreases infarct volume compared to air and NBO. Combination of HBO and NBO decreases infarct volume significantly compared to air, but difference was not significant compared to NBO treated animals (*$p < 0.05$ compared to air; **$p < 0.05$ compared to NBO; ANOVA and post hoc Fisher's protected least significant difference test).

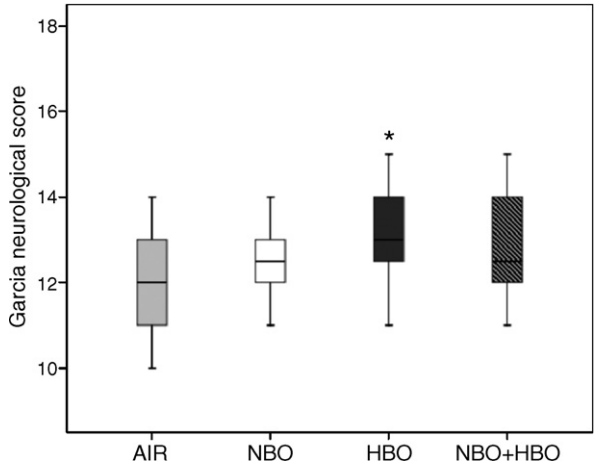

Fig. 3. Neurological deficit graded on the Garcia scale. HBO decreases neurological deficit compared to air and NBO (*$p < 0.05$ compared to air; Mann–Whitney U-test). NBO and combination of NBO and HBO (NBO + HBO) do not improve neurological outcome compared to air ($p > 0.05$; Mann–Whitney U-test).

to most previous studies, the delay until initiation of HBO was sufficiently long in the present study to be of significance for a substantial subgroup of acute stroke patients. Other authors have reported protective effects of HBO in rodent transient ischemia when treatment was started up to 6 h after ischemia-onset [2,14], but in these studies HBO was performed during the reperfusion period after early recanalization. When we examined longer time windows necessitating an MCA occlusion time of 4 or even 5 h, mortality became excessively high in all groups indicating a ceiling effect in the filament model which produces large hemispheric infarcts.

Although there is also accumulating evidence for a protective effect of NBO in cerebral ischemia [6,11,12,21–23], the available data suggest that its time window is short [22], that its effectiveness is inferior to HBO in transient [25,26,28] and limited in permanent focal ischemia [27]. Relative ineffectiveness of NBO compared to HBO in previous experimental studies may have been caused by starting both treatments after an identical interval although NBO could obviously be implemented earlier in the clinical setting. Therefore, our present experiments were designed to account for a 60 min delay of treatment initiation of HBO relative to NBO. NBO was started 30 min after MCAO because this time was effective in one recent study [22] and appears to be the realistic minimum interval that can be achieved in most outpatient treatment situations. NBO was administered for a 5 h period because prolonged treatment was most effective in a well-designed study by Flynn and Auer [6] and in another study using a permanent focal ischemia model [11]. Nevertheless, NBO failed to reduce infarct size and neurological deficit compared to air treated rats in our experiments in contrast to previous reports by other groups [6,11,12,22,23]. A potential explanation for this discrepancy could be the longer MCA occlusion period in our experiments. In this setting, the 30 min time interval until treatment initiation may have been too long as the time from onset of ischemia to initiation of oxygen therapy is of great importance [14,30]. Alternatively, prolonged exposure to NBO for 5 h may have increased production of reactive oxygen species, although recent studies did not find any evidence for enhanced oxygen radical induced cell damage after NBO treatment [15,20,24]. Exposure to normobaric oxygen in these studies, however, was shorter than in our study and did not include the reperfusion period during which oxygen-radical induced damage is of particular importance. Also, rats were anaesthesized only during surgical procedures in our experiments in contrast to previous studies. As inhalative narcotics can alter brain metabolism and infarct size [8] this may offer a further explanation for outcome differences between the studies. Results from clinical studies on hyperoxia in stroke patients are controversial. Ronning and Guldvog [18] observed a worse 1-year-outcome in stroke patients receiving supplementary oxygen. While in this study patients were enrolled in a time window of 24 h, Singhal et al. [21] found an improved clinical outcome and less MRI abnormalities when normobaric hyperoxia was started within 12 h after stroke-onset.

The sequential administration of normobaric and hyperbaric oxygen therapy may be an attractive option in the clinical stroke setting because it combines the advantages of early application of NBO with the apparently more powerful but logistically challenging protective effect of delayed HBO. To our knowledge, this concept so far has only been studied in a study of permanent cortical murine ischemia from our group [27]. Similar to that study, administering NBO prior to HBO resulted in no benefit beyond protection by delayed HBO alone in the present study. However, this hypothesis may have to be retested using a shorter time window than 30 min for NBO.

In conclusion, our findings are of relevance for the potential translation of oxygen therapy from experimental into clinical ischemic stroke because they suggest a more powerful protective effect of HBO than of NBO even when accounting for variables such as delay of treatment-onset and prolonged treatment duration.

Acknowledgments

This work was supported by grants from the Deutsche Forschungsgemeinschaft (VE 196/2-1 and VE196/2-2).

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