Neuroprotection by Oxygen in Acute Transient Focal Cerebral Ischemia Is Dose Dependent and Shows Superiority of Hyperbaric Oxygenation

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Abstract

The neuroprotective effect of oxygen after acute stroke in rats has been shown previously. However, the question of optimal dosing still remains unanswered. Thus, we investigated the use of oxygen at different concentrations by either normobaric oxygenation (NBO) or hyperbaric oxygenation (HBO) at different pressures in a model of transient ischemia/reperfusion in rats. Animals underwent 90 min of middle cerebral artery occlusion (MCAO) followed by 90 min of reperfusion before oxygen treatment. Oxygen was applied either by NBO (100% O_2; 1.0 absolute atmosphere, ATA) or HBO (100% O_2; 1.5, 2.0, 2.5 or 3.0 ATA) for 1 h. Primary endpoints were infarct volume and clinical outcome measured 24 h and 7 days following the MCAO. A statistically significant and long-lasting reduction in infarct volume was seen in the HBO 2.5 ATA and 3.0 ATA groups over a period of 7 days. The reduced infarct volume was accompanied with a statistically significant improvement in clinical outcome in the high-dose oxygen-treated groups. The presented data indicate that oxygen is a highly neuroprotective molecule in transient focal cerebral ischemia in rats, when applied early and at high doses. The effect is dose dependent and shows a superiority of HBO over NBO, when the primary endpoints infarct volume reduction and clinical outcome are analyzed. These data are important for the development of new acute stroke treatment studies in humans.

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Introduction

The hallmarks of ischemic tissue damage, such as ischemic stroke, are oxygen and glucose deprivation leading to rapid ATP decrease and subsequent cell death [1]. Thus, early restoration of blood flow is one important acute treatment strategy to significantly decrease the extent of ischemic tissue damage [2].

In addition to early recanalization procedures, oxygen has been used as a therapeutic agent in a variety of experimental settings in myocardial ischemia [3] and in stroke studies on animals [4]. The use of oxygen as a therapeutic agent in ischemic stroke is often regarded as harmful, due to the concern that higher oxygen concentrations might increase the production of oxygen-derived free radicals. However, previous studies have shown con-

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clusively that the increase in free radicals is not correlated with the amount of oxygen delivered to the ischemic tissue [5, 6].

So far, 2 different ways of oxygen treatment have been described, normobaric oxygenation (NBO) and hyperbaric oxygenation (HBO). They differ with respect to the administered partial pressure of inspired oxygen. The arterial oxygen pressure (P_{aO_2}) at sea level [760 mm Hg, 1 absolute atmosphere (ATA)] in ambient air ventilation (21% O_2) is 102 mm Hg. While the increase in the P_{aO_2} of the blood is limited under NBO (100% O_2, 1 ATA) to 673 mm Hg, HBO can increase the oxygen transport capacity of the blood in dependence on the ambient pressure in a linear fashion. Thus, the P_{aO_2} increases under 3-ATA treatment (100% O_2) to 2,193 mm Hg [7]. Naturally, HBO requires the use of a pressure chamber to achieve the increased ambient pressure.

NBO has been investigated in transient cerebral ischemia in rats. In a model of transient focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO) for 2 h, Singhal et al. [8] reported a significant reduction in infarct volume if NBO was started as early as 15 or 30 min following the MCAO. Furthermore, the neuroprotective effects of NBO treatment during ischemia and reperfusion in transient MCAO have been demonstrated by a total infarct reduction of 70% [9]. Other studies using NBO showed significant clinical improvement and reduced infarct volume in transient MCAO in rats, with the largest reduction of infarct volume occurring when NBO was administered continuously during the MCAO and reperfusion [10].

HBO also attenuated the ischemic brain damage in a variety of ischemic models, mostly in rats [4, 11, 12]. Different stroke models (global ischemia, permanent focal ischemia or transient focal ischemia) in different species (gerbils [13, 14], dogs [15–17], cats [18], rabbits [19] and rats [20–24]) were used to study the effect of HBO. Most of the studies showed a beneficial effect of HBO, despite their heterogeneity. In a previous study, we investigated the optimal timing of HBO in transient MCAO in rats with respect to time windows of clinical settings, and could define a time window of less than 6 h following the MCAO as optimal for the treatment [22].

The neuroprotective effects of oxygen (NBO or HBO) in cerebral ischemia can be summarized as: (1) oxygen appears to be neuroprotective and shows maximum efficacy when administered early in the time course of cerebral ischemia; (2) neither NBO nor HBO appear to cause significant reperfusion injuries induced by oxygen-derived free radicals in a defined time window; (3) NBO

is more feasible than HBO, because no pressure chamber is needed.

As already outlined in a recent review by Helms et al. [4], the optimal dose of oxygen treatment remains unclear from these reports. We therefore designed the present study in order to systematically evaluate the neuroprotective effects of oxygen in a model of transient MCAO, with a dose-escalating design, in rats. We defined infarct volume and clinical outcome as the primary end points of our study, to be determined at 24 h and 7 days following the MCAO. Since we have previously shown a time dependency for HBO in transient MCAO, we used the most efficient time point for the onset of oxygen therapy – 3 h following the MCAO [22].

Materials and Methods

Study Design

The local ethics committee approved all experimental protocols. One hundred and forty-nine male Sprague-Dawley rats were used (weight, 200 g; Charles River, Germany). They were kept under controlled conditions. Animals were randomly assigned to the following groups (n = 10 in each group) receiving either room air or oxygen in a dose-escalating design: A = control group, room air; B = NBO (100% O_2, 1 h); C = HBO 1.5 ATA (100% O_2, 1 h); D = HBO 2.0 ATA (100% O_2, 1 h); E = HBO 2.5 ATA (100% O_2, 1 h); F = HBO 3.0 ATA (100% O_2, 1 h). Oxygen treatment (NBO or HBO) was started 3 h after the MCAO for 1 h. The deeply anesthetized animals were sacrificed 24 h or 7 days after the onset of transient MCAO by cardiac perfusion for infarct volumetry (fig. 1).

Monitoring of Physiological Parameters

Physiological parameters (rectal temperature, arterial pH, P_{aCO_2}, P_{aO_2}, hemoglobin, hematocrit, glucose, potassium, sodium, calcium and chloride) were monitored in each group. Therefore, a polyethylene catheter (pp-50) was inserted through the femoral artery into the abdominal aorta under chloral hydrate anesthesia (400 mg/kg i.p.) 2 days before the MCAO. Blood samples were taken at different time points – (1) before the MCAO; (2) 30 min after the MCAO; (3) 30 min before the HBO treatment; (4) 60 min after the HBO treatment – and analyzed (Radiometer ABL 700, Copenhagen, DK).

Transient Middle Cerebral Artery Occlusion

Animals were anesthetized with chloral hydrate (400 mg/kg i.p.) for all surgical procedures. During surgery, each rat was allowed to breathe normally, and rectal temperature was maintained at 37°C with the use of a heating pad. The right middle cerebral artery was occluded for 90 min with subsequent reperfusion according to the method described by Longa et al. [25]. After 90 min, the filament was withdrawn to allow reperfusion. Animals that did not demonstrate a significant reduction of the regional cerebral blood flow (rCBF) during the MCAO or a rapid restoration of the laser Doppler signal during reperfusion were excluded from the study.
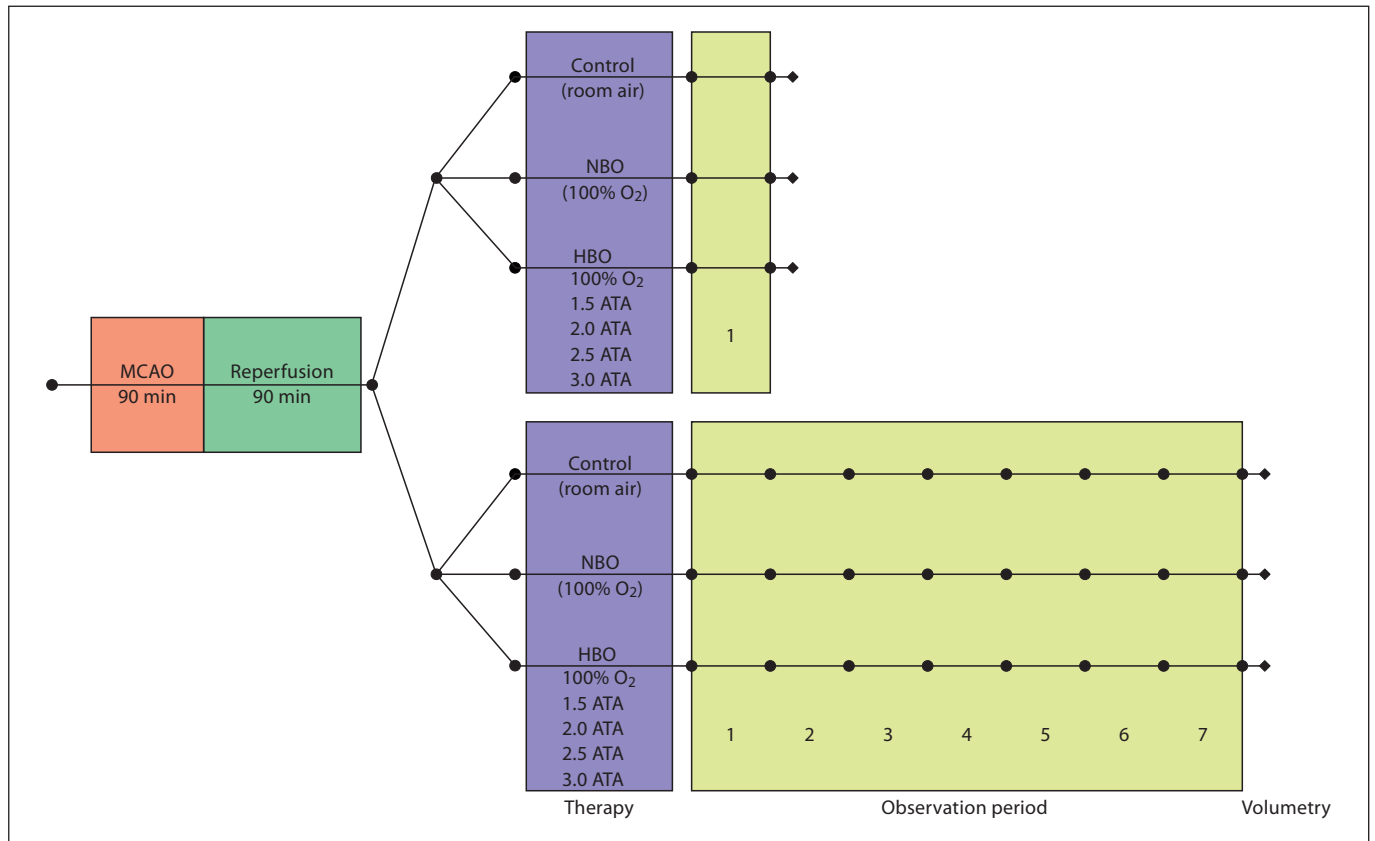
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Fig. 1. Study design. All animals received 90 min of MCAO followed by 90 min of reperfusion prior to randomization into 24-hour or 7-day observation arms. Treatment lasted 1 h in all groups. An observation period followed, to assess clinical scores (Garcia and Bederson) and to detect putative complications. Numbers 1–7 indicate days of observation. Following the observation period, the animals were sacrificed by cardiac perfusion for histological infarct volumetry (◆).

Measurement of the rCBF

Laser Doppler flowmetry (Periflux System, Perimed, Sweden) was used to monitor the rCBF before, during and after the transient MCAO. Flow values were recorded every 10 min. The area selected for rCBF monitoring corresponded to the territory of the occluded middle cerebral artery.

Normobaric Oxygenation

Animals randomized for the NBO treatment were anesthetized and placed in an acrylic cage with a flexible ceiling in order to avoid pressure changes. The air inside the cage was replaced by pure oxygen and kept at 100% O_2 for 1 h (fig. 1).

Hyperbaric Oxygenation

The HBO was performed in an experimental pressure chamber. The spontaneously breathing animals were anesthetized to avoid movement. During the HBO administration, animals were observed through the transparent acrylic glass of the pressure chamber. The HBO was administered at different pressures, ranging from 1.5 to 3.0 ATA for 1 h with 100% oxygen (group C, 1.5 ATA; group D, 2.0 ATA; group E, 2.5 ATA; group F, 3.0 ATA),

which was started 3 h following the MCAO (fig. 1). Arterial blood samples were obtained from a femoral artery catheter for blood gas analysis of the anesthetized animals during the HBO administration, without discontinuation of the HBO. Compression and decompression were achieved within 5 min. After the HBO, the femoral artery catheter was removed. The control group received the same dose of anesthesia at the same time points following the MCAO. Temperature inside the chambers and rectal temperatures were continuously monitored.

Infarct Volume Calculation

Twenty-four hours and 7 days after the MCAO, different rats from each group were deeply anesthetized with chloral hydrate (400 mg/kg) and perfused intracardially with the use of standard protocols. The entire brain was cut into coronal frozen sections (40 μm). Every 20th slice of the brain was mounted on a glass slide and stained with cresyl violet. In total, 14 sections of each brain were stained and analyzed. Sections were digitalized using a scanner and analyzed by 2 blinded investigators using ImageJ (NIH, USA). To eliminate brain edema, the corrected infarct volume was calculated as described in detail by Schäbitz et al. [26].

Fig. 2. Clinical outcome 24 h following the MCAO according to the grading system of Garcia. Clinical improvement (higher score) was seen in the high-dose treatment group F (3 ATA). n = 10 per group; mean ± SEM; ANOVA; * p < 0.05.
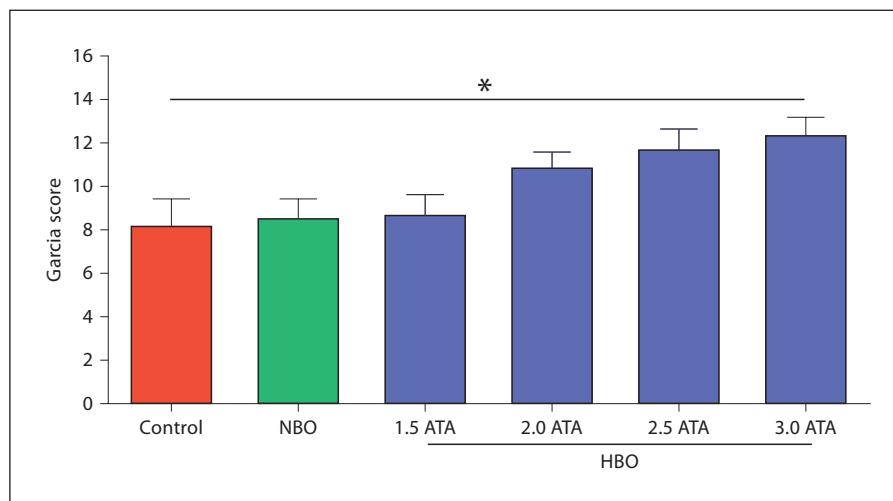
Garcia and Bederson Scores

Two neurological grading systems were used to assess the effects of ischemia. The first grading system was published by Bederson et al. in 1986 [27]. The grading system consists of a scale from 0 to 3: (0) rats with no observable deficit; (1) rats with decreased forelimb resistance to lateral push; (2) rats with forelimb flexion; (3) rats displaying circling behavior alongside the former symptoms.

The second system was introduced by Garcia et al. in 1995 [28]. It consists of 6 different criteria (spontaneous activity, symmetry in the movement of the 4 limbs, forepaw outstretching, climbing, body proprioception and response to vibrissae touch). The individual performance in each test was rated on a 0- to 3-point subscore. The sum of all 6 individual subscores was calculated within a range of 3–18. Thus, the score in healthy rats is 18. Two blinded investigators performed all tests.

Statistical Analysis

Statistical analysis was performed with the use of a 1-way ANOVA, and the post hoc Bonferroni test for pairwise comparisons if a significant difference was found. A probability value of <0.05 was considered significant. We conducted both nonlinear regressions and Spearman rank-sum correlations to test for a relationship between pressure and infarct size or clinical outcome. The tests were run on commercially available software (Prism 4.0c for Mac OS X, GraphPad Software Inc., San Diego, Calif., USA).

Results

Physiological Parameters

NBO and HBO increased P_{aO_2} rapidly out of the range of detection; P_{aCO_2} remained in a normal range over a 60-min exposure to HBO or NBO. All other physiologic parameters (particularly temperature and glucose) re-

mained within the normal range and were not significantly different between the groups. The HBO did not alter the temperature inside the pressure chamber, nor did the HBO change the body temperature of the animals during the HBO. The mortality rate was equal before randomization into the treatment groups. Twelve animals that died prior to randomization were replaced. Another 6 animals died prior to study termination in the 24-hour observation arm, and 11 animals died prior to study termination in the 7-day observation arm and were replaced. There was no difference in mortality rates between the groups. The overall mortality rate in our study was 19.5%.

Regional Cerebral Blood Flow

In transient MCAO, an immediate decrease in the blood flow shows the correct position of the filament at the origin of the MCA. After the occlusion of the common carotid artery, rCBF dropped to 70.33 ± 8.1% in all groups. The MCAO caused a further decrease in rCBF, which fell to 16.36 ± 3.34%. The low blood flow was maintained at less than 20% of the pre-ischemic baseline level during the MCAO. After the withdrawal of the filament, the blood flow increased, followed by persistent hypoperfusion (about 70 ± 11%) until the end of the recording period. No significant difference in rCBF was seen between the control, NBO- or HBO-treated rats.

Neurological Evaluation
Clinical Scoring at 24 h after MCAO
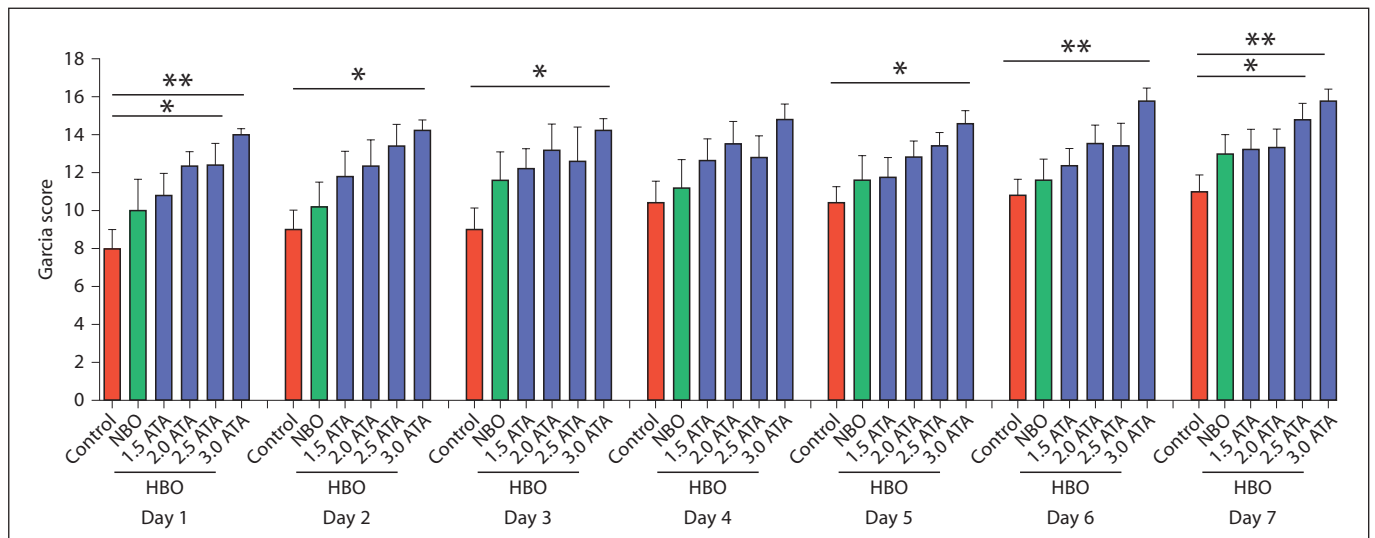Clinical deficits improved with the application of increased oxygen doses, whereas the control group showed

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Fig. 3. Clinical evaluation over a period of 7 days following the MCAO, according to the grading system of Garcia. Clinical improvement (higher score) was seen in the high-dose treatment groups E (2.5 ATA) and F (3 ATA). n = 10 per group; mean ± SEM; ANOVA; * p < 0.05, ** p < 0.01.

severe deficits resulting in low Garcia and high Bederson scores. The control group (group A) reached a Garcia score of 8.2 ± 3.1. NBO-treated animals (group B) were scored at 8.5 ± 2.3. Among the HBO groups, the 3-ATA group (group F) showed the highest score of 12.3 ± 2.1, with a significant difference to the control (p < 0.05). The remaining HBO groups had scores of: 8.7 ± 2.3, group C; 10.8 ± 1.8, group D; 11.7 ± 2.4, group E. Neurological outcome improved with increasing chamber pressure. Significant differences (p ≤ 0.05) were observed between the control group and the HBO 3-ATA group (fig. 2).

The Bederson score showed similar results in groups D, E and F; however, it failed to reach significance.

Clinical Scoring over 7 Days after MCAO

All animals improved with respect to their clinical deficits over time. In general, neurological outcome improved with increasing chamber pressure. Compared to control animals, only animals treated with HBO 2.5 ATA or HBO 3 ATA showed significant clinical improvement over a period of 7 days. As shown in figure 3, animals from groups E and F showed a statistically significant attenuation of neurological deficits with the Garcia score. The control group developed from a score of 8.0 ± 1.0 on day 1 to 11.0 ± 0.9 on day 7. The NBO group scored 10.0 ± 1.6 on day 1 and 13.0 ± 1.0 on day 7. The HBO 1.5-ATA group showed a score of 10.8 ± 1.2 on day 1 and 13.2 ± 1.1 on day 7. HBO 2.0-ATA scores ranged from 12.3

± 0.9 on day 1 to 13.3 ± 1.0 on day 7. The HBO 2.5-ATA group developed from 12.4 ± 1.1 on day 1 to 14.8 ± 0.9 on day 7. Finally, the HBO 3.0-ATA group had a baseline score of 14.0 ± 0.3 on day 1 and a final score of 15.8 ± 0.6 on day 7. Data are given as the mean ± SEM.

Assessment of the clinical development with Bederson scores showed comparable results: group F (HBO 3 ATA) showed a statistically significant improvement over 7 days. However, group E also improved, with a significant difference of p < 0.05, compared to the control group on day 7. In detail, the control group had a baseline score of 2.4 ± 0.3 on day 1 and improved to a score of 1.8 ± 0.2 on day 7. NBO-treated animals had a score of 2.2 ± 0.4 on day 1 and 1.6 ± 0.3 on day 7. HBO 1.5-ATA-treated animals had a score of 1.6 ± 0.25 on day 1 and 1.2 ± 0.2 on day 7. Data are given as the mean ± SEM.

Infarct Volumetry
Infarct Volumetry 24 h after MCAO
In all groups, infarct volume showed good reproducibility and displayed similar trends to those seen in the clinical scores. In the control group, total infarct volume was 244 ± 70 mm³. The average infarct volume from the NBO-treated group was 209 ± 62 mm³, which did not reach significance when compared to the control group. However, significant differences were detected in the HBO 2-ATA group (144 ± 77 mm³; p ≤ 0.05), the HBO 2.5-ATA group (135 ± 54 mm³; p ≤ 0.05) and the HBO

Fig. 4. Quantification of infarct volumes. Cryostat sections were stained with cresyl violet and infarct volumes were calculated after edema correction in a double-blind design at 24 h or 7 days after MCAO. Brain sections were digitalized and the infarct volumes calculated using Image J software. n = 10 per group; mean ± SEM; ANOVA; * p < 0.05, ** p < 0.01. **A** The 24-hour total infarct volumes are displayed. Increasing doses led to enhanced infarct volume reduction. **B** Infarct volumetry obtained 7 days following the MCAO. Total infarct volumes are displayed. The dose-dependent effect of infarct volume reduction was detectable over a period of 7 days. **C** Infarct volumetry obtained 7 days following the MCAO. Total (hemispheric), cortical and subcortical (striatal) infarct volumes are displayed separately. Volumes in both cortical and striatal infarct areas were reduced by HBO treatment.
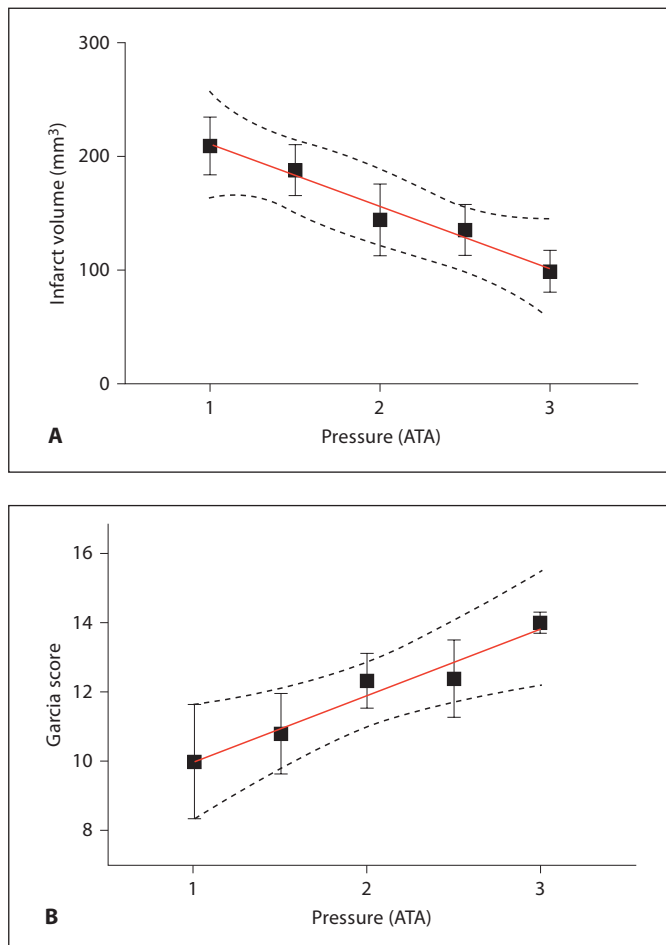
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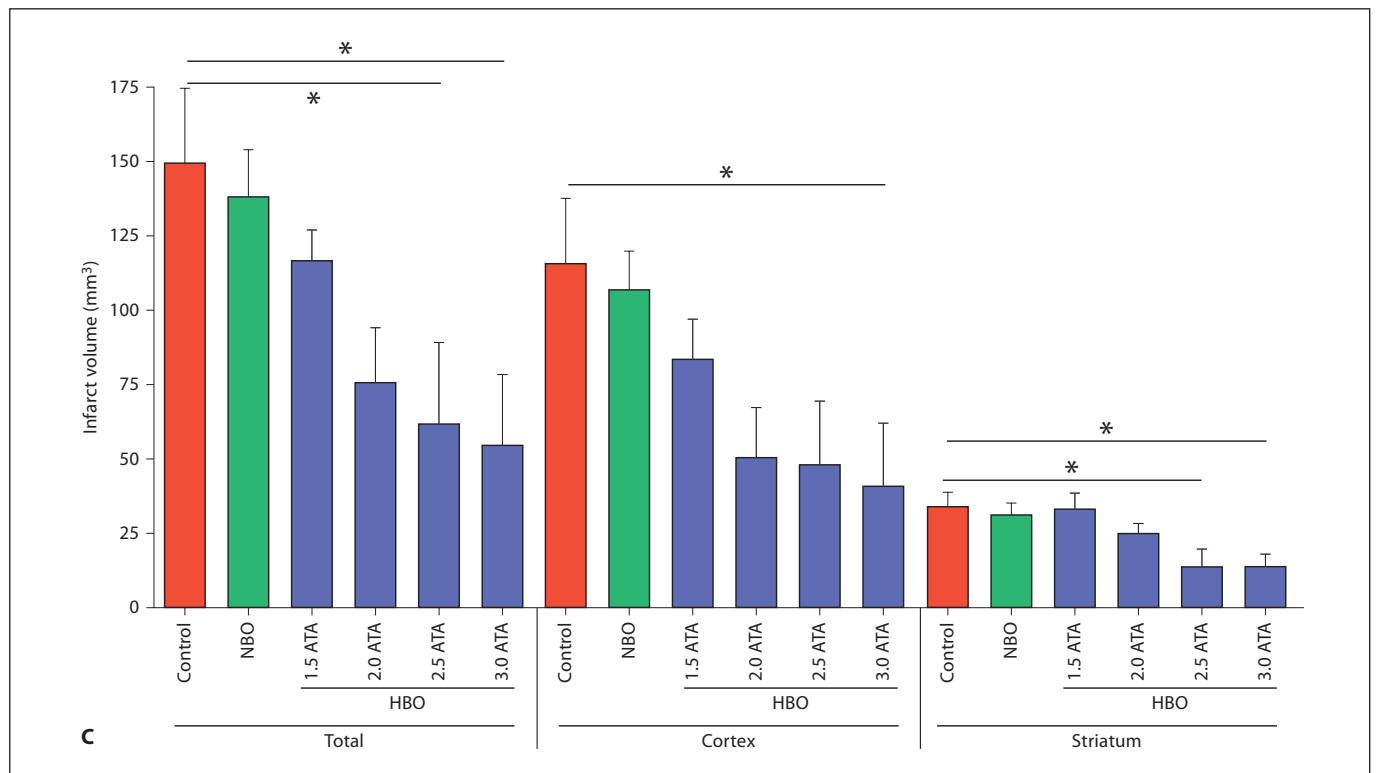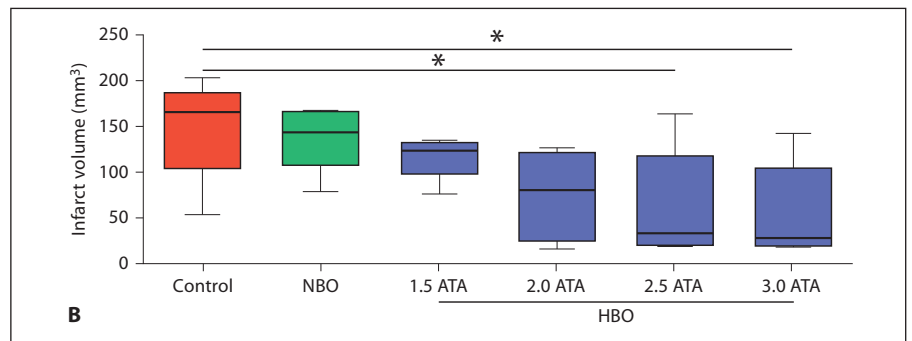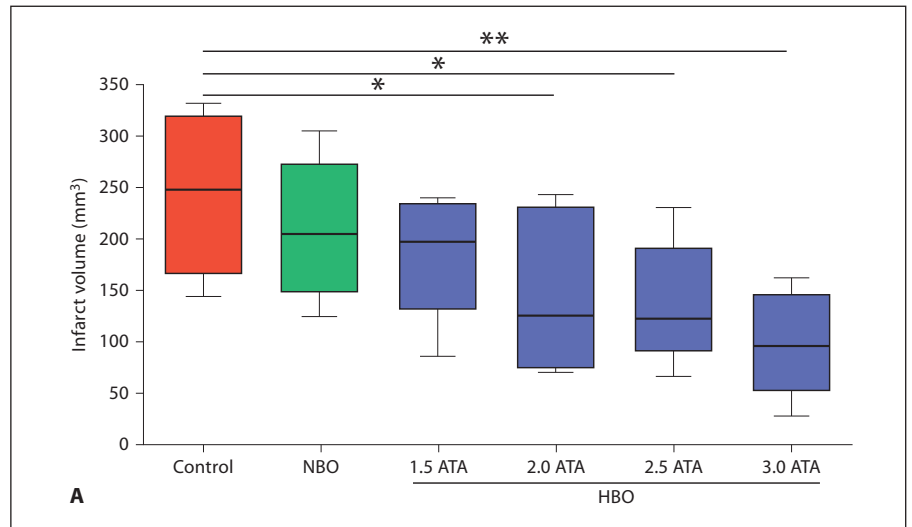
Fig. 5. Regression analysis. The results are given as means ± SEM (n = 10). The dashed lines display the 95% confidence interval. **A** Total infarct volumes were taken as the dependent variable, and pressure from NBO to HBO 3.0 ATA was taken as the independent variable. Regression analysis revealed a highly significant linear correlation of the 2 parameters. The Spearman correlation coefficient was +1.0; p = 0.0083. **B** Clinical outcome as measured by the Garcia score and ambient pressure was analyzed. The regression analysis revealed a highly significant linear correlation of the 2 parameters. The Spearman correlation coefficient was +1.0; p = 0.0083.

3-ATA group (98 ± 45 mm^3; p ≤ 0.01). Again, the most significant reduction of infarct volume was achieved in the HBO 3-ATA group (fig. 4A).

Infarct Volumetry 7 Days after MCAO

Histological analysis of the brain sections obtained 7 days after the MCAO confirmed the neuroprotective effect of high-dose oxygen administration by HBO. Total infarct volume of the control animals (150 ± 25 mm^3)

was reduced in the HBO groups, by increasing oxygen doses, to a minimum of 55 ± 24 mm^3 in the HBO 3-ATA group. Specifically, NBO-treated animals had an average infarct volume of 128 ± 16 mm^3, the HBO 1.5-ATA group had an average infarct volume of 116 ± 10 mm^3, the HBO-2.0 group had an average of 76 ± 19 mm^3 and HBO 2.5 ATA had an average of 62 ± 27 mm^3 (mean ± SEM). Statistically significant reduced infarct volumes were found only in the high-dose oxygen-treatment groups HBO 2.5 and HBO 3.0 ATA (fig. 4B).

Figure 4C shows a subanalysis of total, cortical and striatal infarct volumes. Group A had cortical and striatal infarct volumes of 116 ± 22 and 34 ± 5 mm^3, respectively, while group B had a 107 ± 13-mm^3 cortical and a 32 ± 4-mm^3 striatal infarct volume. The largest reduction in infarct volume was seen in the high-dose treated groups: group E (cortical infarct volume 48 ± 21 mm^3, striatal infarct volume 14 ± 6 mm^3) and group F (cortical infarct volume 41 ± 21 mm^3, striatal infarct volume 14 ± 4 mm^3). Data are given as means ± SEM.

Regression Analysis

A regression analysis was performed in order to analyze a possible correlation between the oxygen dose and the reduction of total infarct volume. The independent value x was pressure (measured in absolute atmospheres) and the dependent value y was infarct volume (measured in cubic millimeters). A linear correlation between infarct volume and applied pressure could be detected (p < 0.0083). The Spearman correlation coefficient was +1.0 (fig. 5A). In a second step, clinical outcome data using the Garcia score and oxygen dose were tested for linear regression. Again, a linear correlation between the Garcia score and applied pressure could be detected (p < 0.0083). The Spearman correlation coefficient was +1.0 (fig. 5B).

Discussion

Possible future treatment of human stroke with HBO needs solid data from animal models to select the best possible conditions. Timing and dosage of HBO are first questions asked when it comes to selection of the optimal conditions for such treatments. In our previous study, we defined an optimal time window for starting HBO treatment as 3 h following MCAO [22]. The present study was aimed at testing the hypothesis of a putative dose response effect of oxygen therapy in acute transient ischemic stroke in rats, while keeping the time point following the MCAO fixed at 3 h. Therefore, animals were treat-

ed with different doses of oxygen, such as NBO and HBO, up to 3 ATA. The primary end points of the study were defined as infarct volume and clinical outcome at 24 h and 7 days following the MCAO.

To the best of our knowledge, this is the first study systematically analyzing the neuroprotective effects of oxygen in acute transient focal ischemic stroke in a dose-escalating design. Our data indicate a strong neuroprotective effect of oxygen therapy in this ischemia/reperfusion model. The infarct volumes, measured histologically, showed a clear trend towards a dose-dependent infarct-volume reduction at 24 h and at 7 days following the MCAO. Statistically significant reductions of infarct volumes were only seen in the high-dose treatment groups starting at 2.0, 2.5 and 3.0 ATA. Histological analysis of infarct volumes at 7 days showed a significant reduction in infarct volumes for the 2.5- and 3.0-ATA groups. We did not use hyperbaric air as a control due to the phenomenon of decompression sickness. Decompression sickness injury of the brain caused by small nitrogen bubbles is a well-known complication, for example in divers. Nitrogen microbubble formation in the CNS may alter the cerebral circulation, cause multiple strokes, endothelial dysfunction and blood-brain barrier opening [7]. In our opinion, the latter effects would have clearly impaired the quality of our study. To avoid decompression sickness, we would have needed to change the hyperbaric protocol in order to include defined decompression stops in the hyperbaric air group. This would have severely limited the comparability of the groups.

In our study, HBO-induced infarct volume reduction was not limited to cortical areas, as usually seen in ischemia studies with neuroprotective substances, but showed also a robust effect in the infarct core. In contrast, NBO did not cause any significant infarct volume reduction. Our data confirm the strong neuroprotective effect of early HBO treatment, as previously published by our group and others [22, 29]. Furthermore, our data are supported by findings of Veltkamp et al. [30], who recently analyzed the effects of NBO and HBO (3.0 ATA) in a model of permanent and transient ischemia in mice. They reported larger infarct volume reductions in HBO-treated animals compared with NBO. The neuroprotective effect of oxygen was more prominent in transient focal ischemia than in permanent focal ischemia. However, in the latter study cerebral ischemia in mice was performed by 2 different methods – coagulation and suture model – which makes a direct comparison more difficult [30].

Earlier reports have shown neuroprotective effects of NBO in experimental stroke. Kim et al. [31] reported decreased infarct volumes in rats following transient ischemia treated with NBO. In a previous study, the same group showed a reduced infarct volume and MRI diffusion abnormalities in NBO-treated animals [8]. These data appear to be at variance with our data, since NBO failed to reduce infarct volume significantly in our study. However, it should be emphasized that Kim et al. [31] and Singhal et al. [8, 9] started NBO therapy 5 and 15 min following the MCAO, respectively, whereas in our experiments oxygen treatment started 3 h following the MCAO for a period of 1 h. Thus, we interpret the lack of efficacy of NBO in our model to be due to differences in the experimental design. We performed a regression analysis of the measured total infarct volume and the applied pressure of oxygen therapy. The results indicate a linear correlation between the oxygen dose applied and the infarct volume, which suggests a neuroprotective effect of oxygen in our model.

The clinical improvement and oxygen dose were significantly correlated in a linear fashion, which further underlines the efficacy of early high-dose oxygen treatment in our model. Our study shows statistically significant clinical improvement in the high-dose-treated groups at 24 h. This clinical improvement was a robust finding, since it could be followed over a period of 7 days. The presented data show a strong and long-lasting neuroprotective effect of HBO in acute experimental stroke, which suggests superiority of HBO compared to NBO. Furthermore, our data suggest an important role for high-dose oxygen therapy in the acute phase of ischemic stroke therapy in rats. In other models of cerebral ischemia, such as global ischemia after cardiac arrest, oxygen itself appears to have a disadvantageous effect upon oxidative energy metabolism in the brain, as recently published by Richards et al. [32]. Due to these fundamental differences in transient global versus focal ischemia models, we suggest careful interpretation of such oxygen treatment studies in acute ischemic events. Thus, each pathogenetic condition for ischemic injury must be investigated carefully for the underlying mechanisms of oxygen-induced neuroprotection, and its potentially harmful effects in animal models, prior to the start of clinical trials. At present, in combination with our previous study [22], we have demonstrated that early high-dose oxygen therapy appears to be beneficial for a long-lasting infarct volume reduction and clinical outcome in rats.

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We consider the present data to be important for the design of future clinical stroke studies, including early oxygenation therapy regimens in combination with recanalization procedures, in order to improve clinical outcome and infarct reduction in humans.

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