Effect of large dose hyperbaric oxygenation therapy on prognosis and oxidative stress of acute permanent cerebral ischemic stroke in rats

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Objective: *To evaluate the therapeutic effect and the oxidative stress effect of 9 and 18 hour hyperbaric oxygenation therapy (HBOT) protocols on the earliest stage of acute permanent middle cerebral artery occlusion (MCAO) in rats.*

Methods: *The permanent MCAO model of rats was used. The animals were randomly divided into 9 and 18 hour HBOT groups, as well as a control group.*

Main outcome measures: *(1) The Garcia neurological grading system was used to assess the therapeutic effect of hyperbaric oxygenation therapy; (2) the infarct volume was calculated with the 2,3,5-triphenyltetrazolium chloride (TTC) pathologic staining and NIH Image J software 24 and 120 hours after MCAO; (3) the level of reactive oxygen species determined by superoxide dismutase (SOD), malondialdehyde (MDA) and nitric oxide (NO) in ischemic brain tissue were separately examined at the 18, 48 and 120 hour post-ischemia time points using spectrophotometry.*

Results: *(1) There were significant improvements in the neurobehavioral outcome of the rats in the 9 and the 18 hour groups, as compared with rats from the control group ($p<0.01$); (2) cerebral infarct volume decreased 63–64% in the rats of 9 hour group and 51–66% in the 18 hour group at the 24 and 120 hour time points, as compared with that of the control group; (3) the SOD levels of the 9 and 18 hour groups were remarkably lower than those of control group after both 18 and 48 hours ($p<0.01$ and $p<0.05$); (4) the MDA level of the 9 and 18 hour groups were both remarkably lower than the control groups, especially at 18 hours ($p<0.05$). Meanwhile, the MDA level in the 9 hour group was remarkably lower than both the 18 hour group and the control group ($p<0.01$ and $p<0.05$); (5) the level of NO in both hyperbaric oxygenation therapy groups were remarkably higher than that of the control at 18 and 48 hour time points ($p<0.01$). While the level in 18 hour group was remarkably lower than that of 9 hour group at 18 hour time point ($p<0.05$). At the 120 hour mark, the NO levels were basically the same in all the three groups.*

Conclusions: *(1) The two protocols of large dose hyperbaric oxygenation therapy are highly efficient in reducing infarct volume and improving neurobehavioral outcome in permanent MCAO rats within the earliest stages of stroke; (2) increased duration of hyperbaric oxygenation therapy does not appear to equate to improved outcomes; in fact, the longer duration may aggravate the oxidative stress in ischemic tissue.* [Neurol Res 2008; **30**: 389–393]

Keywords: Hyperbaric oxygenation therapy; cerebral infarction; neurobehavioral outcome; infarct volume; oxidative stress

INTRODUCTION

Ischemic stroke develops as a result of transient or permanent blocking of an intracranial artery. Previous works have demonstrated that single administration of 2–3 ATA, 40 minutes to 3 hours of hyperbaric oxygenation therapy (HBOT) significantly reduces infarct volume after transient meddle cerebral artery occlusion (MCAO); however, the effect that hyperbaric oxygenation therapy has on permanent MCAO is still controversial[1].

The related literature reveals that the natural pathologic progression of supratentorial non-lacunar large-vessel infarction takes 6–18 hours with an average of 10 hours[2]; therefore, the rapid restoration of oxygenation in infarcted cerebral tissue is one of the most important measures to treat acute ischemic stroke. We hypothesize that the high-dose hyperbaric oxygenation treatment on acute ischemic stroke in the earliest stage

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of stroke may inhibit, and even reverse, the pathologic progression of stroke, thus improving the neurobehavioral outcome of permanent ischemic stroke. We designed two kinds of HBOT protocols involving 9 and 18 hour treatment times. We experimented with the permanent MCAO models and assessed its therapeutic effect through the neurological grading system and the infarction volume measuring protocol with 2,3,5-triphenyltetrazolium chloride (TTC) staining. Meanwhile, we also observed and compared the reactive oxygen species induced by two kinds of oxygen treatment by measuring the levels of superoxide dismutase (SOD), malondialdehyde (MDA) and nitric oxide (NO) of ischemic brain tissue 18, 48 and 120 hours post-MCAO.

MATERIALS AND METHODS

Animals

One hundred and fourteen male Sprague–Dawley rats, aged 2.5 months old, weighing 280 $\pm$ 20 g, were used (the Animal Institute, CACMS, China). The hyperbaric oxygenation device we used was a hyperbaric air cabin in which a self-made pure oxygen animal experimental cabin was employed (China).

Experimental procedures

MCAO models

After being weighed, each rat was deeply anesthetized with chloral hydrate (400 mg/kg) intraperitoneally, and the rats were fixed on the operation table. The body temperature of the rats was maintained at 37°C by an overhead heating lamp. The protocol for the MCAO model was adapted from the protocol developed by Longa *et al.*[3].

Experimental grouping and intervention

The experiment was divided into two parts: First, sixty models were randomly divided into 9 hour hyperbaric oxygenation, 18 hour hyperbaric oxygenation and control group. Then, each group was divided into 24 and 120 hour subgroups, with ten rats in each subgroup. Three hours after surgical initiation of the MCAO, rats in the two HBOT groups stayed in the hyperbaric cabin for a total of either 9 or 18 hours, according to their grouping protocol. The treatment pressure was 2 ATA. Rats in the 9 hour HBOT group inhaled hyperbaric pure oxygen at the first, third, fifth, seventh and ninth hours, and hyperbaric air at the second, fourth, sixth and eighth hours. Rats in the 18 hour HBOT inhaled hyperbaric pure oxygen at the first, third, fifth, seventh, ninth, eleventh, thirteenth, fifteenth and seventeenth hours, and hyperbaric air in alternant hours. Rats were allowed to recover under normal pressure ambience after being taken out of the cabin. Rats in the control group breathed room air. The three 120 hour subgroups were scored at hours 3, 24, 48 and 120. The brain tissues from ischemic side in each subgroup were harvested either 24 or 120 hours after infarction, according to how they were grouped.

Second, 54 models were used for the examination of SOD, MDA and NO level of ischemic brain tissue. They were also randomly divided into 9 and 18 hour groups and a control group. But, every group was divided into three subgroups: 18, 48 and 120 hour subgroups, with six rats in each subgroup.

Observation indexes

Neurobehavioral outcome

The Garcia neurological grading system[4] (grading scales from 3 to 18 with higher scores indicating less disability) was used to assess the outcome of the animals 24, 48 and 120 hours after the stroke.

Measurement of infarct volume

The animals (ten rats in each subgroup) were deeply anesthetized as described above and decapitated after perfusion of the heart with normal saline. The whole brain was removed, chilled and sectioned coronally into five 2 mm thickness slices and was stained with 2% solution of TTC at 37°C for 30 minutes. Each animal's infarct volume was evaluated quantitatively by using the image-analysing software Image J (NIH). To eliminate the error caused by edema in infarction areas, the measurement of infarcted area of brain was adjusted as described in detail by Schäbitz *et al.*[5].

SOD, MDA and NO level

The SOD, MDA and NO levels of ischemic brain tissue were examined at the 18, 48 and 120 hour time points using spectrophotometry.

Statistical analysis

The statistical significance of the data differences between each group was evaluated using one-way analysis of variance. If the disparity was considered statistically significant, the *post hoc* Bonferroni method was used to further measure the inter-group disparity, with SPSS. $p<0.05$ was considered statistically significant.

RESULTS

Neurobehavioral outcome

Three hours after MCAO, the neurological status was equal between the groups. But the Garcia neurobehavioral outcome of both of the HBOT groups was significantly better than that of control group after 24, 48 and 120 hours ($p<0.01$, *Figure 1*). Moreover, the outcome of the 9 hour group was slightly better than the 18 hour group at hour 120, but not statistically significant ($p>0.05$, *Figure 1*) .

Infarct volume

The infarct volume of the HBOT group was significantly smaller than that of the control group at hours 24 and 120 ($p<0.01$, *Figures 2* and *3*). In the 18 hour HBOT group, the infarct volume at hour 120 was

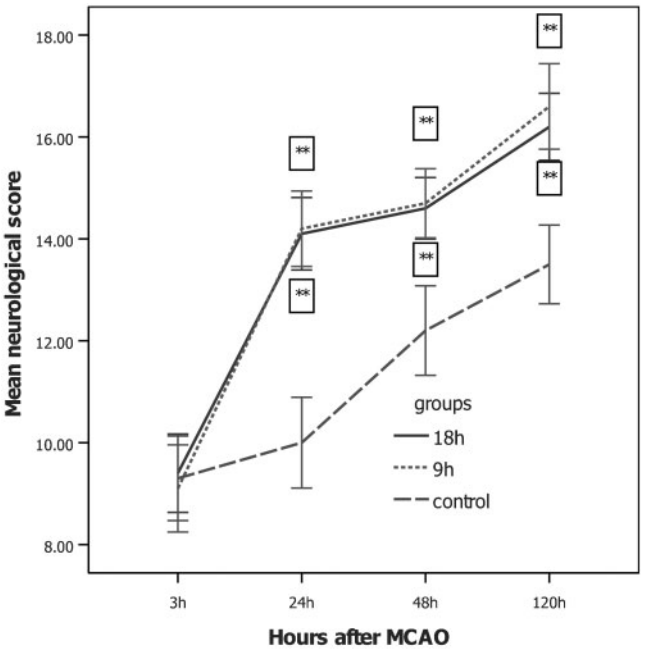

Figure 1: According to the Garcia grading system, both the 9 and 18 hour hyperbaric oxygenation therapy groups exhibited significantly improved neurological function (higher score), at hours 24, 48 and 120, respectively ($p<0.01$). Values are expressed as mean ± SD, error bar: 95% CI

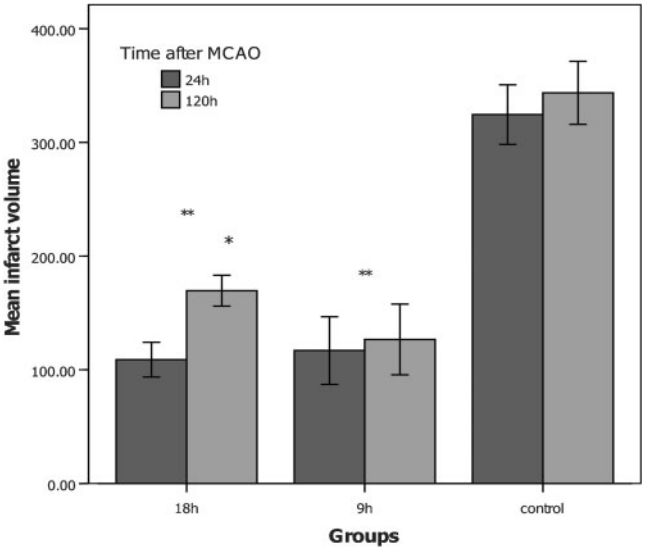

Figure 2: The infarct volumes of 9 and 18 hour hyperbaric oxygenation therapy groups were significantly smaller than that of the control group at hours 24 and 120 ($p<0.01$). In the 18 hour group, the infarct volume of the 120 hour subgroup was significantly larger than that of 24 hour subgroup ($p<0.05$). Error bar: 95% CI

significantly larger than that at hour 24 ($p<0.05$, *Figures 2* and *3*).

Oxidative stress indexes

The SOD levels of 9 and 18 hour HBOT groups were significantly lower than that of the control ($p<0.01$, *Table 1*) at hour 18. At the same time, the SOD level of 18 hour group was lower than that of 9 hour group ($p<0.05$, *Table 1*). The trend of lower SOD levels for the 18 hour group continued for 48 hours post-treatment, unlike the SOD levels of the control group ($p<0.05$, *Table 1*). There was no significant difference in the SOD level between the three groups at hour 120. In contrast, the MDA levels of both HBOT groups were remarkably lower than that of control at hours 18, 48 and 120, especially at hour 18 ($p<0.05$, *Table 1*). Meanwhile, the NO levels of both HBOT groups were remarkably increased compared with that of control at hours 18 and

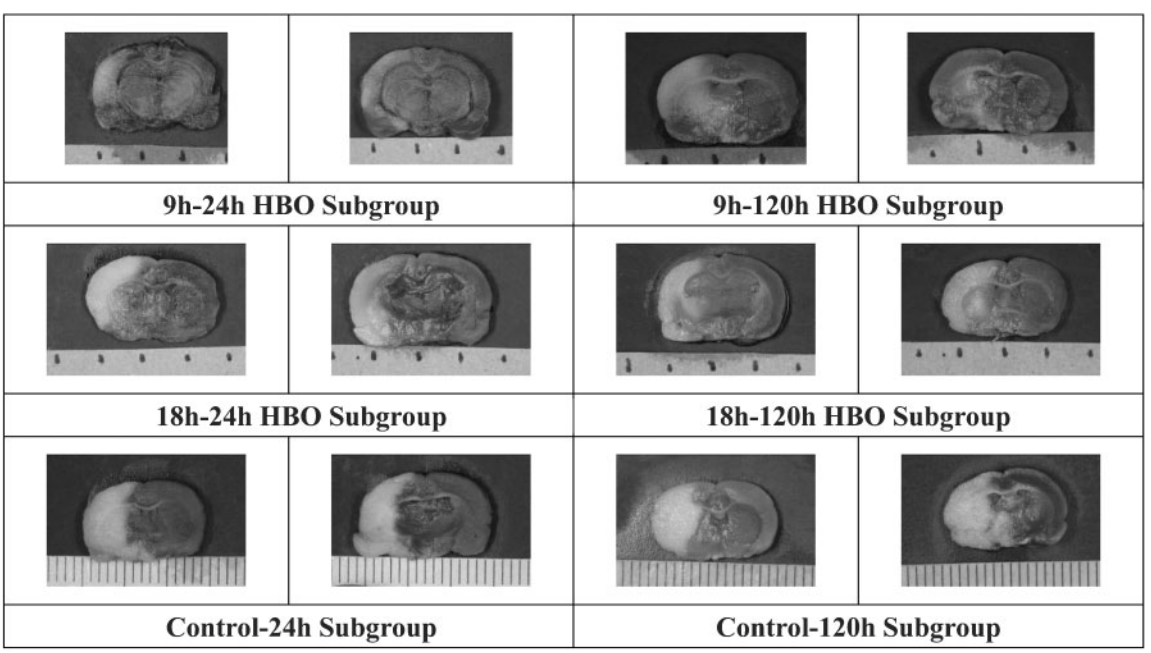

Figure 3: Reprehensive microphotographs showing brain tissue with TTC staining at different ischemic periods in control and hyperbaric oxygenation (HBO) treatment group. The white parts of brain slices indicate infracted tissues

48 ($p<0.01$, *Table 1*). Between the two HBOT groups, the NO level of 18 hour group was also remarkably higher than that of the 9 hour group at hour 18 ($p<0.05$, *Table 1*). After 120 hours of MCAO, there was no significantly difference among the NO levels in the three groups. All of the 114 rats involved in the study entered the stage of final analysis.

DISCUSSION

This study demonstrates that the neurobehavioral outcome of MCAO rats is significantly improved after treatment with HBOT therapy for 9–18 hours. The infarct volumes of 9 and 18 hour groups decreased 63–64 and 51–66%, respectively, as compared with that of the control group. This is different from the reports on single hyperbaric oxygenation treatment of acute permanent MCAO in rats by Hjelde *et al.*[6] and Lou *et al.*[7]. It is understood that reperfusion occurs after a blood vessel that has been occluded for a limited period of time (usually 1–2 hours) is recanalized. After recanalization, the blood supply in the previously ischemic region is either completely or partially recovered. The degree of injury depends on the duration of ischemia and the degree of recanalization. It is greatly different from infarct caused by permanent occlusion of the same vessel. The degree of injury and region of affected brain of the latter are much greater than those of transient MCAO. Also, the duration and stage of the pathologic evolution after infarct are also longer and far more complex. We believe that recanalization is an important reason accounting for different therapeutic effects of HBOT in transient and permanent MCAO. In the transient MCAO, HBOT could act through two ways, both of which improve the blood supply to the penumbra. The first is by recanalizing the vessel and quickly transporting the molecular oxygen in blood to the infarcted area. The other is by permeating and diffusing into the still-viable areas surrounding the infracted tissue, such as the cerebral pia mater vessel, and providing cerebral protection via its anti-oxidative properties[8]. As for permanent MCAO, HBOT could only act through the second way. Single hyperbaric oxygenation therapy of 40–180 minutes is insufficient to inhibit the infarct progression effectively.

This study found that single multi-hour HBOT of focal ischemia in the super early stages of stroke could significantly decrease the infarct volume and improve neural function prognosis. We believe that this effect was related to the therapeutic window (6–18 hours) of the pathologic evolution in cerebral ischemia. The 9 and 18 hour HBOT schemes were adopted in this study with the goal of persistently improving oxygen supply in the ischemic tissue in the early stages of the pathologic evolution of stroke, which can maximally inhibit or even withdraw the pathologic progression of acute cerebral stroke. In the meantime, the rats received an alternative 1 hour of hyperbaric air after receiving every 1 hour of hyperbaric oxygen, both to inhibit the possible side effects of oxygen intoxication and to improve rats' tolerance of HBOT[9]. This study demonstrated that 9 and 18 hour persistent HBOT might have an important inhibiting effect on the pathologic progression of cerebral stroke.

In the past, people considered that ischemia–reperfusion referred only to what occurred when the blood vessel was recanalized, omitting the fact that ischemia–reperfusion also refers to the resolution of hypoxia within the affected cells that needs to occur during this process, in order for the process to be effective. This study demonstrated through SOD levels that oxidative stress increased during the early stages of HBOT; however, the levels of MDA reported appear to suggest that oxidative injury was not aggravated by this therapy. This study also found that there was no significant difference of the neurobehavioral outcome and infarction volume between the two therapy groups at hour 24, though the therapeutic duration was a half discrepancy. Furthermore, the infarction volumes in the 18 hour HBOT group trended towards being larger than that of the 9 hour group at the 120 hour time period; however, this difference did not reach statistical significance ($p>0.05$). Nevertheless, these data suggest that when trying to determine the optimum amount of HBOT required to treat permanent arterial occlusion, longer is not necessarily better. More specific reasons need to be further identified as to why this trend occurs. We believe that it might be related to the aggravation of oxidative stress of ischemic tissue[10]. Therefore, enough importance on the toxicity and side effects of high-dose

Table 1: Comparison of ischemic brain tissue SOD, MDA and NO level among different time subgroups

Group	Oxidative index	18 hours (n=6)	48 hours (n=6)	120 hours (n=6)
9 hour HBO	SOD (U/mg)	64.24 ± 11.32*‡	64.69 ± 8.07	77.55 ± 9.12
	MDA (nmol/mg)	2.63 ± 0.59†	3.09 ± 1.12	2.54 ± 1.30*‡
	NO (μmol/mg)	1.57 ± 0.18*‡	1.73 ± 0.15*	1.28 ± 0.17
18 hour HBO	SOD (U/mg)	54.85 ± 6.95*	62.61 ± 8.00†	77.20 ± 6.16
	MDA (nmol/mg)	2.64 ± 0.95†	3.40 ± 1.44	3.59 ± 1.06
	NO (μmol/mg)	1.78 ± 0.18*‡	1.90 ± 0.21*	1.39 ± 0.17
Control	SOD (U/mg)	70.08 ± 5.86	71.16 ± 8.50	74.78 ± 6.11
	MDA (nmol/mg)	3.93 ± 0.80	3.94 ± 1.12	4.52 ± 1.23
	NO (μmol/mg)	1.31 ± 0.16	1.26 ± 0.13	1.31 ± 0.14

Values are expressed as mean ± SD.
*$p<0.01$ versus control group; †$p<0.05$ versus control group; ‡$p<0.05$ versus 18 hour hyperbaric oxygenation (HBO) group.

persistent HBOT should be attached in the research, as it may increase oxidative stress.

REFERENCES

1 Xiong L, Zhu Z, Dong H, *et al.* Hyperbaric oxygen preconditioning induces neuroprotection against ischemia in transient not permanent middle cerebral artery occlusion rat model. *Chin Med J (Engl)* 2000; **113**: 836–839
2 Saver JL. Time is brain – quantified. *Stroke* 2006; **37**: 263–266
3 Longa EZ, Weinstein PR, Carlson S, *et al.* Reversible middle cerebral artery occlusion. *Stroke* 1989; **20**: 84–91
4 Garcia JH, Wagner S, Liu KF, *et al.* Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats: Statistical validation. *Stroke* 1995; **26**: 627–634
5 Schäbitz WR, Schade H, Heiland S, *et al.* Neuroprotection by hyperbaric oxygenation after experimental focal cerebral ischemia monitored by MRI. *Stroke* 2004; **35**: 1175–1179
6 Hjelde A, Hjelstuen M, Haraldseth O, *et al.* Hyperbaric oxygen and neutrophil accumulation/tissue damage during permanent focal cerebral ischaemia in rats. *Eur J Appl Physiol* 2002; **86**: 401–405
7 Lou M, Eschenfelder CC, Herdegen T, *et al.* Therapeutic window for use of hyperbaric oxygenation in focal transient ischemia in rats. *Stroke* 2004; **35**: 578–583
8 Anderson DC, Bottini AG, Jagiclla WM, *et al.* A pilot study of hyperbaric oxygen in the treatment of human stroke. *Stroke* 1991; **22**: 1137–1142
9 Chavko M, McCarron RM. Extension of brain tolerance to hyperbaric O_2 by intermittent air breaks is related to the time of CBF increase. *Brain Res* 2006; **1084**: 196–201
10 Murakami K, Kondo T, Kawase M, *et al.* Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *J Neurosci* 1998; **18**: 205–213