Effects of hyperbaric oxygen preconditioning on energy metabolism and glutamate level in the peri-infarct area following permanent MCAO

CUI GAO-YU1, DENG CONG-YIN2, ZANG LI-JUN3, LI FEI1, FENG HUA1

1 Department of Neurosurgery, Southwest Hospital, Third Military Medical University, Chongqing, China; 2 Co-first author: the 324th Hospital of PLA, Chongqing, China;

CORRESPONDING AUTHOR: Dr. Feng Hua – fenghua8888@yahoo.com.cn

ABSTRACT
This study aimed to evaluate the effect of hyperbaric oxygen preconditioning (HBO2P) on ischemic metabolites and the glutamate level after permanent middle cerebral artery occlusion (MCAO). HBO2P was administered: five treatments, one treatment per day. The permanent rabbit MCAO models were induced by a modified transorbital approach. The microdialysis procedures were performed in the right peri-infarct area and contralateral area. MCAO decreased glucose levels while increasing lactate, pyruvate and lactate/pyruvate ratios. Early increase of glycerol and glutamate were also shown. HBO2P stabilized the glucose level and decreased the lactate/pyruvate ratios and glycerol in the peri-infarct area. In addition, it inhibited the increase of the glutamate level. Our study demonstrated that MCAO led to the imbalance of brain energy metabolites and excitatory amino acids. The modulation of energy metabolism and glutamate may be one of the factors contributing to the neuroprotective property of HBO2P.

INTRODUCTION
Hyperbaric oxygen preconditioning (HBO2P) has been shown to reduce infarct volumes and improve the neurological outcome in several animal models of cerebral ischemia [1,2,3,4,5]. The same results were also observed in our previous study using the rabbit permanent middle cerebral artery occlusion (MCAO) model [6]. However, the mechanism of HBO2P against cerebral ischemia is not fully understood.

It is well established that brain energy metabolism changes from an aerobic state to an anaerobic respiration state during cerebral ischemia. This leads to an imbalance of glucose and its dominant glycolytic products (lactate, pyruvate and glycerin). Lactate accumulating and brain acidifying play an important role in acidosis neurotoxicity by inducing excitotoxicity during cerebral ischemia [7]. Furthermore, the imbalance of the glucose and its products aggravates the brain damage.

We hypothesized that HBO2P would ameliorate the imbalance of brain energy metabolism against cerebral ischemia. In the present study, microdialysis was introduced to evaluate the effects of HBO2P on the level of glucose, lactate, pyruvate, glycerol and glutamate in the peri-infarct area following permanent MCAO in rabbits.

MATERIALS AND METHODS
All animal procedures were approved by the Ethics Committee for Animal Experimentation and conducted according to the Guidelines for Animal Experimentation of the Third Military Medical University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Animals and groups
Male rabbits (three to four months of age, weighing 2.0-2.5 kg) provided by Experimental Animal Center of the Third Military Medical University were used for the experiments. A total of 72 animals were randomly divided into three groups:

• a sham operation group (n=6);
• MCAO group (n=6) measured at 24 hours, 72 hours, 10 days and 20 days;
• HBO2P plus MCAO group (n=6) measured as above.

HBO2P preconditioning
A research hyperbaric chamber (Binlun Oxygen Chamber Co., Ltd., Yantai, China) was used for HBO2P. Compression was performed at a rate of 1 kg/cm²/minute to 2.5 ATA / 100% oxygen and maintained for 60 minutes. Decompression was performed at 0.2 kg/cm²/minute.
The chamber was flushed with 100% oxygen at a rate of 5 L/minute. For HBO2P, oxygen and carbon dioxide contents were continuously monitored and maintained at ≥98% and at ≤0.03% respectively. Chamber temperature was maintained in the range of 22-25°C. HBO2P was used in five treatments for one treatment per day. The sham operation and MCAO animals were placed in the same chamber for one hour per day for five days in room air without increased pressure. All exposures were begun at 9:00 and 15:00 hours to prevent biological rhythm changes (4).

MCAO model
The permanent rabbit middle cerebral artery occlusion (MCAO) models were induced by a modified transorbital approach [8,9]. After being anesthetized with 2% pentobarbital sodium (40 mg/kg) intraperitoneally, the animals were restrained briefly in the left lateral position with their heads stabilized. A circular incision was made on the right cornea and the whole vitreous body was excised. After orbital tissue retracted laterally, the postorbital bone was drilled, and the dura layer was opened to expose the middle cerebral artery. The middle cerebral artery just distal to the bifurcation was occluded with bipolar coagulation and then transected to prevent recanalization. The craniectomy defect was covered with absorbable gelatin sponge, and the incision was closed using 1-0 silk sutures. Rabbits in the sham group underwent a surgical procedure as above, but the middle cerebral artery occlusion was not taken.

Microdialysis procedures
Microdialysis equipment (CMA/Microdialysis, Stockholm, Sweden) included the microdialysis probe (CMA70), microinjection pump and artificial cerebrospinal fluid. At 12 hours after the MCAO model, animals underwent an MRI scan to determine the position of the ischemic focus and the stereotaxic coordinates of the peri-infarct area (Figures 1A,1B,1C,1D – facing page). An intracerebral cannula guide was implanted into the peri-infarct area at the scheduled time points one hour prior to the procedure, and then the microdialysis probe was inserted.

After equilibrating for 60 minutes by continuously perfusing with artificial cerebrospinal fluid at a flow rate of 3μl/minute and driven by a microinjection pump, the microdialysis samples were continuously collected for one hour and reserved under -20°C. The same microdialysis procedures were performed on the left hemisphere, with stereotaxic coordinates same as in the peri-infarct area. All microdialysis samples were measured in CMA 600 Microdialysis Analyzer [10,11].

Statistical analysis
Results are expressed as mean ± standard deviation (SD). Differences were evaluated by the Students’ paired T-test or ANOVA test. Statistical significance was placed at p<0.05.

RESULTS
In the MCAO group, the significant decrease of glucose concentrations in the peri-infarct area was found at 24 hours, 72 hours and 20 days after MCAO as compared to the left hemisphere. The same results were shown at 72 hours and 20 days after MCAO between the MCAO group and the sham operation group. We found no significant differences in the glucose concentrations between the HBO2P plus MCAO group and the sham operation group. Only at 72 hours after MCAO was the decrease of glucose concentrations in the peri-infarct area revealed, as compared to the left hemisphere (Figure 2A, Page 94).

The MCAO group showed that the lactate concentrations of the peri-infarct area and the left hemisphere were significantly higher than that in the sham operation group at each time point. At 24 hours and 72 hours after MCAO, the lactate concentrations of the peri-infarct area were 7.93 ± 1.69 and 8.38 ± 1.07 respectively – which were significantly higher than that of the left hemisphere – while there were no significant differences between the peri-infarct area and the left hemisphere at 10 days and 20 days. In the HBO2P plus MCAO group, the lactate concentrations of the peri-infarct area increased significantly as compared to that of the sham operation group at each time point, while there were no significant differences between the peri-infarct area and the left hemisphere (Figure 2B, Page 94).

The MCAO group showed that the pyruvate concentrations of the peri-infarct area were significantly higher than that in the sham operation group at each time point. The same results were found in the left hemisphere except at 20 days. In the HBO2P plus MCAO group, the pyruvate concentrations of the peri-infarct area increased significantly as compared to that of the left hemisphere and the sham operation group at each time point.

At 72 hours and 10 days, the pyruvate concentrations of the peri-infarct area in the HBO2P plus MCAO group showed significantly higher levels than that in the MCAO group (Figure 2C, Page 95).

In the MCAO group, the lactate/pyruvate ratios of the peri-infarct area at 72 hours, 10 days and 20 days after MCAO increased significantly as compared to that of the left hemisphere, although there were no significant differences at 24 hours after MCAO. The lactate/
 FIGURE 1 – Non-contrast coronal T2-weighted MR images of A, B, C and D show the different infarct areas at 1 day, 3 days, 10 days and 20 days after the occlusion of MCA respectively. R: the right hemisphere; Arrow: the infarct area.

pyruvate ratio of the peri-infarct area in the MCAO group was higher significantly than that in the sham operation group at 72 hours after MCAO. In the HBO$_2$P plus MCAO group, the lactate/pyruvate ratios of the peri-infarct area decreased significantly as compared to that in the MCAO group at each time point. At 24 hours and 72 hours after MCAO, the lactate/pyruvate ratios of the peri-infarct area in the HBO$_2$P plus MCAO group were 39.15 ± 5.11 and 48.07 ± 5.86 respectively, which were lower significantly than that of the left hemisphere or that in the sham operation group (Figure 2D, Page 95).

In the MCAO group, the glycerol concentration of the peri-infarct area and the left hemisphere were 160.95 ± 48.04 and 116.85 ± 35.80 at 24 hours after MCAO respectively, which was higher significantly than that in the sham operation group.

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FIGURE 2A – Changes in glucose concentrations

Glucose concentration (μmol/L)

FIGURE 2A – The changes in glucose concentrations (mmol/l) in the sham operation group, MCAO group and HBO₂P plus MCAO group.

FIGURE 2B – Changes in lactate concentrations

Lactate concentration (μmol/L)

FIGURE 2B – The changes in lactate concentrations (mmol/l) in the sham operation group, MCAO group and HBO₂P plus MCAO group.

LEGEND – S-R: the right hemisphere in the sham operation group; S-L: the left hemisphere in the sham operation group; MCAO-R: the right hemisphere in the MCAO group; MCAO-L: the left hemisphere in the MCAO group; HBO₂P-R: MCAO-R: the right hemisphere in the HBO₂P plus MCAO group; MCAO-L: the left hemisphere in the HBO₂P plus MCAO group. * P<0.05 vs. the sham operation group; # <0.05 vs. the previous time point; # <0.05 between the right and left hemisphere in same group; † <0.05 vs. the MCAO group.
FIGURE 2C – Changes in pyruvate concentrations

FIGURE 2C – The changes in pyruvate concentrations (μmol/l) in the sham operation group, MCAO group and HBO₂P plus MCAO group.

FIGURE 2D – Changes in lactate/pyruvate ratios

FIGURE 2D – The changes in lactate/pyruvate ratio (μmol/l:μmol/l) in the sham operation group, MCAO group and HBO₂P plus MCAO group.

LEGEND – S-R: the right hemisphere in the sham operation group; S-L: the left hemisphere in the sham operation group; MCAO-R: the right hemisphere in the MCAO group; MCAO-L: the left hemisphere in the MCAO group; HBO₂P-R: the right hemisphere in the HBO₂P plus MCAO group; HBO₂P-L: the left hemisphere in the HBO₂P plus MCAO group. * P<0.05 vs. the sham operation group; # P<0.05 vs. the previous time point; % P<0.05 between the right and left hemisphere in same group; ! P<0.05 vs. the MCAO group.
At 72 hours after MCAO, the glycerol concentrations of the peri-infarct area decreased significantly as compared to that at 24 hours, while it was higher significantly than that of the left hemisphere. At 10 days and 20 days after MCAO, the glycerol concentrations of the peri-infarct area were 90.70 ± 32.94 and 86.09 ± 36.57 respectively, which were no significant differences as compared to that of the left hemisphere or that in the sham operation group.

In the HBO₂P plus MCAO group, the glycerol concentrations of the peri-infarct area decreased gradually; these were lower significantly than that in the MCAO group at each time point, but it was higher significantly than that in the sham operation group at 24 hours after MCAO. At 10 days and 20 days after MCAO, the glycerol concentrations of the peri-infarct area and the left hemisphere in the HBO₂P plus MCAO group all decreased significantly as compared to that in the MCAO group or the sham operation group (Figure 2E, above).

In the MCAO group, the glutamate concentration of the peri-infarct area was 61.86 ± 11.73 µmol/l at 24 hours after MCAO, which increased significantly as compared to that of the left hemisphere or in the sham operation group. At 72 hours after MCAO, the glutamate concentrations of the peri-infarct area reached 97.41 ± 17.24 µmol/l. The glutamate concentrations of the peri-infarct area reduced to 47.82 ± 16.15 at 10 d and 39.54 ± 9.43 at 20 days but were still significantly higher than that of the sham operation group. In the HBO₂P plus MCAO group, the glutamate concentration of the peri-infarct area was 36.20 ± 14.05 µmol/l at 24 hours after MCAO, which decreased significantly as compared to that of the MCAO group. However, it was higher significantly than that of the sham operation group. At 72 hours after MCAO, there was no significant difference in the glutamate concentration of the peri-infarct area between the HBO₂P plus MCAO group and the MCAO group. At 20 days after MCAO, the glutamate concentration of the peri-infarct area was significantly lower than that of the MCAO group. No significant differences were found in the glutamate concentrations of the left hemisphere within the sham operation group, the MCAO group and the HBO₂P plus MCAO group (Figure 2F, facing page).
FIGURE 2F – Changes in glutamate concentrations

FIGURE 2F – The changes in glutamate concentrations (μmol/l) in the sham operation group, MCAO group and HBO2P plus MCAO group.

LEGEND – S-R: the right hemisphere in the sham operation group; S-L: the left hemisphere in the sham operation group; MCAO-R: the right hemisphere in the MCAO group; MCAO-L: the left hemisphere in the MCAO group; HBO2P-R: the right hemisphere in the HBO2P plus MCAO group; HBO2P-L: the left hemisphere in the HBO2P plus MCAO group.

* P<0.05 vs. the sham operation group; # <0.05 vs. the previous time point; ! <0.05 between the right and left hemisphere in same group; ! <0.05 vs. the MCAO group.

DISCUSSION
The major findings in the present study are:
1) MCAO decreased glucose and increased lactate, pyruvate and lactate/pyruvate ratios. Early increases in glycerol and glutamate were shown.
2) HBO2P stabilized the glucose level and decreased the lactate/pyruvate ratios and glycerol in the peri-infarct area. Meanwhile, it attenuated the increase of glutamate level.

The ischemic penumbra or peri-infarct area is the target tissue for intervention. Magnetic resonance imaging (MRI) has been introduced to differentiate the penumbra from the infarct core. T2-weighted and diffusion-weighted MRI sessions can reliably delineate a variable extent of ischemic focus subchronically [12]. The mismatch between diffusion-weighted imaging and perfusion imaging is a surrogate of the ischemic penumbra [13,14]. The fluctuations of the energy metabolism and its products in the peri-infarct area are associated with ischemic neuronal damage. Studies have shown that the changes in glucose, lactate, pyruvate and excitatory amino acids in the extracellular fluid levels reflect intracellular metabolic disturbances produced by cerebral ischemia [15]. However, many related studies focus mainly on the early phase of cerebral ischemia. The long-term variations of the energy metabolism are not clear.

Persson et al. [16] used intracerebral microdialysis on 10 patients with subarachnoid hemorrhage to monitor the disturbances in brain energy metabolism and extracellular levels of excitatory amino acids from six to 11 days after ictus. In the present study, we used the permanent MCAO rabbit model to study the extracellular fluid levels of energy-related substances and glutamate at 24 hours, 72 hours, 10 days and 20 days after MCAO, which provided useful information for ischemia.

The mechanisms of HBO2P against cerebral ischemia remain to be defined. Badr et al. [17] found that the treatment of hyperbaric oxygen (HBO2) within six hours after reperfusion could decrease infarction after two hours of ischemia and 24 hours of reperfusion, and it decreased glucose, pyruvate and glutamate almost to the preocclusion level. Badr et al. [17] suggested that the treatment of
HBO₂ after ischemia might be involved in the mechanisms for the protective effect of HBO₂ in cerebral ischemia by regulation or correction of ischemic striatal metabolites. Recent studies have demonstrated that treatment with HBO₂ can improve oxygen supply of the ischemic penumbra as well as the cellular bioenergetic metabolism [18,19].

The energy-related metabolites are useful extracellular markers of cerebral ischemia [15,16,20,21]. The extracellular levels of glucose appear to be a useful marker of severe ischemia and recirculation [21]. In a transient MCAO and reperfusion model, the significant changes in glucose and lactate levels and the lactate/glucose ratio were observed only in the brain region of the patients with severe ischemia. Only lactate levels increased in probe regions, distinguished by penumbra [15]. As a product of the anaerobic metabolism by the cells, the amount of lactate indicates the degree of ischemia.

Results of experimental studies on ischemia also support that the extracellular levels of lactate and pyruvate levels have been advocated for estimation of the severity and the outcome of cerebral ischemia, and the lactate/pyruvate ratio has proved to be a reliable marker of ischemia [16, 20,22]. Studies showed that pyruvate reduced the cell death and production of reactive oxygen species [23,24], and exogenous pyruvate also had neuroprotective effects [25,26]. An elevated interstitial glycerol level also plays an important role in ischemic injury, which reflects the degree of ischemia [15] and may contribute to the improvement of post-ischemic brain metabolism [27].

Our data display that the significant effects of HBO₂P on increasing the pyruvate level and decreasing the glycerol level may maintain over a long period of time. Furthermore, HBO₂P decrease the lactate/pyruvate ratios in the peri-infarct area, though its role on regulating the lactate level seems comparatively weak. Moreover, HBO₂P can maintain the glucose level in the peri-infarct area almost to the level of the control group.

Excessive release of excitatory amino acids has been known as an important factor in the development of cerebral ischemic injury. Reducing the release of excitatory amino acids has been shown to positively correlate with alleviating ischemic injury [28]. Studies have confirmed that treatment with HBO₂ after ischemia has the effect of reducing the release of glutamate [19,29]. This present study shows that HBO₂P also has the effect on modulating the release of glutamate. However, the detail mechanism is not clear. A recent study found that preconditioning with sublethal ischemia or intermittent normobaric hyperoxia decreased the neurologic deficit score, and infarct volumes were interrelated with up-regulated glutamate transporters [30].

In conclusion, results of the present study revealed that MCAO caused a series changes of the energy metabolism in the peri-infarct area and induced the increase of glutamate levels. HBO₂P played a major role in stabilizing the glucose level and decreasing the lactate/pyruvate ratios, as well as the glycerol and glutamate levels in the peri-infarct area.

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