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The Time Window of Intermittent Hypoxia Intervention after Middle Cerebral Artery Occlusion

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Abstract

It was known that preconditioning hypoxia can reduce the damage caused by ischemia. However, there was no study investigating the effects of intermittent hypoxia post ischemia. The purpose of this study was to investigate the time window for administering the hypoxia for beneficial effects after cerebral ischemic damage. According to the recovery days post transient middle cerebral occlusion (MCAO), the rats were randomly assigned to one of the 4 groups (n = 20 for each group, I, II, III, and IV). Rats were then assigned to one of the 2 subgroups (a and b). Rats in group Ia, IIa, IIIa, and IVa were exposed to 7 days of intermittent hypoxia (12% O_2 for 4 hours per day) after recovery 1, 2, 3, and 7 days from MCAO, respectively. Rats in group Ib, IIb, IIIb, and IVb rested for 7 days in the same hypoxia chamber without hypoxia exposure after 1, 2, 3, and 7 days post MCAO, respectively. The mortality rate of rats received hypoxia after 1 day' and 2 days' recovery post MCAO was 40% (P = 0.087) and 10% (P = 0.5), respectively. The mean infarct volume of rats received hypoxia after 7 days' recovery was significantly less than that of the comparable control group (9.23 ± 0.71% *vs*. 13.32 ± 1.26%; P = 0.013), and no rats died in this group. In summary, intermittent hypoxia intervention for 7 days after 7 days of recovery post ischemia can reduce the infract volume, and does not increase the mortality rate. According to our results, we suggest that 7 days post ischemia may be the suitable time to begin the intermittent hypoxia intervention to enhance the recovery from cerebral ischemia.

Key Words: time windows, intermittent hypoxia, cerebral ischemia

Introduction

The meaning of hypoxia is decreasing in tissue oxygen concentration below normal level (14-16). Several studies have reported that exposure to intermittent hypoxia is beneficial. Gidday *et al*. found that hypoxia prior to ischemia can reduce infarction in neonatal rat brain (5). Bernaudin *et al*. described that normobaric hypoxia increased the tolerance to focal permanent cerebral ischemia in adult mouse brain. They showed that in adult mice exposed to normobaric hypoxia for 1, 3, or 6 h before ischemia, the infarct volume was reduced compared to that of the controls (1). Wang *et al*. found that hypoxic preconditioning can reduce kainic acid-induced oxidative injury in rat hippocampus. In their study, adult female rats were exposed to 380 mmHg in an altitude chamber 15 hours per day for 28 days. Lin *et al*. also reported that intermittent hypoxia for 7 days can attenuate iron-oxidative injury effect in rat brain (7).

Therefore, the infarct volume and oxidative injury induced by ischemia can be effectively reduced by

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preconditioning hypoxia. Can intermittent hypoxia be administered after ischemic damage for beneficial effects as administered prior to ischemic damage? To our best knowledge, there was no study investigating the effects of intermittent hypoxia post ischemia. The purpose of this study was to investigate the time window for administering the hypoxia for beneficial effects after cerebral ischemic damage.

Materials and Methods

Animals

Eighty adult male Sprague-Dawley rats (8 weeks of age, weighing 300~350 g) were used in this study. They were housed in groups of two and maintained under a 12:12-h light/dark cycle with food and water available ad libitum. The institutional animal care and use committee (IACUC) of National Yang-Ming University approved all the experimental protocols.

Middle Cerebral Artery Occlusion (MCAO) Procedure

Middle cerebral artery occlusion procedure leading to focal cerebral ischemia was conducted under chloral hydrate anesthesia (with single 0.5 g/kg *i.p.* bolus in 1 ml of saline) for each rat. Rectal temperature was monitored throughout the surgical procedure and maintained at normothermic (37.0 ± 0.5°C) by a heating blanket (HB 101/2, Debiomed). The right middle cerebral artery (MCA) was exposed using microsurgical techniques. Briefly, a 2 mm burr hole was drilled at the junction of the zygomatic arch and the squamous portion of the temporal bone, following a 2 cm vertical skin incision midway between the right eye and ear and splitting of the temporalis muscle. The right MCA trunk was ligated above the rhinal fissure with 10–0 suture. Complete interruption of blood flow was confirmed by using an operating microscope. Both common carotid arteries (CCAs) were immediately occluded following ligation of MCA with the use of nontraumatic aneurysm clips.

After the predetermined duration of ischemia (60 min), the aneurysm clips and ligation were removed from both CCAs and MCA. Restoration of blood flow in all three arteries was observed directly under the microscope. Free access to food and water were allowed after recovery from anesthesia (8, 23).

Intermittent Hypoxia Intervention Protocol

According to the recovery day after MCAO, the rats were randomly assigned to 4 groups (I, II, III, and IV; n = 20 for each group). In each group, rats were then randomly assigned to one of the two subgroups (a: experimental group, b: control group; n = 10 for each subgroup). Rats in group Ia, IIa, IIIa, and IVa were exposed to 7 days intermittent hypoxia after 1, 2, 3, and 7 days of recovery from post transient MCAO, respectively. Rats in group Ib, IIb, IIIb, and IVb rest for 7 days in the same hypoxia chamber without hypoxia exposure following 1, 2, 3, and 7 day(s) post MCAO, respectively. The intermittent hypoxia was administered for 4 h per day for 7 days in an experimental chamber with 12% oxygen. The mortality rate and infarct volume were used as the outcome measures to document the effects of hypoxia at different time period after recovery from MCAO.

Mortality Rate

We recorded the number of death beginning immediately from post MCAO to the end of the experiment. The data were expressed as number of death and percentages.

Quantitative Analysis of Infarct Volume

After the predetermined time in the study (the next day after completing intermittent hypoxia intervention in the experimental group or the matched day(s) in the control group), rats were sacrificed under pentobarbital anesthesia by intracardiac perfusion with 200 ml of 0.9% NaCl. The brain was removed carefully and dissected into coronal 2-mm sections using a brain slicer. The fresh brain slices were immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in normal saline at 37°C for 30 min, and then fixed in 10% phosphate-buffered formalin at 48°C. The cross-sectional area of infarction in the cerebral cortex of the right MCA territory of each brain slice was measured by an image analyzer, using Image-Pro plus data analysis program (Media Cybernetics, Silver Spring, MD, USA). The damaged areas measured were mainly confined to cerebral cortex including its adjacent caudate nucleus, putamen, and hippocampus (8, 23). Total measured infarct volume (MV) for each brain was calculated by summation of the infarcted area of all brain slices (area of infarct in 2 mm thickness) from the same hemisphere. Both right hemisphere volume (RV) and left hemisphere volume (LV) were also measured and calculated. To compensate for the effect of brain edema on MV in the ischemic hemisphere, the corrected infarct volume was calculated. The corrected infarct volume equals to: $\left[\frac{LV-(RV-MV)}{LV}\right]\times 100\%$ (22).

Statistical Analysis

Data of infarct volume were expressed as mean ± SEM and compared between control and experimental groups by Student's *t*-test. Differences in mortality

Table 1. Numbers of rats died and calculated mortality rate.

	With Hypoxia Intervention x 7d (experimental group: a)		Without Hypoxia Intervention x 7d (control group: b)		
	Number of death	Mortality rate (%)	Number of death	Mortality rate (%)	*P*
Post MCAO Recovery Period					
1d (Group I)	4	40%	0	0	0.087
2d (Group II)	1	10%	0	0	0.5
3d (Group III)	0	0	0	0	–
7d (Group IV)	0	0	0	0	–

rates between control and experimental groups were analyzed using Fisher's exact test. *P* value less than 0.05 was considered significant.

Results

Mortality Rate

Table 1 demonstrated the mortality rates of all groups during experiment. None of the rats in the control groups died during the experimental period. The mortality rate of group Ia (hypoxia administered after 1 day recovery post MCAO) was 40% (number of death = 4) which was higher than that of its control group, but not to reach the significant level ($P = 0.087$). Three rats died at second day post intermittent hypoxia intervention and another rat died at third day post intermittent hypoxia intervention. The mortality rate of group IIa (hypoxia administered after 2 days recovery post MCAO) was 10% (number of death = 1) which was higher than that of the matched control group, but not to a significant level ($P = 0.5$). That rat died at third day post intermittent hypoxia intervention. No rat in group IIIa (hypoxia administered after 3 days recovery post MCAO) and IVa (hypoxia administered after 7 days recovery post MCAO) died during the experimental period.

Infarct Volume

The mean infarct volume of rats survived from hypoxia in group Ia (20.79 ± 3.17%) and IIa (17.80 ± 2.14%) were not different from those of rats in group Ib (18.02 ± 1.07%; $P = 0.434$) and IIb (17.39 ± 1.05%; $P = 0.866$) (Fig. 1A, 1B). No difference in infarct volume rats in group IIIa (13.1 ± 1.29%) and IIIb (16.40 ± 1.19%; $P = 0.077$) was also noted (Fig. 1C). However, the mean infarct volume of rats in group IVa was significantly less than that of their comparable control group (9.23 ± 0.71% *vs.* 13.32 ± 1.26%; $P = 0.013$, Fig. 1D).

Discussion

Our data indicated that intermittent hypoxia intervention administered after 1 or 2 days recovery from post cerebral ischemia may result in high mortality rate. Therefore, it is not suggested to administer the hypoxia immediately after the brain ischemic damage.

The higher mortality rate resulted from early hypoxia may be due to aggravating the consequences of the cerebral ischemia. Within few minutes to hours after ischemia, excitotoxicity is increased by activating calcium influx through activation of glutamatergic receptors. The high Ca^{2+} concentration will lead to mitochondria depolarization, swelling, and even cell death (3, 20). The tissues around the infarct area called penumbra may also start to depolarization. Depolarization in the penumbra area can be recorded for at least 6 to 8 h leading to increase of infarct area (10). Other factors such as nitric oxide synthase (NOS) expressed from macrophages, microglia or neutrophils may also cause destruction of cells with peak at 48 hours after ischemia (13). Intermittent hypoxia was noted to generate the reactive oxygen species (ROS) which are highly reactive in programmed cell death (12, 14, 16). During the acute phase, intermittent hypoxia may aggravate the response of brain to ischemia including increasing the excitoxicity and result in exacerbating the brain damage. In our study, we found that early intermittent hypoxia during acute phase of brain ischemia can lead to higher mortality.

Zhao *et al*. indicated that status of antioxidant was weakened at 6^{th}, 24^{th} and 48^{th} hours after reperfusion in brain ischemic rats. Activities of superoxide dismutase (SOD) returned to normal at 72^{nd} hours after reperfusion, but other antioxidant activities remained in lower value (25). At 72 h post ischemia, the excitotoxic effects were significantly decreased but the antioxidant level was still low. In our study, we found that intermittent hypoxia intervention administered 3 days post cerebral ischemia did not increase the mortality. It did not produce beneficial effect, as indicated by infarct volume.

Our data indicated that intermittent hypoxia administered following 7 days' recovery from post brain ischemia can significantly reduce brain damage,

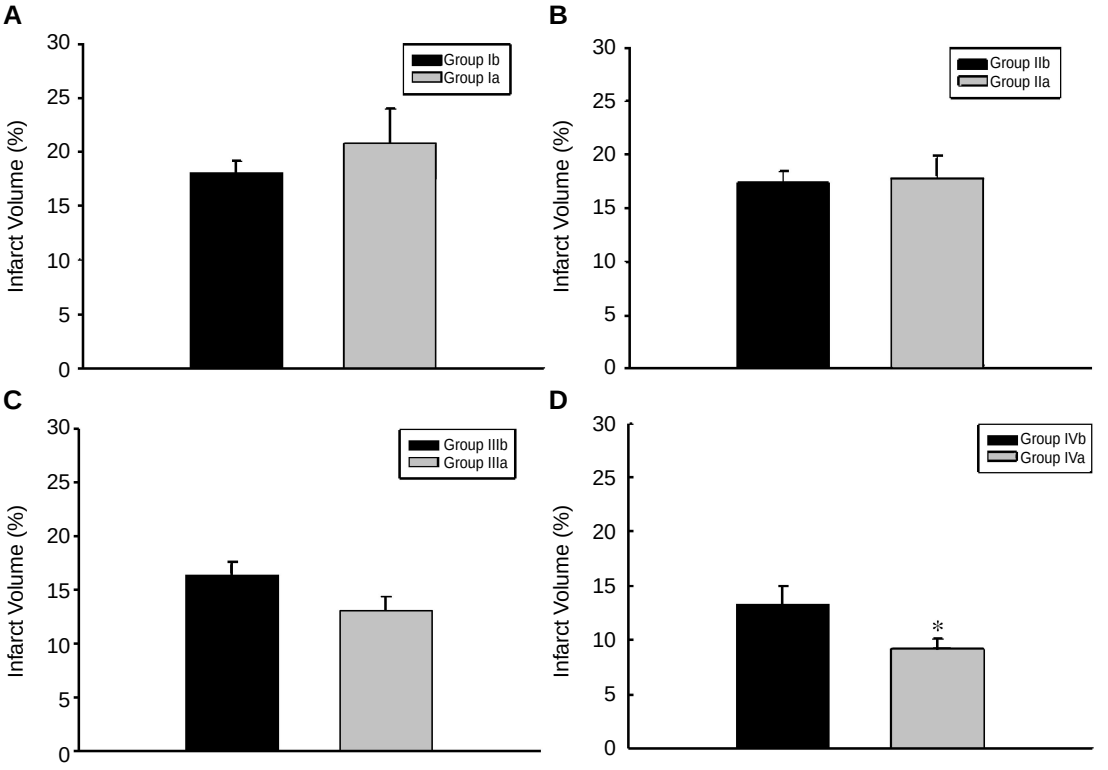

Fig. 1. Infarct volume presented as mean ± SEM for each group after MCAO in rats. Group Ia: hypoxia administered after 1 day recovery from post MCAO; group Ib: rest for 7 days following 1 day recovery from post MCAO. Group IIa: hypoxia administered after 2 days' recovery from post MCAO, group IIb: rest for 7 days following 2 days' recovery from post MCAO. Group IIIa: hypoxia administered after 3 days' recovery from post MCAO; group IIIb: rest for 7 days following 3 days' recovery from post MCAO. Group IVa: hypoxia administered after 7 days' recovery from post MCAO; group IVb: rest for 7 days following 7 days' recovery from post MCAO. *$P < 0.05$ versus the control group.

as noted by decreasing the infarct volume. The inflammation phenomenon reached its peak at the early 2 to 3 days after ischemia and declined with disease processing. (11, 20). Moreover, neural sprouting and synaptogenesis were significantly expressed at day 7 after infarction in rats (21). Lee *et al*. also noted that the level of brain derived neurotrophic factor (BDNF) increased at 7 days after ischemia in rats (6). Gendron *et al*. concluded that erythropoietin (EPO) was upregulated after MCAO, and the effects of EPO can be maintained to 7 days post MCAO by significant alteration of the amounts of red blood cells and hematocrit (4). In addition, the effects of excitotoxicity such as calcium influx, free radicals and NOS may well be significantly reduced after 7 days post ischemia (3, 9, 20). Meanwhile, the effects of spontaneous recovery became domina-nt. At this time, the hypoxia-inducible factor-1α (HIF-1α) induced by hypoxia can play an important role for the regulation of angiogenesis, iron metabolism, cell proliferation and energy metabolism (15, 17). Other protective molecules including BDNF, EPO can also be enhanced by intermittent hypoxia which are beneficial for cell recovery (1, 17). Some studies indicated that BDNF administered after temporary focal cerebral ischemia can reduce infarct size (18, 19). Bernaudin *et al*. also reported that preconditioning normobaric hypoxia significantly reduced the infarct volume caused by cerebral ischemia in association with an increased expression of EPO in the adult mouse brain (1). Hence, 7 days' recovery from post ischemia may be necessary for hypoxia intervention to produce beneficial effects. As shown in our data, intermittent hypoxia intervention after 7 days' recovery from post ischemia could significantly reduce the brain damage.

Rats rested for another 4 days after having being exposed to 7 days of intermittent hypoxia and 3 days' post MCAO were also investigated. The mean infarct volume for this group was 14.29 ± 1.63%. However, the mean infarct volume of rats receiving intermittent hypoxia and 7 days' post MCAO was 9.23 ±0.71%. According to these results, we further confirmed that 7 days' recovery from MCAO was most suitable for following intermittent hypoxia intervention.

The protocols for intermittent hypoxia intervention varied greatly. The O_2 level could range from 10% to

16%, the exposure time from few minutes to 8 hours daily, and the duration from 1 to 30 days (12). Our intermittent hypoxia protocol was mainly modified from Zhang's study (24). In Zhang's study, mice were stimulated by 16% or 10.8% oxygen in hypobaric chamber (4 h/day) for 1, 2, 3, or 4 weeks. For safety concern, we modified the duration of intermittent hypoxia intervention. Chien's study indicated that higher exposure time (12% oxygen, 8 h/day, 0, 7, or 14 days) may induce oxidative stress expression (2). In our laboratory (unpublished), we also found that the infraction volume of rats with higher exposure time (12% oxygen, 8 h/day, 7 days after 7 days' recovery post MCAO) was higher than group IVa (11.40 ± 2.55% *vs*. 9.23 ± 0.71%, $P = 0.437$). According to these results, we used 12% oxygen, 4 h per day for 7 days' intermittent hypoxia *via* intervention protocol.

In summary, intermittent hypoxia intervention for 7 days after 7 days of recovery from ischemia can reduce the infract volume, and does not increase the mortality rate. According to our results, we suggest that 7 days post ischemia may be the suitable time to begin the intermittent hypoxia intervention to enhance the recovery.

Acknowledgments

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