The protective effect of oxygen inhalation on local cerebral ischemia-reperfusion injury in experimental rats

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Abstract: This article is to investigate the effect of 33% O₂ inhalation for different time on neurological function and the expression of vascular endothelial growth factor (VEGF) in the rat model with cerebral ischemia-reperfusion injury. Fifty-four SD rats were randomly and equally into three groups: control group, ischemia-reperfusion group and the oxygen group. By using modified suture embolus method the focal cerebral ischemia-reperfusion model was established. 33% Oxygen was given for 12 hours, 24 hours and 48 hours respectively at 2 hours after ischemia reperfusion in the oxygen group. The nerve functional score was assessed and the expression of VEGF in ischimic brain tissue was evaluated by immunohistochemistry 24 hours after inhalation. The nerve function scores between model group and the oxygen group were significant (P<0.05), pairwise comparison showed that the differences were significant (P<0.05) except the difference between 24h inhalation group and 48h inhalation group (P>0.05). The number of positive cells that expressed VEGF of control group, model group, 12h inhalation group, 24h inhalation group and 48h inhalation group were respectively (7.17±2.14), (19.83±1.17), (26.67±3.14), (35.83±2.79), (37.33±4.84), the difference was significant by using one-way analysis (F=98.82, P<0.001), pairwise comparison showed that the differences were significant (P<0.05) except the difference between 24h inhalation group and 48h inhalation group (P>0.05). Conclusively, the oxygen inhalation has a protective effect on the ischemia-reperfusion injury. Its mechanism may be related to VEGF expression level. 24h oxygen inhalation has a best effect on the ischemia-reperfusion injury.

Keywords: ischemia-reperfusion injury; oxygen; VEGF

Introduction  
Cerebrovascular disease has become the first cause of death in China, and mainly are ischemic. Study have confirmed that Normobaric Hyperoxia (NBO) (21%-94%) can reduce the brain injury and improve the nerve function[1]. Although current guidelines do not support the routine use of in-hospital oxygen, stroke patients receive variable amounts of oxygen in the ambulance at present[2]. Different researches have answers. Liu et al. showed that 4 short cycles of intermittent NBO treatment provided similar neuroprotection at 24 hours and even greater neuroprotection at 72 hours after ischemia onset, when compared to continuous NBO[3]. Singhal et al. reported that NBO therapy for 8 hours via facemask improved scores of stroke scale at 24 hours in NBO-treated patients[4]. But Padma et al. found that a flow of 2 L/min of NBO didn’t improve the clinical mean scores of National Institute of Health stroke scale, modified Rankin scale and Barthel index in stroke patients[5]. The best concentration and duration of oxygen inhalation are still under study.

Vascular endothelial growth factor (VEGF) plays a major role in angiogenesis and the process of formation, and it has the neuroprotective effects[6]. So this study gave 33% oxygen for different time to the focal cerebral ischemia reperfusion in rats, then observed its effect on the expression of VEGF to explore the best time of oxygen inhalation.

Materials and methods
1.1 Experimental animals  
A total of 54 male Sprague-Dawley rats which are 3-4 months old and weighing 250-300g were used. They were SPF level, provided by experiential animal center of Henan province, China. The experimental procedures were approved by the Animal Research Committee of Zhengzhou University, Henan.

1.2 Experiment supplies  
50cm×40cm×25cm glass containers made by research group, cover with 3 circular holes: one hole was made for intake pipe that connected to the oxygen cylinder and nitrogen cylinder; one hole was a vent hole; another hole is connected with the oxygen analyzer. Soda lime granules were laid at the bottom to keep the container dry, and the ordinary squirrel-cage was put into it. Room temperature were kept at (23 ± 2) °C, light was not more than 300 Lux and kept for 12 hours everyday. The nylon fishing wire used for embolizing endovascular bought from the market, whose diameter was 0.26-0.28mm, length was 4.0cm, and the edges of the tip was removed to make it

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smooth. The sign was made from the 19mm to the tip. The wire was put into normal saline after cleaned by alcohol for later using. Rabbit immunohistochemical kit, monoclonal antibody of VEGF, SABC kit and DAB colour-developing agent were purchased from Wuhan boster Biological Products.

1.3 Experimental method

1.3.1 Middle cerebral artery occlusion (MCAO)

The model of middle cerebral artery occlusion was according to the MCAO invented by Zea Longa[7], and slightly improved. Briefly, rats were anaesthetized and a monofilament nylon suture was gently advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the MCA. Reperfusion was performed by withdrawal of the suture two hours after MCAO. Sham-operated animals underwent the same surgical procedure without suture insertion.

1.3.2 Experimental groups and oxygen inhalation

Rats were randomly divided into oxygen inhalation group, MCAO group and Sham-operated group as control, with 18 rats in each group. Oxygen inhalation group received 33% oxygen for 12 hours, 24 hours and 48 hours, while the MCAO control and sham-operated groups received the same time air. The rats died before the time point of Oxygen inhalation or the rats who had complications were removed.

1.4 Observation index

1.4.1 Neurobehavioral evaluation

The neurobehavioral scoring was performed using a six-point scale as was previously described by Zea Longa: normal motor function=0; flexion of contralateral forelimb upon suspended vertically by tail or failure to extend forepaw=1; circling to the contralateral side but have normal posture at rest=2; loss of righting reflex=3; and no spontaneous motor activity =4. If the rats died or the score was 0 or 4 points were eliminated from the study, and supplied randomly. If the rats were found to have brain subarachnoid hemorrhage and other abnormal situations were not involved in statistics.

1.4.2 Detection the expression of VEGF by Immunohistochemistry

The rats were open over 24 hours after they finishing inhalation in order to avoid the impact of ischemia reperfusion. Immunohistochemical SABC method was used: sections were dewaxed routinely and hydrated, endogenous enzyme were inactivated by 3%H2O2, then remediated by microwave, monoclonal antibody of VEGF which was Rabbit anti rat(1:200), 5%BAS, biotinylated antibody which was goat anti-mouse, SABC kit and DAB were used. The colored cells of VEGF were observed by microscope, and the one whose nucleus or cytoplasm granule were brown was positive. The pictures were collected and analysed by NIS-Elements BR software. Five sections of each brain from each group were used for cell counting at ×40 objective in randomly selected 10 non-overlapping fields of vision in the ischaemic cortex.

1.5 Statistical Analysis

The data was showed by (mean ± standard deviation) for descriptive statistics. T test, single factor analysis of variance or rank sum test have been used by SPSS17.0 statistical software.

2 Results

2.1 Neurological function score

The neurological function scores of oxygen group, model group and control group have a significant difference(P<0.05), as showed in the Table 1. A statistically difference(Z=-2.373, P=0.018) was found between inhalation for 48 hours group and 12 hours group; and inhalation for 24 hours group and 12 hours group was statistically difference(Z=-2.815, P=0.005); but there was no difference between inhalation for 48 hours group and 24 hours group(P>0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>Inhalation for 12h</th>
<th>Inhalation for 24h</th>
<th>Inhalation for 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>2.83±0.41*</td>
<td>2.67±0.52*</td>
<td>2.50±0.55*</td>
</tr>
<tr>
<td>Oxygen group</td>
<td>2.17±0.41**</td>
<td>1.50±0.51**</td>
<td>1.33±0.52**</td>
</tr>
</tbody>
</table>

Note: *Results of comparison of neurological function scores with control group, P<0.05; **Results of comparison of neurological function scores with Ischemia-reperfusion group P<0.05

2.2 The results stained by immunohistochemistry

Positive cells of VEGF had a tendency of gradually increasing expression, as showed in Figure 1. There was a statistically significant difference(F=98.82, P<0.001) among each group by single factor analysis of variance, as showed in Table 2. Pairwise comparison showed that the differences were significant(P<0.05) except the difference between inhalation group for 24 hours and inhalation group for 48 hours(P>0.05).
Table 2. Results of comparison of the number of positive cells that expressed VEGF in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Number of positive cells</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>6</td>
<td>7.17±2.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemia-reperfusion group</td>
<td>6</td>
<td>19.83±1.17*</td>
<td>98.82</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Inhalation for 12h group</td>
<td>6</td>
<td>26.67±3.14**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhalation for 24h group</td>
<td>6</td>
<td>35.83±2.79***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhalation for 48h group</td>
<td>6</td>
<td>37.33±4.84***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: *Results of comparison of the number of positive cells with control group, P<0.05; **Results of comparison of the number of positive cells with Ischemia-reperfusion group, P<0.05; ***Results of comparison of the number of positive cells with inhalation for 12h group, P<0.05.

3 Discussion
Cerebrovascular disease has the characteristics of high incidence, mortality and disability rate, and it became the first cause of death in China, ischemic cerebrovascular disease accounted for 80% to 90% of all acute cerebrovascular disease[8]. At present, the treatment of ischemic cerebrovascular disease are mainly two ways: one is thrombolytic therapy; and the other is neuroprotective treatment, which can avoid the death of neurons by preventing ischemia-induced pathology and biochemical reactions[9]. As a part of the neuroprotective treatments, oxygen therapy is mainly to improve tissue oxygen supply, which has long been considered a reasonable treatment for acute cerebral ischemia[10]. Recent animal studies have demonstrated that short-term normobaric hyperoxia (NBO) treatment has a highly neuroprotective function if started early after stroke onset[11]. As an early treatment of acute cerebral ischemia, BO can slow down the necrosis progress of ischemic tissue, save time for combination therapies with further neuroprotectors and to achieve better outcomes[12].

VEGF plays a major regulating role in angiogenesis and the process of formation, it is a kind of nutritional factors commonly existing in the central nervous system. The lack of oxygen can lead to up-regulation of the expression of VEGF in brain tissue[13]. Pathological changes of cerebral ischemia reperfusion is the change of ischemic and hypoxia brain tissue, so it will cause the up-regulation of expression of VEGF. Stroke-induced angiogenesis could help to restore oxygen and nutrient supply for to the affected brain tissue and therefore improve neurological recovery[14]. Yang et al. also confirmed that VEGF can promote angiogenesis and has a neuroprotective effect, it can inhibit apoptosis and oxidative damage, facilitate learning and memory, stimulate synaptic plasticity, promote glial proliferation, neurogenesis and angiogenesis and improve the survival rate[15]. Research shows that the positive expression of VEGF in focal ischemia reperfusion begins in the early stage, and the expression increased with the prolongation of reperfusion time, Which reached the peak at 48 hours after ischemia reperfusion and then declined gradually[16].

All the acute ischemic stroke in oxygen group were given different time inhalation. 24 hours later, all the rats in this study finished the oxygen inhalation, put them to death, and measured the expression of VEGF by immunohistochemistry to avoid the early influence on the expression of VEGF by ischemia and
reperfusion. The study found that VEGF positive particles expressed in the glial cells after cerebral ischemia and reperfusion and VEGF-positive cells increased significantly after oxygen inhalation, so it furtherly proved that oxygen inhalation was effective. Besides, oxygen inhalation could improve the neurological function significantly compared to the model group (P<0.05). It may because that the expression of VEGF began to decline 48 hours after ischemia and reperfusion, but inhalation oxygen can increase the expression of VEGF, and promote the formation of new blood vessels to play a neuroprotective effect. The results of this study also showed that expression of VEGF and neurological function had a significant improvement in oxygen inhalation for 24 hours and 48 hours compared to oxygen inhalation for 12 hours, but the improvement of expression and the neurological function between oxygen inhalation for 24 hours and 48 hours was not statistically significant, so oxygen inhalation for 24 hours may be better, which may be related to the expression increasing of VEGF caused by oxygen inhalation reach to a maximum and no longer increased. But mechanism of up-regulation of VEGF expression caused by oxygen inhalation is not clear, which needed to be further studied.

In summary, 33% \( O_2 \) inhalation can raise the expression of VEGF in brain tissue of acute ischemic stroke infarction model and thus play a neuroprotective role. It will have a better effect with continuous inhalation for 24 hours. While the conclusions of this study is limited to 33% \( O_2 \) inhalation, and only for ischemia-reperfusion model in rats, the effect of inhalation time of other concentrations of oxygen and oxygen inhalation in patients with cerebral infarction remains to be further studied.

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