The Role of Oxygen Radicals in Reducing Cerebral Edema Caused by Normobaric Hyperoxia Pretreatment in Rat Model of Stroke

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Abstract

Background: Recent studies have shown that normobaric hyperoxia compared with normobaric normoxia can reduce the damages resulting from the stroke. The purpose of this study is to investigate the effects of oxygen radicals in reduction of cerebral edema caused by normobaric hyperoxia in rat stroke models.

Materials and Methods: Wistar rats were divided into two main experimental groups and were exposed to 90% oxygen (HO) for 4 hours/day during 6 days; the main control group was placed inside a special chamber and exposed to room 21% oxygen at 1 atmosphere pressure (RA). Then, each group was divided into three subgroups half an hour before placing and treatment in the oxygen chamber, the first, second and third subgroups of both groups were received no substance (RA and HO), saline (RA-S and HO-S), and dimethylthiourea (RA-MT and HO-MT), respectively in order to evaluate the role of oxygen radicals. Then after 24 hours, they were exposed to ischemia through surgically occlusion of middle cerebral artery in order to create brain edema. After 60 minutes of ischemia, the perfusion was reestablished for 24 hours. Then the neurological deficit scores and cerebral edema were analyzed.

Results: Based on Mann–Whitney U test, the median of recovery effect of neurological deficit was significant (p<0.05). The extent of cerebral edema, based on one-way ANOVA test, was also significant (p<0.05). This effect disappeared largely by consumption of dimethylthiourea.

Conclusion: The reduction of cerebral edema resulting from normobaric hyperoxia treatment is largely mediated through oxygen radicals.

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Introduction

Damage from low doses of harmful substances, but under the threshold level to damage cells, induces an adaptive response which protects the brain against other stresses resulted from these damaging stimuli (tolerance) or other damaging stimuli (cross tolerance) [1]. Ischemic tolerance (IT) is one of the most important endogenous mechanisms responsible for tolerance increment of brain tissue against brain damage after stroke cerebral ischemia causes excessive release of excitatory amino acids, activation of their receptors and entrance of calcium into the cells, and thus electrophysiological and metabolic disorders.

Recent studies suggest that ischemic tolerance in the brain can increase the survival of neurons through increasing the synthesis of specific proteins. Among them heat shock protein 70 [2], Bcl2 [3], glutamate carriers [4], superoxide dismutase (SOD) [5], antiapoptotic factors [6], reactive oxygen species [7], NF-kB, and pre-inflammatory cytokines [8] can be cited. Inhibitors of oxidative phosphorylation induce brain tolerance against ischemic process (complete or focal). There are several reports that hyperoxia also leads to ischemic tolerance [9].

One clinical feature of damage of the central nervous system (CNS) after brain ischemia is the development of cerebral edema caused by breaking of the blood-brain barrier (BBB) which can be improved by preconditioning with normobaric hyperoxia [10]. Superoxide dismutase (SOD) enzyme reduces vasogenic brain edema resulted from various damages, which suggests that oxygen radicals play an important role in breaking of the blood-brain barrier. Another clinical feature of damaged CNS is direct damage of nerve cells that induces the release of glutamate due to stimulatory events after cerebral ischemia. This damage also reduces by preconditioning with normobaric hyperoxia through increased expression level of glutamate carriers [11]. Glutamate increases the free calcium concentration which in turn increases calcium-dependent enzymes and leads to free radical production [12]. Recent studies have indicated that damage from stimulation (excitotoxicity) causes neuronal cell death in some neurons. Anti-oxidant enzymes inhibit cell death. Thus, neuronal cell death may play an important role in nerve damage from focal cerebral ischemia model [4]. Therefore, many efforts have been performed to strengthen the anti-oxidant system to prevent brain damage. The anti-oxidant enzymes activity can be increased through different methods of cellular stress such as ischemia and mild blood reperfusion, heat
stress, inflammatory mediators, and hyperbaric oxygenation [9].

Oxygen free radicals produced from hyperbaric oxygen were known as inducers of protection in hippocampus of gerbils [13]. Wad et al. proved that frequent use of hyperbaric oxygen induces resistance against later ischemic injury in CA1 neurons of gerbil’s hippocampus, and the resistance against neuronal ischemic damage with frequent pre-treatment by hyperbaric hyperoxia is probably via induction of HSP72 synthesis. Hyperbaric oxygen protects brain against ischemic damage.

In our laboratory, we have shown in our earlier researches that intermittent and continuous exposure to normobaric hyperoxia induces the ischemic tolerance phenomenon and increases the expression of glutamate carriers, and serum levels of TNF-α and TNF-α converting enzyme (TACE) in the rat brain [8,12]. We have also shown that intermittent normobaric hyperoxia reduces cerebral edema and increases the blood-brain barrier strength [1].

The purpose of this study was to evaluate the role of oxygen radicals in reducing cerebral edema resulted from normobaric hyperoxia pre-treatment in rat stroke model.

Materials and Methods

In this clinical trial study, 61 Wistar rats weighing 250 to 350 g were maintained in a twelve-hour period of darkness-light in 22°C temperature, throughout the study. This research was performed in spring and summer of 2010 in the Heart Physiology Research laboratory of Shahid Beheshti University.

Rats were divided into two main experimental groups (each with 21 rats) and exposed to 90% oxygen (HO) for 4 hours/day during 6 days; the main control group was placed inside a special chamber and exposed to room 21% oxygen at 1 atmosphere pressure (RA).

Then, each group was divided into three subgroups Prior to the treatment and half an hour before placing in the oxygen chamber, the first, second and third subgroups (each with 7 rats) of both groups were respectively received no substance (RA and HO), 5 ml/kg saline (RA-S and HO-S), and 500 mg/kg dimethylthiourea 10% (RA-MT and HO-MT) intraperitoneally in order to evaluate the role of oxygen radicals.

Then after 24 hours, they were exposed to ischemia through surgically occlusion of middle cerebral artery in order to create brain edema. After 60 minutes of ischemia, the perfusion was reestablished for 24 hours. Then, the neurological deficit scores and cerebral edema were analyzed.

In addition, two groups were considered as sham and measuring cerebral blood flow groups (6 rats per group) and exposed to normobaric hyperoxia conditions to measure the water content and cerebral blood flow of the brain.

The hyperoxia chamber: Seven rats were put in a 35×45×65 cm box which all its seams were completely blocked, and had only two channels for air input and output. Soda lime (a carbon dioxide absorber) was put in the box to absorb the produced carbon dioxide and prevent the retention of carbon dioxide in the oxygen chamber. Thus, the possibility of changing the gas concentration inside the box was minimal. To treat animals, pure oxygen (90%) or room air was perfused at 5 liters per minute to the air chamber. The oxygen concentration and temperature were measured by an oxygen-meter which had an oxygen sensor electrode.

Middle cerebral arterial occlusion (MCAO) model: After weighing, the rats were anesthetized with 400 mg per kg body weight chlorate hydrate (Merck, Germany). Surgical modeling of MCAO was done in accordance with the Longa et al. instruction [14]. In summary, under microscopic surgery, a nylon suture was inserted into the right arterial vessel through the ECA (External Carotid Artery) trunk and with closed pterygopalatine, continued through the ICA (Internal Carotid Artery) until reaching up to the right ACA (Anterior Cerebral Artery) Contact of suture with the ACA led to blood flow occlusion from every side to the MCA (Middle Cerebral Artery). This occlusion was identified by the feeling of resistance in the suture advance, after insertion of about 20 mm suture length in ECA trunk. After 60 minutes of ischemia-reperfusion was carried. The temperature of the body was measured through the rectum and maintained at about 37°C.

Behavioral assessment of stroke: Neurological exams were performed after 24 hours of reperfusion. The animals were especially cared from the onset of occlusion until being killed. The neurological findings were classified in 5 scales: the number zero (0) showed no neurological complication; number one (complete failure at the end of front paws) was considered a mild focal neurologic deficit; number two (turning left) moderate focal neurologic deficit; number three (falling to the left) severe focal deficit; the rats scored 4 could not walk spontaneously and had a low consciousness level; and that rats which died within 24 hours after surgery scored 5 if the brain staining revealed its extensive damage and the stroke being the unique cause of death [15, 16].

Measurement of cerebral edema caused by stroke: After beheading the animal, the brain was removed and cerebellum, pons, and olfactory bulb were dissected net weights of cerebral hemispheres (Wet Weight, WW) were measured with a digital scale (FEW, Japan). Then, they were dried in a 127°C oven (Shimazco, Iran) and weighted again (Dry Weight, DW) after 24 hours. Finally, cerebral water content was calculated by [WW-WD/WW] * 100 formula [1].

Laser-Doppler flowmetry: Laser flowmeter (MBF3D, Moor instrument, Axminster, UK) was used to record the regional blood flow (rCBF). The laser flowmeter probe was placed on the brain surface. Using stereotaxic apparatus and low-speed dental driller, a 2 mm diameter hole was perforated above the skull; 1 mm distal and 5 mm right lateral of the bregma point. The probe needle was inserted into the hole and sent to the above of dura matter where blood vessels were seen. The amount of blood flow was measured in steady state and then remeasured after sending the suture into the vessels and...
its value was expressed as the percentage of baseline value. The flowmetry was started 30 minutes before the 60 minutes ischemia and continued 30 minutes after the termination of the ischemia [17].

Statistical analysis: The amounts of brain edema and blood flow were analyzed with one-way ANOVA and neurological deficit scores were analyzed with Mann-Whitney U non-parametric test, through SPSS-11 software and LSD method. The data were shown as Mean±SD, p<0.05 is considered statistically significant.

### Results

Test conditions parameters: Table 1 shows the oxygen content inside the chamber in hypoxic and normobaric normoxic conditions. According to measurements of arterial blood gases (ABG), arterial oxygen pressure in hypoxic condition is much higher than arterial oxygen pressure is normoxic conditions (p=0.001). By sending the suture into the cerebral middle artery, the regional blood flow rate (rCBF) was reduced to 21% (p=0.001) (Fig. 1).

The effects of intermittent normobaric hyperoxia on neurological deficit scores: the median of neurological deficit scores (NDS) was significantly reduced due to exposure to normobaric hyperoxia, however the application of oxygen radical cleaners before hyperoxia, greatly reduced the neuroprotection effects (p=0.010). The median neurological deficit scores in normobaric hyperoxic groups with and without saline and with dimethylthiourea, and in normobaric normoxic groups with and without saline and with dimethylthiourea are shown in table 2 (p<0.03).

Figure 2 shows that normobaric hyperoxia compared with normobaric normoxia (RA) reduced the cerebral edema statistical differences between groups with and without saline and with dimethylthiourea were significant (p=0.001). Statistical difference of mentioned groups compared to normobaric normoxic intact group were also significant (p=0.001). The reduction of cerebral edema, which proves the ischemic tolerance phenomenon of hyperoxia, was consistent with the neurological deficits findings.

### Discussion

Based on the results of this study, it seems that normobaric hyperoxia can effectively decrease the neurological deficit scores and cerebral edema resulting from stroke in the MCAO (middle cerebral arterial occlusion) model. This result is obtained while the application of dimethylthiourea as a cleaner eliminates largely the reduction of brain edema induced by normobaric hyperoxia MCAO model which is developed through the suture insertion is a reliable and repeatable animal stroke model [14].

Body temperature, blood gases, heart rate, and respiratory rate all were within normal limits throughout the experiment. However, in hyperoxic group the oxygen content was increased and respiratory rate was reduced due to high oxygen concentration.

By comparison of the results of this study with other researches in the field of ischemic tolerance, one can say that these results are consistent with the results mentioned in the introduction [1-3]. Based on time and oxygen concentration, the tolerance to ischemia were different in the tested brain tissues. Therefore, the quality and quantity of oxygen administration is important in the induction of ischemia tolerance, and in its side effects and toxicity on the body [9]. Wada et al. showed that oxygen free radicals and Bcl-2 that act as apoptosis inhibitors, may increase after frequent exposure to hyperoxia and lead to increase of neuronal viability [18]. The results also

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**Table 1. ABG tests at the end of pretreatment (N=6)**

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>PO2 (mmHg)</th>
<th>PCO2 (mmHg)</th>
<th>pH</th>
<th>Respiratory rate (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>93±3.54</td>
<td>40±1.01</td>
<td>7.4±0.04</td>
<td>1.43±0.06</td>
</tr>
<tr>
<td>HO</td>
<td>*353±10.32</td>
<td>40±1.09</td>
<td>7.38±0.01</td>
<td>1.3±0.09</td>
</tr>
<tr>
<td>RA-S</td>
<td>98±2.9</td>
<td>39±1.3</td>
<td>7.4±0.02</td>
<td>1.5±0.09</td>
</tr>
<tr>
<td>RA-HO</td>
<td>*364±11.21</td>
<td>40±1.06</td>
<td>7.4±0.01</td>
<td>1.29±0.06</td>
</tr>
<tr>
<td>RA-MT</td>
<td>95±3.06</td>
<td>40±0.9</td>
<td>7.4±0.03</td>
<td>1.51±0.07</td>
</tr>
<tr>
<td>RA-HO-MT</td>
<td>*371±12.01</td>
<td>40.4±0.72</td>
<td>7.39±0.01</td>
<td>1.34±0.08</td>
</tr>
</tbody>
</table>

* p<0.05

**Table 2. The distribution of neurologic deficit score in each experimental group**

<table>
<thead>
<tr>
<th>No</th>
<th>Experimental groups</th>
<th>Neurologic deficit score (N)</th>
<th>Total</th>
<th>Median</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RA</td>
<td>0  1  2  3  4  5</td>
<td>2</td>
<td>1-2 sig</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>HO</td>
<td>5  1  1  0  0  0</td>
<td>7</td>
<td>1-4 sig</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>RA-S</td>
<td>0  2  3  2  0  0</td>
<td>7</td>
<td>2-4 notsig</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>RA-HO</td>
<td>4  2  0  0  0  0</td>
<td>7</td>
<td>0-2-6 sig</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>RA-MT</td>
<td>0  2  2  2  0  0</td>
<td>7</td>
<td>2-3-5 notsig</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>RA-HO-MT</td>
<td>0  2  3  2  0  0</td>
<td>7</td>
<td>2-4-6 sig</td>
<td></td>
</tr>
</tbody>
</table>

N: The number of cases in each groups; sig: significant; notsig: not-significant

**Figure 1.** Regional cerebral blood flow (rCBF) before and during middle cerebral artery occlusion (MCAO), and after reperfusion (* p<0.001, N=6)

**Figure 2.** The graph shows the effects of HO, RA, and MT on brain water content in different experimental groups (* p<0.05, N=7)
showed that treatment with hyperbaric oxygen is more effective than normobaric hyperoxia in the treatment of experimental transient ischemia; the data obtained from stroke volume and neurological deficits indicates this point. Pretreatment with normobaric hyperoxia reduces the extent of stroke and protects the apoptotic cell death in the early and late phases [19]. Yuan et al. showed that treatment with normobaric 95% oxygen delayed and reduced the production of nitric oxide after cerebral ischemia.

According to recent studies, transient ischemic attack which is considered as the ischemia penumbra, is an ideal target for treatment with normobaric hyperoxia, as long as be performed after the onset of neurological deficit [20]. Thus, oxidative conditions and oxygen radicals play an important role in preconditioning of the brain. According to the results of this study and other mentioned studies, one can conclude that oxygen radicals play a pivotal role in creating the phenomenon of ischemia tolerance. So that, removing them greatly reduces the tolerance to ischemia.

Although the results of this study showed that hyperoxia had induced neuroprotection in rat brain through reducing brain edema and neurological deficit scores, it has other effects that can enhance ischemia tolerance in rat brain. These effects are: 1) hyperoxia can lead to angiogenesis and increased vascular density in the volume unit; 2) hyperoxia can block intercellular adhesion molecule-1 (ICAM-1) and inhibit neutrophil aggregation. Therefore, hyperoxia can reduce neutrophil aggregation and brain damage [17]. On the other hand, increased oxygen free radicals and superoxide dismutase is associated with decreased expression of hypoxia-inducible factor-1α (HIF-α) which improve blood-brain barrier function through reduction of vascular growth factor. In addition, oxygen free radicals can cause the phenomenon of ischemia tolerance by increasing TNF-α through TNF-α receptor [11].

Preconditioning through continuous oxygen provision induces neuroprotection in rat brain via providing oxygen free radicals (OFR). Normobaric oxygenation and more effectively hyperbaric hyperoxia protect the brain against permanent cortex ischemia. The results of this study showed that normobaric hyperoxia reduces cerebral edema through production of oxygen radicals. Therefore, application of normobaric hyperoxia or designing substances that can mimic the effects of normobaric hyperoxia in increasing oxygen radicals will create new methods and strategies that will help minimize neural damage during cerebral ischemia or progression of chronic neurodegenerative diseases that are involved in toxic effects of stimulation.

Acknowledgements

This study is extracted from Ms Mahdieh Ashegh Abadi thesis, with the code 600/101 and has been financially supported by Shahid Beheshti University.

Authors’ Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

Funding/Support

Shahid beheshti university, tehran.

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