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Time course of neuroprotection induced by *in vivo* normobaric hyperoxia preconditioning and angiogenesis factors

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ABSTRACT

Objective(s): Every year, a large number of people lose their lives due to stroke. Stroke is the second leading cause of death worldwide. Surprisingly, recent studies have shown that preconditioning with hyperoxia (HO) increases tissue tolerance to ischemia, ultimately reducing damages caused by stroke. Addressed in this study are beneficial contributions from HO preconditioning into reduced harm to be incurred by the attack, as well as its effect on the expression levels of vascular endothelial growth factor (VEGF) and endostatin.

Materials and Methods: A set of experiments was conducted where a number of rats were divided into three groups. The animals in the first group received 90% oxygen for 4 hr a day, for 6 days. The second group was housed in room air and the third group was a sham (surgical stress). After 60 min of ischemia, 24 hr blood flow, neurological deficit score (NDS) and infarct volume (IV) in the group MCAO (Middle Cerebral Artery Occlusion) were investigated. Immediately following a 48 hr HO pre-treatment, sampling was performed to measure the expression levels of VEGF and endostatin.

Results: Preconditioning with alternating HO led to reduced infarct volume and NDS. Moreover, pre-treatment with HO resulted in increased VEGF expression while decreasing endostatin.

Conclusion: Although further studies are deemed necessary to clarify the mechanisms of ischemic tolerance, apparently, somewhat intermittent hyperoxia can be associated with positive impacts by increasing VEGF and decreasing expression of endostatin.

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Introduction

Stroke is one the main causes of death as it brings about brain blood support disorders. On average, one American dies from stroke every 4 min (1). Being so sensitive to lack of oxygen, the brain is significantly and effectively injured in the event of ischemia (2). To relieve the effects of lack of oxygen in the tissues, the creature responds by increasing the oxygenation via angiogenesis induction. In fact, this was shown in the survived people from acute ischemic infarction (3). Significant increases in the oxygen level of penumbra have been seen in treating with high-pressure oxygen or normobaric hyperoxia (NBO) in apoplexy (4). Also, NBO preconditioning has been shown to decrease the infarct volume, neurological deficit score (NDS), brain edema and brain blood barrier permeability (5, 6). On the other hand, NBO could decrease lipid peroxidation, inhibit leukocyte activation and restore blood-brain barrier (7, 8), hence providing a protection against further injuries when blood support in penumbra area is restored (reperfusion injury) (9). HO protective effects induce some adaptive pathways taking cellular and biochemical changes like metabolic hemostasis and gene scheduling. Metabolic changes have transient effects but the change in gene expression is constant and may result in permanent changes (10). In previous studies, this gene expression has been shown to change the glutamate transporter expression, TNF-a, NF-kB and NCX family by preconditioning the rats' brains (10-12). Angiogenesis therapy and new and recovering medical approaches, which may help to treat patients with an ischemic brain have shown that angiogenesis following global ischemia may provide great opportunities to recover from clinical complications after recovering from stroke (13, 14). Angiogenesis plays an important role in neuron healing after the infarction (15, 16), where the angiogenesis stimulant, VEGF represents one of the most important factors (17). The findings indicated that VEGF could protect from neurons in hypoxic injury by inhibiting the activation of caspase3, i.e. it could act as an endogenous neuron-protecting agent in the event of stroke (18). Endostatin, as an anti-angiogenesis parameter, may increase the caspase3 activity followed by apoptosis increment (19, 20). On the other hand, endostatin expressed in the rat brain

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prevented VEGF attachment to endothelial cells and also prevented and inhibited the VEGF attachment to KDR/Flk-1 receptor (21). Though HO could increase ROS production, hydroxyl radicals, and peroxide hydrogen, ROS could help prove the existence of HIF (22-24). This has made HIF one of the best VEGF regulators inducing angiogenesis (23).

In the following paper, the first section comes with a study on time course of neuroprotection induced by NBO in focal cerebral ischemia (estimated IV and neurologic deficit score – NDS). The sec section seeks to identify whether such effects might be associated with changes in the expression of endostatin and VEGF levels in the rat stroke model.

Materials and Methods

Animal and group assignment

All stages with laboratory animals were approved in advance by Ethics Committee of Shahid Beheshti University, Tehran, Iran. Male Dawley-Sprague rats were used in this study weighing approximately 250–350 g. The rats were divided into three groups as follows. The rats in the first group (control) breathed in an atmosphere of 25% oxygen; the sec group comprised 4 subgroups pre-treated in an atmosphere of 90% oxygen. Entitled as 2 HO, 5 HO, 10 HO, and 15 HO, the subgroups were pre-treated with 90% oxygen for 2, 5, 10, and 15 days following the HO, respectively. Then the subgroups were operated for MCAO (Middle Cerebral Artery Occlusion) for 1 hr. 48 hr later, the infarction volume and NDS were analyzed. Called the sham group, the third group included a number of animals exposed to room air and were operated for MCAO but with no suture for the veins, with their brains dissected for analysis. The brains of sham, HO, and intact groups' rats were dissected immediately following anesthesia induction, using chloral hydrate and transcardial reperfusion; subsequently, penumbra, core, and sub-cortex of each hemisphere were dissected.

Environmental chamber

We tried our best to minimize the number of test rats and lower the pain level they were to experience to the lowest possible point. Kept in standard conditions (24 ± 2 ˚C, 12 hr lighting program), the animals were fed adequate amounts of pellets. The tests were conducted following one week of adaptation period with the new environment for the animals who were selected randomly. The hyperoxia box (dimensions: 650 × 350 × 450 mm) had one inlet and one outlet, with all of its interstices covered. Pseudo-lime tablets (as carbon dioxide absorbents) were placed in the box with the rats, so as to minimize gas concentration changes within the box. Pure oxygen (90–95%) was introduced into the box, at 5 lit/min, to increase the oxygen concentration from 21% to 90%, with the inlet immediately closed once the oxygen concentration exceeded 90%. Measured using an oxygen meter with a sensitive electrode to oxygen, the oxygen concentration was managed to range within 90–95%.

Focal cerebral ischemia

The animals were operated for MCAO after inducing anesthesia using chloral hydrate (Merck, Germany) (400 mg/kg BW). Surgery modeling of MCAO was conducted based on the method proposed by Longa *et al* (25). The principal procedure consisted of introducing a 4-0 nylon intraluminal suture into the cervical internal carotid artery (ICA) and advancing it intracranially to block blood flow into the MCA; collateral blood flow was then reduced by interrupting all branches of the external carotid artery (ECA) and all extracranial branches of the ICA. The rat undergoing the test was kept in ischemic condition for 60 min before restarting the blood flow. This method is called tMCAO since it involves the restart of blood flow after 1 hr, i.e. the vessel closure is not permanent. In the course of the experiments, the rat body temperature was controlled by a rectal thermometer, so as to have it kept at 37˚C. Then, all animals were sutured and kept in a sterile cage under appropriate conditions.

Neurobehavioral evaluation

After 24 hr of critical care, the neurological assessment was done. Neurologic findings were ranked into 5 levels following Longa *et al*:

0: animals with no neurologic disorder;

1: animals with minor disorders in forelimb toes;

2: animals with left distortion as an intermediate disorder;

3: animals with left recumbency as an acute disorder;

4: animals with low consciousness and problems in walking;

5: animals that died after the surgery with a high injury to the brain (as confirmed after the staining). In this case, infarction was the main cause of death.

Infarct volume assessment

As explained, in the first phase of the research, anesthesia was induced using chloral hydrate (at 800 mg/kg). The brains were immediately dissected and kept in saline at 4 °C for 5 min. Afterward, each brain was mounted in brain matrix where a 2 mm section was taken from the frontal lobe cranially. The sections were kept in 2% TTC solution (Merck, Germany) for 15 min before being incubated, for essential staining, in an oven. A white color indicated infected regions by ischemia while the red/pink areas were healthy. Eventually, the ischemic area

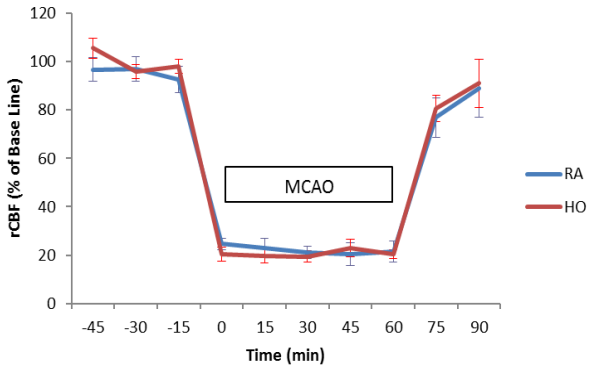

Figure 1. Regional cerebral blood flow (rCBF) before and during middle cerebral artery occlusion (MCAO) and after reperfusion in Room Air(RA) and Hyperoxia (HO) groups (**P*<0.001)

within each section was measured using image tools, with the results multiplied by 2 mm thickness. The eventual results were summed and analyzed using the Swanson method (26).

CIV (Corrected Infarct Volume)= left hemisphere mass – (right hemisphere mass- infarct area mass)

Brain sampling and protein extraction

The brains of all animals were immediately dissected after inducing deep anesthesia (using chloral hydrate) and transcardial reperfusion; afterward, the core, penumbra, and sub-cortex areas were dissected using the proposed method by Lei. The brain tissues were homogenized in a lysis buffer based on the proposed method by Jalalvand (Jalalvand, 2008), so as to extract the cellular fluid. The lysis buffer included 0.5% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 0.03% ethylenediaminetetraacetic acid (EDTA), 1 tablet protease inhibitor cocktail (Roche), 50 mM Tris-HCI with pH 7.0 (homogenization buffer).

Western blot analysis

After the electrophoresis, proteins were transferred to PVDF membrane and the membrane was then shaken at room temperature for 75 min. Once finished with extracting the blocking solution from the membrane, it was immersed in a primary antibody solution which was composed of VEGF (1/500), endostatin (1/1000) and diluted β-actin (1/1000), for 4–24 hr. Upon taking the paper out of the primary antibody solution, it was rinsed three times, each time for 15 min, with TBST. Next, the paper was immersed into secondary antibody solution - which was composed of 1:1000 anti-rabbit for β-actin and 1:1000 anti-mouse for VEGF as well as endostatin - at room temperature for 75 min on a shaker. Eventually, the paper was rinsed with TBST another three times (each time for 15 min).

The appearance of target protein bands was detected with the corresponding antibody using chemiluminescence kits. ECL was used in this test

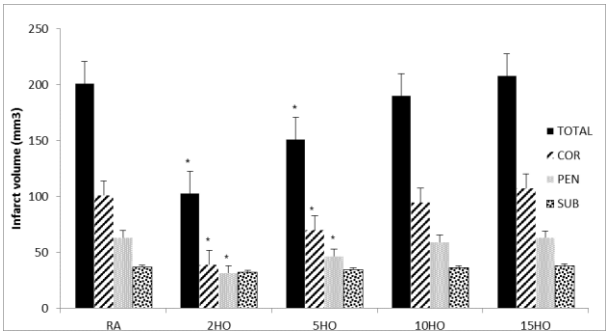

Figure 2A. The graph shows the effects of hyperoxia (HO) on infarct volume in different experimental groups in core, penumbra, sub-cortex, and total areas of the brain. Total infarct volume decreased in 2HO, 5HO (*P*<0.048 and *P*<0.006; respectively) (*P*-value in core and penumbra in 2HO and 5HO respectively: 0.012, 0.019, 0.001, 0.000) (n= 9)

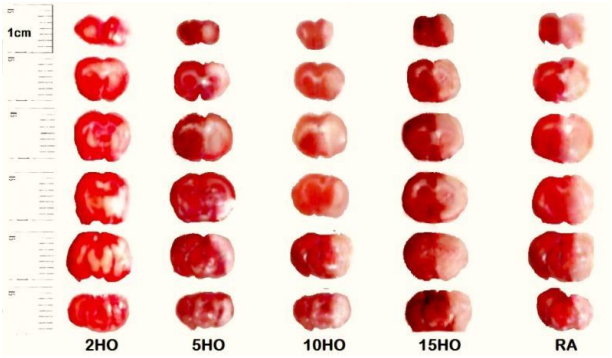

Figure 2B. Images of 2, 3, 5-triphenyltetrazolium (TTC)-stained sections of rat brains after 48 hr of 60-minute middle cerebral artery occlusion (MCAO) model in the hyperoxia-pretreated rats

and the bands appeared on a film. The band densities were measured using Image J software. Thereafter, the films were scanned and uploaded to a computer where the bands were analyzed. VEGF and endostatin expression were normalized to β-actin as a loading control as fold change over control values.

Statistical analysis

Statistical analysis was conducted using SPSS V 19.0 where *P*<0.05 was set as the significance level. All levels were reported in mean±SEM. The infarct volume was measured using image tools. The IV data was analyzed with one-way ANOVA and a method to compare average values, called LSD. The NDS data was analyzed and interpreted with the Man-Whitney method.

Results

Experimental conditions parameters

Arterial blood gas analysis confirmed clinical HO and RA in the pre-treated groups (Table 1). Cerebral blood flow was reduced to less than 25% of the baseline in each group (Figure 1).

Table 1. Arterial blood gas (ABG) tests at the end of pretreatment. Hypeoxia groups(HO) is significantly (*P<0.001) different than room air groups(RA)

Experimental groups	pH	pCO_2 (mmHg)	pO_2 (mmHg)	Respiratory rate (Hz)
RA	7.39±0.02	38.7±0.80	90.8±4.7	1.37±0.05
HO	7.37±0.05	39.8±0.76	371.7±14.59*	1.21±0.03

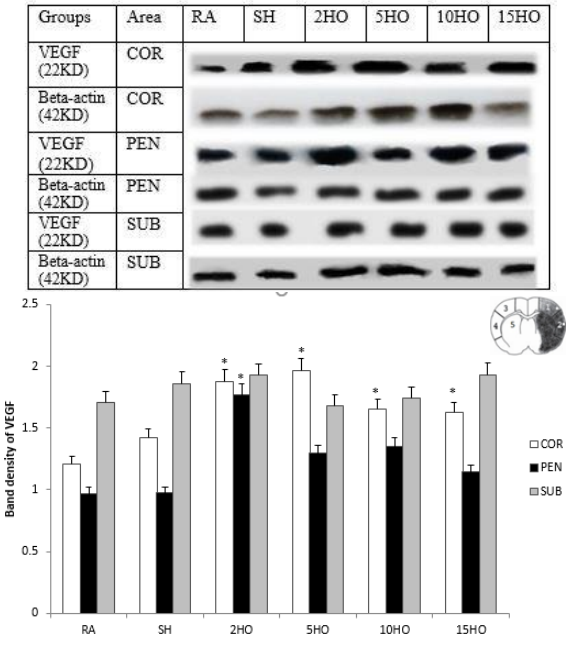

Figure 3. Western blot of VEGF protein in right core, sub-cortex, and penumbra region (regions 3, 4 and 5 in brain section respectively) from rats of hyperoxia (2HO, 5HO, 10HO, and15HO) and room air (RA) and sham subgroups (SH) (A). Western blot analysis of VEGF protein, analysis of bands after normalization with β-actin as a loading control (B). (*P*<0.012). Data were expressed as mean±SEM, *P*<0.05 was considered significant

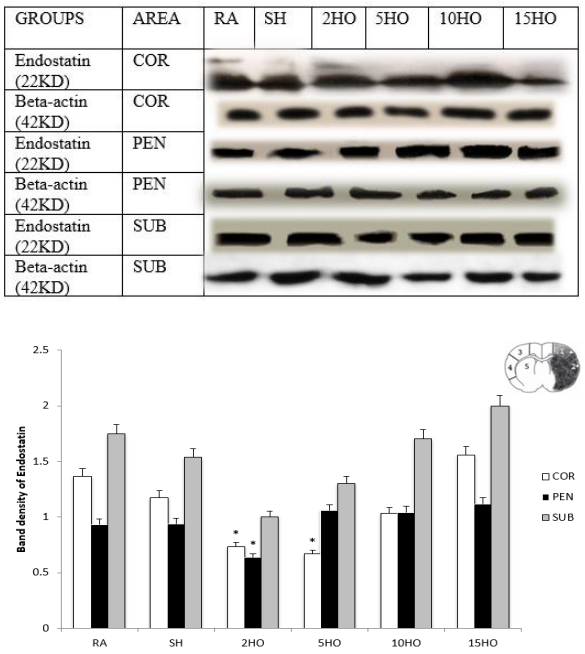

Figure 4. Western blot of endostatin protein in right core, penumbra and sub-cortex region (regions 3, 4 and 5 in brain section respectively) from rats of hyperoxia (2HO, 5HO, 10HO, and15HO) and room air (RA) and sham subgroups (SH) (A). Western blot analysis of VEGF protein, analysis of bands after normalization with β-actin as a loading control (B). (*P*<0.033). Data were expressed as mean±SEM, *P*<0.05 was considered significant

Effects of HO-induced neuroprotection on NDSs and infarct volume

The effects of 2, 5, 10, and 15HO on NDS and IV have also been examined in an animal brain stroke model. Median NDS values decreased significantly with 2HO and 5HO, when compared to RA, being 1 (range: 0–2), 1 (range: 0- 3) and 2 (range: 0-5) in the 2HO, 5HO, and RA groups, respectively (Table 2). The effect of HO on NDS was approved by a significant reduction in infarct volume in 2HO and 5HO (*P*<0.048 and *P*<0.006, respectively; Figure 2). However even though IV exhibited a reduction in 10HO and 15HO, it was not significant.

Normobaric hyperoxia preconditioning effect on VEGF level

VEGF level difference between RA and sham-operated rats was not significant. HO caused VEGF to increase by imposing such an influence on the expression that, in the core area, the level was achieved to 2HO, 5HO, 10HO, and 15HO (*P*<0.002, *P*<0.001, *P*<0.037, and *P*<0.035, respectively; Figure 3). In penumbra, the data showed that VEGF level increased significantly in 2HO group (*P*<0.012; Figure 3) when compared to sham and RA groups. This increment was also observed in 5HO, 10HO, and 15HO, although it was no more significant (Figure 3). HO did not affect VEGF expression significantly in sub-cortex area (Figure 3). The increasing trend was not uniform but followed a random fashion (Figure 3).

Normobaric hyperoxia preconditioning effect on endostatin level

Western blot assays indicated endostatin to be expressed in rat brain. Differences in endostatin level were not significant between RA and sham-operated rats in the penumbra, sub-cortex and core areas (Figure 4). So, experimental conditions failed to affect VEGF and endostatin levels. In core, endostatin was reduced significantly in 2HO and 5HO groups (*P*<0.003 and *P*<0.001, respectively; Figure 4) when compared to the intact (RA) and sham-operated groups (SH). In penumbra, the level of endostatin was observed to decrease significantly in the 2HO subgroup (*P*<0.033; Figure 4) when compared to the RA and sham groups, while such a reduction was not

Table 2. Neurological deficit score (NDS) in each experimental group of normobaric normoxia and hyperoxia.

No.	Groups	Neurologic deficit scores (number)						Premature death (n)	Median	Total	Statistical results
		0	1	2	3	4	5				
1	S-RA	0	1	4	2	1	1	2	2	11	2:5(0.000)
2	S-2HO	4	3	2	0	0	0	2	1	11	1:2(0.001)
3	S-5HO	2	3	2	2	0	0	1	1	10	1:3(0.020)
4	S-10HO	1	2	3	2	1	0	1	2	10	1:4(0.055)
5	S-15HO	0	1	4	2	1	1	1	2	10	1:5(0.086)
6	total	7	10	15	8	3	2	7	8	52	3:5(0.000)

significant in either of 5HO, 10HO, and15HO. In sub-cortex areas, the data indicated an insignificant decrease in expression of endostatin in HO (P<0.067; Figure 4).

Discussion

One of the most important requirements for preconditioning is that the preconditioning should be hard enough to induce a response but not too hard to induce a permanent infarct (27). In ischemic preconditioning (IPC) a sub-lethal infarct would cause a high protection effect against the lethal ischemic infarct (28). Therefore, and based on the above-mentioned parameters, a sub-lethal period (4 hr a day, 6 days) was chosen for IPC to expose the rat to 90% oxygen. In this study, in order to evaluate resistance level of IPC against hyperoxia, infarct volume, endostatin and VEGF expressions were evaluated for 2, 5, 10, and 15-day periods after the last pre-treatment with HO. Furthermore, harmful effects of HO have been long known (29), as they can cause ventilation with a high portion of inhaled oxygen resulting in injuries to the lens of the eye, lungs, heart, brain, and the digestive system and lung toxicity (30, 31), most of which have been related to ROS. Serious HO toxication is usually seen in long exposures (at least 24 hr). So, a 4-hr period was chosen in this study to prevent such injuries.

The surgical approach used in this study was a mimic model of apoplexy by transient middle cerebral artery occlusion (tMCAO). This mimic model of MCAO, as one of the most common apoplexies, allowed faster and easier analysis while minimizing the number of rats required (32, 33). The results indicated that NBO stress is a precondition for IPC that induces some tolerance for two days after the last pre-treatment. The tolerance would diminish within 15 days after the pre-treatment. This could be due to genes expressed in late phases synthesizing proteins (27). Furthermore, ischemic precondition-ing could improve angiogenesis in the first two weeks following the stroke in penumbra (34). Ischemic tolerance occurs together with a sort of protection against perfusion of the arteries during the infarction and the induction of most of the related genes (35). Besides, it increases vascular density within 24 hr of preconditioning (36). Also in hypoxic preconditioning, this could decrease the infarction volume and nervous system disorders while increasing VEGF, resulting in angiogenesis. Such an increase in expression in penumbra was significant for 48 to 72 hr following the precondition-ing (37). On the other hand, hyperbaric oxygen was consequently inhaled in 100% oxygen and 3-atmosphere pressure resulting in BBB destruction (38). In contrast, hyperbaric preconditioning resulted in a decrease in edema, infarction volume, and most importantly, caspase3, the 9 activities followed by apoptosis reduction. Hence, this caused protecting the brain tissue against the ischemia induced by reflow with the inhibition of mitochondrial apoptosis (39). Like hyperbaric hyperoxia, NBO could also protect BBB and improve the results of infarction (5). Since the air's oxygen content could not change the blood oxygen content in a similar way, it might be due to hemoglobin enrichment. However, recent findings showed that the volume and amount of oxyhemoglobin in core and penumbra areas were increased 15 min after the ischemia while the infarct volume was decreasing (4). Also, in case of NBO, it was indicated that it could cause less lipid peroxidation, inhibiting leukocyte activation and restoring BBB (7, 8). Hence, it might provide more protection for the blood flow within the penumbra area and maintain the oxygen level, resulting in a significant decrease in infarction level (40). According to adverse effects of a significant increase in VEGF on BBB permeability (41), it could be concluded that HO preconditioning might lead to some increase in VEGF, preventing BBB destruction. This is in agreement with the results of the current study emphasizing the role of HO preconditioning in providing a neuroprotective effect by regulating VEGF increment. In a recent study where hyperbaric oxygen, normobaric oxygen, and hyperbaric air were compared, it was indicated that hyperbaric groups of rats were provided with some morphological protection of CA1 pyramid neurons, with better behavioral effects compared to the normobaric group (42). On the other hand, the neuroprotective effect of HO preconditioning in long and intermittent pre-treatments was also studied, indicating the nervous system to act well upon intermittent oxygen

exposure (5). Accordingly, the present study was also conducted intermittently. Furthermore, hyper-oxia hyperbaric preconditioning could decrease primary apoptosis and prevent neuron apoptosis possibly via increasing the BDNF level and inhibiting p38 activation (39, 43). In addition, endostatin could decrease Bcl-2 and Bcl-XL genes significantly while being ineffective on the Bax protein level (44). Besides, BDNF could increase the Bcl-2 level (45). Therefore, one can draw that, endostatin might increase apoptosis by increasing caspase3 activity level and decreasing anti-apoptosis proteins, Bcl-2, and Bcl-XL. On the other hand, the current study showed the effect of preconditioning with decreasing the endostatin level; in turn, endostatin is known to inhibit VEGF attachment to endothelial cells (21) and KDR/Flk-1 receptors (21).

Even though HO exposure could induce oxidative stress, it is proven in several studies that ROS represents a major component of oxidative stress (46). The studies indicated that adequate ROS could act as a messenger in physiologic and pathophysio-logic processes (47). Indirectly, ROS could affect hypoxia-inducible factor (HIF) and consequently VEGF expressions (22, 23). Directly, intermittent exposures to HO may induce the expression and activity of HIF (48, 49). The HIF-1 can up-regulate the expression of VEGF (50). Although HO preconditioning induces a neuroprotective effect by regulating VEGF, there are some problems in application as is evidenced in clinical practices (51). For example, it could cause the ventilation with a high portion of inhaled oxygen, resulting in injuries to the lens of the eye, lungs, heart, brain, and the digestive system and lung toxicity. In addition, preconditioning sessions vary in different studies. Therefore, it seems that further studies are needed to find a standard preconditioning protocol in clinical practice.

Conclusion

To sum up, it was concluded in this study that HO could induce a sort of tolerance against brain infarction for the rats, lasting 15 days after the last pre-treatment, with different results in different areas of the brain. The results of the current study indicate that HO succeeded to induce ischemic tolerance by regulating neuroprotective factors like VEGF and endostatin.

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