

# Effect of Normobaric Hyperoxia Preconditioning on VEGF and Endostatin Levels in the Rat Stroke Model

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**Abstract:** Stroke is the second leading cause of death worldwide. It is also the second rationale of disability, after Dementia. Interestingly, pre-conditioning can have a beneficial effect on stroke that increases ischemic tolerance in tissues. In this paper, we have shown that normobaric hyperoxia (HO) protects the rat brain from ischemia injury by changing in VEGF and endostatin levels. Animals were divided into three groups: the first group was exposed to 95% oxygen for 4 hours for 6 days, the second group was in room air, and the third group was sham. MCAO-operated were subjected to 60 minutes of MCAO. After 2 days from pre-treatment and 24 hours reperfusion, neurologic deficit score (NDS) and infarct volume (IV) were evaluated. We have measured VEGF and Endostatin levels of core, penumbra and sub-cortex regions. Pre-conditioning with HO decreased NDS ( $p < 0.002$ ) and IV ( $p < 0.019$ ). Our data indicated that VEGF levels were increased in core and penumbra areas ( $p < 0.000$ ,  $0.015$ , respectively) and Endostatin levels were reduced in penumbra, sub cortex and core of HO subgroups ( $p < 0.004$ ,  $0.007$ ,  $0.000$ , respectively). Although further studies are needed to clarify ischemic tolerance induced by HO, alterations of VEGF and Endostatin levels may have a role in neuroprotective effects.

**Keywords:** Normobaric Hyperoxia, Stroke, Ischemic Tolerance, Endostatin, Vascular Endothelial Growth Factor

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## 1. Introduction

Ischemic stroke is the second leading cause of death [1, 2]. Preconditioning is an attractive experimental strategy to identify endogenous neuroprotective mechanisms which can be therapeutically induced [3]. Sub-lethal injurious stimulus can activate endogenous protective mechanisms and decrease the impact of more severe stimuli, as well [4]. For more details, preconditioning with Hyperoxia (HO) can decrease neurologic deficit score and infarct volume by increased expression of NCX1 as a neuroprotective effect [5]. Vascular endothelial growth factor (VEGF) is known as a protective factor in ischemia [6]. In contrast, Endostatin can induce apoptosis by enhancing caspase-3 activities [7]. Stroke is caused by the interruption of blood supply to the brain [8].

The brain is extremely sensitive to the lack of oxygen. Nevertheless, the patients with stroke can respond by increasing the oxygen delivery to the affected tissue via induction of angiogenesis [9]. It has been reported that angiogenesis has a positive effect on patients surviving after an acute ischemic stroke [10]. VEGF has been known as one of the most essential factors to control angiogenesis [11]. VEGF therapy has been clinically approved in patients with ischemic injury [12]. It can protect neurons from ischemic injury by inhibiting the caspase-3 activity [6]. Endostatin can directly or indirectly inhibit angiogenesis by interaction with VEGF [13]. Moreover, Endostatin can also reduce migration and proliferation of endothelial cells [14, 15]. Endostatin (20 kDa) is expressed in the brain after ischemia [16]. Interestingly, pre-conditioning has a beneficial effect on the stroke. preconditioning synthesizes the specific type of

protein that can affect the tissue over days or weeks [17]. Ischemic preconditioning has been reported in several organs including the brain. Among different types of stress, hypoxia, ischemia, anoxia, oxidative stress, and inhibitors of oxidative phosphorylation are able to induce the tolerance to subsequent cerebral (focal or global) ischemia. Due to the associated toxicity, most of such stimuli do not have a potential to use in the clinical trials [18], accordingly, no safe pharmacologic stimuli have been studied. It has been demonstrated that HO has a similar protection effect in other situations [19], perhaps as through the generation of oxygen-free radicals and hydroxyl radicals [20]. Studies have reported that angiogenesis is able to reduce the extent of brain infarct volume after transient middle cerebral artery occlusion (MCAO) in vivo, suggesting a protective role played by Endostatin and VEGF levels. Preconditioning with HO increased the expression of EAAT1, EAAT2, EAAT3, TNF- $\alpha$  converting enzyme, and serum TNF- $\alpha$  [21]. In this study, in the first part, we studied HO pre-treatment as an ischemic tolerance -inducing factor and MCAO in rats (IV and neurologic deficit score (NDS)). In the second part, we sought to identify whether such effects might be associated with changes in the expression of Endostatin and VEGF levels in the rat stroke model or not.

## 2. Materials & Methods

### 2.1. Animal and Group Assignment

Fifty-six adult male Sprague-Dawley rats (12-14 months old) weighing 250 – 300 g, eight months of age were used. The rats were housed in the room (24°C) with the light cycle from 07:00 a.m. to 19:00 p.m. and dark cycle from 19:00 p.m. to 07:00 a.m. They had free access to the food and water.

Animals were randomly divided into three groups: the first group was exposed to 95% oxygen (HO), the second group was placed in the room air (RA), and the third group was the sham group (SH) (As shown in Figure 1). The first group was divided into 2 sub-groups with an identical duration and condition including HO group and 21% oxygen group. The first group was considered 2 days after pretreatment with HO (4 hours per day for 6 days). The latter was exposed to 21% oxygen (room air). Then Animals were located in an ordinary room air for a further 48 h, after which MCAO-operated subgroups were subject to the MCAO for 60 min. Neurobehavioral scoring and infarct volume evaluations were done 24 hours after reperfusion. The sham group received the same surgery except MCAO. Similarly, after 24-hours pretreatment, the sham group was transcatheterially perfused and sacrificed for assessment of VEGF and Endostatin levels in the core (cor), sub cortex (sub) and penumbra (pen) regions of the right hemisphere.

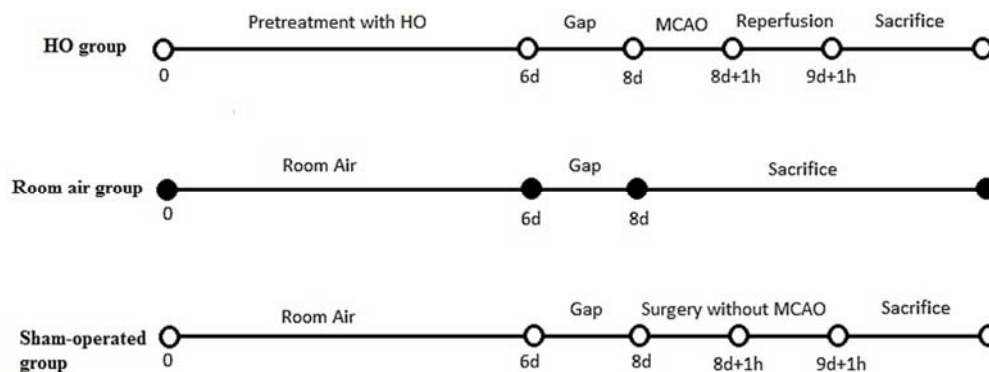


Figure 1. Normobaric hyperoxia (HO) and room air (RA) pre-treatment in various groups are shown by a schematic figure.

### 2.2. Environmental Chamber

The rats were maintained in the animal room. Seven days prior to the study, the animals were adapted to their environment. Environmental chamber comprised an airtight box (650 × 350 × 450 mm) with a gas inlet and outlet port. The internal pressure was continuously monitored by a manometer. Oxygen concentration was well maintained through the inlet and outlet port (approximately 5 L/min oxygen flow) and it was controlled by an oxygen sensor (lutron-Do5510, Taiwan). Carbon dioxide was removed by soda lime (BDH Ltd, Poole, UK) at the bottom of the container. According to the experimental protocol, oxygen concentration was preserved at 95% or 21%.

### 2.3. Focal Cerebral Ischemia

MCAO was done according to longa et al. study [22]. The

rats were weighed and anesthetized using chloral hydrate (Merck, Germany) (400mg/1 kg; ip). In a microscopic surgery, the common carotid and external carotid artery were occluded and a 3 - 0 silicon-coated nylon sutures was interned through a small incision on the carotid branch to internal carotid artery/pterygopalatine bifurcation. The resistance to enter the thread indicated reaching the anterior cerebral artery. Reperfusion was started by withdrawing the suture 60 min after ischemia. Rectal temperature was measured (Citizen-513w, United Arab Emirates), and body temperature sustained at 37°C by surface heating and cooling system through the surgery.

### 2.4. Neurobehavioral Evaluation

The rats were transferred to their cages after withdrawing the suture. Neurobehavioral evaluation was done after 24 h using a 6-point scale according to the Longa et al. 1989 [22].

Neurologic scoring was done as follows: 0: no defect; 1: full defects in the front paws (a minor flaw); 2: rotation towards the left; 3: loss of righting reflex; 4: vigilance is low and the rat cannot walk; 5: the rat died within 24 hours after surgery and the brain was damaged, greatly.

### 2.5. Infarct Volume Assessment

The rats were killed by chloral hydrate (800 mg/kg) and the brains were removed quickly and stored at 4 °C for 10 min in the cold saline. The brain was placed in a brain matrix and cut in the coronal (frontal) plane in 2 mm thick sections. The slices are put in 2, 3, 5-triphenyltetrazolium chloride (TTC) (2%) (Merck, Germany) for 15 minutes at 37 °C. The slices were photographed by a digital camera (Sony, E dual). Software Image Tools was used to analyze the area of the infarct. The corrected infarct volume was calculated as previously described: corrected infarct volume=left hemisphere volume- (right hemisphere volume - infarct volume) [23].

### 2.6. Brain Sampling and Protein Extraction

After pretreatment, room air and sham groups were anesthetized deeply using chloral hydrate. At the same time, they were perfused transcardially with saline. Then, brain slices were removed and divided into three different parts of the brain, including core, penumbra, and sub-cortex. Each part of the brains was separately homogenized by quadrupled of buffer including 0.5% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 0.03% EDTA, NP40 (0.1%, 1 protease inhibitor cocktail tablet (Roche, Germany), 50 mM Tris-HCl (PH 8.0).

### 2.7. Western Blot Analysis

The proteins were transferred to a membrane. The membrane was blocked with a protein-based blocking buffer for an hour at room temperature. The polyvinylidene fluoride (PVDF) membrane is further blocked and incubated with monoclonal primary anti-VEGF antibody (1:500) (ab3109, Abcam, United Kingdom), Endostatin (1:1000) (ab64569, Abcam, United Kingdom), and  $\beta$ -actin (1:1000) (cell signaling technology, USA). Then, PVDF was incubated with

anti-rabbit secondary antibody (1:1000 dilution) (Dakocytomation, Denmark) and anti-mouse (1:1000) (ab6728, Abcam, United Kingdom). VEGF and Endostatin immune-reactive proteins were detected with advanced chemiluminescence and the blots were exposed to the same piece of film. Thereafter, the films were scanned and transferred to a computer and the bands were analyzed. VEGF and Endostatin expressions were normalized to  $\beta$ -actin as a loading control as the fold changed.

### 2.8. Ethical Issues

All animal experiments were conducted with the approval of Shahid Beheshti University Ethics Committee. The experimental protocol was approved by an Institutional Review Committee for the use of Human or Animal Subjects or that procedures in compliance with the Declaration of Helsinki for human subjects, or the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985), the UK Animals Scientific Procedures Act 1986 or the European Communities and the Council Directive of 24 November 1986 (86/609/EEC). We tried to minimize the number of used animals and reduce their suffering, as well.

### 2.9. Statistical Analysis

Data were analyzed using SPSS 19. Infarct volume, rCBF, and arterial blood gases were compared using one-way ANOVA test (least significant difference), and VEGF and Endostatin expression were compared by Tukey post hoc test. NSD was analyzed using the Mann-Whitney U test. Data were expressed as mean  $\pm$  SEM, and  $p < 0.05$  was considered as the significant level.

## 3. Results

### 3.1. Experimental Conditions Parameters

Analysis of the arterial blood gas confirmed clinical 2HO and RA in the pre-treated groups (As shown in Table 1). Cerebral blood flow was reduced to less than 25% of base line in each group (As shown in Figure 2).

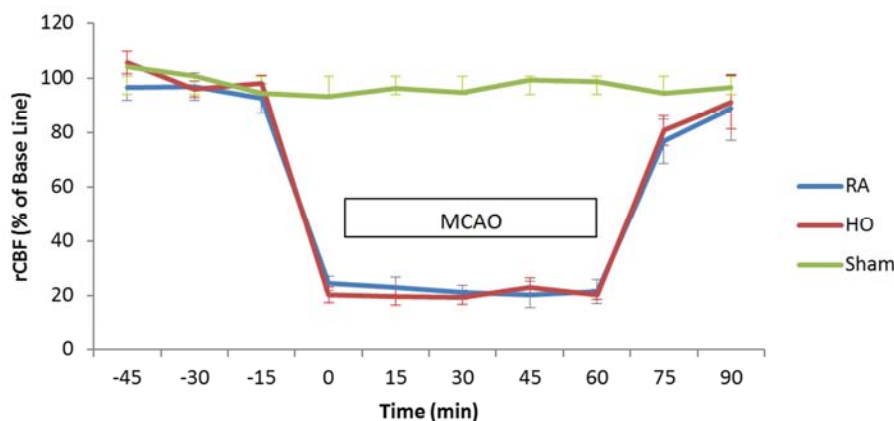


Figure 2. Regional cerebral blood flow (rCBF) before and during MCAO and after reperfusion in RA, Sham and HO groups (\* $p < 0.001$ ).

**Table 1.** Arterial blood gases tests at the end of pre-treatment in HO and RA groups (pre, during and post injury) ( $p < 0.001^*$ ).

Experimental Groups	pH			PCO <sub>2</sub> (mmHg)		
	Before	During	After	Before	During	After
RA	7.382±0.023	7.39±0.01	7.4±0.04	38.2±1.630	39.7±0.52	39.4±1.28
HO	7.382±0.025	7.35±0.04	7.372±0.03	38.2±2.009	39.8±0.93	39.8±1.46

Experimental Groups	PO <sub>2</sub> (mmHg)			Respiratory Rate (Hz)		
	Before	During	After	Before	During	After
RA	96.2±2.925	89.5±5.08	95.2±1.77	1.35±0.022	1.37±0.05	1.438±0.041
HO	96.2±2.517936	365.7±6.49*	98.2±2.95	1.35±0.54	1.19±0.03	1.326±0.055

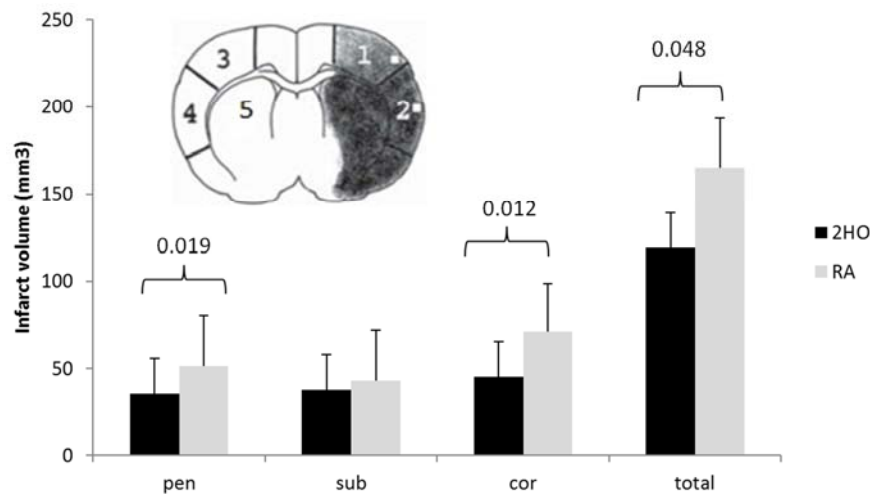
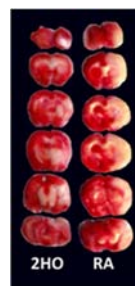
### 3.2. Effects of HO-induced Neuroprotection on NDSs and Infarct Volume

Effects of 2HO on NDS and IV have been examined in an animal brain stroke model. Median NDS were decreased by HO, being 2 (range: 0–2) and 0 (range: 1– 5) in the HO and

RA groups, respectively (As shown in Table 2). The effect of 2HO on NDS was approved by a reduction in infarct volume of the core and penumbra not seen in RA ( $p < 0.012$  and  $0.019$ , respectively; as shown in Figure 3). In the sub-cortex area, IV was reduced. However, it was not significant.

**Table 2.** The distribution of neurologic deficit score in each experimental group. NDS: neurologic deficit score; N: the number of cases in each group; sig: significant; RA: Room Air; HO: Hyperoxia; Median neurologic deficit scores (NDS) were reduced by HO. Therefore, the neurologic deficit scores (NDS) in the MCAO group pre-treated with HO 4 hours for 6 days were reduced in comparison with the vehicle pre-treated MCAO group.

Groups	Neurologic deficit scores (N)						Premature death (N)	median	Total	Statistical results
	0	1	2	3	4	5				
RA	0	1	4	2	0	1	2	2	10	2-1 sig ( $p < 0.002$ )
HO	4	3	1	0	0	0	2	0	10	
total	4	4	5	2	0	1	4	1	20	

**Figure 3.** The graph shows the effects of HO on infarct volume in different experimental group in core, penumbra, subcortex and total infarct volume. Total infarct volume (pen+cor+sub) decreased in 2HO ( $P < 0.048$ ) (and  $P$  value in core and penumbra respectively:  $0.012$ ,  $0.019$ ) ( $n = 9$ ).**Figure 4.** Images of 2, 3, 5-triphenyltetrazolium (TTC)-stained sections of rat brain after 48 hours of 60-minute MCAO model in the HO-pretreated animals.

### 3.3. HO and VEGF Expression

Normobaric hyperoxia is under investigation as a pre-treatment for acute ischemic stroke. In previous studies, normobaric hyperoxia have been shown to be neuroprotective in focal cerebral ischemia (Bigdeli et al 2007). In addition, VEGF protects neurons from ischemic cell death. Here, we provide evidence that normobaric hyperoxia administered before the onset of focal ischemia increases VEGF level. We examined whether exposure to oxygen had an effect on the VEGF and Endostatin levels. Western blot assays indicated that VEGF is expressed in the rat brain (Figure 5). VEGF level was not significant between



RA and sham-operated rats in penumbra, sub cortex and core areas ( $P<0.463$ ,  $P<0.351$ , and  $P<0.226$ , respectively; as shown in Figure 6). So experimental conditions could not affect VEGF and Endostatin levels. VEGF was increased in the 2HO group when compared to the intact (RA) and sham-operated groups (SH) (As shown in Figure 6). Level of VEGF was enhanced in the 2HO group when compared to

the RA and sham groups (As shown in Figure 6). In core and penumbra areas, the data showed Significant increase in the 2HO when compared to sham and RA groups ( $P<0.000$  and  $P<0.015$ , respectively; as shown in Figure 6). In the sub-cortex area, data indicated HO increased expression of VEGF in 2HO but was not significantly ( $P<0.072$ ) (As shown in Figure 6).

VEGF levels	Pen			Sub			Cor		
	RA	SH	2HO	RA	SH	2HO	RA	SH	2HO
VEGF (19-22 kDa)									
Beta-actin (42 KDa)									

Figure 5. Western blots of VEGF protein in penumbra, core and subcortex region and RA and sham group (Sh).

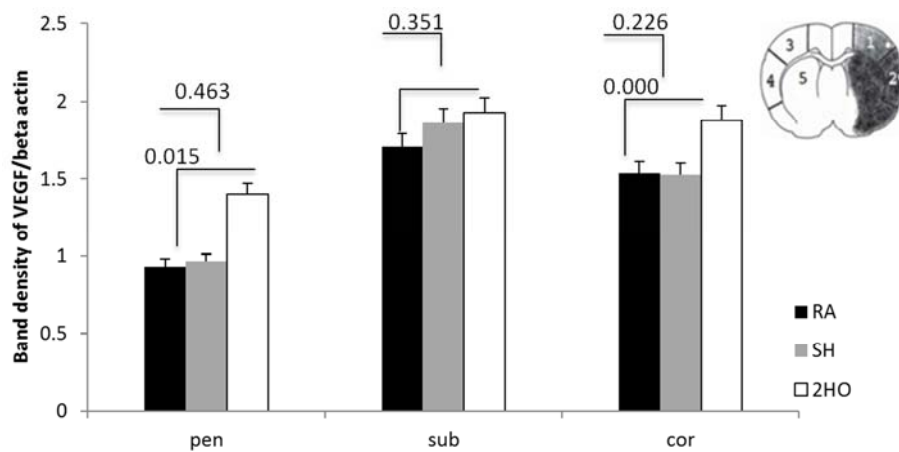


Figure 6. Western blot analysis of VEGF protein, the lower panel shows densitometry analysis of bands after normalization with beta -actin as loading control (\* $P<0.05$ ).

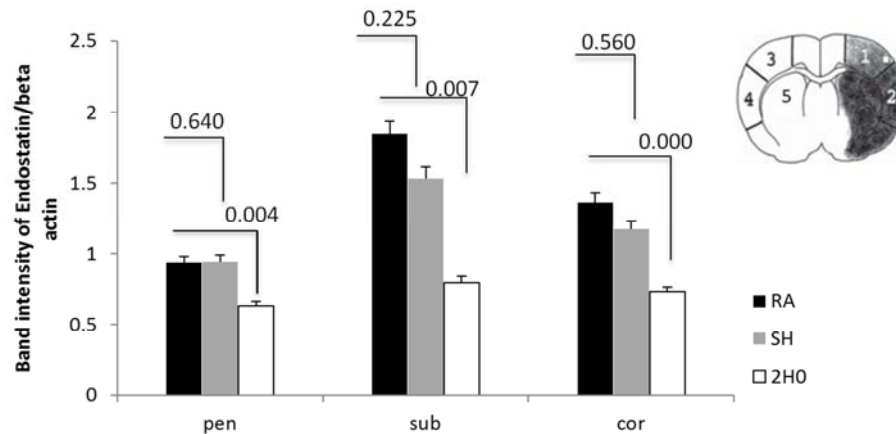
### 3.4. HO and Endostatin Expression

The result of Western blot showed that Endostatin expressed in the rat brain. Levels of Endostatin have been compared with control and sham groups (as shown in Figure 7). Endostatin level was not different between RA and sham-operated rats in penumbra, sub cortex and core areas ( $P<0.640$  and  $P<0.225$ ,  $P<0.560$ , respectively; as shown in

Figure 8). In the core area, Endostatin level was reduced in groups of 2HO when compared with the sham and control groups ( $P<0.000$ ; as shown in Figure 8). In the sub-cortex area, Endostatin level was decreased significantly in 2HO ( $P<0.007$ ; as shown in Figure 8). In the penumbra area, Endostatin level was significantly reduced in 2HO when compared with the sham and control groups ( $P<0.004$ ; as shown in Figure 8).

Endostatin levels	Pen			Sub			Cor		
	RA	SH	2HO	RA	SH	2HO	RA	SH	2HO
Endostatin (22 kDa)									
Beta-actin (42 KDa)									

Figure 7. Western blots of endostatin protein in penumbra, core and sub cortex region and RA and sham group (Sh).



**Figure 8.** Western blot analysis of endostatin protein, the lower panel shows densitometry analysis of bands after normalization with beta -actin as loading control (\* $P < 0.05$ ).

## 4. Discussion

Our data suggest that preconditioning with HO can reduce the infarct volume and NDS and can induce neuroprotective effect by regulating VEGF increment and decreasing the Endostatin level, as well. It has been shown that normobaric hyperoxia improved cerebral blood flow and oxygenation and increased tissue oxygen delivery [24]. Our previous study has shown that HO reduces NDS, infarct volume, blood brain barrier (BBB) permeability, and edema [25]. Moreover, VEGF has a protective effect in stroke [26]. Recent studies also supported that elevating 24-48 h VEGF circulation after acute ischemic stroke, there were no significant difference in using some proteins like interleukin-b or c-reactive protein individually in comparison to combining them with VEGF [27]. In addition, a significant reduction of infarct volume was reported in animals with topical application of VEGF [28]. The application of VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion [29]. Therefore, the neuroprotection effect of VEGF in the ischemic brain is critically dependent on proper dosage of VEGF, route and time of administration, and its combination with other growth factors [30]. Obviously, VEGF can reduce VEGF-induced vascular leakage [31-33]. This fact supported by another study which VEGF an angiogenic factor is up-regulated after ischemic stroke and can induce the development of new collaterals and improve cerebral perfusion. Thus the anti-angiogenic Endostatin may inhibit tissue repair [34, 35]. In contrast, Endostatin can decrease the BBB permeability [36]. Apparently, the intermittent exposures to hyperoxia may produce a temporary 'hypoxic environment' between two hyperoxia exposures which can induce the expression and/or activity of a key oxygen sensor known as hypoxia inducible factor (HIF) [37, 38]. Hyperoxia-induced reactive oxygen species (ROS) formation is able to induce hypoxia-inducible factor-1 (HIF-1) [39-41]. These in turn can regulate a variety of neuroprotection proteins including VEGF [42-44]. The role of Endostatin in the brain is still unknown. It has been

shown that hypoxia can enhance Endostatin levels which in turn increase apoptosis [7]. It is also showed by some research that high serum Endostatin levels in acute phase of ischemic stroke were associated with increase of mortality risk or sever disabilities in patients and reported that Endostatin could be an independent predict of ischemic stroke [35, 45]. Meanwhile, Endostatin can prevent the binding of VEGF to the KDR/Flk-1 receptors and endothelial cells. Endostatin can directly bind to KDR/Flk-1, but not to VEGF [46]. VEGF is stimulated endothelial cell proliferation, and Endostatin antagonizes directly the biological effects of VEGF, although their changes cannot directly affect each other. The level of VEGF was not increased in the subcortical area. There were significant changes in VEGF levels in the core and penumbra areas. It seems that HO exerts its effects partly via increasing the VEGF levels and induction of angiogenesis. HO likely reduces infarct volume by adjusting VEGF levels. The putative beneficial effects of HO were confirmed by a significant reduction in infarct volume in the 2HO group, possibly due to the decrease of BBB permeability. In the core and penumbra areas, VEGF was significantly increased leading to the neuroprotective effect which can be due to the used method. It seems that hypoxia can partly increase apoptosis via increasing Endostatin level [47] and induction of neurodegeneration [7]. Meanwhile, Endostatin prevents the binding of VEGF to the KDR/Flk-1 receptor and endothelial cells. The obtained results indicated that Endostatin levels in all hyperoxia subgroups are associated with ischemic tolerance. It is not identified how VEGF and Endostatin can act to prevent or limit neuronal injury after preconditioning. It can be due to the VEGF signals neuroprotection through the VEGFR-2 receptor in association with both the PI3-K/Akt and the MEK/ERK pathways [48, 49]. Extracellular-signal-regulated kinases (ERKs) are members of the MAP kinase family. ERKs are activated by oxidative stress. Moreover, preconditioning with HO can suppress the activity of p38 MAPK, but induce the expression of brain-derived neurotrophic factor (BDNF), as well [50, 51]. BDNF increases the expression of B-cell lymphoma 2 (Bcl-2) [52].

Bcl-2 proteins can inhibit apoptosis either by direct action on permeability transition pore (PTP) or indirectly via other proteins. Moreover, Endostatin can decrease the expression of Bcl-2 [7], suggesting that HO may have a neuroprotective effect by bcl-2 pathway.

## 5. Conclusion

The result of this study showed that although Endostatin individually may increase the high risk of ischemic stroke, and VEGF depended on some factors like proper dosage, combining with other proteins and growth factors and..., may be able to reduce the tissue damage after ischemia, however, preconditioning with HO increases VEGF, decreases Endostatin levels, and induces ischemic tolerance. This study introduces the new method of therapy for ischemic stroke, we hope that the method of Preconditioning with HO may be a strategy to reduce the amount of brain damage caused by the stroke, but as Endostatin function is not clear exactly so we believe that further studies are needed to clarify and this therapy arrived to the clinic more quickly.

## Abbreviation

ANOVA: analysis of variance; BBB: blood brain barrier;; EDTA: ethylenediaminetetraacetic acid; HO: normobaric hyperoxia; I: intact; 2HO:2 days after pretreatment with HO; IT: ischemic tolerance; IV: infarct volume; LSD: least significant difference; MCA: middle cerebral artery; MCAO: middle cerebral artery occlusion;; NDS: neurologic deficit score; NF-kB: nuclear factor- kappa B; PVDF: polyvinylidene difluoride; RA: room air (normobaric normoxia); SH: sham; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM: standard error of mean; TNF- $\alpha$ : tumour necrosis factor- $\alpha$ ; VEGF: vascular endothelial growth factor.

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