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Original Article

Time course of normobaric hyperoxia preconditioning on NCX2, 3 expression

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Abstract

Introduction: The purpose of this study was to determine Na-Ca exchanger 2, 3 (NCX2, 3) protein level changes during 2, 5, 10, 15 days after induction of normobaric hyperoxia (HO) preconditioning.

Materials and Methods: Rats were divided in two experimental groups. The first group was exposed to 95% inspired HO for 4 h/day for 6 consecutive days (HO). The second group acted as control, and was exposed to 21% oxygen in the same chamber. Each main group was subdivided to middle cerebral artery occlusion (MCAO-operated) and intact (without any surgery) subgroups. After 2, 5, 10 and 15 days from pretreatment, MCAO-operated subgroups were subjected to 60 min of right MCAO. After 24 hours reperfusion, neurologic deficit score (NDS) and infarct volume (IV) were measured in MCAO-operated subgroups. The NCX 2, 3 expression levels of core, penumbra and subcortex regions were assessed in sham-operated and intact subgroups.

Results: Expression of NCX 2, 3 proteins were increased in penumbra (P=0.000, P=0.002), core (P=0.001, P=0.033) and just NCX3 was increased in subcortex (P=0.033) during preconditioning with HO. Neurologic deficit score and infarct volume were decreased with HO preconditioning. These effects of hyperoxia disappeared gradually during 15 days after pretreatment.

Conclusion: Although further studies are needed to clarify the mechanisms of time course of neuroprotection, HO durable effects on NCX2, 3 expression, IV and NDS are consistent with an active role in the genesis of ischemic neuroprotection.

Abbreviation: ANOVA: analysis of variance; BBB: blood brain barrier; EAAT: excitatory amino acid transporter; EDTA: ethylenediaminetetraacetic acid; HO: normobaric hyperoxia; I: intact; I-HO: intact normobaric hyperoxia subgroup; I-RA: intact normobaric normoxia subgroup; IT: ischemic tolerance; LSD: *least significant difference;* MCA: middle cerebral artery; MCAO: middle cerebral artery occlusion; NCX: Na^{+}-Ca^{2+}exchanger; NDS: neurologic deficit score; NF-κB: nuclear factor-kappa B; O-HO: MCAO-operated normobaric hyperoxia subgroup; O-RA: MCAO-operated normobaric normoxia subgroup; *P38MAPK: p38* mitogen-activated protein kinase; PVDF: polyvinylidenedifluoride; RA: room air (normobaric normoxia); S: sham; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM: standard error of mean; TNF-α: tumour necrosis factor- alpha

Keywords:
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Introduction

The sodium-calcium exchanger (often denoted Na^+/Ca^{2+} exchanger, NCX, or exchange protein) is a nine transmembrane segments protein widely distributed in the brain (Blaustein and Lederer 1999) that couples, in a bidirectional way, the movement of Ca^{2+} and Na^+ ions across the cell membrane in the central nervous system (Meyer 1989). In the central nervous system, the Na/Ca exchanger plays a fundamental role in controlling changes in the intracellular concentrations of Na^+ and Ca^{2+} ions that occur in physiologic conditions (Canitano, Papa *et al.*, 2002). There are three different genes for NCX family (NCX1, NCX2 and NCX3), unlike other organs and tissues. In the brain all 3 proteins are present (Lee, Yu *et al.* 1994). These 3 proteins are differentially expressed in distinct regions of the central nervous system where they might underlie different physiological and pathophysiological functions (Yu and Colvin 1997; Canitano, Papa *et al*. 2002; Papa, Canitano *et al*. 2003) Depending on the intracellular concentrations of Ca^{2+}, $[Ca^{2+}]_i$, and Na^+, $[Na^+]_i$, NCX can operate either in the forward mode, coupling the uphill extrusion of Ca^{2+} to the influx of Na^+ ions, or in the reverse mode, mediating the extrusion of Na^+ and the influx of the Ca^{2+} ions (Blaustein and Lederer 1999).

A great number of conflicting reports on the effects of NCX modulation on cell damage, induced by anoxic conditions, have been published (Andreeva, Khodorov *et al*. 1991; Amoroso, De Maio *et al*. 1997; Masada, Hua et al. 2001; Matsuda, Arakawa et al. 2001). The results of these studies provided evidence *in vivo* regarding NCX activation leading to reduction of the extent of brain infarct volume after permanent middle cerebral artery occlusion (MCAO) and its selective pharmacological blockade produced a worsening of the brain lesion, thus suggesting a protective role played by the antiporter during the events leading to brain ischemia (Pignataro*, Gala et al.* 2004). In fact, the inhibition of NCX activity by pharmacological agents, interacting in a quite selective way with the antiporter, leads to a worsening of cell damage, either through an increase of $[Na^+]$ (Amoroso, De Maio et al. 1997) or through an increase of excitatory amino acids release (Amoroso, Sensi *et al*. 1993). It has been found that the lack of NCX2 resulted in an increased infarct volume and increased neuronal loss in a transient focal cerebral ischemia in the mouse brain (Jeon, Chu *et al.* 2008). It should be mentioned that, unlike NCX1 and NCX2, the peculiar capability of NCX3 to maintain $[Ca2+]_i$ homeostasis even when ATP levels are reduced significantly highlights its major role in neuronal preservation during hypoxic conditions (Secondo, Staiano *et al.* 2007).

Several recent reports suggest that ischemic brain damage can be reduced by hyperoxia preconditioning (Zemke*,* Smith *et al.*, 2004). In our laboratory, we have recently shown that pretreatment with intermittent and prolonged normobaric hyperoxia induces different degrees of neuroprotection in rat brain. Intermittent HO induced the genes expression of excitatory amino acid transporters, NF-kB, TNFα, and TNFα convertase enzyme (Bigdeli and Khoshbaten 2008) and the NCX family (NCX1, NCX2 and NCX3) (Mohammadi and Bigdeli 2013). We also reported that decreased infarct volume (IV) and neurologic deficit score (NDS) and increased the expression of NCX1 in rat brain by HO preconditioning was prolonged for 15 days after pretreatment. Here, we sought to identify whether HO preconditioning effects on NCX2, 3 expression might be prolonged as well. Here, we also repeated our investigation that was a study on time course of neuroprotection induced by normobaric hyperoxia on IV and NDS.

Materials and methods

Animals and group assignment

All experimental animal procedures were conducted with the approval of the Ethics Committee of the Shaheid Beheshti University of Iran. Every effort was made to minimize the number of animals used and their suffering. The rats were housed individually at 24°C with food and water provided ad libitum with lights on from 07:00 to 19:00 h (light cycle) and off from 19:00 to 07:00 h (dark cycle). Male Sprague–Dawley (250-380 g) rats were divided in 2 main groups and sham (S) group. Each main group was subdivided to MCAO-

operated (middle cerebral artery occlusion (O)) and intact (I) subgroups for different evaluations (neurologic deficit score (NDS), infarct volume, and NCX expression level).

First main group was placed in an environmental chamber (Bigdeli, Hajizadeh *et al.* 2007) and exposed to a hyperoxic atmosphere (95% oxygen: normobaric hyperoxia groups, or HO) intermittently (for 4 continuous hours of each day for each of six consecutive days, yielding a total hyperoxia exposure of 24 h. first main group was then placed in ordinary room air for a further 2, 5, 10, 15 days (2HO (n=15), 5HO (n=15), 10HO (n=15), and 15HO (n=15) respectively). Second main group was similarly placed in the environmental chamber and exposed to room air equivalent 21% oxygen: normobaric normoxia (room air) groups (RA) for similar time periods. 24 hours after pretreatment, MCAO-operated subgroups (O-HO and O-RA) were subjected to 60 min of MCAO. After twenty four hours reperfusion, neurobehavioral studies and then infarct volume measurement (Bigdeli, Hajizadeh *et al.* 2007) were performed. In sham-operated (S) subgroup, all steps were similar to RA group, except of MCAO. In each intact subgroup, all steps were identical to RA and HO groups who did not undergo surgery (I-RA, and I-HO). Finally 2, 5, 10, 15 days after pretreatment (I-2HO (n=6), I-5HO (n=6), I-10HO (n=6) and I-15HO (n=6)), intact subgroups and sham-operated (S) animals were sacrificed for assessment of NCX2, 3 expression in the core, penumbra and subcortex of right hemisphere.

In a subset of animals, arterial blood gas analysis was performed just prior to removal from the environmental chamber. In addition, middle cerebral artery occlusion was monitored by Laser Doppler flowmeter (MBF3, Moor Instruments, Axminster, UK).

Environmental Chamber

All rats underwent adaptation for 1 week in the animal room. The environmental chamber (Bigdeli, Hajizadeh *et al.* 2007) comprised an air-tight box (650 × 350 × 450 mm) with a gas inlet and outlet port. Internal pressure was continuously monitored by a manometer. Oxygen (95%) or RA (by an aquarium pump) was delivered at a rate of less than 5 L/min through the inlet port. The oxygen concentration inside the container was continuously monitored with an oxygen sensor (Lutron-Do5510, Taiwan) and carbon dioxide cleared using soda lime (BDH Ltd, Poole, United Kingdom) at the bottom of the container. Oxygen concentration was maintained at 95% or 21% according to experimental protocol.

Focal Cerebral Ischemia

The rats were weighed and anesthetized with chloral hydrate (Merck, Germany) (400 mg/1kg). MCAO was performed as described by Longa et al in 1989 (Longa, Weinstein *et al.* 1989). Briefly, under a microscopic surgery, a 3-0 silicone-coated nylon suture was introduced through the external carotid artery stump. The occluder was advanced into the internal carotid artery 20 to 22 mm beyond the carotid bifurcation until mild resistance indicated that the tip was lodged in the anterior cerebral artery and blocked the blood flow to the MCA. Reperfusion was started by withdrawing the suture after 60 minutes of ischemia. Rectal temperature was monitored (Citizen-513w, CITIZEN) and maintained at 37^0 C by surface heating and cooling during surgery.

Blood Flow Laser Doppler

Velocitometry Laser Doppler flowmeter (MBF3D, Moor Instrument, Axminster, United Kingdom) was used for recording regional cerebral blood flow. The probe of laser Doppler flowmeter was positioned to the surface. Using a stereotaxic device and a low-speed dental drill, a burr hole of 2 mm in diameter was made over the skull at 1 mm posterior and 5 mm lateral to the bregma on the right side. A needle-shaped laser probe was placed on the dura away from visible cerebral vessels. Steady-state baseline values were recorded before MCAO and blood flow data

In all cerebral regional were expressed as percentage of their respective basal value (Chen and Cheung 2002). Doppler flux was continuously measured from 30 minutes before MCAO until 30 minutes after reperfusion.

Neurobehavioral Evaluation

After the suture was withdrawn, the rats were returned to their separate cages. At 24 hours later, the rats were

assessed neurologically by a neutral observer. The neurobehavioral scoring was performed using the 6-point scale previously described by Longa and colleagues (Longa, Weinstein *et al.* 1989): normal motor function = 0; flexion of contralateral forelimb on suspension vertically by tail or failure to extend forepaw=1; circling to the contralateral side but normal posture at rest = 2; loss of righting reflex = 3; and no spontaneous motor activity = 4. Death was considered as score 5 only when a large infarct volume was present in the absence of subarachnoid hemorrhage. If the rats died as a result of subarachnoid hemorrhage or pulmonary insufficiency and asphyxia, they were eliminated from the study.

Infarct Volume Assessment

After killing with chloral hydrate (800 mg/kg), the animals were decapitated and the brains rapidly removed and cooled in 4^0C saline for 15 minutes. Eight 2-mm thick coronal sections were cut (Brain Matrix, Tehran, Iran) from the brain, beginning at the olfactory bulb. The slices were immersed in 2% Z2, 3, 5-triphenyltetrazolium chloride solution (Merck, Germany), and kept at 37^0C in a water bath for 15 minutes. The slices were then digitally photographed by a camera (Canon, DSC-W310) connected to a computer. Unstained areas were defined as infarct, and were measured using image analysis software (Image Tools, National Institutes of Health). The infarct volume was calculated by measuring the unstained and stained area in each hemisphere slice (Bigdeli, Hajizadeh *et al.* 2007), multiplying by slice thickness (2 mm), and then summating all of the 8 slices according to the method of Swanson and colleagues (Swanson, Morton *et al.* 1990): corrected infarct volume = left hemisphere volume – (right hemisphere volume - infarct volume).

Brain Sampling and Protein Extraction

At 48 hours after pretreatment, intact (I) and sham (S) animals were killed by chloral hydrate (800 mg/kg) and decapitated for measurement of protein expression. Core, penumbra and subcortex of brain tissue isolated as previously described (Lei, Popp *et al.* 2004). Cells in these areas were removed and homogenized by sonication at 4^0C in 4 volumes of buffer containing 0.5% sodium deoxycholate , 150 mM NaCl, 0.1% SDS, 0.03% EDTA, 1 tablet protease inhibitor cocktail (Roche), 50 mM Tris-HCl with pH 7.0 (homogenization buffer).

Western Blot Analysis

Homogenate containing about 60 µg of total protein from the core, penumbra and subcortex of right hemisphere samples (sham-operated group (S), hyperoxia (I-HO) and RA (I-RA) subgroups) were loaded together with a protein ladder (Thermo Scientific) and the proteins size-separated in %8 SDS-PAGE (polyacrylamide gel electrophoresis) (90 mA). The proteins were blotted onto a PVDF (poly vinylidene fluoride) membrane (Millipore; western blot protocol-Abcam). Blots were blocked at room temperature for 1 hour in blocking reagent (GE Health Care, Amersham) and subsequently incubated with specific primary polyclonal rabbit antibody to NCX2 (1:5000 dilution) (LS-C8A2, LifeSpan, BioSciences), NCX3 (1:5000 dilution) (SLC8A3, LifeSpanBioSciences) and rabbit antibody against β-actin (1:1000 dilution) (Santa Cruz). They were then incubated with secondary anti-rabbit (1:10000 dilution) (Dakocytomation, Denmark) antibody conjugated to horseradish peroxidase for 1 hour at room temperature. NCX2 and NCX3 immunoreactive proteins were detected with advanced chemiluminescence (Enhanced Chemiluminescence, Amersham Biosciences) and film exposure. After scanning and transferring the film images to computer, signal bands were quantified (Image J software). Densitometric analysis of bands after normalization with β-actin as a loading control was calculated as a percent of that seen in the RA group [5].

Statistical Analysis

All the data was described as mean ± SEM. Statistical analysis was performed with SPSS for Windows, version 15.0. The significance of differences among multiple groups (NCXs expression and infarct volume) was compared using one-way analysis of variance (ANOVA). NDS was analyzed using the Mann-Whitney U test. Post-hoc comparison between groups was detected for significance using LSD method. P values less than 0.05 were considered significant.

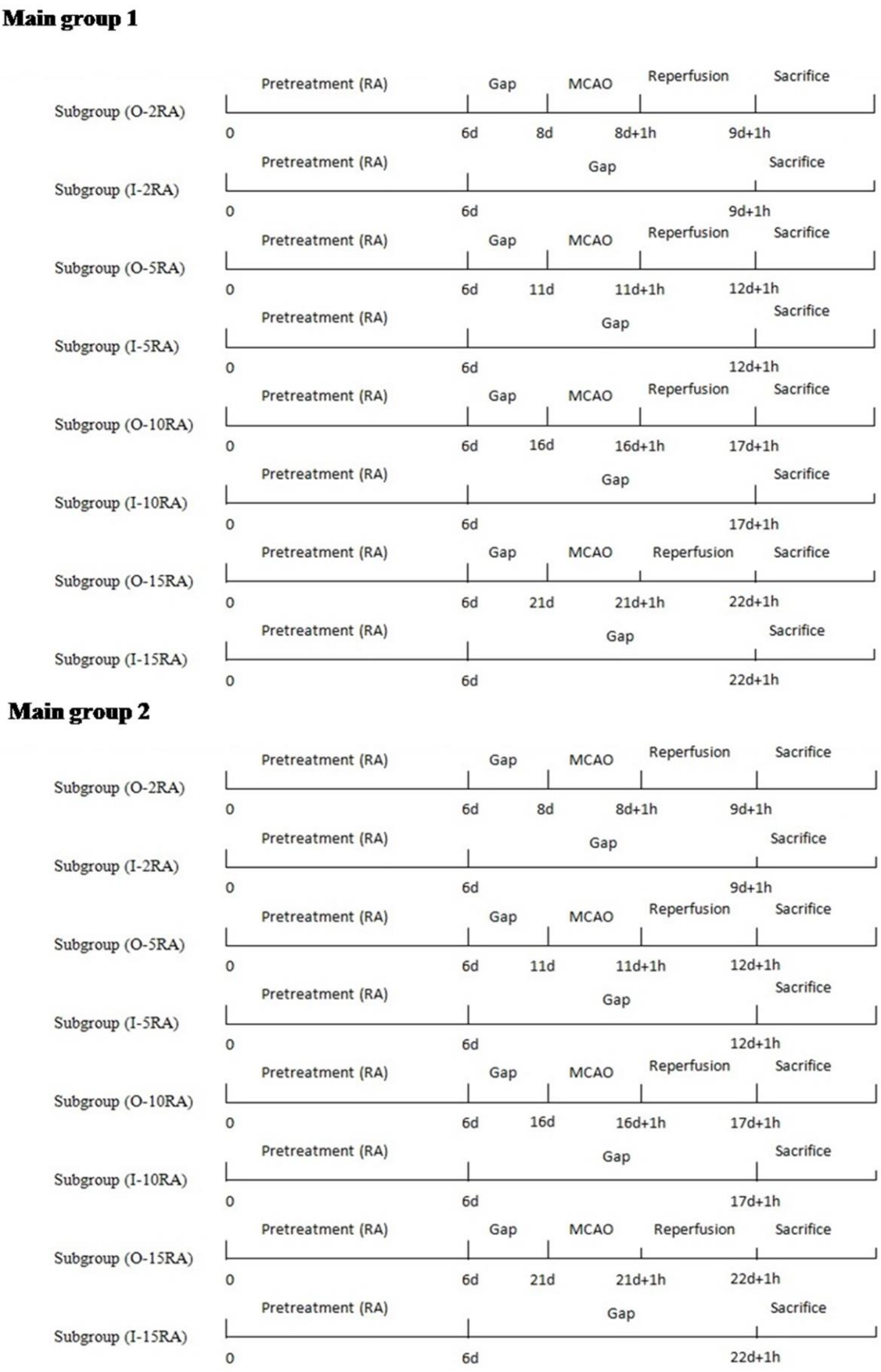


Fig. 1. normobarichyperoxia (HO) and normoxia (RA) pretreatment in various groups are shown by a diagram.

Experimental Groups	pH	PCO_2 (mmHg)	PO_2 (mmHg)	Respiratory Rate (Hz)
RA	7.4±0.02	40.5±0.93	95.9±4.51	1.44±0.1
HO	7.3±0.01	40.6±0.85	361±18.3*	1.31±0.2

Table 1: arterial blood gases tests at the end of pretreatment (P<0.001=S).

No.	Experimental groups	NDS in each groups(N)						Total (N)	Median	Premature death (N)	Statistical results
		0	1	2	3	4	5				
1	O-2HO	6	1	2	0	0	0	9	0	1	1-5 S
2	O-5HO	4	1	1	2	1	0	9	1	2	2-5 S
3	O-10HO	2	3	2	2	0	0	9	2	4	3-5 S
4	O-15HO	0	3	4	1	1	0	9	2	3	4-5 NS
5	O-2RA	0	0	3	2	4	0	9	3	5	4-1 S
6	Total	12	8	12	7	6	0	45	2	12	4-2 NS

Abbreviations: NDS: neurologic deficit score; N: the number of cases in each groups; S: significant, NS: nonsignificant.

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

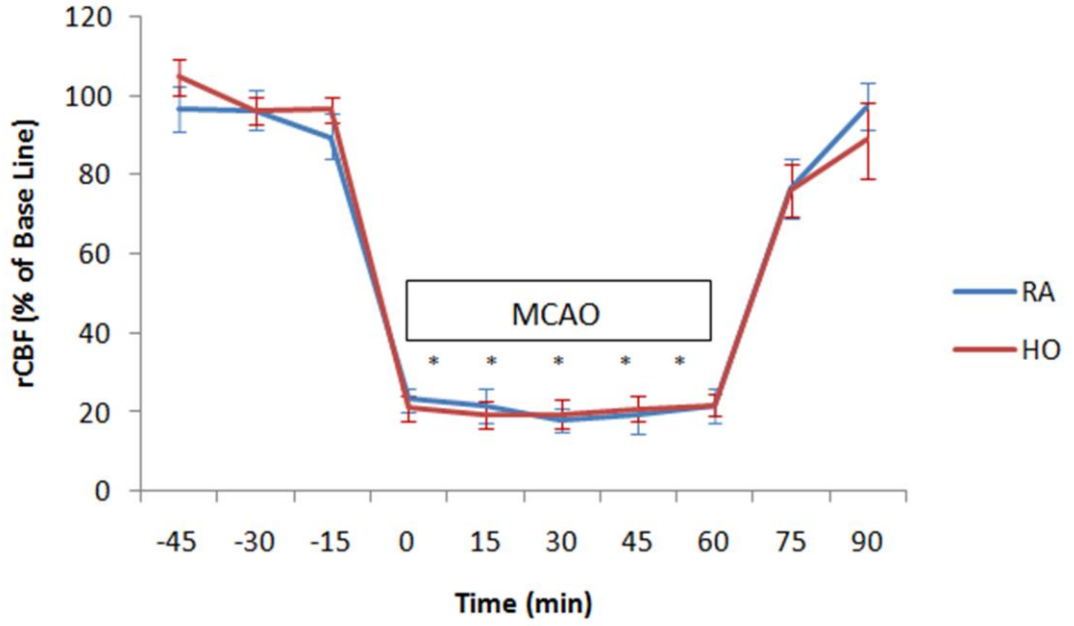
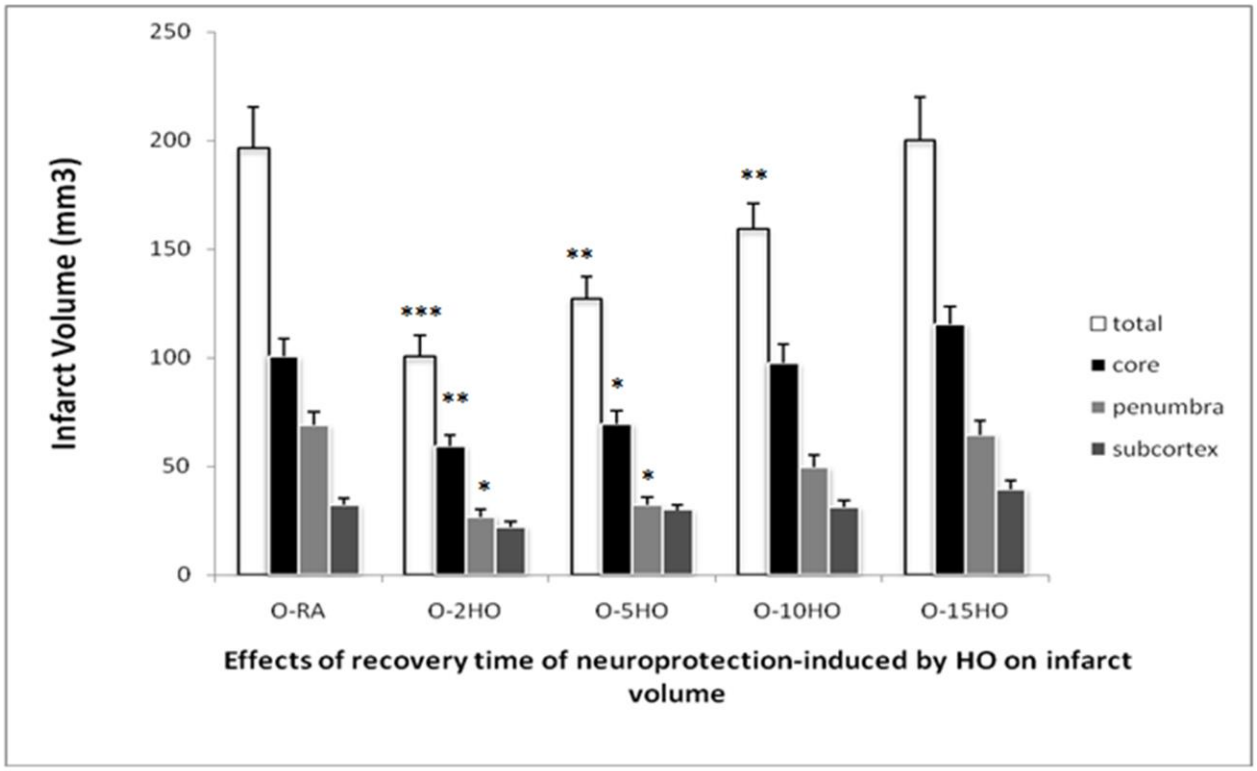


Fig. 2. Regional cerebral blood flow (rCBF) before and during MCAO, and after reperfusion in O-RA and O-HO groups. Signification is in compare with O-RA group (*=P<0.001).

A:



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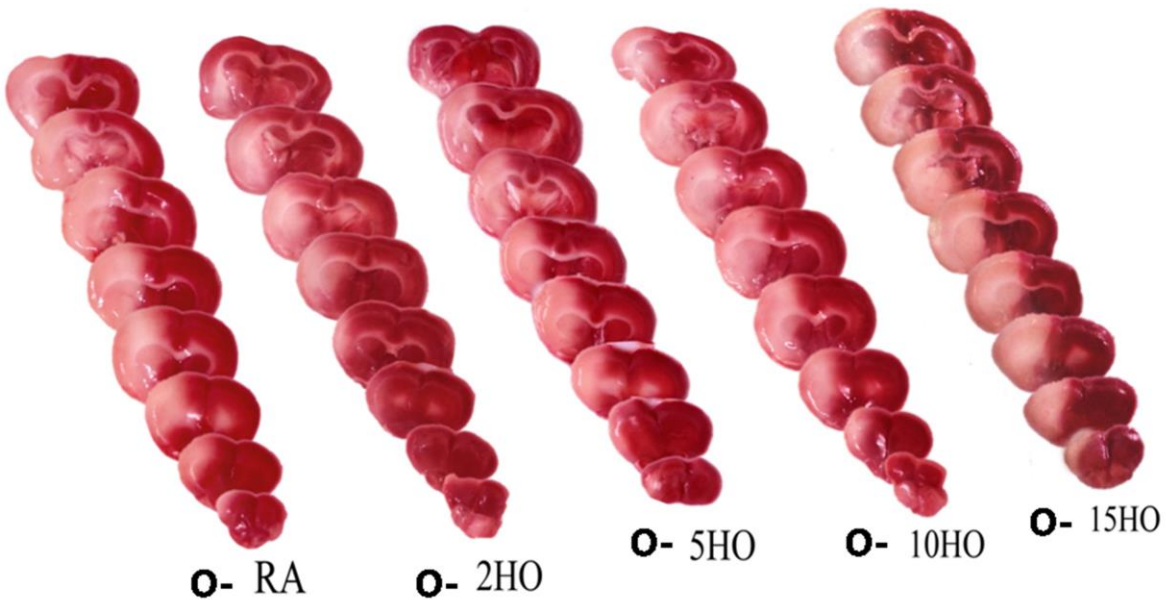


Fig. 3. A: Effects of recovery time of neuroprotection-induced by HO on infarct volume in MCAO (O) subgroups. Signification is in compare with O-RA subgroups (*P<0.05, **P<0.01, ***P<0.001).
B: TTC stained images for each group

Results

Experimental Conditions Parameter

Arterial blood gas analysis confirmed clinical hyperoxia and normoxia in the pretreated groups (table 1). Cerebral blood flow was reduced to less than 24% of base line in each group (Fig 2).

Effects of Recovery time of neuroprotection- induced by measuring HO effects on NDS and infarct volume

Median NDS were reduced by increasing recovery time, and significantly so when O-2HO, O-5HO, and 10HO were compared with O-RA (table 2). The putative beneficial effects of HO were confirmed by a reduction in infarct volume seen in O-2HO and O-5HO significantly (Fig 3). The neuroprotection exerted by HO was mainly seen in the cortex (core and penumbra) (Fig 3). After 15 days recovery period, there was no significant difference in neurologic scores and infarct volume between groups.

Effects of recovery time of neuroprotection-induced by HO on NCX2 expression in core, penumbra and subcortex areas

Western blot analysis showed that NCX2 is expressed in the rat core, penumbra and subcortex. The expression of NCX2 was increased by HO in intact animals (I-2HO), when compared to the intact RA (I-RA) and sham-operated groups (S) (Fig 4). On the other hand, the NCX2 expression of core area in HO group was more than that in RA group in both sham-operated (P=0.002) and intact animals significantly (P=0.001; Fig 4). Therefore, it seems that exposure to HO caused an increase in the expression of NCX2 in core (Fig 4). The expression of NCX2 in core was not different between intact and sham-operated RA rats (Fig 4). Therefore, experimental conditions and RA could not have induced NCX2 expression of core in sham-operated and intact rats.

The increased expression of NCX2 in core decreased gradually during 15 days after pretreatment (I-5HO, P=0.098), (I-10HO, P=0.147) and the expression of the NCX2 in 15 days after pretreatment was not changed significantly (P=0.981) when compared with RA subgroup (I-RA; Fig 4).

The expression of NCX2 in penumbra was increased in HO in intact animals (I-2HO), when compared to the intact RA (I-RA) and sham-operated groups (S) (P=0.00 and P=0.000, respectively; Fig 4). Therefore, it seems that exposure to HO caused an increase in the expression of NCX2 in penumbra (Fig 4). NCX2 expression of penumbra was not different between intact and sham-operated rats in RA subgroups (Fig 4). Therefore, experimental conditions and RA could not induce NCX2 expression of penumbra in sham-operated and intact rats.

The increased expression of NCX2 in penumbra decreased gradually during 15 days after pretreatment ((I-5HO, P=0.014), (I-10HO, P=0.158)) and the expression of the NCX2 in 15 days after pretreatment was not changed significantly (P=0.981) when compared with RA subgroup (I-RA; Fig 4).

The expression of NCX2 in subcortex area was not changed in HO in intact animals (I-2HO), when compared to the intact RA (I-RA) and sham-operated groups (S) (Fig 4). Also, NCX2 expression was not different between intact and sham-operated rats in RA subgroups (Fig 4). Therefore, experimental conditions and RA could not induce NCX2 expression in sham-operated and intact rats.

Effects of recovery time of neuroprotection-induced by HO on NCX3 expression in core, penumbra and subcortex areas

Western blot analysis showed that NCX3 is expressed in the rat core, penumbra and subcortex. The expression of NCX3 was increased in HO in intact animals (I-2HO), when compared to the intact RA (I-RA) and sham-operated groups (S) (Fig 5). On the other hand, the NCX3 expression of core area in HO group was more than that in RA group in both sham-operated (P=0.009) and intact animals significantly (P=0.012; Fig 5). Therefore, it seems that exposure to HO caused an increase in the expression of NCX3 in

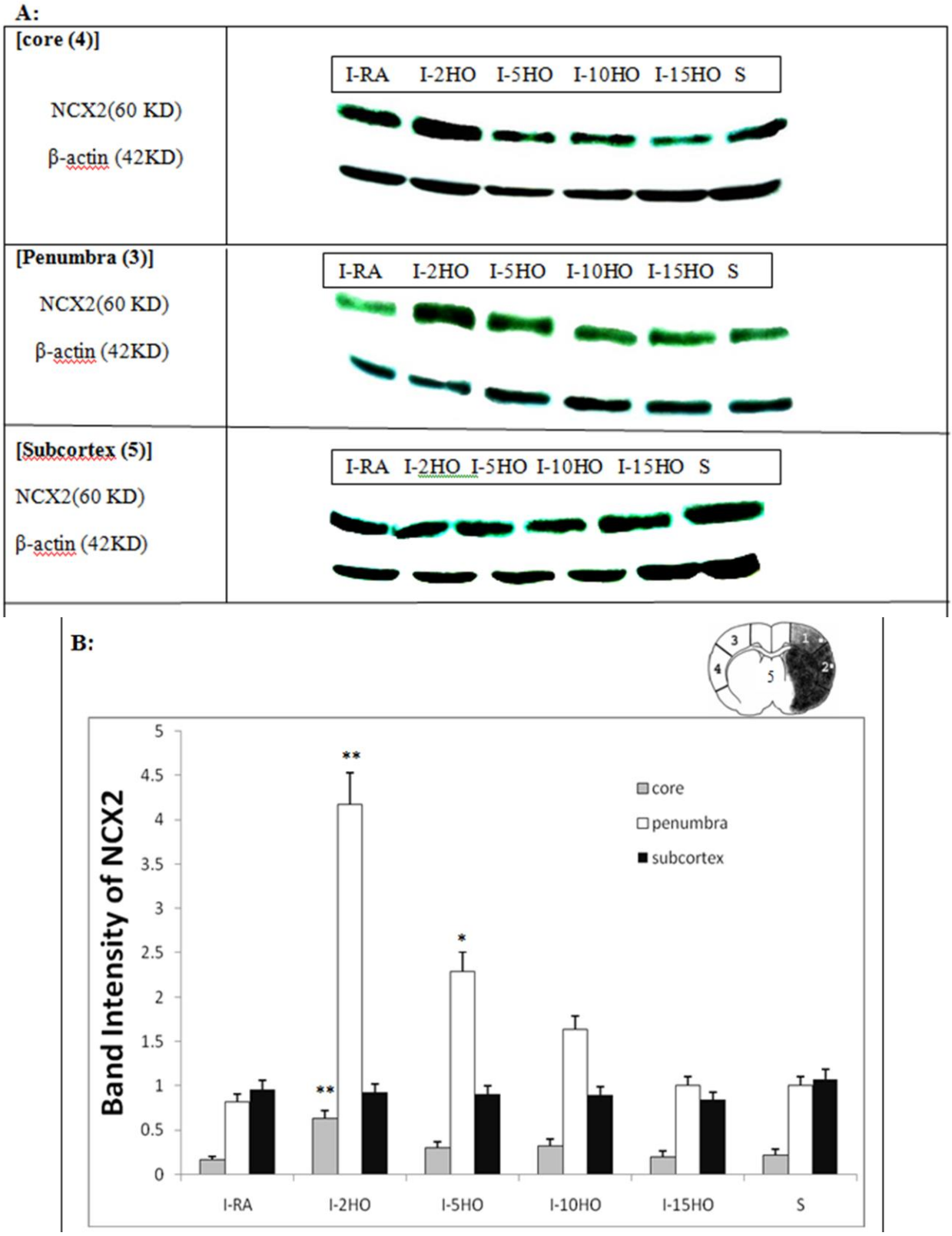
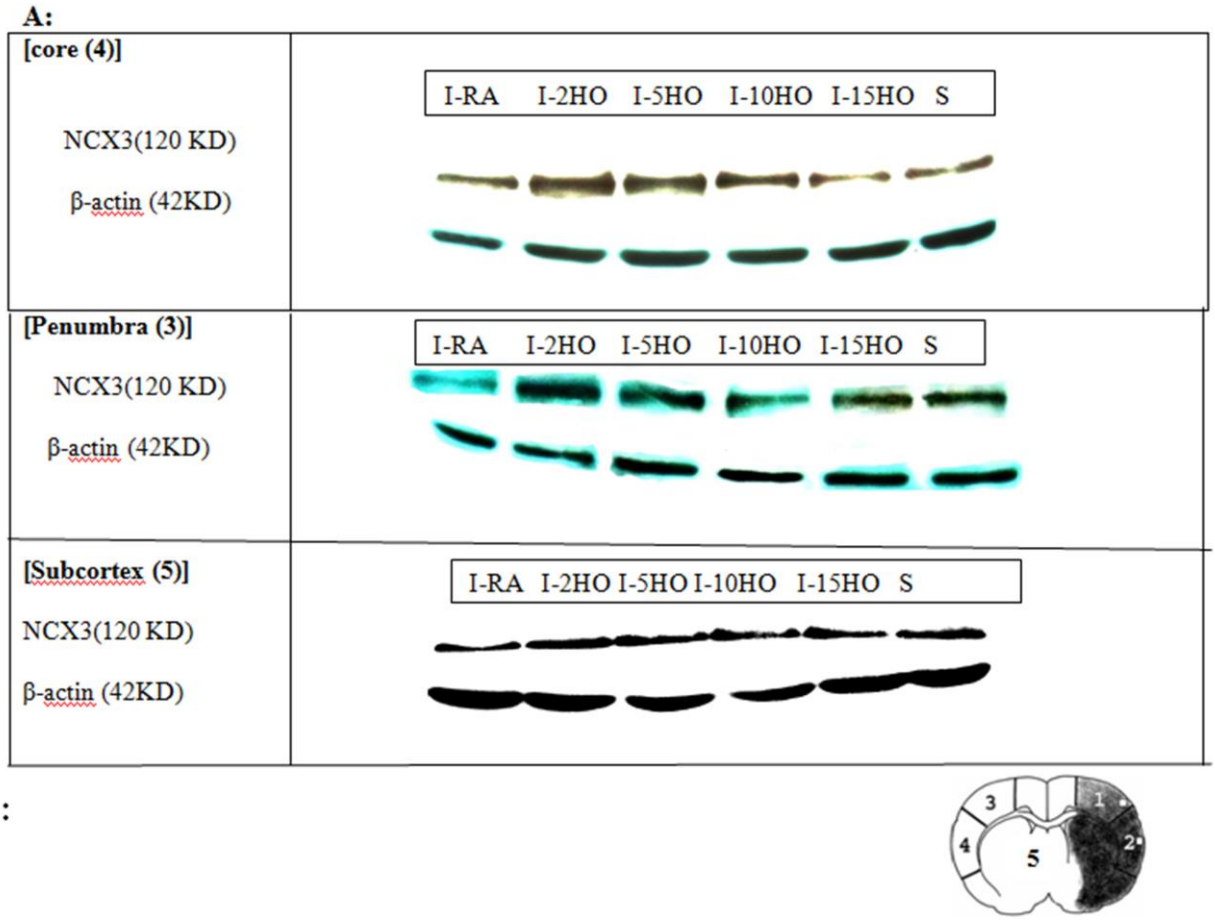


Fig. 4. Western blot of NCX2 protein in core (region 4), penumbra (region 3) and subcortex (region 5) regions from right brain hemisphere in rats of HO (I-2HO, I-5HO, I-10HO and I-15HO) and room air (RA) in intact (I) and sham subgroups (S) (A).Western blot analysis of NCX2 protein, the lower panel shows densitometric analysis of bands after normalization with β-actin as loading control (B). Signification is in compare with I-RA subgroups (*P<0.05, **P<0.01).B: TTC stained images for each group.



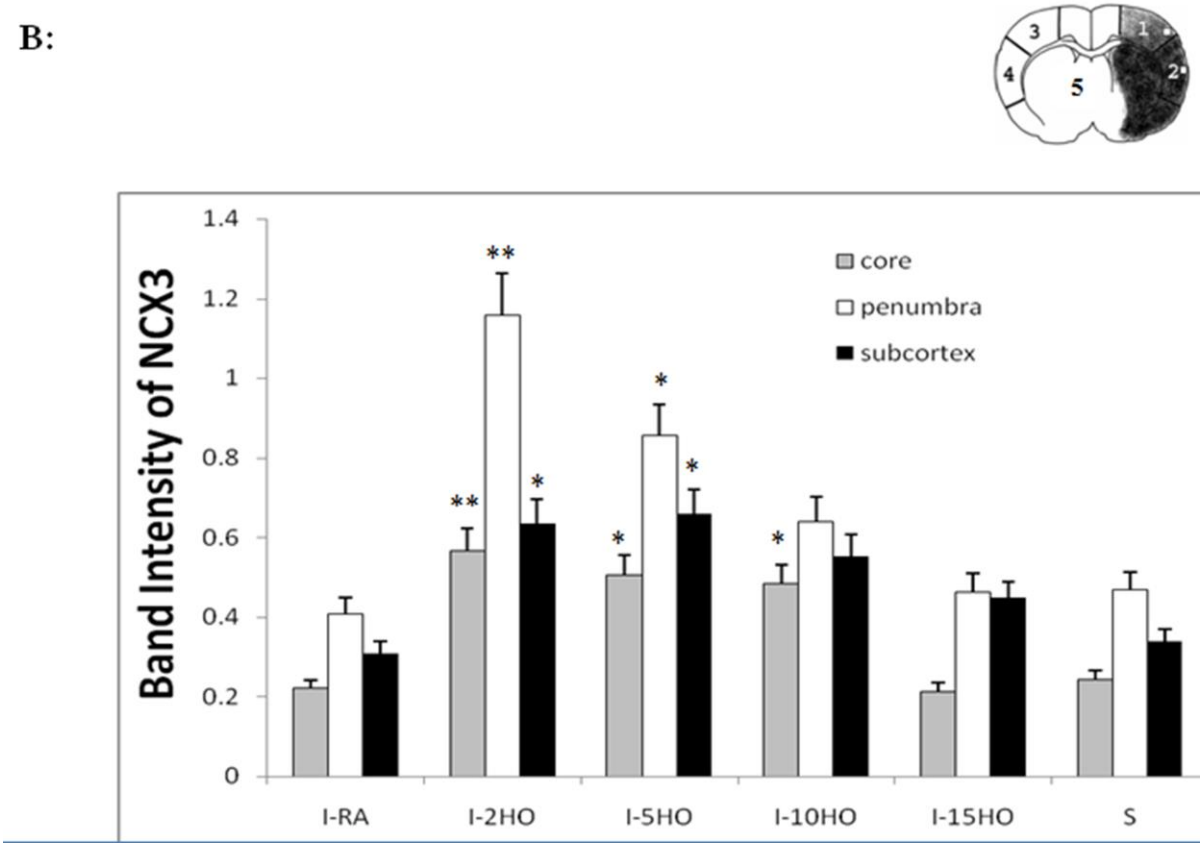


Fig. 5. Western blot of NCX3 protein in core (region 4), penumbra (region 3) and subcortex (region 5) regions from right brain hemisphere in rats of HO (I-2HO, I-5HO, I-10HO and I-15HO) and room air (RA) in intact (I) and sham subgroups (S) (A).Western blot analysis of NCX2 protein, the lower panel shows densitometric analysis of bands after normalization with β-actin as loading control, Signification is in compare with I-RA subgroups (B) (*P<0.05, **P<0.01).

core (Fig 5). The expression of NCX3 in core was not different between intact and sham-operated RA rats (Fig 5). Therefore, experimental conditions and RA could not induce NCX3 expression of core in sham-operated and intact rats.

The increased expression of NCX3 in core partially decreased 5 and 10 days after preconditioning ((I-5HO, P=0.023), (I-10HO, P=0.034)) and the expression of the NCX3 in 15 days after pretreatment was not changed significantly (P=1.000) when compared with RA subgroup (I-RA; Fig 5).

The expression of NCX3 in penumbra was increased in HO in intact animals (I-2HO), when compared to the intact RA (I-RA) and sham-operated groups (S) (P=0.002 and P=0.004, respectively; Fig 5). Therefore, it seems that exposure to HO caused an increase in the expression of NCX3 in penumbra (Fig 5). NCX3 expression of penumbra was not different between intact and sham-operated rats in RA subgroups (Fig 5). Therefore, experimental conditions and RA could not induce NCX3 expression of penumbra in sham-operated and intact rats.

The increased expression of NCX3 in penumbra decreased gradually during 15 days after pretreatment ((I-5HO, P=0.032), (I-10HO, P=0.316)) and the expression of the NCX3 in 15 days after pretreatment was not changed significantly (P=0.990) when compared with RA subgroup (I-RA; Fig 5).

The expression of NCX3 in subcortex was increased in HO in intact animals (I-2HO), when compared to the intact RA (I-RA) and sham-operated groups (S) (P=0.033 and P=0.043, respectively; Fig 5). Therefore, it seems that exposure to HO caused an increase in the expression of NCX3 in subcortex (Fig 5). NCX3 expression of subcortex was not different between intact and sham-operated rats in RA subgroups (Fig 5). Therefore, experimental conditions and RA could not induce NCX3 expression of subcortex in sham-operated and intact rats.

The expression of NCX3 in subcortex increased 5 days after pretreatment (I-5HO, P=0.024) and then gradually decreased in 10 and 15 days after pretreatment (I-10HO, P=0.106). The expression of the NCX3 in 15 days after pretreatment was not changed significantly (P=0.918) in compare with RA subgroup (I-RA; Fig 5).

Discussion

Ischemic Tolerance (IT) induced by HO

Previous studies show that intermittent HO which induces IT plays a significant role in decreasing infarct volume, NDS, brain edema, and BBB permeability (Bigdeli, Hajizadeh *et al.* 2007). Our results confirm these studies and we showed that HO, as an IT factor, decreases NDS and IV in core, penumbra and subcortex regions in Rat brain. These preconditioning effects on NDS and IV were observed in 2, 5 and 10 days after pretreatment and lost 15 days after pretreatment.

As it is shown in fig 3, the core area is more damaged than the penumbra and subcortex areas. Because within the ischemic area the core (ischemia center) has complete blood loss and irreversible cell damage, but the zone around the core which is termed penumbra, has loss of electrical function and blood flow (Astrup, Siesjo *et al.* 1981).

Effects of HO on NCX

NCX is an antiporter membrane protein that removes calcium from cells. It uses the energy that is stored in the electrochemical gradient of sodium (Na^+) by allowing Na^+ to flow down its gradient across the plasma membrane in exchange for the countertransport of calcium ions (Ca^{2+}). The NCX removes a single calcium ion in exchange for the import of three sodium ions (Yu and Choi 1997). The exchanger exists in many different cell types and animal species and is considered one of the most important cellular mechanisms for removing Ca^{2+} (Dipolo and Beaugé 2006). It is usually found in the plasma membranes and the mitochondria and endoplasmic reticulum of excitable cells (Kitagawa, Matsumoto *et al.* 1990; Patterson, Sneyd *et al.* 2007). Three different NCX isoforms (NCX1, 2 and 3) are encoded by distinct genes in mammals (Nicoll, Longoni *et al.*, 1990; Li, Matsuoka *et al.*, 1994). Although NCX isoforms share similar biophysical properties, they show different sensitivity to ATP levels and are differentially expressed both during development and in adults

(Linck, Qiu *et al.* 1998; Papa, Canitano *et al.* 2003).

It has been shown that HO changes NF-kB, EAATs, TNF-α, and TNF-α converting enzyme levels (Bigdeli and Khoshbaten 2008) and NCX family (NCX1, NCX2 and NCX3) (Mohammadi and Bigdeli 2013) expression two days after pretreatment. There is no significant difference between expression of NCXs in sham-operated group and RA subgroup, Therefore. it can be said that HO preconditioning decreases infarct volume and NDS with increasing expression of neuroprotective factors such as NCXs. Findings are consistent with a role for NCXs modulation in generating of IT in the brain as a neuroprotective factor (Pignataro, Tortiglione *et al.* 2004; Jeon, Chu *et al.* 2008). Inhibition of NCX activity by pharmacological agents, interacting in a quite selective way with the antiporter leads to a worsening of cell damage, either through an increase of $[Na^+]_I$ [1] or through an increase of excitatory amino acids release, which occurs through the reverse mode of operation of the glutamate syntransporter system (Amoroso, Sensi *et al.* 1993). oxidants generated during the brief period of highly elevated PO_2 are important for triggering many signaling systems that are more oxidant sensitive, including various kinases, e.g., p38MAPK (Bigdeli 2011). It is relevant to mention that in the early phase of neuronal anoxic insult, the reverse mood of NCX operation elicits a profound Ca^{2+} entry and increases in $[Ca^{2+}]_i$, its effect can be beneficial for neurons, because it contributes to a decrease in $[Na^+]$i overload which consequently, prevents cell swelling and death. On the contrary, in the later phase of neuronal anoxia, when $[Ca^{2+}]$i overload takes place, NCX forward mode of operation contributes to the lowering of $[Ca^{2+}]$i, thus protecting neurons from $[Ca^{2+}]_i$-induced neurotoxicity (Amoroso, De Maio *et al.* 1997).

HO preconditioning produces ROS and likely, ROS provokes a signal transduction pathway and upregulates NCXs expression in some regions of brain, although these patterns of upregulation are different depending on the cerebral regions and NCX isoforms (Mohammadi and Bigdeli 2013).

Our results in this study confirm NCX2, 3 roles as neuroprotective factors that are provoked in HO preconditioning. Increase of NCX2, 3 expression could be one reason in ischemic damage reduction after HO preconditioning. These effects of HO could have durability for 10 days.

Effects of HO on NCX2

NCX2 plays a pivotal protective role in the pathophysiology of cerebral ischemia. Lack of NCX2 resulted in an increased infarct volume and increased neuronal loss in a transient focal cerebral ischemia in the mouse brain. A genetic deletion of NCX2 increased ischemic neuronal injury in vitro and in vivo, due to a dysfunction in the regulation of $[Ca^+]_I$ by extrusion, therefore NCX2 has a neuroprotective role in ischemic cell death in the adult brain (Jeon, Chu *et al.* 2008).

The time course of NCX2 expression suggests that 2 days after pretreatment, NCX2 expression was significantly increased in core and penumbra and this increased expression was reduced gradually up to 15 days after pretreatment, in fact HO pretreatment lasted for 10 days and after 15 days its effects were disappeared.

Some reports suggest that NCX2 exerts is neuroprotective role by exportings Ca^{2+} in ischemia and thus protects neuronal cells from death by reducing $[Ca^{2+}]_i$ in the adult mouse brain (Jeon, Chu *et al.* 2008). Our results confirm this report and reject those findings that say in NCX family, NCX1 and NCX2 merely play neuroprotective roles (Pignataro, Tortiglione *et al.* 2004).

Effects of HO on NCX3

NCX3, one of the three isoforms of the NCX family, is highly expressed in the brain (Molinaro, Cuomo *et al.* 2008). In fact, three NCX gene products display differential sensitivity to intracellular ATP levels (Linck, Qiu *et al.* 1998). whereas ATP is required for NCX1 and NCX2 activity, NCX3 is able to function in the absence of this nucleotide (Secondo, Staiano *et al.* 2007). Indeed, NCX3 independence of ATP is suggestive of its evolutionary role in counterbalancing ATP depletion during brain ischemia (Molinaro, Cuomo *et al.* 2008).

The time course of NCX3 expression suggests 2 days after pretreatment, NCX3 expression was significantly increased in three regions and 15 days after pretreatment it decreased to control level.

Our findings confirm that NCX3 is a neuroprotective

factor (Pignataro *et al.*, 2004), that decreases damages resulted in ischemia.

According our results, NCX3 could be an important neuroprotective factor, because: (1) its expression was increased in all 3 parts of brain (core, penumbra and subcortex); (2) its expression remains high 10 days after pretreatment.

Conclusion

Regarding HO durable effects on NCX2, NCX3, NDS and IV and their coordinated changes during 15 days (2, 5, 10 and 15 days), our data suggest that alteration of NCX2, 3 expression in hyperoxia preconditioning can have an impact on neuroprotection mechanism, and is thus an important implication in the pathogenesis of stroke. Further work is required to extend./confirm these observations. Ultimately, it is hoped that novel cerebroprotective strategies may be developed for those at risk of stroke or in whom cerebral perfusion is electively reduced, at the time of surgery.

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Conflict of interest

All authors declared that there is no conflict of interest.

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