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Delayed Protective Effects of Hyperoxia Against Cardiac Arrhythmias and Infarction in Anesthetized Rats

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Background. **Previous studies have shown that pretreatment with normobaric hyperoxia has cardioprotective effect in isolated rat heart. The present study was designed to test the hypothesis that pretreatment normobaric hyperoxia could induce delayed cardioprotection effect in an *in vivo* regional heart ischemia.**

Materials and methods. **Experiment 1: Rats were exposed to normobaric normoxia or to normobaric hyperoxia (O_2 > 95%) for 15, 30, 60, 120, and 180 min (H15, H30, H60, H120, and H180 groups, respectively). After 24 h, they were subjected to 30 min regional ischemia and 90 min reperfusion. Then, the hearts were harvested for measurement of infarct size. Lead II of electrocardiogram was continuously recorded for analysis of ischemic arrhythmias. Experiment 2: Different oxygen concentrations were tested in the same model of heart ischemia.**

Results. **Compared with normoxia group, infarct size significantly reduced in H120 and H180 groups (from 48.1 ± 4 to 31.4 ± 3.3 and 30 ± 2.4, respectively); 120 and 180 min of >95% hyperoxia significantly reduced the number of ventricular beats (from 314 ± 34.9 to 173 ± 20.3 and 178 ± 15.7, respectively) and incidence of ventricular fibrillation (from 66.8% to 30% and 22.2%, respectively). When the oxygen concentration decreased to 80%, its effect on infarct size was abolished; however, its antiarrhythmic effect persisted. Further reduction of oxygen concentration eliminated both the effects.**

Conclusion. **These results show that hyperoxia pretreatment may induce delayed anti-infarct and antiarrhythmic effects in anesthetized rats. These effects are dependent on the exposure time and oxygen concentration.**

Key Words: **hyperoxia; ischemia; reperfusion; arrhythmia; preconditioning.**

INTRODUCTION

Recent therapeutic methods, such as thrombolysis, coronary bypass graft surgery, and angioplasty have reduced mortality of ischemic heart diseases (IHDs), but many patients may not be suitable candidates for these methods [1]. In addition, sudden cardiac death caused by ischemia or reperfusion-induced arrhythmia is a warning to the development of new antiarrhythmic modes [2, 3]. Finding new strategies for reducing the degree of primary injury and/or the rate of dysrhythmias resulted from an ischemic heart insult could have important clinical consequences and reduce the overall mortality and morbidity of IHDs.

It has been well documented that brief episodes of ischemia-reperfusion (IR), known as ischemic preconditioning (IPC), initiates a protective mechanism reducing injury caused by a subsequent more prolonged ischemic insult [4]. Two phases have been identified for IPC phenomenon: An early phase, which is manifested within minutes after brief IR episodes and lasts 2 to 4 h, and a delayed phase, which starts 24 h after brief

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IR episodes and may last up to 72 h [5]. The delayed phase of IPC has considerable potential for therapeutic applications because it has a sustained nature and protects the heart against stunning, infarction, and arrhythmia [6]. One possible agent involved in delayed preconditioning is reactive oxygen species (ROS) which acts as trigger of the adaptive response [7, 8].

Since in many clinical situations, IPC as an aggressive method is not applicable, developing alternative safe methods for reducing the degree of infarction and/or the incidence of arrhythmias resulted from ischemic insults have clinical importance. It has been indicated in some animal models that short duration pre-exposure to normobaric hyperoxia, as a cause of sublethal oxidative stress, can mimic IPC and protect the isolated heart against infarction [8–11] and reperfusion-induced arrhythmia [9] in a biphasic mode [9, 12, 13]. Recently, our colleagues have shown that preconditioning by normobaric hyperoxia could protect the rat brain [14] and kidneys [15] against subsequent IR injury.

The effects of oxygen pretreatment on infarct size and cardiac arrhythmia are not studied as yet in models of heart ischemic injury in anesthetized animals. Thus, the overall aims of present study were to determine (1) whether normobaric hyperoxia could induce delayed preconditioning-like effects against arrhythmia and infarction in anesthetized rats; and (2) whether these effects depend on the duration of hyperoxia and oxygen concentration.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250 to 300 g were housed under standard conditions with free access to food and water. The investigation was approved by the Animal Ethics Committee of Baqiyatallah University of Medical Sciences.

Surgical Procedure

All animals were anesthetized with sodium pentobarbital (50 $mg \cdot kg^{-1}$, i.p.) and the anesthetic agent was further administered throughout the experimental protocol as needed. The neck was opened with a ventral midline incision and a tracheotomy was performed. The rats were artificially ventilated with room air by a Harvard small animal ventilator (Harvard Apparatus, South Natica, MA) at a rate of 70 to 80 per min and tidal volume of 1 $mL \cdot kg^{-1}$ to maintain blood PO_2, PCO_2, and pH in the normal physiological range. Rectal temperature was continuously monitored and maintained at 37 ± 0.5°C. A standard limb lead II electrocardiogram was monitored and recorded throughout the experiment, using a computerized data acquisition system (ML750 PowerLab/8SP; ADInstruments Pty Ltd, Castle Hill, Australia). Catheters flashed with heparinized saline were inserted into the left carotid artery and tail vein (Angiocat 23; Suppa, Tehran, Iran) for monitoring of blood pressure and administration of saline, respectively. A left thoracotomy was performed in the fourth intercostal space, and pericardium was opened to expose the heart. A 5-0 silk suture was passed around the left main descending coronary artery and the ends of it were threaded through a small polyethylene tube to form a snare. Any animal in which this procedure produced dysrhythmias or a sustained fall in mean arterial pressure to less than 50 mmHg were excluded from the study. Following a stabilization period of 20 min, the snare was tightened and held in place with another tube. Myocardial ischemia was confirmed by regional cyanosis, ST elevation in the electrocardiogram, and a drop in blood pressure. Following 30 min ischemia, reperfusion was established by releasing the snare and similarly confirmed by observation of hyperemia over the heart surface.

Determination of Infarct Size and Area at Risk

After 30 min regional ischemia and 90 min reperfusion, the coronary artery was reoccluded and 2 mL of 2% solution of Evans Blue dye (Sigma, St. Louis, MO) was injected into the tail vein to identify the nonperfused area, also known as area at risk (AAR), from perfused area. The rats were then killed and their hearts were excised and frozen overnight. The atria and right ventricle were removed and the left ventricle (LV) was cut into transverse slices of 2 mm thickness from the apex to the base. The natural uncolored AAR (pink) was separated from the colored nonischemic area (blue). Tissue samples were then incubated with a 1% solution of 2,3,5 triphenyltetrazolium chloride [TTC (Sigma) in 0.1 M phosphated buffer, pH = 7.4] for 20 min at 37°C, and subsequently fixed in 10% phosphate-buffered formalin for 24 h. Viable myocardium is stained red by TTC, whereas necrotic myocardium appears as pale yellow. In each slice, areas at risk and infarcted areas were determined by computerized planimetry using an image analysis software (Image Tool, University of Texas, San Antonio, TX). Infarct size was expressed as percentage of the AAR ([Infarcted area/AAR] × 100).

Assessment of Ventricular Arrhythmias

Ischemia-induced ventricular arrhythmias were determined in accordance with the Lambeth conventions [16]: premature ventricular beat (VEB) as a discrete and identifiable premature QRS complex (premature with respect to the P wave), ventricular tachycardia (VT) as a run of four or more VEB at a rate faster than the resting sinus rate and ventricular fibrillation (VF) as a signal for which individual QRS deflection can no longer be distinguished from one another. Complex forms (bigeminy and salvos) were added to VEB count and not analyzed separately.

Measurement of Plasma Creatine Kinase (CK) and Lactate Dehydrogenase (LDH) Activity

At the end of reperfusion, blood samples were taken and plasma was collected and stored at −40°C. CK and LDH activities were measured by standard kits (Parsazmoon, Tehran, Iran), using an autoanalyzer and expressed as unit per milliliter (U/mL).

Experimental Protocols

In the first series of experiments, the rats in hyperoxic groups were exposed to normobaric hyperoxia ($O_2 > 95\%$) for 15 min (H15 group, $n = 11$), 30 min (H30 group, $n = 12$), 60 min (H60 group, $n = 13$), 120 min (H120 group, $n = 12$), or 180 min (H180 group, $n = 10$) in an air-tight container with a small inlet and outlet. The container was continuously ventilated with nearly pure oxygen. The O_2 concentration of the container was continuously monitored with an oxygen meter (DO-5510, Lutron, Taiwan). All rats were then allowed to breathe normal air for 24 h before their hearts were subjected to 30 min regional ischemia and 90 min reperfusion. The rats in normoxic group (NOR, $n = 14$) were placed in the same container for 60 min with an inflow of normal air (it was determined in pilot studies that different exposure times to normobaric normoxia in this container has no effect on various parameters of ischemic heart injury).

Since 120 min of exposure to $O_2 > 95\%$ was the optimum time for cardioprotection, only this exposure time was considered for further

TABLE 1

Hemodynamic Parameters

Groups	Baseline		Ischemia		Reperfusion	
	HR	MBP	HR	MBP	HR	MBP
Experiment 1						
NOR	360 ± 12	93 ± 4.9	364 ± 9.9	71 ± 4.1**	355 ± 8.5	87 ± 3.3
H15	354 ± 13.7	96 ± 6.0	353 ± 10.4	75 ± 4.9*	348 ± 13.3	88 ± 4.7
H30	349 ± 9.8	95 ± 6.1	377 ± 9.3	75 ± 5.8*	351 ± 8.2	90 ± 5
H60	357 ± 8.5	93 ± 4.0	360 ± 8.2	74 ± 4.48**	363 ± 7.9	85 ± 3.5
H120	383 ± 9.6	90 ± 4.2	376 ± 8.2	70 ± 3.4**	360 ± 9.5	80 ± 3.7
H180	358 ± 7.7	92 ± 4.6	368 ± 6.9	75 ± 3.4**	343 ± 5.7	86 ± 3.9
Experiment 2						
H40%	361 ± 11.4	89 ± 2.6	355 ± 10.8	70 ± 2.6***	358 ± 7.1	83 ± 3.3
H60%	349 ± 11.7	91 ± 3.3	378 ± 13.3	71 ± 4.4**	362 ± 9.6	82 ± 3.9
H80%	342 ± 8.9	88 ± 3.2	354 ± 10.1	69 ± 3.6**	355 ± 7.4	80 ± 3.1

Note. Data are presented as mean ± SEM.

HR = heart rate; MBP = mean arterial blood pressure; NOR = normoxic group and H15, H30, H60, H120, and H180 mean 15, 30, 60, 120, and 180 min of >95% hyperoxia H40%, H60%, H80%, and H95% mean 120 min of 40%, 60%, 80%, and 95% hyperoxia, respectively, and 24 h prior to the subsequent prolonged regional ischemia.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$ compared with the baseline within the same group.

studies on different oxygen concentrations; so the experimental groups in the second series of experiments were as the follows:

(1) NOR ($n = 14$); (2) 120 min of 40% hyperoxia (H40%, $n = 10$); (3) 120 min of 60% hyperoxia (H60%, $n = 13$); (4) 120 min of 80% hyperoxia (H80%, $n = 11$); (5) 120 min of 95% hyperoxia (H95%, $n = 12$).

In additional 27 rats, arterial PO_2 (PaO_2) was checked by a gas analyzer (Ciba-Corning 865 blood gas analyzer, Medfield, MA) in similar conditions to the experimental groups at the end of exposure to the hyperoxic or normoxic conditions ($n = 3$ in each condition).

Statistical Analysis

Data are expressed as mean ± SEM or the percentage of incidence. Statistical comparison of means between groups was made by one-way analysis of variance followed by Tukey's post-hoc test. Within each group, data of homodynamic parameters were compared using one-way repeated measures analysis of variance. The incidences of VF and survival rate were compared using the Fisher exact test. $P < 0.05$ was considered to be significant.

RESULTS

The Effect of the Duration of Hyperoxia (O_2 > 95%) on Ischemic Injury

PaO_2 significantly increased following exposure to hyperoxia but there was no significant difference between PaO_2 values in various exposure times (92.8 ± 3.3, 408 ± 9.4, 449 ± 11.5, 433 ± 27, 444 ± 13.6, and 435 ± 18 in NOR, H15, H30, H60, H120, and H180 groups, respectively).

Hemodynamic Parameters

Table 1 summarizes the hemodynamic data. There were no significant differences at baseline values for heart rate and mean arterial blood pressure among the experimental groups. Ischemia caused a marked reduction in blood pressure without any significant effect on the heart rate. Blood pressure was nearly restored to the baseline level during reperfusion period.

Infarct Size

Figure 1 shows AAR and infarct size following 30 min regional ischemia and 90 min reperfusion. There was no marked difference in AAR among the groups. Infarct size did not reduce in H15 (49.8 ± 2.5), H30 (45.8 ± 2.3), and H60 (37 ± 2.7) groups but it signifi-

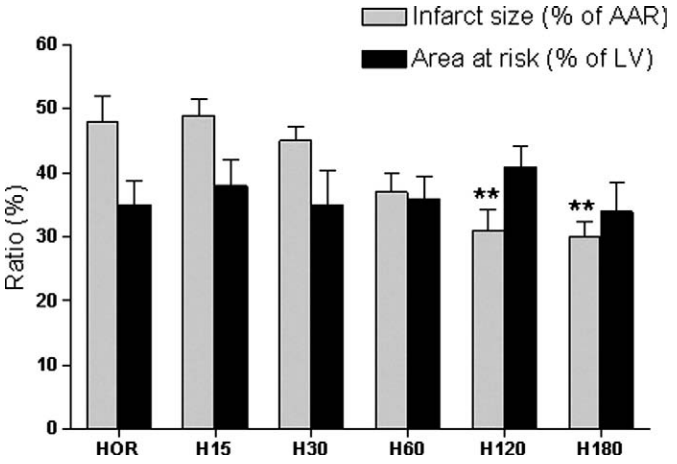

FIG. 1. Infarct size and AAR following 30 min regional ischemia and 90 min reperfusion in anesthetized rats. Left ventricular LV, NOR, and H15, H30, H60, H120, and H180 mean 15, 30, 60, 120, and 180 min of >95% hyperoxia 24 h prior to the subsequent prolonged regional ischemia. Data are mean ± SEM. **$P < 0.01$ *versus* NOR group.

TABLE 2

Plasma LDH Activity and CK Activities at the End of Reperfusion

Groups	LDH (U/mL)	CK (U/mL)
Experiment 1		
NOR	0.62 ± 0.03	1.52 ± 0.06
H15	0.60 ± 0.04	1.049 ± 0.08
H30	0.55 ± 0.02	1.42 ± 0.06
H60	0.49 ± 0.04	1.27 ± 0.07
H120	0.40 ± 0.03**	1.03 ± 0.05*
H180	0.41 ± 0.04**	1.01 ± 0.06*
Experiment 2		
H40%	0.62 ± 0.03	1.52 ± 0.02
H60%	0.58 ± 0.03	1.55 ± 0.02
H80%	0.55 ± 0.02	1.55 ± 0.04

Note. Data are presented as mean ± SEM.

NOR = normoxic group and H15, H30, H60, H120, and H180 mean 15, 30, 60, 120, and 180 min of >95% hyperoxia H40%, H60%, H80%, and H95% mean 120 min of 40%, 6%0, 80%, and 95% hyperoxia, respectively, and 24 h prior to the subsequent prolonged regional ischemia.

* $P < 0.05$.

** $P < 0.01$, compared with the NOR group.

cantly reduced in H120 (31.4 ± 3.3) and H180 (30 ± 2.4) groups compared with NOR group (48.1 ± 4). There was no significant difference between the infarct size in H120 and H180 groups.

Plasma CK and LDH Activities

Plasma CK and LDH activities are shown in Table 2. In rats pretreated with hyperoxia for 15, 30, and 60 min, these parameters did not change significantly compared with NOR group, however rats exposed to hyperoxic environment for 120 and 180 min had plasma CK and LDH activities significantly less than NOR group. There was no significant difference between H120 and H180 groups in this regard.

Ischemia-Induced Arrhythmias

Figure 2 represents the number of VEB (A) and VT (B) episodes and the incidence of VF (C) during the ischemic period. The rate of these arrhythmias in H15 and H30 groups had no significant difference with NOR group. Exposure to hyperoxic environment for 120 and 180 reduced the incidence of these dysrhythmias. The exposure time of 60 min only significantly reduced the incidence of VF. It should be noted that there were no considerable differences in reperfusion-induced arrhythmias in the present work.

Survival at the End of Reperfusion

As Fig. 2D shows, 58%, 64%, and 59% of animals in NOR, H15, and H30 groups completed ischemia-reperfusion protocol, respectively. Survival rate significantly increased in H60 (77%), H120 (92%), and H180 (90%) groups.

Effect of Different Oxygen Concentrations

Since 120 min exposure to hyperoxic environment was the optimum duration in the first series of experiments, this exposure time was chosen to determine whether lower oxygen concentrations could protect the heart against infarction and/or arrhythmia.

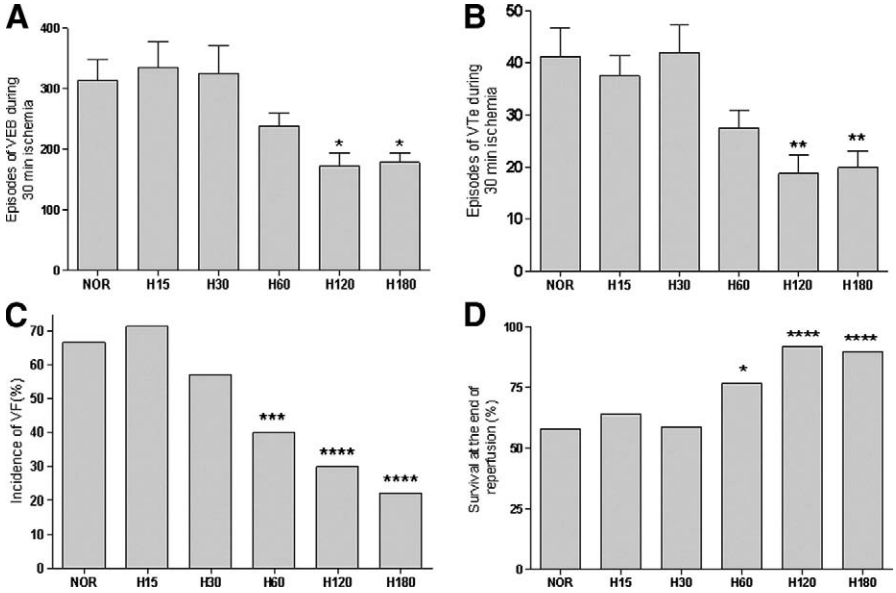

FIG. 2. VEB (A) and VT (B) episodes as mean ± SEM and the incidence of VF (C) during 30 min regional ischemia and survival rate (D) at the end of reperfusion in anesthetized rats. NOR group and H15, H30, H60, H120, and H180 mean 15, 30, 60, 120, and 180 min of >95% hyperoxia 24 h prior to the subsequent prolonged regional ischemia. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ *versus* NOR group.

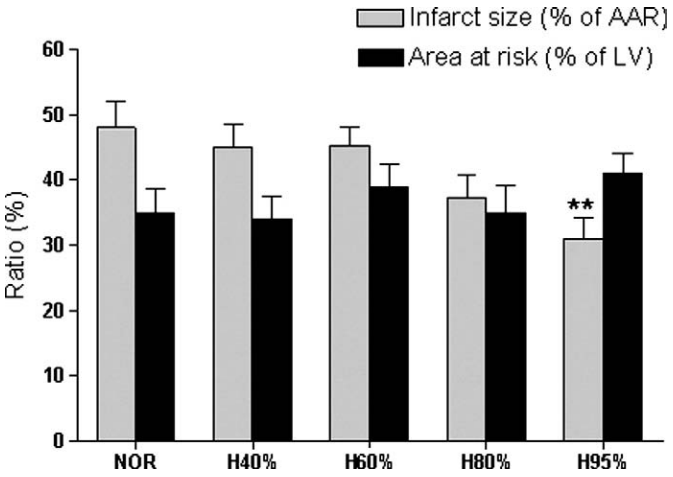

FIG. 3. Effects of different oxygen concentrations on infarct size and AAR 24 h prior to prolonged regional ischemia in anesthetized rats. LV, left ventricular; NOR group, and H40%, H60%, H80%, and H95% mean 120 min of 40%, 60%, 80%, and 95% hyperoxia, respectively, 24 h prior to the subsequent prolonged regional ischemia. Date are mean ± SEM $^{**}P < 0.01$ *versus* NOR group.

PaO_2 significantly increased following exposure to different oxygen concentrations and higher O_2 concentrations, resulting in significantly greater PaO_2 levels (188 ± 7.2, 254 ± 11.5, 323 ± 16.3, and 444 ± 13.6 in H40%, H60%, H80%, and H95%, respectively).

AAR, Infarct Size, CK, and LDH Activities

There was no significant difference in AAR among all experimental groups (Fig. 3). When the oxygen concentration decreased to 80%, 60%, and 40%, the infarct sizes did not have any significant difference compared with NOR group (there was only a nonsignificant decrease in H80% group). There was also no marked difference in plasma CK and LDH activities among the groups (Table 2).

Ischemia-Induced Arrhythmias

As indicated in Fig. 4A–C, episodes of VEB and VT number decreased in H80% group nonsignificantly. The incidence of VF was significantly lower in H80% group (37.5%) compared with NOR group (66.6%). When the O_2 concentration decreased further to 60% and 40%, there was no marked reduction in arrhythmias compared with NOR group.

Survival Rate at the End of Reperfusion

Exposure to 40% and 60% oxygen did not alter the survival rate, however, when the oxygen concentration was increased to 80%, survival at the end of reperfusion increased from 58% in NOR group to 72% in H80% group (Fig. 4D).

DISCUSSION

The main finding of this study in anesthetized rats was that pre-exposure of rats to normobaric hyperoxia ($O_2 > 95\%$) can protect the heart against subsequent ischemia-induced arrhythmia and infarction. These effects were dependent on both duration of hyperoxic exposure and the concentration of oxygen in inspired air. Hyperoxic preconditioning also reduced plasma CK and LDH activities, as indexes of cell death, in a duration- and concentration-dependent manner.

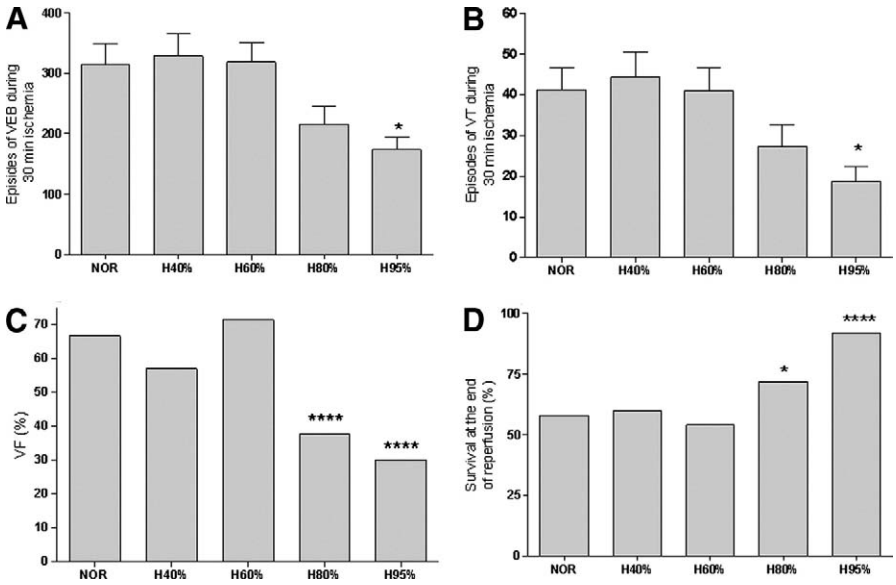

FIG. 4. VEB (A) and VT (B) episodes as mean ± SEM and the incidence of VF (C) during 30 min regional ischemia and survival rate (D) at the end of reperfusion in anesthetized rats. NOR group and H40%, H60%, H80%, and H95% mean 120 min of 40%, 60%, 80%, and 95% hyperoxia respectively 24 h prior to the subsequent prolonged regional ischemia. $^{*}P < 0.05$; $^{****}P < 0.0001$ *versus* NOR group.

ROS generated during brief IR episodes have been recognized as possible trigger factors for initiation of late IPC [17], and studies have demonstrated that antioxidants abolished the development of late preconditioning in pigs and rabbits [18, 19].Similarly, intracoronary infusion of a ROS-generating solution can elicit a late preconditioning response [7]. The mechanisms by which ROS are responsible for this cardioprotective effect are not well recognized, however, it seems that ROS both indirectly, through the activation of protein kinases, and directly lead to the activation of some transcription factors like nuclear factor-kappa B, which in turn trigger genes related to tissue protection, including Bcl-2 [10, 12].

Under normal physiological conditions, 1% to 4% of available oxygen in the body is converted to free radicals. This process becomes greatly accelerated in conditions such as breathing hyperoxic gas mixture, which lead to O_2 tensions higher than normal values [20]. Therefore, being in hyperoxic environment may lead to the observed cardioprotective effects via production of ROS in extraordinary amounts. Although in this study we did not examine any possible underlying mechanism, several studies addressed that hyperoxia induces its protective effects through ROS production [9, 12, 21].

It has been shown that pre-exposure to normobaric hyperoxia could be protective against subsequent brain [14], isolated heart [8, 9], spinal cord [22], and renal IR injury [15]. Most of the previous studies focused on the early phase of hyperoxia-induced preconditioning against heart IR injury [8–11, 23, 24]. There are only some reports in Tahepold *et al.* studies [9] about amelioration of postischemic cardiac function and lower incidence of irreversible reperfusion-induced arrhythmias in hearts isolated from rats that have been in hyperoxic environment for 1 or 3 h, 24 h before ischemia. Tahepold and colleagues have worked on a global model of isolated heart ischemia and have not report in their elegant studies the infarcted area in experimental groups related to the late phase of hyperoxia-induced preconditioning. For the first time, our results show in an *in vivo* model of regional heart ischemia that normobaric hyperoxia can induce a late preconditioning effect against heart IR injury. Consistent with studies using the same animal model of cardiac IR injury, in present study there were no marked reperfusion-induced arrhythmias [3]. Although we could not measure ROS production following hyperoxia pretreatment, it appears hyperoxia increases ROS production reaching the threshold level at 80% to 95% PO_2 for 120 min. Thus, further studies need to determine how high ROS production has to be before triggering the late phase of preconditioning. Recently, Brueckl and coworker using *in situ* lung preparation have shown that hyperoxia increases ROS production in a time- and concentration-dependent manner [20].

Ischemia itself, like reperfusion, can cause lethal ventricular arrhythmias, including, VT and VF [2, 3]. In the present work, we indicated that pretreatment with oxygen could reduce the incidence of ischemic arrhythmias and mortality rate in anesthetized rats. Although these effects were also duration- and concentration-dependent, an oxygen concentration of 80% was sufficient for marked reduction in the rate of severe ischemia-induced arrhythmias.

Most clinical studies have concluded that IPC via cross clamping of aorta have protective effects on human myocardial tissue. For example, Illes and Swoyer [25] observed improved cardiac index and absence of inotropic support after preconditioning. In patients undergoing valve surgery, preconditioning is protective, as this was evident by improved cardiac function and reduced CK release [26]. However it seems that application of brief episodes of IR will never be accepted in clinical practice because most surgeons have an antagonism against repeated episodes of cross-clamping the aorta because of a possible chance of embolism. IPC may also prolong the surgical procedure by 15 to 30 min. In contrast, breathing gas with high oxygen content for 1 to 3 h is clinically acceptable, inexpensive, and may have protective effects on other vital organs, such as kidney and brain tissue.

It has been documented that hyperoxygenation therapy is safe for humans if administrated according to the standard protocols. In this regard, administration of 80% oxygen for less than 24 h is considered safe [27]. Since in rats the first histological findings of oxygen toxicity will appear in the lung tissue only after about 40 h of pure oxygen administration [8], it seems that there is a wide safety window between protective effects of oxygen pretreatment and its toxic effects. This may be also true in humans and remains to be investigated. Hyperoxic preconditioning of vital organs, such as heart, prior to organ transplantation is also a potential clinical application for this benign protocol, because sometimes organ IR injury may not be preventable in the course of transplantation procedure [9].

In conclusion, we report that exposing rats to normobaric hyperoxia 24 h before a subsequent prolonged ischemia leads to a marked protective effect in this *in vivo* rat model of heart IR injury, including decreased cardiac cell death and reduction of sever arrhythmias. These beneficial effects depend both on duration of hyperoxic exposure and the oxygen concentration in the environment.

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