Delayed Cardioprotective Effects of Hyperoxia Preconditioning Prolonged by Intermittent Exposure

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Submitted for publication July 21, 2008

Background. In our previous study, it was indicated that pre-exposing rats to normobaric hyperoxia could induce a late preconditioning against infarction and arrhythmia. In this study, attempts were made to know whether the intermittent pre-exposure to the same environment could prolong the late phase of hyperoxia preconditioning.

Methods. In the first series of experiments, rats were divided into five groups; group 1 was pre-exposed to normal air (NOR) and the other groups to hyperoxic air (O2 > 95%, 120 min once a d) 12, 24, 48, and 72 h (H12, H24, H48, and H72 groups) before 30 min ischemia. In the second series of experiments, rats were pre-exposed to intermittent hyperoxic air (1, 2, or 3 consecutive d) at different times before being subjected to ischemia (H48, H2-48, H2-72, H3-72, and H3-96 groups). The infarct size was measured by triphenyltetrazolium chloride staining, and lead II of electrocardiogram recorded to monitor ischemic-induced arrhythmia.

Results. Compared with NOR group, the infarct size and incidence of arrhythmia were reduced significantly in H24 and H48 groups. When the exposure periods were enhanced to 2 d, the infarct size did not decrease significantly, but the incidence of arrhythmia reduced. When the pre-exposure times were enhanced to 3 d, both the infarct size and incidence of arrhythmia decreased significantly in H3-72 group, but not in H3-96 group.

Conclusion. These results show that the late phase of hyperoxia preconditioning may last for more than 48 h and prolong by intermittent per-exposure to the same environment. © 2010 Elsevier Inc. All rights reserved.

Key Words: hyperoxia; preconditioning; intermittent; infarct; arrhythmia.

INTRODUCTION

Ischemic-reperfusion (I/R) injury is a major complication occurring in heart stroke, coronary artery revascularization, and heart transplantation [1, 2]. Reactive oxygen species (ROS) were initially thought to play a role in the pathogenesis of I/R injury [2–7]. Recent studies involving ischemic preconditioning (IPC) have identified ROS as potential mediators for the cardioprotective effects observed following this phenomenon, as well as other mediators, like adenosine, nitric oxide, etc. [3–6].

Likewise, under normal physiological conditions, 1% to 4% of available oxygen is converted to ROS. ROS level is increased to higher levels in proportion to dose-dependent of oxygen concentration [8]. Several studies have reported that transient pre-exposure to high levels of oxygen concentrations (O2 > 90 %), as a low-graded systemic oxidative stress stimulus, could induce preconditioning-like effects in heart [9–11], brain [12, 13], spinal cord [14, 15], and liver [16], which are comparable to the effects of IPC. Our colleagues have also
shown that pre-exposure to normobaric hyperoxia could induce protective effects on rat kidney and brain [17, 18]. Previously, we have also shown that pre-exposing rats to normobaric hyperoxia (O₂ > 95%) for at least 120 min protects the anesthetized rat hearts against infarction and ischemic arrhythmia in a time- and concentration-dependent manner 24 h later [19]. This is consistent with the works of Tahepold’s group using isolated rat and mouse hearts [20, 21].

The purpose of this study was to investigate (1) how long does the late phase of hyperoxic preconditioning last in the heart of anesthetized rats and to determine (2) whether the intermittent normobaric hyperoxia (pre-exposure to the same hyperoxic environment 24 h prior to the termination of the previous late phase of hyperoxic preconditioning) can potentiate and prolong this phase.

**MATERIAL AND METHODS**

**Animals**

Male Wistar rats (250–300 g) were housed at 12:12-h light-dark cycles inside Baqiyatallah University animal house. They were fed ad libitum and conditioned in this nonstressful environment for at least 1 wk. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and was approved by the ethics committee for animal research at Baqiyatallah University.

**Surgical Preparation**

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Rectal temperature was monitored and maintained at 37.5 °C. The neck was opened with a ventral midline incision and tracheotomy was performed, followed by intubation. The animals were artificially ventilated at 80 strokes/min with a tidal volume of 1 mL/100 g to maintain PO₂, PCO₂, and pH in the normal physiological range. The tail vein was cannulated with an angiocat (yellow color) for the infusion of Evans blue solution. Left carotid artery, likewise, was dissected and cannulated for continuous arterial pressure monitoring. Electrocardiographic leads were attached to subcutaneous electrodes to monitor limb lead II continuously. A left thoracotomy was performed in the fourth intercostal space and the pericardium was opened to expose the heart. A 5-0 silk suture with an atraumatic needle was then passed around the left anterior descending coronary artery (LAD), midway between the atrioventricular groove and the apex, and a snare formed by passing both ends of the suture through a piece of polyethylene tube. After 15 to 20 min stabilization, LAD was occluded by clamping the snare against the surface of the heart (30 min). Ischemia was confirmed by the regional cyanosis downstream of the occlusion, by the ST elevation, and by the reduced blood pressure. Reperfusion (90 min) was confirmed by the lack of cyanosis in that region. Exclusion criteria were dysrhythmia and/or a sustained fall in mean arterial blood pressure below 60 mmHg before occlusion.

**Infarct Size Measurement**

At the end of 90 min reperfusion, the LAD was reoccluded and 2 mL of a bolus of atraumatic needle with an Evans blue solution was injected as a bolus into the tail vein and allowed to perfuse the nonischemic portion of the heart as the blue area. The entire heart was then excised, rinsed of excess blue dye, trimmed of atrial tissue and frozen at –20 °C. The hearts were then sliced into 2 mm thick transverse sections from the apex to the base. The ischemic area or area at risk (AAR) was cut down and incubated in 1% triphenyltetrazolium chloride (TTC) solution in isotonic pH 7.4 phosphate buffer at 37 °C for 20 min. Samples were fixed in 10% formalin for 24 h to enhance the contrast. An image was obtained from both sides of every slice and all calculations from one heart (using Image Tool Software, San Antonio, TX) were averaged into one value for statistical analysis. Infarct size, as the white color area, was expressed as a percentage of AAR (as the red color area).

**Measurement of Plasma Creatine Kinase (CK) Activity**

At the end of reperfusion, the blood samples (2 mL) were collected from carotid artery. Plasma was obtained by centrifugation at 2500 g for 15 min (4 °C). Plasma CK activity was determined by standard kits (Parsazmoon, Tehran, Iran) using an autoanalyzer and expressed as unit per milliliter (U/mL).

**Ischemic-Induced Arrhythmia Analysis**

Arrhythmia were defined according to the Lambeth conventions [22] in which ventricular premature beats (VEBs) were defined as discrete and identifiable premature QRS complex and ventricular tachycardia (VT) as a run of four or more consecutive VEBs. Ventricular fibrillation was defined as a signal where individual QRS deflection could not easily be distinguished from each other and where heart rate could no longer be measured. Complex forms (bigeminy and salvos) were added to VEBs count and not analyzed separately.

**Experimental Protocol**

In our previous study [19], it was observed that 120 min of exposure to normobaric hyperoxia 95% is the optimum time and concentration to induce late hyperoxic preconditioning. These time and oxygen concentrations were selected for the present study as follow:

- **Group NOR:** normoxia (21% O₂ for 120 min) 24 h before ischemia (n = 14)
- **Group H12:** hyperoxia (≥95% O₂ for 120 min) 12 h before ischemia (n = 11)
- **Group H24:** Hyperoxia (≥95% O₂ for 120 min) 24 h before ischemia (n = 12)
- **Group H48:** hyperoxia (≥95% O₂ for 120 min) 48 h before ischemia (n = 9)
- **Group H72:** hyperoxia (≥95% O₂ for 120 min) 72 h before ischemia (n = 12)

Rats in hyperoxic groups were exposed to normobaric hyperoxia (O₂ ≥ 95%) for 120 min in an air-tight chamber with a small inlet and outlet. The chamber was continuously ventilated with nearly pure oxygen. The CO₂ concentration of chamber was continuously monitored with an oxygenmeter (Lutron Do5510; Taiwan). To prevent CO₂ retention, soda lime was placed in the chamber. All rats were then allowed to breathe normal air for 12, 24, 48, and 72 h before their hearts were subjected to 30 min ischemia and 90 min reperfusion. The normoxic group was placed in the same container for 120 min, with an inflow of normal air.

Second series of experiments were designed to investigate whether intermittent hyperoxia preconditioning (pre-exposure to the same normobaric hyperoxia 24 h prior to the end of the late phase of the last preconditioning in which the infarct size was assumed as a gold standard) could potentiate and prolong the protective effects of hyperoxia preconditioning. In these series, each group was chosen according to the results of previous group as follows:

- **Group NOR:** rats were exposed to normoxia for 120 min once per d, 24 h before being subjected to I/R (n = 14)
Group H48; rats were exposed to hyperoxia for 120 min once per d, 48 h before being subjected to I/R (n = 9).

Group H2-48; rats were exposed to hyperoxia for 120 min once per d for 2 consecutive d, 48 h before being subjected to I/R (n = 8).

Group H2-72; rats were exposed to hyperoxia for 120 min once per d for 2 consecutive d, 72 h before being subjected to I/R (n = 7).

Group H3-73; rats were exposed to hyperoxia for 120 min once per d for 3 consecutive d, 72 h before subjected to I/R (n = 7).

Group H3-96; rats were exposed to hyperoxia for 120 min once per d for 3 consecutive d, 96 h before being subjected to I/R (n = 7).

**Statistical Analysis**

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

**RESULTS**

First series of Experiments

**Hemodynamic Parameters**

The hemodynamic parameters are summarized in Table 1. There was no significant difference at baseline values for heart rate and mean arterial blood pressure among all the groups. During ischemic period, the only factor that reduced significantly was the arterial blood pressure in all the groups, which returned approximately to normal values.

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ischemia</th>
<th>Reperfusion</th>
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<tr>
<td></td>
<td>HR (b/min)</td>
<td>MBP (mmHg)</td>
<td>HR (b/min)</td>
</tr>
<tr>
<td>First series experiments</td>
<td></td>
<td></td>
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<tr>
<td>NOR</td>
<td>360 ± 12</td>
<td>95 ± 4.9</td>
<td>364 ± 9.9</td>
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<tr>
<td>H12</td>
<td>359 ± 11</td>
<td>96 ± 3.5</td>
<td>366 ± 13</td>
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<tr>
<td>H24</td>
<td>383 ± 9</td>
<td>89 ± 4.2</td>
<td>376 ± 8</td>
</tr>
<tr>
<td>H48</td>
<td>363 ± 9</td>
<td>89 ± 4.3</td>
<td>374 ± 9</td>
</tr>
<tr>
<td>H72</td>
<td>383 ± 13</td>
<td>92 ± 4.8</td>
<td>353 ± 11</td>
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<tr>
<td>Second series experiments</td>
<td></td>
<td></td>
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<tr>
<td>H2-48</td>
<td>354 ± 12</td>
<td>91 ± 3.8</td>
<td>361 ± 8</td>
</tr>
<tr>
<td>H2-72</td>
<td>342 ± 8</td>
<td>93 ± 4.5</td>
<td>351 ± 7</td>
</tr>
<tr>
<td>H3-72</td>
<td>368 ± 10</td>
<td>89 ± 4.3</td>
<td>382 ± 12</td>
</tr>
<tr>
<td>H3-96</td>
<td>351 ± 14</td>
<td>97 ± 5.1</td>
<td>360 ± 10</td>
</tr>
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</table>

NOR: normoxic groups; H12, H24, H48, and H72 referring to rats exposed to hyperoxic air (O2 > 95%)12, 24, 48, and 72 h later, respectively. H48, H2-48, H2-72, H3-72, and H3-96 referring to rats were pre-exposed to hyperoxia for 1, 2, or 3 d (120 min once a d) at different times before a 30-min ischemia followed by a 90-min reperfusion. 
P < 0.05. 
P < 0.01 compared with NOR.

**Infarct Size and Plasma CK Activity**

Figure 1A represents infarct size as a percentage of area at risk. There was no significant difference in the area at risk among all the groups (data not shown). The infarct size was 48.1% ± 4% and 44.2% ± 4.2% in control and H12 groups, respectively. It was significantly reduced in H24 (31.3% ± 3.3%) and H48 (27.7% ± 2.9%) groups. There was no marked difference among the NOR and H72 (41.8% ± 3.6%) groups. The plasma CK activity was in consistent to infarct size (Fig. 1B). It was reduced significantly in H24 and H48 groups. Plasma CK activity was 1.52 ± 0.06, 1.55 ± 0.05, 1.03 ± 0.05, 1.26 ± 0.04, and 1.54 ± 0.06 U/mL in NOR, H12, H24, H48, and H72 groups, respectively.

**Ischemic I induced Arrhythmia**

The episodes of VEBs decreased from 340 ± 35 and 293 ± 38 in NOR and H12 groups, to 173 ± 20 and 190 ± 23 in H24 and H48 groups, respectively. The difference between NOR and H72 (274 ± 36) groups was...
not significant (Fig. 2A). The episodes of VT only reduced in H24 group. It was 41.3 ± 5.3, 42.4 ± 6.6, 18.8 ± 3.8, 30.2 ± 5.1, and 39 ± 7.7 in NOR, H12, H24, H48, and H72 groups, respectively (Fig. 2B). The incidences of VF were 66.6% in NOR group, 85% in H12 group, and 62.5% in H72 group without any significance, whereas, it had a marked reduction in H24 (30%) and H48 (42.8%) groups (Fig. 2C).

Survival Rate
The survival rate was 58% in NOR and 63% in H12 groups. It was increased to 92% in H24 and to 77.7% in H48 group. There was no difference between NOR and H72 (77.7%) groups (Fig. 2D).

Second Series of Experiments
In these experiments we hypothesized that the intermittent pre-exposure to hyperoxia could potentiate and prolong the late phase of hyperoxia preconditioning. In order to examine this hypothesis, we modified the parameters according to the data obtained from the first series of experiments. For instance, we did not observe anti-infarct effect 72 h after exposures for 2 consecutive d, so the time of exposure was changed from 2 d to 3 d.

Hemodynamic Parameters
Table 1 shows the heart rate and mean arterial blood pressure at the baseline values among all the groups. During ischemic period, the only factor that reduced significantly was the arterial blood pressure in all the groups, which returned approximately to normal values.

Infarct Size
As Fig. 3A shows, there was a significant difference between the infarct size of H48 (27.7% ± 2.9%) and H2-48 (24.2% ± 2.5%) compared with NOR group. There was no marked difference between H48 and H2-48 groups. The infarct size was 36.7% in H72 group. When the pre-exposure times were increased to 3 consecutive d (H3-72 group), the infarct size reduced significantly 72 h later (28.5% ± 3.6%), but not 96 h later (H3-96 group; 46.1% ± 4%). The plasma CK activity had a similar pattern with the changes in the infarct size (Fig. 3B). It was 1.52 ± 0.06, 1.26 ± 0.0, 1.13 ± 0.07, 1.37 ± 0.07, 1.13 ± 0.06, and 1.44 ± 0.08 U/mL in NOR, H48, H2-48, H2-72, H3-72, and H3-96 groups, respectively.

Ischemic-Induced Arrhythmia
Figure 4A–C show the episodes of VEB and VT and the incidence of VF during ischemic period.
episodes of VEBs decreased from 314 ± 25 in NOR group to 190 ± 22, 183 ± 25, and 167 ± 22 in H2-48, H2-72, H3-72, and H3-96 groups, respectively (Fig. 4A). There was no marked difference between NOR and H2-72 (252 ± 22) and H3-96 (279 ± 32) groups.

Although there was no marked significant differences between all the groups in VT episodes, however, the pattern of changes was similar to that of VEB episodes (Fig. 4B). It was 41.3 ± 5.3, 30.25.1, 21.52.9, 30.55.8, 23.44.6 and 35.74.8 in NOR, H48, H2-48, H2-72, H3-72 and H3-96, respectively. Figure 4C demonstrates that VF% decreased significantly in all the groups except H3-96 group. It was 66.6, 42.8, 28, 42.8, 14.2, and 57.1 % in NOR, H48, H2-48, H2-72, H3-72, and H3-96 groups, respectively. VF% had a more reduction in H2-48 and H3-72 groups than H48 group.

Survival Rate

Survival rate was 58% in NOR group and it was increased in all the groups considerably compared with NOR group, except H3-96 group (Fig. 4D). It was 58, 77.7, 88, 78.8, 100, and 64% in NOR, H48, H2-48, H2-72, H3-72, and H3-96 groups, respectively. The survival rate had a significant increase in H2-48 and H3-72 groups than H48 group.

DISCUSSION

Our salient findings of this study were: (1) exposing rats to normobaric hyperoxia for at least 120 min may protect their heart against ischemic-induced arrhythmia and infarction, which lasts for approximately 48 h; (2) intermittent pre-exposure to the same hyperoxic environment for at least 3 consecutive d (120 min, once a d), could prolong this protection. These protections were consistent with plasma CK activity. Increasing the protection of tissues against a prolonged ischemic injury has a great interest in clinical realm. Since Murry et al. in 1986 [23], it has been reported that preconditioning by sublethal stimulus induces a biphasic tolerance against a subsequent lethal insult: with an early phase that lasts for 2 to 3 h and a delayed phase that initiates about 24 h later and continues up to 72 h [19, 24]. Several studies using infusion of low concentrations of oxygen radicals [3, 4, 6, 25] and administration of antioxidants [26–28] have demonstrated that ROS acts as the trigger of preconditioning process. Recent studies have shown that pre-exposure to normobaric hyperoxia, as a low graded systemic oxidative stress, could induce preconditioning like-effects in rat and mouse hearts [9, 11, 29–32]. In our previous study, we demonstrated that pre-exposure to normobaric hyperoxia could enhance the tolerance of myocardium against ischemic induced arrhythmia and infarction 24 h later in anesthetized rats [19]. The results were consistent with the report of Tahepold’s group using isolated rat and mouse heart [20, 21]. In this study, we showed that these protective effects of hyperoxia pre-treatment last for about 48 h in anesthetized rats. There is no study about the length of the late phase of normobaric hyperoxia in different organs, but our study was similar to the study of Choi’s group using hyperbaric hyperoxia in the isolated rat hearts [10].

As it was noted in the previous section, the delayed phase of preconditioning lasts for up to 72 h [10], so potentiating and prolonging the late phase of hyperoxia preconditioning might cause a great interest in clinical medicine. To our knowledge, there is no such study about prolonging the late phase of hyperoxia-induced preconditioning. Recently, Kaljusto and co-workers have indicated that the combination of the late phase of dexamethasone and the early phase of hyperoxia preconditioning improved postischemic heart function, however, did not reduce infarct size compared with
In the second series of our experiments when rats were exposed to hyperoxic environment once a day for 2 consecutive days, the incidence of VF decreased and survival rate increased remarkably, 72 h later. But, the infarct size did not decrease (Fig. 4). When the numbers of exposures were increased to 3 days, both the infarct size and arrhythmia reduced 72 h later. These results indicate that prolonging the protection against infarction or arrhythmia by pretreatment with intermittent hyperoxia depends on the number of exposure.

Pre-exposure to hyperoxia could induce preconditioning effect in other organs, however pre-exposure times are very different for different organs [14, 15, 32, 34]. Most of these studies have used intermittent hyperoxia to induce preconditioning. Our colleagues observed hyperoxia preconditioning effect in rat kidneys when they were exposed to hyperoxic environment at least for 1 h once a day for 5 consecutive days [18]. Zhang et al. observed neuroprotective effect with 24 h consecutive exposure to hyperoxia, but did not observe the same effect with 6 and 12 h of exposure [35]. So, we hope that next studies using the different methods of hyperoxia could find a more optimal time and concentration of oxygen that precondition all organs over the body.

In summary, although prolonged exposure to hyperoxia (more than 24 h) has detrimental effects [8, 36], transient exposure may induce a late preconditioning that lasts for about 48 h, and intermittent pre-exposure to the same hyperoxia might prolong this protection in the hearts of rats.

ACKNOWLEDGMENTS

This work was supported by a grant from Trauma Research Center of Baqiyatallah University of Medical Sciences. The authors thank Professor Alireza Asgari for critical review on the manuscript.

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