

# Pretreatment with hyperoxia reduces *in vivo* infarct size and cell death by apoptosis with an early and delayed phase of protection<sup>☆,☆☆</sup>

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## Abstract

**Objective:** Exposure to normobaric hyperoxia protects the heart against ischemia–reperfusion injury *ex vivo*. In the present study, we investigated the effect of the early and late phase of hyperoxia on *in vivo* myocardial infarction and apoptosis. **Methods:** Rats were exposed to room air preoxygenation ( $O_2 \geq 95\%$ ) followed by regional ischemia (30 min) and 0, 90, 180, and 360 min of reperfusion. Hyperoxic exposure was performed for 120 min either immediately or 24 h before coronary occlusion followed by 360-min reperfusion. Infarct size was evaluated by Evans blue/triphenyltetrazolium chloride staining. Apoptosis in the infarcted area was evaluated by terminal deoxy-nucleotidyl transferase-mediated deoxy uridine triphosphate (dUTP) nick end-labeling (TUNEL). Caspase 3 activity was measured by fluorometric enzyme assay, Bcl-2 and Bax protein expression assessed by western blotting and DNA laddering assessed with DNA gel electrophoresis. **Results:** The infarct size did not increase with increasing duration of reperfusion. However, apoptosis as evaluated by Bcl-2/Bax ratio, caspase 3 activity, and TUNEL-positive cells increased with increasing time of reperfusion. Both early and delayed pretreatment with hyperoxia reduced infarct size ( $p = 0.0013$ ,  $p = 0.046$ ), ameliorated ischemic arrhythmias and increased Bcl-2/Bax ratio ( $p = 0.015$ ,  $p = 0.0159$ ). Only hyperoxia immediately before coronary occlusion decreased caspase 3 activity ( $p = 0.026$ ) and decreased TUNEL-positive staining ( $p = 0.046$ ) with no visible DNA laddering. **Conclusions:** Detection of myocardial apoptosis increased with prolongation of reperfusion time, as opposed to infarct detection where reperfusion was essential to detect infarction, but the infarct size did not increase with time. Pretreatment with hyperoxia significantly decreased infarct size and apoptotic cell death. Pretreatment, immediately before coronary occlusion, was most cardioprotective.

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**Keywords:** Reperfusion; Normobaric hyperoxia; Apoptosis; Bcl-2; Bax; Infarct size

## 1. Introduction

Improved myocardial protection is important in many clinical situations involving open-heart surgery, thrombolysis, and balloon dilatation. Preconditioning is so far the most powerful cardioprotection intervention in experimental studies, and it is also effective in cardiac surgery [1]. In

many clinical situations, mechanical preconditioning is not applicable for several reasons, among them prolongation of the ischemic insult and the risk of embolization from atherosclerotic plaques [1]. Previous investigations in animal models show that short exposure to normobaric hyperoxia mimics preconditioning and protects the isolated heart against ischemic injury and reperfusion-induced arrhythmias in a biphasic mode analogous to early and delayed protection by ischemic preconditioning [2–4]. Pretreatment with normobaric hyperoxia also protects the brain and kidney [5,6] against subsequent ischemia–reperfusion injury. Recently, we performed a dose–response study on hyperoxic pretreatment on *in vivo* myocardial infarction, and found that preexposure to 120 and 180 min normobaric hyperoxia was most protective, reducing infarct size and the incidence

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of ventricular fibrillation [7]. Early signaling of protection of normobaric hyperoxia is so far known to be through mitogen-activated protein kinases, reduction of nuclear factor kappa B activation through increase of myocardial I $\kappa$ B $\alpha$ , involving tumor necrosis factor- $\alpha$  in the signal transduction [3,8,9]. The early and late protection afforded by hyperoxia are likely to signal through different pathways.

Many studies suggest that cardiomyocyte apoptosis may be an important component of the pathogenesis of ischemia–reperfusion injury and heart failure in animals and humans [10–13]. Apoptosis may be implicated in infarct expansion, progression of myocardial dysfunction and ventricular remodeling [2]. In the process of programmed cell death, the Bcl-2 family of proteins plays an important role. The antiapoptotic protein, Bcl-2, stabilizes the mitochondrial membrane, while the proapoptotic member, Bax, permeabilizes it, leading to leakage of cytochrome C [14]. Thus, a high Bcl-2/Bax ratio will favor an antiapoptotic state in the cell. At the end of apoptosis development, DNA is cleaved into small fragments, which can be detected *in situ* (terminal deoxy-nucleotidyl transferase-mediated deoxy uridine triphosphate (dUTP) nick end-labeling, TUNEL) or as DNA laddering on extracted and electrophoresed DNA [14]. The effect of normobaric hyperoxia on apoptosis of cardiomyocytes has not been investigated.

The purpose of this study was threefold: (1) to establish an optimal duration of post-ischemic reperfusion for investigation of apoptosis, (2) to study if normobaric hyperoxia could reduce post-ischemic apoptosis in the optimal model of apoptosis detection, and (3) to compare the effect of hyperoxia given immediately before coronary occlusion versus 24 h earlier.

## 2. Materials and methods

Male Wistar rats weighing 270–320 g (Pasteur, Tehran, Iran) were housed at 12:12-h light–dark cycles. They were fed *ad libitum* and conditioned in a non-stressful environment for at least 1 week prior to experiments. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (1996), and was approved by the ethics committee for animal research at Baqiyatallah University.

### 2.1. Experimental protocol

Rats were kept in a confined environment exposed to either normobaric O<sub>2</sub> ( $\geq 95\%$  O<sub>2</sub>) or air (21% O<sub>2</sub>). Animals were divided into the following seven groups:

- (1) *ISCH*: Rats were kept in room air for 120 min before 30 min regional ischemia without reperfusion ( $n = 12$ ).
- (2) *R90*: Rats were treated as ISCH but with 90-min reperfusion ( $n = 12$ ).
- (3) *R180*: Rats were treated as ISCH but with 180-min reperfusion ( $n = 10$ ).
- (4) *R360*: Rats were treated as ISCH followed by 360-min reperfusion ( $n = 10$ ).
- (5) *HE360*: Rats were exposed to  $\geq 95\%$  O<sub>2</sub> for 120 min immediately before 30-min ischemia and 360-min reperfusion ( $n = 8$ ).

- (6) *HL360*: Rats were exposed to  $\geq 95\%$  O<sub>2</sub> for 120 min 24 h before 30-min ischemia and 360-min reperfusion ( $n = 8$ ).
- (7) *Sham*: Rats kept in room air and exposed to thoracotomy without coronary artery ligation but time-matched to 360-min reperfusion were sampled for comparison in apoptosis analysis ( $n = 5$ ).

### 2.2. Surgical procedure

Rats were anesthetized with sodium pentobarbital (50 mg kg<sup>-1</sup>, i.p.). Rectal temperature was monitored and maintained at about 37.5 °C. A ventral midline incision tracheotomy was performed, followed by intubation. The animals were artificially ventilated at 80 strokes min<sup>-1</sup> with a tidal volume of 1 ml (100 g)<sup>-1</sup> to maintain PO<sub>2</sub>, PCO<sub>2</sub>, and pH in the normal physiological ranges. The tail vein was cannulated with an angiocat (24 g) for infusion of Evans blue solution. The left carotid artery was dissected and cannulated for continuous arterial pressure monitoring. Electrocardiographic leads were attached to subcutaneous electrodes to monitor limb lead II continuously. A left-sided thoracotomy was performed in the fourth intercostal space and the pericardium was opened to expose the heart. A 5/0 silk suture was passed around the left anterior descending (LAD) of coronary artery, 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 15–20 min stabilization, the artery was occluded for 30 min by clamping the snare against the surface of the heart. Ischemia was confirmed by regional cyanosis downstream to the occlusion, by ST elevation on electrocardiogram (ECG) and by reduced blood pressure. Reperfusion was confirmed visually by reversal of cyanosis. Exclusion criteria were dysrhythmia and/or a sustained fall in mean arterial blood pressure below 60 mm Hg before occlusion.

### 2.3. Blood PO<sub>2</sub> measurement

To evaluate arterial O<sub>2</sub> concentration following the placement in the hyperoxic chamber, a gas analyzer (Ciba-Corning 865, Medfield, MA, USA) was used in two separate groups of animals ( $n = 3$  in each) immediately after exposure to 120 min normoxia or the same time of hyperoxia.

### 2.4. Analysis of ischemia-induced arrhythmias

Arrhythmias were defined according to the Lambeth conventions [15] 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 complexes 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.

### 2.5. Tissue preparation

At the end of reperfusion, the LAD was re-occluded and 2 ml Evans blue (1%) injected as a bolus into the tail vein to

perfuse the non-ischemic portion of the heart. The entire heart was then excised, rinsed of excess blue dye, trimmed of atrial tissue, put in a matrix, wrapped in plastic foil and frozen at  $-20^{\circ}\text{C}$  (2 h) for detection of infarct size. The area of the heart unstained by Evans blue was calculated as the area at risk (AAR).

In separate and additional experiments, the infarcted part of the left ventricle was isolated by visual inspection ( $n = 4$  in each group). The tissues were immediately placed in tubes and frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$  until use for detection of apoptosis by immunoblot or enzyme activity assay.

In yet other experiments, the left ventricle was excised and fixed in 4% buffered formaldehyde, and embedded in paraffin. Cross sections,  $5\ \mu\text{m}$  in size, from the apex to the atrioventricular groove, were collected for evaluation of apoptosis by the TUNEL method.

## 2.6. Infarct size measurement

Frozen hearts were divided into 2-mm thick transverse sections from the apex to the base. The AAR was isolated and incubated in 1% triphenyltetrazolium chloride (TTC) solution in isotonic pH 7.4 phosphate buffer at  $37^{\circ}\text{C}$  for 20 min. TTC leaves infarcted tissue unstained (white), while viable tissue is stained (red). Samples were fixed in 10% formaldehyde for 24 h to enhance the contrast. An image was obtained (CanonScan Lid 25) from both sides of each slice and all calculations from one heart (using Image Tool Software) were averaged into one value for statistical analysis. Infarct size, as the white color area, was expressed as a percentage of AAR.

## 2.7. Immunoblotting

Left ventricles were homogenized (T25 basic Ultra-Turrax, Heidolph) at  $4^{\circ}\text{C}$  for 15 s in a lysis buffer containing 50 mM Tris-HCl, 500 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 0.2% Triton X100, pH 7.4 and phenylmethylsulfonylfluoride (PMSF) and centrifuged at  $11\ 000 \times g$  for 15 min. Protein concentration was measured by the Bradford method. As much as 50–80  $\mu\text{g}$  of total protein were mixed with loading buffer (5% beta-mercaptoethanol, 0.05% bromophenol blue, 75 mM Tris-HCl, pH 6.8, 2% sodium dodecylsulfate (SDS) and 10% glycerol), boiled for 5 min, and loaded onto 12.5% SDS-polyacrylamide gel using Mini Protean 3 Cell (BioRad, Hercules, CA, USA). Proteins were transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Seoul, South Korea) in the presence of glycine/methanol transfer buffer (25 mM Tris base, 192 mM glycine, and 20% methanol) in Trans-Blot Semi-Dry Electrophoretic Transfer system (BioRad, Hercules, CA, USA). Membranes were blocked with Western blocking solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Membranes were subsequently exposed to rabbit polyclonal Bcl-2 antibody (1:500), rabbit polyclonal Bax antibody (1:1000) and rabbit polyclonal  $\beta$ -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at  $4^{\circ}\text{C}$ . After washing with Tris-buffered saline Tween-20 (TBST), membranes were incubated with horse-radish peroxidase conjugated goat anti-rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The 3,3',5,5'-tetramethylbenzidine reagent was employed to visualize

peroxidase reaction products. Bcl-2 protein was detected as a 26-kDa band, Bax protein was detected as a 23-kDa band and  $\beta$ -actin as a 43-kDa band. The membranes were scanned (CanonScan Lid 25) and the intensities of bands were determined by the National Institutes of Health Image J program. Bcl-2/Bax band density was related to actin density.

## 2.8. In situ detection of cell death

Hydration was done by transferring the sections through the following solutions: twice to xylene bath for 15 min, and then for 3 min to 100%, 96%, 90%, 80%, 70% ethanol and double-distilled water. Nuclei of tissue sections were stripped of proteins through incubation with  $10\ \mu\text{g}\ \text{ml}^{-1}$  proteinase K (Roche, Boehringer Mannheim, Germany) for 30 min at  $37^{\circ}\text{C}$ . The sections were incubated with 3%  $\text{H}_2\text{O}_2$  for 5 min at room temperature to allow inactivation of endogenous peroxidase and then incubated for 60 min. Apoptotic myocytes were detected by TUNEL assay using In Situ Cell Death Detection Kit (Cat. No. 11684817910, Roche, Boehringer Mannheim, Germany) according to the manufacturer's instructions. Briefly, the enzyme, terminal deoxy-nucleotidyl transferase (TdT) was used for labeling of DNA strand breaks. The signal of TdT-mediated dUTP nick end-labeling (TUNEL) was then detected by an anti-fluorescein antibody conjugated with horse-radish peroxidase, a reporter enzyme that catalytically generates a brown-colored product from DAB substrate K (Roche, Boehringer Mannheim, Germany). The sections were dehydrated in graded alcohols and coverslipped with Methyl Green counterstaining. The sections were washed, dried, and mounted in accumount medium. For each section, digital images were captured by use of a  $\times 40$  objective on an Olympus BX41 microscope connected to a computer with a Olympus DP12 U-TV0.5XC camera. Cells with clear nuclear labeling were defined as TUNEL-positive cells. Apoptotic cells were calculated as following: number of TUNEL-positive cell nuclei divided by the total number of cells per field  $\times 100$ . At least four sections per heart were evaluated. For each section, 12 fields were randomly chosen. As a positive control for the TUNEL assay, DNase I recombinant, grade I K was used (Roche, Boehringer Mannheim, Germany), while incubation with label solution instead of TUNEL reaction mixture served as a negative control.

## 2.9. DNA isolation and gel electrophoresis

Freshly frozen myocardium (20–30 mg) was minced in 600  $\mu\text{l}$  of lysis buffer and quickly homogenized using 30–50 strokes with a microfuge-tube pestle. The tissue was digested with  $100\ \mu\text{g}\ \text{ml}^{-1}$  of proteinase K (Roche, Boehringer Mannheim, Germany) at  $56^{\circ}\text{C}$  for 3–4 h and incubated with RNase A at  $37^{\circ}\text{C}$  for 1 h. After incubation, tissues were precipitated and centrifuged at  $13\ 000 \times g$  for 5 min. Supernatants containing DNA were precipitated with isopropanol. After centrifugation at  $13\ 000 \times g$  for 5 min, the resulting DNA was washed with 75% ethanol and dissolved in DNA hydration solution at 260 nm by spectrophotometry. A total of  $10\ \mu\text{g}$  of DNA was loaded onto 1.5% agarose gel containing  $0.5\ \text{mg}\ \text{ml}^{-1}$  ethidium bromide. DNA electrophoresis was carried out at 80 V for 1–2 h. DNA ladders were

Table 1. Hemodynamic data obtained at the end of the observation period in rats subjected to 30 min regional ischemia followed by reperfusion for 0 min (ISCH), 90 min (R90), 180 min (R180) or 360 min (R360). Pretreatment with normobaric hyperoxia (>95% O<sub>2</sub>) was performed for 120 min either immediately before infarct induction (HE360) or 24 h before (HL360) (n = 8–10 per group).

Groups	Baseline		Ischemia		Reperfusion	
	HR	MBP	HR	MBP	HR	MBP
ISCH	372 ± 41	100 ± 15	380 ± 32	82 ± 12**	—	—
R90	362 ± 45	93 ± 12	378 ± 30	78 ± 8*	355 ± 28	94 ± 10
R180	344 ± 35	88 ± 9	362 ± 35	70 ± 10**	340 ± 25	85 ± 11
R360	355 ± 45	91 ± 10	369 ± 16	75 ± 8**	348 ± 11	95 ± 12
HE360	353 ± 42	107 ± 11 <sup>#</sup>	340 ± 19	99 ± 12 <sup>*,###</sup>	351 ± 11	104 ± 12
HL360	348 ± 23	100 ± 9	350 ± 22	95 ± 8 <sup>*,#</sup>	344 ± 18	97 ± 9

Data are presented as mean ± SD. HR: heart rate; MBP: mean blood pressure.

\* p = 0.05 compared with the baseline within the same group.

\*\* p = 0.01 compared with the baseline within the same group.

<sup>#</sup> p = 0.05 compared with R360.

<sup>###</sup> p = 0.001 compared with R360.

visualized under ultraviolet light (UV Transilluminator) and photographed with a Polaroid camera. DNA was extracted from four hearts in each group.

### 2.10. Caspase 3 activity

Caspase 3 activity was determined by Caspase 3 Activity Assay kit (Cat. No. 2 012 952, Roche, Boehringer Mannheim, Germany) according to the manufacturer's recommendations. Briefly, caspase 3 from cellular lysates is captured by a monoclonal antibody. Following the washing step, substrate is added that is cleaved proportionally to the amount of activated caspase 3. Due to proteolytic cleavage of the substrate, free fluorescent AFC is generated. Free AFC is determined fluorometrically at  $\lambda_{\text{max}} = 505 \text{ nm}$ . The developed fluorochrome is proportional to the concentration of activated caspase 3 and can be quantified by a calibration curve. Results are expressed as fold to sham.

### 2.11. Statistical analysis

Data, unless otherwise stated, are shown as mean ± SD. Infarct size and haemodynamic data were analyzed by a one-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test for comparing means in different reperfusion times, and with unpaired *t*-test for evaluation of hyperoxia. The repeated measurement was used for comparison of haemodynamic data in phases of each group. Density of immunoblot bands, caspase 3 activity and TUNEL assays were analyzed with the Kruskal–Wallis test with Dunns post-test for multiple comparison but the Mann–Whitney test was used for comparison of the hyperoxia group with its control. The incidence of ventricular fibrillation (VF) was compared using Fisher's exact test using SPSS for Windows 16.0 (SPSS Inc., Chicago, IL, USA). A value of  $p \leq 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Hemodynamic parameters

Table 1 summarizes the hemodynamic data, which were collected at the end of either ischemia or reperfusion in the respective groups. At baseline, the mean arterial pressure

was higher in rats pretreated with hyperoxia immediately before infarct induction ( $p = 0.03$  vs the R360 group). Otherwise, there were no significant differences at baseline between groups. Ischemia caused a marked reduction in blood pressure that returned to about normal values during reperfusion, but had no significant effect on heart rate. Pretreatment with hyperoxia immediately and 24 h before occlusion markedly improved arterial blood pressure during ischemia ( $p = 0.0001$  and  $p = 0.002$ , respectively, vs R360). After reperfusion, there were no differences between groups.

Arterial PO<sub>2</sub> in the normoxic group was  $93 \pm 2 \text{ mm Hg}$ , and increased to  $433 \pm 27 \text{ mm Hg}$  in the hyperoxic group immediately after 120 min exposure ( $p = 0.05$ ).

### 3.2. Infarct size

When ischemia was performed in the absence of reperfusion, hardly any infarct was detectable (Fig. 1).

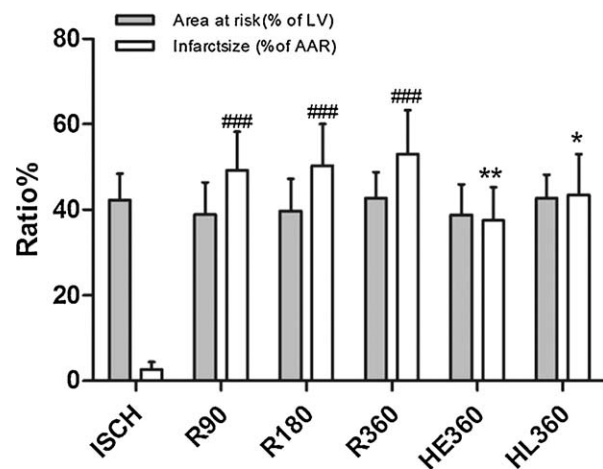


Fig. 1. The effect of time of reperfusion and exposure to hyperoxia on area at risk and infarct size after *in vivo* regional occlusion of the left coronary artery in rats. ISCH, R90, R180 and R360 were rats which were subjected to 0 (ISCH), 90, 180 and 360 min reperfusion after 30 min ischemia, respectively. HE360 and HL360 were rats pretreated with hyperoxia immediately or 24 h before 30 min ischemia followed by 360 min reperfusion. #### denotes  $p = 0.0001$  compared with ISCH group. \* $p = 0.046$  and \*\* $p = 0.0013$  compared with R360 group. There were no differences between R90, R180 and R360 groups. Mean ± SD are shown of  $n = 8–10$  per group. LV: Left ventricle; AAR: area at risk.

Reperfusion for 90 min increased infarct size ( $53 \pm 10\%$ ,  $p = 0.0001$ ). There was no increase of infarct size with increasing time of reperfusion. When rats were treated with hyperoxia immediately before infarct induction, the infarct size was reduced ( $37 \pm 9\%$ ,  $p = 0.0013$  vs R360). Pretreatment with hyperoxia 24 h before occlusion also reduced infarct size ( $44 \pm 10\%$ ,  $p = 0.046$  vs R360). There was no significant difference between HE360 versus HL360 groups.

### 3.3. Ischemia-induced arrhythmias

Arrhythmias occurred during ischemia–reperfusion, and the occurrence was independent of the duration of reperfusion (Fig. 2). Preexposure to hyperoxia both immediately and 24 h earlier reduced the incidence of arrhythmias in the form

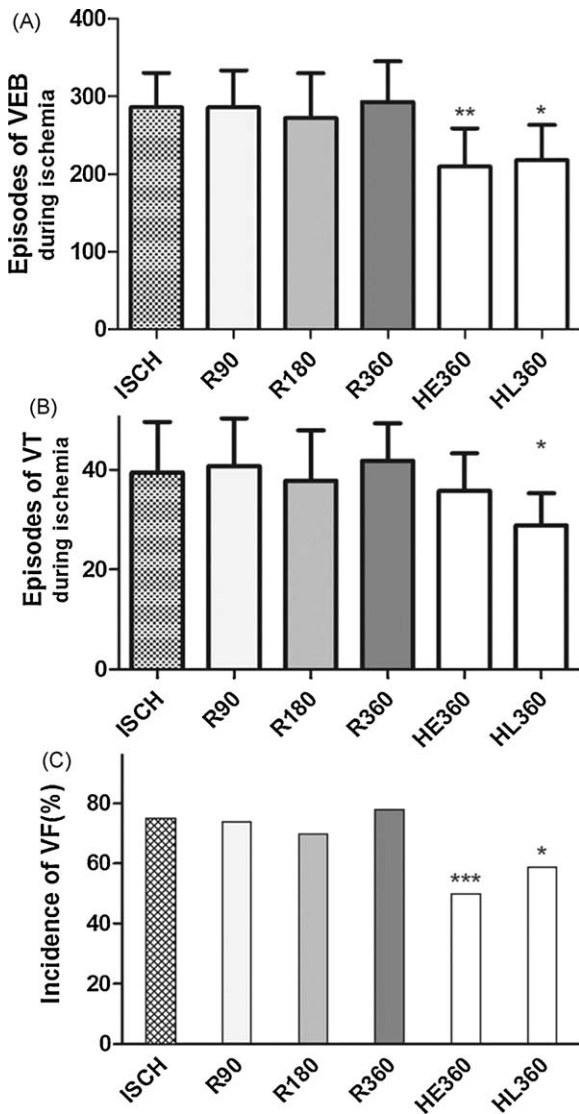


Fig. 2. The effect of time of reperfusion and exposure to hyperoxia on ischemia-induced arrhythmias in open chest rat hearts with 30 min induced regional ischemia and different times of reperfusion as explained in legend to Fig. 1. VEB: ventricular ectopic beats; VT: episodes of ventricular tachycardia; VF: ventricular fibrillation. \* $p = 0.02$ , \*\* $p = 0.0017$  and \*\*\* $p = 0.001$  compared with R360 group. Data in A and B are shown as mean  $\pm$  SD but in C as frequency of the values ( $n = 8–10$  per group).

of VEBs ( $p = 0.0017$  and  $p = 0.02$  in HE360 and HL360 vs R360, respectively). Pretreatment with hyperoxia 24 h before infarct induction reduced VT ( $p = 0.02$  vs R360). Both early and delayed treatment with hyperoxia reduced the incidence of VF ( $p < 0.001$  and  $p < 0.05$  vs R360, respectively). Survival

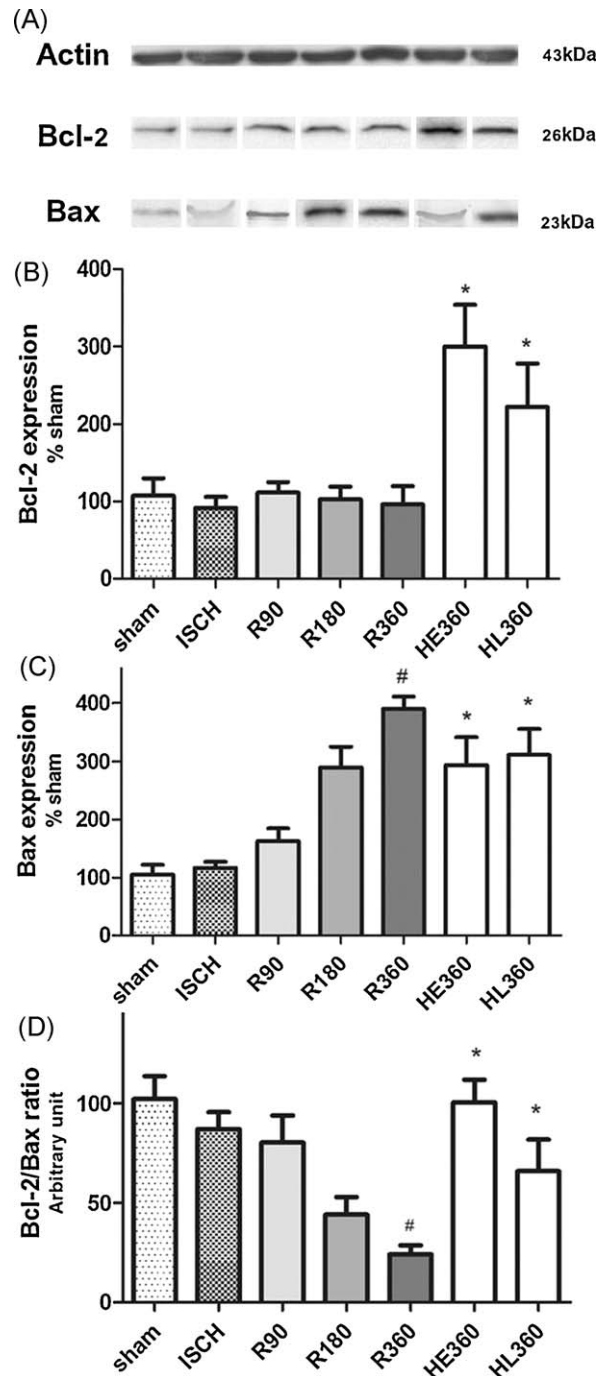


Fig. 3. Expression of Bax and Bcl-2 proteins in the infarcted part of the left ventricle after different times of reperfusion as explained in legend to Fig. 1. (A) Representative immunoblots from all together four different experiments after incubation with a  $\beta$ -actin antibody as internal control, or with anti-Bcl-2 or anti-Bax antibodies. The order of the blots are the same as on the graphs. (B) Blot densitometry of Bcl-2. (C) Densitometry of Bax. (D) The calculated Bcl-2/Bax ratio. Values are shown as mean  $\pm$  SD of 4 per group. # denotes  $p = 0.021$  versus sham. \* $p = 0.0159$  versus R360.



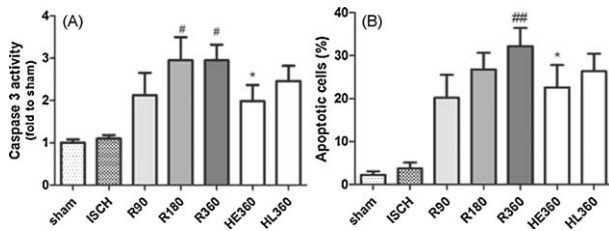


Fig. 4. (A) Comparison of caspase 3 activity after different times of reperfusion and exposure to hyperoxia on infarct size after *in vivo* regional occlusion of the left descending coronary artery in rats as explained in legend to Fig. 1. Values are mean  $\pm$  SD of four independent experiments. # denotes  $p = 0.05$  versus sham. \*denotes  $p = 0.026$  versus R360. (B) Comparison of apoptotic myocytes using the terminal transferase UTP nick end-labeling (TUNEL) assay after different times of reperfusion or hyperoxic pretreatment. Mean  $\pm$  SD of at least three separate experiments are shown. \* $p = 0.05$  versus R360, ## $p = 0.046$  versus sham.

rate at the end of reperfusion did not significantly increase in the HE360 group (89%) or in the HL360 group (87%) compared with the R360 group (84%).

### 3.4. Bcl-2/Bax expression

The expression of the antiapoptotic protein Bcl-2 did not change with time of reperfusion. Hyperoxic pretreatment, both early and late, increased expression of Bcl-2 compared with R360 ( $p = 0.0159$ , respectively, Fig. 3(B)). The expression of the proapoptotic protein Bax gradually increased with time of reperfusion. Both early and delayed hyperoxic pretreatment attenuated this expression compared with R360 ( $p = 0.0159$ , respectively, Fig. 3(C)). The Bcl-2/Bax

ratio gradually decreased with increasing reperfusion time ( $p = 0.021$  after 360 min compared with sham, Fig. 3(D)). A marked elevation of the Bcl-2/Bax ratio was observed after pretreatment with hyperoxia in both immediate and delayed time frames ( $p = 0.0159$ , compared with R360). There was no significant difference between these two kinds of pretreatments.

### 3.5. Caspase 3 activity

There was a gradual increase of caspase 3 activity with time of reperfusion, reaching a difference compared with sham after 180 min of reperfusion ( $p = 0.02$ , Fig. 4(A)). Pretreatment with hyperoxia immediately before infarct induction decreased the activity compared with R360 ( $p = 0.026$ ). Caspase 3 activity was not significantly decreased when hyperoxia was used as pretreatment 24 h earlier.

The percentage of TUNEL-positive cells increased with increasing time of reperfusion, reaching a significant difference from sham after 360 min of reperfusion ( $p = 0.009$ , Fig. 4(B)). Pretreatment with hyperoxia immediately before ischemia reduced the percentage of TUNEL-positive cells compared with R360 ( $p = 0.046$ ), while the reduction afforded by hyperoxia 24 h earlier did not reach significance.

### 3.6. DNA fragmentation

DNA laddering was not apparent in hearts exposed to ischemia only, or ischemia followed by 90 min reperfusion (Fig. 5). When the reperfusion time was increased to either 180 or 360 min, DNA fragments of approximately 200 base pairs of length appeared on the gels. When rats were treated with hyperoxia immediately before ischemia and reperfusion, no DNA laddering was visible (HE360 group). Rats treated with hyperoxia 24 h earlier had faint, but visible DNA fragmentation.

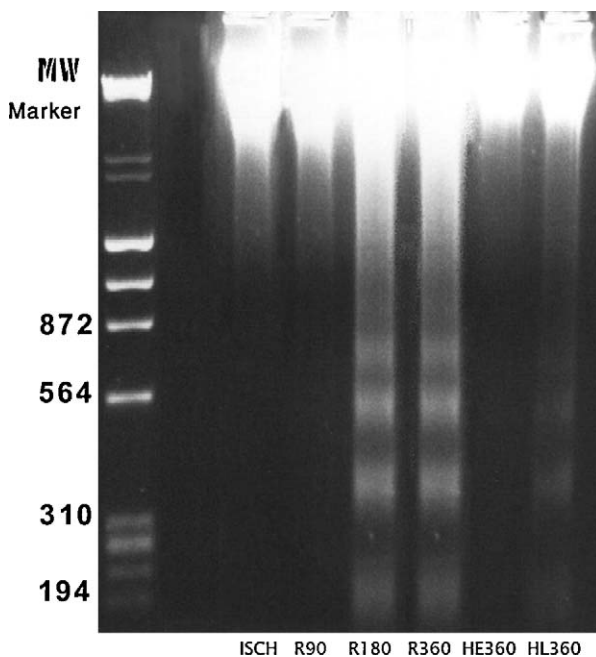


Fig. 5. Detection of DNA fragmentation using agarose gel electrophoresis on DNA extracted from the ischemic area of the left ventricle. A representative gel is shown. When 180 or 360 min reperfusion after 30 min regional ischemia was used, fragmentation of DNA into approximately 200 base pairs were seen on the gel. Pretreatment with hyperoxia immediately before ischemia abolished the DNA fragmentation, while hyperoxic treatment 24 h earlier reduced the laddering. For details see legend to Fig. 1.

## 4. Discussion

The major findings of the present study were: (1) while infarct size was unchanged depending on duration of reperfusion, the degree of apoptosis gradually increased with reperfusion time, (2) pretreatment with normobaric hyperoxia reduced both necrosis and apoptosis, and (3) pretreatment with hyperoxia immediately before ischemia was more protective than pretreatment 24 h earlier.

Myocardial ischemia–reperfusion injury depresses cardiac function and increases morbidity and mortality. Therefore, finding strategies for amelioration or preservation of myocardial cell viability has been a matter of great interest in both clinical and experimental settings [16]. Cell death through necrosis and apoptosis are distinctly different processes, where necrotic death leads to adenosine triphosphate (ATP) depletion, loss of cell membrane integrity, and leakage of intracellular components to adjacent cells causing inflammation [10]. Apoptosis requires energy and is associated with cell shrinkage and phagocytosis without loss of membrane integrity, sparing adjacent tissues from inflammation. In the process of cell death, cells may switch between oncotic and

apoptotic pathways. TTC is a widely used method for evaluation of experimental infarcts, and is based on detecting viable tissue through dehydrogenase conversion of a water-soluble compound into red insoluble formazan precipitate, leaving viable tissue red and dead tissue unstained [17,18]. It does not differentiate between cell death processes. Detection of infarct size through TTC staining has been previously shown by others to depend on time of reperfusion both *in vivo* and *ex vivo*, where reperfusion times of less than 60 min will underestimate the degree of cell death [17,18]. In one previous study on reperfusion times in the *in vivo* model, 180 min of reperfusion was found to be the optimal duration for infarct detection [17]. In the current study, we found that the infarct size was similar independent of reperfusion time ranging from 90 to 360 min, which confirms findings previously generated by others [19,20].

To our knowledge, no systematic evaluation of the reperfusion time for detection of the process of apoptosis has been undertaken in rats. In addition, the proportion of apoptotic death within a TTC-negative area is obscure. We show here that apoptosis can be detected within the infarcted area of the left ventricle, and that the reperfusion time is critical for estimation of apoptosis. The process of apoptosis can be initiated by several pathways, converging into caspase 3 activation [11]. One of the upstream pathways to caspase 3 activation involves the Bcl-2 family, where a shift in the balance between the proapoptotic Bax and the antiapoptotic Bcl-2 is an indirect evidence of programmed cell death. The balance between Bcl-2 and Bax will indicate if the tissue is in a pro- or antiapoptotic balance. The end result is cleavage of DNA into small fragments of about 200 base pairs, detected through *in situ* staining by TUNEL or DNA electrophoresis in the current study. All end-points of apoptosis were dependent on the duration of reperfusion (except caspase 3 activation, which was similar after 180 and 360 min of reperfusion), which is consistent with findings in dogs [12]. We found that Bax expression increased in hearts with prolongation of reperfusion time but Bcl-2 remained unchanged leading to decreasing Bcl-2/Bax ratio.

Several studies have shown that exposure to short periods of hyperoxia evokes protection against ischemia in the heart [2–4,7–9], kidney [6], and brain [5]. Prolonged exposure to a hyperoxic environment (more than 12–24 h) can lead to injury of different organs [5]. We have previously investigated a time course for optimizing the exposure time to hyperoxia in the *in vivo* rat model, and have determined that 120 min exposure to  $\geq 95\%$  O<sub>2</sub> 24 h before coronary occlusion gave optimal protection against arrhythmias and cell death [7]. In the current study, we demonstrate that hyperoxia protects against ischemic injury both when given 24 h before or when given immediately before infarct induction, reducing arrhythmia and cell death as we and others have previously shown in isolated rat and mice hearts [2–5,7,8]. As arrhythmias are an important feature of reperfusion injury in the clinical setting, the anti-arrhythmic effect of hyperoxia may have therapeutic consequences.

Normobaric hyperoxia, when given either early or late, had an antiapoptotic effect. This was evident as a decreased Bax expression and an increased Bcl-2 expression, and improved Bcl-2/Bax ratio. This is in accordance with previous findings from Choi et al., who showed that hyperoxia induced

a nuclear factor kappa B dependent upregulation of Bcl-2 [2]. The effect was most pronounced when hearts were infarcted immediately after hyperoxic treatment. Upregulation of Bcl-2 as an antiapoptotic factor and downregulation of Bax as a proapoptotic factor attenuates apoptosis and prevents ischemia–reperfusion injury [21]. Recent studies have shown that Bcl-2 overexpression can attenuate the generation of reactive oxygen species (ROS) *in vivo* and *in vitro*, and limit post-ischemic necrosis and apoptosis [22]. Hyperoxia used immediately before occlusion reduced caspase 3 activity, reduced the number of TUNEL-positive cells, and reduced DNA laddering, an effect, which was less pronounced when hyperoxia was given as a 24-h pretreatment. At the moment, we cannot offer a clear speculation on why the immediate effects of hyperoxia were more pronounced than the delayed effects. It may be analogous to the more robust protection afforded by classic rather than delayed ischemic preconditioning, in which latter case activation of gene programs and more complex signaling of protection are involved.

The precise intracellular mechanisms of hyperoxic cardioprotection are far from fully elucidated. Hyperoxia elicits myocardial protection through inducing a low-graded oxidative stress, activating nuclear factor kappa B, mitogen-activated protein kinases, and signaling through nitric oxide [2–4,9]. Activation of nuclear factor kappa B may, in a delayed time frame, upregulate cytoprotective enzymes [23]. Tumor necrosis factor-alpha, acting through its receptor I, may play a role in myocardial protection by hyperoxia through improved recovery of contractile function and reduced necrosis in mice hearts [8]. Tumor necrosis factor-alpha is both upstream and downstream to nuclear factor kappa B, and has regulatory effects on apoptosis [23].

In summary, these results may enhance our understanding of the cardioprotective actions of hyperoxic pretreatment on the heart, and may lead to development of new strategies to protect patients. Furthermore, investigations of cardiac apoptosis in *in vivo* models of infarction should include long times of reperfusion to optimize detection.

The use of hyperoxia in clinical settings is attractive. It has been shown that hyperoxygenation therapy is well tolerated by humans, if administered according to standard protocols. In this regard, administration of 80% oxygen for less than 24 h is considered well tolerated [24]. As the first signs of oxygen toxicity appear in lung tissue of rats only after about 40 h of pure oxygen exposure [25], it seems that there is a wide safety window between protective effects of oxygen pretreatment and its toxicity. The advantage of short-term normobaric hyperoxia as a type of preconditioning is that it is non-invasive, simple, and easy to use. Increasing the oxygen uptake will give whole-body effects, and thus potentially affect all organs beneficially. The clinical applications could be far beyond cardiac surgery. However, more experimental work is required in larger animals and in clinical trials to fully understand the clinical benefits of hyperoxia.

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