ELSEVIER

European Journal of Cardio-thoracic Surgery 37 (2010) 942—949

EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY

www.elsevier.com/locate/ejcts

Daily reoxygenation decreases myocardial injury and improves post-ischaemic recovery after chronic hypoxia☆

Giuseppina Milano [a,*], Antonio F. Corno [b], Michele Samaja [c], Sandrine Morel [d], Giuseppe Vassalli [a,e], Ludwig K. von Segesser [a]

[a] *Cardiovascular Surgery, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland*
[b] *Alder Hey Children NHS Foundation Trust, Liverpool, UK*
[c] *Department of Medicine, Surgery and Dentistry, San Paolo Hospital, University of Milan, Milan, Italy*
[d] *Cardiology, University Hospital, Geneva, Switzerland*
[e] *Cardiology, Fondazione Cardiocentro Ticino, Lugano, Switzerland*

Received 9 July 2009; received in revised form 2 October 2009; accepted 7 October 2009; Available online 6 December 2009

Abstract

Objective: In contrast to the clinical evidence, experimental studies showed that chronic hypoxia (CH) confers a certain degree of protection against ischaemia—reperfusion damage. We studied the effects of daily reoxygenation during CH (CHReox) on hearts exposed to ischaemia—reperfusion. We also separated the intrinsic effects on the myocardium of CH and CHReox from those related to circulatory and nervous factors. **Methods:** Fifty-one Sprague-Dawley rats were maintained for 15 days under CH (10% O_2) or CHReox (10% O_2 + 1 h day^{-1} exposure to air). Normoxic (N, 21% O_2) rats were the control. The animals were randomly assigned to one of the three following protocols: (1) protocol A: hearts (n = 7 per group) were subjected to 30-min occlusion of the left anterior descending (LAD) coronary artery followed by 3-h reperfusion, with measurement of the injury by tetrazolium staining; (2) protocol B: the end-diastolic pressure (EDP) and left ventricular developed pressure × heart rate (LVDP × HR) were measured in Langendorff-perfused isolated hearts (n = 5 per group) during 30-min global ischaemia and 45-min reperfusion; and (3) protocol C: hearts (n = 5 per group) were frozen for the determination of levels of endothelial nitric oxide synthase (eNOS) by Western blotting. **Results:** CHReox hearts displayed greater phosphorylation of the eNOS and enhanced plasma level of nitrates and nitrites in comparison to CH hearts ($P < 0.0001$, Bonferroni's post-test). The infarct size was greater in CH than in N hearts ($P < 0.0001$, Bonferroni's post-test) while it was reduced in CHReox in comparison to CH and N hearts ($P < 0.0001$). At the end of reperfusion, EDP was higher in CH than CHReox and N hearts ($P = 0.01$, Bonferroni's post-test) while LVDP × HR was higher in CHReox and N than in CH hearts ($P = 0.03$, Bonferroni's post-test). **Conclusions:** Exposure to CH results in impairment of myocardial tolerance to ischaemia—reperfusion, greater injury and reduced recovery of performance, in agreement with clinical evidence. Infarct size, diastolic contracture and myocardial performance have been reduced, respectively, by 63%, 64% and 151% with daily reoxygenation compared with chronic hypoxia by accelerating intrinsic adaptive changes.

Keywords: Chronic hypoxia; Daily reoxygenation; Cardioprotection; Myocardial infarct

1. Introduction

Children with congenital cyanotic heart defects represent one of the largest patient populations affected by the deleterious consequences of chronic hypoxia (CH). The results of surgical repair of cyanotic heart defects are complicated by myocardial and multi-organ damage as a consequence of acute reoxygenation at the moment of institution of cardiopulmonary bypass with elevated oxygen content, followed by myocardial ischaemia required to arrest the heart for the intra-cardiac repair with the subsequent reperfusion [1]. The sequence of events is therefore hypoxia/reoxygenation, followed by ischaemia—reperfusion.

In contrast to the evidence provided by the clinical observations in congenital heart surgery, some studies have shown that, in other situations, hypoxia confers a certain degree of protection against ischaemia—reperfusion damage. One example comes from populations living at altitude, who are less prone to develop cardiovascular diseases, despite lack of a causal relationship between chronic hypoxia and myocardial protection [2,3]. Furthermore, cardiac protection by hypoxia was frequently reported in experimental studies on animals investigating the manifestations of ischaemia—reperfusion injury, such as myocardial infarct size, [4] post-ischaemic contractile dysfunction [5,6] and occurrence of arrhythmias [4,7].

☆ This study has been supported by Fondation Andreas P. Naef de la Chirurgie Thoracique, Société Académique Vaudoise, Lausanne, Switzerland.
* Corresponding author. Address: Department of Cardiovascular Surgery, Centre Hospitalier Universitaire Vaudois, 46 rue du Bugnon, CH-1011, Lausanne, Switzerland. Tel.: +41 21 3142297; fax: +41 21 3142278.
E-mail address: giuseppina.milano@chuv.ch (G. Milano).

1010-7940/$ — see front matter
doi:10.1016/j.ejcts.2009.10.030

In most of the above studies, the animals have been exposed to 'so-called' chronic hypoxia (CH) in hypoxic chambers intermittently opened to allow procedures such as feeding, weighing and cleaning. The intermittent openings of the chambers were associated with unavoidable and repeated exposures to room air and therefore with episodes of reoxygenation. We have proved [8] that hearts exposed to CH display impaired tolerance to reoxygenation as compared with normoxic hearts (control group) and also to hearts exposed to intermittent reoxygenation (daily exposure to room air) during CH [8], a protocol referred by us as CHReox. CHReox and CH were found to cause different effects on the regulation of sarcK^+_{ATP} and mitoK^+_{ATP} channels [9], mitogen-activated protein kinases (MAPKs) [1], hypoxia-inducible factor-1α [10], apoptosis [10] and nitric oxide (NO) [8], but not on myocardial morphology [11]. Our most important conclusions were that all the previously reported investigations using 'so-called' CH were, in reality, exposing the animals to chronic hypoxia with daily reoxygenation (CHReox). Therefore, the observation that 'hypoxia confers protection against the ischaemia–reperfusion damage' was only due to the fact that it was not CH but CHReox that was responsible for the positive effects of myocardial protection. Therefore, the negative effects of CH on the myocardial function have never been thoroughly investigated, and therefore, they cannot be denied on the base of the reported literature.

So far it has not been determined whether CH and/or CHReox may induce any degree of cardiac protection to prevent or reduce the negative effects of the ischaemia–reperfusion damage.

To answer the above question, this study has been designed to:

(1) Determine the cardiac protection provided by CH and CHReox in an animal model as close as possible to the clinical practice, where hearts of cyanotic children undergo reoxygenation, followed by myocardial ischaemia and reperfusion in chronological sequence; and
(2) Separate the intrinsic effects on the myocardium of CH and CHReox from those related to the circulatory and nervous factors.

2. Materials and methods

2.1. Animals

Fifty-one male Sprague-Dawley rats, weighing 248 $\pm$ 1 g at entry into the study, were divided into three groups (n = 17 per group): control group with normoxia (N, breathing room air at 21% O_2 saturation for 15 days), chronic hypoxia (CH, breathing a normobaric gas at 10% O_2 saturation and 90% N_2 for 15 days) and chronic hypoxia with daily reoxygenation (CHReox, as in CH, but daily exposed to room air 1 h day^{-1} for 15 days). All animals had free access to water and a laboratory diet until 24 h before sacrifice. To achieve true CH without any exposure to room air and therefore to avoid any unwanted reoxygenation, a hypoxic compensation chamber kept at 10% O_2 saturation was used to perform daily operations as cleaning and weighing the animals, as previously described [12].

After the 15 days of observation, all N animals were anaesthetised (10 mg Na-thiopental 100 g^{-1} and 1500 IU heparin i.p.) in room air, while all CH and CHReox animals were anaesthetised in the hypoxic compensation chamber, and therefore maintained in hypoxic environment even during the induction of anaesthesia. CHReox animals were anaesthetised 24 h after the 15th daily exposure to room air, therefore not immediately after exposure to reoxygenation. All hearts were randomly assigned to one of three following experimental protocols:

(1) Protocol A: Hearts were subjected to coronary occlusion and reperfusion;
(2) Protocol B: Hearts were isolated and perfused in a Langendorff system with Krebs–Henseleit buffer;
(3) Protocol C: Hearts were frozen for the biochemical analyses.

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with Swiss law and local ethical committee guidelines for animal research.

2.1.1. Blood

Immediately after heart harvesting, a blood sample was withdrawn into two heparinised tubes as previously reported [8,9].

2.1.2. Protocol A: Coronary occlusion and reperfusion in vivo

The 21 anaesthetised animals (n = 7 for each group) were placed over a heating platform at 37 °C and connected to a mechanical ventilator after tracheotomy (tidal volume 2.5 ml at 50 strokes min^{-1}) using a Harvard Apparatus with either room air or hypoxic atmosphere, respectively, for normoxic and hypoxic groups CH and CHReox (no inhalant anaesthetics). After left thoracotomy, a silk suture (6/0) was passed around the proximal segment of the left coronary artery, and a polyethylene catheter was used to form a snare. All animals were allowed 10 min to reach steady state before beginning regional ischaemia–reperfusion. After stabilisation, LAD coronary artery was snared and myocardial ischaemia was maintained for 30 min by placing a haemostatic clamp on the occluded snare. After 30 min, the heart was reperfused for 3 h by releasing the snare; then the aorta was mounted on a cannula and perfused with 15–20 ml saline at room temperature to wash out the blood. The LAD coronary artery was re-occluded and saturated Evans blue (2 ml) injected to mark the ischaemic zone as tissue area without the blue dye. The heart was frozen in liquid nitrogen and stored at −20 °C until analysis. To investigate the infarct and risk areas, the frozen heart was cut into five to six 1-mm-thick transverse slices from apex to base. The areas at risk were identified as the areas demarcated with Evans blue dye, whereas the non-infarcted and infarcted areas were demarcated after incubation with 1% triphenyltetrazolium chloride (TTC, Sigma) phosphate buffer (pH 7.4) at 37 °C for 20 min and fixed in 10% formalin for 4 days to enhance contrast of the Evans blue and TTC staining. With the use of NIH Image software (National Institutes of Health, Bethesda,

Maryland, USA), the different areas were quantified. The risk areas have been defined as = (non-infarcted + infarcted areas/total ventricular area) × 100. Infarct size has been defined as = (infarcted area/risk area) × 100.

2.1.3. Protocol B: Langendorff-perfused isolated heart in ex vivo model

The 15 anaesthetised animals (n = 5 for each group) were dissected in the compensation chamber at 10% O_2 saturation; then the heart was quickly removed and immersed in a beaker containing de-aerated isotonic saline at 25 °C; the beaker was taken out of the chamber, the aorta immediately cannulated and connected to the perfusion system and the heart perfused at 37 °C with the hypoxic medium containing 6% CO_2 at pH 7.39 ± 0.01 as previously reported [8,9]. Hearts were stabilised for 30 min at 10% O_2 saturation. During this period, the balloon volume was set to an end-diastolic pressure (EDP) of 10 mmHg and kept constant afterwards. At the end of the hypoxic stabilisation, hearts were subjected to 30-min reoxygenation at 100% O_2 saturation, followed by 30-min global ischaemia at 37 °C and 45-min reperfusion. The performance was continuously monitored, but data were reported every 5 min for clarity. We measured EDP, heart rate (HR), peak systolic pressure (PSP), left-ventricle developed pressure (LVDP or PSP−EDP), coronary perfusion pressure, maximal rate of heart contraction ($+dP/dt_{max}$) and relaxation ($-dP/dt_{min}$) and LVDP × HR. At the end of reperfusion, both the atria were resected, the ventricles were weighed and stored at −20 °C until analysis. To measure the infarct size, the ventricles were cut into 1 mm transverse sections from apex to base (five to sixslices per heart). Once thawed, the slices were incubated at 37 °C with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) for 20 min and fixed in 10% formalin for 4 days to enhance the contrast between stained (viable) and unstained (damaged) areas. The infarct size was determined from imaging the slices (NIH Image AutoExtractor 1.51) and expressed as percentage of ventricular size.

2.1.4. Protocol C: eNOS and phosphorylated-eNOS

The frozen tissue of 15 hearts (n = 5 for each group) was homogenised in Ripa buffer solution containing protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cell lysates were centrifuged (15 min at 13 000 rpm, 4 °C). Supernatants were transferred to pre-cooled micro-centrifuge tubes, frozen in liquid nitrogen and stored at −80 °C. Total protein was measured by the BSA protein assay kit (Thermo Scientific). Equal concentrations of protein from each sample (80 μg) were measured by Western blotting using primary antibodies for eNOS (N-20: sc-653) and phosphorylated eNOS (Ser^{1177}) (Santa Cruz Biotechnology) on an 8% denaturating gel, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody B; intensity was quantified by NIH AutoExtractor-1.51 software. The same extract from a normoxic heart was loaded on all blots for quantitative comparisons between blots.

2.2. Statistics

Data are expressed as mean ± SD. Significance level was P = 0.05 (two-tailed). To detect differences among the groups, we performed one- or two-way ANOVA. If this test resulted significant, the differences between selected pairs of data were tested using the Bonferroni's procedure (StatView, Abacus Concepts, Berkeley, CA, USA).

3. Results

3.1. Body weight and blood values

In agreement with previous studies [9] both types of chronic hypoxia (CH and CHReox) led to marked polycythaemia, irrespectively of daily reoxygenation (Table 1). The normal gain in body weight observed in the control normoxic group (N) over the 15-day observation period was significantly decreased by CH; this negative effect was partially reduced by 1 h of daily reoxygenation in CHReox. The heart/body weight ratio was higher for CH than for CHReox rats.

3.2. Daily reoxygenation decreases infarct size after LAD occlusion in vivo

The extension of the area at risk was the same in all groups, indicating that the consequences of the ligature of the LAD coronary artery presented the same degree of severity in all groups (Fig. 1).

The infarct size was greater in CH than N hearts (68.5 ± 2.4% vs 46.5 ± 4.4%, $P < 0.0001$). Daily reoxygenation during hypoxia reduced the infarct size to 23.8 ± 2.8% of the area at risk ($P < 0.0001$ vs CH).

3.3. Daily reoxygenation improves the post-ischaemic recovery of myocardial performance in ex vivo model

We monitored myocardial function in Langendorff-perfused isolated hearts exposed to 30-min global ischaemia and 45-min reperfusion (Fig. 2). During hypoxic baseline perfusion, EDP remained unchanged in all groups. The reoxygenation decreased EDP in all hearts, but more so in CHReox hearts. During the subsequent global no-flow ischaemia, EDP increased, reflecting increasing diastolic stiffness, more severe for N than hypoxic hearts (CH and CHReox). After 45 min reperfusion, final EDP was higher in CH (46.9 ± 4.1 mmHg,

Table 1
Homeostasis and blood values in animals exposed for 15 days to normoxia (N), chronic hypoxia (CH) and chronic hypoxia with daily reoxygenation (CHReox). Data are mean ± SD. ANOVA $P < 0.05$ for all variables.

n	N 12	CH 12	CHReox 12
Initial body weight (g)	248 ± 3	247 ± 8	250 ± 5
Final body weight (g)	340 ± 8	252 ± 10*	269 ± 5*,#
Heart weight (mg)	1203 ± 23	1383 ± 58*	1326 ± 29*,#
Heart weight/body weight (mg g^{-1})	3.54 ± 0.07	5.57 ± 0.22*	5.21 ± 0.09*,#
Haematocrit (%)	51 ± 2	66 ± 2*	68 ± 1*
Haemoglobin (g l^{-1})	158 ± 4	200 ± 7*	218 ± 3*
Red blood cell count (RBC/μl/1000)	8.6 ± 0.4	10.4 ± 0.4*	9.8 ± 0.2*

* $P < 0.05$ versus N.
\# $P < 0.05$ versus CH (Bonferroni's post-test).

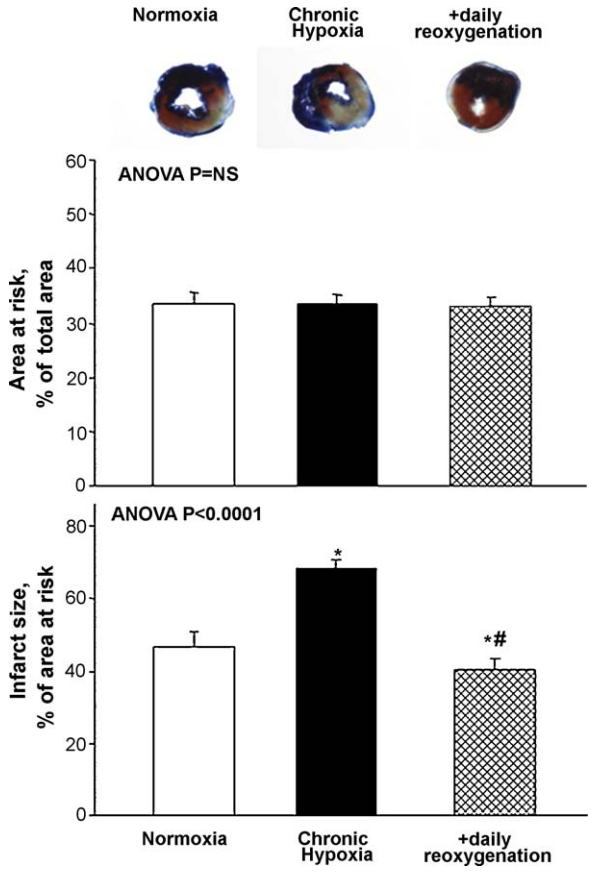

Fig. 1. Daily reoxygenation reduces injury after LAD coronary artery occlusion and reperfusion in vivo. The top panel shows representative sections of hearts taken at the end of the 30-min LAD occlusion followed by 3-h reperfusion in the three groups. White and red areas mark the infarct and vital. Averaged area at risk (middle panel) and infarct size (bottom panel) are shown as mean ± SD (n = 5 per group). The one-way ANOVA P value is therein reported. *, versus normoxia; #, versus chronic hypoxia (Bonferroni's post-test).

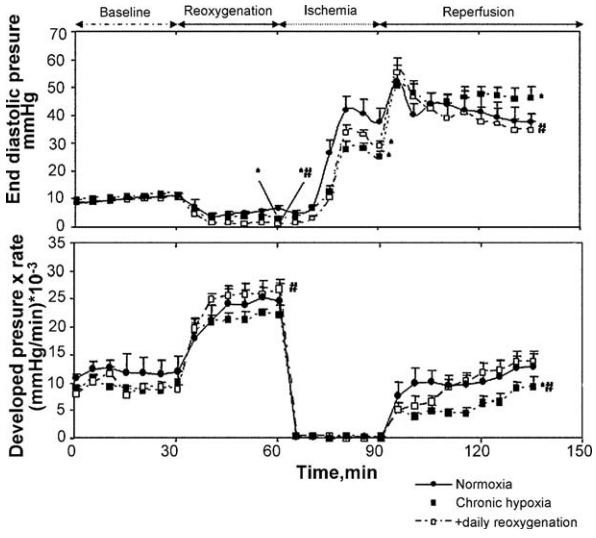

Fig. 2. Daily reoxygenation improves myocardial performance after ischaemia–reperfusion. Time course of the end-diastolic pressure (top panel) and the double product developed pressure × heart rate (bottom panel) during the baseline, reoxygenation, ischaemia and reperfusion sequence (mean ± SD; n = 5 per group). The two-way ANOVA P value is reported. *, P = 0.02 versus normoxia; #, P = 0.03 versus chronic hypoxia (Bonferroni's post-test).

n = 5) than in CHReox and N (33.5 ± 3.2 and 35.1 ± 3.0 mmHg, n = 5 and 5, respectively) hearts.

The changes in myocardial contractility were evaluated by measuring the product LVDP × HR. This parameter was the same in all groups until the end of global ischaemia. After reperfusion, LVDP × HR was higher in CHReox and N (12.8 ± 1.6 and 14.1 ± 2.8 mmHg × 10^3 min^{-1}, respectively)

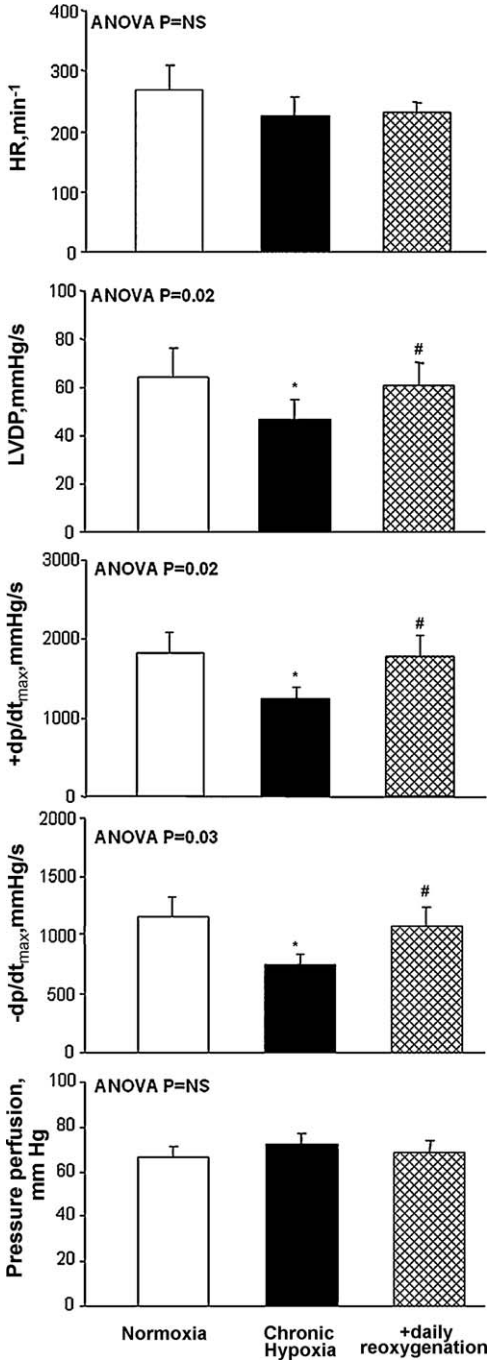

Fig. 3. Daily reoxygenation improves systolic function after ischaemia–reperfusion. Heart rate (HR), left ventricular developed pressure (LVDP), maximal rates of heart contraction (+dP/dt_{max}) and relaxation (−dP/dt_{min}) and coronary pressure at the end of reperfusion (mean ± SD; n = 5 per group). The two-way ANOVA P value is reported. The one-way ANOVA P value is therein reported. *, versus normoxia; #, versus chronic hypoxia (Bonferroni's post-test).

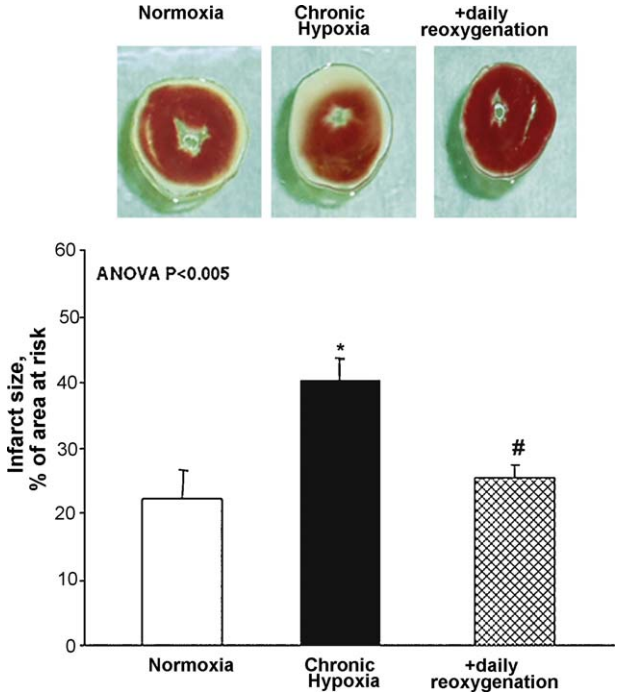

Fig. 4. Daily reoxygenation reduces injury after ischaemia-reperfusion ex-vivo. The top panel shows representative sections of hearts taken for the three groups at the end of the Langendorff experiments, with white areas marking the infarct. The bottom panel shows the infarct size averaged for all hearts and expressed as percentage of ventricular size (mean ± SD; $n = 5$ per group). The one-way ANOVA P value is therein reported. *, versus normoxia; #, versus chronic hypoxia (Bonferroni's post-test).

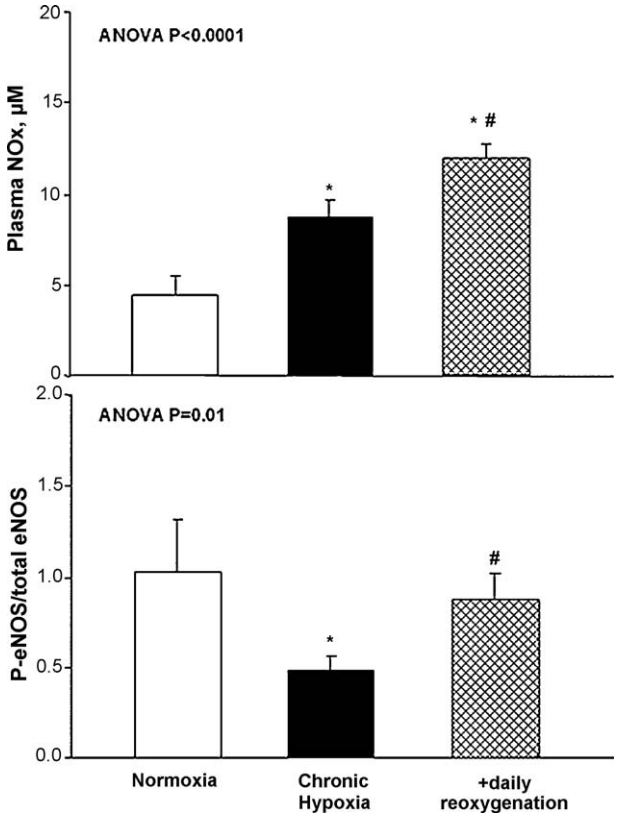

Fig. 5. Daily aeration increases plasma nitrates + nitrites (NO_x, top panel) and phosphorylated eNOS (bottom panel). The intensity of the blots from 5 hearts per group was measured by densitometry, averaged and reported in the bottom panel as phosphorylated/total ratio (mean ± SD; $n = 5$ per group). The one-way ANOVA P value is therein reported. *, versus normoxia; #, versus chronic hypoxia (Bonferroni's post-test).

than in CH hearts (8.5 ± 2.8 mmHg $\times 10^3$ min^{-1}). We have observed the same trend for LVDP, + dP/dt_{max} and $-dP/dt_{min}$ (Fig. 3) at the end of reperfusion. No significant differences were observed for HR and perfusion pressure (Fig. 3).

To provide a further marker of injury, reperfused hearts were harvested to measure the infarct size by a colourimetric technique (Fig. 4). CH hearts displayed the greatest extension of the infarct ($40 \pm 3\%$ vs $25 \pm 2\%$ and $22 \pm 4\%$ in CHReox and N, respectively), especially in the epicardial region, in agreement with previously reported observations [13].

3.4. Daily reoxygenation improves NO signalling

Exposure to CH induced an increase in plasma nitrates + -nitrites (NO_x), a marker of NO production. This increase was higher in CHReox than in CH rats (Fig. 5). The expression level of eNOS was unaffected by both CH and CHReox, but eNOS phosphorylation was halved in CH with respect to N. By contrast, eNOS phosphorylation recovered almost fully in CHReox hearts.

4. Discussion

The results of this experimental study showed that daily reoxyenation during CH markedly limited ischaemia—reperfusion injury both in the in vivo LAD coronary artery occlusion and in the ex vivo Langendorff-perfused heart models. The reduction of myocardial injury was also demonstrated by the improved recovery of the ventricular performance.

4.1. The chronic hypoxia model

We previously reported that 1 h of daily reoxygenation during CH improves tolerance to reoxygenation regardless of blood oxygen content, haemoglobin concentration and ventricular hypertrophy [8,11]. In our original model of chronic hypoxia, animals are cared for and sacrificed under truly hypoxic conditions, constantly preventing any exposure to air.

The CH protocol thus closely mimics the physiological (high altitude) and pathophysiologic (cyanotic congenital heart defects, pulmonary diseases and severe anaemia) settings where tissue oxygenation remains substantially reduced to critical levels.

Virtually all studies employing standard hypoxic chambers report that the so-called 'chronic hypoxia' is characterised by protective effects [4—7]. By contrast, the studies where a real CH was induced by surgical techniques [14—17] or in chambers preventing aeration [8,9,11,17], demonstrated that hypoxia is a risk factor. These apparently contradictory findings can be explained by the observations of the present experimental study. If 'chronic hypoxia' in standard hypoxic chambers is similar to our CHReox protocol considering that

they involve periods of reoxygenation, then it is not surprising that the so-called 'chronic hypoxia' was protective, as opposed to deleterious effects of true CH, in agreement with the clinical observations.

Thus, it is not hypoxia that conferred protection, but daily reoxygenation associated with the use of standard hypoxic chambers. Remarkably, the signal transduction pathways originating from antioxidants, enzymes and stress proteins were found to be critical in generating adverse effects during CH, as opposed to protective effects in intermittent hypoxia, a variant of CHReox [18]. While that study was the first to propose that CH impairs recovery after ischaemia–reperfusion, the present report is the first validation of the above hypothesis.

The main factors accounting for the differences between CH and CHReox may be related to the ox-redox balance and increased oxidative stress associated with reoxygenation [8]. This hypothesis is confirmed by the augmented blood lipid hydroperoxides and malondialdehyde during intermittent hypoxic training, as opposed to attenuation of that increase in regular hypoxic training [19]. However, the previous investigations failed to prove that daily exposure to oxidative stress might induce myocardial protection.

4.2. Myocardial ischaemia/reperfusion injury and function

In this study, we made the original observation that the size of myocardial infarction following LAD occlusion was significantly reduced by daily reoxyenation during exposure to chronic hypoxia than in normoxic hearts (Fig. 1).

A possible explanation could be the increase of the plasmatic NO_x in vivo following repeated episodes of reoxyenation during chronic hypoxia (Fig. 5). Plasma NO_x represents an index of NO production and therefore an increase in NO production in CHReox. NO is involved in the ischaemic preconditioning, where short antecedent ischaemic periods render tissue more resistant to subsequent prolonged and potentially lethal ischaemic insults [20]. NO has been shown to have an important role in ischaemic preconditioning and cardioprotection [21].

The in vivo LAD occlusion model is a close reproduction of the clinical situation encountered when a coronary artery lesion is complicating either the natural history or the surgical repair of a congenital heart disease. However, the identification of the specific role of intrinsic changes in myocardial tissue metabolism may be difficult with that model, because of the presence of potentially confounding factors correlated with the circulation. In the ex vivo Langendorff model, such factors are excluded because enervated hearts are separated from the circulation and perfused with the same medium throughout, thereby emphasising the role of intrinsic factors. The employed ex vivo protocol (hypoxic baseline, reoxygenation, global ischaemia and reperfusion) enables separating the effects due to oxygenation of CH hearts from those due to ischaemia–reperfusion, thereby facilitating the comparison among the three groups of hearts. That protocol also mimics the sequence of events to whom hearts of children with cyanotic congenital heart defects are subjected at the moment they undergo cardiopulmonary bypass and myocardial ischaemia–reperfusion to perform intra-cardiac repair.

With regard to the duration of reperfusion, in this study, in the ex vivo model with chronic hypoxia, the infarct size was reduced by daily reoxygenation after 45 min of reperfusion (Fig. 4). The duration of 45 min has been based on a recent study where the infarct size has been analysed after only 30 min of reperfusion, sufficient to induce myocardial damage [22].

The advantages of employing isolated heart model include accurate grading of hypoxia, no interference from blood–O_2 affinity changes, red cell spacing within capillaries, haemoglobin–O_2 unloading kinetics, lymphocyte-mediated inflammatory processes, hormones, coagulation factors and capillary clogging phenomena. Most of these phenomena are altered by hypoxia, which also increases blood viscosity due to raised haematocrit and induces haematocrit heterogeneity in the various districts of the myocardium.

However, although the crystalloid-perfused heart represents a reasonable approach to the in vivo situation, we agree that doubts remain regarding the validity of the model in situations far from the normal physiology.

Despite the above points, the outcomes of the ex vivo and the in vivo models are remarkably similar, supporting the use of these models for the aims of this study. Moreover, our observation confirmed that cardiac protection was not enhanced by the presence of factors residing in the circulation, but the myocardial tissue itself underwent intrinsic changes enabling greater protection against ischaemia–reperfusion.

A previous report [23] demonstrated that the negative effects of ischaemia-reperfusion can be reduced in children undergoing cardiac surgery, even if in this application on human subjects, the target of ischaemia–reperfusion was remote tissue and not myocardium. These researchers made an important contribution not only to prove the existence of the ischaemia-reperfusion damage with its clinical consequences, but also to provide the clinical confirmation of the validity of several experimental studies.

4.3. Limits of the study

Differing from altitude dwellers with healthy hearts, children with cyanotic congenital heart defects usually present with ventricular hypertrophy, pressure and/or volume overload, heart failure or the combination of the above. Furthermore, the underlying changes of their myocardial structure and function are often aggravated by chronic hypoxia. By contrast, our animals present with structurally normal hearts, and they became hypoxic at 5 weeks of age, and only at this time the deleterious effects of chronic hypoxia affect heart, lungs and other organs and systems. To limit this potentially confounding variable, a model of hypoxia immediately after birth was developed [24]. In this model hypoxic kits are regularly returned to their mother in a normoxic environment for 20 min once or twice a day, thus exposing kits to reoxygenation as in CHReox in the present study.

In this study, as in our previous investigations [9], we demonstrated that reoxygenation alters the pattern created by true chronic hypoxia, for example, the real situation of

children with cyanotic congenital heart defects. Of course, caution should be used when transferring information obtained in experimental studies to the clinical scenario of cyanotic children hypoxic since birth who undergo surgery for the repair of their congenital heart defect. Nevertheless, in the clinical practice, there are situations where, after a period of relatively normal O_2 saturation, a progressive cyanosis develops at few weeks of age because of progressive reduction of the effective pulmonary blood flow, as in patients with tetralogy of Fallot, transposition of the great arteries with ventricular septal defect and pulmonary stenosis, functionally single ventricles with pulmonary stenosis, double discordance (or congenitally corrected transposition of the great arteries) with ventricular septal defect and pulmonary stenosis, double outlet right ventricle with ventricular septal defect and pulmonary stenosis. In all these patients, the degree of dynamic obstruction to the pulmonary blood flow can substantially increase during the first few weeks after birth, leading to a progressive increase of cyanosis. All these situations are not much different from our animals made cyanotic 5 weeks after birth.

The myocardial ischaemia obtained with LAD occlusion is limited to a portion of the left ventricle to mimic a coronary artery lesion complicating the surgical repair of a congenital heart defect. These cyanotic patients are first exposed to reoxygenation at the beginning of cardiopulmonary bypass, and then to myocardial ischaemia because of the aortic cross clamping required to arrest the heart for the intra-cardiac repair, and finally, to myocardial reperfusion after the aortic cross clamp is removed. This scenario is closely mimicked in the present protocol.

Finally, we decided not to pace these hearts, as in previous studies [25], to consider the spontaneous heart rate response as one of the variables resulting from the induced myocardial injury.

5. Conclusions

Exposure to chronic hypoxia for 15 days results in impaired myocardial tolerance to ischaemia/reperfusion, greater injury and reduced recovery of performance, in agreement with clinical evidence. Daily reoxygenation markedly reduces the hypoxia-induced derangements by accelerating intrinsic adaptive changes in the myocardium. Although a causal relationship with NO metabolism still needs to be studied, these findings correlate with enhanced NO signalling via up-regulation of the endothelial isoform of NO synthase.

The observations of this study could possibly improve the results of surgical repair in infants with cyanotic heart defects. The results in paediatric cardiac surgery are still associated with the risk of myocardial and/or multi-organ damage. In our opinion, these are the consequence of acute reoxygenation at the moment of institution of cardiopulmonary bypass with elevated oxygen content, followed by myocardial ischaemia required to arrest the heart for the intra-cardiac repair with the subsequent reperfusion [1]. As in our experimental studies, in the daily clinical practice, the sequence of events remains hypoxia/reoxygenation, followed by ischaemia—reperfusion. The reported findings may be useful to design and evaluate alternative approaches of surgical plan, conduction of cardio-pulmonary bypass and myocardial protection, aimed at protecting immature hearts requiring a period of ischaemia—reperfusion for the surgical repair of their congenital heart defect.

References

[1] Morel S, Milano G, Ludunge KM, Corno AF, Samaja M, Fleury S, Bonny C, Kappenberger L, von Segesser LK, Vassalli G. Brief reoxygenation episodes during chronic hypoxia enhance posthypoxic recovery of LV function: role of mitogen-activated protein kinase signaling pathways. Basic Res Cardiol 2006;101:336—45.
[2] Baibas N, Trichopoulou A, Voridis E, Trichopoulos D. Residence in mountainous compared with lowland areas in relation to total and coronary mortality. A study in rural Greece. J Epidemiol Community Health 2005;59:274—8.
[3] Mortimer E, Monson R, MacMahon B. Reduction in mortality from coronary heart disease in men residing at altitude. New Engl J Med 1977;296:581—5.
[4] Neckar J, Papousek F, Novakova O, Ost'adal B, Kolar F. Cardioprotective effects of chronic hypoxia and ischaemic preconditioning are not additive. Basic Res Cardiol 2002;97:161—7.
[5] Baker J, Curry B, Olinger G, Gross G. Increased tolerance of the chronically hypoxic immature heart to ischemia. Contribution of the KATP channel. Circulation 1997;95:1278—85.
[6] Zhu HF, Dong JW, Zhu WZ, Ding HL, Zhou ZN. ATP-dependent potassium channels involved in the cardiac protection induced by intermittent hypoxia against ischemia/reperfusion injury. Life Sci 2003;73:1275—87.
[7] Asemu G, Papousek F, Ostadal B, Kolar F. Adaptation to high altitude hypoxia protects the rat heart against ischemia-induced arrythmias. Involvement of mitochondrial KATP channels. J Mol Cell Cardiol 1999;31:1821—31.
[8] Milano G, Corno A, Lippa S, von Segesser L, Samaja M. Chronic and intermittent hypoxia induce different degrees of myocardial tolerance to hypoxia-induced dysfunction. Exp Biol Med 2002;227:389—97.
[9] Milano G, Bianciardi P, Corno AF, Raddatz E, Morel S, von Segesser LK, Samaja M. Myocardial impairment in chronic hypoxia is abolished by short aeration episodes: involvement of K + ATP channels. Exp Biol Med (Maywood) 2004;229:1196—205.
[10] Bianciardi P, Fantacci M, Caretti A, Ronchi R, Milano G, Morel S, von Segesser L, Corno A, Samaja M. Chronic in vivo hypoxia in various organs: hypoxia-inducible factor-1alpha and apoptosis. Biochem Biophys Res Commun 2006;342:875—80.
[11] Corno AF, Milano G, Morel S, Tozzi P, Genton CY, Samaja M, von Segesser LK. Hypoxia: unique myocardial morphology? J Thorac Cardiovasc Surg 2004;127:1301—8.
[12] Corno AF, Milano G, Samaja M, Tozzi P, von Segesser LK. Chronic hypoxia: a model for cyanotic congenital heart defects. J Thorac Cardiovasc Surg 2002;124:105—12.
[13] Maxwell MP, Hearse DJ, Yellon DM. Species variation in the coronary collateral circulation during regional myocardial ischemia: a critical determinant of the rate of evolution and extent of myocardial infarction. Cardiovasc Res 1987;21:737—46.
[14] Fujiwara T, Kurtts T, Anderson W, Heinle J, Mayer J. Myocardial protection in cyanotic neonatal lambs. J Thorac Cardiovasc Surg 1988;96:700—10.
[15] Lupinetti FM, Wareing TH, Huddleston CB, Collins JC, Boucek Jr RJ, Bender Jr HW, Hammon Jr JW. Pathophysiology of chronic cyanosis in a canine model. Functional and metabolic response to global ischemia. J Thorac Cardiovasc Surg 1985;90:291—6.
[16] Silverman NA, Kohler J, Levitsky S, Pavel DG, Fang RB, Feinberg H. Chronic hypoxemia depresses global ventricular function and predisposes the depletion of high-energy phosphates during cardioplegic arrest: implications for surgical repair of cyanotic congenital heart defects. Ann Thorac Surg 1984;37:304—8.
[17] Corno A, Milano G, Samaja M, Tozzi P, von Segesser L. Chronic hypoxia: a model for cyanotic congenital heart defects. J Thorac Cardiovasc Surg 2002;124:105—12.
[18] Zhuang J, Zhou Z. Protective effects of intermittent hypoxic adaptation on myocardium and its mechanisms. Biol Signals Recept 1999;8:316—22.
[19] Bailey DM, Davies B, Young IS. Intermittent hypoxic training: implications for lipid peroxidation induced by acute normoxic exercise in active men. Clin Sci (Lond) 2001;101:465—75.

[20] Murry C, Jennings R, Reimer K. New insight into potential mechanisms of ischemic preconditioning. Circulation 1991;84:442—5.
[21] Jones S, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol 2006;40:16—23.
[22] Das A, Xi L, Kukreja RC. Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. J Biol Chem 2008;283:29572—85.
[23] Cheung MM, Kharbanda RK, Konstantinov IE, Shimizu M, Frndova H, Li J, Holtby HM, Cox PN, Smallhorn JF, Van Arsdell GS, Redington AN. Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. J Am Coll Cardiol 2006;47:2277—82.
[24] Baker J, Boerboom L, Olinger G. Tolerance of the developing heart to ischemia: impact of hypoxemia from birth. Am J Physiol 1995;268: H1165—73.
[25] Samaja M, Allibardi S, Chierchia S. Biochemical consequences of electrical pacing in ischemic-reperfused isolated rat hearts. Mol Cell Biochem 1999;194:245—9.