

Effects of Leukocyte-depleted Reoxygenation on Endothelial and Ventricular Function: With Observation of a Short Time Period

Yoshimasa Sakamoto, MD, Liu Hua Wei, MD, Gerald D. Buckberg, MD,
and Helen H. Youg, PhD

Postoperative myocardial global dysfunction causes mortality and morbidity in cyanotic congenital defects. This study investigates whether leukocyte depletion during coronary perfusion following reoxygenation can maintain endothelial and myocyte function, or less oxidant damage in a porcine neonatal model. After 30 minutes hypoxemia, 13 piglets underwent 60 minutes reoxygenation. The aorta was clamped and coronary reperfusion was either with normal blood (N=6), or leukocyte free blood by an inline filter (N=7). Cardiac function and endothelial response were assessed before and after cardiopulmonary bypass (CPB). Contractile recovery was improved by leukocyte depletion. Additionally, the antioxidant reserve capacity reserved more (534 ± 36 versus 772 ± 91 ; $p<0.05$) than reoxygenation without a leukocyte-depleting filter. Leukocyte depletion returned more extreme relaxation to acetylcholine ($71.8\pm20.4\%$ versus $41.2\pm9.8\%$; $p<0.05$), but did not change the endothelium-independent relaxation to sodium nitroprusside in either group. Activated leukocytes release oxygen free radicals that play a role in deterioration of the endothelial/myocardial function after reoxygenation and this deterioration in cyanotic heart diseases may be avoided by the use of the leukocyte-depleting filter. (Ann Thorac Cardiovasc Surg 2002; 8: 343–9)

Key words: reoxygenation, leukocyte depletion, endothelial cell dysfunction

Introduction

Our studies demonstrated that reoxygenation injury caused postoperative myocardial dysfunction and could be ameliorated by changing management during initiation of cardiopulmonary bypass (CPB) in immature cyanotic hearts.^{1,2)} The mechanism of hypoxemic/reoxygenation injury is uncertain. Reoxygenation by conventional hyperoxemic cardiopulmonary bypass after hypoxemia caused profound myocardial global dysfunction

and oxidant damage. Reactive oxygen species may mediate reperfusion/reoxygenation injury, as reoxygenation makes the myocardium, and endothelial cells release a burst of superoxide anion (O_2^-) which becomes a source of toxic oxygen species (hydrogen peroxide, H_2O_2 and hydroxyl radical, OH^\cdot). We suspect hypoxemia/reoxygenation damages endothelial cells, and allows neutrophils to adhere to endothelial surfaces that cause capillary plugging, reduced flow and subsequent oxidant damage. Physiologically, nitric oxide is continuously released to control vascular tone³⁾ and has beneficial properties to inhibit neutrophil adhesion⁴⁾ and platelet aggregation,⁵⁾ and neutralize superoxide radicals.^{6,7)} Recent studies in ischemic tissue show that endothelial function could be maintained by the administration of L-arginine (the precursor of NO).⁸⁻¹⁰⁾ The study tests whether

From Division of Cardiothoracic Surgery, UCLA School of Medicine, Los Angeles, CA, USA

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Address reprint requests to Yoshimasa Sakamoto, MD: Department of Cardiovascular Surgery, Machida City Hospital, 2-15-41, Asahimachi, Machida-shi, Tokyo 194-0023, Japan.

leukocyte-depleted reoxygenation could be beneficial in a similar way.¹¹⁻¹³ We evaluated coronary endothelial function, and global myocardial function, and oxidant damage after reoxygenation of hypoxia in vivo neonatal piglet models, exposed to coronary leukocyte filtration.

Materials and Methods

Preparation

Nineteen immature Yorkshire-Duroc piglets (2 to 3 weeks old, 3 to 6 Kg) were premedicated intramuscularly with 5 mg/Kg of ketamine, anesthetized intraperitoneally with 30 mg/Kg of pentobarbital. Anesthesia was maintained by intermittent 5 mg/Kg bolus intravenous injections of pentobarbital and the lungs ventilated on a volume limited respirator (Servo 900D, Siemens-Elma Solna, Sweden) after tracheotomy and endotracheal intubation. All animals received human care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication, No. 86-23, revised 1985). The ductus arteriosus was ligated with a surgical clip via a left fourth intercostal thoracotomy and the coronary sinus was cannulated through the ligated hemiazygous vein. The heart was exposed by median sternotomy. A saline filled catheter was inserted into the left atrium and connected to a pressure transducer. A thermodilution probe was directed into the main pulmonary artery and connected to a cardiac output computer (Model 9520A, American Edwards Laboratory, Santa Ana, CA). After systemic heparinization (300 units/Kg), an aortic cannula (8F) and a single-stage venous cannula (20F) were inserted into the left subclavian artery and the right atrial appendage. A venting line was inserted in the left ventricle through an apical stab incision. The eight-electrode-equipped conductance catheter (Webster Laboratories, Baldwin Park, CA) and the pressure-transducer tipped catheter (Millar Instruments Inc., Houston, TX) were inserted through the left ventricular apex. The conductance catheter was connected to a Sigma-5-DF signal conditioner processor (Leycom, Oegsteest, The Netherlands). The extracorporeal circuit was primed with packed red blood cells and calcium chloride was added to counteract the citrate. The hematocrit value was kept at 25 to 30% with hetastarch (Hespan, Dupont, Wilmington, Del.) throughout the experiment. A membrane oxygenator (Sarns 16310 membrane oxygenator, Sarns, Ann Arbor,

MI) was used and mean aortic pressure was kept at 50 to 60 mmHg by adjusting perfusion flow around 100 ml/Kg/min.

Experimental protocols

All piglets underwent 90 minutes of CPB, followed by 30 minutes of observation after CPB, at which time final hemodynamic and biochemical measurements were made.

CPB control group: CPB without hypoxemia; six piglets underwent 60 minutes of CPB at PO₂ about 400 mmHg.

Experimental group: (1) CPB with hypoxemia and reoxygenation with normal blood (without leukocyte filter group); six piglets underwent hypoxemia for 30 minutes, which was imposed by adding N₂ to the gas mixture of extracorporeal circuit, producing an arterial PO₂ of around 25 mmHg. After clamping the ascending aorta, reoxygenation was done by perfusing the coronary arteries with normal blood (PO₂: 400 mmHg) via the aortic root needle for 60 minutes.

(2) CPB with hypoxemia and reoxygenation with leukocyte-depleted blood (leukocyte filter group); seven other piglets underwent hypoxemia for 30 minutes. After clamping the ascending aorta, the coronary arteries were perfused with leukocyte-depleted blood via the aortic root needle for 60 minutes.

Measurements

White blood cell counts: Leukocyte depletion was achieved by leukocyte-filters (Pall RC 100, Pall Biomedical Products Corporation, Glen Cove, NY) incorporated between the roller pump and cardioplegic line. Leukocytes were measured within the circuit just before and after the leukocyte filter.

Left ventricular performance: (1) Left ventricular stroke work index (LVSWI); myocardial function was measured before CPB was started and 30 minutes after CPB was discontinued. The starting curve was obtained by infusion of blood from CPB at 5 ml/Kg/min while recording CO, MAP, and LAP. CO was determined by duplicate injections of 1 ml of 4°C saline solution into a central venous catheter. LVSWI was calculated by the following equation;

$$LVSWI (g\cdot m/Kg) = (MAP - LAP) \times CO \times 0.0136 / (HR \times BW)$$
where MAP is mean aortic pressure (mmHg), LAP is mean left atrial pressure (mmHg), CO is cardiac output (ml/min), HR is heart rate (beats/minute), and BW is body

weight (Kg).

(2) Left ventricular end-systolic elastance (Ees): Left ventricular (LV) performance was evaluated by LV pressure-volume loops. LV pressure and conductance catheter signals were amplified and digitalized to inscribe LV pressure-volume loops. A series of declining LV pressure-volume loops were obtained by transient occlusion of the inferior vena cava during a 10-second apnea. The end-systolic pressure volume relationship (ESPVR) was analyzed by a user-interactive videographics program (Spectrum, Bowman Gray School of Medicine, Winston-Salem, NC) on an 383/33 MHz IBM computer (IBM, Armonk, NY), and the LV systolic performance was determined as the slope of linear regression. Postbypass LV contractility was expressed as percent recovery of prebypass control value.

Myocardial oxidant injury: (1) Myocardial conjugated dienes. The level of conjugated dienes (CDs), as a marker of oxidant-mediated lipid peroxidation, was determined in the coronary sinus plasma and aortic blood plasma. The blood samples were centrifuged for 5 minutes at 1,000 g, and plasma was stored in liquid nitrogen. Hydroxyconjugated diene levels were measured spectrophotometrically after chloroform methanol 2:1 vol/vol extraction as described by Lesnfsky and coworkers.¹⁴ The level of CDs were expressed as absorbance at a wavelength of 233 nm per 0.5 ml plasma. Myocardial production of CDs was calculated by the following formula: myocardial production of CDs=(CDcs-CDa)×CBF/heart weight where CDcs and CDa are CD concentration in the coronary sinus and arterial blood corrected by hematocrit, respectively. CBF (coronary blood flow, ml/min) means a flow rate in which the coronary arteries were perfused via a calibrated pump to keep the aortic pressure around 50 mmHg under temporary clamping of the proximal ascending aorta.

(2) Antioxidant reserve capacity; The myocardial endogenous antioxidant state was assessed by determining in vitro lipid peroxidation. The LV endocardial tissue was homogenized and incubated with t-butylhydroperoxide at concentrations varying from 0 to 5 mmol/L for 3 minutes at 37°C. Lipid peroxidation was determined spectrophotometrically by using bituric acid reactive substances at 532 nm. A standard curve was made and lipid peroxidation was described as malondialdehyde (MDA) per gram of protein.

(3) Myeloperoxidase activity; Myeloperoxidase (MPO) level is an index of neutrophil accumulation in myocar-

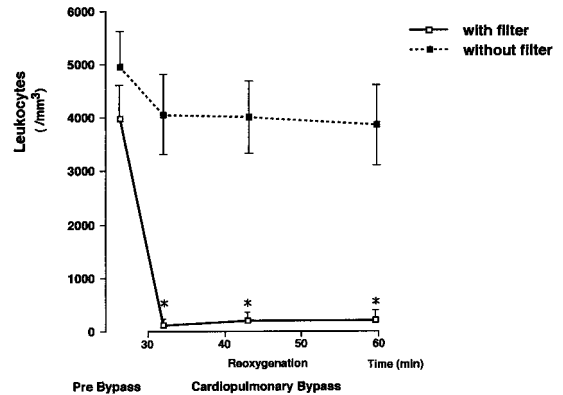


Fig. 1. Leukocyte counts. (*p<0.05 vs. without filter group)

dium and correlates with the number of neutrophils. Samples from the anterior free wall were immediately frozen in liquid nitrogen until analyzed. MPO was determined spectrophotometrically by the method as described before.¹⁵ MPO is expressed as units/g.

In vivo coronary vascular response: The coronary vascular responses to the endothelial receptor-dependent vasodilator acetylcholine (Ach) and the endothelial receptor-independent vasodilator sodium nitroprusside (SNP) were assessed before and after the experiments using a constant flow technique. The aorta was clamped while perfusing the aortic root with blood via a calibrated pump to control the aortic pressure around 50 mmHg (approximately 100 ml/min). Heart rate was atrially paced at 170 beats/min in a beating empty condition. After a few minutes stabilization, 3 ml of Ach (10-4 M) was bolus injected into the clamped aortic root. The proximal aortic pressure was recorded within 60 seconds after injection. After washout and stabilization, SNP (10-5 M) was infused in the same way at least 2 minutes apart. Coronary vascular responses were calculated as percent decrease in coronary pressure compared to baseline pressure responses.

Statistical analysis

All data are reported as mean±standard error of the mean. Statistical analysis of variance (ANOVA) was used for intragroup comparisons. The paired Student's t test was for comparison of variables within experimental groups. All data were analyzed on the software StatView Version2.0 (Abacus Concepts Inc., Berkeley, CA). A p value less than 0.05 was considered significant.

Table 1. Left ventricular performance before and after CPB

	Control		WBC filter		No WBC filter	
	Before CPB	30 min after CPB	Before CPB	30 min after CPB	Before CPB	30 min after CPB
Ees (mmHg/ml)	5.92±1.72	5.68±1.63	5.78±1.50	4.80±0.89	6.06±1.02	2.97±0.40*

Ees, slope of end-systolic pressure volume relation

*p<0.05 vs. before CPB

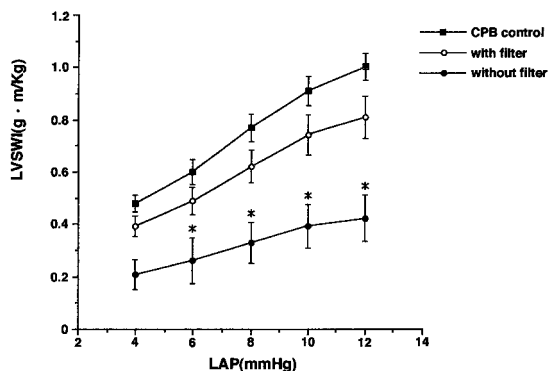


Fig. 2. Left ventricular performance evaluated by volume infusion before hypoxemia (control) and after reoxygenation with or without leukocyte filter. (LVSWI, left ventricular stroke work index; LAP, left atrial pressure. (*p<0.05 vs. CPB control and with filter group)

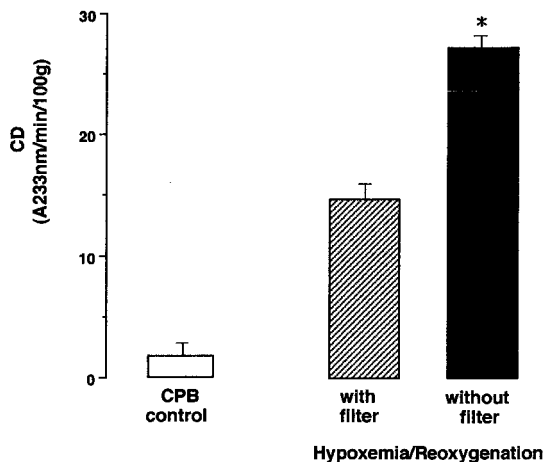


Fig 3. Myocardial conjugated diene (CD) production during reoxygenation. (*p<0.05 vs. CPB control and with filter group)

Results

White blood cell counts

Two minutes after reoxygenation, there was a 97.4% decrease in leukocyte counts in the filter group (3.98±1.1×10³/mm³ before filtration versus 0.11±0.08×10³/mm³ after filtration for the leukocyte filter group). Figure 1 shows how the use of the leukocyte filter markedly lowered coronary perfusing leukocytes during reoxygenation.

LV performance

LVSWI was determined before and after CPB at left atrial mean pressure (LAP) 12 mmHg. In control nonhypoxemic piglets, LVSWI recovered almost 100% after CPB. LVSWI recovered only 53±6% after CPB in the piglets which were reoxygenated with normal blood (without leukocyte filter group). In contrast, in the piglets reoxygenated with leukocyte-depleted blood (leukocyte filter group), LVSWI recovered 81±8% (p=0.02 compared

with the without leukocyte filter group) (Fig. 2). Ees returned to 96±7% after CPB in the control nonhypoxemia group. Percent recovery in Ees of the without leukocyte filter group was 49±5% and that of the filter group was 83±6% (p=0.001) (Table 1).

Conjugated dienes

These myocardial CD levels in the piglets reoxygenated with normal blood (without leukocyte filter group) rose significantly 25 minutes after reoxygenation (3.2±0.8 versus 2.3±0.5; p=0.04) compared with the leukocyte depleted group (Fig. 3).

Antioxidant reserve capacity

Antioxidant reserve capacity was 72% in the piglets reoxygenated with normal blood (without leukocyte filter group) versus the leukocyte depleted group, as malondialdehyde (MDA) increased 16%. Myocardial homogenates were incubated in 2 mmol/l-t-butylhydroperoxide (Fig. 4).

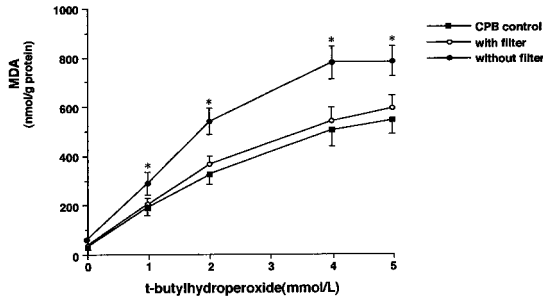


Fig. 4. Antioxidant reserve capacity. LV subendocardial muscle sampled after 30-minute observation after CPB was discontinued. (* $p < 0.05$ vs. CPB control and with filter group)

Myeloperoxidase activity

MPO activity averaged 1.16 ± 0.15 units/g after reoxygenation with normal blood (without leukocyte filter group). In contrast, MPO activity was significantly lower in the control group (0.21 ± 0.03 versus without leukocyte filter group; $p = 0.0001$) and the leukocyte filter group (0.45 ± 0.06 versus without leukocyte filter group; $p = 0.0015$) (Fig. 5).

In vivo coronary vascular responses: (1) Vasodilatation with Ach: Coronary endothelial dependent vasodilatation recovered $71 \pm 20\%$ of baseline in the leukocytes depleted group, compared to only $41 \pm 9\%$ ($p < 0.05$) recovery after normal blood reoxygenation (Table 2).

(2) Vasodilatation with nitroprusside: Coronary endothelial independent vasodilatation recovered more than 70% of baseline in all piglets and was not affected by reoxygenation (Table 2).

Discussion

Previous studies in ischemic hearts have investigated the role of leukocytes using leukocyte filters,^{11,12,16-19} leukocyte-depleted animals,¹⁴ and specific monoclonal anti-

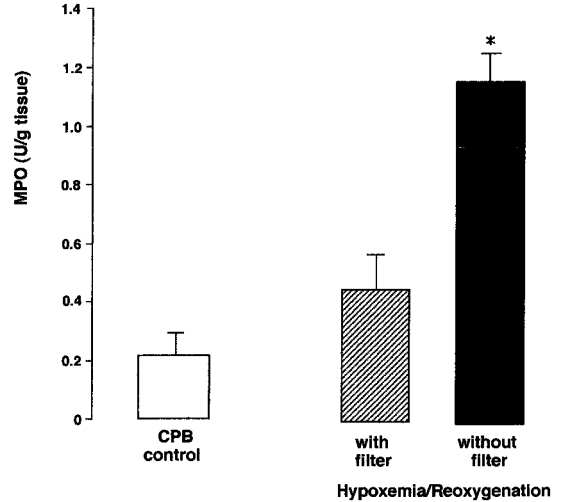


Fig. 5. Myeloperoxidase (MPO) activity, an index of leukocyte accumulation. (* $p < 0.05$ vs. CPB control and with filter group)

bodies against the CD11/CD18 adhesion molecules.^{20,21} Engler et al. reported the accumulation of granulocytes in the myocardium was increased by reperfusion associated with increase in coronary vascular resistance, and reperfusion by leukocyte-depleted blood contributed to improvement of postischemic cardiac dysfunction in a dog model.²² However, several studies suggested reperfusion of leukocyte-depleted blood did not contribute to attenuation of myocardial dysfunction after ischemia/reperfusion injury.^{13,16,23} The contribution of leukocytes to myocardial reperfusion injury remains controversial, and may be related to the degree of leukocyte depletion and the severity of ischemia. Our study investigates the role of leukocytes in the hypoxemia/reoxygenation injury model, using piglet immature hearts. Leukocytes depletion by a coronary reperfusion reduced the total number of leukocytes $>90\%$ of baseline during the initial 15-minute reoxygenation.

Table 2. Percent recovery of coronary vascular response

CVR to	Baseline	% recovery of baseline	
		WBC filter	No WBC filter
Ach (10^{-5} mol/L)	14.0 ± 3.2	71.8 ± 20.4	$41.2 \pm 9.8^*$
SNP (10^{-4} mol/L)	27.5 ± 8.0	77.8 ± 16.2	70.0 ± 19.4

CVR, coronary vascular resistance; Ach, acetylcholine; SNP, sodium nitroprusside

* $p < 0.05$ vs. filter

Reoxygenated damaged endothelium can release superoxide anion (O_2^-) and become potent generators of OH^\bullet , causing lipid peroxidation and protein sulfhydryl oxidation. Subsequent neutrophil activation and accumulation leads to capillary plugging, reduced flow, and release of oxidants that may cause myocardial stunning, with myocardial contractile and further endothelial dysfunction. Leukocyte depletion reduced oxidant damage in this study, as conjugated dienes were less elevated and the antioxidant reserve capacity was preserved. These findings suggest that depletion of white blood cell (WBC) reduced cardiac lipid peroxidation, and the subsequent expenditure of antioxidant, compared with normal blood reperfusion. This refers that leukocytes activation and adherence to the endothelial cells may be an important source of oxygen radicals in hypoxemia/reoxygenation injury.

Intracoronary injection of Ach induced vasoconstriction in atherosclerotic coronary arteries of adult humans,²⁴ but dilates normal coronary arteries of neonatal piglets; this response is decreased after hypoxemia/reoxygenation injury. This deterioration of the coronary responses is reversed by reoxygenation with leukocyte-depleted blood. Tsao et al.²⁵ showed that endothelial cell dysfunction occurred during the early phase of reperfusion (2.5 min) following regional ischemia and progressed with time. The peak of free radical generation is 0.5-10 minutes after the readmission of oxygen but prolongs with reoxygenation. This finding, with WBC depleted blood suggest that neutrophils may be the source of these free radicals. We found reoxygenation by leukocyte-depleted blood did not diminish endogenous antioxidant reserve capacity and increased conjugated diene levels, compared with normal blood reoxygenation (Figs. 3, 4) and myeloperoxidase activity, an indicator of leukocyte adherence and infiltration, was reduced 50% after reperfusion with leukocyte-depleted blood (Fig. 5). Consequently, leukocyte-filtration may play an important role at the early phase of reoxygenation to improve cardiac contractile dysfunction. Our data showed that endothelial dysfunction could be prevented to some extent by reoxygenation with leukocyte-depleted blood. Thus, the endothelial dysfunction leads to increased vasoconstriction, enhanced leukocytes adhesion and accumulation which possibly infiltrated into the myocardium.

In conclusion, this study demonstrates that (1) the coronary endothelial cell is injured in hypoxemia/reoxygenation, (2) these damages are often caused mainly by oxygen free radicals, (3) the occurrence of such an

unintended reoxygenation injury in immature hearts can be avoided to some extent by using a leukocyte depleting filter.

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