STUDIES OF HYPOXEMIC/ REOXYGENATION INJURY: WITHOUT AORTIC CLAMPING

VI. Counteraction of oxidant damage by exogenous antioxidants: *N*-(2-mercaptopropionyl)- glycine and catalase

This study tests the hypothesis that antioxidants administered before reoxygenation can reduce oxygen-mediated damage and improve myocardial performance. Of 25 Duroc-Yorkshire piglets (2 to 3 weeks, 3 to 5 kg) five underwent 60 minutes of cardiopulmonary bypass without hypoxemia (control group), and five others underwent 30 minutes of hypoxemia on cardiopulmonary bypass with a circuit primed with oxygen tension about 25 mm Hg blood followed by reoxygenation on cardiopulmonary bypass (no treatment). In vitro studies were performed to obtain the optimal dosage of the antioxidants *N*-(2-mercaptopropionyl)-glycine and catalase to be used in subsequent in vivo experimental studies; cardiac homogenates were incubated in 0 to 5 mmol/L concentrations of the oxidant *t*-butylhydroperoxide and malondialdehyde production was measured. Fifteen piglets were made hypoxemic on cardiopulmonary bypass for 30 minutes, and the antioxidants *N*-(2-mercaptopropionyl)-glycine at either 30 or 80 mg/kg body weight or *N*-(2-mercaptopropionyl)-glycine, 30 mg/kg body weight, and catalase, 50,000 U/kg body weight, were added to the cardiopulmonary bypass circuit 15 minutes before reoxygenation. Left ventricular contractility, which was expressed as end-systolic elastance, was measured by conductance catheter before hypoxemia and after reoxygenation. Myocardial antioxidant reserve capacity was determined after reoxygenation by incubating cardiac homogenates in the oxidant *t*-butylhydroperoxide and measuring subsequent malondialdehyde elution. The in vitro bioassay studies showed a dose-dependent reduction of lipid peroxidation with *N*-(2-mercaptopropionyl)-glycine, with maximal benefits of a 40% decrease and malondialdehyde elaboration occurring with *N*-(2-mercaptopropionyl)-glycine and catalase compared with untreated cardiac homogenates. Cardiopulmonary bypass (no hypoxemia) caused no oxidant damage or changes in contractile function after cardiopulmonary bypass. Reoxygenation without treatment raised conjugated diene levels 57%,* lowered antioxidant reserve capacity 51%,* and was associated with only 38%* recovery of contractile function ($p < 0.05$ vs control). In contrast, treatment with antioxidants avoided lipid peroxidation, maintained antioxidant reserve capacity, and resulted in a dose-dependent improvement in left ventricular contractility with complete recovery occurring in *N*-(2-mercaptopropionyl)-glycine and catalase-treated piglets (* $p < 0.05$ vs no treatment). This study confirms the occurrence of hypoxemic/reoxygenation injury in immature hearts placed on cardiopulmonary bypass and shows that biochemical and functional damage can be

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counteracted by adding antioxidants to the cardiopulmonary bypass priming fluid. Contractile function improved in a dose-dependent manner, and oxygen-mediated damage could be avoided by mercaptopropionyl glycine/catalase treatment. In vitro studies were helpful to determine drug dosages to be used in in vivo experiments and may be useful to reduce the number of whole animal experiments required to develop optimal dosage regimens. (J THORAC CARDIOVASC SURG 1995;110:1212-20)

A growing body of experimental^{1,2} and clinical^{3,4} evidence indicates that the cyanotic immature heart is susceptible to oxygen-mediated injury that occurs when molecular oxygen is restored. This sequence occurs when cardiopulmonary bypass (CPB) is initiated in cyanotic subjects, and we confirmed this in immature, hypoxemic intact piglets by showing that reoxygenation injury occurred independent of the methods of producing hypoxemia and reoxygenation.⁵ The mechanisms of injury appear to relate to hypoxemic reduction of endogenous antioxidants, coupled with the inability to detoxify the burst of oxygen free radicals that follows reoxygenation.⁶ Hydroxyl ion [$\cdot\text{OH}$] is the most cytotoxic of the different species of reactive oxygen metabolites (hydrogen peroxide [H_2O_2], $\cdot\text{OH}$ and superoxide anion [O_2^-]) that accentuate the chain reaction of lipid peroxidation that follows reoxygenation⁷ and further reduce antioxidant reserve capacity.^{8,9} Antioxidants limit oxygen-mediated damage in studies of reperfusion after myocardial ischemia^{10,11} and reoxygenation after in vitro hypoxemia,¹² but the salutary effects of antioxidants in in vivo hypoxemic conditions are relatively unestablished.

This study was undertaken to test the potential salutary effect of *N*-(2-mercaptopropionyl)-glycine, an $\cdot\text{OH}$ scavenger, and catalase, an H_2O_2 scavenger, in reducing reoxygenation injury in reoxygenation of the infantile cyanotic piglet undergoing CPB. The findings show that (1) antioxidants reduce reoxygenation injury in a dose-dependent fashion both in vitro and in vivo; (2) in vitro bioassay studies are helpful to determine optimal in vivo dosage; and (3) superior results occur when antioxidant therapy is directed against both $\cdot\text{OH}$ and H_2O_2 .

Material and methods

Preparation. Twenty-five immature, 2- to 3-week-old Yorkshire-Duroc piglets (3 to 5 kg) were premedicated with 0.5 mg/kg diazepam intramuscularly, anesthetized with 30 mg/kg pentobarbital intraperitoneally followed by 5 mg/kg intravenously each hour, and the lungs ventilated

on a volume-limited respirator (Servo 900D, Siemens-Elema, Solna, Sweden) via a tracheostomy. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

The experimental preparation, including cannulation for CPB and for monitoring and blood samples, was carried out as previously described.⁵

Measurements. Hemodynamic measurements were made before the start of hypoxemia (control) and 15 and 30 minutes after CPB was discontinued.

Blood samples for the determination of conjugated dienes from coronary sinus blood were taken before the start of CPB (control), 5 minutes after hypoxemia, at the end of hypoxemia, 5 minutes after reoxygenation, before the discontinuation of CPB (30 minutes of reoxygenation), and at the end of the 30-minute observation period.

Arterial blood gas, electrolyte, and hemoglobin measurements were made by a blood gas system (Blood Gas System 288, Ciba-Corning, Medfield, Mass.). Final biochemical measurements were made immediately after final evaluation of cardiac mechanical function by taking transmural left ventricular (LV) biopsy samples after arresting the heart with a 4°C cold blood cardioplegic solution (30 mEq KCl) to stop metabolism and placing them in liquid nitrogen for subsequent analysis.

LV performance was evaluated by inscribing end-systolic pressure volume relationships using a LV five-electrode conductance catheter (Webster Laboratories, Baldwin Park, Calif.) inserted through the LV apex, connected to a Sigma 5-DF Signal conditioner processor (Leycom, Oegstgeest, The Netherlands).¹³ A series of declining LV pressure-volume loops was obtained by vena cava occlusion during a 7-second apnea. The end-systolic pressure volume/dimension relationship was analyzed by a user-interactive videographics program "Spectrum"¹⁴ on a 386/33 MHz IBM PC. Data were expressed as end-systolic elastance (Ees).

Cardiac output was determined by duplicate 1 ml injections of 4°C cold saline solution into the right atrium. The thermodilution probe was placed in the main pulmonary artery and connected to a cardiac output computer (Model 9520A, American Edwards Laboratory, Santa Ana, Calif.). Results were expressed as stroke work index (SWI) in $\text{g} \times \text{m/kg}$ to normalize for body weight. $\text{SWI} = (\text{MAP} - \text{LAP}) \times \text{CO} \times 0.0136 / (\text{heart rate} \times \text{BW})$, where MAP is mean aortic pressure, LAP is mean left atrial

In vitro lipid peroxidation (at 2 mM t-BHP)

Fig. 1. Cardiac antioxidant reserve capacity, expressed as percentage of malondialdehyde production in myocardial homogenates incubated in 2 mmol/L *t*-butylhydroperoxide (hatched line 100%, no treatment). Note: (1) significant reduction of malondialdehyde elution by incubation with malondialdehyde, catalase, or both, (2) absence of effect with SOD; see text for explanation. MDA, Malondialdehyde; *t*-BHP, *t*-butylhydroperoxide; MPG, *N*-(2-mercaptopropionyl)-glycine.

pressure, CO is cardiac output in milliliters per minute, HR is heart rate, and BW is body weight in kilograms.

Systemic vascular resistance index (SVRI) was determined by the following equation: $SVRI \text{ (mm Hg} \cdot \text{min} \cdot \text{ml}^{-1} \cdot \text{kg)} = (\text{MAP} - \text{CVP}) \text{ (mm Hg)} \cdot \text{CO}^{-1} \text{ (ml/min)} \cdot \text{BW (kg)}$ where CVP is central venous pressure.

Conjugated diene levels (a marker of lipid peroxidation) were determined from samples of coronary sinus blood as described by Lefnisky and coworkers.¹⁵

Antioxidant reserve capacity was measured by the method of Godin and coworkers¹⁶ to determine vulnerability of hypoxemic and reoxygenation myocardium to subsequent oxidant stress. Myocardial homogenates were exposed to 0 to 5 mmol/L *t*-butylhydroperoxide for 30 minutes at 37°C with subsequent measure of thiobarbituric acid-reactive substance. Absorbance 532 nm of the organic phase is measured against a reaction blank. Data were expressed as nanomoles malondialdehyde per gram protein.

Each heart was examined postmortem to ensure satisfactory ligation of the ductus arteriosus and the absence of a patent foramen ovale and to inspect for macroscopic damage to abdominal and thoracic structures.

In vitro studies

An in vitro bioassay analysis was performed to potentially reduce the number of experiments needed to determine dose-relation responses in the in vivo model. Myocardial homogenates were incubated with different

concentrations of the antioxidants superoxide dismutase (SOD), *N*-(2-mercaptopropionyl)-glycine, and catalase to determine the dosage that reduced malondialdehyde elaboration maximally on exposure to the oxidant *t*-butylhydroperoxide. This oxidant concentration was selected because it was the minimum concentration that produced significant differences between groups in our previous studies of normal and hypoxemic/reoxygenated tissue.⁵

In vivo studies

Control (no hypoxemia). Five piglets underwent CPB at oxygen tension (Po_2) about 400 mm Hg for 1 hour without hypoxemia.

Experimental (hypoxemia and reoxygenation on CPB). Twenty piglets were placed on CPB with the extracorporeal circuit primed with hypoxemic blood (Po_2 about 25 ± 2 mm Hg). This hypoxemic level was achieved by adding N_2 to the gas mixture. Hypoxemia was maintained for 30 minutes, followed by reoxygenation on CPB for 30 minutes at Po_2 about 400 mm Hg.

NO TREATMENT. Five piglets underwent 30 minutes of hypoxemia on CPB as described previously, followed by 30 minutes of reoxygenation on CPB (no treatment).

ANTIOXIDANT TREATMENT. In 10 piglets *N*-(2-mercaptopropionyl)-glycine was added to the CPB circuit 15 minutes before reoxygenation. The dose was 30 mg/kg in five piglets and 80 g/kg in five others.

Fig. 2. Plasma conjugated diene levels in coronary sinus blood. *Note:* high levels of this marker of lipid peroxidation throughout reoxygenation in untreated piglets (no treatment) compared with normal conjugated diene levels when antioxidants are added to CPB prime.

In five piglets *N*-(2-mercaptopropionyl)-glycine (80 mg/kg body weight) plus catalase (50,000 U/kg body weight) were added to the CPB circuit 15 minutes before the start of reoxygenation.

Final biochemical and functional measurements were made 30 minutes after CPB observation.

Statistics. Data were analyzed with the use of the StatView V2.0 program (Abacus Concepts Inc., Berkeley, Calif.) on a Macintosh IICI computer (Apple, Inc., Cupertino, Calif.). Analysis of variance was used for intergroup comparison, and the paired Student *t* test was used for comparison of variables within experimental groups. The relationship between functional impairment (percent Ees) and lipid peroxidation (CDs) was tested by linear regression analysis. Differences were considered significant at the probability level of $p < 0.05$. Group data were expressed as mean plus or minus standard error of the mean.

Results

In vitro studies

Malondialdehyde elution in untreated myocardial homogenates incubated with 2 mmol/L *t*-butylhydroperoxide was considered to reflect maximum oxidant damage from this oxidant or 100%. SOD did not depress malondialdehyde production, whereas *N*-(2-mercaptopropionyl)-glycine was protective in a dose-dependent fashion (Fig. 1), with maximum

inhibition of malondialdehyde occurring when 1 mmol *N*-(2-mercaptopropionyl)-glycine was combined with catalase, 933 units ($p < 0.05$).

In vivo studies

Biochemical. Reoxygenation caused a 57% increase in coronary sinus conjugated diene levels compared with prehypoxemic control levels ($p < 0.05$, Fig. 2), whereas conjugated dienes remained at control levels in all treated piglets. Reoxygenation caused a 51% reduction in antioxidant reserve capacity in untreated piglets, whereas antioxidant reserve capacity remained within control levels in all the piglets receiving antioxidants, with a trend toward the lowest levels when *N*-(2-mercaptopropionyl)-glycine and catalase were combined (Fig. 3, $p < 0.05$).

Hemodynamics. Cardiac Ees recovered completely in nonhypoxemic piglets undergoing CPB for 60 minutes, whereas Ees was restored to only 38% of control levels ($p < 0.05$) after hypoxemia/reoxygenation in untreated piglets (no treatment, Fig. 4). Recovery improved to 60% and 81% respectively, in a dose-dependent fashion when *N*-(2-mercaptopropionyl)-glycine was added to the pump prime at 30 and 80 mg/kg and returned to 101% of control

Fig. 3. Cardiac antioxidant reserve capacity expressed as production of malondialdehyde after incubation with 5 mmol/L antioxidant *t*-butylhydroperoxide. Note: severely reduced antioxidant reserve capacity (high malondialdehyde production in untreated animals (no treatment) after hypoxemia and reoxygenation and restoration to control levels by antioxidant treatment. *MDA*, Malondialdehyde; *MPG*, *N*-(2-mercaptopropionyl)-glycine.

values when *N*-(2-mercaptopropionyl)-glycine and catalase were combined ($p < 0.05$ vs untreated piglets).

Discussion

This study provides data to confirm the occurrence of a biochemical and functional reoxygenation injury that is avoided by antioxidant treatment. Supplementation of the CPB prime with the $\cdot\text{OH}$ and H_2O_2 scavengers (*N*-(2-mercaptopropionyl)-glycine and catalase) had the same favorable effects on lipid peroxidation and oxidant reserve capacity and functional recovery that occur when these oxidants are given intravenously before reoxygenation.¹⁷ Our in vivo findings of the dose-dependent benefits of antioxidants quantified by the in vitro studies infer that bioassay systems may be useful to determine optimal in vivo dosage regimens.

The experimental model of subjecting immature piglets to hypoxemia/reoxygenation on CPB avoids ischemic changes that follow ventilator hypoxemia.⁵ Acute asphyxia (i.e., ventilator hypoxemia) produces consistent hemodynamic deterioration that often leads to premature institution of CPB,¹⁸ thereby preventing imposition of a standard interval and magnitude of oxidant stress. The additional advan-

tages of the in vivo model are described elsewhere¹⁹ together with the limitations caused by the absence of compensatory adaptations of chronic cyanosis. These limitations notwithstanding, the biochemical and functional changes produced by reoxygenation parallel alterations in isolated hearts^{8,9} and hypoxemic patients undergoing reoxygenation on CPB.^{3,4} The potential oxygen-mediated damaging effects of CPB were present in all subjects; these included activation of leukocytes,²⁰ the arachidonic pathway,²¹ and abnormalities in xanthine metabolism.²² Oxidant damage occurred *only* in untreated piglets that did not receive antioxidant treatment and were absent when 1 hour of CPB was imposed without either hypoxemia or antioxidants.

The adverse effects of the hypoxemia/reoxygenation process have been described previously^{8,9,23} and mimic those of ischemia/reperfusion.²⁴ Our current in vivo findings are consonant with (1) reduced in vitro myocardial endogenous antioxidant depletion during hypoxemia and further lowering during reoxygenation⁸ and (2) evidence of lipid peroxidation (malondialdehyde production) after reoxygenation developing coincident with enzymatic cell damage (creatine kinase release) and myocardial stunning characterized by decreased contractile

Fig. 4. LV contractility expressed as percentage recovery of Ees 30 minutes after CPB was discontinued. Note: severely depressed function after hypoxemia/reoxygenation in untreated animals (no treatment) in contrast to dose-related improvement after treatment with *N*-(2-mercaptopropionyl)-glycine and normal postbypass function after *N*-(2-mercaptopropionyl)-glycine and catalase (*N*-(2-mercaptopropionyl)-glycine and catalase).

recovery.^{9, 25, 26} The predominance of oxidant damage appears to occur with reoxygenation, because hypoxemia does not cause lipid peroxidation; conjugated diene levels remained normal during PO_2 about 25 mm Hg perfusion on CPB (Fig. 2) but expends endogenous antioxidants (i.e., SOD and glutathione). Consequently, the natural barriers to oxidant damage with reintroduction of molecular oxygen become depleted and lipid peroxidation (conjugated diene production) that causes subsequent myocardial functional damage follows reoxygenation.⁹ Exogenous replenishment of antioxidants bolsters this otherwise expended natural defense against oxidant injury, but our studies do not define the sources of reactive oxygen intermediates (i.e., catecholamines,²⁷ prostaglandin pathways,²⁸ cardiac mitochondria,²⁷ or endothelium²⁹) or the site of action (i.e., cytosol or membrane) of the antioxidants. The answers to the aforementioned questions require future studies to determine the sources of cytotoxic O_2 species and site-directed antioxidants to define their foci of action.

Bioassay studies were conducted on normal myo-

cardial homogenates incubated with varying concentrations of the oxidant *t*-butylhydroperoxide to determine if these in vitro and relatively inexpensive analyses would furnish data on antioxidant dosage that could be transferred to the more expensive in vivo model and therefore limit the number of whole animal studies that were needed. Oxidant damage is inevitable in in vitro studies because the myocardium contains a finite concentration of endogenous antioxidants, whereas an unlimited amount of oxidant can be added to overwhelm natural defense barriers. Consequently, all tissues will exhibit evidence of oxidant damage when the oxidant challenge overwhelms the endogenous antioxidant defense mechanisms. Resultant malondialdehyde levels are related inversely to reserve capacity, as shown in Fig. 1. The 100% value indicates how much malondialdehyde is produced in the presence of endogenous antioxidants and how exogenous additives reduce lipid peroxidation. We evaluated SOD to test the system, and it did not reduce lipid peroxidation; the oxidant *t*-butylhydroperoxide was added exogenously, and SOD is not involved. *N*-(2-

Table. Hemodynamics before, during, and after CPB

	Control	No Rx	MPG (30 mg)	MPG (80 mg)	MPG + CAT
Cardiac index (ml/min/kg)					
Start	114 ± 18	112 ± 7	119 ± 14	133 ± 22	136 ± 8
15 min CPB	96 ± 0	112 ± 11	131 ± 19	120 ± 32	96 ± 8
30 min CPB	92 ± 9	126 ± 21	123 ± 22	88 ± 11	79 ± 8*
60 min CPB	90 ± 8	111 ± 20	87 ± 8	76 ± 9	55 ± 14*
30 min off CPB	82 ± 8	74 ± 7	69 ± 8	79 ± 9	132 ± 24†
Stroke work index (g · m/kg)					
Start	0.47 ± 0.06	0.49 ± 0.06	0.47 ± 0.06	0.42 ± 0.09	0.49 ± 0.06
30 min off CPB	0.26 ± 0.04	0.26 ± 0.03	0.21 ± 0.04	0.24 ± 0.06	0.49 ± 0.1†
Heart rate (beats/min)					
Start	186	202 ± 4	202 ± 9	206 ± 13	190 ± 10
30 min off CPB	196	212 ± 7	188 ± 12	205 ± 10	176 ± 10*
Mean aortic pressure (mm Hg)					
Start	70 ± 5	69 ± 3	65 ± 2	58 ± 5	54 ± 2
15 min CPB	63 ± 4	54 ± 2	52 ± 2	52 ± 2	56 ± 8
30 min CPB	62 ± 3	56 ± 4	55 ± 4	53 ± 2	62 ± 3
60 min CPB	65 ± 4	59 ± 3	52 ± 2	52 ± 2	58 ± 2
30 min off CPB	59 ± 4	60 ± 7	55 ± 7	49 ± 4	52 ± 4

MPG, *N*-(2-mercaptopropionyl)-glycine.**p* < 0.05 vs no treatment.†*p* < 0.05 vs all other groups.

mercaptopropionyl)-glycine is an $\cdot\text{OH}$ scavenger and exerted a dose-dependent reduction in oxidant damage. The additional effects of catalase are ascribed to its capacity to alter the peroxidatic reaction as described by Chance and colleagues.³⁰

N-(2-mercaptopropionyl)-glycine and catalase were the only antioxidants tested in these studies and produced near-complete avoidance of reoxygenation injury under the imposed experimental conditions. Other antioxidants (i.e., deferoxamine,³¹ CoQ₁₀,³² allopurinol,³³ or agents directed at other sources of oxygen injury (i.e., white cells,²⁰ arachidonic acid metabolism²⁸) may provide comparable protection. *N*-(2-mercaptopropionyl)-glycine is an $\cdot\text{OH}$ scavenger that may be effective in both the classic Haber-Weiss pathway and the newly described *L*-arginine-nitric oxide pathway of oxidative injury, because *N*-(2-mercaptopropionyl)-glycine also scavenges the toxic intermediate peroxynitrite (OONO⁻).³⁴ Hydroxyl radical ($\cdot\text{OH}$) is more cytotoxic than O₂⁻,³⁵ but there is no satisfactory explanation how extracellularly formed $\cdot\text{OH}$ reaches the cellular target, because it reacts within a few Angstroms of its site of formation.³⁶ Conversely, OONO⁻ is sufficiently stable to diffuse several cell diameters before being protonated to yield $\cdot\text{OH}$ ³⁶ and may therefore be a more immediate source of reoxygenation injury in vivo. However, this is speculative,

because our observations only quantify evidence of lipid peroxidation and myocardial dysfunction.

The benefits of antioxidant pretreatment have been stressed previously by Dhaliwal and colleagues⁹ and Bolli and coworkers¹⁰ in both ischemic and hypoxemic preparations. The concentrations found effective in our bioassay studies are comparable with those reported by Zimmer and Evers³⁷ in the isolated rat heart model exposed to hypoxemia/reoxygenation. The total 80 mg/kg in vivo dose was increased over that used in our previous intravenous studies¹⁷ to overcome the dilutional effects introduced by the priming volume of the CPB circuit. The resultant intravascular concentrations were comparable with those found cardioprotective by Mitsos and colleagues¹⁰ and somewhat higher than those reported by Bolli and coworkers¹¹ in the ischemia/reperfusion model. Our previous studies in the ventilator hypoxemia model showed that *N*-(2-mercaptopropionyl)-glycine and catalase were cardioprotective either as pretreatment (intravenous infusion during hypoxemia) or when added to the extracorporeal prime to ensure their presence at the initiation of reoxygenation. In the present studies of the model of hypoxemia/reoxygenation on bypass, adding *N*-(2-mercaptopropionyl)-glycine and catalase exerted the dual effect of pretreatment (because they were administered during hypoxemia) and en-

sured effective concentration in the CPB circuit at the initiation of reoxygenation.

We believe that this study supplies additional evidence that reoxygenation injury occurs during initiation of CPB in hypoxemic subjects. The importance of considering use of antioxidants is underscored by reports of myocardial depression progressing to stunning (absence of aortic pulsations) in cyanotic infants placed on extracorporeal membrane oxygenation for pulmonary disorders causing hypoxemia³⁸ and preischemic tissue biopsy evidence of lipid peroxidation and diminished endogenous antioxidants in cyanotic children placed on CPB for correction of tetralogy of Fallot.^{3,4} Recognition of this potential source of unintended myocardial damage should allow appropriate preparations to be made to counteract it, because both intravenous pretreatment and supplementation of the prime of the extracorporeal circuit with antioxidants can be accomplished readily in the clinical setting. We hope that these experimental findings provide a stimulus to others to study this issue and test the reproducibility of our findings, because it appears that reoxygenation injury is an avoidable complication of CPB in hypoxemic subjects.

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