STUDIES OF HYPOXEMIC/ REOXYGENATION INJURY: WITHOUT AORTIC CLAMPING

VIII. Counteraction of oxidant damage by exogenous glutamate and aspartate

Previous studies show that (1) hypoxemia depletes immature myocardium of amino acid substrates and their replenishment improves ischemic tolerance, (2) reoxygenation on cardiopulmonary bypass causes oxygen-mediated damage without added ischemia, and (3) this damage may be related to the nitric oxide-L-arginine pathway that is affected by amino acid metabolism. This study tests the hypothesis that priming the cardiopulmonary bypass circuit with glutamate and aspartate limits reoxygenation damage. Of 22 immature Duroc-Yorkshire piglets (<3 weeks old), five were observed over a 5-hour period (control), and five others underwent 30 minutes of CPB without hypoxemia (cardiopulmonary bypass control). Twelve others became hypoxemic by reducing ventilator inspired oxygen fraction to 6% to 7% (oxygen tension about 25 mm Hg) before reoxygenation on cardiopulmonary bypass for 30 minutes. Of these five were untreated (no treatment), and the cardiopulmonary bypass circuit was primed with 5 mmol/L glutamate and aspartate in seven others (treatment). Left ventricular function before and after bypass was measured by inscribing pressure-volume loops (end-systolic elastance). Myocardial conjugated diene levels were measured to detect lipid peroxidation, and antioxidant reserve capacity was tested by incubating cardiac muscle with the oxidant *t*-butylhydroperoxide to determine the susceptibility to subsequent oxidant injury. CPB (no hypoxemia) allowed complete functional recovery without changing conjugated dienes and antioxidant reserve capacity, whereas reoxygenation injury developed in untreated hearts. This was characterized by reduced contractility (elastance end-systolic recovered only $37\% \pm 8\%^*$), increased conjugated diene levels (1.3 ± 0.1 vs $0.7 \pm 0.1^*$), and decreased antioxidant reserve capacity (910 ± 59 vs 471 ± 30 malondialdehyde nmol/g protein at 2 mmol/L *t*-butylhydroperoxide*). In contrast, priming the cardiopulmonary bypass circuit with glutamate and aspartate resulted in significantly better left ventricular functional recovery ($75\% \pm 8\%$ vs $37\% \pm 8\%^*$), minimal conjugated diene production (0.8 ± 0.1 vs $1.3 \pm 0.1^*$), and improved antioxidant reserve capacity (726 ± 27 vs 910 ± 59 malondialdehyde nmol/g protein*) (* $p < 0.05$ vs cardiopulmonary bypass control). We conclude that reoxygenation of immature hypoxemic piglets by the initiation of cardiopulmonary bypass causes myocardial dysfunction, lipid peroxidation, and reduced tolerance to oxidant stress, which may increase vulnerability to subsequent ischemia (i.e., aortic crossclamping). These data suggest that supplementing the prime of cardiopulmonary bypass circuit with glutamate and aspartate may reduce these deleterious consequences of reoxygenation. (J THORAC CARDIOVASC SURG 1995;110:1228-34)

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Recent experimental^{1,2} reports suggest that unintended reoxygenation injury of hypoxemic myocardium occurs during initiation of extracorporeal circulation. The damage is characterized by lipid

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peroxidation and myocardial dysfunction and may contribute to postbypass dysfunction after surgical correction of cyanotic heart defects, because subsequent ischemia is needed for cardiac repair.

Our previous studies show that hypoxemia-related disorders of amino acid metabolism may enhance reoxygenation-induced myocardial dysfunction because (1) hypoxemia depletes the immature myocardium of amino acid substrates needed for energy production,³ (2) replenishment of glutamate and aspartate during hypoxemia improves ischemic tolerance,⁴ and (3) preoxygenation treatment with an intravenous metabolic support solution that includes these amino acids limits oxidant damage and reoxygenation-induced myocardial dysfunction in hypoxemic immature piglets.⁵

Recent studies implicate the L-arginine-nitric oxide (·NO) pathway in the pathogenesis of reoxygenation damage.⁵ Amino acids also affect the L-arginine cationic transport system and may therefore reduce lipid peroxidation by inhibiting L-arginine uptake and subsequent NO synthesis.^{6,7} This study tests the hypothesis that priming the cardiopulmonary bypass (CPB) circuit with glutamate and aspartate will reduce lipid peroxidation and improve post-CPB functional recovery after reoxygenation of the cyanotic immature heart when CPB is initiated.

Material and methods

Preparation. Twenty-two 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" published by the National Academy of Sciences, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). The surgical preparation, including vessel cannulation for CPB and catheter replacement for blood sampling and monitoring, is similar to that described previously.²

Measurements

Hemodynamics. Hemodynamic measurements were made during control conditions, each 15 minutes during hypoxemia, and 15 minutes and 30 minutes after CPB was discontinued. Cardiac output was determined by duplicate injections of 1 ml of 4° C saline solution into a central venous catheter. Cardiac index (CI) and left ventricular stroke work index (LVSWI) were calculated with the following equations:

$$CI \text{ (ml/min/kg)} = CO/\text{body weight (kg)}$$

$$LVSWI \text{ (g} \cdot \text{m/kg)} = (\text{MAP} - \text{LAP})$$

$$\times CO \times 0.0136/(\text{HR} \times \text{body weight [kg]})$$

where MAP is mean aortic pressure, LA is mean left atrial pressure, CO is cardiac output in milliliters per minute, and HR is heart rate.

Left ventricular performance. Left ventricular (LV) pressure-volume and ultrasonic crystal signals from sonomicrometer crystals placed on the anterior and posterior LV epicardial surfaces were amplified and digitized to inscribe LV pressure-volume loops. A series of pressure-dimension loops under variable loading conditions was generated by rapid transient occlusion of the inferior vena cava during a 7-second period of apnea, during control conditions, and 30 minutes after CPB was discontinued.⁸

The end-systolic pressure volume relationship (ESPVR) was analyzed by an interactive videographics data analysis computer program (Spectrum, Bowman Gray School of Medicine, Winston-Salem, N.C.) on an 386/33 MHz IBM computer (IBM, Armonk, N.Y.), and LV performance was described as the slope of linear regression (end-systolic elastance [Ees]), as described previously.⁹ Postbypass LV contractility was assessed by percent recovery of prebypass control value (percent Ees). Volume loading by transfusion to LAP 8 mm Hg was used to compare recovery of LVSWI before and after CPB.

Tissue injury due to oxidants. Myocardial conjugated dienes were measured as a marker of lipid peroxidation. LV endocardial biopsy specimens were frozen immediately and stored in liquid nitrogen; tissue levels of hydroxyconjugated dienes were determined as described by Lesnefsky and coworkers.¹⁰

Antioxidant reserve capacity. In vitro lipid peroxidation was measured by the method of Godin and colleagues¹¹ to assess the antioxidant state or vulnerability to subsequent oxidant stress as described previously.

Experimental Groups

Control. Five normoxemic piglets were anesthetized, instrumented, and observed over a 5-hour period to define the stability of an open-chest preparation and provide baseline control functional and biochemical data.

CPB without hypoxemia or reoxygenation. Five piglets underwent 30 minutes of CPB without hypoxemia, followed by 30 minutes of observation after CPB to determine the effects of CPB per se.

Hypoxemia and reoxygenation. Twelve piglets were made hypoxemic by lowering inspired oxygen fraction (Fio₂) (6% to 7%) to reduce arterial oxygen tension (Pao₂) to about 25 mm Hg for up to 120 minutes, followed by reoxygenation on CPB (Pao₂ > 400 mm Hg). CPB was started before 120 minutes if mean arterial pressure fell below 30 mm Hg and was unresponsive to volume infusion or arterial pH could not be maintained above 7.3 by NaHCO₃ bolus intravenous infusions.

No treatment. Five piglets were reoxygenated on CPB without amino acid supplementation of the prime of extracorporeal circuit.

Table. Changes in heart rate, cardiac index, and left ventricular stroke work index during hypoxia/reoxygenation

	Control	Hypoxia (min)				Reoxygenation 30 min after CPB	% of control
		15	30	60	End		
Heart rate (beats/min)							
CPB	169 ± 11					176 ± 10	
Control	154 ± 9					166 ± 9	
NoRx	170 ± 8	183 ± 9	193 ± 9	198 ± 10	177 ± 7	163 ± 8	
G/A	189 ± 17	190 ± 5	210 ± 7	185 ± 10	169 ± 8	160 ± 6	
Cardiac index (ml/min/kg)							
CPB	83 ± 12*					75 ± 8	(111 ± 24*)
Control	87 ± 8*					80 ± 7	(98 ± 10*)
NoRx	120 ± 12	135 ± 13	148 ± 19	128 ± 21	103 ± 12	70 ± 7	(62 ± 6)
G/A	134 ± 12†‡	133 ± 14	158 ± 30	73 ± 30*	91 ± 21	103 ± 11*†	(77 ± 14)
LVS WI (g · m/kg)							
CPB	0.43 ± 0.09					0.52 ± 0.06*	(126 ± 33*)
Control	0.61 ± 0.09					0.64 ± 0.09	(101 ± 14)
NoRx	0.71 ± 0.12	0.70 ± 0.11	0.79 ± 0.13	0.66 ± 0.14	0.35 ± 0.07	0.29 ± 0.04	(52 ± 11)
G/A	0.57 ± 0.07	0.50 ± 0.08	0.74 ± 0.15	0.43 ± 0.08	0.27 ± 0.08	0.40 ± 0.07	(72 ± 8*)

No Rx, No treatment; G/A, piglets with glutamate/aspartate supplementation; LVS WI, left ventricular stroke work index; CPB, cardiopulmonary bypass.

* $p < 0.05$ vs no treatment.

† $p < 0.05$ vs CPB.

‡ $p < 0.05$ vs control.

GLUTAMATE AND ASPARTATE IN CPB PRIME. In seven piglets the prime of the CPB circuit was supplemented with 5 mmol/L glutamate and aspartate. A 30-minute period of reoxygenation on CPB was followed by 30 minutes of observation after CPB before final functional assessments were performed and specimens for biochemical analyses were obtained. At the end of each experiment, the brain and thoracic and intraabdominal organs were inspected visually to confirm closure of the foramen ovale and arterial ductus.

Statistics. Data were analyzed with the use of the StatView V2.0 program (Abacus Concepts Inc., Berkeley, Calif.) on a Macintosh IIfx computer (Apple, Inc., Cupertino, Calif.). Analysis of variance was used for intergroup comparison and the paired Student *t* test used for comparison of variables within experimental groups. 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

CI and stroke work rose initially after hypoxemia but declined during the second hour; premature institution of CPB because of hemodynamic deterioration was needed in seven of 12 piglets (Table). The average tolerated hypoxemic period was 102 ± 15 minutes, with no difference in mean duration of hypoxemia in piglets reoxygenated on CPB with and without amino acid supplementation of the extracorporeal circuit priming fluid.

Ventricular performance. Post-CPB function was unaltered in piglets undergoing CPB without pre-

ceding hypoxemia (CPB), because Ees recovered to 98% of control values and LVS WI (at 8 mm Hg LAP) recovered to the same level recorded before CPB was started. In contrast, Ees recovered only $38\% \pm 12\%$ of prehypoxemic control values ($p < 0.05$ vs CPB) after reoxygenation of CPB without altering the pump prime (no treatment). Supplementation of the prime with glutamate and aspartate resulted in $75\% \pm 8\%$ recovery of LV contractility ($p < 0.05$ vs no treatment) (Fig. 1). LVS WI recovered only 52% in untreated piglets, whereas glutamate and aspartate supplementation of the CPB prime resulted in 72% recovery ($p < 0.05$ vs no treatment, after transfusion to LAP of 8 mm Hg (Table).

Conjugated dienes. LV conjugated dienes (Fig. 2) were comparable with those in normoxemic instrumented piglets after CPB without hypoxemia. Reoxygenation on CPB (no treatment) increased myocardial tissue conjugation diene levels 89% above control values (0.7 ± 0.1 vs 1.3 ± 0.2 ; $p < 0.05$). Conjugated diene levels after CPB remained in the normal range after the addition of glutamate and aspartate to the CPB prime, with final values significantly lower than in untreated piglets (0.83 ± 0.1 vs 1.3 ± 0.1 ; $p < 0.05$).

Antioxidant reserve capacity. Antioxidant reserve capacity was unaltered by CPB (Fig. 3). Conversely, reoxygenation on CPB reduced antioxidant reserve capacity, as malondialdehyde increased sig-

Fig. 1. LV contractility. LV Ees after discontinuation of CPB, expressed as percent of individual prehypoxemic values. *CPB*: piglets undergoing CPB without hypoxemia; *no treatment*: piglets undergoing reoxygenation without treatment; *Glu/Asp*: piglets undergoing reoxygenation on CPB with glutamate/aspartate supplementation. * $p < 0.05$ vs no treatment; † $p < 0.05$ CPB.

nificantly in myocardial homogenates incubated in the oxidant *t*-butylhydroperoxide. These adverse effects of reoxygenation were limited by adding glutamate and aspartate to the extracorporeal circuit (Fig. 3).

Discussion

These data indicate that supplementation of the CPB circuit prime with glutamate and aspartate reduces the lipid peroxidation and myocardial dysfunction that would otherwise occur when hypoxemic immature hearts are reoxygenated when CPB is started. These findings are consistent with our previous observations.⁵ Prior studies of glutamate and aspartate in immature and adult hearts focused primarily on the capacity of these amino acids to maintain anaerobiosis during ischemia¹²⁻¹⁶ and restore aerobic metabolism by replenishing substrates depleted by hypoxemia and ischemia.^{3, 17, 18} This report provides the first direct evidence that amino acid supplementation limits lipid peroxidation after reoxygenation.

Hypoxemia or ischemia reduces cytosolic concentrations of glutamate and aspartate^{12, 13}; these amino acids are used to produce adenosine triphosphate anaerobically via substrate phosphorylation and thereby become less available for resumption of

aerobic metabolism via the Krebs cycle after reoxygenation. Studies by Pisarenko and coworkers¹² indicate that the pool size of key amino acids influences postischemic recovery of both cellular energy supply and contractile function. Furthermore, it seems that only those amino acids that can be transformed to glutamate and aspartate are effective in maintaining cardiac adenine nucleotides and limiting ischemic injury.^{14, 15}

One recognized aspect of the improved recovery afforded by amino acid supplementation of the extracorporeal circuit may be via the mechanisms of ensuring their availability for aerobic adenosine triphosphate production after reoxygenation.¹⁸ The capacity of amino acids to limit oxidant damage was an unanticipated occurrence that may be related to the less recognized roles of glutamate and aspartate in L-arginine synthesis and L-arginine-NO pathway.

In the past, generation of cytotoxic O₂ radicals has been ascribed predominantly to the Haber-Weiss pathway, in which hydroxyl radical (·OH) is the most deleterious species.¹⁹ More recently, Beckman and colleagues²⁰ suggested that generation of ·OH via the Haber-Weiss pathway may be limited in vivo and proposed that ·NO reacts with superoxide anion (O₂⁻) to produce peroxynitrite ion (OONO⁻) that degrades homolytically to ·OH. ·NO is a physiologic

Fig. 2. Conjugated diene content. Biopsy specimen of LV subendocardial muscle taken 30 minutes after discontinuation of CPB. Dotted bar shows range of values $\pm$ SE (A_{233} /mg total lipid) from normoxemic instrumented control piglets. CPB: piglets undergoing CPB without hypoxemia, no treatment: piglets undergoing reoxygenation without treatment; Glu/Asp: piglets undergoing reoxygenation on CPB with glutamate/aspartate supplementation. * $p < 0.05$ vs no treatment; † $p < 0.05$ CPB.

modulator of vascular tone and white cell and platelet adhesiveness and is known also as endothelial-derived relaxation factor. Reoxygenation is also associated with a burst of $\cdot\text{NO}$,¹ and $\cdot\text{NO}$ has been implicated recently in tissue injury.^{1, 21, 22}

$\cdot\text{NO}$ is produced by the interaction of oxygen and L-arginine, and studies imply that L-arginine uptake by endothelial cells is essential for sustained NO synthesis.²³ L-arginine uptake is regulated by a saturable cationic amino acid transport system sensitive to inhibition by L-orthinine and neutral amino acids.²⁴ Hecker and coworkers⁷ showed that glutamine inhibits endothelial-derived relaxation factor release in bovine endothelial cells. Glutamine synthetase is present in cardiac muscle and converts glutamic acid to L-glutamine,²⁵ and L-glutamine is synthesized also from aspartate via aspartate amino transferase.²⁶ These observations imply that glutamate and aspartate, through their conversion to L-glutamine, may inhibit L-arginine transport and subsequent $\cdot\text{NO}$ production in addition to their favorable effect on cardiac energy metabolism. Unfortunately, we did not measure $\cdot\text{NO}$ production to confirm this suggested mechanism for an antioxidant action of glutamate and aspartate.

Glutamate- and aspartate-treated piglets showed less severe depletion of antioxidant reserve capacity

than did untreated piglets, which suggests better retention of endogenous antioxidants (e.g., superoxide dismutase, glutathione). This may have limited lipid peroxidation after reoxygenation. An alternate explanation is that glutamate/aspartate enhanced the energy state, thereby increasing the rate of restoration of endogenous antioxidants; reduced nicotinamide adenine dinucleotide phosphate supply from reduced nicotinamide adenine dinucleotide via transhydrogenation in mitochondria is essential for maintaining the glutathione pool.²⁷

The glutamate and aspartate dose (5 $\mu\text{mol/L}$) was derived from our previous studies of intravenous metabolic support.^{4, 5} The total dose was modified to account for the priming volume of the extracorporeal circuit. This dose is (1) consonant with the studies,¹⁴ showing a dose-dependent relationship between L-glutamate infusion and oxidative phosphorylation, and (2) does not cause neurotoxicity under nonhypoxemic or ischemic conditions.²⁸ Glutamate and aspartate are neuroexcitatory compounds,²⁸ so that further short- and long-term studies are needed in the hypoxemic setting (with and without circulatory arrest) before they can be considered for intravenous clinical application. Amino acids, however, have been used to surgical advantage in cardioplegic solutions both experimentally²⁹ and clinically³⁰ in immature hearts.^{29, 30}

Fig. 3. Antioxidant reserve capacity. Vulnerability of hypoxemic reoxygenated myocardium to subsequent oxidant stress is determined by exposing tissue to 0 to 4 mmol/L *t*-butylhydroperoxide for 30 minutes at 37° C with subsequent measurement of thiobarbituric acid-reactive substances. *MDA*, Malondialdehyde; *CPB*: piglets undergoing CPB without preceding hypoxemia; *no treatment*: piglets undergoing reoxygenation without treatment, *Glu/Asp*: piglets undergoing reoxygenation on CPB with glutamate/aspartate supplementation. **p* < 0.05 vs. no treatment; †*p* < 0.05 CPB, control. *t-BHP*, *t*-butylhydroperoxide.

The recent extended use of cerebral retroperfusion may limit use of circulatory arrest and increase the potential safety of systemic use of amino acid infusions during repair of congenital defects.

Our findings suggest that supplementation of the CPB prime of the extracorporeal circuit with glutamate and aspartate may reduce oxidant damage and minimize reoxygenation injury, provided subsequent studies document that systemic amino acid administration is not neurotoxic. Alternatively, a myocardial-directed approach (adding glutamate and aspartate to the cardioplegic solution) may be clinically more relevant, because substrate enrichment may add reduction of oxidant damage to the recognized benefits of enhancing metabolic recovery.

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