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14. ABSTRACT

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15. SUBJECT TERMS

Final, Pyruvate, Shock
Report Title

ABSTRACT
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Specific Aim 4: To determine whether pyruvate administration at the time of myocardial reperfusion following cardioplegic arrest restores myocardial energy state, antioxidant defenses, and prevents oxyradical inactivation of metabolic enzymes.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

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### Sub Contractors (DD882)
Inventions (DD882)
a. Specific Aims
The overall goals of this investigation are to test the ability of pyruvate to protect the heart during cardiopulmonary bypass and, thus, hasten recovery of cardiac function post-bypass, and to delineate the mechanisms of pyruvate’s salutary effects. To accomplish these goals, the following specific aims were addressed in patients undergoing elective coronary artery revascularization on cardiopulmonary bypass (Specific aim 1) and in situ swine hearts subjected to cardioplegic arrest (Specific aims 2-4):

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b. Studies and Results
Specific aim 1. Effect of pyruvate fortified cardioplegia on post-surgical cardiac performance (publications 1, 2). A prospective, randomized, semi-blinded trial was conducted in 30 adult patients undergoing elective coronary artery bypass graft surgery. Patients were randomized to receive either a standard, lactate-based cardioplegia solution or a pyruvate-fortified solution during bypass (15 patients per group). Cardiac performance was monitored with continuous cardiac output Swan-Ganz catheters. Coronary sinus blood was sampled immediately before cross clamp and after resumption of spontaneous cardiac rhythm for measurement of protein markers of cardiac injury.

Left ventricular stroke work index did not differ between the two treatment groups before surgery, nor immediately following resumption of cardiac rhythm post-bypass (Figure 1). Later in the post-bypass period, however, contractile function differed markedly between the groups. Stroke work index fell during the first 4 h post-bypass in all 15 patients receiving lactate based cardioplegia, but increased over the same interval in every patient in the pyruvate cardioplegia group. The robust left ventricular performance of the pyruvate group persisted between 4 and 12 h post-bypass; in contrast, stroke work in the lactate group only gradually recovered and remained well below that of the pyruvate group throughout the same period (Figure 1). Pyruvate cardioplegia minimized myocardial injury during aortic cross-clamp (Figure 2): cardiac troponin I increased in every lactate cardioplegia patient post-bypass, but no increase was seen in the pyruvate group.

The improved left ventricular recovery of the pyruvate cardioplegia group minimized requirements for inotropic support at 0-4 h post-bypass (Figure 3). Ten of the 15 lactate patients, but only 4 of the 15 pyruvate patients (P = 0.067) required dobutamine or epinephrine to maintain cardiac index above 2.2 l • min^{-1} • m^{-2}. Importantly, pyruvate-treated patients met the criteria for discharge 5.2 ± 0.1 days post-surgery vs. 6.3 ± 0.3 days in the lactate group (P < 0.002), at a savings of over $3,000 in hospital costs per patient. It should be noted that pyruvate was administered only as a component of cardioplegia to arrest the heart during surgery, so the persistent improvements in cardiac function resulted from cardioprotection during surgery, not from direct actions of pyruvate during post-surgical recovery.

Figure 1. Left ventricular contractile performance pre- and post-cardiopulmonary bypass. Left ventricular stroke work index (LVSWI) was measured at pre-bypass baseline and at 0-12 h post-bypass in patients receiving lactate- (circles) or pyruvate-fortified (squares) cardioplegia solution during bypass. Values are means ± SEM; n = 15 per group; *P < 0.05 vs. pre-bypass.
Pre- and post-bypass myocardial release of cardiac troponin I isoform. Cardiac troponin I, a marker of myocardial injury, was measured in coronary sinus blood collected before cardiopulmonary bypass and immediately after resumption of cardiac contractile activity post-bypass. Results from individual experiments (filled symbols) and means ± SEM (open symbols) are shown for patients (15 per group) receiving lactate cardioplegia (circles) or pyruvate cardioplegia (squares). From publication 1.

Figure 3. Catecholamine inotropic support requirements and post-surgical hospitalization. Panel A: Bars indicate the percentage of patients in lactate (filled bars) and pyruvate (hatched bars) cardioplegia groups requiring dobutamine or epinephrine support to maintain adequate cardiac performance at 0-2 or 2-4 hours post-bypass, and the sum of the 2 intervals. No patient required inotropic support during both time intervals; †P = 0.067 vs. lactate cardioplegia. Panel B: Days of post-operative hospitalization (means ± SEM) in the 2 cardioplegia groups; *P < 0.002 vs. lactate cardioplegia. From publication 1.

Specific aims 2, 3. Pyruvate-fortified cardioplegia suppresses oxidative stress and enhances phosphorylation potential of arrested myocardium (publications 3, 4, 7). Studies in cardioplegically arrested, in situ pig hearts were conducted to delineate the metabolic mechanisms of the beneficial effects of pyruvate-fortified cardioplegia demonstrated in the clinical trial. The use of pigs permitted sampling of myocardium necessary to evaluate the effects of the cardioplegia solutions on myocardial energy and antioxidant metabolism.

Crystallloid cardioplegia solutions containing three different substrate combinations (188 mM glucose alone, glucose + 23.8 mM pyruvate, or glucose + 23.8 mM lactate) were tested in separate experiments. Combining the pyruvate- and lactate-fortified solutions with 4 vol whole blood yielded plasma concentrations of approximately 7 mM pyruvate and 6 mM lactate, respectively. The pyruvate concentration was well within the optimally effective range of 5-10 mM defined in post-ischemic guinea-pig hearts.

Myocardial contents of pyruvate and its metabolites. As expected, pyruvate cardioplegia increased myocardial pyruvate content during arrest (Figure 4A). Pyruvate content fell somewhat during reperfusion with cardioplegia-free whole blood. Lactate also accumulated in the pyruvate-treated myocardium, to the level produced by lactate cardioplegia (Figure 4B). Citrate, a metabolic product of pyruvate carboxylation forming Krebs cycle intermediates, increased threefold in myocardium receiving pyruvate vs. control or lactate cardioplegia (Figure 4C). These results indicate that, as expected, pyruvate in cardioplegia is taken up and
heavily metabolized in the myocardium.

Figure 4. Myocardial pyruvate, lactate, and citrate contents. Pyruvate (Panel A), lactate (Panel B), and citrate (Panel C) contents were measured in snap-frozen left ventricular biopsies taken at 45 min arrest (solid bars) and at 3 min reperfusion (hatched bars) in control, lactate, and pyruvate cardioplegia groups, and at 105 min post-sternotomy in non-arrested sham hearts. *P < 0.05 vs. sham; †P < 0.05 vs. respective values in the same group at 45 min arrest; ‡P < 0.05 vs. sham, control, and lactate. From publication 4.

Myocardial glutathione redox state. An important objective of this research is to determine, for the first time, the impact of cardioplegic arrest, reperfusion and pyruvate treatment on myocardial antioxidant defenses. Glutathione (GSH) is the major intracellular antioxidant in myocardium. Accordingly, glutathione/glutathione disulfide redox state, i.e., GSH/GSSG, provides a global index of the reducing power of the myocardium’s intracellular antioxidant systems (Figure 5). Interestingly, GSH/GSSG increased (Figure 5B), and GSSG content fell (Figure 5A), in the control and especially in the pyruvate cardioplegia groups during arrest, indicating that cardioplegic arrest dampened oxidative stress and its demand on endogenous antioxidants. Nevertheless, oxidative stress associated with cardioplegia-free reperfusion caused a 25% decline in GSH content and sharp declines in GSH/GSSG in all three cardioplegia groups. Thus, myocardial antioxidant defenses were depleted following reperfusion.

Figure 5. Myocardial glutathione redox state. Panel A: Glutathione (GSH: solid bars) and glutathione disulfide (GSSG: hatched bars) contents were measured in left ventricular myocardium at 45 min arrest and 3 min reperfusion in the cardioplegia groups, and at 105 min post-sternotomy in non-arrested sham hearts. GSH and GSSG values are plotted on different scales. Panel B: Glutathione redox state (GSH/GSSG) at 45 min arrest (black bars) and 3 min reperfusion (hatched bars) was computed from GSH and GSSG contents. *P < 0.05 vs. sham; †P < 0.05 vs. respective values in the same group at 45 min arrest; ‡P < 0.05 vs. lactate. From publication 4.

Myocardial energy state. The impact of cardioplegic arrest and reperfusion on myocardial energy state was examined by measuring phosphocreatine (PCr) phosphorylation potential, $\frac{[\text{PCr}]}{[\text{Cr}][\text{Pi}]}$, an index of cytosolic energy state according to the creatine kinase equilibrium. Phosphorylation potential tended to increase in all three cardioplegia groups during arrest (Figure 6A), despite interruption of coronary blood flow. These increases were due largely to reductions in intracellular inorganic phosphate (Pi) concentration ([Pi]; Figure 6B). On reperfusion, [Pi] increased and phosphorylation potential fell in the control and lactate cardioplegia groups, but not in the pyruvate group. In the pyruvate-treated hearts, myocardial phosphorylation potential remained elevated in the face of oxidative stress upon reperfusion. Thus, pyruvate administration during cardioplegic arrest produced persistent enhancement of myocardial energy state, the source of energy for contractile performance and cellular function.

Figure 6. Myocardial phosphorylation potential and inorganic phosphate. Panel A: Phosphocreatine phosphorylation potential ($\frac{[\text{PCr}]}{[\text{Cr}][\text{Pi}]}$) was computed from phosphocreatine (PCr) and creatine (Cr) contents and intracellular inorganic phosphate (Pi) concentration (Panel B) measured in left ventricular myocardium sampled at 45 min arrest (solid bars) and 3 min reperfusion (hatched bars). *P < 0.05 vs. sham; †P < 0.05 vs. respective values in the same group at 45 min arrest; ‡P < 0.05 vs. sham, control, and lactate. From publication 4.

Specific aim 4. Pyruvate mitigates oxidative stress during reperfusion of cardioplegia-arrested myocardium (publication 6). The investigation described above demonstrated that pyruvate-fortified cardioplegia bolstered myocardial antioxidant defenses during cardioplegic arrest, and prevented decline in phosphorylation potential following whole blood reperfusion. However, pyruvate enhancement of antioxidant redox state did not persist beyond reperfusion. Accordingly, we tested whether administration of pyruvate during whole blood reperfusion following cardioplegic arrest could mitigate oxidant stress and bolster myocardial energy state of reperfused myocardium.

Oxidative stress imposed by cardioplegic arrest and reperfusion could inactivate metabolic enzymes and compromise myocardial energy production. The impact of cardioplegic arrest, reperfusion, and pyruvate treatment on a panel of important metabolic enzymes of glycolysis, Krebs cycle, hexose monophosphate pathway and cellular energy transport was determined by measuring enzyme activities in left ventricular myocardium.
Oxidative stress: myocardial 8-isoprostane content and glutathione redox state. Reactive oxygen species (ROS) are produced during ischemia-reperfusion injury and cause cellular damage which likely contributes to myocardial contractile dysfunction. ROS oxidize and inactivate proteins, and by oxidizing phospholipids, alter plasma membrane permeability to electrolytes. Measurements of 8-isoprostane, a product of lipid peroxidation, indicate the degree of oxidative stress. 8-Isoprostane was measured in left ventricular myocardium using an ELISA kit. 8-Isoprostane content did not increase during arrest but doubled within 3 min reperfusion (Figure 7). Pyruvate administration during reperfusion prevented the increase in 8-isoprostane. Thus, reperfusion engendered oxidative stress in myocardium, but pyruvate prevented lipid peroxidation due to this oxidant burst.

Figure 7. Antioxidant redox state and oxidative stress. Glutathione redox state (GSH/GSSG) and 8-isoprostane content were measured in left ventricular myocardium sampled at 45 min arrest (n = 8), at 3 min reperfusion with (n = 6) and without (n = 7) pyruvate infusion, and at 105 min post-sternotomy in non-arrested sham hearts (n = 6). Values in this and the other figures are means ± SEM. P < 0.05 vs. sham; †P < 0.05 vs. reperfusion. From publication 6.

The ratio of glutathione/glutathione disulfide content, a global measure of the redox state of the myocardium’s antioxidant defenses, GSH/GSSG increased during arrest, but fell sharply upon reperfusion, coincident with oxidative stress and lipid peroxidation (Figure 7). Pyruvate administration during reperfusion prevented the decline in GSH/GSSG.

Myocardial water content. Myocardial water content was measured to assess edema formation. Water content did not change during cardioplegic arrest, but increased during reperfusion, from 78.9 ± 0.2 to 81.1 ± 0.1 ml/100 g of myocardium (Figure 8). Pyruvate treatment prevented the increase in myocardial water content during reperfusion.

Myocardial pyruvate and derivatives. Pyruvate and its derivatives were measured in left ventricular myocardium of arrested, reperfused and sham control hearts (Figure 9). Pyruvate content did not increase in the control group during arrest or reperfusion, but sharply increased as expected during pyruvate administration. Cytosolic lactate dehydrogenase converts pyruvate to its reduced congener, lactate. In mitochondria, pyruvate carboxylation generates Krebs cycle intermediates, leading to increased citrate content. Lactate content increased twofold during arrest and another threefold during pyruvate-free reperfusion. Pyruvate treatment unexpectedly prevented lactate accumulation during reperfusion. Citrate content increased during arrest and remained elevated during reperfusion in the control group. Pyruvate administration further increased citrate content. These results disparate effects of pyruvate on citrate vs. lactate contents suggested that pyruvate metabolism was largely compartmented in the mitochondria, where citrate is produced.

Figure 8. Myocardial water content. Tissue water content was determined from masses of fresh and dessicated tissue. P < 0.05 vs. all other groups. From publication 6.

Figure 9. Myocardial pyruvate and its metabolic derivatives. Pyruvate (solid bars), lactate (hatched bars) and citrate (open bars) were measured in left ventricular myocardium. P < 0.05 vs. sham; †P < 0.05 vs. reperfusion. From publication 6.

Myocardial enzymes. Activities of glycolytic (phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase), hexose monophosphate shunt (glucose 6-phosphate dehydrogenase), Krebs cycle (aconitase) and energy shuttling (creatine kinase) enzymes were measured in arrested, reperfused and sham control myocardium. Creatine kinase

Figure 10. Myocardial enzymes. Activities (U/mg protein) of creatine kinase (CK; Panel A), phosphofructokinase (PFK; Panel B), aconitase (Panel C), glucose-6-phosphate dehydrogenase (G6PDH; Panel D), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Panel E), and lactate dehydrogenase (LDH; Panel F) were measured in left ventricular myocardial extracts. P < 0.05 vs. sham; †P < 0.05 vs. reperfusion. From publication 6.
activity (Figure 10A) did not change during arrest but fell 43% within 3 min of reperfusion. Exogenous pyruvate attenuated
creatine kinase inactivation. Like creatine kinase, phosphofructokinase was unaltered during arrest but fell upon reperfusion
(Figure 10B). However, pyruvate did not protect phosphofructokinase activity during reperfusion. Aconitase activity fell 40%
during arrest and, unlike the other enzymes, partially recovered during reperfusion (Figure 10C). Pyruvate markedly increased
aconitase activity, to a level even 30% higher than in sham myocardium. Glucose 6-phosphate dehydrogenase was inactivated
by 26% during arrest, but was unaffected by reperfusion ± pyruvate (Figure 10D). Activities of glyceraldehyde 3-phosphate
dehydrogenase (Figure 10E) and lactate dehydrogenase (Figure 10F) were unaltered by arrest, reperfusion or pyruvate.

Myocardial energy state. Left ventricular myocardial ATP content tended to fall during arrest and declined even further upon
reperfusion (Figure 11). Pyruvate treatment during reperfusion restored ATP content to the level observed in non-arrested
sham controls. To assess the effects of cardioplegic arrest and reperfusion and the impact of pyruvate on myocardial energy
state, phosphocreatine phosphorylation potential was computed from intracellular concentrations of phosphocreatine, creatine
and inorganic phosphate. Phosphorylation potential nearly tripled during arrest but fell to near sham values within 3 min
reperfusion (Figure 11). Pyruvate prevented the fall in phosphorylation potential during reperfusion.

Figure 11. Myocardial phosphorylation potential and ATP content. Phosphocreatine phosphorylation potential ([PCr]/([Cr][Pi]))
was computed from intracellular concentrations of phosphocreatine (PCr), creatine (Cr) and inorganic phosphate (Pi) measured
in left ventricular myocardium. ATP content was measured in the same samples. *P < 0.05 vs. sham; †P < 0.05 vs.
reperfusion. From publication 6.

Interpretation. In this study, oxidative stress was minimal during cardioplegic arrest, but substantial stress accompanied
reperfusion with whole blood. Pyruvate, administered by infusion into the aortic blood used to reperfuse the myocardium,
prevented myocardial lipid peroxidation and rapid decline of GSH/GSSG; thus, pyruvate mitigated reperfusion-induced oxidative
stress. This antioxidant action provided several benefits: pyruvate prevented tissue edema, increased myocardial
phosphorylation potential, and enhanced activity of creatine kinase and aconitase, two oxidant-sensitive enzymes present in the
mitochondria. The two cytosolic enzymes inactivated by arrest and/or reperfusion, glucose 6-phosphate dehydrogenase and
phosphofructokinase, were not responsive to pyruvate treatment. Moreover, pyruvate increased citrate content but did not
increase lactate, indicating that pyruvate metabolism was restricted to the mitochondrial compartment during the first minutes of
reperfusion. To our knowledge, this is the first investigation of alterations in myocardial enzyme activities during and
immediately following cardioplegic arrest. These results indicate that pyruvate may be a beneficial antioxidant and energy-
generating intervention in the setting of cardioplegic arrest.

c. Significance
The most important findings of this project are:
1. The use of pyruvate-fortified cardioplegia solution during cardiopulmonary bypass surgery markedly increased post-surgical
left ventricular performance, thereby lessening requirements for inotropic support, shortening post-surgical hospitalization by ≥1
day, and reducing healthcare costs by ~$3,000.
2. Pyruvate-fortified cardioplegia mitigated myocardial injury more effectively than standard, lactate-based cardioplegia during
cardiopulmonary bypass surgery.
3. Pyruvate-fortified cardioplegic solution minimized oxidative stress in myocardium during cardioplegic arrest and supported
marked enhancement of myocardial energy state following reperfusion, even though the antioxidant effects of pyruvate
cardioplegia did not persist into the reperfusion period.
4. Reperfusion of cardioplegically arrested myocardium produced appreciable oxidative stress that depleted antioxidant
reducing power, inactivated oxidant-sensitive metabolic enzymes, and was associated with myocardial edema.
5. Pyruvate administration during whole blood reperfusion of cardioplegically arrested myocardium suppressed oxidative
stress, maintained glutathione antioxidant redox potential, prevented tissue edema, restored ATP content, and sharply
increased myocardial phosphorylation potential.
6. Pyruvate reactivated the important oxidant-sensitive mitochondrial enzymes creatine kinase and aconitase. These
enzymes are essential for energy production in the heart, and pyruvate protection of these enzymes could support improved
post-bypass cardiac recovery.

d. Plans
The work summarized above strongly supports therapeutic application of pyruvate, in cardioplegia and as a blood additive
during reperfusion of arrested myocardium, to prevent cardiac injury following cardioplegic arrest. Our next objective is to test
pyruvate in the setting of on-pump cardiopulmonary bypass. Pyruvate will be administered in cardioplegia, as in specific aims 2
and 3 above, and, upon release of aortic cross-clamp, by infusion into the blood reperfusing the heart, as in specific aim 4. This
approach will permit study of the long-term consequences of temporary pyruvate treatment during cardiopulmonary bypass.
e. Publications

Abstract

Technology Transfer