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ABSTRACT

Introduction: Cardiac arrest followed by resuscitation and recovery of spontaneous circulation (ROSC) produces systemic ischemia reperfusion (I/R), affecting all internal organs, including the kidney. This type of stress generates both a robust increase in reactive oxygen and nitrogen species (RONS) and an intense inflammatory response, which can result in renal cell death. The glycoprotein erythropoietin (EPO) has been shown to combat renal I/R injury by offering cyto-protection against inflammation and oxidative damage, as well as inhibiting apoptosis. The endogenous intermediary metabolite pyruvate has been observed to stabilize specific genetic machinery responsible for the production of EPO. This study was conducted to test the efficacy of intravenous pyruvate in exploiting these endogenous mechanisms of EPO to protect the kidney from cardiac arrest-induced, I/R injury. Hypothesis: Pyruvate administration during cardiopulmonary resuscitation (CPR), defibrillation, and ROSC will protect the kidneys from I/R injury by suppressing oxidative stress and inflammation via increased EPO production at the renal corticomedullary border. Methods: Yorkshire swine underwent 10 minutes of cardiac arrest, CPR effected by precordial compressions, and defibrillation, and were recovered for either 4 hours (acute) or 3 days (chronic). The animals were randomly assigned to 1 of 4 groups. Two groups underwent the cardiac arrest protocol described

above: one group received intravenous infusion of 2M sodium pyruvate at a rate of 0.1 $mmol \cdot kg^{-1} \cdot min^{-1}$ during CPR and the first 60 minutes of recovery; the other group received an equimolar infusion of NaCl. The other two groups were surgically prepared and infused with NaCl or sodium pyruvate, but were not subjected to cardiac arrest, CPR, or defibrillation. For the acute protocol (n=28), animals were sacrificed 4hr after cardiac arrest, while in the chronic protocol (n=18), animals recovered for 3d before sacrifice. To evaluate the impact of cardiac arrest and pyruvate treatment on renal metabolism and antioxidant defense, proteins were extracted from snap-frozen renal corticomedullary border tissue for spectrophotometric activity assays of a panel of 10 metabolic and antioxidant enzymes; myeloperoxidase (MPO), an enzyme marker of pro-inflammatory leukocytes, was analyzed to assess inflammation. Plasma was sampled before cardiac arrest and at the time of biopsy to measure creatinine concentration, an indirect measure of glomerular filtration rate (GFR). Enzyme-linked immunosorbent assay (ELISA) kits were used to measure EPO content and Kidney Injury Molecule-1 (KIM-1) content, a receptor expressed on renal tubular cells that plays an important role in apoptosis. Tissue sections were stained with hematoxylin and eosin (H&E) and examined under light microscopy to count neutrophils and monocytes and to compare structure integrity across the different treatment groups and protocols. Results: In this study global I/R stress imposed on the kidneys by reversible cardiac arrest did not appreciably alter the activity of the 10 panel enzymes. Despite having no histological evidence of neutrophil infiltration (H&E stained

slides), an increase in renal MPO activity was evident at 4 h recovery in the NaCl group which was prevented by pyruvate treatment ($P<0.05$). There was no evidence of ultrastructural damage to renal cortical and outer medullary structures. There was a noticeable increase in renal EPO content at 4 h ROSC vs. the sham group. An apparent, albeit not statistically significant, increase in KIM-1 content was observed in the two CPR groups vs. the NaCl-infused sham group. Plasma creatinine concentrations did not change appreciably between pre-arrest baseline and 3 d recovery. Interpretation and Conclusion: The I/R stress produced by the present cardiac arrest-resuscitation failed to alter appreciably the activities of the 10 panel enzymes, suggesting the oxidative stress was not sufficient to overwhelm the kidney's endogenous antioxidant defenses. Plasma creatinine concentrations were also stable, implying the GFR was maintained and the glomerular ultrastructures were unaffected by I/R. The increase in MPO activity at 4 h ROSC implied a transient infiltration of inflammatory leukocytes, although none were visible on histological examination. The increase in KIM-1 content, though not statistically significant, suggests modest renal apoptotic activity after cardiac arrest and reperfusion. The transient increase in renal EPO content in the NaCl-infused post-arrest vs. sham pigs supports the possibility that even a brief period of renal ischemia by cardiac arrest can evoke renal EPO production. Collectively, these results indicate the renal I/R imposed by cardiac arrest and resuscitation does not inflict appreciable damage on the kidneys or its enzyme systems, at least within the first 3 d of post-arrest recovery.

Abbreviations: AKI: acute kidney injury; ARF: acute renal failure; CK: creatine kinase; CPR: cardiopulmonary resuscitation; CS: citrate synthase; EPO: erythropoietin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; G6PDH: glucose 6-phosphate dehydrogenase; GFR: glomerular filtration rate; GP: glutathione peroxidase; GR: glutathione reductase; HIF-1: hypoxia-inducible factor 1; I/R: ischemia-reperfusion; KIM-1: kidney injury molecule 1; LDH: lactate dehydrogenase; MPO: myeloperoxidase; PFK: phosphofructokinase; PHD: prolyl hydroxylase; RONS: reactive oxygen and nitrogen species; ROSC: recovery of spontaneous circulation

Intravenous Pyruvate to Prevent Renal Injury Following Cardiac Arrest and Resuscitation

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THESIS

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Chapter 1

Background and Rationale

Ischemia-Reperfusion Injury and Renal Dysfunction

Over 300,000 people die each year from out-of-hospital cardiac arrest in the United States alone. Survival rates are slightly higher for in-hospital cardiac arrest victims, yet even in those patients, the chances of long term survival after hospital discharge are still poor [12]. While the treatment of cardiac arrest with noninvasive cardiopulmonary resuscitation (CPR) may provide adequate blood flow to the heart, perfusion of other organs, especially those in the abdomen, can be severely impaired [14]. CPR is the only available intervention to sustain a victim until cardioversion. Unfortunately CPR does not produce sufficient arterial pressures to adequately perfuse the already ischemic organs [14]. Renal blood flow during CPR can be especially compromised, and even after cardioversion and recovery of spontaneous circulation (ROSC), the kidneys may still be hypoperfused [14]. The sharp drop in mean arterial pressure during cardiac arrest will increase sympathetic activity, causing powerful vasoconstriction of the renal afferent arterioles, further decreasing renal blood flow.

Reperfusion of the kidney, which is necessary for the survival of this ischemic organ, can generate reactive oxygen and nitrogen species (RONS) that

inflict additional cellular damage [29]. According to Ishii *et al* [13], "ischemia-reperfusion injury is a major cause of acute renal failure resulting in death and also impairs short and long term graft function after kidney transplantation." Acute renal failure (ARF) culminates in patient death in more than 50% of cases [33]. The ischemia caused by reduction in renal blood flow below the limits of autoregulation is thought to be the major underlying pathophysiological factor for these high rates of morbidity and mortality associated with ARF [29].

Cellular models have shown that the reperfusion of ischemic tissue can result in a massive increase of RONS within minutes of reperfusion [12]. Hydrogen peroxide and the superoxide anion are generated during reperfusion, and concomitantly the ischemia caused by cardiac arrest induces nitric oxide synthase (NOS), generating excess NO. This NO can condense with superoxide to form peroxynitrite, another damaging RONS that can nitrosylate tyrosine residues in proteins [6]. RONS can also cause carbonylation of proteins, altering their structure and function. Among these oxidatively modified proteins are enzymes involved in intermediary metabolism such as glyceraldehyde 3-phosphate dehydrogenase and pyruvate decarboxylase [23, 23].

Reperfusion also initiates a cascade of intracellular signaling events that can result in renal cell death through a combination of necrosis and apoptosis [29]. The generation of RONS from I/R stress increases the permeability of the

outer mitochondrial membrane, resulting in release of cytochrome C into the cytosol, where it combines with APAF-1, dATP, and procaspase 9 forming an apotosome that cleaves and activates caspase 9 [7]. During ischemia, the lack of O_2 as an electron acceptor decreases electron flux through the mitochondria respiratory chain and dampens H^+ extrusion, thereby decreasing the mitochondrial membrane potential and compromising decreasing ATP production.

The innate immune response is also a major contributing factor to the pathology of acute renal I/R injury. Neutrophils attach to the activated endothelium, particularly in the outer medulla, within 30 minutes of reperfusion. These neutrophils release substances that aggravate the kidney injury such as proteases, ROS, and myeloperoxidase [1, 36].

Erythropoietin and Hypoxia Inducible Factor-1

The glycoprotein erythropoietin (EPO) protects the kidney from I/R injury and can be used in the prevention of acute kidney injury [20, 29]. Erythropoietin, a class I cytokine and erythropoietic hormone, is a 30.4 kD glycoprotein consisting of 165 amino acids. Most (c.90%) of the systemic EPO in adults is produced in the renal cortex and outer medulla by peritubular interstitial fibroblasts (Figure 1.1) [20]. EPO production is regulated by a feedback mechanism mediated by oxygen concentration in the tissues, and EPO is secreted in response to hypoxia [13, 20]. Production of EPO, along with

several other O_2 regulated proteins, is controlled by the oxygen-sensitive transcription factor hypoxia-inducible factor-1 (HIF-1), a heterodimer consisting of two subunits, the O_2-regulated HIF-1α and the constitutively expressed HIF-1β (figure 1.2) [8, 13, 30]. Hypoxia stabilizes the O_2 sensitive α subunit, allowing its subsequent translocation to the nucleus. Once in the nucleus, HIF-1α then dimerizes with the β subunit, forming the transcriptionally active HIF-1. This heterodimer then binds to the hypoxia responsive element (HRE) in the promoter regions and thereby activates the transcription of a host of genes involved in glucose metabolism, angiogenesis and hematopoiesis [8, 13, 26, 30]. The activation of these genes mediates cyto-protective changes such as increased anaerobic glycolysis, which increases O_2-independent ATP production, and angiogenesis, which increases O_2 delivery [31].

Under normoxic conditions, the enzyme prolyl hydroxylase (PHD) hydroxylates two prolyl residues, proline 402 and 564, within the HIF-1α oxygen-dependent degradation (ODD) domain. This hydroxylation chemically tags HIF-1α for the recognition component of an E3 ubiquitin-protein ligase, called the von Hippel-Lindau (VHL) tumor suppressing protein, for polyubiquitination and subsequent proteasomal degradation [8, 25, 27, 30]. This enzymatic hydroxylation is oxygen dependent, but also requires ferrous iron (Fe^{2+}) as an electron donor and α-ketoglutarate as a cofactor [26, 27]. Therefore PHD-catalyzed HIF-1α hydroxylation decreases when cells are deprived of iron, α-ketoglutarate, or oxygen, allowing increased expression of

HIF-1's genes program [30]. A separately controlled O_2 dependent enzyme, asparaginyl hydroxylase, also hydroxylates the HIF-1α subunit at a specific asparagine residue, inhibiting HIF-1 interaction with the HRE; therefore blocking HIF-1 transcriptional activity in normoxic conditions [17].

Erythropoietin and the Erythropoietin Receptor

Erythropoietin stimulates red blood cell production through its interaction with erythroid progenitor cells, mediated by the recognition and binding of EPO to the EPO receptors located on their membranes. This receptor binding prevents these erythroid progenitor cells from undergoing apoptosis, allowing them to mature into erythrocytes, and exemplifying EPO's cytoprotective properties [16]. Later discoveries revealed that EPO's actions are not limited to its classical role of erythropoiesis; indeed, EPO receptors have been found on many cell types outside the hematopoietic system, including endothelial cells, cardiomyocytes, and renal cells. The broad distribution of these receptors suggests that EPO may protect a multitude of non-hematopoietic cells [16]. The inhibition of apoptosis appears to be essential for this cyto-protective effect of EPO [29].

Binding of EPO to its membrane receptor initiates EPO's cytoprotective signaling cascade (figure 1.3). The EPO receptor on the target cell's membrane is a homodimer, and the binding of EPO to its receptor initiates specific intracellular cascades, beginning with a conformational change within the

receptor's protein structure. This structural shift approximates two Janus kinase 2 molecules (JAK2) which are tethered to the cytosolic aspects of the receptor subunits [16, 20]. The apposition of the two JAK2 enzymes facilitates their cross-phosphorylation, which initiates the JAK2/STAT5-signaling axis. This signal induces phosphorylation and homodimerization of the signal transducer and activator of transcription 5 protein (STAT5), which then translocates into the nucleus where it binds to *cis*-acting elements. This action of STAT5 enhances transcription of the antiapoptotic B-cell lymphoma-extra-large molecule (Bcl-Xʟ), which is a member of the Bcl-2 protein family [16, 20, 24]. The activated JAK2 molecules also induce phosphorylation of tyrosine residues in the cytosolic domain of the EPO receptor, allowing the receptor's kinase domain to phosphorylate and activate phosphatidylinositol 3-kinase (PI3-K), which in turn phosphorylates protein kinase B (PKB/Akt). Activated PKB/Akt can then phosphorylate proapoptotic molecules, such as caspase 9, Bad, or glycogen synthase kinase-3β, leading to their inactivation [16, 20, 24].

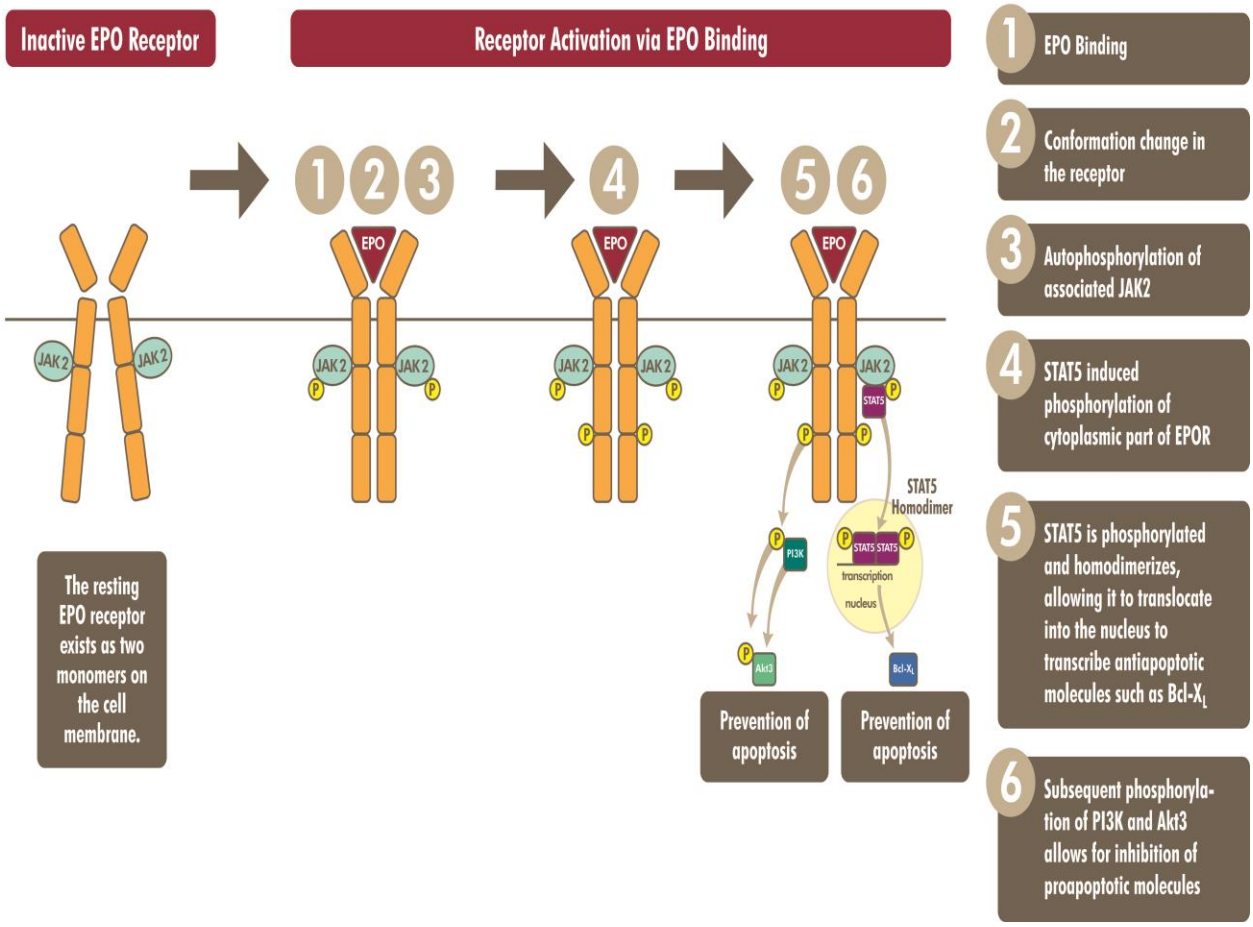


Figure 1.3.*Activation of antiapoptotic signaling through EPO and its receptor.* The binding of the EPO molecule promotes the conformational change that is needed to induce the apposition and transphosphorylation of the tethered JAK2 molecules, as well as the phosphorylation of the cytoplasmic portion of the receptor. These adjacent JAK2 molecules will then phosphorylate the transcription factor STAT5, which then homodimerizes and translocates to the nucleus where it activates gene expression of the antiapoptotic molecule Bcl-XL. Secondly, the phosphorylation of PI3-kinase by the cytoplasmic domain of the EPO receptor allows it to phosphorylate and activate PKB/Akt, which in turn can phosphorylate and inactivate proapoptotic molecules such as GSK-3β, caspase 9, or Bad.

Renal Protection Imparted by Erythropoietin

Many studies have demonstrated that EPO can reduce renal injury in various animal models, particularly models of renal I/R injury [20, 25, 34]. Under hypoxic conditions or acute ischemic renal injury, EPO has been shown to inhibit apoptosis, enhance tubular epithelial regeneration, and promote renal functional recovery [21, 24, 34]. As discussed above, EPO has a profound ability to inhibit cellular apoptosis through its interaction with the EPO receptor. Given that EPO receptors are expressed on renal tubular epithelial cells, an increase in renal EPO production may afford protection against acute renal damage [24, 29]. In addition, intravenous administration of EPO can protect the kidney from the damaging inflammatory response following I/R injury has been demonstrated by [13]. However, in our study we induced the endogenous production of EPO, thereby avoiding the known adverse risks of administering EPO exogenously such as an increase in hematocrit and an increased risk of thrombophilia [20].

Erythropoietin's antioxidant capabilities are due in part to its induction of the powerful, endogenous antioxidative enzymes glutathione peroxidase, catalase, and superoxide dismutase. One of EPO's most important antioxidant mechanisms in renal endothelial cells is its indirect upregulation of heme oxygenase-1 [4, 16]. EPO can also indirectly enhance cellular antioxidative capacity by increasing the number of circulating, young erythrocytes. Young red blood cells contain higher activities of antioxidative enzymes than do

mature red blood cells [16]. The ability of EPO to continually replenish the systemic circulation with erythrocyte antioxidative enzymes is an effective way to indirectly reduce oxidative stress [16]. EPO can also suppress proinflammatory cytokines such as IL-6 and TNF-α, further ameliorating the inflammatory response to I/R injury [13].

Pyruvate: Redox Chemistry and Induction of Erythropoietin Production

Pyruvate is an endogenous alpha-keto carboxylic acid that is both a product of glycolysis and a mitochondrial substrate in aerobic tissue. Pyruvate can serve as a metabolic fuel; indeed, pyruvate oxidation can increase, more effectively than other fuels, the cytosolic energy state of cardiomyocytes [18]. Pyruvate can combat oxidative stress in by two mechanisms: First, pyruvate's α-keto carboxylate structure allows it to neutralize peroxides and peroxynitrite non-enzymatically by means of decarboxylation. Secondly, pyruvate can help maintain cellular antioxidant reserves by the generation of NADPH, thereby providing the reducing power needed to maintain glutathione redox state [17,18, 36]. Mongan *et al* [19] details how millimolar concentrations of pyruvate can improve cellular function in the face of metabolic stress by lowering the cytosolic redox potential ($[NADH]/[NAD^+]$) while also maintaining the cellular phosphorylation potential ($[ATP]/[ADP][P_i]$).

As noted above, the activity of the transcription factor HIF-1 is regulated by oxygen-dependent degradation of its α subunit. Under normoxic conditions

HIF-1α is hydroxylated by the enzyme PHD and tagged for proteosomal degradation in the cytosol [8, 25, 38]. As discussed earlier, PHD requires a molecule of α-ketoglutarate as a co-substrate to catalyze the addition of two hydroxyl groups at proline residues 402 and 564 on the HIF-1α subunit [8, 9, 25]. Pyruvate, a structural analogue of α-ketoglutarate, inhibits PHD by competing with α-ketoglutarate for access to the enzyme's catalytic domain [9, 17, 27]. Specifically, pyruvate occupies the α-ketoglutarate site of PHD, depriving the enzyme of an essential cofactor [17]. This action suppresses proteosomal degradation of the HIF-1α subunit. Hence pyruvate may allow a fully functional HIF-1 transcription factor to activate the EPO gene in the face of normoxic conditions [13, 27]. HIF-1 itself will promote glycolytic metabolism by initiating the transcription of the entire glycolytic sequence of enzymes [38]**.** Thus, the enhancement of HIF-1 by pyruvate constitutes a novel feed-forward signaling mechanism which increases cellular resistance to hypoxia and ischemia [17]. We hypothesize that the cellular protection afforded by pyruvate's enhancement of EPO production will protect the kidney from I/R injury during CPR, defibrillation, and ROSC through dampening of oxidative stress and inflammation.

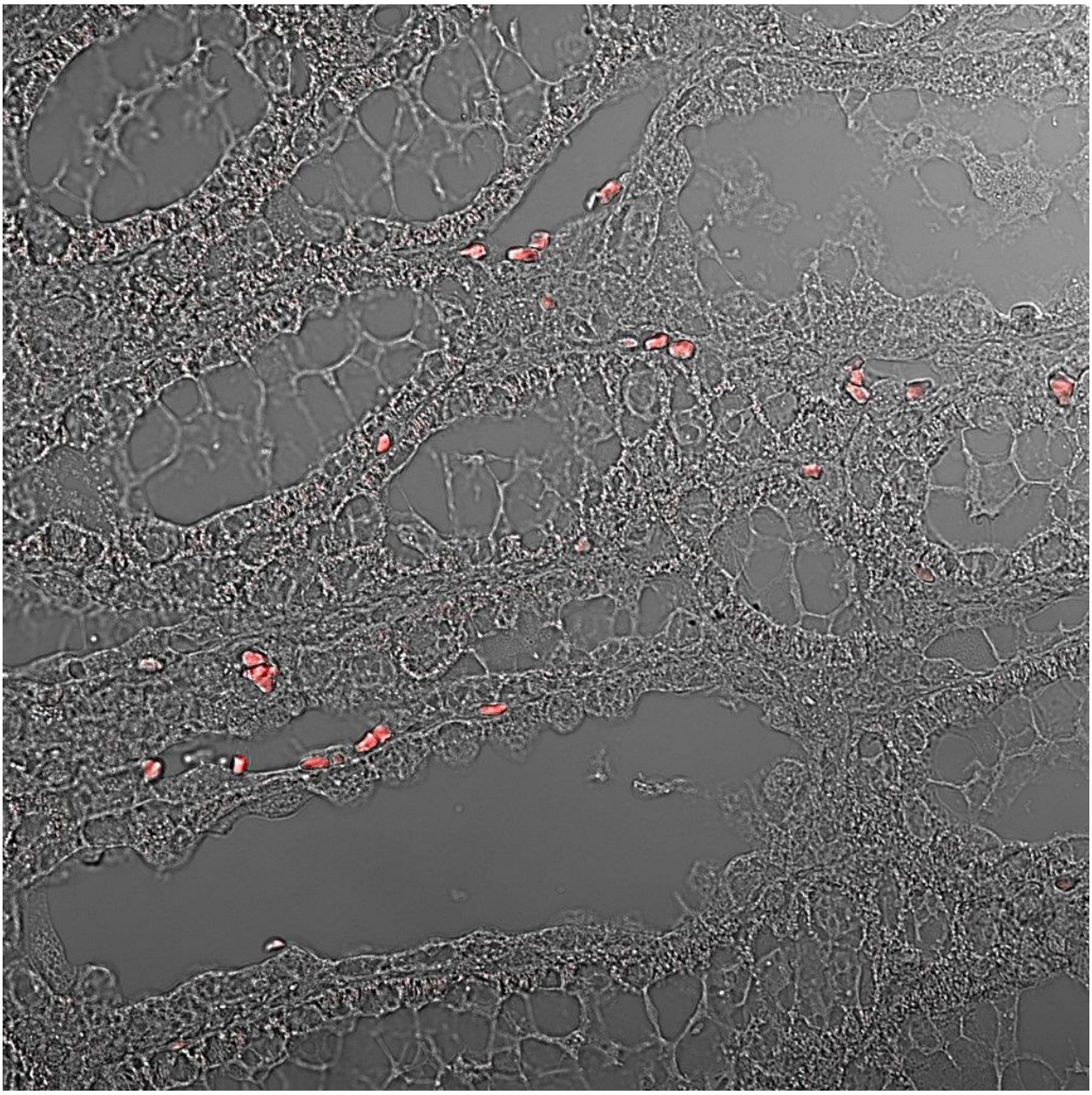


Figure 1.1. *Erythropoietin immunoreactivity in renal peritubular interstitial cells.* In this image, a photomicrograph of renal cortex near the corticomedullary border is overlain with immunofluorescence (red) indicating the presence of EPO in peritubular interstitial cells. Primary antibody: rabbit anti-porcine erythropoietin; secondary antibody: fluorophore-conjugated donkey anti-rabbit IgG. Courtesy of Myoung-Gwi Ryou, Ph.D.

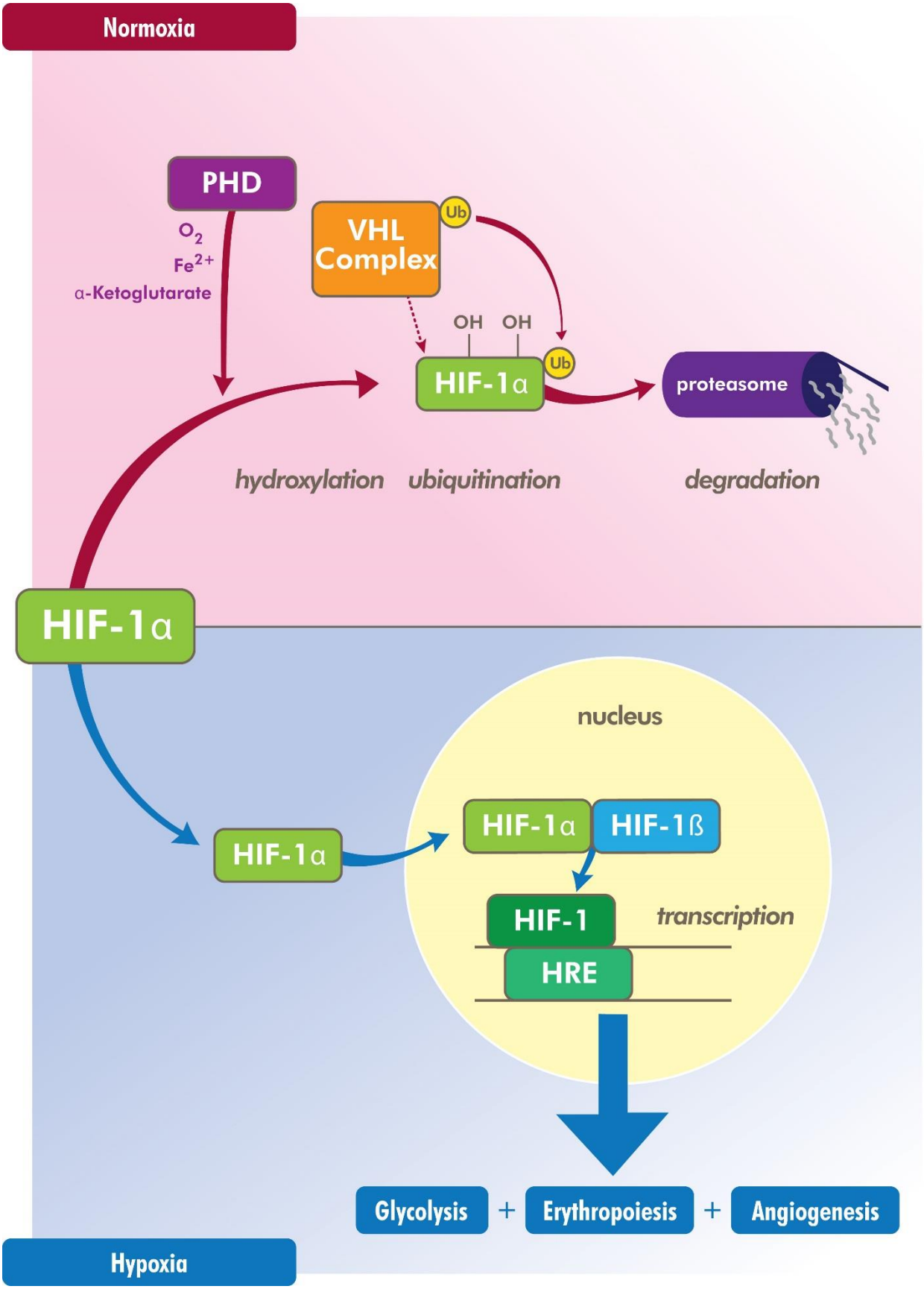

Normoxia
PHD
O_2
Fe^{2+}
α-Ketoglutarate
VHL Complex
Ub
OH OH
HIF-1α
Ub
proteasome
hydroxylation
ubiquitination
degradation
HIF-1α
nucleus
HIF-1α
HIF-1ß
HIF-1
transcription
HRE
HIF-1α
Glycolysis + Erythropoiesis + Angiogenesis
Hypoxia

Figure 1.2. *Mechanism of HIF-1 Regulation***:** The transcription factor hypoxia-inducible factor (HIF-1) controls the gene expression of EPO. HIF-1 is a heterodimer which consists of two subunits, the oxygen sensitive HIF-1α that resides in the cytosol and the constitutively expressed HIF-1β within the nucleus. Normoxic conditions initiate the O_2 dependent enzyme prolyl-hydroxylase (PHD), which, in the presence of O_2, ferrous iron and α-ketoglutarate as cofactors, hydroxylates two proline residues on the HIF-1α subunit, allowing for recognition and polyubiquitination by the von Hippel-Lindau (VHL) tumor suppressing protein, designating the HIF-1α subunit for proteosomal degradation. In hypoxic conditions, the decreased O_2 concentration will restrict PHD activity, and the HIF-1α subunit will avoid hydroxylation and degradation. The intact HIF-1α subunit can now translocate into the nucleus and dimerize with the HIF-1β subunit, forming the functional transcription factor HIF-1. This heterodimer binds to the hypoxia response element (HRE), activating a host of HIF-1-mediated genes that participate in cyto-protection, including EPO.

CHAPTER 2

Experimental Protocol and Methods

Animals

Animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center and was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, 8th edn. Washington, D.C.: National Academies Press; 2011. For this protocol, male and female domestic Yorkshire swine (30-40 kg) were housed for 7 days before experimentation. The pigs consumed a chow diet and water *ad libitum* and were fasted the night before the experiment.

Anesthesia and Surgical Preparations

The pigs were sedated with telazol/xylazine cocktail ($5mg^{-1}$ kg^{-1} *im*), intubated, and maintained under a surgical plane of anesthesia by mechanical ventilation (tidal volume 10-12 ml/kg; 12-14 cycles/min) with 1- 3% isoflurane supplemented with 100% O_2. Physiological signs were monitored to evaluate the anesthetic plane. Fractional isoflurane was increased upon limb withdrawal in response to pinching of soft tissue between hooves, wink reflex in response to contact of the ocular canthus, spontaneous movement of the animal, detection of jaw tone, or increase in heart rate and systemic arterial pressure

independent of treatment. The monitoring of cardiac electrical activity, by standard limb lead II electrocardiography, was done by the use of epidermal electrode patches placed on the limbs. The animals were placed upon a circulating water heating pad on the surgical table, while body temperature was monitored with a rectal thermal probe.

The left femoral artery and vein were exposed and isolated by inguinal incision, electrocautery and blunt dissection. A saline-filled, 7 Fr polyurethane cannula was inserted into the femoral artery, then advanced into the abdominal artery and connected to a pressure transducer (Grass Technologies; West Warwick, RI) interfaced with recording software (Emka Technologies; Falls Church, VA) so that arterial pressures could be monitored throughout each experiment. Blood samples were also taken via this cannula with sterile syringes at designated time points (figure 2.1). A separate 7 Fr cannula was inserted into the femoral vein for medication infusion and to administer drugs to support hemodynamic recovery following cardioversion. The right external jugular vein was also exposed and isolated to allow a pacing wire to be introduced through another 7 Fr cannula. The pacing wire was advanced until the tip contacted the right ventricular endocardium. After the surgical preparations were completed, heparin (500 U/kg) was injected into the femoral vein for the 4 h acute recovery studies; supplemental doses (150 U/kg) were given 2 and 4 h later for anticoagulation maintenance. There was no administration of heparin to pigs undergoing the 3 d chronic protocols due to risk of uncontrolled bleeding after closure of incision sites.

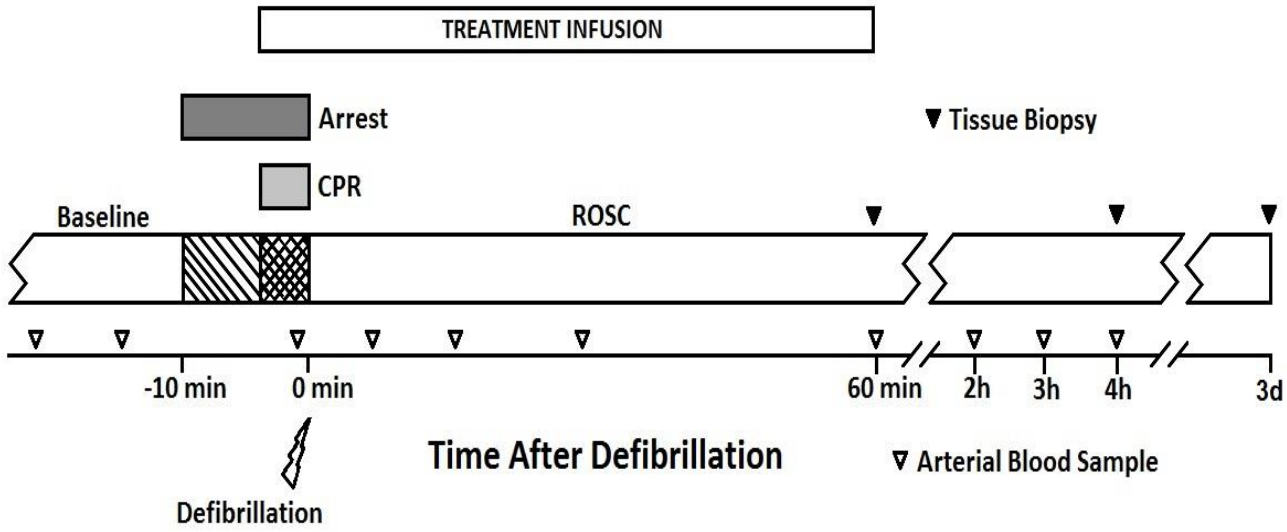


Figure 2.1. *Experimental protocol.* Open triangles indicate collections of arterial blood samples; filled triangles indicate time of sacrifice and tissue sampling. Arterial samples were drawn at these specific time points in the cardiac arrest experiments and at analogous times in the non-arrested sham experiments; for analysis of arterial blood chemistry and plasma carbohydrates. CPR: cardiopulmonary resuscitation; ROSC: recovery of spontaneous circulation.

Cardiac Arrest, Treatment, and Cardiopulmonary Resuscitation

An electrical current (0.5-1s burst of 60 Hz) transmitted via the pacing wire to the right ventricular endocardium induced ventricular fibrillation cardiac arrest. Arterial pressure recordings and standard lead II electrocardiogram were used to verify induction of ventricular fibrillation. Once ventricular fibrillation was verified, mechanical ventilation was immediately

suspended for the remainder of arrest and CPR, and the pacing wire was withdrawn. One minute prior to the initiation of CPR, bicarbonate (0.3-0.4 mEq/kg) was administered into the femoral vein for the purpose of partially offsetting the acidemia that develops during cardiac arrest and CPR. After five minutes and thirty seconds of arrest, infusion of treatment (either 2 M sodium pyruvate or 2 M NaCl) was commenced at a rate of 0.1 $mmol \cdot min^{-1} \cdot kg^{-1}$ by use of an infusion pump (Harvard Apparatus: Holliston, MA). Treatment infusion was begun 30s before starting precordial compressions to ensure delivery treatment to the heart at the onset of CPR.

After six minutes of cardiac arrest, closed chest CPR was administered by applying precordial, manual chest compressions with sufficient force to compress the chest by one-fourth of its anterior-posterior diameter, followed by release and recoil, at a rate of 100 cycles/min, in accordance with current American Heart Association guidelines [43]. The individual administering the CPR was blinded to the treatment. At 7 minutes of cardiac arrest (1 min of CPR) 10 U of vasopressin was injected into the right external jugular vein, and flushed with normal saline (0.9% NaCl), to increase the efficacy of the chest compressions. After 4 min of CPR (10 min of arrest) transthoracic DC counter-shocks were administered with external paddles (LIFEPAK 12, Phsio-Control; Redmond, WA) to attempt cardioversion. Up to three 6-7 J/kg countershocks were applied, followed by an additional three 8-12J/kg countershocks, with 30s of CPR after each unsuccessful countershock, until spontaneous cardiac

rhythm was restored. Efforts to defibrillate were abandoned if cardioversion was not achieved within 6 countershocks.

Recovery of Spontaneous Circulation and Post-Arrest Monitoring

Mechanical ventilation was resumed upon recovery of sinus rhythm. Lidocaine (20 mg in 1 mL of normal saline) was injected into the right external jugular vein, followed by normal saline flush, as a precaution to minimize recurrence of ventricular fibrillation or ventricular tachycardia, and to aid in recovery of a normal QRS waveform complex. If ventricular tachyarrhythmias did occur, an additional 20 mg of lidocaine was administered. A sustained, spontaneous cardiac electrical rhythms, combined with a mean aortic pressure ≥ 60 mmHg, were taken to verify ROSC. Once ROSC was achieved, pigs were recovered for 4 h and 3 d. For the 4 h recovery experiments, a surgical plane of anesthesia was maintained for 4 h ROSC, while electrocardiogram and aortic pressure were monitored. Animals recovered for 3 d were returned to a separate cage for recovery. They received tap water *ad libitum* and standard chow.

Standard lead II electrocardiogram, mean arterial pressure, and heart rate were recorded continuously throughout each experiment. At the designated time points (Figure 2.1) arterial blood was sampled, and arterial chemistries were measured in a blood gas analyzer (Instrumentation Laboratories; Lexington, MA). Another arterial blood sample was centrifuged (8

min., 10.8 rcf), and the plasma supernatant was frozen in liquid N_2 for metabolite measurements.

Tissue Collection and Fixation

At the end of the recovery period, the animal was anesthetized, the heart was exposed via left thoracotomy and pericardiotomy, and the animal was sacrificed by fibrillating the heart with an electrical impulse applied to the epicardium. Kidney biopsies were collected after exposing the kidneys via laparotomy. The renal cortex, along with the corticomedullary border, were snap-frozen with liquid N_2-cooled Wollenberger tongs and stored at -80°C until further analysis. Additional biopsies, collected from the same kidney were fixed in 4% paraformaldehyde and kept at room temperature for 24h, after which they were washed 3 times in 70% ethanol. The flash frozen kidney biopsy was pulverized into a fine powder under liquid N_2 by the use of precooled to the temperature of liquid N_2 mortar and pestle. In a plastic tube, 0.1 g of powdered tissue was combined with1 ml of phosphate buffer (1mM, pH 7.2) and homogenized for 1 min with a Teflon piston. The homogenate was then centrifuged at 100,000 g at 4°C for 22 min. The supernatant was collected, and the pellet was extracted two more times following this same protocol. The three supernatant fractions were combined, divided into aliquots, and stored at -80°C. The total protein concentration of each extract was measured by using

Bradford's colorimetric method [2]. These values were used to normalize data from enzymatic activity assays and ELISAs.

Grouping of Experiments

To test our hypothesis, four groups of pigs were studied, for both acute and chronic protocols:

1. Sham + NaCl: This group underwent the same surgical preparations as CPR groups 3 and 4 described below, but did not undergo cardiac arrest, CPR, cardioversion, or recovery. Group 1 pigs received an intrajugular infusion of 2 M NaCl (0.1 $mmol \cdot min^{-1} \cdot kg^{-1}$) for a period corresponding to the NaCl infusion period in group 3. This group controlled for the effects of the surgical preparation and intravenous NaCl infusion *per se*, and provided the crucial baseline values for the study endpoints, to define the effects of cardiac arrest/CPR/recovery on these endpoints.

2. Sham + Pyruvate: This group also underwent the same surgical preparations as the two CPR groups, but like group 1 did not undergo the cardiac arrest/CPR/recovery protocol. 2 M sodium pyruvate was infused *iv* (0.1 $mmol \cdot min^{-1} \cdot kg^{-1}$) for a period corresponding to the sodium pyruvate infusion period in group 4. This group controlled for the effects of the

surgical preparation and intravenous sodium pyruvate infusion *per se* and provided a surgical control for group 4.

3. Cardiac Arrest/CPR + NaCl: These pigs underwent the cardiac arrest, CPR, and recovery protocol described above. They were infused with 2 M NaCl (0.1 $mmol \cdot min^{-1} \cdot kg^{-1}$) during CPR, cardioversion, and the first 60 min of recovery. This group controlled for the effects of infusing the cation sodium, which accompanies the pyruvate anion in group 4. Also, group 3 demonstrated the effects of cardiac arrest, CPR, and recovery in the absence of pyruvate treatment.

4. Cardiac Arrest/CPR + Pyruvate: This group underwent the same surgical preparation and cardiac arrest/CPR/recovery protocol as group 3. They were infused with 2 M sodium pyruvate (0.1 $mmol \cdot min^{-1} \cdot kg^{-1}$) during CPR, cardioversion, and the first 60 min of recovery.

Colorimetric Enzyme Assays

The activities of a panel of ten enzymes were measured in tissue extracts of renal biopsies collected at 4 h ROSC. In general, the enzyme assays were performed accordingly to the procedures described by Bergmeyer *et al* [37], in a

Shimadzu UV-1800 spectrophotometer. The reaction sequences of these assays are summarized in figure 2.2. This panel includes the glycolytic enzymes hexokinase, phosphofructokinase (PFK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and lactate dehydrogenase (LDH); the Krebs cycle enzymes and citrate synthase (CS) and aconitase; the critical energy transferring enzyme, creatine kinase (CK); and the antioxidant system enzymes glucose 6-phosphate dehydrogenase (G6PDH), glutathione peroxidase (GP), and glutathione reductase (GR). Some of these enzymes, particularly GAPDH, aconitase, and CK, are susceptible to inactivation by RONS [18, 29]; others, e.g. LDH, are considered resistant to RONS. Enzyme activity is expressed as units (U) per mg of total protein in the tissue extract. A unit of enzyme activity is defined as the amount of enzyme required to catalyze the conversion of 1 μmol of substrate to product per minute at a specified temperature. The activities were determined by the rate of appearance or disappearance of the coenzyme, NAD(P)H, monitored by light absorbance at 339nm wavelength, for CS, the appearance of APADH was monitored. Myeloperoxidase (MPO) activity was measured in kidney extract obtained at 4 h and 3 d recovery using a colorimetric assay kit (Northwest Life Science Specialties, Vancouver, WA).

An ELISA kit (Kamiya Biomedical, Seattle, WA) was used to measure EPO content. KIM-1 concentrations were also measured by an ELISA kit (Mybiosource, San Diego, CA). Plasma creatinine concentrations were measured spectrophotometrically in a SPOTCHEM Analyzer by the use of Spotchem™ creatinine reagent strips (scil animal care company, Gurnee, IL).

The creatinine concentrations were measured from plasma samples obtained a few minutes before cardiac arrest and just before the animal is sacrificed at 3 d recovery.

Histology

Renal integrity and infiltration of inflammatory leukocytes were evaluated by light microscopy of the fixed tissue sections. Paraffin-embedded sections (5 μm) of corticomedullary tissues obtained at 4 h and 3 d recovery were stained with hematoxylin and eosin (H&E). When hematoxylin oxidizes, it binds to DNA, coloring the nucleus of blue; while the eosin counterstain colors the intracellular and extracellular proteins, including the cytoplasm, various shades of pink and red. An investigator blinded to the protocol and treatment visually compared the number of neutrophils for the different sacrifice protocols within each treatment group and inspected and evaluated the histological structures.

Statistical Analyses

Data are presented as means ± standard error of means (SEM). All data was analyzed by two factor (protocol, treatment) ANOVA. Treatment effects on MPO activity and EOP content at 4 h ROSC also were analyzed by two-tailed t-test. Statistical significance was accepted at $P<0.05$.

A. Hexokinase

Reaction:

Glucose + ATP $\xrightarrow{HK}$ Glucose-6-Phospate + ADP

Glocose-6-phosphate + $\underline{NADP^+}$ $\xrightarrow[\text{G6-PDH}]{}$ 6-phosphogluconate + $\underline{NADPH^+}$ + H^+

B. Phosphofructokinase

Reaction:

Fructose-6-phosphate $\xrightarrow{PFK}$ Fructose 1,6 bisphosphate

Fructose 1,6 bisphosphate $\xrightarrow[\text{Aldoase}]{}$ Glyceraldehyde-3-phosphate

Glyceraldehyde 3-phosphate + $\underline{2NADH + H^+}$ $\xrightarrow[\text{GOPDH}]{}$ 1,3 bisphosphoglyerate + $\underline{2NAD^+}$

C. Glyceraldehyde 3-Phosphate Dehydrogenase

Reaction:

Glyceraldehyde 3-phosphate + $\underline{NAD^+}$ $\xrightarrow[\text{GAPDH}]{}$ 1,3 bisphosphoglyceate + $\underline{NADH + H^+}$

D. Lactate Dehydrogenase

Reaction:

Pyruvate + $\underline{NADH + H^+}$ $\xrightarrow[\text{LDH}]{}$ Lactate + $\underline{NAD^+}$

E. Citrate Synthase

Reaction:

Malate + $\underline{APAD^+}$ $\xrightarrow[\text{MDH}]{}$ Oxaloacetate + Acetyl CoA + $\underline{APADH}$ $\xrightarrow{CS}$ Citrate + CoA

F. Aconitase

Reaction:

1) Citrate $\xrightarrow[\text{Aconitase}]{}$ Isocitrate

2) Isocitrate + $\underline{NADP^+}$ $\xrightarrow{ICDH}$ 2-oxoglutorate + $\underline{NADPH + H^+}$

G. Creatine Kinase

Reaction:

1) Creatine phosphate + ADP $\xrightleftharpoons{CK}$ Creatine + ATP

2) Glucose + ATP $\xrightarrow{HK}$ ADP + Glucose-6-phosphate

3) Glucose-6-phosphate + $\underline{NADP^+}$ $\xrightleftharpoons{G6PDH}$ Gluconate-6-phosphate + $\underline{NADPH + H^+}$

H. Glucose-6-Phosphate Dehydrogenase

Reaction:

Glucose-6-Phosphate + $\underline{NADP^+}$ $\xrightleftharpoons{G6PDH}$ 6-phosphogluconolactone + $\underline{NADPH + H^+}$

I. Glutathione Peroxidase

Reaction:

$2GSH + ROOH \xrightleftharpoons{GP} GSSG + ROH + H_2O$

$GSSG + \underline{NADPH} + H^+ \xrightleftharpoons{GR} 2GSH + \underline{NADP^+}$

J. Glutathione Reductase

Reaction:

$GSSG + \underline{NADPH} + H^+ \xrightleftharpoons{GR} 2GSH + \underline{NADP^+}$

Figure 2.2. *Enzyme activity assays*. Abbreviations: ADP: adenosine diphosphate; $APAD^+$; oxidized acetylpyridine-adenine dinucleotide; APADH: reduced acetylpyridine-adenine dinucleotide; ATP: adenosine triphosphate; GSH: glutathione (reduced form); GSSG; glutathione disulfide (oxidized form); ICDH: isocitrate dehydrogenase; MDH: malate dehydrogenase; NAD^+: nicotinamide adenine dinucleotide (oxidized form); $NADH+H^+$: nicotinamide adenine dinucleotide (reduced form); $NADP^+$: nicotinamide adenine dinucleotide phosphate (oxidized form); $NADPH+H^+$: nicotinamide adenine dinucleotide phosphate (reduced form); ROH; fatty acyl alcohol; ROOH: fatty acyl peroxide.

CHAPTER 3

Results

Panel of Enzymes

Activities of enzymes mediating cellular metabolism and antioxidant defense were measured in renal cortical biopsies at 4 h recovery. Although none of these enzymes showed any statistically significant effects of ischemia-reperfusion or pyruvate treatment, aconitase (figure 3.2B) demonstrated a trend toward increased activity in the Sham+Pyruvate group when compared to the other three groups. Similar, albeit slightly less obvious, trends were noted for PFK (figure 3.1B), CS (figure 3.2A), and CK (figure 3.2C) activities. No trends were observed for the activities of the other six enzymes: hexokinase (figure 3.1A), GAPDH (figure 3.1C), LDH (figure 3.1D), G6PDH (figure 3.3A), GP (figure 3.3B), and GR (figure 3.3C). Thus, 6 min zero flow ischemia imposed by cardiac arrest followed by 4 min partial reperfusion effected by closed-chest cardiac massage and definitive reperfusion upon cardioversion did not appreciably impair a panel of enzymes including several known targets of reactive oxygen and nitrogen species.

Myeloperoxidase Activity and Renal Cortical Histology

To assess the post-arrest inflammatory response, myeloperoxidase (MPO) activity, a marker of pro-inflammatory leukocytes [35], was measured in renal cortical biopsies. At 4 h recovery renal MPO activity (U/mg) was greater in the CPR+NaCl group (21±5) than the Sham+NaCl (12±4) and CPR+Pyruvate (9±2) groups, but single-factor ANOVA did not reveal statistically significant differences (Figure 3.4A). A sub-analysis of the two CPR groups (figure 3.4B) revealed statistically significant treatment effects, where MPO in the CPR+Pyruvate group was 59% less than that of the CPR+NaCl group (P=0.045), suggesting that pyruvate can reduce the infiltration of inflammatory cells within 4 h of I/R injury. Histologically no neutrophils or monocytes were noted upon visual inspection in any microscopic field in any of the 4 treatment groups at 4 h and 3 d recovery (figures 3.5 and 3.6). There was no evidence of tissue damage within these same groups, and the integrity of all visible structures was maintained.

Creatinine Concentrations

Plasma creatinine concentration, which varies inversely with glomerular filtration rate, was measured 15 min before cardiac arrest and at 3 d recovery to assess stability of the GFR following cardiac arrest and resuscitation (figure 3.7). There were no statistically significant changes in plasma creatinine in pigs

subjected to cardiac arrest-resuscitation with NaCl or pyruvate treatment, or in non-arrest sham pigs studied over the same period. Thus the present cardiac arrest-resuscitation protocol did not produce a discernable effect on the glomerular filtration rate.

Kidney Injury Molecule-1 (KIM-1)

There were no statistically significant differences (P=0.35) in the content of the transmembrane protein KIM-1 (ng/mg protein), a protein product of renal apoptosis, at 3 d recovery (figure 3.8). A slight trend toward an increase in KIM-1 content was noted in the CPR+NaCl (0.69±.08) and the CPR+Pyruvate (0.71±.07) groups when compared to the Sham+NaCl (0.49±.02) groups, suggesting that cardiac arrest, resuscitation, and recovery may increase renal content of a protein marker of kidney injury, but pyruvate treatment did not modulate accumulation of the injury marker.

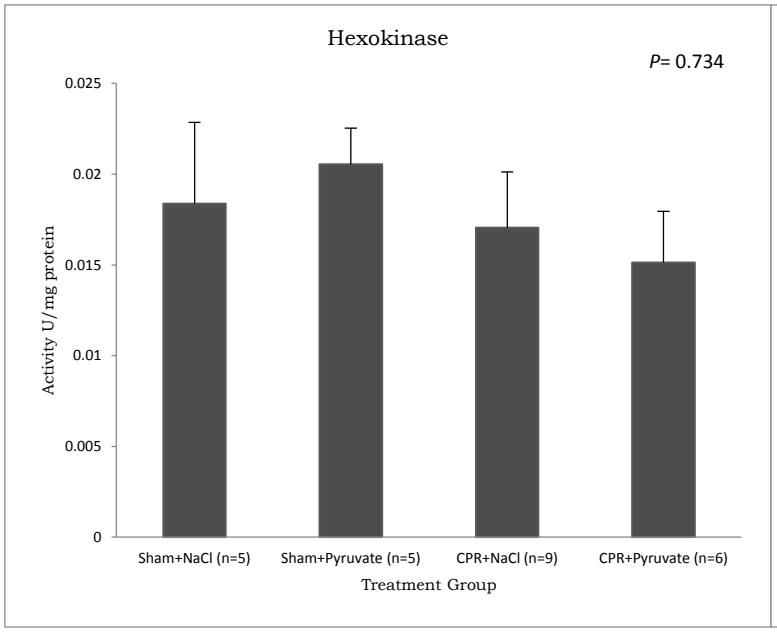
Erythropoietin (EPO)

Cortical peritubular interstitial cells near the corticomedullary border are the principal sources of the erythropoietic cytokine erythropoietin [20]. Accordingly, EPO content (IU/μg protein) was measured in biopsies collected from this region of the kidney at 4 h and 3 d recovery (figure 3.9A). At 4 h recovery, the EPO contents in the CPR+NaCl (4.24±0.6) and the CPR+Pyruvate

(4.29±1.1) groups were higher than that of the Sham+NaCl group (2.64±0.22). A sub-analysis of EPO content of the Sham+NaCl vs. CPR+NaCl groups (figure 3.9B) revealed a statistically significant increase (P=0.036) in the CPR+NaCl group indicating the I/R stress induces EPO production. There was no evidence of further EPO induction by pyruvate treatment. Similarly, there were no statistically significant differences in EPO contents among the three groups at 3 d recovery; indeed, EPO content in the Sham+NaCl (0.93±0.3), CPR+NaCl (1.28±0.1) and CPR+Pyruvate (1.38±0.2) groups were similar. However, the renal cortical EPO contents at 3 d recovery were sharply lower than the respective 4 h recovery values in all three groups. Because the decrease in EPO content was similar in the non-arrest sham group, it could not be ascribed to cardiac arrest and resuscitation *per se*, but rather likely resulted from the conditions of the acute experiment common to all three groups, including isoflurane anesthesia, surgical trauma, and/or hyperoxic ventilation.

Figure 3.1.*Activites of glycolytic enzymes*. Activities were measured in renal cortex at 4 h ROSC. Values are mean ± SEM, number of experiments are shown below each bar. Activities were measured by colorimetric assay [37].

A



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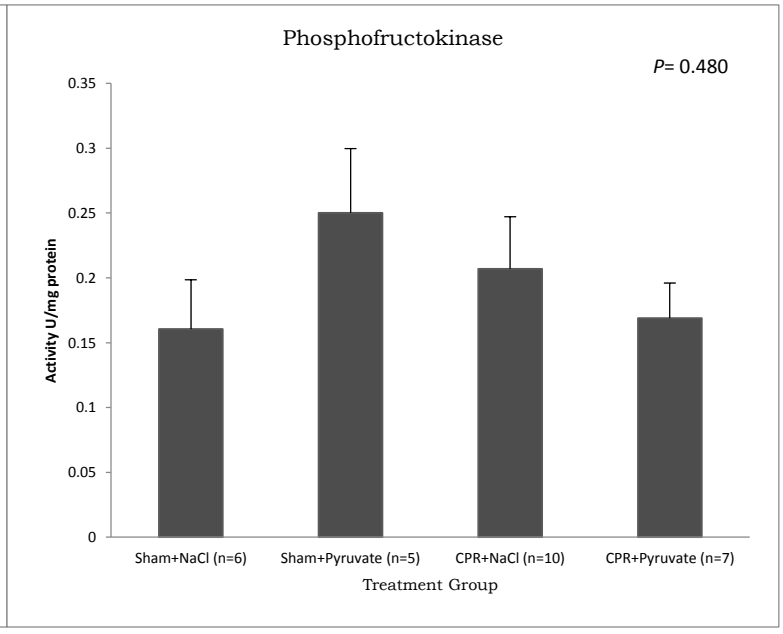

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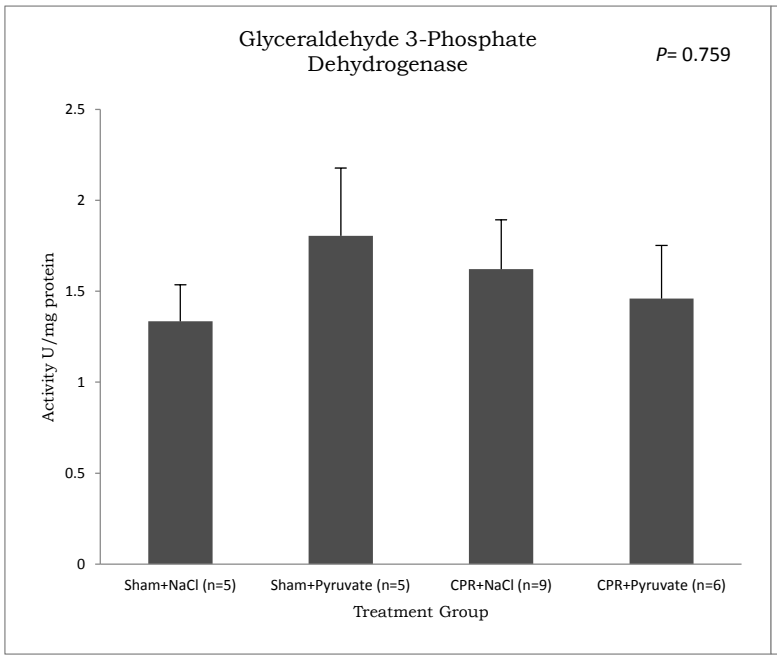

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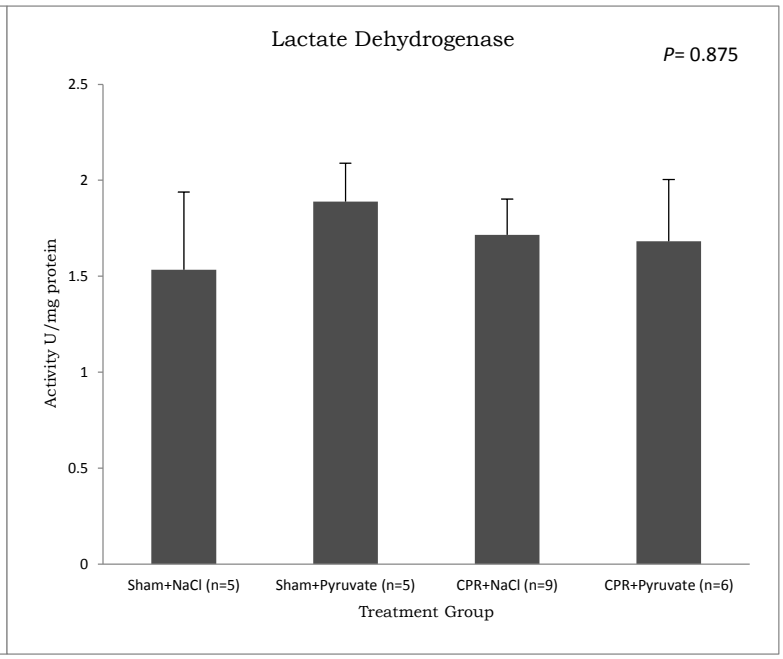
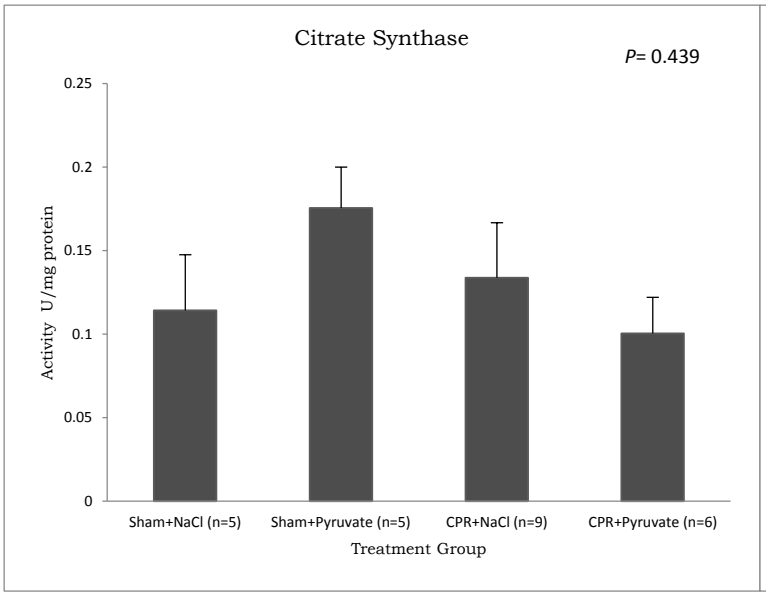


Figure 3.2.*Enzyme activities of the Krebs cycle and energy transfer*. Activities were measured in renal cortex at 4 h ROSC. Values are mean ± SEM, number of experiments are shown below each bar. Activities were measured by colorimetric assay [37].

A



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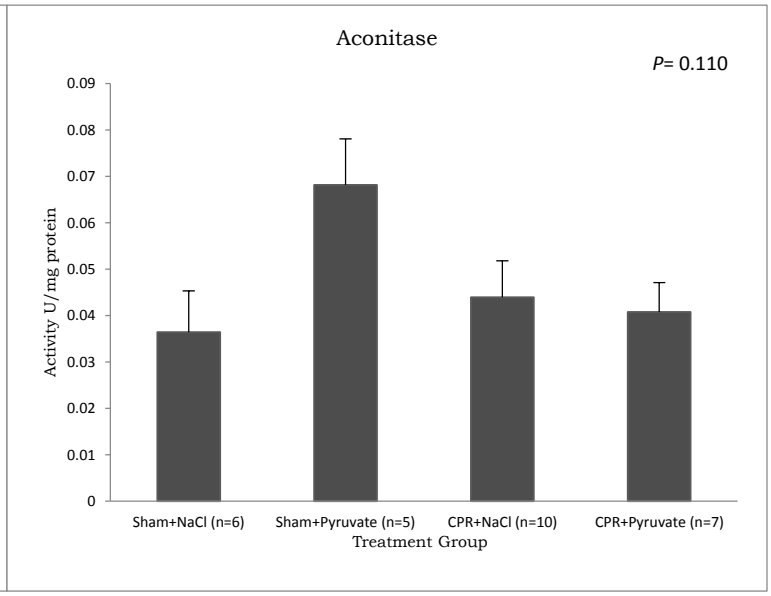

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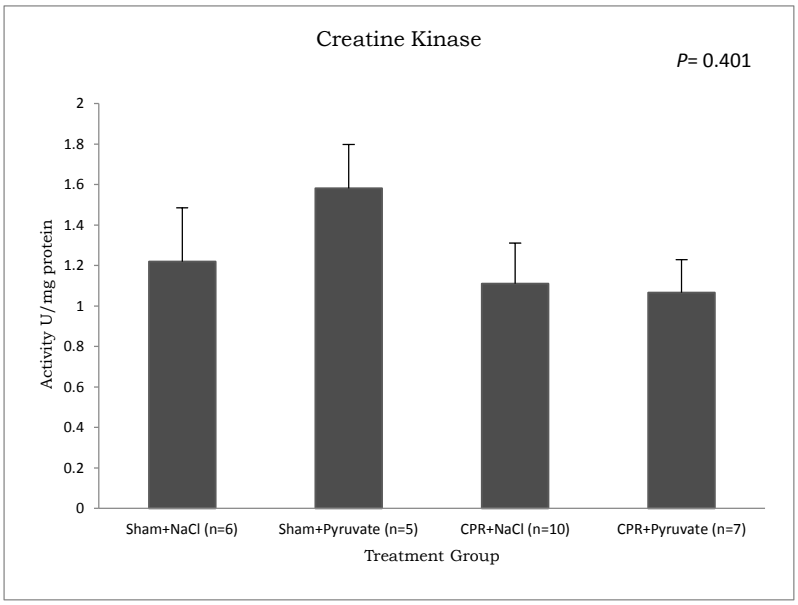
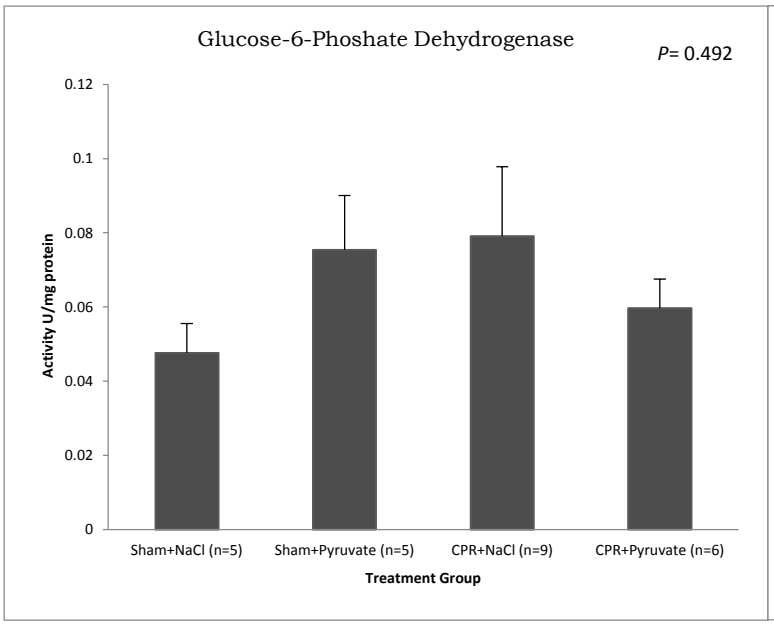


Figure 3.3.*Enzyme activities of antioxidant defense*. Activities were measured in renal cortex at 4 h ROSC. Values are mean ± SEM, number of experiments are shown below each bar. Activities were measured by colorimetric assay [37].

A



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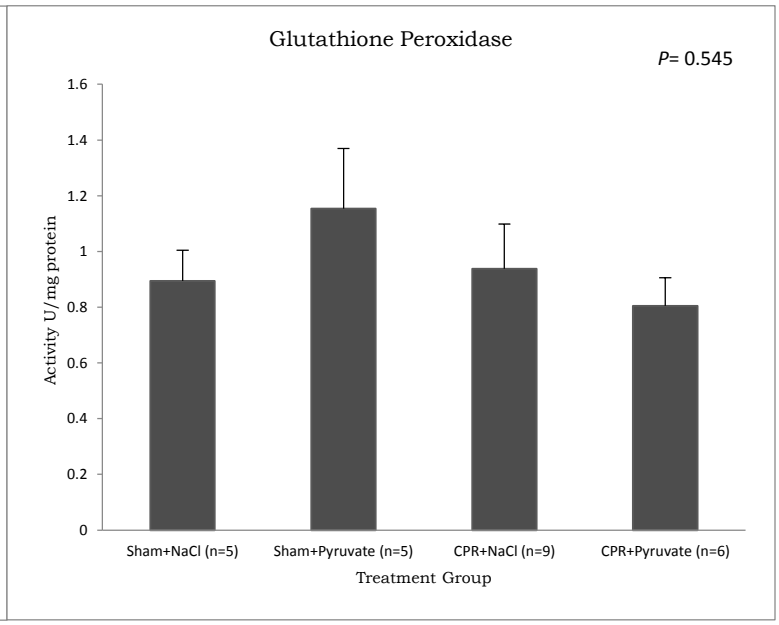

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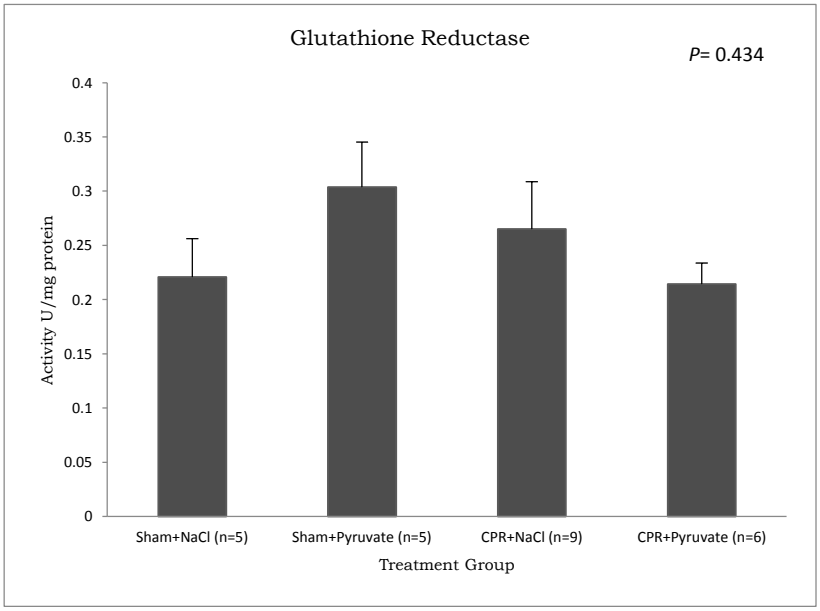
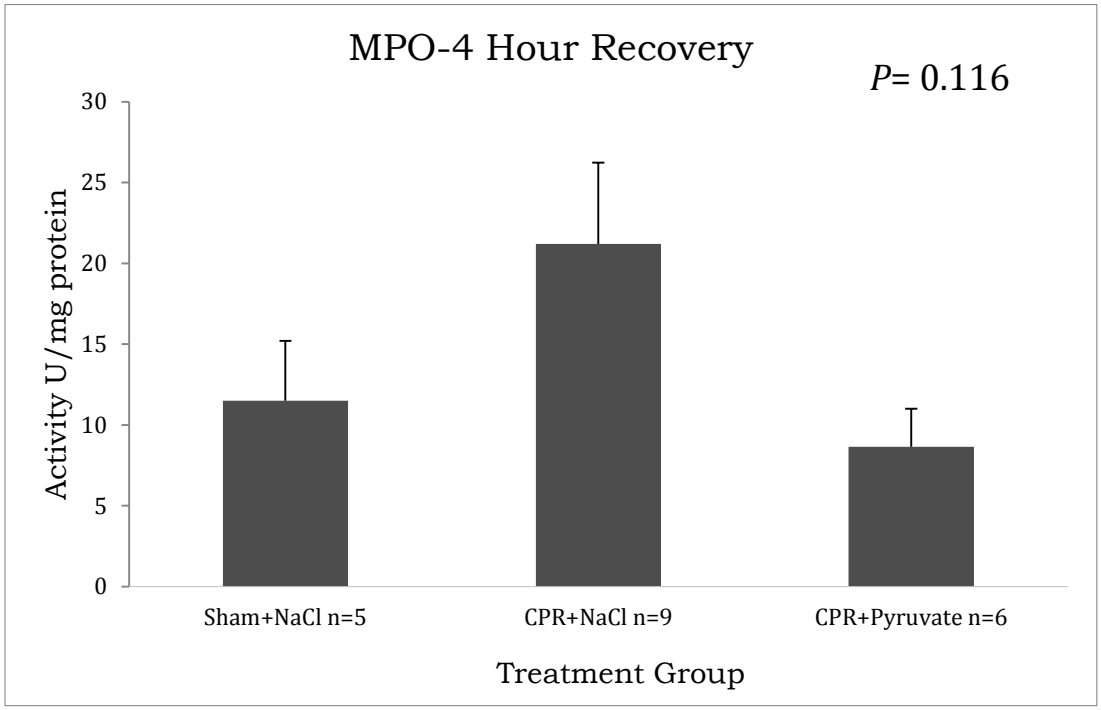


Figure 3.4. Activity or myeloperoxidase (MPO) in renal cortex at 4 h ROSC. Activities were measured by a colorimetric assay kit (Northwest Life Science Specialties, Vancouver, WA). Values are mean ± SEM, number of experiments are shown below each bar.

A



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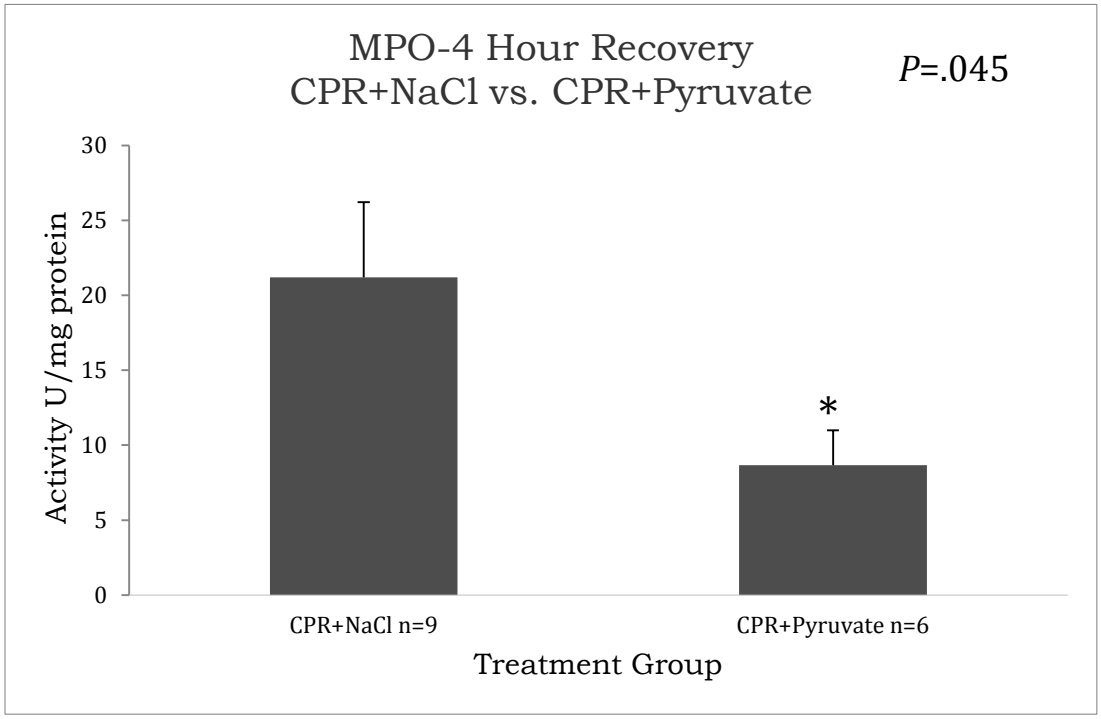


Figure 3.5

Four Hour Recovery (200X)

Top: Sham+NaCl
Bottom: CPR+NaCl

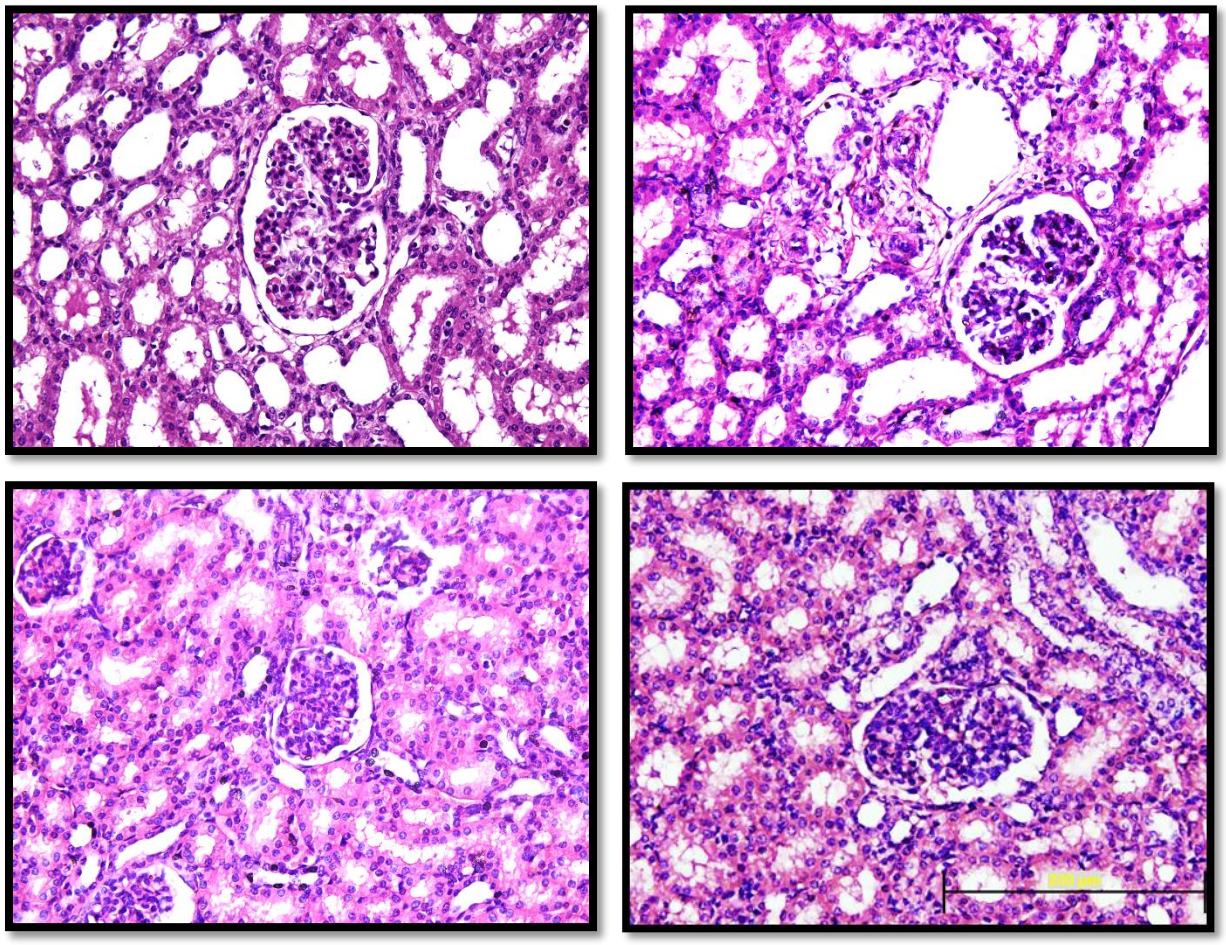


Figure 3.6

Three Day Recovery (200X)

Top: Sham+NaCl
Bottom: CPR+NaCl

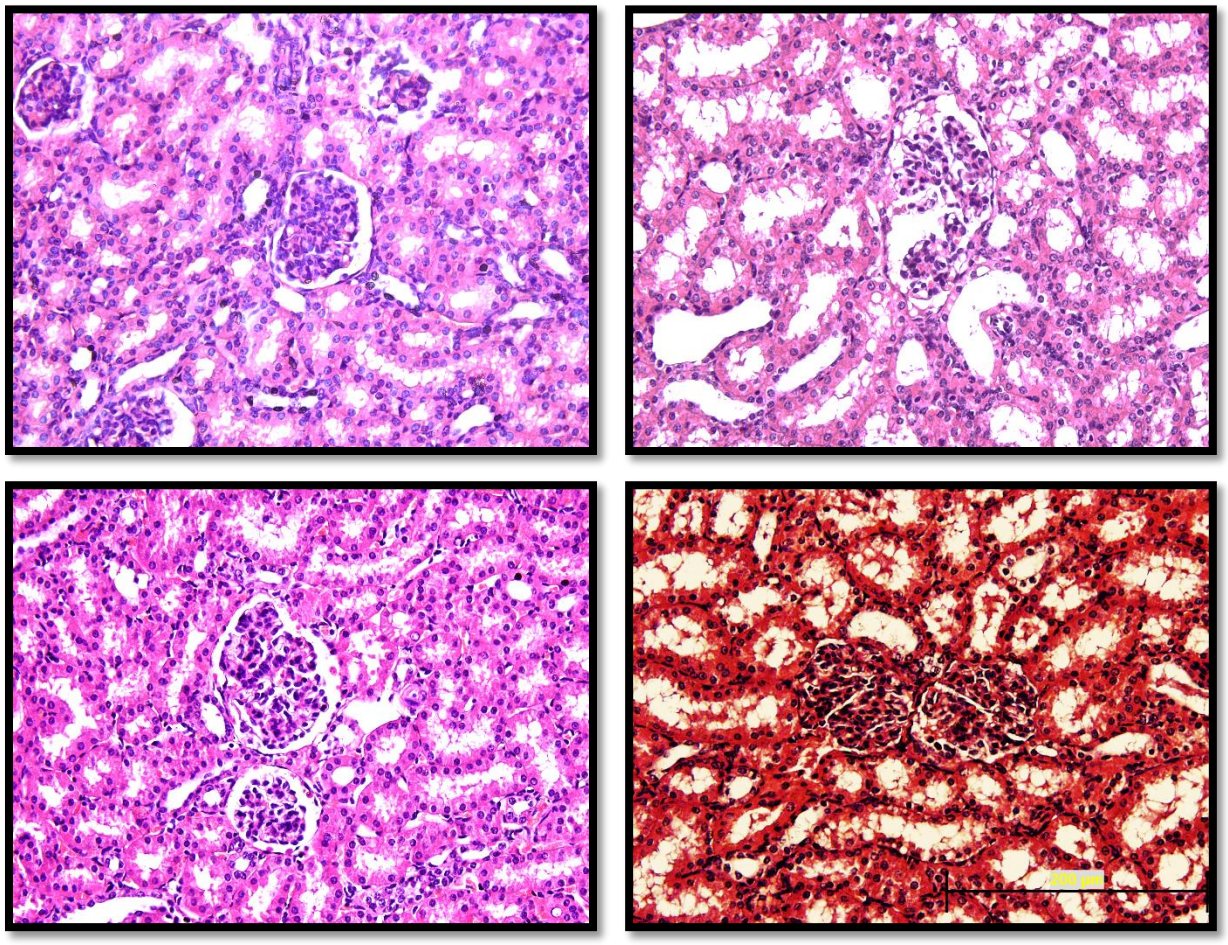


Figure 3.7. Plasma creatinine concentrations of in renal cortex at 4 h ROSC. Concentrations were measured by colorimetric assay using a SPOTCHEM Analyzer (scil animal care company, Gurnee, IL). Values are mean ± SEM, number of experiments are shown below each bar.

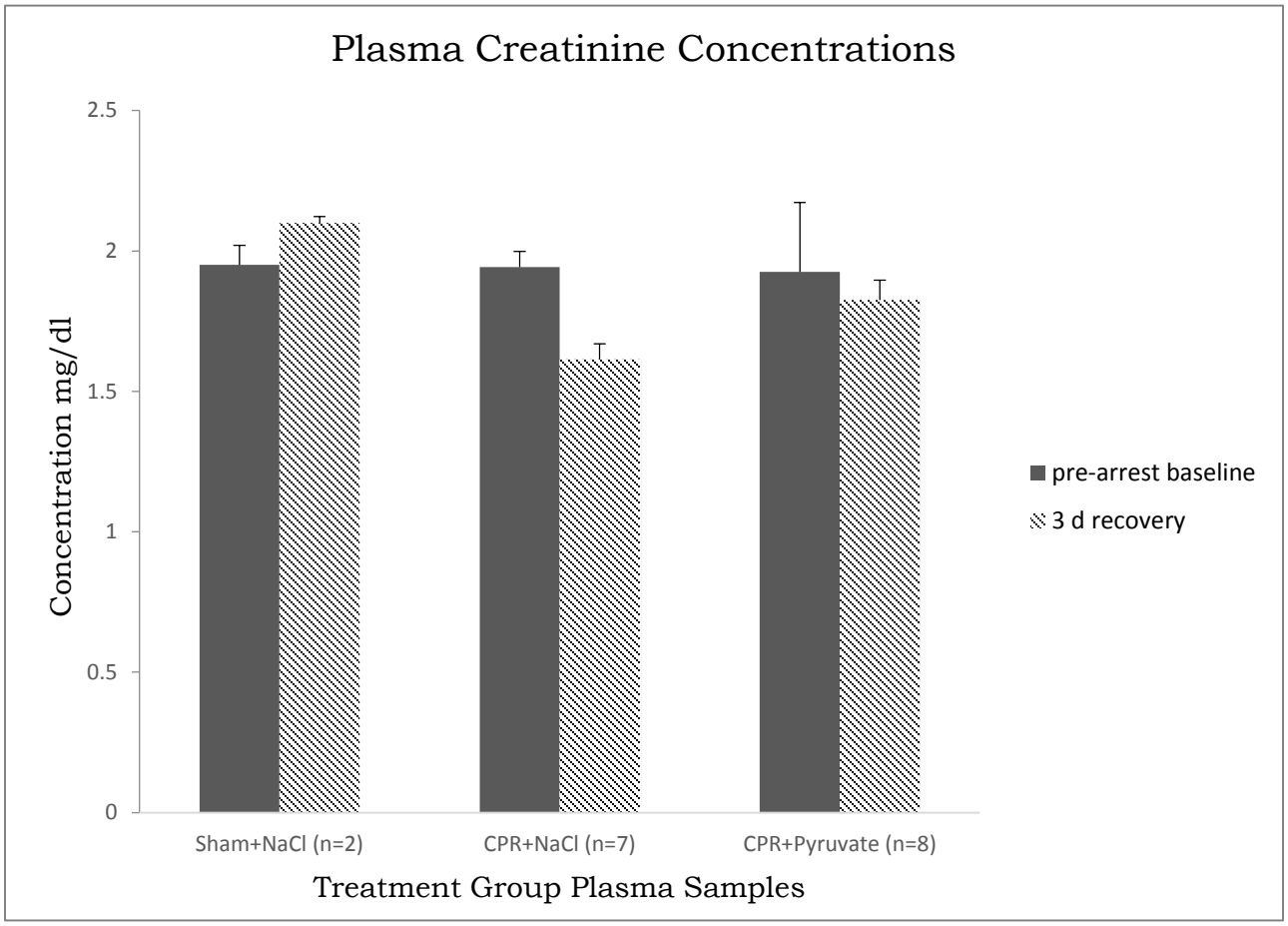


Figure 3.8. Content of Kidney Injury Molecule-1 (KIM-1) in renal cortex at 3 d ROSC. Concentrations were measured by ELISA kit (Mybiosource, San Diego, CA). Values are mean ± SEM, number of experiments are shown below each bar.

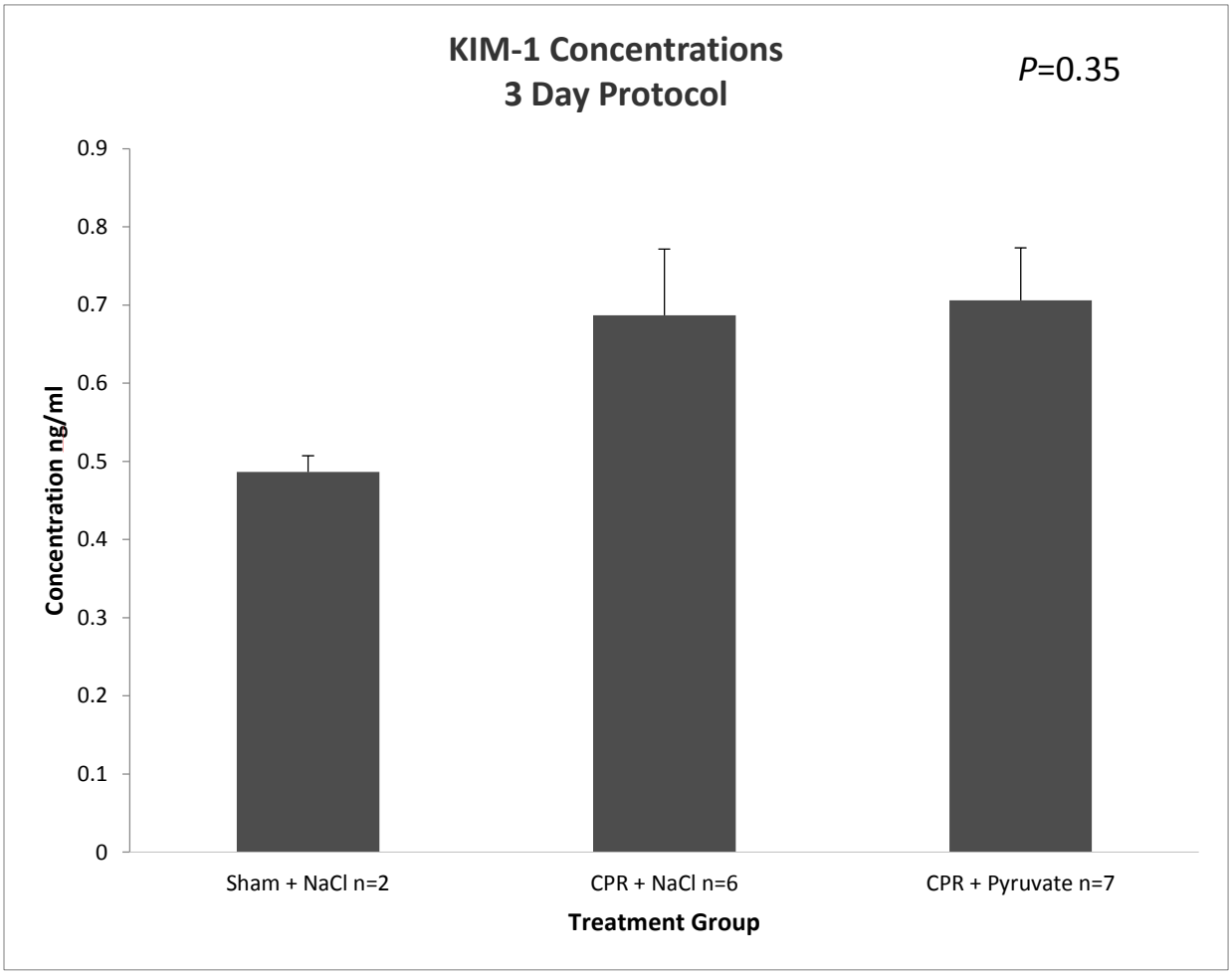
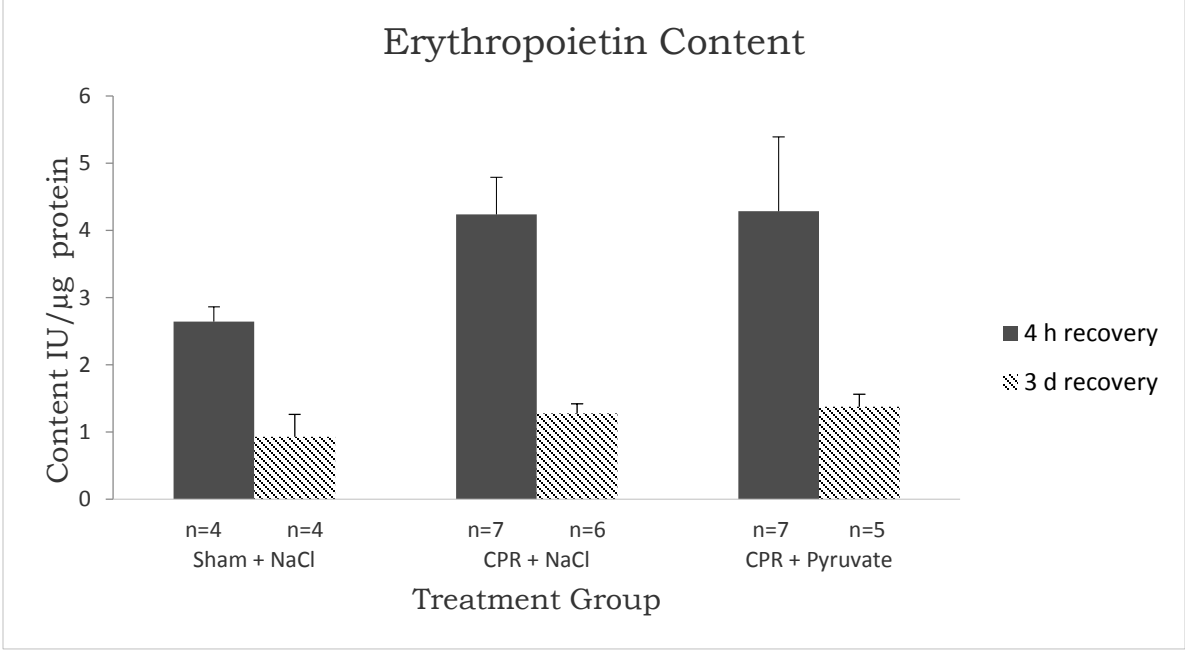


Figure 3.9. Content of erythropoietin (EPO) in renal cortex at 4 h and 3 d ROSC. Content was measured with ELISA kit (Kamiya Biomedical, Seattle, WA). Values are mean ± SEM, number of experiments are shown below each bar. Activities were measured by colorimetric assay [37].

A



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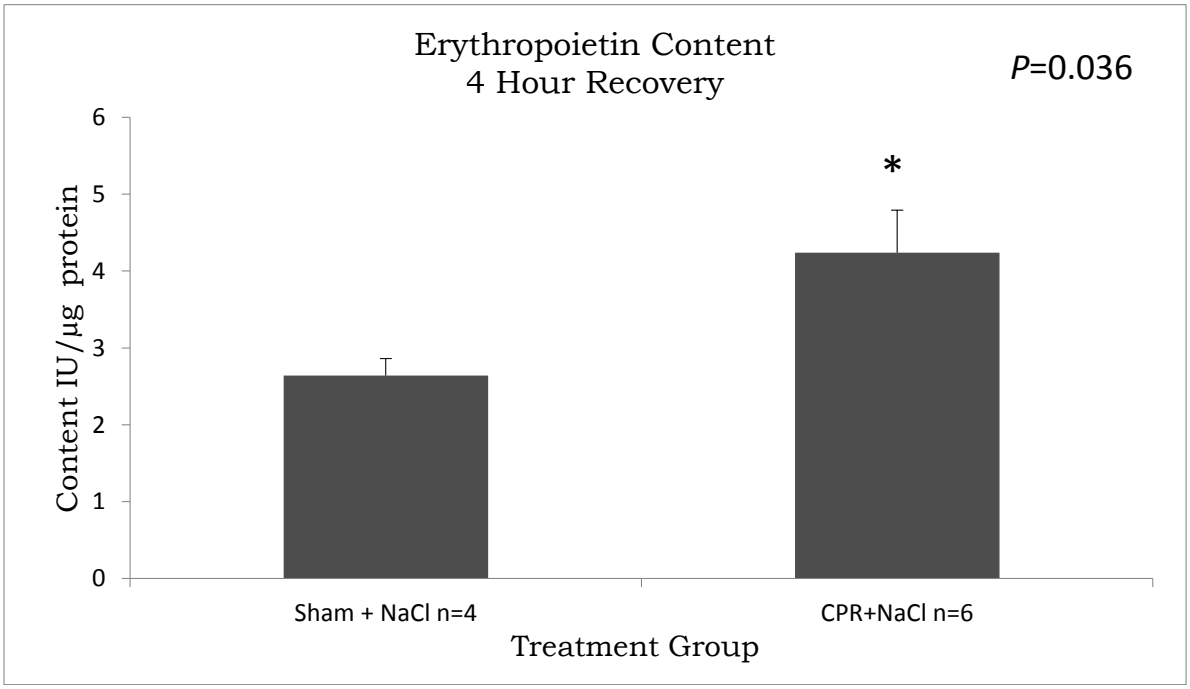


CHAPTER 4

Discussion

In adults, the predominant source of the erythropoietic hormone EPO is the kidneys, where in response to hypoxia specialized fibroblasts nestled in the interstitium surrounding the proximal tubules release the hormone. Upon its release, EPO enters the peritubular capillaries and is carried into the systemic circulation, which delivers the hormone to erythrocytic precursors in the bone marrow. Recently EPO has been found to exert non-erythropoietic effects which are potentially protective to tissues and organs threatened by ischemia-reperfusion [28]. Not only is EPO secretion a normal response to hypoxia; in addition, increased expression of EPO can exert beneficial, non-hematopoietic effects that serve to protect cells from I/R injury [16, 29].

Erythropoietin is a product of a gene expression program driven by an oxygen-regulated transcription factor, HIF. This factor is a heterodimer; its β subunit is constitutively expressed and omnipresent, but the α subunit is rapidly degraded in the presence of normal or elevated O_2 concentrations as a result of hydroxylation of two proline residues within the α subunit. This prolyl hydroxylation, which directs the α subunit into a pathway culminating in its proteosomal degradation, requires O_2, Fe^{2+}, and α-ketoglutarate as substrates. Pyruvate and several other Krebs cycle intermediates that share structural

homology with α-ketoglutarate can interfere with prolyl hydroxylation of HIF-1α [17, 25], thereby stabilizing the subunit in the face of abundant O_2 and, thus, activating expression of HIF-1-dependant genes, including EPO.

The purpose of this study was to test the efficacy of intravenous pyruvate in exploiting endogenous mechanisms of erythropoietin (EPO) to protect the kidney from cardiac arrest-induced I/R injury. In turn, the induction of this endogenous erythropoietin within the kidney may afford renoprotection by generating the protective protein locally within the tissue at risk. I/R stress can produce oxidative stress by creating an imbalance between the formation of RONS and the endogenous systems that detoxify reactive intermediates or repair the resulting damage [4]. These elevated amounts of RONS cause oxidative damage to cell components, including many important metabolic enzymes, thereby decreasing their activity [11, 18, 23]. In this study, a panel of intermediary metabolic and antioxidant enzyme activities, some not previously reported in porcine kidney, was evaluated at 4h recovery following the cardiac arrest-resuscitation or sham protocols. Aconitase and PFK are known to be affected by oxidative and nitrosative stress, while LDH is more RONS-resistant, and therefore suitable as a "housekeeping" enzyme. The fact that there were no statistically significant changes in activity for any of these enzymes among the four treatment groups suggests that the I/R stress was not sufficiently severe to elicit enough RONS production to inactivate these enzymes, or that the endogenous antioxidant defense mechanisms had the capability to minimize the damage. These defenses include RONS scavengers

such as superoxide dismutase, catalase, and glutathione that protect against acute kidney injury [6]. Furthermore, if the I/R injury did not inactivate the three enzymes involved in antioxidant defense, it is conceivable that they offered protection from oxidative damage for the other enzymes, thereby preserving their activities. Since the renal enzymes were not inactivated by cardiac arrest-resuscitation, there was no opportunity for pyruvate treatment to protect the enzymes.

Inflammation has emerged as one of the major pathophysiological pathways mediating I/R injury [3]. It is the oxidative stress ignited by tissue reperfusion that provokes an intense and potentially injurious inflammatory response [35]. We measured changes in MPO activity and conducted histology to examine for leukocyte infiltration. Histologically, no leukocytes were detectable in biopsies taken at 4 h and 3 d post-arrest recovery or the respective sham experiments. Indeed, by 3 d recovery there was no discernable MPO activity. Statistical analysis revealed no statistically significant effects of protocol or treatment on MPO activity at 4 h recovery. Donnahoo *et al* [39] reported low but detectable activities of MPO in sham rats, suggesting that the surgical procedure and anesthesia can in fact create a low level, systemic inflammatory response. A sub-analysis comparing the MPO activities of the CPR+NaCl vs. CPR+Pyruvate groups revealed a statistically significant decrease in MPO activity (P=0.045) in the kidneys of pyruvate treated pigs at 4 h recovery. This finding provides evidence that pyruvate can ameliorate the inflammation associated with I/R injury. Ysebaert *et al.* [35] reported

significant increases in renal MPO activity 1 h after ischemia, reaching its maximum activity after 12 h reperfusion. It should be noted that Ysebaert *et al.* [35] induced renal ischemia by cross clamping the left renal pedicle for 60 min, followed by right nephrectomy at the end of the ischemia, creating a more severe stress than the 10 min ischemia imposed by cardiac arrest. The present histological evaluation also revealed no structural damage in the CPR vs. Sham groups. Thus, our cardiac arrest-resuscitation protocol did not appear to inflict substantial injury on the kidneys.

Erythropoietin is shown to protect kidneys from I/R injury [4, 13, 20]. Our central hypothesis is the administration of pyruvate during renal I/R injury would enhance the kidney's production of EPO by stabilizing HIF-1α, and thereby protect the kidney. As described above, under O_2-abundant conditions renal HIF-1 driven gene expression is suppressed by prolyl hydroxylation of HIF's α subunit. α-ketoglutarate is an essential cosubstrate of the prolyl hydroxylase reaction [9, 27]. Pyruvate is structurally similar to α-ketoglutarate, both being aliphatic 2-oxoacids, and will competitively inhibit PHD [18]. Hence pyruvate has been shown to stabilize HIF-1α in normoxic conditions. This action in turn allows for the transcription of EPO [17,27], one of the first identified products of HIF-1's gene program.

Renal EPO content was measured at both 4 h and 3 d recovery. There were no statistically significant effects of cardiac arrest-resuscitation *per se* or pyruvate treatment on EPO content. A sub-analysis showed a 38% increase in

EPO content in the CPR+NaCl group vs. Sham+NaCl, demonstrating that the ischemic event alone can induce renal EPO production within 4 h ROSC, and, therefore, that HIF-1 activation and EPO transcription and translation can occur within a few hours. There was no effect of pyruvate on EPO production. Renal EPO content at 3 d recovery was sharply lower than at 4 h. Unexpectedly, a similar decrease in EPO content was detected in non-arrested sham pigs. The fact that all three groups of pigs, including the shams, showed similar sharp decreases in EPO content from 4 h to 3 d indicates that the conditions of the experiment, including hyperoxic ventilation, isoflurane anesthesia and surgical trauma, either produced a temporary increase in EPO, and/or suppressed EPO expression sufficiently to lower renal EPO content 3 days later. Further investigation is needed to better understand when EPO production is initiated by an ischemic stimulus, when its production is most elevated, and when its production subsides.

Apoptosis has been proposed as the principal mechanism of death in ischemically injured kidneys [34], and the renoprotective effect of EPO is exerted mainly by inhibition of apoptotic cell death [13, 24]. KIM-1 was one of the first induced molecules identified in the post-ischemic kidney [5]. KIM-1 is a functional phosphatidylserine receptor that is utilized by activated renal proximal tubular epithelial cells for phagocytosis of apoptotic cells and has a central role in clearing apoptotic debris [22]. Vaidya *et al* [32] demonstrated that KIM-1 concentrations were highest 24 h post-I/R, and Rees *et al* [22] showed that phagosomes lined with KIM-1 can be seen 24 and 48 h after acute

renal injury. Since 4 h ROSC may be too soon after cardiac arrest to detect changes in renal KIM-1, we measured KIM-1 content at 3 d recovery.

There were no significant differences in the renal KIM-1 contents among the groups. KIM-1 contents in the CPR+NaCl and CPR+Pyruvate groups were almost identical, but the content in the Sham+NaCl groups was slightly (*c.*30%) lower. On the other hand, KIM-1 is not detectable in normal kidney tissue [32], yet there was a measurable amount in the Sham+NaCl group (0.49±.02 ng/mg protein). This group did not undergo cardiac arrest, so kidneys were not exposed to I/R injury. One reason for this unexpected KIM-1 may be the method of the tissue biopsy. The parent project is investigating the effect of cardiac arrest-resuscitation on the brain, and the kidney is harvested after the brain and heart. Because the heart is excised, the kidneys are subjected to a few min of zero flow prior to excision and biopsy. In this case there is no reperfusion, but perhaps this brief period of ischemia can initiate enough renal cellular apoptosis to express the KIM-1 molecule. On the other hand, it seems unlikely that a protein can be expressed in a few minutes. A more likely explanation for the appearance of KIM-1 in the sham kidneys, is the fact that the sham pigs, like those undergoing cardiac arrest and resuscitation were ventilated with 100% O_2, resulting in systemic hyperoxemia (4.1A). Because O_2 is the precursor of ROS, this hyperoxic ventilation for 4 h may have generated enough ROS in the kidneys to induce KIM-1 formation [40, 41].

Acute kidney injury is characterized functionally by a rapid decline in GFR, and biochemically by a resultant accumulation of creatinine in the circulation over the first few days after the acute insult [6]. Burne-Tanet *et al* [3] further explain that whole body ischemia-reperfusion injury leads to a significant increase in serum creatinine. Burne-Taney *et al* [3] and Vaidya *et al* [32] both show serum and plasma creatinine concentrations highest at 24 h after renal I/R injury. For our creatinine measurement, plasma creatinine concentrations were measured 15 min before cardiac arrest to establish a baseline and again at 3 d recovery, just before sacrifice. There were no significant differences in the concentrations at the two time points among the three treatment groups, which further suggests ischemia-reperfusion in this study did not substantially injure the kidneys.

Limitations

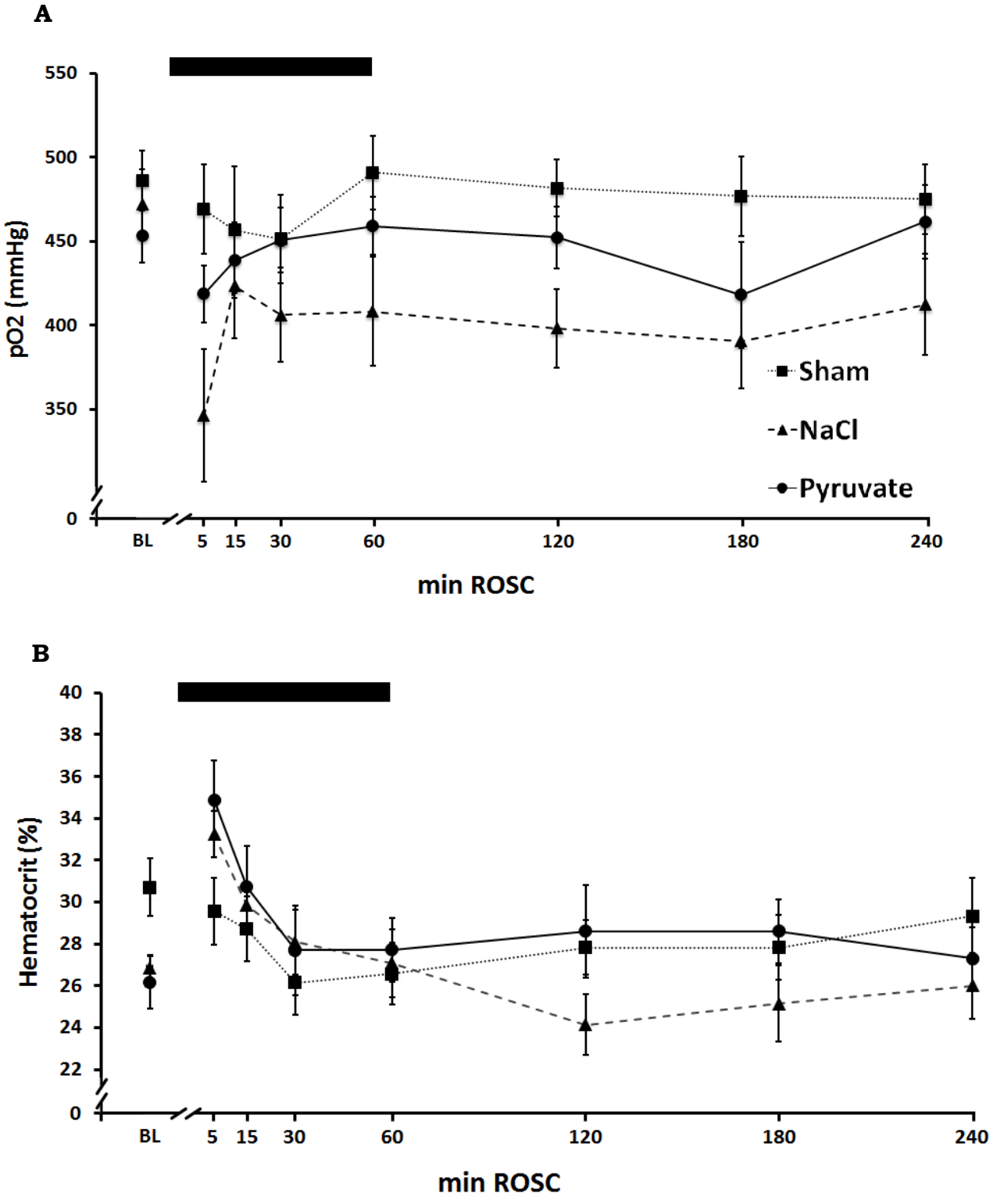
All experiments were performed in anesthetized animals. It could be questioned if this preparation models the effects of cardiac arrest-resuscitation and/or pyruvate treatment in human cardiac arrest victims, most of whom are stricken while awake, and who might not receive effective CPR by bystanders. Also pigs used in this study were smaller than adult humans, so it is possible that their thoracic cage was more flexible than those of adult humans, leading to more effective compressions than would be given by a bystander or even a paramedic treating a human victim. Collectively, these factors could account

for a higher survival rate for the pigs in this study vs. human victims. Similar studies have shown substantial tissue damage from I/R stress, and pyruvate has proven efficacious in large, anesthetized animals [25, 28]. Moreover, a defined cardiac arrest-CPR protocol of 6 minutes pre-intervention arrest and 4 minutes of chest compressions was studied. This duration of ischemia, which was relatively brief compared with most out-of-hospital cardiac arrests, was chosen to ensure a sufficiently high survival rate (*c.*75%) to permit studies of post-arrest recovery. In the clinical setting, the pre-CPR period may be more prolonged, especially for out-of-hospital cardiac arrests. It is possible that injury to the kidneys and/or other internal organs was more severe in the 25% of the pigs that did not survive, creating a 'selection bias' wherein the surviving pigs have modest or no renal injury and, thus, intact renal function. It should be noted that the biopsies were taken after two defined recovery periods, so the complete time course of renal injury following cardiac arrest wasn't examined. Lastly, the pigs were outwardly healthy, free of clinically evident disease, whereas most adult human cardiac arrest victims suffer from a host of chronic diseases, e.g. hypertension, diabetes, coronary atherosclerosis, or cerebrovascular disease.

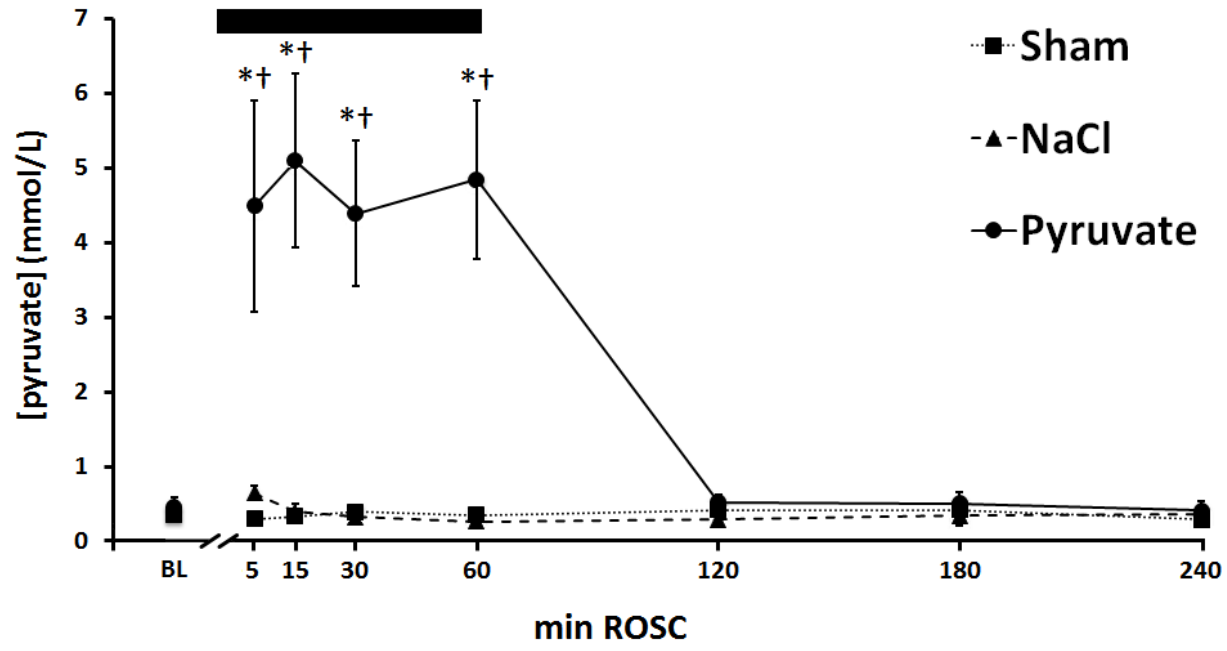
The objective of the parent project is to investigate the deleterious effects of cardiac arrest of the brain and the heart, and the protection offered by the infusion of pyruvate on these organs. That objective drove the experimental design and protocol. The lack of renal damage from the 10 min of arrest suggests a longer period of ischemia is needed to cause significant kidney

injury. However imposing a longer period of cardiac arrest would intensify damage to the heart and brain, thereby increasing the mortality rate, and, thus, increasing the costs and duration of this study.

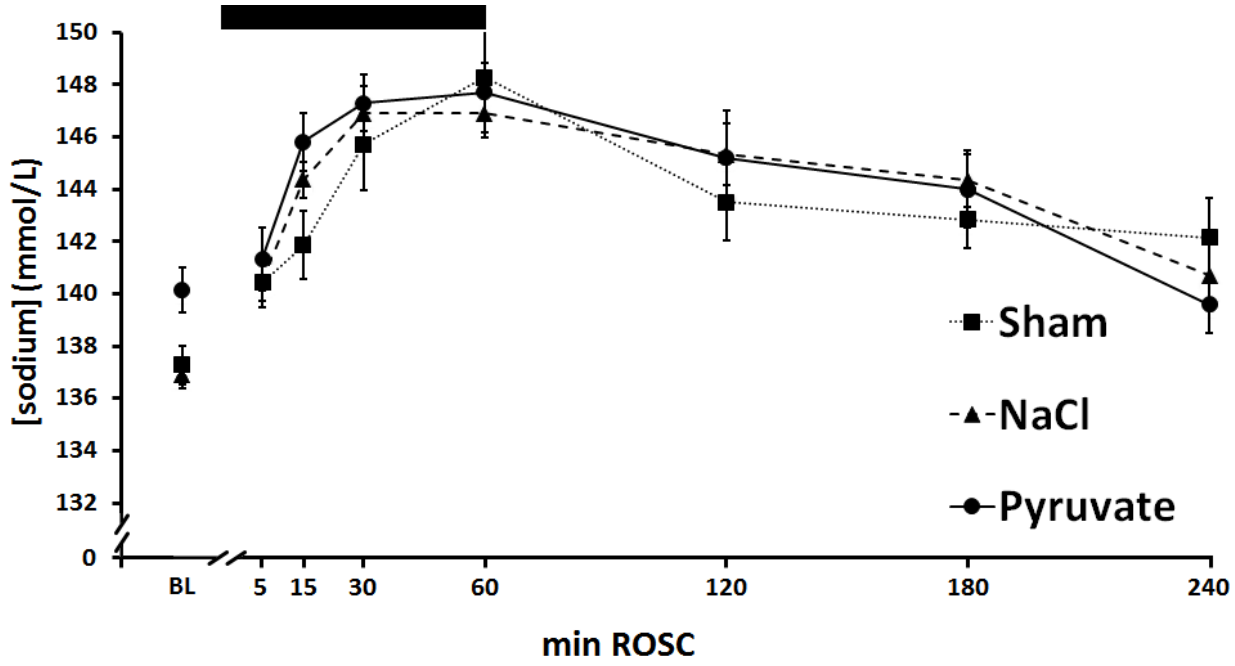
Figure 4.1. Sytemic arterial pO_2, hematocrit, pyruvate concentration, and sodium concentrations during first 4 h of ROSC



C


[pyruvate] (mmol/L)
7
6
5
4
3
2
1
0
*†
*†
*†
*†
Sham
NaCl
Pyruvate
BL
5
15
30
60
120
180
240
min ROSC

D


[sodium] (mmol/L)
150
148
146
144
142
140
138
136
134
132
0
Sham
NaCl
Pyruvate
BL
5
15
30
60
120
180
240
min ROSC

CHAPTER 5

Future Directions

In this study two durations of post-arrest recovery, 4 h and 3 d, were investigated. For further investigation of I/R injury in the kidney, and the protective properties of pyruvate, an intermediate time point, e.g. 24 h recovery, could be examined. This time point would afford the opportunity to detect peak amounts of the biomarkers investigated in this study. Also, measuring contents of HIF-1 by use of immunohistochemistry and immunoblots for the acute protocols, particularly 1 h post recovery while pyruvate infusion is still ongoing, would be of interest.

The method of inducing ischemia and reperfusion can affect the severity of I/R injury. Vaidya *et al.* [32] reported evidence of kidney damage, including remarkable elevations in both KIM-1 and creatinine concentrations, from bilateral clamping of the renal pedicles to induce ischemia for periods up to 45 minutes before releasing the clamp to create the I/R injury. This period of ischemia, longer than that of our study, allows for a robust accumulation of O_2, exposing the kidney to increased levels of RONS. This rapid and intense increase in RONS will very likely modify protein structures, disable enzymes, induce apoptosis, and activate a robust inflammatory response. Finally, the age

of the animals is a factor to consider when evaluating the renal damage caused by cardiac arrest. This study used healthy, juvenile pigs, on average 4-5 months old.

A novel approach to better understanding renal damage after cardiac arrest is to investigate the high incidence of heart disease in hemodialysis patients. These patients have an age adjusted mortality rate that is more than 3 times higher than that of the regular population, and *c.* 50% of these deaths are attributed to cardiac causes, including cardiac arrest [15]. Many pathological factors contribute to the heightened vulnerability of these dialysis patients, such as left ventricular hypertrophy (present in *c.*75% of dialysis patients), rapid pro-arrhythmic electrolyte shifts, and decreased ischemia tolerance [10]. The patients that did experience cardiac arrest were older (66±13 vs. 60±15 years) and were more likely to have diabetes (62% vs. 47%) than non-arrest dialysis patients [15].

Studies of renal disease associated with cardiac causes of death may require more severe ischemic stress on the kidneys, but potentially are of interest. For example, renal failure is associated with increased incidence of cardiac arrest [42]. To produce acute kidney injury, one could produce renal ischemia via bilateral renal pedicle clamping for 45 min. The extent of renal injury would be assessed by measuring plasma creatinine and urine KIM-1 concentrations before and after the insult. After a period of recovery, the animals will be subjected to cardiac arrest for 10 min. Cardioversion and ROSC

will be attempted, and the rate of successful cardioversion will be compared to the animals that did not undergo antecedent renal I/R injury. The duration of renal ischemia, the recovery period, and the arrest time are all factors that can be adjusted. This model will provide a better understanding of how acute kidney injury can affect the vulnerability of the heart to ischemia.

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