ABSTRACT

Cardiac arrest is a leading cause of death in the United States and Western Europe. Cardiopulmonary resuscitation (CPR) is the only means of sustaining the victim until application of defibrillatory countershocks. Although it has been over 50 years since its advent, CPR remains a work in progress. Many initially resuscitated victims later die from the damage sustained from ischemia-reperfusion, and treatments to combat the extensive ischemia-reperfusion injury sustained during cardiac arrest-resuscitation remain elusive. The major mechanism of injury underlying ischemia-reperfusion is the intense overproduction of reactive oxygen and nitrogen species (RONS) that accumulate during reperfusion and compromise normal cell function. RONS formed during resuscitation trigger lipid peroxidation, disable enzymes vital for cell metabolism and survival and, ultimately, induce cell death within affected organs. In order to prevent extensive damage to the central nervous system culminating in permanent neurocognitive disability and death, prospective treatments must possess robust antioxidant properties, traverse the blood-brain barrier between the cerebral circulation and brain parenchyma, and be non-toxic at effective doses.

Pyruvate is a natural intermediary metabolite, energy-yielding substrate and antioxidant. Pyruvate neutralizes RONS, thereby dampening oxidative stress and preventing covalent oxidative modification of enzymes and lipid membranes, and generates ATP to support brain

function. Pyruvate readily traverses the blood-brain barrier and is non-toxic over a wide range of doses, including those previously demonstrated to protect the heart during cardiopulmonary bypass and the brain during stroke, thereby supporting oxygen and fuel delivery to the recovering brain. Moreover, pyruvate has been shown to promote cardiac electromechanical and metabolic recovery following cardiac arrest and open-chest CPR.

This study tested whether infusion of pyruvate during, CPR and early recovery can decrease the biomarkers of oxidative stress after cardiac arrest. Isoflurane-anesthetized pigs were subjected to 6 min electrically-induced, untreated ventricular fibrillation, followed by 4 min closed-chest CPR, defibrillation and either 1 or 4 h recovery. Beginning at 5.5 min arrest, either sodium pyruvate or NaCl control were infused *iv* for the duration of CPR and for the first 60 min after recovery of spontaneous circulation (ROSC). Arterial blood was sampled pre-arrest and at 5, 15, 30, 60, 120, 180, and 240 min ROSC for analyses of blood gases and plasma constituents. At either 1 h (*i.e.* end of treatment infusion) or 4 h ROSC, a craniotomy was performed, the pig was euthanized, the brain was removed, and biopsies from hippocampus and cerebellum were snap-frozen in liquid nitrogen for biochemical analysis.

The first phase of this project tested the hypothesis that intravenous administration of sodium pyruvate during precordial compressions and the first 60 min ROSC restores hemodynamic, metabolic, and electrolyte homeostasis in a closed chest porcine model of cardiac arrest. Resuscitation with pyruvate sharply decreased the incidence of lethal pulseless electrical activity (PEA) following defibrillatory countershocks, and lowered the dosage of vasoconstrictor

phenylephrine required to maintain systemic arterial pressure. Pyruvate also enhanced glucose clearance, elevated arterial bicarbonate, and raised arterial pH.

The second phase of this project tested the hypothesis that pyruvate prevents the decrease in activity of the brain's antioxidant enzymes following cardiac arrest and hyperoxic (100% O_2). Activities of glutathione peroxidase and glutathione reductase were decreased at 60 min ROSC *vs.* sham in both the hippocampus and cerebellum. Pyruvate partially preserved glutathione peroxidase activity at 1 h ROSC, but by 4 h, after 3 h of pyruvate clearance from the circulation, the enzyme's activity fell to the same extent as in NaCl-infused pigs. Interestingly, the glutathione peroxidase/reductase activity fell sharply in non-arrested sham pigs between the time points corresponding to 1 and 4 h ROSC, suggesting that hyperoxia resulting from ventilation with 100% produced sufficient oxidative stress to inactivate the enzymes. Similarly, lactate dehydrogenase activity fell between 1 and 4 h ROSC in hippocampus and especially cerebellum. In sham pigs, lactate dehydrogenase activity decreased from the time points corresponding to 1 and 4 h ROSC, and pyruvate had no effect on lactate dehydrogenase in either region of the brain. Thus, cardiac arrest and hyperoxic ventilation disabled a critical antioxidant system in two ischemia-sensitive brain regions. Pyruvate afforded partial protection of these enzymes which waned after pyruvate cleared from the circulation.

We conclude that 1) Pyruvate infusion during cardiac arrest, CPR and early recovery promotes conversion from ventricular fibrillation to a productive sinus rhythm instead of lethal PEA; 2) Pyruvate hastened glucose clearance, a prognostic measure used clinically; 3) Pyruvate elevated the arterial bicarbonate concentration and raised arterial pH, which combats the acidemia

normally observed following ROSC; 4) Cardiac arrest-resuscitation and hyperoxic ventilation disabled the glutathione peroxidase-reductase system, a critical component of the brain's antioxidant defenses, in hippocampus and cerebellum; and 5) Pyruvate delayed oxidative inactivation of glutathione peroxidase in the cerebellum, but this effect subsided as pyruvate elevated. These investigations demonstrate the therapeutic effects and limitations of pyruvate as a resuscitative treatment to hasten electrocardiographic and metabolic recovery post cardiac arrest.

INTRAVENOUS PYRUVATE TO PROTECT HEART AND BRAIN DURING CLOSED-CHEST RESUSCITATION AND RECOVERY FROM CARDIAC ARREST

DISSERTATION

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DOCTOR OF PHILOSOPHY

By

Brandon Hunter Cherry

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ABBREVIATIONS

4-HNE: 4-hydroxy-2-nonenal

AD: Alzheimer's disease

Bak: Bcl-2 homologous antagonist killer

Bax: Bcl-2-associated X protein

BBB: blood-brain barrier

Bcl-2: B-cell lymphoma 2 family of proteins

BH_4: tetrahydrobiopterin

BL: pre-arrest baseline

Casp-3: Caspase-3

Casp-9: Caspase-9

CoQ: Coenzyme-Q

CPR: cardiopulmonary resuscitation

Cyt C: cytochrome C

ECG: electrocardiogram

eNOS: endothelial isoform of nitric oxide synthase

GPx: glutathione peroxidase

GRed: glutathione reductase

GSH: glutathione

GSSG: glutathione disulfide

H/K: hydrogen/potassium antiporter

HR: heart rate

iNOS: inducible isoform of nitric oxide synthase

LDH: lactate dehydrogenase

MAP: mean arterial pressure

MCT: monocarboxylate transporter

mPTP: mitochondrial permeability transition pore

mtDNA: mitochondrial DNA

NFκB: nuclear factor-kappa B

NO: nitric oxide

O_2^-: superoxide

$ONOO^-$: peroxynitrite

OxS: oxidative stress

PEA: pulseless electrical activity

ROS/RNS: reactive oxygen and nitrogen species

RONS: reactive oxygen and nitrogen species

ROSC: recovery of spontaneous circulation

RPP: heart rate x mean arterial pressure product

SMAC: second mitochondria-derived activator of caspases

TNF-α: tumor necrosis factor alpha

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AWARDS

- Outstanding Integrative Physiology and Anatomy Student, UNTHSC 2014
- Sigma Xi, The Scientific Research Society—Associate Member 2014
- Society for Experimental Biology and Medicine Young Investigator Award 2014
- UNTHSC Cardiovascular Research Institute Award 2013
- UNTHSC Graduate School Association Award—2nd place, poster presentation 2013
- Pre-doctoral Fellow, *Training in the Neurobiology of Aging (T32AG020494)* 2013-2014
- Texas Tech University, College of Arts and Sciences Dean's List 2010
- National Honor Society Member 2006

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INTRODUCTION

Cardiac arrest is a leading cause of death in the United States. Each year, approximately 360,000 Americans suffer out-of-hospital cardiac arrest, yet only ~10% of them survive to hospital discharge. Many victims of cardiac arrest are resuscitated, only to later succumb to brain injury inflicted by ischemia-reperfusion during arrest and the subsequent recovery of cerebral blood flow (Han *et al.*, 2008; Dezfulian *et al.*, 2009; Young *et al.*, 2009). Moreover, many of those victims fortunate enough to survive and be discharged from the hospital must still endure the consequences of permanent brain damage (Adrie *et al.*, 2004; Madl and Holzer, 2004; Idris *et al.*, 2005; Young *et al.*, 2009). Despite such a substantial negative impact on a country in which heart disease remains the number one killer (Heron, 2012), as noted by White *et al.* (White *et al.*, 2000), *"There are as of yet no clinically effective therapeutic protocols for amelioration of brain damage by ischemia-reperfusion."* Moreover, according to the American Heart Association's 2008 Consensus Statement on Resuscitation (Neumar *et al.*, 2008), "*…little evidence exists to suggest that the in-hospital mortality rate of patients who achieve ROSC after cardiac arrest has changed significantly in the past half-century.*"

CARDIAC ARREST AND CARDIOPULMONARY RESUSCITATION

The majority of cardiac arrests are characterized by ventricular fibrillation, which presents on an electrocardiogram as an uncoordinated rhythm referred to as Torsades de Pointes, or "twisting of

spikes" (*cf.* Figure 1). Ventricular fibrillation interrupts coordinated contraction of the myocardium and the forward movement of blood, causing ischemia of the entire body including its vital organs. To achieve recovery of spontaneous circulation (ROSC), cardiopulmonary resuscitation (CPR) is performed by delivering precordial compressions, which only partially restores blood flow, followed by transthoracic countershocks delivered with a defibrillator. These countershocks, convert ventricular fibrillation either to productive electrocardiographic rhythm and forceful contraction of the heart, or to ineffective pulseless electrical activity (PEA), an outcome with an extremely high mortality rate (Warner et al. 1985). Figure 1 shows representative electrocardiographic recordings at baseline, during ventricular fibrillation, and at 5 min and 1 h ROSC.

Pulseless electrical activity

Pulseless electrical activity is a 'non-shockable' cardiac electrical rhythm that does not produce ventricular contraction or forward movement of blood. It is estimated that approximately 60% of out-of-hospital resuscitation attempts result in the development of PEA (Niemann et al. 2001). Survival of cardiac arrest victims with PEA is much lower than those presenting with ventricular fibrillation; indeed, only 2-5% of patients who present with PEA as their initial rhythm survive (Rea et al. 2004; Atwood et al. 2005; Kajino et al. 2008). Even fewer patients in whom ventricular fibrillation converted to PEA following countershocks survive to hospital discharge (Warner et al. 1985).

Hemodynamics and plasma chemistry

A first priority following return of spontaneous circulation (ROSC) is to stabilize metabolic, electrolyte, and hemodynamic variables to prevent refibrillation or development of irreversible PEA or asystole (Mangla et al. 2014). In particular, measurements of blood pH, circulating concentrations of lactate, bicarbonate, sodium, potassium and glucose, and calculated base excess are crucial to evaluate the status of post-arrest patients. During and immediately after cardiac arrest, blood pH falls sharply due to elevated lactate from anaerobic glycolysis and hypercapnia due to inadequate alveolar ventilation. Hypercapnia and respiratory acidosis can be ameliorated by mechanically ventilating patients, but resolving lactate-dependent metabolic acidosis requires bicarbonate supplementation and careful monitoring of arterial blood gas chemistry (Mangla et al. 2014). In patients recovering from cardiac arrest, greater clearance of lactate at 24 h ROSC is reportedly associated with higher survival rates (Donnino et al. 2007). Post-ROSC management of electrolytes, especially potassium, is essential to prevent hyperkalemia-induced electrical disturbances that could re-fibrillate the heart (El-Sherif & Turitto 2011). Finally, unless hypoglycemia is the cause of cardiac arrest, hyperglycemia is typical in resuscitated patients and, if untreated, may contribute to long-term neurological impairment (Mullner et al. 1997; Langhelle et al. 2003; Mangla et al. 2014).

MECHANISMS OF OXIDATIVE BRAIN INJURY FOLLOWING CARDIAC ARREST

A principal cause of cardiac arrest-induced brain damage is the oxidative stress imposed on neurons by reactive oxygen (Cerchiari *et al.*, 1987; Opie, 1991; Becker, 2004; Idris *et al.*, 2005) and nitrogen (Gulyaeva *et al.*, 1996; Lipton, 1999; Love, 1999; White *et al.*, 2000; Dohi *et al.*, 2003; Keynes *et al.*, 2004; Thiyagarajan *et al.*, 2004; Zhu *et al.*, 2004) species (RONS) during

ischemia-reperfusion. RONS accumulate within the affected tissue and cause lipid peroxidation, oxidative inactivation of metabolic enzymes, and mitochondrial disfunction, culminating in cell death and neurocognitive impairment (Cerchiari *et al.* 1987; Grune *et al.*, 1997; Brown and Borutaite, 1999; White *et al.*, 2000; Dohi *et al.*, 2003; Becker, 2004; Zhu *et al.*, 2004; Idris *et al.*, 2005; Nakamichi *et al.*, 2005; Wang *et al.*, 2007). Nitric oxide (NO), generated in brain by three nitric oxide synthase (NOS) isoforms, exerts both protective and harmful effects in the ischemic and reperfused brain, which depend on the specific NOS isoform and the NO concentration (Wink *et al.*, 1993; Dalkara *et al.*, 1994; Lipton *et al.*, 1994; Gulyaeva *et al.*, 1996; Adams *et al.*, 2007). When stimulated by reactive oxygen species and pro-inflammatory cytokines, inducible NOS (iNOS) produces large amounts of NO. Ischemia-reperfusion initiates a burst of superoxide radical production; this superoxide condenses with NO to form cytotoxic peroxynitrite, initiating a cascade of nitrosative stress (Manukhina *et al.*, 2006), which modifies membrane lipids, inactivates metabolic enzymes, and initiates apoptosis (Ye *et al.*, 2007). Additionally, ROS also provoke apoptosis via the mitochondrial permeability transition pore and activation of caspases 9 and 3 (Kirkland and Franklin, 2007; Martin *et al.*, 2007; Franklin, 2011; Pan *et al.* 2012).

PROPOSED TREATMENTS FOR ISCHEMIA-REPERFUSION INJURY

Erythropoietin

Recombinant human erythropoietin (rhEPO) has been proposed as a potential treatment to combat cardiac-arrest induced ischemia-reperfusion injury. The peritubular interstitial cells of the renal cortex produce most endogenous erythropoietin in adults. The primary function of erythropoietin is hormonal regulation of red blood cell production (erythropoiesis); once released from the renal cortex, erythropoietin circulates to the bone marrow, where it serves as the

hormonal signal to drive erythropoiesis by suppressing apoptosis of proerythroid precursor cells. In addition to its principal role within bone marrow, antioxidant effects of erythropoietin have been demonstrated in many models of disease. Specifically, rhEPO has been shown to be neuroprotective in models of global cerebral ischemia, stroke, traumatic brain injury, and cerebral hemorrhage (Byts and Siren, 2009; Siren *et al.*, 2009). However, erythropoietin is a large (34 kD), polyanionic glycoprotein and, thus, does not readily cross the blood-brain barrier, meaning only about 1% of intravenously administered erythropoietin actually reaches the brain (Cerami, 2001; Spandou, 2005; McPherson and Juul, 2008; Siren *et al.*, 2009). Therefore, massive intravenous doses of rhEPO are required to attain the desired protective effects within the brain; such excessive concentrations of circulating erythropoietin raise the risk of thromboembolic complications such as stroke (Siren *et al.*, 2009; Rabie and Marti, 2008) and may stimulate overproduction of erythrocytes, thereby increasing blood viscosity.

Therapeutic hypothermia

A cerebroprotective treatment that has advanced to clinical application is therapeutic hypothermia—that is, lowering the core body temperature of cardiac arrest victims during arrest and cardiopulmonary resuscitation as a means of reducing ischemia-reperfusion induced damage to vital organs. Therapeutic hypothermia is the only intervention that has proven to be clinically effective in minimizing brain injury after cardiac arrest. By slowing cellular metabolism, hypothermia dampens production of RONS, enabling endogenous antioxidant defenses to recover during rewarming (Dohi et al. 2013). In a swine model of cardiac arrest-resuscitation, therapeutic hypothermia maintained blood pressure and cerebral oxygenation after ROSC and prevented organ damage by suppressing oxidative stress (Ostadal et al. 2013). This antioxidant

action of hypothermia during cardiac arrest is partly attributed to protection of respiratory enzymes and upregulation of an antioxidant enzyme, manganese superoxide dismutase (Gong et al. 2012).

TREATMENT WITH PYRUVATE

Pyruvate exerts powerful antioxidant effects to preserve neuronal function and integrity in the face of ischemia-reperfusion (Figure 2). Pyruvate is an endogenously produced intermediary metabolite and antioxidant (O'Donnell-Tormey *et al.*, 1987; Desagher *et al.*, 1997; Bassenge *et al.*, 2000), which has been shown to neutralize RONS in direct, non-enzymatic chemical reactions (Desagher *et al.*, 1997; Bassenge *et al.*, 2000; Mallet, 2000; Mallet and Sun, 2003; Flaherty *et al.*, 2010). This detoxification of RONS prevents lipid peroxidation, inhibition of metabolic enzymes, and the oxidative induction of iNOS (Mallet and Sun, 2003; Chen *et al.*, 2010). The latter effect of pyruvate prevents production of NO, which condenses with superoxide ions to form peroxynitrite ($ONOO^-$), a highly reactive intermediate and precursor of a host of cytotoxic derivatives (Manukhina *et al.*, 2006). Pyruvate's antioxidant actions preserve the metabolic machinery that generates ATP and the NADPH reducing equivalents needed to maintain the redox state of the principal endogenous antioxidant, glutathione (GSH; Mallet and Sun, 2003). Finally, pyruvate itself is a readily oxidized metabolic fuel capable of bolstering the free energy of ATP hydrolysis, the immediate energy source for cellular function (Mallet and Sun, 2003; Sharma *et al.*, 2007). Pyruvate is the final intermediary metabolite of glycolysis and, after being converted to acetyl-CoA, enters the TCA cycle and subsequently fuels oxidative phosphorylation in the electron transport chain to produce ATP when oxygen is readily available. In the absence of oxygen, pyruvate is reduced to lactate by lactate dehydrogenase and

subsequently is either cleared by the kidneys or utilized for gluconeogenesis in the liver, at the cost of ATP.

Of utmost importance for neuroprotection, pyruvate readily traverses the blood-brain barrier via a high-capacity, high-affinity monocarboxylate transporter (Miller and Oldendorf, 1986; Steele, 1986). It has been suggested that treatment with sodium pyruvate may impose a substantial sodium load cause osmotic water shifts resulting in cell shrinkage, and expansion of the extracellular fluid. Thus, ethyl pyruvate, an electroneutral compound containing pyruvate and ethanol moieties linked by an ester bond, was proposed as an alternate treatment to provide the beneficial antioxidant effects of pyruvate while circumventing the osmotic load imposed by the sodium. The principal shortcoming of ethyl pyruvate treatment is release of ethanol within the brain when the ethanol-pyruvate bond is cleared by endogenous esterases to liberate pyruvate (Figure 3). Moreover, ethyl pyruvate is much less soluble than pyruvate in aqueous solution, which limits the concentrations of ethyl pyruvate that can be safely delivered via the systemic circulation (Mallet et al. 2005).

SPECIFIC AIMS AND SUMMARY OF PROTOCOLS AND METHODS

The first specific aim of this project tested the hypothesis that intravenous sodium pyruvate during CPR and the first 60 min ROSC promotes recovery of cardiac electromechanical function and electrolyte homeostasis during the first 4 h ROSC. To address this aim, Yorkshire swine (30±0.7 kg; 41 males, 9 females) were subjected to 6 min pacing-induced ventricular fibrillation and 4 min closed-chest CPR, and then defibrillated by transthoracic countershocks and monitored until 4 h ROSC. Arterial blood samples were collected at predetermined times

throughout the protocol (Figure 4) for measurement of plasma electrolytes and metabolites. Sodium pyruvate or NaCl control were infused *iv* throughout CPR and the first hour ROSC. The α-adrenergic agonist phenylephrine, a systemic vasoconstrictor, was infused *iv* during ROSC as needed to stabilize the systemic arterial pressure. Pyruvate was found to sharply increase the likelihood that transthoracic countershocks delivered at 4 min CPR would convert ventricular fibrillation to a productive electrocardiographic rhythm rather than lethal PEA. Pyruvate-treated pigs required lower dosages of phenylephrine to maintain systemic arterial pressure, and exhibited enhanced glucose clearance, elevated arterial bicarbonate and increased arterial pH *vs.* NaCl-treated sham pigs. Therefore, pyruvate treatment during cardiac arrest-resuscitation hastened restitution of stabilized electrocardiographic and hemodynamic function in swine recovering from cardiac arrest.

The second specific aim tested the hypothesis that pyruvate exerts antioxidant effects on brain during cardiac arrest-resuscitation, specifically by protecting activities of the enzyme components of the glutathione antioxidant system. Using the same experimental design as aim one, pigs were recovered for 1 or 4 h, at which time biopsies of cerebellum and hippocampus were collected and snap-frozen in liquid nitrogen for biochemical analysis. Cardiac arrest sharply lowered glutathione peroxidase and glutathione reductase activities in both the hippocampus and cerebellum; Na-pyruvate preserved glutathione peroxidase activity, particularly in the cerebellum. After pyruvate infusion, the enzyme activities fell to those of the NaCl group by 4 h recovery. Unexpectedly, even in the non-arrested sham pigs, the initially robust activities of both enzymes fell to the post-arrest range between 1 and 4 h hyperoxia. Thus, cardiac arrest compromised the glutathione peroxidase-reductase antioxidant system of

hippocampus and cerebellum, and hyperoxic ventilation blunted activities of these pivotal antioxidant enzymes either with or without antecedent cardiac arrest-resuscitation. Pyruvate protection of these enzymes was not sustained after pyruvate infusion was discontinued.

CLINICAL SIGNIFICANCE

Although it has been more than 50 years since the advent of closed-chest CPR, effective treatments to protect vital organs including brain from cardiac arrest-induced ischemia-reperfusion injury are scant. While therapeutic hypothermia lowers metabolic demand and aids in preventing reperfusion injury, teams of trained professionals and sophisticated equipment are required to induce and manage targeted temperature therapy. A treatment that may be by emergency medical responders is needed to protect the brain during the critical interval when patients are being transported to the hospital. This study tested sodium pyruvate, a blood brain barrier permeable antioxidant and intermediary energy metabolite, in pigs subjected to cardiac arrest and resuscitation emulating the scenario frequently encountered in the field by emergency medical technicians. The following studies provide crucial insight into the potential protective actions of pyruvate during arrest-resuscitation that support its role as a treatment to increase the rate of survival from cardiac arrest, as well as the limitations of pyruvate treatment.

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FIGURE LEGENDS

Figure 1. *Representative electrocardiographic recordings during arrest-resuscitation protocol.* Representative electrocardiographic recordings at pre-arrest baseline, during electrically-induced ventricular fibrillation, 5 min after defibrillation, and at 1 h ROSC. During ventricular fibrillation (VF), the characteristic uncoordinated Torsades de Pointes rhythm prevents coordinated contraction of the myocardium and, thus, interrupt cardiac arrest. Defibrillation restores a productive cardiac rhythm, which stabilizes by 1 h ROSC.

Figure 2. *Cardiac arrest-initiated oxidant cascade.* Cardiac arrest-resuscitation-induced cerebral ischemia-reperfusion generates reactive oxygen species (ROS), which activates the inducible isoform of nitric oxide synthase (iNOS) to overproduce nitric oxide, which then combines with ROS to form peroxynitrite ($ONOO^-$). Peroxynitrite and ROS increase lipid peroxidation and disable metabolic enzymes, which leads to a decrease in ATP production and an increase in the ratio of glutathione to glutathione disulfide (GSH/GSSG). Pyruvate is proposed to mitigate oxidative stress by detoxifying ROS and $ONOO^-$.

Figure 3. *Generation of ethanol by hydrolysis of ethyl pyruvate.* As ethyl pyruvate is hydrolyzed within cells by esterases to release pyruvate, ethanol is produced as a byproduct.

Figure 4. *Timeline of experimental cardiac arrest-resuscitation protocol.* Swine undergo electrically induced ventricular fibrillation, 6 min untreated arrest, 4 min CPR and are recovered for 1 or 4 h ROSC. Arterial samples are collected pre-arrest, during CPR and at 5, 15, 30, 60,

120, 180, and 240 min ROSC (open triangles). Vasoconstrictor phenylephrine is infused (dashed box) during ROSC as needed to maintain systemic arterial blood pressure.

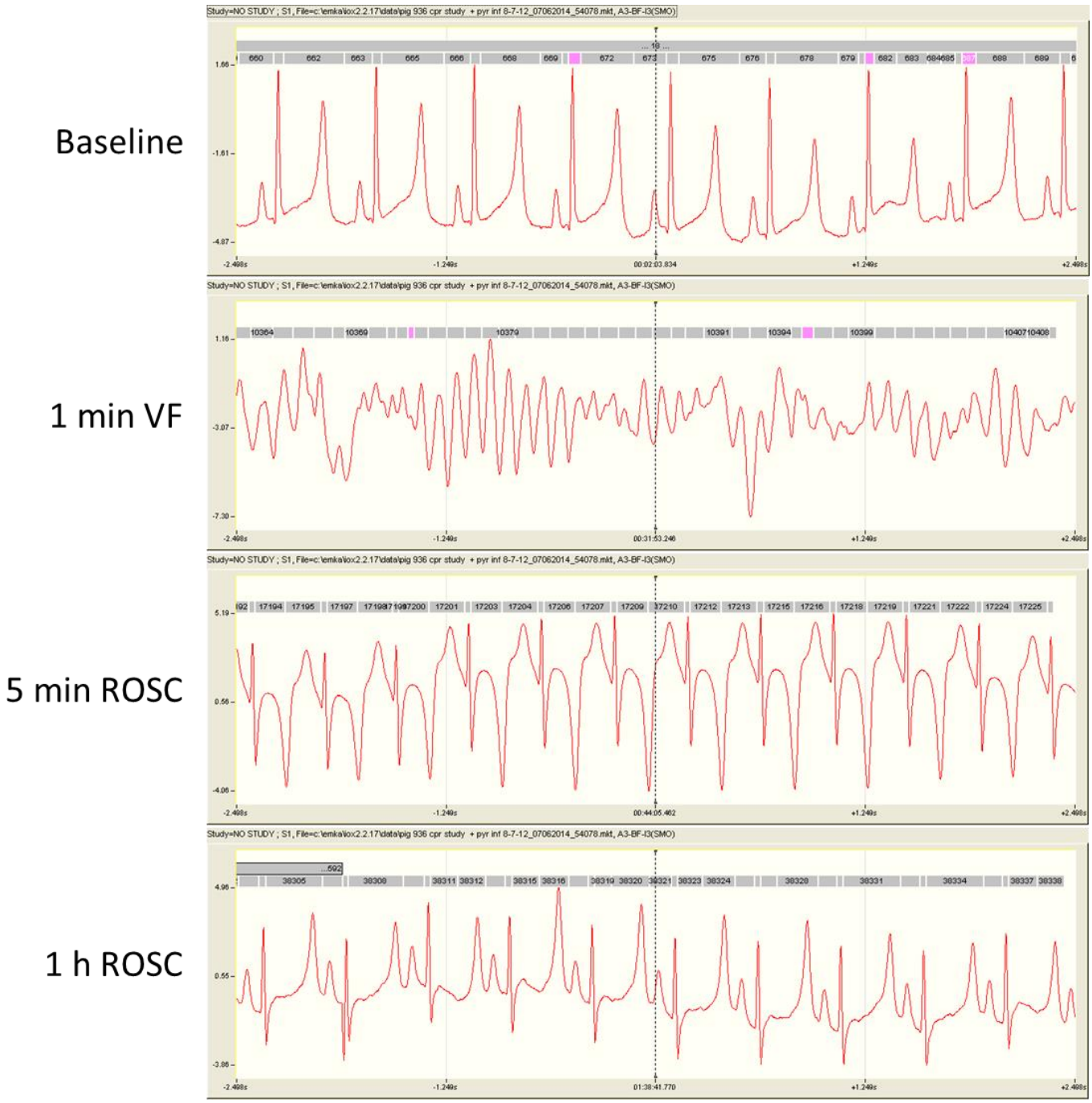

Figure 1

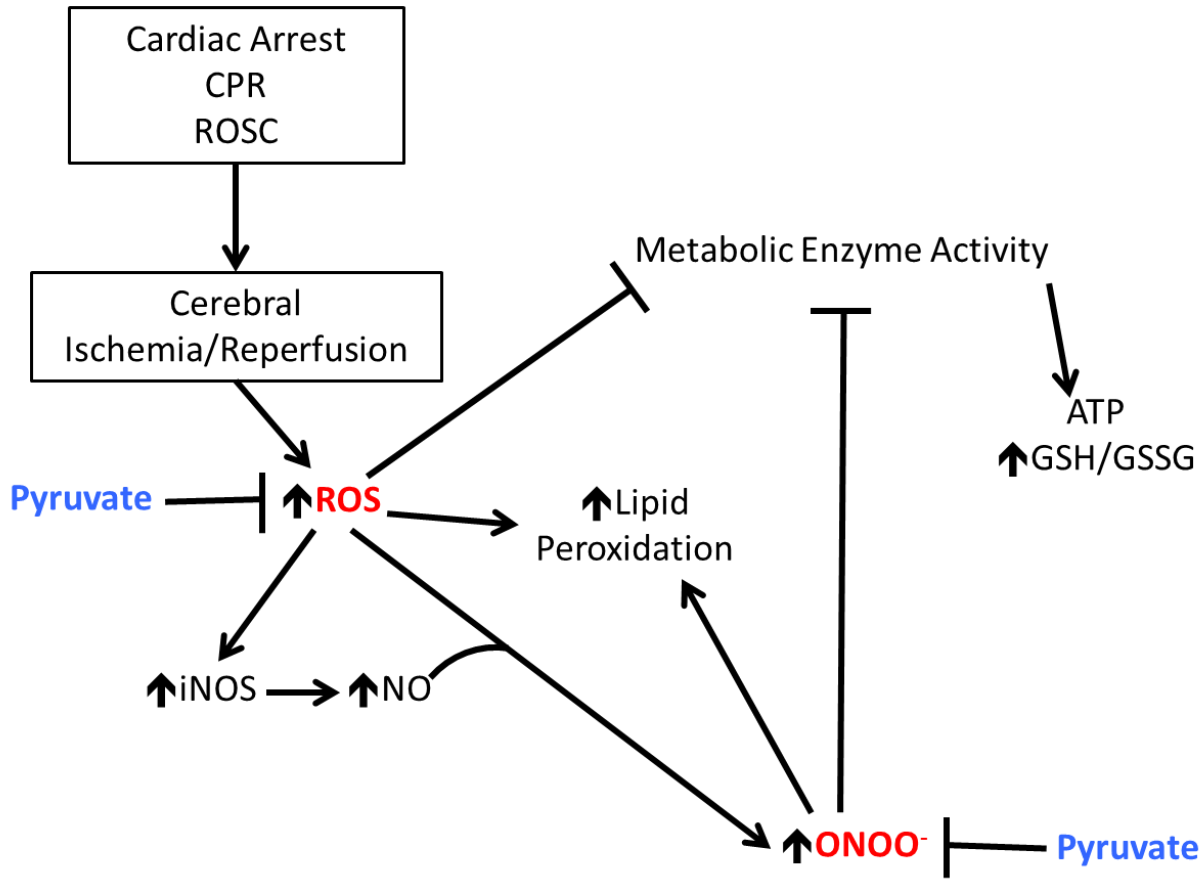
Cardiac Arrest
CPR
ROSC
Cerebral
Ischemia/Reperfusion
Metabolic Enzyme Activity
ATP
↑GSH/GSSG
Pyruvate
↑ROS
↑Lipid
Peroxidation
↑iNOS
↑NO
↑ONOO-
Pyruvate

Figure 2

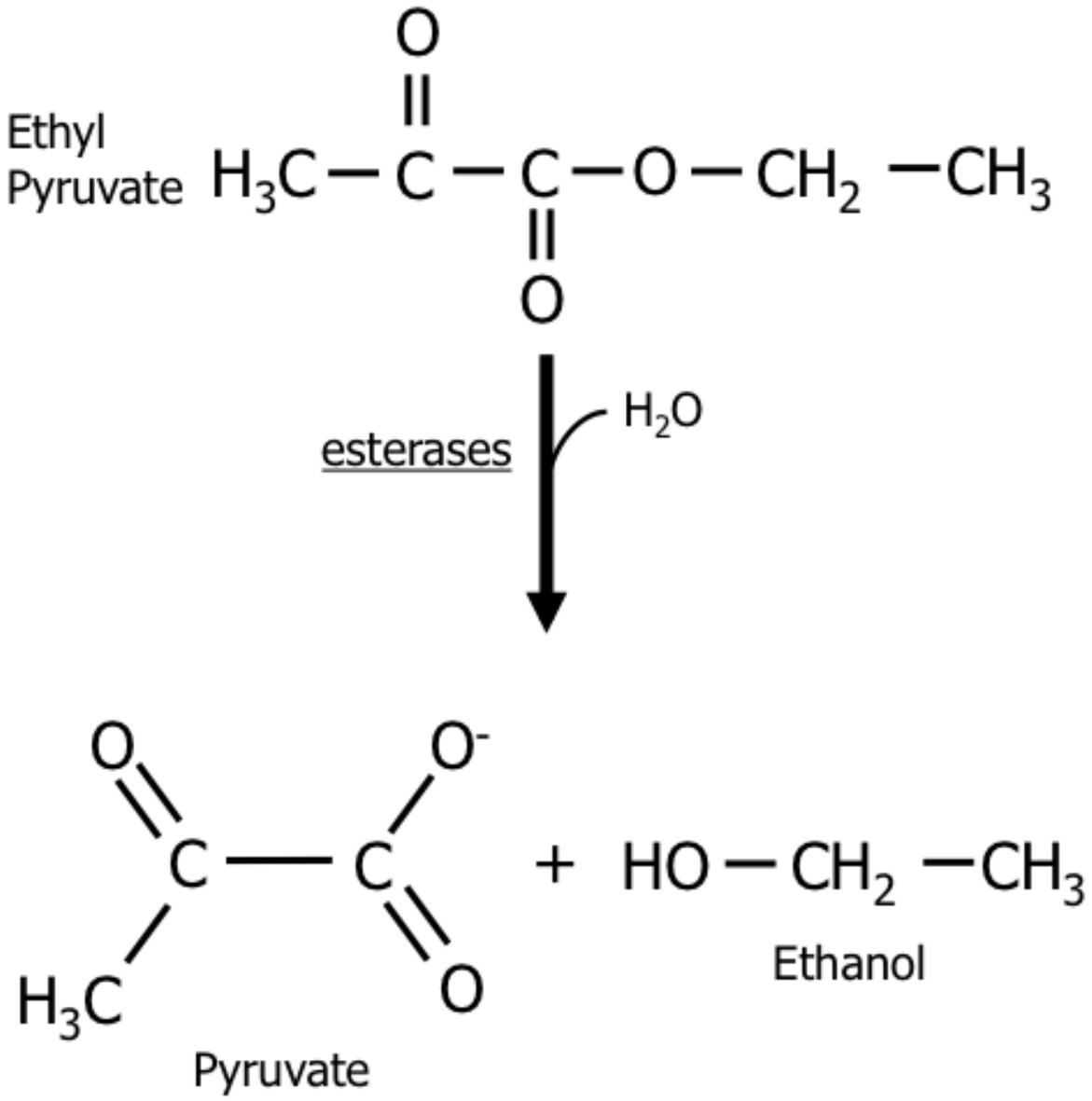

Figure 3

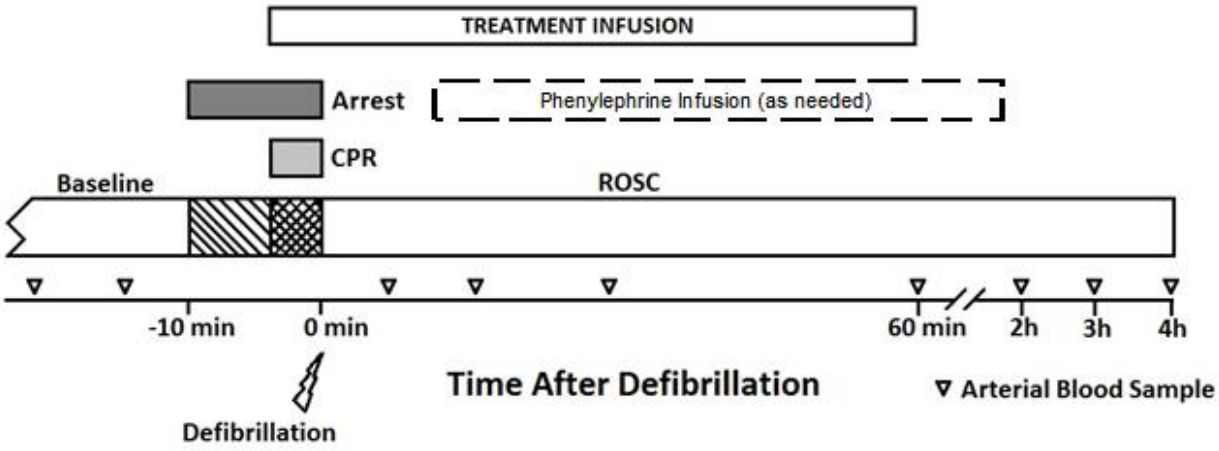
TREATMENT INFUSION
Arrest
Phenylephrine Infusion (as needed)
CPR
Baseline
ROSC
-10 min
0 min
60 min
2h
3h
4h
Defibrillation
Time After Defibrillation
Arterial Blood Sample

Figure 4

CHAPTER II

Methods

Modeling cardiac arrest and resuscitation in the domestic pig

Brandon H. Cherry, B.A.,[1,4,5] Besim Hoxha, M.D.,[6] Anh Q. Nguyen, M.S.,[1,4]

Arthur G. Williams Jr., B.S.,[1,4] Shirley R. Nelson, RLAT,[3] Egeenee Q. Daniels, D.V.M.,[3]

Albert H. Olivencia-Yurvati, D.O.,[2,4] Robert T. Mallet, Ph.D.[1,4,5]

Departments of [1]Integrative Physiology, [2]Surgery and [3]Laboratory Animal Medicine

[4]Cardiovascular Research Institute

[5]Institute of Aging and Alzheimer's Disease Research

University of North Texas Health Science Center

Fort Worth, Texas, USA

[6]Department of Emergency Medicine

Baylor College of Medicine

Houston, Texas, USA

ABSTRACT

Cardiac arrest remains a leading cause of death and permanent disability in the United States and Western Europe. Although many victims are initially resuscitated, most succumb to the extensive ischemia-reperfusion injury inflicted on the internal organs, especially the brain. Many experimental animal preparations have been developed to study the mechanisms of damage to vital internal organs following cardiac arrest and cardiopulmonary resuscitation (CPR), and to develop treatments to interrupt the lethal injury cascades. Porcine models of cardiac arrest and resuscitation offer several important advantages over other species, and outcomes in this large animal are readily translated to the clinical setting. This article summarizes porcine cardiac arrest-CPR models reported in the literature, describes clinically relevant phenomena observed during cardiac arrest and resuscitation in pigs, and presents in detail a porcine model used in the authors' laboratory and optimized to study brain injury and protection during cardiac arrest, CPR and recovery. In isoflurane-anesthetized pigs, ventricular fibrillation cardiac arrest was induced by rapid ventricular pacing and, after 6 min pre-intervention arrest and 4 min CPR, converted to sinus rhythm by direct current countershocks administered with external paddles. Intravenous injection of β-adrenergic (epinephrine) or non-adrenergic (vasopressin) vasoconstrictors increased the arterial pressures generated by precordial compressions. Systemic arterial hypotension, a consequence of post-arrest cardiac stunning, ensued after cardioversion, but could be controlled by intravenous infusion of the α-adrenergic vasoconstrictor phenylephrine. Post-arrest lactic acidosis and hyperglycemia gradually resolved over the first several hours of recovery. Over 80% of the pigs survived the cardiac arrest protocol, permitting study of the animals for several days after arrest. The domestic pig is a suitable large animal model of cardiac

arrest which is responsive to CPR, defibrillatory countershocks and medications, and yields extensive information to benefit human victims of cardiac arrest

Keywords: acidemia; cardiopulmonary resuscitation; countershocks; phenylephrine; vasopressin; ventricular fibrillation

BACKGROUND

Prior to 1960 cardiac resuscitation was administered by direct cardiac massage following thoracotomy. Based on animal experimentation a method of external cardiac massage administered by rapid, forceful compressions and passive recoil of the sternum was developed by Kouwenhoven *et al.* (Kouwenhoven et al. 1957). Although over fifty years have passed since the inception of closed chest cardiac massage, and despite many refinements of this approach in the intervening decades, cardiac arrest remains a leading cause of death and persistent disability in the United States and Western Europe. All too often, victims who are initially resuscitated later succumb to extensive ischemia-reperfusion injury to their vital organs, especially the brain (Dezfulian et al. 2009; Young 2009; Heron 2012; Nolan et al. 2012). Further, many of the 10% of cardiac arrest patients who survive to hospital discharge experience persistent neurocognitive impairment which profoundly impacts their quality of life (Adrie et al. 2004; Young 2009).

Although public health data and anecdotal evidence inform the refinement of resuscitation protocols (Stub et al. 2011), knowledge of the complex mechanisms of internal organ damage,

essential to foster development of effective pharmacological interventions, is incomplete. In the brain, ATP depletion, intracellular Ca^{2+} overload, excessive formation of reactive oxygen and nitrogen derivatives, inflammation and glutamate-induced excitotoxicity conspire to kill neurons and other cells and disrupt the blood-brain barrier (BBB). Currently there are no clinically effective pharmacological treatments to protect the brain during cardiac arrest and cardiopulmonary resuscitation (CPR; Dezfulian et al. 2009). Reliable preclinical models of cardiac arrest and resuscitation are essential to decipher the injury mechanisms and develop treatments to increase survival and improve quality of life after cardiac arrest.

Ischemia-reperfusion damage in the central nervous system is the result of a multifaceted injury cascade. The structural complexity of the brain, which consists of integrated networks of different cell types including neurons, astrocytes, oligodendrocytes, microglia and vascular endothelium, presents fundamental challenges to developing neuroprotective treatments. The brain contains many functional regions of differing susceptibilities to ischemia-reperfusion injury. In order to exert protective actions within the brain parenchyma, pharmacotherapeutic agents must first traverse the BBB, an important permeability barrier to all but small, non-polar compounds. Thus, potential treatments must be able to penetrate the BBB readily and act on multiple injury mechanisms, without producing untoward side effects. Sophisticated animal models are essential to model the composite structure and integrated function of the central nervous system and to evaluate the benefits and potential side effects of prospective treatments for ischemia and other brain disorders. This article describes several porcine models of cardiac arrest and resuscitation, including one utilized in the authors' laboratory.

Modeling cardiac arrest and CPR in the pig

Extensive research has established the domestic pig as an excellent animal model to study the impact of cardiac arrest, resuscitation, and therapeutic interventions on the brain and other internal organs. Several attributes make the domestic pig an ideal animal model for cardiac arrest research (Xanthos et al. 2007; Walters et al. 2011): 1) a large mammal, the pig accommodates extensive instrumentation for blood sampling, monitoring of intravascular and intracardiac pressures, electrocardiography and intravenous administration of medications and experimental treatments; 2) pigs tolerate invasive surgical procedures and rapidly regain consciousness post-anesthesia; 3) resting heart rate, blood pressure, and blood chemistries of pigs and humans are very similar (Breecher & Dworken 1986; AALAS 2009; Merck & Co. 2010); 4) pigs have sufficient blood volume to permit collection of multiple arterial and venous samples for analyses of blood gases and serum chemistry; 5) neurological examinations have been developed to evaluate neurobehavioral function in pigs (Bircher & Safar 1985; Benson et al. 2005); 6) the pig's large chest accommodates forceful precordial chest compressions and application of transthoracic defibrillatory countershocks of electrical energies similar to those used clinically; and 7) pigs have the largest brains among the commonly studied laboratory animals, which provides ample brain tissue for extensive biochemical and histological analyses of specific brain subregions. Porcine models are especially well-suited to study cardiac arrest and CPR, because they are easily tailored to address the aims of each study; specific features include anesthesia regimen, duration of pre-intervention cardiac arrest, method of delivery and duration of CPR, whether or not the pigs are ventilated during CPR, fraction of inspired O_2 (FIO_2) during recovery and the sequence and intensity of transthoracic countershocks (Table1).

Factors to consider when studying cardiac arrest and resuscitation in pigs

The pathophysiological complexities of sudden cardiac death and cardiopulmonary resuscitation challenge the development of animal models that accurately replicate the clinical situation. The primary factors in developing suitable animal models are the study end points and objectives. If the study requires a high survival rate, the period of pre-intervention cardiac arrest may be limited, and aggressive interventions may be implemented during resuscitation and post-arrest recovery to assure survival. The myriad variables in model design and experimental protocol, which mirror the complexity of cardiac arrest and its treatment, complicate the direct comparison of results obtained in different models.

The anesthetic regimen is an important consideration. Invasive surgical procedures will require the induction and maintenance of a surgical anesthetic plane. Anesthetics are injected or, in the case of volatile anesthetics, inhaled. The temporary or persistent effects of the anesthetic on study endpoints, *e.g*. cardiac function, cell death, inflammation or neurobehavioral recovery must be taken into account. For example, the cardiodepressant effects of some volatile anesthetics, *e.g*. halothane and isoflurane may produce hypotension (Newman et al. 1986; Lessard & Trepanier 1991; Matta et al. 1995; Hoffman et al. 2001), yet these anesthetics also exert cardioprotection (Kato & Foex 2002; Landoni et al. 2008); thus, the dosages of these anesthetics must be monitored carefully throughout the experiment.

There are different methods of inducing cardiac arrest. A prevailing method employed by our laboratory is rapid ventricular pacing, *e.g.* with a train of impulses delivered to the right ventricular myocardium via an intravascular pacing wire. Alternatively, high dosages of certain chemicals, *e.g*. bupivacaine (Mayr et al. 2004), may be injected into the right atrium to arrest the heart. The duration of pre-intervention arrest is an important factor, because as this interval is prolonged, cardioversion, survival and good neurological recovery become progressively less achievable.

It is of paramount importance to carefully construct an appropriate CPR protocol specific to the study end points. The frequency and depth of the compressions can profoundly influence outcome (Abella 2005; Idris et al. 2012; Wallace et al. 2013). In some studies, CPR is administered by a pneumatic, piston-driven device (*e.g*. Thumper®), which can be adjusted to deliver forceful compressions at a predetermined frequency and provide consistency of CPR administered across experiments. Alternatively, precordial compressions can be administered manually, modeling the CPR given by a bystander witnessing an out-of-hospital cardiac arrest. Another important consideration is whether or not the animal will be ventilated during CPR and, if so, at what compression:ventilation ratio and FIO_2. In accordance with current recommendations for bystander CPR (Rea et al. 2010), our laboratory suspends mechanical ventilation for the duration of the cardiac arrest and CPR. Finally, a systemic vasoconstrictor may be administered to increase the arterial pressures produced by the compressions. The most widely used vasoconstrictors include epinephrine, a physiological adrenergic agonist, and vasopressin, a non-adrenergic vasoconstrictor which may afford greater survival to hospital discharge than epinephrine, especially in patients with asystole (Wenzel et al. 2004). With

multiple options for each component, it is critical that CPR protocols be carefully considered and structured to provide the best design for each individual study.

Additional factors include the duration of CPR administration before attempting defibrillation, and the sequence of defibrillatory countershocks, *i.e.* whether the shocks will be administered singly, or in a sequence of multiple (often three) countershocks, before checking for cardioversion. The electrical energies of the countershocks must be considered, including the energy of the initial countershock, and, if the initial shock fails to achieve cardioversion, whether or at what progression the intensity will be increased for subsequent countershocks. It must be determined if and for how long CPR will be administered after unsuccessful cardioversion attempts. The criteria for abandoning futile resuscitation efforts must be defined. The cardiocirculatory values that constitute recovery of spontaneous circulation (ROSC), including the presence of an organized electrical rhythm and maintenance of arterial blood pressure above a predetermined target value for a minimum duration (Table 1) must be specified. Core body temperature during cardiac arrest and CPR has a marked effect on mortality and neurological injury; indeed, moderate hypothermia is the only currently approved intervention consistently shown to produce significant clinical benefit (Dumas et al. 2012; Horburger et al. 2012; Wang et al. 2013). Pigs do not thermoregulate effectively while under anesthesia, so typically the animal must be maintained on a heating pad during the cardiac arrest-resuscitation protocol to avoid the impact of hypothermia on study endpoints.

As the period of ROSC progresses, interventions may be necessary to maintain adequate arterial pressure. Intravenous saline solutions may be infused to expand extracellular fluid volume. Vasopressor agents, *e.g*. phenylephrine, may be administered, but it should be recognized that vasopressors may lose their efficacy over time due to desensitization of their membrane receptors (Chalothorn et al. 2002) and, thus, may not be suitable for long-term maintenance of arterial pressure. It may be necessary to adjust tidal volume and frequency of ventilations or administer bicarbonate to compensate for post-arrest acidemia and/or hypercapnia.

The oxygen concentration of medical gases used during resuscitation is an important consideration when designing a model of cardiac arrest-resuscitation. For decades, it has been recommended that patients be ventilated with 100% oxygen during resuscitation to increase oxygen delivery to ischemic tissues (Smith et al. 1968; O'Driscoll et al. 2008). Recently, however, hyperoxic ventilation during resuscitation has been shown to generate RONS and exacerbates oxidative ischemia-reperfusion injury (Richards et al. 2006, 2007; Pilcher et al. 2012). Oxygen toxicity has been studied for years in a perioperative setting, but it was only recently that there has been sufficient clinical evidence for the European Resuscitation Council to recommend that patients should not be ventilated with 100% oxygen after cardiac arrest, but rather with room air supplemented with only enough O_2 to maintain an oxyhemoglobin saturation (spO_2) of 94-98% in an effort to minimize oxidative damage (Balan et al. 2006; Deakin et al. 2010). Thus, when designing a cardiac arrest model, the oxygen concentration used during resuscitation may be adjusted depending on whether the study aims to mimic the current strategy of ventilation with 100% oxygen, or newly recommended strategies such as titration of oxygen administration to maintain a spO_2 of 94-98%.

CARDIAC ARREST MODEL IMPLEMENTED IN THE AUTHORS' LABORATORY

This section describes a porcine model of cardiac arrest, CPR, defibrillation and recovery designed and implemented by the authors to test intravenous interventions to effect cardio- and cerebroprotection following ROSC. Monitored variables and endpoints include systemic arterial pressure, heart rate, electrocardiogram, arterial serum chemistries (pH, PO_2, PCO_2, and concentrations of bicarbonate, electrolytes, lactate and glucose), circulating pro- and anti-inflammatory cytokines and leukocytes, neurobehavioral function, and survival. Biopsies of internal organs including brain (cerebral cortex, cerebellum, hippocampus), heart (left and right ventricular myocardium), kidneys (renal cortex and medulla), lungs and liver are taken for histological examination of tissue structure and inflammation and for measurements of energy metabolites (ATP, ADP, phosphocreatine, creatine, inorganic phosphate), metabolic intermediates, glutathione and glutathione disulfide, and contents and activities of metabolic and antioxidant enzymes, nitric oxide synthase, matrix metalloproteinases and myeloperoxidase. The pig was chosen for these studies because its large size accommodates the extensive instrumentation, multiple blood samples and tissue biopsies necessary to obtain these measurements without having to pool samples from multiple animals. Over 80% of the pigs subjected to the cardiac arrest-resuscitation protocol described below recovered spontaneous circulation and survived to the completion of the experiment.

All surgical and experimental procedures in pigs were conducted in accordance with the *Guide to the Care and Use of Laboratory Animals* (2011) and were approved by the Institutional Animal

Care and Use Committee of the University of North Texas Health Science Center. Before the experiment, domestic Yorkshire swine (25-35 kg, both genders) were housed for 7 d for habituation to the vivarium and project personnel. The pigs consumed a chow diet and water *ad libitum*, and were fasted overnight before the CPR experiment.

Anesthesia and surgical instrumentation

Pigs were premedicated with a telazol/xylazine cocktail (5 mg/kg *im*). After sedation and intubation, a surgical plane of anesthesia was maintained by mechanical ventilation at 15 mL/kg body mass for 12-14 cycles/min with 1-3% isoflurane in 100% O_2. To minimize isoflurane's cardiodepressant actions (Newman et al. 1986; Lessard & Trepanier 1991; Matta et al. 1995; Hoffman et al. 2001) its administration was held to the minimum dosage necessary to maintain a surgical anesthetic plane; in most experiments, this isoflurane dosage was 1-3%. The anesthetic plane was evaluated by monitoring physiological signs. Anesthesia was increased upon detection of jaw tension, limb withdrawal in response to pinching the soft tissue between the hooves, wink reflex in response to delicate contact of the ocular canthus, spontaneous limb movements, and/or treatment-independent increases in heart rate and systemic arterial pressure.

Epidermal electrode patches were placed on the limbs for standard limb lead II electrocardiography. The pigs were placed in a recumbent position on a heated pad and the body temperature monitored via a rectal thermal probe. The left femoral artery and vein were exposed by inguinal incision and blunt dissection. A 7 Fr (2.3 mm diameter) polyurethane catheter was inserted into a branch of the femoral artery and advanced into the abdominal aorta to monitor

arterial blood pressure and to sample arterial blood. Another 7 Fr polyurethane catheter was inserted into a branch of the femoral vein for infusing medications and resuscitative fluids. The right external jugular vein was accessed via a 3 cm cervical incision, and a catheter was inserted and advanced into the right ventricle to allow for advancement of a pacing wire until the tip contacted the endocardium. In 1 and 4 h acute recovery studies, heparin (500 U/kg) was injected into the femoral vein after completion of all surgical preparation; supplemental doses (150 U/kg) were given 2 and 4 h later to maintain anticoagulation.

Cardiac arrest-resuscitation protocol

Figure 1 summarizes the sequence of procedures during a typical cardiac arrest and CPR protocol. To arrest the heart, a 0.5-1s burst of 60 Hz electrical impulses was transmitted to the right ventricle via the pacing wire, causing ventricular fibrillation. Electrical induction of fibrillation was chosen over pharmacological induction because the former better emulates the sudden onset of ventricular fibrillation in human cardiac arrest victims and avoids potential systemic drug effects. Once ventricular fibrillation was confirmed by the characteristic disorganized electrocardiographic activity, monophasic decline in aortic pressure and the absence of an arterial pulse, mechanical ventilation was suspended for the duration of cardiac arrest and CPR. Bicarbonate (0.3-0.4 mEq/kg) was administered *iv* one minute before starting CPR to partially offset the acidemia that developed during cardiac arrest and resuscitation.

After 6 min of cardiac arrest without intervention, closed-chest CPR was applied by manual mid-sternal chest compressions, at a force sufficient to compress the chest by one-fourth of its antero-

posterior diameter, followed by release and recoil, at a rate of 100 cycles/min, in accordance with current American Heart Association guidelines (Meany et al. 2013). The person administering the compressions was blinded to the treatment, arterial pressure, end-tidal PCO_2 and blood gas chemistry. At 7 min arrest (*i.e.*, 1 min CPR) 10 U of vasopressin was injected into the right jugular vein and flushed with normal saline to increase the efficacy of the chest compressions. In our experimental design, mechanical ventilation remained suspended throughout CPR, in accordance with extensive evidence that assisted ventilation during CPR following witnessed cardiac arrest offers little or no neurological or survival benefit (Bohm et al. 2007; Ewy et al. 2007; Iwami et al. 2007; Berger 2008; Nagao 2009).

Intravenous resuscitative solutions of the highest commercially available purity were prepared within 30 min of use. The solid chemicals were dissolved in double-distilled H_2O, sterilized by passage through 0.22 µm membranes (Millipore Stericup® vacuum driven filtration system) and stored on ice in sterile containers prior to infusion. All resuscitative solutions were prepared in a separate room by an investigator not directly participating in the experiment to ensure that personnel conducting the experiments were blinded to the specific treatment. In this study, infusions were initiated at the onset of CPR and maintained until 60 min ROSC (Figure 1).

After 4 min CPR, transthoracic direct current countershocks were administered with external paddles (LifePack 12; Physio-Control) to restore cardiac rhythm. If necessary, up to three 6-7 J/kg countershocks were applied, followed by up to three 8-12 J/kg countershocks, at 30 s intervals with CPR administered between countershocks, until spontaneous cardiac

electromechanical rhythm was restored. Defibrillation attempts were abandoned if cardioversion wasn't achieved within 6 countershocks.

In our model, mechanical ventilation with 100% oxygen was resumed immediately after spontaneous cardiac rhythm was confirmed, at an initial rate of 20-24 cycles/min. Next, lidocaine (20 mg in 1 mL normal saline) was administered via jugular catheter as a prophylactic measure to minimize recurrence of ventricular fibrillation or other arrhythmia such as ventricular tachycardia and to assist in recovery of a normal QRS waveform complex. If a normal QRS complex was not achieved and/or ventricular tachycardia was observed after 30 seconds of spontaneous electrical rhythm, a second 20 mg dose of lidocaine was administered. Mean aortic pressure ≥ 60 mm Hg and a sustained spontaneous cardiac electrical rhythm were taken to indicate ROSC. At 10 min ROSC, the laboratory lights were dimmed and the pupillary reflex tested with a small flashlight. An absent pupillary reflex is a sign of severe brain damage, and none of the 5 pigs with fixed, dilated pupils at 10 min ROSC survived to 4 h ROSC. Ventilatory frequency was lowered in stages to 12-14 cycles/min as arterial PCO_2 and pH recovered.

Post-resuscitation management

After defibrillation and achievement of ROSC, a surgical plane of anesthesia was maintained for 1 or 4 h ROSC while aortic pressure and electrocardiogram were monitored. Blood samples (5 ml) were withdrawn from the left femoral artery into sterile syringes 15 and 5 min before arrest, at 3 min CPR, and at 5, 15, 30, 60, 120, 180 and 240 min ROSC (Figure 1).

By 15-30 min ROSC mean aortic pressure may fall below 60 mm Hg, potentially compromising perfusion of the vital organs. To prevent such systemic hypotension, the α-adrenergic agonist phenylephrine, a potent vasoconstrictor, was infused into the left femoral vein at a rate sufficient to maintain aortic pressure at 65-75 mm Hg. Phenylephrine was dissolved in 500 ml sterile 0.9% NaCl to a concentration of 50 mg/l, and the phenylephrine inflow rate was adjusted in order to achieve stable aortic pressures within the desired range. The phenylephrine infusion was tapered and ultimately discontinued as cardiac function recovered.

Termination of experimental protocol

Pigs were sacrificed at 1 h or 4 h recovery and tissues of internal organs were obtained for histological and biochemical analyses. A surgical plane of anesthesia was induced with telazol/xylazine (5 mg/kg, *im*) and maintained by ventilation with 1-3% isoflurane. Next, the heart was exposed by left lateral thoracotomy through the fourth intercostal space and pericardiotomy, the cranium exposed by incising and reflecting the scalp, and a craniotomy performed to access the brain. The pig was then humanely sacrificed by arresting the heart with electrical current applied to the left ventricular epicardium with a pacing wire. This method of euthanasia is concordant with the recommendations specified in the *American Veterinary Medical Association Guidelines for the Euthanasia of Animals* (2013). The brain was quickly removed and sectioned to obtain samples of cerebral cortex, cerebellum and hippocampus for analysis of tissue injury, inflammation, apoptosis and cytoprotection. Transmural biopsies of left and right ventricular myocardium were taken *in situ* for similar analyses. Two samples were collected from each lung, the liver and a kidney; one sample was fixed and processed for

histological analysis as described below, and the other was snap-frozen in liquid N_2 for analyses of messenger RNA, protein contents and enzyme activities.

Statistics. Mean values ± SEM are reported. Blood pressure, heart rate and serum values in the CPR vs. sham experiments were compared by two factor (treatment, time) ANOVA with repeated measures on time. p values < 0.05 were considered to indicate statistically significant treatment effects.

RESULTS AND DISCUSSION

Selection of vasopressor to enhance CPR

To improve the efficacy of CPR, vasoconstrictors are administered via the jugular vein catheter to increase arterial pressures generated by chest compressions, thereby increasing perfusion of brain and myocardium at the expense of peripheral organs and tissues. Although epinephrine has been used in this manner for decades, its potentially detrimental effects, including increased physiological shunt compromising pulmonary gas exchange (Tang et al. 1991; Thrush et al. 1997), intensified myocardial ATP consumption and oxygen demand (Ditchey & Lidenfeld 1988), and the resultant post-resuscitation myocardial dysfunction (Tang et al. 1995) and ventricular arrhythmias (Niemann et al. 1986; Tovar & Jones 1997) have raised concerns regarding its use for CPR. Preclinical and clinical evidence has shown the non-adrenergic vasoconstrictor vasopressin to be at least as effective as epinephrine at augmenting arterial pressure during precordial compressions, but without epinephrine's untoward effects. In a porcine cardiac arrest model, vasopressin *vs*. epinephrine produced greater myocardial and brain

blood flows and mean arterial pressures during CPR while decreasing perfusion of muscle, fat and small intestine (Lidner et al. 1993). Thus, vasopressin was associated with higher incidence of conversion to productive sinus rhythm (Wenzel et al. 1999), increased post-arrest cardiac function and decreased morbidity and mortality *vs*. epinephrine. We found that vasopressin injected at 60 s CPR improved markedly the quality of CPR, increasing the mean arterial pressures from 25-30 to 50-60 mm Hg within 3 min (Figure 2A). Although epinephrine produced a more abrupt increase in arterial pressure following its injection, within 2 min vasopressin increased mean arterial pressure to a similar extent (Figure 2A), without the ensuing tachycardia observed during the initial 10 min of ROSC after epinephrine-enhanced CPR (Figure 2B).

Number and intensity of defibrillatory countershocks

Sinus rhythm was achieved by a single 200 J countershock in 15 of the 27 pigs, and a second 200 J countershock restored sinus rhythm in another 7 pigs (Figure 3). Five pigs required 3 or more countershocks to achieve cardioversion. The cumulative electrical energy of the countershocks delivered to the pigs was 12.6 ± 1.7 J/kg.

In our experience, the initial CPR regimen, as well as the 20-25 s of chest compressions following unsuccessful countershocks, are essential to achieve ROSC. By affording modest delivery of O_2 and metabolic fuels to the myocardium, these chest compressions may support enough myocardial ATP production to sustain ion transport and repolarize cardiomyocytes, enabling countershocks to restore spontaneous electrical rhythm. Indeed, in a canine cardiac

arrest model, effective CPR afforded partial recovery of myocardial Gibbs free energy of ATP hydrolysis (Sharma et al. 2005), the immediate energy source for cardiac electromechanical activity. A similar protocol of single shocks with intervening chest compressions increased post-arrest survival *vs*. a conventional 3-shock protocol in a porcine model of ventricular fibrillation cardiac arrest (Tang et al. 2008).

Occasionally, pulseless electrical activity (PEA) ensued after countershocks, characterized by spontaneous electrical rhythm but without productive cardiac mechanical activity. In our experience PEA is an ominous finding; typically, even heroic efforts fail to convert PEA to a productive sinus rhythm. None of the 4 pigs developing PEA during resuscitative efforts survived for 4 h ROSC. This situation replicates the clinical setting of out-of-hospital cardiac arrest, where a much lower rate of survival to hospital discharge is achieved in cardiac arrest victims in which PEA is the initial rhythm *vs*. patients with an initial electrocardiographic substrate of ventricular fibrillation (Eisenberg & Mengart 2001).

Phenylephrine infusion to stabilize aortic pressure

Aortic pressures declined after the first 5 min ROSC (Figure 4) as the inotropic actions of epinephrine or vasopressin subsided, in accordance with the reversible myocardial contractile impairment (cardiac stunning) that often follows initial resuscitation from cardiac arrest (Gazmuri et al. 1996; Young 2009). In 17 of the 27 pigs in which ROSC was achieved, mean aortic pressure fell below 60 mm Hg within 10-20 min of cardioversion, prompting intravenous infusion of the α-adrenergic vasoconstrictor phenylephrine to restore and maintain aortic

pressure at 65-75 mm Hg. Phenylephrine proved highly effective; indeed, diluting the vasoconstrictor in 0.9% NaCl to a concentration of 5 mg/l afforded control of the infusion such that aortic pressure could be kept within the desired range. The cumulative phenylephrine dosage of 0.1-0.5 mg/kg was infused over a 30-60 min interval, and was gradually tapered and discontinued as myocardial function recovered from cardiac arrest.

Serum metabolites and acid base values

Arterial lactate concentration (Figure 5A) increased during CPR and the first 15 min ROSC, then subsided as ROSC progressed, but remained elevated *vs.* baseline and contemporaneous sham values for at least 4 h ROSC. Arterial bicarbonate concentration fell sharply during CPR and the first 15 min ROSC, and then gradually recovered to its pre-arrest baseline by 2-4 h ROSC (Figure 5B), although arterial pH recovered only partially (Figure 5C). Arterial glucose concentration tripled immediately after cardiac arrest, and then gradually fell over 4 h ROSC (Figure 5D). This post-arrest hyperglycemia, a result of glycogen mobilization by circulating catecholamines, replicates post-arrest hyperglycemia in human victims (Mullner et al. 1997; Beiser et al. 2009). Moreover, hyperglycemia in resuscitated patients, if untreated, may contribute to long-term neurological impairment (Langhelle et al. 2003; Mangla et al. 2014).

Malignant hyperthermia

A small minority of pigs harbor a genetic lesion in the skeletal muscle sarcoplasmic reticular Ca^{2+} release channels (Fujii et al. 1991; Mickelson & Louis 1996) that predisposes them to

develop malignant hyperthermia (*aka* porcine stress syndrome) when treated with volatile anesthetics (Liou et al. 2000). Malignant hypothermia has no overt clinical symptoms detectable by routine pre-experimental screening. As post-arrest survival and neurobehavioral recovery are negatively correlated with body temperature, an episode of malignant hyperthermia, during which core body temperature may rise above 42˚C, can have disastrous consequences, including systemic hypotension, acidemia, hypercapnia and hyperkalemia that are refractory to conventional interventions. Indeed, neither of the two pigs that developed acute malignant hyperthermia in our study survived to 4 h ROSC, despite aggressive measures including intravenous infusion of ice-cold saline and the K^+ chelator calcium gluconate.

Limitations

An important limitation of this and many other porcine cardiac arrest models is that juvenile, disease-free pigs are generally used. In clinical settings, patients who experience cardiac arrest typically are elderly and suffer from chronic disorders such as hypertension, atherosclerosis, congestive heart failure, diabetes, emphysema or end-stage renal disease. Unlike most porcine preparations, human victims of out-of-hospital cardiac arrest are not anesthetized when they are stricken.

CONCLUSIONS

Over the last few decades the collective efforts of many investigators have fostered the development of sophisticated porcine models of cardiac arrest, CPR and recovery of spontaneous circulation. The domestic pig provides an excellent large animal model of the human cardiovascular system and yields ample tissue to permit extensive analyses of mechanisms of

injury and cytoprotection in the internal organs, such that each experiment generates a wealth of information. Although there is much to consider when constructing an experimental design, the swine model of cardiac arrest-resuscitation is easily tailored to accommodate the desired study end points. For example, by selecting appropriate vasoconstrictors to augment arterial pressures either during cardiac arrest or post-ROSC recovery and by performing a craniotomy before euthanasia, we have refined our porcine model the impact of cardiac arrest-resuscitation on heart and brain as well as to study both cardiovascular and cerebral recovery from cardiac arrest. Thus, the swine model provides unparalleled translational value among current mammalian models of cardiac arrest and CPR, permitting an integrative approach to bridge the gap from bench to bedside.

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FIGURE LEGENDS

Figure 1. *Cardiac arrest/CPR protocol.* The sequence of events in an experiment encompassing cardiac arrest, closed chest CPR, countershock-induced defibrillation and recovery of spontaneous circulation (ROSC) is shown.

Figure 2. *Hemodynamic effects of vasopressor support with epinephrine* vs. *vasopressin during CPR and ROSC.* A: Mean aortic pressure during CPR with either epinephrine (Epi; closed circles) or vasopressin (VP; open circles). The broken vertical line denotes the time of vasopressor injection. B: Heart rate during the first 10 min ROSC after treatment with epinephrine (EPI; solid line) or vasopressin (VP; dashed line). Dotted line: non-arrested sham experiments. *$p < 0.05$ EPI *vs*. VP value at the same time.

Figure 3. *Number of direct current countershocks required to achieve sustained sinus rhythm.* Countershocks were given at 30 s intervals until ROSC was achieved. The first 3 countershocks were each 200 J, and countershocks 4-6 were 300 J. Closed chest compressions were given for 20-25 s between countershocks.

Figure 4. *Mean aortic pressure before cardiac arrest and over 4 h recovery.* Mean values ± SEM from 6 cardiac arrest (closed symbols, dotted line) and 6 non-arrested sham (open symbols, solid line) experiments. *$p < 0.05$ *vs*. sham value at the same time.

Figure 5. *Systemic arterial serum chemistries before and after cardiac arrest and CPR.* A: lactate concentration; B: bicarbonate concentration; C: pH; D: glucose concentration. Mean values ± SEM from 4 cardiac arrest (closed symbols, dotted line) and 4 non-arrested sham (open symbols, solid line) experiments. *$p < 0.05$ *vs*. sham value at the same time.

Table 1. *Details of representative cardiac arrest protocols in pigs.*

Reference	**Lindner *et al.* 1993** [45]	**Idris *et al.* 1994** [57]	**Voelckel *et al.* 1999** [58]	**Mayr *et al.* 2004** [21]	**Tang *et al.* 2006** [48]	**Ewy *et al.* 2007** [35]	**Current Protocol**
Pre-anesthetic, induction anesthetic	Metomidate: 10 mg/kg *im*	Ketamine: 20 mg/kg *im* Thiamylal: 6 mg/kg *iv*	Ketamine: 20 mg/kg *im* Propofol: 6-8 mg/kg *im*	Ketamine: 20 mg/kg *im* Propofol: 1-2 mg/kg *iv* Piritramide: 30 mg *iv*	Ketamine: 20 mg/kg *im* Pentobarbital: 30 mg/kg *iv*	5% Isoflurane *via* nose cone	Telazol/xylazine: 5 mg/kg *im*
Maintenance anesthesia	Metomidate: 0.5 $mg \cdot kg^{-1} \cdot h^{-1}$	Thiamylal: 8-16 $mg \cdot kg^{-1} \cdot h^{-1}$	Propofol: 6-8 mg/kg + Piritramide: 0.3 mg/kg	Isoflurane (1-2%) in 65% nitrous oxide	Pentobarbital: 8 $mg \cdot kg^{-1} \cdot h^{-1}$	Isoflurane (1.5-3%) in 100% oxygen	Isoflurane (1-3%) in 100% oxygen
Method of arrest	Electrical: 50 Hz, 140 mA	Electrical: 5-10 V	Electrical: 50 Hz, 60 V	Pharmacological: 5 mg/kg bupivacaine	Electrical: 1-2 mA	Electrical: right ventricular pacing	Electrical: 60 Hz, 40-60 V
Duration of pre-CPR arrest	12 min	16 min	12 min	6 min	7 min	3, 4, 5 or 6 min	6 min
Method of CPR	Open-chest: 90/min	Closed-chest: 100/min mechanical	Closed-chest: 80/min manual	Closed-chest: 100/min manual	Closed-chest: 100/min mechanical	Closed-chest: 100/min manual	Closed-chest: 100/min manual
Duration of CPR	3 min	10 min	3 min	2 min	1 min (if required)	9, 8, 7 or 6 min (total arrest 12 min)	4 min
Ventilation during CPR?	None	None or F_{IO2} = 0.85	None	F_{IO2} = 1.0	F_{IO2} = 1.0	None or 30:2 via endotracheal tube	None
Countershocks	2·20 + 1·40 J/kg	3, 4, 6 J/kg	3, 6, 6 J/kg	3, 4, 6 J/kg	150, 200, 300, 360 J	150 J	3·6-7 J/kg 3·8-12 J/kg
CPR between countershocks	60 s/3 shocks	3 min/3 shocks	90 s/3 shocks	None	1 min/shock	2 min/shock	60 s/3 shocks

Drugs used to enhance CPR	0.045 mg/kg EPI or 0.8 U/kg AVP	0.05 mg/kg EPI if no ROSC after 3 countershocks	0.4 U/kg AVP	0.4 or 0.8 U/kg AVP, 45 or 200 μg/kg EPI, or both AVP and EPI	None	0.02 mg/kg EPI at 19, 22, 25 min if ROSC not achieved	10 U AVP 20 mg/kg lidocaine
Definition of ROSC	ECG; BP ≥ 90/40 mmHg for ≥5 min	Systolic BP >80 mmHg for >5 min	ECG; BP >80/40 mmHg	ECG; systolic BP ≥80 mmHg for ≥5 min	ECG; Mean aortic BP >60 mmHg for ≥5 min	Peak systolic BP > 50 mmHg, pulse pressure >20 mmHg for 1 min	ECG; Mean aortic BP ≥60 mmHg for ≥5 min
ROSC duration	2 h	5 min	6 h	1 h	3 d	24 h	1 h, 4 h, 7 d
Animals completing protocol	14/14	Ventilated during CPR: 9/12; Non-ventilated: 1/12	12/12	Placebo: 0/7; AVP: 5/7; EPI: 4/7; AVP + EPI: 7/7	36/44	Ventilation during CPR: 18/31; Non-ventilated: 24/33	1 h ROSC: 6/7 4 h ROSC: 18/21 7 d ROSC: 6/8

AVP: vasopressin; BP: systemic arterial blood pressure; ECG: electrocardiogram; EPI: epinephrine; ROSC: recovery of spontaneous circulation.

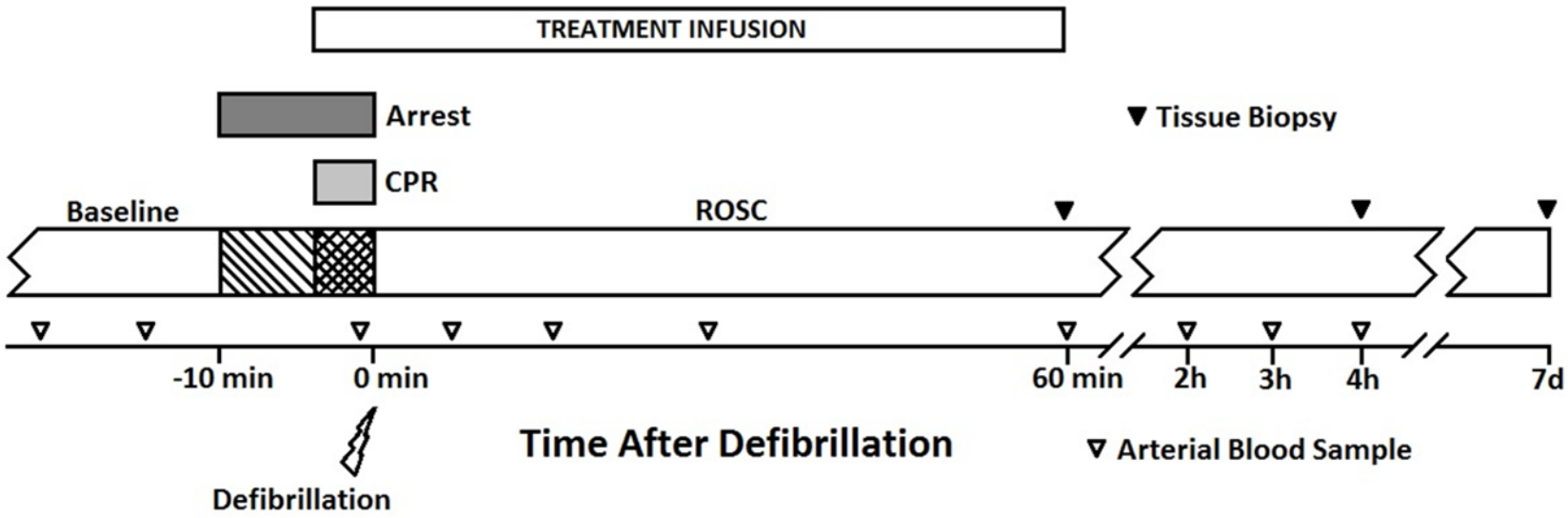
TREATMENT INFUSION
Arrest
CPR
Baseline
ROSC
Tissue Biopsy
-10 min
0 min
60 min
2h
3h
4h
7d
Defibrillation
Time After Defibrillation
Arterial Blood Sample

Figure 1.

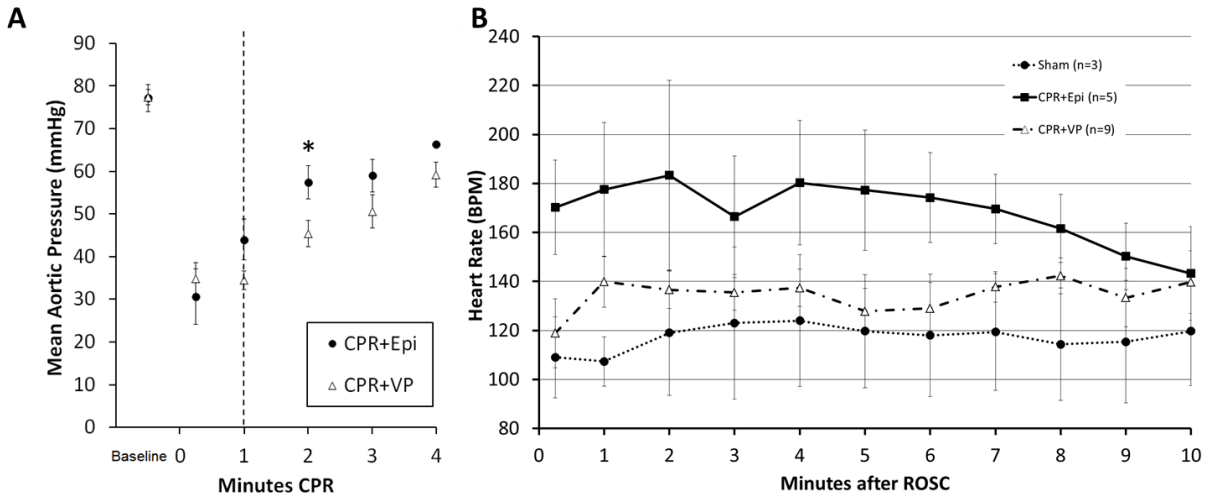
A
Mean Aortic Pressure (mmHg)
90
80
70
60
50
40
30
20
10
0
Baseline
0
1
2
3
4
*
CPR+Epi
CPR+VP
Minutes CPR
B
Heart Rate (BPM)
240
220
200
180
160
140
120
100
80
Sham (n=3)
CPR+Epi (n=5)
CPR+VP (n=9)
0
1
2
3
4
5
6
7
8
9
10
Minutes after ROSC

Figure 2.

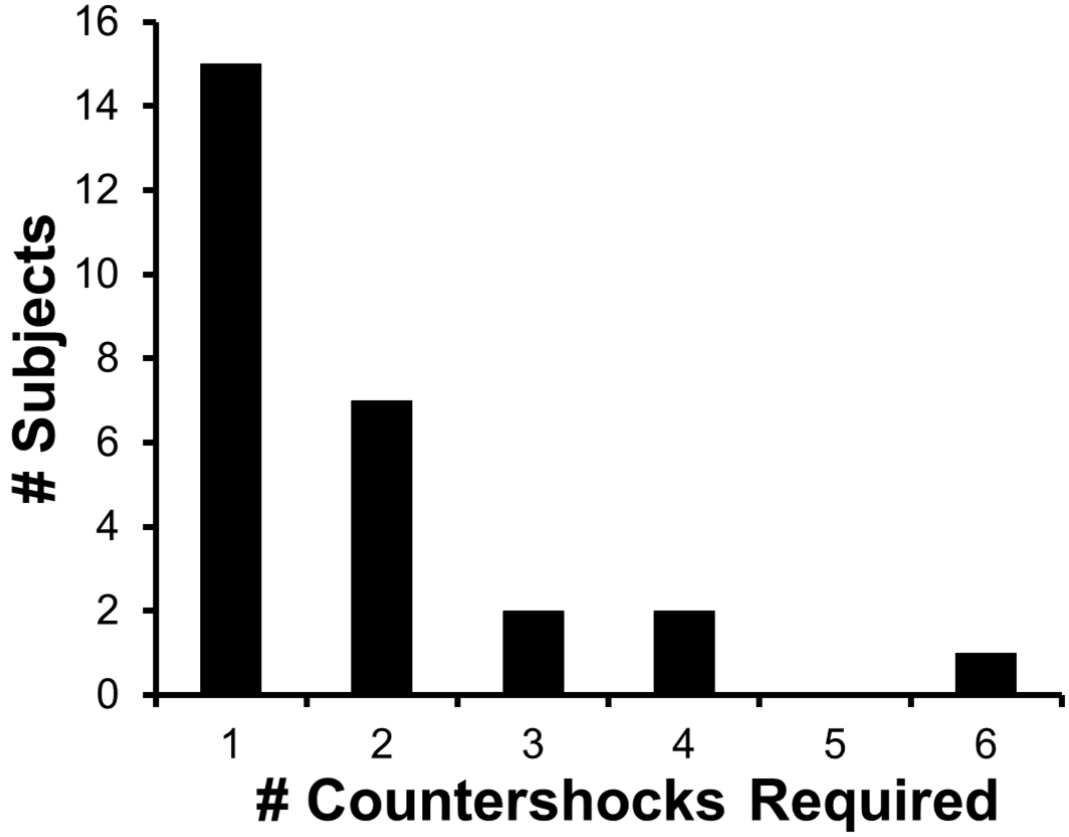

Figure 3.

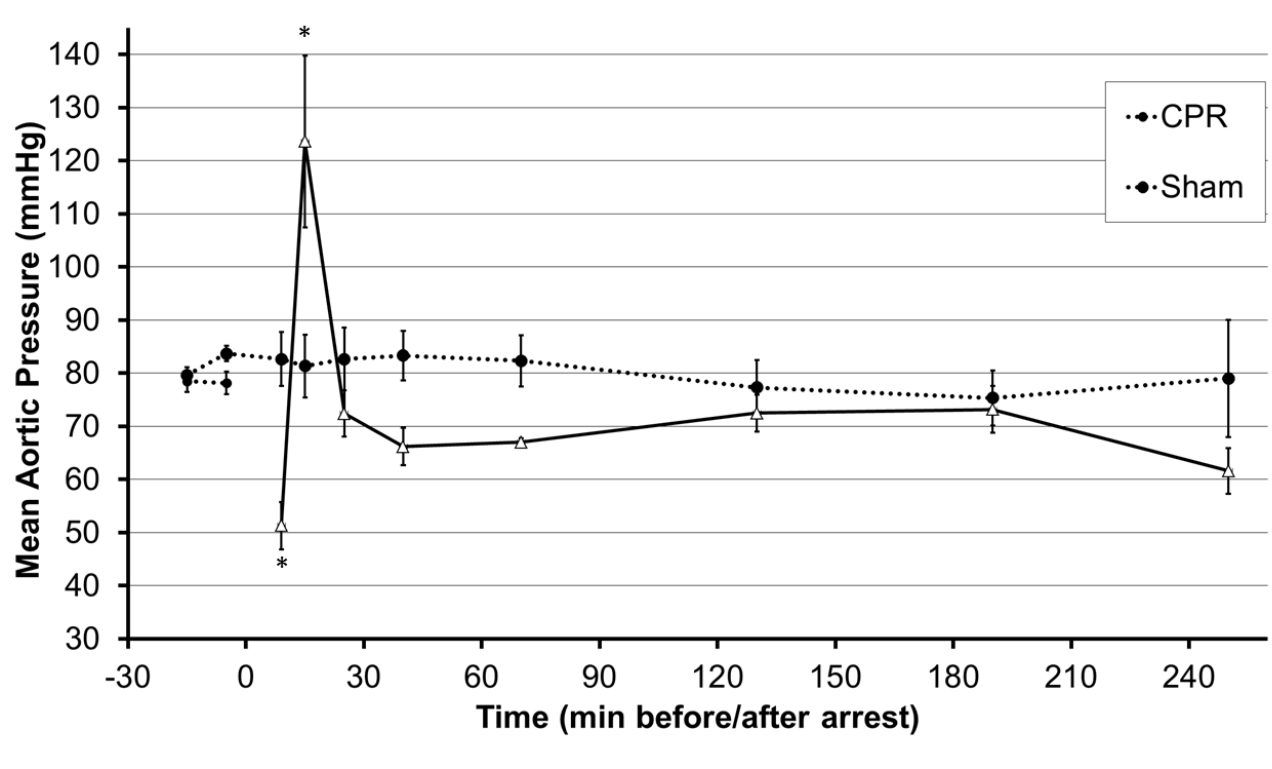
Mean Aortic Pressure (mmHg)
140
130
120
110
100
90
80
70
60
50
40
30
-30
0
30
60
90
120
150
180
210
240
Time (min before/after arrest)
CPR
Sham
*
*

Figure 4.

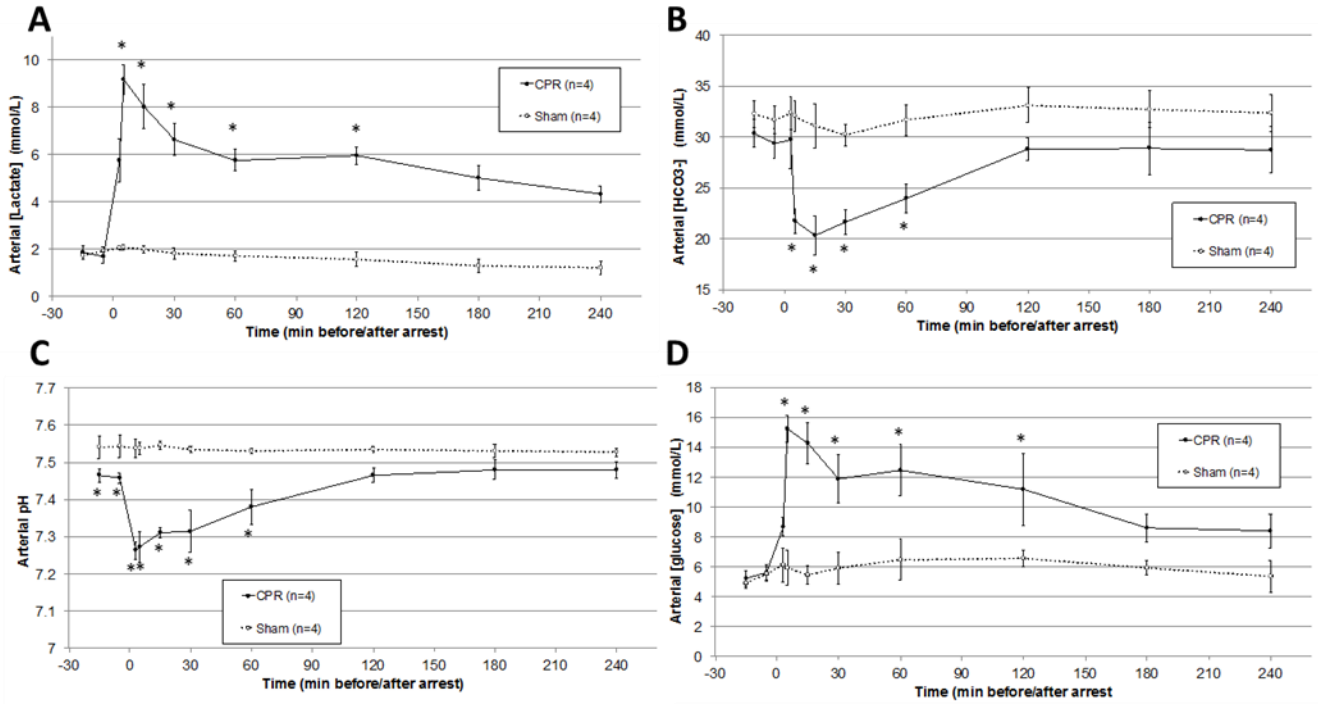
A
Arterial [Lactate] (mmol/L)
Time (min before/after arrest)
CPR (n=4)
Sham (n=4)
B
Arterial [HCO3-] (mmol/L)
Time (min before/after arrest)
CPR (n=4)
Sham (n=4)
C
Arterial pH
Time (min before/after arrest)
CPR (n=4)
Sham (n=4)
D
Arterial [glucose] (mmol/L)
Time (min before/after arrest
CPR (n=4)
Sham (n=4)

Figure 5.

CHAPTER III

Pyruvate stabilises electrocardiographic and haemodynamic function in pigs recovering from cardiac arrest

Brandon H. Cherry,[1,3,4] Anh Q. Nguyen,[1,3] Roger A. Hollrah,[1,3]

Arthur G. Williams Jr.,[1,3] Besim Hoxha,[5] Albert H. Olivencia-Yurvati,[2,3] Robert T. Mallet[1,2,3]

Departments of [1]Integrative Physiology and [2]Surgery

[3]Cardiovascular Research Institute

[4]Institute of Aging and Alzheimer's Disease Research

University of North Texas Health Science Center

Fort Worth, Texas, USA

[5]Department of Emergency Medicine

Baylor College of Medicine

Houston, Texas, USA

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ABSTRACT

Purpose: Cardiac arrest remains a leading cause of death in the United States and Western Europe. Patients who are initially resuscitated often succumb to severe ischaemia-reperfusion injury of the internal organs, and effective treatments remain elusive. Pyruvate, a natural intermediary metabolite, energy substrate and antioxidant, has been found to protect the heart, brain and other internal organs from ischaemia-reperfusion injury. This study tested the hypothesis that pyruvate-enriched resuscitation restores haemodynamic, metabolic, and electrolyte homeostasis following cardiac arrest. *Methods:* Yorkshire swine underwent pacing-induced ventricular fibrillation and, after 6 min pre-intervention arrest, 4 min precordial compressions followed by transthoracic countershocks. After defibrillation and recovery of spontaneous circulation, the pigs were monitored for another 4 h. Sodium pyruvate or NaCl control were infused *iv* (0.1 mmol • kg^{-1} • min^{-1}) throughout precordial compressions and the first 60 min recovery. *Results:* In 8 of the 24 NaCl-infused swine, the first countershock converted ventricular fibrillation to pulseless electrical activity unresponsive to subsequent countershocks, but only 1 of 18 pyruvate-treated swine developed pulseless electrical activity (relative risk 0.17; 95% confidence interval 0.13, 0.22). Pyruvate treatment lowered the dosage of vasoconstrictor phenylephrine required to maintain systemic arterial pressure, enhanced lactate and glucose clearance, elevated arterial bicarbonate, and raised arterial pH. *Conclusion:* Pyruvate treatment during cardiac arrest-resuscitation hastens restitution of stabilised electrocardiographic and haemodynamic function in swine recovering from cardiac arrest.

INTRODUCTION

Although over fifty years have passed since the inception of closed chest cardiac massage, and despite many refinements of this approach in the intervening decades, cardiac arrest remains a leading cause of death and persistent disability in the developed world, and effective treatments are elusive (Neumar et al. 2008). Each year, about 360,000 Americans suffer out-of-hospital cardiac arrest (Go et al. 2013). Many victims are resuscitated, but later succumb to extensive ischaemia-reperfusion injury to their internal organs (Heron 2008; Dezfulian et al. 2009; Young 2009; Nolan et al. 2012). Only ~10% of EMS-treated out-of-hospital cardiac arrest victims survive to hospital discharge (Go et al. 2013).

A first priority following return of spontaneous circulation (ROSC) is to stabilise metabolic, electrolyte, and haemodynamic parameters to prevent refibrillation or shock leading to an irreversible state and resulting in mortality (Mangla et al. 2014). In particular, measurements of blood pH, circulating concentrations of lactate, bicarbonate, sodium, potassium and glucose, and calculated base excess are crucial to evaluate the status of post-arrest patients. Following cardiac arrest, blood pH falls sharply due to elevated lactate from anaerobic glycolysis and hypercapnia due to inadequate alveolar ventilation. Hypercapnia and respiratory acidosis can be ameliorated by mechanically ventilating patients, but resolving lactate-dependent metabolic acidosis requires careful monitoring of arterial blood gas chemistry (Mangla et al. 2014). In patients recovering from cardiac arrest, greater clearance of lactate at 24 h ROSC is reportedly associated with higher survival rates (Donnino et al. 2007). Post-ROSC management of electrolytes, especially potassium, is essential to prevent electrical disturbances that could re-arrest the heart (El-Sherif & Turitto 2011). Finally, unless hypoglycaemia is the cause of cardiac arrest, hyperglycaemia is

typical in resuscitated patients and, if untreated, may contribute to long-term neurological impairment (Mullner et al. 1997; Langhelle et al. 2003; Mangla et al. 2014).

In a canine cardiac arrest model, intravenous administration of sodium pyruvate during cardiac arrest, open chest cardiac compression and the first 25 minutes of ROSC improved post-arrest cardiac electromechanical and metabolic recovery (Sharma et al. 2005). Pyruvate enhanced left ventricular contractility and lusitropy to increase systemic arterial pressure during early ROSC, and hastened electrocardiographic recovery evidenced by resolution of ST segment displacement when compared to NaCl-infused control experiments (Sharma et al. 2005). An important limitation of this study is that open-chest cardiac massage and direct epicardial defibrillatory countershocks were employed, not conventional precordial compressions and transthoracic countershocks. Therefore, we tested the hypothesis that intravenous administration of sodium pyruvate during precordial compressions and the first 60 min ROSC restores haemodynamic, metabolic, and electrolyte homeostasis in a closed chest porcine model of cardiac arrest.

METHODS

Surgical preparation and instrumentation

All surgical and experimental procedures in pigs were conducted in accordance with the *Guide to the Care and Use of Laboratory Animals* (2011) and were approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center. After an overnight fast, juvenile Yorkshire swine (30±0.7 kg; 41 males, 9 females) were sedated with a telazol/xylazine cocktail (5 mg/kg *im*) and intubated, and a surgical plane of anesthaesia was maintained by mechanical ventilation (12-14 cycles/min) with 1-3% isoflurane in 100% O_2. Epidermal electrodes were placed for standard limb lead II electrocardiography. The left femoral

artery and vein were exposed by inguinal incision and blunt dissection. A 7 Fr polyurethane catheter was inserted into a branch of the femoral artery and advanced into the abdominal aorta to monitor arterial blood pressure and sample arterial blood. Another catheter was inserted into a branch of the femoral vein for infusing medications and resuscitative fluids. The right external jugular vein was accessed via a 3 cm cervical incision, and a catheter was installed for insertion of a pacing wire and for infusion of resuscitative treatments. In a subset of experiments (n = 9), a second catheter was inserted into the right external jugular vein and advanced into the jugular bulb. Simultaneous measurements of jugular bulb and aortic pressures permitted assessment of cerebral perfusion pressure during cardiopulmonary resuscitation (CPR).

Cardiac arrest

A pacing wire was inserted into the jugular catheter and advanced into the right ventricle until the tip contacted the endocardium. To arrest the heart, a 0.5-1s burst of 60 Hz electrical impulses was transmitted to the right ventricle via the pacing wire, causing ventricular fibrillation. Once ventricular fibrillation was confirmed by the characteristic disorganized electrocardiographic activity, monophasic decline in aortic pressure and absence of an arterial pulse, mechanical ventilation was suspended for the duration of cardiac arrest and CPR. Bicarbonate (0.3-0.4 mEq/kg) was administered *iv* at 5 min arrest to partially offset the acidaemia that developed during cardiac arrest (Vukmir et al. 1995; Leong et al. 2001).

Cardiopulmonary resuscitation

After 6 min of cardiac arrest, mid-sternal chest compressions were applied manually, at a force sufficient to compress the chest by one-fourth of its antero-posterior diameter, followed by release and recoil, at a rate of 100 cycles/min, in accordance with current American Heart

Association guidelines (Meaney et al. 2013). The person administering the compressions was blinded to intravenous treatments, arterial pressure, end-tidal PCO_2 and blood gas chemistry. At 7 min arrest (*i.e.*, 1 min CPR) vasopressin (10 U) was injected into the right jugular vein to increase the efficacy of the chest compressions (Layek et al. 2014). Vasopressin-enhanced CPR achieved a cerebral perfusion pressure (*i.e.* mean aortic pressure minus jugular bulb pressure) of 69±5 mmHg. Mechanical ventilation remained suspended throughout CPR, in accordance with extensive clinical evidence that assisted ventilation ('rescue breathing') during CPR following witnessed cardiac arrest offers scant neurological or survival benefit (Bohm et al. 2007; Ewy et al. 2007; Iwami et al. 2007; Berger 2008; Nagao 2009).

Resuscitative solutions of the highest commercially available purity (Sigma, St. Louis, MO) were prepared within 30 min of use. Crystalline sodium pyruvate (n = 18) or NaCl (n = 24) were dissolved to 2 M concentration in double-distilled H_2O, sterilized by passage through 0.22 μm membranes (Millipore Stericup® vacuum driven filtration system) and stored on ice in sterile containers prior to infusion. All resuscitative solutions were prepared in a separate room by an investigator not directly participating in the experiment to ensure that experiments were conducted in a blinded fashion. Intravenous infusion of these solutions (0.1 mmol • kg^{-1} • min^{-1}) was initiated at the onset of CPR and maintained until 60 min ROSC.

Sham control pigs (n = 8) were instrumented, anaesthetised, and mechanically ventilated, but not subjected to cardiac arrest, CPR or defibrillation. The sham protocol was the same duration as the cardiac arrest-resuscitation protocol. These pigs received intravenous NaCl infusion for a period corresponding to that of treatment infusions in the cardiac arrest experiments.

Defibrillation and post-resuscitation management

After 4 min CPR, transthoracic direct current countershocks were administered with external paddles (LifePack 12; Physio-Control) to restore cardiac rhythm. If necessary, up to three 6-7 J/kg countershocks were applied, followed by up to three 8-12 J/kg countershocks, at 30 s intervals with intervening CPR, until spontaneous cardiac rhythm was restored. These efforts were discontinued and the experiment terminated if cardioversion was not achieved within 6 countershocks (9 of 45 cardiac arrests).

Mechanical ventilation was resumed immediately after spontaneous cardiac rhythm was confirmed by a rapid increase in mean arterial pressure and a discernible electrocardiographic rhythm. Next, lidocaine (20 mg in 1 mL 0.9% NaCl) was administered via the jugular catheter as a prophylactic measure to minimize recurrence of ventricular tachycardia or ventricular fibrillation, and to hasten recovery of a normal QRS waveform complex. Mean aortic pressure ≥ 60 mm Hg and a sustained spontaneous cardiac electrical rhythm were taken to indicate ROSC. After defibrillation, a surgical plane of anaesthesia was maintained and aortic pressure and electrocardiogram were monitored. Beginning at approximately 15 min ROSC, the α-adrenergic vasoconstrictor phenylephrine was infused into the left femoral vein at a rate sufficient to maintain aortic pressure at 65-75 mm Hg. The phenylephrine infusion was tapered and ultimately discontinued as cardiac function recovered.

At 1 or 4 h ROSC, the heart was exposed by left lateral fourth intercostal thoracotomy and pericardiotomy and the pig was then humanely euthanised by arresting the heart with electrical current applied to the left ventricular epicardium via a pacing wire. This method of euthanasia is

concordant with the recommendations specified in the *American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2013).*

Analysis of arterial blood

Blood samples (5 ml) were withdrawn from the left femoral artery into heparinised sterile syringes 15 and 5 min before arrest, and at 5, 15, 30, 60, 120, 180 and 240 min ROSC. These samples were centrifuged at 10,000 • g for 7.5 min to sediment the formed elements, and then the plasma supernatant was flash-frozen in liquid nitrogen. Plasma samples were thawed and extracted with 1 vol $HClO_4$ to precipitate proteins, and pyruvate concentration was determined colorimetrically in a Shimadzu model UV-1800 dual wavelength UV-vis spectrophotometer (337 nm measuring wavelength, 417 nm reference wavelength, $\varepsilon = 5.65\ M^{-1} \bullet cm^{-1}$). Additional blood samples were collected for measurements of sodium, potassium, lactate, bicarbonate, and glucose concentrations, pH, base excess, and haematocrit in an Instrumentation Laboratory Gem Premier 3000 blood gas analyzer.

Statistical methods

Haemodynamic function and plasma chemistry values in pyruvate-treated, NaCl-treated and sham pigs were compared by two-factor (time, treatment) analyses of variance with repeated measures on time and Student-Newman-Keuls *post-hoc* analyses. Phenylephrine dosages and energy required for defibrillation were compared between pyruvate- and NaCl-treated pigs by Student's t-test. P values <0.05 were taken to indicate statistically significant treatment effects. The incidences of pulseless electrical activity (PEA) in pyruvate- *vs.* NaCl-treated pigs were compared by relative risk and 95% confidence intervals.

RESULTS

Cardiopulmonary resuscitation and defibrillation

Before cardiac arrest, mean arterial pressure (MAP) was 85±3 mmHg in the pigs destined to receive sodium pyruvate, and 82±2 mmHg in the pigs which subsequently received NaCl (P=0.72.; Figure 1). MAP values achieved by precordial compressions did not differ between sodium pyruvate *vs.* NaCl treatment; at 4 min CPR, MAP was 78±7 and 67±5 mmHg in pyruvate- and NaCl-treated pigs, respectively (P=0.19; Figure 1). In 8 of 24 NaCl-treated swine, the first countershock converted ventricular fibrillation to PEA unresponsive to subsequent countershocks, but only 1 of 18 pyruvate-treated swine was converted to PEA (relative risk 0.17; 95% confidence interval 0.13, 0.22). Intravenous treatment did not have a statistically significant effect on the number of countershocks required to achieve ROSC (NaCl 1.4 ± 0.18; pyruvate: 2.0 ± 0.37; P=0.18) or on the electrical energy requirement to restore spontaneous rhythm (NaCl 14.8 ± 2.9 J • kg^{-1}; pyruvate: 8.3 ± 1.3 J • kg^{-1}; P=0.20). Overall, pyruvate infusion increased the likelihood of cardioversion to a productive cardiac rhythm, but did not affect the electrical energy required to achieve defibrillation.

Haemodynamic function

After defibrillation mean arterial pressure abruptly and transitorily increased in both CPR groups *vs.* sham pigs (Figure 2A). At 5 min ROSC, MAP in pyruvate-treated pigs (134±8 mmHg) was higher (P=0.03) than that of NaCl-infused pigs (112±16 mmHg), but by 15 min MAP had returned to baseline in both groups and remained similar thereafter (P=0.05). Heart rate (HR; Figure 2B) was increased with NaCl (149±9 min^{-1}) and pyruvate (138±13 min^{-1}) at 5 min ROSC

vs. sham (P=0.16 and 0.02, respectively), then gradually returned to baseline by 4h ROSC. As a surrogate measure of myocardial ATP demand (Laurent et al. 1956), heart rate • pressure product (Figure 2C) was calculated from haemodynamic measurements. The heart rate • pressure product increased following defibrillation with both treatments *vs.* sham, and returned to baseline by 15 min ROSC, suggesting that pyruvate had no effect on myocardial ATP demand. As an index of haemodynamic stability following ROSC, we measured the dose of the vasoconstrictor phenylephrine required to stabilize MAP. The pyruvate-treated pigs required less phenylephrine (Figure 2D) to maintain MAP at 65-75 mm Hg (0.04±0.01 mg • kg^{-1}) than did the NaCl-treated pigs (0.14±0.05 mg • kg^{-1}; P=0.006). Thus, pyruvate-treated pigs exhibited a briefly higher MAP that stabilized by 15 min, and required less pharmacological intervention to stabilize blood pressure for the 4 h following ROSC.

Arterial plasma carbohydrates

To ensure that *iv* infusion achieved a circulating concentration of pyruvate within range previously demonstrated to be cardio- and cerebroprotective (Sharma 2005, 2008), we measured plasma pyruvate concentrations spectrophotometrically. Sodium pyruvate infusion achieved a stable arterial pyruvate concentration of 4-5 mM (Figure 3A). The excess pyruvate cleared within 60 min post-infusion. Arterial lactate (Figure 3B) was sharply increased at 5 min ROSC in both pyruvate-treated (8.5±0.6 mM) and NaCl-treated (7.3±0.3 mM) pigs (P<0.001 *vs.* sham). Arterial lactate concentration remained elevated until pyruvate infusion was discontinued, and then fell sharply, whilst lactate concentration gradually subsided in NaCl-treated pigs (Figure 3B). As an index of redox state following cardiac arrest (Bassenge et al. 2000), we calculated the plasma lactate/pyruvate ratio. In NaCl-treated pigs the arterial lactate/pyruvate concentration

ratio increased from 4±1 pre-arrest to 24±6 at 15 min ROSC (P<0.001 *vs.* sham and pyruvate-treated pigs) and remained elevated until 4 h ROSC (Figure 3C). NaCl infusion increased the lactate/pyruvate ratio above that of pyruvate-infused and sham pigs. After infusion, the ratio was not different between groups. Arterial glucose concentration increased sharply after cardiac arrest (P<0.001 *vs.* sham) and remained elevated above the sham value, especially in the NaCl-infused pigs, until treatments were discontinued (Figure 3D). Glucose concentration remained higher in NaCl- *vs.* pyruvate-treated pigs at 15-60 min ROSC; after treatment, plasma glucose concentrations gradually returned to that of the sham controls by 4 h ROSC. Overall, pyruvate infusion achieved a therapeutically effective plasma pyruvate concentration and improved the redox state of the animal by decreasing the lactate to pyruvate, and promoted glucose clearance.

Arterial electrolytes and blood gases

To determine if *iv* Na-pyruvate or NaCl infusion imposed hypernatraemia on the recovering pigs, arterial sodium concentration was analyzed. Arterial sodium concentration (Figure 4A) increased during the first 30-60 min of ROSC, *i.e.* during NaCl or sodium pyruvate infusion. Similar changes were seen in NaCl-infused sham pigs. Arterial potassium concentration (Figure 4B) was elevated in both pyruvate- and NaCl-treated *vs.* sham pigs (P<0.001) at 5 min ROSC, but by 15 min, pyruvate-treated pigs had lower potassium concentrations than NaCl-treated and sham pigs (P=0.002), and this effect persisted until 4 h ROSC. Thus, treatment infusion did not impose a persistent hypernatraemia and pyruvate prevented the acute hyperkalaemia observed with NaCl treatment.

Arterial blood pH (Figure 4C) fell by *c.* 0.25 in both pyruvate- and NaCl-treated pigs at 5 min ROSC *vs.* the respective pre-arrest values; thus, cardiac arrest produced acidaemia, as expected. Arterial pH returned to baseline by 1 h ROSC in pyruvate-treated pigs, but recovered more gradually in NaCl-treated pigs. Arterial pH continued to increase after pyruvate infusion and plateaued at 2-3 h ROSC (P=0.003 *vs.* NaCl-treated pigs). Arterial base excess/deficit, an index of buffering capacity by the circulation, paralleled arterial pH; thus, both pyruvate- and NaCl-treated pigs had base deficits *vs.* shams at 5 (P<0.001) and 15 min (P<0.001) ROSC (Figure 4D). Contributing to the early base deficit, the arterial bicarbonate concentration (Figure 4E) fell in both pyruvate- and NaCl-treated pigs *vs.* the respective pre-arrest baselines (P=0.01 and <0.001, respectively) and contemporary sham values (P=0.009 and 0.001, respectively). However, by 1 h ROSC, pyruvate-treated pigs had persistently elevated base excesses *vs.* sham and NaCl-treated pigs (P=0.01 and <0.001, respectively). Pyruvate treatment restored arterial bicarbonate to baseline by 30 min ROSC, and by 1 h had persistently elevated bicarbonate concentration; in contrast, in NaCl-treated pigs arterial bicarbonate didn't recover until 3 h ROSC. A transitory increase in haematocrit (Figure 4F) was observed after cardiac arrest in both NaCl- and pyruvate-treated pigs (P<0.001 *vs.* baseline). Thus, pyruvate augmented the buffering capacity of arterial blood by persistently increasing circulating bicarbonate concentration.

DISCUSSION

Administration of sodium pyruvate during CPR and the first 60 min ROSC sharply lowered the conversion of ventricular fibrillation to PEA and decreased phenylephrine dosages necessary to maintain haemodynamic stability during ROSC. Pyruvate also hastened post-arrest clearance of arterial glucose, a prognostic indicator of survival (Mangla et al. 2014), and although pyruvate

increased arterial lactate during infusion, it lowered the arterial lactate/pyruvate concentration ratio, an effect which may minimise reactive oxygen species formation and promote recovery (Bassenge et al. 2000). Pyruvate also promoted the alkalinisation of blood, an action which could afford recovery from metabolic acidosis imposed by cardiac arrest. Finally, pyruvate lowered plasma K^+ concentration, which may avoid pro-arrhythmic hyperkalaemia and re-arrest of the heart. Thus, intravenous pyruvate infusion during cardiac arrest-resuscitation hastened restitution of electrocardiographic, haemodynamic and metabolic function in swine recovering from cardiac arrest.

Pulseless electrical activity

Pulseless electrical activity is a 'non-shockable' cardiac electrical rhythm that does not produce ventricular contraction or forward movement of blood. It is estimated that approximately 60% of out-of-hospital resuscitation attempts result in the development of PEA (Niemann et al. 2001). Survival of cardiac arrest victims with PEA is much lower than those presenting with ventricular fibrillation; indeed, only 2-5% of patients who present with PEA as their initial rhythm survive (Rea et al. 2004; Atwood et al. 2005; Kajino et al. 2008). Even fewer patients in whom ventricular fibrillation converted to PEA following countershocks survive to hospital discharge (Warner et al. 1985). In this study, none of the 9 pigs that developed PEA after the first countershock could be restored to spontaneous circulation. On the other hand, pyruvate treatment sharply lowered the incidence of PEA *vs.* NaCl treatment. A proposed mechanism underlying development of PEA following ischaemia and attempted defibrillation is depletion of myocardial ATP and phosphocreatine (Niemann et al. 2001; Choi et al. 2013). Ventricular fibrillation is energetically costly, and the likelihood of post-countershock PEA increases as

myocardial ATP reserves are expended. Thus, the increased frequency of defibrillation to a productive electrocardiographic rhythm in pyruvate- *vs.* NaCl-treated pigs may be attributed to pyruvate's enhancement of myocardial energy state during CPR and ROSC (Sharma et al. 2005), which increases ATP supply for cardiac electromechanical recovery (Bünger et al. 1989; Mallet & Sun 1999).

Post-arrest haemodynamic recovery

Pyruvate treatment produced a temporarily higher MAP than did NaCl, which could possibly be ascribed to increased energy supply for cardiac work afforded by mitochondrial ATP production (Bünger et al. 1989; Mallet & Sun 1999), or potentiation of catecholamine actions within the myocardium (Tejero-Taldo et al. 1998), as circulating catecholamine activity is likely elevated after cardiac arrest. Collectively, these factors lowered the requirement for vasoconstrictor phenylephrine to maintain MAP in pyruvate- *vs.* NaCl-treated pigs recovering from cardiac arrest.

Plasma carbohydrates

Elevated arterial lactate is considered clinically adverse, but that produced by pyruvate treatment is an exception, since it results from pyruvate conversion to lactate *via* the lactate dehydrogenase equilibrium, not from an underlying pathology. Furthermore, the excess lactate rapidly cleared after infusion was discontinued. More rapid clearance of glucose during the first hour of recovery in pyruvate- *vs.* NaCl-infused pigs suggests enhanced metabolic recovery with pyruvate infusion, especially since the enhanced glucose clearance occurred despite infusion of a competing metabolic fuel, pyruvate (Mullner et al. 1997). This disappearance of glucose could

be attributed to the ability of pyruvate to activate pyruvate dehydrogenase secondary to direct suppression of pyruvate dehydrogenase kinase [Cooper et al., 1974]. The latter enzyme phosphorylates and inactivates pyruvate dehydrogenase, an effect which is overcome by pyruvate's inhibition of the kinase, permitting oxidation of glucose-derived pyruvate in the Krebs cycle. Additionally, in a stroke model of focal ischemia-reperfusion, pyruvate activated Akt (Ryou et al. 2012), which increases glucose uptake *via* glucose transporter 4 exocytosis (Ishiki and Klip 2005). The effects of pyruvate on glucose clearance after cardiac arrest, therefore, may be at least partly attributed to these mechanisms.

Post-arrest hypernatraemia

Infusion of 2M sodium, either as pyruvate or chloride salt, effected hypernatraemia which largely subsided by 3h post-infusion. The treatments imposed a sodium load totaling 6 mmol sodium • kg^{-1}. If the *c*. 30 kg pigs of this study contain *c*. 20 L total body water, the 200 mmol infused Na^+ would produce *c*. 10 mEq/L increase in plasma Na^+ after osmotic equilibration, which approximates the transitory hypernatraemia which plateaued at 30-60 min infusion. Importantly, sodium pyruvate administration did not impose persistent hypernatraemia.

Haematocrit

Cardiac arrest provoked an appreciable, albeit transitory, increase in haematocrit of approximately 0.06-0.08. Hyperadrenergic conditions provoke contraction of the porcine spleen, increasing the amount of circulating erythrocytes to augment oxygen delivery to tissues (Swindle 2007). The current results show cardiac arrest-resuscitation also temporarily raised the haematocrit. The subsequent decline in haematocrit during ROSC likely reflected haemodilution

due to osmotic expansion of extracellular fluid volume secondary to sodium infusion, and administration of phenylephrine in 0.9% NaCl.

Acid-base responses to pyruvate infusion

Elevated arterial pH and base excess in pyruvate- *vs.* NaCl-treated pigs is most likely the result of pyruvate-mediated electrolyte shifts. Pyruvate enters cells via high-capacity monocarboxylate transporters (Figure 5) which co-transport pyruvate with hydrogen ions, thereby lowering the extracellular H^+ concentration and generating an outward transmembrane H^+ gradient. This gradient would favour the exchange of extracellular K^+ for intracellular H^+ via the H^+/K^+ antiporter, thereby helping to resolve post-arrest hyperkalaemia. The pyruvate-driven shift of H^+ into cells would shift the carbonic anhydrase equilibrium in favour of H^+ and HCO_3^- formation, thereby increasing extracellular HCO_3^- concentration. Moreover, pyruvate has been shown to preserve function of the Na^+,K^+ ATPase, which, by transporting K^+ into cells contributes to the decreases in extracellular K^+ concentration (Wang et al. 2003). Collectively, this decrease in circulating potassium would prevent pro-arrhythmic hyperkalaemia that could re-arrest the heart (El-Sherif & Turitto 2011).

Limitations

The experimental model differed in several important respects from clinical cardiac arrest. Experiments were conducted in healthy, juvenile swine free of the chronic diseases that afflict cardiac arrest patients. Cardiac arrest was induced in fully anaesthetised animals, isoflurane is a known cardioprotectant (Kato & Foex 2002; Landoni et al. 2008). These juvenile swine had compliant chest walls that facilitated delivery of effective chest compressions. Chest

compressions were begun at 6 min cardiac arrest, and defibrillation attempted at 10 min arrest to ensure a high survival rate, whereas the durations of cardiac arrest and CPR often are more prolonged in human victims, especially those suffering out-of-hospital cardiac arrest, and are associated with higher mortality.

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FIGURE LEGENDS

Figure 1. *Mean arterial pressure during CPR.* Intrajugular administration of the peripheral vasoconstrictor vasopressin at 1 min CPR (vertical dashed line) increases the efficacy of precordial chest compressions to raise mean arterial pressure. BL: pre-arrest baseline.

Figure 2. *Hemodynamic function during recovery from cardiac arrest*. A: Mean arterial pressure (MAP); B: heart rate (HR); C: Heart rate • mean arterial pressure product (RPP); D: Total phenylephrine dosage to maintain MAP at 60-75 mm Hg during recovery. On this figure and Figures 3 and 4, the horizontal bar denotes the period of treatment infusion. *P<0.05 *vs.* sham; †P<0.05 *vs.* CPR + NaCl.

Figure 3. *Arterial plasma carbohydrates and redox state.* A: Lactate concentration; B: pyruvate concentration; C: lactate/pyruvate concentration ratio; D: glucose concentration. *P<0.05 *vs.* sham; †P<0.05 *vs.* CPR + NaCl.

Figure 4. *Arterial electrolyte profile and haematocrit.* A: Arterial Na^+ concentration; B: K^+ concentration; C: pH; D: bicarbonate concentration; E: arterial base excess/deficit; F: haematocrit. *P<0.05 *vs.* sham; †P<0.05 *vs.* CPR + NaCl.

Figure 5. *Proposed mechanisms of pyruvate-induced alkalaemia and hypokalaemia.* Plasmalemmal monocarboxylate transporters (MCT) co-import pyruvate and H^+ which lowers extracellular H^+ concentration. The resultant transmembrane H^+ gradient K^+ import by the hydrogen/potassium antiporter (H/K). Because pyruvate preserves function of the Na^+/K^+ ATPase, potassium is also imported by this transporter.

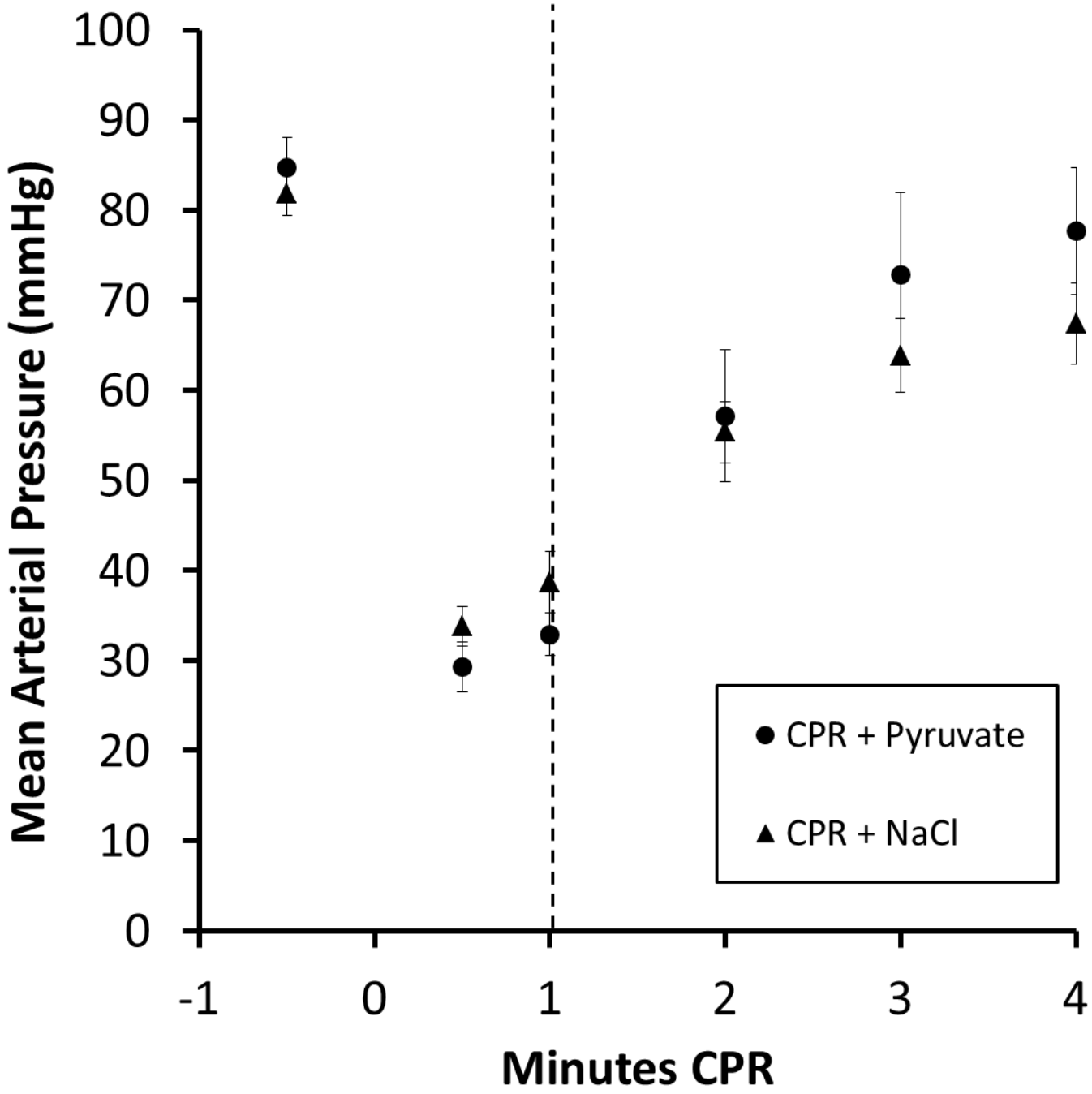
Mean Arterial Pressure (mmHg)
100
90
80
70
60
50
40
30
20
10
0
-1
0
1
2
3
4
Minutes CPR
CPR + Pyruvate
CPR + NaCl

Figure 1

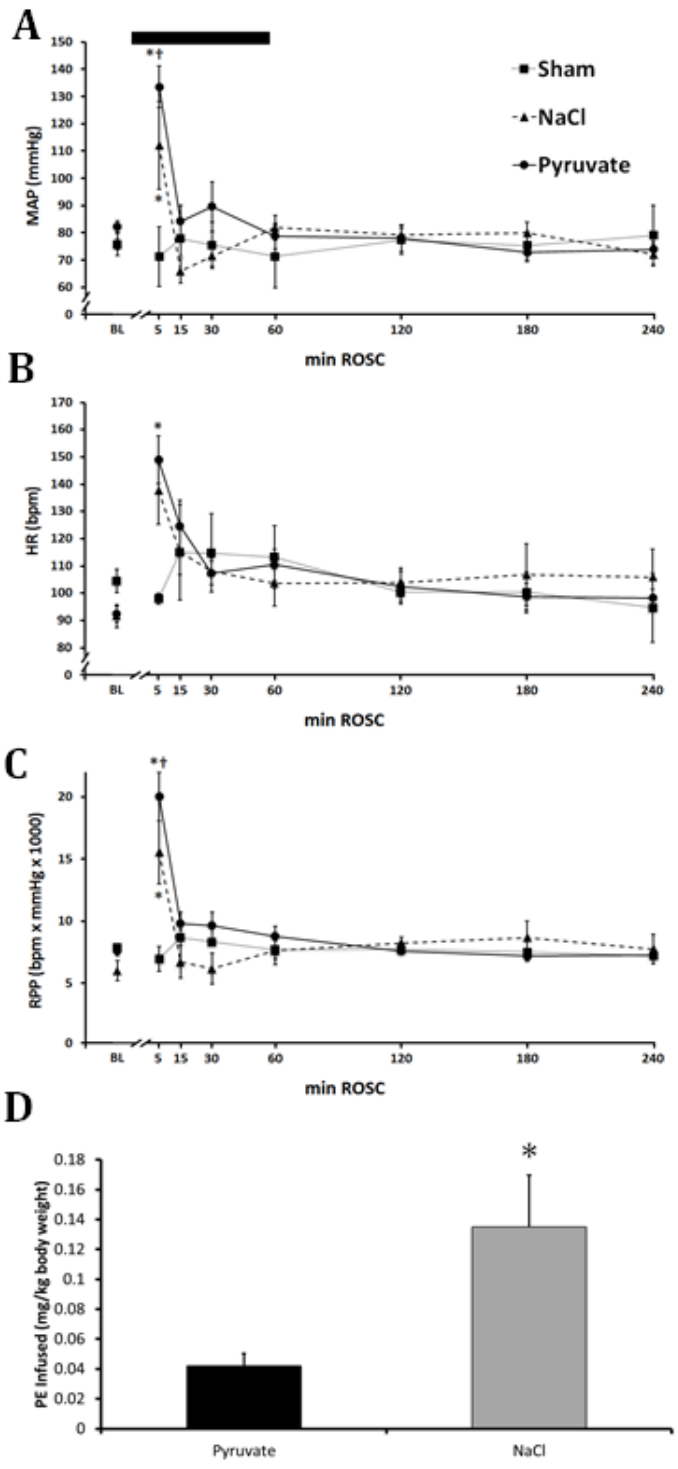
A
MAP (mmHg)
Sham
NaCl
Pyruvate
BL
5
15
30
60
120
180
240
min ROSC
B
HR (bpm)
min ROSC
C
RPP (bpm x mmHg x 1000)
min ROSC
D
PE Infused (mg/kg body weight)
Pyruvate
NaCl

Figure 2

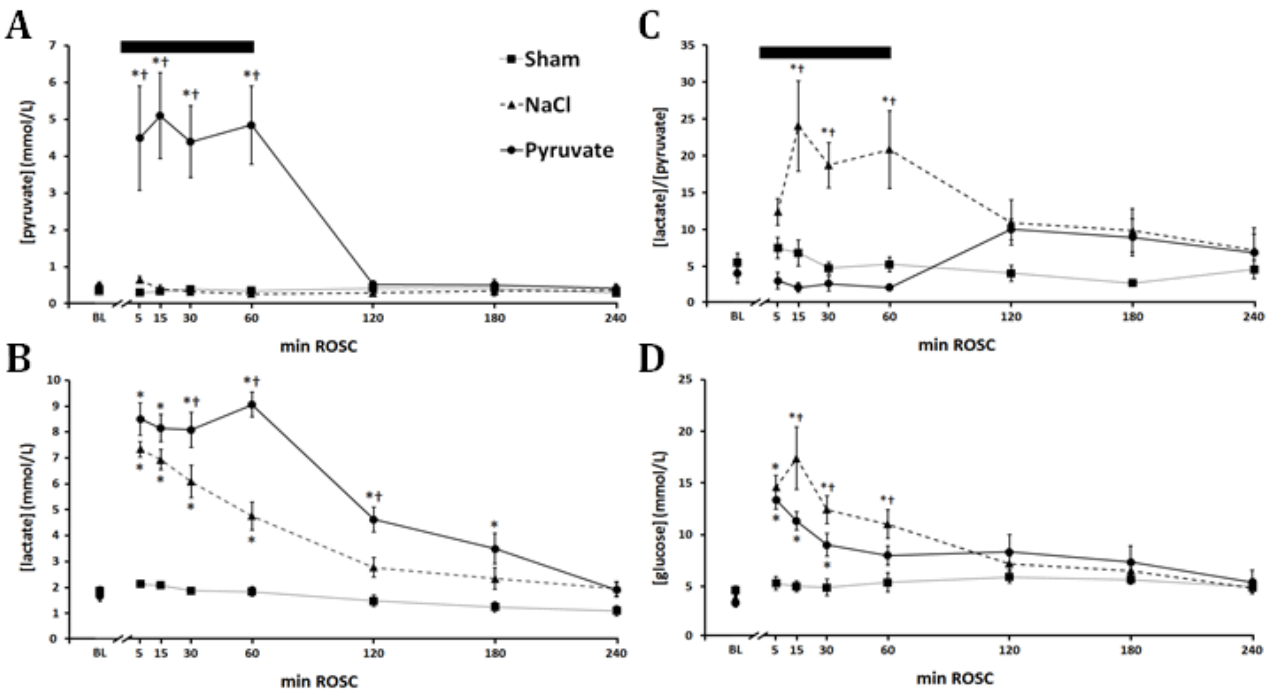
A
B
C
D
Sham
NaCl
Pyruvate
[pyruvate] (mmol/L)
[lactate] (mmol/L)
[lactate]/[pyruvate]
[glucose] (mmol/L)
min ROSC
BL
5
15
30
60
120
180
240

Figure 3

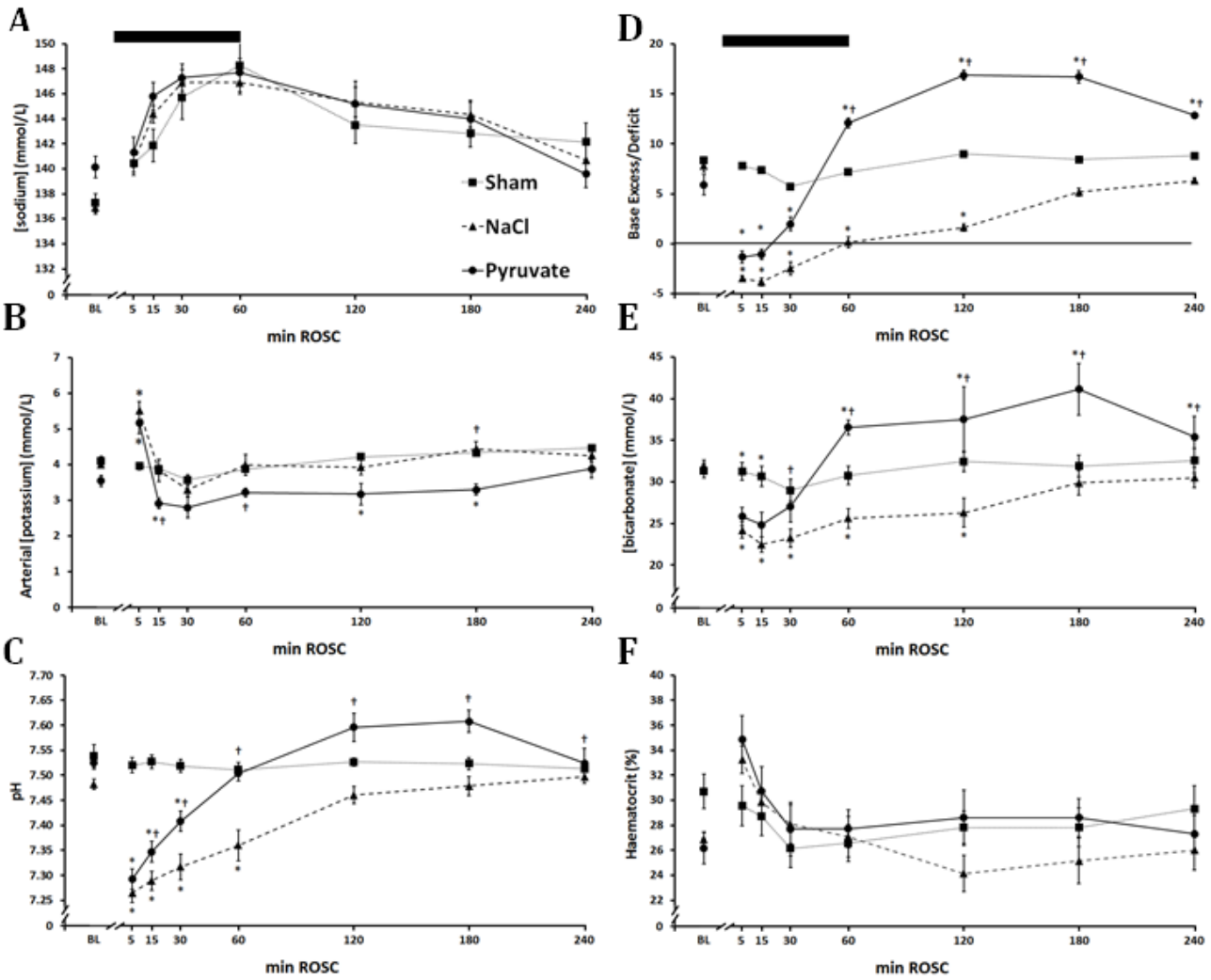
A
[sodium] (mmol/L)
Sham
NaCl
Pyruvate
BL
5
15
30
60
120
180
240
min ROSC
D
Base Excess/Deficit
B
Arterial [potassium] (mmol/L)
E
[bicarbonate] (mmol/L)
C
pH
F
Haematocrit (%)

Figure 4

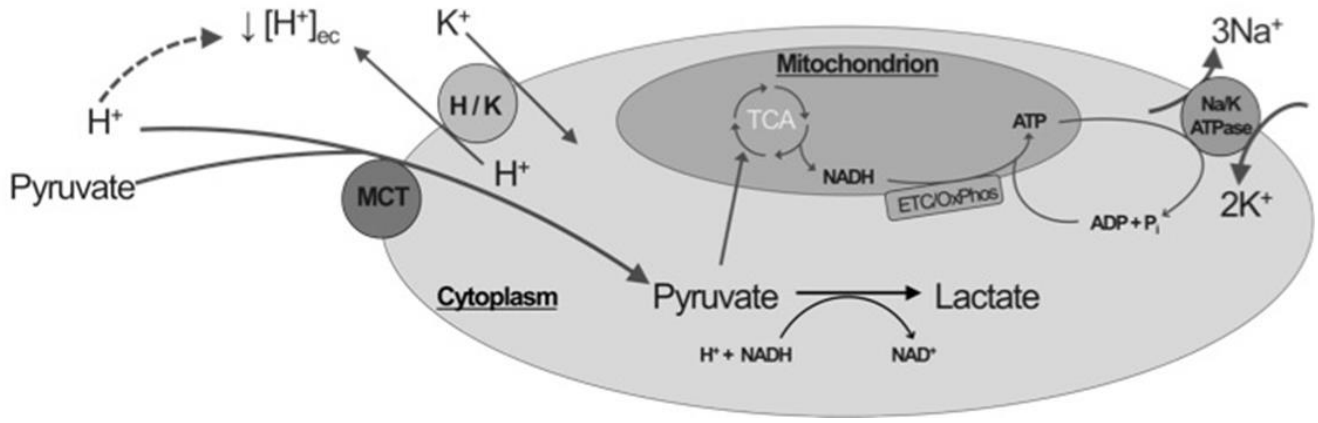
↓ [H+]ec
K+
3Na+
Mitochondrion
H+
H / K
TCA
ATP
Na/K ATPase
Pyruvate
MCT
H+
NADH
ETC/OxPhos
2K+
ADP + Pi
Cytoplasm
Pyruvate
Lactate
H+ + NADH
NAD+

Figure 5

CHAPTER IV

Impact of cardiac arrest and hyperoxia on the glutathione peroxidase/reductase system in porcine brain

Brandon H. Cherry,[1,3,4] Anh Q. Nguyen,[1,3] Roger A. Hollrah,[1,3]

Arthur G. Williams Jr.,[1,3] Besim Hoxha,[5] Albert H. Olivencia-Yurvati,[2,3] Robert T. Mallet[1,2,3]

Departments of [1]Integrative Physiology and [2]Surgery

[3]Cardiovascular Research Institute

[4]Institute of Aging and Alzheimer's Disease Research

University of North Texas Health Science Center

Fort Worth, Texas, USA

[5]Department of Emergency Medicine

Baylor College of Medicine

Houston, Texas, USA

ABSTRACT

Cardiac arrest and resuscitation and the resulting ischemia-reperfusion elicit profound overproduction of reactive oxygen and nitrogen species (RONS) in brain. Hyperoxic ventilation during resuscitation and recovery intensifies this RONS assault. The principal antioxidant defense against RONS is the glutathione peroxidase-reductase system, yet this system itself is a target of RONS. Pyruvate has been shown to preserve endogenous antioxidants during oxidative stress. Therefore, this study examined the effects of hyperoxic ventilation, with and without pyruvate treatment, on the activities of the glutathione peroxidase-reductase system in the cerebellum and hippocampus, two brain regions particularly susceptible to oxidative injury, during recovery from cardiac arrest-resuscitation. Domestic swine were subjected to 6 min cardiac arrest, 4 min closed-chest CPR (100 compressions/min), defibrillation (transthoracic countershocks) and either 1 h or 4 h recovery, and were ventilated with 100% oxygen throughout recovery. Sodium pyruvate (n=12) or NaCl control (n=12) were infused iv (0.1 mmol • kg^{-1} • min^{-1}) during CPR and the first 60 min recovery. A non-arrested sham group (n=7) received intravenous NaCl. Cardiac arrest sharply lowered glutathione peroxidase and glutathione reductase activities in both the hippocampus and cerebellum; pyruvate partially preserved glutathione peroxidase activity, particularly in the cerebellum. After pyruvate infusion, the enzyme activities fell to those of the NaCl group by 4 h recovery. In the non-arrested sham pigs, activities of both enzymes fell to those of the post-arrest groups between 1 and 4 h hyperoxia, indicating that hyperoxia disabled the enzymes independently of cardiac arrest. Thus, cardiac arrest compromised the glutathione peroxidase-reductase antioxidant system of hippocampus and cerebellum, and hyperoxic ventilation prevented post-arrest recovery of these pivotal antioxidant enzymes.

Keywords: cardiocerebral resuscitation, hyperoxia , pyruvate ventricular fibrillation,

INTRODUCTION

Although over fifty years have passed since the advent of closed chest cardiac massage, cardiac arrest remains a leading cause of death and disability in the developed world (Heron 2012). Many victims are initially resuscitated, but later succumb to extensive injury to their vital organs (Dezfulian et al. 2009; Young et al. 2009; Heron 2012; Nolan et al. 2012). Effective treatments to combat the extensive ischemia-reperfusion injury that ensues after return of spontaneous circulation (ROSC) are elusive (Neumar et al. 2008). A key perpetrator in the ischemia-reperfusion injury sustained following cardiac arrest and cardiopulmonary resuscitation (CPR) is the oxidative stress imposed on internal organs, especially brain (Idris et al. 2005). Reperfusion provokes the formation and accumulation within the brain of reactive oxygen (Becker 2004; Idris et al. 2005) and nitrogen species, *i.e.* RONS (White 2000; Thiyagarajan et al. 2004; Zhu et al. 2004), leading to lipid peroxidation, inactivation of metabolic enzymes, and mitochondrial dysfunction, ultimately culminating in cell death and resultant neurological impairment (Richards et al 2006; Brücken et al 2010).

For over 40 years, ventilation with 100% oxygen during resuscitation has been recommended to increase oxygen delivery to ischemic tissues after cardiac arrest (Smith et al. 1968; O'Driscoll et al. 2008). Recently, however, this strategy has been debated based on evidence that ventilation with 100% oxygen during resuscitation and ROSC may intensify production of RONS, thereby

exacerbating the oxidative damage inflicted upon reperfusion of the brain (Richards et al. 2006, 2007; Pilcher et al. 2012). Prevention of the explosive production of RONS and the ensuing oxidative damage of brain holds powerful therapeutic significance.

Augmentation of antioxidant defenses by exploiting endogenous genetic responses to oxidative stress has received much recent attention (Genc et al. 2010; Zhang et al. 2013). For example, RONS-dependent activation of the transcription factor nuclear factor erythroid 2-related factor (Nrf-2) elicits the expression of a host of phase II defense enzymes that afford antioxidant cytoprotection (Zhang et al. 2010, 2013; Nguyen et al. 2014; Levonen et al. 2014). Thus, it is reasonable to suggest that oxidative stress may provide a stimulus to upregulate endogenous antioxidant defenses and thereby aid recovery from cardiac arrest and the resultant ischemia-reperfusion injury. Pyruvate, an intermediary metabolite and energy substrate, has also proven to be neuroprotective during brain ischemia-reperfusion (Mongan et al 2001; Sharma et al. 2003, 2008; Ryou et al. 2012; Nguyen et al 2014). Additionally, pyruvate prevents inactivation of the myocardial glutathione peroxidase-reductase antioxidant system (Sharma et al. 2007) and reduces oxidative stress following cardioplegic arrest (Knott et al. 2005). Therefore, this study sought to examine the effects of cardiac arrest-resuscitation, hyperoxic ventilation and pyruvate administration on the glutathione peroxidase-reductase antioxidant defense system in hippocampus and cerebellum. We hypothesized that pyruvate treatment would preserve activity of the glutathione peroxidase-reductase system and in the face of intense RONS formation.

METHODS

Surgical preparation and instrumentation

All surgical and experimental procedures in pigs were conducted in accordance with the *Guide to the Care and Use of Laboratory Animals* (2011) and were approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center. Juvenile Yorkshire swine (30±0.7 kg) were fasted overnight, sedated with a telazol/xylazine cocktail (5 mg/kg *im*), intubated and mechanically ventilated (12-14 cycles/min) with 1-3% isoflurane in 100% O_2 to maintain a surgical anesthetic plane. Epidermal electrodes were placed for standard limb lead II electrocardiography. After inguinal cutdown and blunt dissection, a 7 Fr polyurethane catheter was inserted into a branch of the femoral artery and advanced into the abdominal aorta to monitor arterial blood pressure. For infusion of medications and resuscitative fluids, a catheter was also inserted into a branch of the femoral vein. A third catheter was implanted via the right external jugular vein for insertion of a pacing wire and for infusion of resuscitative treatments.

Cardiac arrest and cardiopulmonary resuscitation

A 0.5-1s burst of 60 Hz electrical impulses was transmitted via pacing wire to the right ventricular endocardium to arrest the heart. Once ventricular fibrillation was confirmed by the characteristic disorganized electrocardiographic activity, monophasic decline in aortic pressure and absence of an arterial pulse, mechanical ventilation was suspended for the duration of cardiac arrest and CPR. After 6 min of cardiac arrest, precordial compressions were applied

manually at a rate of 100 cycles/min. The person administering the compressions was blinded to intravenous treatments, arterial pressure, end-tidal PCO_2 and blood gas chemistry. At 1 min CPR (*i.e.* 7 min arrest), vasopressin (10 U) was injected into the right jugular vein to increase the efficacy of the chest compressions (Layek et al. 2014).

Resuscitative solutions of the highest commercially available purity (Sigma, St. Louis, MO) were prepared within 30 min of use. 2 M sodium pyruvate (n = 12) or NaCl (n = 12) were prepared in a separate room by an investigator not directly participating in the experiment and sterilized by passage through 0.22 µm membranes (Millipore Stericup® vacuum driven filtration system). Intravenous infusion of these solutions (0.1 mmol • kg^{-1} • min^{-1}) was initiated at the onset of CPR and maintained until 60 min ROSC.

Defibrillation and post-resuscitation management

After 4 min CPR, transthoracic direct current countershocks were administered with external paddles (LifePack 12; Physio-Control) to restore cardiac rhythm. If necessary, up to three 6-7 J/kg countershocks were applied, followed by up to three 8-12 J/kg countershocks, at 30 s intervals with intervening CPR, until spontaneous cardiac rhythm was restored. These efforts were discontinued and the experiment terminated if cardioversion was not achieved within 6 countershocks.

Immediately after spontaneous cardiac rhythm was confirmed by a rapid increase in mean arterial pressure and a discernible electrocardiographic rhythm, mechanical ventilation with

100% oxygen was resumed. Next, lidocaine (20 mg in 1 mL 0.9% NaCl) was administered via the jugular catheter as a prophylactic measure to minimize recurrence of ventricular tachyarrhythmias and hasten recovery of a normal QRS waveform. After defibrillation, a surgical plane of anesthesia was maintained with 1-3% isoflurane and aortic pressure and electrocardiogram were monitored. Beginning at approximately 15 min ROSC, the α-adrenergic vasoconstrictor phenylephrine was infused *iv* and titrated to maintain aortic pressure at 65-75 mm Hg, then tapered and ultimately discontinued as cardiac function recovered.

At 1 or 4 h ROSC, the heart was exposed by left lateral thoracotomy in the fourth intercostal space and pericardiotomy, and the pig was then euthanized by arresting the heart with electrical current applied via a pacing wire to the left ventricular epicardium. This method of euthanasia is concordant with the recommendations specified in the *American Veterinary Medical Association Guidelines for the Euthanasia of Animals* (2013). Sham control pigs were instrumented, anesthetized, and mechanically ventilated, but not subjected to cardiac arrest, CPR or defibrillation. Sham animals were ventilated for a duration corresponding to 1 h (n = 3) or 4 h (n = 4) ROSC.

Tissue biopsy and protein extraction

To minimize the interval between euthanasia and brain biopsy, a craniotomy was performed to expose the brain. At the time of euthanasia, the entire brain was removed and immediately placed on ice-cold aluminum foil. Biopsies from hippocampus and cerebellum were carefully dissected and snap-frozen with liquid nitrogen-precooled Wollenberger tongs. Biopsies were

pulverized to a fine powder in liquid nitrogen using a mortar and pestle, then homogenized in phosphate buffer (0.1 M KH_2PO_4, pH 7.2) containing protease inhibitor cocktail (5 µL/mL buffer; Sigma, St. Louis, MO). Homogenate was then centrifuged (112,000 • g, 4°C) for 20 min, the supernatant collected, the pellet resuspended and homogenized in phosphate buffer, and centrifuged again to collect the supernatant. After the supernatants were combined, protein concentration of the extract was assayed (Bradford 1976) using bovine serum albumin standard (Thermo Scientific, Rockford, IL).

Enzyme activites

Glutathione peroxidase activity was measured using a commercially available 96-well microplate assay kit (Cayman Chemical, Ann Arbor, MI), in which glutathione peroxidase is coupled to glutathione reductase-catalyzed oxidation of NADPH to $NADP^+$ monitored at 340 nm wavelength. In the same fashion, glutathione reductase activity was also measured using a microplate kit (Cayman Chemical, Ann Arbor, MI). Lactate dehydrogenase (LDH) activity was measured spectrophotometrically using a commercially available kit (abcam, Cambridge MA) in which the formation of NADH from lactate oxidation is couple to the formation of a proprietary that absorbs light at 450 nm.

Statistical methods

Brain enzyme activities in pyruvate-treated, NaCl-treated and sham pigs were compared by two-factor (time, treatment) analyses of variance with repeated measures on time and Student-

Newman-Keuls *post-hoc* analyses. Values of $p<0.05$ were taken to indicate statistically significant effects of time or treatment. Mean values ± SEM are shown in the figures.

RESULTS

Glutathione peroxidase and reductase activities in brain are impaired by cardiac-arrest resuscitation and hyperoxic ventilation

In NaCl- and pyruvate-treated pigs who were subjected to cardiac arrest-resuscitation, hippocampus glutathione peroxidase activity at 1 h ROSC in the hippocampus (Figure 1A) was only 20% of the value in sham pigs, but the difference was not statistically significant ($p=0.144$). Glutathione peroxidase activity fell drastically in the sham pigs between the time points corresponding to 1 h and 4 h ROSC, to a value similar to that of the NaCl-infused cardiac arrest pigs. In the pyruvate-treated pigs subjected to cardiac arrest, at 1 h ROSC glutathione peroxidase activity was not significantly different from that of sham ($P=0.283$) and NaCl-treated post-arrest ($p=0.202$) pigs. After pyruvate infusion was discontinued, glutathione peroxidase activity fell even further ($p=0.111$ *vs.* 1 h ROSC) and was similar to that of the NaCl resuscitated pigs (Figure 1A). The effects of cardiac arrest-resuscitation and pyruvate treatment were more striking in the cerebellum (Figure 1 B). Here, glutathione peroxidase activity fell 91% *vs.* the contemporaneous sham value ($p<0.001$). Pyruvate treatment partially protected the enzyme, such that its activity was 4-fold higher than that of the NaCl-treated cardiac arrest group ($p=0.012$). As in the hippocampus, cerebellar glutathione peroxidase activity fell in the sham group between the 1 h and 4 h time points, to a value similar to that of the 4 h ROSC value in the NaCl-treated cardiac arrest group. Although cerebellar glutathione peroxidase activity at 4 h

ROSC in the pyruvate group exceeded that of the corresponding sham and post-NaCl cardiac arrest pigs, the differences were no longer statistically significant. Thus, glutathione peroxidase activity was severely compromised by cardiac arrest-resuscitation and did not recover during subsequent ROSC under hyperoxic conditions; pyruvate treatment patially protected the enzyme, particularly in the cerebellum, but this protection waned after pyruvate infusion was discontinued; and glutathione peroxidase activity fell in the non-arrested shams as hyperoxic ventilation was extended to 4 h. Glutathione reductase activity fell appreciably in both hippocampus (Figure 2A) and cerebellum (Figure 2B) at 1 h ROSC *vs.* the respective sham activities. In the sham experiments, an additional 3 h hyperoxia decreased glutathione reductase activity to values similar to the 4 h ROSC values of the NaCl- and pyruvate-treated pigs. Pyruvate treatment did not appreciably preserve glutathione reductase activity at 1 h or 4 h ROSC.

Hyperoxia impairs lactate dehydrogenase in hippocampus and cerebellum

Lactate dehydrogenase activity in the hippocampus did not fall appreciably at 1 h ROSC *vs.* the respective sham value, and was not affected by pyruvate treatment. However, by 4 h lactate dehydrogenase activity fell sharply in all 3 groups, suggesting the enzyme was impaired by the ongoing hyperoxia. Lactate dehydrogenase activities in cerebellum were generally lower than those of hippocampus. However, pyruvate treatment increased cerebellar lactate dehydrogenase activity to a value similar to that of the hippocampus of pyruvate-treated pigs. Nevertheless, lactate dehydrogenase activity fell to that of the NaCl-treated pigs and 4 h shams after pyruvate infusion was terminated. Thus, lactate dehydrogenase activity was only modestly affected by

cardiac arrest-resuscitation, but like the glutathione peroxidase-reductase system, lactate dehydrogenase in hippocampus and cerebellum was impaired by subsequent hyperoxia.

DISCUSSION

A pivotal component of the endogenous antioxidant defenses, the glutathione peroxidase/reductase system is crucial to cell survival in brain in the face of oxidative stress. Glutathione peroxidase defends cells from oxidative stress by transferring electrons from glutathione (GSH) to reduce hydrogen peroxide to water and organic peroxides to their conjugate alcohols. In these reactions, GSH is oxidized to glutathione disulfide (GSSG). Glutathione reductase then transfers electrons from NADPH to reduce GSSG to GSH. These reactions serve to defend the brain parenchyma from the toxic effects of oxidative stress.

Ischemia-reperfusion imposed by cardiac arrest-resuscitation provokes intense formation and accumulation in brain and other tissues of reactive oxygen (Becker 2004; Idris et al. 2005) and nitrogen species, or RONS (Thiyagarajan et al. 2004; Zhu et al. 2004). Accumulation of RONS within affected tissue leads to lipid peroxidation, and covalent modification and/or inactivation of enzymes essential for cell survival. The injury cascades triggered by this oxidative stress substantially impact the hippocampus and cerebellum, two regions highly susceptible to oxidative ischemia-reperfusion injury (White et al. 2000; Dohi et al. 2003; Becker 2004; Zhu et al. 2004), manifesting as neuronal death and neurocognitive impairment and is characterized by cell death. The excess reactive oxygen species aslo activate the inducible nitric oxide synthase isoform (iNOS), which then overproduces nitric oxide (Manukhina et al. 2006; Kalyanaraman

2013). The nitric oxide produced by overactive iNOS combines with superoxide formed from uncoupled eNOS to form peroxynitrite, which inactivates proteins essential for cellular function and converts antioxidant glutathione to non-antioxidant S-nitrosyl glutathione (Manukhina et al. 2006; Kalogeris et al. 2012; Kalyanaraman 2013).

Recently, ventilating resuscitated patients with 100% oxygen has been the subject of considerable debate as mounting evidence suggests that the resultant hyperoxia may lead to overproduction of RONS (Richards et al. 2006, 2007; Pilcher et al. 2012) and, thus, exacerbate brain injury after cardiac arrest. Brücken *et al.* demonstrated that resuscitation with 100% oxygen dramatically increased the number of necrotic neurons in porcine brain after cardiac arrest, but without producing neurocognitive impairment (Brücken et al. 2010). In a secondary analysis of previously published data from a multicenter cohort, Kilgannon *et al.* reported an association between patients presenting with post-resuscitation hyperoxemia and risk of in-hospital death (Kilgannon et al. 2011). Moreover, a recently published review of post-ROSC hyperoxia (Wang et al. 2014) shows that hyperoxia is correlated with increased in-hospital mortality after resuscitation. Thus, hyperoxia after global ischemia-reperfusion has detrimental effects that are attributable to increased RONS, which compound of the harmful effects of cardiac arrest-resuscitation.

The sharp decrease in cerebellar glutathione peroxidase activity (Figure 1) after cardiac arrest and resuscitation may reflect the intensity of the RONS production under these conditions, which could potentially overwhelm endogenous antioxidant defenses including the peroxidase-

reductase system. Glutathione peroxidase is inactivated by oxidative stress, especially when accompanied by hyperoxia (Ansari et al. 2008; Kuo et al. 2011; Eynan et al. 2014). Glutathione peroxidase is disabled by protein carbonylation and nitrosylation of tyrosine residues during oxidative stress (Ansari et al. 2008). Although antioxidant pyruvate during CPR and the first hour ROSC temporarily protected glutathione peroxidase within the cerebellum, the enzyme activity nevertheless fell over the 3 h following treatment infusion. Similarly, glutathione reductase activity was markedly reduced following cardiac arrest-resuscitation, but pyruvate treatment did not protect glutathione reductase. Remarkably, activities of the glutathione peroxidase/reductase system had fallen to the same extent in the sham pigs after 4 h hyperoxic ventilation as in the post-resuscitation pigs. This outcome suggests that the hyperoxia produced by 100% oxygen had imposed oxidative stress sufficiently severe to not only prevent recovery of glutathione peroxidase/reductase activity after cardiac arrest, but also to independently impair the antioxidant system. Thus, hyperoxic ventilation alone can inactivate glutathione peroxidase/reductase, and this effect exacerbates the negative impact of cardiac arrest on this critical antioxidant system. Moreover, pyruvate protection of glutathione peroxidase did not persist in the face of continued hyperoxia as the metabolite cleared from the circulation.

Lactate dehydrogenase is a pivotal enzyme in cellular metabolism. Under aerobic conditions lactate dehydrogenase initiates lactate oxidation, enabling lactate to be oxidized as energy substrate in the brain. Under anaerobic conditions, the enzyme generates lactate from pyruvate and concomitantly oxidizes NADH to NAD^+ thereby enabling continued anaerobic glycolysis. Although lactate dehydrogenase is generally considered to be resistant to oxidative stress (Bogaert et al. 1994), lactate dehydrogenase activity within the brain, and specifically the

hippocampus, may be modulated by tyrosine nitration (Kuo et al. 2000), S-glutathionylation (Townshend et al. 2006), and S-nitrosylation (Zahid et al. 2014). The present study provides evidence supporting the notion that oxido-nitrosative stress decreases lactate dehydrogenase activity. The reduction in LDH activity within hippocampus, and to a lesser degree cerebellum, between 1 and 4 h ROSC can be attributed to the oxidative stress imposed by hyperoxia, because a similar decline was detected in the sham experiments. In the cerebellum, pyruvate treatment increases LDH activity at 1 h ROSC. A similar but less substantial pyruvate enhancement of lactate dehydrogenase was demonstrated in canine myocardium after cardiac arrest, and the pharmacological antioxidant N-acetylcysteine had a similar effect (Sharma et al. 2007). Thus, pyruvate's preservation of cerebellar lactate dehydrogenase is likely due to the metabolite's ability to directly neutralize RONS and prevent oxidative modification of the enzyme.

Limitations

The experimental model differed in several important respects from clinical cardiac arrest. Experiments were conducted in healthy, juvenile swine that were free of chronic diseases that afflict most cardiac arrest patients. Cardiac arrest was induced in fully anesthetized animals. Chest compressions were begun at 6 min cardiac arrest, and defibrillation attempted at 10 min arrest to ensure a high survival rate, whereas the durations of cardiac arrest and CPR often are more prolonged in human victims and are associated with much higher mortality. Additionally, the time required between induction of ventricular fibrillation for euthanasia and removal of the brain and flash-freezing of biopsies imposed further ischemia, which was considered negligible. This study examined the impact of cardiac arrest-resuscitation, hyperoxia and pyruvate on the

glutathione peroxidase-reductase system. Although these enzymes are central components of the brain's antioxidant defenses, there are a host of antioxidant enzymes and substrates that merit investigation. Also, acute changes in glutathione peroxidase and reductase over the first 4 h ROSC were examined, but the effects of prolonged recovery and/or hyperoxia, including potential phase II gene responses to RONS, remains unknown.

CONCLUSIONS

Cardiac arrest-resuscitation partially disabled the glutathione peroxidase/reductase system in porcine hippocampus and cerebellum. These enzymes did not recover and in some cases were further impaired as ROSC progressed. Pyruvate temporarily preserved cerebellar glutathione from cardiac arrest-resuscitation, but this effect subsided in the face of continued hyperoxia exposure between 1 and 4 h ROSC. Even in non-arrested pigs, the activities of glutathione peroxidase and reductase fell sharply over the course of the sham protocol, suggesting that hyperoxic ventilation alone imposes sufficient oxidative stress to disable this antioxidant system. For the first time, this study demonstrates, in a model of cardiac arrest and closed-chest CPR, reduction in the activities of endogenous antioxidant defenses in the brain as the result of ischemia-reperfusion, and furthermore, that hyperoxic ventilation alone was enough to compromise antioxidant defenses and lactate dehydrogenase in hippocampus and cerebellum. Clinically, ischemia-reperfusion injury sustained during cardiac arrest creates a cascade of damage that culminates in long-term neurological impairment, and in most cases, death. Intravenous sodium pyruvate effected partial protection of glutathione peroxidase, but this

salutary effect subsided after pyruvate treatment was terminated. Thus, temporary pyruvate may be of limited neuroprotective benefit in the setting of persistent hyperoxia.

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FIGURE LEGENDS

Figure 1. *Pyruvate partially protects glutathione peroxidase activity*. A: Glutathione peroxidase activity in hippocampus sharply decreased at 4 h (grey bars) *vs.* 1 h (black bars) ROSC; B: Glutathione peroxidase activity in cerebellum sharply decreased at 4 h (grey bars) *vs.* 1 h (black bars) ROSC, and pyruvate partially prevented the fall in activity. All values are reported as U/mg protein ± S.E.M. *p<0.05 *vs*. 1 h; † p<0.05 *vs*. sham; ‡ p<0.05 *vs.* NaCl. NaCl: pigs subjected to cardiac arrest, CPR and resuscitation with *iv* NaCl; Pyruvate: pigs subjected to cardiac arrest, CPR and resuscitation with *iv* Na-pyruvate.

Figure 2. *Glutathione reductase activity in cerebellum and hippocampus at 1 and 4 h ROSC.* A: Glutathione reductase activity in hippocampus sharply decreased at 4 h (grey bars) *vs.* 1 h (black bars) ROSC; B: Glutathione reductase activity in cerebellum sharply decreased at 4 h (grey bars) *vs.* 1 h (black bars) ROSC. All values are reported as U/mg protein ± S.E.M. *p<0.05 *vs*. 1 h; † p<0.05 *vs*. sham. NaCl: pigs subjected to cardiac arrest, CPR and resuscitation with *iv* NaCl; Pyruvate: pigs subjected to cardiac arrest, CPR and resuscitation with *iv* Na-pyruvate.

Figure 3. *Lactate dehydrogenase activity in cerebellum and hippocampus at 1 and 4 h ROSC.* A: LDH activity in hippocampus sharply decreased at 4 h (grey bars) *vs.* 1 h (black bars) ROSC in NaCl-treated pigs; B: LDH activity in cerebellum sharply decreased at 4 h (grey bars) *vs.* 1 h (black bars) ROSC in pyruvate-treated pigs, however pyruvate increased activity at 1 h. All values are reported as U/mg protein ± S.E.M. *p<0.05 *vs*. 1 h; † p<0.05 *vs*. sham; ‡ p<0.05 *vs.*

NaCl. NaCl: pigs subjected to cardiac arrest, CPR and resuscitation with *iv* NaCl; Pyruvate: pigs subjected to cardiac arrest, CPR and resuscitation with *iv* Na-pyruvate.

Figure 4. *Glutathione peroxidase-reductase antioxidant system.* Glutathione peroxidase uses glutathione (GSH) to Reduce H_2O_2 to H_2O, yielding glutathione disulfide (GSSG). Glutathione reductase reduces GSSG to GSH by oxidizing NADPH to $NADP^+$. GPx: glutathione peroxidase; GRed: glutathione reductase.

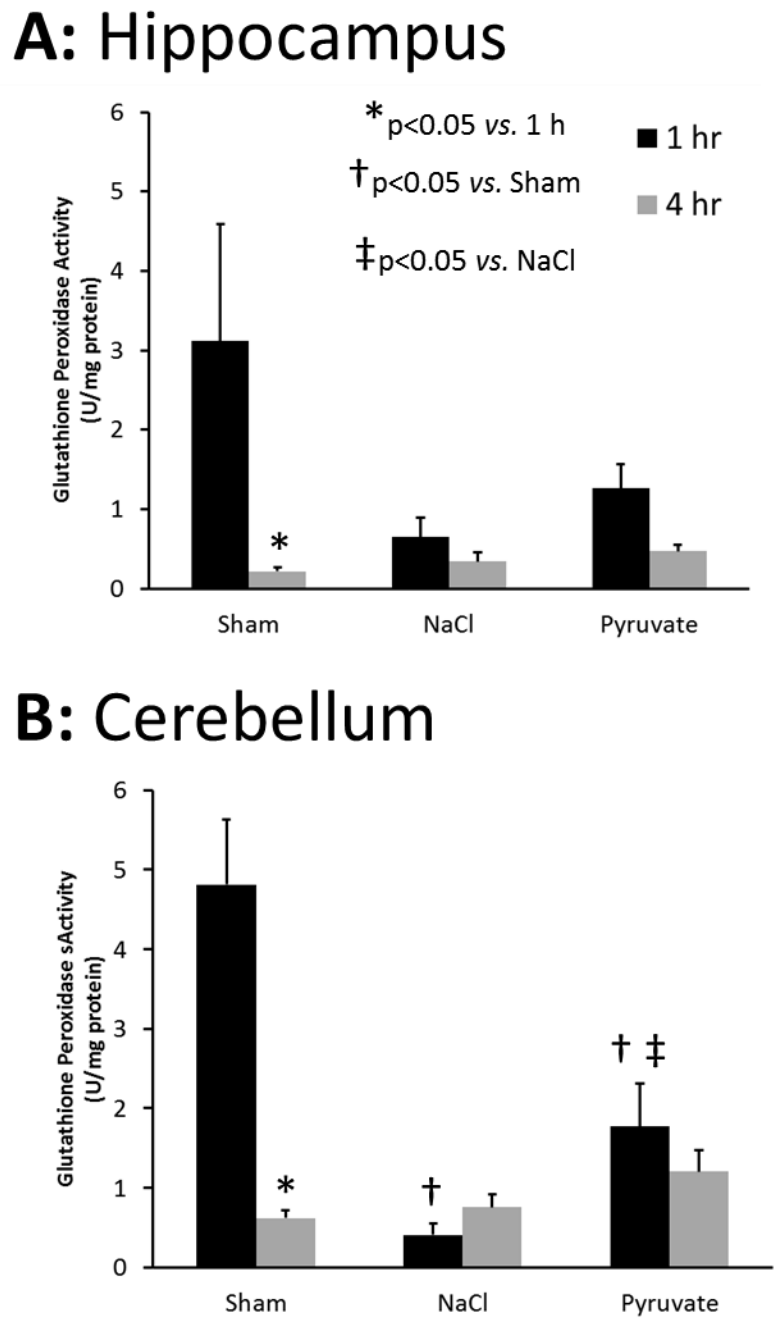
A: Hippocampus
*p<0.05 vs. 1 h
†p<0.05 vs. Sham
‡p<0.05 vs. NaCl
1 hr
4 hr
Glutathione Peroxidase Activity (U/mg protein)
Sham
NaCl
Pyruvate
B: Cerebellum
Glutathione Peroxidase sActivity (U/mg protein)
Sham
NaCl
Pyruvate

Figure 1

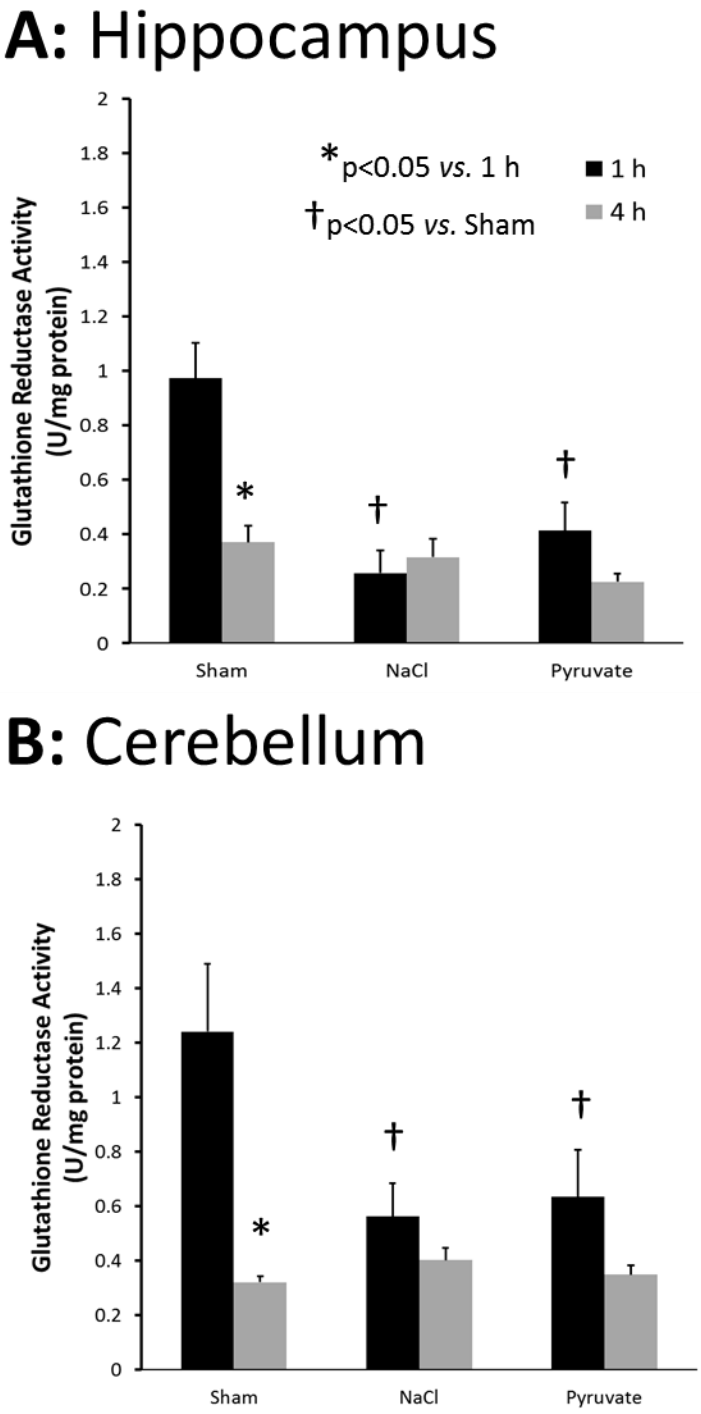
A: Hippocampus
*p<0.05 vs. 1 h
†p<0.05 vs. Sham
1 h
4 h
Glutathione Reductase Activity (U/mg protein)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sham
NaCl
Pyruvate
B: Cerebellum
Glutathione Reductase Activity (U/mg protein)
Sham
NaCl
Pyruvate

Figure 2

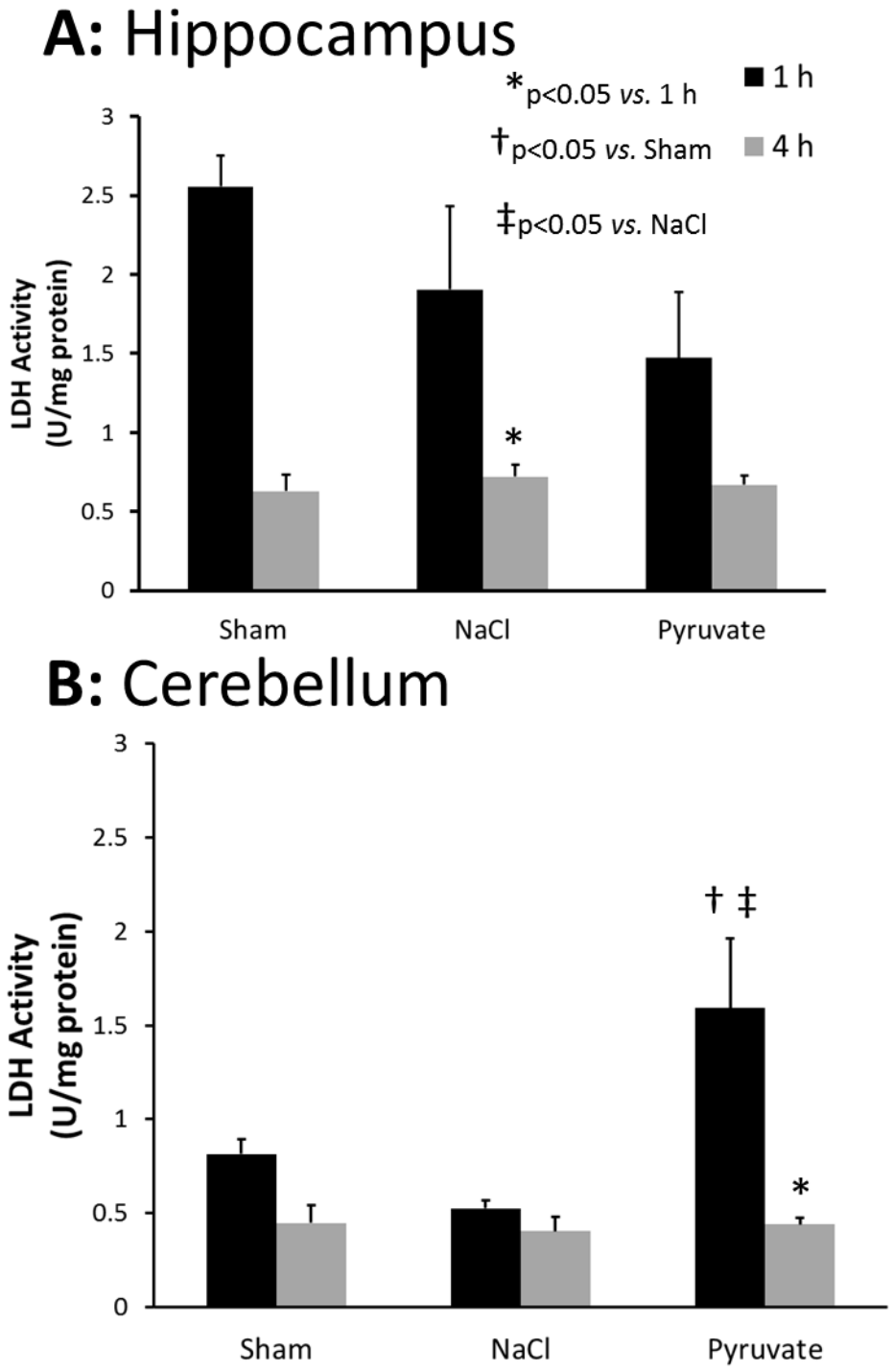
A: Hippocampus
*p<0.05 vs. 1 h
†p<0.05 vs. Sham
‡p<0.05 vs. NaCl
1 h
4 h
LDH Activity (U/mg protein)
3
2.5
2
1.5
1
0.5
0
*
Sham
NaCl
Pyruvate
B: Cerebellum
LDH Activity (U/mg protein)
3
2.5
2
1.5
1
0.5
0
† ‡
*
Sham
NaCl
Pyruvate

Figure 3

Figure 4

CHAPTER V

Conclusions

This investigation focused on the impact of pyruvate's electrocardiographic, metabolic, and antioxidant effects in a porcine model of cardiac arrest and cardiopulmonary resuscitation. In the studies presented here, we conclude that: 1) *iv* infusion of pyruvate during cardiac arrest, CPR and early ROSC prevented development of lethal pulseless electrical activity as the initial rhythm following defibrillation; 2) Once ROSC was achieved, pyruvate hastened clearance of glucose to promote metabolic stability; 3) Pyruvate increased the arterial bicarbonate concentration and pH to combat post-resuscitation acidemia; 4) Cardiac arrest-resuscitation partially disabled glutathione peroxidase-reductase activity; 5) Pyruvate dampened oxidative inactivation of glutathione peroxidase, an endogenous antioxidant defense crucial to prevention of oxidative ischemia-reperfusion injury; and 6) Hyperoxic ventilation alone was sufficient to sharply decrease activities of the glutathione peroxidase-reductase system. Thus, while pyruvate promotes ROSC and metabolic stability following cardiac arrest, it may be of limited neuroprotective benefit in the setting of persistent hyperoxia.

CHAPTER VI

Future Directions

Long-term effects of pyruvate treatment post-ROSC

As more is learned about the immediate neuroprotective effects of pyruvate (*i.e.* during early ROSC), the effects of infusion later in recovery remain to be determined. Studies, therefore, are needed to examine the potential neuroprotective effects of pyruvate at 3 d and 7 d ROSC. Additionally, since pyruvate's protective effects on cerebellar glutathione peroxidase-reductase activity dissipated after treatment infusion was discontinued, it stands to reason that longer infusion of pyruvate may exert more robust preservation of enzyme activity. Therefore, studies must be conducted to determine the effects of longer duration infusion on the brain.

Oxidative stress

Further characterization of the temporal and spatial impact of post-resuscitation oxidative stress on brain is necessary. It is likely that all regions of the brain do not respond the same to cardiac arrest-resuscitation, and pyruvate may afford greater protection to some regions of the brain than others. Indeed, the present study revealed discrete differences in enzyme activities and the effects of cardiac arrest-resuscitation in hippocampus *vs.* cerebellum. More sensitive measures of oxidative stress would greatly improve our understanding of exactly how the reactive oxygen and nitrogen species are damaging brain tissue. 4-hydroxy-2-nonenal (4-HNE) is one of the major end products of lipid peroxidation, and has been shown to affect events of the cell cycle in a concentration-dependent manner (Awasthi et al. 2004). At higher concentrations, 4-HNE

signals apoptosis, which kills brain neurons after cardiac arrest. Given its role in cell signaling, 4-HNE is a more reliable indication of lipid peroxidation and apoptotic signaling than conventional measurement of 8-isoprostane or malondialdehyde. Measurements of 4-HNE in post-resuscitation brain would reveal if lipid peroxidation is a key contributor to the cell death associated with ischemia-reperfusion. Additionally, measurements of the markers of oxidative stress in sham and NaCl/pyruvate-treated pigs ventilated with 21% instead of 100% oxygen would obviate the effects of hyperoxia revealed by this study and would allow for a more clear understanding of the oxidative effects on the brain of cardiac arrest alone. Finally, comparison of pyruvate with other antioxidants such as N-acetylcysteine would allow us to determine whether direct or downstream antioxidant effects of pyruvate are predominant following ischemia-reperfusion.

Protection of other vital organs from cardiac arrest

Through its antioxidant and anti-inflammatory actions, pyruvate could prevent cardiac arrest-resuscitation-induced ischemia-reperfusion injury in other vital organs such as lung and kidney. Pulmonary function following resuscitation is critical to maintain blood and tissue oxygenation to support long-term recovery. Pyruvate could potentially dampen pulmonary edema resulting from post-resuscitation inflammatory responses and thereby promote recovery. Another organ susceptible to injury from cardiac arrest is the kidney. Renal clearance of waste products following cardiac arrest and resuscitation is crucial to restore metabolic and electrolyte homeostasis. Since that the main source of endogenous erythropoietin is the peritubular interstitial cells of the renal corticomedullary border, pyruvate could upregulate erythropoietin production via the hypoxia-inducible factor (HIF) pathway and thereby potentially exploit the

antioxidant (Kumral et al. 2005; Genc et al. 2006; Zhang et al. 2010) and anti-inflammatory (Kadri et al. 2000; Villa et al. 2003; Li et al. 2008) actions of the hormone.

Clinical Trials

Our laboratory has previously demonstrated pyruvate's ability to prevent ischemia-reperfusion injury in skeletal muscle (Flaherty et al. 2010; Gurji et al. 2014) and the heart (Flaherty et al. 2010; Gurji et al. 2013) following hypovolemia, the heart during cardiopulmonary bypass (Knott et al. 2006; Ryou et al. 2009, 2010), and the brain following stroke (Ryou et al. 2012). Pyruvate is non-toxic at a wide range of doses, and is stable in crystalline state. Randomized clinical trials of resuscitation with and without pyruvate infusion would provide insight into the translatability of research conducted in animal models of ischemia reperfusion. Pyruvate could be stored in its crystalline form in a vial that would be included with standard emergency medical supplies to be resuspended and added to a bag of sterile water at onset of out-of-hospital resuscitation. Since pyruvate is non-toxic and an endogenous intermediary metabolite, it would be inexpensive to provide to emergency medical personnel for trials.

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Appendix

Neuronal Injury from Cardiac Arrest: Aging Years in Minutes

Brandon H Cherry,[1,3,4] Nathalie Sumien,[2,3] Robert T Mallet[1,3,4]

Departments of [1]Integrative Physiology and [2]Pharmacology and Neuroscience, [3]Institute for Aging and Alzheimer's Disease Research, and [4]Cardiovascular Research Institute, University of North Texas Health Science Center, Fort Worth, TX, USA

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ABSTRACT

Cardiac arrest is a leading cause of death and permanent disability. Most victims succumb to the oxidative and inflammatory damage sustained during cardiac arrest/resuscitation, but even survivors typically battle long-term neurocognitive impairment. Although extensive research has delineated the complex mechanisms that culminate in neuronal damage and death, no effective treatments have been developed to interrupt these mechanisms. Of importance, many of these injury cascades are also active in the aging brain, where neurons and other cells are under persistent oxidative and inflammatory stress which eventually damages or kills the cells. In light of these similarities, it is reasonable to propose that the brain essentially ages the equivalent of several years within the few minutes taken to resuscitate a patient from cardiac arrest. Accordingly, cardiac arrest-resuscitation models may afford an opportunity to study the deleterious mechanisms underlying the aging process, on an accelerated time course. The aging and resuscitation fields both stand to gain pivotal insights from one another regarding the mechanisms of injury sustained during resuscitation from cardiac arrest and during aging. This synergism between the two fields could be harnessed to foster development of treatments to not only save lives, but also to enhance the quality of life for the elderly.

INTRODUCTION

Cardiac arrest remains a leading cause of death and persistent disability in the United States. In its 2014 update on heart disease and stroke statistics, the American Heart Association estimated that approximately 380,000 out of 424,000 (~90%) Americans who experience out-of-hospital cardiac arrest annually do not survive (Go et al. 2014). Only 23% (97,520) of all cardiac arrest victims present to emergency medical services personnel with a shockable cardiac rhythm, and most who are initially resuscitated later succumb to extensive ischemia-reperfusion injury to the brain and other vital organs (Dezfulian et al. 2009; Heron 2012; Nolan et al. 2012; Young 2009; Go et al. 2014). Moreover, approximately half of the *c.* 10% of cardiac arrest victims who do survive to hospital discharge experience persistent neurocognitive impairment manifested as memory and sensorimotor deficits that profoundly impact their quality of life (Adrie et al. 2004; Moulaert et al. 2009; Wachelder et al. 2009; Young 2009; Go et al. 2014).

While many studies have examined the complex mechanisms of brain damage following cardiac arrest-initiated ischemia-reperfusion injury, the precise cascade of events culminating in neurocognitive impairment remains to be completely delineated. It is known that ATP depletion, intracellular Ca^{2+} overload (Bano and Nicotera 2007; Li et al. 2007), reactive oxygen and nitrogen species (Calapai et al. 2000), inflammation and glutamate-induced excitotoxicity (Conroy et al. 1999; Backstrom et al. 2003) initiated by cardiac arrest and resuscitation collectively inflict lethal damage to neurons, oligodendrocytes, microglia and the cerebrovascular endothelium, and disrupt the blood-brain barrier (BBB). Despite mounting knowledge of the mechanisms of brain injury, currently there are no clinically proven

pharmacological treatments to protect the brain during cardiac arrest and cardiopulmonary resuscitation (CPR) (Dezfulian et al. 2009).

Similar to long-term recovery from cardiac arrest and CPR, the principal mechanisms of neurocognitive impairment in the aging brain have yet to be assembled into a coherent cascade of events that would allow for development of efficacious preventative therapies. However, it is becoming increasingly evident that, many age-related changes leading to neuronal damage and death parallel those observed during and following cardiac arrest. Specifically, accumulation of reactive oxygen and nitrogen species, as well as proinflammatory cytokines and markers of inflammation have all been observed in brain aging studies (Hagen 2003; Kregel and Zhang 2007; Cortese et al. 2011). Moreover, as the brain ages calcium mismanagement and mitochondrial dysfunction also contribute to the death and dysfunction of neurons and other cells within the most vulnerable regions such as the hippocampus (Landfield 1988; Foster and Norris 1997; Toescu et al. 2004). The progression of neuronal impairment and cell death observed during aging is, however, a much slower process than the injury cascade that follows cardiac arrest, CPR, and post-arrest recovery. The purpose of this review is to highlight parallel mechanisms of brain damage and neuronal death that ensue following cardiac arrest and in the aging brain. Despite their different time courses, mechanistic information gained from studying the two conditions could be harnessed to synergistically advance both fields and to develop treatments targeting specific components in these neurodegenerative pathways to provide more robust protection of patients from neurocognitive impairment and/or death.

OXIDATIVE STRESS

Oxidative injury during cardiac arrest and cardiopulmonary resuscitation

A major culprit in the ischemia-reperfusion injury sustained following cardiac arrest and CPR is the oxidative stress imposed on the brain (Idris et al. 2005; Wang et al. 2007). Intense formation and accumulation of reactive oxygen (Opie 1991; Cerchiari et al. 1987; Becker 2004; Idris et al. 2005) and nitrogen species, *i.e.* ROS/RNS (Lipton 1999; Love 1999; White et al. 2000; Dohi et al. 2003; Keynes and Garthwaite 2004; Thiyagarajan et al. 2004; Zhu et al. 2004) within the affected tissue leads to lipid peroxidation, inactivation of metabolic enzymes, and mitochondrial dysfunction, which collectively ignite a cascade of cell death that manifests as neurocognitive impairment once brain regions such as the hippocampus and cerebellum—which are highly susceptible to oxidative damage—are substantially impacted (Cerchiari et al. 1987; Brown and Borutaite 1999; White et al. 2000; Dohi et al. 2003; Becker 2004; Zhu et al. 2004). When its co-factor, tetrahydrobiopterin (BH_4), is oxidized by superoxide and hydrogen peroxide, the endothelial isoform of nitric oxide synthase (eNOS) becomes uncoupled and no longer generates nitric oxide (NO), instead producing the superoxide anion (Figure 1) (Manukhina et al. 2006; Kalyanaraman 2013). The resultant excess of reactive oxygen species (ROS) activates the inducible NOS isoform (iNOS), which then overproduces NO (Manukhina et al. 2006; Kalyanaraman 2013). iNOS-generated NO combines with superoxide from uncoupled eNOS to form peroxynitrite, which nitrosylates tyrosine residues, thereby inactivating proteins essential for cellular energy metabolism and function, and nitrosylates the pivotal intracellular antioxidant glutathione to form non-antioxidant *S*-nitrosyl glutathione (Manukhina et al. 2006; Kalogeris et al. 2012; Kalyanaraman 2013). Figure 1 summarizes this vicious cycle of peroxynitrite generation.

During ischemia, the intracellular accumulation of protons from anaerobic glycolysis and ATP hydrolysis causes an abrupt drop in cytosolic pH (Kalogeris et al. 2012). To minimize intracellular acidification, the Na^+/H^+ exchanger expels H^+ from cells in exchange for Na^+ (Baines 2010). Intracellular Na^+ ions are then exchanged for extracellular Ca^{2+} by Na^+/Ca^{2+} countertransport (Kalogeris et al. 2012). Upon reperfusion, washout of extracellular H^+ by restored circulation increases the H^+ gradient across the cell membrane and accelerates the actions of the Na^+/H^+ and Na^+/Ca^{2+} exchangers, exacerbating the intracellular Ca^{2+} overload (Baines 2010; Kalogeris et al. 2012). In combination with excess Ca^{2+}, the reperfusion burst of ROS/RNS triggers integration of the pro-apoptotic Bcl2 family proteins, Bax and Bak, into the outer mitochondrial membrane (Baines 2010). The pore formed by Bax/Bak enables efflux of small mitochondrial proteins such as cytochrome c, second mitochondria-derived activator of caspases (SMAC), and endonuclease-G (Baines 2010). In the cytosol, SMAC and cytochrome c combine with apoptotic protease activating factor 1 (APAF1), forming an apoptosome which activates caspases -9 and -3 (Baines 2010). In concert with caspase-mediated pro-apoptotic signaling, Bax/Bak permits endonuclease-G efflux from mitochondria; this enzyme enters the nucleus and fragments genomic DNA, a pivotal event in apoptotic cell death (Baines 2010). The post-ischemic Ca^{2+} overload and ROS/RNS burst in the mitochondrial matrix also open a large, non-selective channel in the inner mitochondrial membrane, the mitochondrial permeability transition pore (mPTP). Opening of mPTP collapses the proton electrochemical gradient required for oxidative phosphorylation, further draining cellular ATP reserves already depleted by ischemia (Baines 2010; Halestrap 2010; Kalogeris et al. 2012). Figure 2 summarizes the cascade by which this intense oxidative insult ultimately opens both the Bax/Bak and mPTP pores, thereby activating caspases -9 and -3, DNA fragmentation and mitochondrial rupture

culminating in apoptosis of neurons and astroglia (Kirkland and Franklin 2003; Baines 2010; Halestrap 2010; Franklin 2011; Martin et al. 2011).

Oxidative injury during aging

The "oxidative stress theory" of aging identifies the accumulation of oxidative damage caused by ROS/RNS over the course of the aging process as pivotal to the progressive decline of biological function and shorter lifespan (Kregel and Zhang 2007). According to this paradigm, ROS as well as RNS accumulate due to an imbalance between their production and detoxification by endogenous redox systems, *e.g*. the glutathione peroxidase/reductase and thioredoxin/peroxiredoxin systems (Hagen 2003; Kregel and Zhang 2007). This oxidative imbalance potentiates deleterious protein oxidation, lipid peroxidation, and apoptotic cell death (Blumberg 2004; Stadtman 2004; Matsuzawa and Ichijo 2005).

One line of evidence supporting the "oxidative stress theory" of aging stems from measurements of the common biomarkers of antioxidative capacity—that is, the degree to which the body is able to neutralize existing and *de novo* oxidative stress via its endogenous antioxidant defense mechanisms. Among the most widely accepted measures of *in vivo* redox state are the ratios of reduced to oxidized glutathione (GSH/GSSG) and nicotinamide adenine dinucleotide phosphate ($NADPH/NAPD^{+}$) (Kregel and Zhang 2007). Of these redox systems, GSH/GSSG can be taken as a global measure of the collective poise of endogenous antioxidant defenses, as the glutathione reductase/peroxidase system is linked, via redox cycles, to the other cellular antioxidant systems (Schafer and Buettner 2001). A progressive decline in GSH/GSSG with advancing age has been identified (Droge 2002), suggesting either a decreased ability of cells to

neutralize oxidative stress, increased formation of ROS/RNS, or perhaps impaired GSH generation as the result of decreased glutathione reductase activity. In support of the latter possibility, several studies have reported oxidative inactivation of metabolic enzymes and membrane lipid peroxidation in aging brain (Beckman and Ames 1998; Bokov et al. 2004; Poon et al. 2004; Rodrigues Siqueira et al. 2005; Kregel and Zhang 2007). A major source of oxidant accumulation with age is mitochondrial dysfunction leading to overproduction and release of ROS/RNS into the cytosol (Sohal et al. 1995; Giulivi 1998). The ROS/RNS then uncouple eNOS, causing the enzyme to overproduce superoxide (O_2^-), which activates iNOS and drives the production of cytotoxic peroxynitrite in a manner similar to that of cardiac arrest induced brain ischemia-reperfusion (Manukhina et al. 2006; Ungvari et al. 2010; Kalyanaraman 2013). Collectively, cytosolic ROS/RNS accumulation activates death cascades mediated by caspases -9 and -3 (Kirkland and Franklin 2003; Franklin 2011; Martin et al. 2011). Finally, it is important to note that chronic oxidative stress with advancing age has been implicated in the pathogenesis of age-related neurodegenerative disorders (Volicer and Crino 1990; Dexter et al. 1994).

Oxidative damage to mitochondrial DNA

According to the mitochondrial theory of aging, the accumulation of mutations in mitochondrial DNA (mtDNA) over the lifetime leads to bioenergetic impairment and contributes substantially to aging (Linnane et al. 1989; Lee and Wei 2007). Because of its close proximity to the respiratory chain, the mitochondrial genome is particularly susceptible to oxidative damage from excessive ROS/RNS produced during ischemia-reperfusion (Richter 1995; Chen et al. 2001) and accumulated during aging (Mecocci et al. 1993; Ozawa 1995; Barja and Herrero 2000). Moreover, mtDNA is much more vulnerable to oxidative modification than nuclear DNA (Ames

et al. 1993; Richter 1995), due to its lack of protection by histones and the limited ability to repair mtDNA, compared with nuclear DNA (Croteau et al. 1999; Lee and Wei 2007). Modification of mtDNA by ROS/RNS may alter genes expressing protein components of the respiratory chain, which creates a vicious cycle of ROS/RNS over-production and further cell damage, eventually culminating in apoptosis (Murakami et al. 1998; Chen et al. 2001; Lee and Wei 2007). Indeed, disturbances of mitochondrial gene expression which disable oxidative phosphorylation within the CA1 neurons of the hippocampus have been demonstrated during reperfusion, and ultimately culminate in cell death (Abe et al. 1996).

Neurocognitive impairment from oxidative stress

Survivors of cardiac arrest often endure cognitive and behavioral impairments such as deficits in long-term memory and executive function (Parnia et al. 2007). For example, between 2 months to 1 year after resuscitation, patients' ability to recall memory is impaired (Grubb et al. 1996). Computed tomography and magnetic resonance imaging revealed that oxidant-induced tissue atrophy following cardiac arrest extended beyond the hippocampus to involve the frontal and temporal lobes, which relates to the disrupted executive function in cardiac arrest survivors (Grubb et al. 2000; Nunes et al. 2003). In a longer-term study that compared cardiac arrest survivors to patients who survived myocardial infarction without cardiac arrest, memory scores in both groups declined with age, but the cardiac arrest survivors performed significantly worse on recall memory assessments *vs.* myocardial infarction survivors 3 years after the ischemic event (Drysdale et al. 2000). This impairment demonstrates that neurocognitive function may be sufficiently compromised to severely impact quality of life (O'Reilly et al. 2003). Similarly, memory and executive function become impaired during aging. As oxidative stress accumulates

with advancing age, the brain deterioration eventually causes neurocognitive impairments resembling to those that follow cardiac arrest-resuscitation (Mahncke et al. 2006; Kim & Oh 2013).

Oxidative stress has been implicated in the pathogenesis of neurodegeneration and neurocognitive impairment after cardiac arrest (Liu et al. 1998; Parnia et al. 2007; Fiskum et al. 2008) and during aging (Dexter et al. 1994; Volicer and Crino 1990). To test the hypothesis that overproduction of ROS/RNS produces neurocognitive impairment, Vereczki *et al.* compared resuscitation of dogs with 100% oxygen *vs.* room air (*c.* 21% oxygen) and found that hyperoxic resuscitation increased hippocampal tyrosine nitration—a marker of oxidative cell injury—and intensified post-ischemic impairment of hippocampus-dependent functions (Matsuzawa and Ichijo 2005; Vereczki et al. 2006; Kregel and Zhang 2007).

Summary

A common mechanism underlying brain deterioration both following cardiac arrest-resuscitation and during aging is the accumulation of ROS/RNS, which act to modify mtDNA, disrupt cellular function, initiate apoptosis and ultimately impair neurocognitive function. An important difference between aging *vs.* post-cardiac arrest is the time course of ROS/RNS accumulation—that is, alterations in ROS/RNS concentrations occur within minutes following resuscitation from cardiac arrest, but develop over many years during aging. In both cases, the accumulated ROS/RNS attack mitochondrial and nuclear DNA, inactivate enzymes catalyzing energy metabolism, compromise ATP production and impair the glutathione peroxidase/reductase and thioredoxin/peroxiredoxin antioxidant systems. Additionally, oxidative stress in both settings

provokes opening of the Bax/Bak and mitochondrial permeability transition pores, which respectively release cytochrome c into the cytosol and dissipate the electrochemical gradient required for oxidative phosphorylation. Cytochrome-c release initiates activation of caspases -9 and -3 and eventually apoptotic cell death within the affected brain tissue. Cell death caused by oxidative stress in both post-resuscitation and aging results in recall memory loss and deficits in executive function. Thus, aging and cardiac arrest-resuscitation produce remarkably similar cascades of oxidative stress, cell death, and neurocognitive impairment, albeit over vastly different time courses.

IMMUNE RESPONSE

Immune response to cardiac arrest and resuscitation

The sterile inflammatory response—*i.e*. that in the absence of microorganisms—to ischemia and reperfusion during cardiac arrest and resuscitation is initiated in an effort to repair damaged tissue. In a manner similar to the response directed against invading pathogens, ischemia increases neutrophil recruitment and production of cytokines, chemokines, and other pro-inflammatory stimuli with in the brain (Kalogeris et al. 2012; Kvietys and Granger 2012). Activated neutrophils infiltrate the ischemic brain parenchyma and initiate damage by releasing ROS/RNS, hydrolytic enzymes, and pore-forming molecules onto targeted cells (Kalogeris et al. 2012). The neutrophil-generated ROS/RNS promote leukocyte adhesion to post-capillary venules and their infiltration of the tissue, intensifying post-ischemic injury (Kalogeris et al. 2012; Kvietys and Granger 2012). In the brain capillary endothelium, xanthine oxidase and other ROS-forming enzymes perturb nitric oxide production and induce endothelial expression of leukocyte-specific adhesion molecules to promote adhesion of innate immune cells (Kalogeris

et al. 2012; Kvietys and Granger 2012). Moreover, during this time, other perivascular cells in the brain including macrophages and mast cells are activated and begin to release inflammatory mediators such as TNF-α, platelet-activating factor, leukotriene B4, and other cytokines to promote leukocyte adhesion to the post-capillary endothelium (Kalogeris et al. 2012). Collectively, these maladaptive responses to ischemia and reperfusion of the brain and capillary endothelium exacerbate the oxidative injury inflicted by cardiac arrest and provoke endothelium-dependent microcirculatory dysfunction, which disrupts delivery of nutrients and clearance of waste products after resuscitation(Jerome et al. 1995; Kalogeris et al. 2012; Kvietys and Granger 2012).

Release of TNF-α triggers an extrinsic apoptotic pathway in post-ischemic tissue that activates caspase-8, which then cleaves and activates caspase-3, leading to cleavage of cellular proteins and death of affected cells (Kroemer et al. 2007; Broughton et al. 2009). An intrinsic pathway is activated by oxidant-induced Bax and Bak integration into the outer mitochondrial membrane, allowing the aforementioned cytochrome-c release and activation of the caspase-9 and -3 cell death cascade (Kroemer et al. 2007; Broughton et al. 2009). Moreover, necrosis induced by ischemia-reperfusion also activates the complement system (Hill and Ward 1971; Rossen et al. 1994; Frangogiannis et al. 2002; Ioannou et al. 2011). The classical, alternative, and mannose-binding lectin complement pathways have all been implicated in ischemia-reperfusion injury (Kalogeris et al. 2012). The activated complement system recruits neutrophils and macrophages to the site of injury and also causes direct cell lysis by formation of a plasma membrane attack complex (Kalogeris et al. 2012). Thus, cardiac arrest leads to brain damage through a multifaceted mechanism of inflammation and cytotoxic pore formation.

Immune response to aging

Increased basal inflammation is considered an underlying mechanism of aging (Sierra et al. 2014). There is an age-related increase in proinflammatory cytokines in the aging brain, including IL-1β, IL-6, and TNFα (Krabbe et al. 2004; Diniz et al. 2014). Additionally, the chronic oxyradical burden that accompanies aging causes stress to the endoplasmic reticulum of microglia, which in turn provokes NF-κB activity to exacerbate the inflammatory response to aging (Hasnain et al. 2012). In accordance with the "oxidative stress theory" of aging, it is conceivable that the chronic accumulation of oxidants would potentiate an immune response similar to that ensuing after the acute, rapid accumulation of ROS/RNS following cardiac arrest-induced ischemia-reperfusion.

As endogenous antioxidant defenses are gradually depleted in the aging brain, sustained activation of perivascular macrophages by ROS/RNS would provoke these cells to infiltrate the brain parenchyma and cause damage and cell death. Specifically, by releasing ROS, proteolytic enzymes, and inflammatory cytokines(*e.g.* IL-1β, IL-6, and TNFα), these activated macrophages initiate mechanisms that incorporate Bax and Bak into the outer mitochondrial membrane (Kroemer et al. 2007; Broughton et al. 2009). The resulting cytochrome c release activates the intrinsic, caspase-mediated apoptotic pathway which eventually destroys the affected neurons, astrocytes and microglia (Kroemer et al. 2007; Broughton et al. 2009). When TNF-α released by the macrophages triggers the extrinsic apoptotic pathway, caspases -8 and -3 are activated, leading to further cell death (Kroemer et al. 2007; Broughton et al. 2009). The microcirculatory dysfunction summarized above exacerbates these intrinsic and extrinsic apoptotic pathways

(Jerome et al. 1995; Kalogeris et al. 2012; Kvietys and Granger 2012). During aging, chronic apoptosis of this nature would lead to degeneration of brain tissue in the regions that are particularly susceptible to oxidative stress, culminating in neurocognitive impairment. Additionally, recent studies have revealed that triggering of the innate immune system provokes an exaggerated local immune response within the hippocampus of aged rats (Barrientos et al. 2006; Cortese et al. 2011). In this experimental model, *Escherichia coli* were injected into the peritoneum of aged and young rats in order to activate the innate immune system. In response to signals triggered by this immune activation, aged rats showed a more intense inflammation within the brain than the young rats, exemplified by persistently increased hippocampal production of the pro-inflammatory cytokine interleukin-1β (Barrientos et al. 2006). This exaggerated inflammatory response did not affect short-term memory, but did produce substantial deficits in hippocampus-dependent long-term memory (Barrientos et al. 2006).

Summary

Cardiac arrest-resuscitation and aging trigger an immune response that provokes brain degeneration, particularly in the regions most susceptible to oxidative stress and oxidant-induced inflammation. In both cases, and by similar mechanisms, this response is exaggerated by excessive production of ROS/RNS and leads to activation of perivascular inflammatory macrophages. Once activated, these cells release pro-inflammatory cytokines to recruit neutrophils to the site of injury and provoke leukocyte adhesion to the post-capillary endothelium. The neutrophils release proteolytic enzymes and ROS/RNS to cause apoptosis of the injured cells, and the accumulated leukocytes then clear the cellular remnants, leading to degeneration of the regions affected by ischemia-reperfusion and aging. One susceptible region—the hippocampus—is pivotal in neurocognitive functions such as learning and memory.

Ischemia-reperfusion and aging impose oxidative stress, initiating an inflammatory response leading to tissue degeneration and neurocognitive impairment (Figure 2).

ANTIOXIDANT THERAPIES

Numerous preclinical and clinical studies have examined the potential neuroprotective effects of various antioxidants during both cardiac arrest (Table 1) and aging (Table 2). However, many agents that exerted robust neuroprotection in preclinical studies failed to protect when used in clinical trials. Thus, clinically-effective pharmacological treatments to mitigate oxidative stress and brain injury from cardiac arrest-resuscitation and/or aging remain elusive.

Antioxidant therapy following cardiac arrest-resuscitation

In rats subjected to ventricular fibrillation and CPR, treatment with ascorbic acid (vitamin C) following cardiac arrest reduced lipid peroxidation and mitochondrial oxidative stress (Tsai et al. 2011), but failed to preserve left ventricular distensibility during CPR and negatively impacted resuscitability (Motl et al. 2012). The study of Motl *et al.* (2012) suggests that scavenging ROS may disrupt protective oxidant-mediated signaling.

Erythropoietin minimizes ischemia-reperfusion injury of brain by stabilizing mitochondrial function and preventing formation of ROS (Nguyen et al. 2014). Erythropoietin induces key components of the brain's antioxidant defenses, such as glutathione *S*-transferase, NAD(P)H:quinone oxidoreductase-1 and heme oxygenase-1 (Zhang et al. 2010). Erythropoietin, however, does not readily traverse the blood brain barrier, so massive doses are required for neuroprotection, greatly increasing the risk for thrombosis and stroke (McPherson et al 2008).

Therapeutic hypothermia is the only intervention that has proven to be clinically effective in minimizing brain injury from cardiac arrest-induced ischemia-reperfusion. By slowing cellular metabolism, hypothermia dampens production of ROS and fortifies endogenous antioxidant defenses during rewarming (Dohi et al. 2013). In a swine model of cardiac arrest-resuscitation, therapeutic hypothermia maintained blood pressure and cerebral oxygenation after ROSC and prevented organ damage by suppressing oxidative stress (Ostadal et al. 2013). This antioxidant action of hypothermia during cardiac arrest is partly attributed to protection of respiratory enzymes and upregulation of an antioxidant enzyme, manganese superoxide dismutase (Gong et al. 2012).

Antioxidant therapy in aging

Ascorbic acid content of brain is lower in demented elderly individuals and an analysis of 894 patient records revealed that dementia patients taking pharmacological dosages of vitamin C were less likely to develop significant cognitive decline (von Arnim et al. 2012). However, supplementation with vitamin C has failed to prevent cognitive decline with aging (Arzi et al. 2004; Galasko et al. 2012). Similarly, α-tocopherol (vitamin E) had no impact on steady-state oxidative damage (Sumien et al. 2003) and short-term supplementation with vitamin E did not reverse preexisting age-related cognitive impairments in mice (Sumien et al. 2004), but did slow the decline in cognitive function in Alzheimer's disease patients (Dysken et al. 2014). On the other hand, co-administration of vitamin E with other antioxidant vitamins (vitamin C, coenzyme Q, α-lipoic acid) improved cognitive function in aged mice (Arzi et al. 2004; Macdonald et al. 2005) and elderly human subjects (Galasko et al. 2012). Finally, high-dose coenzyme Q_{10} was

shown to preserve spatial learning and decrease protein oxidation in brain mitochondria of aged mice when given for a short duration (Shetty et al. 2013).

Summary

Although antioxidant therapies have not proven unequivocally effective against oxidant-induced damage and neurological impairment following cardiac arrest-resuscitation and during aging, treatments are still being developed. Therapeutic hypothermia greatly reduces post-resuscitation oxidative stress and coenzyme Q administration has shown promise in preservation of cognitive function during aging, but it has been recently suggested by Ghosh *et al.* that the reactive oxygen species generated during both cardiac arrest-resuscitation and aging may be formed downstream of more impactful therapeutic targets. Accordingly, induction of antioxidant defenses upstream of RONS production, *e.g.* by activating NAD(P)H production or induction of antioxidant gene expression may afford more robust protection of cognitive function than simple antioxidant treatments (Ghosh et al. 2014a; 2014b).

CONCLUSIONS AND COMMENTARY

In accordance with the "oxidative stress theory" of aging, it is apparent that many components of the pathogenesis of damage and death in the aging brain are common to the mechanisms of brain injury following cardiac arrest-resuscitation, particularly those mechanisms mediated by ROS/RNS. The principal difference between these two forms of neurodegeneration and neurocognitive impairment is their respective time courses. That is, the years of ROS/RNS accumulation and the resulting mitochondrial and cellular dysfunction that provoke inflammatory responses and cell death in the aged brain occur within a matter of minutes

following cardiac arrest and resuscitation. Accordingly, a collaborative effort to resolve the mechanisms of injury in these neurodegenerative scenarios could potentially enhance our understanding of both the pathobiology of aging and of brain resuscitation following cardiac arrest. Indeed, cardiac arrest-resuscitation may provide an accelerated model of the brain aging process, affording more efficient development of treatments to target the common elements of these injury cascades to ultimately promote patient survival and quality of life.

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FIGURE LEGENDS

Figure 1. *Uncoupling eNOS initiates a vicious cycle of nitrosative stress.* Oxidation of its cofactor, tetrahydrobiopterin (BH_4), uncouples the endothelial isoform of nitric oxide synthase (eNOS), which then generates the superoxide anion (O_2^-). Superoxide then combines with nitric oxide (NO) produced by adjacent, still-coupled eNOS to form the powerful oxidant peroxynitrite ($ONOO^-$), which in turn up-regulates nuclear factor kappa B (NFκB). NFκB activates expression of the inducible nitric oxide synthase (iNOS), which produces massive amounts of NO that combine with eNOS-generated O_2^- to intensify $ONOO^-$ formation.

Figure 2. *Mechanisms of oxidative and inflammatory injury during aging and recovery from cardiac arrest*. The figure summarizes mechanisms of brain injury common to cardiac arrest-resuscitation and aging, albeit over entirely different time courses. Casp-9: caspase-9; Casp-3: caspase-3; Cyt C: cytochrome c; GSH/GSSG: concentration ratio of reduced (GSH) to oxidized (GSSG) glutathione; iNOS: incucible isoform of nitric oxide synthase; mPTP: mitochondrial permeability transition pore; NO: nitric oxide; ROS: reactive oxygen species; $ONOO^-$: peroxynitrite.

Table 1. *Preclinical and clinical studies of interventions to protect the brain from cardiac arrest-resuscitation*

Reference	Trial type	Species	Treatment	Factor(s) Tested	Findings
Undén et al. 2013	Preclinial	Rat	Erythropoietin	Ischemia-reperfusion injury of brain	Post-ischemic treatment with erythropoietin is not neuroprotective in a cardiac arrest model.
Ostadal et al. 2013	Preclinical	Swine	Hypothermia	Oxidative stress	Therapeutic hypothermia aided in maintenance of blood pressure and cerebral oxygenation, and prevented oxidant-induced organ damage after cardiac arrest
Dohi et al. 2013	Clinical	Human	Hypothermia	Oxidative stress	Hypothermia downregulated ROS production and fortified endogenous antioxidant systems during resuscitation
Motl et al. 2012	Preclinical	Rat	Vitamin C	Resuscitability	Vitamin C failed to preserve ventricular distensibility and impaired resuscitability
Gong et al. 2012	Preclinical	Swine	Hypothermia	Oxidative stress	Hypothermia decreased production of ROS, preserved function of mitochondrial respiratory enzymes and upregulated the antioxidant MnSOD and Nrf2
Tsai et al. 2011	Preclinical	Rat	Vitamin C	Oxidative stress	Vitamin C (100 mg/kg body wt) decreased lipid peroxidation and respiratory dysfunction following cardiac arrest

Table 2. *Preclinical and clinical studies of interventions to slow the neurodegenerative effects of aging*

Reference	Stage	Subject	Treatment	Factor(s) Tested	Findings
Dysken et al., 2014	Clinical	Human	Vitamin E	Cognitive function in AD	Among patients with mild to moderate AD, those who received vitamin E (2000 IU/d) showed slower decline in cognitive function
Shetty et al. 2013	Preclinical	Mouse	CoQ	Cognitive function	Protein oxidation was decreased and spatial learning impairment was not as severe in aged mice supplemented with high-dose CoQ
von Arnim, et al., 2012	Clinical	Human	N/A	Serum antioxidant concentrations	Vitamin C and β-carotene concentrations were lower in demented *vs* control subjects
Galasko et al., 2012	Clinical	Human	Vitamin C + Vitamin E + α-lipoic acid Coenzyme-Q	CSF biomarkers of AD and OxS	Vitamin C (500 mg) + Vitamin E (800 IU) + α-lipoic acid (900 mg) administered daily for 16 weeks did not influence biomarkers of AD, but did reduce oxidative stress. However, this treatment may accelerate cognitive decline
Lloret et al., 2009	Clinical	Human	Vitamin E	Cognitive function	Vitamin E lowers oxidative stress and maintains preserves function in some AD patients, but in patients for whom vitamin E did not prevent oxidative stress, supplementation caused detrimental effects to cognition
Pérez et al., 2009	Preclinical	Mouse	N/A	Overexpression of antioxidant enzymes	Overexpression of copper zince superoxide dismutase, catalase, and/or manganese superoxide dismutase was insufficient to extend lifespan
Mcdonald et al., 2005	Preclinical	Mouse	Coenzyme-Q + Vitamin E	Cognitive function	Aged mice given daily supplements of CoQ (123 mg/kg body wt) with (+)-α-tocopherol (200 mg/kg body wt) showed enhanced learning
Maxwell et al., 2005	Prospective analysis	Human	Vitamin C and Vitamin E	Risk of cognitive decline	Population-based prospective 5-year study shows that patients who take antioxidant vitamins were less likely to develop significant cognitive decline

Arzi et al., 2004	Preclinical	Mouse	Vitamin C and Vitamin E	Cognitive function	Separately, vitamin C and E had no effect on cognitive function in aged mice. Combined, improved cognitive function in aged but not young mice. Synergistic effect of combined administration proposed to be regeneration of α-tocopherol by vitamin C
Sumien et al., 2004	Preclinical	Mouse	Vitamin E	Cognitive function	Short-term supplementation of vitamin E (1.65 g/kg body wt) did not reverse preexisting age-related impairments in cognitive function
Sumien et al., 2003	Preclinical	Mouse	Vitamin E	Oxidative damage	Supplementation with vitamin E had little or no impact on the steady-state degree of cellular oxidative damage

AD: Alzheimer's disease; OxS: oxidative stress

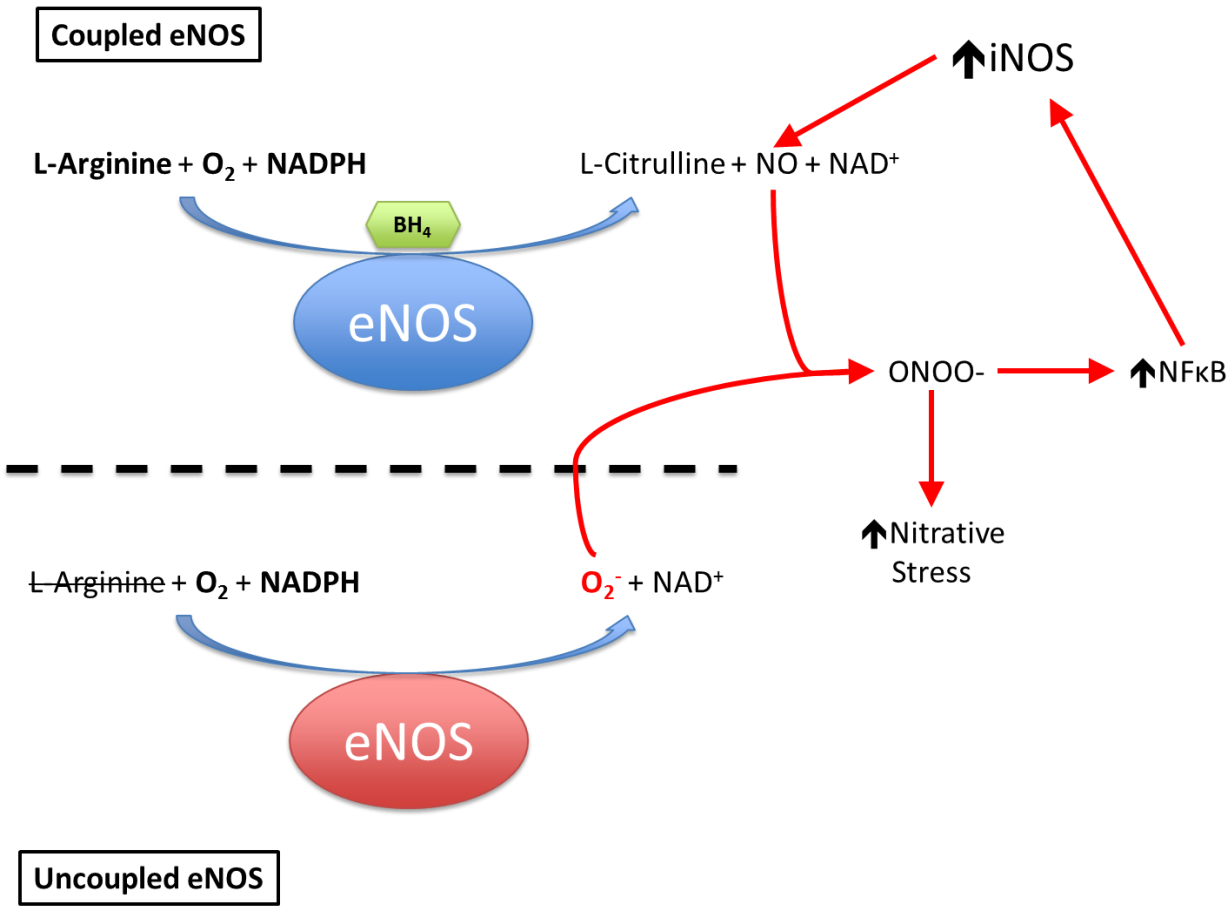
Coupled eNOS
↑iNOS
L-Arginine + O2 + NADPH
L-Citrulline + NO + NAD+
BH4
eNOS
ONOO-
↑NFκB
↑Nitrative Stress
L-Arginine + O2 + NADPH
O2- + NAD+
eNOS
Uncoupled eNOS

Figure 1

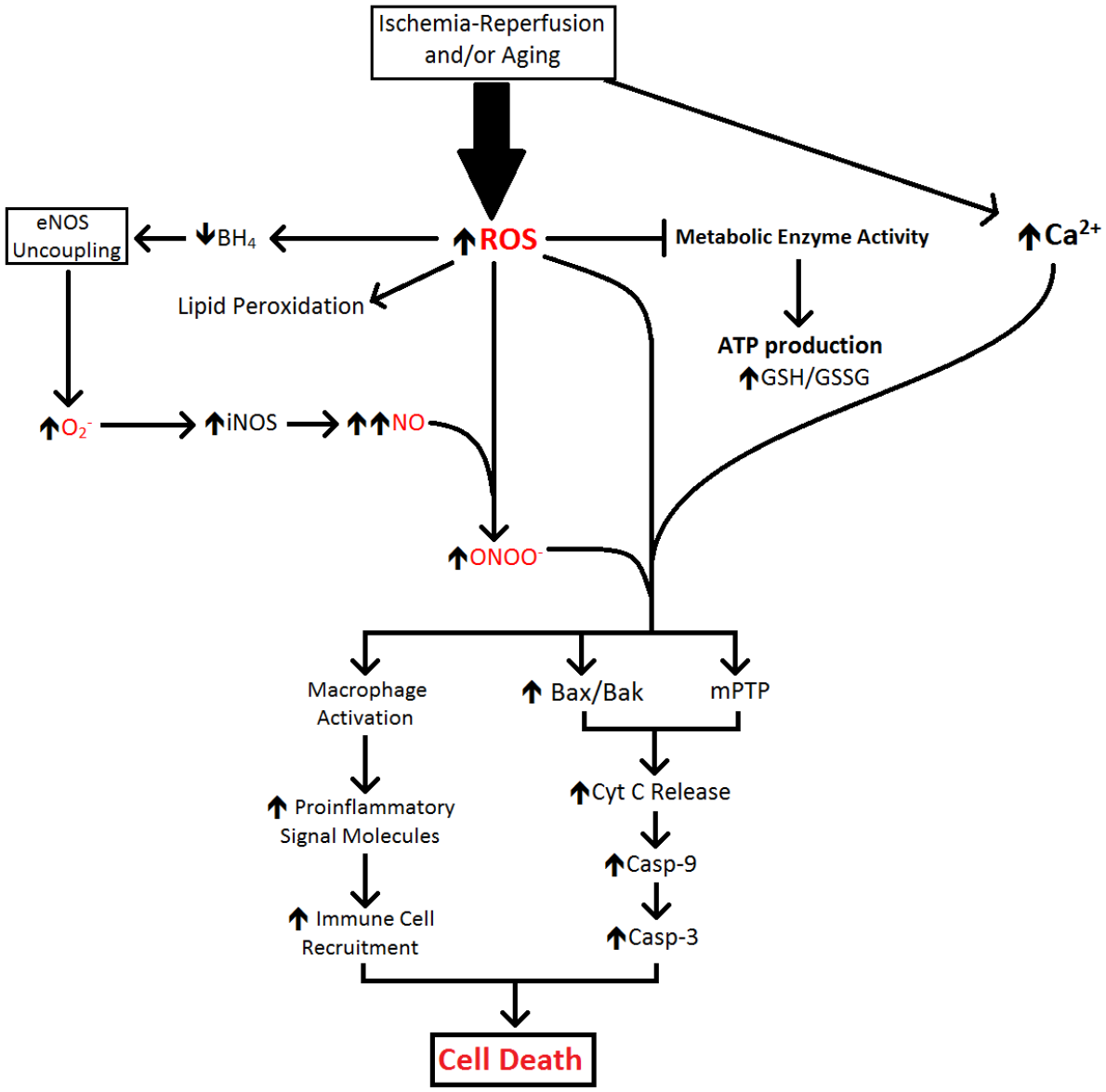
Ischemia-Reperfusion and/or Aging
eNOS Uncoupling
↓BH4
↑ROS
Metabolic Enzyme Activity
↑Ca2+
Lipid Peroxidation
ATP production
↑GSH/GSSG
↑O2-
↑iNOS
↑↑NO
↑ONOO-
Macrophage Activation
↑ Bax/Bak
mPTP
↑ Proinflammatory Signal Molecules
↑Cyt C Release
↑Casp-9
↑ Immune Cell Recruitment
↑Casp-3
Cell Death

Figure 2

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