"We are what we repeatedly do. Excellence, therefore, is not an act but a habit"

Aristotle

University of Alberta

The Role of Cyclosporine Treatment in Cardioprotection during Resuscitation of Asphyxiated Newborn Piglets

by

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Dedication

I'd like to dedicate this PhD thesis to those that have been part of my whole journey and those who company I've been blessed with along the way. Firstly, I dedicate this thesis to my wife, whose love and support, and most importantly patience has allowed me to complete this work. With her in my life, my perspective has changed, and I have forever been changed for the better. I thank her for being my companion during this work, but also for being there during all of our accomplishments and challenges. I will forever be in her debt for giving me the two greatest gifts I could ask for……Saava & Akaash. Though they don't realize it yet, it has been my greatest joy over these three years, to see them grow, laugh and change. I never knew how quickly my life would be forever changed, and how blissful each day could be. I sincerely hope that one day you realize how much you truly mean to me.

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Abstract

Asphyxiated neonates often have myocardial depression which is a significant cause of morbidity and mortality. Cardioprotective effects of cyclosporine have been observed in adult animal models and human trials with myocardial infarction. However, the cardioprotective effect of cyclosporine in neonates has not yet been studied. We hypothesize that cyclosporine will improve cardiac function, reduce myocardial injury and preserve cardiac mitochondria during resuscitation of asphyxiated newborn piglets. In addition, we hypothesize that early cyclosporine treatment will lead to superior cardiac recovery compared to delayed treatment. In the first experiment, thirty-six piglets (1-4 days-old) were acutely instrumented for continuous monitoring of cardiac output and systemic hemodynamics. After stabilization, normocapnic alveolar hypoxia (10-15% oxygen) was instituted for 2h followed by reoxygenation (100% FiO_2, 0.5h; 21% FiO_2, 3.5h). Piglets were block-randomized to receive intravenous boluses of cyclosporine A (2.5, 10 or 25 mg/kg) or in saline (control) at 5 minutes of reoxygenation (n=8/group). Plasma troponin, left ventricle injury markers were determined. HPLC determined pharmacokinetic parameters. In the second experiment, thirty piglets were similarly instrumented with hypoxia and 6h reoxygenation. Piglets were block-randomized to receive cyclosporine (10 mg/kg) early (at 5min) or delayed (at 120min) during reoxygenation, or placebo at both 5min and 125 min reoxygenation (n=8/group). Statistical

analyses were performed using ANOVA. In the first experiment, hypoxic piglets had cardiogenic shock and acidosis. Cyclosporine treatment caused bell-shaped improvements in cardiac function and systemic oxygen delivery ($p<0.05$ vs. controls). Lower troponin levels were associated with cyclosporine exposure (AUC) below 10umol*h/L. Myocardial lactate and cytochrome-c were higher in controls than cyclosporine-treated (10 mg/kg) piglets. Severe damage was observed in mitochondria of control piglets but attenuated with cyclosporine treatment. In the second experiment, early and delayed cyclosporine treatment significantly improved cardiac output and attenuated lactate levels vs. controls. Cardiac function was superior in piglets treated earlier compared to delayed treatment. Firstly, our results demonstrate that the post-resuscitation administration of cyclosporine improves cardiac function, attenuates myocardial injury and preserves cardiac mitochondrial integrity in asphyxiated newborn piglets following resuscitation. Secondly, our results suggest that both early and delayed cyclosporine treatment improves cardiac recovery following asphyxia-reoxygenation, with modest additional benefit of early treatment.

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Abbreviations

ADP	Adenosine 5-diphosphate
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
AMP	Adenosine 5-monophosphate
ANT	Adenine Nucleotide Translocase
ARF	Acute Renal Failure
AST	Aspartate aminotransferase
ATP	Adenosine 5-triphosphate
BPD	Bronchopulmonary Dysplasia
Ca	Calcium
CAFI	Carotid Artery Flow Index
$CADO_2$	Carotid Artery Oxygen Delivery
CAT	Catalase
CI	Cardiac index (cardiac output / kg)
Cl^-	Chloride ion
CNS	Central Nervous System
CPB	Cardiopulmonary Bypass
cTnI	Plasma Cardiac Troponin I
Cyp-A	Cyclophilin-A
CyP-D	Cyclophilin-D
D°	Systemic Oxygen Delivery
DIC	Disseminated Intravascular Coagulation
EKG	Electrocardiogram
FiO_2	Fractional inspired oxygen concentrations
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
HIE	Hypoxic Ischemic Encephalopathy

H_2O_2	Hydrogen Peroxide
HOCl	Hypochlorous acid
H-R	Hypoxia-Reoxygenation
IMM	Inner mitochondrial membrane
I-R	Ischemia-Reperfusion
i.v.	Intravenous
LPO	Lipid Hydroperoxides
MAP	Mean arterial pressure
MPO	Myeloperoxidase
MPTP	Mitochondrial Permeability Transition Pore
Na/Ca	Sodium/Calcium
NAD(P)H	Nicotine Adenine Dinucleotide Phosphate
NAG	N-acetyl-beta-D-glucosaminidase
Na/H	Sodium/Hydrogen
Na-K ATPase	Sodium-Potassium ATPase
NEC	Necrotizing Entercolitis
NICU	Neonatal Intensive Care Unit
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
O_2^-	Superoxide radical
$^1O_2$	Singlet oxygen
OFR	Oxygen Free Radical
OH^-	Hydroxyl radical
OMM	Outer mitochondrial membrane
$ONOO^-$	Peroxynitrite anion
PAP	pulmonary artery pressure
PCI	Percutaneous Coronary Intervention
PDA	Patent Ductus Arteriosus
PGE_2	Prostaglandin
PiC	Mitochondrial Phosphate carrier
PPHN	Persistent Pulmonary Hypertension

PPIase	peptidyl-prolyl cis-trans isomerase
R	Radical
RA	Renal Artery
$RADO_2$	Renal Artery Oxygen Delivery
RAFI	Renal Artery Flow Index
RDS	Respiratory Distress Syndrome
RNS	Reactive Nitrogen Species
ROP	Retinopathy of Prematurity
ROS	Reactive Oxygen Species
SH	sulfhydryl group
SMAFI	Superior Mesenteric Artery Flow Index
$SMADO_2$	Superior Mesenteric Artery Oxygen Delivery
SOD	Superoxide dismutase
SVI	Stroke Volume Index
$SysDO_2$	Systemic Oxygen Delivery
$SysVO_2$	Systemic Oxygen Consumption
TBI	Traumatic Brain Injury
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
V°	Systemic Oxygen Consumption

Chapter 1

Perinatal Asphyxia

Introduction

Asphyxia is derived from the Greek word meaning “a stopping of the pulse”. The modern definition of asphyxia states it as a condition of impaired gas exchange leading, if it persists, to progressive hypoxemia and hypercapnia with a significant metabolic acidosis (1)(2). Metabolic acidosis associated with asphyxia may be confirmed via an umbilical artery base deficit greater than 12 mmol/L (2). Ultimately the pathogenesis involves inadequate oxygen delivery to meet the metabolic demands of the newborn. Specifically, the deficit of oxygen in the blood, secondary to inadequate gas exchange, progresses and leads to conversion of aerobic to anaerobic metabolism, and resultant acidosis.

The essential components of asphyxia are variable in the literature. Clinically it remains a difficult condition to categorize due to a lack of a standard definition. Components, which are generally agreed upon consist of profound acidemia (pH<7), decreased Apgar score, clinical neurological sequelae and evidence of multi-organ system dysfunction in the immediate neonatal period (3). Martin-Ancel et al defined asphyxia based on four traditional markers, of which three must be present. These consist of fetal scalp blood pH and umbilical cord arterial pH of less than 7.2, Apgar scores less than 4 at one minute and/or less than 7 at five minutes and greater than one minute of positive pressure ventilation (PPV) required prior to maintaining sustained respirations (4). Low and colleagues have stated that

umbilical artery base deficit less than 12 mmol/L confirms asphyxia exposure, however this does not classify the severity of the exposure (2). This dilemma stems from inability to determine duration and nature of exposure (intermittent vs. continuous). Thus Low suggests that the classification of severity be defined by newborn encephalopathy and other organ system complications (1). The American Academy of Pediatrics considers a neonate to be asphyxiated if the following conditions are satisfied: (i) Umbilical cord arterial pH less than 7; (ii) Apgar score of 0 to 3 for longer than 5 minutes; (iii) Neonatal neurologic manifestations (e.g. seizures, coma or hypotonia); (iv) Multisystem organ dysfunction involving the cardiovascular, gastrointestinal, hematologic, pulmonary or renal systems (5).

Epidemiology

In 2005, Lawn et al reported that 4 million of the 130 million newborn infants born worldwide each year die within the first 4 weeks of life (6)(7). Lawn et al estimated that 23% of all newborn deaths were caused by birth asphyxia in 2005 (7). The prevalence of perinatal asphyxia differs between term and preterm births. At term, the estimated prevalence is 25 per 1000 live births, of which 15% are classified as moderate to severe. With preterm births, the prevalence is estimated to be 75 per 1000 live births, with 50% classified as moderate to severe. In developing countries these figures are likely underestimated, due to a large proportion of births and deaths

occurring in non-hospitalized settings. Aside from mortality associated with perinatal asphyxia, morbidity is a major concern. With long-term neurological sequelae and multi-organ damage, the estimated prevalence is nearly impossible to approximate.

Risk Factors

Perinatal asphyxia may occur during the antepartum, intrapartum or postpartum periods or combinations thereof. Dilenge et al suggest that asphyxia occurs primarily during the antepartum period in 50% of cases, the intrapartum period in 40% of cases and postpartum in 10% of cases (8). Antepartum risk factors can be further divided into 4 categories: prior stillbirth or neonatal death, maternal medical complications, obstetric complications and fetal complications (Table 1-1). The postpartum period consists of asphyxia primarily from cardio-respiratory complications. However, these complications may originate in either the antepartum or intrapartum periods.

Circulatory Effects

During normoxemia only a small fraction of the cardiac output is distributed to the brain (3%), heart (2.6%), small bowel (2.6%), kidneys (2.3%) and adrenals (0.006%) in fetal sheep (9). The human fetus has similar oxygen requirements with cerebral blood flow comprising a larger fraction of cardiac output. Normal distribution of blood flow within the fetus or neonate

changes dramatically during asphyxia. The fetal response to asphyxia involves centralization of blood flow, increasing blood flow to the brain, heart and adrenals (10). Concurrently, blood flow is decreased to the lungs, kidneys and intestine. Circulatory adjustments to hypoxia involve local vascular effects, adrenergic stimulation and chemoreceptor stimulation. The effects can be inhibitory or excitatory in nature; therefore the net effect is determined by the magnitude of each component (11). Circulatory centralization is accompanied by metabolic centralization, which maintains oxygen delivery to the brain and heart to ensure cell function and survival of the neonate (12).

Local Vascular Effects

The local vascular effects of hypoxia tend to be inhibitory in nature. The response to hypoxia tends to reduce arterial blood pressure. Typically, when hypoxic blood is perfusing a vascular bed, the blood vessels within the bed tend to dilate. It must be noted that hypoxia must be severe before vasodilation can be detected. The vasodilatation in the blood vessels is not uniform, but rather selective. The coronary and cerebral vessels respond predominately with dilatation. The vessels to the limbs and renal vessels have a minimal response (11). This selective response seems beneficial because it redistributes blood flow to coronary and cerebral vasculature. This differential effect on blood vessels appears to be related to organ specific metabolic requirements, therefore the selective inhibition of

vasoconstriction, allows redistribution of flow to areas that are most metabolically active.

Adrenergic Stimulation

Adrenergic stimulation is modulated by the sympathetic nervous system and catecholamine release. Iwamoto et al suggested that in fetal sheep, hypoxemia leads to arterial hypertension, redistribution of blood flow and an increase in circulating concentration of catecholamines (13). Further studies in hypoxic fetal sheep have demonstrated the importance of the alpha-adrenergic system in maintaining the redistribution of cardiac output (14). The circulating catecholamines released by the adrenal glands, supply adequate quantities, allowing fetal sheep to tolerate hypoxemia. An animal study by Iwamoto et al subjected chemically sympathectomized fetal sheep to hypoxemia. The catecholamine levels were adequately responsive to allow the fetal sheep to tolerate the hypoxemia. However, the arterial pressure did not increase in this study, which suggested importance of the sympathetic nervous system in mediating vasoconstrictor responses in the peripheral, mesenteric, splanchnic and splenic vascular beds.

Chemoreceptor Stimulation

Chemoreceptors are located peripherally in the carotid and aortic bodies. Hypoxia has an excitatory effect on chemoreceptors, which subsequently increases the arterial pressure. The primary response of

arterial chemoreceptors is an increase in arterial pressure, while secondarily blood flow is redistributed. Animal experiments have demonstrated that an increase in arterial pressure is primarily due to an increase in peripheral vascular resistance, and secondarily due to the inotropic response of the myocardium (11). The peripheral vascular resistance is selectively increased, with marked vasoconstriction in skeletal muscle. In experimental animal models, stimulation of carotid chemoreceptors by hypoxia produced marked reflex vasoconstriction in skeletal muscle and vasodilatation in coronary muscle (15). Interestingly, cerebral blood flow has not consistently been shown in experimental models to be related to arterial chemoreceptor influence. Chemoreceptor response can be modulated further by asphyxia. The three factors that have been specifically implicated in modulating chemoreceptor response are (i) Acidosis; (ii) Catecholamines; (iii) Systemic hypotension. Both acidosis and hypercapnia will augment the response to the chemoreceptor reflex (11). It has been shown with catecholamines, that stimulation of beta-adrenergic receptors may also augment the response leading to an increase in arterial pressure. Additionally, when hypotension is induced with hypoxia, arterial baroreceptors are also stimulated and the response is synergistically augmented (11).

Multi-organ Effects

The fetus's survival depends on its ability to respond to hypoxic conditions. However, as hypoxia progresses to asphyxia, these compensatory

mechanisms may become overwhelmed and fail. Centralization of blood flow to the heart and brain via local vascular effects, adrenergic stimulation and chemoreceptor stimulation preserve oxygenation to vital organs. Hypoxia coupled with acidemia and hypotension lead to the breakdown of protective mechanisms and multi-organ damage may ensue. Cardiovascular compensation initially maintains central nervous system (CNS) integrity by increasing cerebral blood flow with concurrent increased oxygen extraction. However, experiments in fetal lambs have demonstrated decreased cerebral blood flow and oxygen metabolism secondary to fetal cardiovascular decompensation (16). Ikeda et al reported in near-term fetal lambs that the duration of hypotension highly correlated with the severity of histological damage to the brain (16). Low et al suggest that cerebral dysfunction may occur beyond a threshold metabolic acidosis of base deficit greater than 12 mmol/L from the umbilical artery (2). Severe CNS injury may be accompanied by severe injury to other organs (3) (Table 1-2). In the study by Martin-Ancel et al, 72 newborns with perinatal asphyxia were assessed and 82% were found to have damage to one or more organs involved. Furthermore, these authors reported that Apgar score at 5 minutes as the best perinatal marker for identifying multi-organ damage (3).

Brain

Neurological sequelae following birth asphyxia have a reported incidence ranging from 1-5% of live births (17). Hypoxic-ischemic injury is

the most important cause of brain injury in the newborn, and has consequences that can persist through the infant's lifetime (18). Asphyxia, hypotension and cerebral hypo-perfusion, results in brain damage. Cerebral autoregulation, which serves as a protective mechanism, may be abolished after just 20 minutes of hypoxia (19). Initial cardiovascular compensation attempts to maintain CNS integrity during asphyxia. Concurrently, there is also increased cerebral blood flow and oxygen extraction. Gunn and colleagues' experiments on fetal sheep suggested that increased histological brain damage was not determined by the degree of hypoxia, but rather by the occurrence of hypotension (20). Interestingly though, hypotension can be induced by hypoxia. The study by Gunn et al further suggests that impaired cerebral blood flow is critical in provoking and localizing neuronal injury. Liu and colleagues studied forty newborn infants with hypoxic-ischemic injury, and correlated increasing cardiac dysfunction with severe encephalopathy (21).

Perinatal asphyxia is the primary cause of hypoxic ischemic encephalopathy (HIE). The incidence of HIE is approximately six in 1000 term infants, with incidence of death or severe neurological deficit being one in 1000 neonates (21). The severity of encephalopathy is dependent on the degree and duration of asphyxia. The encephalopathy can range from Mild (jitteriness, irritability), Moderate (lethargy, seizures, abnormal tone) to Severe (coma, abnormal tone, multiple seizures) (1). Perinatal hypoxic-

ischemic cerebral injury is also the most clearly recognized cause of cerebral palsy (22). It is believed to be an evolving process initiated in-utero extending to the neonatal period. Approximately 14.5% of the cases of cerebral palsy can be attributed to hypoxic-ischemic brain injury (23).

Heart

In asphyxiated neonates, myocardial ischemia is often secondary to hypoxia. This syndrome of hypoxemia-related myocardial dysfunction occurs in about 30% of asphyxiated infants (3). The initial compensatory mechanisms attempt to centralize blood flow in order to maintain myocardial oxygen requirements and cardiac output remains relatively constant. Ley et al demonstrated an increase in coronary blood flow following acute asphyxia induced by total cord occlusion in fetal sheep (24). This initial increase in coronary blood flow can be sustained for a limited time with ongoing exposure to asphyxial conditions. The hemodynamic changes caused by umbilical cord occlusion are characterized by immediate bradycardia and an initial increase in arterial pressure, which is then followed by arterial hypotension (24).

Severe asphyxia in neonates may lead to myocardial damage and cardiovascular collapse. Barberi et al reported that 38% of severely asphyxiated newborn infants did not survive a week. Of those infants that survived, cardiac ischemia was demonstrated by ischemic EKG changes,

depressed left ventricular function and marked cardiac enzyme (i.e.. CK-MB) increases (25). Myocardial ischemia, secondary to perinatal asphyxia, seems to occur despite preferential myocardial perfusion. Furthermore, the combination of EKG, echocardiogram and serum enzyme results, correlate with the severity of myocardial damage (25). Echocardiography is able to identify myocardial dysfunction by either demonstrating depressed left ventricular function or moderate-to-severe tricuspid regurgitation secondary to pulmonary hypertension. Costa et al observed asphyxiated neonates to have a significantly higher Troponin T levels than healthy newborn controls. However, the cutoff value for Troponin T (cTnT) remains to be determined (26).

Kabra et al observed myocardial dysfunction in all 50 newborns exposed to moderate and severe birth asphyxia (27). Myocardial damage can lead to transient alterations in function, or to complete cardiovascular collapse. Low et al classified these cardiovascular complications in neonates as Mild (bradycardia or tachycardia), Moderate (hypertension vs. hypotension) and Severe (Abnormal Echocardiogram or EKG) (1,2).

Lungs

Asphyxia places the neonate at risk for a variety of pulmonary complications. This includes Meconium Aspiration Syndrome, Respiratory

distress syndrome, Persistent Pulmonary Hypertension (PPHN), pulmonary hemorrhage and pulmonary edema.

Acute Respiratory Distress Syndrome (RDS), also known as Hyaline Membrane Disease, can occur following asphyxia, though is primarily seen in premature infants. The primary cause of RDS is surfactant deficiency. In conditions of hypoxia and acidemia, surfactant synthesis may be partially disrupted. The resultant alveolar atelectasis, hyaline membrane formation and interstitial edema lead to the lungs being less compliant (28). Inadequate function of the lungs leads to hypoxia, while inadequate ventilation leads to hypercapnia and acidosis. It is believed the hypoxia and acidosis induces pulmonary vasoconstriction, which increases pulmonary vascular resistance. As this progresses, intrapulmonary shunting and right-to-left shunting across the ductus arteriosus and foramen ovale may occur (28). Resultant decreased pulmonary perfusion increases ischemic injury, which subsequently worsens pulmonary edema.

Persistent Pulmonary Hypertension of the Newborn (PPHN) occurs in 1 per 500-1500 live births. This disease is defined by the persistence of fetal circulatory pattern of right-to-left shunting through the patent ductus arteriosus and foramen ovale after birth, due to high pulmonary vascular resistance secondary to a number of etiologies. These include birth asphyxia, meconium aspiration pneumonia, early-onset sepsis, RDS, hypoglycemia and

polycythemia. The common etiology in PPHN remains hypoxemia. The newborn can present with cyanosis and tachycardia. The disease can also be resistant to supplement oxygen in a small percentage of cases (28).

Intestine

Necrotizing enterocolitis (NEC) in the neonate is the most common gastrointestinal emergency. NEC presents as various degrees of mucosal or transmural necrosis of the small intestine. Even though the underlying etiology is believed to be multifactorial, asphyxia is considered an important risk factor. Incidence of NEC in the intensive care unit ranges between 1-5% (28). Though considered to be a disease primarily of premature infants, NEC may also occur in term infants. The spectrum of the disease ranges from mild to severe illness with perforation, peritonitis, shock and death (28).

Bell et al presented a staging system for NEC based on clinical and radiographic data. This study classified 48 neonates in a prospective fashion according to this staging system for NEC. Intervention was based on the Stage of NEC, with Stage 1 (Suspect) being provided supportive care and diagnostic evaluation. Stage 2 (Definite), were treated medically with both parenteral nutrition and antibiotic therapy, while Stage 3 (Advanced) required operative intervention (29).

The etiology of NEC remains multifactorial, however it has been postulated that both hypoxemia and the physiological response to hypoxemia may play a key role. Edelstone et al observed in neonatal lambs, that the intestine is able to meet oxygen requirements over a range of hypoxemia levels, until a critical low level is reached (30). At this point, the intestine switches to anaerobic metabolism and oxygen supply to the neonatal intestinal tract is unable to satisfy oxygen demand (30). In the fetus, the intestines will receive approximately four times greater oxygen supply than demand (30). Thus, during initial hypoxemia, intestinal perfusion does not change; rather the fetal intestines are able to maintain oxygen supply by increasing oxygen extraction. However, as hypoxemia progresses in the neonatal lamb, intestinal perfusion decreases and ischemia ensues.

Neonates exposed to asphyxia compensate by centralizing blood flow to vital organs such as the heart and brain as a protective mechanism. This results in reduced intestinal perfusion. Lambert et al assessed 30 term newborns that developed NEC and compared them to 5847 normal newborn infants as controls, and reported admission to NICU, as a significant risk factor (31). They speculated that the combination of reduced mesenteric perfusion and exposure to artificial formula, were predisposing factors. Gellen et al reported in neonatal piglets that asphyxia induced severe intestinal ischemic injury including intestinal hemorrhage and coagulative necrosis (32). Asphyxia in this study induced NEC-like intestinal injury,

which the authors concluded to be secondary to the severe vasomotor change that resulted in a 60% decrease in blood flow through the superior mesenteric artery (32). These findings were assessed in term infants by Koc et al using a transcutaneous doppler ultrasound and correlated with umbilical artery pH values. This study associated a reduction of intestinal circulation with umbilical cord blood acidemia (pH<7.2) and hypoxemia (33).

Hepatic

Under normal conditions, oxygen consumption of the liver accounts for 20% of the total fetal oxygen consumption (34). The umbilical vein from the placenta is the main source of oxygenated blood to the fetal liver. During acute hypoxia, the total liver blood flow decreases by 20%. Blood flow to the right lobe decreases twice as much as the left lobe of the liver (34). In 8 fetal lambs, Rudolph et al observed a 50% decrease in oxygen delivery to the fetus and a 73% decrease to the liver when umbilical flow was reduced by 50% secondary to compression (35). The oxygen-rich blood from the umbilical vein is shunted from the liver through the ductus venosus to other vital organs. Interestingly, hepatic oxygen consumption was maintained by increased oxygen extraction (35).

The liver also serves as a store for glycogen, which can be degraded to raise circulating glucose concentrations in fetal lambs when hypoxemia is induced (36). The liver demonstrates no net uptake of glucose during

hypoxia. Instead, other sources are used to support oxidative metabolism. This utilization of other substrates may thus spare glucose for organs such as the brain (34). It is hypothesized that glucose production by the liver is from glycogenolysis. Furthermore, during hypoxemia in fetal lambs, the liver produces 45% of the total glucose available to the fetus. (35).

Godambe et al investigated 70 newborns exposed to asphyxia and observed significantly elevated ALT levels when compared to controls. Also, increased prothrombin time was seen in asphyxiated newborns (37). These findings support observations made by other authors that suggest ischemic damage to the liver may occur following asphyxia. Specifically, the liver exhibits a reduced ability to produce coagulation factors and cellular damage as shown by a rise in aminotransferases. Similarly, Karlsson et al assessed 26 asphyxiated newborns and reported elevations in AST or ALT, compatible with hypoxic hepatitis/shock liver (38).

Herzog et al completed a retrospective review on 181 asphyxiated newborns and reported that the rate of transient neonatal cholestasis was 8.5% and 33% for appropriate birth weight for gestational age and small for gestational age newborns, respectively (39). Cholestasis, of any etiology, in non-asphyxiated infants was lower at 3.9%. Fortunately, transient cholestasis of the newborn is self-limiting and only requires substitution of lipid-soluble vitamins (39). Vajro supports these results in their case report,

which suggests asphyxia as a potential causal factor in newborns that develop transient neonatal cholestasis (40).

Renal

Acute Renal Failure (ARF) is a common clinical condition in the neonatal intensive care (41). Renal failure in the newborn is classified as pre-renal, intrinsic renal disease including vascular insults and obstructive uropathy. Intrinsic renal failure can be acquired in the postnatal period secondary to hypoxic-ischemic injury or cortical necrosis. Neonates with severe asphyxia have a higher incidence of ARF than neonates with moderate asphyxia. Specifically, cortical necrosis is associated with perinatal anoxia and other hypoxic-ischemic insults. These infants have a worse prognosis, which typically requires short or long-term dialysis therapy (42).

Asphyxia has been recognized as an etiology of ARF. Olavarria et al prospectively followed 21 term infants with asphyxia, and reported a 9% incidence of intrinsic ARF (43). Furthermore, 48% of the asphyxiated group demonstrated functional oliguria but did not by definition develop acute renal failure (43). Luciano et al evaluated 23 severely asphyxiated neonates (>32 weeks GA) with ARF developing in 30% (44). They also reported significantly decreased blood flow through the renal artery, assessed by Doppler renal flow systolic velocity, in asphyxiated neonates with renal

involvement (44). A previous study on asphyxiated newborn piglets also observed decreased renal blood flow and increased vascular resistance (45).

Coagulation

Hemostasis in humans is an intricate process with many cells and factors involved. Two of the most important aspects of coagulation are platelets and coagulation factors. Platelets are small cells without a nucleus and are a product of bone marrow megakaryocyte maturation and differentiation. For hemostasis, both adequate quantity and adequate function are required (46).

Castle et al prospectively studied 807 infants admitted to the regional neonatal intensive care unit and reported that thrombocytopenia developed in 22% of the infants (47). Of the neonates with thrombocytopenia, hemoglobin concentrations were higher and presence of birth asphyxia was more frequent. This study suggests that a strong link between thrombocytopenia and birth asphyxia exists, however, the underlying mechanism is undetermined at this time (47). Previously, Meberg also reported transitory thrombocytopenia in newborn mice after intrauterine hypoxia (48). The newborn mice were observed to be both polycythemic and had a postnatal transitory thrombocytopenia lasting seven days. The author suggests these findings may be explained by a competitive mechanism on

common stem cells, which secondary to hypoxia, shunt hematopoietic cells towards erythropoiesis rather than thrombopoiesis (48).

The most common coagulation disorder in perinatal asphyxia is disseminated intravascular coagulation (DIC). Chessells et al studied 9 severely asphyxiated infants of which 2 demonstrated thrombocytopenia (22%) and 4 demonstrated hypofibrinogenemia (44%) (49). Of the 9 severely asphyxiated infants, four died; however no infants demonstrated a clinically bleeding tendency.

Conclusion

Perinatal asphyxia may have detrimental neurological sequelae and multi-organ consequences. Asphyxiated neonates remain at an increased risk of mortality and morbidity. The long-term sequelae from neurological injury signify the importance of understanding and effectively treating this condition. Animal and clinical studies have greatly increased our knowledge, however much work remains. Preventing and treating hypoxemia-related organ damage in the neonate continues to be a challenging endeavor.

Table 1-1: Antepartum and Intrapartum Risk Factors

Perinatal Asphyxia Risk Factors	
Antepartum Risk Factors	**Intrapartum Risk Factors**
Maternal Hypertension	Placenta previa
Maternal Diabetes	Prolonged labour
Maternal Sexually transmitted disease	Abruptio placenta
Substance abuse	Fetal distress
Chronic placental insufficiency	Prolapsed cord
Premature rupture of membranes	Chorioamnionitis
Maternal bleeding	Prolonged labour

Table 1-2: The Multi-organ Effects of Neonatal Asphyxia

Effects of Neonatal Asphyxia	
Central Nervous System	Hypoxic-ischemic encephalopathy Infarction Intracranial hemorrhage Seizures Cerebral edema Hypotonia or hypertonia
Cardiovascular System	Myocardial ischemia Poor contractility Cardiac stunning Tricuspid insufficiency Hypotension
Pulmonary	Pulmonary hypertension Pulmonary hemorrhage Respiratory distress syndrome
Renal	Acute tubular or cortical necrosis
Adrenal	Adrenal hemorrhage
Gastrointestinal	Bowel perforation Bowel ulceration with hemorrhage Necrosis Necrotizing enterocolitis
Liver	Ischemic hepatitis Cholestasis
Metabolic	Inappropriate secretion of antidiuretic hormone Hyponatremia Hypoglycemia, Hypocalcemia Myoglobinuria
Hematology	Thrombocytopenia Disseminated intravascular coagulation

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Chapter 2

Oxidative Stress of the Immature Myocardium During Resuscitation of Asphyxiated Newborns

Adapted from:

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Abstract

Perinatal asphyxia contributes to over one million newborn deaths worldwide annually, and may progress to multi-organ failure. Cardiac dysfunction, of varying severity, is seen 50-70% of asphyxiated newborns. Resuscitation is necessary to restore oxygenation to deprived tissues, including the heart. However, reoxygenation of asphyxiated newborns may lead to generation of reactive oxygen species (ROS) and further myocardial damage, termed reperfusion injury. The newborn heart is especially vulnerable to oxidative stress and reperfusion injury due to immature antioxidant defense mechanisms and increased vulnerability to apoptosis. Currently, newborn myocardial protective strategies are aimed at reducing generation of ROS through controlled reoxygenation, boosting antioxidant defenses, and attenuating cellular injury via mitochondrial stabilization.

Introduction

Perinatal asphyxia contributes to over one million newborn deaths worldwide annually (1). In developed countries, asphyxia affects 3-5 neonates per 1000 live births (2). It is commonly described as a condition of impaired gas exchange leading, if it persists, to progressive hypoxemia, hypercapnia and eventually metabolic acidosis (3). Asphyxia in newborns is associated with multi-organ dysfunction and potentially cardiovascular collapse. An estimated 50-70% of asphyxiated newborns suffer from mild to severe cardiac dysfunction (4). Following in-utero hypoxemia, immediate resuscitation/reoxygenation at birth is typically required to support the newborn, consisting of oxygen therapy. However, reestablishment of tissue oxygenation/perfusion also has undesirable consequences, especially to the immature newborn heart. Specifically, the newborn myocardium has been shown to be particularly vulnerable to apoptosis (5). Furthermore, the generation of reactive oxygen species (ROS) during reperfusion/reoxygenation may contribute to apoptotic or necrotic cardiac cell death. Conversely, the newborn myocardium has also been suggested to be tolerant to ischemia-hypoxia related to increased glycolytic capacity (6).

Oxidative stress during reoxygenation as a result of ROS may contribute significantly to the ultimate extent of cardiac injury. ROS are normally generated in ordinary physiologic conditions and are balanced by endogenous anti-oxidant mechanisms. However, during hypoxia-reoxygenation, the excessive production of ROS and their high reactivity

result in damage to cellular lipids, proteins and DNA (Figure 2-1). The rapid burst of ROS overwhelms the endogenous anti-oxidant systems. These ROS originate from a number of sources, including mitochondria, xanthine oxidase and activated neutrophils. In this review, we will explore the mechanisms associated with the generation of ROS and associated myocardial damage. In addition, we will assess the evidence of potential protective strategies to attenuate myocardial injury associated with oxidative stress in asphyxiated newborns.

Hypoxia-Reoxygenation

Hypoxic conditions during pregnancy may arise during the antepartum, intrapartum or postpartum periods. Specifically hypoxic conditions arise during the antepartum period in 50% of cases and during the intrapartum period in 40% of cases (7). Multiple animal models focusing on the mother, placenta-uterine interface and neonate have been utilized to advance our understanding of asphyxia and related physiologic consequences. These include maternal models of hypoxemia, utero-placental models of insufficiency, cord compression and newborn asphyxia. Newborn asphyxia models include induction of alveolar hypoxia, pneumothorax or intratracheal meconium administration (8). One challenge in studying clinical asphyxia in newborns is that the timing of the insult and duration of insults is difficult to identify. Furthermore, the mechanisms involved in multi-organ damage have not been entirely delineated. Regardless of the underlying

mechanisms, impaired tissue oxygenation limits aerobic metabolism, and eventually anaerobic metabolism ensues. This leads to inefficient energy production and accumulation of cellular byproducts, which may lead to cellular failure. Restoring oxygen to oxygen-deprived tissues is paramount to prevent further ischemic/hypoxic myocardial injury. However, the return of oxygen to hypoxic-ischemic tissues may lead to ROS generation and further tissue damage.

Reactive Oxygen Species & Generation of Oxidative Stress

Mitochondria

ROS are formed as intermediates of cellular metabolism. Typically, ROS refers to a group of active oxygen species including superoxide ion ($\bullet O_2$), hydrogen peroxide (H_2O_2) and hydroxyl ion ($\bullet OH$). They can be derived from a variety of sources including mitochondria; endoplasmic reticular and nuclear membrane electron transport processes (9). The most important source of physiologic generation of superoxide ion may be the mitochondria. Typically, the mitochondrial reduce the majority of O_2 inspired by cells to form H_2O via the electron transport chain. The process of aerobic metabolism efficiently produces ATP for cellular function. During this process, a small number of electrons can leak from the mitochondrial electron transport chain to oxygen prematurely forming superoxide (10). It is estimated that 1-3% of all electrons leak in the transport chain instead of contributing to the reduction of oxygen to water (10).

Superoxide specifically is made from both complexes I and III of the mitochondrial electron transport chain. Complex I-dependent superoxide, which involves iron-sulphur centers, is released into the mitochondrial matrix. Conversely, Complex III dependent superoxide, involving ubiquinone and cytochrome b, leaks extramitochondrially. The superoxide radical is too strongly charged to cross the inner mitochondrial membrane. The conversion of oxygen to superoxide is significant considering mitochondria consume 90% of inhaled oxygen, thus serving as a rich source of ROS. It is approximated that 1-2% of oxygen reduced in mitochondria is converted to superoxide (11). The production of superoxide is further increased during hypoxia-reperfusion. This may be related to the mitochondria containing iron and copper, which facilitate the conversion of hydrogen peroxide to oxidizing hydroxyl radicals via the Haber-Weiss reaction (11,12). The conversion of hydrogen peroxide to hydroxyl radicals potently damage lipids, proteins and nucleic acids. Specifically, Gutteridge et al (13) observed in-vitro the production of hydroxyl radicals in the presence of iron salts from superoxide and hydrogen peroxide and suggested that the most toxic effects of superoxide are due to the formation of hydroxyl radicals.

Hypoxanthine-Xanthine Oxidase System

Another important source of ROS production is the hypoxanthine-xanthine oxidase system. As tissues are exposed to lower oxygen concentrations during ischemia, ATP is utilized and eventually converted to

hypoxanthine, which accumulates in the cells. Concurrently, xanthine dehydrogenase is converted to xanthine oxidase via calcium and protease, and also accumulates in the hypoxic tissues. Xanthine oxidase and xanthine dehydrogenase are interconvertible forms of the same enzyme, known as xanthine oxidoreductase. Xanthine oxidase uses molecular oxygen provided during reoxygenation/reperfusion of hypoxic-ischemic tissues to convert hypoxanthine into xanthine, creating a burst of superoxide radicals and uric acid in the process (12). McCord assessed the accumulation of xanthine oxidase in the heart, liver and intestine and observed rapid doubling times of 8 minutes, 30 minutes and 10 seconds respectively within these tissues during ischemia. Thus prolonged ischemia creates ideal conditions for superoxide generation upon reperfusion.

Phagocytic Cells – NADPH Oxidase System

Leukocytes, monocytes and eosinophils are all phagocytic cells capable of producing oxidative bursts. These cells specialize in producing ROS, as part of the host's inflammatory response against pathogens. Specifically activated neutrophils produce large quantities of superoxide and other ROS via the phagocytic isoform of nicotine adenine dinucleotide phosphate oxidase (NADPH oxidase).

NADPH oxidase facilitates the reaction between NADPH and O_2, leading to the formation of superoxide anion and $NADP^+$. Superoxide can react with itself in the presence of superoxide dismutase (SOD) generating

hydrogen peroxide. Activated neutrophils also generate singlet oxygen by reaction involving NAD(P)H oxidase or myeloperoxidase (10). Myeloperoxidase (MPO) is secreted by neutrophils into the extracellular medium, where it is able to catalyze two-electron oxidation of chloride ion (Cl^-) by hydrogen peroxide to yield the potent cytotoxic oxidants hypochlorous acid (HOCl) and N-chloramines (14). MPO levels have been observed to be increased during ischemia-reperfusion. Specifically, Grishman et al reported increased MPO levels during reperfusion of intestinal mucosa, indicating a role for neutrophils in ischemia-reperfusion injury. Brown et al (15) observed attenuated intestinal mucosal injury during reperfusion using a leukocyte-free solution compared to normal blood, supported these results.

Nitric Oxide and Peroxynitrite

Nitric oxide (NO) is a labile gas which is generated by NO synthase. Under physiological conditions, NO is primarily generated in cardiomyocytes and vascular endothelial cells (16). During the initial phase of reperfusion, there is a rapid production of NO from the myocardium (17). According to Wang and Zweier, during reperfusion, the rapid production of NO and superoxide occurs concurrently, followed by reaction generating peroxynitrite. During myocardial reperfusion, the increased generation of NO is able to outcompete SOD for superoxide, which predominately forms peroxynitrite (18). Peroxynitrite in the cell leads to damage of lipids, proteins, carbohydrates and nucleic acids. Specifically, peroxynitrite reaction

leads to lipid peroxidation and nitration of tyrosine residues (19,20). In an *in-vivo* isolated rat heart model of ischemia-reperfusion, Wang & Zweier (17) observed increased peroxynitrite production using electron paramagnetic resolution spin trap techniques.

Endogenous Protective Mechanisms in the Newborn

Endogenous protective mechanisms are present in the newborn to scavenge ROS and disable them. These inherent defense mechanisms against oxidative stress include enzymes, metal chelators and various antioxidants. The main enzymatic antioxidants include SOD, catalase (CAT) and Glutathione peroxidase (GPx), while non-enzymatic antioxidants include glutathione, ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), carotenoids (Vitamin A), flavonoids and uric acid. Under normal conditions, a balance exists between these antioxidants. Disruption of this balance can exacerbate oxidative stress.

Superoxide Dismutase (SOD)

SOD is an endogenous enzyme that catalyzes the conversion of two superoxide anions into hydrogen peroxide and oxygen. SOD belongs to the family of metal containing enzymes, which depend on manganese, copper or zinc at the active site to function. Generation of hydrogen peroxide in the place of the more potent superoxide ion relatively reduces oxygen toxicity. Furthermore, the presence of SOD increases the rate of this detoxifying

reaction 10 000-fold over the non-catalyzed reaction. As an inducible enzyme, the quantity of SOD is increased with exposure to higher oxygen concentrations (10).

Catalase

Catalase is a well-known heme-containing protein that scavenges hydrogen peroxide in humans. It remains the most efficient enzyme to convert hydrogen peroxide into O_2 and water (21). The reaction involves two-stages in which hydrogen peroxide is oxidized and reduced. In addition, following the enzymatic reaction of catalase, no further ROS are generated. Furthermore, the stable and rigid structure of catalase makes it relatively resistant to pH changes. Catalase has been shown to attenuate apoptosis secondary to oxidative damage (22) and is most abundant in the liver and blood.

Glutathione Peroxidase (GPx) and Glutathione

Glutathione (GSH) is non-enzymatic antioxidant that is converted to glutathione disulphide (GSSG) by Glutathione Peroxidase (GPx). The conversion of GSH to GSSG scavenges ROS leading to their reduction. GSH is abundantly present in the nuclei, mitochondria and cytosol of cells. Though GSH is produced in cytosol, it requires active transport into the mitochondria (10). With plentiful GSH present within cells, the GSH/GSSH ratio serves as a measure of cellular oxidative stress (23). GSH has multiple protective roles,

including serving as a cofactor for detoxifying enzymes such as GPx. Secondly; GSH scavenges singlet oxygen and the hydroxyl radical directly. Thirdly, GSH acts to regenerate Vitamin C and E to their active (reduced) forms. Furthermore, GSH/GSSG couple serves as the major cellular redox buffer. Thus, during increased oxidative stress, the concentration of GSSG increases. To maintain high ratios of reduced GSH compared to oxidized GSSG, GSH reductase actively and inducibly transforms GSSG back to GSH.

Vitamins C & E

Vitamin E encompasses groups of tocopherols and tocotrienols, of which α-tocopherol has the greatest biological activity. In appropriate conditions, Vitamin E can serve as an antioxidant, aiding in the detoxification of lipid peroxides and peroxynitrites (24). Vitamin E is adapted to protecting membrane integrity due to its fat-soluble nature. Vitamin C, also known as ascorbic acid, preserves Vitamin E during oxidative stress and protects lipoproteins (25). Furthermore, Vitamin C can neutralize hydrogen peroxide (26). In combination with GSH, these antioxidants serve to protect against lipid peroxidation secondary to ROS damage.

Myocardial Injury and Reactive Oxygen Species

The generation of ROS has been implicated in the damage of asphyxiated newborn myocardium following reperfusion/reoxygenation. Reperfusion or restoration of oxygen and blood flow is essential; otherwise

recovery of the myocardium is not possible. Unfortunately, the massive burst of ROS generated during reperfusion of ischemic-hypoxic cardiac tissue, damages lipid membranes, proteins and nucleic acids in cardiomyocytes. The newborn myocardium compared to the adult myocardium is more vulnerable to apoptosis, thus is at increased risk from ROS induced damage (5).

Zweier applied electron paramagnetic resolution spin trapping techniques to directly measure ROS in the myocardium during reperfusion using isolated rabbit hearts (27). They observed increased levels of superoxide anion and hydroxyl radical in the reperfused myocardium. Furthermore, they reported a direct relationship between oxidative stress and cardiac contractile dysfunction. Contractile function was then improved with abolishment of oxidative stress. Bolli et al (28) also used electron paramagnetic resolution in an *in-vivo* canine model that directly measured ROS generation during reperfusion following regional myocardial ischemia secondary to coronary occlusion. Similarly, they observed increased ROS production in the reperfused myocardium, which correlated with increased contractile dysfunction. The prolonged cardiac dysfunction following ischemia-reperfusion was termed “myocardial stunning”, with ROS proposed to be key mediators (29). Wang and Zweier (17) also demonstrated increased NO and peroxynitrite formation in isolated reperfused rat hearts using electron paramagnetic resolution spin trapping techniques. Overall ROS formation, cellular calcium overload and mitochondrial dysfunction have

all been proposed as possible mechanisms that cause reperfusion/reoxygenation myocardial injury.

Newborn Heart – Immature Antioxidant Capacity

The newborn heart is unique compared to its adult counterpart. As the fetus transitions to the neonatal life, there is a rapid change in environment conditions (i.e. O_2 levels) and physiological circulation with the first breath. In utero the fetus is exposed to relatively hypoxemic conditions (PO2 20-25 mmHg) compared to the newborn (21% O2) (30). According to Turrens et al, the generation of superoxide and hydroxyl radical increases linearly with increasing PO2 (31,32). This may be related to the relatively hyperoxic extrauterine environment and high metabolic rate and subsequent increased mitochondrial work. On the other hand, according to animal studies by Frank & Groseclose, there is a marked increase in antioxidant enzyme activity during the final 10-15% of gestation (33). To determine antioxidant levels in the newborn, Friel et al (34) measured levels of lipid peroxidation, SOD, catalase and ability to resist oxidative stress (ferric reducing ability of plasma) in full-term healthy infants during the first 12-months of life. They observed significantly increased levels of lipid peroxidation during first 4-months of life. These levels eventually reached normal adult values by 6-months of age. SOD and catalase also increase significantly from 1-month of age to 3-months. Furthermore, ferric reducing ability of plasma levels, as marker of ability to resist oxidative stress were

elevated at 1 month and declined thereafter, but remained above adult levels at 12 months.

Interestingly, premature newborns are considered to be even more susceptible to hyperoxia, which may be related to further decreased anti-oxidant levels. According to Yam et al (35), activities of SOD, catalase and GPx in the lungs were lower in premature rats fetuses compared to newborn rats. Furthermore, premature animals have been observed to have limited ability to increase antioxidant activity when exposed to oxidative stresses (36). Specifically in the heart, Hayashibe et al (37) observed a mild, but significant increase in GPx activity in rat hearts from day 20 to 22 gestation. However, levels of other antioxidants remain unchanged. These authors suggest that different organ systems mature at different rates during gestation, therefore are vulnerable to oxidative stress differently. In clinical studies immaturity of antioxidant defenses in premature infants have been suggested by decreased levels of cord blood SOD and lower plasma GSH concentrations (38,39).

Newborn Myocardial Protective Strategies

Controlled reoxygenation with Ambient Air

Once asphyxiated conditions are suspected in the fetus, immediately delivery and resuscitation are needed. Resuscitation of the asphyxiated newborn historically was carried out with 100% O_2. Rapid reversal of hypoxemia with oxygen therapy is one of the most important aspects of newborn resuscitation. A number of animal experiments have compared

21% O_2 to 100 O_2 used in resuscitation. In newborn piglet models of asphyxia-reoxygenation, Haase et al (40) observed similar cardiac recovery following oxygen therapy with 21, 50 and 100% O_2. Fugelseth et al (41), also reported similar cardiac recovery and injury following hypoxemia and reoxygenation with either 21% O_2 or 100% O_2 for 30 min in newborn piglets. Despite these findings of similar cardiac recovery, Kondo et al (42) observed increased ROS production on the lung surface following resuscitation with 100% O_2 compared to room air in newborn piglets.

Overall, *in-vivo* animal experiments suggest equivalent resuscitation with 21% compared to 100% O_2, with attenuated ROS production. These findings were evaluated in human pilot clinical study, in which 42 asphyxiated newborns were resuscitated with 21% O_2 and 42 with 100% O_2 (43). These authors reported similar outcomes between groups, with regard to acid base status, normalization of heart rate and time to first breath. However, asphyxiated newborns treated with room air had significantly improved Apgar scores at 5 minutes. This pilot study was then followed by a large multicenter clinical trial called Resair 2 (44), which included 609 neonates. This study reported room air to be equivalent to 100% O_2 resuscitation, along with significantly more neonates in the room air group with greater Apgar scores at 5 minutes. Interestingly, the time to first breath or first cry was shorter in the room air resuscitated neonates, which may indicate oxygen related depression of ventilation. Mortality was 5% lower in the room air resuscitated newborns, however was not statistically significant.

Vento et al (45) measured oxidative stress markers in 40 asphyxiated term neonates randomized to receive room air or 100% O_2 reoxygenation. Reduced to oxidized GSH ratio (a marker of oxidative stress) was significantly lower in the room air compared to the 100% O_2 resuscitated newborns, and these findings persisted as long as 28 days postpartum. Interestingly, in the newborns resuscitated with 100% O_2, antioxidant enzymes SOD and catalase were elevated following birth and remained elevated at 28 days post-partum, suggesting that antioxidant defenses may have been surpassed and not resolved 28 days later. A systematic review and pooled analysis by Davis et al (46) of five clinical trials and 1302 newborns resuscitated with either room air or 100% oxygen revealed a significant benefit for newborns resuscitated with room air (RR 0.71, CI 0.54 to 0.94). Saugstad et al (47) reported similar findings in a meta-analysis of five studies with 881 depressed newborns requiring resuscitation. The reported reduced mortality in depressed term newborn resuscitated with room air (OR 0.59, CI 0.40 to 0.87). A meta-analysis by Rabi et al (48) also reported a lower mortality rate in depressed newborn resuscitated with room air at 1-week and 1-month of life. Based on animal studies and human trials, controlled reoxygenation of asphyxiated newborns with room air (21% O_2) has been adopted into resuscitation guidelines in many countries worldwide (49).

Exogenous Antioxidant Therapy

Endogenous antioxidant defenses in the newborn (previously discussed) can become overwhelmed during hypoxia-reoxygenation. It has been proposed that exogenous administration of these antioxidants or other agents may combat the increased oxidative stress during resuscitation. Cheung et al (50) treated isolated rat hearts subjected to ischemia-reperfusion with GSH, and observed improved mechanical function and reduced production of peroxynitrite compared to non-treated hearts. Similarly, Shalfer et al (51) demonstrated in isolated rabbit hearts that reperfusion in the presence of SOD and catalase significantly enhanced left ventricular functional recovery compared to control hearts subjected to global ischemia.

Despite *in-vitro* evidence suggesting a role for exogenous antioxidant therapy, delivery of such treatment intracellularly remains an obstacle. N-acetylcysteine (NAC) has been proposed as a possible antioxidant therapy *in-vivo*. However, currently NAC is primarily used as a pharmacological therapy to restore GSH and cysteine, which are lost secondary to acetaminophen toxicity. NAC serves as a precursor of cysteine and GSH. In addition NAC can scavenge ROS, which is likely related to it being a thiol similar to GSH (52). However, the ability of NAC to directly scavenge ROS is substantially less compared to SOD, catalase and GPX (53). Therefore, NAC principally functions by replenishing cysteine, which is necessary for GSH synthesis (54). Cuzzocrea et al (55) treated rats subjected to shock with NAC and observed a protective effect, which they suggested may be related to peroxynitrite-

related pathways. Liu et al (56) randomized asphyxiated newborn piglets to receive NAC infusion or saline (placebo) for 24 hours during resuscitation with room air. The authors observed improved cardiac output and decreased troponin and lipid hydroperoxides (marker of oxidative stress) levels in NAC treated piglets compared to controls. Despite these encouraging findings suggesting NAC may be protective to the newborn heart through amelioration of oxidative stress via replenishment of reduced GSH, confirmation by clinical trials are needed.

Mitochondrial Modulators and Mitochondria Permeability Transition Pore

Mitochondria serve as cellular powerhouses, which drive cellular function through ATP production via oxidative phosphorylation. It also has been established that mitochondria play a key role in cellular death. Specifically, the extent of mitochondrial damage is associated with cellular apoptosis and necrosis. The cascades of events leading to apoptosis have been reviewed elsewhere (57), however we will focus on the mitochondrial permeability transition pore (MPTP) and its role in cellular myocardial death following hypoxia-reoxygenation or ischemia-reperfusion.

Al-Nasser and Crompton (58) initially suggested the presence of a reversible non-selective Ca^{2+}-activated pore in isolated liver mitochondria, which opened in response to increased matrix calcium. During myocardial ischemia-reperfusion, there is increased uptake of calcium into the tissue, resulting in mitochondrial calcium overload (59). Crompton et al (60), then

used isolated rat mitochondria to determine the presence of a reversible non-selective Ca^{2+}-activated pore in the heart. Furthermore, they observed that calcium and oxidative stress act synergistically to promote formation of this membrane pore during ischemia-reperfusion. They concluded that progression of ischemia-reperfusion injury is likely related to pore formation and resultant uncoupling of mitochondrial energetics. Controversy remains regarding the exact structure of the pore, however increasing evidence supports the involvement of cyclophilin-D (CyP-D) (61), adenine nucleotide translocase (ANT) (62). However, the key role of CyP-D was suggested following experiments on CyP-D knock-out mice (63). Specifically, CyP-D knockout mice mitochondria were highly resistant to calcium-induced pore opening and oxidative stress.

The inhibition of the MPTP has been suggested to limit ischemia-reperfusion injury to the heart or at least prevent the transition of reversible injury to irreversible myocardial damage. Halestrap et al were the first to establish that MPTP formation occurs during reperfusion rather than during ischemia (64). Furthermore, in subsequent experiments using radioactive tracers, Halestrap correlated MPTP closure with cardiac recovery following ischemia-reperfusion injury. Supportively, Hausenloy et al demonstrated decreased myocardial injury and increased resistance to oxidative stress in isolated perfused rat hearts following MPTP inhibition (65). Thus there may be a potential role of mitochondrial modulators or MPTP inhibitors in limiting reperfusion injury.

Cyclosporine A was initially observed to be potent inhibitor of MPTP opening by Crompton et al in 1988 (66). Griffiths et al reported that in-vivo cyclosporine A treatment of isolated rat hearts minutes before induction of ischemia-reperfusion restored myocyte ATP/ADP ratios to pre-ischemic values (67). Furthermore, these authors observed a reduction of myocardial reperfusion injury in the cyclosporine A treated group. Lim et al also observed that inhibition of the MPTP with cyclosporine A treatment in wild type mice resulted in reduction in myocardial infarct size (68). In contrast, CyP-D-deficient mice undergoing similar ischemia-reperfusion demonstrated no reduction in infarct size. It was hypothesized that cyclosporine's immunosuppressive activity, through inhibition of calcineurin in lymphocytes may have a role in reperfusion injury. However, Leshnower et al demonstrated a reduction in infarct size (by 20%) and apoptosis index in rabbit hearts following ischemia-reperfusion with cyclosporine treatment, but not with another calcineurin inhibitor (FK506) (69). Recently a pilot adult human randomized controlled study assessed whether the administration of cyclosporine at the time of percutaneous coronary intervention (PCI) would limit the size of infarct during acute myocardial infarction (70). Patients treated with cyclosporine A prior to PCI were observed to decreased biomarkers of myocardial injury and significant reduction in infarct size. Overall, adult animal models and human trials support the use cyclosporine A as a cardioprotective agent in adult myocardial ischemia-reperfusion injury.

The evidence supporting the potential protective effect of cyclosporine A in the resuscitation of asphyxiated newborns is limited. Currently, no human newborn trials have been conducted. In a newborn piglet model of asphyxia-reoxygenation (71), intravenous cyclosporine treatment minutes' following the onset of reoxygenation was observed to improve cardiac function and attenuate myocardial injury. Furthermore, mitochondrial morphology was better preserved in cyclosporine treated piglets compared to controls. Though further investigation of underlying mechanisms and optimal timing of the administration of cyclosporine are warranted, these initial findings are optimistic.

Therapeutic Hypothermia

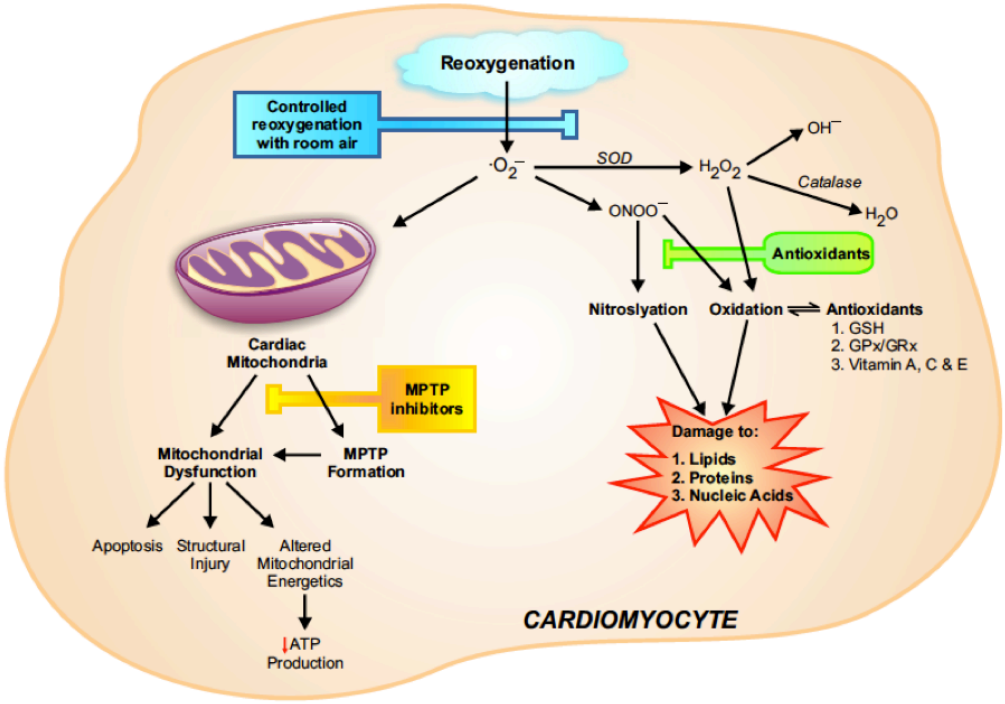
Therapeutic hypothermia has been investigated in a number of newborn animal models including rats and piglets. Following an initial report that demonstrated the efficacy of therapeutic hypothermia on attenuating cerebral injury in asphyxiated newborn piglets, Zhu et al reported reduced apoptotic cell death in newborn rats undergoing hypothermia following carotid artery ligation compared to controls at 72h (72). In human trials, therapeutic hypothermia after asphyxia complicated by hypoxic-ischemic encephalopathy has further been shown to be effective in reducing the neonatal mortality rate (73). In a recent randomized controlled trial, whole-body hypothermia reduced the mortality rate in asphyxiated newborns from 66% (controls) to 51% (hypothermia group) (73). These authors reported

that in newborns with hypoxic-ischemic encephalopathy, treatment with whole-body hypothermia within 6 hours of birth of seven infants would prevent one infant from dying or surviving with major disability. Regarding the effect of hypothermia in myocardial injury, the benefit is less obvious with therapeutic hypothermia being associated with bradycardia, arrhythmia and hypotension requiring vasopressive and or inotropic support (74,75). Indeed, the role of hypothermia in pediatric cardiac surgery, a practice since the report by Bigelow et al in 1953, has recently been challenged with the growing evidence of effectiveness and advantages of warm blood cardioplegia (76). Nonetheless, plasma troponin I concentration in asphyxiated newborn infants have been shown to correlate with clinical grade of hypoxic-ischemic encephalopathy (77). Recently using the porcine model of hypoxic-ischemic encephalopathy, Liu et al observed decreased plasma troponin I concentrations and fewer pathological lesions of myocardial ischemia in newborn piglets treated with therapeutic hypothermia (78). Shao et al have suggested that therapeutic hypothermia may protect cardiomyocytes by enhancing nitric oxide generation and attenuating ROS generation (79). Further clinical trials are needed to determine if the benefit of therapeutic hypothermia in attenuating the myocardial injury and enhancing cardiac functional recovery exists in asphyxiated newborns.

Conclusions

Perinatal asphyxia remains a significant cause of mortality and morbidity in newborns worldwide. Resuscitation of the asphyxiated newborn following delivery is necessary to reconstitute oxygenation to deprived myocardium and other vital tissues. During this reoxygenation process, ROS may be generated through multiple mechanisms. The immature endogenous antioxidant defense mechanism in the newborn heart may become overwhelmed, and oxidative cellular damage may ensue. Myocardial protective strategies during resuscitation, such as controlled reoxygenation with room air have been effective in reducing morbidity in asphyxiated newborns. Additional strategies aimed at improving antioxidant defenses and preventing MPTP formation have demonstrated improvement in cardiac function and reduction in myocardial injury in large animal models. Future clinical trials in asphyxiated newborns are warranted to determine future clinical applicability.

Figure 2-1. Cardiomyocyte injury mechanisms secondary to reactive oxygen species and potential protective strategies.



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Chapter 3

The Role of Cyclosporine in the Treatment of Myocardial Reperfusion Injury

Adapted from:

Gill RS, Bigam D, Cheung PY. *The Role of Cyclosporine in the Treatment of Myocardial Reperfusion Injury*. Shock 2012; 37: 341-347.

Abstract

Myocardial injury in adults, pediatric and newborn patients is a leading cause of mortality and morbidity. Though the underlying etiologies are different among patient populations, the sequence of initial ischemic-hypoxic injury followed by secondary myocardial reperfusion injury is relatively consistent. Overall infarct size is important because it is believed to be a key determinant of mortality. The detrimental effects of myocardial reperfusion have been proposed to be at least partially related to the formation of mitochondrial permeability transition pore (MPTP). The MPTP is a non-specific pore, which forms during myocardial reperfusion and allows the release of apoptotic signaling molecules and may also lead to cellular necrosis. Cyclosporine A has been shown to be a potent inhibitor of the MPTP, leading to its study as a potential treatment to limit myocardial reperfusion injury. Multiple adult animal models have demonstrated the protective effects of cyclosporine in ischemia-reperfusion. A recent human pilot clinical trial also reported reduced myocardial injury and infarct size in patients treated with cyclosporine intravenously prior to percutaneous coronary intervention for ST-elevation myocardial infarction. Despite the paucity of evidence of cyclosporine A demonstrating myocardial protective in pediatric and newborn patients, the existing animal experimental results are promising.

Introduction

Myocardial injury in adults is the leading cause of mortality worldwide (1). Initial injury to the myocardium occurs following coronary occlusion, followed by secondary injury during reperfusion. Though it is important to reestablish oxygenation of ischemic myocardial tissue, the generation of oxidative stress may occur and lead to myocardial injury (2). Overall infarct size is important because it is believed to be a key determinant of mortality (3). However, cardiac injury is not limited to adults with coronary artery disease. In pediatric patients, the myocardium following cardioplegic arrest with cardiopulmonary bypass surgery is exposed to similar reperfusion conditions with resultant myocardial dysfunction secondary to apoptosis (4,5). Furthermore, 50% to 80% of asphyxiated newborns demonstrate signs of myocardial injury or dysfunction (6), which may be worsened following reoxygenation or reperfusion (7).

The mitochondrial permeability transition pore (MPTP), a non-specific pore, has been suggested to play a key role in myocardial reperfusion injury through signaling cellular apoptosis and necrosis (8,9) (Figure 3-1). The opening of this pore in heart mitochondria leads to uncoupling of oxidative phosphorylation, ATP depletion and cell death (10-13). Cyclosporine A, a commonly used immunosuppressive in organ transplantation, has been shown to inhibit calcium-induced permeability transition in isolated mitochondria (14,15). Yellon and Hausenloy suggested that based on animal studies, cyclosporine might reduce final myocardial

infarct size by 50% by inhibiting the MPTP (1,16,17). In this review we explore the relationship between myocardial reperfusion injury and the MPTP using clinically relevant models of coronary arterial occlusion, cardiopulmonary bypass and neonatal asphyxia. Furthermore, we review the evidence suggesting cyclosporine as a pharmacological intervention against reperfusion injury through its actions on the MPTP.

Mitochondrial Permeability Transition Pore and Myocardial Reperfusion Injury

Crompton et al recognized that damage to the post-ischemic heart during reperfusion might be related to the opening of a non-specific pore (14,18). Subsequently, this non-specific pore was named the MPTP, which is a channel that forms within the inner mitochondrial membrane and allows passage of molecules smaller than 1.5 kDa (19,20). It is proposed that MPTP opening results in two major consequences; firstly it allows small molecular weight solutes to move freely into the mitochondria matrix with resultant mitochondrial swelling. This swelling then leads to unfolding of cristae and release of intermembrane proteins (i.e. cytochrome c) that signal apoptosis (21). According to Halestrap et al the key factors associated with MPTP opening are mitochondrial calcium overload, oxidative stress, mitochondrial depolarization and adenine nucleotide depletion (21). These factors are all the conditions present during reperfusion following an ischemic event (19-

21). Thus the potential relationship of the MPTP and myocardial reperfusion injury has made it a target of interest for cardioprotection.

Cyclosporine and the Mitochondrial Permeability Transition Pore

The major components of the MPTP remain a topic of debate as further research continues (11,12). Currently, the core components of the MPTP are proposed to be adenine nucleotide translocase (ANT) and mitochondrial cyclophilin (CyP)-D (12) (Figure 1). CyP-D has peptidyl-prolyl cis-trans isomerase (PPIase) activity, which in response to the onset of reperfusion leads to conformation changes in other less clearly defined membrane proteins and the eventual formation of the pore. The role of CyP-D in the formation of the MPTP is strongly supported by experiments in which Cyp-D knockout mice demonstrated high resistance to MPTP opening (13,22). The role of ANT has been less clearly delineated. Although most models suggest that CyP-D binds to ANT leading to a conformation change to induce pore formation (11,15,20), mice lacking ANT1 and ANT2 have been shown to still progress to pore formation (23).

Cyclosporine A is known to have a high affinity for the cyclophilin protein family. Cyclosporine has been previously shown to bind with to CyP-A and contribute to immunosuppression in renal transplant patients. Discovery of cyclosporine's affinity for CyP-D has led to research into its potential for cardioprotection. Cyclosporine A was initially shown to inhibit

calcium-induced permeability transition in isolated cardiac mitochondria (14,15). Subsequently Nazreth et al demonstrated cyclosporine as a potent inhibitor of MPTP opening in an isolated cardiomyocyte model (24). They reported an increase in isolated myocyte viability following hypoxia-reoxygenation with cyclosporine treatment. Subsequently, Griffiths and Halestrap demonstrated cyclosporine-induced cardioprotection on isolated rat hearts following 30 min of ischemia and 15 min of reperfusion (25). They reported that treatment with cyclosporine restored ATP/ADP ratio to pre-ischemic values and significantly improved left ventricular pressure during reperfusion. Xu et al cultured myocytes subjected to anoxia and reoxygenation to demonstrate that cyclosporine-treatment increased the number of viable cells, reduced cytochrome-c release and apoptosis based on TUNEL assay (26). In further studies, Griffiths and Halestrap delineated the timing of MPTP formation, which was shown to occur during reperfusion, and not the initial ischemic period (9). These authors subjected isolated perfused rat hearts to ischemia (30 min) followed by 15 min of reperfusion, and reported an increased yield of intact mitochondria with cyclosporine treatment compared to controls. Additional experiments with mitochondrial fractions from isolated hearts subjected to ischemia-reperfusion demonstrated an increased uptake of radiolabeled glucose with cyclosporine treatment. These findings suggested that MPTP closure with cyclosporine treatment would trap increased levels of radiolabeled glucose within the mitochondrial matrix (9). These finding in either myocytes or isolated

perfused hearts demonstrated the potent ability of cyclosporine to inhibit MPTP opening during reperfusion *in vitro*. Subsequently, experiments were performed to assess whether improvement in cardiomyocyte mitochondrial health may lead to attenuation of myocardial injury during reperfusion. Specifically, studies were performed to examine the effect of MPTP inhibition in animal models of myocardial injury associated with reperfusion/reoxygenation. In addition to cyclosporine, other inhibitors of MPTP opening have been developed with better specificity (Debio-025) or lack of immunosuppressive property (NIM811). For example, Debio-025, also known as alisporivir, was developed and has been investigated as a novel cyclophilin-binding agent to inhibit the human immunodeficiency virus type 1 (27). Altering the structure of cyclosporine to produce Debio-025 removes the calcineurin-binding domain, which was shown to abolish its immunosuppressive capacity (28-30). In addition, Debio-025 was shown to have an increased infinity for cyclophilin (27). The structure of NIM811 was also altered at the calcineurin-binding domain of CsA. In *in vivo* experiments using a graft-versus-host rat model, NIM811 was reported to have no immunosuppressive activity, while cyclosporine administration in this model inhibited lymph node enlargement (31). In addition, Rosenwirth et al observed increased serum creatinine and decreased creatinine clearance in rats following oral administration of cyclosporine A over 10 days. However, in rats treated with NIM811, both markers of renal injury remained unchanged (31), suggesting less nephrotoxicity than cyclosporine A.

The Protective Effect of Cyclosporine in Adult Ischemia-Reperfusion Models

Myocardial reperfusion injury following reestablishment of myocardial perfusion following an ischemic cardiac event leads to secondary injury. The initial ischemic damage to the adult heart occurs during coronary artery occlusion. Specific regions of the myocardium undergo irreversible damage, however other regions may have potentially viable tissue if apoptotic events can be attenuated. Argaud et al utilized an adult rabbit model, in which the left circumflex coronary artery was occluded for 30 min followed by 4 h of reperfusion (32) (Table 3-1). Cyclospoirne A or NIM811 (a non-immunosuppressive analog of cyclosporine) treatment was given intravenously either 10 min prior to sustained ischemia or 1 min before reperfusion. They reported a significant reduction in infarct size (reduced area of necrosis to area at risk ratio) with cyclosporine A and NIM811 treatment at both time points. Area of necrosis (as % of area at risk) was reduced with CsA and NIM811 treatment at reperfusion to 24% and 25% respectively, compared to 60% in controls. There was no difference in infarct size between cyclosporine and NIM811 treated groups at 10 min prior to ischemia or 1 min before reperfusion. Myocardial apoptosis (TUNEL) was also attenuated, evidenced by significantly reduced number of TUNEL-positive cardiomyocytes in both cyclosporine and NIM811 treated rabbits compared to controls. However, there was no difference between

cyclosporine and NIM811 treated groups at 10 min prior to ischemia or 1 min before reperfusion. Nevertheless, NIM811 is an attractive treatment option because of the proposed reduction in side effects compared to cyclosporine. Argaud et al also reported the need for a higher Ca^{2+} load to open MPTP in isolated mitochondria that were treated with NIM811 (33).

Leshnower et al also used a similar protocol utilizing adult rabbits with temporary left circumflex coronary artery occlusion (34). However, they questioned whether cyclosporine's calcineurin-inhibition might be responsible for its presumed cardioprotection and thus compared cyclosporine treatment to FK506 (calcineurin inhibitor) treatment. FK506 is also a potent calcineurin inhibitor used in solid-organ transplant, however directly binds FK506-binding proteins to inhibit calcineurin. These authors subjected adult rabbits to 30 min of ischemia and 3h of reperfusion and observed a decreased infarct size in only the cyclosporine treated group, with FK506 treated rabbits being similar to placebo-treated controls. On further examination of isolated cardiomyocytes at the end of the experiment, they reported a significantly reduced apoptotic index in the cyclosporine treated group compared to both placebo-treated controls and FK506 treated rabbits. Lim et al experimented on 8 to 10 week old wild-type mice that were subjected to 30 min of ischemia followed by 120 min of reperfusion. In wild-type mice, cyclosporine treatment (10 mg/kg i.v.) just prior to reperfusion, reduced infarct size (defined as % of area at risk) compared to placebo-treated controls following I-R (35). More importantly, when the protocol of I-

R was followed in CyP-D -/- knockout mice, infarct size was no longer attenuated with cyclosporine treatment, suggesting a critical role for MPTP formation in myocardial reperfusion injury.

Gomez et al subjected 8-week-old mice to left anterior coronary arterial occlusion for 25 min followed by 24h of reperfusion, and treated them with an MPTP-specific inhibitor (Debio-025) and followed them for 30 days (36). The mice treated with Debio-025 demonstrated improved 30-day survival compared to controls (survival rate: 89% vs. 58%), which they attributed to improved left ventricular contractile function. Specifically, mice treated with Debio-025 had improved left ventricular ejection fraction (77% vs. 62%) and reduced left ventricular end-diastolic diameter compared to controls on echocardiography at 30 days. This was the first study using a specific MPTP inhibitor to suggest that functional preservation of cardiac function may lead to improved survival.

A pilot clinical trial was performed by Piot et al to use cyclosporine-treatment following myocardial infarction in humans (37). They randomly assigned 58 patients presenting with acute ST-elevation myocardial infarct to either receive cyclosporine i.v. bolus (2.5 mg/kg) or saline (controls). A significant reduction in infarct size 5-days post-MI as seen with cardiac magnetic resonance imaging was found in the cyclosporine-treated group. Although this data is preliminary and requires confirmation in larger clinical trials, it further supports the potential preventative role for cyclosporine in myocardial reperfusion injury.

Multiple animal studies and a pilot clinical trial (Table 3-1) have suggested that cyclosporine may be a protective treatment in adult myocardial injury. Despite these favorable results, cyclosporine treatment was assessed in a large swine model of I-R (38). Thirty-six adult swine underwent 45 min of left anterior descending (LAD) coronary arterial occlusion, followed by cyclosporine A (10 mg/kg) treatment immediately prior to 4h of reperfusion. In contrast to previous animal studies, cyclosporine treatment at reperfusion did not limit myocardial infarct size. This may be related to the high mean plasma concentration of cyclosporine in the adult swine was 4.0 umol/L at 45 min post-administration. Comparatively, in the human adult trial by Piot et al, which demonstrated decreased infarct size; cyclosporine plasma levels were 2.5 umol/L at 20 min post-administration (37). In previous experiments in rats, cyclosporine treatment has been shown to have a narrow therapeutic window. Griffiths and Halestrap reported improved left ventricular pressure in isolated rats heart following I-R at cyclosporine 0.2 umol/L, however this protective effect was reversed with CsA 1.0 umol/L (25). The optimal dosing and therapeutic range has not been clearly defined in large animal or human models and likely is dependent on the differing rate of metabolism of cyclosporine among species. Furthermore, delineation of an optimal single intravenous dose of cyclosporine is also challenging considering the variability in therapeutic index seen in chronic use of cyclosporine A in solid-organ transplant patients. Further research is needed to define optimal therapeutic levels considering

the pharmacokinetics and pharmacodynamics of cyclosporine given as a single intravenous bolus.

The optimal timing of cyclosporine treatment in adult myocardial reperfusion injury has been suggested to be just prior to coronary revascularization. Despite equivocal reduction in infarct size and myocardial apoptosis observed with cyclosporine treatment 10 min prior to ischemia and 1 min prior to reperfusion in rabbits subjected to ischemia-reperfusion (32). In clinical practice, myocardial infarction secondary to coronary occlusion only allows pretreatment prior to revascularization with percutaneous coronary intervention. Pretreatment prior to ischemia, would not be clinically realistic. In the trial by Piot et al, cyclosporine was administered less than 10 min prior to intervention would thus theoretically allow adequate uptake by cardiomyocytes prior to reperfusion (37).

The Role of Cyclosporine in Pediatric Cardiopulmonary Bypass

Liu et al recently suggested age-associated differences in the inhibition of MPTP opening by cyclosporine, leading to decreased cardioprotection with aging when compared young (3-5 months) vs. old (20-24 months) rats (39). Cardiopulmonary bypass (CPB) is a vital component of pediatric cardiac surgery for congenital cardiac malformation repair. Once the pediatric patient is placed on CPB, the heart is arrested with the assistance of a cardioplegic solution, at which point the myocardium is

exposed to global ischemia. Unlike adult coronary arterial disease that affects specific regions of myocardium defined by the arterial territory, pediatric ischemic-reperfusion injury is diffuse. Even though cardioplegic solution and cooling is used to limit myocardial injury, reperfusion injury may occur once CPB is weaned. Oka et al placed 14-day-old newborn piglets on CPB and assessed markers of apoptosis and mitochondrial calcium tolerance in cardiomyocytes either pretreated with cardioplegic solution with cyclosporine (10 mg/kg) or cardioplegic solution alone (40) (Table 3-1). They reported improved mitochondrial structure and function in newborn (14 days) piglets given cardioplegic solution with cyclosporine during CPB. Despite a paucity of research assessing the potential protective effect of cyclosporine A during CPB, these results suggest that inhibition of MPTP formation may lead to improved mitochondrial integrity.

The Potential Benefit of Cyclosporine in Resuscitation of Asphyxiated Newborns

Neonatal asphyxia is defined as progressive, prolonged hypoxemia and hypercapnia leading to metabolic acidosis, which may result in multi-organ damage. Neonatal asphyxia affects over 1 million newborns worldwide (41), with over 50% to 80% demonstrating signs of cardiac dysfunction (6). Hence myocardial dysfunction is associated with significant mortality and morbidity in newborns. In addition, the immature newborn heart is unique compared to the adult heart. The neonatal heart has a greater volume of non-

contractile tissue mass, cardiac output that is heart rate-dependent and an increased vulnerability to apoptosis (42,43). Thus following hypoxemia, which leads to global ischemia, the newborn heart is more susceptible to diffuse injury during reoxygenation. We used an acute newborn piglet model of asphyxia-reoxygenation in which piglets were treated with cyclosporine i.v. at the onset of reoxygenation using various doses (44) (Table 3-1). All cyclosporine-treated (2.5, 10, 25 mg/kg) piglets demonstrated improved cardiac output and systemic oxygen delivery compared to controls. Cyclosporine-treatment at 2.5 and 10 mg/kg reduced myocardial injury evidenced by reduced plasma troponin and left ventricle lactate compared to controls. We also observed improved mitochondrial morphology with cyclosporine treatment. Although these initial findings are encouraging, longer studies are needed to confirm that these protective effects are sustained.

The therapeutic window is the newborn is less clearly defined than in adults. Pretreatment with cyclosporine is not feasible, as intravenous access to the asphyxiated fetus is not possible until actual delivery and establishment of intravenous access. Furthermore, many asphyxiated newborn infants are born in rural or peripheral locations without neonatal intensive care teams. Therefore, there may be a delay of approximately 2h (personal observation) before advanced resuscitative medications can be administered. Further research is needed to define the window of opportunity and the optimal time of administration in which cyclosporine

treatment may be cardioprotective during resuscitation of asphyxiated newborns.

The Adverse Effects of Cyclosporine

The adverse effects of cyclosporine administration should not be overseen. With cumulative dosing of cyclosporine in pediatric patients following renal transplantation, potential toxicities have been reported including seizures, gastrointestinal problems, gingival hyperplasia, arrhythmias, neuropathy, tremors and hypertrichosis. In particular, cyclosporine (and other calcineurin inhibitors) administration results in endothelial dysfunction (as in nephrotoxicity and transplant vasculopathy), which is related to impaired nitric oxide bioavailability, increased endothelin-1 sensitivity and oxidative injury (8-isoprostane levels) (45,46). It remains unclear if similar potential complications of cyclosporine administration may be observed with administration of a single dose of cyclosporine. The adult animal models of myocardial reperfusion injury discussed above did not assess renal function or injury. However, Cibulsktye et al specifically assessed the pharmacokinetic profile and renal toxicity in adult swine following single intravenous cyclosporine administration (47). They reported similar pharmacokinetic profile of intravenous CsA administration in swine to human renal transplant patients (CsA 3, 6 and 9 mg/kg i.v.). More importantly, they did not observe any deleterious effects of single intravenous cyclosporine administration on renal blood flow or

relative glomerular filtration rate. In asphyxiated newborn piglets, we also observed no significant adverse effect on renal perfusion, function or injury following a single dose of cyclosporine and 4h of reoxygenation (44). Nonetheless, caution should be exercised.

Summary

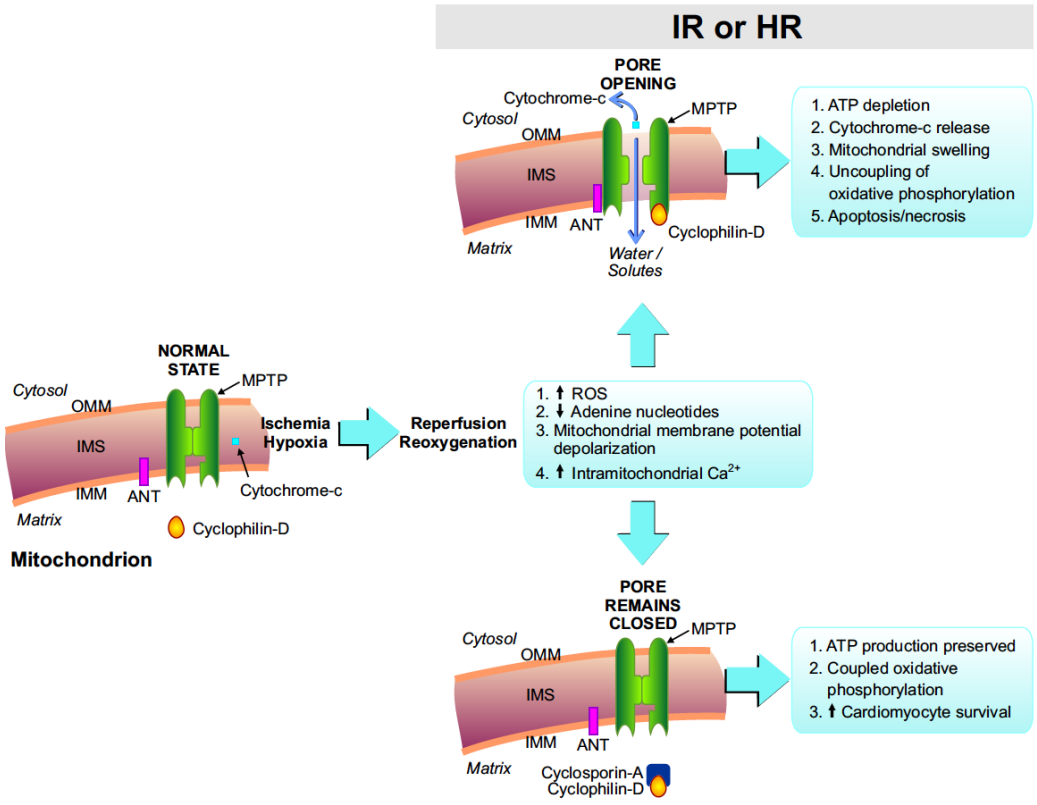
Cyclosporine A holds encouraging potential for cardioprotection during treatment of adults with coronary thrombosis during myocardial infarction, pediatric patients undergoing cardiac surgery and in asphyxiated newborns undergoing resuscitation. Despite different etiologies of ischemic-hypoxia myocardial injury, and the differing maturity of the myocardium, cyclosporine treatment has demonstrated protective effects. However, further clinical trials are needed to clarify the therapeutic threshold and optimal dosing of cyclosporine A.

Table 3-1. In-vivo models of cyclosporine A use in ischemia-reperfusion

Author, Year	Subjects	Model	Length of I-R	Dose of IV CsA (mg/kg)	Timing of CsA dose	1° Outcome	2° Outcome
Argaud, 2005 (32)	Adult rabbits	Circumflex artery occlusion	I=30min R=4h	10	(1) 10 min before ischemia (2) 1 min prior to reperfusion	Reduced infarct size (AN/AR)	Apoptotic cell death reduced (% apoptosis of cardiomyocytes)
Argaud, 2005 (33)	Adult rabbits	Left Circumflex Coronary Artery	I=30min R=4h	(NIM811) 5	1 min prior to reperfusion	Reduced infarct size (AN/AR)	Increased Ca^{2+} load to open MPTP
Gomez, 2007 (36)	8 wk old mice	Left Anterior occlusion	I=25min R=1d or 30d	(Debio-025) 10	5 min prior to reperfusion	Improved 30d survival (% survival rate)	Improved LV ejection fraction
Lim, 2007 (35)	8-10 wk old mice	LAD occlusion	I=30min R=2h	10	At reperfusion	Reduced infarct size (AN/AR)	
Leshnower, 2008 (34)	Adult rabbits	Circumflex Coronary Artery occlusion	I=30min R=3 h	25 (over 1h)	Infusion during ischemia and portion of reperfusion	Infarct size decreased (AN/AR)	Apoptotic cell death decreased (apoptosis index%)
Piot, 2008 (37)	Adult humans	STEMI and PCI	<12 hours CP	2.5	Less than 10 min prior to reperfusion	Reduced infarct size (on MRI)	Decreased serum CK levels
Karlsson, 2010 (38)	Adult Swine	LAD Coronary Artery occlusion	I=45min R=2h	10	3 min prior to infusion	No change in infarct size (AN/AR)	
Liu, 2011 (39)	3-5 mo & 20-24 mo old rats	LAD occlusion	I=30min R=2h	10	5 min prior to reperfusion	Reduced infarct size (AN/AR) in young rats only	NAD+ levels preserved in young rats only
Oka, 2008 (40)	14 d piglets	CPB	Aorta CC time= 60min	10 + CCP	Following Aorta CC	Less Cytochrome-c release	Decreased apoptosis (TUNEL)
Gill, 2011 (44)	1-4 d piglets	Alveolar hypoxia	H=2h R=4h	2.5, 10, 25	5 min after reoxygenation	Improved cardiac output (% baseline)	Reduced troponin and LV lactate

I = ischemia; H = hypoxia; R = reperfusion/reoxygenation; CsA = cyclosporine A; i.v. = Intravenous; LV = left ventricle; Ca^{2+} = calcium; MPTP = mitochondrial permeability transition pore; LAD = left anterior descending; STEMI = ST-elevation myocardial infarction; PCI = percutaneous coronary intervention; CPB = cardiopulmonary bypass; TUNEL = terminal dUTP nick end labeling; CK = creatinine kinase; CC = cross clamp; AN = area of necrosis; AR = area at risk; MRI = magnetic resonance imaging

Figure 3-1. Proposed working model of mitochondrial permeability transition pore (MPTP) formation during reperfusion/reoxygenation after ischemia/hypoxia, and inhibitory effects of cyclosporine A (48).



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Chapter 4

Neonatal Piglet Model for Perinatal Asphyxia (Part I)

Introduction

Perinatal asphyxia in human newborns can lead to a range of neurological and cardiovascular sequelae (1). Neurological sequelae can range from minor learning disabilities to seizures and coma. Cardiovascular damage may be mild, or severe with heart failure. Therefore to study the effects of asphyxia a surrogate animal model is needed. Animal models enhance our understanding of the physiological and pathophysiological consequences of asphyxia and allow us to assess the benefits and risks of interventional strategies. The species used should preferably be of appropriate size and weight to increase its similarity to the human infant. The neonatal piglet seems to be the most appropriate animal species, with a size of approximately 1.5 to 2 kg at birth and development age similar to a 36-38 week old human fetus (1). Further structural and functional resemblances between humans and pigs will be discussed in detail in this chapter.

Cardiovascular System

Swine, also called *Sus scrofa domestica* descend from European wild boar domesticated 9000 years ago (2). Swine are good models for animal research because of their uniform and predictable size for each breed and the short cycle of reproduction. Currently, swine models are increasingly used because of physiological characteristic similarities to humans (3). In terms of the cardiovascular system, the coronary blood flow in swine is almost

analogous to humans. Swindle et al suggest that the coronary system is similar to 90% of the human population in anatomy and function (4). In addition, there are no pre-existing collateral vessels in the myocardium and the blood supply to the conduction system is via the posterior septal artery. Therefore conduction is right side dominant, similar to humans. These similarities have allowed swine to be used as experimental models for coronary blood flow, infarct production and hemodynamic studies. The comparable histological myocardial anatomy is also useful when assessing infarction. However, blood vessels and atria are more friable in swine, especially neonates, making the instrumentation more challenging (4).

Gastrointestinal System

The gastrointestinal tract of swine has anatomic differences from human but analogous physiologic function (4). There are vascular differences in the small intestine with vascular arcades forming in the muscularis mucosa rather than in the mesentery. The swine colon is also spiral and located in the left upper quadrant. No discernible appendix exists in the pig. The splanchnic blood flow characteristics are analogous to the human thereby serving as an excellent model for neonatal necrotizing entercolitis.

Pulmonary System

The pulmonary vascular reactivity to gas exchange in response to global and regional hypoxia in newborn piglets is similar to the human

neonates (5). They both share morphological and functional characteristic within the pulmonary vascular bed. Pulmonary vasoconstriction occurs in response to alveolar hypoxia. The hypoxic pulmonary vasoconstriction serves a mechanism to match perfusion with ventilation, to optimize gas exchange. Redding et al also reported in piglets that anesthesia does not significantly reduce pulmonary vascular responsiveness to alveolar hypoxia (6).

Hemodynamics and Laboratory Values

There has been extensive research conducted in establishing baseline hemodynamic and serum values in newborn piglet models to compare various interventions. Hannon presented hemodynamic profiles of conscious newborn swine with heart rate ranging between 180-250 beats per minute and mean arterial pressure ranging around 58-74 mmHg (7). We observed similar hemodynamic profiles in newborn piglets in our experiments. Schmidt et al reported normal blood values found in clinically healthy normal pigs. Their reported normal hemoglobin at birth was 12.2 +/- 0.3g/dl (8). Furthermore, blood chemistry is described for newborn swine with normal values of sodium, potassium and chloride being 142 +/- 1, 5.0 +/- 0.2 and 109 +/- 2 respectively (9).

Anesthesia

Induction of anesthesia in the newborn piglet often requires inhaled muscle relaxants such as halothane or isoflurane because of the lack of superficial veins for intravenous sedation (10). Sedation may be maintained with intravenous medications once access is established. Muscle relaxants can be given intravenously to prevent undesired movements. Succinylcholine should be avoided because it may trigger malignant hyperthermia (10), however pancuronium serves as a useful option. According to Manohar et al the use of inhaled anesthetics such as halothane alter hemodynamics with the swine. Cerebral vasodilatation ensues along with hemodynamic shifts in myocardial, renal and splanchnic flow. Thus, following induction of anesthesia, halothane should be discontinued.

In summary, the neonatal piglet model is an excellent model to study perinatal asphyxia in human neonates.

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Chapter 5

Neonatal Piglet Model for Perinatal Asphyxia (Part II)

Adapted from:

Cheung PY, **Gill RS**, Bigam DL. *A Swine Model of Neonatal Asphyxia.* Journal of Visualized Experiments 2011 Oct 11; (56). Pii: 3166. Doi: 10.3791/3166.

Abstract

Annually more than 1 million neonates die worldwide as related to asphyxia. Asphyxiated neonates commonly have multi-organ failure including hypotension, perfusion deficit, hypoxic-ischemic encephalopathy, pulmonary hypertension, vasculopathic enterocolitis, renal failure and thrombo-embolic complications. Animal models are developed to help us understand the patho-physiology and pharmacology of neonatal asphyxia. In comparison to rodents and newborn lambs, the newborn piglet has been proven to be a valuable model. The newborn piglet has several advantages including similar development as that of 36-38 weeks human fetus with comparable body systems, large body size (~1.5-2 kg at birth) that allows the instrumentation and monitoring of the animal and controls the confounding variables of hypoxia and hemodynamic derangements.

We here describe an experimental protocol to simulate neonatal asphyxia and allow us to examine the systemic and regional hemodynamic changes during the asphyxiating and reoxygenation process as well as the respective effects of interventions. Further, the model has the advantage of studying multi-organ failure or dysfunction simultaneously and the interaction with various body systems. The experimental model ***is a non-survival procedure that*** involves the surgical instrumentation of newborn piglets (1-3 day-old and 1.5-2.5 kg weight, mixed breed) to allow the establishment of mechanical ventilation, vascular (arterial and central venous) access and the placement of catheters and flow probes (Transonic

Inc.) for the continuously monitoring of intra-vascular pressure and blood flow across different arteries including main pulmonary, common carotid, superior mesenteric and left renal arteries. Using these surgically instrumented piglets, after stabilization for 30-60 minutes as defined by <10% variation in hemodynamic parameters and normal blood gases, we commence an experimental protocol of severe hypoxemia which is induced via normocapnic alveolar hypoxia. The piglet is ventilated with 10-15% oxygen by increasing the inhaled concentration of nitrogen gas for 2h, aiming for arterial oxygen saturations of 30-40%. This degree of hypoxemia will produce clinical asphyxia with severe metabolic acidosis, systemic hypotension and cardiogenic shock with hypoperfusion to vital organs. The hypoxia is followed by reoxygenation with 100% oxygen for 0.5h and then 21% oxygen for 3.5h. Pharmacologic interventions can be introduced in due course and their effects investigated in a blinded, block-randomized fashion.

Protocol Text

1. Anesthesia

1.1. Set the flow rate of the anesthetic machine at 2L/min. Connect the exhaust to vacuum suction.

1.2. Charge face mask with anesthetic gas (Isoflurane) at 5% (~3 min).

1.3. Newborn piglets will be induced with inhaled Isoflurane 5% in 100% oxygen (~3 min).

1.4. Maintain anesthesia at 2-3% of Isoflurane. Fine adjustment of Isoflurane by 0.5% as appropriate, however, it may range from 0.5 to 5% depending on the condition of piglets.

1.5. Once the vascular access has been established, the inhalational anesthesia can be switched to intravenous anesthesia using fentanyl (5-50 mcg/kg/h) and midazolam (200-500 mcg/kg/h) infusions. Pancuronium (50-100 mcg/kg/h) may be required to control excessive muscle movements during the surgery, whilst the ability to observe animal's state is preserved for the adjustment of anesthetic medications.

1.6. The piglet is monitored by pulse oximetry (percutaneous oxygen saturation at 95-100%) and ECG (heart rate at 130-170 beats/min).

1.7. The piglet's rectal temperature is maintained at 38-40°C with heating blanket and radiant warmer.

1.8. The anesthetic state of piglet is being regularly evaluated throughout the experimental period using neurological (pupil size, tearing, body movements), behavioral (agitation), cardiovascular (tachycardia and hypertension) and respiratory (tachypnoea) parameters as appropriate. Minimal paralysis is given. Previous experience of anesthesia in piglets with and without paralysis would be useful for evaluation.

1.9. The protocol is a non-survival procedure with euthanization of the animal at the end of experiment with an overdose of pentobarbital (100 mg/kg) intravenously.

2. Surgical placement of vascular catheters at the groin (Figure 5-1)

2.1. Make a long 2-3cm incision in the right groin.

2.2. Dissect 1cm of the right femoral venous and 1cm right femoral artery. Put two 3-0 strings around each vessel.

2.3. Right femoral venous catheterization: Ligate the distal of the vein. Insert an Argyle™ catheter (3.5 or 5 French, double- lumen)(Covidien, Mansfield, MA) to 15cm and this will place at the right atrium. Tie both strings to secure the catheter. The catheter can be used for maintenance fluid and medications infusion (secondary port) and central venous/right atrial pressure measurement (primary port).

2.4. Right femoral arterial catheterization: Ligate the distal of the artery. Lift up the proximal string to stop the blood flow. Insert an Argyle™ catheter

(3.5 or 5 French, single-lumen) to 5cm. This will place the arterial catheter at the infra-renal aorta for continuous mean arterial pressure measurement and blood sampling. Tie both strings to secure the catheter.

2.5. Close the skin.

3. Establish mechanical ventilation (Figure 5-2)

3.1. Make a long 2-3cm horizontal incision in the neck.

3.2. Dissect and expose 1cm of the trachea. Put two 1-0 strings around the trachea.

3.3. Insert an endotracheal tube (3.0 or 3.5) at 1cm into the trachea. Connect to a ventilator and commence mechanical ventilation. Secure the endotracheal tube.

3.4. Dissect and expose the common carotid artery. Encircle the vessel with a transit time ultrasound flow probe (2SB or 2RB, Transonic Systems Inc., Ithica, NY) to continuously measure the blood flow.

4. Placement of flow probes at superior mesenteric (Figure 5-3) and left renal (Figure 5-4) arteries

4.1. Extra doses of fentanyl (5-10 mcg/kg) and acepromazine (0.01-0.02 mg/kg) are required prior to skin incision.

4.2. Make a long subcostal-flank incision and carefully dissect muscle layers.

4.3. Expose the abdominal aorta.

4.4. Minimize vascular handling (vasospasm) and lymphatic injury.

4.5. Dissect 0.5-1cm superior mesenteric artery and put a Transonic® flow probe (3SB) around it.

4.6. Dissect 0.5-1cm left renal artery and put a Transonic® flow probe (2SB) around it.

4.7. Close the skin and secure the flow probe.

5. Placement of pulmonary artery catheter (Figure 5-5) and flow probe (Figure 5-6)

5.1. Extra doses of fentanyl (5-10 mcg/kg) and acepromazine (0.01-0.02 mg/kg) are required prior to skin incision.

5.2. Lie the animal at the right lateral position.

5.3. Thoracotomy at the left 4th intercostal space.

5.4. Watch out for the internal mammary artery and vein, ligate if needed.

5.5. Use a dental swab to press down the left lung and increase oxygen as needed.

5.6. Open the pericardium.

5.7. Identify the ductus arteriosus which runs from the pulmonary artery to the aorta.

5.8. Ductus arteriosus may be ligated by placing a clip or by a thick “3-O silk” tie at its origin.

5.9. Free the main pulmonary artery and pass a vascular sling using a thick “0” tie.

5.10. Perform a purse string (5-0 prolene) suture at the base for the placement of pulmonary artery catheter.

5.11. Insert a 20G Angiocath® (with 3 side holes at less 1 cm from the tip of the catheter) through the purse string to a maximum of 1 cm.

5.12. Check for free flow of venous blood.

5.13. Connect to pressure transducer, check for pulmonary artery pressure and waveform.

5.14. Tighten the purse string and secure the pulmonary catheter.

5.15. Place a Transonic® flow probe (6SB) around the main pulmonary artery.

5.16. Cover the wound with moist saline gauze.

5.17. Place ultrasonic gel between the flow probe and artery to allow for optimal signal transduction.

6. Hypoxia and reoxygenation protocol

6.1. Decrease the inspired oxygen concentration to 10% by increasing the concentration of inhaled nitrogen gas to induce hypoxemia.

6.2. Adjust the inspired oxygen concentration between 10% and 15% to obtain a PaO_2 of 20-40 mmHg or SaO_2 of 30-40% for 2h.

6.3. Perform arterial blood analysis to assess $PaCO_2$ and adjust ventilator rate accordingly.

6.4. With the induction of hypoxemia, the first hour is dedicated to steadily inducing a tachycardic (and cardiac output) response.

6.5. Continue to monitor for changes in blood flow at the common carotid, superior mesenteric and left renal arteries.

6.6. During the second hour of hypoxia, the hypoxic stress is increased to steadily lower cardiac output to 30-40% of baseline, mean arterial pressure to 30-35 mmHg and arterial pH 6.95-7.05.

6.7. Hypoxic stress may be prematurely terminated or extended by 15 min as appropriate.

6.8. Increase inspired oxygen concentration abruptly to 100% abruptly by discontinuing nitrogen gas, while continuing pure oxygen.

6.9. Monitor cardiac output, mean arterial pressure and other hemodynamic parameters for rapid recovery.

6.10. Resuscitation with 100% oxygen can be continued for 0.5h. Following this time period, reduce the inspired oxygen concentration quickly to 21%.

6.11. Continue reoxygenation with 21% oxygen for the remaining period of experiment. The inspired oxygen concentration can be titrated to 25% if needed.

6.12. Fluid boluses of 10 ml/kg Ringer's lactate solution may be needed as appropriate during the experimental period. Its use has to be protocolized.

Discussion

The current experimental protocol has an advantage to examine the systemic and regional hemodynamic changes in neonatal subjects during the hypoxia and reoxygenation process. We can also examine the respective effect of interventions used to improve the cardiovascular function during recovery. We and others have reported the experience and findings in the study of neonatal asphyxia regarding the effects in cardiovascular (1), pulmonary (2), neurologic (3), gastrointestinal (4), hepatic (5), renal (6), adrenal (7) and hematologic (8) systems. While it is important to understand the cardiovascular function with information based on continuous data measurements, it is technically challenging if not impossible to surgically instrument small-sized animals such as rodents or guinea pigs. Recent advancement in technologies such as ultrasonography and real-time imaging may however overcome some of these challenges. Nonetheless, large-sized

animals also allow the simultaneous collection of biological samples including plasma and tissue samples during the experimental period. This additional biological sampling will allow biochemical assays and histologic examination, which help the understanding of the patho-physiology and pharmacology of hypoxia and reoxygenation. While the primary objective of *in vivo* animal models may be the study of patho-physiologic function of a single body system, it is important to understand it in the context of organ-organ interaction. For example, the interaction between the cardiac function and pulmonary hypertension or hepatic dysfunction is important in a multi-organ dysfunction as that of neonatal asphyxia (9). The newborn lamb is an alternative to swine in the common animal models used to study neonatal asphyxia. The precocious development and limited litter size of newborn lambs may however restrict a more generalized use than newborn piglets, which correspond to that of 38 weeks gestation human fetus and have approximately 10 per litter (10,11). Nonetheless, newborn piglets are the most frequently used animals after rodents in the study of neonatal asphyxia.

However, there are limitations of this swine model of neonatal asphyxia, in addition to the challenge related to the translation of findings generated from animal studies to human. The effect of anesthesia and surgical stress as to the acute setting may be minimized with an adequate stabilizing period, appropriate use of anesthetic medications, refined surgical techniques as well as the inclusion of sham-operated control animals for comparison. Prolonging the experimental period beyond days is needed to

investigate if any acute hemodynamic effect will persist in the long term. Indeed, we have been succesful in modifying the experimental protocol to extended subacute (e.g. 48-72 hours) (12), survival (5-7 days) (13) as well as chronically instrumented studies. In these prolonged protocols, careful hypoxia and intensive medical and nursing care are important to minimize the mortality and morbidity. Furthermore, the ligation of patent Ductus Arteriosus is important to our use of pulmonary arterial flow as a surrogate of cardiac output although there is minimal flow across the Ductus during hypoxia and reoxygenation in these newborn piglets. The accuracy of estimating pulmonary vascular resistance will improve with the cannulation of left atrium for the simultaneous measurement of the left atrial pressure. In comparison with our hypoxia protocol, combining hypercapnia with severe hypoxia will better simulate clinical asphyxia. Apart from alveolar hypoxia as in the current protocol, other approaches to induce hypoxia include the creation of pneumothorax (14), halting mechanical ventilation (15) and the addition of carotid artery occlusion for cerebral ischemia. We attempt to make the hypoxia and reoxygenation clinically relevant. The experiment includes 2h of hypoxia which is approximate to the duration required for emergency cesarean section for fetal distress without clinical bleeding based on personal observation. The resuscitation is initiated with 100% oxygen for 30 min, instead of 60 min in our previous studies. This is to limit the hyperoxia which remains a common practice in many community hospitals prior to the arrival of neonatal transport team. Initial reoxygenation with

21% oxygen will follow the recently updated guideline on the use of supplemental oxygen in neonatal resuscitation (16).

Figure 5-1. Groin incision with the placement of femoral arterial and venous catheters

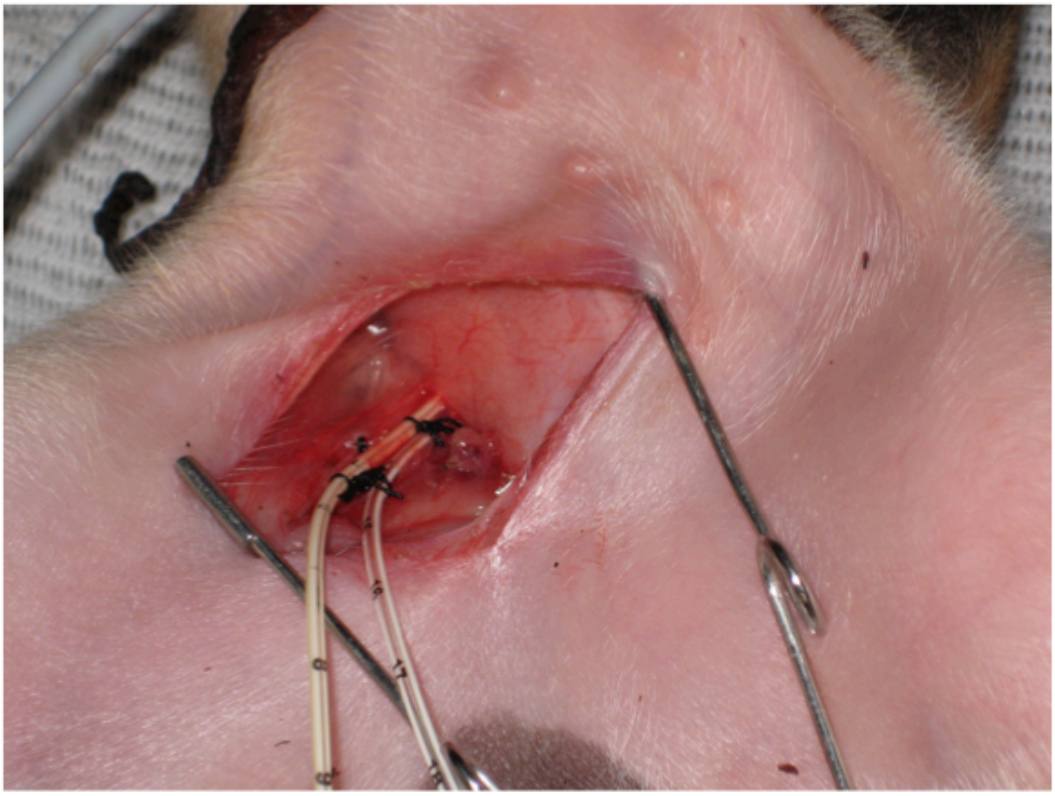


Figure 5-2. Neck incision with the placement of an endotracheal tube and a flow probe around the common carotid artery

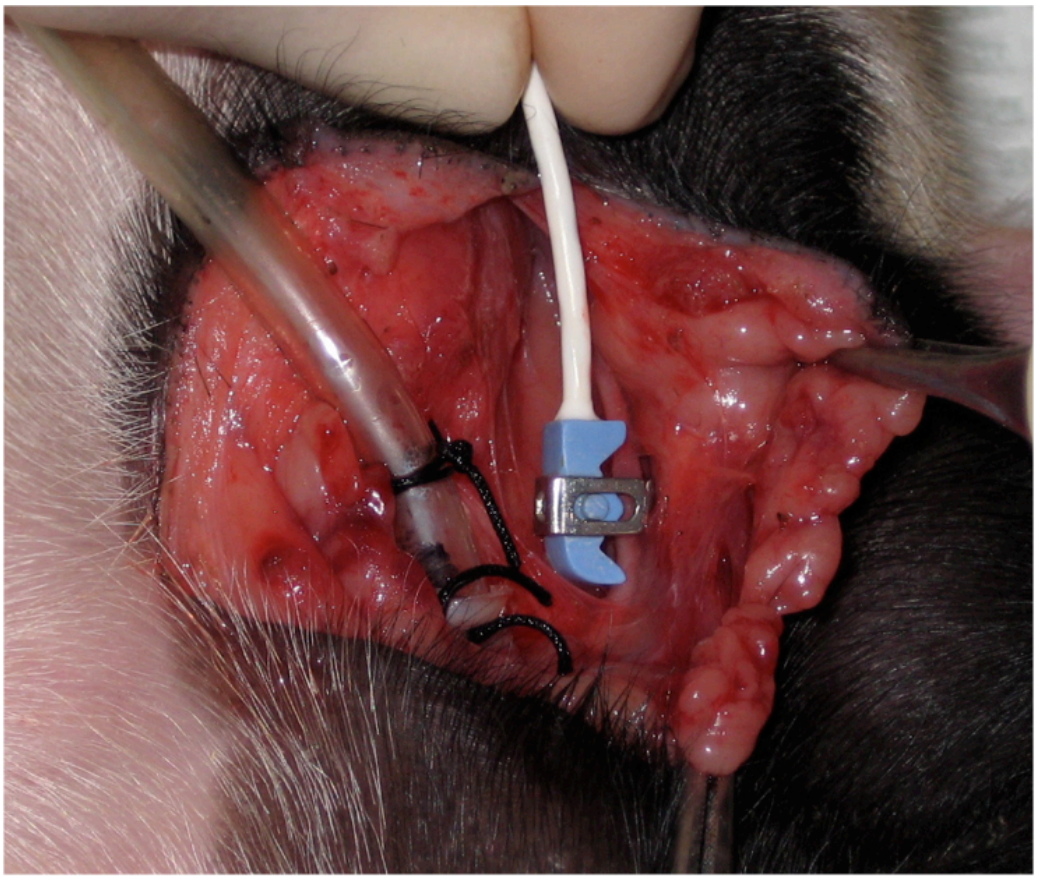


Figure 5-3. Flank incision with the isolation of the superior mesenteric artery

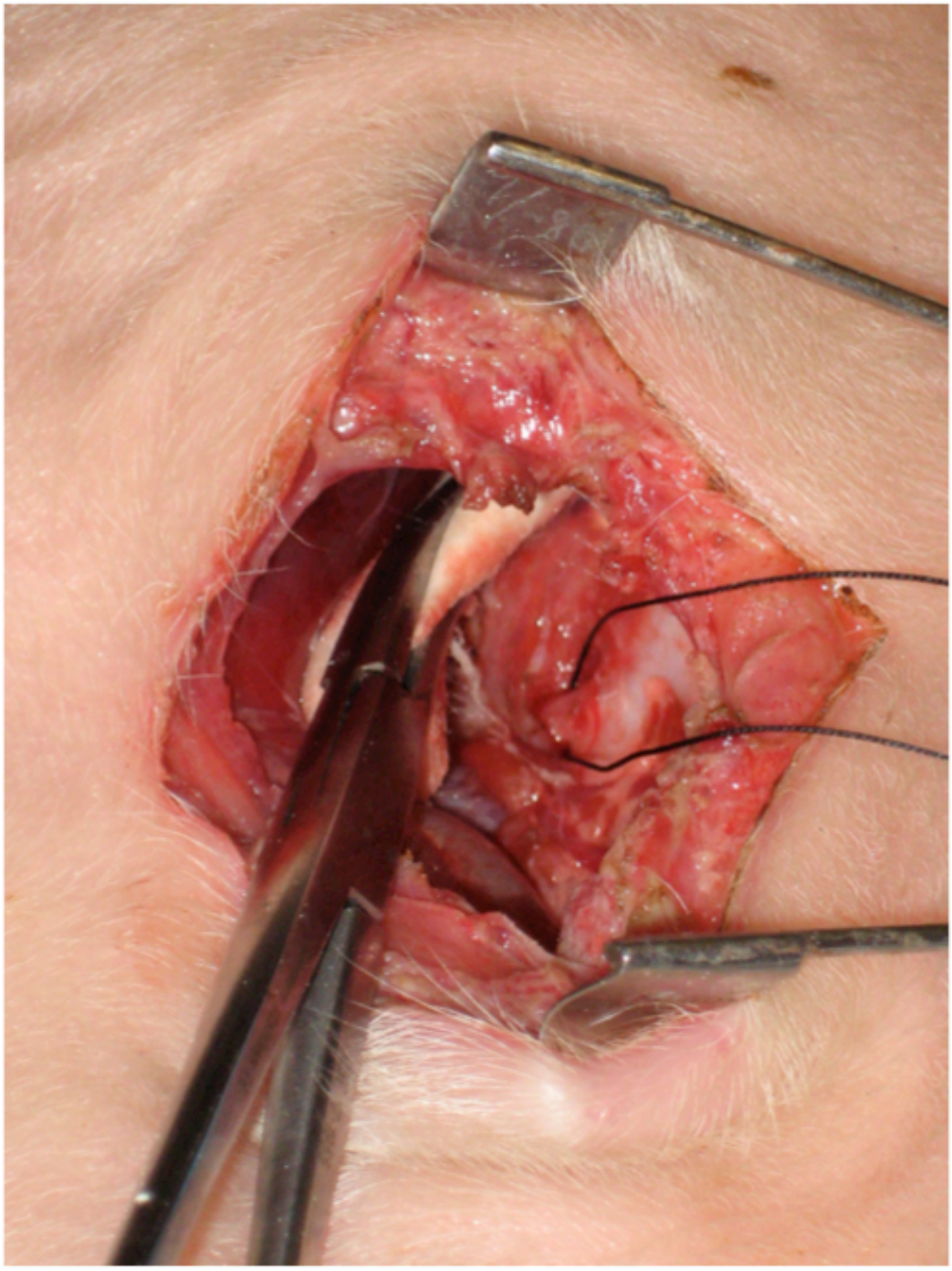


Figure 5-4. Flank incision with isolation of left renal artery.

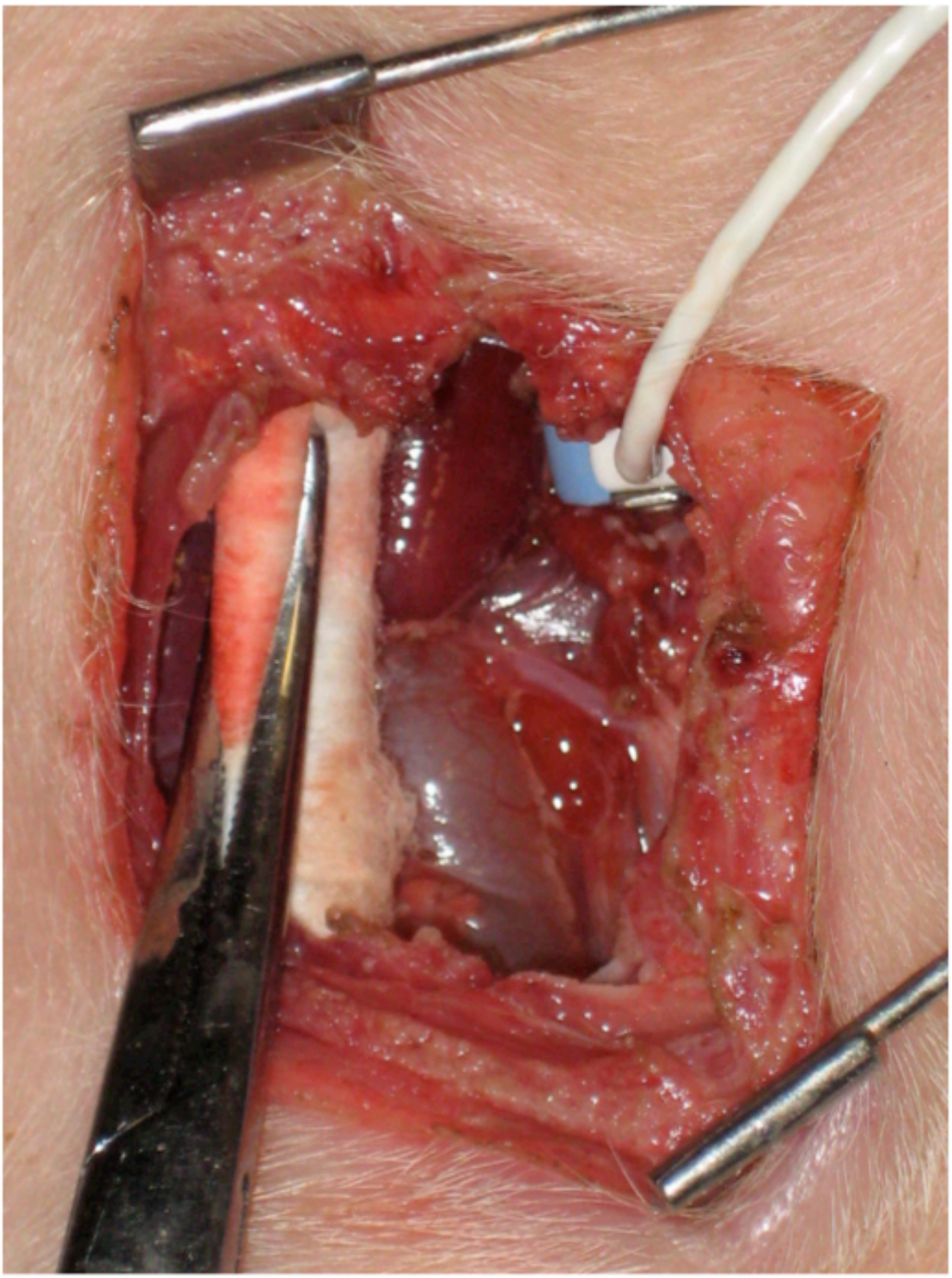


Figure 5-5. Thoracotomy with the placement of pulmonary artery catheter.

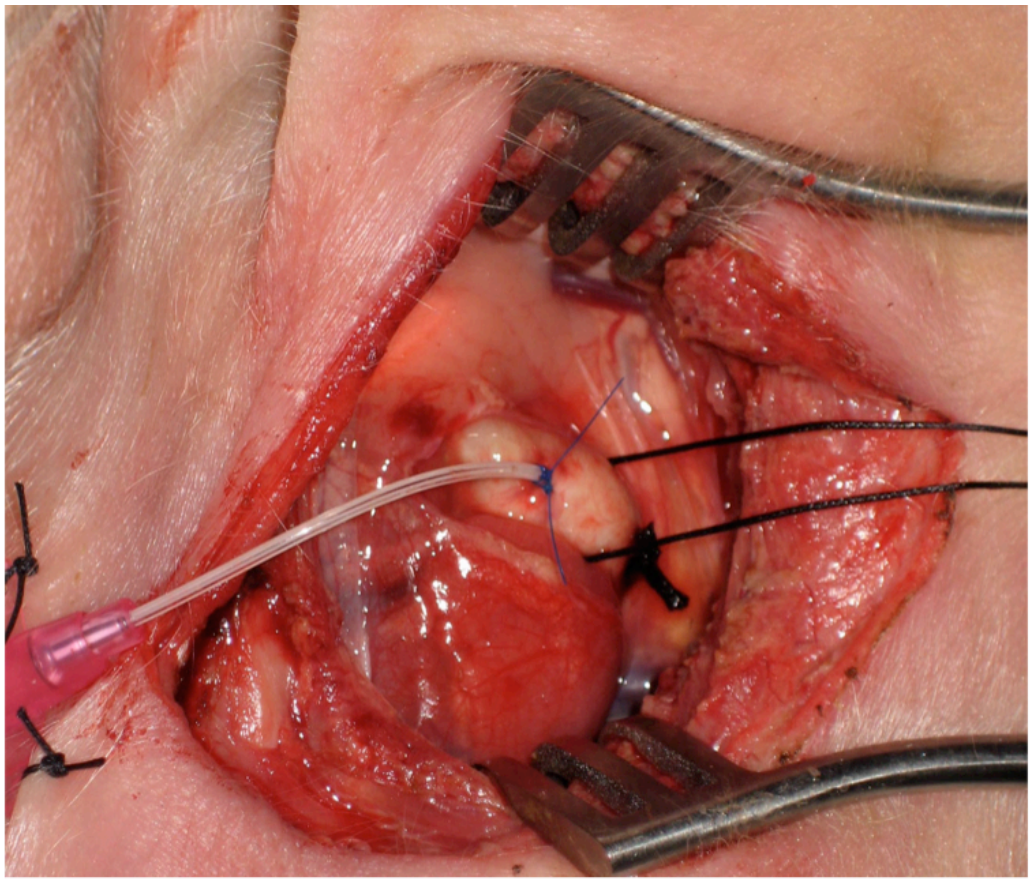
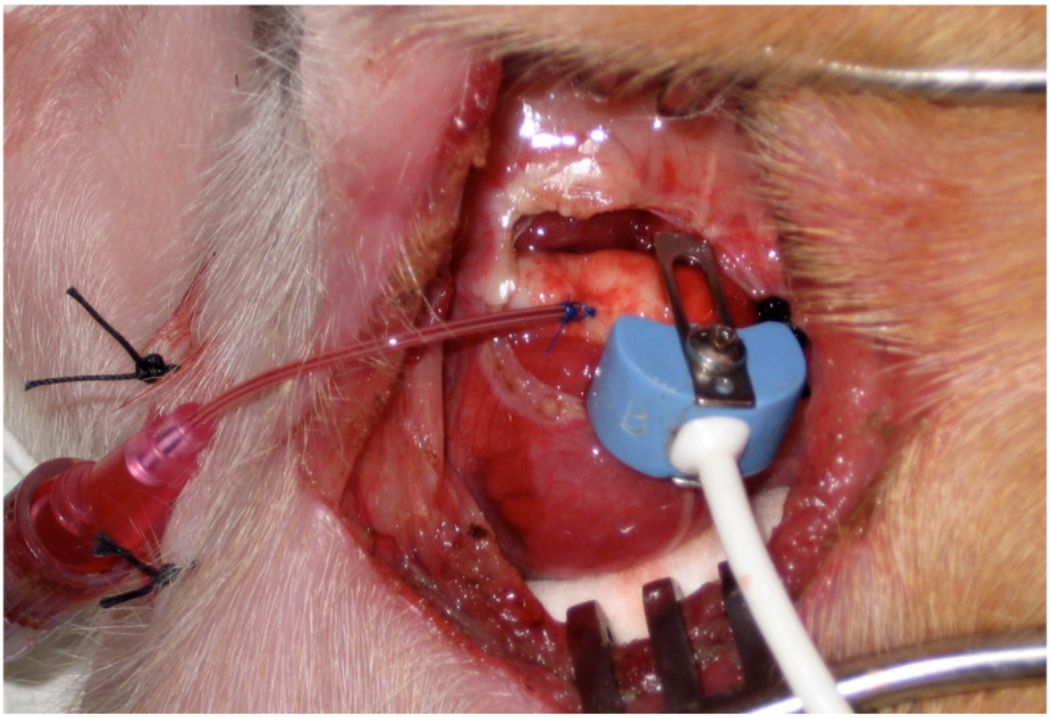


Figure 5-6. Thoracotomy with the placement of a Transonic® flow probe around main pulmonary artery.



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Chapter 6

Cyclosporine Treatment Improves Cardiac Function and Systemic Hemodynamics during Resuscitation in a Newborn Piglet Model of Asphyxia: A Dose-Response Study

Adapted from:

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Abstract

Background

Asphyxiated neonates often have myocardial depression which is a significant cause of morbidity and mortality. Cardioprotective effects of cyclosporine have been observed in adult patients and animals with myocardial infarction. However, the cardioprotective effect of cyclosporine in neonates has not yet been studied. We hypothesize that cyclosporine will improve cardiac function and reduce myocardial injury in asphyxiated newborn piglets.

Methods

Thirty-six piglets (1-4 days-old, weighing 1.4-2.5 kg) were acutely instrumented for continuous monitoring of cardiac output and systemic arterial pressure. After stabilization, normocapnic alveolar hypoxia (10-15% oxygen) was instituted for 2h followed by reoxygenation with 100% oxygen for 0.5h, then 21% for 3.5h. A non-asphyxiated, sham-operated group was included (n=4) to control for effects of the surgical model. Plasma troponin and myocardial lactate concentrations were determined as well as morphological examinations. Piglets were block-randomized to receive intravenous boluses of cyclosporine A (2.5, 10 or 25 mg/kg) or normal saline (control) at 5 minutes of reoxygenation (n=8/group).

Results

Hypoxic piglets had cardiogenic shock (cardiac output 40-48% of baseline), hypotension (mean arterial pressure 27-31 mmHg) and acidosis (pH 7.04).

Cyclosporine treatment caused bell-shaped improvements in cardiac output, stroke volume and systemic oxygen delivery ($p<0.05$ vs. controls). Plasma troponin and left ventricle lactate were higher in controls than that of 2.5 and 10 mg/kg cyclosporine-treated groups ($p<0.05$). Although histological features of myocardial injury were not different among groups, severe damage was observed in mitochondria of control piglets but attenuated in that of cyclosporine (10 mg/kg) treatment.

Conclusions

Post-resuscitation administration of cyclosporine causes preservation of cardiac function and attenuates myocardial injury in newborn piglets following asphyxia-reoxygenation.

Introduction

Asphyxia is a global issue, with over 1 million newborn deaths per year worldwide (1). Hypoxemia-related myocardial dysfunction occurs in over 30% of asphyxiated neonates (2). Thus, myocardial dysfunction following asphyxia of the neonate ~~is~~ may lead to significant morbidity and mortality (3). Myocardial ischemia, secondary to asphyxia, seems to occur despite preferential myocardial perfusion. This may be demonstrated by elevated cardiac enzymes and troponin, ventricular dysfunction or complete cardiovascular collapse (4). In neonates, currently no treatment is available to protect the vulnerable myocardium following asphyxia. Inotropic support to improve cardiac function is limited by the structure and properties of the neonatal heart. Compared to adult hearts, the neonatal heart has reduced receptiveness to beta-adrenergic agents, greater volume of non-contractile myocardial tissue mass, cardiac output that is heart rate-dependent because of a limited capacity to augment stroke volume (5), and an increased vulnerability to apoptosis (6).

Cyclosporine is known for its use as an immunosuppressive agent in organ transplantation. The pathway for immunosuppression is based on its high affinity for binding to cyclophilin-A. The discovery that cyclosporine also binds to cyclophilin-D, a major protein in the formation of mitochondrial permeability transition pore (MPTP) (7), has led to its study as a cardioprotective agent in reperfusion injury. In reperfusion injury leading to

apoptotic cardiac cell death, the formation of the MPTP is believed to be a key mechanism in which cyclosporine reduced injury in isolated rat hearts (8). The key feature in transition from reversible cell damage to irreversible damage appears to be mitochondrial dysfunction related to opening of the MPTP (8). The cardioprotection of cyclosporine was further supported by experiments in wildtype mice given cyclosporine upon reperfusion and subsequent myocardial infarct size reduction compared to cyclophilin-D deficient mice (9). Cyclophilin-D, a key component of the MPTP (10, 11) is thus believed to be the molecular target of cyclosporine in the amelioration of post-ischemic reperfusion injury.

The cardioprotective benefits of cyclosporine in humans have been demonstrated in adult patients undergoing percutaneous coronary intervention for ST-elevation myocardial infarction (12). An intravenous bolus of cyclosporine given prior to reperfusion of the myocardium reduced the myocardial infarct size at five days. *However, the potential cardioprotective effects of cyclosporine in neonates, whose myocardium is immature, have not yet been studied.* Furthermore, controversy remains regarding the optimal dosing for cardioprotection (13). Therefore, the objectives of the present study were to determine whether cardiac function, systemic hemodynamics and oxygen metabolism, and markers of myocardial injury were affected by cyclosporine used during resuscitation of newborn

piglets with asphyxia. We hypothesize that cyclosporine will improve cardiac function and reduce myocardial injury in asphyxiated newborn piglets.

Methods

All experiments were conducted in accordance with the guidelines and approval of the Animal Care and Use Committee (Health Science), University of Alberta. Thirty-nine newborn mixed breed piglets 1 to 4 days of age weighing 1.4 to 2.5 kg were obtained on the day of experimentation from a local farm.

Animal preparation

As discussed in Chapter 5.

Experimental Protocol

The piglets were block-randomized to 4 groups (n=8 per group) that underwent hypoxia-reoxygenation. A fifth sham-operated group of piglets (n=4) underwent complete instrumentation without H-R and delivery of medications.

In the 4 groups, hypoxemia was induced via normocapnic alveolar hypoxia. These piglets were ventilated with a FiO_2 of 0.10 to 0.15 by increasing the inhaled concentration of nitrogen gas relative to oxygen for 2h, aiming for arterial oxygen saturations of 30 to 40%. It has been shown in

previous studies that this degree of hypoxemia in the newborn piglet model will produce clinical asphyxia with severe metabolic acidosis and systemic hypotension (14, 15). This was followed by reoxygenation with 100% oxygen for 0.5 h and then 21% oxygen for 3.5 h. At 5 min of reoxygenation, piglets received a blinded treatment either with cyclosporine A as an intravenous bolus (2.5, 10, 25 mg/kg) or saline (control). Cyclosporine A treatment was given at 5 min reoxygenation to simulate the clinical setting, in which intravenous access is obtained in the neonate prior to administering resuscitative medications.

Medication Preparation and Delivery

Blinding was maintained by reconstituting all doses of cyclosporine A with normal saline to a standard total volume (5 ml) immediately before administration (12). The medication was given intravenously over 2 min. A laboratory technician uninvolved in the experiment prepared the medications. Cyclosporine A was diluted from stock solution (50 mg/ml) containing ethanol vehicle, with 10 times difference in ethanol concentration between the high and low dose cyclosporine treatment groups.

Hemodynamic measurements and oxygen transport

Hemodynamic recording for data analysis was carried out at specified time points: baseline (0 min), 60 and 120 min of hypoxia, 130 (10-min reoxygenation) and 150 min (30-min reoxygenation) reoxygenation with

100% FiO_2, then at 180, 240, 300 and 360 min for reoxygenation with 21% FiO_2. All recordings were calculated as a mean over 2 min of recording. Hemodynamic variables were calculated as shown in a previous study (16).

At the specified time points, both arterial and venous blood samples were taken for blood gases, hemoglobin levels and co-oximetry. The systemic oxygen delivery (D°), renal artery oxygen delivery and systemic oxygen consumption (V°) were calculated using standard formulas (17).

Arterial blood samples (1 ml) were taken at predetermined intervals coinciding with hemodynamic measurements, centrifuged at 15,000 rotations per min (rpm) for 10 min. The supernatant was then collected and frozen at -80 C for determination of plasma lactate. Of the piglets blood volume, less than 5% was collected for blood work.

At the end of the study, piglets were euthanized with 100 mg/kg pentobarbital i.v. Samples of left ventricle, left papillary muscle and inferior pole of right kidney were fixed in 10% formalin for histological analysis. A second sample of left ventricle and right kidney was also taken and snap frozen in liquid nitrogen and stored in -80 C for biochemical analysis.

Determination of Plasma and Left Ventricle Lactate

Blood was collected in heparin tubes and plasma was prepared by centrifugation at 10,000g for 15 min and stored at -80°C. The plasma lactate concentration was determined by a nicotinamide adenine dinucleotide (NAD) enzyme-coupled colorimetric assay with spectrophotometry at 340 nm (18) at each coinciding time point with hemodynamic measurements.

Myocardial tissues were homogenized with 10 µl/mg of 50 mM phosphate buffer containing 0.5 mM EGTA. Left ventricle lactate was assayed by enzymatic spectrometric methods to measure the absorbance of NADH at 340 nm. The protein content was determined by bicinchoninic acid assay kit (Sigma).

Determination of Troponin

Plasma cardiac troponin I (cTnI) concentration was measured using a commercially available ELISA kit (Life Diagnostics, #2010-4-HS) at baseline and at the end of 4 h of reoxygenation.

Determination of N-acetyl-beta-D-glucosaminidase (NAG) activity/Creatinine Ratio

Urine was collected at baseline, 2h of hypoxia, 4h of reoxygenation and stored at -80°C. The NAG concentration was determined by colorimetric assay, with photometry measured at 580 nm. Creatinine concentration was

determined using spectrophotometry at 495 nm. NAG/Creatinine ratio was then calculated (19).

Histopathology

Left ventricle and left papillary muscle samples preserved in formalin were prepared for histological assessment using hematoxylin and eosin staining. An independent pathologist (AT) blinded to treatment group evaluated histological damage of the specimens and assigned a grade based on the Rose classification of myocardial injury (20). Renal samples were also preserved and prepared similarly and assessed by an independent pathologist (AT) based on a modified clinical classification.

Transmission Electron Microscopy

Myocardial samples were taken at the end of the experiment from the control, cyclosporine-treated (10 mg/kg) and sham-operated groups. The myocardial samples were cut to 1-2mm^3 and placed in a fixative (2.5% glutaraldehyde, 4% paraformaldehyde, 1mM $CaCl_2$, 4% sucrose in 0.1M sodium cacodylate buffer, pH 7.4) overnight at 4°C. The fixed samples were briefly rinsed in 0.075M sodium cacodylate buffer, post-fixed in 1% OsO_4 in 0.05M sodium cacodylate buffer for 2 hours at 4°C, dehydrated in ethyl alcohol (50, 70, 80, 90, 95, 100%) and propylene oxide followed by thermally polymerized in a mixture of Araldite512 and Embed812 for 48 hours at 60°C. Ultrathin sections of 60-70nm were cut, stained with 7% uranyl acetate in

50% ethyl alcohol and Reynold's lead citrate, and examined under a Philips 410 transmission electron microscope equipped with a charge-coupled device camera (Megaview III, Soft Imaging System, Olympus, Canada) at 80Kv and iTEM software (Olympus Soft Imaging Solutions, Co., Olympus, Canada).

Statistical Analysis

Results are expressed as mean±standard error of mean. Hemodynamic and oxygen transport variables were analyzed by two-way repeated measures ANOVA. We used the Student-Newman-Keuls method where appropriate for pairwise comparisons in post hoc testing. Biochemical markers were analyzed by two-way repeated measures ANOVA or one-way ANOVA as appropriate. If the normality test failed, ANOVA on ranks (Kruskal-Wallis) was performed. Significance was defined as $p<0.05$.

Results

The piglets were 2.3±0.2 day-old and weighed 1.9±0.04 kg with no significant differences among groups. The mortality rate was 8% (3/39 piglets), secondary to surgical complications (n=2) and severe hypoxia (n=1). There was no significant difference between groups at normoxic baseline in arterial blood gases (Table 6-1) and all hemodynamic variables (Table 6-2). Sham-operated animals were stable throughout the experimental period.

Systemic hemodynamic responses during hypoxia and reoxygenation

Severe normocapnic alveolar hypoxia resulted in cardiogenic shock within cyclosporine-treated and control groups with decreased cardiac index (CI) to 40-48% of normoxic baseline at 2h ($p<0.05$, vs. sham-operated piglets) (Figure 6-1A). The CI recovered within 10 min of reoxygenation and then gradually deteriorated over 240 min of reoxygenation to 57±8% of baseline in the control group (Figure 6-1A). Cyclosporine treatment with 2.5, 10 and 25 mg/kg significantly improved CI to 88±8%, 100±7% and 85±11% of baseline at 240 min of reoxygenation, respectively. Cyclosporine treated-group at 10 mg/kg had higher CI than that of controls at 30, 60, 180 and 240 min of reoxygenation (all $p<0.05$)(Figure 6-1A).

At 2h of hypoxia, *there was no difference in heart rate between cyclosporine-treated and control groups ($p<0.05$).* As shown in Figure 6-1B, the control group steadily increased heart rate over the 4h of reoxygenation; however, no significant difference among groups was observed.

All groups had decreased stroke volume index (SVI) after 2h of hypoxia ($p<0.05$ vs. sham-operated group)(Figure 6-1C). Upon reoxygenation, SVI of cyclosporine-treated and control groups improved rapidly and then steadily decreased over the course of reoxygenation. Cyclosporine at 10 mg/kg significantly improved SVI compared to the control group at 180 min reoxygenation (Figure 6-1C, 82±8% vs. 55±9% of baseline, respectively). At 240 min reoxygenation, SVI of 10 mg/kg cyclosporine

treatment was higher than that of controls (80±10% vs. 51±9% of baseline, respectively, $p<0.05$), but lower than that of sham-operated group (108±12% of baseline), which was significantly higher than all groups undergoing H-R (Figure 6-1C).

Severe hypotension developed during hypoxia with mean arterial pressure of 27-31 mmHg at 2h of hypoxia (Figure 6-2A). The mean arterial pressure recovered after 10 min of reoxygenation (48-60 mmHg) and gradually deteriorated over the course of reoxygenation with no significant difference among groups. There was pulmonary hypertension at 2h of hypoxia ($p<0.05$, vs. sham-operated group), which gradually resolved during reoxygenation with no significant difference among groups (Figure 6-2B).

Systemic oxygen transport during hypoxia and reoxygenation

Systemic oxygen delivery (D°) decreased significantly in all H-R groups (vs. sham-operated piglets) and normalized rapidly upon reoxygenation (Figure 6-3A). As reoxygenation continued, D° gradually deteriorated in the control group, but not in cyclosporine-treated groups (Figure 6-3A). At 180 min of reoxygenation, both cyclosporine 10 mg/kg and 25 mg/kg treated groups had higher D° than the control group (90±6% and 84±8% vs. 62±10% of baseline, respectively, $p<0.05$). All cyclosporine-treated groups had significantly higher D° at 240 min of reoxygenation than that of controls (73±7%, 84±6%, 76±14% respectively for 2.5, 10 and 25

mg/kg cyclosporine-treated groups vs. 51±9% of baseline of controls, $p<0.05$). Sham-operated piglets maintained D° near 100% of baseline throughout the experiment.

After 2h of hypoxia, systemic oxygen consumption (V°) decreased significantly in all hypoxia-reoxygenation groups (Figure 6-3B). V° increased steadily following reoxygenation in the H-R groups reaching peak recovery at 60 min of reoxygenation. During the final 3h of reoxygenation, there was gradual deterioration of V° in control piglets whereas the V° was maintained in the cyclosporine-treated groups with significant differences noted at 240 min of reoxygenation (72±12% vs. 88±5%, 104±3% and 93±8% of baseline for control, 2.5, 10 and 25 mg/kg cyclosporine-treated groups, respectively, all $p<0.05$). There was no significant difference among cyclosporine-treated groups.

After 2h of normocapnic alveolar hypoxia, all groups had significant metabolic acidosis (pH 7.04±0.02) and elevated plasma lactate concentrations (vs. sham-operated group, both $p<0.05$) (Table 6-1). Both arterial pH and plasma lactate returned back to normal after 4h of reoxygenation (Table 6-1). There was no significant difference between cyclosporine-treated and control groups in acid-base balance and plasma lactate concentrations during hypoxia-reoxygenation.

Markers of myocardial injury

Plasma troponin I levels were similar at baseline in all groups. After 2h of hypoxia and 240 min of reoxygenation, all hypoxia-reoxygenation groups had higher plasma troponin levels compared to sham-operated group ($p<0.05$)(Figure 6-4A). Among the hypoxia-reoxygenation groups, plasma troponin levels at 240 min of reoxygenation of piglets treated with cyclosporine at 2.5 mg/kg and 10 mg/kg, but not 25 mg/kg, were significantly lower than that of the control group.

After 2h of hypoxia and 240 min of reoxygenation, the left ventricle myocardial lactate levels of cyclosporine-treated groups of 2.5 and 10 mg/kg were lower than that of control piglets (both $p<0.05$), which was significantly higher than that of sham-operated group (Figure 6-4B).

There was significant negative correlation between CI at 240 min reoxygenation with both plasma troponin I levels ($r = -0.54$, $P < 0.001$) and left ventricle myocardial lactate ($r = -0.52$, $P < 0.001$) among all groups. As markers of myocardial injury, plasma troponin I at the end of experiment was significantly positively correlated with left ventricle myocardial lactate ($r = -0.52$, $P < 0.01$).

Samples of both the left ventricle and papillary muscle were assessed using the Rose Criteria for myocardial histological injury (19). There was no difference in histopathological score among groups (data not shown).

Ultrastructural changes in mitochondria were evident when comparing sham-operated, control and cyclosporine-treated heart groups. Mitochondria in sham-operated group maintained well their structural integrity of cristae and electron density in matrix (Figure 6-5A). In control group most of mitochondria lost their structural integrity of cristae and electron density in matrix (asterisks) (Figure 6-5B). Interestingly, in cyclosporine-treated group (10mg/kg), although mitochondria were fragmented and their sizes were smaller than sham-operated and control groups (data not shown) structural integrity of cristae in mitochondria as well as electron density in mitochondrial matrix were well recovered (Figure 6-5C).

Renal perfusion and injury

After 2h of hypoxia, all hypoxia-reoxygenation groups had significantly lower renal artery flow index and oxygen delivery compared to sham-operated group (Figure 6-6A and 6-6B). During reoxygenation, renal perfusion improved in all hypoxia-reoxygenation groups with significant differences between 10 mg/kg cyclosporine-treated and control groups at 240 min of reoxygenation (renal artery flow index: 104±18% vs. 67±7% of

baseline, respectively; renal artery oxygen delivery: 90±19% vs. 60±9% of baseline, respectively)(Figure 6-6).

Urinary NAG/creatinine ratio, a marker of hypoxia-reoxygenation induced renal tubular impairment, was measured at the end of the 6h experiment. The NAG/creatinine ratios were significantly higher in all hypoxia-reoxygenation groups than that of sham-operated piglets with no difference among cyclosporine-treated and control groups (Figure 6-6C).

Acute renal injury was assessed using a modified histological scoring criteria (21). There was no difference in histopathological score among groups (data not shown).

Discussion

This is the first study to demonstrate that cyclosporine treatment during the resuscitation of asphyxiated newborn piglets at early reoxygenation causes bell-shaped improvements in CI and SVI, with the optimally effective dose being 10 mg/kg. Furthermore, cyclosporine treatment was correlated to reduced myocardial injury evidenced by lower plasma troponin and myocardial lactate levels and attenuated mitochondrial damage in cardiomyocytes compared to the control group.

The improvement in cardiac function is likely secondary to the preservation of cardiomyocytes. These findings are supported by *in vitro* experiments using isolated rat hearts subjected to 30 min ischemia and 15 min reperfusion, in which Griffiths and Halestrap demonstrated that cyclosporine administration substantially improved left ventricular pressure (8). They proposed that cyclosporine's protective effect is by inhibiting the formation of MPTP resulting in the restoration of ATP/ADP ratio and AMP levels. However, they hypothesized that cyclosporine was unable to completely restore left ventricular pressure because other mechanisms such as oxygen free radical damage may be involved in ischemia-reperfusion injury (8). Halestrap et al further clarified the role of MPTP formation during reperfusion by perfusing [^{3}H]-2-deoxyglucose into rat hearts undergoing ischemia-reperfusion (22). The opening of MPTP during reperfusion was inhibited by cyclosporine administration (22). Further studies in surviving mice with myocardial ischemia (23) and cyclophilin-D knockout mice (9) confirmed the cardioprotection of cyclosporine. In adult patients undergoing percutaneous coronary intervention for ST-elevation myocardial infarction, cyclosporine treatment (2.5 mg/kg) prior to reperfusion reduced infarct size at 5 days post-event, but not plasma troponin levels, compared to the control group (12). Interestingly, Piot et al found that creatine kinase-MB levels were decreased in the cyclosporine-treated group (12). However, Bes et al observed a 67% reduction of troponin I concentrations *in vitro* in ischemic-reperfused cardiomyocytes (24).

The neonatal heart differs from the adult heart with a greater proportion of non-contractile myocardial tissue, decreased responsiveness to beta-adrenergic agents and a limited capacity in stroke volume augmentation (5). Furthermore, the immature myocardium is vulnerable to ischemia-reperfusion and hypoxia-reoxygenation injury secondary to mitochondrial dysfunction and/or immature oxygen scavenging mechanisms (25). The neonatal heart has also been suggested to be more vulnerable to apoptotic pathways, where Karimi et al demonstrated an increased apoptotic signaling in newborn lamb myocardium than adult myocardium in hypoxia-reoxygenation injury secondary to cardioplegic arrest (25). Indeed, anti-apoptotic intervention has been suggested as a promising approach to limit myocardial injury and potentially promote survival of the myocardium (5). To our knowledge, the effect of cyclosporine treatment in hypoxia-reoxygenation injury of asphyxiated neonatal myocardium has not been examined. In this *in vivo* study, we demonstrated the cardioprotective effect of cyclosporine in the newborn piglets with decreased levels of myocardial injury markers including the plasma troponin I and left ventricular tissue lactate levels in cyclosporine-treated groups (2.5 mg/kg and 10 mg/kg) compared to controls. Histopathological assessment of both the left ventricle and papillary muscle was similar between the cyclosporine-treated and control groups. The duration of hypoxia-reoxygenation may have been too short to allow clear delineation of myocardial damage, although a reduction

in infarct size was found in rabbits with transient coronary artery occlusion at 4h of reoxygenation (26). We thus qualitatively assessed mitochondrial morphology in cardiomyocytes using transmission electron microscopy. In the left ventricular myocardium, cyclosporine-treated piglets had mitochondria that closely resembled those of the sham-operated group, whereas there were severely damaged mitochondria found in the cardiomyocytes of control piglets (Figure 5). These findings are consistent with that in rabbits undergoing 30 min of ischemia and 3h of reperfusion using a coronary ligation model (27). We speculate that improved mitochondrial morphology in our cyclosporine-treated piglets correlates with the significantly improved cardiac function and biochemical markers of myocardial injury in the cyclosporine-treated groups. It remains unclear whether the greater proportion of non-contractile tissue in the newborn heart contributes to reperfusion injury, and whether is effected by cyclosporine treatment during resuscitation. Furthermore, the benefit of preservation of this non-contractile heart tissue is undetermined.

The clinical use of cyclosporine in solid organ transplant is limited in particular by its nephrotoxicity. Cyclosporine has been shown to cause distal tubular acidosis in uninephrectomized rats (28). Zahmatkesh et al also demonstrated that administration of cyclosporine in uninephrectomized rats decreased creatinine clearance and increased NAG activity (29). In this study, the improved CI after 4h of reoxygenation was associated with significantly

improved renal artery flow and oxygen delivery in the 10 mg/kg cyclosporine-treated group. These findings suggest that systemic hemodynamic improvement associated with cyclosporine administration actually improves renal perfusion in asphyxiated newborn piglets. This is in contrast to findings by English et al who found vasoconstriction of afferent arterioles with cyclosporine treatment for immunosuppression in rats (30). Furthermore, they concluded that vasoconstriction of afferent arterioles was the major pathogenetic factor rather than direct tubular injury. Regardless of the mechanism of renal injury, the renal perfusion was not adversely affected by cyclosporine treatment. The lack of significant aggravation of renal injury after hypoxia-reoxygenation with the administration of cyclosporine is further supported by our findings in NAG/creatinine ratio, which is a widely used specific marker of renal tubular injury (31, 32), and the histopathological examination. Cyclosporine at 25 mg/kg however had the highest NAG/creatinine ratio, demonstrating nephrotoxicity at this extremely high dose. Nonetheless, these findings suggest that cyclosporine treatment at 2.5 and 10 mg/kg confers significant cardioprotection without significantly increased renal injury in newborn piglets. This may be explained by the administration of a single intravenous bolus of cyclosporine rather than cumulative doses. However, with cumulative dosing of cyclosporine in pediatric patients following renal transplantation, other potential toxicities have been reported including seizures, gastrointestinal problems, gingival hyperplasia, arrhythmias, neuropathy, tremors and hypertrichosis (33). It

remains unclear if similar potential complications of cyclosporine administration may be observed with administration of a single dose of cyclosporine, thus caution is recommended.

The interpretation of our findings requires caution because of limitations related to the acute setting of a swine model of hypoxia-reoxygenation. Further studies are needed to investigate if the acute improvement in cardiac function will persist in the long term. Despite the challenge to translate the findings from animal studies to human, newborn piglets have been shown to closely resemble newborn humans in the physiologic response to asphyxia (34, 35). We induce severe hypoxia with normocapnia, which simulates clinical asphyxia. Further neonatal asphyxia is different from asphyxia during the fetal-to-neonatal transition. Nonetheless, it is our attempt to make the hypoxia-reoxygenation protocol clinically relevant. The experiment includes 2h of hypoxia, which is approximate to the duration required for emergency cesarean section for fetal distress without clinical bleeding based on personal observation. The resuscitation is initiated with 100% oxygen for 30 min, instead of 60 min in our previous studies. This is to limit the hyperoxia, which remains a common practice in many community hospitals prior to the arrival of neonatal transport team.

In conclusion, this is the first study to demonstrate that post-resuscitation administration of cyclosporine causes bell-shaped preservation

of cardiac function with an effective dose of 10 mg/kg in newborn piglets following asphyxia-reoxygenation without worsening renal injury.

Table 6-1: Arterial blood gases and plasma lactate concentrations during hypoxia and reoxygenation.

	Normoxic Baseline	End of hypoxia	30 min Reoxygenation	4h Reoxygenation
pH				
Controls	7.41 ± 0.06	7.08 ± 0.07*	7.19 ± 0.09*	7.31 ± 0.12
CsA 2.5 mg/kg	7.39 ± 0.08	7.02 ± 0.15*	7.14 ± 0.16*	7.35 ± 0.08
CsA 10 mg/kg	7.43 ± 0.05	7.05 ± 0.17*	7.16 ± 0.17*	7.35 ± 0.04
CsA 25 mg/kg	7.40 ± 0.05	7.02 ± 0.1*	7.09 ± 0.08*	7.34 ± 0.05
PaO_2 (mmHg)				
Controls	74 ± 13	37 ± 8*	348 ± 96*	63 ± 16
CsA 2.5 mg/kg	83 ± 9	36 ± 6*	383 ± 86*	63 ± 4
CsA 10 mg/kg	79 ± 13	36 ± 8*	413 ± 36*	64 ± 4
CsA 25 mg/kg	75 ± 5	39 ± 6*	409 ± 28*	78 ± 22
$PaCO_2$ (mmHg)				
Controls	39 ± 3	39 ± 5	37 ± 4	39 ± 4
CsA 2.5 mg/kg	39 ± 4	42 ± 4	39 ± 5	42 ± 3
CsA 10 mg/kg	37 ± 4	39 ± 5	36 ± 5	41 ± 3
CsA 25 mg/kg	38 ± 3	38 ± 4	40 ± 3	41 ± 3
HCO_3 (mM)				
Controls	25 ± 3	11 ± 2*	14 ± 2*	20 ± 5
CsA 2.5 mg/kg	24 ± 3	10 ± 4*	13 ± 4*	22 ± 4
CsA 10 mg/kg	25 ± 2	10 ± 4*	14 ± 5*	22 ± 2
CsA 25 mg/kg	24 ± 2	9 ± 2*	12 ± 2*	21 ± 2
Lactate (mM)				
Controls	3.9 ± 0.6	13.8 ± 3.9*	11.3 ± 2.9*	5.5 ± 3.3
CsA 2.5 mg/kg	3.7 ± 0.7	14.6 ± 4.3*	11.3 ± 3.3*	3.2 ± 2.2
CsA 10 mg/kg	3.7 ± 0.7	14.0 ± 4.9*	11.7 ± 4.3*	3.1 ± 1.0
CsA 25 mg/kg	3.5 ± 0.7	14.2 ± 2.7*	11.8 ± 1.8*	2.6 ± 0.6

CsA = Cyclosporine *P<0.05 vs. normoxic baseline

Table 6-2: Hemodynamic measurements and oxygen transport at normoxic baseline in piglets treated with different cyclosporine concentrations (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4).

Variables	Cyclosporine 2.5 mg/kg	Cyclosporine 10 mg/kg	Cyclosporine 25 mg/kg	Controls	Sham-operated
Cardiac Index (mL/kg/min)	187±16	164±9	167±12	211±14	189±31
Heart Rate (beats/min)	167±7	162±9	175±9	181±12	204±17
Stroke Volume Index (mL/kg/beat)	1.1±0.1	1.0±0.1	1.0±0.1	1.2±0.1	0.9±0.1
Mean Arterial Pressure (mmHg)	78±4	72±2	73±3	68±4	68±3
Pulmonary Arterial Pressure (mmHg)	29±1	28±2	27±2	27±1	23±2
Systemic Oxygen Delivery (O_2 mL/kg/min)	20±1	18±1	18±1	20±1	19±3
Systemic Oxygen Consumption (O_2 mL/kg/min)	7.7±0.5	7.6±0.4	7.5±0.4	8.5±0.9	9.4±1.6
Renal Artery Flow Index (mL/kg/min)	13±2	12±2	13±2	14±2	11±4
Renal Artery Oxygen Delivery (O_2 mL/kg/min)	1.4±0.3	1.3±0.3	1.4±0.2	1.3±0.2	1.1±0.4

Figure 6-1. Cardiac Function. (A) Cardiac index, (B) heart rate, and (C) stroke volume index during 4h of reoxygenation with different cyclosporine concentrations (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls, #P<0.05 vs. all hypoxia-reoxygenation groups. Downward pointing black arrow represents administration of Cyclosporine A.

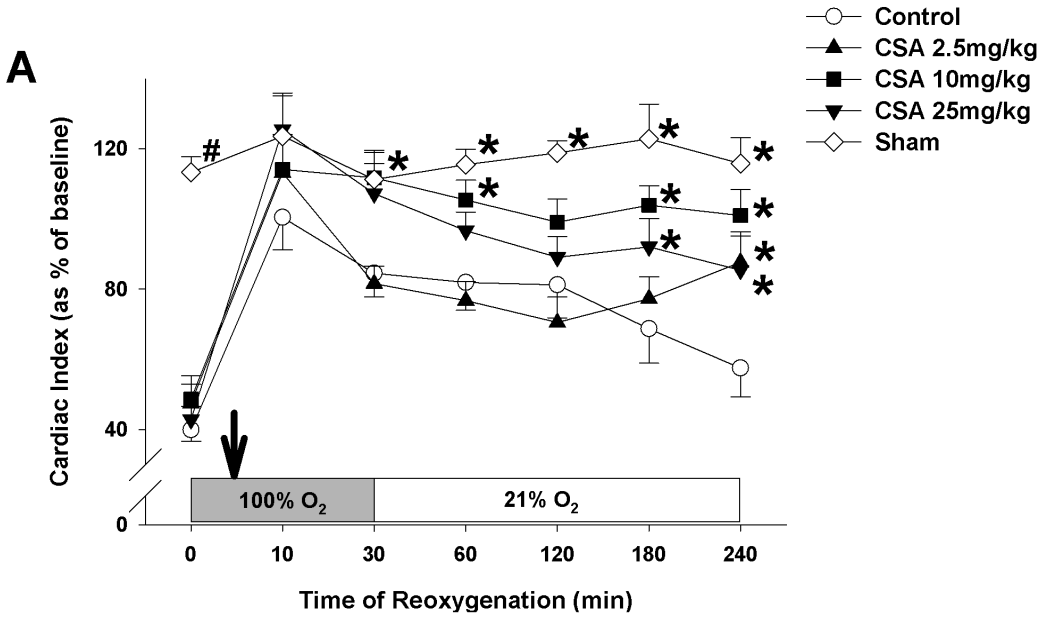


Figure 6-1 continued. Cardiac Function. (A) Cardiac index, (B) heart rate, and (C) stroke volume index during 4h of reoxygenation with different cyclosporine concentrations (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls, #P<0.05 vs. all hypoxia-reoxygenation groups. Downward pointing black arrow represents administration of Cyclosporine A.

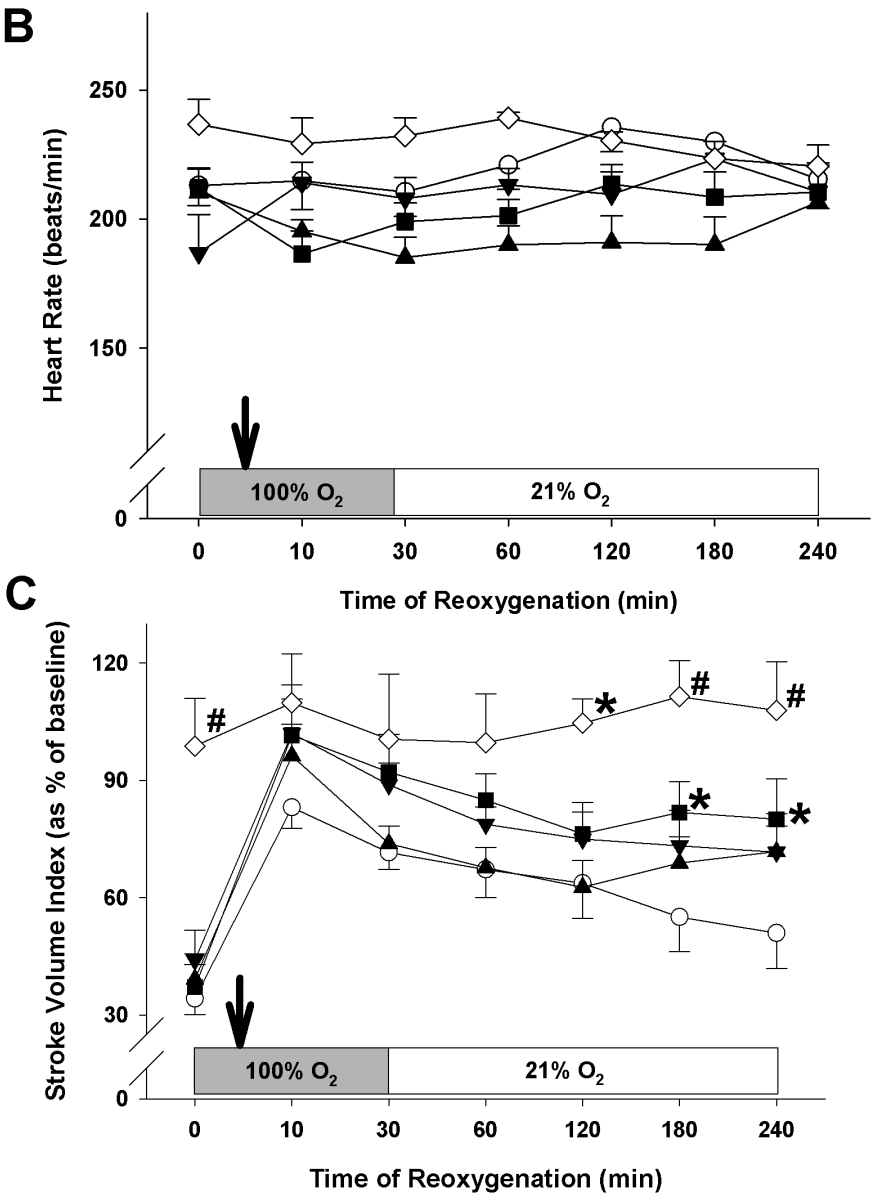


Figure 6-2. Systemic and pulmonary artery pressures. (A) Mean arterial pressure and (B) pulmonary arterial pressure during 4h of reoxygenation with different cyclosporine concentrations (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls. Downward pointing black arrow represents administration of Cyclosporine A.

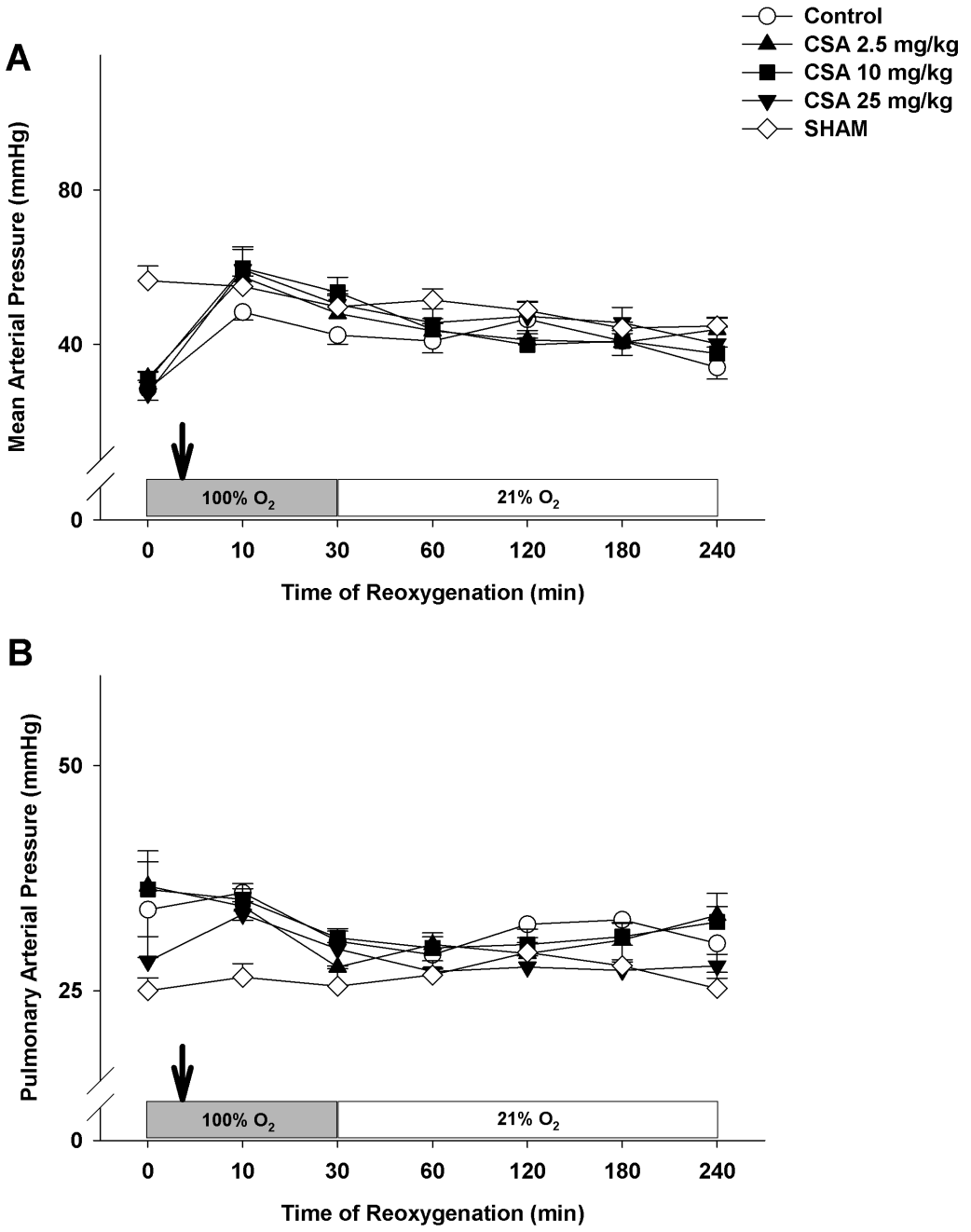


Figure 6-3. Systemic oxygen transport. (A) Systemic oxygen delivery and (B) systemic oxygen consumption during 4h of reoxygenation with different cyclosporine concentrations (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls, #P<0.05 vs. all hypoxia-reoxygenation groups. Downward pointing black arrow represents administration of Cyclosporine A.

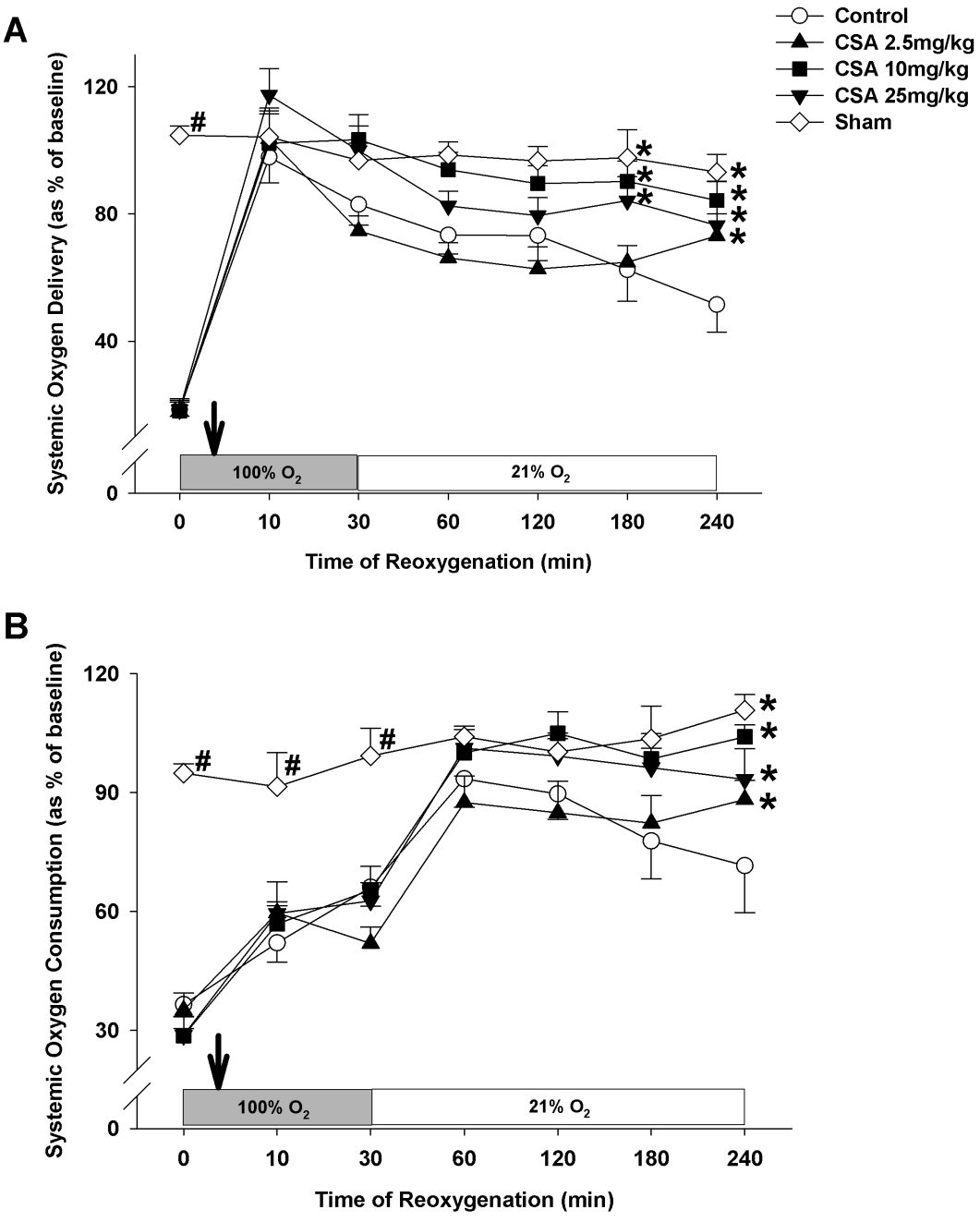


Figure 6-4. Markers of myocardial injury. (A) Plasma troponin I levels and (B) left ventricle myocardial lactate levels in piglets after 2h hypoxia and 4h reoxygenation with different cyclosporine concentrations (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls, #P<0.05 vs. all hypoxia-reoxygenation groups.

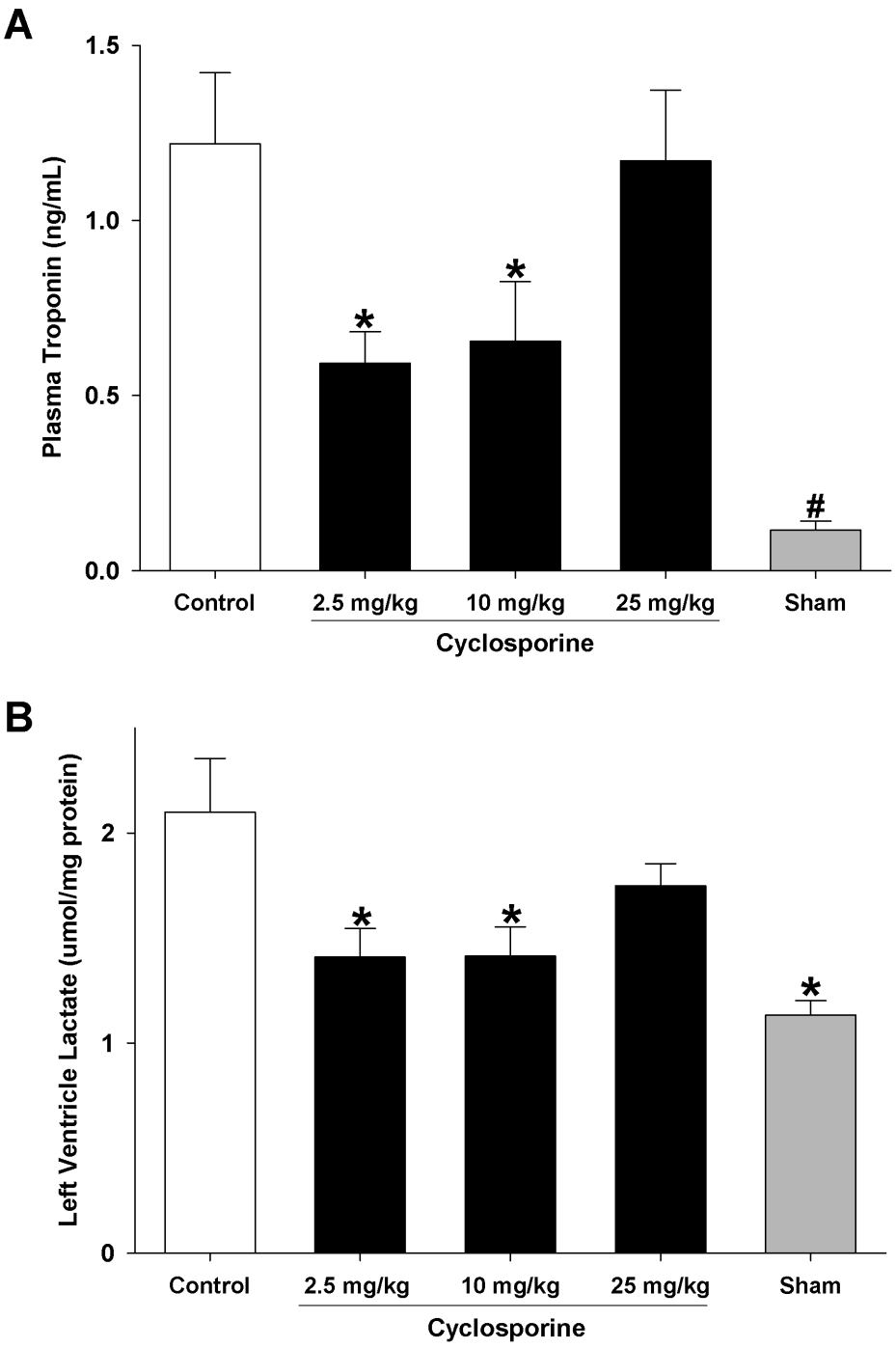


Figure 6-5. Representative ultrastructural images of mitochondria in cardiomyocytes. In sham-operated group cristae and matrix in mitochondria showed well-maintained structural integrity and electron density (A). Cristae and matrix in mitochondria, in control group, lost their integrity and electron density (asterisks) (B). Mitochondria in cyclosporine-treated group showed integrity of cristae and electron density in matrix same as sham-operated group (C). A scale bar is 500 nm for all images.

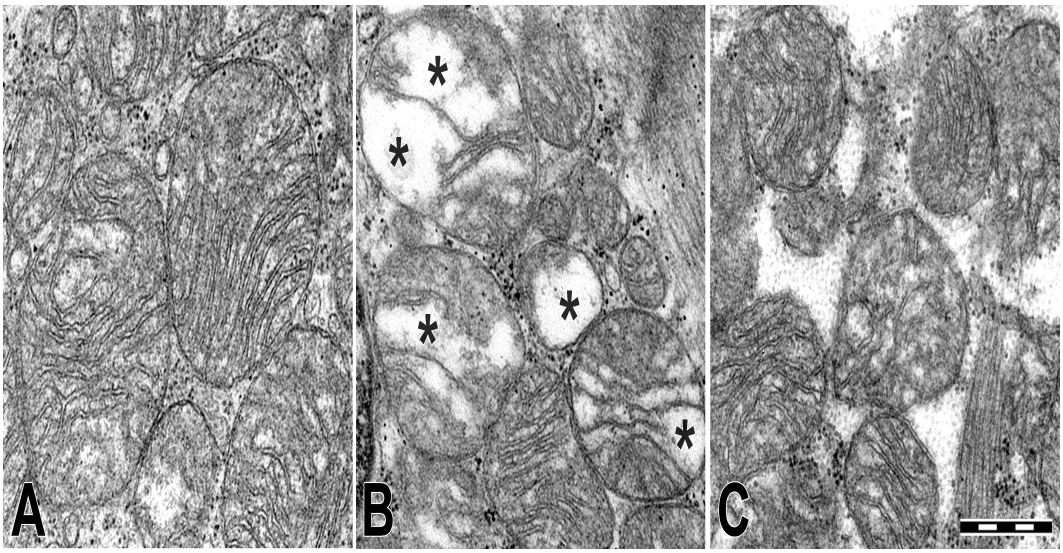


Figure 6-6. Renal perfusion and function. (A) Renal artery flow index, and (B) renal artery oxygen delivery during 4h reoxygenation, and (C) N-acetyl-beta-D-glucosaminidase (NAG)/creatinine ratio in urine after 4h reoxygenation with different cyclosporine concentrations (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls, #P<0.05 vs. all hypoxia-reoxygenation groups. Downward pointing black arrow represents administration of Cyclosporine A.

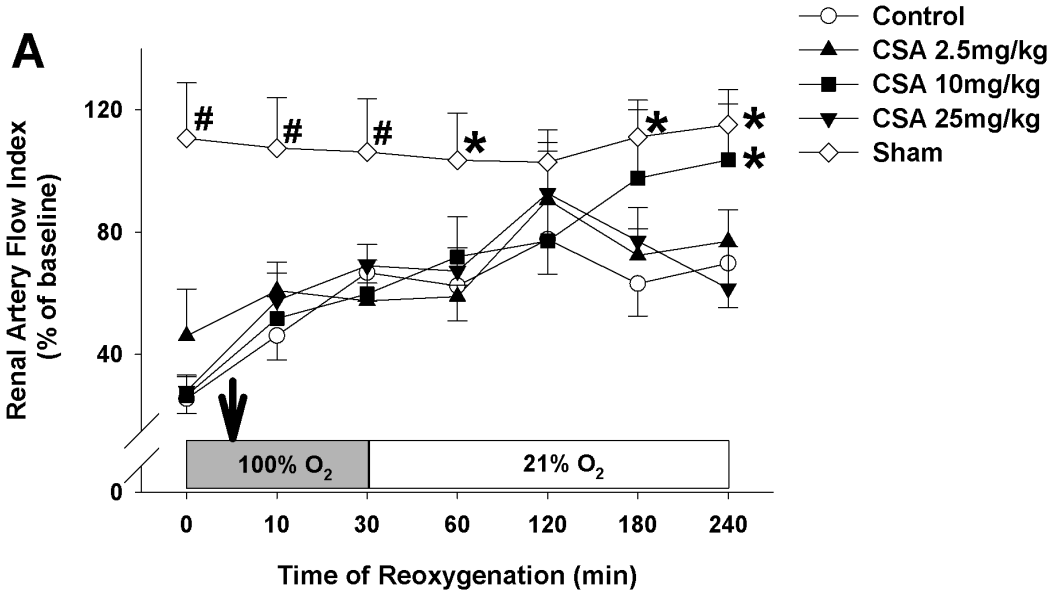
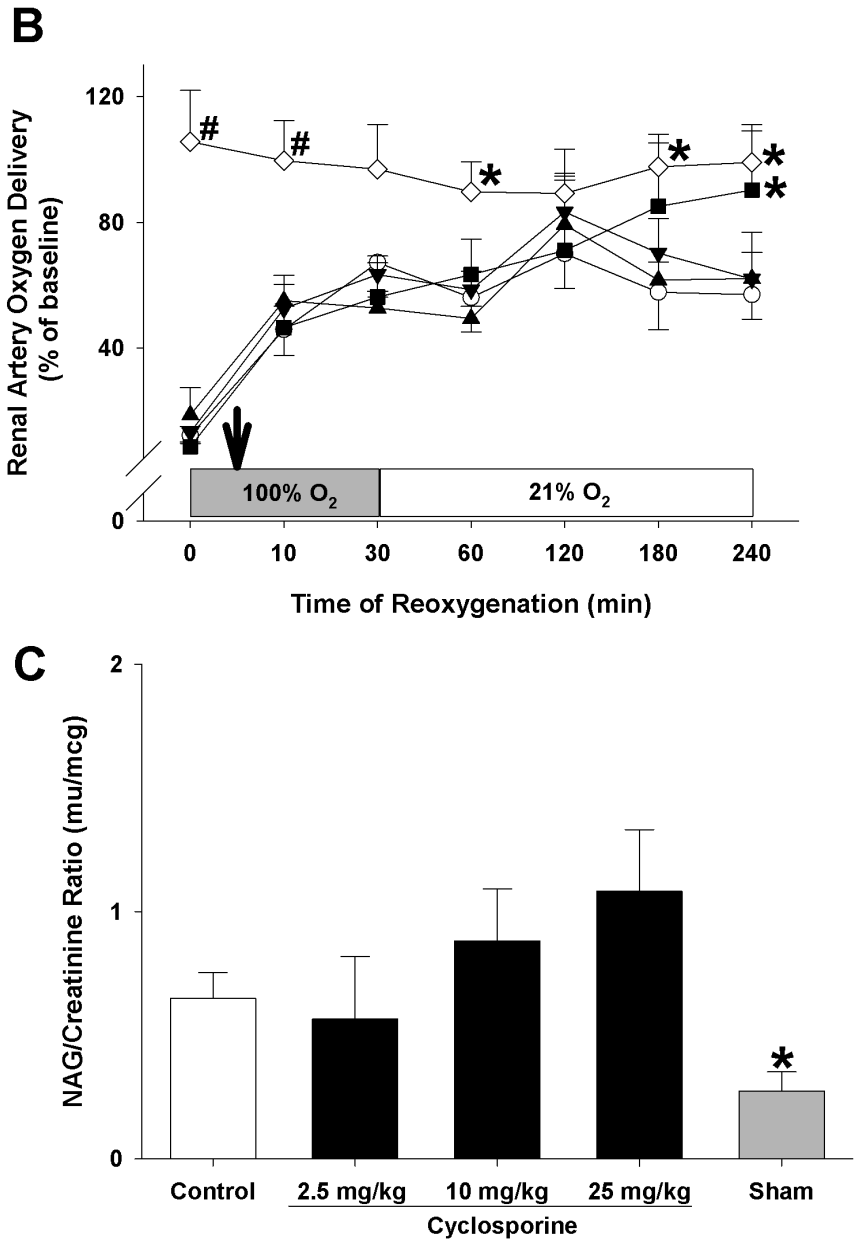


Figure 6-6 continued. Renal perfusion and function. (A) Renal artery flow index, and (B) renal artery oxygen delivery during 4h reoxygenation, and (C) N-acetyl-beta-D-glucosaminidase (NAG)/creatinine ratio in urine after 4h reoxygenation with different cyclosporine concentrations (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls, #P<0.05 vs. all hypoxia-reoxygenation groups. Downward pointing black arrow represents administration of Cyclosporine A.



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Chapter 7

Cyclosporine Treatment Improves Mesenteric Perfusion and Attenuates NEC-like Intestinal Injury in Asphyxiated Newborn Piglets During Reoxygenation

Adapted from:

Gill RS, Manouchehri N, Lee TF, Cho WJ, Thiesen A, Churchill T, Bigam DL, Cheung PY. *Cyclosporine Treatment Improves Mesenteric Perfusion and Attenuates NEC-like Intestinal Injury in Asphyxiated Newborn Piglets during Reoxygenation*. Intensive Care Medicine 2012; 38: 482-490.

Abstract

Background

Asphyxia-related intestinal injury in neonates may present similar to necrotizing entercolitis (NEC) and is partially associated with hypoxia-reoxygenation injury. Cyclosporine has been shown to reduce myocardial cell death following ischemia-reperfusion. We hypothesize that cyclosporine treatment may attenuate NEC-like intestinal injury in asphyxiated newborn piglets during reoxygenation.

Methods

Twenty piglets (1-4 days-old) were acutely anesthetized and instrumented for continuous monitoring of systemic hemodynamics and superior mesenteric arterial (SMA) flow. After stabilization, normocapnic alveolar hypoxia (10-15% oxygen) was instituted for 2h followed by reoxygenation with 100% oxygen for 0.5h, then 21% for 3.5h. The piglets were blindly block-randomized to receive cyclosporine (10 mg/kg) or placebo (normal saline) boluses at 5 minutes of reoxygenation (n=8/group). A sham-operated group was included (n=4) and received no hypoxia-reoxygenation. Intestinal samples were collected for tissue lactate and histological assessment (Park's criteria).

Results

At 2h of hypoxia, piglets had cardiogenic shock (cardiac output 45% of baseline), hypotension (mean arterial pressure of 30mmHg), acidosis (pH=7.04) and decreased superior mesenteric perfusion (all $p<0.05$ vs. sham-

operated group, ANOVA). Cyclosporine treatment increased SMA flow (114±6% vs. 78±19% of baseline of controls, respectively) with improved SMA oxygen delivery and intestinal tissue lactate (all $P<0.05$). Some control piglets had NEC-like injuries including pneumatosis intestinalis, which were attenuated in cyclosporine-treated piglets ($P<0.05$ vs. controls).

Conclusions

This is the first study to demonstrate that post-resuscitation administration of cyclosporine improves mesenteric perfusion and attenuates NEC-like intestinal injury in newborn piglets following asphyxia-reoxygenation.

Introduction

Perinatal asphyxia occurs in 3% of neonates and contributes to significant morbidity and mortality of critically ill neonates. Among the morbidities, gastrointestinal complications are common and asphyxiated neonates may present with intestinal lesions similar to that of necrotizing enterocolitis (NEC) (1). NEC, a common gastrointestinal emergency in neonates (2, 3), is a neonatal inflammatory bowel disease that has significant mortality and morbidity rates (2-5). Treatment of NEC-like intestinal injury in asphyxiated neonates consists of bowel rest, antibiotics and surgical resection. At this time clinically there is no preventive treatment available.

The etiology of NEC is complicated and multi-factorial including intestinal immaturity as that of premature neonates, abnormal bacterial colonization (6-8), and hypoxic-ischemic injury. Indeed hypoxia-ischemia is considered as an important cause for the development of intestinal injury in both NEC and asphyxiated neonates, based on animal studies. It has previously been shown that intestinal ischemia-reperfusion with oxygen free radicals-related injury is pathologically similar to NEC (9). Interestingly, the intestinal injury has been described to result in a final common pathway ending with intestinal apoptosis and necrosis (10-12).

Cyclosporine A has been shown to reduce myocardial cell death following ischemia-reperfusion in isolated rat hearts (13). Cyclosporine A is

postulated to be a potent inhibitor of the formation of the mitochondrial permeability transition pore (MPTP), which is believed to be a key event in the progression to cellular death (14, 15). Furthermore, experiments on rat intestine suggest that cyclosporine A treatment may also protect enterocyte mitochondria from oxidative stress (16).

The potential protective effects of cyclosporine A on neonatal intestine following asphyxia have yet to be fully studied. Therefore, our objective was to determine the effects of cyclosporine A treatment on mesenteric perfusion and intestinal injury developed after the resuscitation of newborn piglets with severe asphyxia. We hypothesize that following asphyxia-reoxygenation of newborn piglets; cyclosporine A treatment will improve mesenteric perfusion and reduce intestinal injury.

Materials and Methods

Animals

All experiments were conducted in accordance with the guidelines and approval of the Animal Care and Use Committee (Health Sciences), University of Alberta. Twenty newborn mixed breed piglets, 1-4 days of age and weighing 1.4-2.5 kg, were obtained on the day of experimentation from a local farm.

Anesthesia

As discussed in Chapter 5.

Surgical Procedure

As discussed in Chapter 5.

Monitoring and Stabilization

The piglets were allowed to recover from surgical instrumentation until baseline hemodynamic measurements were stable. Ventilator rate was adjusted to maintain the P_aCO_2 35-45 mmHg. This was monitored with periodic arterial blood gas analysis. Heart rate, MAP, SMA blood flow, cardiac output and oxygen saturation were continuously monitored and recorded throughout the experiment.

Experimental Protocol

The piglets were block-randomized to two groups (n=8 per group) that underwent hypoxia-reoxygenation (H-R) (H-R control group and cyclosporine-treated group). A third sham-operated group of piglets (n=4) underwent surgical instrumentation without H-R and delivery of medications to serve as a control for the surgical model.

Normocapnic alveolar hypoxia was induced. Piglets were ventilated with an inspired oxygen concentration 10-15% by increasing the inhaled concentration of nitrogen gas relative to oxygen for 2h, aiming for arterial

oxygen saturations of 30-40%. We previously showed that this degree of hypoxemia in the newborn piglet model will produce clinical asphyxia with severe metabolic acidosis, cardiogenic shock and systemic hypotension (17). This was followed by reoxygenation with 100% oxygen for 0.5h and then 21% oxygen for 3.5h to mimic a clinical resuscitation scenario. At 5 min of reoxygenation, piglets received a blinded treatment with cyclosporine A as an intravenous bolus (10 mg/kg) or normal saline (placebo-treated H-R control group). Cyclosporine A treatment was given at 5 min reoxygenation to simulate the clinical setting, in which intravenous access is obtained in the neonate prior to administering resuscitative medications. Cyclosporine intravenous dosing was based on literature search and pilot dose-response experiments indicating 10 mg/kg as the optimal dose to attenuate myocardial injury in the newborn piglet.

Medication Preparation and Delivery

Blinding was maintained by reconstituting cyclosporine A (50 mg/ml) and normal saline in a standard volume (5 ml) immediately before administration (18). The medication was given intravenously slowly over 2 min. A laboratory technician uninvolved in the experiment mixed the medications.

Hemodynamic Measurements and Oxygen Transport

Hemodynamic recording for data analysis was carried out at specified time points: baseline (0 min), 60 and 120 min of hypoxia, 10 and 30 min of reoxygenation (at 100% oxygen), then at 60, 120, 180 and 240 min of reoxygenation (at 21% oxygen). All recordings were calculated as a mean over 2 min of recording. Hemodynamic variables were calculated as shown in a previous study (9).

At the specified time points, both arterial and venous heparinized blood samples were taken for blood gas analysis, co-oximetry, hemoglobin and lactate levels. SMA oxygen delivery ($SMADO_2$) was calculated using standard formula (9). Of the piglets blood volume, less than 5% was collected for blood work.

Small Bowel Tissue Collection

At the end of the study, piglets were euthanized with i.v. pentobarbital (100 mg/kg). Samples of the terminal ileum were fixed in 10% formalin for histological analysis. A second set of samples of terminal ileum were also taken and snap frozen in liquid nitrogen and stored in -80 °C for biochemical analysis.

Determination of Small Intestine Tissue Lactate and Lipid Hydroperoxides (LPO)

Intestinal tissue was homogenized in 0.1 M phosphate buffer saline (pH 7.4). For intestinal tissue lactate determination, intestinal tissue was assayed by enzymatic spectrometric methods to measure the absorbance of NADH at 340 nm. LPO were extracted with chloroform and then determined by LPO assay kit (Cayman, MI, USA) with spectrophotometry at 500 nm. The protein content was determined by bicinchoninic acid assay kit (Sigma).

Histopathology

Terminal ileum samples preserved in formalin were prepared for histological assessment using hematoxylin and eosin staining. An independent pathologist (AT) blinded to treatment group evaluated histological damage of the specimens and assigned a grade based on the Park's classification of intestinal injury (20).

Transmission Electron Microscopy

Intestinal samples were taken at the end of the experiment from the control, cyclosporine-treated and sham-operated groups. The intestinal samples were cut to 1-2 mm^3 and placed in a fixative (2.5% glutaraldehyde, 4% paraformaldehyde, 1mM $CaCl_2$, 4% sucrose in 0.1M sodium cacodylate buffer, pH 7.4) overnight at 4°C. The fixed samples were briefly rinsed in 0.075M sodium cacodylate buffer, post-fixed in 1% OsO_4 in 0.05M sodium cacodylate buffer for 2h at 4°C, dehydrated in ethyl alcohol (50, 70, 80, 90, 95, 100%) and prophylene oxide followed by thermally polymerized in a

mixture of Araldite512 and Embed812 for 48h at 60°C. Ultrathin sections of 60-70nm were cut, stained with 7% uranyl acetate in 50% ethyl alcohol and Reynold's lead citrate, and examined under a Philips 410 transmission electron microscope equipped with a charge-coupled device camera (Megaview III, Soft Imaging System, Olympus, Canada) at 80Kv and iTEM software (Olympus Soft Imaging Solutions, Co., Olympus, Canada).

Statistical Analysis

Results are expressed as mean±SEM. Hemodynamic and oxygen transport variables were analyzed by two-way repeated measures ANOVA. Biochemical markers were analyzed by two-way repeated measures or one-way ANOVA. We used the Fisher LSD method where appropriate for pairwise comparisons in post hoc testing. If the normality test failed, ANOVA on ranks (Kruskal-Wallis) was performed. Correlation between variables was performed using Pearson Moment test. Significance was defined as $P<0.05$.

Results

The piglets were 2.3±0.2 days old and weighed 1.9±0.04 kg with no differences among groups. There was no significant difference among groups at normoxic baseline in arterial blood gases (Table 7-1) and all hemodynamic variables (Table 7-2).

Arterial pH and Plasma Lactate

After 2h of normocapnic alveolar hypoxia, there was significant metabolic acidosis (pH 7.04±0.02 of H-R groups,) and elevated plasma lactate (vs. sham-operated group, all $P<0.05$) (Table 7-1), with no significant difference in pH or plasma lactate between the cyclosporine-treatment and H-R control groups. Both arterial pH and plasma lactate returned back to normal following 4h of reoxygenation similarly in cyclosporine-treated and H-R control groups (Table 7-1).

Systemic Hemodynamic Response during Hypoxia and Reoxygenation

At the end of 2h of normocapnic alveolar hypoxia, MAP and cardiac output in piglets that underwent H-R decreased (28-31 mmHg and 40-47% of normoxic baseline, respectively, vs. baseline, $P<0.05$)(Table 7-2). Thus, cardiogenic shock was achieved in both H-R groups at the end of 2h hypoxia with a significant metabolic acidosis (Table 7-1). Upon resuscitation, MAP recovered steadily and similarly between both H-R groups during 4h of reoxygenation. Cardiac output recovered rapidly following reoxygenation at 30 min, followed by steady deterioration in the H-R groups, with greater deterioration of the H-R control group (Table 7-2). At the end of 240 min of reoxygenation, cyclosporine-treated group had significantly improved cardiac output (vs. the H-R control group, $P<0.05$).

There were fluctuations of heart rate for both cyclosporine-treated and H-R control groups, however no significant difference between groups during hypoxia-reoxygenation.

Mesenteric Perfusion

All groups had similar mesenteric perfusion (SMA flow index and $SMADO_2$) at baseline (Table 7-2). Following 2h of hypoxia, SMA flow index was significantly decreased in all H-R piglets (vs. respective baseline and sham-operated group, both $P<0.05$) (Figure 7-1A). SMA flow index of both H-R groups similarly recovered to baseline in the first 30 min of reoxygenation. During subsequent period of reoxygenation, there were significant differences in SMA flow recovery between cyclosporine-treated and H-R control groups (at 180 min: 114±6% vs. 78±19% of baseline; at 240 min: 97±8% vs. 60±19% of baseline; respectively, $P<0.05$)(Figure 7-1A).

After 2h of hypoxia, $SMADO_2$ significantly decreased in both H-R groups (vs. respective baseline and sham-operated group, both $P<0.05$) (Figure 7-1B). $SMADO_2$ recovered rapidly upon reoxygenation, followed by a steady deterioration in H-R groups. The deterioration in $SMADO_2$ of H-R control piglets was greater but this was not statistically significant from that of cyclosporine-treated piglets.

Tissue Markers of Intestinal Cellular Injury

Small intestine tissue lactate, a marker for tissue perfusion and anaerobic metabolism, was measured following H-R. The intestinal lactate level of cyclosporine-treated group was lower than that of H-R control group ($P<0.05$)(Figure 7-2). The intestinal lactate correlated significantly with SMA flow index and $SMADO_2$ ($r=-0.6$ and $r=-0.6$, both $P<0.01$, respectively) (Figure 7-3A).

At the end of the experiment, intestinal LPO, a marker for cellular damage secondary to oxidative stress, was analyzed. H-R control piglets had higher intestinal LPO levels than that of sham-operated and cyclosporine-treatment piglets with modest significance ($P=0.08$; $\beta=0.65$ and $P=0.07$; $\beta=0.81$, respectively) (Figure 7-4). Intestinal LPO modestly correlated with $SMADO_2$ but not SMA flow index ($r=-0.5$, $P=0.05$; $r=-0.4$, $P=0.1$, respectively) (Figure 7-3B). Intestinal LPO was positively correlated with intestinal lactate ($r=0.6$, $P<0.005$).

Histological Injury of Small Intestine

Gross pathological evidence of pneumatosis intestinalsis was observed in segments of the terminal ileum in 3 piglets in the H-R control group (n=8). No gross evidence of pneumatosis was observed in any piglet in the cyclosporine-treated group (n=8). Microscopically, all specimens were graded by the Park's Criteria for intestinal tissue injury, as shown in Figure 7-5. Figure 7-5A demonstrates healthy intestinal specimen from sham-

operated piglets with intact villi including the epithelial layer of the intestine. H-R control piglet intestine seen in Figure 7-5B, showing extensive mucosal injury with denuded and fragmented villi and transmural extension of the intestinal injury. In contrast, cyclosporine-treated (10 mg/kg) intestine seen in Figure 7-5C have relatively well preserved villi with prominent lymphoid aggregates in the mucosal layer. As seen in Figure 7-5D, the H-R control group had significantly greater intestinal injury than the cyclosporine-treated group (Park's grade of 4.3±0.6 vs. 2.5±0.5, $P<0.05$). Both SMA flow index and $SMADO_2$ correlated significantly with the Park's grading with r=-0.6 and r=-0.7, respectively (both $P<0.005$)(Figure 7-3C).

Transmission Electron Microscopy of Enterocytes

Ultrastructural changes in mitochondria showed between sham-operated, control and cyclosporine-treated group. Mitochondrial structural integrity of cristae and electron density in the matrix was maintained in the sham-operated group (Figure 7-6A). In the H-R control group, most of mitochondria lost their structural integrity of cristae and electron density in matrix (Figure 7-6B). Interestingly, in the cyclosporine-treated group, structural integrity and electron density in the mitochondrial matrix was well preserved (Figure 7-6C).

Discussion

In this study, we have demonstrated that during resuscitation of asphyxiated newborn piglets, cyclosporine treatment at reoxygenation attenuates histological intestinal injury according to Park's criteria (20), along with biochemical markers of intestinal injury (tissue lactate and LPO levels). Furthermore, post-resuscitation cyclosporine treatment improves mesenteric perfusion as shown by the SMA flow and oxygen delivery.

We observed NEC-like intestinal injury, such as pneumatosis intestinalsis in only 3 of 8 controls, but not in cyclosporine-treated, piglets. Intestinal injury occurs during the original hypoxic insult and secondarily during reoxygenation. Increased production of oxygen free radicals during reperfusion has been implicated to be involved in the pathogenesis of NEC (21). Oxidative stress has also been reported to be a factor in intestinal injury following ischemia-reperfusion (22). Among the multi-factorial etiologies (23), perinatal asphyxia is considered an important cause of NEC. The potential relationship between systemic hypoxemia and NEC was first recognized by Singleton and colleagues (24). Furthermore, Lloyd and colleagues assessed infants with gastrointestinal perforation and found an 80% incidence of asphyxial complications (25).

Previous experiments on newborn piglets have reported increased oxidative stress with increased $SMADO_2$ (9). However, our findings suggest that cyclosporine-treatment protects enterocytes from increased oxidative

stress despite greater $SMADO_2$. Mitochondrial damage has been postulated to occur through release of oxygen free radicals leading to opening of the MPTP (26, 27). In the myocardium, opening of the MPTP is believed to be a key mechanism in cellular apoptosis and necrosis (15, 26, 28). The proposed mechanism involves the opening of the MPTP, leading to uncoupling of oxidative phosphorylation and inner membrane swelling following reperfusion (29). This allows release of apoptotic signaling molecules such as cytochrome c (26), or ATP depletion and necrotic cell death (29). Cyclosporine has been shown to be a potent inhibitor of MPTP opening (27, 30). The role of MPTP has been studied in both *in vivo* and *in vitro* in relation to cardiomyocytes (13, 26, 30-32). Despite its role being less extensively investigated in intestinal reperfusion injury, cyclosporine may have a similar role in apoptotic and necrotic death of enterocytes following ischemia-reperfusion injury. This theory is supported by the preservation of intestinal mitochondrial integrity in the cyclosporine treated piglets compared to the control group. Increased mitochondrial swelling was visible in the intestinal mitochondria of control piglets, likely secondary to opening of the MPTP.

We also observed improved mesenteric perfusion in piglets treated with cyclosporine compared to the control group. Nowicki and colleagues previously demonstrated persistently decreased gastrointestinal blood flow following severe hypoxemia and recovery in newborn piglets (33). The gastrointestinal blood flow of newborn piglets remained significantly

depressed following 65 min of recovery (32% of baseline) (33). Similarly in our newborn piglets, SMA flow decreased to and remained at 60% of baseline in the control group after 4h of reoxygenation. However cyclosporine-treated piglets had recovery of SMA flow to near baseline levels. Improvement in SMA perfusion is partially attributable to improvement in cardiac output, related to cardioprotective effect of cyclosporine (13). Attenuation of intestinal injury with improved microcirculation by cyclosporine may also explain the improved SMA perfusion of cyclosporine-treated piglets. Although the relationship between mesenteric perfusion and intestinal injury is not certain, we found interesting and significant correlations between mesenteric perfusion and markers of intestinal injury (intestinal lactate, LPO and histological grading). Nonetheless, cyclosporine has a potential benefit to protect the small intestine of neonatal subjects against H-R or ischemic-reperfusion injury. Further investigations on the mechanistic action of cyclosporine in intestinal protection are needed.

Although our study demonstrated NEC-like injury in the piglet intestine, it cannot be directly correlated with NEC in the human neonate, because the pathogenesis of NEC is complicated and remains undefined (6-8). It must be noted that the newborn piglets are full-term and have a developmental age similar to a 36-38 week gestation human infant (34), while the majority of NEC is seen in premature infants (35). The newborn piglet model most closely mimics the human neonate's response to asphyxia-

reoxygenation (34, 36). However, there are limitations in its clinical translation including the absence of hypercapnia and 30 min of hyperoxic resuscitation in anesthetized, surgically instrumented newborn subjects. These factors are not uncommon in clinical scenario but may alter the hemodynamic responses (37), oxidative stress (38, 39) and thus the resultant intestinal injury (9). In addition, our animal model is unable to take into the account infectious and immunologic aspects related to NEC-like injury. Thus, we cannot comment on the potentially beneficial role of probiotic therapy (40). Nonetheless, our newborn piglet model demonstrates NEC-like intestinal injury that closely approximates the clinical setting. Lastly, though our findings demonstrate attenuation of intestinal injury with cyclosporine treatment in the short term, further studies are needed to investigate if this acute improvement will persist in the long term.

In conclusion, cyclosporine treatment attenuates NEC-like intestinal injury in asphyxiated newborn piglets following reoxygenation. The protective mechanism of cyclosporine may be similar to that seen in cardiomyocytes, however further studies are needed to delineate its underlying mechanisms.

Table 7-1: Arterial blood gases

	Normoxic Baseline	End of hypoxia	30 min Reoxygenation	240 min Reoxygenation
pH				
Control	7.41 ± 0.06	7.08 ± 0.07*	7.19 ± 0.09	7.31 ± 0.12
CsA 10 mg/kg	7.43 ± 0.05	7.05 ± 0.17*	7.16 ± 0.17	7.35 ± 0.04
Sham	7.39 ± 0.03	7.39 ± 0.02	7.42 ± 0.02	7.41 ± 0.04
PaO2 (mmHg)				
Control	74 ± 13	37 ± 8*	348 ± 96	63 ± 16
CsA 10 mg/kg	79 ± 13	36 ± 8*	413 ± 36	64 ± 4
Sham	67 ± 5	68 ± 3	68 ± 4	76 ± 16
PaCO2 (mmHg)				
Control	39 ± 3	39 ± 5	37 ± 4	39 ± 4
CsA 10 mg/kg	37 ± 4	39 ± 5	36 ± 5	41 ± 3
Sham	37 ± 1	41 ± 1	40 ± 3	37 ± 1
HCO3- (mM)				
Control	25 ± 3	11 ± 2*	14 ± 2	20 ± 5
CsA 10 mg/kg	25 ± 2	10 ± 4*	14 ± 5	22 ± 2
Sham	23 ± 1	25 ± 2	26 ± 2	24 ± 2
Plasma Lactate (mM)				
Control	3.9 ± 0.6	13.8 ± 3.9*	11.3 ± 2.9	5.5 ± 3.3
CsA 10 mg/kg	3.7 ± 0.7	14 ± 4.9*	11.7 ± 4.3	3.1 ± 1.0
Sham	3.8 ± 1.0	2.8 ± 0.5	2.4 ± 0.5	2.0 ± 0.2

* $P < 0.05$ vs. normoxic baseline (RM ANOVA)

Table 7-2. Hemodynamic Variables During Hypoxia and Reoxygenation

	Normoxic Baseline	End of hypoxia	30 min Reoxygenation	240 min Reoxygenation
Heart Rate (beats/min)				
Control	181 ± 12	213 ± 8	211 ± 9	216 ± 11
CsA 10 mg/kg	162 ± 9	212 ± 8	199 ± 7	211 ± 10
Sham	204 ± 17	237 ± 10	232 ± 7	221 ± 8
Mean Arterial Pressure (mmHg)				
Control	68 ± 4	28 ± 3*	42 ± 2	34 ± 3
CsA 10 mg/kg	72 ± 2	31 ± 2*	54 ± 4	38 ± 2
Sham	68 ± 3	57 ± 4	50 ± 3	45 ± 2
Cardiac Output (mL/min/kg)				
Control	211 ± 14	83 ± 8*	178 ± 17	127 ± 21
CsA 10 mg/kg	164 ± 9	79 ± 10*	184 ± 16	166 ± 14
Sham	189 ± 31	214 ± 36	214 ± 42	213 ± 25
SMA Flow (ml/min/kg)				
Control	39 ± 6	15 ± 3*	39 ± 5	23 ± 5
CsA 10 mg/kg	36 ± 4	20 ± 2*	41 ± 4	34 ± 4
Sham	33 ± 6	42 ± 8	44 ± 9	44 ± 6
SMA Oxygen Delivery (mL O_2/min/kg)				
Control	3.8 ± 0.6	0.6 ± 0.2*	3.8 ± 0.6	2.0 ± 0.5
CsA 10 mg/kg	3.8 ± 0.3	0.8 ± 0.1*	4.0 ± 0.2	3.1 ± 0.4
Sham	3.3 ± 0.4	3.8 ± 0.5	3.8 ± 0.4	3.5 ± 0.3

*P < 0.05 vs. normoxic baseline (RM ANOVA)
All results are shown as Mean ± SEM

Figure 7-1. Mesenteric perfusion. (A) Superior mesenteric flow index (as % of baseline) and **(B)** Superior mesenteric oxygen delivery (as % of baseline) in piglets after 2h hypoxia and 4h reoxygenation with either saline (controls) or cyclosporine-treatment (n=8 per group). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls, #P<0.05 vs. all hypoxia-reoxygenation groups. Downward pointing black arrow represents administration of cyclosporine. SMA = superior mesenteric artery.

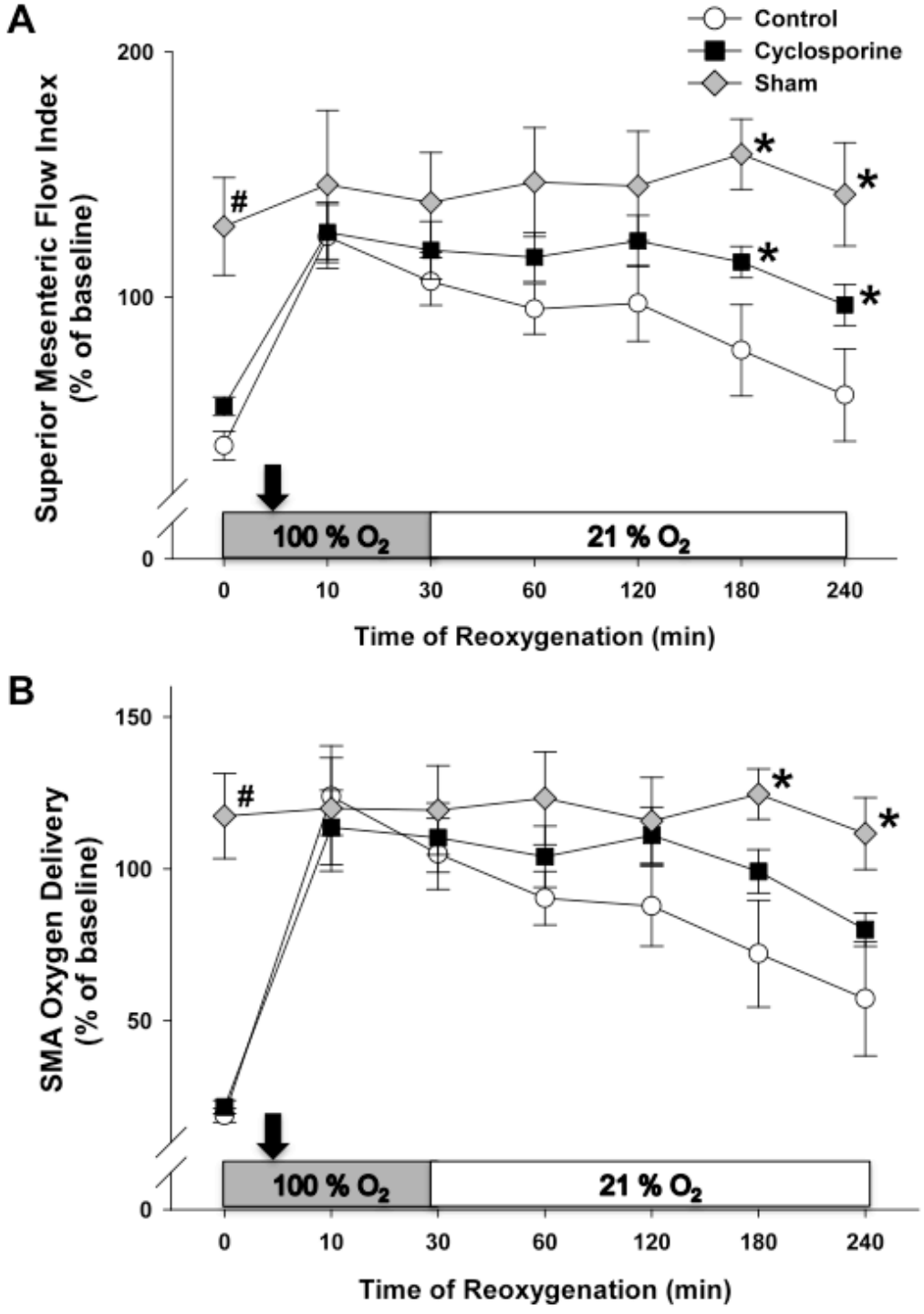


Figure 7-2. Tissue markers of intestinal injury. Intestinal lactate levels in piglets after 2h hypoxia and 4h reoxygenation with either saline (controls) or cyclosporine-treatment (n=8 per group). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls.

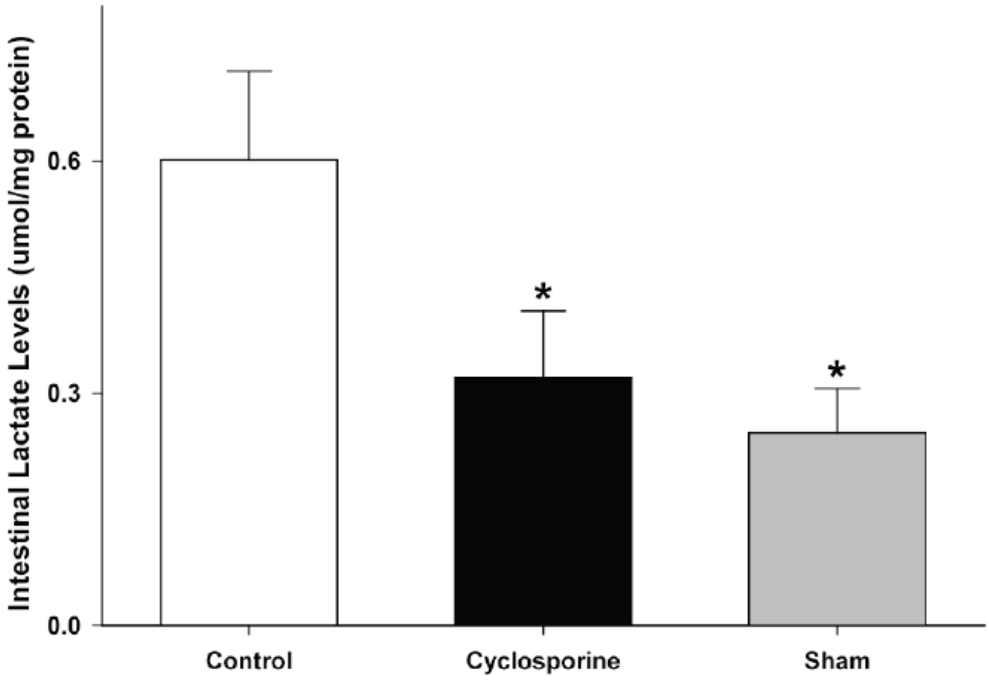


Figure 7-3. Relationship between mesenteric oxygen delivery and intestinal injury. Correlation between superior mesenteric oxygen delivery and **(A)** intestinal lactate, **(B)** intestinal lipid hydroperoxides, **(C)** histological grade (Park's criteria). SMA = superior mesenteric artery.

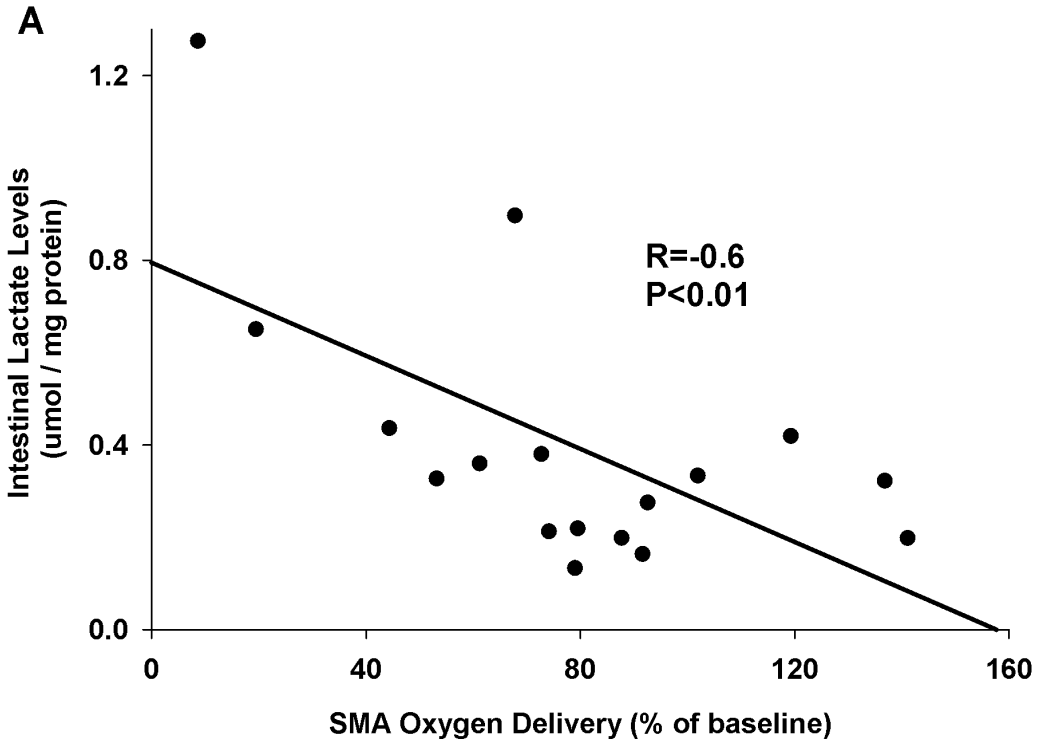
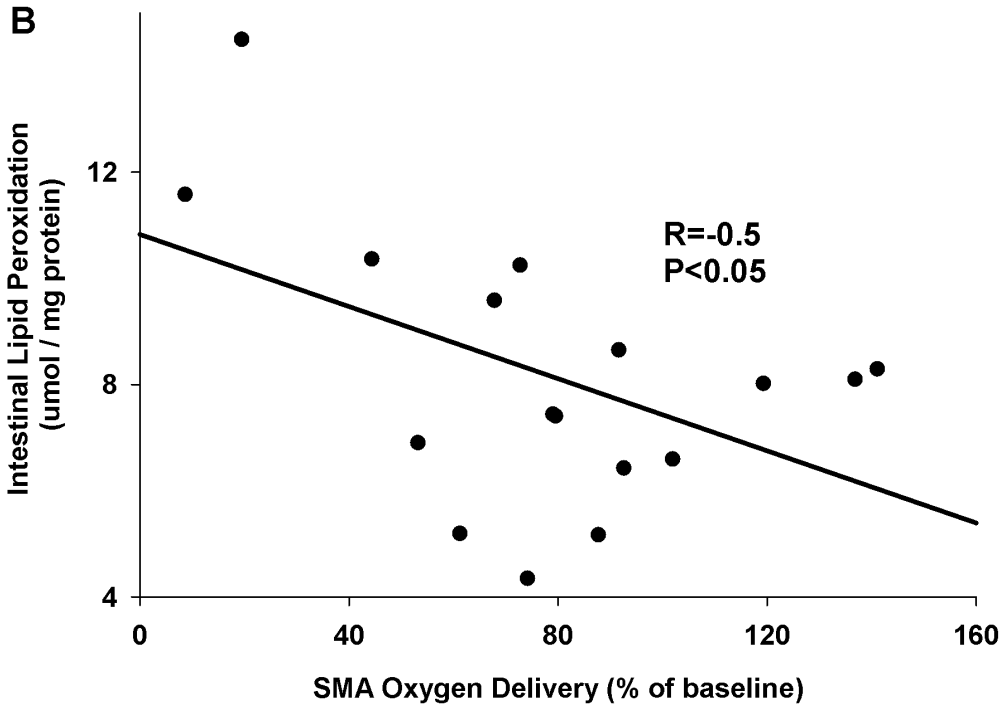


Figure 7-3 continued. Relationship between mesenteric oxygen delivery and intestinal injury. Correlation between superior mesenteric oxygen delivery and **(A)** intestinal lactate, **(B)** intestinal lipid hydroperoxides, **(C)** histological grade (Park's criteria). SMA = superior mesenteric artery.



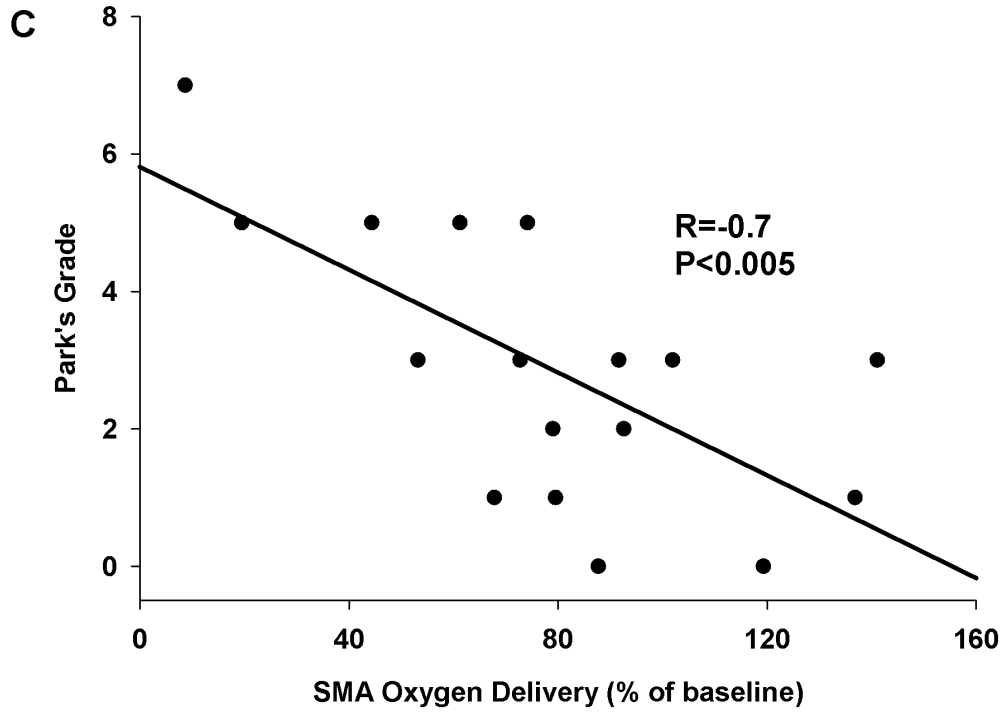


Figure 7-4. Lipid hydroperoxides levels in piglets after 2h hypoxia and 4h reoxygenation with either saline (controls) or cyclosporine-treatment (n=8 per group). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4).

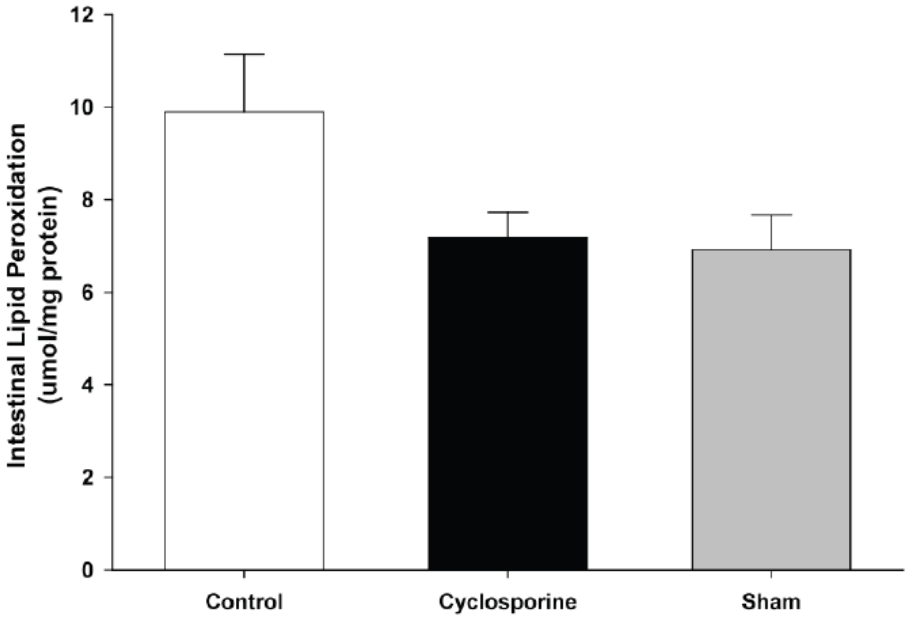


Figure 7-5. Intestinal histology. Representative histological features (hematoxylin and eosin stain) for piglets in **(A)** sham-operated, **(B)** control **(C)** cyclosporine-treatment groups. Black arrow points toward transmural extension of intestinal injury. White arrow points are prominent lymphoid aggregates. **(D)** Histological grade (Park's criteria) in piglets after 2h hypoxia and 4h reoxygenation with either saline (controls) or cyclosporine-treatment (n=8 per group). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). Horizontal line indicates mean grade of the group. *P<0.05 vs. controls.

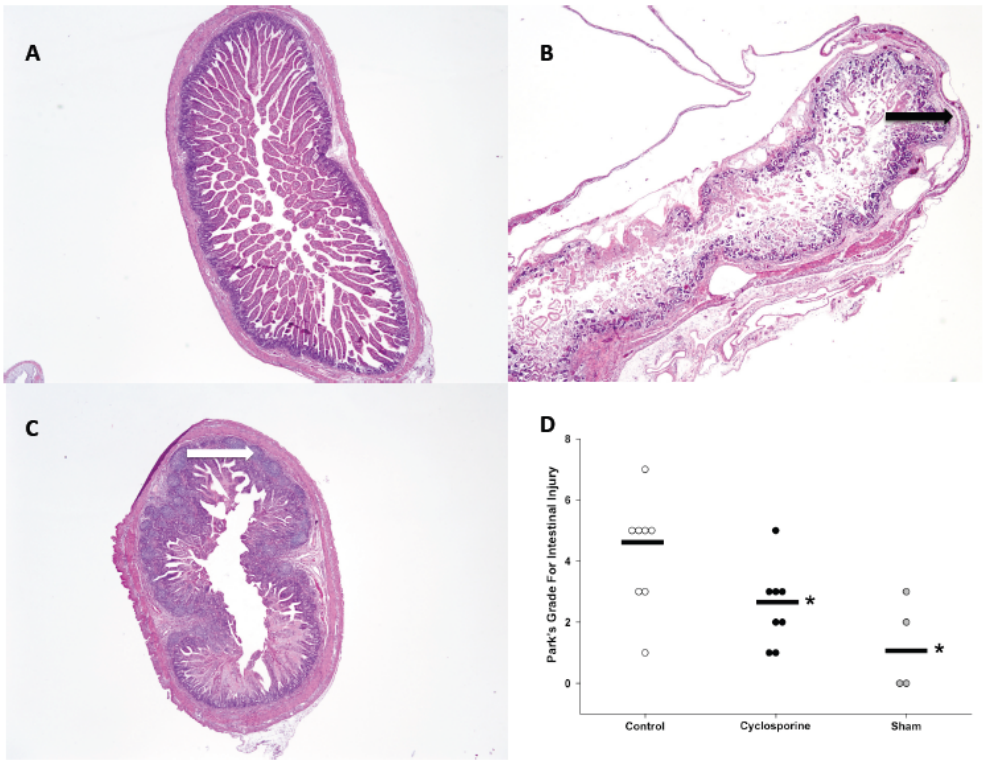
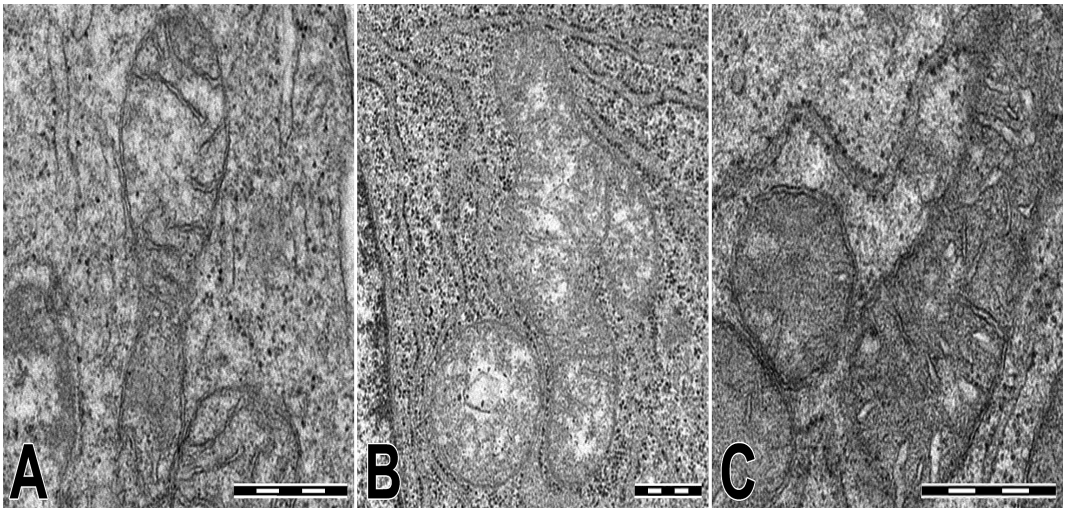


Figure 7-6. Representative ultrastructural images of mitochondria in the small intestine. (A) In sham-operated group cristae and matrix in mitochondria showed well-maintained their structural integrity and electron density. **(B)** In control group, cristae and matrix in mitochondria lost their integrity and electron density. **(C)** Mitochondria in cyclosporine-treated group showed integrity of cristae and electron density in matrix same as sham-operated group. A scale bar is 500nm for all images.



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Chapter 8

Cyclosporine Treatment Reduces Oxygen Free Radical Generation and Oxidative Stress in the Brain of Hypoxia-Reoxygenated Newborn Piglets

Adapted from:

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Abstract

Background

Oxygen free radicals have been implicated in the pathogenesis of hypoxic-ischemic encephalopathy (HIE). It has previously been shown in traumatic brain injury animal models that treatment with cyclosporine reduces brain injury. However, the potential neuroprotective effect of cyclosporine in asphyxiated neonates has yet to be fully studied. Using an acute newborn swine model of hypoxia-reoxygenation (H-R), we evaluated the effects of cyclosporine on the brain, focusing on hydrogen peroxide (H_2O_2) production and markers of oxidative stress.

Methods

Piglets (1-4d, 1.4-2.5kg) were block-randomized into three H-R experimental groups (2h hypoxia followed by 4h reoxygenation)(n=8/group). At 5min after reoxygenation, piglets were given either i.v. saline (placebo, controls) or cyclosporine (2.5 or 10 mg/kg i.v. bolus) in a blinded-randomized fashion. An additional sham-operated group (n=5) underwent no H-R. Systemic hemodynamics, carotid arterial blood flow (transit-time ultrasonic probe), cerebral cortical H_2O_2 production (electrochemical sensor), cerebral tissue glutathione (ELISA) and cytosolic cytochrome-c (western blot) levels were examined.

Results

Hypoxic piglets had cardiogenic shock (cardiac output 40-48% of baseline), hypotension (mean arterial pressure 27-31 mmHg) and acidosis (pH 7.04) at

the end of 2h of hypoxia. Post-resuscitation cyclosporine treatment, particularly the higher dose (10 mg/kg), significantly attenuated the increase in cortical H_2O_2 concentration during reoxygenation, and was associated with lower cerebral oxidized glutathione levels. Furthermore, cyclosporine treatment significantly attenuated the increase in cortical cytochrome-c and lactate levels. Carotid blood arterial flow was similar among groups during reoxygenation.

Conclusions

Conclusively, post-resuscitation administration of cyclosporine significantly attenuates H_2O_2 production and minimizes oxidative stress in newborn piglets following hypoxia-reoxygenation.

Introduction

Asphyxia contributes to over 1 million neonatal deaths per year worldwide, with hypoxic-ischemic encephalopathy (HIE) being the most common morbidity in survivors (1). In the US, HIE is estimated to occur in 1 to 4 cases per 1000 live births (2). Globally, approximately 10% to 60% of these neonates with HIE will die, with more than 25% of survivors developing long-term neurodevelopmental complications (3,4).

Although the exact mechanisms have not been fully elucidated, Rodrigo et al proposed that oxygen free radicals (OFR) played an important role in reperfusion/reoxygenation injury following asphyxia (5). Excess production of OFR such as superoxide anion, hydroxyl radical, hydrogen peroxide (H_2O_2) and nitric oxide (NO) has been reported during ischemia-reperfusion or hypoxia-reoxygenation (H-R). These OFR and their metabolites cause cellular damage and cell death by oxidizing proteins, inducing lipid peroxidation and damaging DNA. There is evidence that mitochondrial dysfunction plays an essential role in both apoptotic and necrotic cellular death in HIE (6,7). Mitochondrial damage secondary to oxidative stress during reperfusion/reoxygenation has been proposed to be associated with opening to the mitochondrial permeability transition pore (MPTP), which uncouples oxidative phosphorylation and leads to mitochondrial swelling. Swelling may lead to rupture of the outer

mitochondrial membrane and release of apoptotic signaling molecules such as cytochrome-c (6,7,8).

Cyclosporine A has been shown *in vivo* to reduce swelling of isolated brain mitochondria (9). Furthermore, experimental studies show that cyclosporine A inhibits opening of the MPTP by binding to cyclophilin-D (10). In animal models of traumatic brain injury, cyclosporine treatment has been shown to reduce axonal injury (11) and attenuate lipid peroxidation (12). Only a few studies have been carried out to examine the effectiveness of cyclosporine treatment in neuroprotection of the immature newborn brain. A significant improvement against ischemic/hypoxic-induced brain injury following cyclosporine treatment has been reported in both fetal and newborn rats using models of in utero ischemia and carotid/cerebral artery ligation, respectively (8,13,14). In contrast, Puka-Sundvall et al. reported that cyclosporine did not provide neuroprotection after hypoxia-ischemia in newborn rats (15). Despite these controversial observations, the neuroprotective effects of cyclosporine have *not* been assessed in large-sized newborn animals that underwent global H-R as that in the clinical scenario. Our objective was to test the hypothesis that post-resuscitation cyclosporine treatment would attenuate cerebral OFR (H_2O_2) production and oxidative stress-related injury in newborn piglets during asphyxia-reoxygenation.

Methods

All experiments were conducted in accordance with the guidelines and approval of the Animal Care and Use Committee (Health Science), University of Alberta. Twenty-eight newborn mixed breed piglets, 1 to 4 days of age, weighing 1.4 to 2.5 kg were obtained on the day of experimentation from a local farm.

Animal preparation

Animal preparation as discussed in Chapter 5. In addition, the piglet was then placed in the prone position with the head mounted in a stereotaxic holder. Using bregma as the reference point, a stainless steel guide cannula (19-gauge) was implanted in the right frontoparietal cortex using the following co-ordinates: AP=6.5 L=4 H=6 mm. The co-ordinates for the cerebral cortex were based on an atlas constructed with several pilot studies. This cerebral region was chosen because significant histological and biochemical injury was observed in previous studies (17,18,19). The guide cannula was fixed on the skull with dental cement.

The piglets were allowed to recover from surgical instrumentation until baseline hemodynamic measures were stable. Ventilator rate was adjusted to maintain the P_aCO_2 35-45 mmHg as determined by periodic arterial blood gas analysis during experimentation. Heart rate, MAP and CABF were continuously monitored and recorded throughout the experiment.

Experimental Protocol

The piglets were block-randomized to 3 groups (n=8 per group) that underwent H-R. A fourth sham-operated group of piglets (n=4) underwent complete instrumentation without H-R.

In the 3 H-R groups, hypoxemia was induced via normocapnic alveolar hypoxia. These piglets were ventilated with a FiO_2 of 0.10-0.15 by increasing the inhaled concentration of nitrogen gas relative to oxygen for 2h, aiming for arterial oxygen saturations of 30-40%. It has been shown in previous studies that this degree of hypoxemia in the newborn piglet model will produce clinical asphyxia with severe metabolic acidosis and systemic hypotension (20,21). This was followed by reoxygenation with 100% oxygen for 0.5 h and then 21% oxygen for 3.5 h. At 5 min of reoxygenation, piglets received a blinded treatment either with cyclosporine as an intravenous bolus (2.5 or 10 mg/kg) or saline (H-R control). Cyclosporine A treatment was given at 5 min reoxygenation to simulate the clinical setting, in which intravenous access is obtained prior to administering resuscitative medications to the neonate.

Blinding was maintained by reconstituting all doses of cyclosporine and normal saline in a standard volume (5 ml) immediately before administration. The medication was given intravenously over 2 min. A

laboratory technician, who was not involved in the experiment, prepared and administered the medications.

At the end of the study, piglets were euthanized with 100 mg/kg pentobarbital i.v. The whole brain was removed immediately and placed in 50 mL ice-cold 2-methylbutane for 10 min. After discarding 2-methylbutane, the brain was stored in -80^{o}C for further biochemical analysis.

Hemodynamic measurements

Hemodynamic recording for data analysis were carried out at specified time points: baseline (0 min), 60 and 120 min of hypoxia, 130 (10-min reoxygenation) and 150 min (30-min reoxygenation) reoxygenation with 1.0 FiO_2, then at 180 (60-min reoxygenation), 240 (120-min reoxygenation), 300 (180-min reoxygenation) and 360 min (240-min reoxygenation) for reoxygenation with 0.21 FiO_2. All recordings were calculated as a mean over 2 min of recording. At the specified time points, both arterial and venous blood samples were taken for blood gases, hemoglobin levels and co-oximetry.

Cerebral cortical hydrogen peroxide measurement

The change in cortical H_2O_2 during H-R was measured directly by electrochemical H_2O_2 sensor (HPO-100, World Precision Instruments Ltd.). After completion of surgery, the H_2O_2 sensor was inserted through the guide

cannula into the cortex area. The sensors were connected to a computer-controlled data acquisition system (Apollo 4000, World Precision Instruments Ltd., Sarasota, FL). The signal outputs were recorded continuously throughout the experimental period. Immediately before and after each experiment, the H_2O_2 sensor was calibrated with 1 mM H_2O_2 in phosphate buffer (10 mM, pH 7.4). The phosphate buffer was pre-warmed to 38±1°C to adjust the temperature deviation. The mean value was used for converting the signal outputs. The relative change in cortical H_2O_2, expressed in μM, was calculated with reference to the normoxic baseline after stabilization.

Determination of cortical glutathione and lactate

A block of cortical tissue (5 x 5 x 5 mm^3) from the left side of the cortex corresponding to the cannulation area of the right side was dissected. Part of the tissue was then homogenized with 5 μl/mg of 50 mM phosphate buffer containing 1 mM EDTA (pH 7.0) and stored at -80°C until biochemical analyses. The cortical level of GSH/GSSG was measured using commercially available assay kits (Cayman Chemical, #703002). Brain lactate was assayed by enzymatic spectrometric methods to measure the absorbance of NADH at 340 nm. The protein content was determined by bicinchoninic acid assay kit (Sigma).

Determination of cytosol cytochrome-c

Using polytron tissue grinder, part of the cortical tissue (~100 mg) obtained as described above was homogenized with 250 µL cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM NaPP, 250 mM sucrose, and 1 mM dithiothreitol) supplemented with Complete protease inhibitor (Roche Diagnostic GmbH, Germany). The homogenates were centrifuged at 5,000g for 10 min. Protein extract (50 µg) was mixed with loading buffer and denatured by boiling for 5 min before loading on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After electrophoresis, proteins were transferred to a 0.2-µm polyvinylidene difluoride membrane (Bio-Rad), and the latter were then blocked with 10% fat-free dry milk in Tween-Tris-buffer saline (TTBS) for 30 min at room temperature. After brief washing with TTBS, the membrane was incubated with a polyclonal antibody to cytochrome-c (dilution 1:1,000; Biovision) overnight at 4°C cold room. After washing four times (5 min per wash) with TTBS to remove the unbound antibody, the membrane was then incubated in TTBS for 1 h at room temperature with horseradish peroxidase conjugated to goat anti-rabbit IgG (dilution 1:1,000; EY Laboratories). Membranes were washed four times again with TTBS. Bound proteins were detected using chemiluminescence reagents (ECL plus; Amersham Biosciences) and visualized by exposing to x-ray film (Biomax MR, Kodak Photo Film). The film was developed by Kodak X-OMAT 1000A processor (Eastman Kodak Company). The x-ray films were scanned using PowerLook 1000 scanner (UMAX) and bands were analyzed using Quantity One 1-D

analysis software (Bio-Rad). The integrated areas of bands were determined and expressed as a percentage of a standard sample ran on the same membrane. The amount of protein to be used for detection was normalized using the ß-actin as loading control.

Statistical Analysis

All results are expressed as mean±SEM. Two-way repeated measures and 1-way analysis of variance and Krushal-Wallis test were used to study the differences between groups for parametric and non-parametric, respectively. *Post-hoc* testing with Fisher Least Significant Method was performed for pairwise comparisons with the H-R control group as appropriate. Correlation between variables was studied by Pearson Moment or Spearman Rank Order test as appropriate. Statistical analyses were performed using SigmaPlot® (SPSS v11.0). Significance was set at $p<0.05$.

Results

The piglets aged 2.3±0.2 day and weighed 1.9±0.04 kg with no significant differences among groups. Sham-operated animals were stable throughout the experimental period (Table 8-1).

Effects of cyclosporine on physiological parameters

MAP significantly decreased to 40% of the normoxic baseline value after 2h of hypoxemia ($p<0.05$) (Table 8-1). After the immediate recovery

upon reoxygenation, MAP of H-R controls deteriorated and remained lower than the normoxic baseline value throughout the reoxygenation period ($p<0.05$) (Table 8-1). The heart rate of hypoxic piglets was higher than the baseline value at the end of hypoxia and remained high throughout the reoxygenation period (Table 8-1). The temporal changes in MAP and heart rate during hypoxia and reoxygenation of cyclosporine-treated groups were not different from those observed in the H-R control group (Table 8-1).

The baseline value of CABF was 17.9±1 mL/min/kg (n=28) with no difference among groups. The CABF of H-R controls was significantly lower than the baseline value at the end of hypoxia and remained low throughout reoxygenation (Fig. 8-1). Treating H-R piglets with cyclosporine did not significantly affect CABF (Fig. 8-1).

After exposure to hypoxia for 2h, the arterial pH, PO_2 and base excess levels of piglets decreased significantly below their baseline value (Table 8-1). After reoxygenation, all these parameters in blood gas recovered gradually towards the respective values at normoxic baseline. There were no significant differences in arterial blood gas between saline and cyclosporine-treated groups throughout hypoxia and reoxygenation (Table 8-1).

Effects of cyclosporine on cerebral cortical hydrogen peroxide production

Fig. 8-2 demonstrates the temporal changes in cortical H_2O_2 concentration during H-R. Cortical H_2O_2 concentrations in the H-R control and cyclosporine-treated groups maintained near baseline levels during hypoxia. After reoxygenation, the H_2O_2 concentration in H-R controls increased gradually within the first hour and then became significantly greater than the normoxic baseline for the remainder of experimental period (Fig. 8-2). Post-resuscitation administration of cyclosporine at 10 mg/kg, but not 2.5 mg/kg, abolished the increase in cortical H_2O_2 concentration during reoxygenation.

Effects of cyclosporine on cortical cytosol cytochrome-c

The cytosol cytochrome-c level in the cerebral cortex of H-R controls was significantly higher than that of sham-operated group (Fig. 8-3). Treating the piglets with cyclosporine caused a dose-related reduction in cytochrome-c levels, with a significant difference observed between piglets treated with 10 mg/kg cyclosporine and H-R controls.

Effects of cyclosporine on cortical glutathione and lactate levels

In the cerebral cortical tissue following H-R, there was an increase in GSSG levels in the H-R control group (vs. sham-operated piglets, $p<0.05$)(Table 8-2). Cortical GSSG levels were significantly reduced in piglets treated with 10 mg/kg cyclosporine compared to H-R controls and were similar to levels of sham-operated piglets. Total GSH levels were not different among groups (Table 8-2). These changes in GSSG and GSH levels resulted in

a significant increase in glutathione redox (GSSG:GSH) ratio in the H-R controls, but not in cyclosporine-treated groups (Table 8-2). The H-R control piglets had significantly higher cerebral cortical lactate levels than that of sham-operated piglets at the end of experiment (Fig. 8-4). Treating the animal with 10 mg/kg cyclosporine significantly reduced the cortical lactate level compared to H-R controls (Fig. 8-4).

Overall, the cumulative cortical H_2O_2 concentration was significantly correlated with cytosol cytochrome-c level ($r=0.45$, $p<0.05$) and cortical GSSG ($r=0.51$, $p<0.01$), but not cortical GSH level or GSSG:GSH redox ratio. The cumulative cortical H_2O_2 concentration also correlated positively cortical lactate levels ($r=0.77$, $p<0.01$). No significant correlation was found between cortical H_2O_2 concentration and simultaneous CABF, heart rate or MAP, cortical cytochrome c, GSH and lactate levels (data not shown).

Discussion

This is the first study to demonstrate that post-resuscitation cyclosporine treatment attenuates (1) cortical H_2O_2 concentration and oxidative stress, (2) cortical lactate and cytosol cytochrome-c levels in newborn piglets during reoxygenation after a severe hypoxic insult, with no associated changes in carotid hemodynamics. These findings support the therapeutic potential of cyclosporine as a neuroprotective agent of

cyclosporine in neonatal asphyxia with its attenuation of H-R induced cerebral damage.

Although several studies have evaluated the effectiveness of cyclosporine treatment following hypoxic-ischemic injury to the immature brain (8,13,14,15), none of these studies examined the effectiveness of cyclosporine treatment on OFR generation. Using specific electrochemical H_2O_2 sensors, we were able to directly monitor the change in cortical H_2O_2 production continuously during H-R. The cortical H_2O_2 concentration remained near baseline during hypoxia. Upon resuscitation, the cortical H_2O_2 concentration of H-R control piglets increased gradually during the early period of reoxygenation and then became markedly elevated at 1h post-reoxygenation. Interestingly, this observation is different from previous reports of a rapid surge of OFR, particularly nitric oxide (NO), immediately after reoxygenation/reperfusion (22,23). We are not certain about the etiology but speculate that the slow rise of cortical H_2O_2 concentration could be related to the competitive reactions of NO and superoxide dismutase for superoxide anions. Beckman et al showed a three-fold difference in rate constants between the NO reaction and enzymatic dismutation with superoxide anions (6.3 vs. 2.3 x 10 -6 M/s, respectively) (24). Further, it has been recently shown that reduction of NO may lead to enhanced formation of H_2O_2 (25).

Post-resuscitation cyclosporine treatment significantly attenuated the *in vivo* rise in H_2O_2 similar to levels observed in the sham-operated piglets. In regard to tissue markers of cortical oxidative stress, we observed increased GSSG levels and GSSG/GSH ratio in the cortex of H-R control piglets following reoxygenation were significantly attenuated by cyclosporine treatment. Further, a positive correlation was found between the cumulative cortical H_2O_2 and GSSG concentrations. A significant reduction in OFR has been reported previously in isolated mitochondria from ischemic cells following treatment by cyclosporine or its non-immunosuppressive analog (26,27,28). Furthermore, cyclosporine has been shown to inhibit H_2O_2 generation in isolated brain and liver mitochondria exposed to excess calcium (29). Mitochondria are a major source of OFR generation during reoxygenation/reperfusion. OFR produced by mitochondria may lead to the release of calcium from the endoplasmic reticulum, resulting in mitochondrial calcium over-loading, MPTP opening and further OFR production (7,29,30,31,32). Cyclosporine has been shown in *in vitro* experiments to maintain mitochondrial homeostasis by binding to cyclophilin D and preventing MPTP opening. Interestingly, the opening of MPTP itself may induce OFR production at complex I of the respiratory chain (33). It has been previously reported that MPTP opening may enhance cytochrome-c dislocation from the mitochondrial inner membrane and its subsequent release into the cytosol (6), (7,8). Therefore, we suggest that observed reduction in H_2O_2 production following cyclosporine treatment in

the present study may be related to cyclosporine's inhibitory effect on MPTP opening. This speculation is supported by our observations on cytosol cytochrome-c levels and the significant correlation between cortical H_2O_2 concentrations and cytochrome-c levels.

Our speculation that cyclosporine treatment elicits its protective effects by maintaining mitochondria homeostasis is further supported by reduced cortical lactate, in the absence of carotid hemodynamic effects. Despite preserved regional blood flow and brain tissue oxygen tension, increases in brain extracellular lactate have been reported in patients with head injury (34,35). These authors suggested that the increase in lactate concentration may be due to inefficiency of mitochondrial oxidative metabolism. A recent study indicated that the opening of MPTP can stimulate glycolytic lactate production through the inhibition of malate/aspirate shuttle system (36). A significant increase in cortical lactate level was noted in H-R controls in our study, which was reduced by treating the piglet with cyclosporine. Similar reductions in brain lactate levels by cyclosporine treatment after hypoxia-ischemia has also been reported recently in newborn rats (13). These results indicate that cyclosporine may attenuate mitochondrial metabolic impairment in newborn subjects. Interestingly, we did not observe any associative improvement in carotid hemodynamics. However, cyclosporine or its non-immunosuppressive analogs have been shown recently to improve cerebral blood flow in rats after cortical

spreading depression (37). These differences may be related to methodology for blood flow assessment, cyclosporine dosage, and the animal model used.

Taken together, our findings support the likelihood that cyclosporine may exert its neuroprotective effects by preventing MPTP opening, with reduced oxidative stress and preserved energy homeostasis, in a newborn piglet model of H-R. Further research is warranted to confirm if the post-resuscitation administration of cyclosporine provides neuroprotection against oxidative stress-related injury in asphyxiated neonates.

Table 8-1. Changes in mean arterial pressure (MAP), heart rate and arterial blood gases during hypoxia and reoxygenation.

	Normoxic Baseline	End of hypoxia	Reoxygenation 30 min	Reoxygenation 2 h	Reoxygenation 4h
MAP (mmHg)					
H-R Control	68 ± 5	28 ± 3§	47 ± 4§	42 ± 3§	34 ± 3§
CsA 2.5 mg/kg	78 ± 3	31 ± 2§	48 ± 5§	41 ± 3§	44 ± 3§
CsA 10 mg/kg	72 ± 2	31 ± 2§	54 ± 6§	40 ± 2§	38 ± 2§
Sham-operated	68 ± 3	57 ± 4*	50 ± 3	49 ± 2	45 ± 2*
Heart Rate (bpm)					
H-R Control	181 ± 12	213 ± 8	211 ± 9	225 ± 9#	216 ± 11
CsA 2.5 mg/kg	167 ± 7	210 ± 9§	185 ± 8	191 ± 10	207 ± 14
CsA 10 mg/kg	162 ± 9	212 ± 8§	199 ± 7§	214 ± 8	211 ± 10§
Sham-operated	204 ± 17	237 ± 10	232 ± 7	231 ± 3	221 ± 8
pH					
H-R Control	7.41 ± 0.06	7.08 ± 0.07§	7.19 ± 0.09§	7.31 ± 0.07	7.31 ± 0.12
CsA 2.5 mg/kg	7.39 ± 0.08	7.02 ± 0.15§	7.14 ± 0.16§	7.35 ± 0.09	7.35 ± 0.08
CsA 10 mg/kg	7.43 ± 0.05	7.05 ± 0.17§	7.16 ± 0.17§	7.35 ± 0.07	7.35 ± 0.04
Sham-operated	7.39 ± 0.02	7.39 ± 0.02*	7.42 ± 0.02*	7.39 ± 0.02	7.41 ± 0.04
PaO_2 (mmHg)					
H-R Control	74 ± 13	37 ± 8§	348 ± 96§	62 ± 6	63 ± 16
CsA 2.5 mg/kg	83 ± 9	36 ± 6§	383 ± 86§	63 ± 8	63 ± 4
CsA 10 mg/kg	79 ± 13	36 ± 8§	413 ± 36§	69 ± 6	64 ± 4
Sham-operated	67 ± 5	68 ± 3*	68 ± 4*	77 ± 18	76 ± 17
Base excess (mmol/L)					
H-R Control	0.1 ± 3	-17 ± 3§	-13 ± 3§	-6 ± 3	-5 ± 2
CsA 2.5 mg/kg	-1.0 ± 3	-18 ± 5§	-14 ± 6§	-5 ± 5	-2 ± 5
CsA 10 mg/kg	0.5 ± 2	-17 ± 6§	-14 ± 6§	-4 ± 4	-2 ± 2
Sham-operated	-2.0 ± 2	-0.3 ± 1*	1.5 ± 3*	-0.9 ± 3	-0.8 ± 2

§P<0.05 vs. normoxic baseline (2-way repeated measures ANOVA)
*P<0.05 vs. H-R controls (2-way repeated measures ANOVA)

Table 8-2. Effects of cyclosporine on cerebral cortical glutathione levels after hypoxia-reoxygenation (H-R).

	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSSG:GSH Ratio
H-R Control	229.3±20.4	27.2±1.5	0.12±0.01
CSA 2.5 mg/kg	272.7±35.8	24.6±3.4	0.09±0.01*
CSA 10 mg/kg	303.5±54.1	16.8±2.1*	0.06±0.01*
Sham-operated	330.1±47.3	18.2±3.5*	0.06±0.01*

*P<0.05 vs. H-R controls (One-way ANOVA)

Figure 8-1. Temporal changes in carotid blood flow (CCAF) during 4h of reoxygenation with different doses of cyclosporine (2.5 [▲] or 10 mg/kg [■]). Control piglets received no cyclosporine (●). Sham-operated piglets underwent no hypoxia-reoxygenation (○). †p<0.05 vs. H-R control (2-way repeated measures ANOVA); *p<0.05 vs. H-R controls at simultaneous time point (1-way ANOVA), § p<0.05 vs. baseline (1-way ANOVA).

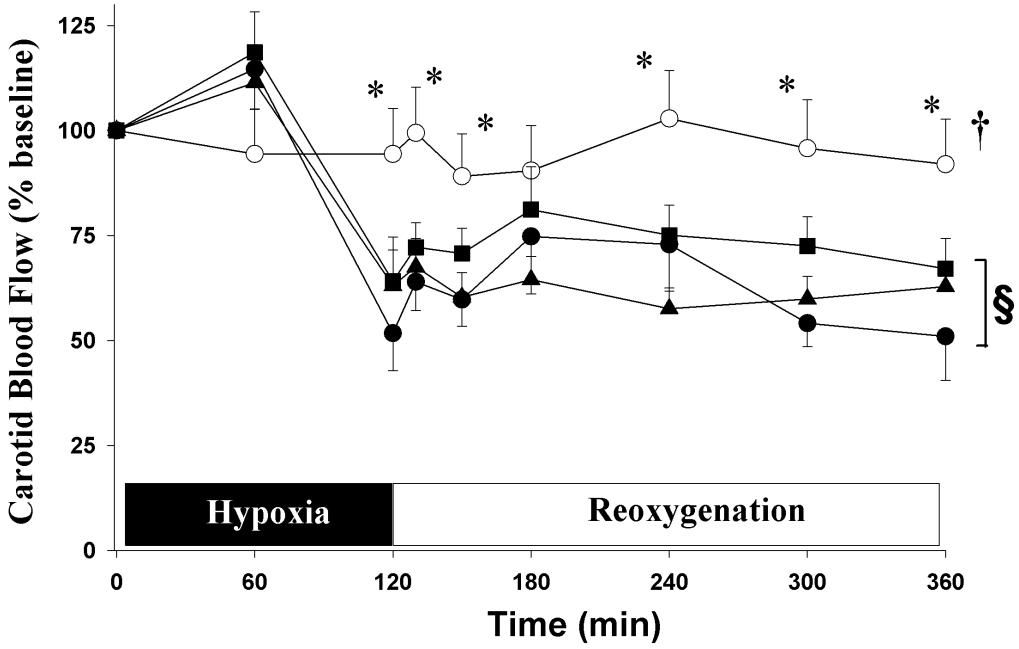


Figure 8-2. Temporal changes in cortical hydrogen peroxide (H_2O_2) concentration in sham-operated piglets (○, without hypoxia and reoxygenation), hypoxic piglets receiving either saline (●, H-R control) or cyclosporine 2.5 (▲) or 10 mg/kg (■) 5 min after reoxygenation. †p<0.05 vs. H-R control (2-way repeated measures ANOVA); *p<0.05 vs. H-R controls at current time point (1-way ANOVA),), § p<0.05 vs. baseline (1-way ANOVA).

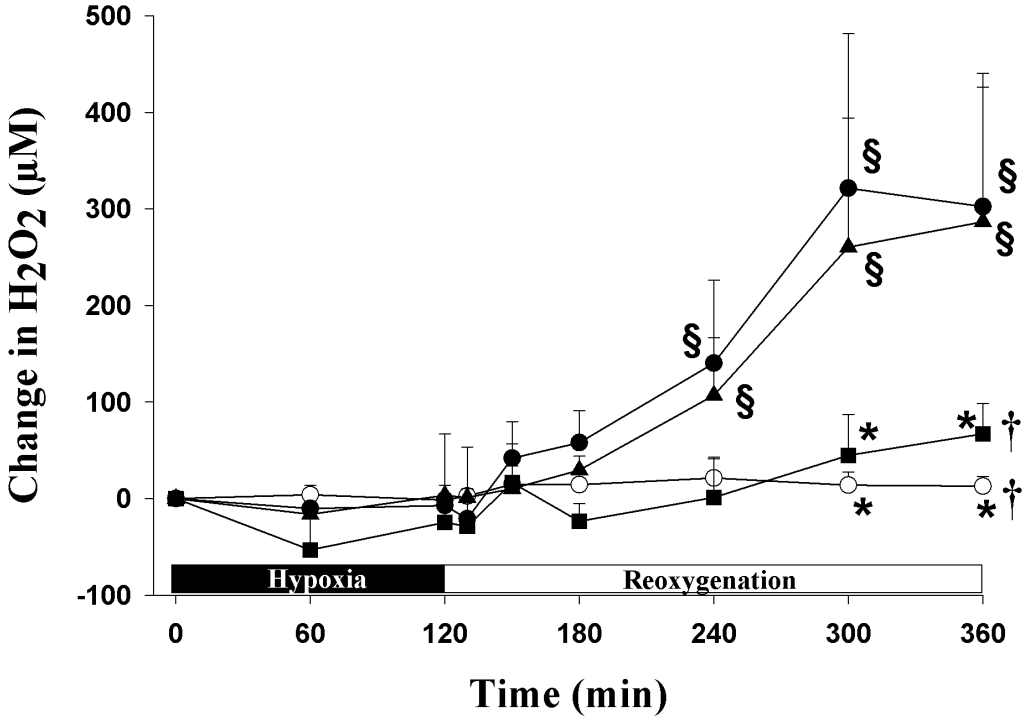


Figure 8-3. Representative western blots and levels of cytochrome-c (15 kDa) in brain cortical tissue of hypoxic piglets after hypoxia-reoxygenation, which received either saline (control) or cyclosporine 2.5 (CSA 2.5) or 10 mg/kg (CSA 10) 5 min after reoxygenation (n=8 each). Sham piglets had no hypoxia and reoxygenation (n=4). $p<0.05$ vs. H-R controls (1-way ANOVA).

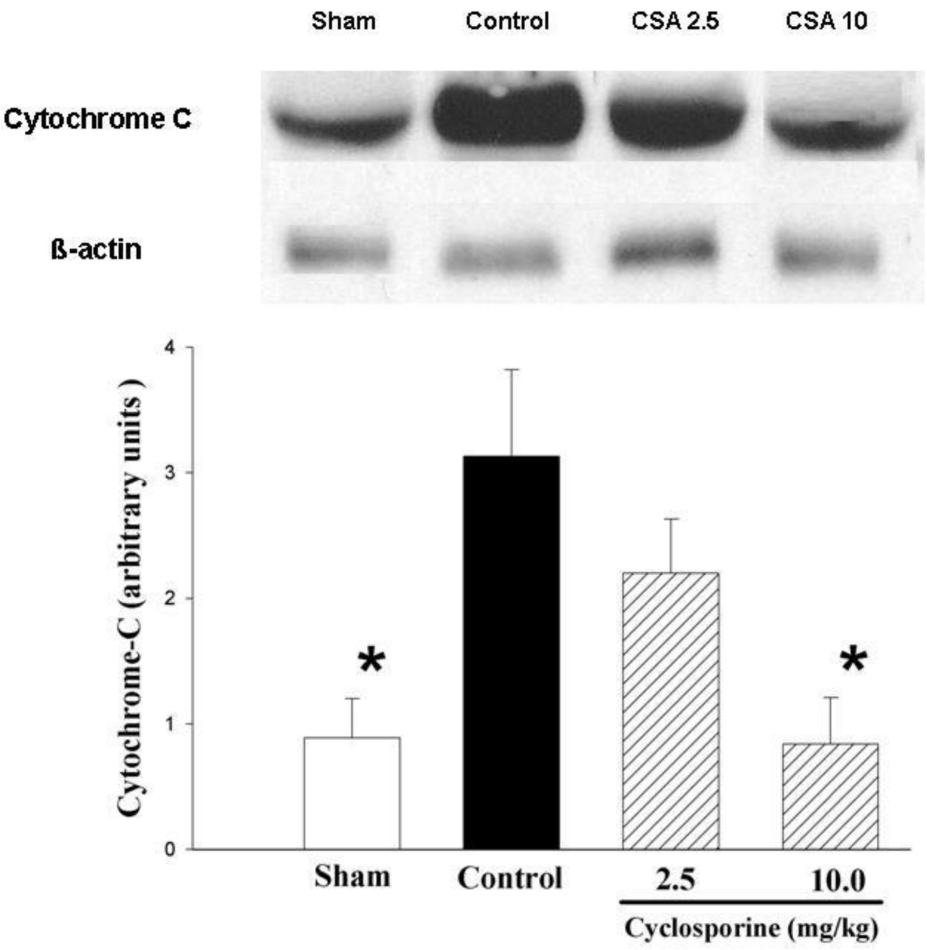
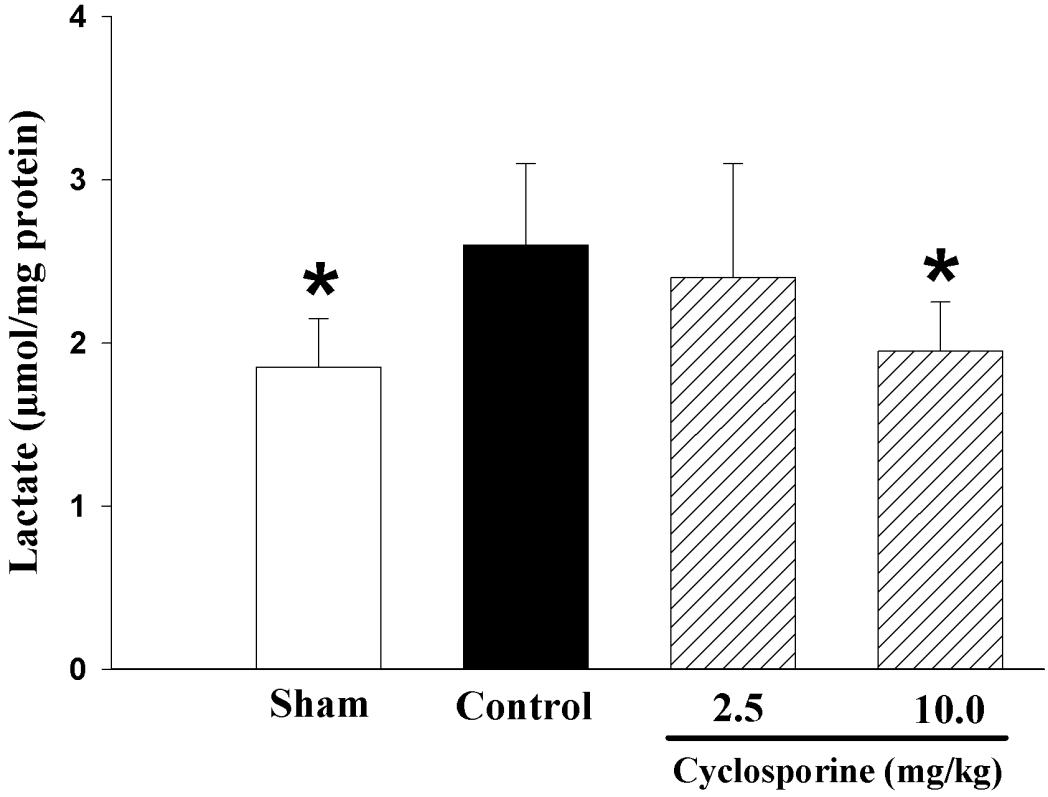


Figure 8-4. Changes in cerebral cortical lactate levels of hypoxic piglets after hypoxia-reoxygenation, which received either saline (control) or cyclosporine 2.5 (CSA 2.5) or 10 mg/kg (CSA 10) 5 min after reoxygenation (n=8 each). Sham piglets had no hypoxia and reoxygenation (n=4). * $p<0.05$ vs. H-R controls (1-way ANOVA).



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Chapter 9

Post-resuscitation Cyclosporine Treatment Attenuates Myocardial Injury and Preserves Cardiac Mitochondrial Integrity in Newborn Piglets with Asphyxia-Reoxygenation

Adapted from:

Gill RS, Lee TF, Manouchehri N, Liu JQ, Lopaschuk G, Bigam DL, Cheung PY. *Post-Resuscitation Cyclosporine Treatment Attenuates Myocardial Injury and Preserves Cardiac Mitochondrial Integrity in Newborn Piglets with Asphyxia-Reoxygenation*. 2012 (submitted).

Abstract

Background

Cardiovascular dysfunction occurs in the majority of asphyxiated neonates and has been suggested to be a major cause of neonatal morbidity and mortality. We previously demonstrated that cyclosporine A treatment during resuscitation can significantly improve cardiovascular performance in asphyxiated newborn piglets. However, the mechanisms through which cyclosporine elicits its protective effect in neonates have not yet been fully characterized. We hypothesize that cyclosporine A treatment will attenuate myocardial injury and preserve cardiac mitochondrial integrity during the resuscitation of asphyxiated newborn piglets.

Methods

After acute instrumentation, piglets received normocapnic alveolar hypoxia (10–15% oxygen) for 2h followed by reoxygenation with 100% (0.5h), then 21% (3.5h) oxygen. At 4h of reoxygenation, plasma troponin, left ventricle myocardial levels of lipid hydroperoxides, ATP, AMP, cytochrome-c and mitochondrial aconitase activity were determined. Piglets were randomized to receive an intravenous bolus of cyclosporine A (10 mg/kg) or normal saline (placebo, control) at 5 mins of reoxygenation (n = 8/group). Sham-operated piglets (n=8) underwent no asphyxia-reoxygenation.

Results

Asphyxiated piglets treated with cyclosporine had lower plasma troponin and myocardial lipid hydroperoxides levels (vs. controls, both $P<0.05$,

ANOVA). Cyclosporine treatment also improved mitochondrial aconitase activity and cardioenergetics, and attenuated the rise in cytosol cytochrome-c (vs. controls, all $P<0.05$). The improved mitochondrial integrity significantly correlated with cardiac output ($P<0.05$, Spearman test),

Conclusions

We demonstrate that the post-resuscitation administration of cyclosporine attenuates myocardial injury and preserves cardiac mitochondrial integrity in asphyxiated newborn piglets following resuscitation.

Introduction

Perinatal asphyxia occurs in approximately 3% of neonates worldwide and contributes to over one millions deaths annually (1). Cardiovascular dysfunction is estimated to occur in 50% to 80% of asphyxiated neonates (2). Generally, myocardial injury may present as decreased cardiac output, decreased stroke volume, or altered contractility (3-5). Compared to adult myocardium, the immature myocardium of neonates is at increased risk to dysfunction, secondary to a greater volume of non-contractile tissue, limited ability to augment stroke volume and increased susceptibility to apoptosis (6,7).

Mitochondria are key energy providers for myocardial contractility, and as such any mitochondrial dysfunction may lead to cardiac functional impairment. Recently, substantial evidence indicates that cardiac mitochondria also serve as critical mediators of apoptotic and necrotic cell signaling pathways following ischemia-reperfusion (8,9). It has been proposed that the formation of the mitochondrial permeability transition pore (MPTP) plays a key role in myocardial reperfusion injury (10,11). The MPTP is a non-specific pore that is proposed to form under conditions of mitochondrial calcium overload, oxidative stress, mitochondrial depolarization, and adenine nucleotide depletion (12,13). These same conditions are typically present during reperfusion subsequent to an ischemic event (12-14) Opening of the MPTP has been suggested to lead to

uncoupling of oxidative phosphorylation, ATP depletion, and eventual cell death (9, 15, 16).

Cyclosporine A has been shown to be a potent inhibitor of MPTP formation during myocardial reperfusion following ischemia (17). Cyclosporine A binds to cyclophilin-D, a key protein in the formation of the MPTP (18), presumably leading to attenuated myocardial injury following ischemia-reperfusion. In addition, cyclosporine treatment has been reported to protect cardiomyocytes in in-vivo adult animal models (17,19,20). However, the potential for cyclosporine treatment to mitigate myocardial and cardiac mitochondrial injury following resuscitation of asphyxiated neonates has yet to be explored. Previously, we demonstrated that post-resuscitation cyclosporine treatment causes a dose-related improvement in cardiac output, stroke volume and systemic oxygen delivery in asphyxiated newborn piglets (21). Therefore, our objectives were to further examine the beneficial effects of cyclosporine treatment on myocardial injury and mitochondrial integrity in a neonatal swine model of asphyxia-reoxygenation. We hypothesized that post-resuscitation CsA administration would attenuate myocardial injury and preserve mitochondrial integrity of asphyxiated newborn piglets.

Methods

All experiments were conducted in accordance with the guidelines and approval of the Animal Care and Use Committee (Health Science),

University of Alberta. Twenty-four newborn mixed breed piglets 1 to 4 days of age weighing 1.4 to 2.5 kg were obtained on the day of experimentation from a local farm.

Experimental Protocol

The experimental animal preparation of this nested study has been previously described (21). Piglets were block-randomized into 2 experimental groups (n=8 per group) that underwent hypoxia-reoxygenation (H-R) in a blinded fashion. A third sham-operated group of piglets (n=8) underwent complete instrumentation without H-R or delivery of medications.

In the H-R groups, hypoxemia was induced via normocapnic alveolar hypoxia. These piglets were ventilated with a FiO_2 of 0.10 to 0.15 by increasing the inhaled concentration of nitrogen gas relative to oxygen for 2h, aiming for arterial oxygen saturations of 30 to 40%. It has been shown in previous studies that this degree of hypoxemia in the newborn piglet model produces clinical asphyxia with severe metabolic acidosis and systemic hypotension (22,23). This was followed by reoxygenation with 100% oxygen for 0.5 h and then 21% oxygen for 3.5 h. At 5 min of reoxygenation, piglets received, in a blinded treatment manner, either cyclosporine as an intravenous bolus (10 mg/kg) or saline (placebo, control). cyclosporine treatment was given at 5 min reoxygenation to simulate the clinical setting, in which intravenous access would be obtained in the neonate prior to

administering resuscitative medications. Cyclosporine A dosing of 10 mg/kg was used as it has been shown to improve cardiac function significantly in our previous study (21). Blinding was maintained by reconstituting cyclosporine and normal saline in a standard volume (5ml) immediately before administration. The medication was given intravenously over 2 min. A laboratory technician uninvolved in the experiment prepared the medications. Cyclosporine was diluted with physiological saline from stock solution (50 mg/ml).

At the end of experiment (6h), piglets were euthanized with an overdose of pentobarbital (100 mg/kg). Samples of left ventricle myocardium were harvested, snap frozen in liquid nitrogen and stored in -80^{o}C for biochemical analysis.

Determination of Troponin

Plasma cardiac troponin I concentration was measured using a commercially available ELISA kit (Life Diagnostics, #2010-4-HS). Plasma levels were determined at baseline and at the end of the experiment.

Myocardial Lipid Peroxidation

Myocardial tissue was homogenized in 0.1 M Phosphate buffer saline (pH 7.4). Lipid hydroperoxides (LPO) were then extracted with chloroform. LPO was then determined by a LPO assay kit (Cayman, MI, USA) with spectrophotometry at 500nm.

Cytochrome-c Western blot analysis

Cytochrome-c released from within the mitochondria into the cellular cytosol was used as an early indicator of mitochondria injury. Using a polytron tissue grinder, the myocardial tissue (~200 mg) obtained was homogenized with 250 μL cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM NaPP, 250 mM sucrose, and 1 mM dithiothreitol) supplemented with complete protease inhibitor (Roche Diagnostic GmbH, Germany). The homogenates were centrifuged at 5,000g for 10 min. Protein extract (50 μg) was mixed with loading buffer and denatured by boiling for 5 min before loading on to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After electrophoresis, proteins were transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad), and the latter were then blocked with 10% fat-free dry milk in Tween-Tris-buffer saline (TTBS) for 30 min at room temperature. After brief washing with TTBS, the membrane was incubated with a polyclonal antibody to cytochrome-c (dilution 1:1,000; Biovision) overnight at 4°C cold room. After washing four times (5 min per wash) with TTBS to remove the unbound antibody, the membrane was then incubated in TTBS for 1 h at room temperature with horseradish peroxidase conjugated to goat anti-rabbit IgG (dilution 1:1,000; EY Laboratories). Membranes were then washed four times with TTBS. Bound proteins were detected using chemiluminescence reagents (ECL plus; Amersham Biosciences) and

visualized by exposing the membranes to x-ray film (Biomax MR, Kodak Photo Film). The film was developed by a Kodak X-OMAT 1000A processor (Eastman Kodak Company). The x-ray films were scanned using a PowerLook 1000 scanner (UMAX), and bands were analyzed using Quantity One 1-D analysis software (Bio-Rad). The integrated areas of bands were determined and expressed as a percentage of a standard sample ran on the same membrane. The amount of protein to be used for detection was normalized using the ß-actin as loading control.

Mitochondrial Aconitase Activity Assay

Mitochondrial functional activity was estimated by the mitochondrial enzyme aconitase, which is an important enzyme in Kreb's cycle. Myocardial tissue was homogenized in the aconitase assay buffer and centrifuged at 700g for 10 min at 4^{o}C. The supernatant was then centrifuged at 10, 000 x g for 30 min at 4^{o}C. The mitochondrial pellet was then resuspended in assay buffer. Activity was determined by an Aconitase assay kit (Cayman, MI, USA) with spectrophotometry at 340nm. The protein content was determined using a bicinchoninic acid assay kit (Sigma).

Mitochondrial Energetics

Myocardial tissue was homogenized in 6% perchloric acid and 0.5 mM EGTA. Samples were then centrifuged at 12,000 g for 5 min at 4^{o}C. The supernatant was then placed in autosampler vials and pH was adjusted to 5-7

with 5M K_2CO_3. Samples were then centrifuged at 10, 000 g for 2 min at 4°C, and the supernatant was transferred to a clean autosample vial. 10 µL of sample was subjected to ultra-performance liquid chromatography as previously described (24). Adenosine triphosphate (ATP) and adenosine monophosphate (AMP) levels were then calculated.

Caspase-3 Activation

Myocardial tissue was homogenized in 0.1 M Phosphate buffer saline (pH 7.4). Caspase-3 activation was then determined by Capase-3 Fluorescence assay kit (Cayman, MI, USA) with spectrophotometry at 485nm and 535nm. The protein content was determined using a bicinchoninic acid assay kit (Sigma).

Apoptosis-inducing Factor

Using polytron tissue grinder, part of the left ventricle (~100 mg) was homogenized with 250 µL cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM NaPP, 250 mM sucrose, and 1 mM dithiothreitol) supplemented with Complete protease inhibitor (Roche Diagnostic GmbH, Germany). The homogenates were centrifuged at 5,000g for 10 min. Protein extract (50 µg) was mixed with loading buffer and denatured by boiling for 5 min before loading on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After electrophoresis, proteins were transferred to a 0.2-µm polyvinylidene difluoride membrane

(Bio-Rad), and the latter were then blocked with 10% fat-free dry milk in Tween-Tris-buffer saline (TTBS) for 30 min at room temperature. After brief washing with TTBS, the membrane was incubated with a polyclonal antibody to apoptosis-inducing factor (dilution 1:1,000; Cell Signal Technology) overnight at 4°C cold room. After washing four times (5 min per wash) with TTBS to remove the unbound antibody, the membrane was then incubated in TTBS for 1 h at room temperature with horseradish peroxidase conjugated to goat anti-rabbit IgG (dilution 1:1,000; EY Laboratories). Membranes were washed four times again with TTBS. Bound proteins were detected using chemiluminescence reagents (ECL plus; Amersham Biosciences) and visualized by exposing to x-ray film (Biomax MR, Kodak Photo Film). The film was developed by Kodak X-OMAT 1000A processor (Eastman Kodak Company). The x-ray films were scanned using PowerLook 1000 scanner (UMAX) and bands were analyzed using Quantity One 1-D analysis software (Bio-Rad). The integrated areas of bands were determined and expressed as a percentage of a standard sample ran on the same membrane. The amount of protein to be used for detection was normalized using the ß-actin as loading control.

Statistical Analysis

Results are expressed as mean ± the standard error of the mean. Biochemical and mitochondrial integrity markers were analyzed by two-way repeated measures ANOVA or one-way ANOVA as appropriate. If the

normality test failed, ANOVA on ranks (Kruskal-Wallis) was performed. Post hoc testing with Tukey or Dunnett methods was used as appropriate. Spearman rank order correlation was used. Significance was defined as $p<0.05$.

Results

The piglets used in this study were 1 to 4 days old, with no significant differences in body weight and age among groups. There were also no significant differences between groups at normoxic baseline in hemodynamic variables and pH. Severe normocapnic alveolar hypoxia resulted in cardiogenic shock and metabolic acidosis within the H-R groups, with decreased cardiac output to 44±4% of normoxic baseline and pH 7.04±0.02 at 2h ($p<0.05$ vs. sham-operated piglets). Compared with the H-R control group, post-resuscitation cyclosporine treatment significantly improved cardiac output and systemic oxygen delivery in asphyxiated newborn piglets (21).

Markers of Myocardial Cellular Injury

Plasma cardiac troponin I levels were not different among groups at the beginning of experiment (Figure 9-1). At the end of reoxygenation, the plasma troponin I levels of both H-R groups were significantly higher than that of sham-operated group, which was not different from baseline.

cyclosporine treatment attenuated the rise in plasma troponin levels compared to the control piglets ($p<0.05$)(Figure 9-1).

At the end of experiment, the left ventricle myocardial LPO levels, a marker of oxidative damage, were significantly higher in control piglets than that of cyclosporine-treated and sham-operated piglets, which were not different (Figure 9-2).

Markers of Mitochondrial Functional Integrity

The cytochrome-c levels in the left ventricle of H-R controls were higher than that of the sham-operated group by the end of reoxygenation ($p<0.05$) and was significantly reduced in piglets treated with cyclosporine (Figure 9-3). Control piglets had significantly lower myocardial aconitase activity than those of cyclosporine-treated and sham-operated piglets, which were not different ($p<0.05$)(Figure 9-4). Left ventricle myocardial ATP levels were significantly greater in cyclosporine-treated piglets compared to controls (Figure 9-5A). AMP levels were lower in cyclosporine-treated piglets than in control piglets, although this difference was not significant (Figure 9-5B). In contrast, the myocardial ATP/AMP ratio was significantly greater in cyclosporine-treated piglets compared to controls (Figure 9-5C).

In the left ventricle, cytochrome-c levels were significantly correlated with ATP levels and ATP/AMP ratio ($r=-0.6$ and -0.5, respectively). There were modest, non-significant correlations between ATP/AMP ratio and

aconitase activity and between left ATP levels and plasma troponin levels (r=0.4 and r=-0.4, respectively, both p=0.08). Cardiac output was significantly correlated with plasma troponin levels, myocardial aconitase activity and LPO levels (r=-0.6, 0.5 and -0.5, respectively), but not with the cytochrome-c and ATP levels and ATP/AMP ratio.

Marker of Apoptosis

Left ventricle caspase-3 activation at the end of the 6h experiment was similar among shams and H-R piglets (P>0.05). Casapse-3 activation among H-R piglets was similar between controls and those treated with cyclosporine following 2h of hypoxia and 4h of reoxygenation (control = 5.7±2.0 units/mg protein vs. cyclosporine-treated = 5.0±2.1 units/mg protein, p>0.05). The myocardial tissue content of apoptosis-inducible factor was similar at the end of 6h experiment among shams and H-R piglets (data not shown).

Discussion

In this study we showed that post-resuscitation administration of cyclosporine in asphyxiated newborn piglets attenuates myocardial cellular injury, as demonstrated by reduced plasma troponin I and left ventricle LPO levels. The cardioprotective effect of cyclosporine A treatment may be related to its preservation of mitochondrial integrity as evidenced by attenuated

cytosolic cytochrome-c release and preserved aconitase enzymatic activity with improved cellular energetics (ATP/AMP ratio).

Despite multiple adult animal models of ischemia-reperfusion with cyclosporine treatment, there is a paucity of studies on cyclosporine using newborn animal models of H-R. Adult animal models have demonstrated a reduction of myocardial infarct size with cyclosporine treatment following ischemia-reperfusion (17,19,20). However, these models used isolated coronary occlusion to induce ischemia, with subsequent removal of the obstruction to allow reperfusion. This isolated coronary arterial occlusion does not simulate the global myocardial ischemia observed in asphyxiated neonates during hypoxia, nor the global myocardial reperfusion injury during reoxygenation/resuscitation. Furthermore, the neonatal myocardium has been shown to be more susceptible to apoptosis during reperfusion compared to their adult counterparts (6). Interestingly, Liu et al recently demonstrated age-associated differences in the inhibition of MPTP opening by cyclosporine, leading to increased cardioprotection in young (3-5 months) rats compared to old (20-24 months) rats (25). Our study is the first to simulate the clinical setting of newborn asphyxia-reoxygenation using a large animal model and testing the potential protective effects of post-resuscitation cyclosporine treatment.

We previously demonstrated that cyclosporine treatment (at 10 mg/kg) improved cardiac index and lowered plasma troponin levels of asphyxiated newborn piglets (21). We speculated that cardiac function and

injury are related to mitochondrial health of cardiomyocytes. Specifically, cardiac mitochondria have previously been shown to be a key source of reactive oxygen species (ROS) during reoxygenation/reperfusion (26). Furthermore, ROS generation may lead to increased calcium loading of the mitochondria, and further ROS production (27). Excess production of ROS can overwhelm endogenous antioxidant defense systems and lead to damage of lipid membranes (28). Alternately, *in vitro* experiments have suggested that Ca^{2+}accumulation may be reduced by inhibiting the MPTP (29). Supporting this concept, Batandier et al observed increased ROS production in mitochondria in which MPTP formation occurred (30). Similarly, we observed lower left ventricular LPO levels, a marker of oxidative stress, in the cyclosporine-treated piglets after H-R. The protection of cyclosporine in cardiomyocytes against H-R may be secondary to the attenuation of oxidative stress and the preservation of mitochondria through MPTP inhibition.

We speculate that cyclosporine treatment exerts its protective effects by maintaining mitochondrial integrity. *In vitro* CsA has been shown to preserve mitochondrial integrity by binding to cyclophilin D and preventing MPTP opening (18). Supporting the findings by Xu et al in neonatal cardiomyocytes (29), we also observed attenuated cytosolic cytochrome-c in the left ventricle of cyclosporine-treated piglets, which is typically released from the mitochondria through MPTP opening. Furthermore we observed an increase of myocardial aconitase activity of cyclosporine-treated piglets compared to controls. Aconitase activity has been previously shown in *in*

vitro experiments to decline during cardiac reperfusion (31). Aconitase is known to be susceptible to inactivation by ROS, based on *in vitro* experiments (32,33) These findings thus suggest that cyclosporine A treatment may maintain mitochondrial integrity in the newborn piglets with attenuated cytochrome-c release and preserved aconitase activity.

Left ventricle ATP levels and ATP/AMP ratio were significantly higher in asphyxiated newborn piglets treated with cyclosporine A than those of control piglets. Griffiths et al observed similar improvements in cardiac energetics in isolated rat hearts following ischemia-reperfusion (34). These authors reported recovery of the ATP/ADP ratio following 30 min of ischemia and 15 min of reperfusion to pre-ischemic values with cyclosporine treatment. However, ATP levels only partially recovered with the short period of reperfusion. This may be related to loss of total adenine nucleotides, which occurs during ischemia (35). Interestingly, in our newborn piglets treated with cyclosporine A, myocardial ATP levels and the ATP/AMP ratio were greater than controls and sham-operated piglets following 4h of reperfusion/reoxygenation. We speculate that this may be related to an appropriate increase in ATP levels following significant myocardial stress. The depressed myocardial ATP levels and elevated myocardial AMP levels in control piglets may represent uncoupling of cardioenergetics in injured and failing myocardium. Depressed myocardial ATP levels have previously been reported in failing myocardium secondary to dilated cardiomyopathy (36). Nonetheless, the improved cardiac

energetics further support preserved mitochondrial integrity, attenuated cardiomyocyte injury and improved cardiac function.

Our study has a few important limitations. Firstly, the duration of the experiment is 6h, thus our findings represent cardiac and mitochondrial injury in the early phase of reperfusion. As such, we did not observe an increase in markers of apoptosis such as caspase-3. This was not unexpected, as in previous experiments using this newborn piglet model, elevation of activated caspase-3 was observed after 48h of reoxygenation. Therefore, it is currently unknown if the protective effects of CsA treatment will persist. Secondly, despite the observations of attenuated myocardial injury and preserved cardiac mitochondrial integrity in the newborn piglets, we did not directly observe MPTP formation. Instead, we used surrogate markers of mitochondrial integrity and oxidative stress to assess the protective effects of cyclosporine A on the neonatal heart. However, previous *in vitro* experiments provide strong evidence for the primary mechanism of cyclosporine action in ischemia-reperfusion myocardial injury. Thirdly, though newborn piglets have been shown to similar to human newborn infants in anatomy and physiology, it remains challenge to translate findings from animal studies (37). Lastly, the duration of hypoxia in our experiment is an approximation of the clinical setting based on personal observations. However, has been previously shown to produce clinically relevant asphyxia (21-23).

In conclusion, this is the first study to demonstrate that cyclosporine A treatment during resuscitation of asphyxiated newborn piglets attenuates myocardial injury and preserves cardiac mitochondrial integrity.

Figure 9-1. Marker of myocardial injury. Plasma troponin I concentration in piglets at start of experiment (0 min) and after 2h hypoxia and 4h reoxygenation (360 min). Cyclosporine treated piglets received 10 mg/kg of intravenous cyclosporine A. Control piglets received no cyclosporine and sham-operated piglets underwent no hypoxia-reoxygenation. (n=8 in each group)*P<0.05 vs. control piglet group.

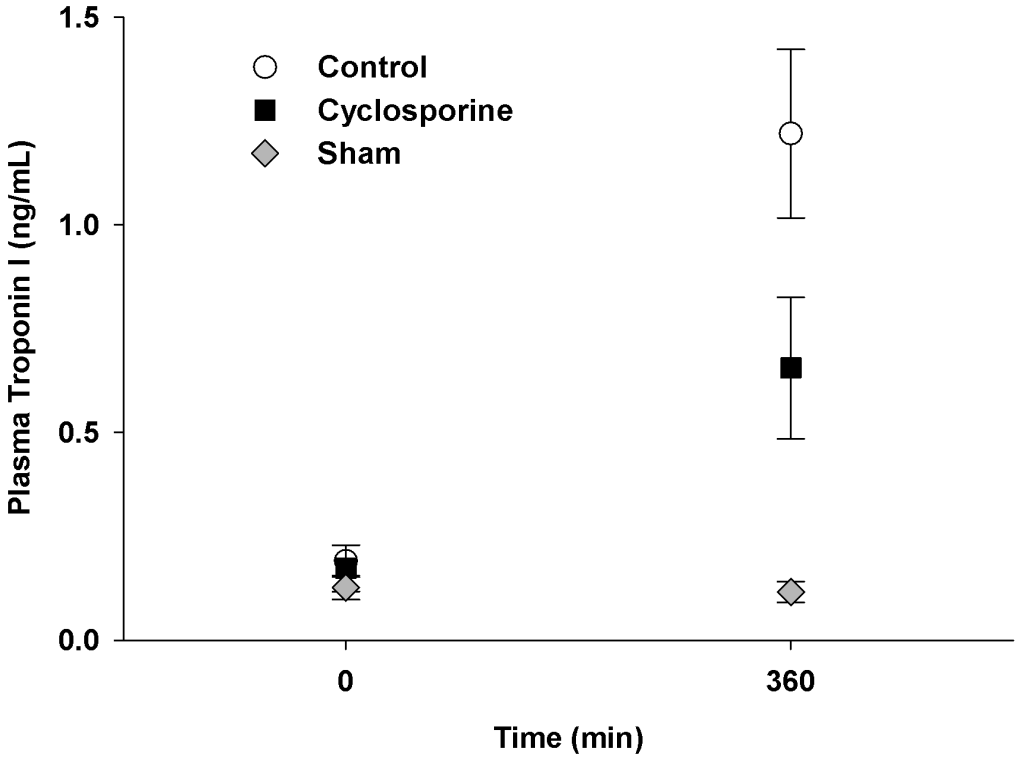


Figure 9-2. Marker of oxidative stress. Left ventricle lipid peroxidation levels following 2h of hypoxia and 4h reoxygenation. Cyclosporine treated piglets received 10 mg/kg of intravenous cyclosporine A. Control piglets received no cyclosporine and sham-operated piglets underwent no hypoxia-reoxygenation. (n=8 in each group)*P<0.05 vs. control piglet group.

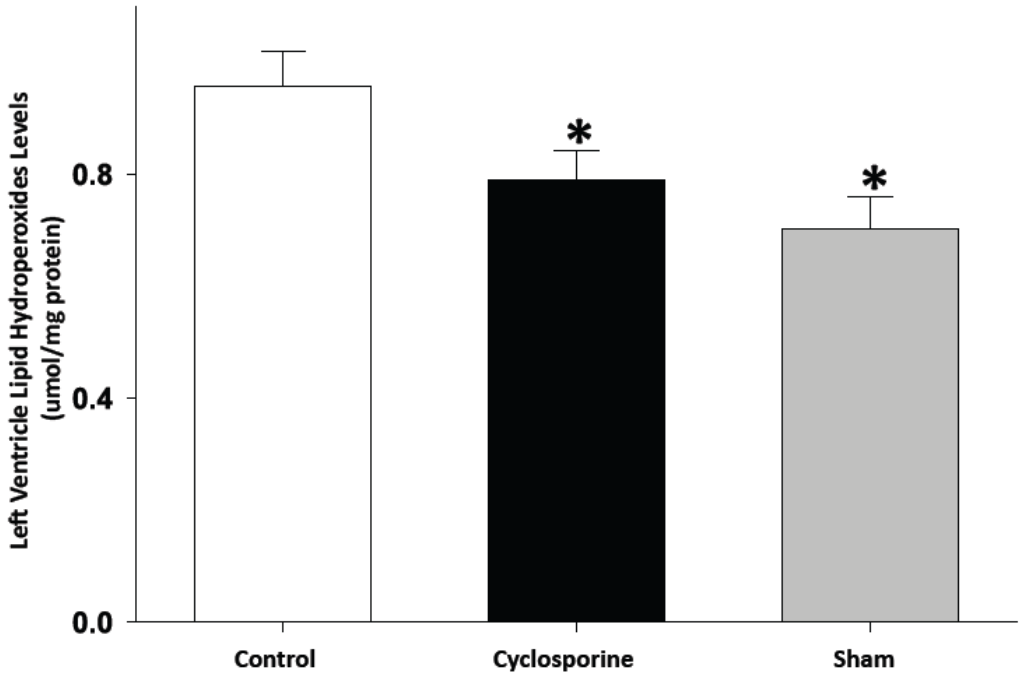


Figure 9-3. Cardiac mitochondria, cytosol cytochrome-C levels following 2h hypoxia and 4h reoxygenation. Cyclosporine treated piglets received 10 mg/kg of intravenous cyclosporine A. Control piglets received no cyclosporine and sham-operated piglets underwent no hypoxia-reoxygenation. (n=8 in each group)*P<0.05 vs. control piglet group.

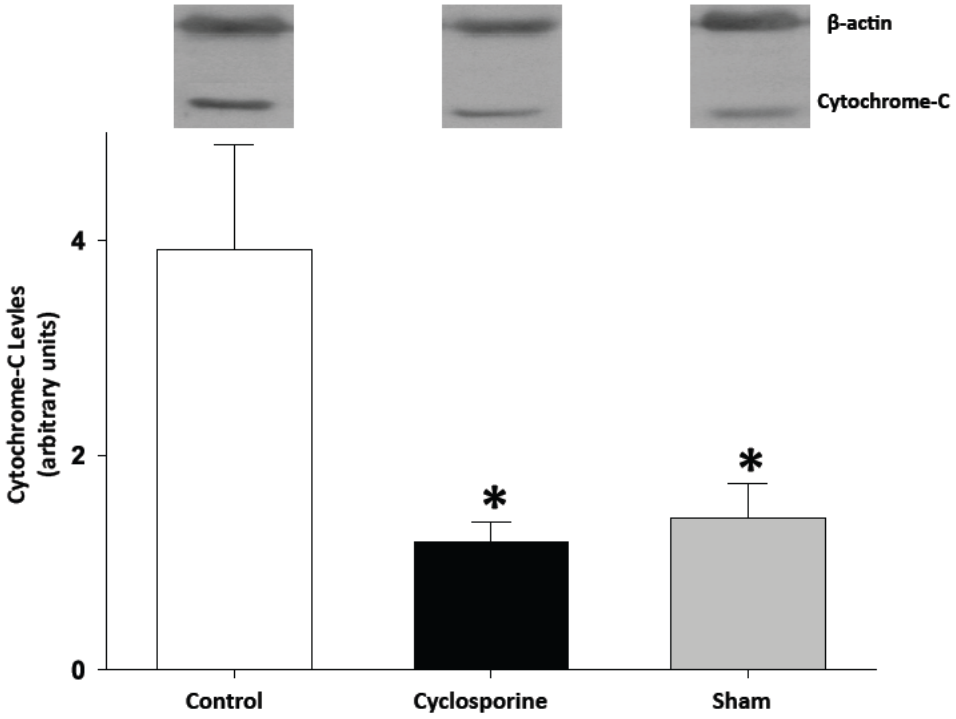


Figure 9-4. Cardiac mitochondria, aconitase activity following 2h hypoxia and 4h reoxygenation. Cyclosporine treated piglets received 10 mg/kg of intravenous cyclosporine A. Control piglets received no cyclosporine and sham-operated piglets underwent no hypoxia-reoxygenation. (n=8 in each group)*P<0.05 vs. control piglet group.

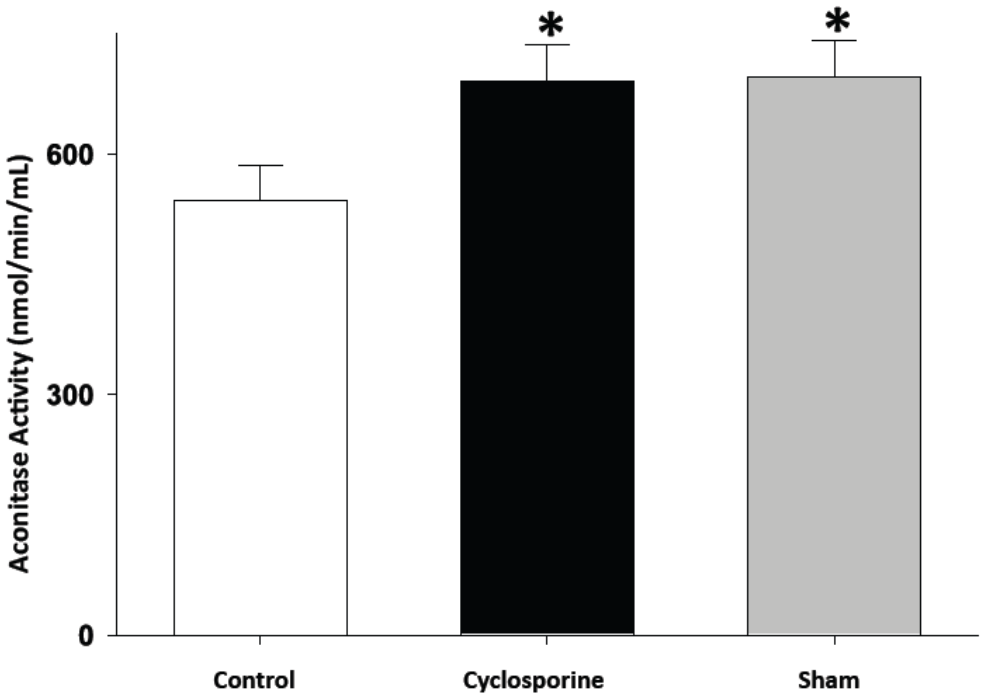


Figure 9-5. Left ventricular energetics. (A) Adenosine triphosphate (ATP) levels (B) Adenosine monophosphate (AMP) levels (C) ATP/ATP Ratio following 2h hypoxia and 4h reoxygenation. Cyclosporine treated piglets received 10 mg/kg of intravenous cyclosporine A. Control piglets received no cyclosporine and sham-operated piglets underwent no hypoxia-reoxygenation. (n=8 in each group) *P<0.05 vs. control piglet group.

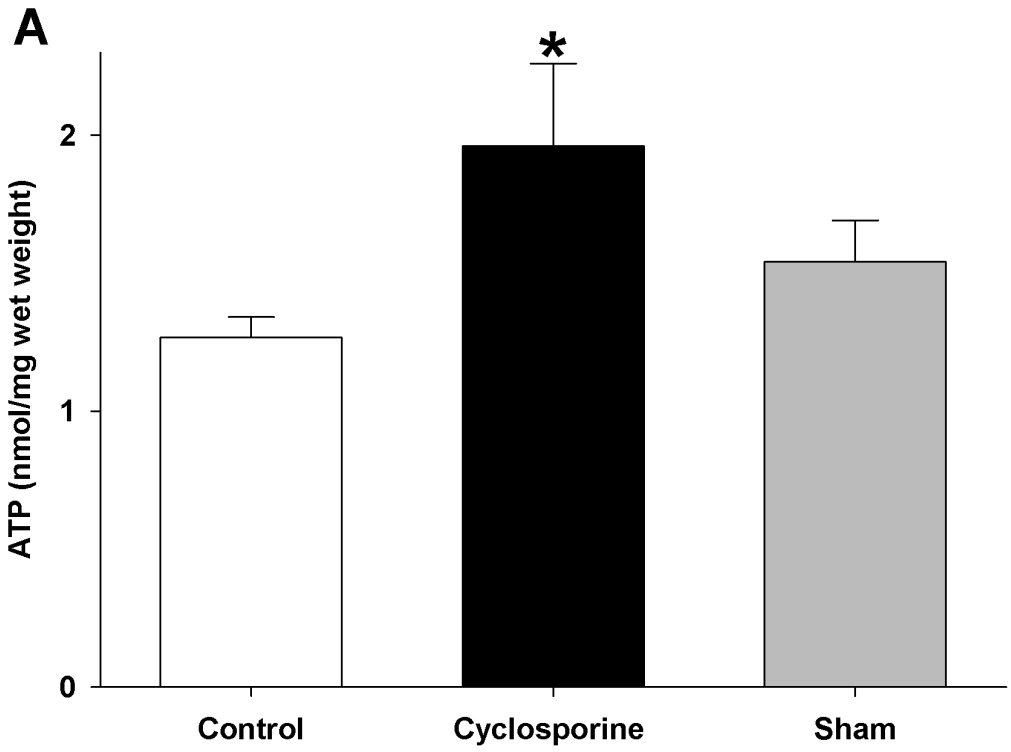
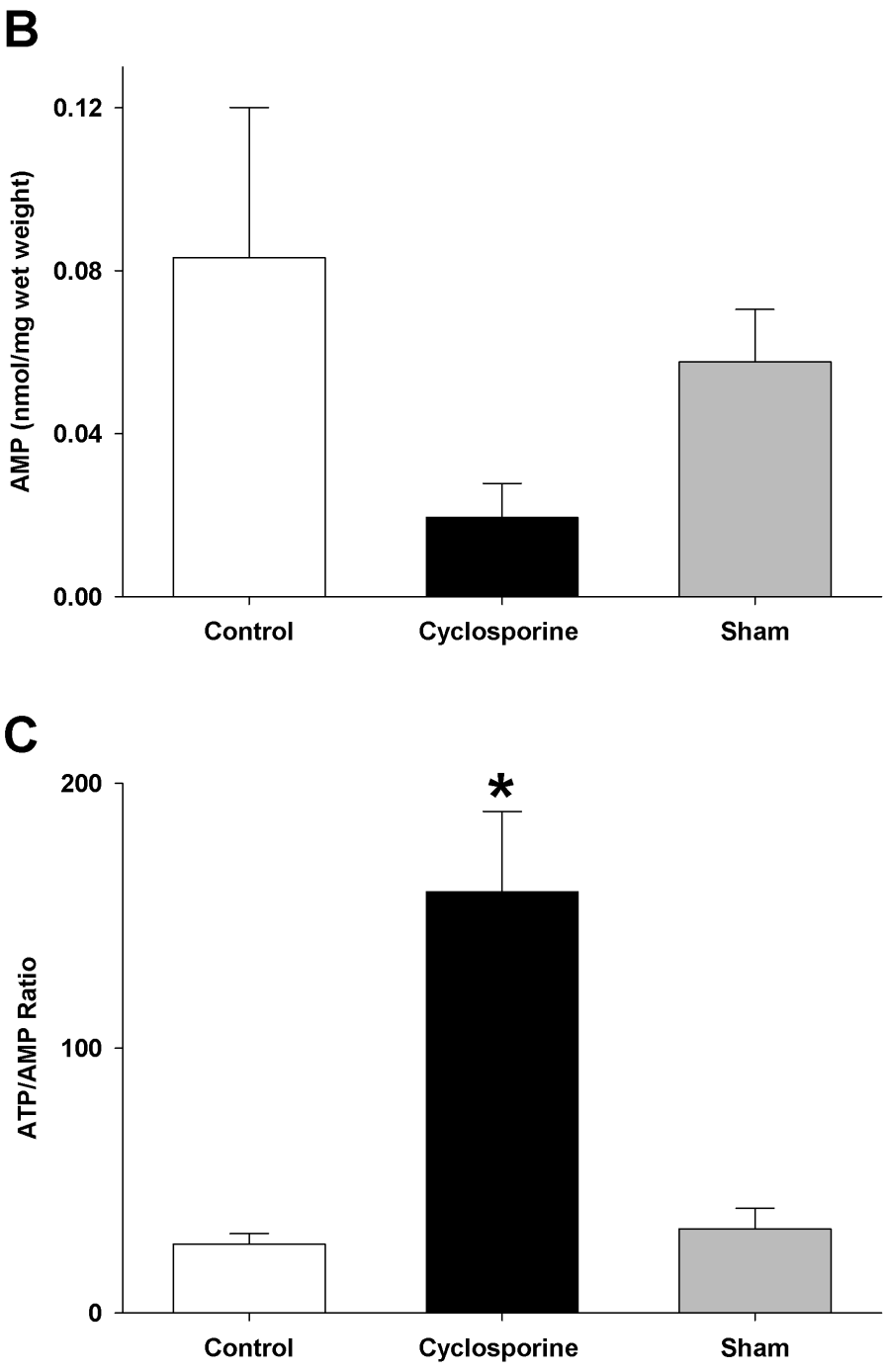


Figure 9-5 continued. Left ventricular energetics. (A) Adenosine triphosphate (ATP) levels (B) Adenosine monophosphate (AMP) levels (C) ATP/ATP Ratio following 2h hypoxia and 4h reoxygenation. Cyclosporine treated piglets received 10 mg/kg of intravenous cyclosporine A. Control piglets received no cyclosporine and sham-operated piglets underwent no hypoxia-reoxygenation. (n=8 in each group) *P<0.05 vs. control piglet group.



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Chapter 10

Pharmacokinetic Characterization of Intravenous Cyclosporine Treatment for Cardioprotection during Resuscitation of Asphyxiated Newborn Piglets

Adapted from:

Gill RS, Brocks DR, Churchill T, Lee TF, Bigam DL, Cheung PY. *Pharmacokinetic Characterization of Intravenous Cyclosporine Treatment for Cardioprotection during Resuscitation of Asphyxiated Newborn Piglets*. Pediatr Crit Care Med 2012 (In Revision).

Abstract

Background

Cyclosporine treatment, as a single intravenous bolus, during resuscitation has been shown to attenuate myocardial injury in asphyxiated newborn piglets. However, the pharmacokinetics of cyclosporine treatment for cardioprotection in newborn piglets has not been studied. Our objectives were to assess the pharmacokinetics of a single intravenous cyclosporine treatment during resuscitation of asphyxiated newborn piglets and compare these parameters to healthy newborn piglets.

Methods

Thirty-two piglets (1-4 days-old) were acutely instrumented. Following stabilization, normocapnic alveolar hypoxia was instituted for 2h followed by 4h of reoxygenation. Piglets were block-randomized to receive a single intravenous bolus of cyclosporine at 2.5, 10 or 25 mg/kg (n=8/group). An addition group of piglets (n=8) underwent no hypoxia-reoxygenation, however received cyclosporine at 10 mg/kg. Plasma troponin levels were determined. High-performance liquid chromatography (HPLC) was utilized to determine plasma cyclosporine concentrations at various time points during 4h of reoxygenation. Noncompartmental methods were used to calculate the pharmacokinetic parameters. Cyclosporine concentrations and pharmokinetics parameters were analyzed by one-way ANOVA.

Results

Severe normocapnic hypoxia resulted in cardiogenic shock and hypotension, with at 2h, with similar hemodynamic recovery at 240 min of reoxygenation. The plasma $AUC_{0\text{-}4h}$ was significantly greater in the cyclosporine 25 mg/kg treated group compared to 2.5 and 10 mg/kg treated groups (both $P<0.001$). Plasma $AUC_{0\text{-}4h}$ and C_{max} in piglets treated with cyclosporine at 25 mg/kg was associated with increased plasma troponin levels, a marker of myocardial injury, relative to piglets treated with 2.5 and 10 mg/kg. There was no difference in pharmacokinetic parameters between healthy and asphyxiated piglets treated with cyclosporine.

Conclusions

This is the first study to demonstrate the pharmacokinetics of cyclosporine treatment during resuscitation of asphyxiated newborn piglets. In addition, cyclosporine exposure following treatment at 2.5 and 10 mg/kg is associated with decreased myocardial injury.

Introduction

According to the World Health Organization, asphyxia accounts for 23% of all newborn deaths worldwide annually (1). Asphyxiated newborns may present with cardiac dysfunction in over 50% cases (2). Furthermore, resuscitation with reoxygenation may lead to generation of reactive oxygen species (ROS) and resultant myocardial injury through apoptotic and necrotic cellular pathways. Recently treatment with intravenous cyclosporine A during reoxygenation of asphyxiated newborn piglets has been shown to improve cardiac function and attenuate myocardial injury compared to placebo-treated controls (3). Nevertheless, cyclosporine has been shown to have a narrow therapeutic index in in-vitro studies. In isolated rat hearts subjected to ischemia-reperfusion, cyclosporine A improved left ventricular recovery at 0.2 μmol/L (4), however the protective effect was abolished at 1.0 μmol/L. Interestingly, Karlsson et al also observed a lack of protective effect of cyclosporine at 10 mg/kg in adult swine subjected to ischemia-reperfusion (5). These authors speculated that these results might have been related to high plasma concentrations of cyclosporine in these adult swine, however no pharmacokinetic characterization was performed.

Cyclosporine A pharmacokinetic characterization has been studied in adult animal models and human clinical studies for its use in solid-organ transplant immunosuppression (6, 7). However, the pharmacokinetic characteristics of a single intravenous cyclosporine treatment for

cardioprotection in asphyxiated newborns have not been assessed. In addition, Liu et al recently reported that the protective effects of cyclosporine might be age-related (8). This suggests that the pharmacokinetics may also be different among different age groups. Prior to progressing to a clinical trial, further understanding of the effects of administrating cyclosporine A for resuscitation in asphyxiated newborns is needed. The purpose of this study was three-fold. Firstly to describe the pharmacokinetics of a single intravenous cyclosporine treatment during resuscitation of asphyxiated newborn piglets. Secondly, we intended to explore the association between cyclosporine exposure and myocardial injury in these piglets. Lastly, we aimed to compare the pharmacokinetic profiles of intravenous cyclosporine administered in healthy and asphyxiated newborn piglets. We hypothesize that the pharmacokinetics of intravenous cyclosporine will be similar in healthy and asphyxiated newborn piglets.

Methods

All experiments were conducted in accordance with the guidelines and approval of the Animal Care and Use Committee (Health Science), University of Alberta. Thirty newborn mixed breed piglets 1 to 4 days of age (1.5-2.5 kg) were obtained on the day of experimentation from a local farm.

Experimental Protocol and Medication

Animal preparation has been previously described (3). Piglets were block-randomized to 3 groups (n=8 per group) that underwent hypoxia-reoxygenation (H-R) (3). A fourth sham-operated group (n=6) underwent the same duration of experimentation without H-R.

As we have previously reported, in the 3 H-R groups, hypoxemia was induced via normocapnic alveolar hypoxia (3). These piglets were ventilated with a FiO_2 of 0.10 to 0.15 by increasing the inhaled concentration of nitrogen gas relative to oxygen for 2h, aiming for arterial oxygen saturations of 30 to 40%. This was followed by reoxygenation with 100% oxygen for 0.5 h and then 21% oxygen for 3.5 h. At 5 min of reoxygenation, piglets received treatment with cyclosporine A as an intravenous bolus at a blinded dose (2.5, 10 or 25 mg/kg).

Blinding was maintained by reconstituting all doses of cyclosporine A and normal saline in a standard volume (5 ml) immediately before administration (9). The medication was given intravenously over 2 min. A laboratory technician uninvolved in the experiment prepared the medications. Cyclosporine A was diluted from stock solution (50 mg/ml) containing ethanol vehicle. Cyclosporine A treatment was given at 5 min reoxygenation to simulate the clinical setting, in which intravenous access is obtained in the neonate prior to administering resuscitative medications. A fourth “healthy” sham-operated group, received cyclosporine A (10 mg/kg)

at an equivalent time point of the total experimental period as the H-R groups (at 125 min).

Determination of Plasma Cyclosporine Concentrations

Arterial blood samples were taken at predetermined intervals: baseline (0 min), 60 and 120 min of hypoxia, 130 (10-min reoxygenation) and 150 min (30-min reoxygenation) reoxygenation with 100% FiO_2, then at 180, 240, 300 and 360 min for reoxygenation with 21% FiO_2. Samples were centrifuged at 15,000 rotations per min (rpm) for 10 min. The supernatant (100 uL) was then collected and frozen at -80 °C for determination of cyclosporine levels. Of the piglets blood volume, less than 5% was collected for blood work.

Determination of plasma cyclosporine A levels was performed by using high-pressure liquid chromatography (HPLC). Cyclosporine D (CsD) was used as a reference standard to calculate CsA levels within plasma samples. 80uL of Cyclosporine D (10 ug/mL) was added to 80uL of the plasma sample. Mixed samples were then centrifuged at 20 000 g for 10 min to remove cellular debris. The supernatant was initially purified through addition and mixing of 200uL HCL (0.18 N) and 900uL ethyl ether. Following this, the supernatant was added to 250uL of NaOH (0.1 N) and extraction was repeated with ethyl ether. The supernatant was then centrifuged under vacuum until dry; 50 µL of acetonitrile:water (80:20) was added to the tubes

to resuspend any CsA in the sample; 20 µl sample was injected into the HPLC system for analysis. Cyclosporine A & D in the samples (and standards) were separated on a Waters Symmetry C18, 250mm column with an HP1050 quaternary pump/autosampler in conjunction with an HP1040 Diode Array Detector. Analysis of the spectra was performed with Chemstation software for the HP1050 setup. Separation was achieved at a column temperature of 75^{o}C, flow rate of 1.0 ml/min and UV detection at 205 nm. Separation was achieved with a method involving: 0 to 12 min with 80:20 ACN:water; 12.1 to 18 min with 100% ACN; 18.1 to 23 with 80:20 ACN:water. Typical retention times for cyclosporine A and D were 9.4 and 12.1 min, respectively. Unknown peak areas were compared to CsA and CsD standards for subsequent calculations.

Pharmacokinetic Analysis

Noncompartmental methods were used to calculate the pharmacokinetic parameters. The terminal elimination rate constant (λz) was calculated by subjecting the plasma concentrations in the terminal phase to linear regression. The terminal phase half-life (t½) was calculated by division of 0.693 by λz. The area under the plasma concentration vs. time curve (AUC) from time of dosing to the last measured time point (AUC_{0-t}), and extrapolated to infinity ($AUC_{0-\infty}$) were calculated using the combined log-linear trapezoidal rule from time 0 h postdose to the time of the last measured concentration, plus the quotient of the last measured

concentration divided by λz. The clearance (CL) was calculated as the quotient of dose to AUC0-∞ and the steady state Vd (Vdss) as CL×AUMC/ AUC0-∞, where AUMC is the area under the first moment plasma concentration vs. time curve, from time of dosing to infinity.

Determination of Troponin

Plasma cardiac troponin I (cTnI) concentration was measured using a commercially available ELISA kit (Life Diagnostics, #2010-4-HS) at the end of 4 h of reoxygenation.

Statistical Analysis

Results are expressed as mean±standard error of mean. Cyclosporine concentration and pharmokinetics parameters were analyzed by one-way ANOVA. Post hoc testing was done where appropriate using Fisher's method. When normality failed, Kruskal-Wallis test was used. Student t-test was used as appropriate for comparisons between two independent groups. Fisher Exact test was used as appropriate for comparisons between categorical variables. Significance was defined as $p<0.05$.

Results

Hypoxia-Reoxygenation Piglets Treated with Cyclosporine A at 2.5, 10 or 25mg/kg

Piglets were 1-4 days old and weighed 1.5 to 2.5 kg, with no significant differences among H-R groups. Severe normocapnic hypoxia resulted in cardiogenic shock and hypotension, with no differences among groups at 2h. All H-R piglets were then reoxygenated and treated with cyclosporine, with similar hemodynamic recovery at 240 min of reoxygenation. However, all H-R piglets treated with cyclosporine had significantly improved hemodynamic recovery compared to placebo-treated controls, as previously reported (3).

All H-R piglets tolerated treatment with cyclosporine. Figure 10-1A depicts the best-fit curves for the mean plasma concentration versus time of reoxygenation for cyclosporine treatment with 2.5, 10 and 25 mg/kg groups, respectively. Figure 10-1B depicts the curves for the mean plasma concentrations versus time of reoxygenation for cyclosporine treatment on a log scale. The results of pharmacokinetic analysis for cyclosporine treatment groups (2.5, 10 and 25 mg/kg) are shown in Table 10-1. The plasma $AUC_{0\text{-}4h}$ was significantly greater in the cyclosporine 25 mg/kg treated group compared to 2.5 and 10 mg/kg treated groups (both $P<0.001$). The plasma $AUC_{0\text{-}4h}$ of the cyclosporine 10 mg/kg group was also significantly greater that the 2.5 mg/kg treatment piglets ($P<0.001$). The CL was significantly lower in the cyclosporine 25 mg/kg group compared to both cyclosporine 2.5 and 10 mg/kg groups. The $t_{1/2}$ of cyclosporine A was similar in all H-R groups. As seen in Figure 2A & 2B, mean plasma $AUC_{0\text{-}4h}$ and C_{max} increased

non-linearly with increasing intravenous cyclosporine A dosing above 10 mg/kg doses. Plasma $AUC_{0\text{-}4h}$ and C_{max} in piglets treated with cyclosporine A at 2.5 and 10 mg/kg were associated with lower plasma troponin levels, a marker of myocardial injury, compared to piglets treated with 25 mg/kg (Figure 10-3A & 10-3B). Specifically, $AUC_{0\text{-}4h}$ ≤10 umol*h/L was significantly associated with plasma troponin levels ≤ 1.1 ng/mL ($P<0.03$). Also, C_{max} ≤ 9 umol/L was significantly associated with plasma troponin levels ≤ 1.1 ng/mL ($P<0.03$). All of the piglets with $AUC_{0\text{-}4h}$ ≤10 umol*h/L, C_{max} ≤ 9 umol/L and plasma troponin levels ≤ 1.1 ng/mL were those treated with cyclosporine at 2.5 and 10 mg/kg.

Healthy vs. H-R Piglets Treated With Cyclosporine A

As depicted in Figure 10-4, the curves for the mean plasma concentration versus time of reoxygenation for cyclosporine (10 mg/kg) treatment in healthy and asphyxiated piglets was similar. The pharmacokinetic analysis of cyclosporine treatment in healthy and asphyxiated piglets is shown in Table 10-2. There was no significant differences in $AUC_{0\text{-}4h}$, $AUC_{0\text{-}\infty}$, CL, Vss or $t_{1/2}$ among healthy and asphyxiated piglets treated with cyclosporine.

Discussion

This experimental study describes the plasma pharmacokinetics of cyclosporine A following a single intravenous bolus during resuscitation of

asphyxiated newborn piglets, to further our understanding of cyclosporine treatment to attenuate myocardial injury. The $AUC_{0-\infty}$ increased as the dosage of cyclosporine was increased. This was similar to previous pharmacokinetic analysis of cyclosporine given as an intravenous infusion over 1 h in adult swine (33±3kg) (10). Though 1st order elimination of cyclosporine is comparable in different aged piglets, cyclosporine metabolism and tissue distribution has previously been shown to be altered by age in other species. For example, Molpeceres et al demonstrated different cyclosporine concentrations over 48hr in 10 and 40 week old rats following a 10 mg/kg intravenous dosage (11). Furthermore, these authors noted differences in tissue distribution based on the age and gender of the rats. Sangalli et al suggested that differences between cyclosporine pharmacokinetics might be related to related to differences in body weight, and specifically liver weight (12). However, they noted that certain species have an increased capacity to metabolize cyclosporine independent of body weight, and they did not specifically study swine in their experiments. Therefore, with similar size and physiology of human newborns and newborn piglets, our findings in newborn piglets may be representative of the human infant, however may not be directly transferable.

Although there were increases in AUC seen with higher dose levels, the AUC was observed to increase disproportionate to the increase in dose, especially comparing the 10 and 25 mg/kg doses. This was reflected in the CL

which decreased with increasing cyclosporine dosage in the newborn piglets. These findings at the doses ranging from 2.5 to 10 mg/kg were in line with the relative increases in blood CL seen in adult swine given intravenous infusions of cyclosporine ranging from 3 to 9 mg/kg (10), but they did not administer higher doses as we did in this study. Our results are in line with those in rats which had decreased CL in adult rats treated with 30 mg/kg intravenous cyclosporine over 2 min, compared to treatment with 1.2 and 6 mg/kg (13). These findings suggest a saturation of clearance processes involving cyclosporine elimination at doses in excess of 10 mg/kg.

Cyclosporine treatment has been previously shown to attenuate myocardial injury at 2.5 and 10 mg/kg, when given as an intravenous bolus immediately following the start of reoxygenation (3). Specifically, treatment at these doses lowered the troponin I plasma levels in newborn piglets following asphyxia-reoxygenation. In addition, treatment with cyclosporine at 25 mg/kg resulted in similarly increased troponin levels as placebo-treated controls in our previous experiment (3). Supportively, in this experiment, it appears that plasma troponin levels in newborn piglets were associated with $AUC_{0\text{-}4h}$ or exposure to cyclosporine. Specifically, lower troponin levels (myocardial injury) were associated with $AUC_{0\text{-}4h}$ of cyclosporine treatment at 2.5 and 10 mg/kg. These findings suggest that as the dose of cyclosporine increases beyond its narrow therapeutic index, the protective effect of cyclosporine is abolished, and may in fact be harmful.

While the therapeutic range of cyclosporine A is not defined in asphyxiated newborns, we have tried to use plasma concentrations of CsA to clarify this.

Griffiths et al observed similar findings using adult isolated rat hearts subjected to ischemia-reperfusion (4). Cyclosporine A improved left ventricular recovery at 0.2 μmol/L in the rat hearts, however the protective effect was abolished at 1.0 μmol/L. Karlsson et al also observed a lack of cardioprotection in adult swine treated with cyclosporine prior to reperfusion (5). These authors measured plasma concentration at 45 min following cyclosporine intravenous administration, and observed a mean concentration of 4.0 μmol/L. In our newborn piglets, the cyclosporine plasma concentrations were near 2.0 μmol/L or lower at 45 min, in groups treated with cyclosporine at 10 mg/kg or 2.5 mg/kg, respectively. Poit et al observed reduced myocardial injury in adult patients with myocardial infarction treated with cyclosporine and percutaneous coronary intervention (9). These authors reported blood concentrations of cyclosporine of 0.67 μmol/L (800 μg/L) at 3h post intravenous bolus. We observed similar plasma concentrations of cyclosporine at 3h in asphyxiated newborn piglets treated with 2.5 and 10 mg/kg. Interestingly, Karlsson et al, recently reported no differences in myocardial infarct size in adult swine (33-49 kg) treated with cyclosporine treatment at 2.5 mg/kg, despite blood concentrations of approximately 1.0 umol/L at 30 min post intravenous cyclosporine bolus in the adult swine (14). These findings support the notion that the therapeutic

index of cyclosporine is narrow, and may be age-related (8). Therefore, a thorough understanding of the pharmacokinetics of cyclosporine is needed to guide resuscitation of asphyxiated newborns in the clinical setting.

Pharmacokinetics of cyclosporine in humans can be variable depending on the health of the patient, with the efficiency of liver metabolism being of primary importance. Therefore we compared the pharmacokinetics of cyclosporine treatment in healthy and asphyxiated newborn piglets. We assumed that asphyxiated newborn piglets might develop some liver dysfunction as a result of overall multi-organ dysfunction seen in previous experiments. Interestingly, we observed similar pharmacokinetics of cyclosporine in healthy and asphyxiated newborn piglets. This is in contrast to human adult studies, in which whole blood pharmacokinetic parameters (CL and V_{ss}) were different between healthy volunteers and renal transplant patients (10, 15-17). Importantly, these findings suggest that cyclosporine treatment at 10 mg/kg in asphyxiated piglets is tolerated, and metabolism is relatively preserved. This may be related to the relatively rapid recovery of hepatic perfusion observed during resuscitation of asphyxiated newborn piglets (18). Therefore the use of cyclosporine as a resuscitative intravenous medication given quickly over 2 min, in this patient population may be feasible.

Though this study demonstrates important pharmacokinetic information for the cardioprotective role of cyclosporine in asphyxiated newborn piglets, there are some limitations to consider. The duration of our experiment was 6h, with plasma cyclosporine concentrations assessed following reoxygenation for a total of 4h. This duration of measurement is relatively short compared to other studies in rats and adult humans, and it is possible that the terminal phase half-life is underestimated; this would cause some underestimation of AUC and overestimation of clearance. However, this is the first attempt at assessing pharmacokinetics of cyclosporine in the neonatal population. For ethical reasons, cyclosporine pharmacokinetics cannot currently be readily assessed in healthy human infants, as was possible in human adults. Therefore, though our experimental duration is relatively restricted, it does provide important information on cyclosporine pharmacokinetics in healthy and asphyxiated newborn piglets in the short term. Despite the challenge to translate the findings from animal studies to human, newborn piglets have been shown to closely resemble 36-38 gestation age newborn humans (19). Furthermore, The improved appreciation of pharmacokinetics of cyclosporine, and their relation to myocardial injury and similar profile in healthy and asphyxiated piglets, may allow a clinical trial to be feasible.

Conclusion

In conclusion, this is the first study to demonstrate the pharmacokinetics in plasma of cyclosporine during resuscitation of asphyxiated newborn piglets. In addition, cyclosporine exposure following treatment at 2.5 and 10 mg/kg attenuates myocardial injury. Lastly, the capacity of asphyxiated newborn piglets to metabolize and clear cyclosporine remains preserved. These findings cumulatively suggest that a clinical trial may be appropriate.

Table 10-1. Cyclosporine plasma pharmacokinetic parameters after single intravenous doses in asphyxiated newborn piglets during reoxygenation

Pharmacokinetic Parameters	Cyclosporine 2.5 mg/kg	Cyclosporine 10 mg/kg	Cyclosporine 25 mg/kg	ANOVA on Ranks
$AUC_{0\text{-}4h}$ (umol·h/L)	1.2 ± 0.4	6.6 ± 1.2	30 ± 8.0	P<0.001
$AUC_{0\text{-}\infty}$ (umol·h/L)	1.6 ± 0.8	9.2 ± 1.7	47 ± 10*	P<0.001
CL (L/h/kg)	1.6 ± 0.7	0.9 ± 0.2	0.5 ± 0.1#	P<0.002
Vss (L/kg)	7.5 ± 4.3	5.5 ± 2.1	3.7 ± 1.2	NS
$t_{1/2}$ (h)	2.3 ± 1.0	2.5 ± 0.6	3.0 ± 0.4	NS
MRT (h)	5.7 ± 3.8	6.1 ± 2.9	8.2 ± 2.4	NS

* P<0.05 vs. cyclosporine 2.5 mg/kg group; # P<0.05 vs. Cyclosporine 2.5 & 10 mg/kg groups; AUC = area under the cyclosporine concentration vs. time curve; CL = clearance; Vss = steady state distribution; $T_{1/2}$ = half life; MRT = mean residual time; NS = not significant

Table 10-2. Plasma pharmacokinetic parameters of single cyclosporine (10 mg/kg) intravenous bolus in asphyxiated and healthy newborn piglets

Pharmacokinetic Parameters	Healthy newborn piglets	Asphyxiated newborn piglets	ANOVA on Ranks
$AUC_{0\text{-}4h}$ (umol h/L)	7.5 ± 1.6	6.6 ± 1.2	NS
$AUC_{0\text{-}\infty}$ (umol h/L)	12 ± 2.7	9.6 ± 1.3	NS
CL (L/h/kg)	0.7 ± 0.2	0.9 ± 0.1	NS
Vss (L/Kg)	3.1 ±1.1	3.2 ± 1.3	NS
$T_{1/2}$ (h)	3.3 ± 1.8	2.8 ± 1.2	NS
MRT (h)	4.8 ± 2.5	3.6 ±1.5	NS

AUC = area under the cyclosporine concentration vs. time curve; CL = clearance; Vss = steady state distribution; $T_{1/2}$ = half life; MRT = mean residual time; NS = not significant

Figure 10-1. (A) Plasma concentration vs. time curves of cyclosporine A with respect to time of reoxygenation following cyclosporine intravenous dosages of 2.5, 10 or 25 mg/kg. **(B)** Plasma concentration vs. time curves of cyclosporine A with respect to time of reoxygenation following cyclosporine intravenous dosages of 2.5, 10 or 25 mg/kg with log-scale on y-axis.

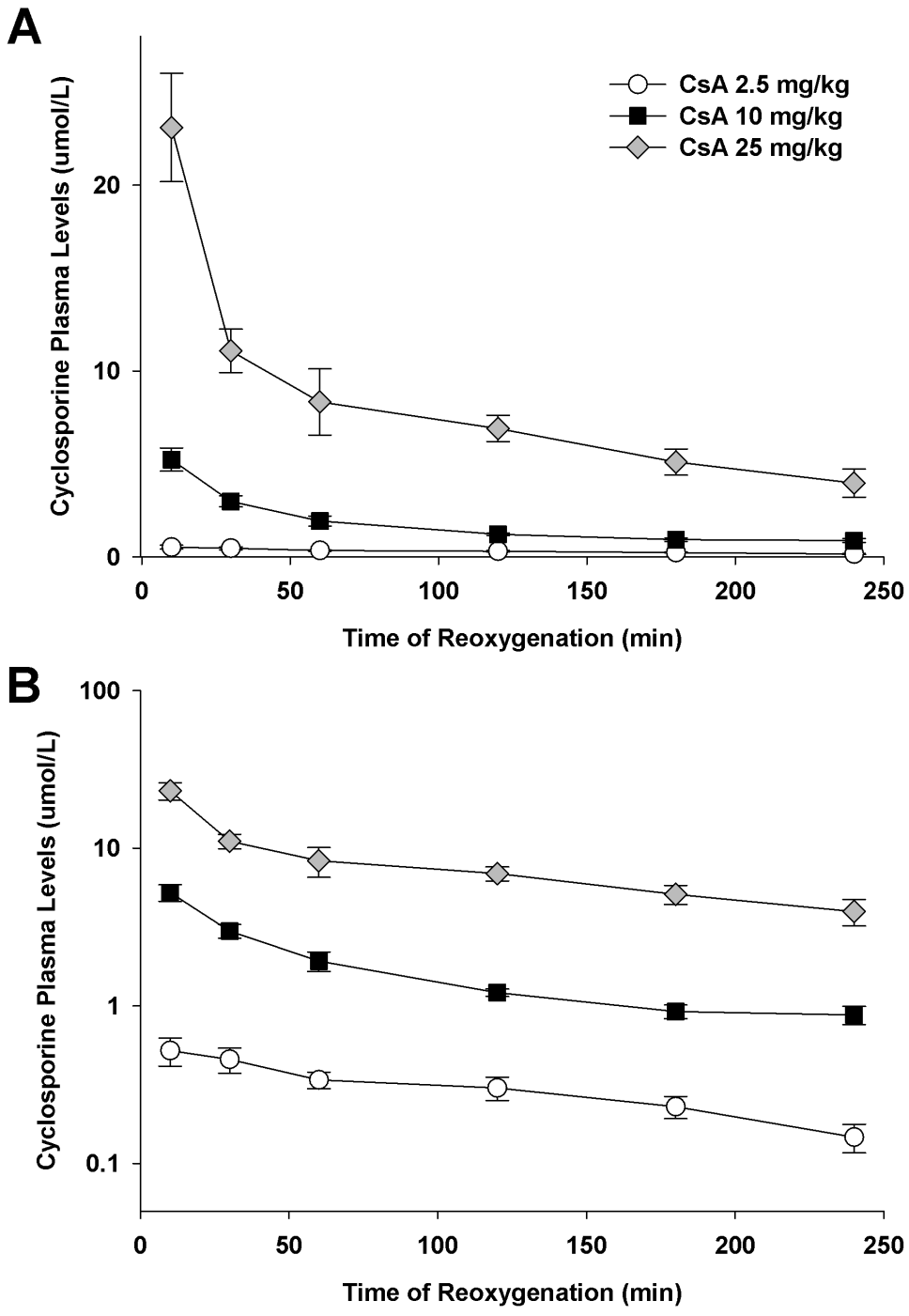


Figure 10-2. (A) Plasma AUC_{0-4} vs. cyclosporine intravenous dosing (mg/kg). **(B)** Plasma C_{max} vs. cyclosporine intravenous dosing (mg/kg).

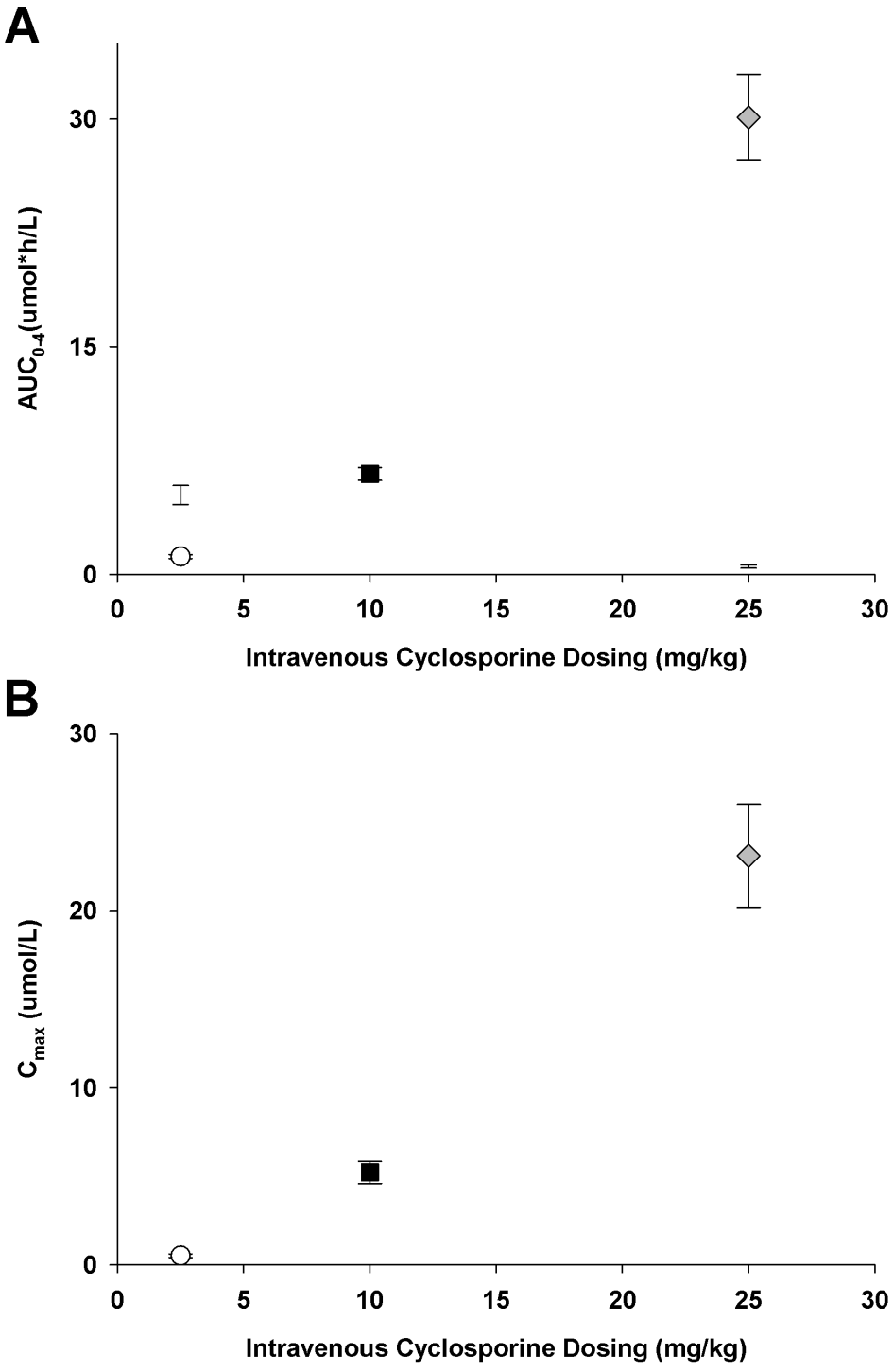


Figure 10-3. (A) Plasma troponin levels (ng/mL) following hypoxia-reoxygenation in newborn piglets compared to AUC_{0-4} curves for cyclosporine intravenous dosages of 2.5, 10, 25 mg/kg. **(B)** Plasma troponin levels (ng/mL) following hypoxia-reoxygenation in newborn piglets compared to C_{max} curves for cyclosporine intravenous dosages of 2.5, 10, 25 mg/kg.

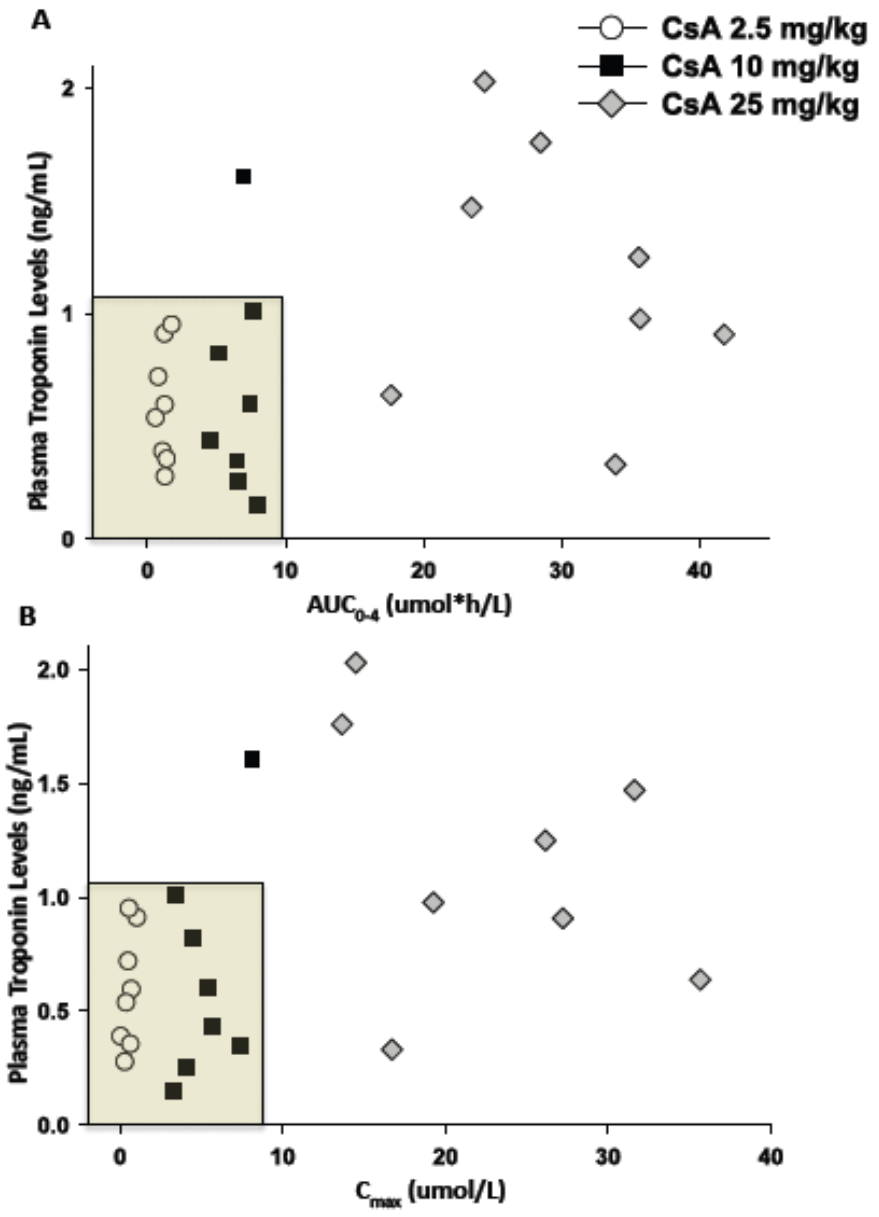
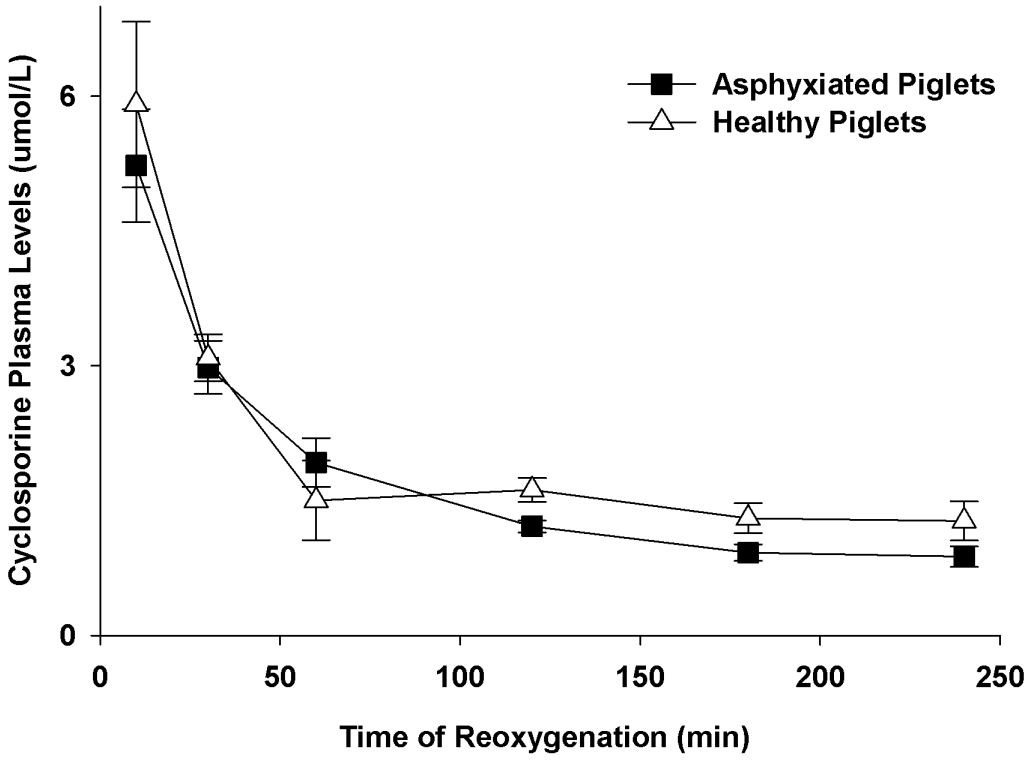


Figure 10-4. Cyclosporine plasma levels during reoxygenation in asphyxiated and healthy newborn piglets.



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Chapter 11

Early versus delayed cyclosporine treatment in cardiac recovery and intestinal injury during resuscitation of asphyxiated newborn piglets

Adapted from:

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Abstract

Background

Recently, we have demonstrated that treating asphyxiated newborn piglets with intravenous cyclosporine immediately following resuscitation can improve cardiac function and attenuate intestinal injury. However, immediate treatment may not be feasible for a large portion of newborns delivered in peripheral or rural hospitals. Therefore, our objective was to determine if delayed cyclosporine treatment was still effective in protecting asphyxiated newborn piglets. We hypothesize that both early and delayed treatment with cyclosporine A would improve cardiac recovery during resuscitation of asphyxiated newborn piglets.

Methods

Thirty piglets (1-4 days-old) were instrumented for continuous monitoring of cardiac output and regional hemodynamics. After stabilization, normocapnic alveolar hypoxia (10-15% oxygen) was instituted for 2h followed by reoxygenation with 100% oxygen for 0.5h, then 21% for 5.5h. Piglets were block-randomized to receive either early (at 5 min of reoxygenation) or delayed (at 120 min reoxygenation) intravenous bolus of cyclosporine A (10 mg/kg) or normal saline (control) at identical times during reoxygenation (n=8/group). Myocardial and intestinal lactate concentrations as well as histological examinations were determined.

Results

Hypoxic piglets had cardiogenic shock (cardiac output 52±1% of baseline), hypotension (mean arterial pressure 32±1 mmHg) and acidosis (pH 6.98±0.1). Although both early and delayed cyclosporine treatment improved cardiac output ($P<0.05$ vs. controls), only early cyclosporine treatment improved stroke volume and systemic oxygen delivery ($p<0.05$ vs. controls). Left ventricle and intestinal lactate were higher in controls than in both cyclosporine-treated groups ($p<0.05$). Early, but not delayed, cyclosporine treatment also attenuated intestinal injury compared to controls ($P<0.05$).

Conclusions

This is the first study to demonstrate both early and delayed cyclosporine treatment during resuscitation improves cardiac recovery in asphyxiated newborn piglets. However, early treatment with cyclosporine may offer superior cardioprotection and attenuates H-R intestinal injury.

Introduction

Asphyxia is estimated to be the primary cause of death in 23% of the estimated annual four million newborn deaths worldwide (1). In survivors, cardiovascular dysfunction of varying severity is estimated to occur in 50% to 80% (2). This may be related to the immature newborn heart adapting to new circulatory patterns and becoming overwhelmed with additional hypoxic stress. Furthermore, the immature newborn heart has been shown to have an increased vulnerability to oxidative stress and apoptosis during resuscitation and/or reoxygenation (3, 4). Concurrently, hypoxic-ischemic stress of the gastrointestinal system may lead to intestinal injury resembling necrotizing enterocolitis in up to 20% of asphyxiated newborns (5). Currently, no standard clinical treatments exist to protect the immature newborn heart and intestine from hypoxia-reoxygenation injury.

Cyclosporine treatment is proposed to minimize the formation of mitochondrial permeability transition pore (MPTP) in cardiomyocytes during reperfusion, hence limiting apoptotic and necrotic cell death (6). Specifically, cyclosporine has been suggested to bind to cyclophilin-D, preventing the interaction of cyclophilin-D and less well-defined membrane proteins to form the MPTP (7). The cardioprotective effects of cyclosporine treatment have been reported in an adult pilot clinical trial, in which intravenous cyclosporine treatment prior to revascularization of occluded coronary arteries reduced myocardial infarct size (8). Recently, we have demonstrated improved cardiac function and mesenteric perfusion with

attenuated myocardial and intestinal injuries in asphyxiated newborn piglets treated with a single intravenous cyclosporine bolus immediately following reoxygenation (9,10). However, the administration of advanced resuscitative medications during resuscitation of human neonates is usually delivered by specialized neonatal intensive care (NICU) teams. Immediate treatment may not be feasible for a large portion of newborns delivered in rural or peripheral sites, where there may have a delay prior to treatment by the NICU team. Thus, optimal timing of cyclosporine treatment in asphyxiated newborns is important and currently unknown.

Therefore, our objective was to determine the optimal timing of intravenous cyclosporine treatment during the resuscitation of asphyxiated newborn piglets. In addition, we aimed to determine if delayed cyclosporine treatment during resuscitation of asphyxiated newborn piglets would improve cardiac function and intestinal recovery. We hypothesized that early and delayed cyclosporine treatment during resuscitation of asphyxiated newborn piglets would improve cardiac recovery and attenuate intestinal injury.

Methods

Thirty newborn mixed breed piglets 1 to 4 days of age weighing 1.5 to 2.5 kg were obtained on the day of experimentation from University swine research centre. All experiments were conducted in accordance with the

guidelines and approval of the Animal Care and Use Committee (Health Science), University of Alberta.

Animal Preparation

Animal preparation has been previously described (9). Briefly, following induction of anesthesia, mechanical ventilation was commenced. Fractional inspired oxygen concentrations (FiO_2) were continuously measured and maintained between 0.21 and 0.25 to keep arterial oxygen saturations between 90% and 100%. Intravenous fluids consisting of 5% dextrose in water at 10 ml/kg/h and 0.9% NaCl at 2 ml/kg/h were used to maintain glucose levels and hydration. Anesthesia was maintained with intravenous fentanyl 5-20 microgram/kg/h and midazolam 0.2 to 1 mg/kg/h. and pancuronium 0.05 to 0.1 mg/kg/h. Additional intravenous doses of. fentanyl (10 mcg/kg) and acepromazine (0.25 mg/kg) were also given as needed. Piglet body temperature was maintained at 38.5 to 39.5 °C using overhead warmer and a heating pad.

A 5-French Argyle double-lumen catheter was inserted into the femoral vein, up to the level of the right atrium for administration of fluids and medications. A 5-French Argyle single-lumen catheter was inserted into the femoral artery to the distal aorta and attached to a pressure transducer for continuous systemic measurement of arterial pressure to determine mean arterial pressure (MAP). Heart rate and MAP were measured with a Hewlett Packard 78833B monitor (Hewlett Packard Co., Palo Alto, CA).

Endotracheal intubation via a tracheostomy was performed, and pressure-controlled ventilation (Sechrist infant ventilator model IV-100; Sechrist Industries, Anaheim, CA) was commenced at a respiratory rate of 16-20 breaths/min and pressure of 19/4 cm H_2O. A left flank incision was used to open the retroperitoneum. The superior mesenteric artery (SMA) was encircled with a 3-mm transonic flow probe (3SB) to measure SMA blood flow. In the third intercostal space a left anterior thoracotomy was preformed and a 6-mm transonic flow probe (6SB; Transonic Systems Inc, Ithica, NY) was placed around the main pulmonary artery to measure blood flow, which served as the surrogate of cardiac output. The ductus arteriosus was ligated. A 20G Arrow® angiocatheter (Arrow International, Reading, PA) was inserted for the continuous measurement of pulmonary artery pressure. Transonic flow probes and pressure transducer outputs were digitized and recorded by a converter board in a computer equipped with custom Asyst programming software (Data Translation, Ontario, Canada).

The piglets were allowed to recover from surgical instrumentation until baseline hemodynamic measures were stable. Ventilator rate was adjusted to keep the P_aCO_2 35 to 45 mmHg as determined by periodic arterial blood gas analysis. Heart rate, MAP, cardiac output, SMA blood flow and oxygen saturation were continuously monitored and recorded throughout the experiment.

Experimental Protocol

The piglets were block-randomized to 3 groups (n=8 per group) that underwent hypoxia-reoxygenation (H-R). A fourth sham-operated group of piglets (n=6) underwent complete instrumentation without H-R and delivery of cyclosporine.

Normocapnic alveolar hypoxia was induced in all H-R piglets. These piglets were ventilated with a FiO_2 of 0.11 to 0.15 by increasing the inhaled concentration of nitrogen gas relative to oxygen for 2h, aiming for arterial oxygen saturations of 40 to 50%. It has been shown in previous studies that this degree of hypoxemia in the newborn piglet model will produce clinical asphyxia with severe metabolic acidosis and systemic hypotension (9, 11). This was followed by reoxygenation with 100% oxygen for 0.5 h and then 21% oxygen for 5.5 h. At 5 min and 120 min of reoxygenation, piglets received a blinded treatment either with cyclosporine A as an intravenous bolus (10 mg/kg) or saline (control). Cyclosporine A treatment was given at 5 min reoxygenation to simulate the clinical scenario when intravenous access is obtained in the neonate for the administration of resuscitative medications. In another experimental group, cyclosporine A was given at 120 min reoxygenation to simulate the clinical setting in which resuscitation by the mobile NICU team or transfer to a NICU requires an estimated two hours (120 min) following newborn delivery at a peripheral or rural hospital (personal observation).

Medication Preparation and Delivery

Blinding was maintained by reconstituting cyclosporine A with normal saline to a standard total volume (5 ml) immediately before administration (9). The medication was given intravenously over 2 min. A laboratory technician uninvolved in the experiment prepared the medications. Cyclosporine dosing of 10 mg/kg was based on previous dose-finding experiments (9).

Hemodynamic Measurements and Oxygen Transport

Hemodynamic recording for data analysis was carried out at specified time points: baseline (0 min), 60 and 120 min of hypoxia, 130 (10-min reoxygenation) and 150 min (30-min reoxygenation) reoxygenation with 100% FiO_2, then at 180, 240, 250, 270, 300, 360, 420 and 480 min for reoxygenation with 21% FiO_2. All recordings were calculated as a mean over 2 min of recording. Hemodynamic variables were calculated as shown in a previous study (11).

At the specified time points, both arterial and venous blood samples were taken for blood gases, hemoglobin levels and co-oximetry. The systemic oxygen delivery (DO_2), mesenteric oxygen delivery (SMA DO_2) and systemic oxygen consumption (VO_2) were calculated using standard formulas (12).

Arterial blood samples (1 ml) were taken at predetermined intervals coinciding with hemodynamic measurements, centrifuged at 15,000 rotations per min for 10 min. The supernatant was then collected and frozen

at -80 °C for determination of plasma lactate. Of the piglets blood volume, less than 5% was collected for blood work.

At the end of the study, piglets were euthanized with i.v. 100 mg/kg pentobarbital. Sample of left ventricle and intestine were immediately harvested, snap-frozen in liquid nitrogen and stored in -80 °C for biochemical analysis.

Determination of Plasma, Left Ventricle and Small Intestinal Lactate

Blood was collected in heparin tubes and plasma was prepared by centrifugation at 10,000g for 15 min and stored at -80 °C. The plasma lactate concentration was determined by a nicotinamide adenine dinucleotide (NAD) enzyme-coupled colorimetric assay with spectrophotometry at 340 nm at each coinciding time point with hemodynamic measurements.

Tissue lactate, a marker for tissue perfusion and anaerobic metabolism, was measured following H-R. Left ventricle myocardial and small intestinal (ileum) tissues were homogenized with 10 µl/mg of 50 mM phosphate buffer containing 0.5 mM EGTA. Left ventricle and intestinal lactate were assayed by enzymatic spectrometric methods to measure the absorbance of NADH at 340 nm. The protein content was determined by bicinchoninic acid assay kit (Sigma).

Histopathology

Samples of left ventricle and small intestine (ileum) collected at the end of the experiment were fixed in 10% formalin for histological analysis. Samples were processed for histological assessment using hematoxylin and eosin staining. An independent pathologist (CS) blinded to all groups evaluated and graded the histological damage of specimens based on the Rose classification for myocardial injury and Park classification for intestinal injury (13, 14).

Statistical Analysis

Results are expressed as mean±standard error of mean. Hemodynamic and oxygen transport variables were analyzed by two-way repeated measures ANOVA. We used the Student-Newman-Keuls method where appropriate for pairwise comparisons in *post hoc* testing. Biochemical markers were analyzed by two-way repeated measures ANOVA or one-way ANOVA as appropriate. If the normality test failed, ANOVA on ranks (Kruskal-Wallis) was performed. Pearson moment correlation was used. Significance was defined as $p<0.05$.

Results

The piglets were 2.4±1.1 day-old and weighed 1.8±0.3 kg with no significant differences among groups. There was no significant difference among groups at normoxic baseline in arterial blood gases (Table 11-1) and

all hemodynamic variables (Table 11-2). Sham-operated animals were stable throughout the experimental period (data not shown).

Cardiac Function and Injury during Hypoxia-Reoxygenation

Severe normocapnic alveolar hypoxia resulted in cardiogenic shock within H-R groups with decreased cardiac index (CI) to 52±1% of normoxic baseline at 2h ($p<0.05$, vs. sham-operated piglets) (Figure 11-1). The CI recovered within 10 min of reoxygenation and then gradually deteriorated over 360 min of reoxygenation to 62±5% of baseline in the control group (Figure 11-1). As shown in Figure 11-1, cyclosporine treatment significantly improved CI at 180, 240 and 300 min of reoxygenation. At 360 min of reoxygenation, CI was improved in early and delayed cyclosporine treatment (95±4% and 79±6% of baseline vs. controls, $p<0.05$ and $p=0.1$, respectively). As compared with delayed treatment group, the CI of early cyclosporine treatment group was higher at 360 min of reoxygenation ($p=0.05$).

As shown in Figure 11-2, there was no difference in heart rate between cyclosporine-treated and control groups at the end of hypoxia and throughout the reoxygenation period. At 2h of hypoxia, severe hypotension developed in all H-R groups (MAP of 32±1 vs. 65±3 mmHg of sham-operated group, respectively). The MAP bounced back after 10 min of reoxygenation (65±4 mmHg) in all H-R groups and gradually deteriorated over the course of reoxygenation with no significant difference among groups (Figure 11-3). Pulmonary arterial pressure increased at 2h of hypoxia in all H-R groups

(34±1 vs. 24±1 mmHg of sham-operated group). The pulmonary arterial pressure gradually decreased during reoxygenation in all H-R groups with no significant differences among groups (Figure 11-4).

All H-R groups had decreased stroke volume index after 2h of hypoxia ($p<0.05$ vs. sham-operated group)(Figure 11-5). Upon reoxygenation, they all improved rapidly and then steadily decreased over the course of reoxygenation with significant differences between sham-operated and control piglets at 300 and 360 min of reoxygenation (300 min: 92±11% vs. 60±7% of baseline; 360 min: 91±12% vs. 59±7% of baseline, respectively, both $p<0.05$)(Figure 11-5). Early but not delayed cyclosporine treatment significantly improved stroke volume index compared to the control group at 360 min of reoxygenation (84±6% and 77±9% vs. 59±7% of baseline, respectively).

After 2h of hypoxia and 360 min of reoxygenation, the left ventricle myocardial lactate levels of early and delayed cyclosporine-treated groups were lower than that of control piglets (both $p<0.05$) (Figure 11-6). Further, there was significant negative correlation between CI at 360 min reoxygenation with left ventricle myocardial lactate level ($r=-0.54$, $p<0.005$) among all groups. Samples of the left ventricle were assessed using the Rose Criteria for myocardial histological injury (10). There was no difference in histopathological scores among groups (data not shown).

Systemic Oxygen Transport during Hypoxia-Reoxygenation

Systemic DO_2 decreased significantly in all H-R groups (vs. sham-operated piglets) during hypoxia and normalized rapidly upon reoxygenation (Figure 11-7A). As reoxygenation continued, systemic DO_2 gradually deteriorated in the control group, but not in cyclosporine-treated groups (Figure 11-7A). At the early stage of reoxygenation, both control group and delayed cyclosporine treatment group has similar deterioration of systemic DO_2. However, systemic DO_2 was modestly improved after receiving cyclosporine in the delayed treatment group. At 180 min of reoxygenation, both early and delayed cyclosporine treated groups had higher systemic DO_2 than the control group (85±6% and 86±7% vs. 64±6% of baseline, respectively, $p<0.05$). The early cyclosporine-treated group had significantly higher systemic DO_2 at 300 and 360 min of reoxygenation than that of controls (300 min: 81±5% vs. 55±5%; 360 min: 82±5% vs. 55±6%, respectively, both $p<0.05$).

After 2h of hypoxia, systemic VO_2 decreased significantly in all H-R groups (Figure 11-7B). Systemic VO_2 increased steadily following reoxygenation in the H-R groups reaching peak recovery at 60 min of reoxygenation. During the final 4h of reoxygenation, there was gradual deterioration of systemic VO_2 in control piglets whereas the systemic VO_2 was maintained in the early cyclosporine-treated piglets with significant differences noted at 300 min and 360 min of reoxygenation (at 300 min: 106±7% vs. 75±8% of baseline; at 360 min: 111±8% vs. 80±10% of baseline, both $p<0.05$). The improvement in systemic VO_2 of delayed cyclosporine-

treated group was modest and did not differ significantly from those of control and early cyclosporine-treated groups.

All H-R groups had significant metabolic acidosis (pH 6.98±0.1) and elevated plasma lactate concentrations at the end of hypoxia (vs. sham-operated group, both $p<0.05$) (Table 11-1). Both arterial pH and plasma lactate returned back to normal after 6h of reoxygenation (Table 11-1). There was no significant difference between cyclosporine-treated and control groups in acid-base balance and plasma lactate concentrations during H-R.

Mesenteric Perfusion and Intestinal Injury during Hypoxia-Reoxygenation

SMA flow index and DO_2 significantly decreased in all H-R piglets after 2h of hypoxia (SMA flow index: 50±4% vs. 81±6% of baseline in sham-operated piglets; $SMADO_2$: 20±2% vs. 86±9% of baseline in sham-operated piglets). The mesenteric perfusion of all H-R groups recovered similarly in the first 60 min of reoxygenation (SMA flow index and DO_2: 105±9% and 92±8% of baseline, respectively). Thereafter, all H-R groups had steady deterioration of mesenteric perfusion throughout the remaining experimental period. The improvement in mesenteric perfusion of both cyclosporine-treated groups was modest but did not differ significantly from that of controls at 360 min of reoxygenation (SMA flow index: 102±13% and 80±12% vs. 77±20% of baseline; SMA DO_2: 88±10% and 71±12% vs. 71±21% of baseline, respectively). At 360 min of reoxygenation, the SMA DO_2

of delayed cyclosporine-treated and control groups, but not early cyclosporine-treated piglets, remained significantly different from their respective baselines.

The intestinal tissue lactate levels of both cyclosporine-treated groups were lower than that of control group ($p<0.05$)(Figure 11-8). Intestinal histological specimens were microscopically graded by Park's Criteria for intestinal injury (11), as shown in Figure 11-9. Cyclosporine treatment attenuated the intestinal injury with significantly lower Park's grade of early cyclosporine-treated piglets than that of controls (1.4 ± 0.6 vs. 4.9 ± 0.9, $p<0.02$), whereas the improvement by delayed cyclosporine treatment was modest (2.9 ± 0.7, $p=0.1$). Histological intestinal injury (Park's grade) was positively correlated with intestinal lactate level $r=0.42$ ($p<0.03$).

Discussion

This is the first study to demonstrate that both early and delayed cyclosporine treatment during the resuscitation of asphyxiated newborn piglets improves cardiac functional recovery and systemic oxygen transport. However, in comparison to delayed cyclosporine treatment, administering cyclosporine early during resuscitation of asphyxiated newborn piglets may offer marginally superior cardioprotection and it has the added benefit of attenuating intestinal injury.

In contrast to adult myocardial infarction secondary to coronary occlusion, newborn myocardium suffers from global hypoxic-ischemic injury

secondary to asphyxia. In adult animal models and pilot human trials (8, 15-17), cyclosporine treatment is administered just prior to revascularization of the coronary arteries by percutaneous coronary intervention. In contrast, asphyxiated newborns are unique in that pretreatment is not directly feasible with the fetus in-utero. Thus timing of administration of resuscitative medication is restricted to post-delivery and insertion of intravenous access. We have previously demonstrated that cyclosporine treatment immediately following resuscitation with reoxygenation improves cardiac function [9] and attenuates intestinal injury (10) in asphyxiated newborn piglets. Consistently, we also observed improved cardiac function and reduced intestinal injury with early cyclosporine treatment in the present study. The improvements in cardiac function and attenuation of intestinal injury have been proposed to be related to preservation of cardiomyocytes and enterocytes.

As significant improvement of left ventricular function and ATP/ADP levels in isolated rat heart treated with cyclosporine treatment, Griffiths et al suggested that the transition from reversible to irreversible myocardial injury might involve mitochondrial dysfunction related to the MPTP (18). In agreement with their suggestion, we also observed preserved cardiac mitochondrial morphology (9) and functional integrity (unpublished data) following early cyclosporine treatment during resuscitation of asphyxiated newborn piglets.

The role of MPTP in reperfusion injury of enterocytes is less well investigated. We previously observed preserved intestinal mitochondrial morphology with early cyclosporine treatment during resuscitation of asphyxiated newborn piglets (10). Supportively, Madesh et al also suggested that CsA might be protective of enterocytes mitochondria from oxidative stress (19). In addition, enterocyte mitochondria have been shown to be sensitive targets of damage during ischemia-reperfusion (20). However, to the best of our knowledge, the optimal timing cyclosporine treatment has not been specifically assessed in adult or newborn intestinal ischemia-reperfusion models. We hereby showed that cyclosporine attenuated intestinal H-R injury, which contributes to up to 20% of morbidity in asphyxiated neonates, with significant improvements in perfusion, lactate and histological features in early treatment.

According to Halestrap et al the key factors associated with MPTP opening are mitochondrial calcium overload, oxidative stress, mitochondrial depolarization and adenine nucleotide depletion (21). Cromptom et al have also suggested the importance of oxidative stress in the activation of the MPTP during reperfusion (22). The depletion of oxygen during hypoxia and the conversion of xanthine to hypoxanthine, lead to ideal conditions for a rapid burst of ROS upon reoxygenation (23). Therefore, the rapid burst of ROS during early phase of reperfusion/ reoxygenation may stimulate MPTP formation soon after resuscitation. We previously observed concurrent increase in left ventricle lipid hydroperoxides and cytochrome-c following H-

R in newborn piglets, with attenuation of both these levels with early cyclosporine treatment (24) Taken together, this may at least in part explain the better protective effects with early treatment of cyclosporine, compared to delayed treatment, during resuscitation of asphyxiated newborn piglets.

Although the formation of MPTP has been demonstrated to occur during reperfusion (25), the exact duration of MPTP opening during reperfusion/reoxygenation remains unclear. In clinical practice, there may be a delay of up to 2h, before specialized NICU care is available. Therefore, determining the optimal timing of cyclosporine administration during resuscitation of asphyxiated newborn piglets would provide important information regarding the therapeutic window. Although early cyclosporine treatment is optimal, our results demonstrated that a benefit exists with delayed treatment compared to no treatment at all. Similar to our observations, Sullivan et al also reported that early cyclosporine treatment (15 min post injury) provided better neuroprotection than late treatment (24 h post injury) in rats following traumatic brain injury (26). By treating the mice with Debio-025 (non-immunosuppressive analog of cyclosporine A) following myocardial infarction, Gomez et al suggested that limiting infarct size might be the best strategy to reduce post-ischemic cardiac failure and improve overall survival (27). Therefore, it is possible that modest improvement in cardiac function and myocardial oxygen transport with delayed cyclosporine treatment may lead to improved outcomes. However,

further research is needed to substantiate these claims in the newborn population.

Although our findings suggest that a clinical trial including asphyxiated neonates with early and delayed cyclosporine treatment may be feasible, important limitations exist. Firstly, translation of animal studies to human infants is always challenging, however newborn piglets are the most similar surrogate model for human newborns, with similar anatomy and physiology (28). Secondly, the acute setting of our swine model allows for only a limited time (8h) of observation. Further investigations are necessary to determine whether improved cardiac recovery with early and delayed cyclosporine treatment will persist in the long term. Thirdly, there is a paucity of literature on the pharmacokinetics of CsA in newborns, thus the therapeutic window and therapeutic index remains undefined. The delay in administration of resuscitative medication by the NICU team was estimated to be 2h. This is an approximation based on our personal observations, however may be variable based on the regional resources available. Furthermore, future studies should address if repeated administration of cyclosporine in asphyxiated neonates during recovery is effective and safe.

In conclusion, this is the first study to demonstrate early and delayed cyclosporine treatment during resuscitation improves cardiac recovery and myocardial oxygen transport in asphyxiated newborn piglets. However, early treatment with cyclosporine may offer superior cardioprotection and attenuates intestinal injury in H-R piglets.

Table 11-1: Arterial blood gases

	Normoxic Baseline	End of hypoxia	120 min Reoxygenation	360 min Reoxygenation
pH				
Sham	7.43 ± 0.02	7.40 ± 0.03	7.40 ± 0.02	7.36 ±0.02
Control	7.48 ± 0.02	6.99 ± 0.04*	7.38 ± 0.02	7.42 ± 0.06
Early CsA	7.49 ± 0.01	7.00 ± 0.05*	7.33 ± 0.03	7.36 ± 0.01
Delayed CsA	7.43 ± 0.02	6.95 ± 0.06*	7.36 ± 0.05	7.36 ± 0.03
PaO2 (mmHg)				
Sham	73 ± 3	68 ± 3	66 ± 2	68 ± 1
Control	79 ± 4	42 ± 4*	72 ± 5	87 ± 8
Early CsA	83 ± 7	39 ± 1*	68 ± 4	68 ± 5
Delayed CsA	78 ± 5	44 ± 5*	71 ± 4	74 ± 4
PaCO2 (mmHg)				
Sham	34 ± 2	39 ± 1	38 ± 1	41 ± 1
Control	36 ± 1	38 ± 2	39 ± 1	39 ±1
Early CsA	32 ±2	39 ± 2	41 ± 2	42 ± 1
Delayed CsA	39 ± 2	39 ±1	38 ±1	40 ± 1
HCO3- (mM)				
Sham	24 ± 1	24 ± 1	24 ± 1	23 ± 1
Control	27 ± 2	9 ± 1*	23 ± 1	23 ± 1
Early CsA	27 ± 2	9 ± 1*	21 ± 2	23 ± 1
Delayed CsA	26 ± 1	8 ± 1*	22 ± 2	22 ± 2
Plasma Lactate (mM)				
Sham	3.3 ± 0.4	2.3 ± 0.3	1.7 ± 0.3	1.9 ± 0.3
Control	4.5 ± 0.6	17 ± 1.2*	6.1 ± 0.4	4.4 ± 0.8
Early CsA	4.2 ± 0.4	15 ± 1.3*	4.9 ± 0.4	2.8 ± 0.2
Delayed CsA	4.8 ± 0.2	18 ± 1.3*	7.0 ± 0.7	4.2 ± 0.9

* $P < 0.05$ vs. normoxic baseline (RM ANOVA) and Sham piglets at end of hypoxia

Table 11-2: Hemodynamic measurements and oxygen transport at normoxic baseline in piglets treated with early or delayed cyclosporine intravenously (10mg/kg) (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=6).

Variables	**Early Cyclosporine (10 mg/kg)**	**Delayed Cyclosporine (10 mg/kg)**	**Controls**	**Sham-operated**
Cardiac Index (mL/kg/min)	148 ± 13	165 ± 13	170 ± 10	170 ± 20
Heart Rate (beats/min)	209 ± 18	206 ± 14	193 ± 4	196 ± 15
Stroke Volume Index (mL/kg/beat)	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Mean Arterial Pressure (mmHg)	83 ± 5	78 ± 3	75 ± 3	74 ± 3
Systemic Oxygen Delivery (O_2 mL/kg/min)	15 ± 1	17 ± 2	19 ± 2	16 ± 2
Systemic Oxygen Consumption (O_2 mL/kg/min)	5.7 ± 0.4	6.7 ± 1.0	6.0 ± 0.3	7.1 ± 1.0
SMA Flow Index (mL/kg/min)	30 ± 4	27 ± 4	34 ± 3	37 ± 3
SMA Oxygen Delivery (O_2 mL/kg/min)	3.2 ± 0.4	2.8 ± 0.3	3.7 ± 0.4	3.6 ± 0.4

SMA = superior mesenteric artery

Figure 11-1. Cardiac Function. Cardiac Index during 6h of reoxygenation with early or delayed cyclosporine (10 mg/kg) treatment (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=6). *P<0.05 vs. controls, $^{\phi}$P=0.05 vs. delayed cyclosporine treated group, $^{\#}$P<0.05 vs. all H-R groups. Downward pointing black arrow represents "early" administration of Cyclosporine A. Downward pointing dark grey arrow represents "delayed" administration of Cyclosporine A.

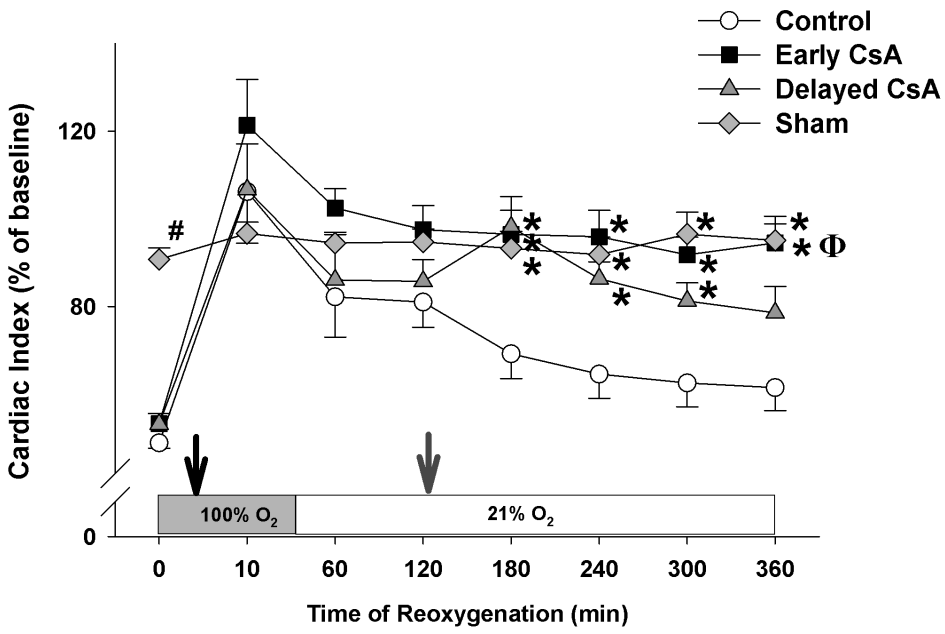


Figure 11-2. Heart rate and during 6h of reoxygenation with early or delayed cyclosporine (10 mg/kg) treatment (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=6). *P<0.05 vs. controls, #P<0.05 vs. all H-R groups. Downward pointing black arrow represents "early" administration of Cyclosporine A. Downward pointing dark grey arrow represents "delayed" administration of Cyclosporine A.

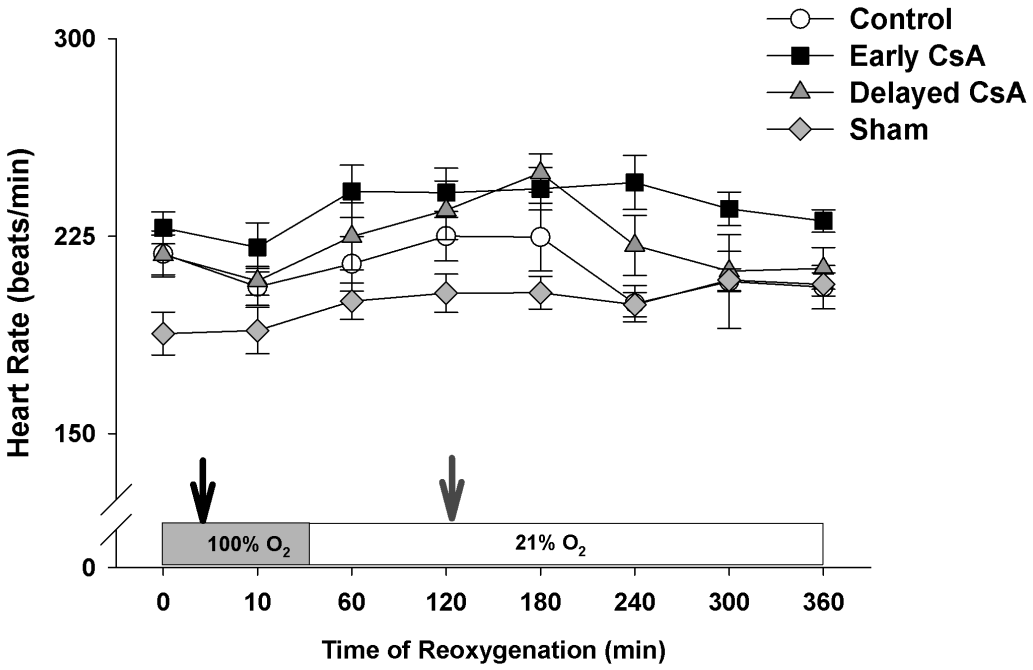


Figure 11-3. Mean arterial pressure during 6h of reoxygenation with early or delayed cyclosporine (10 mg/kg) treatment (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=6). *P<0.05 vs. controls. Downward pointing black arrow represents "early" administration of Cyclosporine A. Downward pointing dark grey arrow represents "delayed" administration of Cyclosporine A.

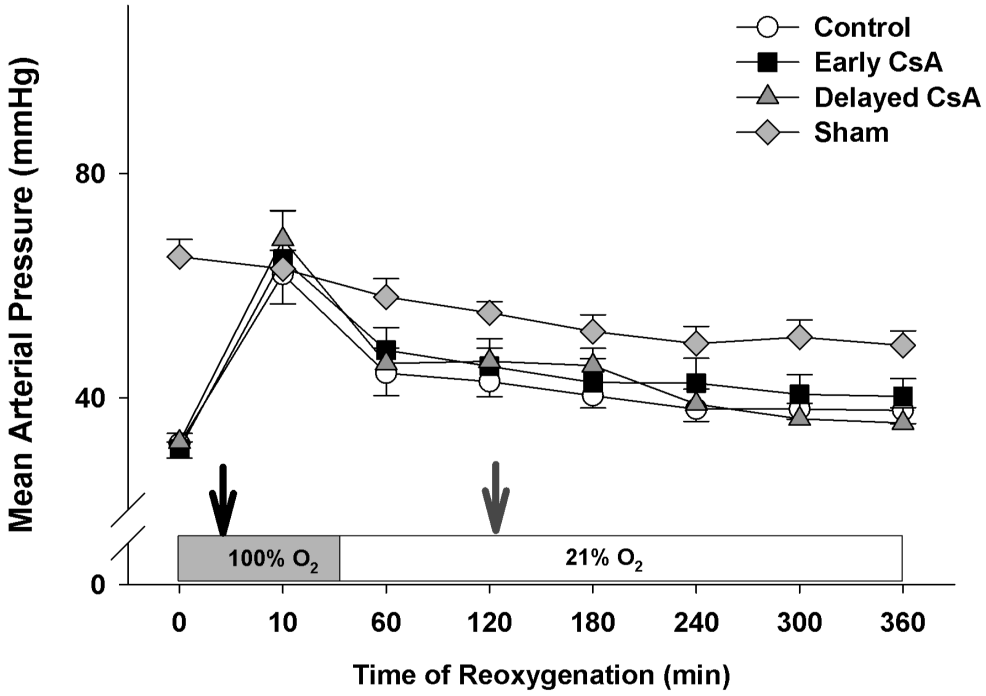


Figure 11-4. Pulmonary artery pressure during 6h of reoxygenation with early or delayed cyclosporine (10 mg/kg) treatment (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=6). *P<0.05 vs. controls. Downward pointing black arrow represents "early" administration of Cyclosporine A. Downward pointing dark grey arrow represents "delayed" administration of Cyclosporine A.

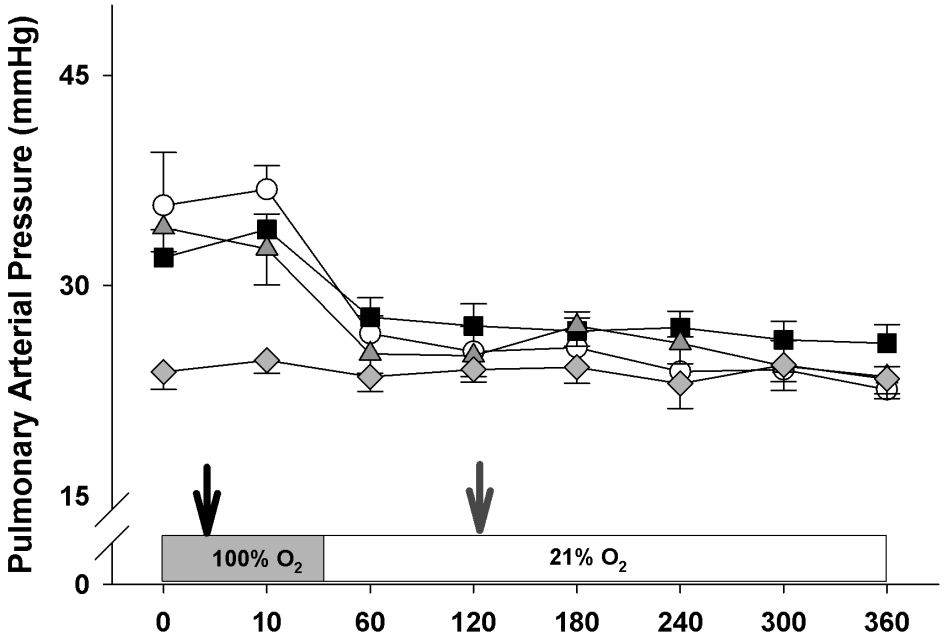


Figure 11-5. Stoke volume index during 6h of reoxygenation with early or delayed cyclosporine (10 mg/kg) treatment (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=6). *P<0.05 vs. controls, #P<0.05 vs. all H-R groups. Downward pointing black arrow represents "early" administration of Cyclosporine A. Downward pointing dark grey arrow represents "delayed" administration of Cyclosporine A.

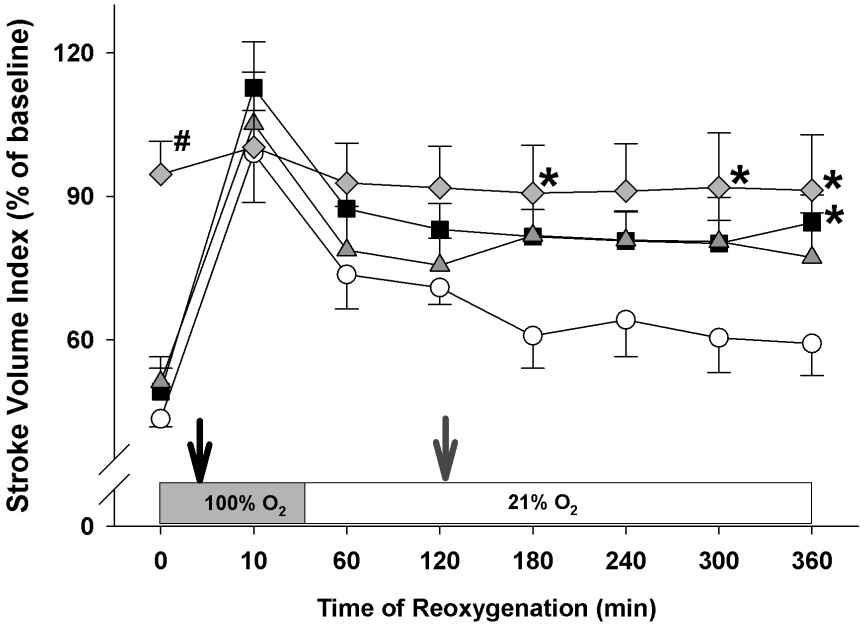


Figure 11-6. Left ventricle lactate levels in piglets after 2h hypoxia and 6h reoxygenation with early and delayed cyclosporine treatment. Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=6). *P<0.05 vs. controls.

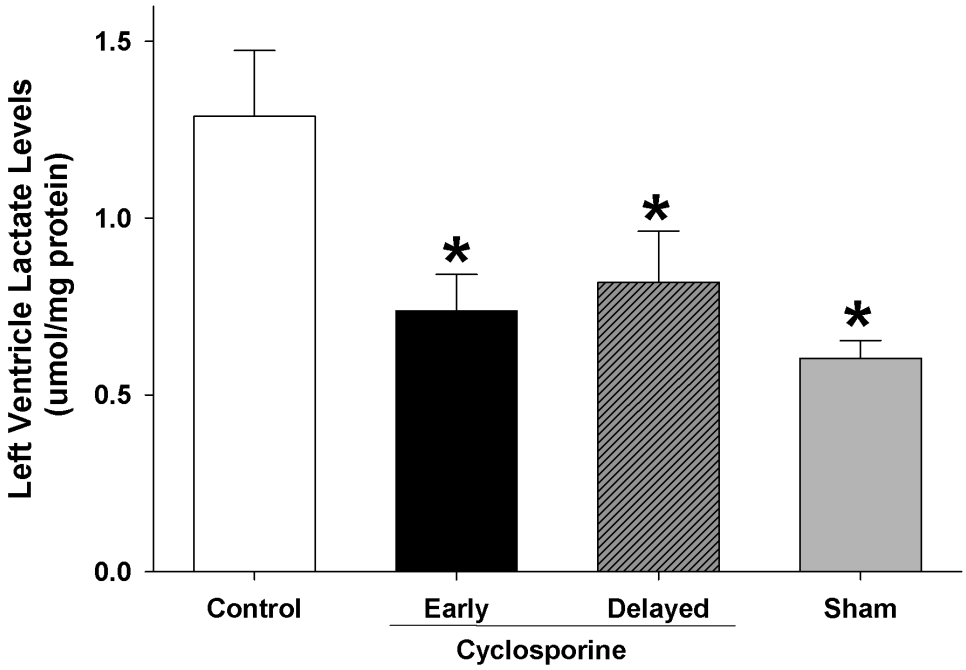


Figure 11-7. Systemic oxygen transport. (A) Systemic oxygen delivery and (B) systemic oxygen consumption during 6h of reoxygenation with early or delayed cyclosporine (10 mg/kg) treatment (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=4). *P<0.05 vs. controls, #P<0.05 vs. all H-R groups. Downward pointing black arrow represents "early" administration of Cyclosporine A. Downward pointing dark grey arrow represents "delayed" administration of Cyclosporine A.

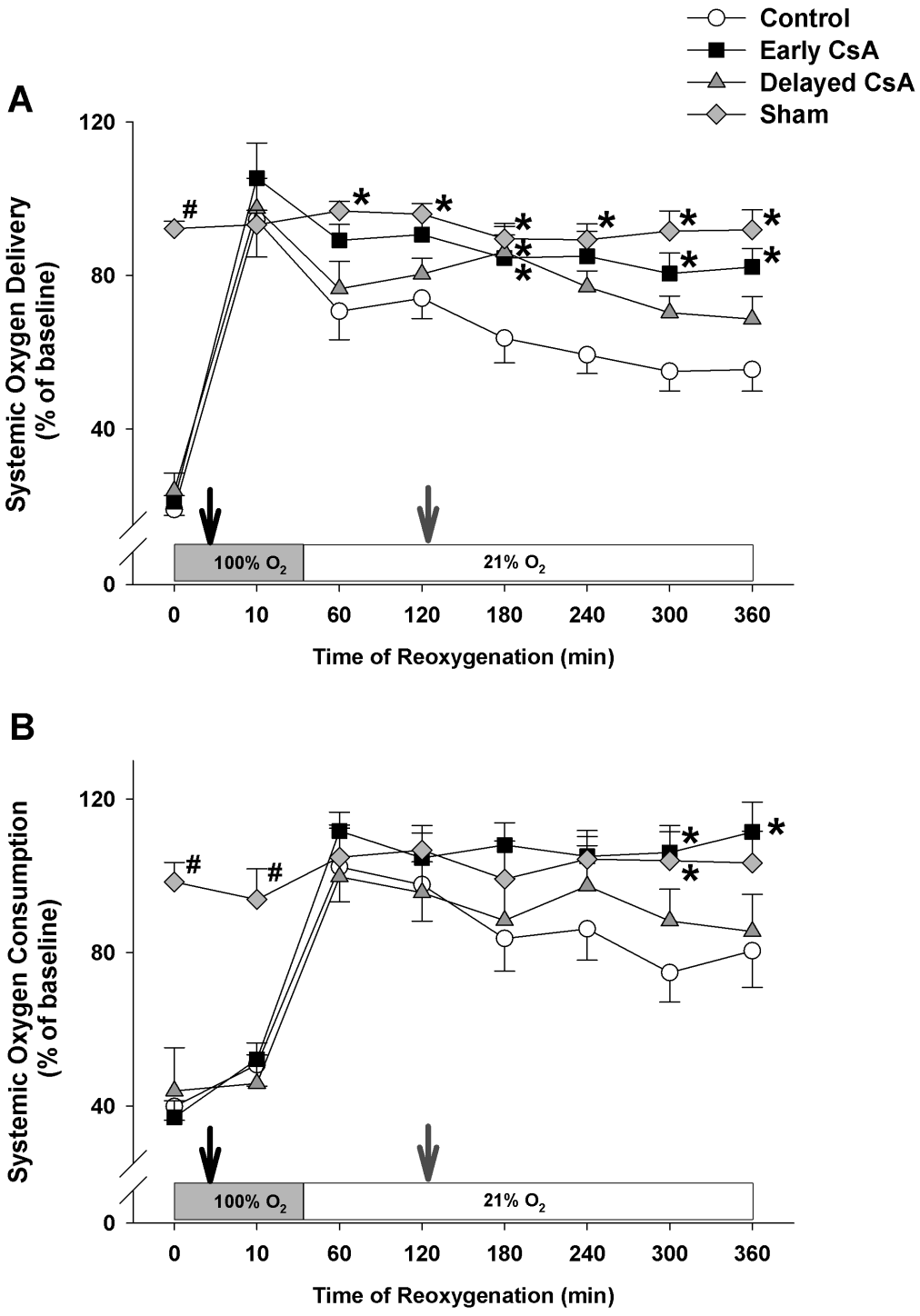


Figure 11-8. Intestinal Lactate Levels. Intestinal lactate levels in piglets after 2h hypoxia and 6h reoxygenation with early or delayed cyclosporine treatment (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=6). *P<0.05 vs. controls.

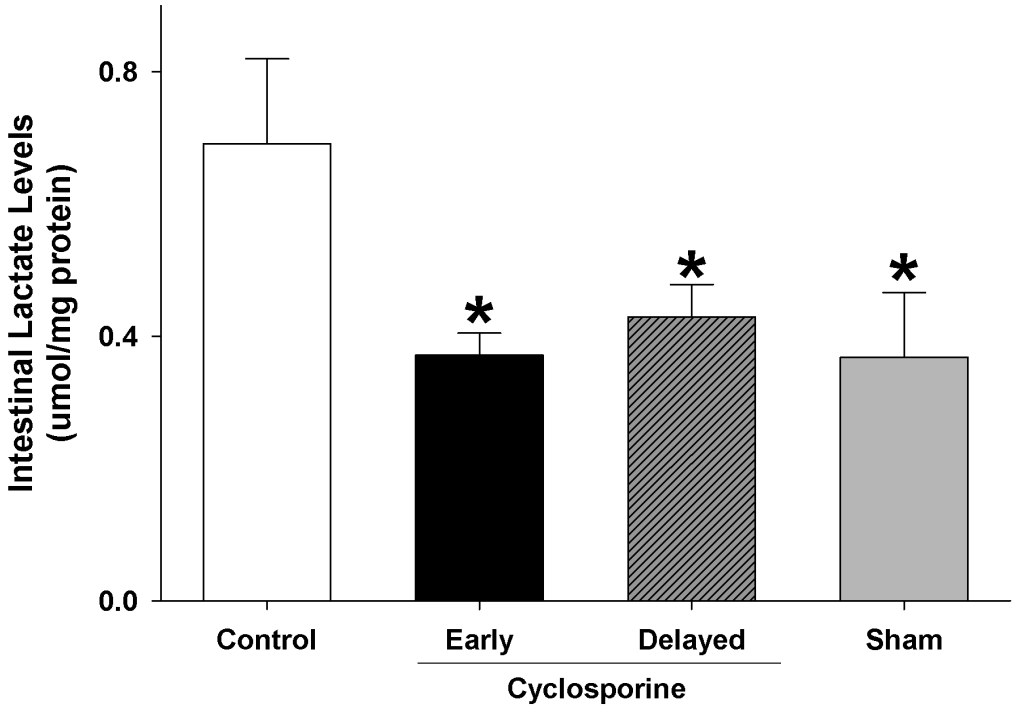
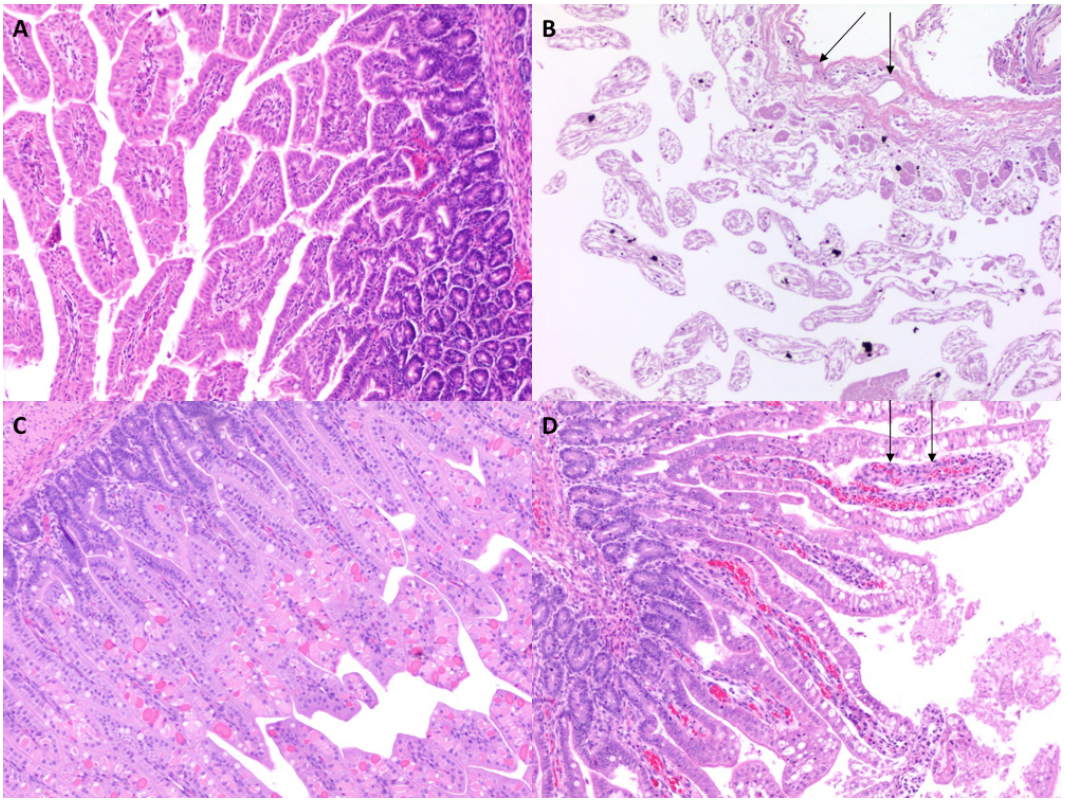


Figure 11-9. Intestinal histology. Representative histological features (hematoxylin and eosin stain) for piglets in **(A)** sham-operated, **(B)** control, **(C)** early cyclosporine treatment, and **(D)** delayed cyclosporine treatment groups following 2h hypoxia and 6h of reoxygenation with early or delayed cyclosporine (10 mg/kg) treatment (n=8 in each group). Control piglets received no cyclosporine (n=8). Sham-operated piglets underwent no hypoxia-reoxygenation (n=6). **Figure 4A** represents healthy intestinal specimen from sham-operated piglets (Parks grade = 1.7±0.8) which shows an intact villous, finger-like morphology with viable enterocytes and no ischemia changes in the submucosal layers, including submucosa, muscularis propria and tunica adventitia. **Figure 4B** represents control piglets (Parks Grade = 4.9±0.9) and reveals transmural necrosis with infarction of all layers of the bowel and formation of soma bullae in proximity and in the muscularis propria (arrows). **Figure 4C** represents early cyclosporine treated piglets (Park's Grade = 1.4±0.6), and shows villous morphology with single cell necrosis and protein accumulation, with no abnormalities seen in the submucosal layers. **Figure 4D** represent delayed cyclosporine treated piglets (Park's Grade = 2.9±0.7) and shows epithelial lifting along villous sides (arrows) indicating ischemia related change of the subepithelial tightness, while the submucosal layers show no ischemia changes. Original magnification: 100x.



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Chapter 12

The Role of Cyclosporine Treatment During the Resuscitation of Asphyxiated Newborn Piglets: Summary & Future Directions

Asphyxia remains a global health concern in the newborn population. Once asphyxia is diagnosed, the newborn is delivered and resuscitation is needed to save the newborn. Cardiac dysfunction and injury may occur in over 50% of these newborns following resuscitation. In newborn infants, this myocardial dysfunction may range from mild to severe, with possible cardiovascular collapse. Typically, postnatal management consists of supporting the failing myocardium with inotropic or vasopressive agents to maintain tissue perfusion to vital organs (brain, heart, etc.). However, recently the concept of preventing myocardial injury or preserving cardiac tissue in asphyxiated newborns has emerged. It is hypothesized that preserving precious cardiomyocytes during resuscitation, may lead to superior cardiac function and overall outcome. This seems a reasonable theory, considering reperfusion injury has been suggested to account for over 50% of the final injury to the myocardium. Therefore, limiting reperfusion injury may be a practical therapeutic goal. As detailed in Chapter 2, a similar strategy of limiting oxidative stress with room air resuscitation, rather than 100% oxygen, in asphyxiated newborns, has been associated with less myocardial injury and a lower mortality rate. Recently, substantial evidence indicates that cyclosporine has certain protective effects against hypoxic/ischemic injury in various adult models. We explored the possibility that cyclosporine treatment during resuscitation may also attenuate reperfusion/reoxygenation injury in asphyxiated newborn piglets and improve cardiac function and mitochondrial integrity.

The preceding chapters presented our findings following cyclosporine treatment during the resuscitation of asphyxiated newborn piglets. As outlined in Chapter 3, the formation of the cardiac mitochondrial permeability transition pore (MPTP) has been demonstrated during reperfusion/reoxygenation, leading to mitochondrial swelling, release of cytochrome-c and eventual cellular death. The strong inhibition of MPTP activation by cyclosporine in both *in vitro* and *in vivo* studies has suggested that it may have certain role in the clinical setting. Though a pilot human clinical trial has shown that cyclosporine treatment following myocardial infarction (secondary to coronary occlusion) just prior to revascularization of coronary vessels in adults may limit final infarct size, no such data exists in human newborns. Furthermore, the newborn myocardium is unique compared to its adult counterparts, in that it may be more vulnerable to apoptosis. Prior to clinical trials in the newborn population, animal studies are needed.

We used a newborn piglet model, in which piglets undergo alveolar hypoxia for 2h to simulate asphyxia in the clinical setting, in the newborn infant. Following hypoxia, the newborn piglets are reoxygenated for 4-6h. As detailed in Chapter 6, a single intravenous cyclosporine bolus immediately following reoxygenation did improve cardiac function of asphyxiated

newborn piglets. However, only piglets treated with cyclosporine at 2.5 and 10 mg/kg demonstrated attenuated myocardial injury (as indicated by plasma troponin levels). Further, piglets treated with cyclosporine at 10 mg/kg had significantly increased stroke volume at the end of 4h of reoxygenation. Interestingly, we also observed improved renal blood flow in piglets treated with cyclosporine at 10 mg/kg without increased tubular injury.

In the acute setting cyclosporine treatment during resuscitation improved cardiac function without worsening renal injury. Based on the above observations, we explored the effects of cyclosporine on cardiac mitochondrial integrity. Asphyxiated newborn piglets treated with the optimal dose of intravenous cyclosporine (10 mg/kg), also demonstrated lower oxidative injury markers in the myocardium. In addition, cytochrome-c release from the mitochondria, an early marker of apoptosis, was also attenuated in the cyclosporine treated piglets. Markers of mitochondrial enzymatic function, specifically aconitase activity were improved with cyclosporine treatment compared to controls. Overall ATP production and ATP/AMP ratio was also improved in the hearts of piglets treated with cyclosporine. Though we did not observe the direct activation of MPTP, these surrogate markers strongly suggest that cyclosporine treatment preserves mitochondrial functional integrity, resulting in better cardiac function.

Similar to those reported in adult swine, we also found that cyclosporine has a narrow therapeutic index, and beyond this, the cardioprotective effect of cyclosporine is lost. Therefore, we further examined the pharmacokinetics of cyclosporine after bolus injection following resuscitation of asphyxiated newborn piglets (Chapter 8). We observed similar pharmacokinetic profile of intravenous cyclosporine in asphyxiated and healthy newborn piglets and cyclosporine treatment with 25 mg/kg was outside the therapeutic index. Interestingly, there was a positive correlation between decreased myocardial injury based on plasma troponin levels and cyclosporine plasma concentration. These results suggested that the capacity to metabolize cyclosporine seems to be preserved in asphyxiated piglets. Therefore, the use of a single intravenous bolus of cyclosporine is well-tolerated. With no clinical data available on the use of intravenous cyclosporine in human newborn infants, this study provided insightful information on the pharmacokinetic profile in this patient population.

As discussed in Chapter 11, the therapeutic window of cyclosporine treatment during the resuscitation of newborn piglets is undefined. Following delivery of the asphyxiated fetus, resuscitation is usually started with 21% oxygen therapy. Intravenous access via the umbilical vein is needed prior to administration of resuscitative medications. Therefore the earliest administration of intravenous cyclosporine is a few minutes

following the onset of resuscitation. Administration of advanced resuscitative medications is performed by an experienced neonatal intensive care (NICU) team. Asphyxiated newborns delivered in rural or peripheral sites may have delay of up to 2h prior to receiving advanced resuscitative medications. Therefore, we used this premise to compare early and delayed cyclosporine treatment during resuscitation of asphyxiated newborn piglets. Interestingly, both early and delayed cyclosporine treated piglets had improved cardiac function compared to controls. However, early treatment with cyclosporine demonstrated a modest improvement in cardiac function compared to delayed treatment. These findings suggest that the therapeutic window may be a few hours following birth, however, earlier treatment with cyclosporine appears to be better in the short-term.

Overall, our results demonstrate that cyclosporine treatment as single intravenous bolus (10 mg/kg) during resuscitation of asphyxiated newborn piglets improves cardiac function, attenuates myocardial injury and preserves mitochondrial functional integrity. The optimal timing to administer cyclosporine is likely as soon as intravenous access is available. However, delayed administration may still be beneficial. These findings suggest that a clinical trial should be feasible. However, a number of limitations exist. Firstly, the duration of our experiments was between 6h to 8h, which is relatively short. It remains unknown if the observed cardioprotective effect of cyclosporine in asphyxiated newborn piglets will

persist. As myocardial injury and final infarct size may occur 24h to 48h following the initial hypoxic event, our experiments can only suggest an early cardioprotective effect of cyclosporine treatment. The acute duration of the experiments limits the clinical applicability of our results, which also limits direct translatability. However, we provide strong evidence that cyclosporine treatment may be beneficial in the short-term. Further, studies using chronically instrumented animals with survival models are likely needed to clarify the intermediate effects of cyclosporine treatment. Secondly, it remains a challenge to translate findings from *in vivo* animal studies to clinical trials. Though the newborn piglets are similar to human newborn infants in anatomy and physiology, direct translation is challenging. In addition, a majority of asphyxiated newborns with cardiac dysfunction are likely to have hypoxic-ischemic encephalopathy. Our model does not assess the neurological sequelae following asphyxia-reoxygenation. Long-term neurological development is an important end point that cannot be assessed in a non-survival acute swine model. Lastly, we initiated resuscitation with 100% oxygen for 30 min, instead of with room air as is currently recommended.

Though a clinical trial with intravenous cyclosporine treatment during resuscitation of asphyxiated newborn infants is the ultimate goal, further animal studies remain. Despite the fact that we did not any observe obvious side effects in our newborn piglet model, neuro- and nephrotoxicity of

cyclosporine with cumulative dosing in solid organ transplant has been extensively reported. The use of analogs of cyclosporine has been suggested to have equal or greater affinity for cyclophilin-D in the mitochondria, without the associated renal toxicity. However, these analogs have not been assessed in newborn animal models. The next phase of preclinical experiments should compare the cardioprotective effects of cyclosporine and one of its non-immunosuppressive analogs (NIM811) during resuscitation of asphyxiated newborn piglets. If NIM811 is also shown to have similar cardioprotective properties in newborn piglets, further experiments should focus on determining if the cardioprotective effects continue in a chronic newborn piglet experiment (>72h). These chronic experiments would clarify if the myocardial injury following hypoxia-reoxygenation is reduced and functional myocardium is preserved with cyclosporine treatment. In addition, these and other chronic experiments need to assess the neurological development in the asphyxiated newborn animal models. Nevertheless, intravenous cyclosporine treatment appears to have certain potential to preserve myocardium during resuscitation of asphyxiated newborn. An eventual clinical trial to determine the efficacy of intravenous cyclosporine during resuscitation of asphyxiated newborns remains our aspiration.