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The Use of Doxycycline in Asphyxiated Newborns

by

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Dedicated to my son,
Benjamin William LaBossiere

All Our Dreams Come True If We Have the Courage to Pursue Them

- Walt Disney -

Abstract

Asphyxia is a significant cause of newborn morbidity and mortality. Multiorgan injury and dysfunction is a common finding, mediated in part through an increase in matrix metalloproteinase (MMP) activity. We hypothesized administration of doxycycline, a known MMP inhibitor, would improve systemic and regional hemodynamics as well as attenuate myocardial, renal and intestinal injury and dysfunction in a clinically translatable newborn swine model of hypoxia-reoxygenation.

Newborn piglets were subjected to hypoxia-reoxygenation and received normal saline (control) or one of three doses of doxycycline five minutes into resuscitation. Doxycycline improved recovery of cardiac index, stroke volume index, systemic arterial pressure (SAP), systemic oxygen delivery and consumption with no effect on heart rate. Pulmonary artery pressure (PAP) and PAP/SAP were reduced, while renal artery flow index and oxygen delivery were improved. Markers of myocardial, renal and intestinal injury were attenuated with doxycycline and associated with a reduction in tissue oxidative stress and total MMP-2 activity. We conclude that in a clinically translatable newborn swine model of hypoxia-reoxygenation, early administration of doxycycline during resuscitation improves systemic and regional hemodynamics, and attenuates myocardial, renal and intestinal injury.

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Abbreviations

ANOVA	analysis of variance
$CADO_2$	carotid artery oxygen delivery
CAFI	carotid artery flow index
CAVRI	carotid artery vascular resistance index
CI	cardiac index
cTnI	cardiac troponin I
H-R	hypoxia-reoxygenation
IFABP	intestinal fatty acid binding protein
LPO	lipid hydroperoxides
MMP	matrix metalloproteinase
NAG	N-acetyl-β-D-glucosaminidase
$PaCO_2$	arterial partial pressure of carbon dioxide
PaO_2	arterial partial pressure of oxygen
$RADO_2$	renal artery oxygen delivery
RAFI	renal artery flow index
RAVRI	renal artery vascular resistance index
RONS	reactive oxygen and nitrogen species
SAP	systemic arterial pressure
SEM	standard error of the mean
$SMADO_2$	superior mesenteric artery oxygen delivery
SMAFI	superior mesenteric artery flow index
SMAVRI	superior mesenteric artery vascular resistance index
SVI	stroke volume index
$SysDO_2$	systemic oxygen delivery
$SysVO_2$	systemic oxygen consumption

Chapter 1

Neonatal Asphyxia

Introduction

Oxygen, a highly reactive non-metallic element, makes up only 21% of our atmosphere and yet is vital for human survival. It is the fuel that drives various metabolic and physiologic processes, and the body cannot be sustained for long periods without it. Indeed, the human body will experience significant damage, and eventually death, within minutes of oxygen deprivation.

Definition

Asphyxia is a broad term with many definitions that are generally applied to situations in which the body 'lacks oxygen.' The Greek meaning is translated as 'the stopping of the pulse,' an apt definition given the terminal consequence of untreated asphyxia. A common lay understanding is given in the Merriam-Webster dictionary, which defines asphyxia as a lack of oxygen or excess of carbon dioxide in the body that results in unconsciousness and often death and is usually caused by an interruption in breathing or inadequate oxygen supply.

Within the medical community, a proper definition of asphyxia is less agreed upon. Due to uterine contractions during the birthing process, all fetuses experience asphyxia to some extent.(1) However the vast majority of these fetuses will not experience any organ injury as a consequence of this event. Therefore, a condition of impaired gas exchange leading to hypoxemia (low oxygen content in the blood) and hypercapnia (high or excess carbon dioxide), as asphyxia is typically described, will often have no pathological significance.(2) However, persistent and severe asphyxia can result in organ damage and death, and this

persistent impairment in gas exchange will also cause for, through numerous mechanisms, an accumulation of fixed acids resulting in a metabolic acidosis. Thus, clinically significant asphyxia can be defined as a condition of impaired gas exchange resulting in the development of hypoxemia, hypercapnia and a metabolic acidosis.(2)

Although clinically significant asphyxia, particularly if severe and/or left untreated, usually results in damage to organs and potentially death, this is not always the case. A neonate could have signs of impaired gas exchange with an associated metabolic acidosis but no evidence of organ injury or dysfunction. To the layperson, the term asphyxia is often associated with a poor prognosis and/or long term complications, therefore labeling a child as asphyxiated under the aforementioned definition could invoke unneeded stress and hardship for parents. Thus, the medico-legal ramifications of mistakenly diagnosing a child as being asphyxiated must also be considered. In light of this, the clinical definition of neonatal asphyxia at birth, as per the American Academy of Pediatrics and the International Cerebral Palsy Task Force is as follows (the neonate must meet all of the following criteria): 1) umbilical cord pH <7 (metabolic or mixed), 2) APGAR score (Table 1-1) between 0 to 3 persisting longer than 5 minutes, 3) manifestations of neurologic injury (seizures, coma, hypotonia, etc.), and 4) multisystem organ dysfunction (injury to an additional organ system: cardiovascular, renal, intestinal, etc.).(1)

Epidemiology

Determining the number of neonatal deaths attributable to asphyxia is difficult as there is no universal consensus on a definition for this condition, and the majority of neonatal deaths occur in low to middle income countries that typically lack accurate reporting systems. As such, the incidence of neonatal asphyxia is likely underestimated.(3,4) Although rates of neonatal death have declined since the 1960's, it is estimated that there are approximately 4 million neonatal deaths worldwide, corresponding to roughly 30 neonatal deaths per 1000 live births.(5) Industrialized nations have significantly lower rates, owing to better access to superior health care. In Canada, the rate of neonatal deaths is approximately 4.6 deaths per 1000 live births.

Asphyxia is one of the leading causes of neonatal deaths worldwide.(6) Lawn et al looked at 192 countries, and based on mortality data and multi-cause modeled estimates, determined that asphyxia was responsible for 23% of all neonatal deaths, with infection (29%) and complications of preterm birth (29%) comprising the other two major causes.(5) It has also been noted that a reduction in neonatal deaths has come largely by improving the management of neonatal infections and complications of preterm birth, although advances in neonatal resuscitation have improved mortality (and potentially morbidity) related to asphyxia as well. Overall mortality in neonatal asphyxia is typically estimated between 10% and 20%, but can be as high as 60% depending on the severity of organ damage.(7) Morbidity related to neonatal asphyxia is much harder to estimate but likely much more prevalent.

Risk Factors

Multiple factors can contribute to the development of neonatal asphyxia, working either alone or in combination. Risk factors are generally grouped into three categories, with antepartum causes accounting for approximately 50% of asphyxia events, intrapartum 40%, and postpartum 10%.(8) (Table 1-2)

Examples of antepartum risk factors include maternal hypertension, sexually transmitted diseases, chronic placenta insufficiency, and premature rupture of the membranes. Regarding antepartum causes, Chandra et al found that pregnancy induced hypertension, fetal growth retardation (small for dates baby), and placental abruption all increased the risk of asphyxia.(9) Prior C-section, maternal age ≥ 35, prior neonatal death, and uterine rupture have also been described as antepartum risk factors.(10)

Examples of intrapartum causes include prolonged second stage of labour, vaginal breech delivery, elective Cesarean section delivery, chord prolapse, the use of general anesthesia, thick meconium and all cause abnormal fetal heart rate during delivery.(9,10)

Postpartum causes are much more rare and less described in the literature, and are typically related to congenital disorders of the cardio-respiratory system (congenital heart disease, congenital diaphragmatic hernia, etc), acquired infections, or neurologic disorders.(11)

Multiple Organ Effects

Asphyxia induces multiple changes to normal neonatal physiology. Bradycardia (low heart rate) occurs within minutes of an asphyxiating event,

resulting in a decreased cardiac output, hypotension (low blood pressure) and severe metabolic acidosis. Initial physiological responses are aimed at conserving adequate perfusion to organs most vital to survival including the brain, heart, and adrenal glands, typically at the expense of decreased blood flow to the other organs. This is accomplished through a variety of mechanisms that include increased shunting of blood through the ductus venosus, ductus arteriosus, and foramen ovale.(12) Thus, neonatal asphyxia has the potential to cause damage and dysfunction to many organs and may induce this injury in multiple organ systems concurrently. (Table 1-3) Up to 80% of neonates experiencing clinically significant asphyxia will exhibit some degree of organ damage.(13) The central nervous system, by definition, is always involved. Other systems that can potentially be involved include the cardiovascular, pulmonary, hepatic, renal and gastrointestinal system.

Central nervous system

Neurological injury from hypoxia, termed hypoxic-ischemic encephalopathy (HIE), is the most frequent and often devastating sequelae encountered in neonatal asphyxia. Mortality and morbidity rates range between 10% - 60% and 30% - 100% respectively, depending on the severity of neurological injury.(7)

HIE is a result of a deprivation of oxygen and glucose in the neural tissues resulting from a combination of hypoxia and ischemia, both which are present in asphyxia. The initial physiologic adaptation to asphyxia is to shunt cardiac output to the brain, heart and adrenal glands. Although initial cerebral blood flow can

increase by 30% - 175%, prolonged asphyxia ultimately leads to a decreased cardiac output and hypotension resulting in decreased cerebral perfusion.(7) The lack of oxygen and glucose converts normal cellular aerobic metabolism to less efficient anaerobic metabolism. As energy stores fall, a host of pathophysiologic events occur that lead to damage and necrosis of the nervous tissues. This includes a failure in ATP dependent transmembrane pump activity resulting in alteration of transmembrane cellular charge and the accumulation of calcium in the cytosol that leads to activation of phospholipases, proteases, and endonucleases. This results in tissue damage and necrosis through the destruction of vital intracellular proteins, interference of oxidative phosphorylation, and generation of oxygen free radicals.(7) Restoration of cerebral blood flow and oxygenation will reverse these pathophysiologic changes relatively quickly, however, a delayed secondary injury results during the resuscitation (reoxygenation) of the neonate due to a variety of mechanisms, including the generation of oxygen free radicals and the activation of matrix metalloproteinases (MMP).(14,15)

Historically, cerebral palsy has been the most commonly associated long–term neurological sequelae of neonatal asphyxia. Mounting evidence now suggests that a fairly high percentage of children with neurological injury due to an asphyxiating event have abnormal neurological conditions other than cerebral palsy. In a retrospective study of 40 children with a known neonatal asphyxiating event and documented neurological outcomes, Al-Macki et al described the spectrum of possible abnormal outcomes with neonatal asphyxia. 58% of the

children met criteria for cerebral palsy, compared to the remaining 42% who had a variety of other abnormal neurological conditions.(16) These conditions included global developmental delay, mental retardation, language impairments, autistic spectrum disorder, and epilepsy. In their study, the authors found that there are certain features in the neonatal period that were associated with the development of cerebral palsy. These features included a higher grade of encephalopathy (Table 1-4), increased number of seizures or use of anti-seizure medication, abnormal imaging of the CNS system, and an abnormal neurological exam. Other studies have also observed that the incidence of neurological deficits depends on certain factors during the asphyxiating event. In their systematic review of the long-term developmental outcome of asphyxiated term neonates, Dilenge et al found that the incidence of long-term neurological deficits was 0% for neonates with grade 1 encephalopathy, 30% to 50% for grade 2, and >90% for grade 3.(8) Interestingly, the authors also identified that the vast majority of studies focus on the development of moderate to severe long-term deficits, and that little is known in terms of minor neurological deficits such as learning disabilities and behavior disorders. Presumably asphyxiated neonates are at risk for the development of these disorders as well, and they may in fact be more common in occurrence compared to their more severe counterparts. One recent systematic review found that approximately 37% of neonates with HIE experience at least one neurobehavioral sequelae, with multiple sequelae being common. Effects on cognition (general developmental delay/learning disabilities) were most common (present in 45% of children who develop sequelae), followed by

cerebral palsy (29%), visual impairment/blindness (26%), gross motor impairments (17%), epilepsy (12%), deafness/hearing loss (9%) and behavioral problems (1%).(17)

Cardiac

Cardiovascular dysfunction, first described during the 1960's as a sequelae of neonatal asphyxia, is increasingly being recognized as a relatively common and potentially severe consequence of hypoxic-ischemic damage to the myocardium.(18,19) The degree of injury can range from a reversible, transitory myocardial ischemia to a more severe and permanent acute myocardial infarction. Clinical presentation therefore varies considerably, including having no findings (asymptomatic), respiratory distress with tachypnea, murmurs (typically tricuspid valve regurgitation) and extending to more severe findings of congestive heart failure and contractile dysfunction resulting in cardiogenic shock with resultant systemic hypoperfusion.(18,20) Although myocardial damage secondary to ischemic injury in adults is well understood and easily diagnosed, the diagnosis in neonates is much more difficult. This, coupled with the varying clinical picture of cardiac dysfunction described above, has made the prevalence of cardiac involvement in neonatal asphyxia difficult to quantify. Prevalence rates in the literature range from approximately 30% to as high as 80% depending on the definition of cardiac involvement used by the authors. (18,20)

Many methods to diagnosis ischemic injury in neonatal asphyxia have been studied and are used in clinical practice to varying degrees, including the use of biochemical markers such as troponin I or T (TnI, TnT) and creatine kinase-

MB isoenzyme (CK-MB), electrocardiogram (ECG) and echocardiogram (Echo) findings. In adults plasma troponin I levels have been validated as a marker of myocardial injury and as a reliable prognosticator of future outcomes.(21,22) In neonates, however, no true consensus exists regarding which markers should be utilized in the diagnosis of myocardial injury in the asphyxiated neonate. In their study of 50 newborn infants with varying degrees of hypoxia (Group 1 – healthy, Group 2- mild respiratory distress with moderate hypoxia, Group 3- neonates meeting criteria for asphyxia), Barberi et al found that elevation in CK-MB, Grade 3 and 4 ECG findings (Table 1-5), and Echo findings of left ventricle fractional shortening and reduced peak aortic velocity (indicative of left ventricular dysfunction) where observed in asphyxiated neonates (Group 3).(23) Furthermore, the degree of CK-MB elevation correlated with a poorer prognosis. The authors concluded that severely asphyxiated neonates demonstrate biochemical, ECG and echo findings that reflect ischemic myocardial injury, and that an interplay of these modalities can reliably detect and grade myocardial damage. In slight contrast, Rajakumar et al studied 60 neonates (30 healthy controls, 30 with asphyxia) and found that the specificity and sensitivity of TnT in diagnosing and quantifying the degree of myocardial injury in neonatal asphyxia was 82.6% and 97.3% respectively, much better than CK-MB which had a specificity and sensitivity of 56.5% and 75.7% respectively. The authors also found that increasing levels of TnT correlated with the severity of cardiac dysfunction as well as mortality, and that no such relationship existed with CK-MB.(24) In another study of 13 asphyxiated neonates, Trevisanuto et al observed

that TnI is also a good marker of myocardial injury in asphyxiated neonates, with a specificity and sensitivity of 97% and 77% respectively,(25) a finding supported by another study of 34 asphyxiated neonates with varying degrees of HIE that found higher TnI levels were a significant predictor of mortality verses CK-MB which did not demonstrate any predictive value.(26) Despite these findings, however, the use of currently used cardiac biomarkers to diagnose cardiac injury in newborns is debated, as highlighted by a recent review in which the authors conclude that there is insufficient evidence at present to promote the use of current cardiac biomarkers in newborns.(27) To summarize the current understanding, diagnosis of myocardial injury in neonatal asphyxia is difficult and likely requires a multi-model approach including biochemical, ECG and echo findings. TnI and TnT appear to be the best available biochemical markers of myocardial injury in neonatal asphyxia at present compared to the traditionally used CK-MB enzyme, but though routinely used, their clinical utility in newborns is debatable. CK-MB has been largely discarded as a marker for myocardial injury during asphyxia by most centers as levels can vary considerably with gestational age, sex, mode of delivery and birth weight.(25)

Although cardiac dysfunction does contribute to increased mortality in neonatal asphyxia, some studies suggest that normal cardiac function is typically restored in asphyxiated neonates who survive.(24) Yet evidence is emerging which suggests that cardiac injury may contribute to the development and/or aggravation of other conditions observed in neonatal asphyxia, specifically HIE. As previously described, HIE severity can vary in neonatal asphyxia and is a

common cause of significant short and long term morbidity as well as increased mortality. The primary mechanism in the development of HIE is brain hypo-perfusion through a variety of mechanisms as described, including hypo-perfusion secondary to decreased cardiac output. In a retrospective study of 60 neonates with hypoxic ischemic encephalopathy, Shastri et al observed that TnI levels correlated strongly with the clinical grade of HIE as well as with the duration of inotropic support (which in their study reflected the degree of myocardial injury). Thus, the degree of myocardial injury correlated with the severity of HIE.(28) Liu et al prospectively followed 40 newborns with HIE and 30 healthy controls for the first 14 days of life, assessing myocardial function and cerebral hemodynamic parameters throughout the study. They found that the severity of myocardial dysfunction correlated with the grade of encephalopathy the neonate would suffer.(29) The conclusions from theses studies and others highlight the importance of preserving cardiac function and treating cardiac dysfunction in asphyxiated infants.

Pulmonary

The neonatal pulmonary circulation undergoes a significant, near instantaneous change, at birth. The cumulative effects of these changes are to convert the pulmonary circulation from a high resistance state *in utero* to a low resistance circuit to enable adequate perfusion.(30) The hypoxia and acidosis present in neonatal asphyxia increases the pulmonary vascular resistance, thus modifying the normal physiological adaption that occurs at birth. This modification results in cardiac dysfunction and further exacerbates hypoxia due to

a right to left shunting of deoxygenated blood into the systemic circulation (persistence of fetal circulation), clinically referred to as persistent pulmonary hypertension of the newborn.(30)

Persistent pulmonary hypertension of the newborn primarily effects term, or near term neonates, with an estimated incidence of 2 per 1000 live births.(31) Persistent pulmonary hypertension of the newborn can be associated with meconium aspiration syndrome and respiratory distress syndrome, and includes a spectrum of mild to severe respiratory distress symptoms in terms of clinical presentation. Hyypoxia remains a common cause of persistent pulmonary hypertension of the newborn. Other causes include meconium aspiration pneumonia, early sepsis, hypoglycemia, and polycythemia. Mortality rates were traditionally quite high, up to 34% in the 1980's, though with improvements in management and respiratory support, current mortality from persistent pulmonary hypertension of the newborn is estimated to be lower than 10%.(31)

Hepatic

Within minutes of an asphyxiating event liver perfusion substantially decreases due to the initial circulatory responses that during an asphyxiating episode that includes shunting of the blood to 'more vital' organs such as the brain, heart, and adrenal glands. Although intuitively hepatic injury can be expected, and despite the increasing literature to support this claim, hepatic injury is typically not considered in the multi-organ involvement criteria for neonatal asphyxia.(12)

Ischemic hepatitis is typically seen in the adult population and occurs when there is a reduced blood flow to the liver secondary to cardiac failure or reversible hypotension. In association with a decreased cardiac output (and in the absence of a viral hepatitis), ischemic hepatitis is defined by an early, sharp rise in serum transaminase (alanine aminotransferase, aspartate aminotransferase) levels which will resolve within several days to a week once perfusion is restored.(32) Hypoxemia is a much more rare cause of this condition.(12) The criterion for hepatic injury in neonatal asphyxia therefore varies between study groups. In general, hepatic injury can be defined by an increase in transaminase levels >100 IU/L at any time during the first week after birth.(33)

In an attempt to characterize hepatic injury during neonatal asphyxia, Tarcan et al, using an alanine transaminase level of greater >100 U/L (greater than twice the upper limit of normal) to define hepatic injury, retrospectively studied 56 newborns with perinatal asphyxia. They found that 39% of the newborns had hepatic injury, and this injury was associated with a higher mortality rate, with 22.7% deaths occurring in neonates with hepatic injury compared to 2.9% in those without.(12) Additionally, the authors found that factors strongly associated with hepatic injury included fetal distress, thrombocytopenia, convulsions, pathological findings on central nervous system imaging, and presence of intrauterine growth retardation.(12) Another study followed 26 asphyxiated neonates with the objectives to assess for the occurrence of hypoxic hepatitis, the temporal transaminase levels following asphyxia and whether the degree of hepatic injury correlated with the severity of central nervous system injury. The authors

observed hepatic injury in two-thirds of the asphyxiated neonates. Furthermore, they found that elevation in AST and ALT levels resembled the pattern seen in classic hypoxic hepatitis seen in adults. Finally, they observed a correlation between transaminase levels and the extent of central nervous system injury.(32)

Renal

The neonatal kidney is physiologically different from an adult kidney and highly susceptible to hypo-perfusion injury during asphyxia. Differences include a higher renal vascular resistance, lower glomerular filtration rate, decreased intercortical perfusion, decreased re-absorption of sodium in the proximal tubules, and a higher plasma renin activity compared to adults.(34) Taken together, neonatal kidneys are more susceptible to acute tubular or cortical necrosis when hypo-perfused as compared to adults.

Causes of neonatal acute kidney injury and failure include respiratory distress syndrome, dehydration, sepsis, asphyxia, congestive heart failure, and nephrotoxic drugs. One of the most common causes of acute kidney injury in the neonate is asphyxia. A retrospective study on 151 neonates with acute kidney injury found that perinatal asphyxia was the most commonly associated risk factor found in neonates with acute kidney injury (present in 29.8% of patients).(34) However, the incidence of acute kidney injury in neonatal asphyxia is difficult to quantify given there is no agreed upon definition at present.(35) In adults, acute kidney injury is typically defined by an elevation in the serum creatinine and/or a decreased (oliguria) or absent (anuria) urine output. In neonates the criteria have not been well established. Some authors advocate for the use of both parameters

in the neonate. Shah et al in assessing multi-organ dysfunction in neonates with post-asphyxia hypoxic-ischemic encephalopathy used oliguria/anuria and elevated serum creatinine to define kidney injury during neonatal asphyxia.(33) However, many authors disagree, and have found that serum creatinine is not a good marker for renal dysfunction as neonatal levels of serum creatinine often reflect maternal levels (which can take weeks to resolve) and are typically elevated only with significant kidney injury.(36) Furthermore, oliguria/anuria is also considered a poor marker of renal dysfunction, as up to 50% of neonatal renal failure occurs without a decrease in urine output.(35)

Excreted low molecular weight proteins such as β_2-microglobulin, retinol binding protein, and N-acetyl-β-D-glucosaminidase (NAG) have been proposed as a way to detect renal tubular dysfunction, and are among other biomarkers currently being investigated.(35,36) Aggarwal et al performed a prospective case control study on 25 asphyxiated neonates matched to 25 healthy neonatal controls and found that excretion of β_2-microglobulin and NAG was higher in asphyxiated neonates on day 2 and 4 post-birth. 68% of asphyxiated neonates in their study had AKI kidney injury with only 42% of those neonates having oliguria.(37) Despite the lack of an agreed upon definition, most studies report an incidence of acute kidney injury in approximately 30%-56% of asphyxiated neonates.(35)

Data describing morbidity and mortality from acute kidney injury in neonatal asphyxia is also extensively lacking. Factors associated with a worse prognosis include oliguric renal failure and the need for dialysis.(35) Mortazavi et

al observed a 20.5% mortality rate in neonates with all cause acute kidney injury.(34)

Intestinal

Necrotizing enterocolitis is a severe inflammatory disorder of the intestine that occurs in approximately 1/1000 live term births and in up to 7% of premature babies. Histologically the affected intestine is characterized by severe inflammation and coagulation necrosis. Clinically the condition can result in gastrointestinal perforation and sepsis that can be fatal. In survivors, long-term complications such as short bowel syndrome, abnormal growth, and neuro-developmental delays are observed. Necrotizing enterocolitis remains the leading cause of death in the neonatal intensive care unit.(38)

In 1969, Lloyd attempted to link the intestinal circulation to the pathogenesis of necrotizing enterocolitis.(39,40) He noted a strong association between severe neonatal asphyxia and gastrointestinal perforation, and hypothesized that intestinal hypo-perfusion (ischemic-reperfusion injury) as a result of asphyxia resulted in intestinal damage that ultimately resulted in gastrointestinal perforation. The redistribution of cardiac output, the shunting of blood to the brain, heart and adrenal glands seen in asphyxia became known as the "diving reflex," modeled after diving mammals that also demonstrated such a circulatory redistribution during deep dives.(39)

For a time after this discovery neonatal asphyxia was thought to be a major factor in the development of necrotizing enterocolitis. However subsequent animal and human studies have failed to validate this claim, as the ischemia that

leads to necrotizing enterocolitis has not been significantly associated with neonatal asphyxia.(39) Today necrotizing enterocolitis remains a poorly understood entity. Pathogenesis is likely multi-factorial in nature, involving an over-reactive immune system that targets the intestine leading to increased inflammation, intestinal permeability, bacterial translocation, hypoxic-ischemic injury, and eventually sepsis.(38) Although hypoxic-ischemic injury is generally accepted as a contributor to the pathogenesis of necrotizing enterocolitis, the timing of the hypoxia-ischemia during in the pathophysiologic spectrum is uncertain.(41)

Young et al in their review describes the current understanding of how ischemia-reperfusion relates to neonatal intestinal injury. Intestinal coagulation necrosis seen on histology may arise from factors that disrupt endothelial function, altering the ratio of nitric oxide (a vasodilator) and peptide endothelin-1 (a vasoconstrictor) resulting in increased vasoconstriction leading to intestinal ischemia. These factors include hypoxic-ischemia injury, release of pro-inflammatory mediators, and persistent low flow states.(42) Although ischemic-reperfusion injury likely plays a role in the pathogenesis of necrotizing enterocolitis, asphyxia itself, previously considered a significant contributor, appears to be a lesser factor in the development of necrotizing enterocolitis. This statement is supported by the results of a recently published experiment performed by Mannoia et al, who prospectively followed 55 premature neonates and assessed the development of intestinal ischemia by measuring a urinary marker for this condition, intestinal fatty acid binding protein (iFABP). The

authors observed that the degree of iFABP elevation was significantly associated with the development of necrotizing enterocolitis, however, asphyxia was not.(43)

Current Management of the Asphyxiated Neonate

The current management of the asphyxiated neonate varies by center, with new methods of intervention such as hypothermia still under debate. At present, other than hypothermia, the treatment provided to the asphyxiated neonate is mostly supportive in nature, that is, aimed at correcting the asphyxiated state and minimizing further damage to the organs with the hope of facilitating repair and recovery. Neonatal asphyxia can involve multiple organ systems, thus supportive care to the neonate, including monitoring for and intervention of altered physiologic homeostasis, is complex in nature and demands the resources of a neonatal intensive care unit.

Respiratory care in the form of machine ventilation is primarily aimed at correcting hypoxia and hypercapnia. Oxygenation and ventilation parameters are continuously monitored in the neonatal intensive care unit. The present recommendation is to resuscitate with 21% oxygen and titrate up as necessary to achieve oxygenation, while adequate ventilation is defined by avoiding hypo-or-hypercapnia.(14) The shift in guidelines from 100% oxygen use to 21% oxygen owes to the observation that neonates resuscitated with a fraction of inspired oxygen (FIO_2) of 100% have higher mortality rates than those resuscitated with an FIO_2 of 21%, a finding that reflects an increased production of reactive oxygen and nitrogen species (RONS), collectively referred to as increased oxidative stress, with higher levels of FIO_2.(44)

Cardiovascular support is initiated in response to evidence of myocardial dysfunction. Reduced myocardial contractility resulting in hypotension is treated with inotropic and chronotropic drugs, such as dopamine or dobutamine, which improve myocardial contractile function. Hypotension secondary to hypovolemia is treated with fluid administration.

Metabolic disturbances are frequent in the asphyxiated newborn, including acidosis, electrolyte abnormalities and hypoglycemia. Correction of these metabolic derangements, including the direct replacement of electrolyte, fluid, or glucose, is crucial to avoiding further injury and to facilitate recovery. Dialysis may be necessary depending on the degree of renal injury and the severity of metabolic disturbances.

HIE can result in seizure activity, and current understanding is that these seizures are not only a reflection of hypoxic-ischemic neuronal injury, but may also exacerbate further injury to the brain.(14) Although there is no current evidence to suggest the use of prophylactic anticonvulsant use in HIE, anticonvulsants including phenobarbital and lorazepam, are used in response to demonstrated seizure activity. Unfortunately the success of these drugs are limited, with monotherapy success rates at approximately 50% and increasing only to 60% with the addition of a second anticonvulsant medication.(14) Newer anticonvulsants are being studied, however, randomized control trials are lacking at present and they are therefore currently not recommended in HIE.

A common theme in the treatment of the asphyxiated neonate is the failure to prevent organ injury from occurring in the first place, or to attenuate the degree

of injury, instead adopting a supportive role and responding to altered physiological states (seizures, myocardial contractile dysfunction, renal injury, etc) accordingly. Treatments that attempt to prevent injury and/or attenuate the degree of injury are currently being investigated. One such example is the use of hypothermia in HIE. Hypothermia is aimed at reducing or preventing the secondary brain injury seen in ischemic-reperfusion through the reduction of metabolic demands, reduced accumulation of excitotoxic amino acids, and suppression of reactive oxygen and nitrogen species formation and the inflammatory cascade.(14) Current 'pro-active' therapies for other organ systems involved in neonatal asphyxia are non-existent.

Conclusions

Neonatal asphyxia is a significant concern and among the leading causes of newborn morbidity and mortality worldwide. Even in developed countries with advanced health care systems, asphyxia remains a burden to the health of newborns. In Canada alone, the incidence of birth asphyxia is approximately 2.4 per 1000 live births.(45) Management of asphyxia in the newborn is challenging for a variety of reasons: multiple organ systems are typically involved, consensus guidelines for the diagnosis of organ injury in newborns are scarce, and treatments are largely reactive, failing to prevent or attenuate injury that occurs during the resuscitation of an asphyxiated neonate. There exists a great potential and need, then, to research methods of diagnosing organ injury and dysfunction in asphyxiated newborns, and to design and test novel interventions aimed at attenuating or preventing organ injury from occurring in the first place.

Table 1-1: Determinates of the APGAR score

A score of 0, 1 or 2 is assigned to each clinical sign. The score is the sum of the values obtained at 1 and 5 min, with a score of 7 or greater indicating excellent health.(1)

	0	1	2
Heart Rate	Absent	Below 100	Above 100
Respiratory Effort	Absent	Slow, irregular	Good, crying
Muscle Tone	Flaccid	Some Flexion of Extremities	Active motion
Reflex Irritability	No Response	Grimace	Vigorous Cry
Color	Pale	Cyanotic	Completely Pink

Table 1-2: Risk factors associated with neonatal asphyxia (9-11,46-49)

Antepartum	Intrapartum	Postpartum
Maternal chronic illness	Prolonged second stage of labour	Fetal central nervous system disorders (e.g. congenital muscular dystrophy)
Maternal hypertension	Breech delivery	Cardiovascular disorders (e.g. congenital heart disease)
Pregnancy induced hypertension	Instrument-assisted delivery	Respiratory disorders (e.g. respiratory distress syndrome)
Maternal Infections (including sexually transmitted disease)	Premature rupture of the membranes (PROM)	Drug intoxication
Chronic placenta insufficiency	Presence of meconium	Infection (e.g. meningitis)
Maternal age < 16 or >35	Elective C-section	Child abuse
Previous fetal/neonatal death	Umbilical chord prolapse	
Fetal growth retardation	Use of general anesthesia	
Multiple gestations	Abnormal fetal heart rate	
Maternal substance abuse	Uterine rupture	
Fetal malformation	Abruptio placentae	
No antenatal care	Placenta previa	
Vaginal Bleeding		

Table 1-3: Acute and chronic consequences of asphyxia in the neonate (7,11,12,16,18,19,30)

Central nervous system	Hypoxic-ischemic encephalopathy Cerebral palsy Seizure disorders Global developmental delay Language impairment Autistic spectrum disorder
Cardiovascular system	Myocardial stunning Myocardial infarction Congestive heart failure Persistent fetal circulation
Respiratory system	Persistent pulmonary hypertension of the newborn Respiratory distress syndrome
Hepatic system	Hypoxic hepatitis
Renal system	Acute tubular necrosis Acute cortical necrosis Chronic kidney disease
Gastrointestinal system	Necrotizing enterocolitis

Table 1-4: – The Sarnat and Sarnat post-anoxic encephalopathy classification scheme (14)

	Grade 1	Grade 2	Grade 3
Level of Consciousness	Hyper-alert	Lethargic/Obtunded	Stuporous
Muscle Tone	Normal	Mild Hypotonia	Flaccid
Posture	Mild Distal Flexion	Strong Distal Flexion	Intermittent Decerebration
Stretch Reflexes	Overactive	Overactive	Decreased/Absent
Moro Reflex	Strong; Low threshold	Weak/Incomplete; High Threshold	Absent
Suck Reflex	Weak	Weak/Absent	Absent
Autonomic Function	Generalized Sympathetic	Generalized Parasympathetic	Both systems decreased
Seizures	None	Common: focal or multifocal	Uncommon

Table 1-5 – ECG criteria for myocardial ischemia in neonates (50)

	Grade 1	Grade 2	Grade 3	Grade 4
T- Wave	Flat/Inverted in 1 or 2 Leads	Flat/Inverted in ≥3 Leads (Except AVR)	Flat/Inverted in ≥3 Leads (Except AVR) and either	Classic segmental infarction pattern
ST	Normal	Normal	Depression or elevation > 2mm in ≥2 chest leads or >1mm in ≥2 Standard Leads	Markedly elevated or complete left bundle branch block
Q-Wave	Normal	Normal	Duration >0.02 seconds or amplitude >25% of R wave in 1 anterior or 3 related chest leads	Duration >0.02 seconds or amplitude >25% of R wave in 1 anterior or 3 related chest leads

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Chapter 2

Hypoxia-Reoxygenation

Introduction

Organ injury in asphyxia is complex, owing to a variety of pathologic mechanisms. These mechanisms can be broadly classified into two distinct temporal insults: injury that occurs due to the hypoxic event itself, with a second injury occurring during reoxygenation of the hypoxic neonate. These temporal mechanisms are collectively referred to as hypoxia-reoxygenation (H-R) injury, a term often used interchangeably with neonatal asphyxia.

Hypoxia

There are immediate physiologic changes that take place during the acute phase of asphyxia as the body attempts to preserve 'function' in organs considered vital to neonate survival. This is accomplished through hemodynamic and metabolic 'centralization', as blood flow (and the oxygen/nutrients contained within) is directed preferentially to the heart, brain and adrenal glands at the expense of other organ systems.(1,2) As asphyxia progresses, oxygen demand in these 'centralized' organs surpasses delivery, aerobic metabolic processes progressively decline while anaerobic metabolism increases. Peripherally, anaerobic metabolism is already well established as hypoxia is further exacerbated by the decreased perfusion resulting from the initial 'centralizing' adaptation. As anaerobic metabolism is less efficient than aerobic metabolism, cellular energy sources fall, and cell energy demand eventually surpasses supply, resulting in cellular organ dysfunction and ultimately cell death. Furthermore, plasma levels of lactic acid, a byproduct of anaerobic metabolism, increase with several pathophysiologic consequences, including a decrease in peripheral

vascular resistance which results in the loss of preferential perfusion to the brain, heart and adrenal glands and further exacerbates injury to these organs.(2)

The degree of myocardial injury and dysfunction contributes significantly to mortality during the hypoxic insult.(2) As production of adenosine triphosphate (ATP), the primary energy source of cells, falls during hypoxia cellular supply-demand mismatch steadily rises leading to cellular (and thus contractile) dysfunction. Furthermore, decreased ATP synthesis can lead to cell death through the induction of apoptotic pathways.(3) Dysregulation of Na^+/H^+ as well as Na^+/Ca^{2+} exchangers also occurs resulting in increased intracellular Ca^{2+} accumulation and the activation of Ca^{2+} dependent proteases and phospholipases.(4) This results in the destruction of vital intracellular proteins and inhibition of oxidative phosphorylation resulting in cellular dysfunction and necrosis. An increased level of adenosine, produced from the cardiac myocyte and endothelial cells during hypoxia, reduces the sensitivity of the myocardium to adrenergic stimulation and further worsens contractile function. Although several neural and humoral changes, including an increase in sympathetic activity and release of the positive inotrope apelin, occur to counteract a decrease in myocardial contractility, these counteracting mechanisms eventually fail as myocycte injury and contractile dysfunction progress.(3)

In the brain glucose is the primary energy source for the neurons. As in the heart, a transition from aerobic to anaerobic metabolism occurs leading to increase lactate levels and a decrease in ATP production resulting in a supply-demand mismatch. As neuronal metabolism fails there is an increase in the release

of inflammatory cytokines and excitatory neurotransmitters (such as glutamate), voltage-gated calcium channels are opened and N-methly-D-aspartate (NMDA) receptors are stimulated.(2,5) This leads to a variety of pathologic changes in the cell including increased concentration of calcium in the cytoplasm and activation of apoptotic pathways, ultimately leading to neuronal death.

Similar metabolic derangements with subsequent organ injury and dysfunction are observed in other organ systems as well. Glomerular filtration rate decreases in the kidneys as hypoxia progresses and an abnormal release of adrenal hormones occurs, as characterized by an increase in catecholamine (norepinephrine, epinephrine) production and a decreased release of cortisol.(2) Similar physiologic adaptations are observed in these organ systems as well, and much like in the heart and brain, these adaptations will ultimately fail and organ injury and dysfunction will progress in a relatively short period of time if the hypoxic insult is not corrected.

Reoxygenation

Hypoxia can rapidly cause significant organ injury and dysfunction, ultimately leading to death if left untreated. As the causes of asphyxia are varied, so too are the interventions aimed at reversing tissue hypoxia; however, the ultimate goal in all cases is to restore oxygen to the system. Though this is a relatively intuitive concept, past experience has demonstrated that this goal is not so simple. Since the 1950's it has been observed that tissue injury is often worsened during reoxygenation after hypoxia, and this phenomenon was justifiably referred to as the "oxygen paradox."(6) Before 2010 it was a common

practice to resuscitate asphyxiated neonates with a fraction of inspired oxygen (FIO_2) of 100%, as it was believed that higher concentrations of oxygen would more promptly reverse tissue hypoxia and halt its deleterious effects. However, recent evidence has demonstrated that there is not only a higher degree of organ injury when an asphyxiated neonate is resuscitated with 100% oxygen compared to room air (7) but that there is an increase in mortality as well.(8-10) Current clinical guidelines (as of 2010) therefore recommend the use of room air when resuscitating asphyxiated neonates.(11)

It is known today that the injury observed during reoxygenation of the hypoxic tissues is related primarily to the generation of reactive oxygen and nitrogen species (RONS) and studies have demonstrated an increase in RONS generation (increased oxidative stress) when neonates are resuscitated with higher FIO_2's.(7,12,13)

Reactive oxygen and nitrogen species

RONS are partially reduced derivatives of molecular oxygen and nitrogen that have varying degrees of chemical reactivity and which have a variety of biological targets. Although RONS do have important physiologic functions within the human body, RONS also negatively interact with a host of biological targets which include lipids, proteins and DNA, and these pathologic interactions can ultimately result in cellular dysfunction.(14)

RONS have been implicated in the pathogenesis of various neonatal diseases including chronic lung disease, retinopathy of prematurity, and some

inflammatory conditions.(15) As mentioned, RONS are now understood to play a significant role in the pathogenesis of neonatal asphyxia. In their study of 50 human term newborns with perinatal asphyxia, Kumar et al observed higher degrees of oxidative stress compared to eight healthy infants serving as controls. The authors concluded that RONS may play a significant role in the pathogenesis of perinatal asphyxia, a claim that is consistent with the current literature.(16)

There exist many different RONS, yet only a small number are important to biological systems, including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and peroxynitrite ($ONOO^-$).(17,18) These RONS can be generated in a variety of cells through many different pathways, the most important being reactions catalyzed by the enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase (XO) as well as through the generation of nitric oxide (NO) via three isoforms of nitric oxide synthase (NOS).(14,18,19) NADPH oxidase uses NADPH as an electron donor, catalyzing a one-electron reduction of molecular oxygen to form $O_2^{\cdot -}$. (Figure 1-1)(18,19) The oxidation of xanthine by molecular oxygen to form $O_2^{\cdot -}$ and uric acid is performed through the catalyst XO. (Figure 1-1) (18,20) Both of these pathways produce $O_2^{\cdot -}$ as the predominate RONS which can then be involved in further subsequent reactions that produce other biologically relevant RONS. One of the body's natural defenses against $O_2^{\cdot -}$ is the enzyme superoxide dismutase (SOD), which exists in three different forms (extracellular, nuclear, and mitochondrial).(14) SOD catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2, (18,19) and the generated H_2O_2 then has the potential to react with iron (Fe^{2+}) or copper (Cu^+)

to form the highly reactive oxygen species $OH^{\cdot}$, referred to as the Fenton reaction. (Figure 2-2) (18,20) $OH^{\cdot}$ can also be generated in a Haber-Weiss reaction, which combines the reduction of iron (Fe^{3+}) or copper (Cu^{2+}) by $O_2^{\cdot -}$, which generates Fe^{2+}or Cu^{+} for use in a Fenton reaction. (Figure 2-2)(20) Finally, $OH^{\cdot}$ can be generated from a reaction between $ONOO^{-}$ and H^{+} to form peroxynitrous acid (ONOOH), which decomposes to $OH^{\cdot}$ and $NO_2^{\cdot}$.(21)

NO plays a significant role in the formation of the RONS $ONOO^{-}$ during reoxygenation. NO is generated through three different isoforms of NOS which include endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). NO production varies by organ system and in response to a variety of stimuli. For example, NO production in the normal heart is tightly regulated, resulting in a small quantity of NO existing in the cells at any given time. Low levels of NO in the heart have a multitude of beneficial physiologic functions including promoting coronary vasodilatation, decreasing intracellular calcium levels and regulating cellular metabolism by reversibly inhibiting mitochondrial respiration. During reoxygenation after a hypoxic event, the observed increase in intracellular calcium stimulates eNOS in a variety of cardiac cells resulting in a large production of NO in a relatively brief period of time.(14) There is evidence to suggest as well that iNOS, which is activated by various cytokines independent of intracellular calcium levels, may also play a significant role in the synthesis of high levels of NO in reoxygenation injury. NO rapidly reacts with the $O_2^{\cdot -}$ that is also being produced in high quantity during reoxygenation and this reaction forms $ONOO^{-}$.(Figure 2-3)(18) Normally in the heart SOD converts the $O_2^{\cdot -}$ to H_2O_2,

and although NO has a greater affinity for $O_2^{\cdot -}$ than SOD, the low levels seen in normal physiological states prevents NO from outcompeting SOD and therefore H_2O_2 is preferentially produced. During reoxygenation, however, super-physiological levels of NO are present and thus outcompete SOD for $O_2^{\cdot -}$ resulting in preferential production of $ONOO^-$.(14)

The pathophysiologic consequences of RONS generation during reoxygenation have been intensely studied in the heart as well. During reoxygenation of the hypoxic myocardium, there is a significant increase in the generation of RONS, often referred to as an 'oxygen burst.' The generated RONS then proceed to interact with numerous biological constituents resulting in a host of pathophysiological processes. These include, for example, the inactivation of key enzymes involved in intermediary metabolic processes, the inactivation of a variety of phosphatases leading to alterations in signal transduction pathways, and generation of single-strand breaks in the DNA.(18) Furthermore, it is now believed that one of the most significant RONS mediated mechanisms of organ injury which occurs during reoxygenation is mediated through the activation of matrix metalloproteinases (MMP).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a group of zinc dependent endopeptidases best known for their ability to proteolyse a wide variety of extracellular matrix proteins throughout the body. First discovered in 1962 by Gross and Lapiere (22) who observed that proteolytic activity was required for a

tadpole to reabsorb its tail, there are now 28 known MMPs. MMPs were historically classified according to the extracellular matrix substrates they proteolysed, however MMPs are now known to target non-extracellular substrates as well as having vital non-proteolytic intracellular functions.(23) Therefore today MMPs are more commonly referred to by a numerical definition. (Table 2-1) (4)

MMPs perform a wide variety of physiological functions in the human body (24), for example, MMPs are known to play an important role in embryonic heart development, angiogeneis, and adaptive vascular remodeling during exercise and pregnancy.(23) In contrast, MMPs have also been implicated in the pathogenesis of many disease processes including asphyxia, fibrotic disease, arthritis and other inflammatory diseases, stroke, atherosclerosis, ischemic heart disease, sepsis, heart failure, and cancer pathogenesis.(24,25)

Structurally MMPs possess several common characteristics including a zinc dependent catalytic domain and a highly conserved autoinhibitory propetide domain with a PRCGVPD amino acid sequence covering the active site.(4) The autoinhibitory domain binds to the catalytic site via a bond formed between a key cysteine residue in the domain and zinc cation within the active site. This prevents access of substrate to the active site and thus the MMP is inactive, and indeed MMPs are primarily synthesized as inactive zymogens. MMP-2 specifically is synthesized in an inactive 72 kDa form, often referred to as “pro-MMP-2.” Regulation of MMP activity is complex and mediated through various mechanisms. Regulation can occur at the level of transcription, and increased MMP-2 transcription occurs in response to many different stimuli and during

multiple disease processes including hypoxia, the release of cytokines such as interleukin-1, 6, and tumor necrosis factor alpha, as well as an increase in hormone levels such as estrogen and melatonin.(4,26) Post-transcriptional regulation involves the stabilization of MMP mRNA transcripts and is achieved through various exogenous and endogenous factors, including androgens, glucocorticoids, growth factors (epidermal growth factor, platelet derived growth factor) and chemokines (transforming growth factor beta - TGF-β).(4) Post-translational modification occurs primarily through four mechanisms: 1) MMP interactions with endogenous inhibitors, 2) proteolysis, 3) conformational modifications in response to variety of stimuli (such as oxidative stress) and through 4) phosphorylation of specific residues.

Many MMPs, including MMP-2, posses a flexible hinge region following the catalytic domain which can serve as a binding site for other proteins that can therefore alter the activity of the MMP. One such group are the four endogenous tissue inhibitors of MMPs (TIMPs), labeled 1 through 4. They are found throughout the body to various degrees, with all four being present in cardiac myocytes.(4) The TIMPs are comprised of two domains, a large N-terminal containing the MMP inhibitor sequence, and a smaller C-terminal domain. MMP inhibition occurs through TIMP binding in a 1:1 stoichiometric ratio, and in general the TIMPs do not exhibit any high degree of specificity for a particular MMP, though TIMP-2 has a higher preference for MMP-2.(4,25) Regulation of TIMPs are less well understood than for MMPs, but it is believed that increased MMP activity *in vivo* reflects an imbalance in the ratio of MMP to TIMP's, and

evidence suggests that TIMP levels/activity are also directly altered in pathologic conditions as well, such as an increase in cellular oxidative stress, resulting in decreased TIMP efficacy.(27,28)

A second post-translational modification occurs through direct proteolytic cleavage of the autoinhibitory domain, which disrupts the bond between the key cysteine residue in the domain and the zinc cation in the active site, thus exposing the active site and resulting in an active 64 kDa MMP-2 form.(25) This occurs in response to multiple stimuli and as a result of the actions of various proteases, including neutrophil elastase, thrombin, plasmin and other activated MMPs.(29) A main mechanism of proteolytic activation of MMP-2 involves a complex interaction between 72 kDa MMP-2, TIMP-2 and membrane type-1 MMP (MT1-MMP, also referred to as MMP-14). It is believed that TIMP-2 acts as a bridge between 72 kDa MMP-2 and MT1-MMP at the cell surface. Though TIMP-2 inhibits the MT1-MMP to which it is bound, adjacent free MT1-MMP is free to proteolyze 72kDa MMP-2 bound within the MMP-2 – TIMP-2 – MT1-MMP complex, yielding active 64 kDa MMP-2.(26,29) Interestingly, it has been demonstrated that this mechanism of activation is enhanced by oxidative stress, likely through an increased expression/activation of MT1-MMP at the cell surface. (30)

More recent evidence suggests that increased oxidative stress, and more specifically increased levels of peroxynitrite, can also induce a conformational change in 72 kDa MMP-2, which exposes the catalytic active site resulting in active 72 kDa MMP-2.(4) Inactive 72 kDa MMP-2 reacts with peroxynitrite in the

presence of cellular glutathione, resulting in the formation of a glutathione disulfide S-oxide bond to the key cysteine residue of the autoinhibitory domain.(25,31) This process, referred to as S-glutathiolation, disrupts the thiolate bond between this key cysteine residue and the zinc cation in the catalytic domain, causing a conformational change in the protein that exposes the active site.

Another mechanism in the regulation of MMP-2 activity is through its phosphorylation. It has been demonstrated that MMP-2 activity is diminished with phosphorylation, and conversely increased with dephosphorylation.(32) At present, however, only some of the phosphorylation sites on MMP-2 are known, though researchers are looking for other phosphorylation sites and the exact kinases and phosphorylases that mediate this mechanism of regulation.

Oxidative stress, MMP-2 activity and hypoxia-reoxygenation

The mechanism(s) by which enhanced oxidative stress and increased MMP activity mediate the development of organ injury and dysfunction in H-R is a relatively new and exciting concept. Yet there exists today a significant body of work that has provided insight into these mechanisms, though most studies have used primarily ischemia-reperfusion (I-R) models of injury (a similar, albeit distinct mechanism of injury to H-R). Furthermore, an exciting revelation has arisen from this increased understanding in the form of novel therapeutic intervention(s) for human diseases of H-R/I-R, and indeed evidence exists which demonstrates the therapeutic potential of MMP inhibition in these models of injury. Currently, a detailed understanding of the mechanism(s) in which

enhanced oxidative stress and increased MMP activation mediate organ injury is greatest for myocardial injury in H-R/I-R. Though the number of studies addressing the mechanism(s) of injury to other organ systems in the context of H-R/I-R is increasing, these mechanisms are less well understood at present.

The extent of myocardial injury in H-R/I-R has been found to correlate with the duration of hypoxic/ischemic insult, with longer insults increasing the potential for irreversible myocardial injury.(25) Studies have demonstrated increased MMP-2 levels and activity in ischemic hearts during reperfusion, and that MMP-2 activity is correlated with increasing levels of oxidative stress (particularly levels of peroxynitrite). Furthermore, this increase in MMP-2 activity correlates with the degree of cardiac dysfunction.(14) Lalu et al studied fifteen adult human patients with stable angina undergoing coronary artery bypass grafting (CABG). Cross clamping of the aorta during the procedure results in ischemia to the heart followed by reperfusion after the clamp is removed, thus ischemic-reperfusion injury to the heart occurs during a CABG procedure. The authors found that during reperfusion there was an increase in MMP-2 and MMP-9 activities in the myocardium, and that the degree of activity correlated positively with the duration of ischemia (cross clamp time) and negatively with cardiac mechanical function.(33)

It is now recognized that activated MMP-2 can induce a host of pathophysiological events within the cardiac myocyte. Activated MMP-2 is thought to disrupt cardiac endothelial integrity, and some evidence suggests that MMP-2 can induce apoptotic and/or necrotic pathways within the cell, potentially

through effects on poly (ADP-ribose) polymerase (PARP) or glycogen synthase kinase-3β (GSK-3β), although the exact mechanism(s) of these effects are still unknown. Additionally, there is evidence to suggest that MMP-2 mediated myocardial injury in hypoxic-reoxygenation injury can also involve effects on the mitochondria, including structural changes to the mitochondria, impaired respiration, and increased lipid peroxidation which may ultimately lead to cellular necrosis or apoptosis.(4)

A more well understood mechanism of MMP-2 mediated cardiac injury/dysfunction in H-R/I-R is the proteolytic cleavage of integral sarcomeric proteins within the myocycte. MMP-2 is found in various locations throughout the cardiac myocyte including the nucleus, mitochondria, caveolae, and sarcomere. Within the sarcomere, which is a complex cellular structure that forms the basis for the contractile function of the myocyte, MMP-2 is co-localized with various proteins including titin, α-actinin, myosin light chain1 and 2 (MLC-1 and MLC-2) and troponin I (TnI).(4) Activated MMP-2 can degrade these proteins, disrupting normal sarcomeric function and leading to cardiac contractile dysfunction. Wang et al found, using isolated perfused rat hearts subjected to I-R, that MMP-2 is co-localized with TnI and that inhibition of MMP-2 during I-R prevented the degradation of TnI and improved cardiac mechanical functional recovery.(34) In a study of H-R injury using a newborn piglet model, Doroszko et al found levels of myocardial MLC-2 decreased in piglets subjected to H-R (versus control piglets), and that this was associated with a decrease in contractile function. They also found that *in vitro* exposure of MLC-2 to peroxynitrite

resulted in nitration of MLC-2 tyrosine residues that increased the susceptibility of MLC-2 to MMP-2 degradation. The authors concluded that MLC-2 degradation contributes to cardiac systolic dysfunction observed during H-R, and suggested that inhibition of MMP-2 degradation of MLC-2 could potentially reduce the cardiac injury and subsequent contractile dysfunction observed in H-R injury, though an MMP inhibitor was not tested in their study.(35) Dorszko et al further demonstrated that MMP-2 has a similar relationship and effect on MLC-1; MMP-2 is co-localized with MLC-1 within the myocyte, peroxynitrite mediates nitration of MLC-1 that modifies the structure of MLC-1 leading to increased degradation by MMP-2, and MMP-2 activity correlates negatively with levels of cardiac MLC-1.(36) Using a combination of *in vitro* experiments with rat hearts and human heart biopsies, Ali et al observed that MMP-2 and titin are co-localized within the myocyte sarcomere and demonstrated titin degradation by MMP-2 in a concentration-dependent manner that was prevented by MMP inhibitors. Furthermore, in isolated rat hearts subjected to I-R, the authors observed increased degradation of titin, and MMP inhibitors not only prevented titin degradation but improved the recovery of myocardial contractile function.(37)

There is increasing evidence for the role of enhanced MMP expression and activity in mediating injury to other organs as well. MMPs are found throughout the kidney and have been implicated in a variety of renal pathologies.(38) For example, an increase in MMP-2/MMP-9 expression has been observed in I-R injury, and it is believed that the activated MMPs mediate renal injury, in part,

through cleavage of cell adhesion molecules found within the vascular endothelium, glomerulus, and tubular epithelial cells, resulting in enhanced vascular and tubular permeability.(38) Increased MMP-2/MMP-9 expression/activity in the liver, brain, and lungs has also been demonstrated in animal models of H-R/I-R, though the mechanism(s) leading to organ injury are presently not well understood.(39-41)

Conclusions

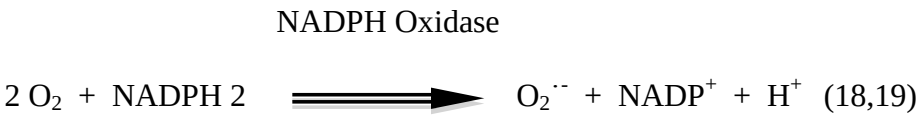
The mechanism(s) of injury and organ dysfunction in H-R are complex, and in some regards remain poorly understood. Yet we now have a better understanding of why organ injury and dysfunction can be worsened during the resuscitation of an asphyxiated neonate. There is a large body of evidence demonstrating enhanced oxidative stress and increased MMP activity during H-R, and that the degree of MMP activity positively correlates with organ injury and dysfunction. The mechanism(s) of injury mediated by increased MMP activity have been best characterized in myocardial injury, and include induction of apoptosis/necrosis pathways and the direct proteolytic cleavage of vital sarcomeric proteins. This understanding has already affected current clinical practice guidelines in the resuscitation of asphyxiated neonates, namely in the recommended use of room air during resuscitation versus traditionally used 100% oxygen. Yet MMPs have also emerged as a novel therapeutic target for interventions aimed at attenuating or preventing organ injury and dysfunction in H-R, and ongoing studies, including the one in this thesis, are attempting to assess the beneficial effects of MMP inhibitors in H-R.

Table 2-1: Comparison of the traditional classification of MMPs to the current numerical classification

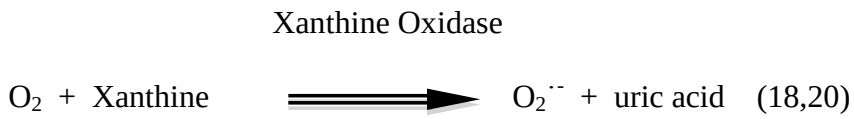
Matrix metalloproteinase numerical classification	Matrix metalloproteinase traditional classification
2, 9	Gelatinases
1, 8, 13	Collagenases
3, 10	Stromelysins
7, 26	Matrilysins
12	Metalloelastases
14, 15, 16, 23, 24, 25	Membrane type
20	Enamelysin

Figure 2-1: Reactions that generate superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2)

Reduction of molecular oxygen to superoxide anion ($O_2^{\bullet -}$) by NADPH oxidase



Reduction of molecular oxgen to superoxide anion ($O_2^{\bullet -}$) by xanthine oxidase



Generation of hydrogen peroxide (H_2O_2) through the dismutation of superoxide anion ($O_2^{\bullet -}$) by superoxide dismutase (SOD)

Figure 2-2: Some reactions that generate hydroxyl radical ($OH^{\cdot}$)

Fenton reaction

$$H_2O_2 + Fe^{2+}\ (Cu^{+}) \longrightarrow OH^{-} + OH^{\cdot} + Fe^{3+}\ (Cu^{2+})$$ (18,20)

Haber-Weiss reaction (20)

First reaction: $O_2^{\cdot -} + Fe^{3+}\ (Cu^{2+}) \longrightarrow O_2 + Fe^{2+}\ (Cu^{+})$

Fenton reaction: $H_2O_2 + Fe^{2+}\ (Cu^{+}) \longrightarrow OH^{-} + OH^{\cdot} + Fe^{3+}\ (Cu^{2+})$

Net reaction: $O_2^{\cdot -} + H_2O_2 \longrightarrow OH^{-} + OH^{\cdot} + O_2$

Formation and decomposition of peroxynitrous acid (21)

Formation of peroxynitrous acid: $ONOO^{-} + H^{+} \longrightarrow ONOOH$

Decomposition of peroxynitrous acid: $ONOOH \longrightarrow OH^{\cdot} + NO_2^{\cdot}$

Figure 2-3: Generation of peroxynitrite ($ONOO^-$)

$O_2^{\cdot -}$ + NO ⟹ $ONOO^-$ (14,18)

$O_2^{\cdot -}$: generated from NADPH oxidase, Xanthine oxidase, mitochondrial respiration

NO: generated from various NOS enzymes

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Chapter 3

Doxycycline

Introduction

Tetracyclines are a family of natural and semi-synthetic broad-spectrum antibiotics first isolated from *Streptomyces aureofaciens* in 1947.(1) The antimicrobial effect of tetracyclines occurs through the inhibition of protein synthesis, which is mediated by binding to the 30S subunit of the bacterial ribosome. Two of the more commonly used tetracycline drugs at present are doxycycline and minocycline, both of which are approved for the treatment of various infections including anthrax, chlamydial, community-acquired pneumonia, Lyme disease, cholera, syphyllis, Yersinia pestis, periodontal infections, and rickettsial diseases such as Rocky Mountain Spotted Fever.(2,3)

Structure and Pharmacokinetics

Though tetracyclines have been around since the 1950's, pharmacockinetic information is sparse, with even less known about the pharmocodynamic properties of this class of antibiotic. The drugs themselves can be broadly classified into three groups; group one being the first generation drugs (example: tetracycline) with poorer absorption and more bacterial resistance, group two being newer second generation drugs (doxycycline and minocycline) which are almost completely absorbed (3-5X more lipophilic than first generation tetracyclines), have better tissue penetration, longer half-lives and less side effects compared to first generation drugs, and group three comprised of the newest drugs currently being tested (example: aminomethylcyclines).(3,4)

Doxycycline is composed of a four-ring core with various side group attachments. (Figure 1) The oxygen-rich lower half of the molecule is vital to the

antimicrobial properties of the drug and also accounts for its ability to bind with divalent cations. Doxycycline is almost completely absorbed from the GI tract, exclusively in the duodenum, with a bioavailability of more than 80% – 100% and an absorption half-life of approximately 0.85 ± 0.41 hours.(4) The absorption of doxycycline is less affected by the presence of food in the gastrointestinal tract compared to older tetracycyline drugs, and these factors combined allow for lower doses to achieve therapeutic effect and improved convenience in taking the drug leading to an increase in therapeutic compliance.(3) Doxycycline is not metabolized in the body and no significant metabolites have been discovered. 80-90% of the drug is protein bound with a volume of distribution of approximately 50-80L (0.7 L/Kg) indicating that the drug has excellent tissue penetration.(4) Peak concentrations are around 2-3 hours and excretion occurs equally through biliary and renal routes, with an elimination half-life of approximately 12-25 hours (depending on the dose, method of delivery and the study referenced).(4)

Non-antimicrobial effects of doxycycline

It has been observed that tetracycline drugs have non-antimicrobial effects as well. These include anti-inflammatory, anti-apoptotic and anti-oxidant effects as well as the ability to act as inhibitors of matrix metalloproteinases.(2,5)

Anti-inflammatory

The anti-inflammatory profile of tetracycline drugs is extensive and includes regulation of inflammatory cytokines and inhibition of phospholipase A, neutrophil aggregation/adherence and lymphocyte proliferation.(1,2) Most of the

current evidence, however, has highlighted that the anti-inflammatory effects of doxycycline are mediated primarily through regulating levels of pro-inflammatory cytokines. Solomon et al studied the anti-inflammatory effect of doxycycline in a pro-inflammatory human cultured corneal epithelium model. Treatment with doxycycline decreased the bioactivity of the pro-inflammatory cytokine IL-1β as effectively as corticosteroid with relatively no adverse effects, and the authors concluded that doxycycline may be an ideal drug to use in a wide spectrum of ocular inflammatory diseases.(6) In a mouse model of septic shock, Milano et al found that intraperitoneal administration of doxycycline or tetracycline significantly protected the mice from a lethal intraperitoneal injection of bacterial lipopolysaccharides (LPS), an effect mediated through a decrease in levels of the pro-inflammatory cytokines TNF-α and IL-1α, as well as through an inhibition of iNOS.(7) Clinically, the anti-inflammatory effects of tetracyclines have been utilized in the field of dermatology, where tetracyclines (primarily doxycycline and minocycline) are used in the treatment of acne and rosacea, two conditions mediated in part by pro-inflammatory mechanisms.(1,3)

Anti-apoptotic

Apoptosis is a complicated process of controlled cell death that can occur through numerous pathways and in response to a variety of stimuli. There is increasing evidence suggesting that tetracycline drugs have anti-apoptotic effects, which are mediated through multiple mechanisms. Yeh et al found that administration of doxycycline suppressed doxorubicin induced apoptosis in

mouse testes through inhibition of cytochrome c release, caspase-3 cleavage and activation.(8) In a more recent study, Lai et al demonstrated inhibition of doxorubicin induced apoptosis in the hearts of mice receiving doxycycline which was mediated through the upstream stabilization of p53/Apaf-1, the regulation of downstream Bcl-2 family proteins, inhibition of mitochondria-dependent apoptotic mediators, and neutralizing ER stress signaling effectors.(9) Though the mechanisms regulating the anti-apoptotic effects of tetracyclines are becoming clearer, more work is required to determine if and how these effects can be utilized therapeutically in clinical practice.

Anti-oxidant

The most debated non-antimicrobial effect of tetracyclines is their potential to act as RONS scavengers. The anti-oxidant effect of tetracyclines is thought to be related to the presence of a multiple-substituted phenol ring in the drugs structure, which is similar to the structure of the well-known anti-oxidant vitamin E.(1) Though debated, there is increasing evidence that RONS scavenging is a true effect of certain tetracyclines, including doxycycline. In a cell culture study, Nungavaram et al found that administration of doxycycline inhibited the conversion of osteoblast procollagenase to active collagenase by reducing the concentration of the RONS hypochlorous acid.(10) A more recent *in vivo* study by Castro et al found in a rat model of hypertension that doxycycline attenuated hypertension, protected against hypertension-induced oxidative stress, reduced MMP activity and improved NO levels in aortic endothelial cells. The

authors concluded that doxycycline attenuated hypertension induced endothelial dysfunction in the rat aorta by reducing oxidative stress, improving NO bioavailability and inhibiting matrix metalloproteinase activity.(11) As with anti-apoptotic effects, further research is required to determine the clinically applicability of RONS scavenging effects by tetracyclines.

Matrix metalloproteinase inhibition

The discovery that tetracyclines could inhibit matrix metalloproteinases (MMP) originated with the hypothesis that enhanced periodontal breakdown in diabetics was secondary either to alterations in oral microflora or to an altered host response, such as a change in collagen turnover. Through a series of experiments using minocycline in diabetic rats, Golub et al discovered that collagenase activity was reduced with minocycline treatment, yet interestingly this was not related to an effect on the oral microflora as was initially hypothesized.(5,12) Subsequent experiments were performed and it was eventually discovered that tetracyclines directly inhibit collagenases as well as other MMPs.

The mechanism(s) of MMP inhibition have not been fully elucidated, though it is currently believed that tetracyclines exert their effect through direct inhibition of the MMP enzyme itself and indirectly through inhibition of MMP expression at the nuclear level.(1) Direct inhibition is thought to occur through chelation of the zinc cation in the enzyme's catalytic domain, owing to the affinity of tetracyclines to bind divalent cations as previously discussed. The strength of

the tetracycline-metal ion interaction depends on the particular tetracycline and metal ion. By binding the zinc cation, tetracycline drugs essentially occupy the catalytic site of the MMP and therefore prevent substrate from contacting it.

Doxycycline is the most potent inhibitor of MMPs within the tetracycline family (5) due to its increased affinity for binding to zinc relative to the other tetracyclines.(2) Furthermore, doxycycline is at present the only tetracycline approved by the Federal Drug Administration and Health Canada for clinical use as an MMP inhibitor; low dose (sub-antimicrobial) doxycycline marketed as Periostat has been approved for the treatment of periodontitis and rosacea, conditions mediated in part by enhanced activity of MMPs.(1)

Doxycycline in hypoxia-reoxygenation injury

As described in Chapter 2, multiple studies have demonstrated an increase in MMP-2 activation in hypoxia-reoxygenation (H-R) and ischemic-reperfusion (I-R) models of injury, and these and other studies have also found that doxycycline reduces MMP-2 activation and attenuates organ injury and dysfunction. While the majority of studies have focused on the cardioprotective effects of doxycycline in H-R and I-R injury, evidence is emerging that MMP-2 inhibition by doxycycline is beneficial in other organ systems as well.

Cheung et al measured the release of MMP-2 in the coronary effluent of isolated, perfused rat hearts during aerobic perfusion and reperfusion after ischemia. The authors observed an enhanced release of MMP-2 into the coronary effluent during reperfusion after ischemia as compared to during aerobic perfusion. This enhanced release of MMP-2 correlated negatively with functional

mechanical recovery during reperfusion. Furthermore, treatment with doxycycline inhibited MMP-2 activity that was associated with an improvement in mechanical heart function.(13) Fert-Bober et al had similar results in their study using isolated perfused rat hearts, which assessed the effect(s) MMP-2 activation has on cardiac function, protein release and coronary endothelium integrity during I-R injury. They found that MMP-2 activity was increased in heart tissue at the end of ischemia and that the level of activity was correlated with ischemic time. Cardiac mechanical dysfunction worsened with increasing MMP-2 activity, as did the extent of endothelial damage. It was observed that cardiac mechanical dysfunction and endothelial integrity improved with inhibition of MMP-2 by doxycycline. The authors concluded that MMP inhibition reduces endothelial damage in addition to attenuating cardiac mechanical dysfunction that occurs from I-R injury.(14) A study by Donato et al compliments the findings of the two aforementioned studies, and found in isolated rabbit hearts subjected to I-R that administration of doxycycline two minutes into reperfusion inhibited MMP-2 and significantly reduced myocardial infarct size.(15)

Increased MMP activity and the protective effects of doxycycline administration have been highlighted in other organ systems as well. In a rat model of renal I-R injury, the administration of doxycycline attenuated renal injury through a reduction in pro-inflammatory cytokines, increasing levels of TIMP-1, and inhibiting MMP-2 activity.(16) These findings are supported by a study from Kucuk et al who also demonstrated in a rat model of renal I-R injury that pre-treatment with doxycycline was renal-protective through MMP-2

inhibition, in addition to anti-inflammatory and anti-oxidant effects.(17) Wang et al studied I-R injury in the rat brain and concluded that doxycycline protected against blood-brain-barrier injury in I-R through MMP-2/MMP-9 inhibition and the up-regulation of tight junction proteins.(18)

Use of tetracyclines in children and neonates

The use of tetracyclines in children younger than eight years old is at present contraindicated due to fear of irreversible staining of the teeth and an increased risk of benign intracranial hypertension, though the later resolves with discontinuation of the drug.(3,19) The mechanism by which tooth staining occurs is due to the affinity of tetracyclines to bind divalent cations, including the calcium that is being incorporated into the enamel during tooth development. Tetracyclines bound to the calcium are incorporated into the developing teeth as a fluorescent pigment resulting in staining of the teeth.(19) This fear of irreversible staining of the teeth arose from studies done in the 1960's which found that the vast majority of children treated with tetracycline experienced some degree of tooth staining, with higher total dosage (and not the duration of treatment) increasing the risk and degree of staining, though long term effects (staining of the permanent teeth for example) were not explored.(20) However, these findings have been challenged over the years by new evidence that has lead to controversy surrounding the use of tetracyclines in children.

A retrospective review performed by Grossman et al in the early 1970's refuted many of the findings in the earlier studies. They authors correlated tooth staining with a history of tetracycline use in 160 patients under their care and

found negligible tooth staining in children treated with short courses (<6 days) of tetracycline. Furthermore, the authors challenged the earlier notion that total drug dosage, and not duration of treatment, increased the risk and degree of tooth staining. The authors of this study in fact found the opposite, that duration of therapy was the significant risk factor determining presence and degree of staining. In their conclusions, the authors also speculated that doxycycline, which has less affinity for binding to calcium than other tetracyclines, may further reduce the risk of staining.(21) This later assertion has been confirmed in multiple subsequent studies. Lochary et al in a retrospective study of ten children treated with short course doxycycline for Rocky Mountain Spotted Fever found no clinically significant staining of the teeth.(22) Volovitz et al in their asthma clinic performed a blinded, 'randomized' (patients were not randomized to treatment group; controls were randomly selected from the clinic population) controlled study involving 31 children aged 2-8 who received doxycycline treatment in their pediatric asthma clinic and 30 randomly selected children with similar asthmatic symptoms who had not been treated with doxycycline. A dentist blinded to group allocation examined all 61 children looking for evidence of tooth staining, and none was detected in any of the children in either group, leading the authors to conclude that use of doxycycline in young children (aged 2-8) is not associated with tooth staining.(23) Furthermore, the claim that tooth staining by tetracycline drugs is permanent has also been questioned by recent evidence. Ayaslioglu and Cebecioglu examined four separate patients (aged 12-28 years old) treated with doxycycline for brucellosis and found that all experienced some tooth staining but

the staining was temporary and eventually reversed, thus leading to the conclusion that although staining may be more common than originally believed, the condition is fortunately reversible.(24)

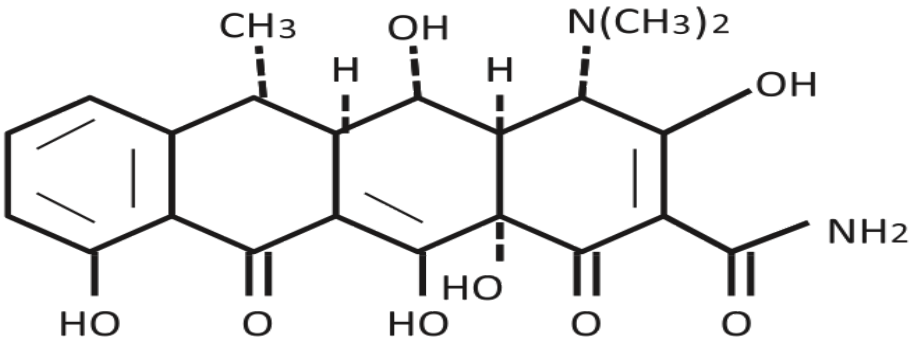
Despite these findings, in the absence of large double-blinded, randomized controlled trials doxycycline still remains contraindicated in younger children. Some believe that the controversy surrounding the use of doxycycline in young children has been sufficiently addressed (25), while others maintain that better studies are still required before definitive changes can be made in clinical practice guidelines.(19) For now doxycycline remains the drug of choice in this age group only for the treatment of certain infectious conditions including tickborne rickettsial disease such as Rocky Mountain Spotted Fever and lyme disease and for more severe, albeit rare infections like anthrax.(25-27)

Conclusions

The non-antimicrobial effects of tetracyclines were an unexpected though interesting finding. The ability to exploit these effects clinically is currently being explored, and in some cases, has already been established in clinical practice guidelines, such as MMP inhibition with low dose doxycycline in peridontitis. The non-antimicrobial effects and excellent safety profile of doxycycline make it an ideal therapeutic intervention to study in H-R, in which injury is mediated through pro-inflammatory mediators, increased oxidative stress, enhanced apoptosis and increased MMP activity. Application of doxycycline into the clinical management of neonatal asphyxia, however, is hindered by the controversy surrounding the risk of irreversible teeth staining when used in this

age group. Several factors must be considered in this debate, however. The original studies that highlighted this undesirable side effect studied tetracycline itself and not doxycycline, which has a lower affinity for binding calcium and, at least theoretically, a lower probability of being incorporated into developing teeth. Secondly, more recent evidence has questioned the original findings, highlighting that staining may be less common than initially believed, duration of treatment (and not total dose) is the most significant risk factor for presence and degree of staining, that staining is not clinically significant when doxycycline is used, and that staining, if present, is reversible. Third, it is highly unlikely that even if there were some staining of the primary teeth at a young age that this would affect the color of the permanent teeth that develop later in childhood. Despite these arguments, in todays medico-legal environment it is unlikely that tetracyclines, and doxycycline specifically, will be adopted into more clinical practice guidelines for children younger than eight in the absence of large double-blinded, randomized controlled trials demonstrating the safety of this drug in this age group. Yet given the many ideal qualities the drug has which are particularly suited for the treatment of hypoxia-reoxygenation, doxycycline is at present the most clinically translatable MMP inhibitor for use in the management of asphyxiated neonates.

Figure 3-1: Structure of doxycycline (1)



DOXYCYCLINE

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Chapter 4

Animal Model for Studying Newborn

Hypoxia-Reoxygenation

Introduction

There exists a great need to study novel treatments for neonatal asphyxia; this, however, is quite difficult for several reasons. One cannot precisely identify the timing of injury and the severity of injury can only be assessed by indirect methods.(1) Furthermore, designing and testing new therapeutic interventions for human disease(s) is complicated. In order to do this, you must first have a thorough understanding of the physiology/pathophysiology involved in a particular disease process. Novel treatments can then be designed from this knowledge; however, the safety, efficacy, and ultimate clinical protocols of a novel treatment have to be ascertained before testing in humans can occur, as ethical issues often limit the study of new treatments in humans. Thus animal models serve as useful surrogates in the study of human disease, and indeed much of our understanding of neonatal asphyxia has been obtained through research utilizing animals. Animals offer not only the advantage of determining the underlying physiologic and pathophysiologic processes that govern a particular disease, but through replication of these disease processes in animals, allow for testing of novel treatments. By testing new treatments in animal models, one can determine the safety and efficacy of the intervention, and if both are favorable, begin to establish treatment protocols that can be implemented in human trials.

Animal models of newborn asphyxia

The causes of neonatal asphyxia are varied, and there are both acute and chronic consequences of this condition.(1,2) Thus designing a single animal model to encompass the varied nature of this clinical condition is difficult, if not

impossible, as the spectrum of injury in asphyxia can vary with the underlying cause of asphyxia as well as with the timing and duration of the insult. A variety of animal models have been designed to test various aspects of neonatal asphyxia including physiological responses, acute and chronic injury patterns, and the safety and efficacy of novel interventions, among others. Animal models most commonly utilize rats, followed by swine, with sheep being the third most common.(3-5) Each model carries with it specific advantages and disadvantages in terms of the information that can be derived, and careful selection of the type of model used to answer the question(s) of interest is vital. For example, large animal models (sheep, swine) offer the advantage of more sophisticated instrumentation, allowing for ongoing assessments of various physiological parameters, and are also superior for studying acute and sub-acute endpoints of asphyxia.(3,5) However, smaller litter sizes, costs and the higher complexity of the models can significantly limit sample size.(4) Small animal models (rat, mouse) on the other hand, allow for larger samples sizes and a greater number of survivors, which is more beneficial when studying histological and biochemical responses to asphyxia, as well as for long-term physiological and behavioral outcome studies.(3-5)

There are obvious drawbacks in using animal models to design and test new treatments for human disease, as humans and animals differ in many regards, most notably in their anatomy and physiology. Furthermore, human diseases can differ considerably from those observed in animals, and even similar disease processes can have vastly different pathophysiologic mechanisms governing

them. In order to study human disease processes and test novel interventions in animals, with the ultimate goal of translating findings to human clinical practice, the ideal animal model should then reflect, as closely as possible, normal human anatomy and physiology, as well as have similar physiologic/pathophysiologic mechanisms involved in the disease process.

Swine neonatal asphyxia models

Sus scrofa domestica, commonly referred to as swine, are descendents of domesticated European wild boar. Swine are an excellent animal model for studying newborn asphyxia. Their size permits the ability to surgical instrument the animal for continuous monitoring of a variety of variables (blood flow through specific vessels, blood pressure, pulmonary artery pressures, oxygen saturation, etc.), and a thorough understanding of swine physiology, including normal hemodynamic and biochemical parameters, is available. Furthermore, many swine organ systems, including the cardiovascular, renal, hepatic, and intestinal system, have a remarkable morphological and functional similarity to humans. Finally, the pathological mechanisms mediating asphyxia, as well as the associated physiological responses, are similar between human neonates and newborn piglets.(4,6)

Surgical instrumentation of the animal during asphyxia is vital to understanding the pathophysiologic mechanism belying the event and to monitor for the potential benefit(s) of intervention. For example, the use of transit-time ultrasound flow probes placed around selected vessels allows for a continuous assessment of blood flow through the vessel. In the context of neonatal asphyxia,

for example, one could continuously assess blood flow to the brain (carotid artery flow probe), intestine (superior mesenteric artery flow probe), kidney (renal artery flow probe), and lungs (pulmonary artery) to elucidate the changes in organ perfusion (brain, intestine, kidney) and cardiac output (pulmonary artery flow) occurring during an asphyxiating event. Furthermore, if changes are observed, you could test the beneficial effect of a novel intervention, such as improving organ perfusion or increasing cardiac output with administration of a novel drug. These flow probes are widely used in research and have been validated by numerous studies.(7) Surgical access also allows for ease in obtaining samples (for example, arterial or venous blood) and administration of drug or other interventions.

The continuous observation of various physiologic parameters is central to understanding the pathophysiology of neonatal asphyxia as well as to observe any beneficial effect of interventions. In order to accomplish this, normal physiologic parameters of the animal must first be understood. These have been obtained in swine, and fortunately those interested in using a swine animal model can easily reference normal biochemical and hemodynamic value ranges specific to the breed of pig used. For example, Friendship et al published an extensive review of the hematology and biochemistry reference values for swine from Ontario in 1984.(8)

Swine share remarkable anatomical and physiologic similarities with humans. The cardiovascular system in swine is very similar to that seen in humans. The gross and histological anatomy of the heart, coronary and

conduction system, blood supply, and damage/wound-healing characteristics are analogous to the human cardiac system.(9) The swine heart of a 40-50 kg pig is approximately the same size as a human adult heart, and growth of the heart and great blood vessels from 6 weeks to 6 months of age is essentially identical to that in human infants from birth to sexual maturity. Hemodynamic function is similar to humans as well.(9,10) The swine renal system, both anatomically and physiologically, is more similar to humans than any other common species of animals.(10) Like in humans, the kidney exhibits a multirenculate, multipapillate form with true calyces, differing from humans only with respect to the blood supply to the renal parenchyma, having a longitudinal rather than transverse division.(9) The gross anatomy and physiology of the swine liver, including metabolic function, is so similar to humans that swine have served as one of the standard animal models for hepatic transplantation research.(9) The gastrointestinal tract in swine is, interestingly, quite anatomically different from that in humans, including a spiral colon, vascular differences in the small intestine and the presence of the torus pyloricus, a muscular out-pouching of the gastric pylorus. Physiologically, however, the pig gastrointestinal system is analogous to humans in terms of splanchnic blood-flow characteristics and digestive function.(9)

Humans and pigs share many similarities with regards to the pathophysiological mechanisms involved in asphyxia and the physiological responses that occur, including patterns of organ injury. As in humans, birth is an abrupt change for piglets, and rapid physiologic changes are necessary to go from

oxygen supply via the umbilical cord to breathing independently. As in humans, some degree of asphyxia during this process is normal.(11) Severe or prolonged asphyxia can, however, have very severe consequences. Despite being relatively mature at birth, piglets, like human neonates, are very susceptible to prolonged or severe asphyxia.(12) Prolonged asphyxia can result in hypoxia, hypercapnia, and a metabolic acidosis through similar mechanisms to human neonates. Diagnosis of asphyxia in the fetal pig shares some similarities with humans as well, including a form of the APGAR score that measure five variables including respiratory rate, heart rate, muscle tone, skin color, and attempt to stand.(12) Compensatory responses, including the shunting of blood to more vital organs such as the brain, heart and adrenal glands are also seen in the asphyxiated piglet. Alward et al observed 6-96 hour old piglets that were asphyxiated and found decreased blood flow to the intestine at 90 minutes but an increased blood flow to the adrenal glands at 60 minutes and normal flow at 90 minutes. Although they did not measure flow to the heart and brain directly, they surmised given their findings that blood was preferentially being shunted to these organs as well.(13) Preferential shunting of blood to more vital organs during asphyxia in swine is also supported in the extensive review of perinatal asphyxia in pigs and humans by Alonso-Spilsbury et al.(12) Consequences of severe, prolonged or untreated asphyxia are also similar between fetal pigs and human neonates, including severe and often irreversible brain damage, persistent pulmonary hypertension of the newborn and death.(12)

Experimental Design and Methods

Animal care/preparation

All experiments for this project conformed to the regulations of the Canadian Council of Animal Care (Revised 2000) and were approved by the Animal Care and Use Committee at the University of Alberta.

Mixed-breed newborn piglets aged 1-5 days old were obtained from the University of Alberta farm on the day of the experiment. Animals rested under heat lamps for 30-45 minutes prior the surgery to allow for acclimatization.

Anesthesia, analgesia and sedation

As in humans, swine are sensitive to physiological stress, thus necessitating adequate anesthesia during surgical intervention and hypoxia-reoxygenation procedures. Animals were initially anesthetized with inhaled 5% isoflurane, with subsequent downward titration to maintain adequate sedation. Though suggested as the best method of anesthesia in swine(14), isoflurane can, in a dose-dependent manner, cause cardiovascular depression manifested by a decrease in cardiac output and systemic arterial pressure.(15) Thus once intravenous access was achieved, isoflurane was replaced by fentanyl and midazolam infusion for maintenance of anesthesia. Fentanyl is a potent opioid analgesic with some sedative properties, which can be given as a bolus or continuous infusion and that has a fast onset and offset of action, allowing for relatively accurate titrations.(15) Respiratory depression is a side effect, particularly at higher doses, and therefore the lowest doses (range 5 – 50

μg/kg/hour) required for adequate pain and sedation were used. Midazolam is a relatively fast acting benzodiazepine with sedative and anxiolytic properties. Cardiopulmonary depression (decreased cardiac output, hypotension) is a possible side effect of the drug, though this effect is thought to be infrequent and minimal when present.(16-18,18) Nevertheless, dosages (0.1 – 0.5 mg/kg/hour) were titrated to the lowest dose required to achieve adequate sedation. Pancuronium (0.05 – 0.1 mg/kg/hour), a long acting, non-depolarizing neuromuscular blocking agent was used to induce muscle relaxation to support coordinated ventilatory support during hypoxia-reoxygenation. Pancuronium can stimulate cardiac muscarinic receptors resulting in an increase in heart rate, cardiac output and systemic arterial pressure and confound the results of the study.(19) Dosage was titrated to the lowest dose required to achieve muscle relaxation; at no time was pancuronium administered for behaviors interpretable as pain.

Surgical Procedure

Following anesthesia, piglets were surgically instrumented for continuous monitoring of normal vital signs and various systemic and regional hemodynamic parameters. The same individuals performed surgical instrumentation for all piglets. Surgery started with a left femoral cut-down for placement of a double lumen femoral venous line for measurement of central venous pressure (CVP), drug and fluid administration. A single lumen femoral arterial line was placed for systemic arterial pressure (SAP) measurements and collection of arterial blood for biochemical analysis. Maintenance fluids consisting of 10% dextrose in water at

10cc/kg/hr and 0.9% NaCl at 2cc/hr were infused for glucose replacement and hydration, respectively. A tracheostomy tube was then placed through a midline neck incision followed by initiation of pressure-controlled mechanical ventilation (Sechrist infant ventilator model IV-100, Sechrist Industries Inc., Anaheim, CA) with pressures of 20/4 cm H_2O at an initial rate of 20 breaths/minute. The left carotid artery was then freed circumferentially and encircled with a 2-mm ultrasonic flow probe (2SS; Transonic Systems Inc., Ithaca, NY) for continuous measurement of left carotid artery blood flow. A suprapubic catheter was placed through a vertical midline extra-peritoneal incision for collection of urine. A left-flank incision was then made and the superior mesenteric artery (SMA) and renal artery were isolated through a retroperitoneal dissection and encircled with 3-mm (3SB) and 2-mm (2SB) ultrasonic flow probe for continuous measurement of SMA and renal artery blood flow, respectively. We then proceeded with a left antero-lateral thoractomy. The ductus arteriosus was carefully dissected circumferentially and ligated. A 20-G angiocatheter (Arrow International, Reading, PA) was inserted into the pulmonary artery and secured for measurement of pulmonary artery pressure (PAP) and central venous blood sampling. Finally, a 6-mm transonic flow probe (6SB) was placed around the main pulmonary artery for continuous measurement of pulmonary artery blood flow, a surrogate of cardiac output (CO) in our model. All incisions were closed or covered to minimize evaporative loss of heat and fluid. Immediately following surgery a 20cc/kg Ringers lactate bolus was administered to replace evaporative losses during the surgical procedure.

Following surgery the piglets entered a stabilization period. Heart rate, SAP, CVP, SpO_2 and carotid, renal, superior mesenteric and pulmonary artery blood flow were continuously monitored. Inspired oxygen concentration (FiO_2) measured by an Ohmeda 5100 oxygen monitor (Ohmeda Medical, Laurel, MD) was maintained at 0.21 to 0.25 for SpO_2's between 90% and 100%. Piglet temperature was kept between 39^{o}C and 40^{o}C throughout the experiment using an overhead warmer and heating pad. Arterial blood gas analysis was performed intermittently throughout the experiment, and the ventilator rate was adjusted as required to maintain arterial CO_2 levels ($PaCO_2$) between 35 to 45 mmHg. Piglet stabilization lasted for approximately one hour with an end-point defined as the time when baseline hemodynamic measurements changed less than ±10% over a twenty minute period.

Use of ultrasonic flow probes

Ultrasonic flow probes were used to measure arterial flow in the pulmonary, carotid, left renal and superior mesenteric arteries. Using wide-beam illumination, plane ultrasound waves are passed back and forth between two transducers that are intersecting the flowing blood within the artery in the upstream and downstream directions. The speed of the ultrasound wave as it passes from one transducer to the other is affected by the velocity of blood flow, and the flow meter derives an accurate measure of blood volume flow from the 'transit-time' (the time it took for the ultrasound signal to pass from on transducer

to the other).(20) Flow probes were regularly calculated as per the manufacture's recommendations.

A variety of methods are available for the measurement of arterial blood flow, including thermodilution methods (for example, the Swan Ganz pulmonary artery catheter to measure cardiac output), radioactive or colored microspheres, Doppler, and transit time flow probes.(21) Thermodilution, microsphere and Doppler measurements have significant disadvantages including the inability for continuous measurement of flow and the sensitivity of readings to changes in hematocrit and temperature.(21) Transit time flow probes circumvent these disadvantages and have been validated for the accurate measurement of flow in a multitude of studies, though they carry the significant disadvantage of requiring surgery for placement, thus limiting their use to animal models.(21,22)

Experimental design and hypoxia-reoxygenation protocol

Piglets were block-randomized into four treatment groups; a control group that was administered normal saline or a 3, 10 or 30mg/kg doxycycline group. (Figure 4-1) Doxycycline dosages were determined based on pharmacokinetic data, previous animal studies and clinical therapeutic dosage.(23-28) Sample size calculations were based on prior experience in our lab (expected difference between means = 50 ± 30 (SD)), and with a power of 80 (P = 0.8) and alpha error of 5% ($\alpha = 0.05$), indicated that 7 animals per group (n = 7/group) were required for the experiment to be appropriately powered.

The protocol for hypoxia-reoxygenation has been described previously.(14) Following a one hour surgical stabilization period, a two hour normocapnic hypoxic period commenced. Hypoxia (blood oxygen saturation (SaO_2) of 40-50%) was achieved by lowering the fraction of inspired oxygen (FiO_2) to 0.11-0.15 through the addition of nitrogen gas into the ventilator system. A two hour period of hypoxia was chosen to reflect the average duration of asphyxia experienced by neonates in delivery rooms in Edmonton (personal communication with Dr. Po-Yin Cheung, Neonatologist). Based on previous experience in our lab, this degree of hypoxia is sufficient to produce a metabolic acidosis (~pH = 7.05) and cardiogenic shock (cardiac output ≤ 60% of baseline; systemic artery pressure ≤ 30 mmHg) in addition to multisystem organ injury and dysfunction (neurologic, myocardial, intestinal, renal, etc) which aligns with the currently accepted clinical definition of neonatal asphyxia from the American Academy of Pediatrics and the International Cerebral Palsy Task Force.(29)

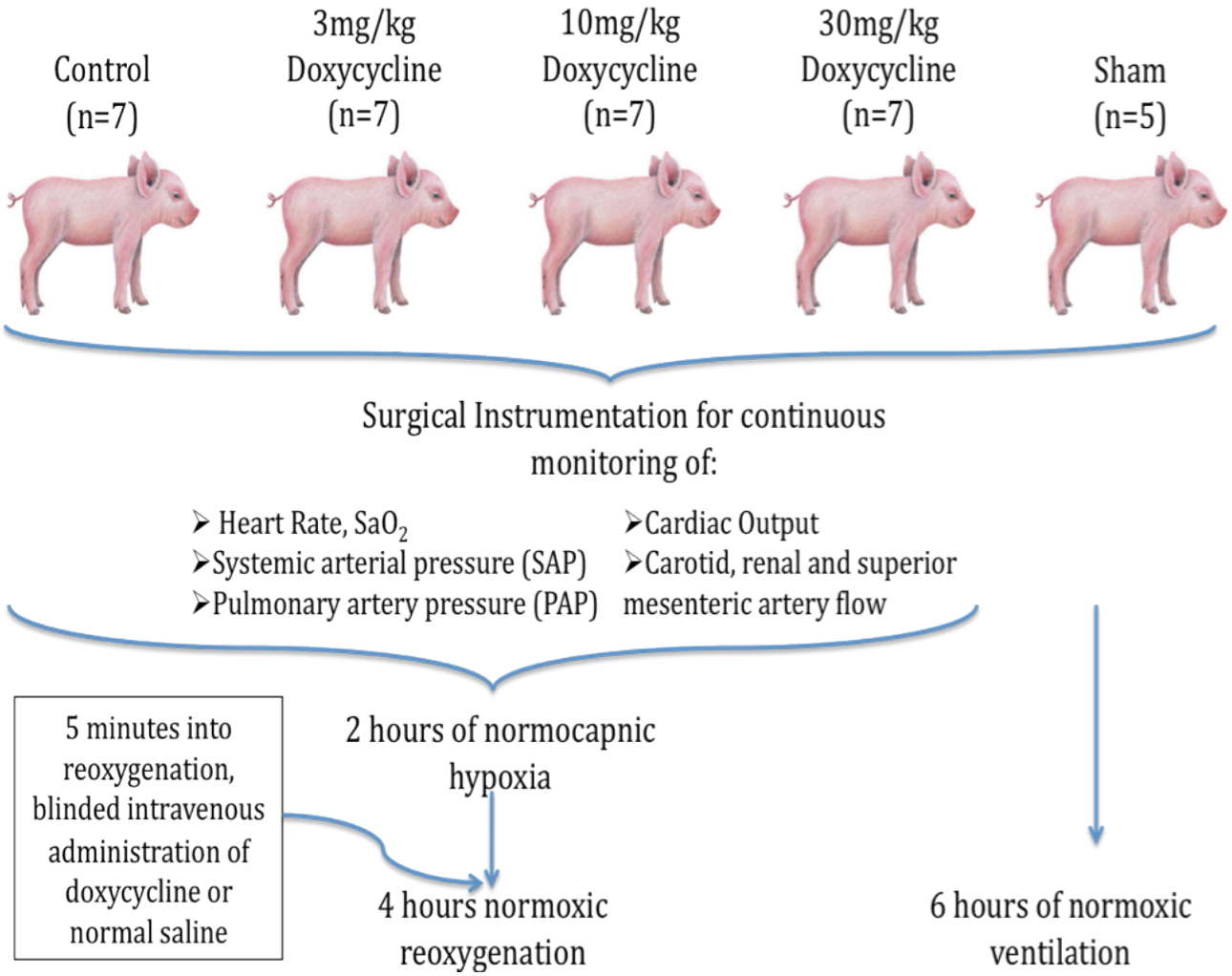
Piglets were resuscitated with 21% oxygen for a four hour period following asphyxia. The use of 21% oxygen complies with current treatment guidelines, which recommend initial resuscitation with 21% oxygen and titrating oxygen content up as necessary to achieve oxygenation.(30,31) Five minutes into reoxygenation piglets received an intravenous bolus of 10 ml normal saline (control group) or 3, 10 or 30 mg/kg doxycycline (reconstituted with normal saline to a total volume of 10 ml) given over 5 minutes. Five minutes was chosen to reflect true clinical conditions of neonatal resuscitation: the time it takes to get intravenous access and prepare the drug for administration. The administration of

saline or doxycycline was blinded to group allocation. Five sham-operated piglets were ventilated at a FiO_2 of 0.21 throughout the six hour period. Piglets were euthanized at the end of the procedure with 100mg/kg iv pentobarbital. An autopsy was performed, and tissue samples (heart, intestine, liver, lungs, kidney, brain, aorta, and pulmonary artery) were preserved in formalin for histology and snap frozen in liquid nitrogen for future biochemical analysis.

Conclusions

Taken together, neonatal pigs serve as an excellent model to study neonatal asphyxia, allowing for the greatest potential of transferability of findings to human neonates. Neonatal pigs have similar anatomy and physiology, particularly with regards to organ systems commonly affected by asphyxiating events in humans (heart, liver, kidney, and intestine), and normal hemodynamic and biochemical parameters have been established for reference. Neonatal pigs are large enough to allow for surgical instrumentation and thus continuous monitoring of various parameters throughout the study, in addition to allowing for sampling and intervention. Finally, neonatal pigs experience asphyxia in a similar way to humans, including the patterns of injury and the pathophysiological mechanisms underlying them.

Figure 4-1: Experimental design



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Chapter 5

Post-resuscitation Administration of Doxycycline Preserves Cardiac Contractile Function in Hypoxia-Reoxygenation Injury of Newborn Piglets

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A version of this chapter has been submitted for publication to Critical Care Medicine. Joseph R. LaBossiere, Jean-Sebastian Pelletier, Mohammad A.M. Ali, Aducio Thiesen, Richard Schulz, David L. Bigam and Po-Yin Cheung.

Introduction

Asphyxia is a significant cause of neonatal morbidity and mortality, accounting for a third of all neonatal deaths worldwide.(1,2) Multisystem organ injury and dysfunction is often present in surviving neonates.(3,4) Damage to the myocardium is unfortunately common, occurring in up to 80% of asphyxiated neonates, and contributes significantly to the morbidity and mortality associated with this condition.(5-7)

Myocardial injury in asphyxiated neonates ranges from a potentially reversible stunning of the myocardium to a irreversible myocardial infarction, and is associated with a variety of clinical presentations including arrhythmias, murmurs, congestive heart failure, and cardiogenic shock.(7) This injury is caused by two distinct temporal insults; injury from hypoxia itself and injury from reoxygenation of the hypoxic myocardium.(5,8) Re-introduction of molecular oxygen reactivates and overwhelms the electron transport chain in the mitochondria leading to an increased production of reactive oxygen and nitrogen species (RONS), including peroxynitrite.(8) RONS then activate matrix metalloproteinase (MMP)-2, a ubiquitous zinc dependent endopeptidase co-localized with various proteins in the cardiac sarcomere. Activated MMP-2 proteolyzes specific cardiac sarcomeric proteins, including troponin I (cTnI), resulting in contractile, and ultimately, cardiac dysfunction.(9,10) Diagnosis of myocardial injury and compromise in the neonate is difficult. Although increased levels of cardiac biomarkers in the plasma, such as cTnI, have been validated to reliably assess cardiomyocyte injury in the adult population, there is insufficient

evidence at present to promote their use in newborns.(11) Furthermore, treatment of the cardiac injury and dysfunction is largely supportive in nature, and aimed at maximizing functional output from the injured myocardium using inotropic and chronotropic agents.(5) With an understanding of the mechanisms leading to myocardial injury during reoxygenation, MMP-2 inhibition has emerged as a novel target for new interventions aimed at attenuating or preventing cardiac injury from occurring in the first place during the resuscitation of an asphyxiated neonate. Previous *ex vivo* studies have demonstrated the cardioprotective effects of MMP-2 inhibition in ischemic-reperfusion injury.(12-14) However, demonstration of this effect in clinically translatable animal models of hypoxia-reoxygenation is lacking.

The objective of this study is therefore to assess the effects of doxycycline, a drug with well recognized non-antimicrobial actions as an MMP inhibitor (15), has on cardiac injury and functional recovery in a clinically translatable swine model of neonatal hypoxia-reoxygenation. We hypothesized that early intravenous administration of doxycycline during the resuscitation of asphyxiated newborn piglets would attenuate cardiac injury and improve cardiac functional recovery. We also assessed the validity of plasma cTnI as a marker of myocardial injury and dysfunction in asphyxiated neonates.

Methods

Surgical procedure and experimental protocol

Refer to Chapter 4 for details.

Hemodynamic Analysis

Heart rate, central venous pressure, systemic arterial pressure (SAP), pulmonary artery pressure (PAP) and pulmonary artery flow (a surrogate of cardiac output) were continuously monitored and analyzed at pre-determined time points. Intermittent arterial and mixed venous blood samples were taken. Cardiac index (CI), stroke volume index (SVI), systemic oxygen delivery and consumption were obtained from standard formulas.(21)

Determination of plasma cTnI levels

Plasma cTnI levels were determined using an enzyme-linked immunosorbant assay kit (Life Diagnostics, Inc., Cat. No. 2010-4-HS).

Heart tissue processing

The entire left ventricle was removed and the apex placed in formalin for histology, with the remainder snap frozen in liquid nitrogen for biochemical analysis. For biochemical analyses, the left ventricle tissue was homogenized with 10μl/mg (tissue wet weight) of 50mM phosphate buffer containing 1mM EDTA (pH 7.0). Protein content was quantified using a bicinchoninic acid assay kit (Sigma-Aldrich Canada Ltd., Oakville, ON).

Determination of lactate, oxidative stress, cTnI and MMP-2 activity in cardiac tissue

Cardiac tissue lactate and lipid hydroperoxides levels were determined by a nicotinamide adenine dinucleotide enzyme coupled colorimetric assay (22)) and an enzyme-linked immunosorbant assay kit (#705003, Cayman Chemical Company, Ann Arbor, MI), respectively. Cardiac tissue cTnI levels were quantified by Western blot. Gelatinolytic activity of MMP-2 was quantified with zymography.(23) Hypoxia-reoxygenation induced activation of 72 kDa MMP-2 through glutathiolation (S-glutathiolated 72 kDa MMP-2) was determined by co-immunoprecipitation of left ventricle homogenates using specific antibodies against glutathione and MMP-2.(24)

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Hemodynamic variables were compared among groups and within specific time points using two-way and one-way analysis of variance (ANOVA) followed by Student Newman Keuls *post hoc* testing, respectively. Biochemical variables were compared between groups using one-way ANOVA followed by Fisher *post hoc* testing. Hemodynamic and biochemical correlations were determined using the Pearson method (SigmaPlot v11, Systat Software Inc, San Jose, CA). Significance was defined as $p<0.05$.

Results

Piglets were 2.6 ± 0.2 days old, weighing 1.90 ± 0.04 kg (n = 33), with no differences between groups. There was no difference in baseline hemodynamic and biochemical parameters between groups ($p>0.05$) (Tables 5-1 and 5-2).

At the end of normocapnic hypoxia, piglets in the treatment groups had evidence of cardiogenic shock (CI = 58±1% of baseline) (Figure 5-1), hypotension (SAP 31±1 mm/Hg) (Figure 5-4) and metabolic acidosis (pH 7.04±0.02) (Table 5-2) compared to sham-operated piglets (109±11% of baseline, 59±2 mm/Hg, and 7.37±0.02 respectively) (all $p<0.05$), with no difference between treatment groups.

Hemodynamic parameters in hypoxic-reoxygenated piglets treated with doxycycline

CI and SVI were significantly improved in asphyxiated piglets that received 10 mg/kg and 30 mg/kg doxycycline compared to controls (CI: 74 ± 4% and 84 ± 3% of baseline vs. 65 ± 3% of baseline in controls; SVI: 69 ± 5% and 78 ± 5% of baseline vs. 50 ± 5% of baseline in controls, respectively)(Figures 5-1 and 5-2). Heart rate was not different among groups (Figure 5-3). Heart rate was not significantly elevated in any of the groups compared to controls throughout reoxygenation, and was lower in all groups compared to controls at the end of reoxygenation.

At 4 hours of reoxygenation SAP was significantly improved in the 30 mg/kg doxycycline group (46 ± 3 mmHg vs. 35 ± 2 mmHg in controls; Figure

5-4), whereas PAP was lower in the 3 mg/kg doxycycline group compared to control (27 ± 2 mmHg vs. 30 ± 2 mmHg, respectively; Figure 5-5). PAP/SAP ratio was lower in the 3 mg/kg and 30 mg/kg doxycycline-treated groups compared to the control group (0.74 ± 0.07 and 0.70 ± 0.06 vs. 0.85 ± 0.07 of controls, respectively; Figure 5-6). Associated with the improved CI, systemic oxygen delivery was increased in the 10 mg/kg and 30 mg/kg doxycycline-treated groups (68 ± 3% and 76 ± 3% of baseline vs. 56 ± 4% of baseline in controls, respectively; Figure 5-7). Systemic oxygen consumption was also improved in the 10 mg/kg and 30 mg/kg doxycycline groups compared to control piglets (84 ± 8% and 101 ± 11% of baseline vs. 69 ± 4% of baseline in controls, respectively; Figure 5-8).

Biochemical markers in hypoxic-reoxygenated piglets treated with doxycycline

At the end of experiment, the control group had significantly higher plasma cTnI levels than that of sham-operated piglets. cTnI was attenuated in piglets treated with 10 mg/kg and 30 mg/kg doxycycline, with significantly lower levels observed only in piglets treated with 30 mg/kg doxycycline compared to controls.(Figure 5-9). Piglets treated with 30 mg/kg doxycycline had significantly higher left ventricular tissue cTnI and lower lactate levels than those of controls (Figures 5-10 and 5-11, respectively), with no significant differences observed in 3 mg/kg and 10 mg/kg doxycycline groups. Plasma cTnI and myocardial lactate were negatively correlated with CI and SVI at 4 hours of reoxygenation (r = -0.47

to -0.52, $p < 0.01$)(Figures 5-12 A and B; Figures 5-13 A and B, respectively). Plasma cTnI correlated positively with myocardial tissue lactate ($r = 0.50$, $p < 0.01$), (Figure 5-14A) and negatively with myocardial tissue cTnI levels ($r=-0.41$, $p=0.02$)(Figure 5-14B).

Myocardial tissue lipid hydroperoxide levels were attenuated in a dose-dependent manner in doxycycline treated piglets, with significantly lower levels in the 10 mg/kg and 30 mg/kg doxycycline-treated groups compared to controls (Figure 5-15). There was also a dose-dependent decrease in total MMP-2 activity as determined by gelatin zymography in the myocardium of piglets treated with doxycycline compared to controls (Figure 5-16), with a significant decrease noted in 64 kDa MMP-2 activity (0.83 ± 0.12 AU and 0.77 ± 0.15 AU of 10 mg/kg and 30 mg/kg doxycycline-treated groups vs. 1.57 ± 0.30 AU of controls, respectively) but not in 72 kDa and 75 kDa MMP-2 activities. Hypoxia-reoxygenated piglets treated with either doxycycline or normal saline had increased S-glutathiolated 72 kDa MMP-2 compared to sham-operated piglets, with no significant difference observed between the treatment groups (Figure 5-17).

Histology

There were no histological differences in the heart tissue observed between any of the groups (data not shown).

Discussion

To our knowledge, this is the first study to test the cardioprotective effects of MMP inhibition in the resuscitation of hypoxic newborns. In a clinically translatable animal model of neonatal hypoxia-reoxygenation resulting in cardiogenic shock, we demonstrated that the post-resuscitation administration of doxycycline improved cardiac function during reoxygenation, which was associated with the inhibition of MMP-2 activation and the attenuation of myocardial injury.

cTnI is a vital sarcomeric protein regulating actin-myosin interactions that has been found to be proteolyzed by MMP-2 in ischemic-reperfusion injury (25,26). The plasma level of cTnI is validated as a marker of myocardial injury and a predictor of adverse outcomes in the adult population.(27,28) However, the utility of cTnI in diagnosing myocardial injury and the prognostication of outcomes in neonates is less clear.(11) Some studies suggest that plasma levels of cTnI reflect the degree of cardiac injury and provide a means to prognosticate morbidity and mortality in asphyxiated human neonates, specifically with regards to the degree of hypoxic-ischemic encephalopathy present.(6,29-31) However, there is a lack of evidence demonstrating that plasma cTnI levels reflect actual myocardial injury and dysfunction in neonates.(11) Plasma cTnI was elevated in control piglets subjected to hypoxia-reoxygenation compared to Sham-operated piglets, which was attenuated with 10 mg/kg and 30 mg/kg doxycycline, though significantly lower levels were observed only in the latter dose. Finally, we demonstrate a significant positive correlation between plasma cTnI and

myocardial tissue lactate levels, and significant negative correlations between plasma cTnI levels and cardiac function (CI and SVI) at recovery.

In addition to the preservation of myocardial tissue cTnI levels, we found significantly lower levels of myocardial tissue lactate in the 30 mg/kg doxycycline-treated group compared to control, suggesting less cardiac injury and improved oxyidative metabolism in these piglets. Myocardial oxidative stress, defined by the amount of lipid hydroperoxides in the tissue, was also significantly reduced with doxycycline treatment. The mechanisms for these changes are not clear and it is not known if it is related to improved myocardial perfusion with doxycycline treatment. Interestingly, tetracyclines have been described to have possible anti-oxidant effects (32) which could explain the reduction in markers of oxidative stress observed in piglets treated with doxycycline.

Increased myocardial MMP-2 activity was observed in control piglets with hypoxia-reoxygenation, which is consistent with previous studies.(33,34) MMP-2 activation occurs through various mechanisms, including direct proteolytic cleavage of an autoinhibitory polypeptide domain that covers the catalytic site (to 64 kDa MMP-2), as well as through S-glutathiolation mediated by peroxynitrite and glutathione, inducing a conformational change that exposes the catalytic site (S-glutathiolated 72 kDa MMP-2).(35) Within the catalytic site is a Zn^{2+} cation that is vital to the proteolytic activity of MMP-2. Doxycycline, through its affinity for binding divalent cations is thought to inhibit MMPs by binding to the Zn^{2+} in the catalytic site.(9,15) Thus doxycycline inhibits both active forms of MMP-2.

Compared to control, MMP-2 activity was significantly reduced in all doxycycline treatment groups in a dose-dependent manner.

In addition to improved systemic hemodynamics with increased SAP, CI and systemic oxygen delivery, the administration of doxycycline may also benefit pulmonary hemodynamics with a lower PAP/SAP ratio. Hypoxic neonates are at risk for pulmonary hypertension (also known as persistent fetal circulation) with a right to left shunting of blood in the cardiopulmonary circulation. The PAP/SAP ratio indicates the potential for pulmonary to systemic circulatory shunting; when the PAP/SAP ratio approaches 1, high PAP can force deoxygenated blood through the ductus arteriosus of the neonate and into the systemic circulation, worsening the systemic hypoxemia. Our findings suggest a unique beneficial effect of doxycycline in neonates at risk for pulmonary hypertension.

Prior studies demonstrating attenuation of cardiac injury with improvement in functional recovery through MMP-2 inhibition have primarily utilized *ex vivo* models of ischemic-reperfusion injury.(14,36,37) Although cardiac injury and dysfunction due to MMP-2 activation has been demonstrated in piglets subjected to hypoxia-reoxygenation, the authors did not test the effect of MMP inhibition in their model.(33,34) We designed the current study to test this and to simulate the resuscitation of severely hypoxic neonates who have significant hypotension and cardiogenic shock. Fluid bolus and drug administration at 5 minutes into resuscitation reflects clinical practice during neonatal resuscitation.

Although there are limitations in animal studies and experimental protocols of hypoxia-reoxygenation, piglets are an excellent model for studying neonatal asphyxia due to facile instrumentation for collection of biological specimens and data and a thorough understanding of swine physiology and anatomy, which is similar to humans.(38-40) Importantly, newborn piglets and human neonates share similar pathophysiological responses to asphyxia.(41) As piglets were observed for four hours of recovery after resuscitation, it is not certain if the improved hemodynamic recovery will be maintained beyond the acute period. Gelatin zymography has inherent limitations as it assesses MMP-2 activity *in vitro* but not *in vivo,* and therefore does not reflect the balance between MMPs and their endogenous tissue inhibitors. Finally, the use of doxycycline deserves consideration, as tetracyclines are typically not used in children under the age of eight for fear of causing cerebral pseudoedema and irreversible staining of the teeth.(42,43) However, the evidence describing these side effects of doxycycline use in neonates is weak. Some evidence suggests that it safe to use doxycycline in children under the age of 8, and doxycycline is in fact the drug of choice when indicated in specific bacterial infections in neonatal and pediatric populations.(17,44,45) Furthermore, doxycycline is the only systemic MMP inhibitor approved for use in humans (treatment of peridontitis and rosacea), and importantly, is the only intravenous MMP inhibitor available for clinical use at present.(16,18,46) Although this makes doxycycline, in our opinion, the most clinically applicable MMP inhibitor at present for use in neonatal asphyxia,

further investigations are needed to establish the efficacy and safety profile of doxycycline in neonates.

In summary, our study is the first to demonstrate in a clinically translatable swine model of neonatal hypoxia-reoxygenation that post-resuscitation administration of doxycycline attenuates cardiac injury and improves cardiac functional recovery resulting in improved cardio-pulmonary and systemic hemodynamics. These findings are associated with a reduction in intracellular oxidative stress and MMP-2 activity. Finally, we have shown that serum cTnI correlates negatively with myocardial tissue cTnI and cardiac functional recovery, and positively with myocardial tissue lactate, thus supporting the role of serum cTnI as a clinically valid marker of cardiomyocyte injury and cardiac dysfunction in neonatal asphyxia.

Table 5-1 – Baseline hemodynamic parameters under normoxic baseline conditions.

	Sham-operated (n = 5)	Control (n = 7)	3 mg/kg Doxycycline (n = 7)	10 mg/kg Doxycycline (n = 7)	30 mg/kg Doxycycline (n = 7)
Cardiac Index (ml/min x kg)	168 ± 9	192 ± 8	209 ± 18	186 ± 7	215 ± 26
Heart Rate (beats/min)	204 ± 8	195 ± 4	206 ± 10	205 ± 10	213 ± 5
Stroke Volume Index (ml/beat x kg)	0.82 ± 0.02	0.98 ± 0.04	1.01 ± 0.08	0.91 ± 0.05	0.93 ± 0.11
Systemic Arterial Pressure (SAP) (mmHg)	66 ± 5	76 ± 4	70 ± 3	70 ± 2	80 ± 5
Pulmonary Artery Pressure (PAP) (mmHg)	24 ± 2	30 ± 2	27 ± 2	30 ± 2	28 ± 2
PAP/SAP Ratio	0.36 ± 0.03	0.39 ± 0.01	0.40 ± 0.02	0.43 ± 0.02	0.36 ± 0.03
Systemic Oxygen Delivery (ml O_2/min x kg)	18 ± 1	20 ± 1	21 ± 1	20 ± 1	21 ± 2
Systemic Oxygen Consumption (ml O_2/min x kg)	8 ± 1	8 ± 1	8 ± 1	7 ± 0	7 ± 1

Table 5-2 – Arterial blood gas parameters at baseline, end of hypoxia and end of reoxygenation.

Group	Baseline 0 min	End of Hypoxia 120 min	End of Reoxygenation 240 min
pH			
Sham-operated	7.41 ± 0.01	7.37 ± 0.02	7.33 ± 0.02
Control	7.39 ± 0.02	7.05 ± 0.03*	7.30 ± 0.02
Doxycycline 3 mg/kg	7.39 ± 0.02	7.02 ± 0.02*	7.30 ± 0.01
″ **10** ″	7.38 ± 0.02	7.06 ± 0.04*	7.35 ± 0.03
″ **30** ″	7.39 ± 0.02	7.07 ± 0.04*	7.34 ± 0.02
P_aO_2 (mmHg)			
Sham-operated	74 ± 2	75 ± 4	73 ± 2
Control	69 ± 1	41 ± 3*	71 ±2
Doxycycline 3 mg/kg	68 ± 2	38 ± 2*	67 ± 2
″ **10** ″	64 ± 2	32 ± 2*	65 ± 3
″ **30** ″	67 ± 2	40 ± 3*	65 ± 3
P_aCO_2 (mmHg)			
Sham-operated	37 ± 2	42 ± 2	42 ± 2
Control	38 ± 1	37 ± 2	42 ± 1
Doxycycline 3 mg/kg	38 ± 2	42 ± 1	42 ± 1
″ **10** ″	41 ± 1	41 ± 1	41 ± 1
″ **30** ″	39 ± 1	39 ± 1	42 ± 1
HCO_3^- (mmol/L)			
Sham-operated	23.5 ± 0.4	23.2 ± 0.5	21.0 ± 0.5
Control	22.9 ± 0.7	9.6 ± 0.6*	19.6 ± 0.9
Doxycycline 3 mg/kg	23.6 ± 0.6	9.4 ± 0.8*	19.5 ± 0.6
″ **10** ″	23.7 ± 1.2	10.2 ± 1.0*	22.1 ± 1.6
″ **30** ″	23.6 ± 0.9	9.8 ± 0.8*	22.0 ± 0.8
Lactate (mmol/L)			
Sham-operated	2.6 ± 0.5	1.8 ± 0.5	2.2 ± 0.8
Control	2.8 ± 0.4	12.3 ± 1.7*	3.5 ± 0.6
Doxycycline 3 mg/kg	2.9 ± 0.2	10.7 ± 1.5*	3.2 ± 0.5
″ **10** ″	3.3 ± 0.4	10.4 ± 1.9*	2.7 ± 0.4
″ **30** ″	2.9 ± 0.5	11.0 ± 1.9*	2.3 ± 0.4

*** p<0.05 vs. sham-operated group**

Figure 5-1: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on cardiac index in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

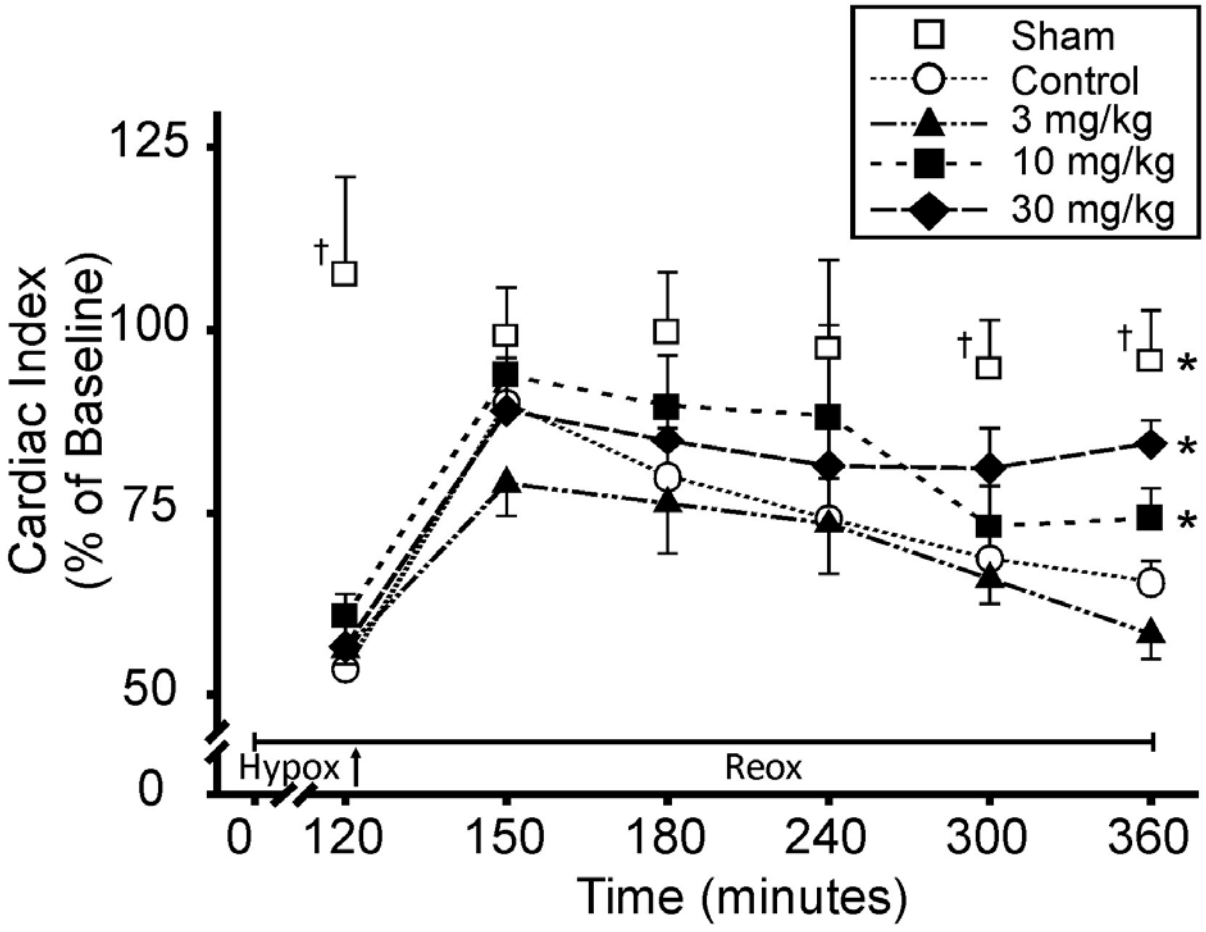


Figure 5-2: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on stroke volume index in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

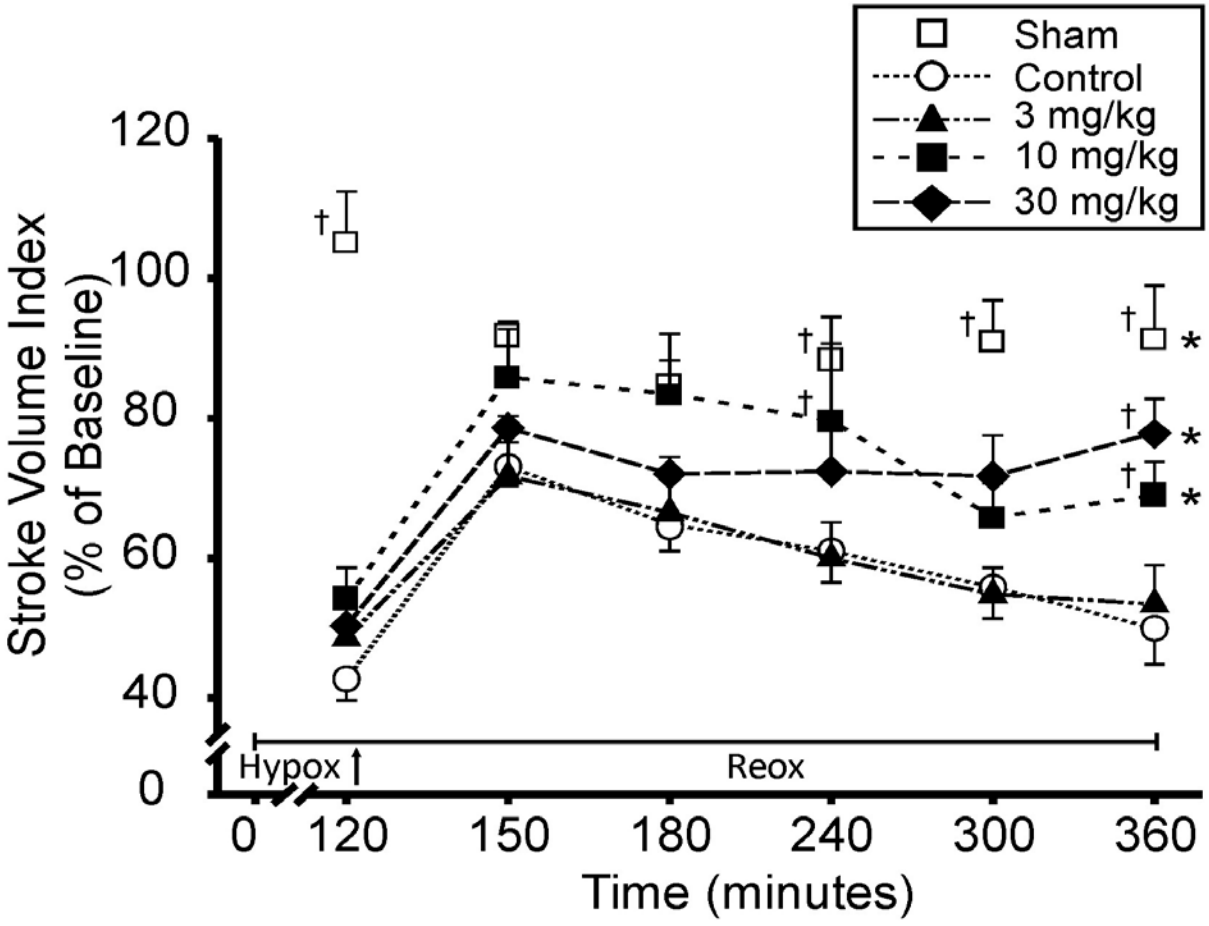


Figure 5-3: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on heart rate in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

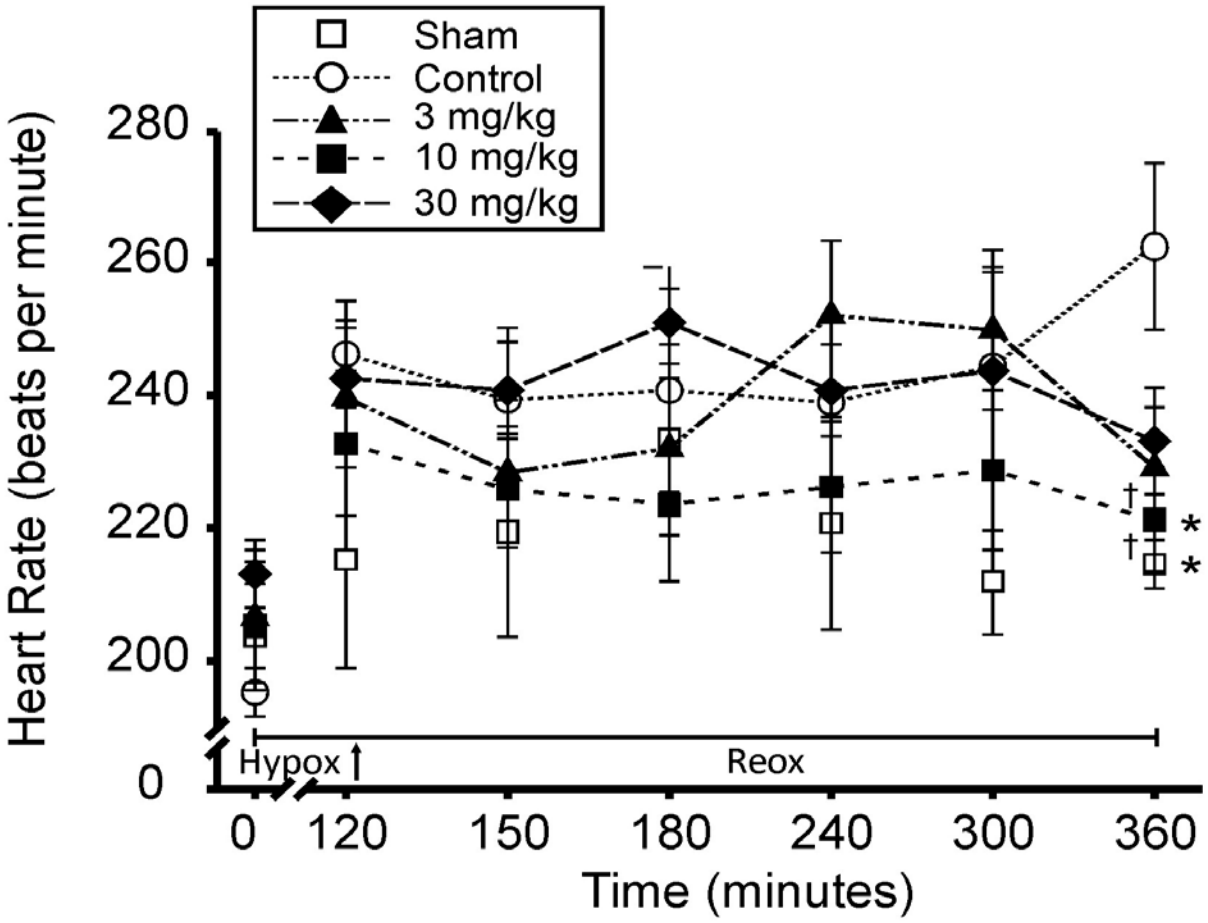


Figure 5-4: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on systemic arterial pressure in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

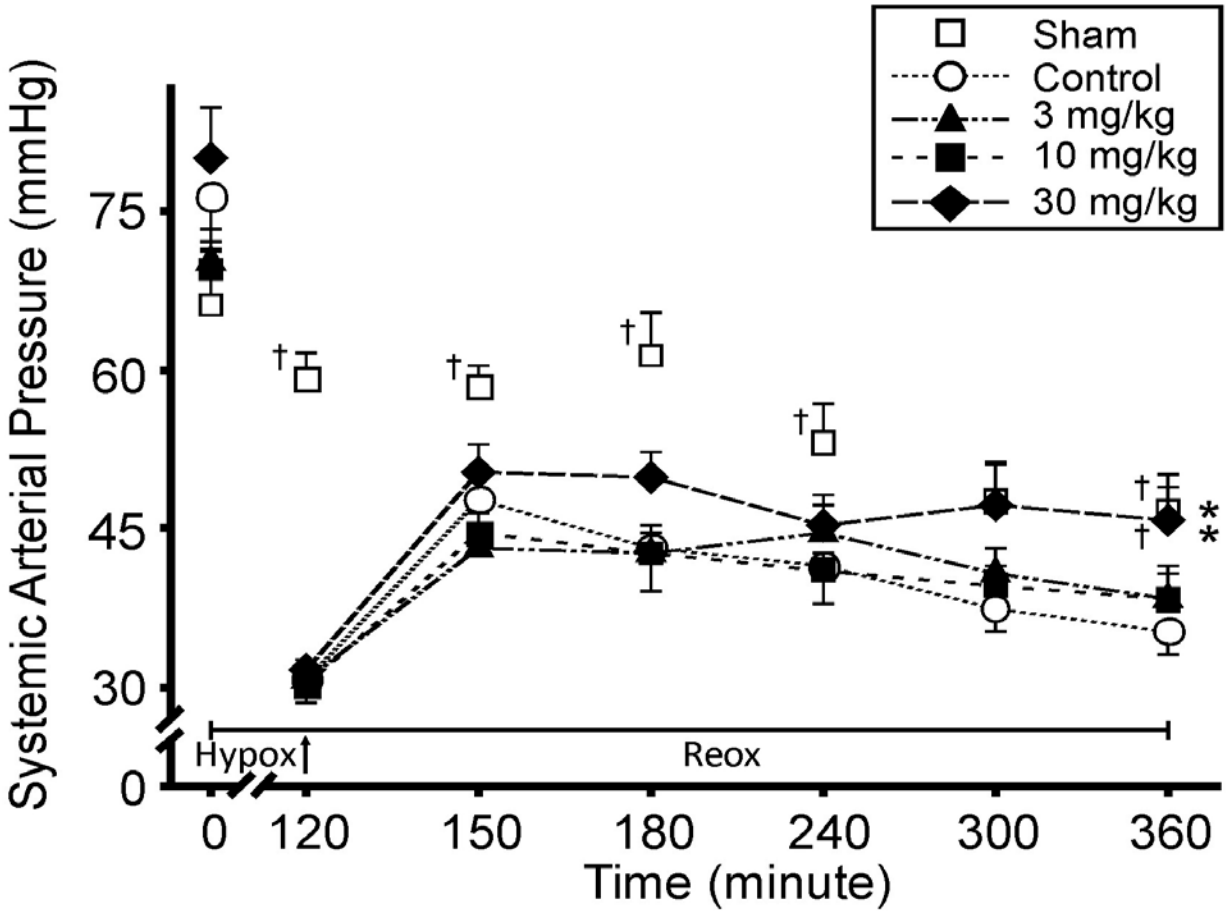


Figure 5-5: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on pulmonary artery pressure in piglets exposed to hypoxia. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

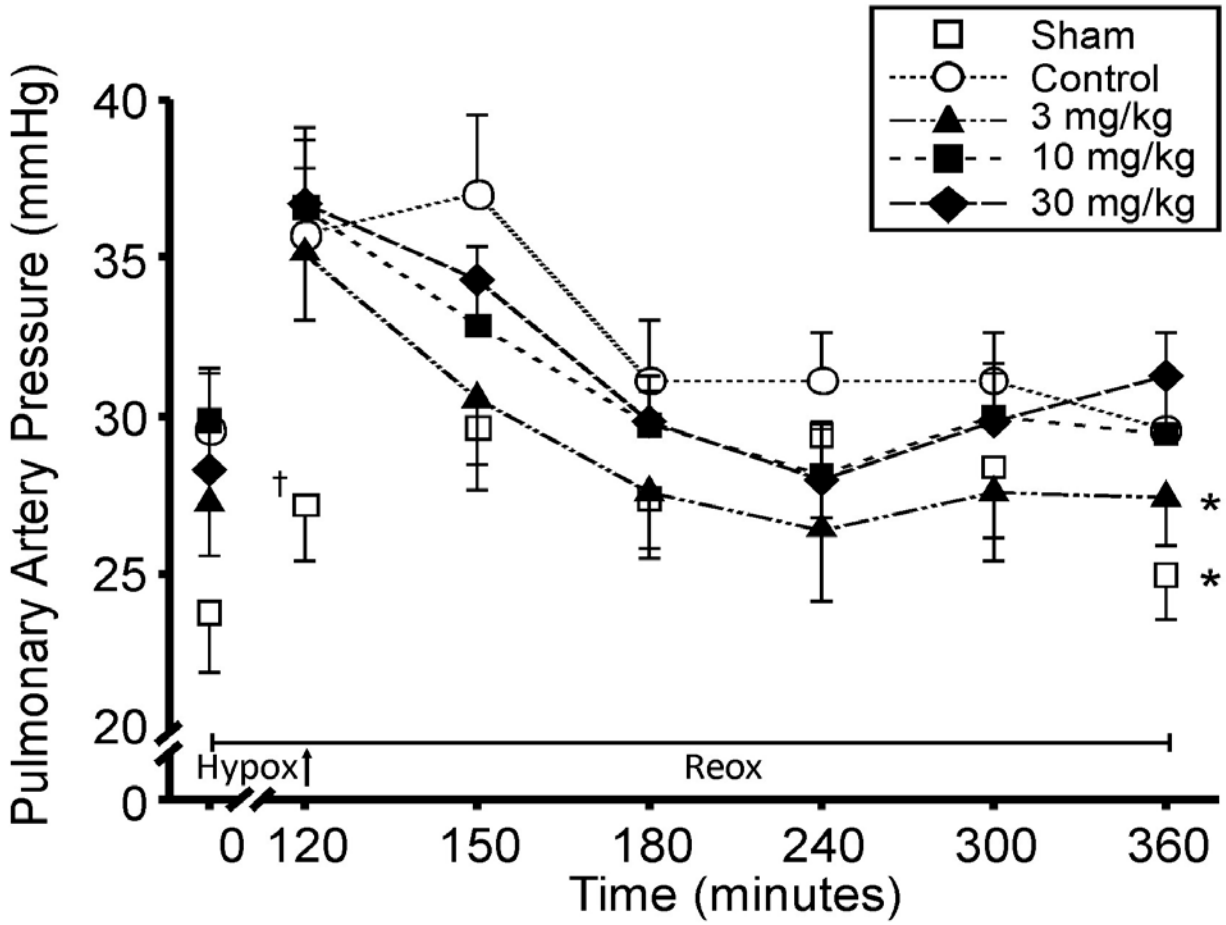


Figure 5-6: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on pulmonary artery to systemic artery pressure ratio in piglets exposed to hypoxia. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

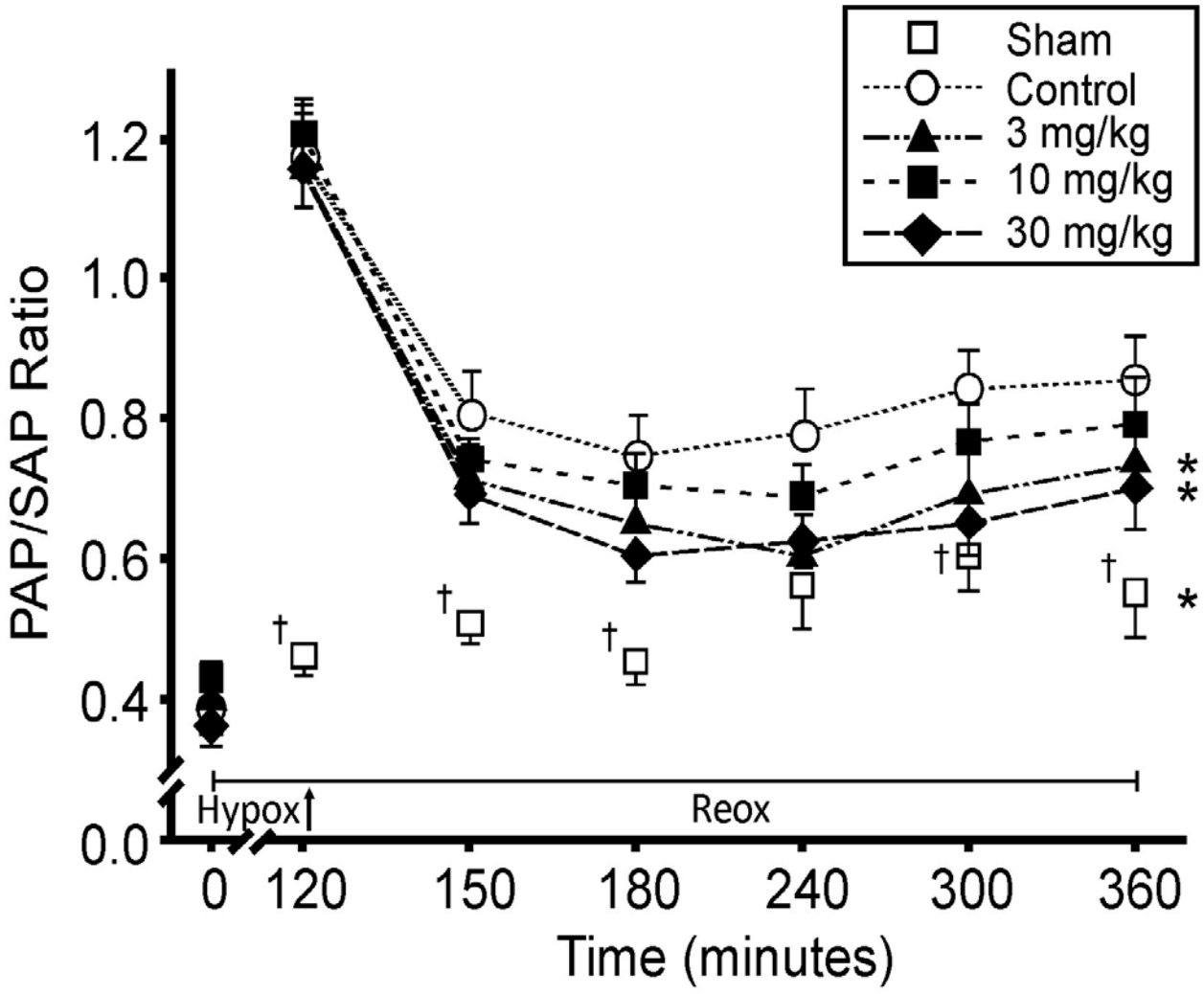


Figure 5-7: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on systemic oxygen delivery for piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

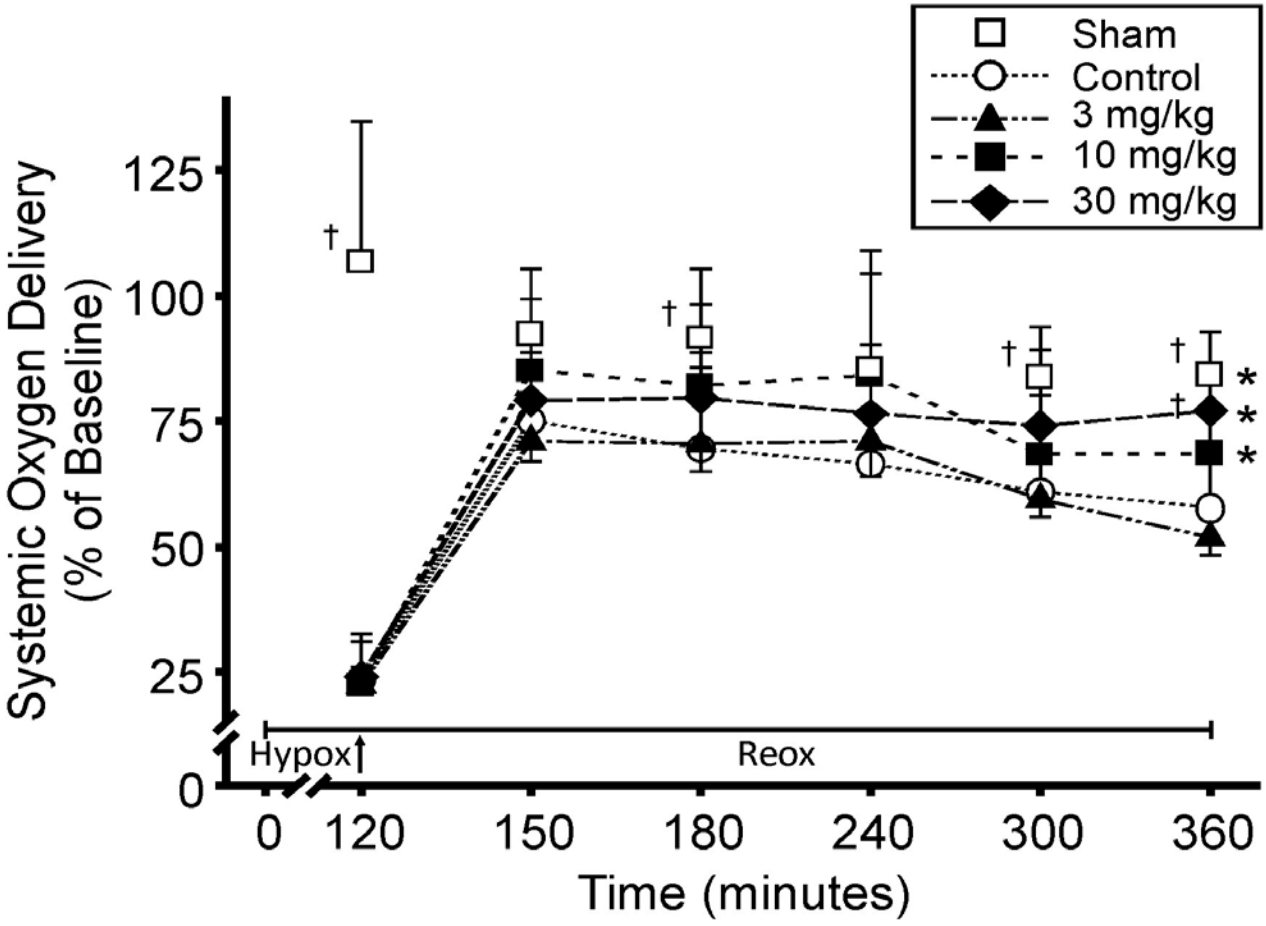


Figure 5-8: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on systemic oxygen consumption for piglets exposed to hypoxia. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

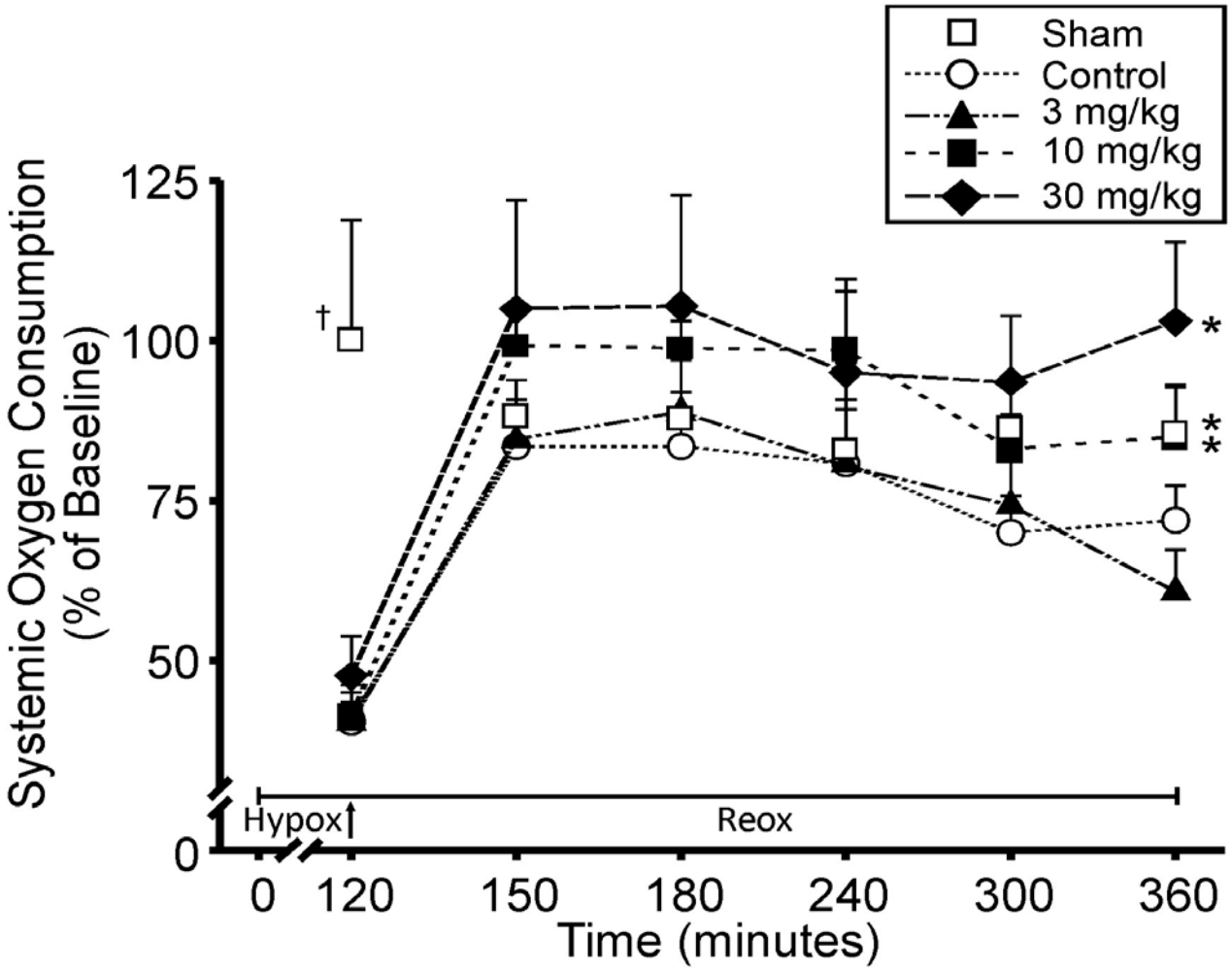


Figure 5-9: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on plasma troponin I levels in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

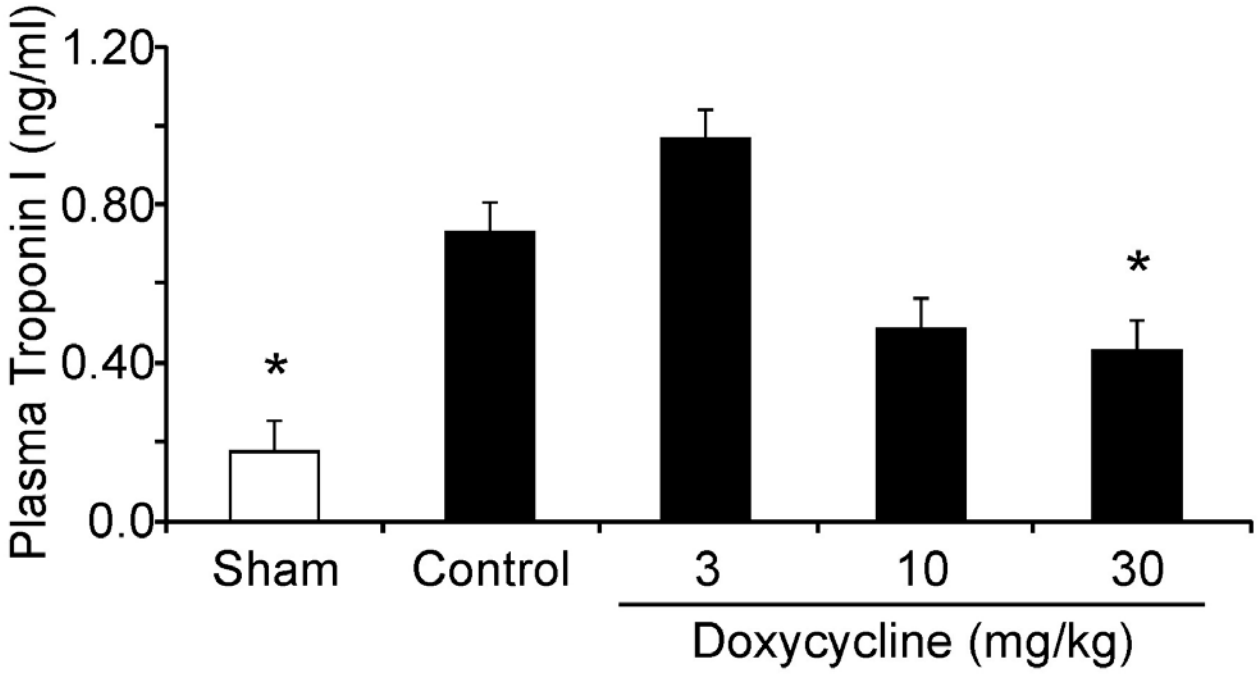


Figure 5-10: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on myocardial tissue troponin I levels in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

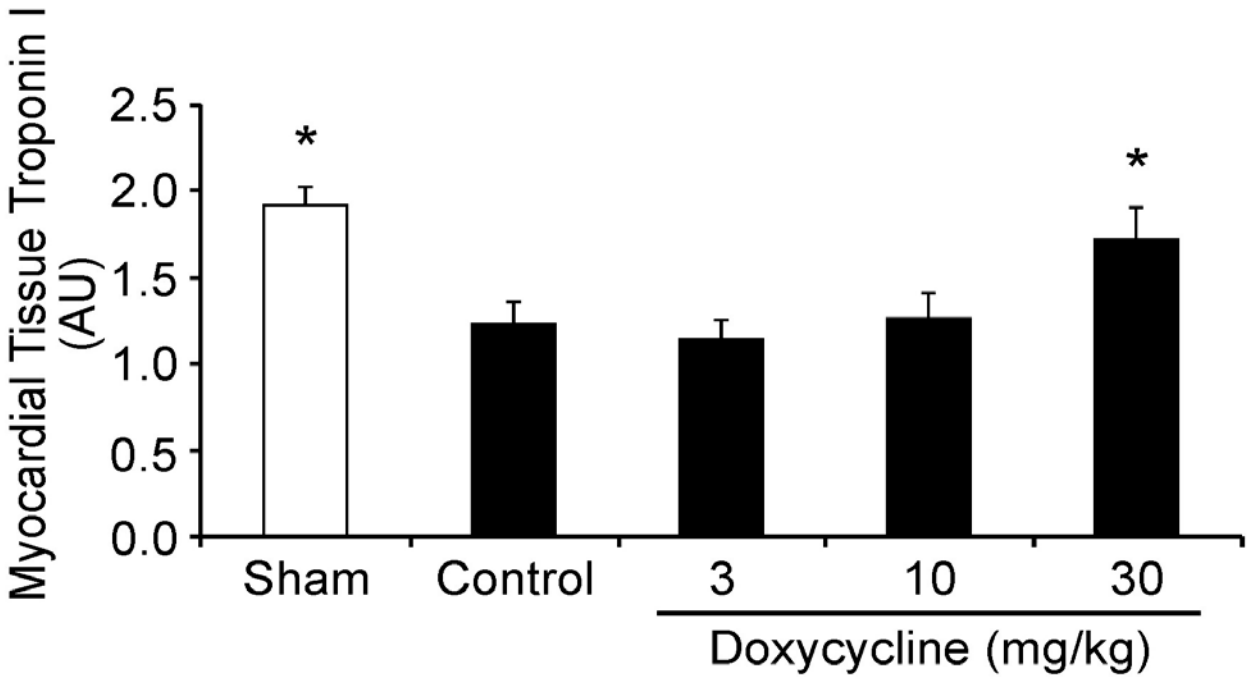


Figure 5-11: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on myocardial tissue lactate levels in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

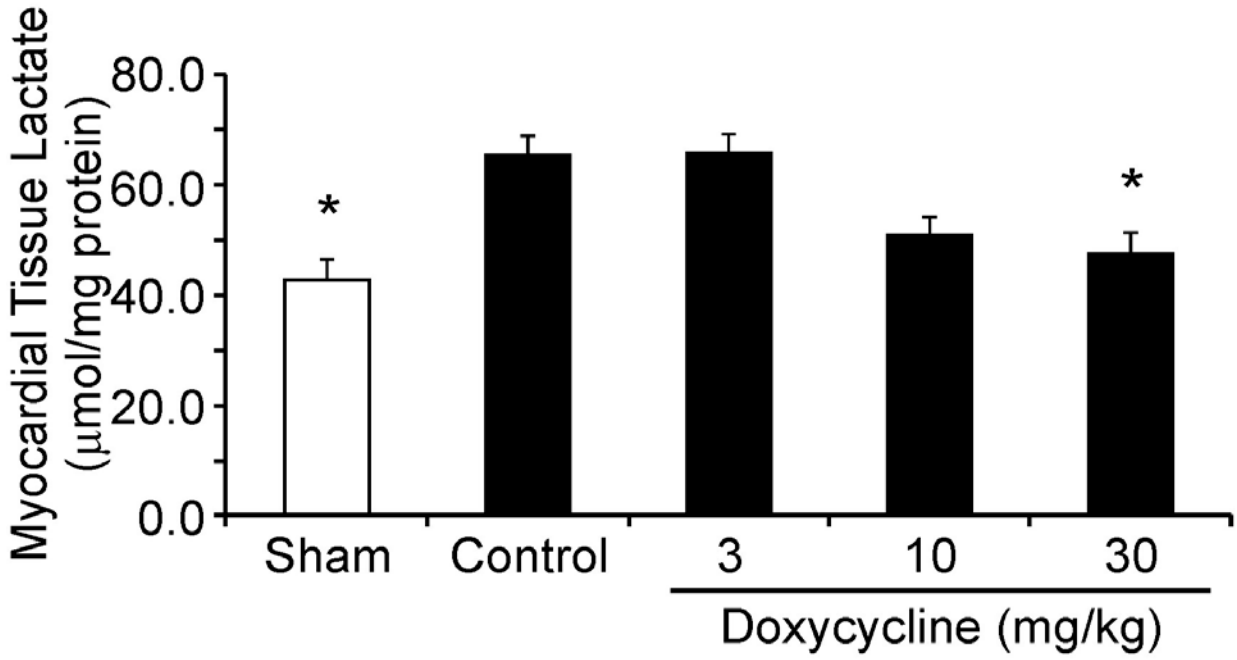
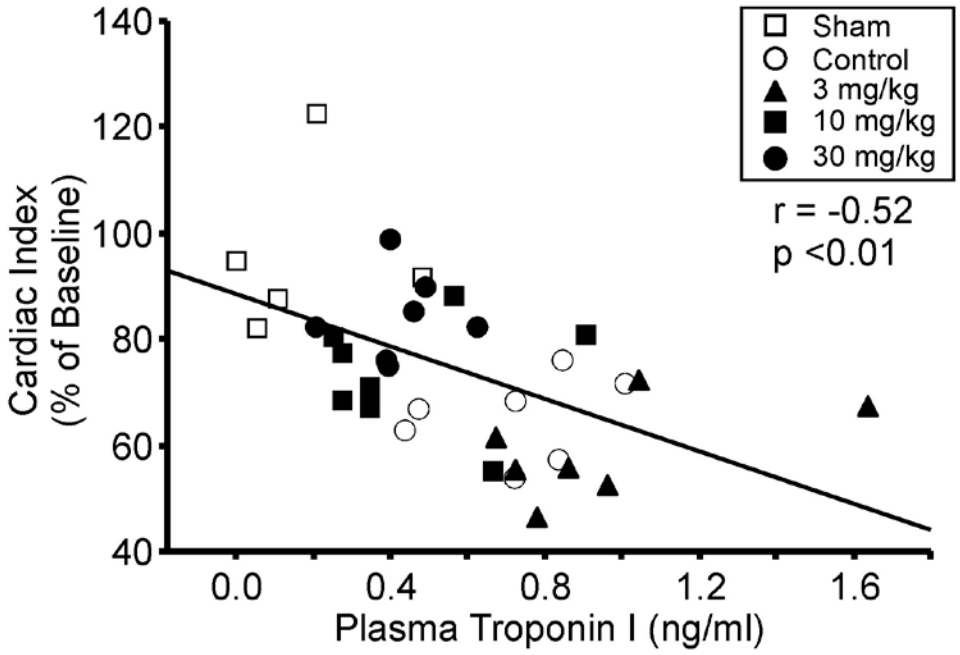


Figure 5-12: Plasma troponin I negatively correlates with cardiac index and stroke volume index in piglets treated with saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) during hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R.

A. Plasma Troponin I vs. Cardiac Index



B. Plasma Troponin I vs. Stroke Volume Index

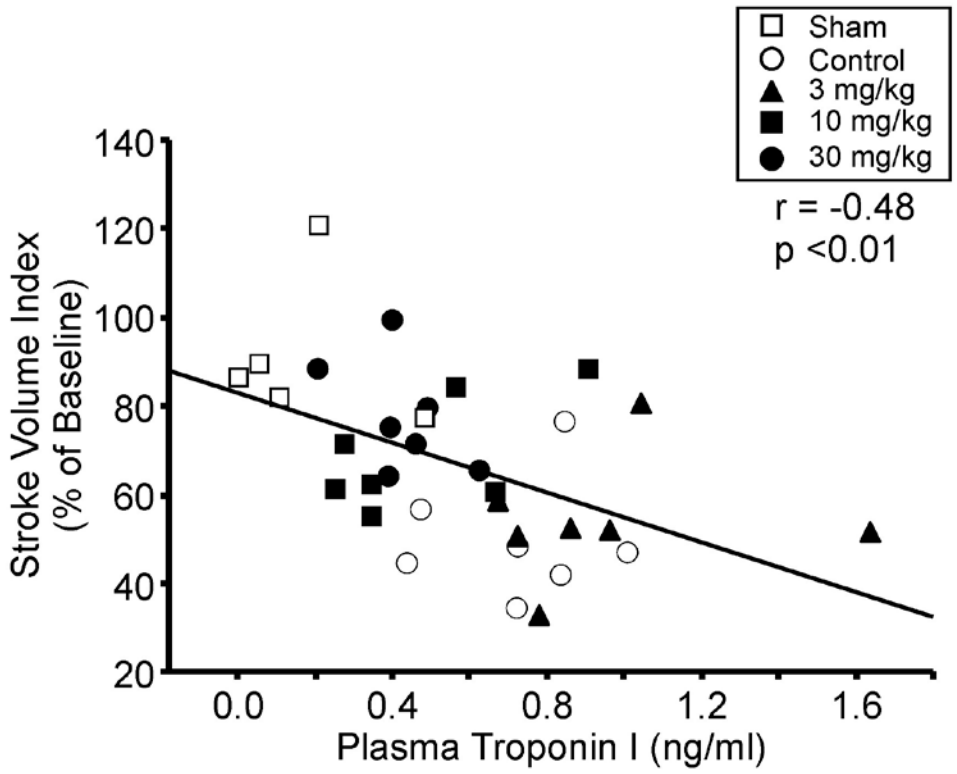
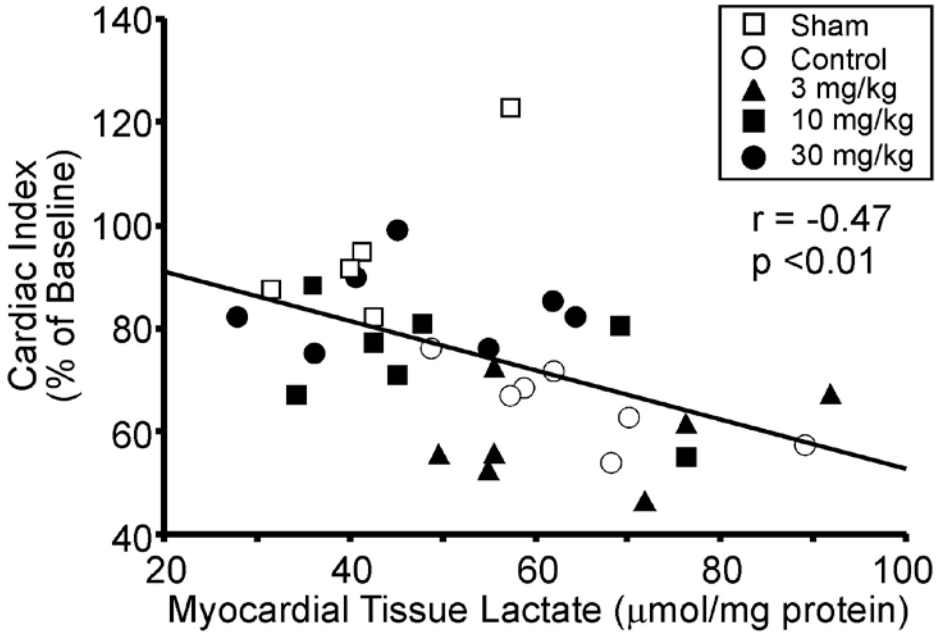


Figure 5-13: Myocardial tissue lactate negatively correlates with cardiac index and stroke volume index in piglets treated with saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) during hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R.

A. Myocardial Tissue Lactate vs. Cardiac Index



B. Myocardial Tissue Lactate vs. Stroke Volume Index

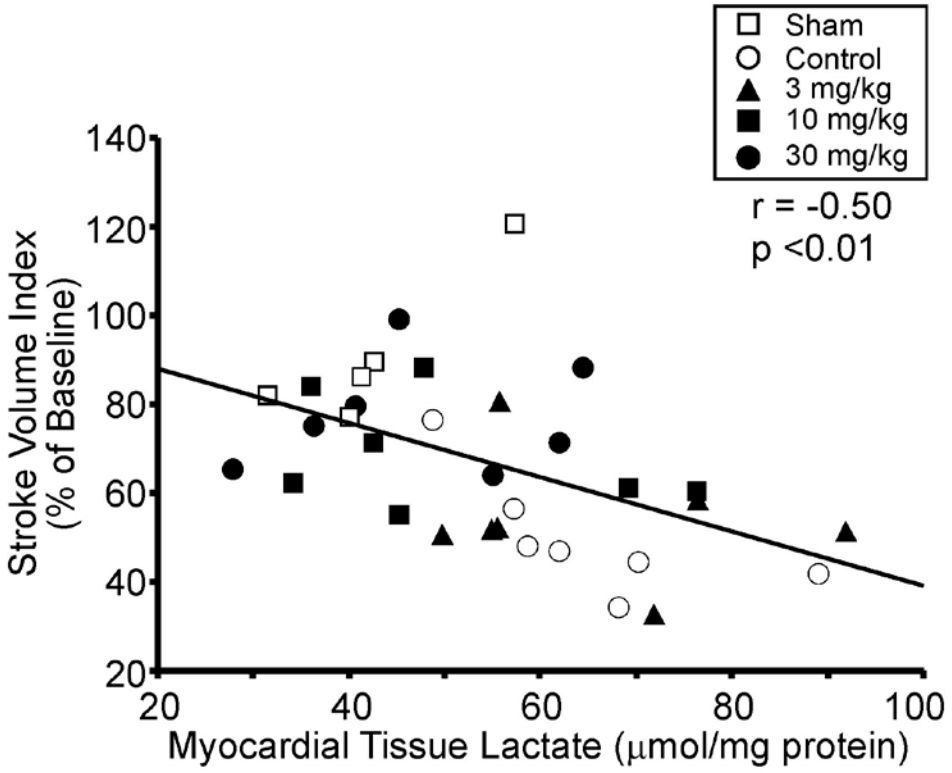
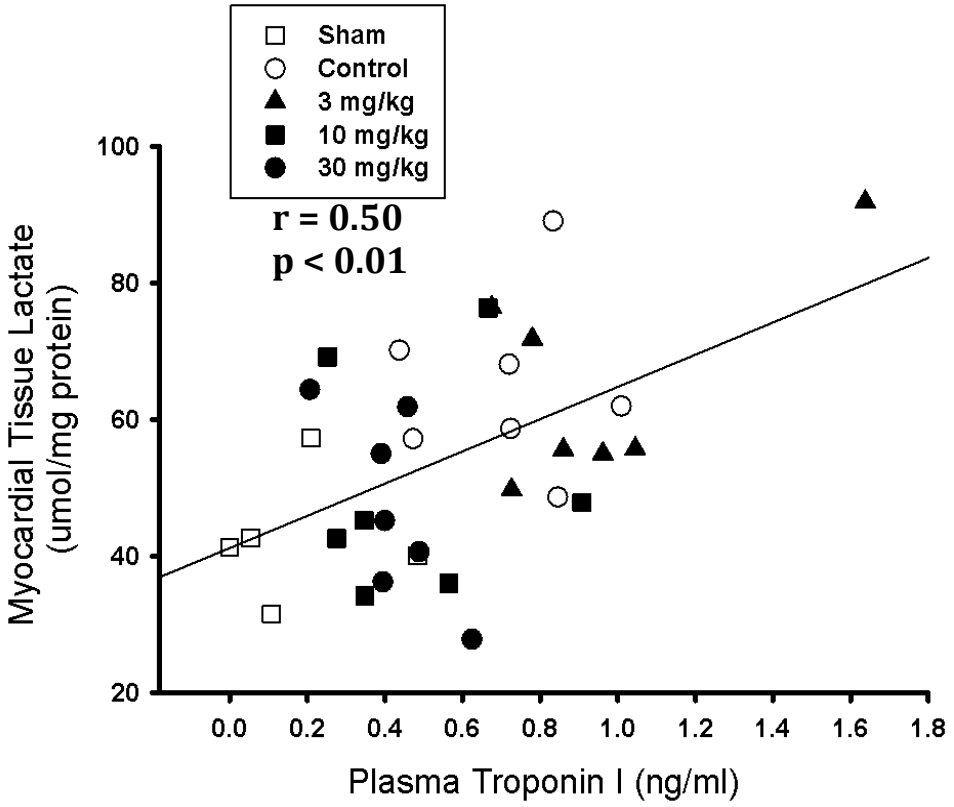


Figure 5-14: Plasma troponin I positively correlates with myocardial tissue lactate and negatively with myocardial tissue troponin I in piglets treated with saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) during hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R.

A. Plasma Troponin I vs. Myocardial Tissue Lactate



B. Plasma Troponin I vs. Myocardial Tissue Troponin I

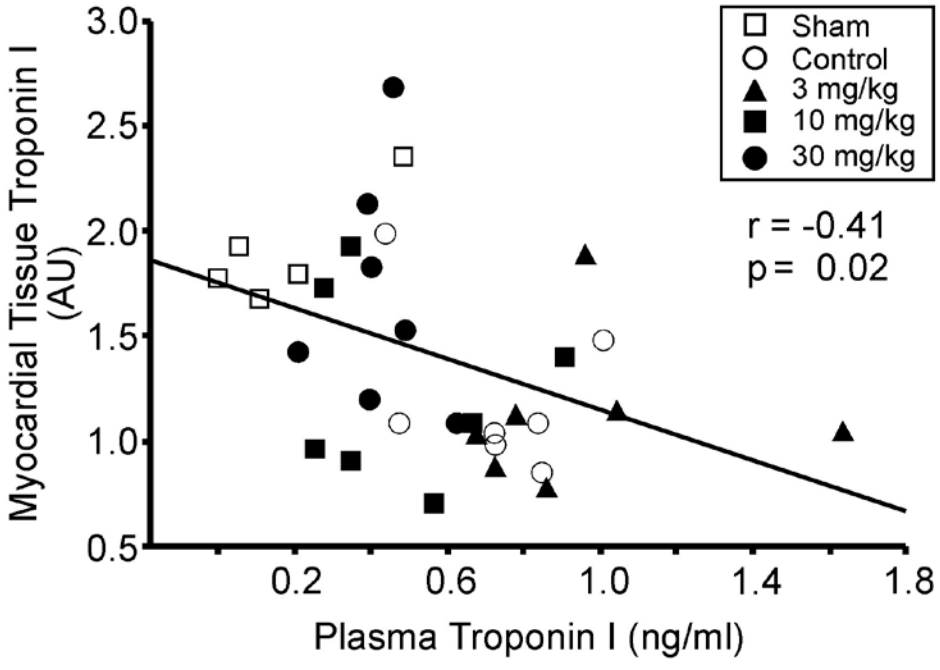


Figure 5-15: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on myocardial lipid hydroperoxide levels in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

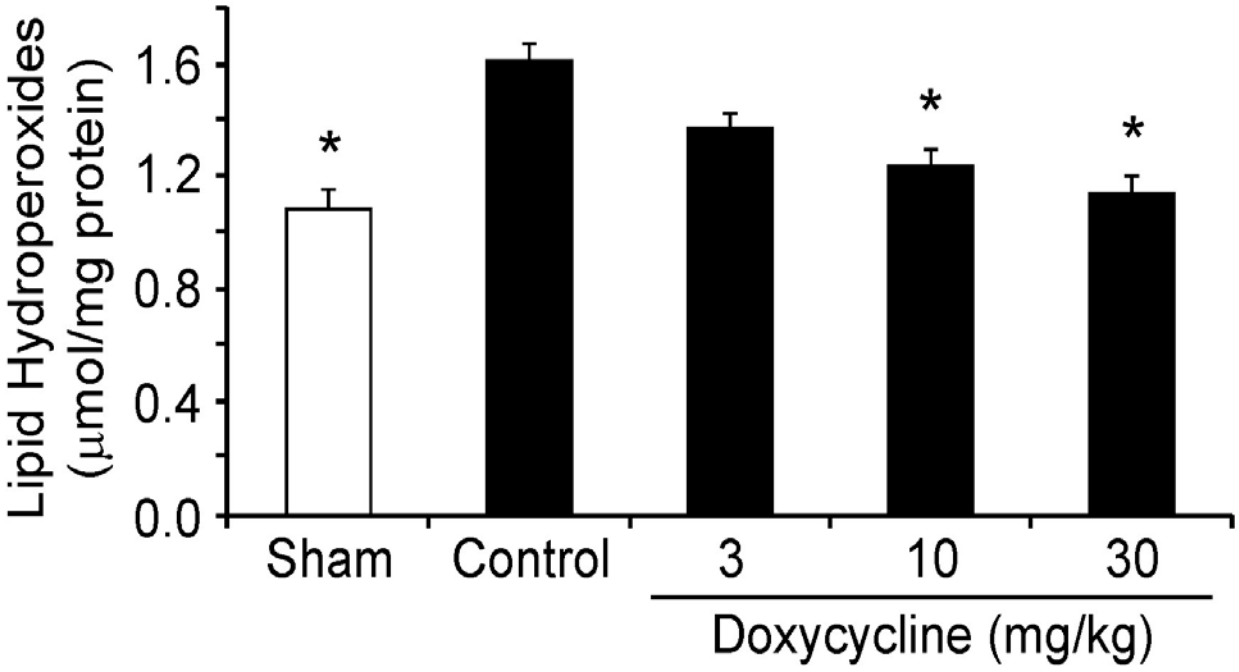


Figure 5-16: : The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on myocardial MMP-2 activity in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

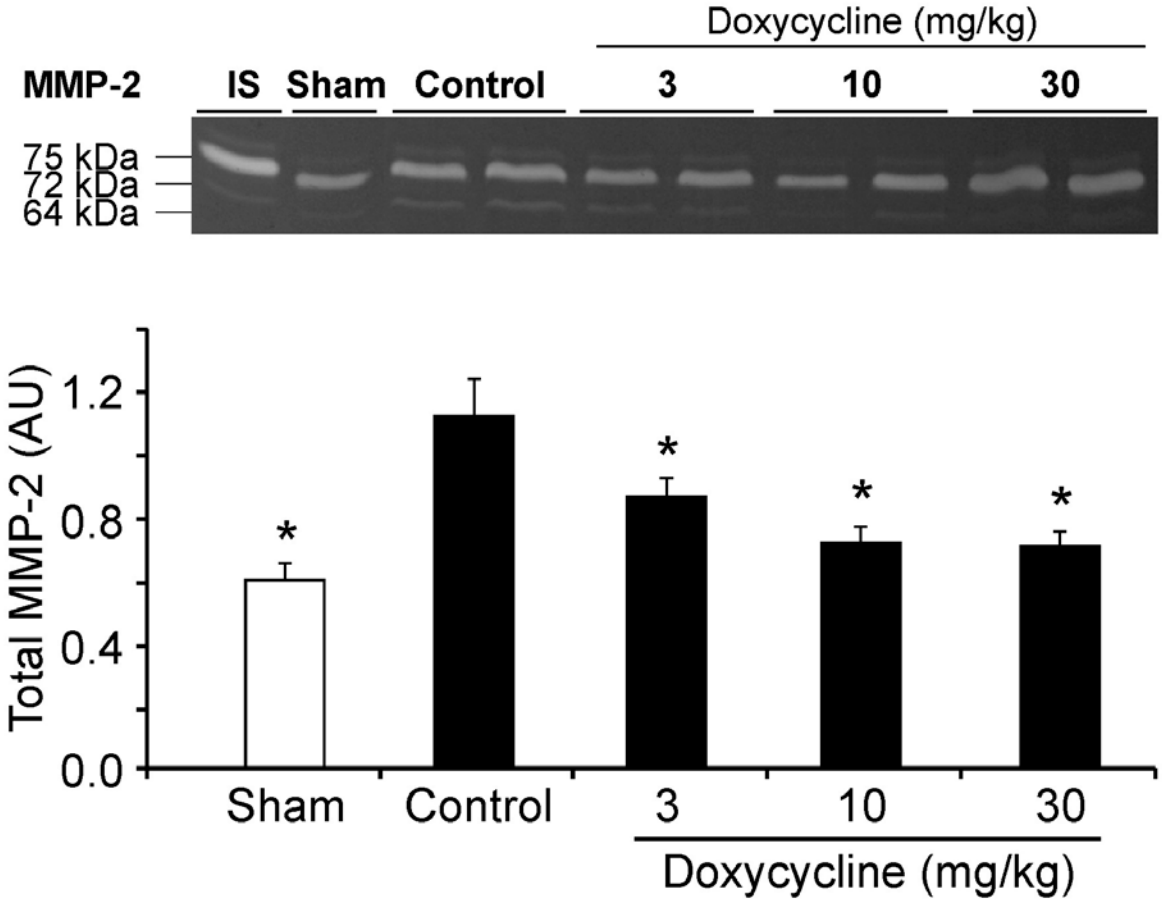
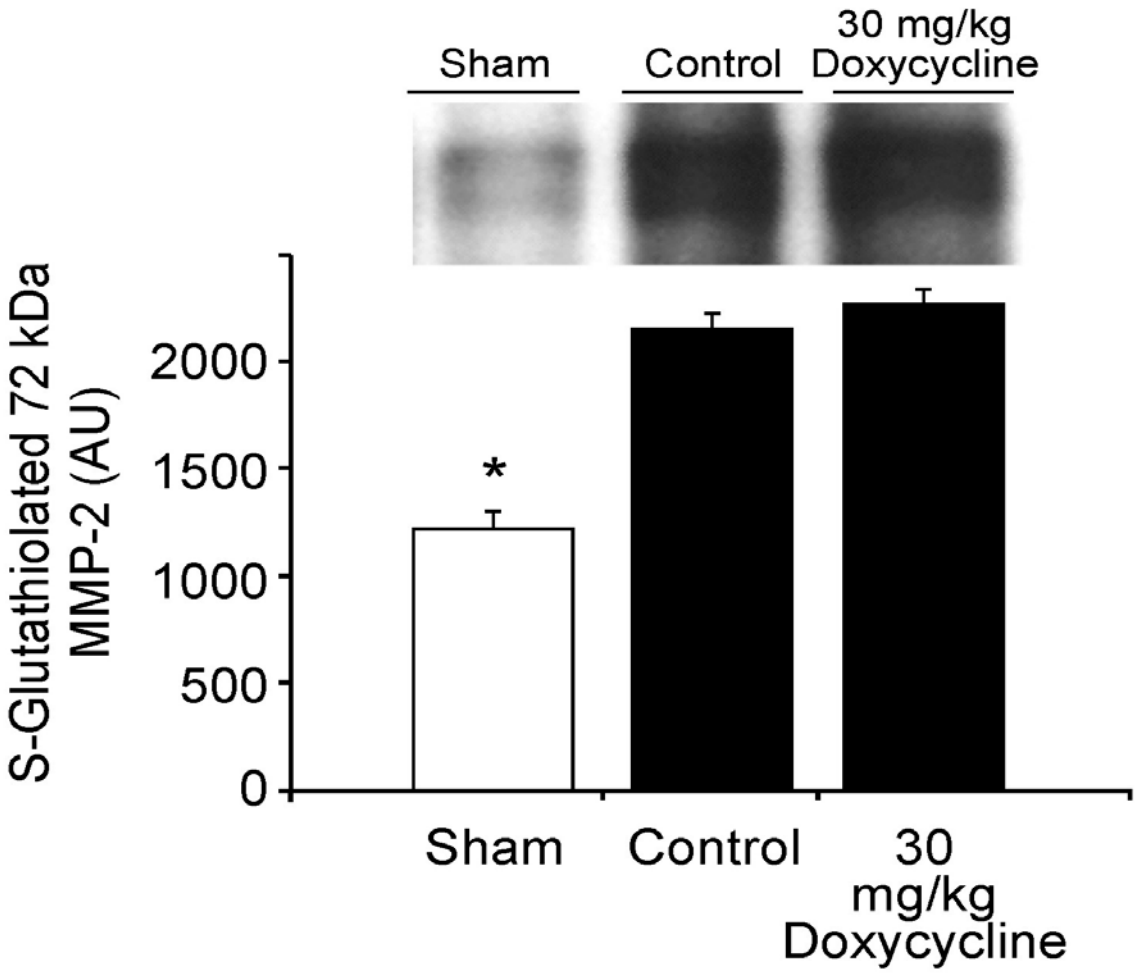


Figure 5-17: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on levels of myocardial S-glutathiolated MMP-2 in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.



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Chapter 6

Doxycycline Attenuates Renal Injury in a Swine Model of Neonatal Hypoxia-Reoxygenation

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A version of this chapter will be submitted for publication. Joseph R. LaBossiere, Jean-Sebastian Pelletier, Aducio Thiesen, Richard Schulz, David L. Bigam and Po-Yin Cheung.

Introduction

Asphyxia accounts for approximately 23% of the estimated 4 million neonatal deaths worldwide each year and is associated with significant morbidities in surviving neonates.(1-4) Though significant, these numbers likely underestimate the burden of this condition as the majority of cases occur in developing countries lacking accurate registration systems.(5,6) The causes of neonatal asphyxia are varied and are grouped into antepartum, intrapartum, or postpartum causes.(7) Regardless of mechanism, asphyxia can result in significant injury to a variety of organ systems, including the kidneys.(8)

Asphyxia remains the leading cause of acute kidney injury (AKI) in the neonatal period and is present in 30-70% of asphyxiated neonates, though these numbers likely reflect an underestimate as there are no consensus diagnostic criteria for AKI in the neonate.(9-11) Initial kidney injury in asphyxia is due to a hypoxic insult as more vital organs such as the heart, brain and adrenal glands are preferentially perfused at the expense of other organ systems.(11,12) Further injury occurs during reoxygenation of the asphyxiated neonate due to an increased production of reactive oxygen and nitrogen species (RONS) which have a host of pathological effects, such as damage to cellular proteins, lipids and DNA.(13) Thus renal injury in asphyxia reflects both the hypoxia itself and a secondary insult during reoxygenation of the hypoxic tissues, collectively referred to as a hypoxic-reoxygenation injury (H-R).

It has been shown that increased oxidative stress can lead to the activation of matrix metalloproteinases (MMP), a family of zinc dependent endopeptidases with a variety of intra – and extracellular proteolytic substrates.(14)
MMPs are found throughout the kidney and there is emerging evidence that MMP activation contributes to a variety of renal pathologies as well, including AKI in ischemia-reperfusion (I-R), which is a similar albeit distinct mechanism of injury to hypoxia-reoxygenation.(15,16) The renal protective effects of doxycycline, the most potent MMP inhibitor within the tetracycline class of antibiotics (17), have been demonstrated in rat models of I-R renal injury.(15,18) The clinical translatability of findings from these studies to asphyxiated neonates is limited, however, by the animal model design which utilizes I-R injury, pre-treats animals with MMP inhibitors prior to (or at the onset) of ischemia, and delivers the drug either orally or intraperitoneally. The mechanism of injury in neonatal asphyxia, however, is H-R where hypoxia is usually well established before treatment is initiated and drug delivery is limited to intravenous and/or inhalational administration. Therefore, clinically translatable studies focusing on the potential renal protective role of MMP inhibition in H-R injury are lacking.

The objective of this study is to assess the effect(s) of doxycycline on renal hemodynamics and injury using an *in vivo* newborn swine model of H-R injury designed to reflect true clinical conditions of neonatal asphyxia. We hypothesize that administration of doxycycline early into reoxygenation of asphyxiated piglets will improve renal hemodynamic parameters and attenuate renal injury.

Methods

Surgical procedure and experimental protocol

Refer to Chapter 4 for details.

Hemodynamic analysis

CO and renal artery flow were averaged over a two minute period and recorded at pre-determined time points throughout the experiment. Cardiac index (CI), renal artery flow index (RAFI) and renal artery oxygen delivery ($RADO_2$) were obtained from standard calculations.

Preparation of kidney tissue

Right kidneys were removed post-mortem and used for biochemical and histological analysis to remove the potential confounding effect(s) of surgical manipulation on the left kidney. The apex of the lower pole was placed in formalin, prepared for light microscopy (hemotoxylin and eosin staining) and sent to a single pathologist (AT) blinded to the group allocation for histological analysis. The remainder of the kidney was snap frozen in liquid nitrogen. For use in biochemical assays, frozen tissues were homogenized with 10µl/mg 50mM phosphate buffer containing 1mM EDTA (pH 7.0), and protein content was then quantified using a bicinchoninic acid assay kit (Sigma-Aldrich Canada Ltd., Oakville, ON).

Determination of kidney injury

Urine creatinine (Cr) levels were measured using a commercially available QuantiChrom assay kit (DICT-500; Bioassay Systems, Hayward, California). N-acetyl-D-glucosaminidase (NAG) activity was measured in the urine by a commercially available colorimetric assay kit (no. 875406; Roche, Indianapolis, Indiana) and normalized to urine creatinine. Renal tissue lactate levels were determined by a nicotinamide adenine dinucleotide enzyme coupled colorimetric assay as previously described.(22)

Measurement of kidney oxidative stress and MMP-2 activity

Kidney tissue lipid hydroperoxides (LPO) levels were quantified using a commercially available assay kit (Cayman Chemical Company, Ann Arbor, MI., Item No. 705003). Gelatinolytic activity of total MMP-2 was quantified using gelatin zymography as previously described.(23)

Statistical Analysis

Results are given as the mean ± standard error of the mean (SEM). Hemodynamic and biochemical variables were compared using two way and one way ANOVA, followed by Student Newman Keuls and Fisher *post hoc* testing, respectively. (SigmaPlot v11, Systat Software Inc, San Jose, CA) Correlations were determined using the Pearson method. Significance was defined as $p < 0.05$.

Results

A total of 33 piglets were used and had a mean age and weight of 2.6 ± 0.2 days and 1.9 ± 0.04 kg, respectively. There were no significant differences in age, weight, baseline hemodynamic and biochemical parameters between groups (data not shown). ($p > 0.05$).

Hemodynamic

At the end of hypoxia PaO_2 and pH were significantly lower and lactate significantly higher in piglets subjected to H-R compared to Sham-operated piglets (H-R: 38 ± 2 mmHg, 7.05 ± 0.01 and 11.1 ± 0.4 mmol/L; Sham-operated: 75 ± 4 mmHg, 7.37 ± 0.02 and 1.8 ± 0.5 mmol/L for PaO_2, pH and lactate respectively). ($p < 0.05$) Cardiogenic shock was induced in all piglets subjected to H-R with a mean CI and SAP between groups of 58 ± 1% of baseline and 31 ± 1 mm/Hg, respectively. ($p < 0.05$ compared to Shams: 109 ± 11% of baseline and 59 ± 2 mm/Hg for CI and SAP). CI was significantly improved in piglets treated with 10 mg/kg and 30 mg/kg doxycycline versus controls, with an improvement in SAP observed only in piglets treated with 30 mg/kg doxycycline. No difference in heart rate between any of the groups was observed during reoxygenation, and all groups had a lower heart rate (significantly so in Sham-operated and 10 mg/kg doxycycline groups) compared to controls at the end of reoxygenation. (Table 6-1) Both RAFI and $RADO_2$ were greatly reduced by the end of hypoxia in all treatment groups compared to Shams. Piglets treated with 30 mg/kg doxycycline experienced a significantly higher recovery of both parameters by the end of

reoxygenation compared to controls. (59 ± 14% and 32 ± 11% of baseline recovery verses 24 ± 6% and 21 ± 5% of baseline recovery, respectively). (Figure 6-1 and 6-2, respectively) RAFI/CI ratios were similar in all groups at baseline (5.7 ± 0.3%), but were significantly reduced in piglets subjected to H-R (1.1 ± 0.2%) compared to Shams (3.2 ± 0.5%; $p < 0.05$) at the end of hypoxia. The RAFI/CI ratio was significantly higher in the 30 mg/kg doxycycline group compared to controls at the end of reoxygenation (4.8 ± 0.02% versus 1.6 ± 0.005%, respectively). (Table 6-1)

Biochemical

Control piglets had a higher level of urinary NAG activity compared to Shams at the end of reoxygenation (3.69 ± 0.54 µmol/mg Cr verses 2.14 ± 0.35 µmol/mg Cr, respectively), which was reduced in a dose-related manner in doxycycline treated piglets compared to controls. ($p=0.065$). (Figure 6-3) NAG activity was found to negatively correlate with recovery of RAFI. (Figure 6-4) Renal tissue lactate levels were significantly elevated in control piglets compared to Shams (4.29 ± 0.28 µmol/mg protein verses 6.72 ± 0.79 µmol/mg protein, respectively), which was reduced in a dose dependent manner in piglets treated with doxycycline, with significantly lower levels observed in 10 mg/kg and 30 mg/kg doxycycline groups (4.92 ± 0.53 µmol/mg protein and 4.72 ± 0.49 µmol/mg protein, respectively). (Figure 6-5) Renal tissue lactate levels were found to positively correlate with urine NAG activity, (Figure 6-6) though no significant correlation was observed between lactate and RAFI. (Data not shown).

Control piglets subjected to H-R had higher levels of kidney tissue LPO compared to Shams (2.27 ± 0.21 μmol/mg protein and 0.17 ± 0.03 versus 1.33 ± 0.08 μmol/mg protein and 0.13 ± 0.02, respectively), which was reduced in a dose-dependent manner in doxycycline groups compared to controls. (p=0.083) (Figure 6-7) Total MMP-2 activity was higher in control piglets (vs Shams) (1.2 ± 0.1 arbitrary units (AU) versus 0.6 ± 0 AU, respectively), and significantly reduced in a dose-dependent manner in all doxycycline treatment groups compared to controls (1.0 ± 0.1 AU, 0.9 ± 0.1 AU and 0.7 ± 0 AU for 3 mg/kg, 10 mg/kg, and 30 mg/kg doxycycline groups, respectively). (Figure 6-8) A significant negative correlation was observed between total MMP-2 activity and RAFI. (Figure 6-9)

Histology

There were no histological differences on light microscopy observed between any of the groups.

Discussion

Although improvements have been made in the management of acutely ill neonates, asphyxia morbidity and mortality rates remain high. This reflects, in part, the lack of interventions aimed at preventing or attenuating organ injury from occurring in the first place during hypoxia-reoxygenation. Our study was designed to reflect true clinical conditions encountered by practicing physicians and tested a novel intervention aimed at attenuating renal injury in asphyxiated neonates. We observed that early administration of doxycycline during resuscitation improved renal perfusion (RAFI and $RADO_2$), with reduced markers of renal injury including urinary NAG, tissue lactate, LPO, and total MMP-2 activity.

CI was significantly improved in 10 mg/kg and 30 mg/kg doxycycline groups compared to controls, and this presumably reflects the improvement in renal hemodynamics observed. The RAFI/CI ratio significantly decreased in piglets exposed to H-R, suggesting an element of renal artery vasoconstriction during hypoxia. A non-changing RAFI/CI ratio during recovery suggests that RAFI is primarily dependent on CI Interestingly, the RAFI/CI ratio increased in piglets treated with 30 mg/kg doxycycline to almost baseline levels, an effect not observed in other treatment groups. Though not definitive, this finding may suggest that improvement in RAFI may reflect not only doxycyclines systemic hemodynamic effects and improved CI, but a local vasodilatory effect as well. There is some evidence suggesting that MMP activation can contribute to renal vessel pathology (24), however no applicable conclusions can be drawn based on

the current evidence at this time. Further investigation into the role increased MMP activation has on renal vessel physiology in H-R injury is required.

The diagnosis of AKI in the neonatal period is difficult, and has traditionally been defined by low urine outputs (olgiuria/anuria) and/or a high serum creatinine levels.(25) This criteria, however, has several limitations. Non-oliguric AKI is common in the neonate(9,26) and serum creatinine levels often reflect maternal levels (which can take weeks to resolve) and are typically elevated only with significant kidney injury.(25) New markers of AKI including urinary NAG activity are currently being investigated and validated for clinical practice.(9,25) NAG is a lysosomal enzyme present in cells of the proximal tubules, and increased activity in the urine reflects proximal tubular injury.(27) NAG has been found in prior studies to be a reliable marker of tubular injury during asphyxia, and it's activity appears to correlate with the severity of asphyxia.(27,28) We observed an increase in NAG activity in control piglets verses Shams, which was attenuated in a dose-related manner in piglets treated with doxycycline, though observed differences were not statistically significant at $p<0.05$. Urine NAG activity positively correlated with renal tissue lactate levels and was negatively correlated with RAFI. Renal tissue lactate levels were significantly elevated in control piglets compared to Shams, and were attenuated in a dose-depenendent manner with administration of doxycycline, with significantly lower levels observed in the 10 and 30 mg/kg doxycycline groups.

We then attempted to elucidate the possible mechanism in which doxycycline exerts its beneficial renal effects by assessing the degree of cellular

oxidative stress and MMP-2 activity in renal tissues. We observed higher levels of lipid hydroperoxides in the kidneys of control piglets compared to Shams, which was attenuated in a dose-related manner by doxycycline, though observed differences were not statistically significant at $p<0.05$. Total MMP-2 activity followed a similar pattern: a significantly higher level of total MMP-2 activity in the control group compared to Shams and a dose dependent attenuation of total activity such that all doxycycline groups had significantly lower levels of MMP-2 activity compared to control. In addition, we observed a significant negative correlation between total MMP-2 activity and RAFI.

It appears then that AKI in H-R injury may be mediated in part by increased oxidative stress resulting in increased MMP-2 activity in kidney tissue. Our conclusions on the mechanism of injury and improvement with doxycycline administration are limited, however. Though gelatin zymography is considered the a good test for assessing MMP activity at present, it is inherently limited in that MMP activation is typically higher in the assay compared to *in vivo*, and changes in activity cannot be directly linked to the presence or absence of an inhibitor.(29) Furthermore, the exact mechanism(s) in which increased MMP-2 activity leads to kidney injury in H-R cannot be inferred from our study. One possible mechanism suggested by prior studies using I-R small animal models of AKI is that activated MMPs target cell adhesion molecules in the renal tubular cells leading to renal tubular injury and dysfunction.(16,30) This mechanism is consistent with our findings of increased MMP-2 activation and renal tubular injury in H-R piglets, both of which are attenuated by treatment with doxycycline.

However we cannot, based on our results alone, rule in or out this or other potential pathophysiologic mechanisms that may mediate AKI in H-R injury. Further investigation into the role of MMP activation in the development of AKI in asphyxiated neonates is required.

Although previous studies have demonstrated the renal protective effects of doxycycline in rat I-R models of injury (15,18), they do not reflect actual conditions of asphyxia in human neonates. Our study utilized an *in vivo* newborn swine model of H-R and utilized the intravenous route for drug delivery, as asphyxiated neonates are commonly intubated or unconscious and cannot be given oral medication. Delivery of drug at five minutes into reoxygenation was chosen to reflect the time it would take to gain intravenous access and prepare drug during the initial resuscitation of an asphyxiated neonate. Though animal models provide certain limitations when translating findings to humans, swine are an excellent model for studying neonatal asphyxia. They permit complex surgical procedures allowing for extensive data collection, have well known hemodynamic and biochemical parameters, have similar anatomy and physiology to humans, and newborn piglets even experience asphyxia in a similar manner to human neonates.(31-33)

A possible limitation of our study is the use of doxycycline as an MMP inhibitor for use in neonates, as tetracyclines in general have traditionally been contraindicated due to fears of irreversible staining of the teeth and potential to cause pseudotumor cerebri.(34,35) The use of doxycycline specifically is more controversial, however, given the paucity of prospective clinical controlled trials

testing the use of the drug in younger children and neonates. Some evidence suggests that use of doxycycline is safe in children under the age of eight, particularly if there is a short duration of therapy, and doxycycline is considered the first line of therapy in specific infectious conditions in this age group.(36-39) Furthermore, doxycycline is an already established MMP inhibitor in clinical practice for treating peridontitis and rosacea (periostat), is the only intravenous MMP inhibitor approved for medical use, and has well established pharmacokinetic/pharmacodynamic data.(20,40,41) Though we feel that this makes doxycycline the most clinical transferable MMP inhibitor for use in neonates at present, studies addressing the safety of doxycycline in this patient population are needed. We are also limited by the duration of the experiment, as we followed the piglets for a total of six hours. Our conclusions therefore reflect acute changes in renal hemodynamics and injury, and we cannot speculate on long-term improvement(s) in both parameters. Our findings do highlight, however, that significant renal hemodynamic dysfunction and tissue injury occur early in H-R and validate early therapeutic intervention. Finally, our model utilizes a specific form of neonatal asphyxia and does not represent all of the numerous etiologies that cause asphyxia in the human neonate.

To our knowledge, this is the first study to test the effect(s) of early administration of doxycycline during resuscitation on renal hemodynamics and injury in an *in vivo* clinically translatable newborn swine model of hypoxia-reoxygenation injury. We demonstrate that administration of doxycycline early in the resuscitation of asphyxiated piglets improves renal artery flow index and renal

artery oxygen delivery, which primarily reflects improved cardiac output but may also relate to local vasodilatory effects of doxycycline. Asphyxiated piglets treated with doxycycline showed less evidence of acute kidney injury, lower cellular oxidative stress and less MMP-2 activity, suggesting that AKI in the asphyxiated neonate may be mediated in part by increased cellular oxidative stress and subsequent activation of MMP-2. Our findings demonstrate that MMP inhibition can attenuate AKI and improve renal hemodynamics in neonatal H-R injury.

Table 6-1 – Hemodynamic parameters at baseline, end of hypoxia, 30 minutes into reoxygenation and end of reoxygenation

Group	Baseline 0 min	End of Hypoxia 120 min	Reoxygenation 150 min	End of Reoxygenation 360 min
Heart Rate (beats per min)				
Sham	204 ± 8	215 ± 17*	220 ± 16	215 ± 4*
Control	195 ± 4	246 ± 8	239 ± 11	263 ± 12
Doxycycline 3 mg/kg	207 ± 10	240 ± 11	228 ± 11	229 ± 9
“ 10	205 ± 10	233 ± 11	226 ± 9	221 ± 8*
“ 30	213 ± 5	243 ± 9	241 ± 7	233 ± 8
Systemic Arterial Pressure (mmHg)				
Sham	66 ± 5	59 ± 2*	58 ± 2*	47 ± 3*
Control	76 ± 4	31 ± 2	48 ± 5	35 ± 2
Doxycycline 3 mg/kg	70 ± 3	31 ± 1	43 ± 2	38 ± 2
“ 10	70 ± 2	30 ± 1	45 ± 2	38 ± 3
“ 30	80 ± 5	32 ± 1	50 ± 3	46 ± 3*
Cardiac Index (ml/(min x kg))				
Sham	168 ± 10	182 ± 25*	167 ± 14	161 ± 14*
Control	186 ± 9	99 ± 6	166 ± 10	122 ± 9
Doxycycline 3 mg/kg	204 ± 19	114 ± 10	161 ± 19	119 ± 12
“ 10	183 ± 7	110 ± 3	170 ± 8	135 ± 8*
“ 30	214 ± 26	122 ± 17	189 ± 21	178 ± 18*
Renal Artery Flow Index (ml/(min x kg))				
Sham	9 ± 3	6 ± 1*	7 ± 1*	3 ± 1
Control	9 ± 2	1 ± 0.3	4 ± 1	2 ± 1
Doxycycline 3 mg/kg	10 ± 2	1 ± 0.4	3 ± 1	2 ± 1
“ 10	11 ± 2	1 ± 1	5 ± 2	4 ± 2
“ 30	13 ± 2	2 ± 1	9 ± 3*	8 ± 3*
RAFI/CI (%)				
Sham	5.3 ± 1.9	3.2 ± 0.5*	4.2 ± 1.0	2.4 ± 0.9
Control	5.2 ± 1.0	1.1 ± 0.3	2.7 ± 0.6	1.6 ± 0.5
Doxycycline 3 mg/kg	5.2 ± 1.3	0.8 ± 0.4	2.1 ± 0.7	1.9 ± 0.6
“ 10	6.2 ± 1.2	0.9 ± 0.6	2.5 ± 0.8	3.5 ± 1.9
“ 30	6.6 ± 0.9	1.6 ± 0.6	5.1 ± 2.0	4.8 ± 1.8*

*** p<0.05 vs. control group**

Figure 6-1: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on renal artery flow index in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

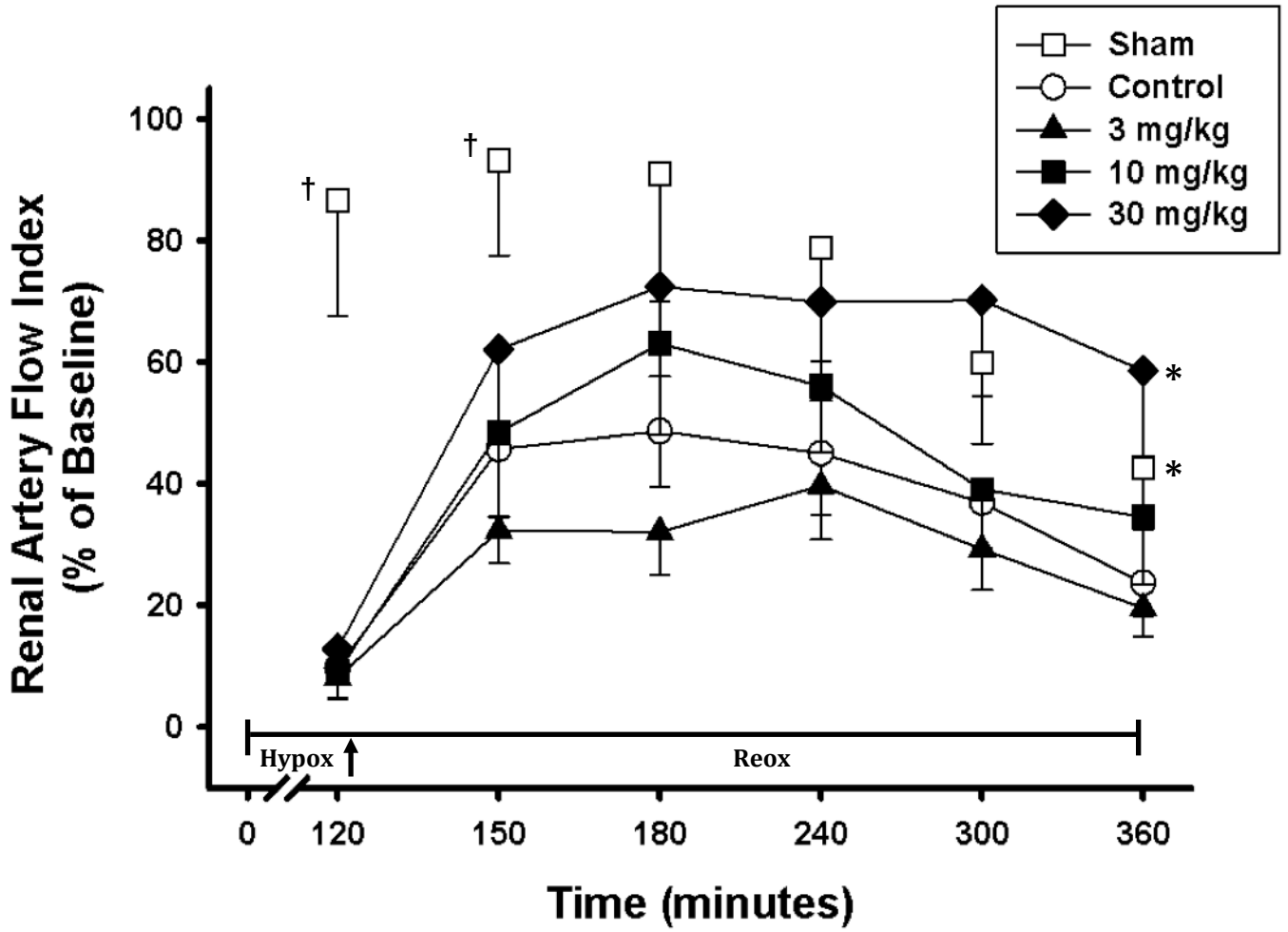


Figure 6-2: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on renal artery oxygen delivery in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

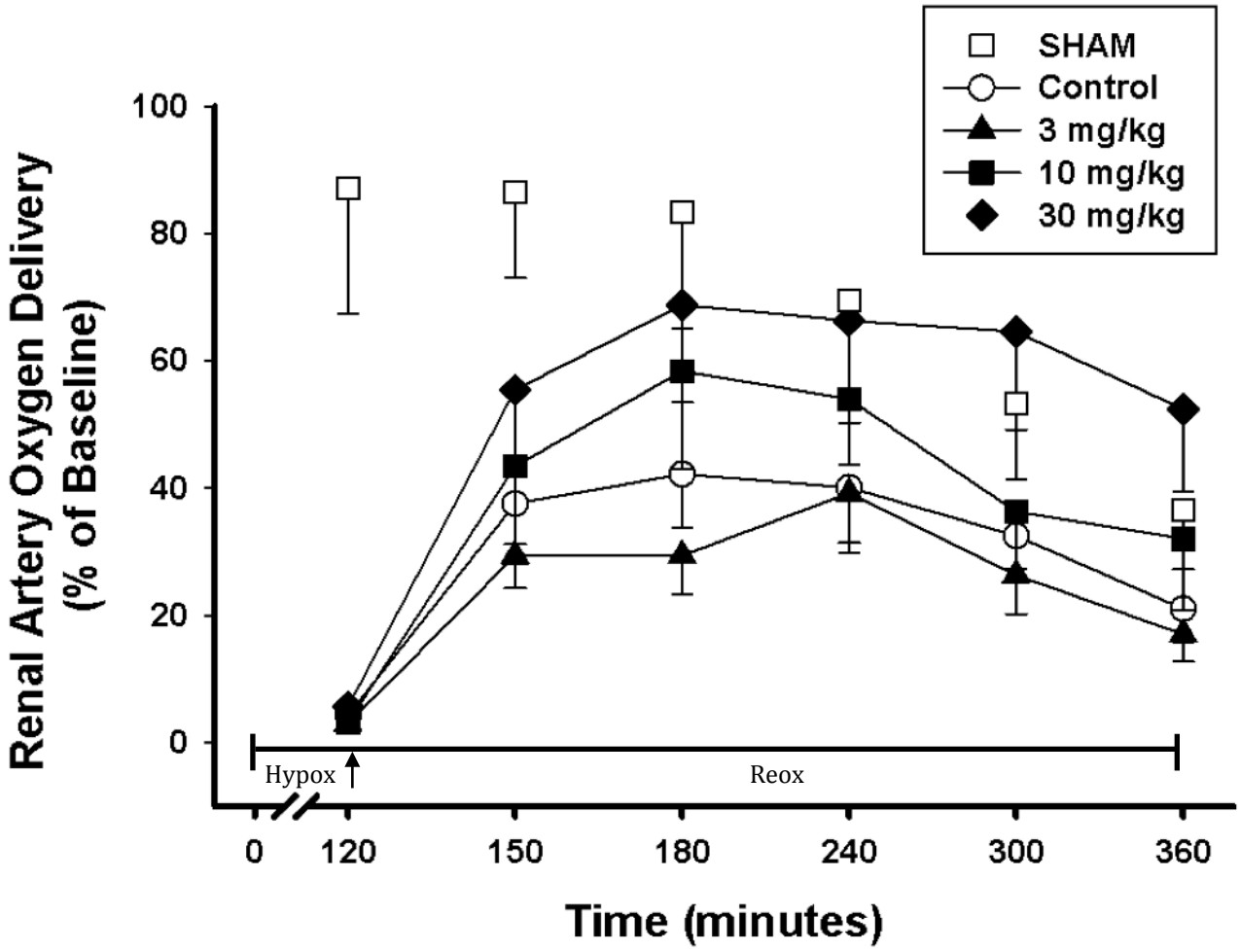


Figure 6-3: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on urine NAG activity in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

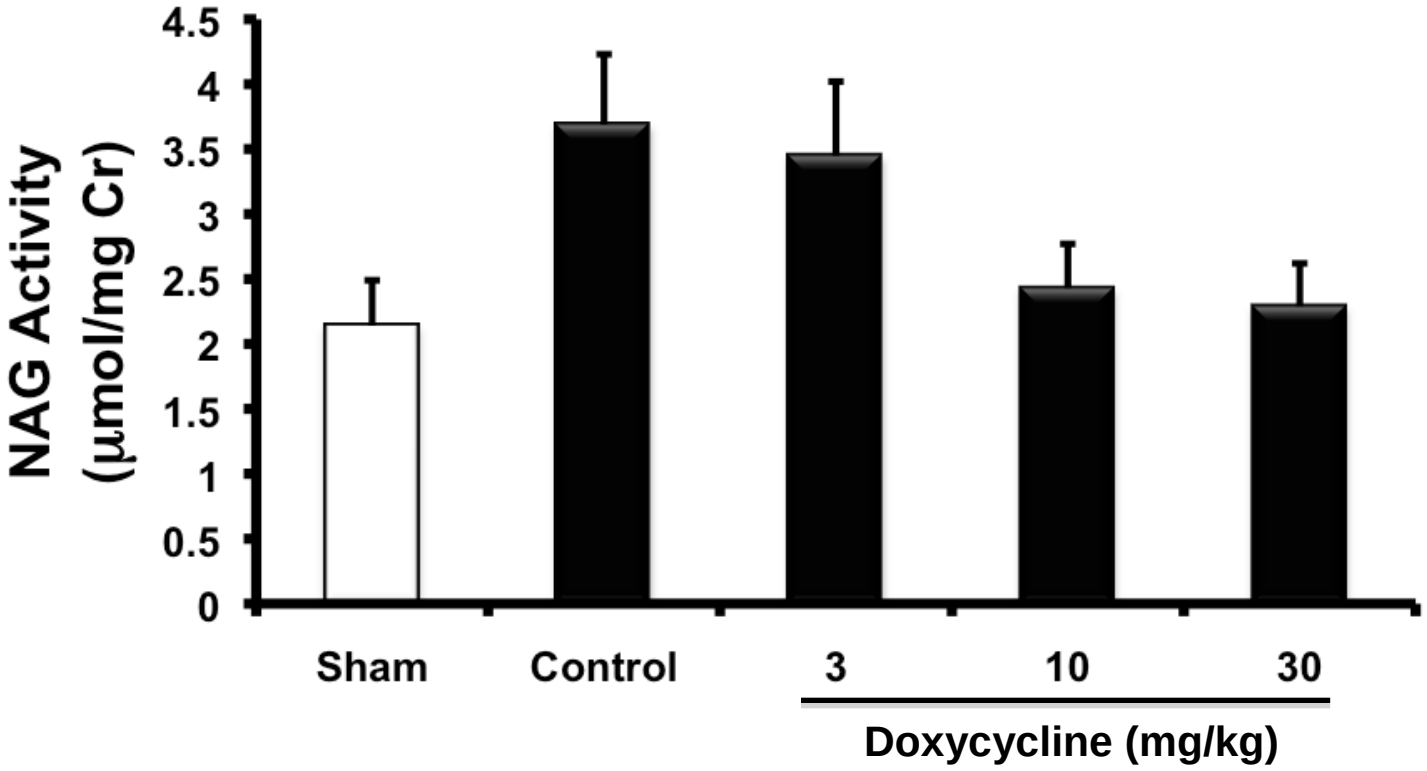


Figure 6-4: Urine NAG activity negatively correlates with renal artery flow index in piglets treated with saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) during hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R.

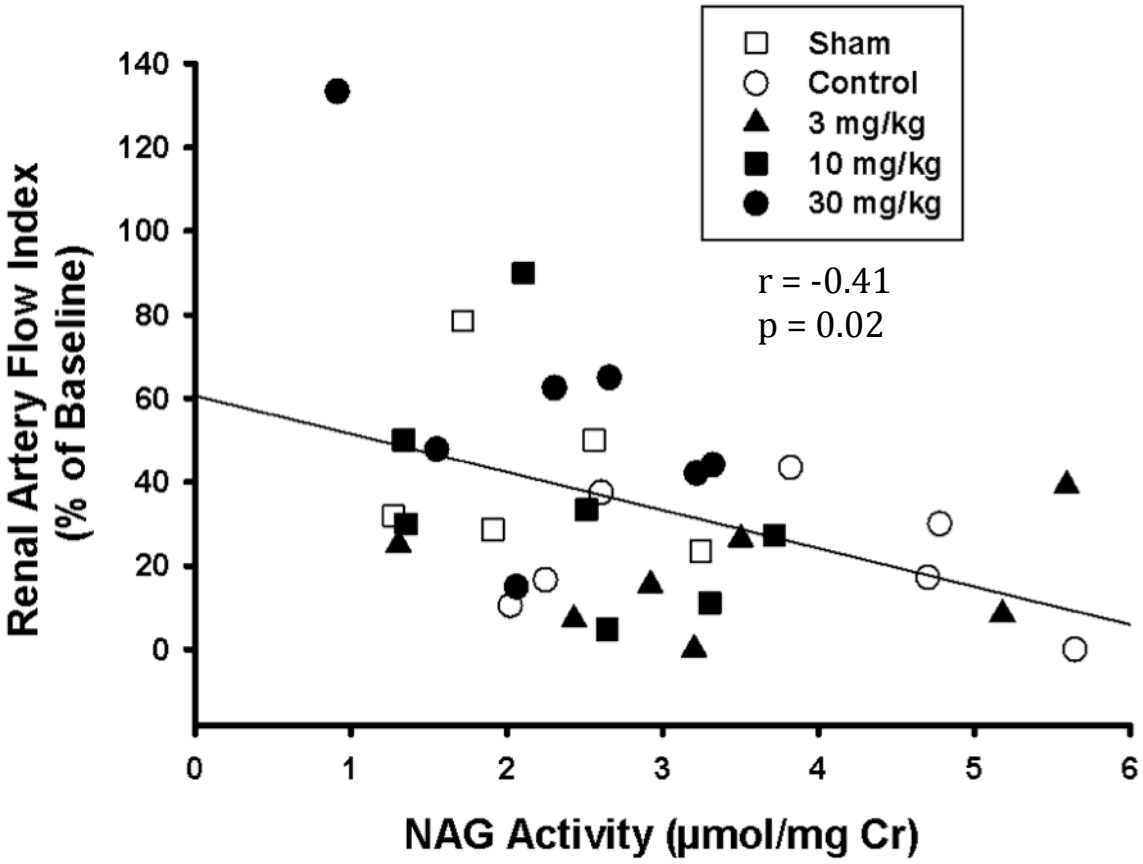


Figure 6-5: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on renal tissue lactate levels in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

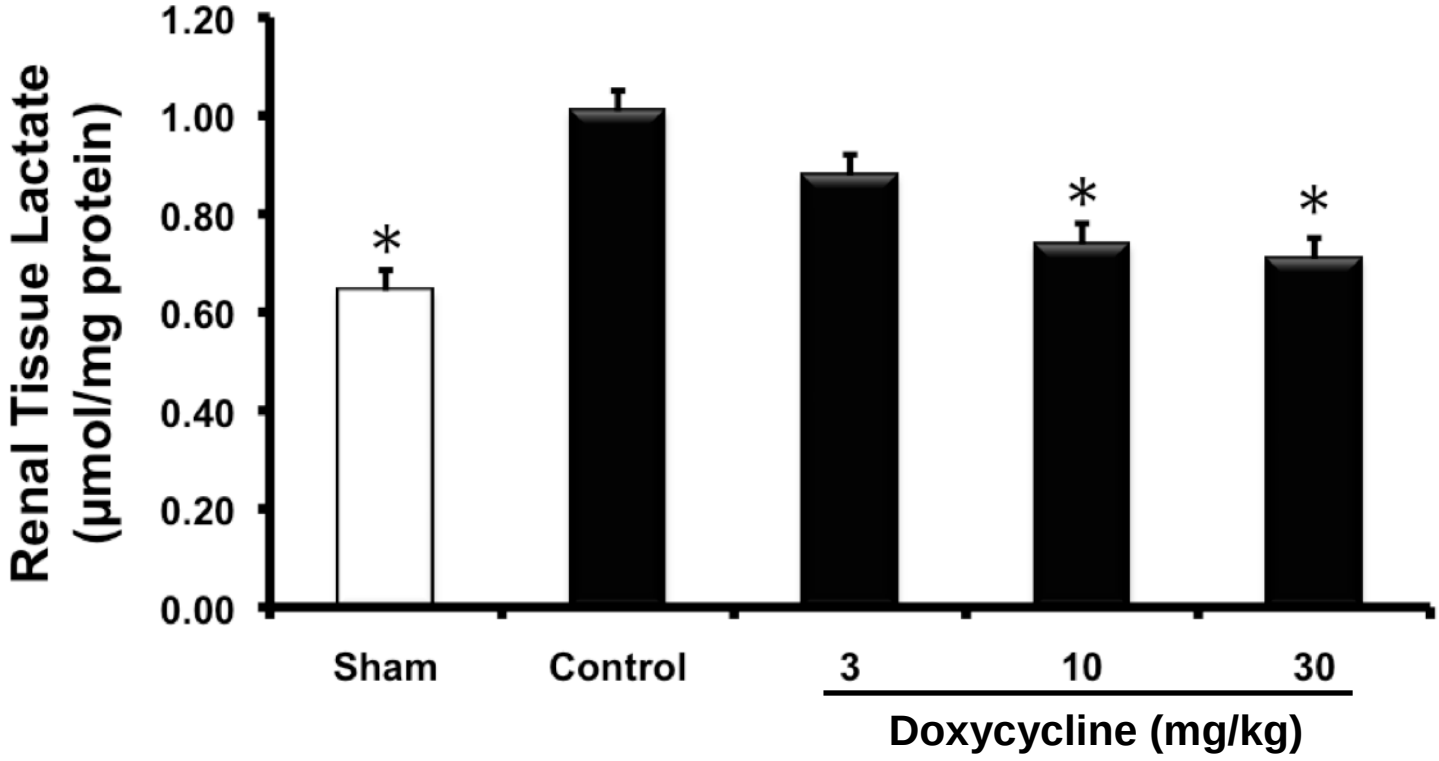


Figure 6-6: Urine NAG activity positively correlates with renal tissue lactate levels in piglets treated with saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) during hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R.

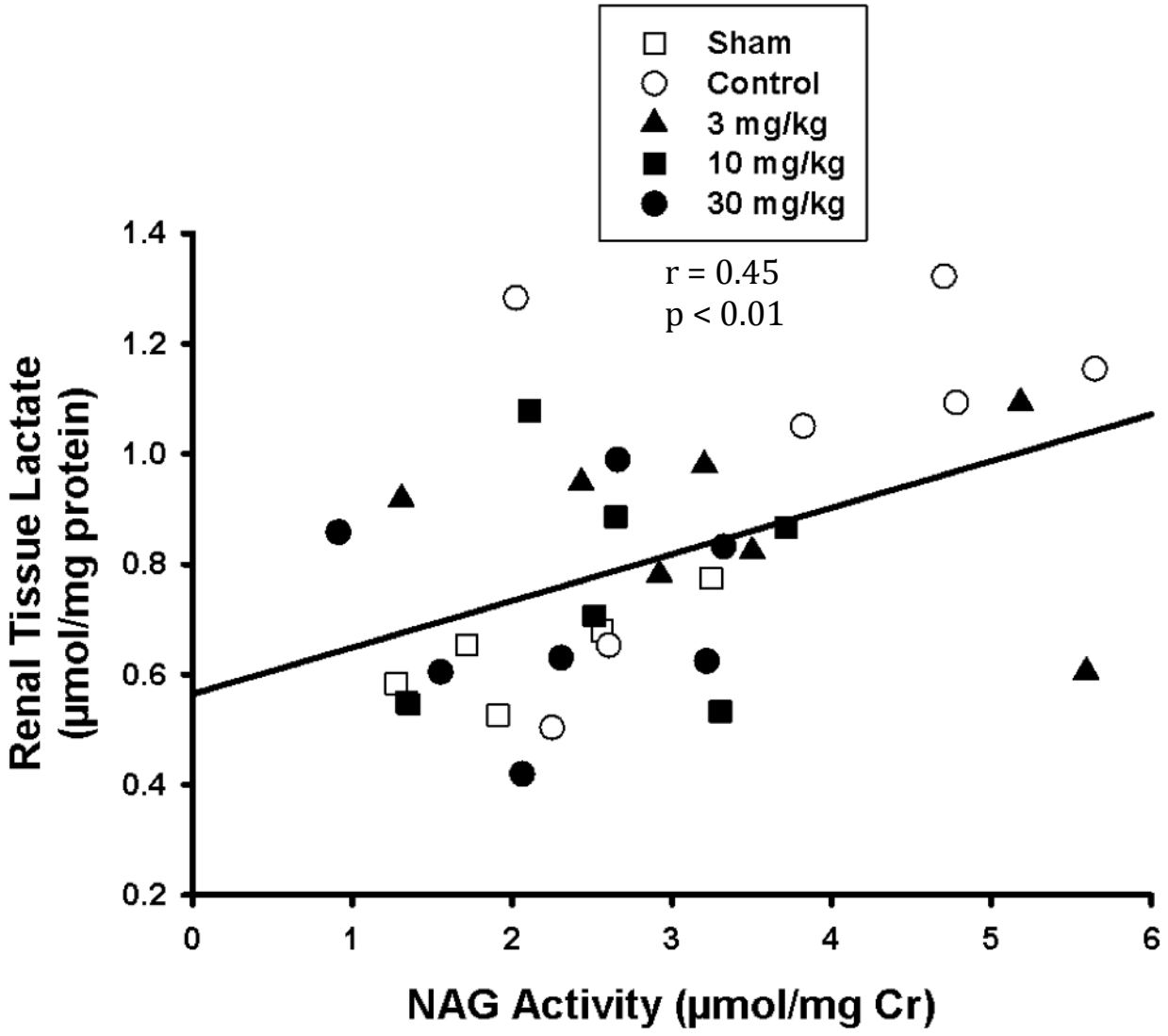


Figure 6-7: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on renal tissue lipid hydroperoxide levels in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

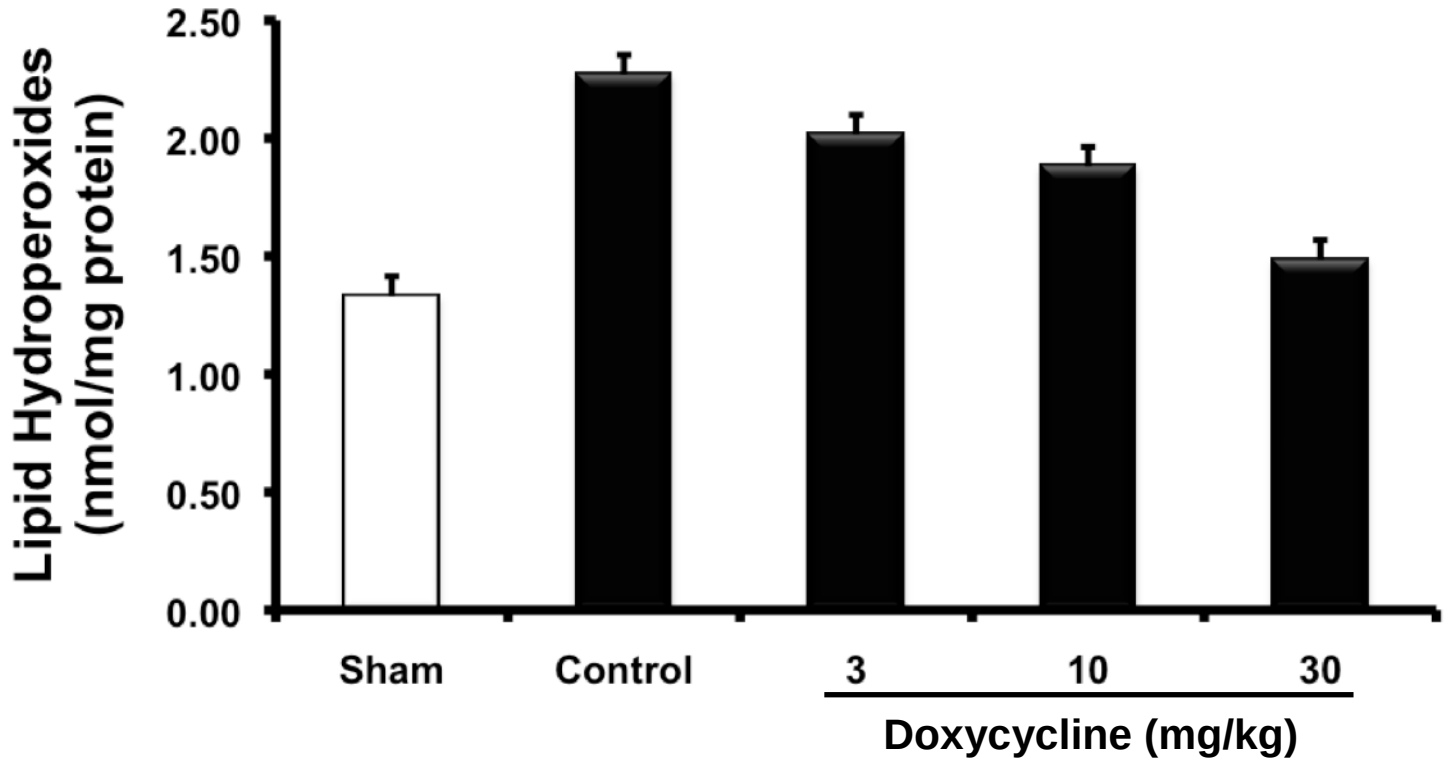


Figure 6-8: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on renal tissue MMP-2 activity in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

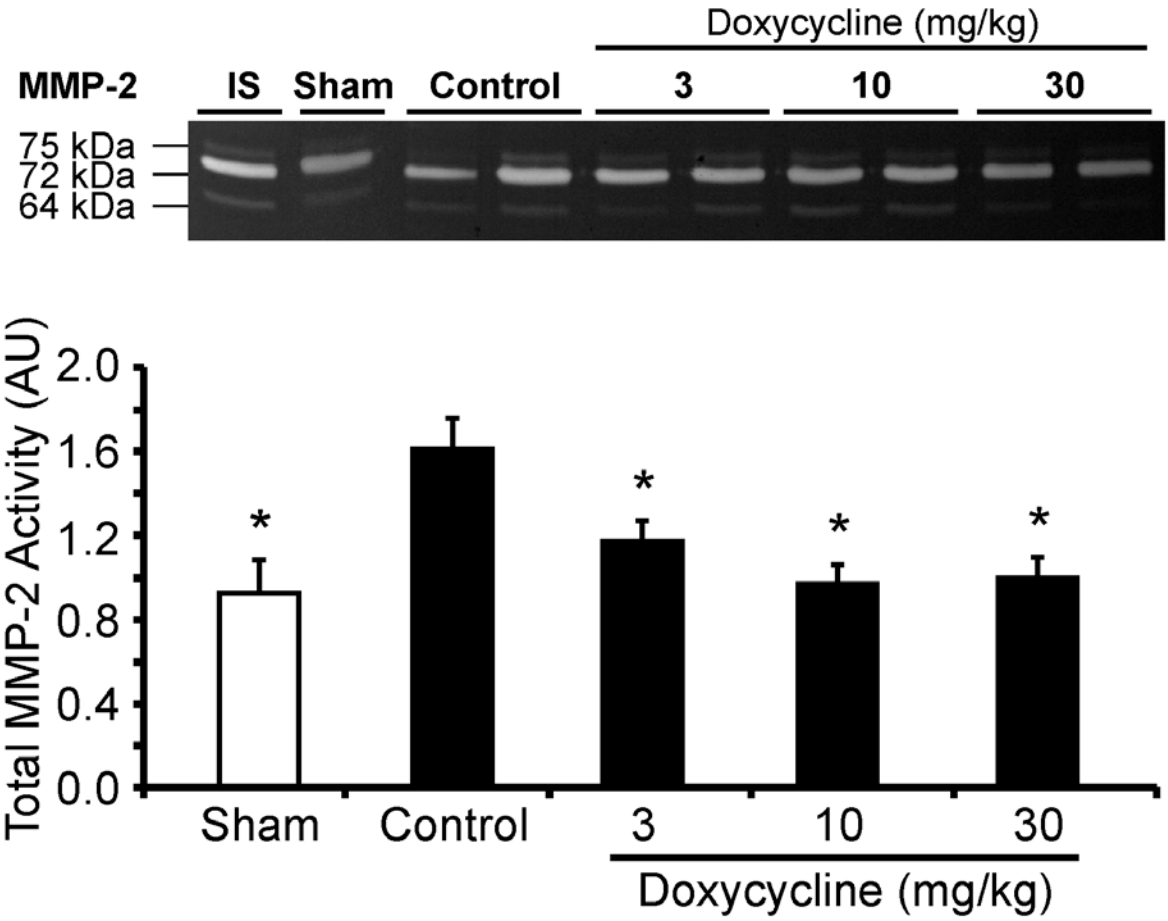
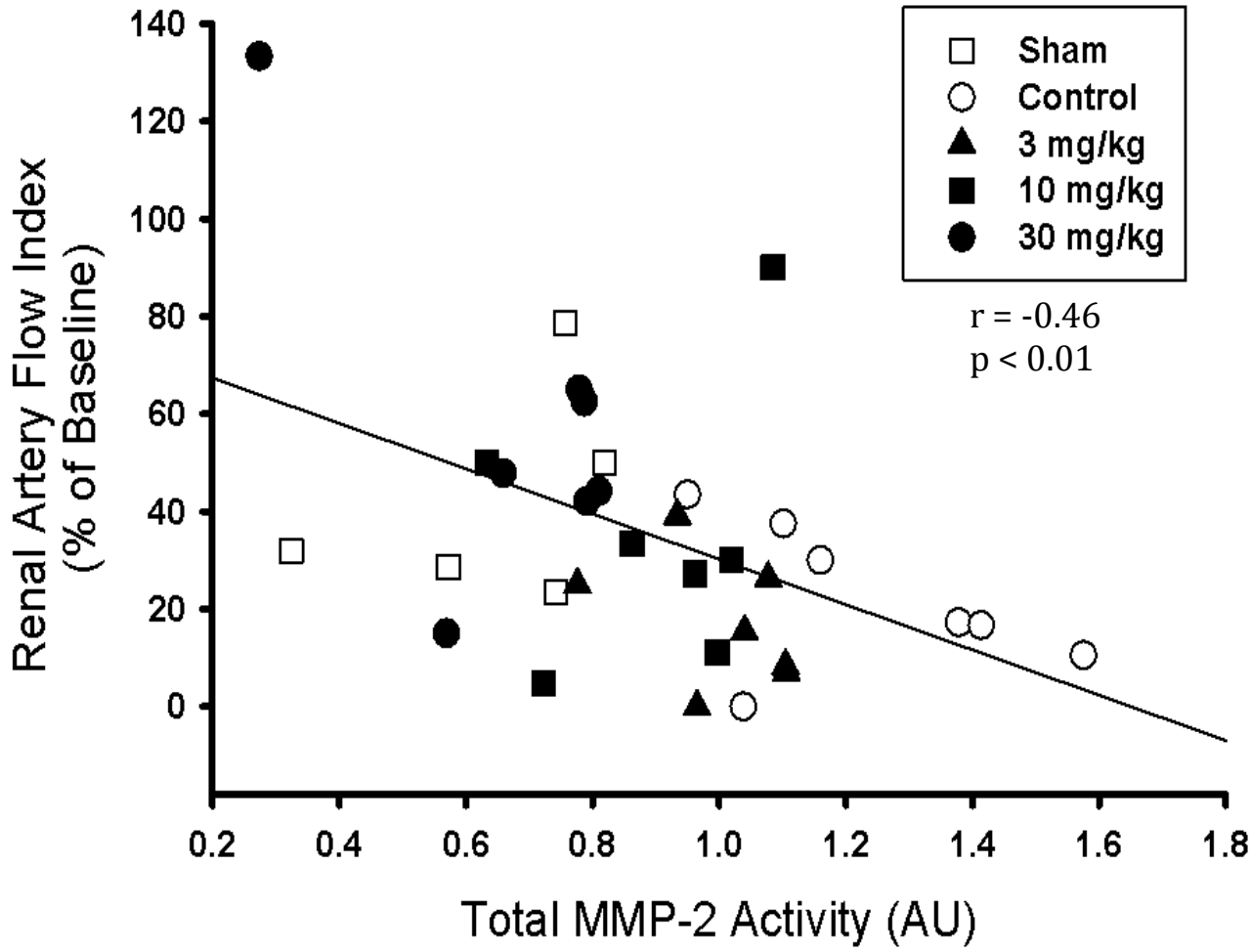


Figure 6-9: Renal tissue MMP-2 activity negatively correlates with renal artery flow index in piglets treated with saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) during hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R.



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Chapter 7

The Effect of Doxycycline Administration on Regional Hemodynamics and Intestinal Injury in a Piglet Model of Neonatal Asphyxia

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A version of this chapter will be submitted for publication. Joseph R. LaBossiere, Jean-Sebastian Pelletier, Aducio Thiesen, Richard Schulz, David L. Bigam and Po-Yin Cheung.

Introduction

Neonatal asphyxia can lead to multisystem organ injury, mediated in part by an initial shunting of blood and nutrients to more vital organs (the heart, brain and adrenal glands) at the expense of other organ systems.(1) First described by Lloyd in the late 1960's, many adopted the view that intestinal hypoperfusion (ischemic-reperfusion injury) which occurred during asphyxia increased the risk of developing necrotizing enterocolitis (NEC), a severe inflammatory disorder of the intestine which today persists as the leading cause of death in the neonatal intensive care unit.(2-4)

The pathogenesis of NEC today, however, is regarded as considerably more complex and involves multiple mechanisms of injury including enhanced release of pro-inflammatory cytokines and changes in levels of vasoactive peptides in addition to ischemia-reperfusion.(5,6) One mechanism in particular that is gaining more attention is the activation of matrix metalloproteinases (MMP) in the intestinal tissue. An increase in oxidative stress and activation of MMPs resulting in organ injury and dysfunction has been observed in ischemic-reperfusion injury to the heart (7,8) and kidneys (9). There is increasing evidence that enhanced oxidative stress and increased MMP activity mediates intestinal injury in NEC, as well as in other ischemic injuries to the intestine including ischemic colitis, though the exact mechanism(s) in which MMPs mediate this injury are yet to be determined.(10-13) Though some have speculated on the therapeutic potential of MMP inhibition on intestinal injury in asphyxiated newborns, studies testing this hypothesis are lacking.

The objective of this study therefore, was to determine the effect of doxycycline, a known MMP inhibitor, on changes in regional blood flow and intestinal injury in a clinically translatable piglet model of neonatal asphyxia.

Methods

Surgical procedure and experimental protocol

Refer to Chapter 4 for details.

Systemic and regional hemodynamic flow analysis

Systemic arterial pressure (SAP) and pulmonary artery (a surrogate of cardiac output), carotid artery and superior mesenteric artery flow were continuously monitored and analyzed at pre-determined time points. Intermittent arterial and mixed venous blood samples were taken. Cardiac index (CI), carotid artery flow index (CAFI) and superior mesenteric artery flow index (SMAFI) were calculated by dividing the respective flows by the weight of the piglet. Carotid artery oxygen delivery ($CADO_2$), carotid artery vascular resistance index (CAVRI), superior mesenteric artery oxygen delivery ($SMADO_2$) and superior mesenteric artery vascular resistance index (SMAVRI) were obtained from the calculations described in chapters 5 and 6, with the only change being the flow parameter used in the calculation.

Intestinal tissue processing

Approximately four inches of distal ileum was removed at autopsy: a small sample was placed in formalin, prepared for light microscopy (hemotoxylin and eosin staining) and sent to a single pathologist blinded to the treatment group allocation for histological analysis. The remainder of the tissue was snap frozen in liquid nitrogen for biochemical analysis. For biochemical analyses, the intestinal

tissue was homogenized with 10 μl/mg (tissue wet weight) of 50 mM phosphate buffer containing 1 mM EDTA (pH 7.0). Protein content was quantified using a bicinchoninic acid assay kit (Sigma-Aldrich Canada Ltd., Oakville, ON).

Measuring serum intestinal fatty acid binding protein (IFABP)

Serum intestinal fatty acid binding protein (IFABP) levels were measured using a commercially available enzyme-linked immunosorbant assay kit (#HK406-02, Hycult biotech, Uden, The Netherlands)

Determination of lactate, oxidative stress and MMP-2 activity in intestinal tissue

Intestinal tissue lactate and lipid hydroperoxides levels were determined by a nicotinamide adenine dinucleotide enzyme coupled colorimetric assay (14) and an enzyme-linked immunosorbant assay kit (#705003, Cayman Chemical Company, Ann Arbor, MI), respectively. Gelatinolytic activity of MMP-2 in the intestinal tissue was quantified with zymography.(15)

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Hemodynamic variables were compared among groups and within specific time points using two-way and one-way analysis of variance (ANOVA) followed by Student Newman Keuls *post hoc* testing, respectively. Biochemical variables were compared between groups using one-way ANOVA followed by Fisher *post hoc*

testing. Hemodynamic and biochemical correlations were determined using the Pearson method (SigmaPlot v11, Systat Software Inc, San Jose, CA). Significance was defined as $p<0.05$.

Results

After stabilization and prior to the initiation of hypoxia cardiac index was 191 ± 8 ml/(min x kg) and systemic arterial pressure 72 ± 2 mmHg. Carotid hemodynamics were CAFI 23 ± 1 ml/(min x kg), $CADO_2$ 2.3 ± 0.1 mL O_2/(min x kg) and CAVRI 3.1 ± 0.1 (mmHg x min x kg)/mL. Intestinal parameters were SMAFI 42 ± 2 ml/(min x kg), $SMADO_2$ 4.4 ± 0.2 ml O_2/(min x kg) and SMAVRI 1.8 ± 0.2 (mmHg x min x kg)/ml. There were no significant differences in hemodynamic variables among the groups at baseline.

Systemic, carotid and intestinal hemodynamic variables during hypoxia-reoxygenation

Cardiac index and systemic arterial pressure were significantly lower in H-R piglets at the end of hypoxia compared to Shams (CI 58 ± 1% of baseline and SAP 31 ± 1 mm/Hg vs 109 ± 11% of baseline and 59 ± 2 mm/Hg, respectively). CI was significantly improved in piglets treated with 10 mg/kg and 30 mg/kg doxycycline versus controls by the end of reoxygenation, with an improvement in SAP observed only in piglets treated with 30 mg/kg doxycycline. CAFI was lower in all H-R piglets at the end of hypoxia compared to Shams, though the difference was not statistically significant.(Figure 7-1) Piglets treated with 30 mg/kg doxycycline had greater preservation of CAFI throughout reoxygenation compared to controls, approaching levels seen in Sham pigs by the end of reoxygenation, however this difference was not statistically significant. H-R piglets had a significantly lower $CADO_2$ compared to Shams at the end of

hypoxia, which recovered be the end of reoxygenation.(Figure 7-2) There were no differences in CAVRI observed between groups at any time point during the experiment. (Figure 7-3) SMAFI was slightly lower in H-R piglets compared to Shams at the end of hypoxia, though this difference was not statistically significant. (Figure 7-4) There was no difference in SMAFI between groups throughout reoxygenation. $SMADO_2$ was significantly lower in H-R piglets at the end of hypoxia but recovered by the end of reoxygenation. (Figure 7-5) No differences in SMAVRI were observed between groups at any time point throughout the experiment. (Figure 7-6)

Intestinal biochemical parameters at the end of reoxygenation

Control piglets had significantly higher levels of serum IFABP compared to Shams at the end of reoxygenation (2137 ± 232 pg/ml vs 666 ± 309 pg/ml, respectively), which was significantly attenuated in piglets treated with 30 mg/kg doxycycline compared to controls (1216 ± 364 pg/ml).(Figure 7-7) Intestinal tissue lactate followed a similar pattern, and was elevated in controls versus Shams (37 ± 6 μmol/mg protein vs 19 ± 3 μmol/mg protein, respectively), which was reduced in a dose-dependent manner in piglets treated with doxycycline (p=0.110). (Figure 7-8) Intestinal tissue LPO levels were higher in control piglets compared to Shams (1.59 ± 0.23 μmol/mg protein vs 0.91 ± 0.09 μmol/mg protein respectively), with reduced levels observed in piglets treated with doxycycline (p=0.078). (Figure 7-9) Intestinal tissue total MMP-2 activity was significantly higher in control piglets verses Shams) (1.6 ± 0.2 arbitrary units (AU) versus 0.9

± 0.2 AU, respectively), and significantly reduced in all doxycycline treatment groups compared to controls (1.2 ± 0.1 AU, 1.0 ± 0.1 AU and 1.0 ± 0.1 AU for 3 mg/kg, 10 mg/kg, and 30 mg/kg doxycycline groups, respectively). (Figure 7-10)

Histology

There were no histological differences on light microscopy observed in the intestinal tissue between any of the groups.

Discussion

The objective of this study was to ascertain the effect MMP inhibition has on changes in regional blood flow and intestinal injury in a clinically translatable swine model of neonatal asphyxia. As it is believed that blood flow is directed to more 'vital' organs during asphyxia, regional blood flow was measured both to the brain (carotid artery flow) and the intestine (superior mesenteric artery flow) throughout the experiment in an attempt to observe this adaptation.

Carotid artery flow, though slightly decreased at the end of hypoxia, was relatively maintained during the hypoxic event despite the presence of significant cardiogenic shock, thus supporting the notion that early adoptive mechanisms in asphyxiated neonates are initiated to preserve blood flow to the brain. However, carotid artery flow progressively declined in piglets subjected to hypoxia-reoxygenation during the reoxygenation phase, except in piglets treated with 30 mg/kg doxycycline which had carotid artery flows similar to Sham piglets by the end of reoxygenation. Though the difference in carotid artery flow was not significant between controls and 30 mg/kg doxycycline piglets (due to the large standard deviation encountered in the data for the 30 mg/kg doxycycline group), this finding does suggest that administration of doxycycline early in the resuscitation of asphyxiated piglets may preserve blood flow to the brain during reoxygenation. It has been established that myocardial dysfunction has a significant impact on perfusion to the brain in asphyxiated neonates.(16,17) Thus, improvement in carotid artery flow in this study likely reflects attenuation of myocardial injury and dysfunction as evidenced by the significant increased in the

recovery of cardiac index (refer to Chapter 5 for full details) in piglets treated with doxycycline. An unexpected finding in the study was flow through the superior mesenteric artery during H-R, which did not significantly change throughout H-R in any group. This finding conflicts with the understanding that blood flow is shunted away from less vital organs (including the intestine) during asphyxia to preserve flow to the heart, brain and adrenal glands in asphyxiated neonates.

IFABP is a 15 kDa cytoplasmic protein found within small intestine enterocytes, and increased levels of IFABP in the serum have been demonstrated to be a sensitive marker of intestinal injury.(18-20) Level of serum IFABP were significantly elevated in control piglets compared to Shams and were attenuated in piglets treated with 10 mg/kg and 30 mg/kg doxycycline, though significantly lower levels were observed only in the latter. Intestinal tissue lactate levels followed a similar pattern, with higher levels noted in controls versus Shams which was decreased in a dose-dependent manner with administration of doxycycline, though observed differences were not statistically significant at $p<0.05$.

The activation of MMPs through an increase in oxidative stress has been demonstrated in multiple studies (21-23), and it has been suggested that an increase in matrix metalloproteinase activity contributes to intestinal injury in NEC.(10,12) Furthermore, an increase in MMP-2 and MMP-9 activity has been observed in the intestines of human patients with ischemic colitis.(13) To ascertain if this mechanism contributes to the intestinal injury observed in this

study, markers of oxidative stress and MMP-2 activity were quantified in the intestinal tissue. Lipid hydroperoxides are a marker of cellular oxidative stress; intestinal tissue levels were elevated in control piglets compared to Shams and were attenuated with administration of doxycycline, though observed differences were not statistically significant at $p<0.05$. Intestinal tissue total MMP-2 activity followed a similar pattern, with significantly higher activity observed in control piglets versus Shams and attenuation of activity in a dose-dependent manner with doxycycline administration such that all three doxycycline groups had significantly lower levels compared to controls.

In this study, piglets subjected to H-R demonstrated evidence of intestinal injury that was attenuated with administration of doxycycline during reoxygenation. A possible mechanism contributing to injury pathogenesis is an increase in cellular oxidative stress and subsequent activation of MMP-2, though the exact mechanisms in which these events mediate injury cannot be deduced from this study. Furthermore, it does not appear that intestinal injury is due to a decrease in blood flow to the intestine (ischemic-reperfusion injury), although intestinal oxygen delivery was significantly reduced during hypoxia, presumably resulting in a hypoxia-reoxygenation injury to the tissue.

In conclusion, administration of doxycycline in newborn piglets subjected to H-R preserved carotid artery flow during reoxygenation but had no effect on superior mesenteric artery flow. Intestinal injury was attenuated with doxycycline administration, and this was associated with a decrease in intestinal tissue oxidative stress and MMP-2 activity. To our knowledge this the first study to

study the effect MMP inhibition has on regional blood flow and intestinal injury in a clinically translatable swine model of neonatal asphyxia.

Figure 7-1: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on carotid artery flow index in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

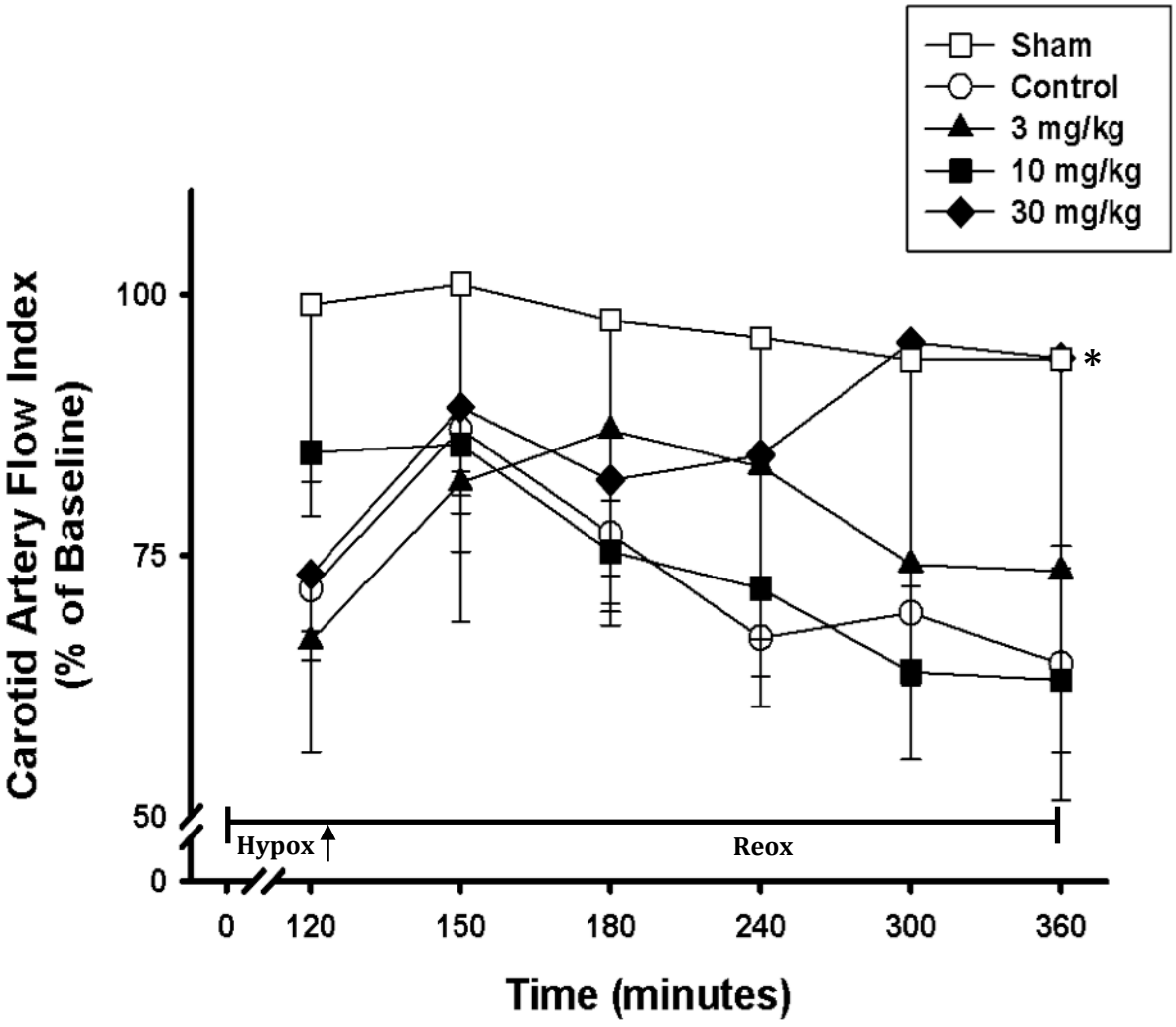


Figure 7-2: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on carotid artery oxygen delivery in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

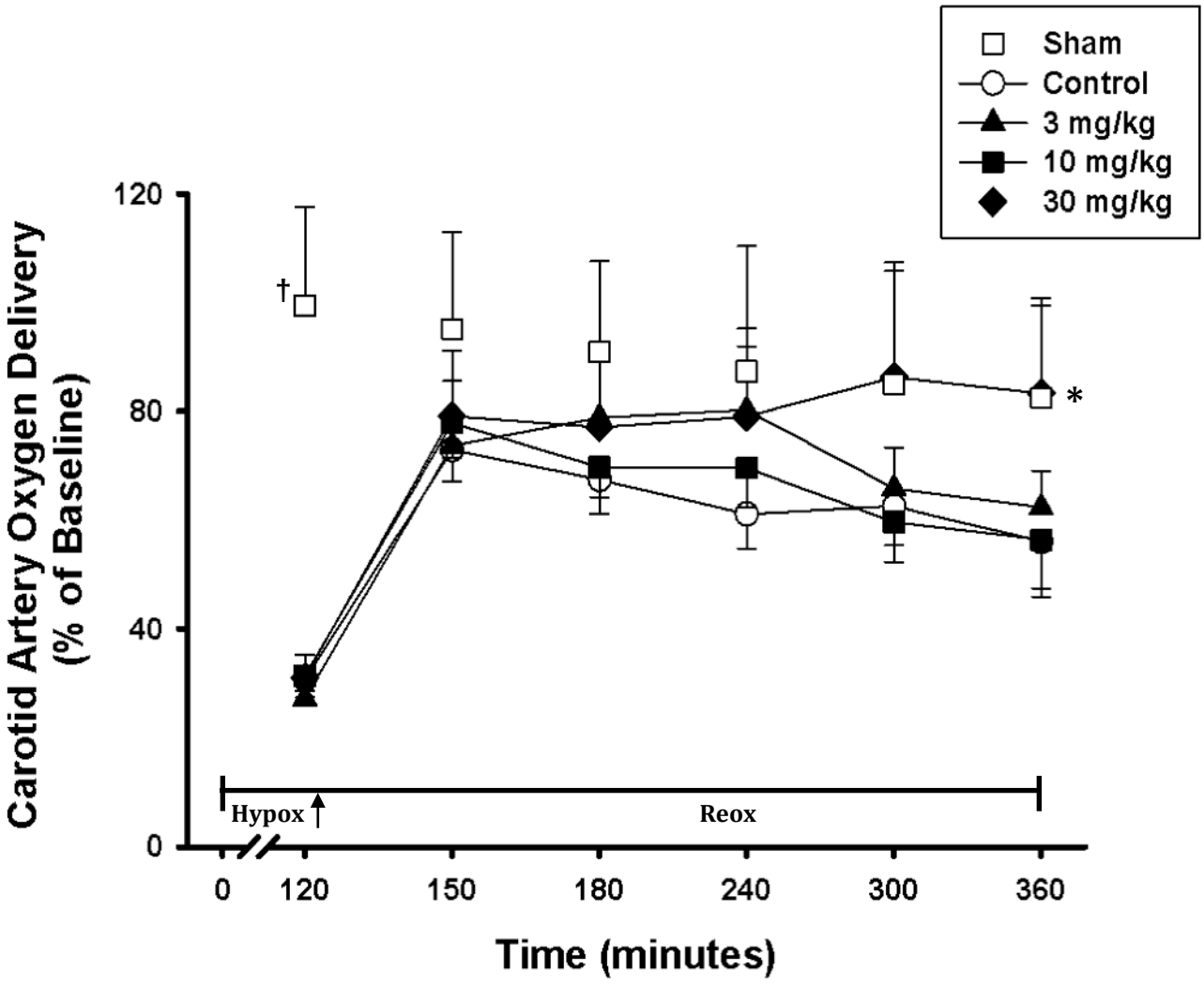


Figure 7-3: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on carotid artery vascular resistance index in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

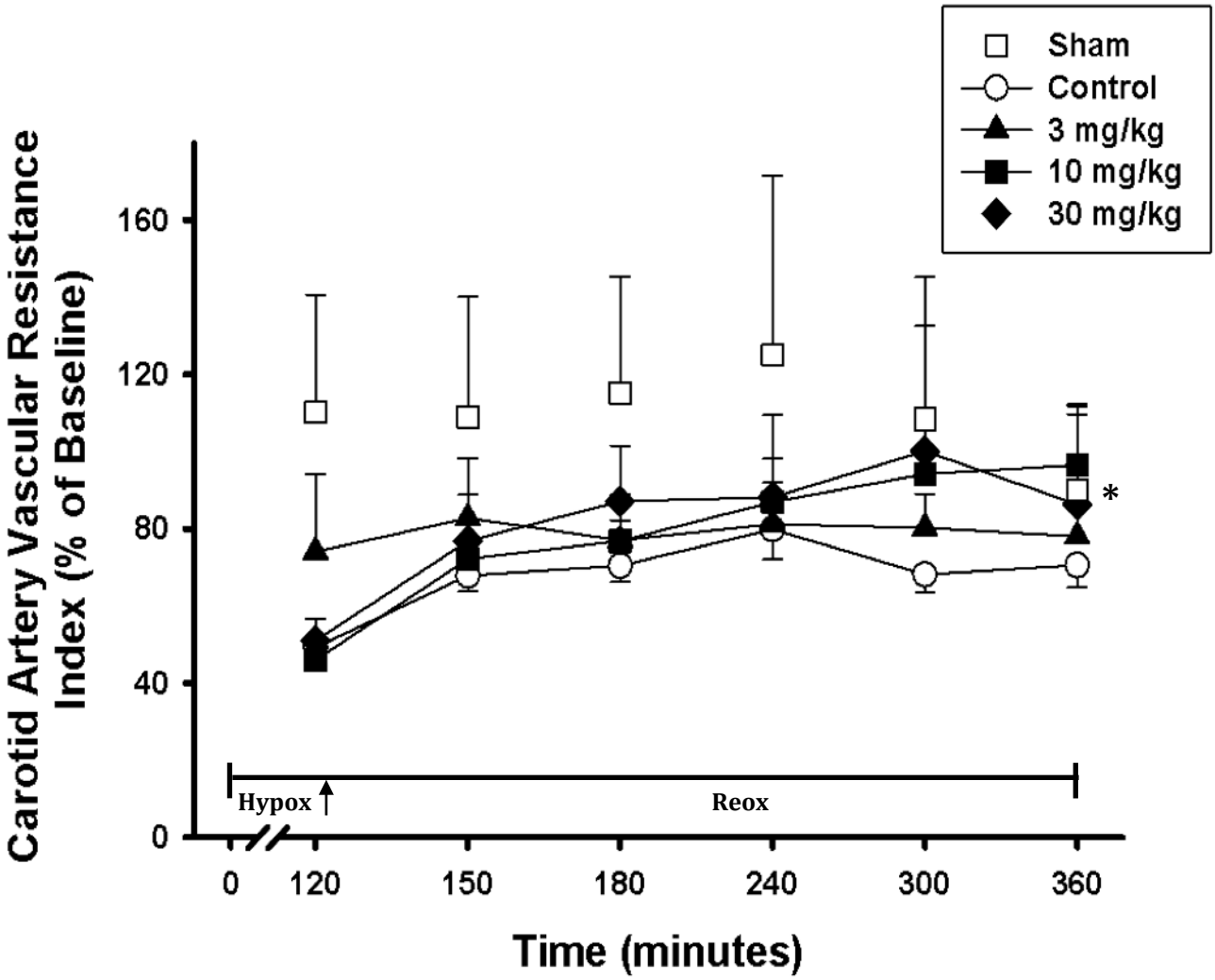


Figure 7-4: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on superior mesenteric artery flow index in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

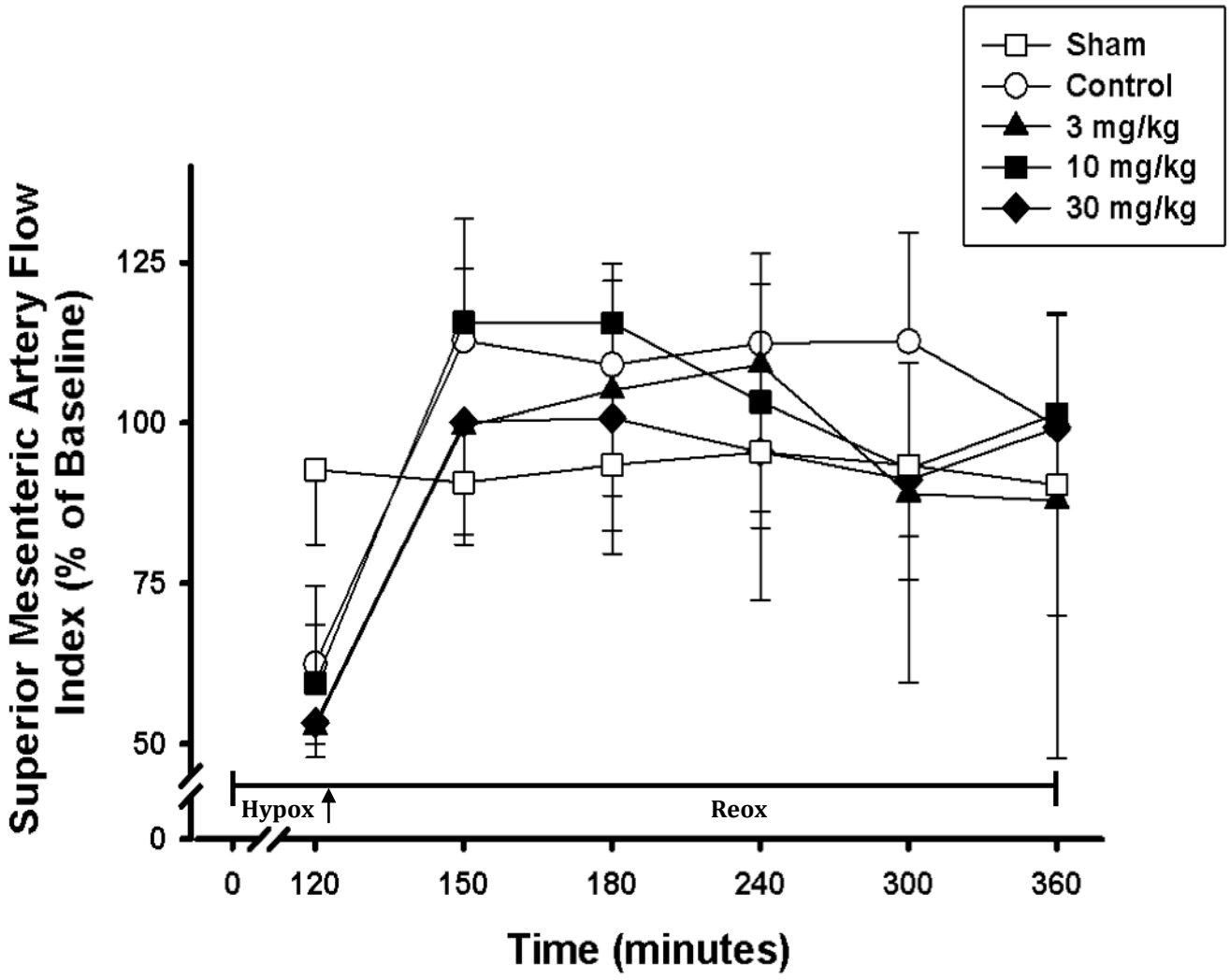


Figure 7-5: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on superior mesenteric artery oxygen delivery in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

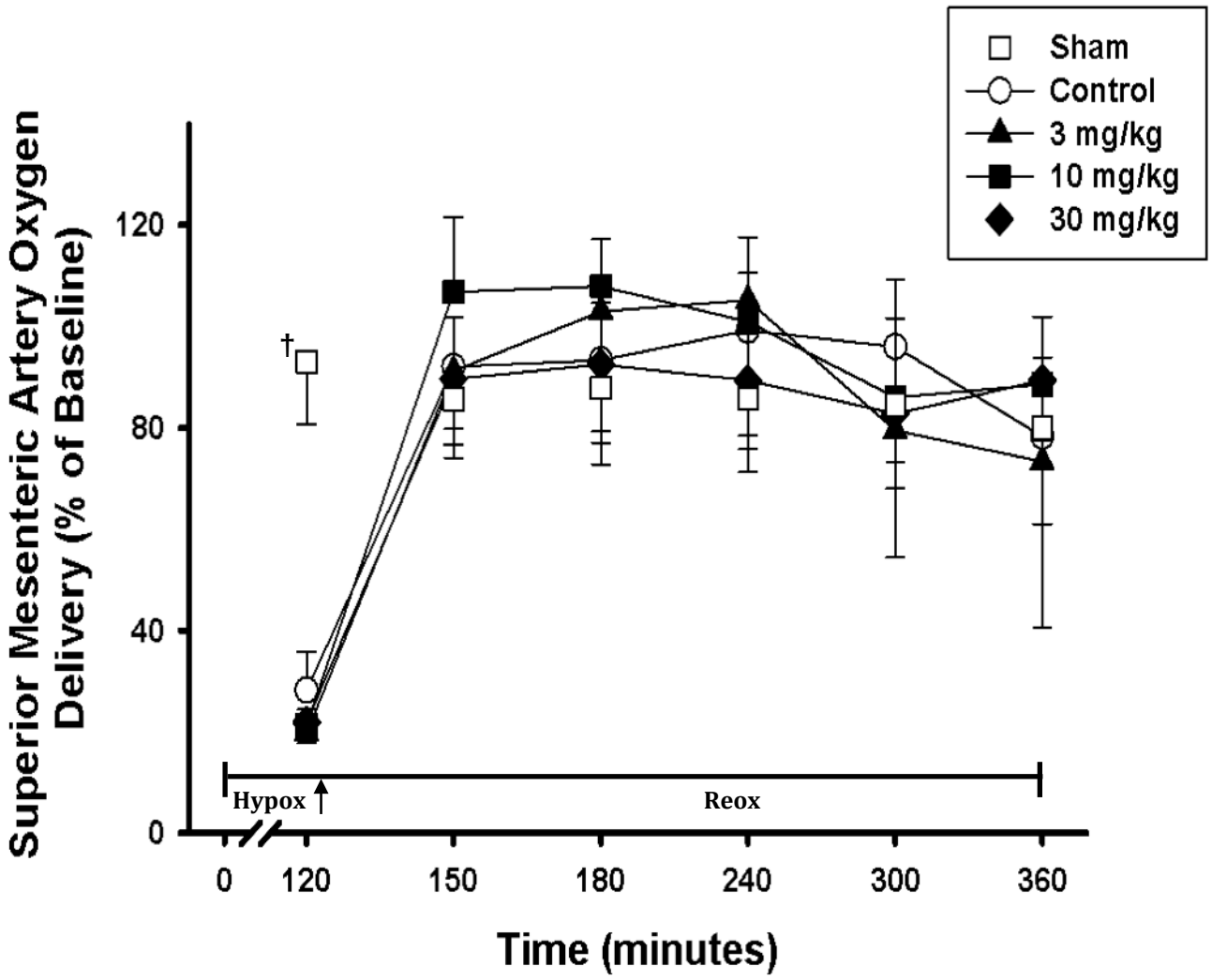


Figure 7-6: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on superior mesenteric artery vascular resistance index in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation, †p<0.05 vs. control group at the specific time-point. Values are given as Mean±SEM; Hypox = 2 hours of hypoxia; Reox = 4 hours of reoxygenation; ↑ saline or doxycycline administration at 5 minutes into reoxygenation.

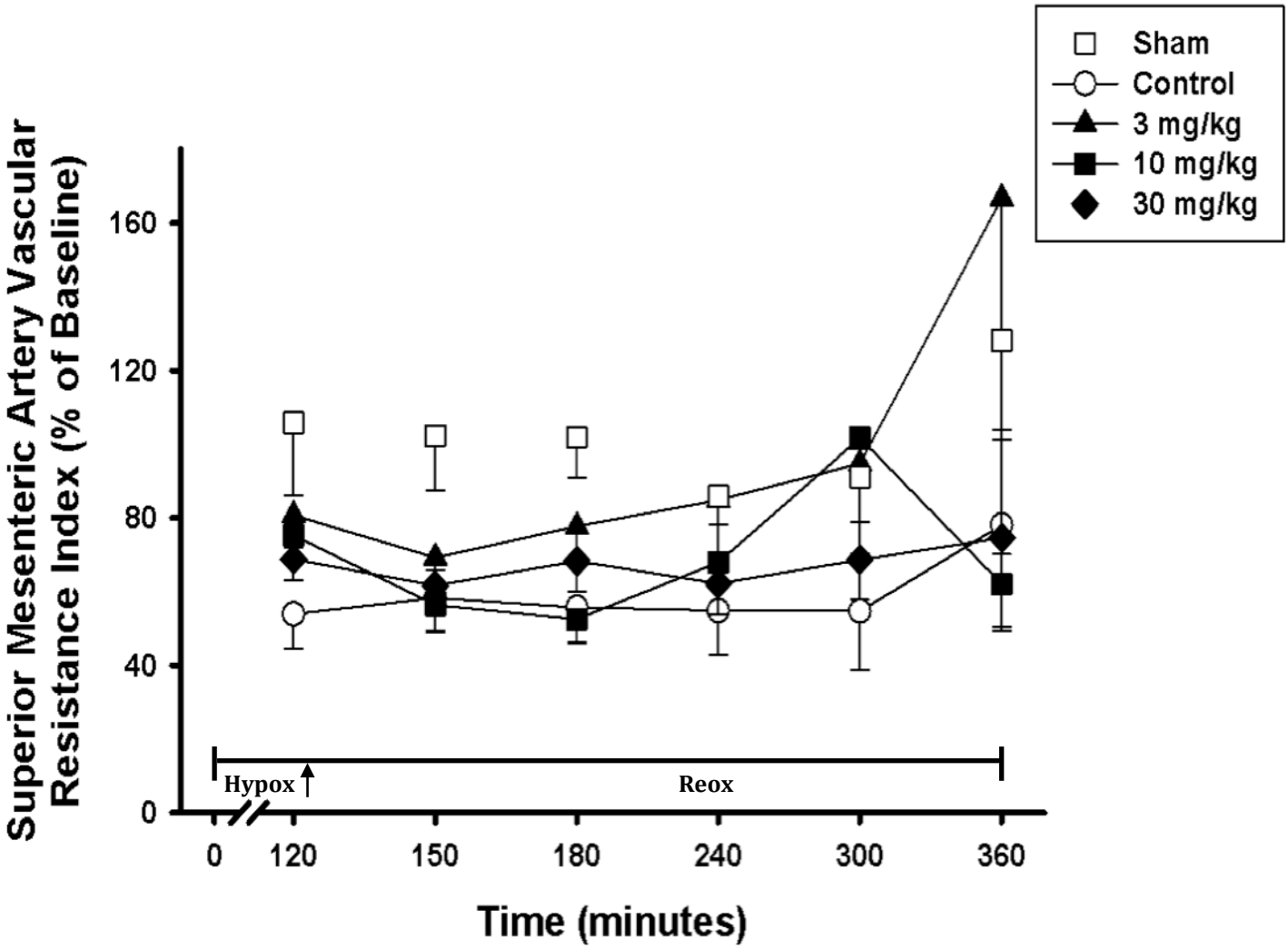


Figure 7-7: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on serum IFABP levels in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

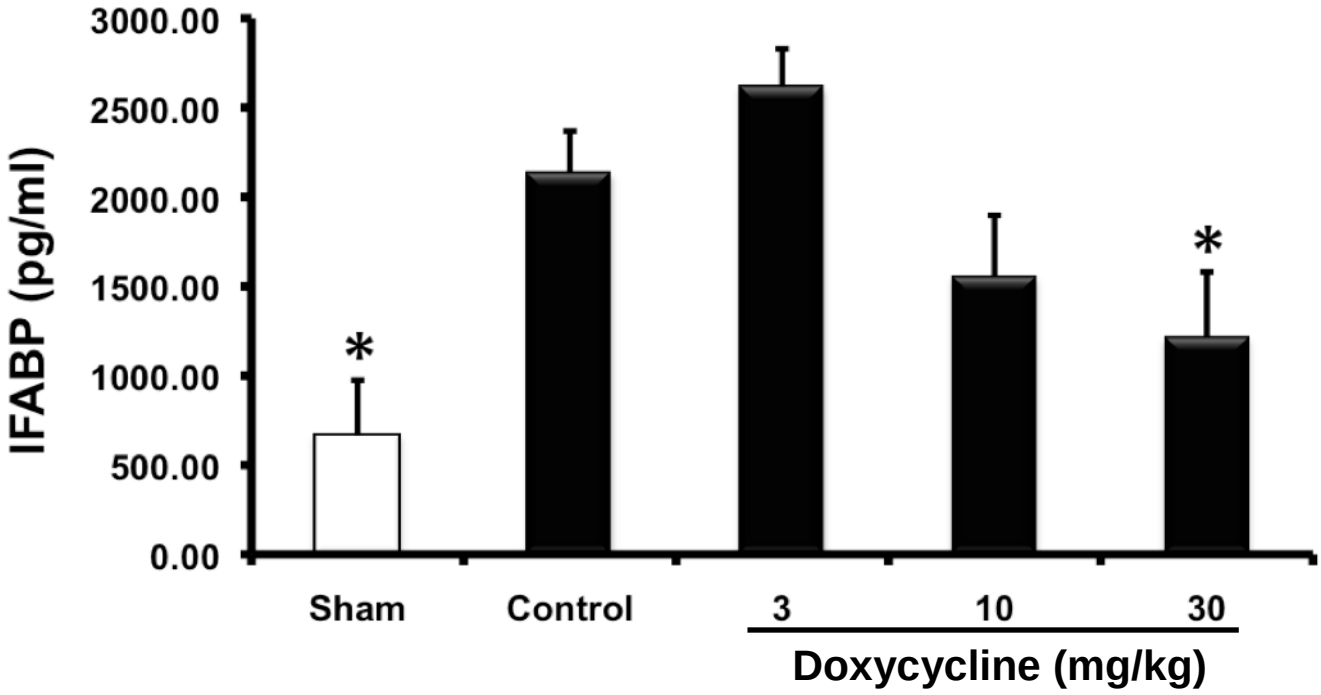


Figure 7-8: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on intestinal tissue lactate levels in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

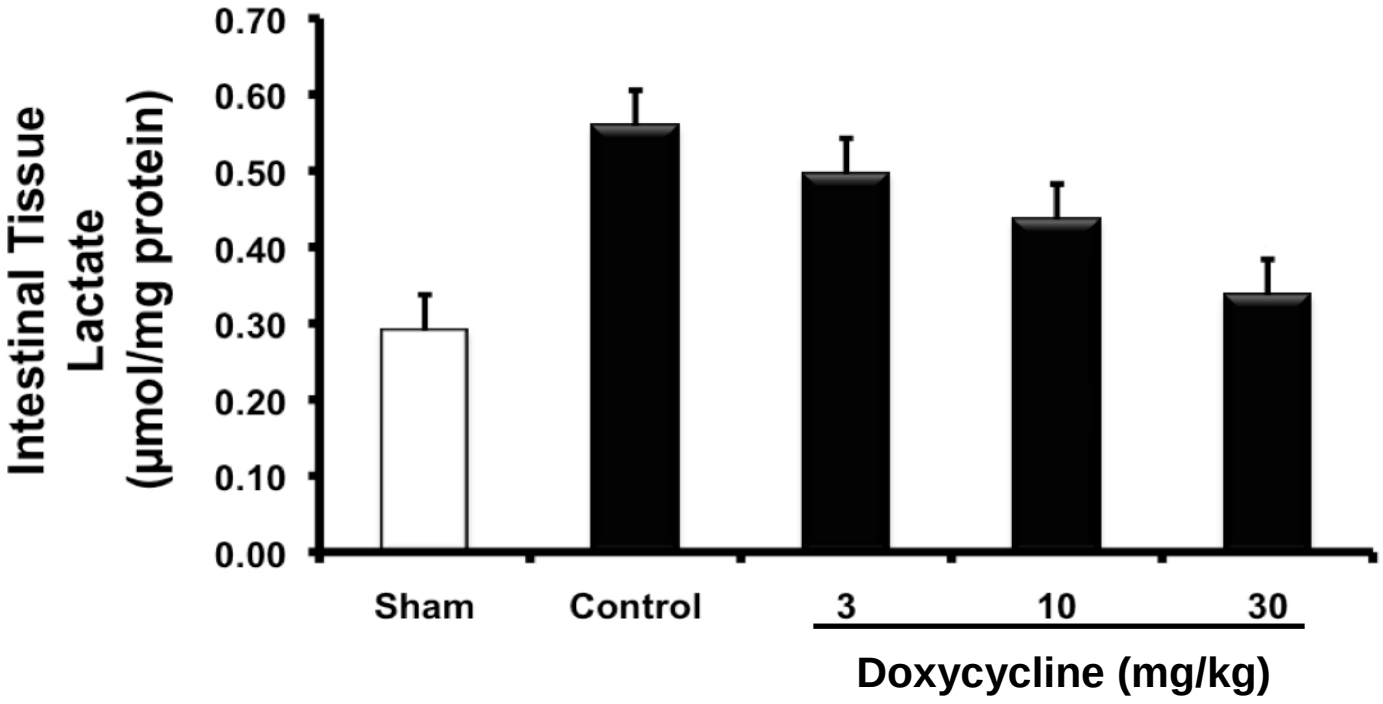


Figure 7-9: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on intestinal tissue lipid hydroperoxide levels in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.

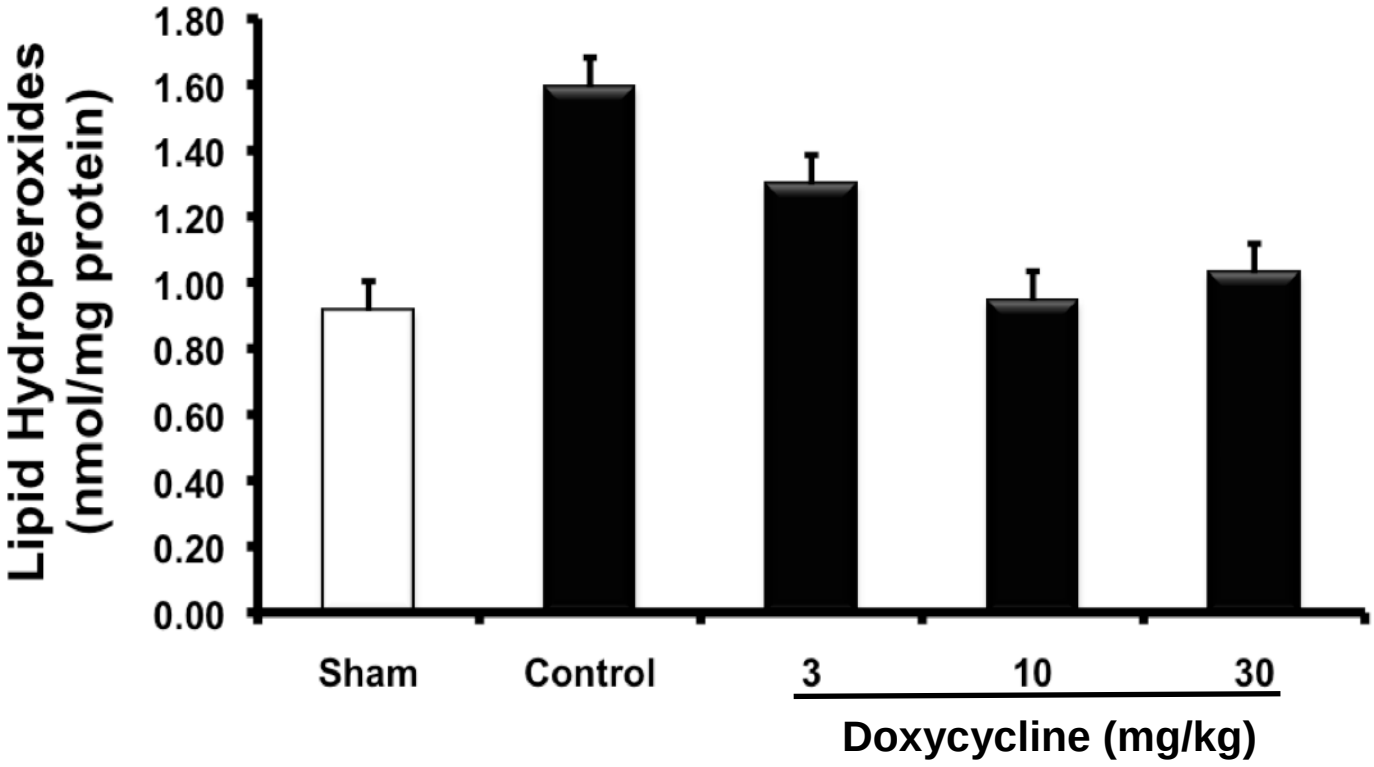
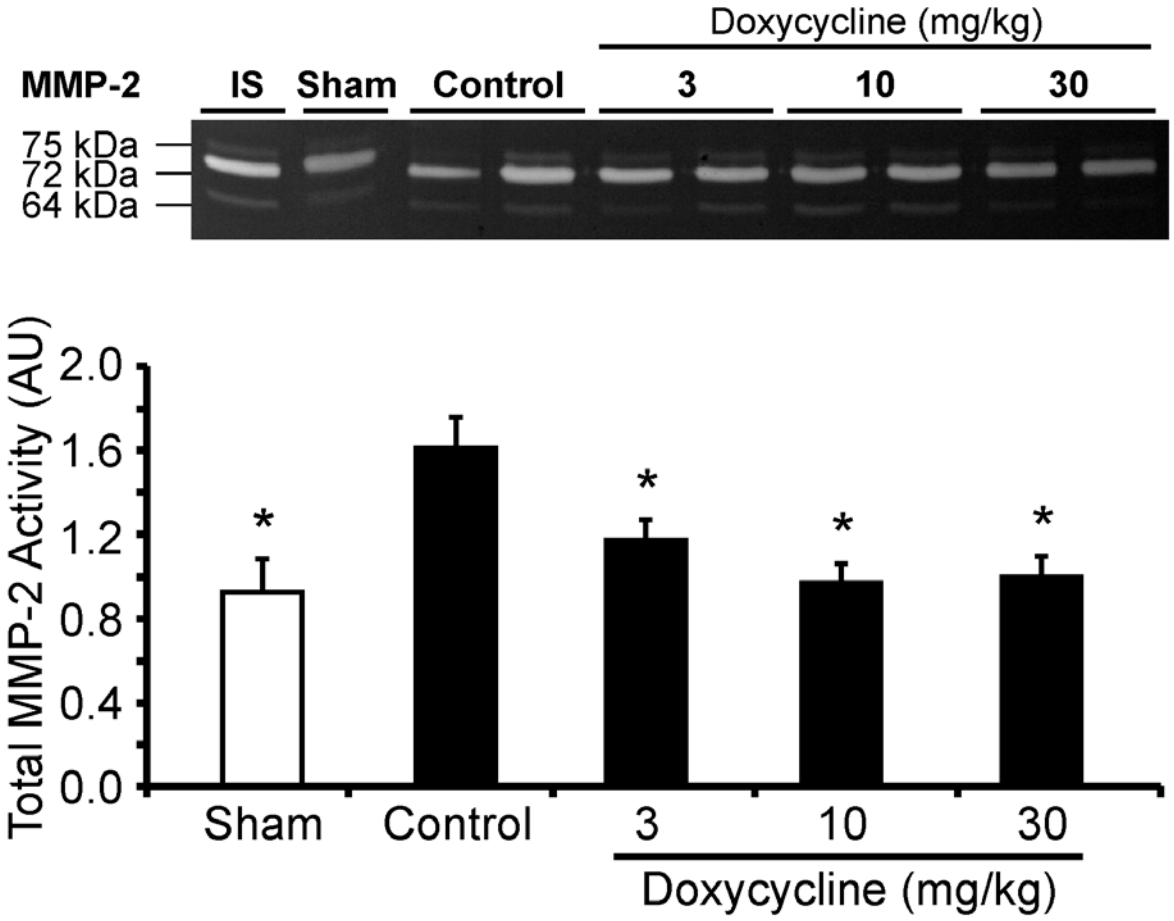


Figure 7-10: The effect saline (control, n=7) or doxycycline (3, 10 or 30mg/kg; n=7/group) administration has on intestinal tissue MMP-2 activity in piglets exposed to hypoxia-reoxygenation. Sham-operated (n=5) piglets received no H-R. *p<0.05 vs. control group during reoxygenation. Values are given as Mean±SEM.



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Chapter 8

Conclusions and Future Directions

Conclusions

Asphyxia is a significant cause of neonatal morbidity and mortality, accounting for a third of all neonatal deaths worldwide. Surviving neonates often experience multiorgan injury and dysfunction, and sequelae from these injuries can persist for the life of the neonate. As there are numerous pathological mechanisms mediating injury in multiple organ systems, management of asphyxiated neonates is complex and best performed in a dedicated neonatal intensive care unit. Though treatment has been advanced greatly in the last decade, current therapies follow a similar theme of treating altered physiologic states and supporting injured organ systems. Thus interventions that attenuate or prevent organ injury and dysfunction from occurring in the first place in asphyxiated neonates are lacking.

Organ injury and dysfunction during asphyxia is due to two distinct temporal insults: the hypoxic injury itself, and less intuitively, there is a second injury that occurs during reoxygenation. This was an unexpected finding which become known as the ‘oxygen paradox’. It is now known that injury during reoxygenation occurs, in part, due to an increase in the production of RONS, which can have a host of pathological effects within the organs. One effect is the activation of MMPs, which through a variety of mechanisms will lead to cellular, and overall, organ injury and dysfunction. From this knowledge, MMP inhibitors have been proposed as a novel therapeutic intervention aimed at attenuating or preventing organ injury and dysfunction from occurring during the resuscitation of asphyxiated neonates.

Tetracycline antibiotics, in addition to their antimicrobial properties, have well known non-antimicrobial effects, including the inhibition of MMPs. Doxycycline is the most potent inhibitor of MMPs amongst the tetracyclines, has a well known pharmacokinetic profile, can be administered intravenously, and is already approved for clinical use as a MMP inhibitor in the treatment of peridontitis.

This project ascertained the effect administration of doxycycline has on systemic and regional hemodynamic parameters, as well as on myocardial, renal and intestinal injury in a clinically translatable swine model of neonatal asphyxia.

Our findings demonstrate that administration of doxycycline five minutes into the resuscitation of asphyxiated newborn piglets improved the recovery of cardiac index without any chronotropic effect, thus indicating an improvement in stroke volume index (contractility). This translated into improved systemic arterial pressure and systemic oxygen delivery. These improved systemic hemodynamic effects were associated with an attenuation in myocardial injury, as evidenced by higher myocardial tissue troponin I levels and lower plasma troponin I and myocardial tissue lactate levels in piglets treated with doxycycline. We further demonstrated that these effects by doxycycline are mediated, in part, through a reduction in myocardial tissue oxidative stress, evidenced by lower lipid hydroperoxide (LPO) levels in the tissue, and an inhibition of myocardial tissue MMP-2 activity. We also found significant negative correlations between plasma troponin I and myocardial tissue troponin I, cardiac index, and stroke volume index, as well as a significant positive correlation with myocardial tissue lactate,

which serves to validate plasma troponin I levels as a marker of myocardial injury and dysfunction in the neonate. Finally, we demonstrate that doxycycline may also benefit pulmonary hemodynamics as well, as reflected in a lower pulmonary artery to systemic artery pressure ratio (and thus a lower risk of right to left shunting of hypoxic blood into the systemic circulation) in piglets treated with doxycycline, which is advantageous for neonates at risk for pulmonary hypertension.

Findings from this study also highlight the effect doxycycline has on regional hemodynamics and peripheral organ injury. We observed a significant improvement in renal artery flow index and renal artery oxygen delivery in piglets treated with doxycycline, which likely reflects improvement in cardiac output but may also relate to a local vasodilatory effect by doxycycline. As with the myocardium, the improvements in renal hemodynamics were associated with attenuation in renal injury, evidenced by lower kidney tissue lactate levels and a reduction in urine N-acetyl-D-glucosaminidase activity. There was also less oxidative stress (lower LPO levels) and lower MMP-2 activity in the renal tissue. Carotid artery flow was relatively maintained during hypoxia, and was preserved during reoxygenation in piglets treated with doxycycline, though findings were not statistically significant compared to control piglets. Doxycycline had no effect on intestinal hemodynamic parameters; however, intestinal injury was attenuated in piglets treated with doxycycline, evidenced by lower levels of intestinal tissue lactate and serum intestinal fatty acid binding protein. Intestinal tissue LPO levels and MMP-2 activity were also lower in piglets treated with doxycycline. Though

intestinal injury does not appear to be related to changes in intestinal perfusion (ischemia-reperfusion injury), it may be related to a local hypoxia-reoxygenation injury as superior mesenteric artery oxygen delivery decreased significantly during hypoxia but recovered during reoxygenation.

To conclude, this study has demonstrated that early administration of doxycycline during the resuscitation of asphyxiated newborn piglets significantly improves systemic and regional hemodynamic parameters, attenuates myocardial, renal and intestinal injury, and exerts its beneficial effects, in part, through the inhibition of MMP-2 activity. Further clinical studies, both large animal, and eventually, human, are required to confirm these observations with the intent of one day translating this novel therapeutic intervention to the treatment of asphyxiated human neonates.

Future Directions

Though observations from this study, which was designed as a clinically translatable model of human neonatal asphyxia, suggests a therapeutic benefit with the administration of doxycycline, further work is required before applying these results into human clinical practice.

The study examined systemic and regional hemodynamic parameters during six hours of H-R and highlighted early myocardial, renal, and intestinal injury and dysfunction that is attenuated with doxycycline. Studies using a chronic animal model of hypoxia-reoxygenation injury would better determine if the beneficial effects of doxycycline persist beyond the acute period, and may allow for the assessment of long-term sequelae. At a cellular level, it would be

interesting to perform electron microscopy on the various organ tissues assessed in this project to determine the degree of cellular injury that occurs during H-R, and whether injury at the cellular level is attenuated with doxycycline. Furthermore, assessing the level and activity of the tissue inhibitors of matrix metalloproteinases would provide further insight into the regulation of the MMPs during H-R, and how this is affected by doxycycline. Finally, it would be interesting and beneficial to assess the activity of other MMPs implicated in H-R injury in asphyxiated neonates, such as MMP-9 in the heart, for example.

There persists some controversy regarding the use of doxycycline in the neonate, despite recent evidence dispelling these concerns. In the absence of large randomized double-blinded controlled trials, it is unlikely that the use of doxycycline in neonates will be unanimously agreed upon. Doxycycline is at present the only clinically approved MMP inhibitor that can be given intravenously, and additionally has other non-antimicrobial effects that could be beneficial in asphyxia including anti-inflammatory, anti-apoptotic and reactive oxygen species scavenging effects. It is for these reasons that I believe that doxycycline is, at present, the drug with the most potential to improve outcomes in asphyxiated neonates. If and when other intravenous MMP inhibitors are designed and approved for clinical practice, a comparison between doxycycline and these drugs will be of interest, as it is unlikely that the beneficial effects of doxycycline observed in this study are related only to MMP inhibition alone, and doxycycline may still be the ideal drug of choice in asphyxiated neonates. Thus

studies addressing the safety of doxycycline use in neonates should be performed as well.

This study has demonstrated the therapeutic potential of doxycycline administration in the treatment of asphyxiated neonates, however, questions remain to be answered and further study will be required if doxycycline is to one day be approved for use in the treatment of asphyxiated human neonates.