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Combination Vasoactive Medication Use in Asphyxiated Newborn Piglets

by

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To those who have taught me stuff.

Abstract

With asphyxia, newborns may suffer cardiogenic shock with myocardial dysfunction and dysregulation of vasomotor tone resulting in multiorgan dysfunction. Vasoactive medications are often administered with limited evidence directing clinicians regarding the use of high-dose monotherapy with dopamine relative to combination treatment with dopamine and a second different agent. We hypothesized that the treatment of hypoxia-reoxygenated newborn piglets with combinations of vasoactive medications would improve systemic and regional hemodynamics. Instrumented newborn piglets were subjected to hypoxia-reoxygenation with subsequent infusion of high-dose dopamine or moderate-dose dopamine and one of epinephrine, milrinone or levosimendan. Treatment with high-dose dopamine improved systemic and mesenteric perfusion. The addition of low-dose epinephrine showed some benefits regarding pulmonary hypertension and should a non-catecholamine agent be added to dopamine, milrinone is preferred to levosimendan given benefits to mesenteric perfusion. We conclude that the selection of appropriate

vasoactive medical therapy should be directed by the clinical effects desired.

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Abbreviations

ANOVA	analysis of variance
ATP	adenosine – 3, 5 – triphosphate
CAFI	carotid artery flow index
CAVRI	carotid artery vascular resistance index
CADO2	carotid artery oxygen delivery
cAMP	cyclic adenosine monophosphate
CI	cardiac index
CVP	central venous pressure
DOPA	high-dose dopamine group
D+E	dopamine with epinephrine group
D+L	dopamine with levosimendan group
D+M	dopamine with milrinone group
FiO2	fraction of inspired oxygen
GSH	glutathione (reduced)
GSSG	glutathione disulphide (oxidized)
H-R	hypoxia-reoxygenation
MAP	mean arterial pressure
mmHg	millimeters of mercury
PaO2	partial pressure of oxygen – arterial
PAP	pulmonary artery pressure
PDE	phosphodiesterase
PVRI	pulmonary vascular resistance index
RAFI	renal artery flow index
RAVRI	renal artery vascular resistance index
RADO2	renal artery oxygen delivery
ROS	reactive oxygen species
SaO2	oxygen saturation – arterial
SMAFI	superior mesenteric artery flow index
SMAVRI	superior mesenteric artery vascular resistance index
SMADO2	superior mesenteric artery oxygen delivery
SVI	stroke volume
SVRI	systemic vascular resistance index
SysDO2	systemic oxygen delivery
SysVO2	systemic oxygen consumption

Chapter 1: Asphyxia

The neonatal period commences at birth and continues for approximately one month. With birth, the newborn undergoes a transition from the fluid-filled placental environment to our air-filled environment where the pulmonary system assumes the role of gas exchange, in lieu of the placenta. The majority of neonates make this transition without adverse event. Often, neonates require some form of resuscitation. This may take the form of minor interventions to intensive care. The following four chapters will focus on the problem of neonatal asphyxia. The disease process, current treatments, evidence in the use of vasoactive medications and justification for the methods used to study asphyxia in a porcine model will be discussed.

Definition

Defining the term asphyxia is a matter under continuous academic debate. Classically, intrapartum brain damage was labeled as the etiologic factor in the development of particular central nervous systems disorders such as cerebral palsy and mental retardation.[1] This "damage" or traumatic phenomenon fell out of favour and was ultimately replaced with the terms perinatal or birth asphyxia. In Greek, asphyxia or *asphygmos*, literally means *without pulse*. Increasing focus has tailored the definition to include various biochemical indices and clinical sequelae. Asphyxia is a "condition of impaired blood gas exchange leading, if it persists, to progressive hypoxemia and hypercapnia."[2]

The following criteria are to be observed for the proper use of the term asphyxia in neonates:[3]

i. Metabolic or mixed acidosis – umbilical cord arterial pH < 7
ii. Apgar score of 0 to 3 for ≥ 5 minutes (see Table 1.1)
iii. Neurologic manifestations (seizures, coma or decreased tone)
iv. Multisystem organ dysfunction

Epidemiology

Every year, nearly 4 million neonates die worldwide.[4] Of the 4 million or so United States births in 2005, the neonatal mortality rate measured 4.5 per 1000.[5] Perinatal mortality, defined as the combined death of the neonates delivered at greater than 28 weeks gestation and less than 7 days of age and fetal deaths, has been

steadily improving yet continues to present a significant challenge. Perinatal mortality rates measured approximately 6.7/1000 births. The late fetal mortality rate, measuring deaths at greater than 28 weeks gestation, is 3.1/1000. Thus, a large proportion of neonatal mortality occurs during the first week of life.

Table 1.1 – Apgar score[6]

SIGN / SCORE	0	1	2
Heart rate	Absent	<100	>100
Respirations	Absent	Slow, irregular	Good, crying
Irritability	Nil	Grimace	Vigorous,crying
Tone	Flaccid	Limited flexion	Active
Colour	Cyanotic	Pale, acrocyanosis	Pink

Perinatal and neonatal asphyxia are significant contributors to these mortality figures. In a 2005 World Health Organization report, asphyxia was said to account for 23% of neonatal deaths.[7] Intrapartum asphyxia alone accounted for a perinatal mortality rate of 4.8 per 1000 births in the South African Perinatal Care Survey.[8]

Etiology

Asphyxia has a plethora of causes. The underlying factor is a hypoxic insult to the fetus or neonate. Compromise of gas exchange, whether placental or pulmonary, leads to hypoxemia with subsequent hypoxia, hypercarbia and acidosis. Causes can be temporally divided between prenatal and perinatal periods, the latter being far more common. Likewise, causes may also be divided between obstetric or maternal factors, many of which are related to placental insufficiency, and fetal or neonatal factors.[9] Table 1.2 summarizes the main causes of asphyxial injury to the neonate. Placenta, cord and membrane complications accounted for 4% while maternal complications accounted for 6.3% of all infant deaths according to the 2006 United States Summary of Vital Statistics.[5]

A significant risk factor for birth asphyxia is low birthweight. Almost three quarters of neonates less than 1 kilogram suffered asphyxia in comparison to only 1% of babies between 3 and 4 kilograms in a study of obstetric and newborn complications.[10] However, mortality was significantly worse when term neonates

were subject to such an insult, demonstrating less resilience to hypoxic stress.

Table 1.2 – Causes of perinatal asphyxia

MATERNAL	NEONATAL
Hypoxemia	Congenital abnormality
Hypotension / Shock	Birth trauma
Uterine tetany	Cyanotic heart disease
Placental abruption	Pulmonary disease
Other antepartum hemorrhage	Severe anemia
Umbilical cord compression	Shock – septic or hypovolemic
Pre-term labour	Congenital diaphragmatic hernia
Gestational hypertension	Intrauterine growth restriction
Infection	

Biochemistry of Asphyxia

Underlying the clinical definition of asphyxia is the requirement that the fetus or neonate must have suffered a hypoxic insult. Hypoxemia describes a low concentration of oxygen in the blood. In contrast, hypoxia is a condition of low tissue oxygenation. Hypercapnia, an elevated carbon dioxide concentration, reflects either an increase in the production of carbon dioxide or a reduction in ventilation of the lungs or gas exchange at the placenta. The result of these conditions is a significant metabolic acidosis.[11]

In the normally oxygenated neonate, energy is derived through metabolism of glucose. Glycolysis in the cytosol yields pyruvate that, following conversion to Acetyl-CoA, enters the mitochondrial citric acid cycle and through a number of enzymatically catalyzed steps is subsequently oxidized. Electrons are transferred to cytochrome oxidase and oxidative phosphorylation results in a generation of adenosine triphosphate, ATP, a high-energy molecule. The final electrons are transferred to oxygen and water is released.

Alternatively, under hypoxic conditions, oxygen is inadequately supplied to allow the citric acid cycle to continue. Precursors of aerobic metabolism accumulate and pyruvate is then predominantly metabolized anaerobically to lactate. Incorporated in

this process is the rapid hydrolysis of ATP, releasing protons which acidify the blood (lower pH). The lactate produced is taken up by the liver and converted to pyruvate. Pyruvate, if sufficient energy is available, is used to re-synthesize glucose through the gluconeogenic pathway. Given a lack of ATP, this cannot be sustained.

Free radicals and oxidative stress

Free radical biochemistry involves molecules with unpaired electrons.[12] These molecules, also known as reactive oxygen or nitrogen species, are normal products of metabolism.[13] They display both harmful and beneficial properties. The noxious effects stem from an overproduction of radicals, often in the absence of sufficient anti-oxidants. The resulting imbalance of pro-oxidants to antioxidants has been implicated in tissue injury following asphyxia, ischemia-reperfusion and hypoxia-reoxygenation. Under such circumstances, the redox, or reduction-oxidation, regulation system fails.[14]

The superoxide radical ($O_2^{-\bullet}$), a molecular oxygen molecule with one additional electron, is a reactive species of particular interest.[15] The mitochondrial electron transport chain normally reduces molecular oxygen to form water. However, a number of electrons do instead 'leak' to create the anion radical, superoxide, at semiubiquinone on the mitochondrial cell membrane. Other methods of oxygen radical formation are enzymatically driven. Excessive ATP consumption during hypoxia or ischemia leads to the accumulation of purines, xanthine and hypoxanthine.[16] These are metabolized by xanthine oxidase when oxygenation is restored, releasing large amounts of superoxide and peroxides. Peroxides can also be formed via metal ion-catalysis. The hydroxyl radical is one such molecule, derived from the Fenton and Haber-Weiss reactions, both influenced by the presence of superoxide.[16] In the cytosol, peroxisomes create radicals through a hydrogen peroxide intermediate. Damaged peroxisomes leak peroxide to the cytosol and interactions with other molecules produces further radicals. Nitric oxide, formed by nitric oxide synthase, is perhaps the most commonly studied reactive nitrogen species. It has multiple physiological effects in the nervous and immune systems as well as a vasodilatory effect on vasculature.[17] During severe inflammatory

processes, superoxide and nitric oxide react to create the peroxynitrite anion, a strong oxidizing agent, damaging both DNA and lipids.[18] Cuzzocrea et al. summarize peroxynitrite's toxicity in tabular format similar to Table 1.3.

Radicals damage cells both directly and via intracellular signaling pathways. Deoxyribonucleic acid, DNA, is susceptible to hydroxyl radical damage both along the de-oxyribose backbone and at the nucleotide bases.[19] Lipids, essential for maintaining the architecture and function of membranes, are particularly susceptible to free radical injury and reactions are typically chain-connected. Major products include malondialdehyde and 4-hydroxy-2-nonenal.[20] Proteins are less involved, however, injury may occur should the focus of radical reaction be very specific, allowing a greater concentration of damage.

Table 1.3 – Peroxynitrite Biochemical Impact[21]

MECHANISM	ACTION	TARGET
Oxidation	Surfactant Damage	Lung
Peroxidation	Lipid Damage	Systemic
Oxidation	GSH Depletion	Systemic
Sulfhydryl group alteration	Inhibition of mitochondrial activity	Systemic
Nitrotyrosine formation	Superoxide dismutase inhibition; DOPA production inhibition	Neurons
Oxidation, deamination	DNA damage	Systemic

Two main mechanisms are employed in protecting tissue from free radical-mediated injury: prevention and consumption. The first, prevention, involves minimizing the generation of free radicals and requires efficient electron transfer. Also, metal ions that catalyse the creation of radicals can be sequestered. The body's intrinsic ability to do so involves binding metal ions to protein, transferrin and ferritin for example. Free radicals can also be consumed by various substances with 'anti-oxidant' properties, whether enzymatic or otherwise. Superoxide dismutase, catalase and glutathione peroxidase are examples of such enzymes. Superoxide dismutase assists in the dismutation (converting a radical substrate to a non-radical product) of superoxide radicals to peroxide. Catalase and glutathione peroxidase, in turn, decompose peroxides.

Non-enzymatic substances with anti-oxidant properties include α-tocopherol (vitamin E), ascorbic acid (vitamin C), uric acid and glutathione.

Glutathione or γ-glutamyl-cysteinyl-glycine (GSH) is an endogenous antioxidant protein in the cytosol of cells. Through oxidation to glutathione disulphide (GSSG), GSH maintains redox homeostasis. It scavenges superoxide and hydroxyl radicals, and, through the action of glutathione peroxidase, reacts with peroxides.[22] Increased levels of GSSG are indicative of oxidative stress.[23] Ultimately, the reduced form is regenerated by riboflavin co-factored GSH reductase activity. GSH is also actively involved in the regeneration of other antioxidants, namely, vitamins C and E.[24]

The study of reactive oxygen species in asphyxia is evolving. Long-chain polyunsaturated fatty acids, such as arachidonic and docosahexanoic acids, play a key role in fetal and neonatal development.[25] These lipids are particularly susceptible to oxidative injury given their high-energy double-bonds. Their levels are dependent on the intrauterine supply of essential fatty acids such as linoleic and linolenic acids. Research has shown reductions in plasma arachidonic and linoleic acids following asphyxia.[26] Oxidative injury of these lipids leads to the generation of lipid peroxides, radicals and aldehydes. Indeed, lipid peroxides have been measured *in vivo* and elevated levels of malondialdehyde are correlated with poor outcomes.[22,27] In response, anti-oxidant activity is upregulated with elevated levels of superoxide dismutase, catalase and glutathione peroxidase activity.

Organ dysfunction and asphyxia

Asphyxia challenges the survival of the neonate with variable effects on different organ systems. The initial depression of the cardiovascular system leads to subsequent dysfunction of end organs. Prior to labour, the fetus employs the placenta for gas exchange. Oxygenated blood returns to the fetal circulation via the umbilical vein. Greater than 50% of this blood bypasses the liver via the ductus venosus.[28] Both the fetus and neonate can selectively distribute vascular flow to specific organs under conditions of stress. This ensures adequate perfusion of vital structures such as the heart, brain and adrenal glands. Perfusion of ‘non-essential’ organs is compromised as a result. Nevertheless, with the

progression of asphyxia, ultimately all organ systems are affected as the neonate continues to decompensate. Following the severe hypoxic insult, a multitude of complications can be appreciated, as listed in Table 1.4.[29]

Table 1.4 – Organ system effects of asphyxia[30]

SYSTEM	COMPLICATION
CNS	Hypoxic ischemic encephalopathy Cerebrovascular accident/infarction Intraparenchymal hemorrhage Seizures Cerebral edema
CV	Myocardial infarction Valvular insufficiency Hypotension
Resp	Pulmonary hypertension Pulmonary hemorrhage Respiratory distress syndrome Meconium aspiration
GI	Enteric perforation Necrotizing enterocolitis Gastrointestinal hemorrhage
Renal	Acute kidney injury
Adrenal	Hemorrhage
Hematologic	Disseminated intravascular coagulopathy Thrombocytopenia

Cardiovascular Effects

29% of asphyxiated neonates suffer hypoxia-related myocardial dysfunction.[30] Carotid arterial chemoreceptors respond to hypoxemia by initially providing both vagal and α-adrenergic stimulus. This results in bradycardia and increased systemic arterial pressure. Tissue perfusion is regulated primarily by local vasodilators such as nitric oxide and adenosine. With persistent severe asphyxia, the adrenergic stimulus ultimately fails to correct hypotension and systemic vascular resistance changes with initial increases followed by eventual decreases. Pulmonary vascular resistance simultaneously worsens yielding pulmonary hypertension and heart strain.[31] Under such circumstances, flow across the

ductus arteriosus and foramen ovale shunts de-oxygenated blood to the systemic circulation.

Herpin et al demonstrated increased concentrations of the catecholamines epinephrine and norepinephrine in piglets suffering asphyxia.[32] Epinephrine both increased the contractility of the newborn myocardium and increased the heart rate. Multiple animal studies assessing the effects of asphyxia on the cardiovascular system have shown consistent results. In the newborn piglet, hypoxia reproducibly causes hypotension, pulmonary hypertension and reduction in cardiac index.[33,34,35] Fugelseth added that hypoxia might also cause increased incompetency of the tricuspid valve.[36] Hypoxia induces a metabolic acidosis with reduction in the base excess.[30,37] This acidosis impairs the catecholamine response, yielding reduced contractility and hypotension. In regards to regional perfusion, renal, carotid and mesenteric flows are typically depressed in studies of newborn piglet hypoxia, leading to reduced oxygen delivery.

Pulmonary hypertension is a concern following asphyxia in the newborn. Perfusion of the pulmonary circulation is adapted so as to match ventilation. Reduction in pulmonary flow with increases in pulmonary vascular resistance and pressures are evident under hypoxic states. This increased right-heart afterload is likely involved in the development of tricuspid valve incompetence, as described by Fugelseth,[37] along with ischemic dysfunction of the papillary muscles. Elevation of pulmonary arterial pressures may also affect the flow of blood through the heart. Suprasystemic pulmonary pressures during hypoxia and subsequent treatment reverses blood flow across physiologic shunts such as the foramen ovale and ductus arteriosus.

Human observational studies have replicated the findings of animal experiments. Following hypoxic injury, 60 to 80% of neonates develop cardiovascular complications with the requirement of inotropic support, elevation of cardiac enzymes or the presence of an electrocardiographic abnormality.[38,39] Pulmonary complications include development of persistent pulmonary hypertension, often requiring inotropic support.[31]

Clinically, myocardial dysfunction can be difficult to recognize. Dysfunction can be evident in normotensive patients and hypotension may present with normal cardiac output.[40] Naturally, this delays diagnosis and thus, treatment also. Newborns may

display an initial tachycardia to preserve cardiac output in response to hypoxia. A number of studies have assessed the use of multiple parameters to diagnose heart strain in the neonate. Martin-Ancel and colleagues found 19% of asphyxiated neonates developed electrocardiographic abnormalities.[31] Barberi et al. found electrocardiogram (ECG) abnormalities only in severely asphyxiated neonates with Apgar at 5 minutes < 7 and pH < 7.2, parameters identical to those used in Martin-Ancel's study.[41] Echocardiograms of these neonates demonstrated reduced aortic flow and decreased contractility. Furthermore, elevations in assays of three, albeit non-specific, enzymes: creatine kinase (CK), CK – MB isozyme (CK-MB) and lactate dehydrogenase (LDH), were appreciated.

Troponin, an inhibitory protein on the actin filament of striated muscle has three subunits: T, I and C. Troponins T and I are primarily cardiac-specific and have been deemed useful for assessing myocardial damage. Moller and colleagues found troponin T to have greater sensitivity than CK or creatinine for neonatal myocardial injury.[42] They found it to be significantly increased in asphyxiated neonates suffering heart failure in particular. Further studies by Günes et al. and Szymankiewicz et al. also demonstrated a troponin T rise with asphyxia.[43,44] The former group, extending data collection to two weeks of life found no persistent difference in troponin T levels, suggesting the myocardial injury was reversible. They failed to reveal any echocardiographic differences from non-hypoxic neonates. The latter group, however, went on to find increased incidence of tricuspid insufficiency not unlike the animal findings of Fugelseth's group. Costa and colleagues also saw frequent abnormal findings on echocardiography in 29 asphyxiated neonates with Apgars < 3 at 1 minute and < 6 at 5 minutes.[45] Systolic and diastolic functions were generally preserved but asphyxia led to reductions in left ventricular output and stroke volume when compared with controls. Tricuspid insufficiency was universally seen in asphyxia cases and troponin T levels were significantly elevated. Whereas troponin T has been studied extensively in the past decade, there exists a paucity of studies investigating the effect of asphyxia on troponin I levels. Trevisanuto et al. found elevated troponin I levels in the serum of a small group of asphyxiated neonates when compared to a threefold larger control group.[46] There was no correlation between troponin I and traditional markers asphyxia such as Apgar scores, serum pH, ALT,

AST and electrocardiographic findings such as QT interval prolongation. To establish reference limits, Baum et al. analyzed the blood of over 800 healthy newborns for troponin T and I.[47] Both levels were increased in comparison to reference limits in adults.

Cerebral Effects

Untreated, asphyxia ultimately leads to neuronal injury. Approximately 70% of neonates suffering asphyxia develop central nervous system abnormalities.[31,40] The normally functioning brain derives energy from aerobic metabolism of glucose producing ATP through oxidative phosphorylation in the cytochrome chain.[48,49] Much of this energy is harnessed to power the Na^+/K^+ pump at the neuronal cell membrane. Hypoxia is said to cause an initial energy deficient insult, *primary energy failure*, as well as a delayed *secondary failure* of energy metabolism.[49] With primary energy failure, oxygen is not present in the cytochrome chain to reduce oxygen to water. Instead, the electrons build up in the cytochrome chain and ATP production is limited. This initial drop in ATP levels is buffered through the creatine kinase enzyme with phosphocreatinine as a substrate. Pyruvate instead undergoes anaerobic metabolism to lactate.

The inadequate ATP supply leads to Na^+/K^+ pump failure and increases in cytosolic sodium and water. The *N*-methyl-*D*-aspartate (NMDA) receptor is stimulated by glutamate in the synaptic cleft. This receptor signals calcium influx. Energy failure inhibits reuptake of glutamate, leading to overstimulation of the NMDA receptor and excessive calcium influx. The combined elevation in cytosolic calcium, sodium and water yields cytotoxic edema. Mueller-Burke et al. demonstrated the NMDA receptor upregulation with hypoxic-ischemic injury of the basal ganglia of piglets.[50] The observed changes in neuronal tissue were appreciated between 3 and 6 hours post-insult.

Secondary energy failure follows restoration of oxygenation.[49] Depletion of high-energy phosphates (phosphocreatine) predisposes to further cellular edema via similar energy-deplete mechanisms. Stimulation by excitatory neurotransmitters such as glutamate and aspartate prompts paroxysmal electrical activity and seizures. Intracellular enzymes, phospholipases, nucleases and proteases, are activated secondary to elevated intracellular calcium

concentrations, causing cellular injury and necrosis in severe insults, and signaling apoptosis in less severe insults.[50]

This cellular injury is determined by the lipid composition of the cell membrane, the rate of lipid peroxidation, the level of antioxidant defenses and the degree of neurotransmitter modulation on receptors such as NMDA.[51] Free radical formation, secondary to elevated intracellular calcium, is mediated through activation of phospholipase A_2. This promotes the cyclo- and lipoxygenase pathways. Xanthine oxidase, stimulated by proteases, metabolizes purines accumulated during ischemia with resultant oxygen free radical production. Nitric oxide synthase yields nitric oxide and together with reactive oxygen species, peroxynitrite is formed. The cascade-like process is further catalyzed by phospholipase-C-mediated production of inositol trisphosphate and mobilization of intracellular calcium stores, with further release of excitatory neurotransmitters.

The neonate brain is rich in polyunsaturated fatty acids, an ideal substrate for free radical-mediated damage. Term infants are particularly susceptible to lipid peroxidation in comparison to those born preterm.[52] Imbalance between the cell's anti-oxidant defenses and free radical production leads to disruption of the cell membrane and cell death. Cytokine (interleukin-1 and TNF-α)-mediated inflammatory cytotoxicity has also been implicated in neuronal cell injury.[52,53]

In animal studies, hypoxic-ischemic exposure has led to variable cortical and midbrain effects.[54] Recurrent insults ultimately impart a cumulative effect and more severe and prolonged hypoxia tends to worsen prognosis. In 1976, Sarnat and Sarnat established staging criteria for the severity of hypoxic-ischemic encephalopathy (HIE) (See Table 1.5).[55] Andres et al. demonstrated a correlation between umbilical artery pH and neurologic outcome in a retrospective analysis of neonates with pH < 7.[56] Those suffering HIE showed median pH of 6.69, though, only 2 neonates were so diagnosed, in comparison to 6.93 in those without.
Interestingly, many of the neonates with pH < 7 went on without short or long term morbidity. It seems that mild to moderate acidemia (pH ~ 7 – 7.2) may also be protective.[57] Hermansen argues that the hypercarbia associated with the respiratory component of acidosis acts as a potent cerebral vasodilator. Similar increases in cerebral flow are seen with lactic or metabolic

acidemia.[58] Also, offloading of oxygen from hemoglobin is improved secondary to the Bohr effect.

Table 1.5 – Sarnat and Sarnat HIE Staging[56]

CRITERION	MILD	MODERATE	SEVERE
Level of Conciousness	Hyperalert	Lethargic or obtunded	Stuporous
Neuromuscular:			
Muscle tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreased / absent
Segmental myoclonus	Present	Present	Absent
Complex reflexes:			
Suck	Weak	Weak / absent	Absent
Moro	Strong	Weak	Absent
Oculovestibular	Normal	Overactive	Weak / absent
Tonic neck	Slight	Strong	Absent
Autonomic function:	Sympathetic	Parasympathetic	Depressed
Pupils	Mydriasis	Miosis	Variable; poor light reflex
Heart rate	Tachycardia	Bradycardia	Variable
Secretions – Bronchial and salivary	Sparse	Profuse	Variable
GI motility	Normal / Decreased	Increased	Variable
Seizures	None	Common; focal / multifocal	Uncommon
EEG Findings	Normal	Early: low-voltage continuous delta and theta Later: periodic pattern Seizures: focal 1 to 1.5 Hz spike and wave	Early: periodic pattern with isopotential phases Later: isopotential
Duration	< 24Hrs	2 to 14 days	Hours to weeks

Multiorgan Dysfunction (MOD)

Multiorgan dysfunction is one of the four features required for the diagnosis of asphyxia.[5] Given the redistribution of flow to vital organs including the brain, heart and adrenal glands, should an insult be of enough severity to injure these organs, one should expect dysfunction of less essential organs also. In a study assessing long-term neurologic outcomes in asphyxiated neonates, Perlman et al. found that greater than 65% of patients suffered injury to more than one organ.[50] A later study often quoted in the literature examined the frequency and severity of MOD following asphyxia.[31] Here, Martin-Ancel and colleagues studied 72 neonates born with blood pH < 7.2 and Apgars < 4 and < 7 at 1 and 5 minutes, respectively. Only 18% of neonates had no evidence of organ dysfunction. The authors concluded that insults of enough severity to cause dysfunction of one organ system usually also affected other organs simultaneously. 26% of neonates developed single system failure in comparison to 56% who developed MOD. This latter group universally demonstrated signs of HIE and 19% of neonates also developed clinical seizure activity. Renal injuries were observed in 42% of neonates and gastrointestinal in 29%, though, no cases of necrotizing enterocolitis were reported.

Shah et al. found no association between MOD and long-term neurologic outcomes when infants with 'post-asphyxial HIE' were assessed in regards to several criteria.[39] Elevations in serum levels of the hepatic aminotransferases were indicative of liver injury; anuria, oliguria or elevations in serum creatinine served diagnostic for renal insult; and cardiac and pulmonary dysfunction were classified as hypotension requiring inotropic support and need for > 4hrs of positive pressure ventilation, respectively. 130 neonates were retrospectively reviewed. All showed signs of MOD. 38% were free of and 62% developed severe long-term adverse outcomes. Hankins et al performed a similar analysis of 46 patients.[40] Prospective data collection revealed hepatic injury in 80% and renal injury in 72% of neonates diagnosed with HIE secondary to asphyxia. An additional observation of thrombocytopenia was found in 54% of neonates. The authors elaborated on Martin-Ancel's conclusion by stating, "acute intrapartum asphyxia sufficient to result in neonatal encephalopathy will most often result in multiple organ system injury."

Renal Effects

The historical estimate of acute kidney injury (AKI) incidence in the neonatal intensive care unit (NICU) is 8-24%.[59] Agras et al. found the incidence to be 3.4%, with asphyxia as the predominant etiology in 40% of cases. 22% of these ultimately died and 18% required hemodialysis. Karlowicz observed the incidence of AKI in 33 severely asphyxiated neonates to be 61% and more often than not, non-oliguric (>1ml/kg/hr).[60] Their moderately asphyxiated group did not develop AKI. Gupta et al. seconded these findings in a prospective study.[61] Here, 47% of asphyxiated neonates developed AKI, predominantly non-oliguric, and AKI was correlated with HIE stage.

Kidney injury has been correlated with neurologic outcomes.[62] Nouri et al. studied 87 term neonates with HIE and 15 developed AKI. The majority of these cases were transient in nature. Long-term neurologic outcomes were worse, however, in those with AKI, with one third showing persistent neurologic abnormalities between 6 and 18 months compared with one tenth of those without.

Gastrointestinal Effects

In 1969, J.R. Lloyd observed an increased incidence of intestinal perforation in asphyxiated neonates.[63] There is however limited evidence to substantiate hypoxia-ischemia as a primary etiology of necrotizing enterocolitis (NEC).[64] Nitric oxide (NO), endothelin-1 (ET-1) and local myogenic responses regulate the basal vascular resistance of the neonatal gut.[65] As the neonate grows, the adrenergic autonomic nervous system plays a more important role. With birth, the intestinal vascular resistance decreases allowing improved blood flow and oxygen delivery. Faced with hypotension however, the newborn fails to reduce vascular resistance further so as to maintain perfusion. In the context of hypoxia, vascular resistance instead increases, predisposing to intestinal ischemia. This is due, in part, to loss of NO as a free radical vasodilator, given that oxygen is required as a substrate for its formation.

NEC is thought to develop in response to an initial mucosal injury. Enteral feeds promote bacterial growth and invasion with a subsequent inflammatory response. The cytokine and reactive

oxygen and nitrogen species released ultimately lead to vasoconstriction and tissue ischemia.[66] An imbalance, due to endothelial cell dysfunction, of ET-1 vasoconstriction to NO vasodilatation has been implicated in the pathogenesis of NEC.[67] Ito et al. displayed an enhanced response of the intestinal microcirculation to ET-1 in rats when initially fed then subjected to regular asphyxia and hypothermic stresses.[68]

In a pig pneumothorax model, Gellen and coworkers demonstrated reduced mesenteric arterial flow.[69] The induced changes in intestinal morphology with increased vascular permeability led to NEC-like lesions. Part of this may be due to oxygen free radical mediated injury. Indeed, Haase et al. demonstrated decreased levels of intestinal glutathione (GSH) following reoxgenation of hypoxic piglets.[70]

The majority of NEC is seen in the preterm newborn. Lambert et al. analyzed possible etiologies of NEC in term neonates and found 27% of those affected had suffered hypotension.[71] Reduced perfusion and formula feeds were felt most contributory in NEC development.

The liver may be injured following neonatal asphyxia. Hepatic insult in the form of elevated liver transaminases was associated with increased mortality in a recent Turkish study.[72] Transient neonatal cholestasis has also been described in the asphyxiated population.[73,74] This spontaneously resolving condition is diagnosed following exclusion of diseases such as biliary atresia and α-1-antitrypsin deficiency. Herzog et al. retrospectively identified the disorder in 11% of asphyxiated neonates at their centre between 1989 and 1993.[75] Inefficient enterohepatic circulation and reduced enteral nutrition combined with fetal stress and impaired intestinal perfusion were believed to be the cause, although the exact etiology has yet to be determined.

Conclusion

In spite of many advances in its prevention and in the resuscitation of the newborn, asphyxia continues to affect many neonates. Intensive care provides supportive care to these neonates, yet the consequences can range from transient hypotension and hypoxia to multisystem organ dysfunction and death. Often, those neonates surviving do so suffering variable

disabilities. In the following two chapters, current treatments of asphyxia will be discussed.

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Chapter 2: Current Evidence in the Management of Asphyxiated Newborns

Neonates suffering hypoxic injury are susceptible to multi-system organ dysfunction. Resuscitative efforts range from simple administration of oxygen to intensive care with cardiovascular support. The use of medications in the treatment of asphyxiated neonates has been studied extensively in both animal and human trials. Many of the interventions attempted in the past, however, carry with them risks that are detrimental to the overall health of the neonate. In this chapter, current practices in the care of the asphyxiated neonate will be reviewed including treatment with oxygen, antioxidants, cooling and neuroprotective agents.

Neonatal Resuscitation Program

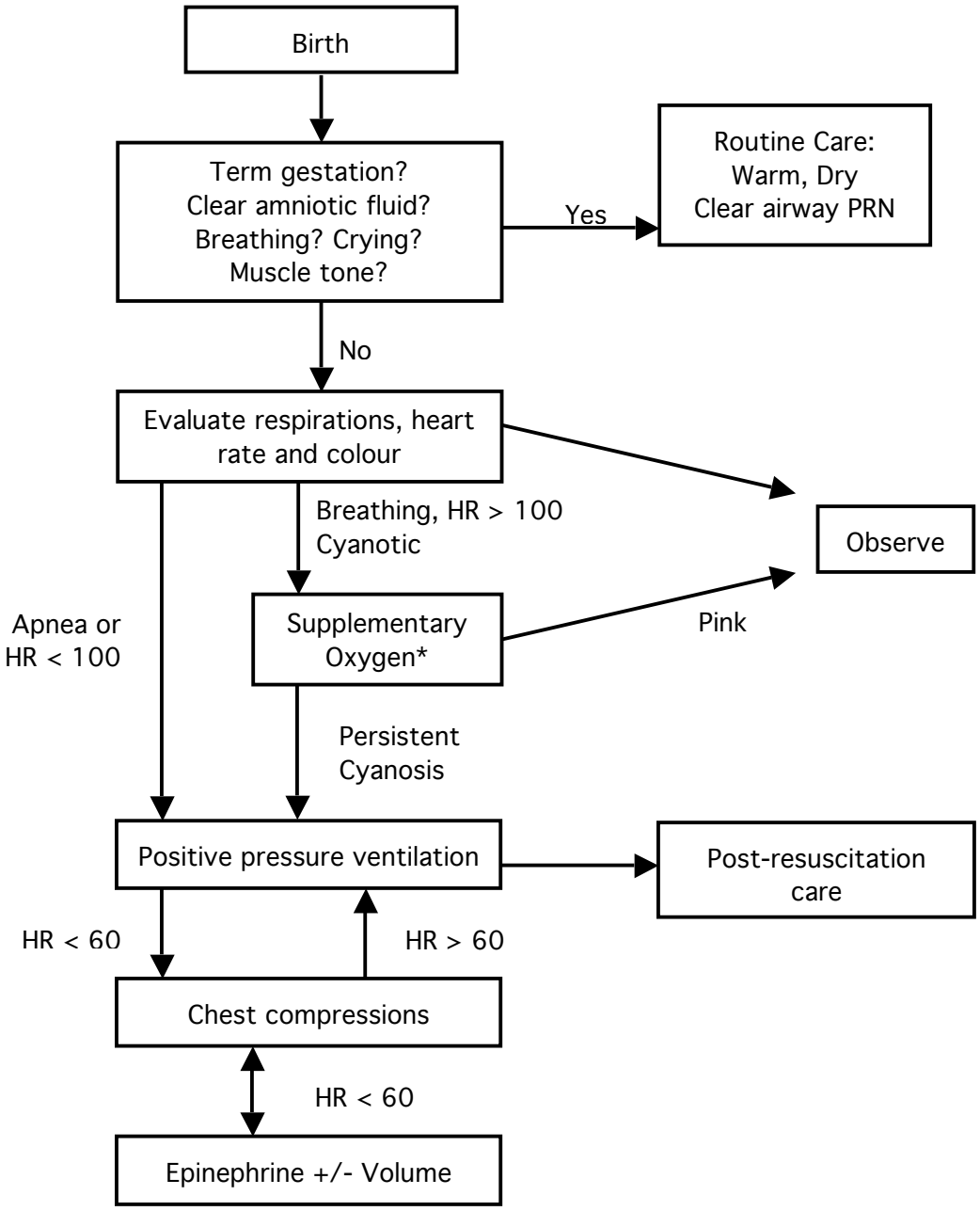
Given the nature of asphyxia and its poor prognosis for the newborn, attempts have been made to standardize management. Namely, the Neonatal Resuscitation Program (NRP) serves to guide the health care practioner in the resuscitation of a neonate.[1](See Figure 2.1) The NRP has been shown to reduce the incidence of asphyxia and improve hospital length of stay and Apgar scores.[2,3]

Neonates expected to require some form of resuscitation include those born prior to full-term, those with evidence of meconium staining or infection, those with poor muscular tone and finally those neonates failing to breathe or cry spontaneously.[1] Initial resuscitation involves a sequence of interventions: stimulation, ventilation, chest compressions and volume administration and/or vasoactive medications. Throughout resuscitative efforts, the neonate is reassessed at 30-second intervals.

Temperature control is invaluable in the management of any neonate. Neonates have a body-surface-area-to-weight ratio far larger than adult counterparts. As such, they face a larger surface from which to suffer insensible heat loss. Hypothermia is associated with several problems. The cardiovascular system is affected with increases in blood pressure and QT interval; decreases in heart rate; and ultimately, dysrhythmias if severe.[4] Hematologic and metabolic disturbances have also been described. As such, preventative measures have been described to minimize this risk to the neonate. These include radiant heat, adequate wrapping and skin-to-skin contact with the mother.[1] On the other hand, hyperthermia may

also be detrimental, with increased incidence of cerebral injury and seizures.

Figure 2.1 – American Neonatal Resuscitation Program Algorithm[1]



* *Canadian guidelines recommend resuscitation with room air and not supplemental oxygen.*

Meconium stained neonates have been subject to multiple perinatal interventions with the aim of optimizing respiratory gas exchange. These include intrapartum suctioning and endotracheal intubation with subsequent withdrawal under suction. Newer evidence from well-designed studies, in turn, has refuted these management strategies.[5] Given a vigorous neonate, that is, one with reassuring clinical findings, endotracheal intubation and suctioning provides little benefit.[6,7]

The use of oxygen administration has been debated largely in reference to tissue damage, reactive oxygen species and circulation. The topic of oxygen in the asphyxiated neonate will be discussed in detail later in this review. The American NRP suggests supplemental oxygen be administered concomitantly with positive-pressure ventilation or when central cyanosis is evident. Positive-pressure ventilation should be provided at a rate of 40 to 60 breaths per minute in neonates who are apneic or gasping, bradycardic (heart rate < 100 beats per minute) or persistently cyanotic despite non-invasive measures.

Endotracheal intubation is indicated under the following circumstances: ineffective bag-mask ventilation, chest compressions, need for tracheal suctioning and for resuscitative medication administration. Chest compressions are indicated for severe bradycardia (heart rate < 60 beats per minute).

Medication administration described in the NRP guidelines consists of epinephrine, buffering agents, narcotic antagonists and volume. In addition, euglycemia should be maintained. Finally, a new treatment modality, selective or systemic hypothermia, has not yet been recommended in the routine care of the asphyxiated neonate. Further clinical trials are required to establish its role.

Oxygen

Resuscitation of the asphyxiated neonate often begins with administration of oxygen and effective ventilation, as dictated in resuscitation guidelines. To this date, the optimal fraction of inspired oxygen has yet to be determined. In 1999, the International Liason Committee on Resuscitation's (ILCOR) Pediatric Working Group recommended the use of 100% oxygen for neonatal resuscitation.[8] Newer 2005 ILCOR recommendations make note of the potential toxicity of hyperoxia.[9] The goal of providing assistance

in oxygenation and ventilation is both to prevent death of the neonate and minimize long-term morbidity, in particular, neurodevelopmental morbidity. Lately, an increasing body of evidence is attempting to show the benefits of resuscitation with lower oxygen content. The main reasoning behind this focus is the minimization of oxygen free radicals and their damaging effects.

Herpin et al. demonstrated a 75% relative risk reduction in early mortality in low-birth-weight newborn piglets treated with 40% oxygen for a short time period.[10] Upon administration of oxygen, arterial partial pressure of oxygen rose. This, in turn, is assumed to contribute to oxidative metabolism and production of ATP. Accordingly, lactate levels were improved in oxygenated piglets when compared to controls. Oxygenated piglets were also subjectively assessed as more vigorous and active than controls and less susceptible to hypothermia. The latter was attributed to improved maintenance of shivering thermogenesis in the state of reduced lactate and increased ATP levels.

Despite the inclination and data to show that oxygen administration can prove helpful in resuscitating the asphyxiated neonate, multiple studies have published data suggesting the relative benefit of room-air resuscitation. 100% oxygen resuscitation is said to delay recovery, increase mortality and oxidative stress, and affect the heart and brain, according to a review by Saugstad in 2005.[11] Supplying the neonate with an abundance of oxygen leads to the overproduction of reactive oxygen species. Kondo et al. demonstrated elevated concentrations of reactive oxygen species in the lungs of apneic piglets following resuscitation with 100% oxygen.[12] The room-air group revealed no change in pre-apneic to post-resuscitation radical levels. In another study, cerebral production of reactive oxygen species was measured in the sagittal sinus.[13] Here, Kutzsche et al. demonstrated elevated hydrogen peroxide levels in the piglets resuscitated with pure oxygen rather than room air. Vento et al. have shown increased oxidative stress in human neonates resuscitated with 100% oxygen in place of room air.[14,15,16,17] A significant increase in oxidized glutathione was appreciated in 100% oxygen groups, as was increased superoxide dismutase activity. Also, 100% oxygen treatment was associated with persistently elevated superoxide dismutase and catalase levels four weeks after resuscitation. This,

in turn, speaks to the chronicity of damage possible from hyperoxygenation.

Further studies assessed the microcirculation of the brain under hypoxic-ischemic conditions and following reperfusion.[18,19,20,21] Resuscitation with room-air led to a slower resolution of depressed cerebral flow and systemic blood pressure. Metabolically, however, conflicting evidence was appreciated. Similar hypoxanthine and glutamate levels were found in experimental groups in all but one study. This study suggested a less favourable outcome for room-air resuscitated neonates owing to higher amino-acid levels in the striatum, greater microcirculatory hypoperfusion and lower mean arterial pressures.[19]

To further clarify the role for oxygen therapy and better limit its deleterious effects, its duration of administration was examined. Solås and colleagues provided 100% oxygen during resuscitation for durations of 5 and 20 minutes and compared outcomes with room-air resuscitated piglets.[22] Pure oxygen administration, equally efficient whether short or long duration, showed improved hemodynamic status in terms of mean arterial pressure and cerebral microcirculation, yet, no difference in biochemical status of cerebral tissues was appreciated. As such, one could infer that the provision of pure oxygen could at least be limited temporally.

Cerebral circulation aside, oxygen serves other vasoactive roles. In regards to pulmonary hemodynamics, Medbo et al. failed to reveal any differences in the pulmonary vascular resistance (PVR) and pulmonary artery pressures (PAP) between hypoxic piglets treated with 21% or 100% oxygen.[23] Both groups documented a rise in both PVR and PAP upon commencing resuscitation and these values gradually tapered down thereafter. A later study by Fugelseth et al. reproduced these pulmonary vascular findings.[24] Cheung et al. further examined systemic and regional vascular indices in response to varying degrees of reoxygenation.[25] No difference in the resolution of initially elevated PAP or pulmonary-to-systemic mean arterial pressure (MAP) ratio (PAP/MAP) was found between 21% and 100% oxygen groups. Regional hemodynamic recovery was also equal among groups. Cardiac index, stroke volume and systemic oxygen delivery, however, were all lower and cardiac troponin higher in the 100% oxygen group. Vento et al. also found more of an elevation in cardiac troponin levels with pure oxygen resuscitation in their human-based clinical study.[14]

Multiple studies, on the other hand, have shown that the elevation of troponin values, markers for cardiac myocyte injury, was no different in piglets resuscitated with 21% or 100% oxygen.[23, 24, 26] Still, Cheung et al. showed evidence of increased myocardial necrosis and oxidative stress, as measured by oxidized myocardial glutathione levels, in piglets treated with 100% oxygen in lieu of room air in a short-term survival model of hypoxia-reoxygenation.[27]

Hepatic hemodynamic recovery is similar given 21% or 100% oxygen resuscitation, yet, again, 100% may lend to more oxidative tissue injury.[28] Cheung et al. have described similar effects regarding the kidney.[27] Vento et al. described increased injury to proximal renal tubular cells upon hyperoxygenation following hypoxia by measuring urinary levels of N-acetyl-glucosaminidase, a lysosomal enzyme.[14] Finally, the intestine is also believed to be affected in varying degrees dependent on the inspired oxygen fraction. Haase et al. demonstrated elevated levels of oxidized glutathione in the intestine of newborn pigs subject to the pure oxygen arm of a hypoxia-reoxygenation protocol.[29] There was also gross evidence of intestinal injury in 2 piglets.

In 1993, a pilot study by Ramji et al. introduced the notion of room-air resuscitation to human clinical trials.[30] For more than a decade thereafter, numerous human studies have assessed the same clinical questions of whether treatment is optimized with room air or pure oxygen. The same group in a multicenter trial published in 2003 revealed no significant differences in outcomes of 431 neonates resuscitated with either 21% or 100% oxygen.[31] In 1998, the Resair 2 Study was published.[32] Data was collected prospectively from the resuscitation of 609 neonates, many of whom were in developing countries. Again, results were unable to substantiate the hypothesis that either room air or oxygen is better for resuscitation of neonates. Room air-treated neonates did, however, show trends toward quicker recovery, with improved 1-minute Apgar scores and times to first breath and cry. A Cochrane Library systematic review by Tan et al. cautions the interpretation of these three studies.[33] The patient populations came from developing countries. As such, the overall antenatal/perinatal care and equipment limit its applicability to worldwide resuscitation. Also, a significant proportion (26%) of neonates allocated to the room air group did in fact receive 100% oxygen as a back-up during the resuscitation. Several methodological issues: inadequate

blinding, randomization, and double counting in two trials, prevented the authors from concluding that room air was superior to pure oxygen in newborn resuscitation.

In a 2004 review by Saugstad, five clinical trials assessing room air versus pure oxygen resuscitation of over 1500 asphyxiated neonates were summarized.[34] Findings were similar in a 2005 systematic review by Saugstad and colleagues.[35] The neonatal mortality rates for the 21% and 100% groups were 8% and 13% respectively. Saugstad likens this benefit to a "rescue" of 200,000 of the 4 million neonates affected by asphyxia yearly worldwide. He further summarized both animal and human study results as shown in Table 2.1.

Table 2.1 – Summary of differences between room air and pure oxygen resuscitation[35]

PARAMETER O_2	21%	100%
Cerebral blood flow	↑↑	↑↑
Cerebral microcirculation	↑	↑↑
Brain cell injury	↑	↑↑
Pulmonary circulation	↑↑	↑↑
Myocardial damage	↑	↑
Oxidative stress	-	↑↑
Neonatal survival	↑	-

Antioxidants

Given the potential for free radical-mediated tissue injury and the imbalance, following hypoxia-reoxygenation, of pro-oxidant to antioxidant molecules, the use of antioxidant medications in resuscitation of asphyxiated neonates has become a topic of interest. The aim of such treatment is to neutralize oxidant molecules and boost endogenous antioxidant activity.[36] Many treatments have been described and investigated. These include endogenous antioxidant-mimicking substances as well as exogenous chemicals.

Studies of superoxide dismutase, the enzyme tasked with degeneration of the superoxide anion radical, have shown conflicting results in the prevention of tissue injury secondary to reactive oxygen species.[37] Reasons for this may be the conversion

of superoxide to hydrogen peroxide, which, under any circumstance of decreased metabolism of the latter, leads to accumulation of the hydroxyl radical and further oxidative effects. Also, recombinant superoxide dismutase is not known to cross cell membranes.[20] This would limit its anti-oxidant effects should there be a predominant intracellular oxidative injury. In turn, smaller low-molecular weight enzymes synthesized may preclude these limitations. Indeed, superoxide dismutase mimetics have shown some positive effects and, according to the review by Cuzzocrea et al., may prove helpful in the prevention of ischemia-reperfusion injury of the myocardium following infarction.[37] Similar issues exist in the use of a synthetic catalase enzyme.

Peroxynitrite anion decomposition is another target for anti-oxidant treatments. Stern et al. initially studied catalysts of this process.[38] Here, iron porphyrin complexes markedly reduced peroxynitrite lifespan with a conversion of nitrite to the benign anion nitrate. The study of these catalysts presents a new promising strategy and new agents that catalyse the isomerization of peroxynitrite to nitrate are being sought.[39] Szabo et al. studied a new agent in this class, FP-15, in regards myocardial ischemia.[40] Treatment of pigs whose left anterior descending coronary artery was ligated showed a reduction in infarct size and reactive hyperemia.

N-acetylcysteine (NAC), a thiol molecule known primarily for treatment of acetaminophen induced hepatic injury, has been studied extensively in the area of free-radical scavenging. The cysteine sulfhydryl side chain is a component of glutathione, the main intracellular antioxidant. The *N*-acetylated form, or NAC, is much more stable than cysteine alone and allows transport into cells for subsequent hydrolysis and release of cysteine.[41, 42] NAC is known to serve both an indirect and direct antioxidant role.[43] The former involves an increase in reduced glutathione, by conversion of NAC to cysteine. The latter comprises an intrinsic reducing capacity given its reduced sulfhydryl group, able to donate an electron for the reduction of oxidized molecules.

In regards to ischemia-reperfusion, multiple studies have published data on the effects of NAC administration, with inconclusive results potentially related to administration differences among studies and duration of assessment.[41,42] Vassilev et al. studied the effects of NAC in pigs suffering septic shock, and found

no effect on systemic nor regional hemodynamics.[44] However, there was evidence of increased glutathione concentration in hepatic venous samples. Alternatively, Johnson et al. did show improvement in systemic and regional hemodynamics, namely, cardiac index, stroke volume, mean arterial pressure, oxygen delivery and renal artery flows, with NAC treatment, this, in hypoxic neonate piglets resuscitated with 100% oxygen.[45] Again, reduced glutathione content, in myocardium and kidney, were increased in the treatment group. Given the conflicting results further research is needed to establish the role of NAC in reperfusion and its effect on the myocardium.[46,47]

Many studies have described strategies for prevention of reperfusion-related injury employing NAC as a common experimental variable. Sener et al. and Chen et al. found reduced oxidative stress in the ischemia-reperfusion of the liver following NAC administration.[48,49] Glantzounis et al. further demonstrated improved liver perfusion, function and oxygenation with NAC in a rabbit liver model.[50] Sehirli, in an ischemia-reperfusion model of the rat kidney, showed decreased glutathione and increased malondialdehyde and myeloperoxidase levels, all of which were attenuated with NAC therapy.[51] Nitescu et al. and Di Giorno et al. revealed the benefits of NAC in improving renal function and decreasing renal inflammation after ischemia-reperfusion.[52,53] Reduced glutathione levels in the NAC-treated animals were similar to shams and significantly higher than untreated rats. Cuzzocrea et al. demonstrated similar benefits of NAC in the reperfusion of the small intestine in regards to oxidative stress.[54] Byrka-Owczarek et al. additionally showed improvement in mesenteric blood flow with NAC administration but, similar to Hazinedaroglu et al., failed to reveal a reduction in mucosal histologic injury.[55,56]

Other antioxidant regimens have been examined. Benders et al. failed to show a benefit with allopurinol treatment in asphyxiated neonates.[57] Alternatively, Marro et al. focused on purine metabolism and hypoxia and hypothesized that increases in intracerebral adenosine provide neuroprotection. Adenosine, a neuromodulator, plays a protective role in ischemia through microcirculatory vasodilatation and inhibition of excitatory neurotransmitters. Marro found that allopurinol functions as an inhibitor to the xanthine oxidase pathway and, ultimately, increases tissue levels of the purines adenosine and inosine.[58] Gunes et al have also studied the

effect of allopurinol, on nitrogen reactive species in particular, and found decreases in serum levels of these and improved neurologic outcomes.[59]

In summary, antioxidants have shown inconclusive results in hypoxia-reoxygenation and ischemia-reperfusion models. Nevertheless, given their relative inexpense and limited side effects, there may be a role for their use. This needs to be further elucidated with clinical trials. Much of the data seems to indicate that the methods of administration, in regards to timing and dosing, have an effect on the drugs' benefit. Future research will likely determine the ultimate role of these medications.

Cooling

Peripartum asphyxia may lead to neuronal damage and hypoxic-ischemic encephalopathy (HIE) with devastating results. A paucity of treatments are available to limit the damage of HIE. The study of hypothermia in neuronal protection aims to improve outcomes in HIE. Hypothermia reduces the proportion of apoptotic cells and limits metabolism by decreasing excitatory amino acid and free radical production.[60,61] Many experiments have shown the reduction in energy utilization and histologic protection from a 2 to 3 degree temperature decrease following HIE insult.[62,63] Induced hypothermia is generally well tolerated. Adverse cardiovascular effects include bradycardia and hypotension. Many of these effects are short-lived and reversible upon rewarming.[64] Nevertheless, the study of selective head cooling aims to limit the systemic effect of hypothermia by targeting the brain alone. However, Van Leeuwen et al. determined that only by reduction of the systemic body temperature to 34 degrees Celsius was it possible to cool deep brain structures.[65] Ideally, hypothermia should be implemented within 6 hours of hypoxic insult.[66]

A Cochrane Collaboration review in 2005 by Jacobs et al. as well as a similar review by Edwards et al. found that cooling decreased mortality in neonates with moderate to severe encephalopathy without worsening neurodevelopmental disability to 18 months of infancy.[67,68] Limitations included the inability to blind physicians and nurses to the treatment group. Also, disability was classified differently among the studies included and duration of assessment also differed. Despite this, the trials reviewed were well

designed and performed and were based on 638 infants. The authors' comment that further ongoing studies will add another 800 or so infants to the analysis and perhaps strengthen the conclusions. One such study protocol has been published, the TOBY Study.[69] Here, the cohort of patients will have longer follow-up to age 6 years. This and two other large studies, the ICE and nnn-Hypothermia trials, are expected to publish data in 2009-2011. Given the limited nature of the evidence and the requirement for further study in this area, current practice does not include the use of cooling, whether systemic or selective, in neonates suffering perinatal asphyxia.[70] However, given the consistency of results trending towards benefit, Hoehn et al. advocate the introduction of therapeutic hypothermia for neonatal encephalopathy.[71]

Magnesium and anticonvulsants

Magnesium, an abundant cation, is often used in gestating mothers with hypertension suffering adverse effects or those undergoing preterm labour. Its neuroprotective effects have been studied extensively, with conflicting data.[72] The mechanism of magnesium action in neuroprotection is felt to be as an NMDA-receptor antagonist, blocking calcium influx and thus stabilizing the cell membrane, reducing oxidation and decreasing excitatory amino acid production. Khashaba studied the effects of asphyxia on cerebral excitatory amino acids and the effect of magnesium therapy on these.[73] Magnesium failed to attenuate the release of these damaging chemicals. Spandou et al. argued that the small dose given and inappropriate timing might have been responsible. In their study, Spandou et al. concluded that magnesium administered early and in longer doses benefited newborn rats suffering from mild to moderate asphyxia and called for further studies to analyze the effect in regards to severity of hypoxic insult and medication timing and dosing.[74] In fact, a small Japanese randomized controlled trial assessed the effect of magnesium sulphate infusion commenced within the first day of life to asphyxiated neonates and found short-term improvement in electroencephalographic and neuroradiologic findings as well as earlier tolerance to oral feeding.[75] However, the treated neonates in this trial also received low-dose infusion of dopamine, confounding the results.

One effect of cerebral hypoxia and encephalopathy is seizures, which increase cerebral metabolic demands and can further exacerbate injury. Anticonvulsants have been studied in this field to assess their role in improving neurologic outcomes following asphyxia in the neonate. A 2007 Cochrane review assessed the prophylactic use of anticonvulsants on morbidity and mortality.[76] Here, trials included were insignificantly powered and definitions of HIE were different among trials, owing to its clinical diagnosis. Yet, the authors concluded that there was limited evidence to suggest the use of anticonvulsants in asphyxiated newborns other than those suffering from prolonged or frequent seizures. Further research is required to define the role of these agents, in particular, in regards to side effects and whether the reduction in seizure activity will improve neurodevelopmental outcomes.

Conclusion

This chapter reviewed a multitude of treatments targeted at improving the outcomes for asphyxiated newborns. To date, much of the evidence for such treatments is limited, and some, still purely experimental. An additional treatment strategy for neonates suffering from cardiac depression and systemic and regional perfusion deficits involves the use of vasoactive medications. The effects of these agents have been studied in both animal models and human trials and will be presented in the following chapter.

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Chapter 3: Vasoactive Medications and Asphyxia in the Newborn

The need for medications in neonatal resuscitation is rare.[1] Generally, these are reserved for severely ill neonates such as those suffering prolonged hypoxia or bradycardia. Medications used initially include epinephrine and fluids followed, potentially, by narcotic antagonists and further vasoactive medicines. The International Liaison Committee on Resuscitation in 2005 summarized only the use of epinephrine in initial neonatal resuscitation.[2] The committee recommended the use of low-dose (0.01-0.03 mg/kg; 0.01 mg/kg only in Canada) epinephrine and warned against the use of high doses (0.1 mg/kg). Following resuscitation, variations exist in neonatal intensive care units, both in the prevalence of hypotension and the administration of vasopressors.[3] In this chapter, evidence in the use of various agents, namely, dopamine, epinephrine, dobutamine, milrinone and levosimendan will be presented.

In the clinical setting, direct measurement of regional blood flow is not feasible. Instead, blood pressure serves as a substitute for assessing systemic circulatory function. Blood pressure is a product of cardiac output and systemic vascular resistance. The former depends on heart rate, stroke volume and myocardial contractility, the latter on vascular tone and hematocrit or blood viscosity.

Institution of supportive therapy for hypotension requires assessment of end organ perfusion in addition to blood pressure. The clinician may therefore assess parameters such as urine output and tissue oxygenation. In the initial phase after hypoxia, the neonate uses neurohormonal compensatory mechanisms to maintain blood pressure. As such, compensated shock may go unnoticed. Thereafter, untreated, the neonate will decompensate with a noticeable reduction in blood pressure. Without intervention, irreversible cellular damage will result.[4] Another difficulty in using blood pressure as a surrogate for measurement of organ perfusion is the lack of consensus for a normal physiologic range.[5] As such, different measures have been examined in systemic blood flow assessment.

Kluckow and Evans suggested the use of superior vena cava flow as a measure of the neonate's systemic blood flow and response to therapy.[6] Many studies in the past decade have used this measure to assess the neonates' response to inotropes, particularly in the preterm population. Ultimately, the clinical

assessment of the neonate requires the interpretation of both blood pressure and other parameters. The optimal values to assess, however, continue to be debated.[7]

Vasoactive medications have a long history of use in the critical care setting. However, there is limited evidence in their use in hypotensive neonates, even less so in term neonates.[8,9] In particular, there is a paucity of data in regards to the effects of inotropes and the use of combinations of these agents. As with all interventions in medicine, there are possible adverse, undesired effects in the use of vasoactive medications. The improvement in myocardial function and systemic blood pressure may come at the expense of regional perfusion due to vasoconstriction. Also, oxygen demand increases, placing added strain on the myocardium. Vasodilation must be balanced so as to preserve diastolic blood pressure and coronary perfusion. The ultimate goal is to identify a treatment or combination of treatments that maximize function in the hypoxic, stressed neonate, while limiting the detrimental effects of therapy.[10] As stated by Seri, the prescription of a formula for the treatment of shock in the neonate is not a simple task.[11] These effects have been assessed, in part, by newborn animal studies. Some of the data, however, have proven contradictory. Reasons for this may be different protocols for both the institution of hypoxia and/or administration of inotropes. Nevertheless, the animal-based studies have laid the groundwork for human-based trials of inotrope efficacy in the treatment of asphyxiated newborns.

Pharmacology and Chemistry of Vasoactive Medicines

Adrenergic agonist medications are used to mimic the sympathetic nervous system and are thus termed, sympathomimetic. *Inotropes* are agents that increase the contractility of heart tissue. *Vasopressors* cause vasoconstriction and thus alter vascular tone. *Chronotropes* affect the rate at which the heart contracts and *lusitropes* alter the diastolic function of the heart. Dopamine, dobutamine, epinephrine, norepinephrine, milrinone and levosimendan can be classified by the preceding mechanisms of action.

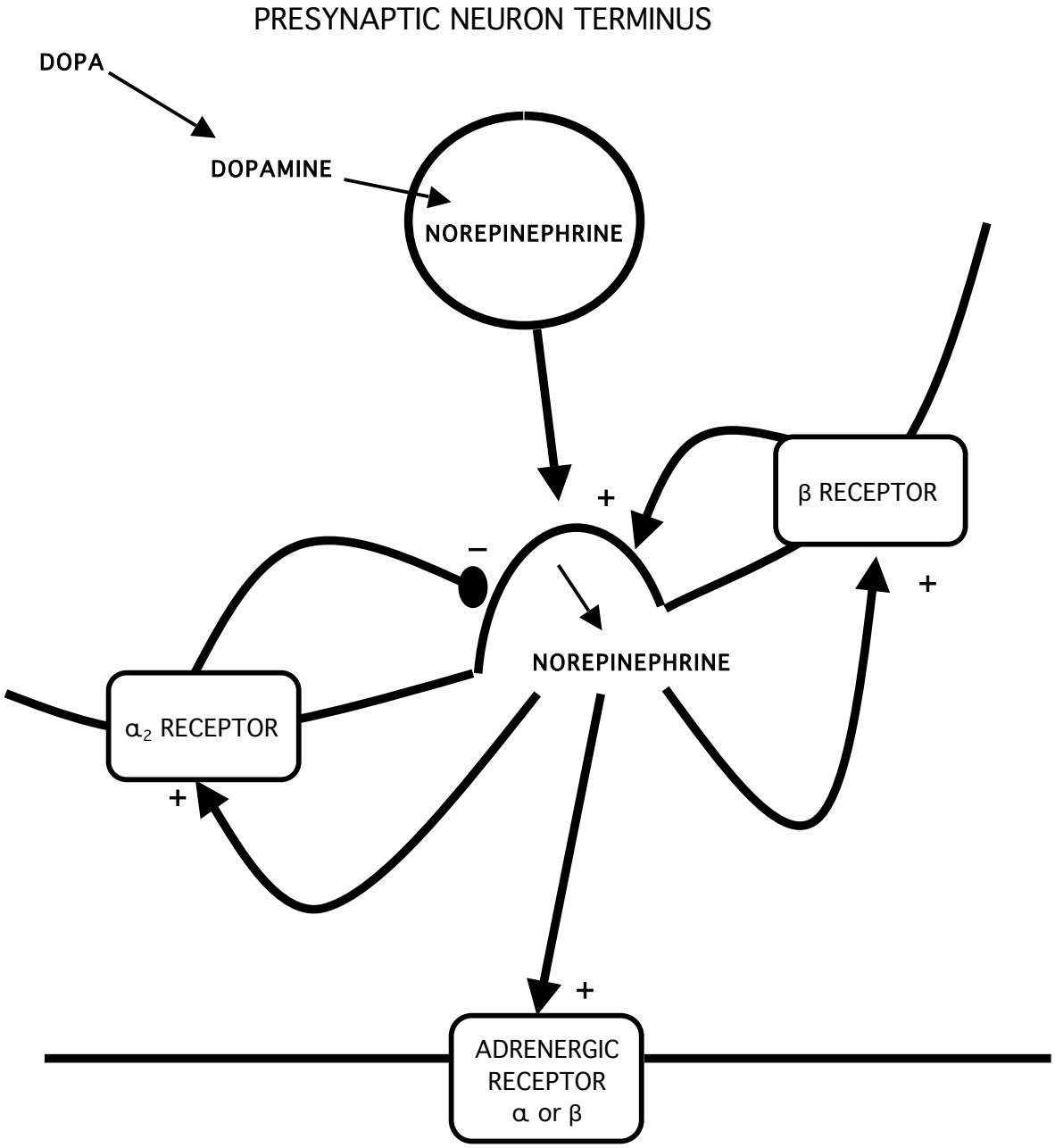
Catecholamines are regulated by the sympathetic nervous system and adrenal medulla.[12] The sympathetic nervous system primarily uses norepinephrine (noradrenaline) as its postganglionic

neurotransmitter. In the neuron ending, tyrosine is converted in stepwise fashion to dopamine and then norepinephrine. From the vesicle where it is stored, norepinephrine is exocytosed from the nerve ending through fusion of the vesicle with the cell membrane. (See Figure 3.1) This is triggered by calcium influx from the action potential.

Norepinephrine then stimulates the adrenergic receptors of the post-synaptic cell. Alternatively, epinephrine (adrenaline), is released to the bloodstream from the adrenal medulla and functions as a systemic hormone. Most available adrenergic agonists are structurally based on epinephrine and norepinephrine.

As mentioned above, catecholamines act on adrenergic receptors or adrenoreceptors. Different receptors provide different second messengers and thus different responses. Adrenoreceptors were initially described as alpha (α) and beta (β) by Ahlquist in 1948. This categorized their effects, either excitatory or inhibitory, respectively, on smooth muscle. The heterogeneous nature of these receptors has since been further described. β_1-receptors are located in the heart to stimulate increased force and rate of contraction. Contrarily, β_2-receptors, in smooth muscle of vascular beds, bronchial airways and the gastrointestinal and genitourinary tracts, control relaxation. A third receptor, β_3, is still being studied and is found primarily in adipose tissue to promote lipolysis. Some studies have reported the significance of β_3-receptor activation in failing myocardium and have shown its activation and upregulation to depress ventricular contraction and relaxation.[13,14,15] On the other hand, alpha receptors are divided into two sub-types, α_1 and α_2. Stimulation of the former contracts vascular smooth muscle as well as somewhat improves contractility of the heart. The latter has a limiting effect on the release of norepinephrine from the nervous system (See Figure 3.1). A separate dopaminergic system, with DA_1 and DA_2 receptor subtypes, can also regulate hemodynamics. DA_1 receptor activation institutes vasodilatation of renal, mesenteric, cerebral and coronary vasculature.[16] DA_2 receptor activation inhibits norepinephrine release from the nerve terminals.

Figure 3.1 – Neuroeffector junction and catecholamines[12]



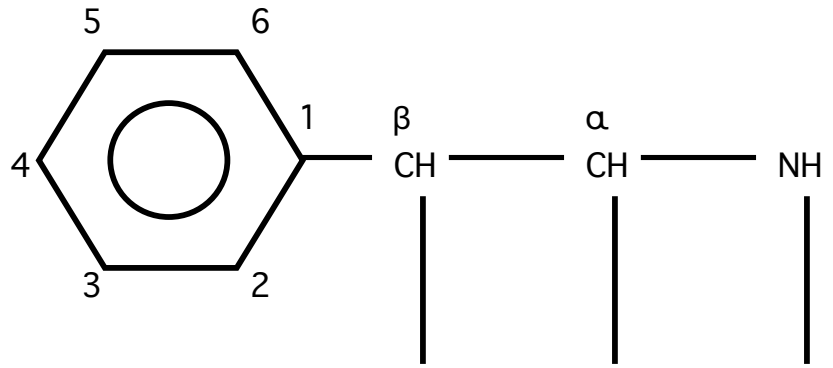
Elevated calcium levels induce fusion of the vesicle with neuronal cell membrane and exocytosis of norepinephrine for interaction with post-synaptic α and β receptors. Excess NE is cleared via a cocaine sensitive transporter. Also, NE acts on presynaptic α and β receptors to inhibit or enhance release, respectively.

β-phenylethylamine is seen as the parent compound to most sympathomimetic agents (Figure 3.2).[12,17] It consists of a benzene ring with ethylamine side chain. Substitutions made off the benzene ring and side chain give molecules with different spectrums of α and β adrenergic activity. The catecholamines used most often are commonly hydroxylated at positions 3 and 4 of the ring. Further substitutions at the amino group with larger alkyl groups increases β activity. The hemodynamic response of autonomic stimulation of α and β receptors is summarized in Table 3.1.

At the cellular level, upon activation of the cell surface receptor, α or β, coupling with G proteins then effects a response.[17] With α_1 stimulation, the G_q coupled protein activates phospholipase C, catalyzing the formation of inositol trisphosphate (IP_3). IP_3, in turn, mobilizes intracellular calcium stores and activates calcium dependent protein kinases. Vasopressors increase systemic blood pressure by causing peripheral vasoconstriction via alpha-adrenergic receptors. α_2-stimulation inhibits adenylyl cyclase activity via the G_i regulatory protein, the end effect of which is reduced intracellular cyclic adenylate monophosphate, cAMP.

Contrarily, β-receptor activation stimulates protein G_s-mediated enhancement of adenylyl cyclase activity. cAMP is synthesized from adenosine triphosphate, ATP, and functions as a second messenger to effect various intracellular responses. Elevated cAMP levels stimulate calcium release from the sarcoplasmic reticulum, enabling more strength from the actin-myosin muscle complex and improved contractility. Catecholamines – dopamine, epinephrine, dobutamine – increase cAMP levels through this beta-adrenergic mechanism.[18,19] In the failing heart, Cheng et al. and Moniotte et al. demonstrated upregulation of β_3-receptors with decreased contractility via coupling to the G_i regulatory protein and inhibition of adenylyl cyclase.[14,15]

Figure 3.2 – Chemical structures of sympathomimetics[12]



				BENZENE RING SUBSTITUTIONS:
Phenylethylamine	H	H	H	NIL
Epinephrine	OH	H	CH_3	3-OH, 4-OH
Dopamine	H	H	H	3-OH, 4-OH
Dobutamine	H	H	$CH\text{-}CH_3$	3-0H, 4-0H

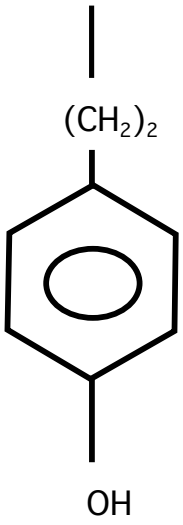


Table 3.1 – Response of cardiovascular effector organs to α and β-adrenergic stimulation[12,17]

ORGAN SYSTEM	EFFECT	RECEPTOR
Heart		
Sinus Node	Tachycardia	$\beta_1 > \beta_2$
Atria	Increased contractility	$\beta_1 > \beta_2$
Atrioventricular Node	Increased automaticity and conduction velocity	$\beta_1 > \beta_2$
Ventricle	Increased contractility	$\beta_1 > \beta_2$
Vasculature - Arterial		
Coronary	Constriction + Dilatation ++	α_1, α_2 β_2
Skeletal Muscle	Constriction Dilatation ++	α_1 β_2
Cerebral	Mild constriction	α_1
Pulmonary	Constriction + Dilatation	α_1 β_2
Abdominal Visceral	Constriction +++ Dilatation +	α_1 β_2
Renal	Constriction ++ Dilatation ++	α_1, α_2 β_1, β_2
Vasculature - Venous		
	Constriction Dilatation	α_1, α_2 β_2

A second class of inotropes, phosphodiesterase (PDE) inhibitors, prevent the depletion of cytoplasmic cAMP.[12] The end result is similar to increased adenylyl cyclase activity with positive inotropy. The effect on the peripheral vasculature is smooth muscle relaxation and, thus, vasodilatation. The result is decreased afterload and preload from dilatation of resistance and capacitance vessels, respectively. PDE inhibitors, due to the combination of these effects, are therefore classified as *inodilators*. Milrinone is a selective inhibitor of PDE III. This is a cyclic GMP inhibited PDE. Myocardial contractility is stimulated by milrinone and relaxation is accelerated. Systemic and pulmonary vascular resistance is reduced with similar reductions in right and left heart filling pressures. With a relatively lengthy half-life of 30 minutes to 1 hour and its vasodilative properties, milrinone's use in patients with low mean arterial pressure may be limited.

A newer class of medications can be classified as calcium-sensitizers. Levosimendan, one such agents, functions to improve contractility and reduce afterload via binding to troponin C and opening of adenosine triphosphate-dependent K^+-channels in vascular smooth muscle, respectively.[20,21]

Feldman, in 1993, proposed a classification system for inotropic agents based on their mechanisms of action (See Table 3.2).[22] In the following work, evidence for the use of inotropes in the newborn asphyxiated population will be presented. Agents will be summarized separately, although the articles reviewed are often the same, comparing one agent to another.

Table 3.2 – Classification of Inotropic Agents[22]

Class	Effect
I	Increase intracellular cAMP
II	Affect sarcolemmal ions, pumps, channels
III	Release sarcoplasmic reticulum calcium or increased sensitivity of contractile apparatus to calcium
IV	Multiple mechanisms of action

Dopamine

Dopamine has been studied as a cardiovascular supportive agent in neonates for more than a quarter century. It is the most frequently used vasoactive agent for the treatment of neonatal hypotension.[23, 24] It has both α- and β-adrenergic as well as dopaminergic effects which are dose-dependent. Dopamine, administered exogenously exerts its effects both directly and through conversion to norepinephrine in the sympathetic nervous system.[25] It is known to have differing effects in different patients and as such, its use is typically titrated to achieve the desired response.[26]

Dose-response and regional hemodynamic studies have been undertaken in the use of dopamine in experimental animals. Ferrara et al. assessed regional flows in normoxic term neonatal piglets infused with different dopamine doses.[27] They demonstrated increased cardiac outputs and improved small intestinal flows with high doses (15mcg/kg/min). Cheung et al. also studied the effects of dopamine in normoxic piglet systemic and regional perfusion and oxygenation.[28] Here, they demonstrated increases in systemic and pulmonary arterial pressures as well as cardiac index with infusions of high-dose dopamine (32mcg/kg/min). Detrimental effects were found neither on systemic and pulmonary vascular resistance indices nor on oxygen extraction. Dopamine had no effect on hepatic arterial or portal venous flows or on mesenteric vascular resistance. However, the effects of dopamine were considerably different when the same group repeated investigations in hypoxic piglets, as will be described shortly.

The next step in the analysis of dopamine in this population was to implement hypoxia. O'Laughlin et al. compared the effects of various sympathomimetics on cardiac output in hypoxic lambs.[29] They demonstrated improved cardiac output with dopamine yet a reduced response with higher doses, owing to reduction in heart rate and increase in vascular resistance. Barrington et al., in a comparison of dopamine and epinephrine in anesthetized piglets, conducted an analysis of the hemodynamic effects of the drugs under normoxia and hypoxia as a follow-up to the previously mentioned study by Cheung.[30] In the normally oxygenated group, again, only higher doses (16mcg/kg/min) of dopamine increased systemic arterial pressure. These doses were also found to

decrease systemic vascular resistance. Dopamine had no effect on myocardial oxygen extraction, where the increased myocardial oxygen demand was met with similar increases in coronary blood flow. Under hypoxia, however, dopamine failed to increase systemic arterial pressure or alleviate pulmonary hypertension. Systemic and pulmonary vascular resistance were reduced at high doses (32mcg/kg/min). Cardiac index was unchanged with dopamine, the authors hypothesizing that myocardial oxygen supply was limited in the setting of hypoxia, leaving no further effect for dopamine on extraction and thus myocardial function. High doses of dopamine reduced the pulmonary/systemic vascular resistance ratio given predominant α-adrenergic selective vasoconstrictive effects. Finally, dopamine failed to alter regional perfusion of the cerebral vasculature during normoxia and hypoxia. The study is not without its flaws. The protocol used only 7 piglets in the normoxic arm and 6 in the hypoxic arm. It called for only a short 20-minute hypoxic insult without reoxygenation followed by inotrope infusion in sequentially increasing doses at 15-minute intervals, randomized to which medication was administered first. This complicates interpretation in that the medications may have had cumulative effects with prior doses. Also, given the treatment during hypoxia, results are difficult to generalize to the human population, given that reoxygenation is necessary, first and foremost. Finally, the parameters assessed were limited to systemic and pulmonary hemodynamics; regional circulation, aside from carotid, was not examined. As such, further research in the past decade has more clearly elucidated dopamine's effects in the hypoxic piglet.

Cheung and Barrington again published in 2001 with a study design assessing regional as well as systemic and pulmonary circulation in hypoxic piglets treated with dopamine and epinephrine.[31] Here, sample sizes were still limited, with 6 piglets in each drug group; though, hypoxia was more prolonged at one hour. Dose administration was randomized, however, each piglet ultimately received all three doses of one of the agents, again, complicating generalizability due to potential cumulative effects of the inotropes. Pulmonary artery pressures were in fact elevated with all doses of dopamine; systemic pressure and cardiac index were, again, unchanged. Mesenteric resistance decreased and given the stable cardiac index, hepatic flows increased with high-dose dopamine. The authors comment that this may be secondary to

hypoxia induced downregulation of mesenteric α-receptors or increased stimulation of dopaminergic receptors. With splanchnic oxygen extraction unaffected, hepatic oxygen delivery was improved.

Obaid et al. later studied these effects further.[32] This design called for "pressure-driven" dosing of dopamine and epinephrine in acutely hypoxic piglets resuscitated with oxygen prior to inotrope infusion. Here, piglets were subjected to hypoxic stress and following two hours of initial resuscitation, the inotrope was started at low doses and later, titrated to achieve systemic arterial pressure near baseline, hence, pressure-driven dosing. The initial low-dose infusion had no effect and pressure-driven doses only limited effects on systemic hemodynamics. The pulmonary-to-systemic arterial pressure ratio was reduced significantly compared to controls. Dopamine was also found to improve superior mesenteric artery flow at pressure-driven doses, similar to the 2001 study by Cheung,[31] yet, renal and common carotid artery flows were unchanged. Obaid's study again uses different doses of inotrope in the same piglets, however, experimental groups received either epinephrine or dopamine, not both.

Early human trials in the use of dopamine focused on improvement of systemic blood pressure. DiSessa et al. found it improved both cardiac and renal function in a small study of "asphyxiated" neonates.[33] They studied term (>35 week gestation) babies greater than 2 kilograms with five-minute Apgar scores less than 6, a lax definition of asphyxia, and found an improvement in systemic blood pressure in comparison to placebo-treated neonates. The results must be taken lightly given the small sample size of 12 patients and the loss to follow-up of a quarter of those, limiting inferences in regards to long-term outcomes. Further limiting the study is a lack of assessment of many crucial parameters such as end-organ perfusion and damage. Unfortunately, a systematic review published by Hunt et al. in the Cochrane register in 2002 regarding the use of dopamine in asphyxiated neonates failed to identify any eligible trials other than the DiSessa study.[34] As such, Hunt concluded that the use of dopamine in suspected asphyxiated neonates to improve mortality and neurologic outcomes lacks evidence-based support.

The balance of dopaminergic-mediated vasodilation and adrenergic-mediated vasoconstriction determines how a neonate

may respond to escalating doses of dopamine.[35] It is hypothesized that as the dose increases, a predominance of α-mediated effect will increase both pulmonary and systemic vascular resistance and thus, despite an increase in blood pressure, limit cardiac output and organ flow. Indeed, the increase in pulmonary vascular resistance may exacerbate the pulmonary hypertension associated with asphyxia. No evidence assesses this issue in term neonates. Repetto et al. observed an increased pressor response to dopamine at doses far in excess of 20 mcg/kg/min with improved blood pressures and urine output, this last observation arguing against reduced renal flow.[36] Only 6 of the 223 neonates were diagnosed with asphyxia, however.

The preterm neonate has immature receptor responses to adrenergic and dopaminergic agents. As such, analysis of dopamine's effects in preterm neonates would have limited significance in the treatment of term newborns. Therefore, the focus on preterm studies in this review will be limited. There is a plethora of literature in the use of dopamine for treatment of hypotension in preterm newborns.[37,38,39,40,41] Subhedar summarizes the findings on this topic well.[42] The majority of very low-birthweight infants respond to doses of dopamine < 10 mcg/kg/min. Pellicer et al. studied preterm, low-weight, hypotensive neonates and found dopamine in low to moderate doses increased mean blood pressure and improved cerebral blood flow.[43] In a later publication, the same group reproduced these results, there, displaying dopamine's lower chronotropic effects in comparison to epinephrine.[44] The regional effects of dopamine administration, including dopaminergic vasodilation and potential α-adrenergic overstimulation, have also been studied in the preterm population. Seri et al. assessed the effects of dopamine in non-hypotensive preterm neonates and found appropriate vasodilatory dopaminergic response on renal blood flow but not on mesenteric flow.[45] Here, dopamine had no effect on cerebral flows, in contrast to Pellicer and Valverde's hypotensive samples.

In conclusion, dopamine has potential benefits in the treatment of neonates and given the ease of administration and dosing, is often used in clinical practice. However, the evidence in its use is poor. Anecdotal and limited evidence of vasoconstrictive side effects at high-doses may promote the addition of a second agent to improve or counter these effects. Further studies are

required to elucidate the drug's ideal role in neonatal cardiovascular support.

Epinephrine

Epinephrine, an endogenous catecholamine released from the adrenal medulla in times of stress, has direct stimulatory effects on both α and β adrenoreceptors. Administered exogenously, at lower doses, epinephrine is believed to predominantly effect β-receptors, causing increased cardiac contractility and reduction of afterload via β_2-mediated vasodilatation. With increased doses, α-receptors play a greater role and subsequent vasoconstriction takes effect. Epinephrine is frequently used in neonatal resuscitation and for refractory hypotension in spite of limited clinical studies to support its use.[46]

Barrington studied the circulatory effects of epinephrine in anesthetized normoxic piglets.[47] Doses in the range of 0.2 to 3.2 mcg/kg/min were assessed in this study, as they would in multiple other studies examining the therapeutic effects of epinephrine. Low-doses of epinephrine substantially increased cardiac output with increased mean arterial pressures and decreased vascular resistance, both pulmonary and systemic. However, with escalating doses, cardiac output dropped significantly in the face of elevated pulmonary and systemic vascular resistance, the latter more so than the former. Cheung et al. elaborated on the results of this study by adding the regional perfusion effects in a similar model, this time administering doses in random order to piglets.[48] Here, interestingly, cardiac index was improved at high doses yet pulmonary arterial pressure was significantly elevated. Again, both systemic and pulmonary vascular resistance worsened at high doses as did mesenteric vascular resistance, limiting portal venous flows and yielding hyperlactatemia, despite stable mesenteric oxygen extraction. Bigam et al. studied the effects of epinephrine infusion on piglets subject to hemorrhagic shock.[49] Here, only high dose epinephrine, 3.2 mcg/kg/min had restrictive effects on mesenteric and renal flows, in both normo- and hypovolemic groups. To determine the significance of these results in asphyxia management, hypoxic studies would have to assess similar parameters.

Barrington et al. again assessed epinephrine's systemic and pulmonary effects on piglets, this time, comparing it with dopamine under hypoxic and normoxic conditions.[30] Here, as mentioned in the dopamine section of this review, piglets, either non-reoxygenated hypoxic or normoxic, were subjected to varying doses of inotrope, thus limiting conclusions given the potential additive effects of inotrope at the different doses. Findings in normoxia were unchanged. Under hypoxia, mean arterial pressure was improved at high doses of 1.6 – 3.2 mcg/kg/min yet cardiac index was unchanged across the spectrum of doses. Again, pulmonary to systemic vascular resistance ratio decreased and at low doses $\leq$ 0.8 mcg/kg/min, pulmonary arterial pressure decreased. The authors concluded that the inotropic response, in particular, with respect to pulmonary hemodynamics, was preferable to that of dopamine under hypoxia. Cheung again furthered these results by adding regional assessment under hypoxia.[31] Results in regards to central hemodynamics, however, were contradictory to those in the Barrington study. Here, cardiac index improved with epinephrine, yet mean arterial pressure was unchanged and pulmonary arterial pressure instead increased with high doses. Low doses of epinephrine were associated with a beneficial reduction in systemic vascular resistance, as well as increase in hepatic arterial flow. Albeit, portal venous and total hepatic flows were unchanged, as was mesenteric vascular resistance. Cheung summarized the findings by stating epinephrine's preferential effects on systemic and pulmonary hemodynamics in comparison to dopamine, though, dopamine exhibited favourable effects on regional flow.

In the past decade, similar analyses were performed under hypoxia-reoxygenation protocols. Cheung et al. published further work comparing moderate-dosed infusions of epinephrine (1 mcg/kg/min) to a combination of low-dose epinephrine (0.2 mcg/kg/min) and dopamine (10 mcg/kg/min).[50] Epinephrine infusion improved cardiac index and reduced pulmonary vascular resistance in comparison with non-inotrope controls, yet pulmonary artery pressure was similar. Carotid, mesenteric and renal artery flow indices were not significantly different between treatment groups and controls. Interestingly, plasma lactate levels were not elevated with epinephrine treatment. In conclusion, the study revealed arguments against the hypothesis that infusion of epinephrine, at least in the short-term, results in excessive

vasoconstriction with subsequent impaired tissue perfusion and hyperlactatemia. Obaid continued this analysis in studying pressure-driven doses of dopamine and epinephrine following hypoxia-reoxygenation.[32] Pressure-driven epinephrine dosing (mean 1.1 mcg/kg/min) provided similar improvements in cardiac index, mean arterial pressure and pulmonary vascular resistance. Also revealed were improved mesenteric flows at low doses (0.2 mcg/kg/min) and carotid flows at all doses. Renal flows and plasma lactate levels were unaltered compared with controls. Obaid took the analysis further by demonstrating an increase in myocardial oxidative injury, a potentially harmful effect on the already stressed heart.

Human-based trials in the use of epinephrine in asphyxiated or hypotensive term neonates are non-existent. There is some literature in the preterm population. Heckmann found improved mean arterial pressures in very-low-birthweight preterm neonates treated with epinephrine following failure to respond to dopamine infusions.[51] A number of the 31 subjects however were also concomitantly receiving dopamine. Epinephrine administration in this population was associated with significant acidosis, potentially precluding its use. In the same two preterm trials mentioned in the dopamine section of this review, Pellicer and Valverde found improved mean arterial pressure and cerebral flows with epinephrine administration, at the expense of hyperlactatemia and hyperglycemia.[43,44]

As with dopamine, further studies are required to establish the role of epinephrine infusions for sick neonates. Human-based clinical trials would be a start. Extension of animal studies to longer-term administrations would also improve conclusions.

Dobutamine

Dobutamine is a synthetic sympathomimetic developed in 1971 to improve cardiac contractility in patients with low cardiac output congestive heart failure.[52,53] Stimulation of β_1-adrenoreceptors potentiates cardiac inotropy while β_2-agonism provides vascular vasodilatation, thus reducing systemic vascular resistance. Dobutamine provides limited α-adrenergic effects and therefore lacks any substantial vasoconstrictive effects. The result of the increased myocardial contractility is increased oxygen demand, ultimately, balanced by increased coronary flow.[54]

O'Laughlin et al. studied dobutamine in their hypoxemic lamb model for systemic hemodynamic effects of inotropes.[29] Dobutamine was found to significantly increase cardiac output by 58%. Ferrara et al. replicated this effect in term and preterm neonate piglets.[27] They also showed a dose-dependent increase in heart rate and cerebral flow. There were, however, reduced mesenteric flows at higher doses, 15 μg/kg/min, despite some evidence for the opposite at low doses of 5 μg/kg/min.

Cheung et al. studied the effects of varying doses and durations of dobutamine infusion in normoxic term neonate piglets.[55] Piglets were studied 2 days following instrumentation for systemic and regional hemodynamics. Doses from 5 to 50 μg/kg/min were randomly given to each piglet for 15 minutes followed by 2-hr infusions of 10 μg/kg/min. Dose-dependent increases in cardiac index were observed. This was due mostly to chronotropy and not inotropy, with little to no effect on stroke volume. At higher doses, the pulmonary-to-systemic mean blood pressure ratios were increased with unchanged pulmonary and reduced systemic vascular resistance. Regional flows were unaffected over the short infusions. With the 'prolonged', 2-hr, infusion, stroke volume ultimately improved and the tachycardic effects were shown to be transient. Both systemic and pulmonary vascular resistance lessened and mesenteric and renal perfusion was improved.

Al-Salam et al. then assessed dobutamine's effects in a hypoxia-reoxygenation model.[56] Piglets were randomized to receive a fixed-dose infusion of dobutamine following reoxygenation. All doses reduced pulmonary vascular resistance and a mild reduction in pulmonary hypertension was seen, potentially reducing right-to-left shunting. High dose, 20 μg/kg/min, improved cardiac index with improvements in stroke volume, leaving mean arterial pressure unaffected. In contrast to a study by Penny et al. where increased oxygen consumption and thermogenesis consumed the increases in systemic oxygen delivery,[57] Al-Salam's study did not reveal any changes in oxygen consumption or extraction.[56] In regards to regional perfusion in this hypoxia-reoxygenated group, no dose of dobutamine was associated with changes in plasma lactate and carotid and renal flows were unaltered. At high-dose, transient improvements in mesenteric flow were observed.

Smolich et al. demonstrated variable reductions in pulmonary vascular resistance of newborn lambs of different ages subjected to dobutamine infusion.[58] The effects were less significant in 1-2 day olds in comparison with 1 week old and 6-8 week old lambs. Reductions in systemic vascular resistance were similar among age groups.

Human studies of dobutamine use in the neonate, as with dopamine and epinephrine studies, have focused mostly on the drug's use in the preterm population. Devictor et al. found dose-dependent effects in full-term severly asphyxiated neonates, with improvements in cardiac output secondary to its chronotropic effects, and an increased aortic flow velocity, despite unchanged stroke volume.[59] In an unasphyxiated population of term and preterm neonates requiring inotropic support for cardiogenic shock, Martinez et al. used variable doses of dobutamine to obtain improvements in cardiac output that were unrelated to heart rate, which was not significantly changed.[60] They too, as did Smolich, hypothesized that higher doses of drug were required in the neonatal population, in part, due to limited sensitivity of the immature cardiovascular system to dobutamine. In preterm neonates with myocardial dysfunction, sepsis-related in some, Robel-Tillig and colleagues found dobutamine at a mean rate of 9.1 μg/kg/min to provide near-immediate improvement in cardiac function with improved stroke volume.[61] Following 8-10 hrs of infusion, cerebral, mesenteric and renal flows were all improved.

Rozé et al. compared dobutamine infusions to dopamine in hypotensive preterm infants.[62] Dobutamine failed to improve the mean arterial pressure to above the target value of 31 mm of Hg in 60% of patients. It did however improve cardiac output by 21% in comparison to reduced values for the dopamine group. Using low superior vena cava flow instead of mean arterial pressure as the variable of interest, Osborn et al. randomized preterm infants to dopamine or dobutamine infusions and assessed longer-term outcomes.[63] Dobutamine was associated with reductions in late cerebral periventricular hemorrhage, albeit, overall hemorrhage rates were not different.

Two Cochrane systematic reviews have been published on the use of dobutamine in neonates, both for the preterm population. Subhedar assessed hypotensive neonates treated with dopamine and dobutamine.[64] There were no differences in mortality,

periventricular hemorrhage or leukomalacia. Dobutamine was associated with increases in cardiac output but had limited effect in treating hypotension, as found in the Rozé study. The authors conclude that there are limited studies in dose-responsiveness for dobutamine and larger doses may be needed for significant effects on blood pressure. Osborn echoed his sentiments from his previously mentioned study in a 2007 review,[65] assessing dopamine and dobutamine use for preterm infants with low systemic flow. There were also no differences in rates for necrotizing enterocolitis. Long-term outcomes were similar in regards to cerebral palsy, deafness and renal impairment.

Milrinone and Levosimendan

Milrinone, a phosphodiesterase III inhibitor improves cardiac contractility and reduces afterload. Studies on its use in the neonate are limited, with the majority of literature focusing on the treatment of adult chronic congestive heart failure.[66] The vast majority of studies regarding its effects in neonates aims at low cardiac output following congenital heart surgery. Milrinone has a shorter half-life than other PDE III inhibitors, facilitating titration of the drug's effects in less stable patients. Levosimendan also has limited evidence in the newborn population. Studies on its use are mostly limited to case reports or series.[67,68,69,70]

Ross-Ascuitto et al. began studying the effects of milrinone on neonatal piglets almost two decades ago.[71] Their ischemia model isolated piglet hearts, assessing peak arterial pressures, coronary flow and heart rate. Milrinone showed chronotropic effects and increased coronary flow in both ischemic and non-ischemic groups. In the ischemic hearts, systolic pressure was improved at all doses. In a model for low-cardiac output following infant cardiopulmonary bypass, Stocker et al. compared milrinone with levosimendan, a calcium sensitizing inotrope.[72] Piglets underwent bypass with aortic cross-clamping. Following cross-clamp removal, cardiac output decreased in control subjects. Infusion of both drugs, starting 2 hrs following clamp removal, maintained the cardiac output. Milrinone, in particular, prevented the rise in systemic and pulmonary vascular resistance but failed to improve contractility, an effect seen in the levosimendan group. Yet, complicating analysis in this study was

that all piglets were concomitantly receiving low-dose infusions of dopamine.

In the hypoxia-reoxygenation asphyxiated population, research has only just begun to assess milrinone's therapeutic effects. Joynt et al. have published two studies investigating the dose-response effects of milrinone in hypoxia-reoxygenated piglets.[73,74] In an acute setting, term piglets were made hypoxic and acidotic, resuscitated with oxygen and subsequently randomized to receive varying doses of milrinone infusion for 2 hrs. Three dosing regimens, low, medium and high, were assessed, all with initial bolus followed by infusion. Milrinone was found to have beneficial effects. It improved cardiac output beyond baseline at high dose (75 μg/kg then 0.75 μg/kg/min), and maintained it at low and medium doses. This was in comparison to a drop in cardiac output in the control group. Systemic and pulmonary vascular resistance increases were alleviated with milrinone administration and systemic oxygen delivery was improved. There was, however, no difference in troponin or lactate measurements between milrinone groups and untreated controls. In regards to regional perfusion, mesenteric blood flow was assessed. Following initial reoxygenation, milrinone reverted superior mesenteric artery flow to baseline levels and improved oxygen delivery. The effect was dose-dependent, with higher plasma levels of drug correlating with improved flow, reduced resistance and reduced intestinal oxidized glutathione. Nevertheless, histologically, there was no difference in tissue injury between milrinone and control groups.

There are no human-based studies assessing the use of milrinone in asphyxiated neonates. Chang et al. did examine the effect of milrinone on neonates suffering low cardiac output in a post-surgical setting.[75] All subjects were simultaneously receiving dopamine. Milrinone reduced mean and pulmonary arterial pressures and vascular resistance indices while improving cardiac index. No inference was made in this study on the relative contributions of milrinone to the inotropic or vasodilatory effects.

Paradisis et al. focused their investigations on the use of milrinone in neonates at risk for low systemic blood flow.[76, 77] Their's was therefore, a preventative and not a treatment model. Based on a previous pharmacokinetic model to establish ideal dosing, 90 preterm infants were randomized to receive milrinone or placebo. The dosing regimen consisted of a loading dose of 0.75 μg/kg/min

for 3 hrs followed by infusion at 0.2 μg/kg/min for 18 hrs. Neonates were assessed for maintenance of superior vena cava flow above a target 45 ml/kg/min. In comparison to controls, milrinone was not shown to change outcomes. In addition, milrinone treated patients suffered chronotropic effects from the drug and closure of the ductus arteriosus was slower. Despite this, there were no significant changes in secondary outcomes of intracerebral hemorrhage, necrotizing enterocolitis or chronic lung disease. Hypotheses for the lack of effect included myocardial immaturity and patency of the ductus arteriosus. Both of these limitations underscore the necessity for further studies in milrinone's use in term neonates.

Levosimendan use has been associated with a reduction in the need for other inotropic agents.[67,68] Its use has also been described for the treatment of newborns experiencing pulmonary hypertension.[70,72,78,79] Nevertheless, the future of levosimendan use in the neonatal population is uncertain and further research is necessary to determine its role.

Norepinephrine

The use of norepinephrine in ill neonates is extremely rare.[25] Benefits may be seen for states of excessive peripheral vasodilatation and hypotension such as septic shock. Hypothetically, given norepinephrine's lack of significant β_2-adrenergic vascular effect, increases in systemic vascular resistance may impair cardiac output.[80] Derleth published an abstract of his centre's experience with norepinephrine infusions in ill neonates.[81] Of the surviving neonates, approximately 25% suffered intestinal perforation, possibly secondary to issues with mesenteric flow. Tourneux et al. observed the use of norepinephrine in term neonates suffering from septic shock and hypotension despite infusions of dopamine or dobutamine.[82] Norepinephrine significantly increased mean arterial pressure and improved urine output, oxygen delivery and plasma lactate concentrations.

There is no evidence in the literature for use of norepinephrine in asphyxiated neonates or those with cardiogenic shock.

Conclusion

Use of inotropes in the neonate requires assessment of systemic and regional hemodynamics and correction of possible hypovolemia. Inotropes have shown beneficial increases in cardiac output and variable effects on regional perfusion. Unfortunately, not a great deal of the literature focuses on the problem assessed by this review, asphyxia in the term neonate. Much, instead, focuses on post-operative cardiogenic failure and the preterm population in particular. Inotropes have been studied primarily as single agents and rarely in combination, as is often used clinically. Further research is required to interpret the ideal approach to vasoactive drug therapy of the neonate suffering shock secondary to asphyxia.

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Chapter 4 – The Piglet and the Hypoxia-Reoxygenation Model

Animal models are used in biomedical research to provide a thorough understanding of disease states. The goal of such models is threefold: to contribute to our knowledge of underlying mechanisms of injury; to improve our understanding of progression of this injury and its outcome; and, to provide a template upon which therapeutic strategies may be tested.[1] In this chapter, evidence for the use of the prescribed model to study inotrope use in asphyxiated newborns will be presented.

The Neonatal Swine

Multiple species have contributed to further our understanding of perinatal hypoxic organ injury. None, however, can be considered a perfect representation of the human response. Past studies have used such species as non-human primates, sheep, rats, dogs, rabbits and pigs. Small animal studies such as those using the rat are commonly used for neurologic outcomes of asphyxia. Efforts in the past century often used non-human primates for this study, given their unequalled conformity to the human response.[2] Yet, use of higher order species such as these is challenged by costs and ethical considerations. Some argue that non-neurologic consequences of asphyxia are better studied in species other than primates.[3]

In choosing an animal for study, one must consider the normal course of disease in the species including biochemical, physiologic and pathologic responses and their correlation with humans.[2] Roohey et al. describes the use of large animal models for the study of acute and subacute metabolic and physiologic endpoints.[4] Alternatively, they advise the use of rodents and primates for long-term neurobehavioural assessment. Roughly a quarter, each, of all studies reviewed by Roohey and colleagues used rodents, sheep or piglets.[4] 71% were acute models with outcomes assessed prior to 24 hours. Use of primates offered testing of higher cerebral functions yet the authors offer caution regarding their potentially higher newborn cerebral maturity in comparison to humans. The same considerations apply to the use of sheep, along with the absence of standard neurological assessment measures in this species. Rodent models are limited by rapid postnatal maturation. Also, all small animal studies have limited utility in assessing multiple organ function.

The porcine model of hypoxic stress has served well for acute and subacute models. Pigs are of the order *Artiodactyla* and family *Suidae*.[5] Logistically, piglets are easily available and relatively cheap. Piglets offer similar responses in cerebral blood flow and metabolism to sheep and lambs.[6] Other similarities include size, omnivorous feeds, hemodynamic parameters and the structure and function of the gastrointestinal, endocrine, immunologic, integumentary, renal and cardiovascular systems.[7]

Despite the many similarities between pigs and humans, gestation differs, with implantation of the embryos on the 14th day following fertilization and total gestation of approximately 16 weeks compared with day 6 and 40 weeks in humans, respectively.[8] Litter sizes offer 12 to 18 piglets, allowing study of multiple subjects from the same parentage and also allowing the option of pairing experimental and control groups based on parentage.[9] Piglet weights however, ~1.4 kg, are generally smaller than humans.

Piglets are also relatively anemic and hypoxemic in comparison to humans. The initially elevated hematocrit at birth trends downward during the first post-natal week. Leukocyte levels are obviously variable due to multiple factors. Table 4.1 summarizes the mean hematologic and biochemical indices of neonatal piglets.

Pigs serve as a strong physiological model for human infants given the above hematologic and biochemical similarities and nearly identical anatomy. Coronary arterial anatomy is shared, as is the pulmonary vasculature. Gastrointestinal and genitourinary systems are also more
similar than other animal models, except for the porcine spiral colon, where the large bowel is coiled intraperitoneally.[10]

The fetus at term gestation has a mean blood volume of 86 to 116 ml/kg, 33% of which is in placental circulation.[11] 60% of umbilical venous return bypasses the liver through a series of shunts termed the ductus venosus. Studies of term fetal pigs reveal combined ventricular cardiac outputs in the range of 1 L / minute. 4.7% of flow is directed cerebrally, 4.2% to the coronary vasculature, 2.4% to each kidney and 4.9% toward the intestinal mesentery. 28% of cardiac output is for placental perfusion.

Hypoxic pulmonary vasoconstriction is variable among piglets.[12] Perfusion is matched to ventilation to ensure optimal gas exchange. The response is not linear, however. As alveolar hypoxia worsens, pulmonary vascular resistance increases to a greater

extent, increasing pulmonary arterial pressures to 80 to 140% above normoxic values.

Table 4.1 – Hematologic and biochemical indices in neonatal piglets[13,14,15]

PARAMETER	TIME	VALUES
Red Blood Cell Count	36 hours	4 – 6.2 X 10^6 / μL
Hemoglobin	36 hours	9.6 – 12.8 g / dL
Leukocyte Count	Birth	5.7 – 6.8 X 10^3 / μL
Platelets	Birth	258 – 416 X 10^3 / μL
	48 hours	193 – 289 X 10^3 / μL
Calcium	Birth	8.3 – 11.5 g / dL
Magnesium	Birth	1.9 – 2.7 g / dL
pH	1 to 3 days	7.39 – 7.42
HCO_3	1 to 3 days	22.1 – 25 mmol / L

A major limitation of past porcine research in asphyxia is the paucity of chronic asphyxia models, offering limited histopathologic evidence of brain injury.[6] Also, swine mature more rapidly than humans, necessitating accurate post-natal age determination for use of animals in study. Finally, just as in sheep, no standard neurologic outcome assessment is available, limiting their use for long-term behavioural outcome studies.[4]

Glauser promoted the use of piglets as the prototype for infants in pediatric research based on their similarities.[9] These allow better extrapolation of findings to human clinical settings. A "research program using a piglet model, when conducted in tandem with a clinical neonatal research program, could provide all aspects of a well-designed investigation."[7]

Anesthesia of the Neonatal Swine

Anesthesia of the swine, as in the human, may involve the use of sedatives, volatile and intravenous anesthetics. Depth of

anesthesia may be determined by monitoring heart rate, blood pressure and wakefulness, and euthermia should be maintained.

Piglets may prove difficult to anesthetize for a multitude of reasons.[16] Resistance to handling is often encountered. Pigs also lack easily accessible peripheral veins for administration of intravenous agents. Finally, the pig airway is more difficult to control extrinsically than that of the human. The latter two points promote the use of volatile anesthetic for initial induction and tracheostomy for definitive airway control.

Inhalational anesthesia provides rapid induction of anesthesia in newborn pigs.[17] Isoflurane is a volatile agent used as such with a minimum alveolar concentration (minimum concentration in the lungs to prevent movement in 50% of patients in response to a painful stimulus) of 1.5 volume %.[16] The pungent odour precludes its use in humans secondary to induced laryngospasm, yet, the drug is well tolerated in pigs.[18] Isoflurane increases cerebral blood flow, preserves renal flow and reduces intestinal perfusion.[19] Hickey et al. demonstrated dose-dependent coronary vasodilating properties of isoflurane in adult pigs.[20] Heerdt et al. then showed an isoflurane induced reduction and increase in left and right ventricular afterload, respectively, owing to alteration of autonomic activity.[21] Ventricle contractility and relaxation are also impaired by isoflurane.[19,22] In comparison with halothane, isoflurane better preserves blood pressure and is less catecholamine sensitizing to the myocardium, limiting the development of catecholamine induced dysrhythmias.[17] Despite the potential influence of isoflurane on hemodynamics, the drug's low solubility ensures rapid clearance and fast recovery with reversal of effects. These effects of isoflurane are controlled for in the proposed experimentation given the conversion to intravenous anesthesia and a generous recovery time prior to the collection of data.

Intravenous anesthesia may include the use of benzodiazepines or propofol in the typical intensive care setting. Total intravenous anesthesia effectively blocks the adrenergic response to surgical stresses and blunts endogenous catecholamine responses.[23] There are no studies assessing the effects of midazolam in the porcine species. A recent rodent model did however demonstrate reduced expression of cerebral endothelial intercellular adhesion molecules (ICAM-1 and P-selectin) with midazolam following hypoxia-reoxygenation.[24] This anti-

inflammatory neuroprotective effect of midazolam is assisted by release of γ-aminobutyric acid (GABA), with known inhibitory effects of cerebral activity. Whether the demonstrated effects are generalized to all benzodiazepines is unknown.

Propofol has been studied in few porcine trials. Graham et al. showed a 25% mean arterial pressure (MAP) reduction with propofol in newborn pigs,[25] though, the typical cardiac depressant effects were not appreciated, failing to show any load-independent ventricular dysfunction. Kurita et al. demonstrated, in asphyxiated adult pigs anesthetized with propofol and fentanyl, a narcotic, an inability of the anesthesia to suppress the animal's endogenous catecholamine response.[23] Nevertheless, MAP reductions failed to respond adequately to catecholamines when propofol is infused, often necessitating the use of exogenous sympathomimetic.[26]

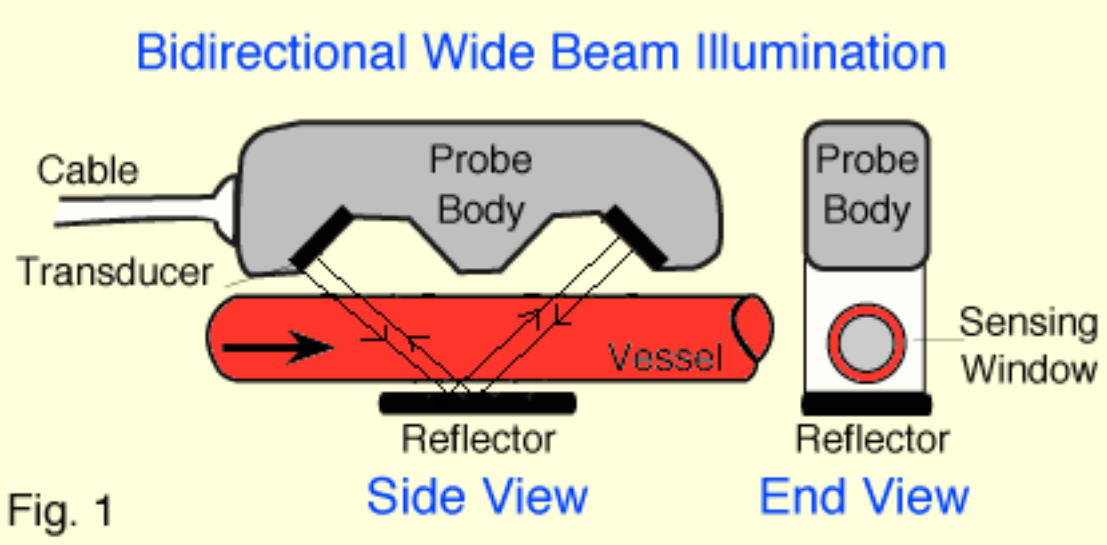
Typical experimentation with porcine species also commonly uses animal tranquilizers such as acetylpromazine and azaperone.[27]

Ultrasonic Transit-Time Technology

For measurement of regional perfusion in animal models, ultrasonic flow measurement has proved invaluable. Transonic®, an American company, has manufactured flowmeters for nearly three decades.[28] Various Transonic probes are used in our hypoxia-reoxygenation model. Figure 4.1 illustrates a typical probe positioned around a vessel.

The probe body's transducer emits a wide-beam ultrasound wave that is reflected after crossing the vessel and returns to the opposite transducer. The total transit time is measured. Both upstream and downstream transit times are measured. The shift in these is proportional to blood velocity and distance across which flow is encountered by the ultrasound wave. The measurement is insensitive to the angle at which the probe lies over the vessel. To determine the mean blood velocity, the measures of velocity from across the ultrasound beam width are summed and divided by the overall area encountered or vessel width.

Figure 4.1 – Transonic probe encircling vessel[28]



To ensure maximum accuracy of this system, the acoustic properties of the coupling media and tissue must be stable. The coupling medium, usually ultrasound gel, must be free of air bubbles and care must be taken to remove any fatty tissue from around the vessel. Also, the temperature should not fluctuate.

Transit-time technology provides continuous dynamic monitoring of vascular flow. Traditional methods for cardiac output and regional flow assessment included the injection of radiolabeled microspheres. This allows only intermittent assessment of flow.

Biochemical Testing

Biochemical analysis of specimens, both serum and tissue, enables determination of lactate, troponin and reactive oxygen species levels. The following describes the protocols used for measurement of serum lactate, tissue lactate and left ventricle troponin. All samples are kept on ice during preparations for measurement to ensure metabolic inactivity.

Serum Lactate

For the experiments proposed, serum lactate levels were analyzed immediately using the ABL 700 blood gas analyzer. The following assay is provided as a template on which determination of tissue lactate was performed following homogenization (see ***Tissue Lactate***).

A glycylglycine buffer was first made dissolving 4.75 g of glycylglycine, free base (Calbiochem cat #3630) and 0.88 g L-glutamic acid (Alfa Aesar CAS #6106-04-3) in 30 ml of distilled water. Next, sodium hydroxide was added to the solution until a pH of 10.0 is observed. The overall solution was then diluted to a total volume of 60 ml with distilled water.

Next, a glycylglycine "cocktail" was developed, the total volume of which was determined by the number of wells to be read. For each well, the following was added to solution and stored on ice:

- 100 μl glycylglycine buffer solution
- 50 μl distilled water
- 20 μl NAD solution (see below)
- 2 μl glutamate-pyruvate transaminase (Roche Applied Science cat #1070371127001 (10 mg/ml)

Nicotinamide adenine dinucleotide (NAD) was made into solution using 0.210 g of NAD crystal (β-Nicotinamide adenine dinucleotide hydrate, from yeast, 98% purity – Sigma cat #N7004) and adding 6 ml of distilled water. Addition of this active redox agent required maintaining the solution on ice so as to prevent electron transfer and metabolic activity.

Using a 96-well plate (Corning Costar #3635 – Fisher Scientific cat #CS-003635), 50 μl of sample was added in duplicate or triplicate. In the first column, 50 μl of distilled water alone was

added to each well to be used as a blank. Next, 172 μl of glycylglycine cocktail was added to each well. The plate was transferred to the absorption reader and read at 340 nm. Measurements were performed at 5-minute intervals until 3 consecutive readings differed by no more than 0.05 absorbance units. This served as the baseline absorbance.

Ensuring the stock solution was kept cool, 2 μl of lactate dehydrogenase (LDH: L-lactate dehydrogenase – Roche Applied Science cat #10127876001 – 5 mg/ml) was added to each well. Absorbance readings were then measured, again, at 5-minute intervals until absorbance was stable within 0.05 units over three readings. This served as the sample absorbance.

For calculation, readings were corrected by subtracting the blank column baseline measure from all readings at baseline. Blank readings were then subtracted from all sample absorbance readings. Lactate was then calculated from the following formula:

$$C = [V*MW*\Delta A]/[\varepsilon*d*v*1000]$$

Where V = final sample volume = 224 μl
MW = molecular weight of lactate
ΔA = sample absorbance – baseline absorbance
ε = absorption coefficient of NADH at 340 nm = 6.3 L/Mmol/cm
d = light path = 1 cm
v = sample volume = 50 μl

Tissue Lactate

The following protocol allowed the measurement of lactate from solid sample tissue. The first steps involved crushing of tissue and homogenization into liquid form. Next, the assay was performed allowing measurement of lactate levels indirectly using an absorbance reader and the aforementioned protocol.

Two solutions were first constituted. The perchloric acid (PCA) solution was made using 2.5 ml of 60% PCA stock and adding 22.5 ml of distilled water. 4.75 mg of EGTA was then added to solution yielding 6% PCA and 0.5 mM EGTA. The second solution was formed using 3.46 g of potassium carbonate (K_2CO_3) diluted in 4.3 ml of distilled water giving 5 M K_2CO_3.

Next, 50 mg of crushed tissue was added to flat-bottomed centrifuge tubes. 500 μl of PCA solution was added and samples were then homogenized using a homogenizer. Samples were left on ice until centrifugation at 11,000 RPM for minutes. The supernatant was then transferred to a separate centrifuge tube and 5M K_2CO_3 solution was added in a ratio of 1 μl K_2CO_3 : 10 μl supernatant. Samples were left on ice for 30 minutes.

Samples were again centrifuged at 11,000 RPM for 2 minutes and the supernatant was again collected. 50 μl of supernatant was needed for the assay. Any excess was stored at – 80 °F.

Supernatant was then used to run the assay as described in the aforementioned serum lactate section. The blank column wells were filled with 45.5 μl of PCA solution and 4.5 μl of K_2CO_3 solution.

Cardiac Troponin-I

The assay for measurement of cardiac troponin-I (Pig Cardiac Troponin-I ELISA Kit, Life Diagnostics Inc., Cat #2010-4-HS) used two purified cardiac troponin-I antibodies. Samples were incubated for one hour following which, unbound antibody was removed and tetramethylbenzidine (TMB) added with subsequent measurement of absorbance.

Preparation for the assay commenced with dilution of the provided wash solution with distilled water to a total volume of 1 L from 50 ml. The remainder of kit solutions were allowed to reach room temperature prior to use. Lyophilized stock troponin was reconstituted into 400 μl of distilled water for a concentration given on the vial. If not used, this was stored in a refrigerator or freezer.

7 centrifuge tubes were prepared, labeled 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.039 ng/ml. Into the tube labeled 2.5 ng/ml, stock troponin diluent (supplied) in the volume indicated of the troponin stock vial was added. Next, the indicated volume of diluted troponin stock solution was added and gently mixed. This tube now contained the 2.5 ng/ml standard. The remaining 6 tubes had 0.25 ml of troponin diluent added. The remaining standards were made at the labeled concentrations using serial dilution. Standards were used within 30 minutes of preparation.

Using a supplied Anti-cardiac troponin-I antibody coated 96-well plate, 100 μl of troponin horse radish peroxidase conjugate

solution was added to each well. 100 μl of standard at each concentration was placed into corresponding wells. 100 μl of supernatant from homogenized samples was then added to the wells. The plate was then incubated on an orbital shaker at 150 RPM for 60 minutes.

The plate was then emptied into a bio-waste container. 400 μl of wash solution was used to rinse each well. Next, 100 μl of TMB reagent was added to each well and mixed for 5 seconds. The plate was again incubated on an orbital shaker, this time for 20 minutes. Samples turned blue. 100 μl of stop solution (1M hydrochloric acid) was added to each well until all blue was turned yellow.

Absorbance was then read at 450 nm. Should absorbance have exceeded the high standard, samples were all diluted accordingly using the troponin diluent. Values below the lowest standard were assigned a value of 0.

Glutathione and Malondialdehyde

Tissue glutathione (GSH) was measured using a commercially available glutathione assay kit (Cayman Chemical, Ann Arbor, Michigan, Catalog #703002). Frozen tissue was crushed and homogenized in buffer containing 0.2M 2-(*N*-morpholino)ethanesulphonic acid, 50mM phosphate and 1mM EDTA at pH 6-7. Following centrifugation, supernatant was deproteinated with 10% metaphosphoric acid and 4M triethanolamine. Colorimetric microplate assay was then performed after the addition of glutathione reductase, glucose-6-phosphate dehydrogenase, $NADP^+$, 5,5'-dithio*bis*-2-nitrobenzoic acid. Absorbance was read at 405 nm after 25 minutes using the aforementioned spectrophotometer. To measure oxidized GSH (GSSG), reduced GSH was derivatized to GSSG using 2-vinylpyridine. The assay was then carried out using this sample. Oxidative status of GSH was determined through interpretation of the GSSG/GSH ratio.

Tissue malondialdehyde (MDA) was measured to assess the effect of oxidative stress. Frozen tissues were homogenized 1:10 in phosphate-buffered saline and fluorescence assays were compared with standard concentrations of MDA as described by Ohkawa.[29]

Graphing

All assays were finalized by plotting the absorbance readings on a graph against the standard concentrations. From this, a standard linear relationship was obtained from which the sample concentrations of biochemical molecule could be determined using the measured sample absorbances.

Tissue Histology

To determine the extent of tissue injury, specimens of ventricle and small intestine were analyzed histologically. Rose et al. described the histologic changes in the ventricle following experimental acute myocardial infarction in a canine model.[30] The following studies used a modified set of criteria that could be interpreted from hematoxylin and eosin stained slides. The criteria were:

1) edema
2) congestion
3) myofibre waviness
4) myofibre cytoplasm crossbanding
5) polymorphonuclear leukocyte infiltration

Each criterion was scored from 0 (normal) to 3 (severely abnormal).

Small intestinal tissue samples were examined for evidence of histologic ischemia-reperfusion injury using a scale described in a rat model by Park et al.[31] Intestinal cross-sections stained with hematoxylin and eosin were graded as follows:

0) normal mucosa
1) subepithelial space at villus tip
2) extended subepithelial space
3) subepithelial space along villus sidewall
4) denuded villi
5) loss of villus tissue
6) infarction of crypt layer
7) transmucosal infarction
8) transmural infarction

Conclusion

The study of human diseases requires the development of reliable models. Naturally, asphyxia cannot be instituted in human studies and therefore, subject recruitment and homogeneity would serve as potent inhibitors to data collection and analysis. The swine model proposed has been published on many occasions and provides a platform on which to study the effects of asphyxia in newborns. The limitations of an animal model and the differences between human and animal physiology must nevertheless be considered when drawing conclusions for application in the clinical environment.

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Chapter 5:

A Comparison of Combination Dopamine and Epinephrine Treatment with High-Dose Dopamine Alone in Asphyxiated Newborn Piglets after Resuscitation

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Glutathione, troponin-I and tissue lactate assays were performed by Yingqian Li. Malondialdehye assays were performed by Jacek Studzinsky.

Introduction

The World Health Organization attributes 23% of the 4 million neonatal deaths each year to asphyxia.[1] Neonatal survival is challenged by asphyxia with variable effects on different organ systems. When 72 asphyxiated neonates were followed prospectively, evidence of multi-organ dysfunction was appreciated with abnormalities of the central nervous, cardiovascular, gastrointestinal and renal systems.[2]

Following asphyxia, neonates develop shock with reduced cardiac output, systemic hypotension and pulmonary hypertension.[3,4,5] Subsequent regional hypoperfusion compromises cerebral, gastrointestinal and renal function. Vasoactive medications – inotropes and vasopressors – have long been used to treat shock in neonates despite a paucity of evidence on their effects, particularly in regards to organ blood flow and tissue perfusion.[6,7]

Dopamine, the most frequently used vasoactive agent in neonates, exerts dose-dependent α and β-adrenergic and dopaminergic effects.[8] Treatment with high doses of dopamine ($\geq$20 mcg/kg/min) is often avoided given potentially excessive α-adrenergic stimulation with vasoconstriction and increased afterload.[7,9,10] An alternative approach for neonates proving refractory to initial dopamine infusion (up to a moderate dose of 10 mcg/kg/min) advocates the addition of a second vasoactive/inotropic agent in lieu of increasing dopamine doses. To our knowledge, there is no evidence directly comparing these two treatment strategies.

Epinephrine may be considered as an additional agent in treating neonatal hypotension refractory to initial dopamine infusion.[11] In asphyxiated newborn term piglets, low-dose epinephrine (0.2 mcg/kg/min) added to dopamine (10 mcg/kg/min) enhances cardiac output and blood pressure to a similar extent as high-dose epinephrine (1 mcg/kg/min) alone.[12] Furthermore, in the hypotensive low-birthweight preterm population, Seri and Evans demonstrated a normalization of blood pressure and improvement in urine output with the addition of epinephrine to dopamine infusion.[13] A few preterm neonate studies have also used dopamine as a background infusion or a combination of agents when there was no response to monotherapy, however, these encompass heterogeneous etiologies of hypotension.[14,15,16]

Using an established swine model of neonatal hypoxia-reoxygenation (H-R), we primarily compared the effect of dopamine and epinephrine combination treatment to high-dose dopamine alone on cardiac output and blood pressure. Secondary outcomes assessed were: (1) carotid, mesenteric and renal circulatory effects, (2) effects on oxygen transport, and (3) degree of histologic H-R injury. We hypothesized that monotherapy with high-dose dopamine would have detrimental effects on cardiac output and regional circulation when compared with dopamine and epinephrine combination therapy in neonatal H-R.

Methods

Animals – Mixed breed (Duroc / Large White) 1-4 day-old piglets, weighing 1.5-2.5 kg, were obtained from the university swine research farm on the morning of experimentation. The following protocol was approved by the University Animal Care and Use Committee and adheres to the Canadian Council on Animal Care guidelines (2000).

Anesthesia – Isoflurane (5%) was used to induce anesthesia, thereafter, titrated between 1 and 3% for maintenance. Pulse oximetry (Nellcor, Hayward, CA) was applied to monitor oxygen saturation and a rectal thermometer was placed for temperature measurement. Throughout experimentation, piglets were maintained at a temperature of 38.5 to 40°C using both a heating underpad and overhead warmer. Following definitive airway placement and vascular access, inhalational anesthesia was discontinued. Mechanical ventilation was started with pressure control of 20/4 cm H_2O and rates of 18-20 breaths/min (Sechrist Infant Ventilator Model IV-100, Sechrist Industries Inc., Anaheim, CA) with fractionated inspired oxygen concentrations (F_iO_2) of 0.21-0.25. Oxygen saturation was maintained at 88-100%. F_iO_2 was measured using a MiniOx III oxygen monitor (Catalyst Research, Owings Mills, MD). Anesthesia was maintained with a combination of intravenous midazolam (0.2-1 mg/kg/h) for sedation and fentanyl (5-20 mcg/kg/h) for analgesia. Pancuronium (0.05-0.1 mg/kg/h) was administered for paralysis during surgery. Boluses of fentanyl (10 mcg/kg) and acepromazine (0.25 mg/kg) were used as needed. 5% dextrose (20 ml/h) and 0.9% normal saline (4 ml/h) were infused with boluses of lactated Ringer's solution given as indicated.

Surgical Instrumentation – The right femoral artery and vein were exposed through a groin incision. The femoral vein was cannulated with a 5-Fr dual-lumen catheter (Sherwood Medical Co., St. Louis, MO) advanced 13-15 cm to the right atrium to administer fluid and medications, and measure central venous pressure (CVP). A 5-Fr single-lumen catheter was then inserted in the femoral artery and advanced 5 cm to the infrarenal aorta for mean arterial pressure (MAP) monitoring. Next, via a transverse neck incision, a 3.5 mm inner diameter endotracheal tube was inserted through a tracheotomy to allow mechanical ventilation.

The left common carotid artery was encircled with a calibrated 2-mm flow probe (2SS, Transonic Systems Inc., Ithaca, NY). A left subcostal incision was used for exposure of the retroperitoneum where the left kidney was reflected anteriorly and superior mesenteric artery (SMA) exposed. Here, a 3-mm flow probe (3SB) was placed and the left renal artery was then encircled with a similar 2-mm (2SB) flow probe. Following a left anterior thoracotomy, the pericardium was opened and ductus arteriosus ligated. The pulmonary artery was then cannulated using a 20-gauge angiocatheter (Insyte-W, Becton Dickinson Infusion Therapy Systems Inc., Sandy, UT) used for pulmonary artery pressure (PAP) monitoring. A 6-mm flow probe (6SB) was then placed around the pulmonary artery to measure pulmonary artery flow as a surrogate for cardiac output.

Stabilization and Monitoring – Following instrumentation, piglets received a bolus of 10 ml/kg lactated Ringer's solution and were allowed to recover for 60 min. Arterial $PaCO_2$ of 35-45 mmHg was maintained throughout experimentation. Heart rate and blood pressures were monitored continuously using a Hewlett Packard 78834A monitor (Hewlett Packard Co., Palo Alto, CA). Hemodynamic readings were digitized (Data Translation, ON, Canada) and recorded in a personal computer equipped with Asyst programming software.

Hypoxia-Reoxygenation (H-R) Protocol – Piglets were block randomized to either a surgical sham group or one of three H-R groups. Sham-operated piglets (sham, n=6) were maintained at F_iO_2 of 0.21-0.25 for the duration of experimentation (6h). H-R piglets underwent normocapnic alveolar hypoxia, through addition of inhaled nitrogen, reducing F_iO_2 to 0.10-0.15 for 2h. PaO_2 was maintained at 20-40 mmHg during hypoxia to produce cardiac dysfunction and hypotension as previously described.[17,18,19,20] Subsequently, piglets were resuscitated with 100% oxygen for 30 min and kept at F_iO_2 0.21-0.25 for the remainder of experimentation (3.5h). A 10 ml/kg bolus of lactated Ringer's solution was administered during reoxygenation prior to drug infusion.

At 2h of reoxygenation, H-R piglets received infusions of either placebo (0.9% saline solution – control) or study drug at a constant rate in a blinded, randomized fashion (n=7/group). Drug groups studied included: DOPA group -high-dose dopamine (20

mcg/kg/min; Baxter Corporation, Toronto, ON, Canada), or D+E combination group - dopamine (10 mcg/kg/min) and epinephrine (0.1 mcg/kg/min; Erfa Canada Inc., Westmount, QC, Canada). A laboratory technician prepared drug solutions into standard volume syringes for blinding purposes. The dosages were derived in part from previous studies in a similar swine model of neonatal H-R and the epinephrine dosage was decided in consideration of its concomitant administration with dopamine.[12,19]

Hemodynamic and Oxygen Measurements – Heart rate, MAP, PAP and central venous pressure were recorded continuously and analyzed at set intervals from normoxic baseline through hypoxia and reoxygenation and at every 30 min of placebo or drug treatment. The data were averaged as means over a 2 min recording at each time point. Also, systemic and pulmonary arterial blood was sampled for blood gas analysis using ABL 700 blood gas analyzer and OSM3 Hemoximeter (Radiometer, Copenhagen, Denmark).

Biochemical Analysis and Histopathology – Arterial samples were analyzed for lactate using the ABL 700 analyzer and plasma was stored at -80°C. Plasma levels of porcine cardiac-specific troponin-I at baseline, 2h of reoxygenation and 2h of drug treatment were analyzed using enzyme-linked immunosorbent assay (#2010-4, Life Diagnostics, West Chester, PA).

At the end of experimentation, piglets were euthanized using intravenous pentobarbital (100 mg/kg). Necropsy was performed immediately for retrieval of left ventricular tissue and terminal ileum. A small sample was stored in 10% formalin solution and the remaining tissue was snap frozen in liquid nitrogen and stored at -80°C. The formalin-preserved specimen was then processed for hematoxylin and eosin staining. Three separate pathologists (M.B., J.S., D.R.) blinded to treatment allocation interpreted histologic findings using previously described scoring systems for H-R injury.[21,22]

For measurement of tissue lactate, frozen myocardial and intestinal tissue was crushed and homogenized in 6% perchloric acid (PCA)/ 0.5mM EGTA on ice. Samples were centrifuged and supernatant collected. 5M potassium carbonate was then added slowly in a 1μL:10μL of supernatant ratio. Following precipitation over 30 min, samples were again centrifuged and supernatant collected for NAD enzyme-coupled colorimetric microplate assay.

After the addition of glycylglycine buffer, NAD, double-distilled water, glutamate-pyruvate transaminase and lactate dehydrogenase, absorbance was read at 340 nm using a microplate spectrophotometer (Spectramax 190; Molecular Devices, Sunnyvale, CA).

Measurement of tissue glutathione (GSH) was performed using a commercially available glutathione assay kit (Cayman Chemical, Ann Arbor, Michigan, Catalog #703002). Frozen tissue was crushed and homogenized in buffer containing 0.2M 2-(*N*-morpholino)ethanesulphonic acid, 50mM phosphate and 1mM EDTA at pH 6-7. Following centrifugation, supernatant was deproteinated with 10% metaphosphoric acid and 4M triethanolamine. Colorimetric microplate assay was then performed after the addition of glutathione reductase, glucose-6-phosphate dehydrogenase, $NADP^+$, 5,5'-dithio*bis*-2-nitrobenzoic acid. Absorbance was read at 405 nm after 25 minutes using the aforementioned spectrophotometer. To measure oxidized GSH (GSSG), reduced GSH was derivatized to GSSG using 2-vinylpyridine. The assay was then carried out using this sample. Oxidative status of GSH was determined through interpretation of the GSSG/GSH ratio.

For measurement of tissue malondialdehyde (MDA), frozen tissues were homogenized 1:10 in phosphate-buffered saline and fluorescence assays were compared with standard concentrations of malondialdehyde as described by Ohkawa.[23]

Statistics – Data were analyzed using SigmaPlot 11 software (Systat Software Inc., 2008). Analysis of variance (one-way and two-way repeated measures) was used where appropriate for parametric data, with *post hoc* analysis by Student-Newman-Keuls test. For nonparametric data, Kruskal-Wallis test was performed with Dunn's method for *post hoc* intergroup comparison. For practical interpretation of drug effect after infusion, percentage change respective to pre-drug baseline was used to analyze data during the drug infusion phase. Correlations between variables were performed with Pearson Moment or Spearman test as appropriate. Significance was defined as $p<0.05$. Results are expressed as mean±standard error of mean.

Results

Of 29 piglets instrumented, 2 were excluded for complications related to surgery (1) and hypoxia (1), leaving 27 piglets for analysis. Animals were aged 2.2±0.2 days and weighed 1.9±0.1 kg. There was no significant difference in gender, hemodynamic and physiologic parameters recorded after stabilization among experimental groups.

Hypoxia – Following 2h of hypoxia (PaO_2 40±1%)(Table 5.1), all H-R piglets were in cardiogenic shock exhibiting significant hypotension (MAP: 28±1 mmHg), pulmonary hypertension (PAP: 32±2 mmHg) and reduced CI (36±2% of normoxic baseline) in comparison with sham (all $p<0.05$)(Table 5.2). Corresponding reductions in systemic oxygen delivery and consumption were also found (data not shown). H-R piglets showed significant decreases in carotid, superior mesenteric and renal oxygen perfusion (22±2%, 15±1% and 21±2% of respective normoxic baselines; $p<0.05$ vs. sham). Severe metabolic acidosis was present with significantly elevated plasma lactate levels in H-R piglets (Table 5.1). No differences were found between H-R groups regarding hemodynamic and biochemical parameters at the end of hypoxia.

Reoxygenation – After 10 min of 100% oxygen resuscitation (PaO_2 351±15 mmHg), hemodynamic parameters improved significantly (data not shown) but then deteriorated over the following 2h. In H-R piglets, MAP was 43±2 mmHg and CI 73±4% of normoxic baseline (Table 5.2). Carotid, superior mesenteric and renal oxygen perfusion was 56±4%, 86±7% and 79±8% of respective normoxic baselines after 2h of reoxygenation and prior to medication delivery. All H-R piglets had reduced serum bicarbonate and elevated plasma lactate (Table 5.1) and troponin I levels ($p<0.05$ vs. sham). Of note, MAP of D+E piglets was significantly lower than that of control piglets ($p<0.05$)(Table 5.2) and plasma troponin was also higher than other H-R groups ($p<0.1$ vs. control and DOPA).

Systemic/Pulmonary Hemodynamic Effects of Medication Infusion – Both DOPA and D+E groups increased heart rate over 2h of infusion (vs. control, $p<0.05$) (Figure 5.1A). The effect of these agents on SVI was modest with transient increases seen early during infusion (Figure 5.1B). The combined effect offered an increase in CI over the duration of infusion in D+E

and DOPA piglets (vs. control, $p<0.05$, Figure 5.1C). MAP decreased further in control piglets, whereas it increased in D+E and DOPA piglets (vs. control, $p<0.05$)(Figure 5.2A). There was no change in SVRI during drug infusion (data not shown). PAP was unchanged during drug infusion (Figure 5.2B). D+E treated piglets decreased PAP/MAP ratio during infusion, relative to controls ($p<0.05$)(Figure 5.2C).

Regional Hemodynamic Effects of Medication Infusion – Both DOPA and D+E groups improved CAFI ($p<0.05$ and $p=0.05$ vs. control, respectively) and SMAFI (both $p<0.05$)(Figure 5.3A). There were no associated changes in carotid vascular resistance. However, mesenteric vascular resistance decreased significantly in the DOPA group ($p<0.05$ vs. control; $p=0.07$ vs. D+E)(Figure 5.3B). There were no differences in RAFI or renal vascular resistance among H-R groups during drug infusion.

Oxygen Delivery and Metabolic Effects of Medication Infusion – H-R piglets in DOPA and D+E groups had higher systemic oxygen delivery than that of controls ($p<0.05$), with no effect on systemic oxygen consumption (Figure 5.4A). Carotid and mesenteric oxygen delivery declined and remained unchanged, respectively, in control piglets. However, corresponding to the improvements in regional flow, DOPA piglets improved carotid and superior mesenteric oxygen delivery ($p<0.05$ vs. control), whereas D+E group had modest respective improvements ($p<0.1$ vs. control)(Figure 5.4B). There was no effect found on renal oxygen delivery. Plasma lactate levels of H-R groups improved with no difference among groups over the 2h infusion.

During the course of experimentation, plasma lactate levels correlated modestly with CI ($r=-0.4$, $p<0.05$). Interestingly, the correlation was not significant in the control or DOPA groups, whereas significant correlation was found in the D+E group ($r=-0.6$, $p<0.01$). Systemic oxygen delivery correlated with plasma lactate ($r=-0.5$, $p<0.01$), particularly in the D+E and DOPA groups ($r=-0.6$, $p<0.01$ and $r=-0.5$, $p<0.01$, respectively) but not in controls.

Tissue Oxidative Stress Markers, Plasma Troponin Levels and Histologic Scores of H-R Injury – Ventricular lactate correlated with systemic oxygen delivery ($r=-0.6$, $p<0.01$) in the H-R groups. Intestinal lactate correlated with SMAFI and mesenteric oxygen delivery following 2h of infusion ($r=-0.4$, $p=0.05$ and $r=-0.5$, $p=0.02$, respectively). Plasma troponin was greater in

D+E piglets following the last 2h of study ($p<0.05$ vs. control and DOPA). Myocardial and intestinal tissue lactate, glutathione and MDA levels were not different among groups. Ischaemic-looking colon was found in 2 cases each in the control and DOPA groups and 3 in the D+E group at the time of necropsy. Despite these findings, there were no significant differences in histopathologic findings of H-R injury in the left ventricle and gut (data not shown). However, the degree of left ventricle histologic injury did correlate moderately with plasma lactate levels at the end of experimentation ($r=0.4$, $p<0.05$).

Discussion

Following asphyxia of the neonate, shock with hypotension is often treated with vasoactive and/or inotropic medications.[24] Despite of the lack of correlation between blood pressure and perfusion, it is common to use blood pressure as the target parameter of cardiovascular supportive therapy for critically ill neonates. However, upward titration of drug doses in pursuit of "normal" blood pressures may pose a concern given the potential for detrimental excessive vasoconstriction and impairment of tissue perfusion.[25] Based on the findings in this swine model of neonatal H-R injury, we suggest that monotherapy with high-dose dopamine may be preferred over combination therapy with dopamine and epinephrine given improvements in mesenteric vascular resistance, and vice versa for efforts to stabilize or alleviate the risk of increasing unbalanced pulmonary-systemic arterial pressure (PAP/MAP) ratio.

The treatment of piglets after H-R with either high-dose dopamine alone or combination dopamine and epinephrine improved myocardial function with chronotropic and transient inotropic effects. The improvement in CI with high-dose dopamine was not appreciated in previous studies using a similar model,[19] nor was the predominant chronotropic effects of both regimens.[12] This may be secondary to underlying variability in large animal studies. Also, the current study utilized a significant protocol change with the use of only 30 minutes of pure oxygen resuscitation in lieu of a full hour. There were no differences in effect on systemic vascular resistance, further substantiating arguments against the detrimental vasoconstrictive effects of high-dose dopamine in neonatal H-R.[26] Similar to the findings of the only term neonate trial studying dopamine, MAP was improved in both treatment groups.[27] Interestingly, at the doses studied, CI was improved without significant increase in afterload so as to worsen plasma lactate. Therefore, either regimen is safe, at least during the initial stages of therapy, regarding their vasoconstrictive effects and overall tissue perfusion. Further studies need to be conducted, however, to determine if this effect holds with prolonged infusion.

The failure to show a difference in plasma lactate levels may be secondary to the limited 2h time frame of study. The trend for increased lactate levels in the combination treatment piglets may

indeed be proven valid if observation were carried further.[16] Furthermore, safety from myocardial injury may also be in question given that analysis of plasma troponin levels was complicated by non-significant but large inter-group differences prior to administration of the blinded medications. Nonetheless, with 2h infusion of either high-dose dopamine or combination dopamine and epinephrine, there were no significant differences in myocardial lactate, glutathione or MDA levels among groups.

Given that blood pressure and tissue perfusion are not synonymous, assessment of other parameters is required to determine the status of asphyxiated neonates.[28] In this study, we observed significant improvements in systemic oxygen delivery with either treatment, with no differences in oxygen consumption relative to controls. End-organ perfusion similarly improved with benefits to carotid and mesenteric perfusion in both groups. Indeed, we demonstrated that improvements in mesenteric perfusion were associated with reduction of intestinal tissue lactate, a marker of anaerobic metabolism. However, treatment with high-dose dopamine alone significantly reduced mesenteric vascular resistance relative to controls and modestly when compared with combination treatment. As a result, high-dose dopamine may be preferable to the combination of dopamine and epinephrine should mesenteric perfusion and intestinal injury be of concern.

The net effect observed on PAP/MAP ratio was different between dopamine and combination groups, the former stabilizing the ratio relative to placebo controls, and the latter reducing it at the specified doses. Higher doses previously studied did not show this effect.[29] With these results, one should consider the combination of agents when managing neonates with persistent fetal circulation given preferable effects on the risk for shunting of deoxygenated blood.

The study is limited by the lack of improvement in systemic oxygen consumption following improvement in oxygen delivery in the drug treatment groups. One may infer that the piglets were not adequately stressed given that oxygen metabolism was at maximal levels prior to drug infusion, offering no room for further improvement. This is different from prior studies using a similar H-R model[20] and may be related to the aforementioned differences in the reoxygenation protocol. Further, this study uses a neonatal model to draw inferences regarding perinatal care. Differences in

adrenoreceptor expression over the early life of the neonate may complicate application of results in the perinatal setting. Finally, we used infusions of drug at fixed doses. Clinically, these agents tend to be titrated to effect. Given the logistical implications with blinding and the relatively short duration of treatment, thereby limiting the time for variable doses to effect a response, this was not possible in our study.

TABLE 5.1 – Arterial Blood Gas and Acid-Base Status

Time	Baseline 0min	End-Hypoxia 120min	Pre-Drug 240min	End-Drug 360min
pH				
Sham	7.47±0.04	7.44±0.02	7.46±0.01	7.45±0.02
Control	7.45±0.02	7.02±0.02*	7.38±0.02	7.41±0.02
D+E	7.44±0.02	7.04±0.03*	7.40±0.01	7.36±0.02*
DOPA	7.47±0.04	7.06±0.03*	7.39±0.04	7.37±0.03*
HCO_3 (mmol/L)				
Sham	27.1±1.9	27.2±1.5	28.3±1.0	27.4±1.2
Control	26.7±1.1	9.2±0.4*	22.5±0.9*	25.0±1.4
D+E	26.4±4.4	9.7±0.7*	21.5±1.2*	22.1±1.0*
DOPA	26.7±1.9	10.5±0.9*	22.0±1.5*	22.6±1.3*
PaO_2 (mm Hg)				
Sham	87.1±5.8	71.5±3.0	70.5±2.9	67.1±1.8
Control	83.6±3.3	38.3±2.2*	65.2±1.1	70.1±2.2
D+E	79.6±7.7	42.0±4.6*	68.3±6.4	78.7±5.7
DOPA	89.1±6.9	39.9±4.4*	70.5±4.1	70.1±1.3
LACTATE (mmol/L)				
Sham	3.7±0.4	2.6±0.3	1.8±0.3	1.7±0.2
Control	2.8±0.2	13.6±1.1*	4.1±0.4*	2.0±0.1
D+E	3.2±0.4	14.0±1.0*	4.7±0.8*	3.2±0.6
DOPA	3.0±0.2	13.0±0.8*	3.4±0.3*	1.8±0.3

* $p<0.05$ vs. Sham

TABLE 5.2 – Systemic Hemodynamic Parameters

	Baseline	End-Hypoxia	Pre-Drug	End-Drug
Time	0min	120min	240min	360min
Heart Rate (beats/min)				
Sham	173±11	171±8	195±11	207±11
Control	176±6	208±11*	228±14	206±6
D+E	181±12	208±14*	237±9*	269±12*†
DOPA	174±12	233±7*	219±11	247±20*†
Mean Arterial Pressure (mmHg)				
Sham	77±3	63±4	54±2	48±2
Control	77±5	29±1*	48±4	41±2
D+E	74±4	26±1*	38±3*†	43±4
DOPA	75±4	29±1*	42±2*	44±1
Pulmonary Arterial Pressure (mmHg)				
Sham	24±1	24±1	26±1	29±2
Control	25±2	30±2*	26±1	26±1
D+E	25±1	32±4*	26±1	29±2
DOPA	23±1	34±4*	24±1	26±2
PAP/MAP Ratio				
Sham	0.32±0.04	0.38±0.03	0.50±0.04	0.62±0.06
Control	0.33±0.03	1.03±0.03*	0.56±0.02	0.65±0.04
D+E	0.34±0.02	1.20±0.10*	0.70±0.07*	0.69±0.09
DOPA	0.31±0.02	1.18±0.09*	0.57±0.02	0.59±0.03
Cardiac Index (ml/kg/min)				
Sham	224±30	189±30	199±26	176±21
Control	229±15	78±5*	178±14	176±12
D+E	205±28	75±11*	152±19	176±24
DOPA	188±15	70±10*	136±14	177±15

* $p<0.05$ vs. Sham

† $p<0.05$ vs. Control

FIGURE LEGENDS

FIGURE 5.1

Changes in (A) heart rate, (B) stroke volume index (SVI) and (C) cardiac index (CI) during infusion of combination dopamine and epinephrine (grey) and high-dose dopamine (black) in H-R piglets in comparison with controls (white). Stroke volume index and cardiac index are expressed as mean percentage change over duration of infusion.

* $p<0.05$ vs. control (2-way repeated measures ANOVA).

FIGURE 5.2

Changes in (A) mean arterial pressure (MAP), (B) pulmonary artery pressure (PAP) and (C) PAP/MAP ratio during infusion of combination dopamine and epinephrine (grey) and high-dose dopamine (black) in H-R piglets in comparison with controls (white). PAP/MAP ratio is expressed as mean percentage change over duration of infusion.

* $p<0.05$ vs. control (2-way repeated measures ANOVA).

FIGURE 5.3

Changes in (A) carotid, superior mesenteric (SMA) and renal arterial flows and (B) respective vascular resistance during infusion of combination dopamine and epinephrine (grey) and high-dose dopamine (black) in H-R piglets in comparison with controls (white). Data are expressed as mean percentage change over duration of infusion.

* $p<0.05$ vs. control; † $p=0.05$ vs. control; # $p=0.07$ vs. combination dopamine and epinephrine group (2-way repeated measures ANOVA).

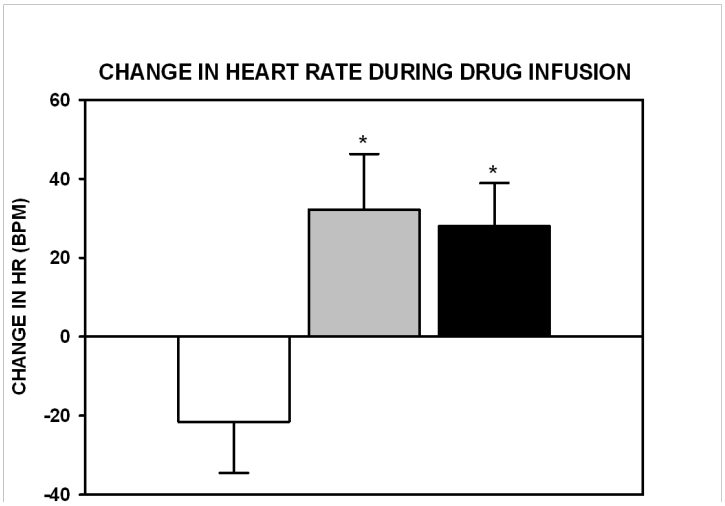
FIGURE 5.4

Changes in (A) systemic (SYS) oxygen delivery (DO_2) and consumption (VO_2) and (B) carotid, superior mesenteric and renal DO_2 during infusion of combination dopamine and epinephrine (grey) and high-dose dopamine (black) in H-R piglets in comparison with controls (white). Data are expressed as mean percentage change over duration of infusion.

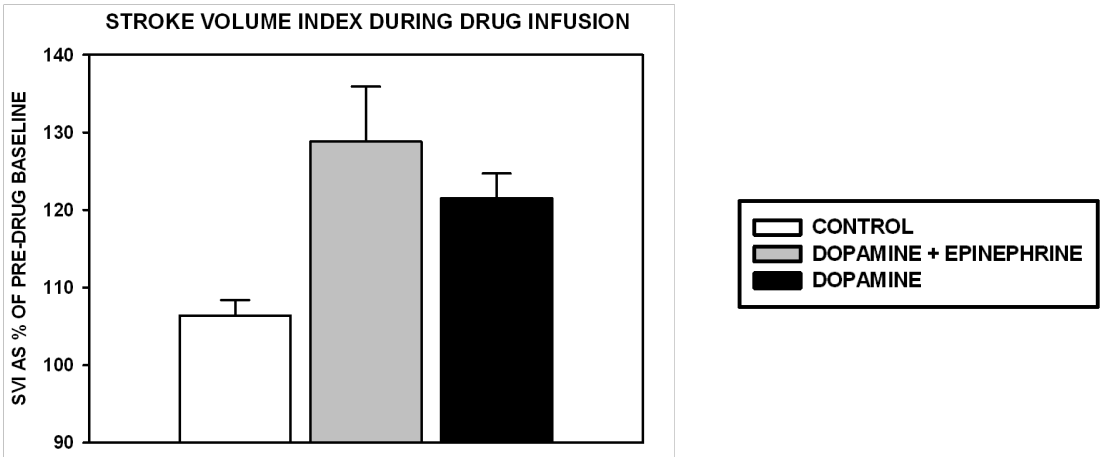
* $p<0.05$ vs. control; † $p<0.1$ vs. control (2-way repeated measures ANOVA).

FIGURE 5.1 –

5.1A:



5.1B:



5.1C:

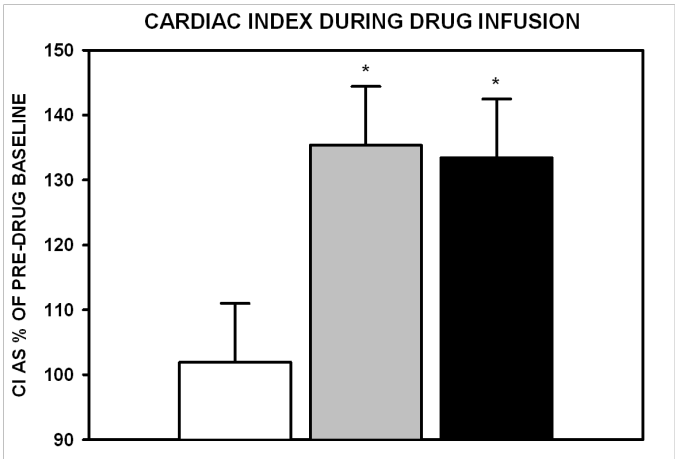
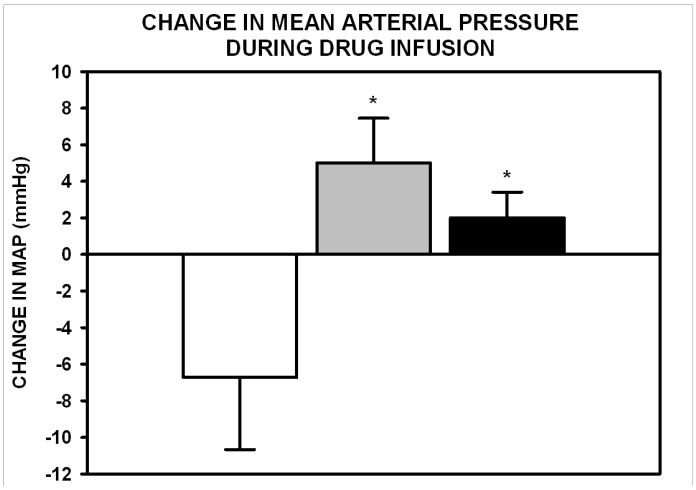


FIGURE 5.2 –

5.2A:



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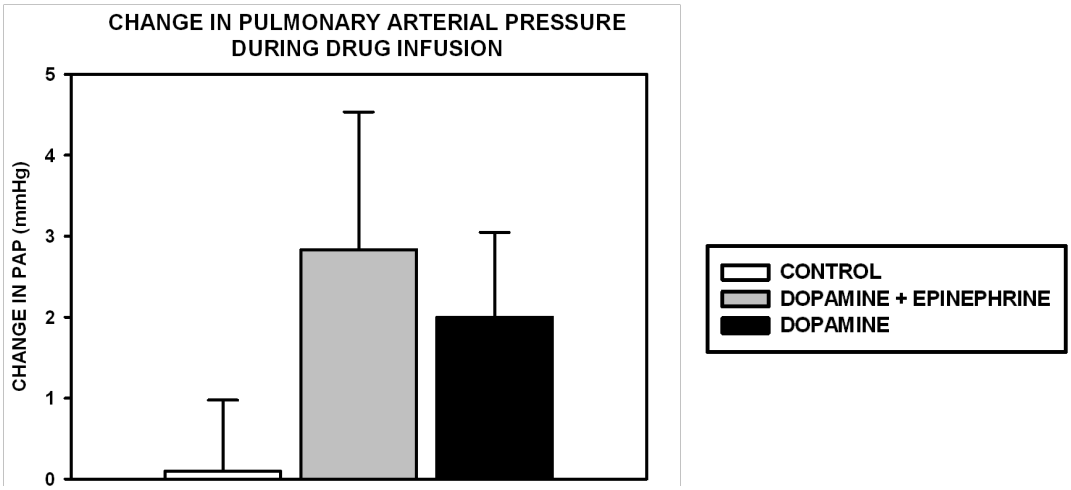

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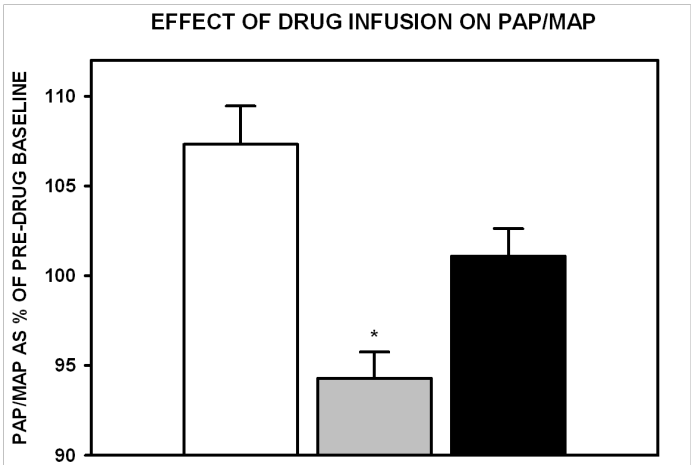
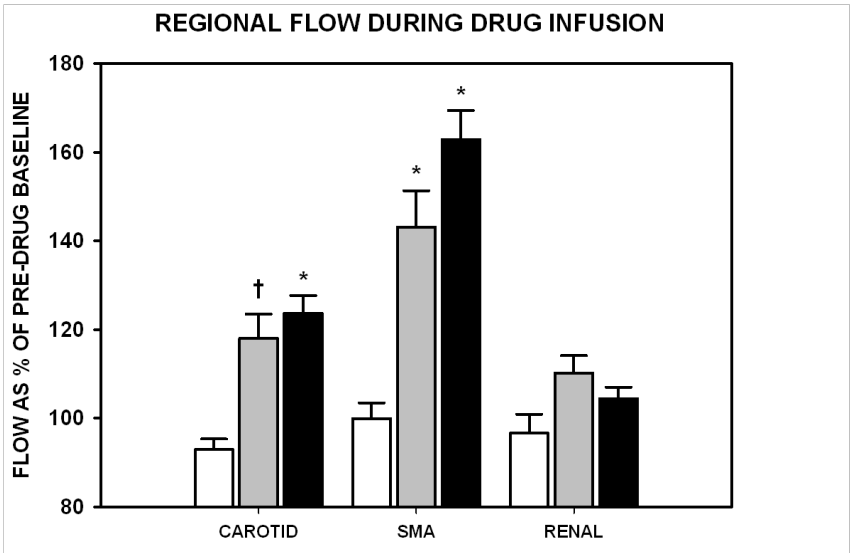


FIGURE 5.3 –

5.3A:



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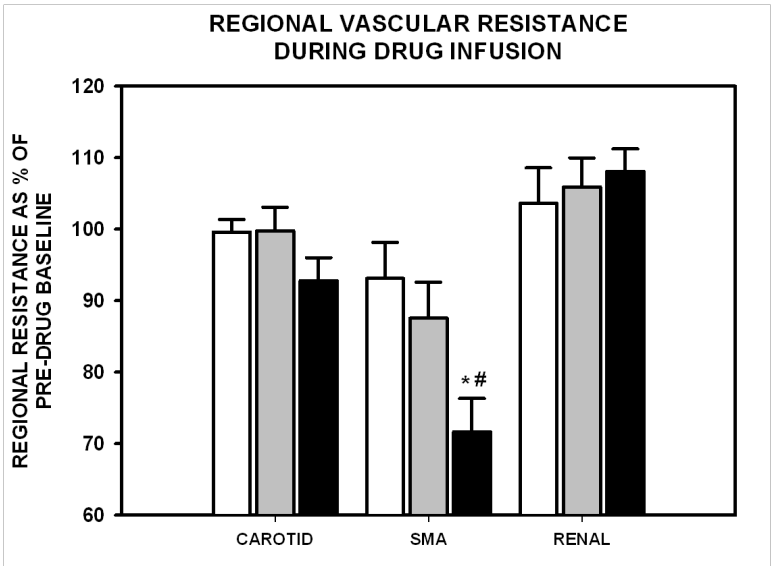
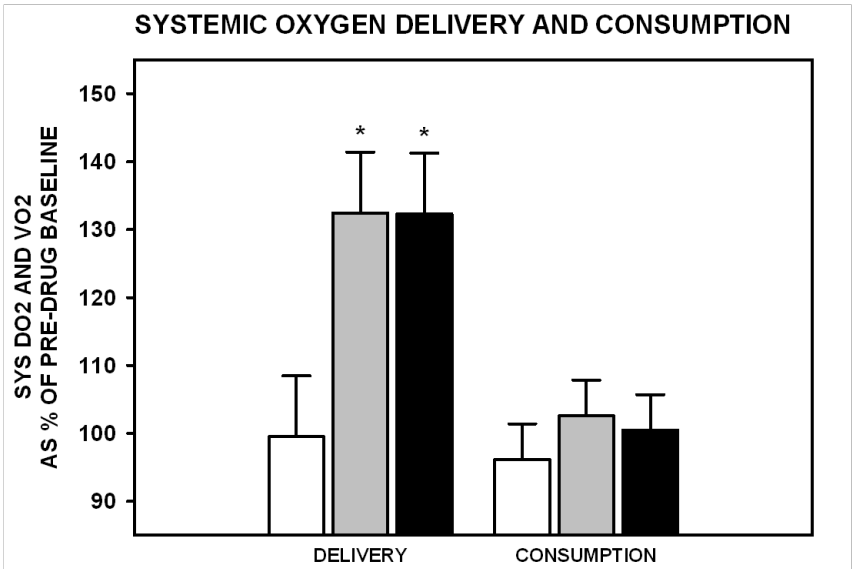


FIGURE 5.4 –

5.4A:



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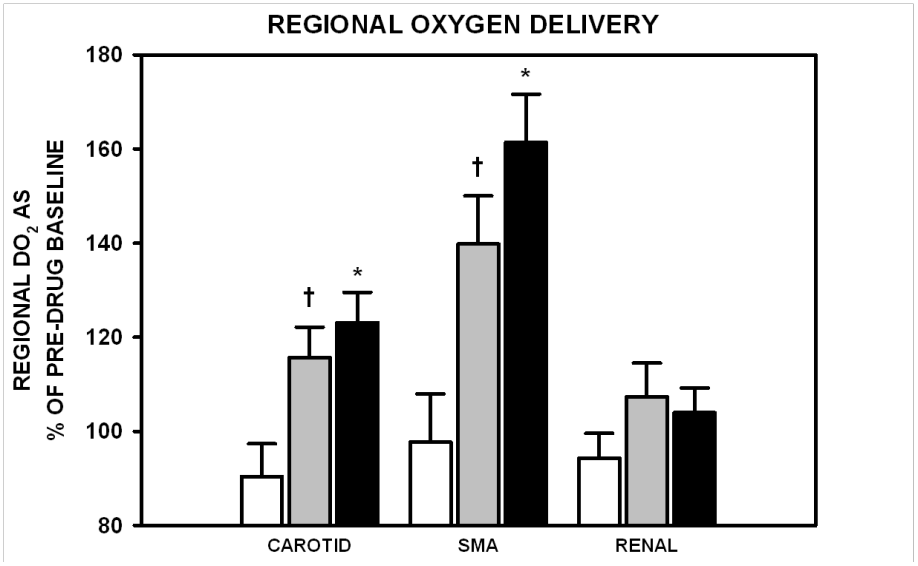

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Chapter 6:
Milrinone Preferred to Levosimendan for Mesenteric Perfusion in Hypoxia-Reoxygenated Newborn Piglets on Dopamine

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Glutathione, troponin-I and tissue lactate assays were performed by Yingqian Li. Malondialdehye assays were performed by Jacek Studzinsky.

Introduction

Asphyxia of the neonate is often complicated by cardiovascular dysfunction.[1,2] Neonates may exhibit hypotension, pulmonary hypertension and shock with end-organ dysfunction.[3,4] Catecholamines like dopamine, epinephrine and dobutamine are used to support the cardiovascular status of these neonates. Dopamine is often used as the initial vasoactive agent infused following resuscitation.[5] High doses of dopamine are typically avoided in neonatal shock given concerns of severe vasoconstriction and tachycardia.[6] It is common to add an inotrope in combination with dopamine infusion. To our knowledge, there is no evidence directly comparing combination treatment strategies.

Following resuscitation, the newborn myocardium may be stunned secondary to reperfusion injury.[7] The commonly used catecholamines have α and β-adrenergic effects. To improve contractility and perfusion, some clinicians may add noncatecholamine agents. Milrinone is a phosphodiesterase III (PDE III) inhibitor with inotropic, lusitropic and vasodilatory properties.[6] Levosimendan is a calcium-sensitizing agent that is still novel to neonatal medicine. Levosimendan functions to improve contractility and reduce afterload via binding to troponin C and opening of adenosine triphosphate-dependent K^+-channels in vascular smooth muscle, respectively.[8,9]

Inotropic medications are frequently used in the setting of newborn shock from asphyxia, albeit, without strong evidence on their use,[4] nor their circulatory effects in regional vasculature. In this study, we used an animal model of neonatal hypoxia-reoxygenation (H-R) to compare the effects on hemodynamics of adding milrinone or levosimendan to a background infusion of dopamine. Additional outcomes assessed include the effect on oxygen metabolism and histologic tissue injury. We hypothesize that the addition of milrinone to dopamine will have beneficial effects on regional perfusion not appreciated with levosimendan combination.

Methods

Animals – With approval of the University Animal Care and Use Committee and following the 2000 Canadian Council on Animal Care guidelines, mixed breed (Duroc / Large White) 1-4 day-old piglets, weighing 1.5-2.5 kg, were obtained from the university swine research farm for experimentation.

Anesthesia – Inhalational anesthesia was induced with 5% isoflurane that was then titrated between 1 and 3% for maintenance. Oxygen saturation was monitored using pulse oximetry (Nellcor, Hayward, CA) and temperature measurement was obtained from a rectal thermometer and maintained from 38.5 to 40°C with a heating underpad and overhead warmer. Once vascular access was established, intravenous anesthesia was substituted for isoflurane with a combination of intravenous midazolam (0.2-1 mg/kg/h) for sedation and fentanyl (5-20 mcg/kg/h) for analgesia. Pancuronium (0.05-0.1 mg/kg/h) was administered for paralysis during surgery. Boluses of fentanyl (10 mcg/kg) and acepromazine (0.25 mg/kg) were used as needed. 5% dextrose (20 ml/h) and 0.9% normal saline (4 ml/h) were infused with boluses of lactated Ringer's solution administered as indicated. Piglets were ventilated mechanically through a definitive airway using pressure control of 20/4 cm H_2O and rates of 18-20 breaths/min (Sechrist Infant Ventilator Model IV-100, Sechrist Industries Inc., Anaheim, CA) with fractionated inspired oxygen concentrations (FiO2) of 0.21-0.25, measured through a MiniOx III oxygen monitor (Catalyst Research, Owings Mills, MD). Oxygen saturations were maintained at 88-100%. Piglets were ventilated to $PaCO_2$ of 35-45 mmHg throughout the protocol.

Surgical Instrumentation – The right femoral vein and artery were cannulated with 5-Fr dual-lumen and single-lumen catheters (Sherwood Medical Co., St. Louis, MO), respectively. The venous line was advanced 13-15 cm to the right atrium to administer fluid and medications, and measure central venous pressure (CVP). The arterial line was positioned in the infrarenal aorta for mean arterial pressure (MAP) monitoring. An airway was established for ventilation using a 3.5 mm inner diameter endotracheal tube inserted through a tracheotomy.

Flow probes (Transonic Systems Inc., Ithaca, NY) were placed at the following positions: a) left common carotid artery (2SS) b)

superior mesenteric artery (SMA) (3SB) via flank through retroperitoneal dissection c) left renal artery (2SB). The ductus arteriosus was ligated through a left thoracotomy and the pulmonary artery was cannulated using a 20-gauge angiocatheter (Insyte-W, Becton Dickinson Infusion Therapy Systems Inc., Sandy, UT) for pulmonary artery pressure (PAP) monitoring. The pulmonary artery was also encircled with a flow probe (6SB) for measurement of pulmonary artery flow as a surrogate for cardiac output.

Stabilization and Monitoring – A bolus of lactated Ringer's solution (10 ml/kg) was administered and 60 min of recovery time provided prior to induction of hypoxia. Heart rate and blood pressures were continuously monitored using a Hewlett Packard 78834A monitor (Hewlett Packard Co., Palo Alto, CA). Flow probe measurements were digitized (Data Translation, ON, Canada) and recorded with Asyst programming software on a personal computer.

Hypoxia-Reoxygenation (H-R) Protocol – Block randomization was used to a surgical sham group or one of three H-R groups. Sham-operated piglets (sham, n=6) were maintained at FiO2 of 0.21-0.25 throughout experimentation (6h). By addition of inhaled nitrogen reducing FiO2 to 0.10-0.15 for 2h, H-R piglets underwent normocapnic alveolar hypoxia with PaO_2 of 20-40 mmHg to produce cardiac dysfunction and hypotension as previously described.[10] Piglets were then resuscitated with 100% oxygen for 30 min and a 10 ml/kg bolus of lactated Ringer's solution. FiO_2 was then maintained at 0.21-0.25 (3.5h).

Following 2h of reoxygenation, H-R piglets received blinded infusions of either placebo (0.9% saline solution – control, n=6) or study drug at a constant rate. Drug groups studied included: D+M-combination dopamine (10 mcg/kg/min; Baxter Corporation, Toronto, ON, Canada) and milrinone (50 μg/kg bolus then 0.5 μg/kg/min infusion; Apotex Inc., Toronto, ON, Canada)(n=6), or D+L-combination dopamine (10 mcg/kg/min) and levosimendan (24 μg/kg bolus then 0.2 μg/kg/min infusion; Abbott Laboratories S.A., Madrid, Spain)(n=6). Drug doses were determined following clinical practice and past studies in our laboratory.[11,12,13] Drug solutions were prepared into standard volume syringes by a laboratory technician not involved in the experimental care of the animals.

Hemodynamic and Oxygen Measurements – Heart rate, MAP, PAP and CVP were recorded continuously and analyzed at set intervals through hypoxia and reoxygenation and at every 30 min during the final 2h of experimentation. Hemodynamic data was recorded as a mean over 2min at each time point prior to systemic and pulmonary arterial blood sampling for blood gas analysis using ABL 700 blood gas analyzer and OSM3 Hemoximeter (Radiometer, Copenhagen, Denmark).

Biochemical Analysis and Histopathology – Arterial samples were analyzed for lactate using the ABL 700 analyzer and plasma levels of porcine cardiac-specific troponin-I were calculated from plasma frozen at baseline, 2h of reoxygenation and 2h of drug treatment using enzyme-linked immunosorbent assay (#2010-4, Life Diagnostics, West Chester, PA).

Piglets were ultimately euthanized using intravenous pentobarbital (100 mg/kg) and, immediately, necropsy was performed for collection of left ventricular and distal small intestinal tissue. Tissue was stored in 10% formalin solution prior to processing into histological slides with hematoxylin and eosin staining. Three separate pathologists (M.B., J.S., D.R.) blinded to treatment allocation interpreted histologic findings using previously described scoring systems for H-R injury.[14,15]

Additional myocardial and intestinal tissue was snap frozen in liquid nitrogen and kept at -80^{o}C. Tissue lactate was determined using NAD enzyme-coupled colorimetric microplate assay. Tissue was crushed and homogenized in 6% perchloric acid (PCA)/ 0.5 mM EGTA on ice, centrifuged and supernatant collected. 5M potassium carbonate was then added slowly in a 1μL:10μL of supernatant ratio. Following precipitation over 30 min, samples were again centrifuged. To the supernatant, glycylglycine buffer, NAD, double-distilled water, glutamate-pyruvate transaminase and lactate dehydrogenase were added and absorbance was read at 340 nm using a microplate spectrophotometer (Spectramax 190; Molecular Devices, Sunnyvale, CA).

Tissue glutathione (GSH) was measured using a commercially available glutathione assay kit (Cayman Chemical, Ann Arbor, Michigan, Catalog #703002). Frozen tissue was crushed and homogenized in buffer containing 0.2M 2-(*N*-morpholino)ethanesulphonic acid, 50mM phosphate and 1mM EDTA at pH 6-7. Following centrifugation, supernatant was deproteinated

with 10% metaphosphoric acid and 4M triethanolamine. Colorimetric microplate assay was then performed after the addition of glutathione reductase, glucose-6-phosphate dehydrogenase, $NADP^+$, 5,5'-dithio*bis*-2-nitrobenzoic acid. Absorbance was read at 405 nm after 25 minutes using the aforementioned spectrophotometer. To measure oxidized GSH (GSSG), reduced GSH was derivatized to GSSG using 2-vinylpyridine. The assay was then carried out using this sample. Oxidative status of GSH was determined through interpretation of the GSSG/GSH ratio. Tissue malonyldialdehyde (MDA) was measured to assess the effect of oxidative stress. Frozen tissues were homogenized 1:10 in phosphate-buffered saline and fluorescence assays were compared with standard concentrations of MDA as described by Ohkawa.[16]

Statistics –SigmaPlot 11 software (Systat Software Inc., 2008) was used for statistical analysis. Continuous hemodynamic data were compared with analysis of variance (one-way and two-way repeated measures) with *post hoc* analysis by Student-Newman-Keuls test. Nonparametric data were analyzed with Kruskal-Wallis test and Dunn's method for *post hoc* intergroup comparison. To focus on drug effect after infusion, percentage change respective to pre-drug baseline was used to analyze data during the drug infusion phase (final 2h). Correlations between variables were performed with Pearson Moment or Spearman test as appropriate. Significance was defined as $p<0.05$ and results are expressed as mean±standard error of mean.

Results

24 of 28 piglets instrumented were analyzed following exclusions for complications related to surgery (1) and hypoxia (3). Animals were aged 2.2±0.2 days and weighed 1.9±0.1 kg. Piglets in the D+L group weighed more than other groups (2.2±0.1 kg; $p < 0.05$ vs. all other). Following stabilization, hemodynamic and metabolic parameters were not different across experimental groups.

Hypoxia and Reoxygenation – After 2h of hypoxia (PaO_2 33±1%)(Table 6.1), H-R piglets demonstrated systemic hypotension, pulmonary hypertension and decreased stroke volume and cardiac index (CI)(all $p<0.05$ vs. sham – Table 6.2). Corresponding reductions were appreciated in systemic oxygen delivery and consumption ($p<0.05$ vs. sham, data not shown). The perfusion of carotid and renal circulation were significantly reduced (both $p<0.05$ vs. sham), though, likely due to atypically low baseline mesenteric flows in sham piglets, only a trend toward reduced mesenteric perfusion was found in H-R piglets following hypoxia (data not shown). Severe metabolic acidosis with associated hyperlactatemia was present in all H-R piglets (both $p<0.05$ vs. sham – Table 6.1).

After 2h of reoxygenation, H-R piglets had MAP of 46±2 mmHg ($p>0.05$ vs. sham). Stroke volume was depressed (58±3% of normoxia baseline; D+M and D+L $p<0.05$ vs. sham) though, no differences were appreciated in CI, systemic oxygen delivery or consumption relative to sham. Carotid perfusion was reduced in H-R piglets (60±4% of normoxia baseline; $p<0.05$ vs. sham), whereas, mesenteric and renal perfusion (84±8% and 97±11% of normoxia baselines, respectively) was not significantly different from sham. Metabolic acidosis and hyperlactatemia persisted following reoxygenation (both $p<0.05$ vs. sham – Table 6.1). Troponin levels were also elevated in all H-R piglets following reoxygenation ($p<0.05$ vs. sham – Table 6.1).

Systemic and Pulmonary Hemodynamic Effects – D+M and D+L piglets increased heart rate during drug infusion ($p<0.05$ vs. control, Figure 6.1A). D+M piglets had nearly significant increases in stroke volume over the course of infusion ($p=0.075$ vs. control) whereas D+L only showed inotropic effects very early during infusion. The combined effect offered an increase in CI for

both D+M and D+L ($p<0.05$ vs. control, Figure 6.1B/C), but MAP did not change. No disparate effect on PAP was appreciated either, though, pulmonary vascular resistance decreased with medication infusion in both groups ($p<0.05$ vs. control). Considering MAP and PAP together, there were no effects on the PAP/MAP ratio during the drug infusion period. Systemic vascular resistance was reduced ($p<0.05$ vs. control) yielding improvements in systemic oxygen delivery with D+M and D+L treatment ($p<0.05$ vs. control), whereas, no effect on oxygen consumption was appreciated (Figure 6.2).

Regional Hemodynamic Effects – Treatment with D+M and D+L improved carotid perfusion with increased CAFI and carotid oxygen delivery with reduced carotid vascular resistance (all $p<0.05$ vs. control – Figure 6.3). D+M but not D+L piglets had increases in SMAFI and mesenteric oxygen delivery (both $p<0.05$ vs. control) with no effect on vascular resistance (Figure 6.3). There were no differences appreciated in effect on renal perfusion.

Biochemical Effects – D+M piglets' myocardium had lower GSSG/GSH ratio ($p=0.051$ vs. control, Figure 6.4). Interestingly, in D+L piglets alone, the myocardial GSSG/GSH ratio correlated strongly with CI ($r=0.9$, $p=0.01$). There were no differences in small intestine GSSG/GSH or tissue MDA. All H-R groups attenuated plasma lactate levels with no difference from sham ($p>0.05$) showing that at the doses studied, the treatments have no detrimental effects on tissue oxygenation and metabolism. H-R piglets also had similar pH, troponin, degree of histologic ischemia-reperfusion injury and left ventricle and small intestinal lactate.

Discussion

Asphyxiated neonates may present with myocardial depression and reduced tissue perfusion following resuscitation. There is limited evidence on the use of vasoactive medications in newborns,[4] particularly following asphyxia. We demonstrated that the addition of milrinone or levosimendan to a background moderate-dose dopamine infusion in H-R piglets improves systemic circulation with increases in CI and oxygen delivery. Similarly, carotid perfusion improved with the combination therapies administered. Milrinone, in particular, provided two benefits not observed with levosimendan addition. Milrinone combination treatment improved mesenteric perfusion and attenuated oxidative stress in the newborn piglet myocardium.

The majority of neonatal literature on milrinone use focuses on the treatment of low cardiac output state following bypass surgery and pulmonary hypertension.[17,18,19,20] Chang et al. demonstrated improvement in heart rate and cardiac index with the administration of milrinone in post-bypass surgery infants receiving dopamine infusion.[17] No increases in myocardial oxygen consumption were seen and atrial filling pressures were decreased, potentially due to improved relaxation of the relatively less compliant myocardium following reperfusion. Reduction of vascular resistance and afterload also contribute to circulatory improvements with milrinone.[21] Similar findings were recognized in a recent animal model where the increase in afterload while on dopamine following bypass surgery was prevented through administration milrinone or levosimendan, thereby preserving cardiac output.[19] Again, there were no detrimental effects on myocardial oxygen consumption. In patients with acute heart failure, levosimendan administration improved ejection fraction and abated the need for other inotropes.[22,23] Nevertheless, the majority of evidence regarding levosimendan use in the newborn population is limited to case reports or series.[22,23,24,25]

In past studies, we have shown that milrinone, administered alone, improves cardiac output and regional perfusion in hypoxia-reoxygenated piglets.[10,11,12] We have also demonstrated improvement in cardiac output with afterload reduction following levosimendan treatment of hypoxia-reoxygenated newborn piglets.[13]

This study revealed similar findings on systemic circulation with chronotropic effects and improved cardiac output with both treatment regimens. Additionally, vascular resistance was decreased through the addition of both milrinone and levosimendan, thereby reducing myocardial wall stress and limiting any effect on myocardial oxygen consumption. Indeed, no differences were appreciated in myocardial lactate levels or on systemic oxygen consumption. Not appreciated in our previous study of milrinone administered alone,[10] D+M piglets' myocardium exhibited less oxidative stress, as shown by the GSSG/GSH ratio, indicative of reduced reperfusion injury and presumably, preserved contractility. Further corroborating this benefit with D+M was the worsening of oxidative stress (GSSG/GSH ratio) with CI improvement in the D+L piglets. However, MDA levels were similar across groups, indicating limited effect on the production of that particular free radical. Troponin also was no different across H-R groups. This may be due to the temporal delay in detection of elevated troponin levels. Indeed, troponin values increased only following the reoxygenation period. As such, longer duration of data collection would be necessary to determine if milrinone addition is more protective to the myocardium.

Both milrinone and levosimendan have been described in the treatment of newborns with pulmonary hypertension.[19,20,25,26,27] We observed a reduction in pulmonary vascular resistance with the infusion of both regimens. Despite this and the reduction in right-heart afterload, no effect on pulmonary arterial pressure was appreciated. Although our piglets exhibited significant pulmonary hypertension during hypoxia and early reoxygenation, these increases were attenuated prior to infusion of medications.

To our knowledge, none of the clinical studies of milrinone and levosimendan use have examined the regional effects of these agents. Administered alone in H-R newborn piglets, milrinone benefits the mesenteric and carotid circulation.[10,11,12] Levosimendan administered as the sole agent in a similar animal model, on the other hand, has no effect on the regional circulation.[13] Used in combination with dopamine, both agents improved carotid perfusion. Similar to monotherapy studies, addition of milrinone to dopamine also improved mesenteric circulation. There were, however, no differences in intestinal lactate between H-R groups

and all groups similarly reduced plasma lactate to normal levels following 4 hours of reoxygenation.

Levosimendan appears to be a reasonable agent to add to moderate-dose dopamine in asphyxiated newborns suffering shock. Benefits to systemic circulation were expressed without detrimental effects on systemic oxygen metabolism; however, worsening of oxidative stress in the myocardium is a possibility. Addition of milrinone, instead, provides similar systemic effects and also improves perfusion of the gut. Moreover, milrinone combination treatment has favourable effects on the myocardium, with less oxidative stress. Further studies are required to assess the use of these agents over a longer duration of infusion. Given the improvements in myocardial oxidation status with milrinone, perhaps improvements in cardiac indices will prove more substantial after prolonged study.

TABLE 6.1 – Arterial Blood Gas, Plasma Lactate and Troponin

	Baseline	End-Hypoxia	Pre-Drug	End-Drug
Time	0 min	120 min	240 min	360 min
pH				
Sham	7.47±0.04	7.45±0.02	7.46±0.01	7.45±0.02
Control	7.44±0.02	7.02±0.02*	7.38±0.02*	7.40±0.02
D+M	7.46±0.03	7.01±0.03*	7.39±0.01*	7.40±0.02
D+L	7.39±0.01	7.03±0.02*	7.36±0.01*	7.36±0.02*
PaO_2 (mmHg)				
Sham	87±6	72±3	71±3	67±2
Control	83±4	38±3*	66±1	80±10
D+M	78±4	38±6*	71±7	79±7
D+L	71±2*	37±3*	64±2	72±3
HCO_3 (mmol/L)				
Sham	27±2	27±2	28±1	27±1
Control	26±1	9±1*	22±1*	25±2
D+M	27±1	10±1*	23±1*	24±1
D+L	23±1	9±1*	22±1*	22±1*
LACTATE (mmol/L)				
Sham	3.7±0.4	2.6±0.3	1.8±0.3	1.7±0.2
Control	2.7±0.2	13.2±1.2*	4.1±0.4*	2.0±0.2
D+M	3.3±0.4	14.0±0.8*	4.2±0.3*	2.4±0.3
D+L	3.3±0.3	13.6±0.8*	3.2±0.4*	2.1±0.3
TROPONIN (μg/L)				
Sham	0.13±0.01	0.14±0.01	0.12±0.01	0.11±0.01
Control	0.11±0.03	0.21±0.07	0.66±0.16*	0.67±0.16*
D+M	0.10±0.03	0.13±0.03	0.62±0.18*	0.72±0.17*
D+L	0.12±0.04	0.18±0.04	0.79±0.19*	0.79±0.18*

* $p < 0.05$ vs. Sham

TABLE 6.2 – Systemic Hemodynamic Parameters

	Baseline	End-Hypoxia	Pre-Drug	End-Drug
Time	0min	120min	240min	360min
HEART RATE (beats/min)				
Sham	173±11	171±8	195±11	207±11
Control	178±8	210±10*	229±16	209±6
D+M	166±4	224±9*	232±15*	251±13*#
D+L	190±15	215±5*	229±9	253±19*#
MAP (mmHg)				
Sham	77±3	63±4	54±2	48±2
Control	75±6	29±1*	48±4	41±2
D+M	75±6	32±1*	45±4	38±3
D+L	71±4	29±1*	44±5	41±3
PAP (mmHg)				
Sham	24±1	24±1	26±1	29±2
Control	25±2	30±2*	27±1	27±1
D+M	27±1	38±2*	27±1	28±1
D+L	25±2	34±4*	27±3	26±2
CARDIAC INDEX (ml/kg/min)				
Sham	224±30	189±30	199±26	176±21
Control	229±18	76±6*	179±17	176±14
D+M	217±24	72±9*	141±10	183±15†
D+L	208±16	76±9*	148±19	177±17†

* $p < 0.05$ vs. Sham

# $p < 0.05$ vs. Control

† change over infusion from respective pre-drug baseline, $p < 0.05$ vs. Control (Fig 1C)

FIGURE LEGENDS:

FIGURE 6.1

Changes in (A) heart rate, (B) stroke volume index and (C) cardiac index during infusion of placebo control (white), dopamine with milrinone (grey) and dopamine with levosimendan (black). Stroke volume index and cardiac index are expressed as mean percentage change over duration of infusion.

* $p < 0.05$ vs. control (2-way repeated measures ANOVA).

$p = 0.075$ vs. control (2-way repeated measures ANOVA).

FIGURE 6.2

Changes in (A) systemic oxygen delivery and (B) consumption during infusion of placebo control (white), dopamine with milrinone (grey) and dopamine with levosimendan (black). Data are expressed as mean percentage change over duration of infusion.

* $p < 0.05$ vs. control (2-way repeated measures ANOVA).

FIGURE 6.3

Changes in carotid and mesenteric (A) flow, (B) oxygen delivery and (C) vascular resistance during infusion of placebo control (white), dopamine with milrinone (grey) and dopamine with levosimendan (black). Data are expressed as mean percentage change over duration of infusion.

* $p < 0.05$ vs. control (2-way repeated measures ANOVA).

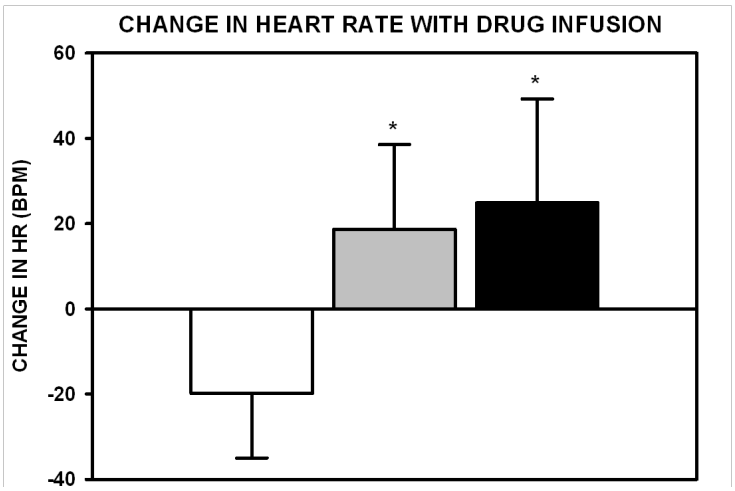
FIGURE 6.4

Effect of infusion of placebo control, dopamine with milrinone (D+M) and dopamine with levosimendan (D+L) on myocardial (left ventricle) GSSG/GSH ratio. A lower ratio indicates less oxidative stress.

* $p = 0.051$ vs. control (oneway ANOVA).

FIGURE 6.1

6.1A:



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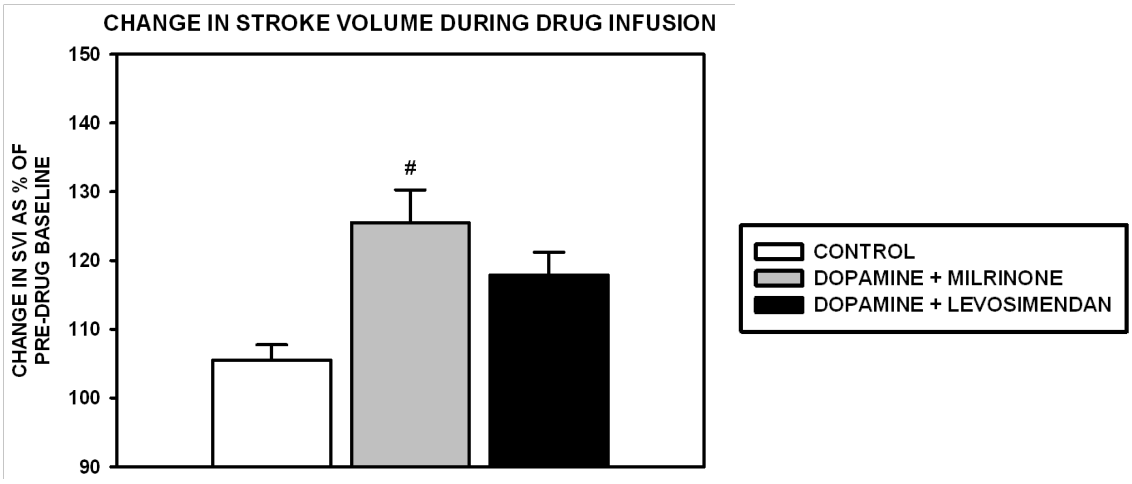

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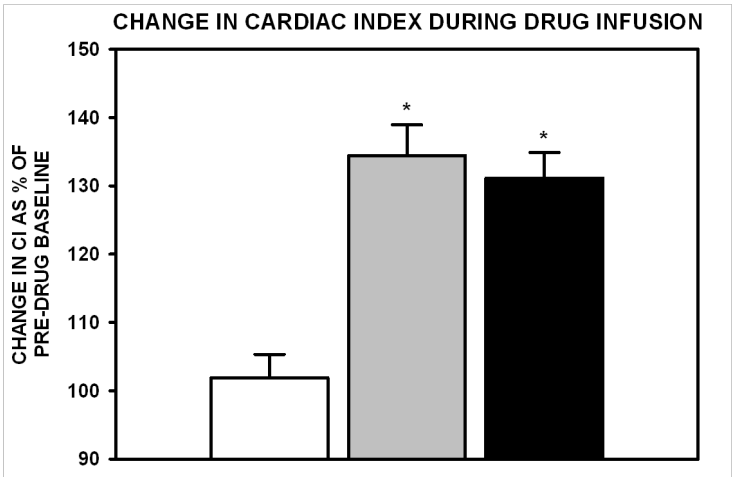
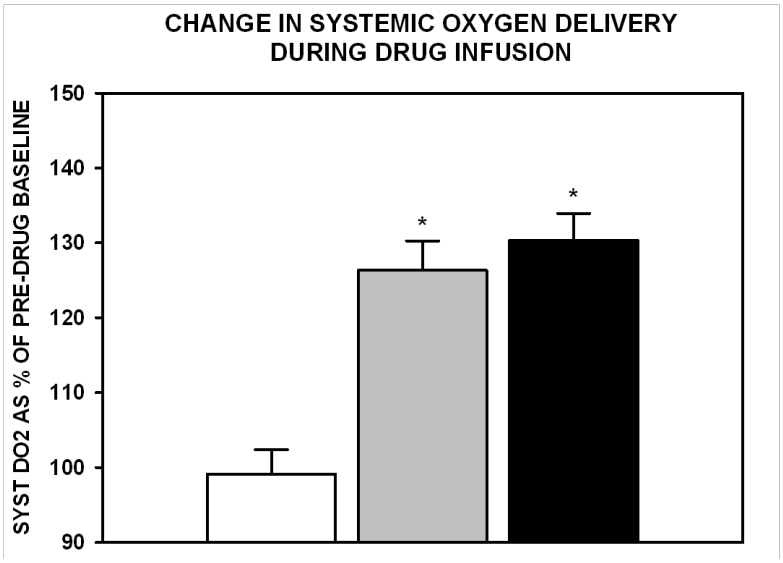


FIGURE 6.2

6.2A:



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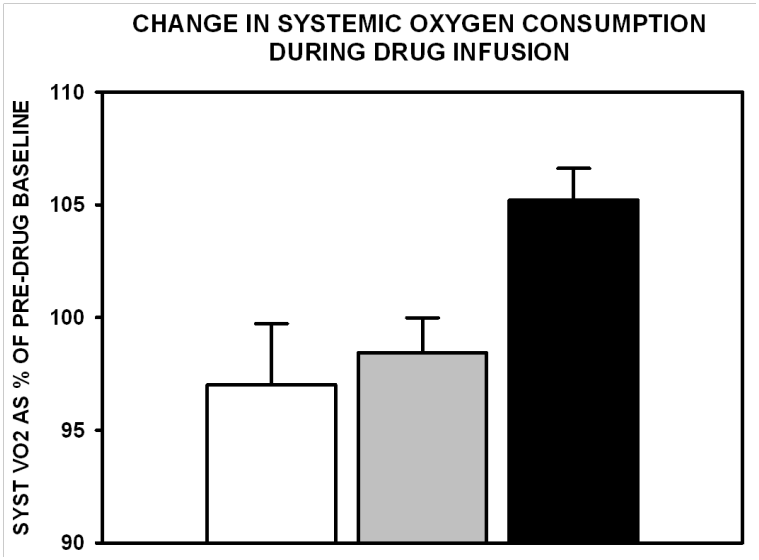
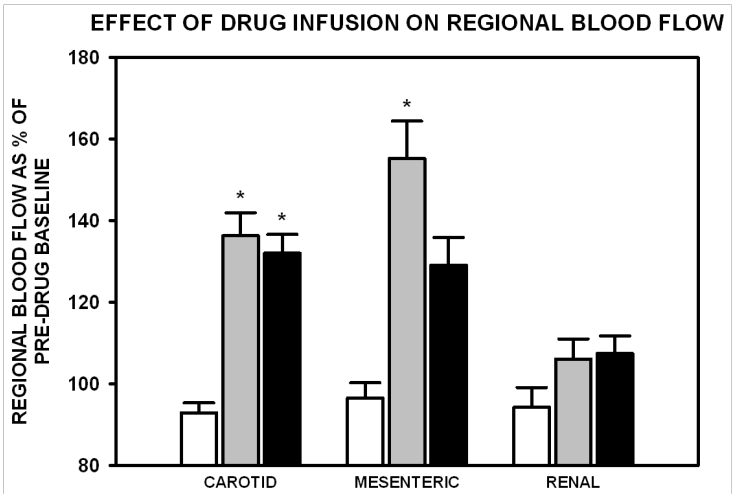


FIGURE 6.3

6.3A:



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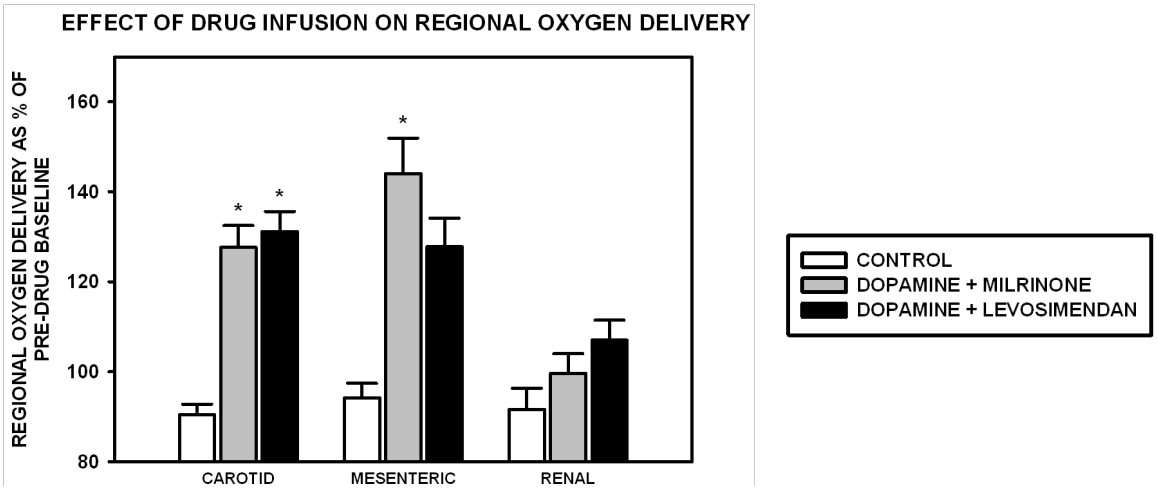

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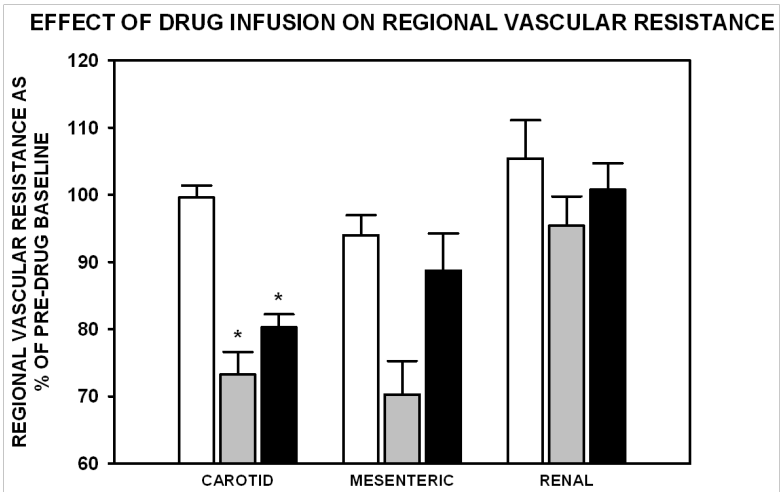
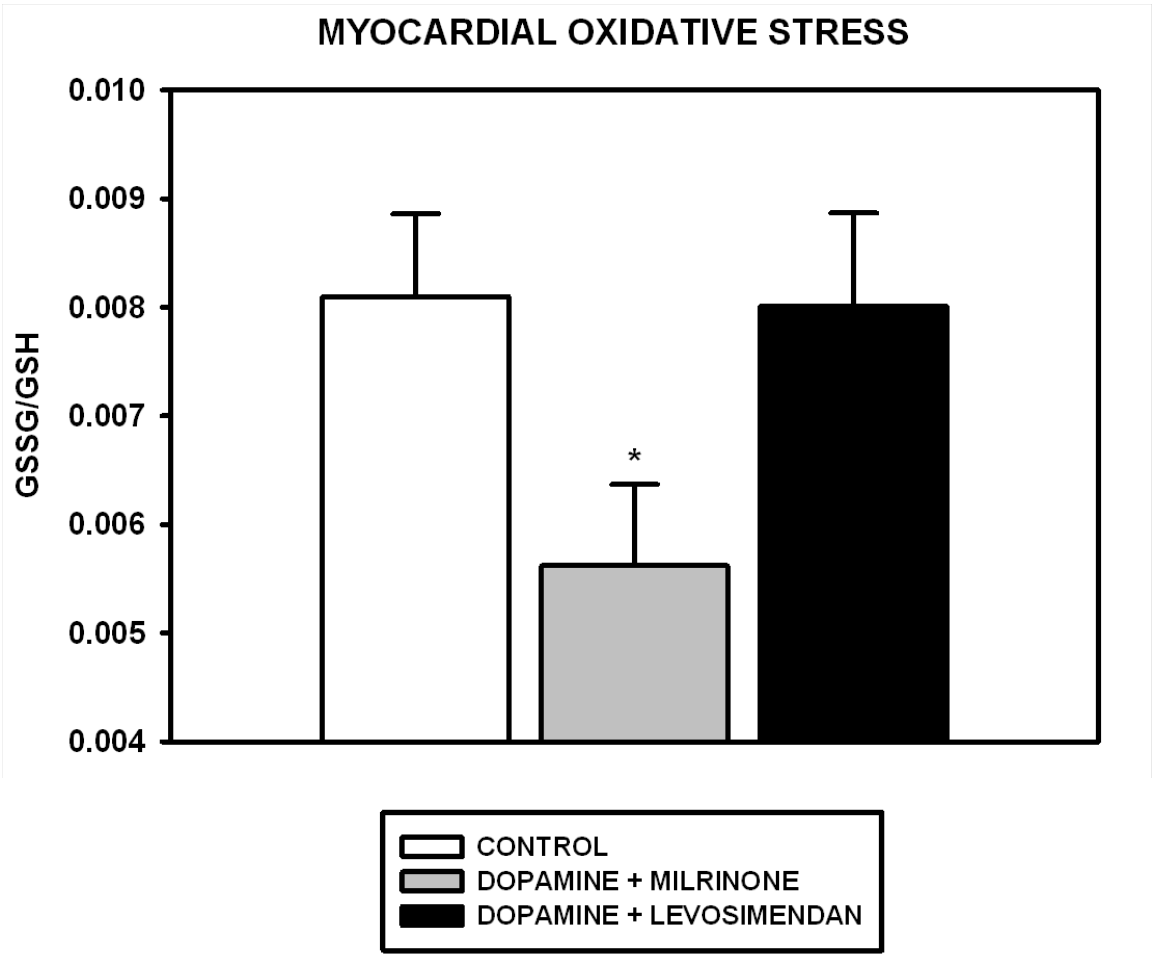


FIGURE 6.4

6.4:


MYOCARDIAL OXIDATIVE STRESS
GSSG/GSH
0.010
0.009
0.008
0.007
0.006
0.005
0.004
*
CONTROL
DOPAMINE + MILRINONE
DOPAMINE + LEVOSIMENDAN

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Chapter 7: Additional Discussion and Conclusion

The preceding chapters presented evidence in the use of high-dose dopamine and combinations of dopamine with epinephrine, milrinone and levosimendan in asphyxiated newborn piglets. High-dose dopamine was found to improve systemic hemodynamics while increasing perfusion of the carotid and mesenteric vascular territories. The combination of dopamine and epinephrine also improved systemic hemodynamics and reduced the ratio of PAP:MAP. Neither of the treatment strategies had attenuating effects on systemic or pulmonary vascular resistance. Alternatively, treatment with dopamine and a non-catecholamine agent, milrinone or levosimendan improved both the aforementioned vascular resistances (see Figure 7.1). This vasodilatory effect and afterload reduction, in particular, combined with the increase in cardiac contractility with milrinone combination treatment underscores the drug's classification as an inodilator.

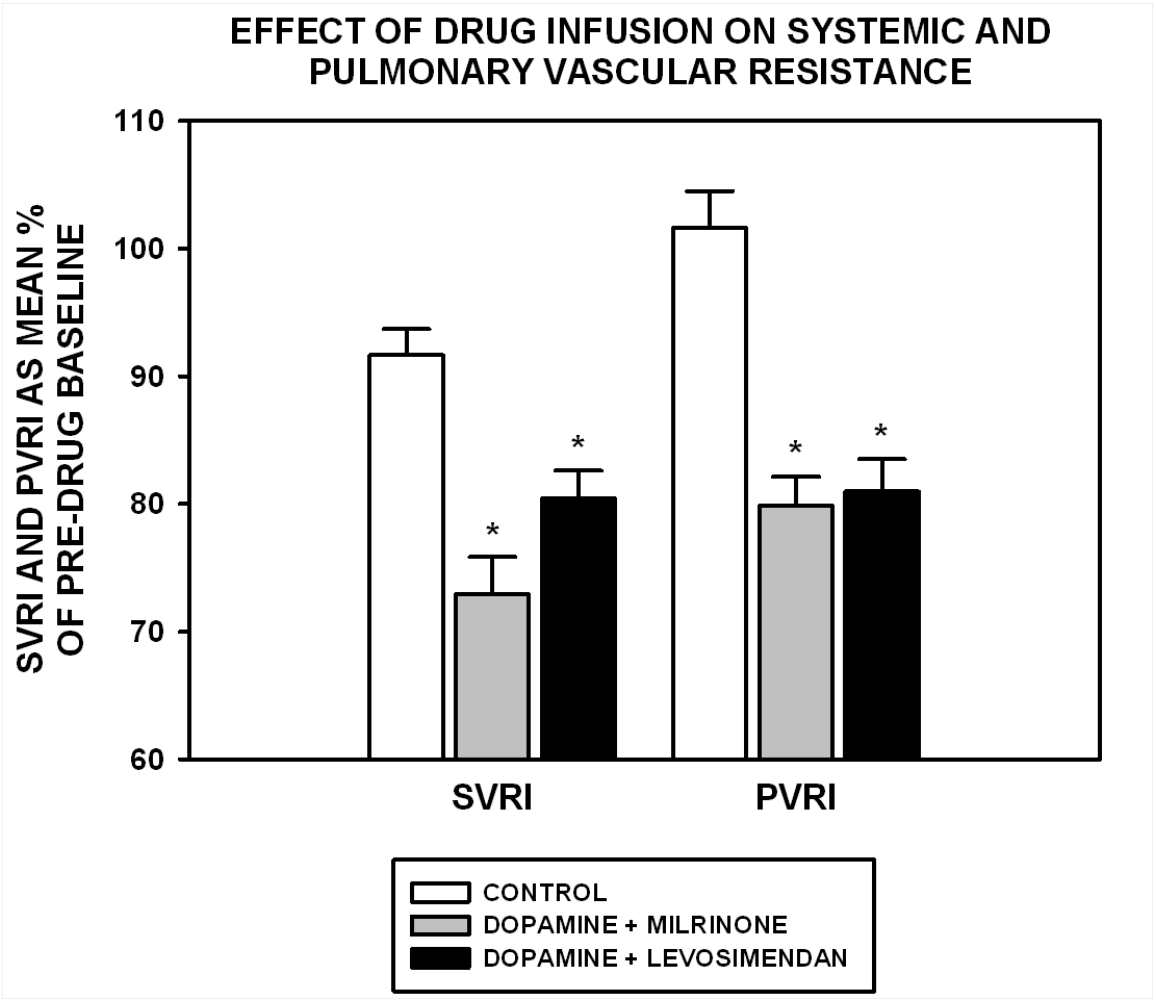
To understand better the relationship between biochemical parameters, correlation coefficients were determined. There were no significant correlations between plasma troponin or lactate, ventricular lactate, glutathione ratio or histologic injury in either of the studies presented. Similarly negative findings were appreciated in the analysis of intestinal lactate, glutathione ratio and histologic injury. The median scores for histologic ischemia-reperfusion injury were not different between groups.

Dobutamine, an agent commonly used to treat cardiovascular dysfunction, was also studied in the set of experiments performed for this work. Unfortunately, complications related to the extent of hypoxia and the development of acidosis precluded the analysis of data from this study group. Further studies are required to establish the role of dobutamine addition to dopamine infusion in the treatment of asphyxiated newborn piglets.

Typical cardiovascular support in a neonatal intensive care unit demands the titration of vasoactive agents over short to extended periods of time. The aforementioned studies focused on the acute infusion of these medications. The total drug infusion period was limited to only two hours. Further studies are required to determine if the effects appreciated evolve or continue with longer durations of therapy. Logistically, such studies are far more demanding. Measures must be taken to ensure adequate ventilation and hydration, and maintenance of the biochemical status of the newborn piglets under study. Early attempts at this proved

FIGURE 7.1

7.1:



* p < 0.05 vs. Control

unsuccessful prior to the commencement of the experiments for this thesis. Also, vasoactive agents are used clinically at a variety of doses, not fixed levels as studied for the purpose of this work. The titration of infusions to effect would require a more substantial follow-up with, again, longer durations of recording in order to document the effect from any change in dose. Nevertheless, such data would prove invaluable to further understand the benefits and detriments of such invasive therapies.

Our research utilizes an animal neonatal model to study a human perinatal problem. Underlying this are two limitations that must be considered prior to drawing inferences regarding the ultimate care of patients. Using animals to study human disorders has proven useful in all fields of medical research. Swine offer marked anatomical similarity to humans and their large size enables the collection of multiple parameters from individual animals allowing more complete analysis. Nevertheless, the effect of such interventions may differ when examined in the human population. Also, vasoactive medications exert their effects via the stimulation of various receptors at the cellular level. Studies have documented differences in the expression of these receptors in animals over the early weeks of life. As a result, the effect of vasoactive agents may prove different following a few days of life when compared with the initial perinatal period.

Asphyxia of the newborn is a global problem with limited literature available to guide therapy. Newborns suffering complications with oxygenation at the time of birth exhibit dysfunction of the cardiovascular system with potentially morbid implications for end-organ systems. Central nervous system dysfunction and gastrointestinal and renal system injury as a result of asphyxia lead to the use of a great deal of health care resources. Research into the ideal treatment of this problem provides valuable information to direct neonatal care.

With the research presented in this dissertation, we have demonstrated meaningful evidence regarding the use of vasoactive medications to support asphyxiated neonates. High-doses of any vasoactive agent, in particular, dopamine, may be complicated by the potential for detrimental side effects. Past studies have demonstrated increases in vascular resistance and heart strain with high doses of dopamine. Contrary to the indication for administering dopamine, such effects would limit tissue perfusion.

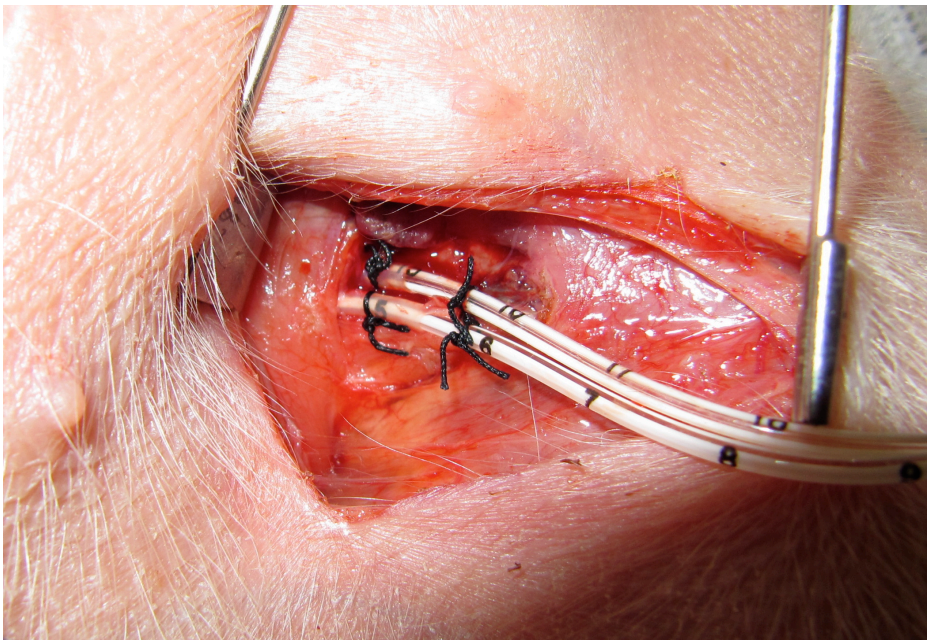
To date, neonatal intensive care practice is highly variable regarding the infusion of vasoactive agents. Two basic strategies exist: a single-agent approach where the initial agent is titrated upward to high-doses and a multiple-agent approach where different agents are used at less extreme doses to obtain a combination of effects. We showed that the infusion of high-dose dopamine (20 mcg/kg/min) had beneficial effects on both the systemic and regional circulation of asphyxiated newborn piglets. Improvements were observed on cardiac output and vascular resistance with concomitant increases in the perfusion of both cerebral and gastrointestinal vascular regions. Such conclusions quell the belief that dopamine use alone at high doses may be detrimental to newborn health. In fact, with the marked reduction of mesenteric vascular resistance and equivocal effects on intestinal morphologic and biochemical status, we recommend high-dose dopamine use in the face of clinical concern regarding gastrointestinal injury.

Two agents less commonly used in the neonatal intensive care setting are milrinone and levosimendan. These agents provide improvements in cardiac output via non-adrenergic mechanisms. However, the majority of literature on their use focuses on a problem different than that studied in this work. Clinicians favoring the approach of administering combinations of agents for asphyxiated newborns may use these agents to target the depressed cardiovascular function and stunned myocardium. In comparing these two agents when added to a background infusion of dopamine, the most likely initially infused agent, we have shown differing effects. Although the systemic circulation benefits equivocally from either agent, we appreciated significant improvements in the mesenteric perfusion with the addition of milrinone and not levosimendan. These conclusions are similar to those drawn from studies in our lab assessing their use in isolation. Additionally, treatment with milrinone improved the oxidative status of the newborn piglet myocardium. Lower oxidized-to-reduced gluathione ratio is representative of reduced levels of reactive oxygen species consuming one of the major intrinsic myocardial antioxidants, glutathione. Despite the absence of effect on tissue lactate levels and plasma troponin, this may have implications on newborn myocardial function after prolonged use.

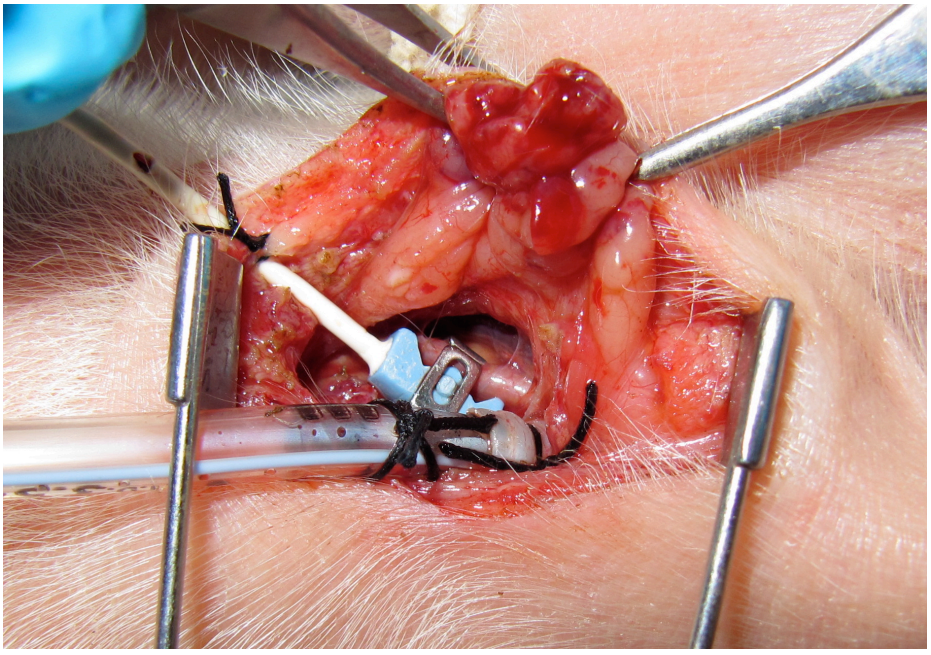
This work has demonstrated variability in the effects of different vasoactive medications commonly used in the neonatal intensive care setting. Despite the known limitations of this short-term animal model, we were able to draw valuable inferences that may better direct clinicians confronting this global clinical problem.

APPENDIX 1: INSTRUMENTATION

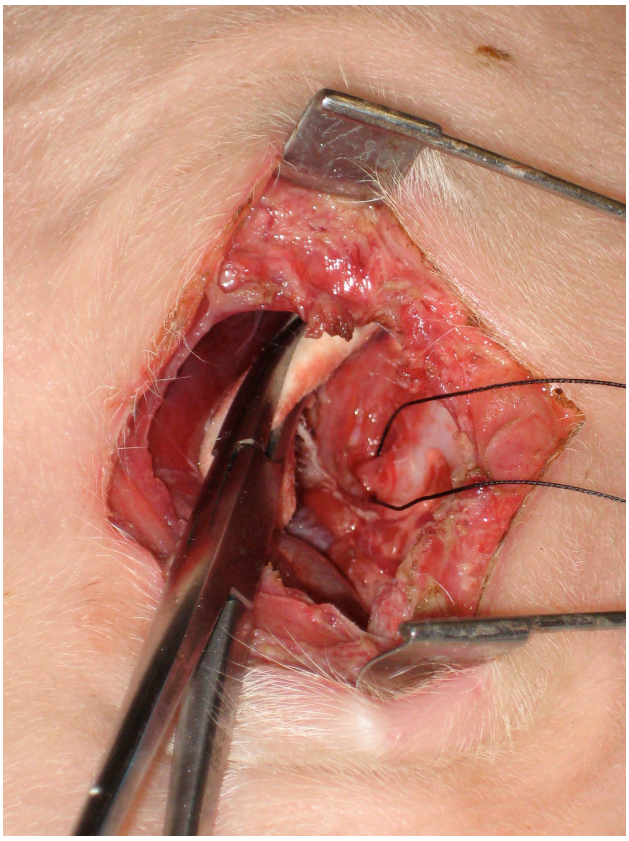
FEMORAL VASCULAR ACCESS



TRACHEOSTOMY AND COMMON CAROTID ARTERY FLOW PROBE



RETROPERITONEAL EXPOSURE – SUPERIOR MESENTERIC ARTERY



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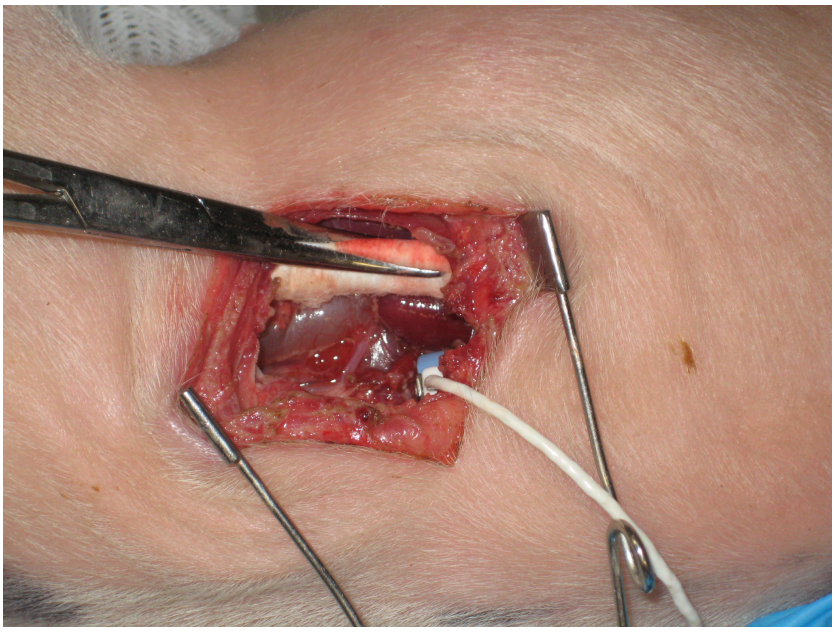
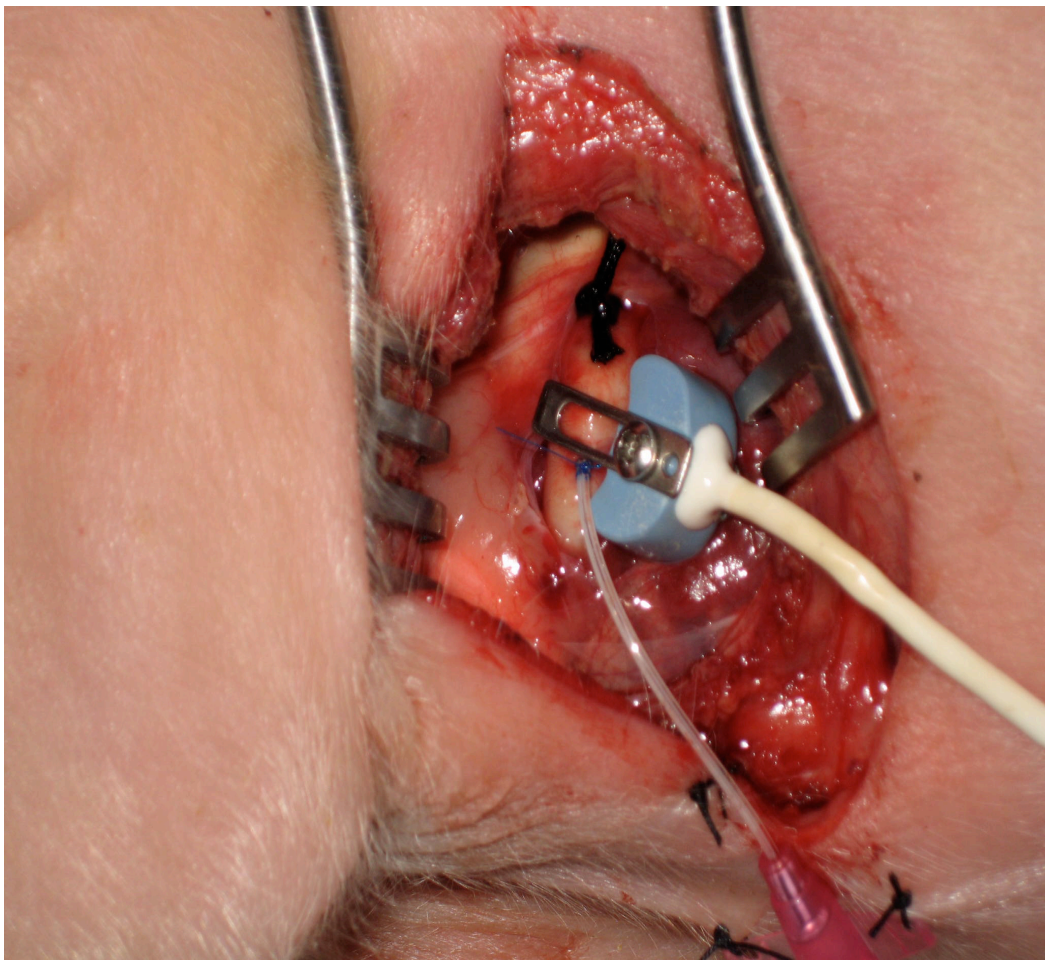


THORACOTOMY WITH PULMONARY ARTERY FLOW PROBE, PULMONARY ARTERY CATHETER AND LIGATION OF DUCTUS ARTERIOSUS



APPENDIX 2 – HYPOXIA-REOXYGENATION PROTOCOL

Animal Preparation:

Instrumentation	60 – 120 minutes
Post-operative recovery	40 – 60 minutes

Data Collection:

TIME (MIN)	ACTION
0	Start hypoxia
	– FiO2 0.10-0.15
120	End hypoxia
	Start reoxygenation – FiO2 1
150	Reduce FiO2 to 0.21-0.25
240	Start of drug infusion
360	End of drug infusion
	Euthanasia of piglet
	Necropsy and tissue collection

APPENDIX 3 – CALCULATIONS

Cardiac index (CI)

Pulmonary artery flow / weight

Stroke volume index

CI / HR

Systemic vascular resistance index

(MAP – CVP) / CI

Carotid artery flow index

Carotid artery flow / weight

Superior mesenteric artery (SMA) flow index

SMA flow / weight

Renal artery flow index

Renal artery flow / weight

Systemic oxygen delivery

$(CI/1000)(SaO_2/100)(1.34)(Hgb)$

Systemic oxygen consumption

$(CI/1000)((SaO_2 - \text{Mixed venous } SO_2)/100)(1.34)(Hgb)$

Regional oxygen delivery

$(\text{Regional flow index}/1000)(SaO_2/100)(1.34)(Hgb)$

Regional vascular resistance index

(MAP – CVP) / Regional flow index