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# Effect of Procedural Pain on Purines, MDA and Allantoin in Premature Infants

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LOMA LINDA UNIVERSITY  
School of Medicine  
in conjunction with the  
Faculty of Graduate Studies

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The Effect of Procedural Pain on Purines, MDA and Allantoin  
in Premature Infants

by

Megan Sue Holden

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A Dissertation submitted in partial satisfaction of  
the requirements for the degree of  
Doctor of Philosophy in Biochemistry

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June 2013

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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## ABBREVIATIONS

$^1\text{H-NMR}$	Proton nuclear magnetic resonance
2-AP	2-Aminopurine
ABST	2,2'-azino-bis(3-ethylbenzthioazoline-6-sulphonic acid)
ADP	Adenosine diphosphate
AGA	Appropriate for gestational age
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHT	Butylated hydroxyl toluene
BUN	Blood urea nitrogen
CPAP	Continuous positive airway pressure
Cr	Creatinine
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
EGA	Estimated gestational age
F	Fructose
$\text{FiO}_2$	Fraction of inspired oxygen
G	Glucose
GCMS	Gas chromatography mass spectrometry
GLUT	Glucose transporter
$\text{H}^+$	Hydrogen ion
$\text{H}_2\text{O}_2$	Hydrogen peroxide

HCl	Hydrochloric acid
HClO <sub>4</sub>	Perchloric acid
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HIE	Hypoxic ischemic encephalopathy
HPLC	High pressure liquid chromatography
IMP	Inosine monophosphate
ISF	Interstitial fluid
IV	Intravenous
K <sub>2</sub> CO <sub>3</sub>	Potassium carbonate
LGA	Large for gestational age
MDA	Malondialdehyde
MMDA	Methyl malondialdehyde
MRI	Magnetic resonance imaging
MTBSTFA	N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide
NADH	Reduced Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NHPA	3-nitro-4-hydroxyphenylacetic
NICU	Neonatal intensive care unit
NIPPV	Noninvasive positive pressure ventilation
NMDA	N-methyl-D-aspartate
NNS	Non-nutritive sucking
NOS	Nitric oxide synthase

NS	Not Significant
O <sub>2</sub>	Oxygen
O <sub>2</sub> <sup>-</sup>	Superoxide anions
PaO <sub>2</sub>	Partial pressure of oxygen in arterial blood
P <sub>i</sub>	Inorganic Phosphate
PIPP	Premature infant pain profile
PN	Poor nipple
PNHB	Poor nipple plus hyperbilirubinemia
PNRD	Poor nipple plus early respiratory disease
PO <sub>2</sub>	Partial pressure of oxygen
RA	Room air
RD	Respiratory disease
RDS	Respiratory distress syndrome
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SGA	Small for gestational age
SGLT	Sodium-glucose cotransporter
SNAPPE-II	Score for neonatal acute physiology-perinatal extension II
TCA	Tricarboxylic acid cycle
TDP	Tissue damaging procedure
UV-Vis	Ultraviolet-visible light
$\chi^2$	Chi-square
XO	Xanthine oxidase



## ABSTRACT OF THE DISSERTATION

The Effect of Procedural Pain on Purines, MDA and Allantoin in  
Premature Infants

by

Megan Sue Holden

Doctor of Philosophy, Graduate Program in Biochemistry  
Loma Linda University, June 2013  
Dr. Penelope Duerksen-Hughes, Chairperson

Identifying and measuring the biochemical effects of procedural pain in infants is a continuing challenge for clinicians. To address this issue we evaluated the relationship between procedural pain, ATP utilization (hypoxanthine and uric acid), and oxidative stress (MDA and allantoin) in premature infants. Through these studies we found that 1) a single heel-lance significantly increases plasma uric acid in rabbit kits, 2) tape removal increases MDA and prevents a decrease in uric acid in the plasma of premature infants, and 3) a single dose of oral sucrose before a heel-lance is sufficient to significantly increase plasma markers of ATP utilization and oxidative stress. These studies indicate that, in premature infants, ATP utilization and oxidative stress are altered by procedural pain and that oral sucrose, a common analgesia, may increase ATP breakdown.

Next, we examined clinical factors that need to be considered when using the urinary concentrations of hypoxanthine, xanthine, uric acid, and allantoin to evaluate the biochemical effects of procedural pain. Specifically, we determined the effects of gestational age, weight for gestational age, and neonatal morbidity on the urinary concentration of purines and allantoin. We found infants born at earlier gestational ages had significantly higher urinary purine and allantoin concentrations compared to infants

born at later gestations, after 31 weeks. Weight for gestational age also altered the urinary concentration of purines with small for gestational age infants having significantly lower urinary hypoxanthine compared to appropriate and large for gestational age infants. In addition, respiratory morbidity significantly increased urinary purines, but not allantoin, within the late preterm population. These data suggest that gestational age, weight for gestational age, and neonatal morbidity alter the urinary concentration of purine metabolites. Allantoin was also found to be significantly altered by gestational age. Based upon these data, we recommend conducting age-matched as well as weight for age-matched studies when using urinary purines and allantoin to evaluate the relationship between procedural pain and hypoxia on ATP utilization and oxidative stress. Lastly, it is important to stratify subjects based on health status or morbidity, due to the significant effects of respiratory diseases on ATP use and oxidative stress.

## CHAPTER ONE

### INTRODUCTION

#### **Infants Feel Pain**

Before the 1980's it was commonly believed that pain perception in neonates was blunted or absent. Neonates were subjected to routine painful procedures and even surgery with no anesthesia or analgesia. In 1987, however, Anand *et al.* reported that even premature infants have the anatomical structures and functional requirements necessary to perceive pain [1]. Further investigations on the development of pain pathways revealed that, due to immature descending pain inhibition pathways, neonates are likely to feel pain more intensely than adults and that this hypersensitivity is exacerbated in premature neonates [2, 3].

In addition to feeling pain more intensely, infants who are exposed to repeated painful stimuli are more likely to exhibit signs of hyperalgesia, a prolonged and exaggerated response to stimuli [3-6]. Research indicates that an insufficient release of inhibitory neurotransmitters contributes to hyperalgesia in premature infants [3]. Tissue injury can further promote hyperalgesia by stimulating an inflammatory response that, because of nerve ending proliferation in the developing newborn, results in hyperinnervation and increased pain perception [7-10].

It has also been suggested that infants can learn to anticipate pain. Goubet *et al.* demonstrated that premature infants who had their leg held up for 10 seconds prior to a heel stick showed significantly higher heart rate during leg lift on day five than on day

one [6]. Studies conducted by Taddio *et al.* revealed that full-term infants who were born to diabetic mothers and were therefore exposed to repeated heel lances in the first 24 to 36 hours of life learned to anticipate pain [4]. This study also noted a heightened pain response during venipuncture for infants who were born to diabetic mothers and exposed to repeated heel lances. Taken together, these studies, in addition to several others, clearly established that neonates perceive, feel, and may even remember or anticipate pain [11-13]. Despite the knowledge that infants feel pain, developing effective interventions and identifying the biochemical consequences pain in infants is a continuing challenge for clinicians.

### **Painful Procedures In The NICU**

Infants admitted to the neonatal intensive care unit (NICU) are repeatedly subjected to noxious stimuli as part of their routine care. In 2003 it was reported that infants in the NICU were exposed to roughly  $14 \pm 4$  painful procedures per day and that pharmacologic, as well as non-pharmacologic, analgesia was rarely used before an invasive procedure [14]. This information was reaffirmed in 2008 when Carbajal *et al.* reported that NICU patients were exposed to an average of  $12 \pm 8$  painful procedures per day and that many of these procedures were performed without analgesia [15]. These procedures included, but were not limited to: suctioning, heel-lance, tape removal, IV line insertion/removal, intubation/extubation, lumbar puncture, and gastric tube insertion. While these procedures are necessary, the metabolic and physiological consequences of pain caused by these routine procedures in infants need to be examined further.

In addition to examining the metabolic and physiological consequences of pain, the efficacy of standard NICU pain management techniques needs to be examined. One of the most common pain interventions for minimally invasive routine painful procedure in the NICU is oral sucrose. Numerous studies report reductions in behavioral pain scores when oral sucrose is administered prior to heel lance [16, 17]; however, recent studies indicate that oral sucrose does not significantly reduce pain induced activity in neonatal brain or spinal cord nociceptive circuits suggesting that oral sucrose may not be effective at reducing pain [18]. The ability of oral sucrose to normalize the biochemical changes that result from procedural pain needs to be examined.

### **Pain Response In Neonates**

It is well documented that exposure to painful procedures can result in acute metabolic and physiological changes like increased heart rate, tachycardia, decreased oxygen saturation, decreased transcutaneous PaO<sub>2</sub> (partial pressure of oxygen in arterial blood), increased movement, altered somatosensory cortex hemodynamics, and increased cortisol [19-25]. In addition to the immediate alterations in physiological state observed to be associated with pain, research indicates that repeated painful procedures might have long-term consequences. Brummelte *et al.* reported that greater neonatal procedural pain may contribute to impaired brain development in premature infants [26]. Studies have also reported higher pain response later in life in infants that were circumcised or exposed to repeated painful procedures [27-29]. While research supports the idea that pain during the neonatal period results in immediate and long term changes, little is known about the

effects of procedural pain on adenosine triphosphate utilization (ATP) and oxidative stress.

### **A Theoretical Link Between Pain And Atp Depletion**

As described previously, common responses to painful procedures in neonates often involve increased heart rate and decreased oxygen availability. Theoretically, this combination of events could result in a hypoxic-like state where ATP utilization surpasses ATP production and purine degradation products are formed. Research indicates that increased heart rate can significantly increase ATP utilization [30]. Because the main source of ATP is oxidative phosphorylation, increased cardiac ATP utilization is associated with increased myocardial oxygen consumption [31]. In order to accommodate for the increase in oxygen demand, cardiac output increases to ensure adequate oxygen delivery to the working tissues [32]. If oxygen consumption is not matched by oxygen delivery, cellular hypoxia can occur. In premature infants, tachycardia has the potential to decrease cardiac output by decreasing stroke volume [32, 33]. Specifically, tachycardia decreases stroke volume by limiting the time available for ventricular filling during diastole. In addition, reductions in oxygen saturation are often seen in infants experiencing pain. This combination of events has the potential to create a hypoxic-like state where ATP production cannot match ATP utilization. This would be reflected in plasma and urine by increased purine production.

In addition to variations in heart rate, hypoxemia also has the potential to reduce ATP production. In patients with sleep apnea, plasma uric acid levels positively correlate with the number of obstructive respiratory episodes and oxygen desaturations during

sleep [34]. Furthermore, hypoxemia was shown to significantly decrease ATP and increase inosine monophosphate in isolated rat hearts without significantly altering phosphocreatine [35]. This study shows a relationship between hypoxemia and ATP breakdown; however, plasma levels of ATP breakdown products were not evaluated. In infants, it was shown that a significant inverse relationship exists between PaO<sub>2</sub> and uric acid (serum and urine) [36]. Taken together, these studies indicate that reductions on oxygen saturation have the potential to alter the production of purine nucleotides; however, the effects of transient decreases in oxygen saturation on purine production are unknown.

### **The Purpose Of These Studies**

Based upon the physiological responses observed to be associated with procedural pain, we hypothesized that markers of hypoxia, a condition associated with ATP depletion, could be used to evaluate the biochemical effects of procedural pain on ATP utilization and oxidative stress in premature infants. We began our studies by reviewing literature on well-established markers of hypoxia in order to determine which ones would be best for evaluating ATP utilization and oxidative stress in small volumes of plasma and urine (**Chapter 2**). Next we determined the most efficient means of quantifying the chosen markers (**Chapter 3**). Then we determined the effect of acute pain on hypoxia associated plasma markers of ATP catabolism (purines) and oxidative stress (MDA, allantoin) in premature infants. To accomplish this we first developed an animal model to measure the effect of a single heel-lance on plasma uric acid concentrations (**Chapter 4**). An animal model was chosen to control for the NICU environment and to enable the

researchers to measure the direct effect of a heel lance on plasma uric acid. Then we measured the changes in plasma uric acid and MDA in premature infants exposed to a common painful NICU procedure, tape removal (**Chapter 5**). Finally, we used our markers to evaluate the efficacy of a commonly used intervention, oral sucrose (Sweet-Ease), on relieving pain caused by a single heel lance (**Chapter 6**). These studies revealed that 1) biochemical markers of hypoxia can be used to evaluate the effects of acute pain on ATP catabolism and oxidative stress, 2) even acute pain can cause significant changes in these markers, and 3) markers of ATP catabolism and oxidative stress may be useful in evaluating the efficacy of analgesia.

In the next set of studies we evaluated the clinical factors that may affect the utility of these compounds as urinary markers of pain (**Chapter 7**). Specifically, we determined the effect of gestational age, weight for gestational age, and common neonatal morbidities on the urinary concentrations of purines and allantoin in premature infants. Late preterm infants were chosen to evaluate the effects of morbidity for several reasons. First, this population is less likely to have central catheter lines when compared to more premature infants. Second, research has already established the effects of morbidity on purine concentrations in infants with younger gestational ages; however, the effect of both mild and severe forms of morbidity on the urinary concentrations of purines and allantoin on late preterm infants is unknown. Third, the late preterm population is understudied and often clinically managed as if they were term. Lastly, we hypothesized that the urinary excretion of purine and allantoin would be inversely related to gestation and wanted to control for this in our morbidity studies. These studies revealed that gestational age, weight for gestational age, and neonatal morbidity alter the urinary



excretion of purine and allantoin and need to be accounted for when using these compounds to evaluate the metabolic consequences of pain in premature infants.

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CHAPTER TWO  
BIOCHEMICAL MARKERS OF NEONATAL HYPOXIA

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## **Abstract**

Neonatal hypoxia is a clinical condition with detrimental biochemical and clinical outcomes including production of reactive oxygen and nitrogen species, ATP depletion, developmental abnormalities and growth retardation. Diagnostic approaches for hypoxia are largely based on nonspecific clinical criteria such as Apgar score, umbilical cord pH, and fetal heart rate monitoring. Since our understanding of the biochemical processes of hypoxia has improved, several biochemical markers have been developed. This review highlights the use of hypoxanthine, xanthine, uric acid, xanthine oxidase, malondialdehyde (MDA), nitrotyrosine and lactate as markers of hypoxia in animal models, preterm neonates, and full-term neonates.

## Background

Hypoxemia refers to low oxygen concentration in the arterial blood, while hypoxia suggests inadequate oxygen availability to the tissues. Hypoxia frequently occurs in the neonatal intensive care unit (NICU) and is a consequence of neonatal diseases, such respiratory distress syndrome, persistent pulmonary hypertension of the newborn, apnea of prematurity, bronchopulmonary dysplasia, hemorrhagic diseases and congenital heart disease. Respiratory distress that result in hypoxia occur in 10% of term newborn [1]. This incidence increases as gestational age decreases [2]. Upon admission to the NICU, infants are handled up to 110 times/day [3]. Of these handling episodes, 75% result in oxygen desaturation [4]. Owing to the lack of reliable methods to quantify hypoxia at the bedside, it is not known if these hypoxemic events result in cellular hypoxia. Hypoxia may be the result of:

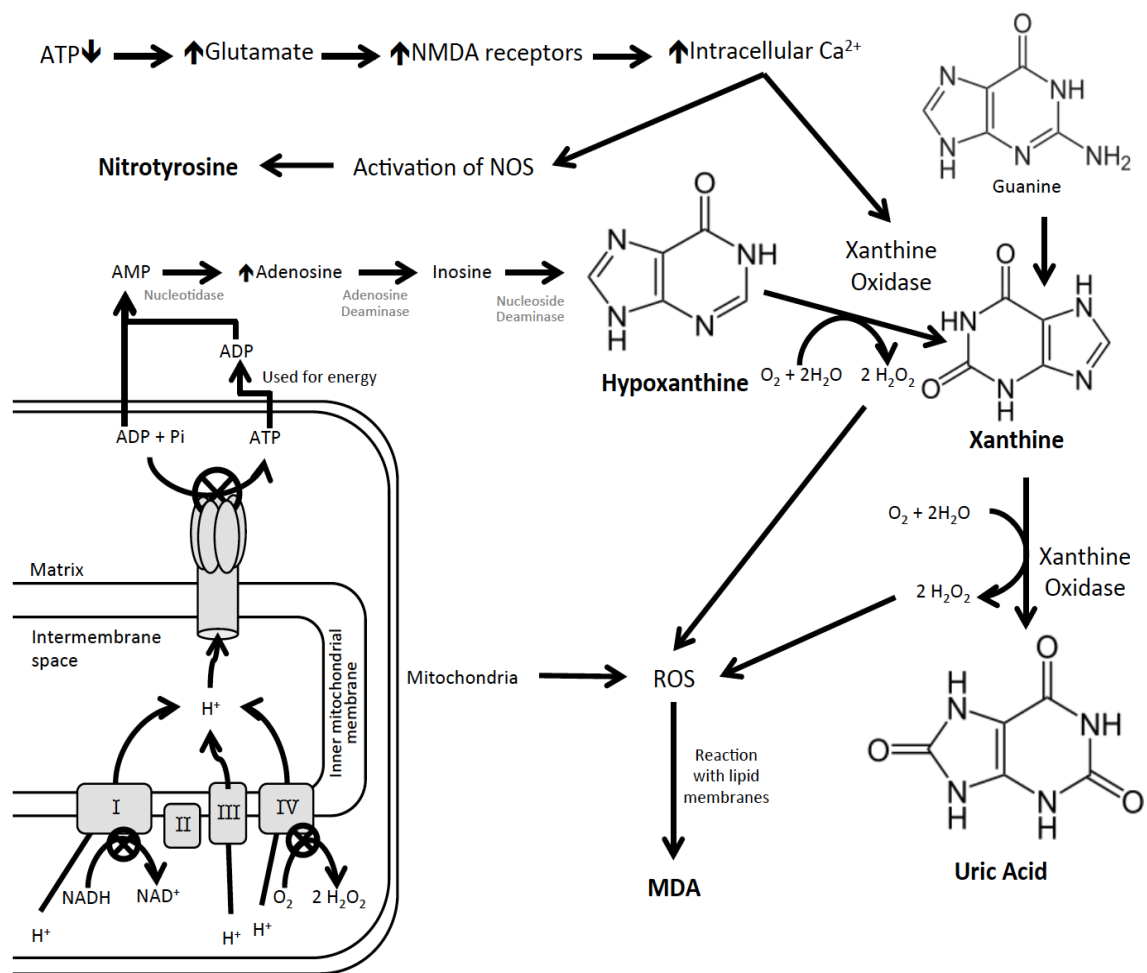
- Low  $PO_2$  of the inspired air or poorly oxygenated lungs
- Decreased hemoglobin
- Deficiencies in oxygen transport and/or utilization

Alveolar hypoxia represents the condition of reduced alveolar  $PO_2$ , often owing to some restriction in gas exchange. This can elicit a compensatory vasoconstriction of the arterial vessels leading to the affected alveoli, thus minimizing the perfusion of under-oxygenated alveoli. Hypoxemic hypoxia is the form of hypoxia that is caused by hypoxemia despite adequate tissue perfusion. Sometimes also referred to as hypoxic hypoxia, this condition arises as a result of a reduction in the delivery of oxygen to the pulmonary capillary system, ultimately leading to low intracellular oxygen

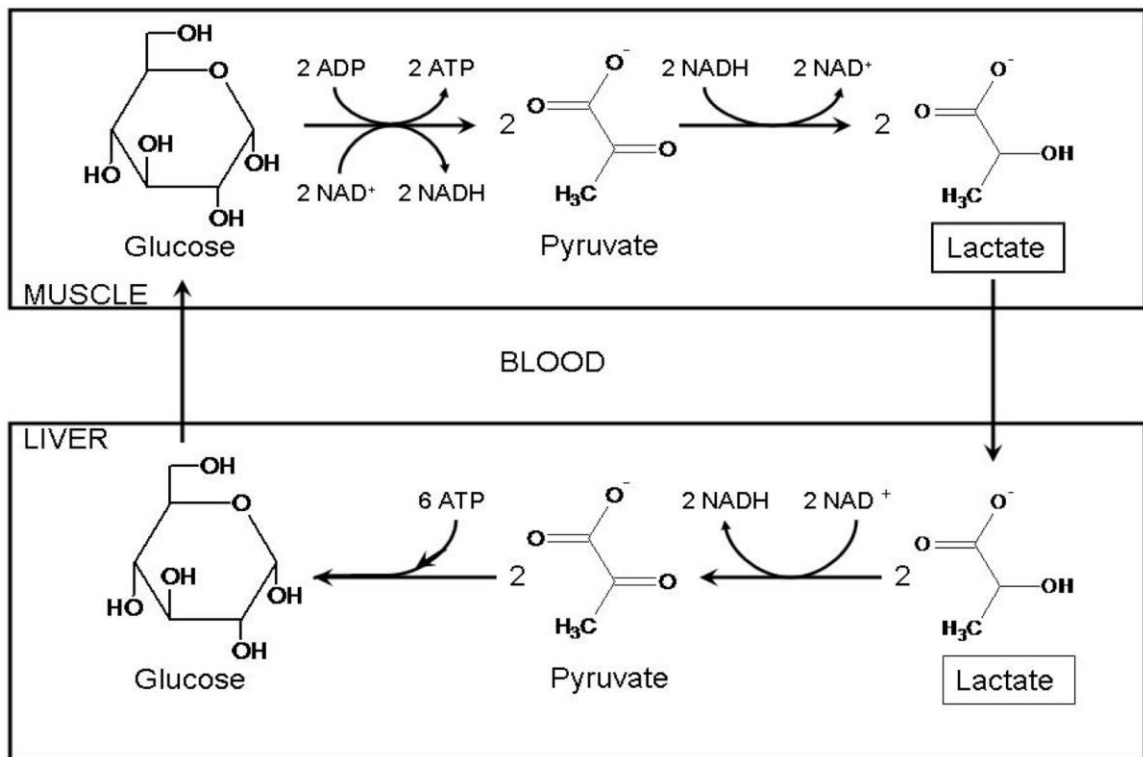
concentrations. This represents the most common form of hypoxic insult in neonates and tends to exacerbate numerous diseases, abnormalities and developmental problems [5-8]. Furthermore, neonatal hypoxia is associated with a high morbidity and mortality rate. In order to identify a condition that can have its effects anywhere in the body, it is important that the condition be categorized according to its biochemical effects on the body.

The detrimental biochemical consequences of hypoxia are due to the processes that occur during anaerobic metabolism (**Figure 1**). In times of oxidative stress, metabolism is forced to switch from aerobic to anaerobic and steady-state concentrations of ATP will decrease [9]. The decrease in ATP is due to a lack of oxidative phosphorylation coupled with continued utilization of ATP for energy. One of the many functions of oxidative phosphorylation is to generate a proton gradient to drive the enzyme, ATP synthase, to synthesize ATP from ADP and inorganic phosphate [10, 11]. Since oxygen is required for oxidative phosphorylation to generate the proton gradient that drives ATP synthase, insufficient amounts of oxygen results in reduced or halted ATP production. The combination of inhibited ATP production and lack of oxygen puts the body in an energy-deprived state. To compensate for this, molecules containing high-energy phosphate bonds, such as ATP, are broken down in order to provide energy for normal cellular processes [9]. Furthermore, the body will switch to anaerobic metabolism, converting pyruvate to lactate in order to re-generate  $\text{NAD}^+$ , enabling glycolysis to continue producing ATP (**Figure 2**). However, the resulting ATP is rapidly consumed. Thus, even with continued ATP production via anaerobic glycolysis, there is a steady decrease in cellular energy levels accompanied by increased release of free adenosine. The free





**Figure 1: Formation of the Biochemical markers of hypoxia.** NMDA: *N*-methyl-D-aspartate; MDA: Malondialdehyde; NOS: Nitric Oxide Synthase; Pi: Inorganic Phosphate; ROS: Reactive Oxygen Species.



**Figure 2: Cori Cycle.** During anaerobic metabolism, pyruvate is converted into lactate to regenerate  $\text{NAD}^+$  in order to enable glycolysis to continue.

adenosine will be degraded to inosine, hypoxanthine, xanthine, and finally to uric acid. The latter two steps are catalyzed by xanthine oxidoreductase, a molybdopyranopterin-containing enzyme, that may be present in two distinct functional forms, xanthine dehydrogenase or xanthine oxidase [12, 13]. Under normoxic conditions, the enzyme form responsible for the degradation of hypoxanthine to xanthine and xanthine to uric acid is xanthine dehydrogenase [14]. Xanthine dehydrogenase is dependent on  $\text{NAD}^+$ , one of the major byproducts of oxidative phosphorylation [15]. Anaerobic glycolysis during hypoxia leads to increased consumption of  $\text{NAD}^+$ , while reduced oxidative phosphorylation effectively decreases  $\text{NAD}^+$  production. Together these processes result in reduced levels of  $\text{NAD}^+$ . Under these hypoxic conditions, xanthine dehydrogenase is converted into xanthine oxidase [16]. Xanthine oxidase is activated during reperfusion and performs the same purine degradation steps as xanthine dehydrogenase, with the exception that xanthine oxidase uses oxygen instead of  $\text{NAD}^+$  as an oxidant [16]. A side effect of this reliance on oxygen is the production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, as well as reactive nitrogen species (RNS), such as peroxynitrite. In addition to the conversion of xanthine dehydrogenase into xanthine oxidase, reduced levels of  $\text{NAD}^+$  also lead to the inhibition of the citric acid cycle, shifting pyruvate conversion to lactate, accompanied by a temporary compensatory  $\text{NAD}^+$  return [17].

In the brain, hypoxia-induced membrane depolarization triggers an increase in intracellular calcium, facilitated by a glutamate-induced increase in NMDA receptors [18-20]. High intracellular calcium levels also contribute to the conversion of xanthine dehydrogenase to xanthine oxidase, and the subsequent production of ROS and RNS [9,

21]. These proposed mechanisms are reinforced by data that demonstrate that the metabolic changes induced by hypoxia result in an increase in the concentration of ROS [22-24]. The major producer of ROS during hypoxia is xanthine oxidase, but it has been proposed that the mitochondria, nicotinamide adenine dinucleotide phosphate oxidase and free fatty acids may also contribute to the generation of ROS [21, 25-27]. Owing to the numerous biochemical changes that occur as a result of the hypoxia-induced transition from aerobic to anaerobic metabolism, there are a growing number of potential biochemical markers that may be useful for quantitative clinical evaluation of the severity of sustained hypoxic stress or trauma. This review will not discuss hypoxia markers detected by medical imaging or UV-Vis spectroscopy.

### **Clinical Markers Of Hypoxia**

The currently accepted method of diagnosing fetal/perinatal hypoxia involves a combination of nonspecific indicators: Apgar score, umbilical cord pH, Sarnat staging, and fetal heart rate monitoring. The Apgar score evaluates five elements, each on a scale of 0-2, at 1 and 5 minutes after birth. These five elements are heart rate, respiratory effort, muscle tone, reflex irritability, and color [28]. However, it has been demonstrated that several obstetric risk factors other than hypoxia are associated with a low 5-min Apgar score in term infants and that the Apgar score has low interobserver reliability [28-30]. Therefore, while this method may provide a general diagnosis of physiologic stress, it does not correlate well with the severity of the hypoxic insult. Furthermore, several authors have shown the limited utility of the Apgar score in preterm infants [31, 32].

Fetal heart rate monitoring is utilized widely for the assessment of fetal wellbeing, but it can result in false-positive diagnosis of fetal asphyxia [33]. Another tool utilized clinically is Sarnat staging, a method for defining the stages of encephalopathy in asphyxiated neonates, without electroencephalography. This method demonstrates a good correlation between stages of encephalopathy and outcome, but without adequate predictive value for survival [34-36]. Furthermore, the inability to define clear stages of encephalopathy in preterm infants greatly reduces the usefulness of Sarnat staging for this vulnerable age group [37]. For all of these reasons, a combination of methods is used to diagnose hypoxia. Even with a combination of diagnostic methods, the level of hypoxia experienced by a neonate can be difficult to evaluate because it ranges from minor to severe and its effects can present in the form of a wide array of symptoms.

Several factors, from the mother as well as the fetus, can contribute to the risks of perinatal hypoxic stress. Chronic hypertension, infection, chronic substance abuse, pre-existing diabetes mellitus, intrauterine growth restriction and pre-eclampsia are some of the maternal factors that have been linked to neonatal hypoxia [38-41]. Other factors include prolapsed umbilical cord, prolonged labor, nuchal cord, and placental abruption [42-44]. With such a vast range of possible etiologies, it is not surprising that studies have shown this condition to be the third leading cause of infant mortality in low risk women in the United States [45]. Among surviving neonates, severity of hypoxia is thought to correlate with neurological development and overall growth. Hypoxia has been linked to developmental malformations in the brain, heart, stomach, liver, and other organs [46-48]. Despite the fact that hypoxia is a major contributor to neonatal mortality by direct or indirect means, diagnostic methods remain generally qualitative and

nonspecific [49]. In order to successfully identify and measure the severity of neonatal hypoxia, reliable quantifiable biochemical markers need to be evaluated for implementation in the clinical setting.

### **Biochemical Markers**

There are several biochemical markers of hypoxia that are currently being evaluated to aid in the clinical diagnosis of hypoxia. The ultimate goal of these biochemical markers is not only to quantify hypoxia, but also to predict the severity of hypoxic insult. This would allow immediate identification and treatment of high-risk neonates. Some of the most promising candidate markers for quantification of the severity of hypoxia and prediction of its sequelae are purine degradation products, ROS and lactate levels.

### **Detecting Hypoxia Using Purine Biochemical Markers**

Purine metabolites were linked to hypoxia as early as 1963 when Berne observed elevated levels of inosine and hypoxanthine in the myocardium of cats and dogs exposed to hypoxia [50]. Since then, a number of studies evaluated the usefulness of purine metabolites from urine, serum, cerebrospinal fluid (CSF) or other tissues in animal models as well as humans. Several studies demonstrated the reliability of purines as biochemical markers for the detection of hypoxia in neonates, children and adults [51-55]. Furthermore, the enzyme responsible for the final two degradation steps of purine metabolism, xanthine oxidase, is also being evaluated as a potential biochemical marker for hypoxia. Since xanthine oxidase is the predominant form of xanthine oxidoreductase

under conditions of hypoxia-reperfusion, as opposed to xanthine dehydrogenase which is present under normoxic conditions, it has the potential to be a useful biochemical marker of hypoxia.

### Purine Metabolites In Animal Models

Several studies of hypoxia in mammals including sheep, rats, pigs, and rabbits have demonstrated elevated levels hypoxanthine, xanthine, and uric acid in response to oxidative stress (**Table 1**). It was shown in the fetal sheep that, during times of severe hypoxia, the concentration of hypoxanthine in the CSF increases [56]. This same study also demonstrated that serum hypoxanthine and xanthine in the fetal sheep increased in response to mild and severe hypoxia [56]. In the adult rat, increased serum levels of uric acid were reported as early as 24 h after a hypoxic insult [57]. In the pig and piglet models, hypoxia-induced rises in purine degradation products were observed in serum, urine, and CSF samples [58]. Further work with pigs and piglets also demonstrated increased hypoxanthine, xanthine, and uric acid in response to hypoxia in the vitreous humor [59]. These animal models demonstrate that purine metabolites may serve as useful biochemical markers of hypoxia in a number of mammals, and further efforts to study the relationship between the severity of hypoxia and these biochemical markers are needed. Physiological relevance could be strengthened by examination of correlation of purine metabolites with brain white matter injury in the various animal models. Previous studies demonstrated that many neurodevelopmental aspects of nonhuman mammals parallel human brain development, and that the brain development of some animal

**Table 1: Biochemical markers of Hypoxia in Animals.** CSF: cerebrospinal fluid; <sup>1</sup>H-NMR: proton nuclear magnetic resonance; MDA: Malondialdehyde

Biochemical marker	Species	Method Of Measurement	Condition Of Animal	Source Of Sample	Recorded Level	Ref.
Hypoxanthine	Fetal Sheep	<sup>1</sup> H-NMR [65]	Severe Hypoxia	CSF	53 µmol/L	[56]
				Arterial Blood	58.1±25.6 µmol/L	
				Arterial Blood	14.3±11.3 µmol/L	
	Pig	HPLC[66, 67]	Hypoxemic	CSF	39.9 µmol/L	[58]
				Serum	103.6 µmol/L	
				Urine	87.1 µmol/L	
Xanthine	Fetal Sheep	<sup>1</sup> H-NMR [65]	Severe Hypoxia	Arterial Blood	12.1±6.5 µmol/L	[56]
				Arterial Blood	4.1±0.8 µmol/L	
				Arterial Blood	4.1±0.8 µmol/L	
	Pig	HPLC[66, 67]	Hypoxemic	CSF	10.6 µmol/L	[58]
				Serum	48.1 µmol/L	
				Urine	12.6 µmol/L	
Uric Acid	Rat	Modified Phosphotungstate Method[68]	Hypoxic	Serum	3.21±1.0 mg/dL	[57]
			Normoxic	Serum	0.98±1.0 mg/dL	
	Pig	HPLC[66, 67]	Hypoxemic	CSF	16.3 µmol/L	[58]
				Serum	208 µmol/L	
				CSF	3.1 µmol/L	
	Xanthine Oxidase	Piglet	Radioactivity Of Uric Acid From The Conversion of C <sup>14</sup> -Xanthine	Hypoxic	Plasma	4-18 µU/ml
Normoxic				Plasma	Undetectable	
				Plasma	Undetectable	
MDA	Rat Pup	Methods Developed By Ohkawa <i>et al</i> [70] & Lowry <i>et al</i> [71]	Hypoxic	Intestinal Tissue	3.826±0.425 nmol/mg protein	[72]
			Normoxic	Intestinal Tissue	2.164±0.229 nmol/mg protein	
Nitrotyrosine	Rat	Optical Density Of Western Blot Bands	Hypoxic	Striatum Supernatant	133.5±16.8%	[73]
			Normoxic	Striatum Supernatant	104.3±10.0%	



models at full-term coincide with brain development of preterm human neonates [60].

This supports the use of animal models to evaluate the extent of brain damage as a result of hypoxic insult.

Several animal models were employed to examine the relationship between hypoxia and the expression of xanthine oxidase activity. These include piglets, rats, and Chinese hamsters. In 1995, increased circulation levels of xanthine oxidase were reported, from non-measurable to detectable, in five of 19 piglets, after experimental ischemia followed by reoxygenation (**Table 1**) [61]. However, this study also indicated that the xanthine oxidase levels did not correlate with documented brain damage [61]. In a rat liver and cultured liver cells, xanthine oxidase activity was shown to have a positive correlation with duration and severity of hypoxia [62, 63]. Increased xanthine oxidase activity was also observed in Chinese hamster V79 cells, which were exposed to hypoxia followed by reoxygenation [64]. These animal models demonstrate that purine biochemical markers of hypoxia are not limited to one species, and efforts to correlate the brain injury with the biochemical markers of hypoxia found in serum, urine, and CSF should be further evaluated.

#### Purine Metabolites In Hypoxic Full-Term Neonates

Increased purine degradation products were demonstrated in full-term neonates that experienced periods of hypoxia (**Table 2**). In 1982 Harkness *et al.* reported a noninvasive way to measure hypoxanthine and xanthine in the urine of full-term neonates [53]. It was demonstrated that hypoxanthine and xanthine levels were higher in neonates following an apneic attack and that the severity of neurological damage correlated with

excreted hypoxanthine and xanthine [53]. Increased hypoxanthine and xanthine were also documented in serum of asphyxiated full-term infants [74]. In addition, full-term neonates were reported to have elevated hypoxanthine, xanthine and uric acid in CSF as a result of hypoxia [54]. In this latter report, hypoxanthine was the better indicator of brain damage [54]. Additional studies have also demonstrated elevated levels of serum hypoxanthine in 48% of full-term, hypoxic neonates [75]. There are few data to substantiate xanthine as a helpful biochemical marker of hypoxia in full-term neonates, but this may be due to relatively low concentration of xanthine compared to hypoxanthine or uric acid [76]. Elevated urine or serum uric acid concentrations were observed in hypoxic full-term neonates on day one of life and, to a lesser extent, on day 3 of life [77]. Uric acid:creatinine ratio was proposed as a reliable biochemical marker for the diagnosis of perinatal asphyxia and for the determination of the stage of hypoxic ischemic encephalopathy [78]. Despite the correlation between increased uric acid and hypoxia, some studies indicated that uric acid may not be as useful in predicting the severity of the resultant brain injury, based on observations that mean uric acid levels do not significantly differ between infants with and without severe brain injury or between those with severe, moderate or mild hypoxic ischemic encephalopathy [89, 90]. Nevertheless, hypoxanthine or xanthine measurements in serum or plasma show promise in predicting the amount of injury caused by hypoxic insult.

The role of xanthine oxidase in the hypoxic full-term neonate is unclear because well-conducted studies that report measurements of this enzyme are still lacking. However, there are some reports of functional xanthine oxidase measurements in adults and in neonates with sepsis, clearly under stressful conditions (**Table 2**). Increased

**Table 2. Biochemical markers of Hypoxia in Full Term Neonates.** CSF: cerebrospinal fluid; GCMS: Gas Chromatography Mass Spectrometry

Biochemical Marker	Method Of Measurement	Condition Of Patient	Source Of Sample	Recorded Level	Ref.
Hypoxanthine	HPLC[79]	Hypoxic	CSF	25.3 µmol/L	[80]
		Normoxic	CSF	1.8-5.5 µmol/L	
Xanthine	HPLC[79]	Hypoxic	CSF	35.8 µmol/L	[80]
		Normoxic	CSF	0.9-9.1 µmol/L	
Uric Acid	HPLC[79]	Hypoxic	CSF	70 µmol/L	[80]
		Normoxic	CSF	30±25 µmol/L	
Xanthine Oxidase	Auto-analyzer Hitachi 747 Uricase Enzymatic Kinetic Method	Mild Hypoxia	Urine	0.95±0.55 Uric Acid/Cr	[81]
		Moderate Hypoxia	Urine	2.16±0.34 Uric Acid/Cr	
		Severe Hypoxia	Urine	3.33±0.73 Uric Acid/Cr	
Xanthine Oxidase	Horecker & Heppel's Method[82]	Septic	Serum	5.27 (2.07) µmol/min/ml	[83]
		Control	Serum	3.43 (1.27) µmol/min/ml	
MDA	Seethambaram & Das' s Method[84]	Septic	Serum	9.5 (1.6) µmol/hr/dl	[83]
		Control	Serum	7.0 (0.78) µmol/hr/dl	
Nitrotyrosine	Thiobarbituric Acid Reaction[85]	Delivery Room Resuscitation	Serum	6.96±8 1.12 µmol/L	[86]
			Urine	4.67±8 1.50 µmol/L	
		Control	Serum	5.26±8 0.63 µmol/L	
			Urine	2.45±8 0.56 µmol/L	
Nitrotyrosine	Immunohistochemical Staining	Asphyxiated	Thalamus	70%	[87]
			Olives	68%	
			Cerebellum	33%	
			Pons	29%	
Nitrotyrosine metabolites	GCMS	Normoxic	Urine		[88]
3-nitro-4-hydroxyphenyl-acetic acid (NHPA)				31.47± 7.27 ng/ml	
para-hydroxyphenyl-acetic acid (PHPA)				2.12±0.79 mg/ml	

xanthine oxidase activity was reported in neonates with sepsis, but this study sampled patients irrespective of gestational age and specifically excluded asphyxiated infants [91]. Elevated plasma xanthine oxidase activity was also reported in adults with acute respiratory distress syndrome [92]. Higher levels of xanthine oxidase in both maternal and fetal plasma were reported in pregnancies complicated by pre-eclampsia [93]. Much more work is needed before xanthine oxidase can be considered as a potential biochemical marker of hypoxia in full-term neonates.

#### Purine Metabolites In Preterm Neonates

Purine metabolites, measured in urine or serum, were proposed to be helpful biomarkers of hypoxia in preterm neonates. Urinary uric acid and xanthine, but not hypoxanthine, were reported to be significantly elevated in the hypoxic neonates on day 1 and 2 of life when compared to normoxic neonates [76]. More recently, however, it was demonstrated that serum hypoxanthine levels were increased in hypoxic preterm infants at birth and continued to be elevated until 7 days of life (**Table 3**) [94]. Buonocore *et al.* further substantiated the reliability of purine degradation products as biochemical markers of hypoxia in preterm neonates, reporting increased serum levels of hypoxanthine, xanthine and uric acid (**Table 3**) [102]. Subsequently, a correlation was documented between serum levels of xanthine or hypoxanthine and periventricular-intraventricular hemorrhage, a condition that affects 15-20% of preterm infants [103]. A group of investigators also suggested the use of xanthine oxidase as a biochemical marker of hypoxia in the preterm neonates. A study involving normoxic and hypoxic preterm infants demonstrated that the xanthine oxidase activity in arterial blood was elevated 4 h

**Table 3. Values of biochemical markers of hypoxia in preterm neonates.** ABST: 2,2'-azino-bis(3-ethylbenzthioazoline-6-sulphonic acid); GCMS: Gas Chromatography Mass Spectrometry. after birth in the hypoxic poor outcome group when compared with control groups [104].

Biochemical Marker	Method Of Measurement	Condition Of Patient	Source Of Sample	Recorded Level	Ref.
Hypoxanthine	HPLC	Hypoxic	Cord Blood	3.9 ± 2.5 µg/ml	[95]
		Normoxic	Cord Blood	1.6 ± 1.4 µg/ml	
	HPLC	Hypoxic	Plasma	3.89± 0.46 µg/ml	[96]
		Normoxic	Plasma	1.13± 0.16 µg/ml	
Xanthine	HPLC	Hypoxic	Plasma	1.16± 0.2 µg/ml	[96]
		Normoxic	Plasma	0.56± 1.71 µg/ml	
Uric Acid	HPLC	Hypoxic	Plasma	51.77± 4.01 µg/ml	[96]
		Normoxic	Plasma	21.69± 3.3 µg/ml	
Xanthine Oxidase	Spectrophotometric Monitoring & Conversion of Hypoxanthine To Urate & Oxidation Of The Chromogen ABTS	Hypoxic	Plasma	4.7±0.6 mU/ml	[97]
		Normoxic	Plasma	1.1±0.2 mU/ml	
MDA	HPLC[98]	Hypoxic	Plasma	0.52±0.20 nmol/ml	[83, 99]
Nitrotyrosine	Seethambaram & Das' s Method[84]	Hypoxic	Plasma	9.19 (1.28) µmol/hr/dl	
	Solid-phase, Antibody-Capture Immunoradio-chemical Assay[100]	Developed Bronchopulmonary Dysplasia	Plasma	0.05-1.3 ng/mg protein	[101]
		Did Not Develop Bronchopulmonary Dysplasia	Plasma	Non Detectable– 0.25 ng/mg protein	
Nitrotyrosine Metabolites	GCMS	Normoxic	Urine		[88]
3-nitro-4-hydroxyphenyl-acetic acid (NHPA)				14.58± 4.49 ng/ml	
para-hydroxyphenyl-acetic acid (PHPA)				0.99±0.38 mg/ml	

As was the case with full-term infants, there is little data on the levels of xanthine oxidase in the preterm neonates, but the information gained thus far suggests that xanthine oxidase may be an important marker of hypoxia.

### **Detecting Hypoxia Using ROS & RNS Biochemical Markers**

Products of short-lived ROS or RNS, specifically malondialdehyde (MDA) and nitrotyrosine (**Figure 1**) may also serve as biochemical markers of hypoxic stress. MDA is a thiobarbituric acid–reacting substance that is formed by the action of ROS on lipid membranes [49, 105, 106]. Because hypoxia and reoxygenation was shown to generate ROS, and MDA measurement is a commonly used indicator of oxygen induced radical damage [105, 107], it was proposed that MDA could be used as a biochemical marker of hypoxia reoxygenation and may even correlate with the severity of cellular or tissue damage and outcome. Other lipid peroxidase products, such as lipid hydroperoxides, have been shown to increase in response to acute cerebral ischemia complicated by hypoxia and reperfusion, but MDA was shown to increase by a greater percentage than lipid hydroperoxides [108]. Nitrotyrosine is generally formed by the reaction of peroxynitrite with free or bound tyrosine residues, but can also be formed as a byproduct of myeloperoxidase released from neutrophils during an inflammatory response [109]. Nitric oxide can react with a suitable ROS, such as superoxide anions ( $O_2^{\cdot-}$ ), to form peroxynitrite [110, 111]. However, nitric oxide formation is dependent on nitric oxide synthase, an enzyme that is induced during the inflammatory response [109] and activated by elevated intracellular calcium levels [21]. In adults, investigators showed that CSF nitrotyrosine levels can be used as a reliable predictor of poor neurological

outcomes, making nitrotyrosine of key interest in correlating severity of hypoxia damage with outcome [112].

### ROS & RNS In Animal Models

Several animal studies using mouse, rat or goat models, demonstrated the reliability of MDA as a marker of hypoxia and reoxygenation. Representative reported values of MDA levels are presented in **Table 1**. In the adult mouse model, it was shown that severe, chronic intermittent hypoxia results in a 60% increase in liver MDA while moderate, chronic intermittent hypoxia results in a 25% increase in liver MDA levels [113]. These data indicate that MDA may be helpful in determining the severity of hypoxia. A study on necrotizing enterocolitis showed that rat pups exposed to hypoxia and reoxygenation exhibited significantly elevated intestinal tissue MDA in comparison to rat pups exposed to normoxic conditions [6]. MDA levels in other tissues as well as body fluids were also studied. Elevated serum levels were observed in rats exposed to hypoxia and reoxygenation [114]. Similarly, elevated plasma levels of MDA were observed in fetal goats subjected to cord occlusion [115].

Although several studies demonstrate the usefulness of MDA as an indicator of hypoxia, there are some factors that need to be taken into consideration when utilizing MDA as a biochemical marker of hypoxia and reoxygenation. A study by Yamada *et al.* reported that fetal plasma MDA levels were significantly elevated after the initiation of maternal oxygen supplementation [115]. Furthermore, Loiseaux-Meunier *et al.* demonstrated elevated levels of MDA in adults that received hyperoxia oxygen therapy [116]. This suggest that oxygen supplementation in the absence of hypoxia can also increase plasma MDA levels.

The effects of hypoxia on nitrotyrosine levels were also studied in several animal models including rabbit and rat (**Table 1**). Western blot, immunohistochemical, and HPLC techniques were commonly employed to demonstrate elevated production of nitrotyrosine after hypoxic exposure. A group of investigators, employing HPLC with electrochemical detection, found increased nitrotyrosine formation from protein bound tyrosine in brain tissue of fetal rabbits following repetitive uterine ischemia-reperfusion [117]. Nitrotyrosine quantitation was further substantiated by the observation that nitrotyrosine levels were reduced following the administration of a selective neuronal nitric oxide synthase inhibitor to fetus [117]. Similarly, nitrotyrosine formation was evaluated immunohistochemically and by Western blotting in striatal samples from rats exposed to hypobaric hypoxia. Increased nitrotyrosine levels accompanied increased levels of neuronal, constitutive and endothelial nitric oxide synthase in rat caudate putamen [118]. Since nitrotyrosine is elevated under conditions of oxidative stress and since it is also a known mediator of myeloperoxidase, an enzyme present under inflammatory conditions [108], it may be helpful to use nitrotyrosine as a marker of hypoxia induced inflammation cell damage. Elevated nitrotyrosine was colocalized in the brains of hypoxic rats with several indicators of cellular injury, including a loss of microtubule-associated protein-2, presence of active caspase-3, nuclear translocation of apoptosis-inducing factor and evidence of specific DNA strand breaks [119]. Colocalization of nitrotyrosine with markers of cell damage strengthens its potential utilization as a clinical indicator of brain damage that occurs after hypoxia.



## ROS & RNS Markers In Full-Term Neonates

Biochemical markers of ROS and RNS were employed in several studies as indicators of hypoxia in neonates (**Table 2**). Kumar *et al.* compared serum and urinary concentrations of MDA between full-term neonates that received delivery-room resuscitation and a healthy group of control neonates [120]. They observed elevated levels of serum and urinary MDA in delivery room resuscitated neonates, and noted that infants with clear signs of fetal and neonatal distress (meconium staining, hypoxic ischemic encephalopathy or death), exhibited the highest levels of MDA [120]. Similar increases in MDA levels, as a result of hypoxia, were also demonstrated in the plasma of blood samples taken from placental part of the umbilical cord [121]. While some data regarding urinary and serum levels of MDA are available for hypoxic and normoxic neonates, little is known regarding the MDA levels in the CSF. More studies using MDA measurements are required to definitively characterize lipid peroxidation due to oxidative stress in neonates. Furthermore, there is evidence that factors other than hypoxia can affect the levels of MDA. Kahw *et al.* recorded significantly elevated levels of plasma MDA in umbilical cord and maternal blood samples after the administration of maternal oxygen supplementation [122]. It has also been observed that small-for-gestational-age term infants, born to undernourished mothers, had higher serum MDA levels than average for gestational age infants born to healthy mothers[123]. More studies are required to characterize factors that alter MDA in biologic fluids and as a potential marker of cellular damage induced by oxidative stress.

Elevated nitrotyrosine was documented in human neonatal brain (**Table 2**). A study of 22 full-term asphyxiated neonates demonstrated increased nitrotyrosine staining

in brain tissue samples [124]. Nitrotyrosine was also found in the spinal cord of asphyxiated full-term neonates. A study of 18 asphyxiated neonates demonstrated positive nitrotyrosine staining in five, with the highest nitrotyrosine staining in an infant who died shortly after the hypoxic-ischemic insult [125]. Further work is needed to obtain information regarding nitrotyrosine levels in CSF, serum, and urine of full-term infants with or without hypoxia.

### ROS & RNS Markers In Preterm Neonates

Malondialdehyde levels were measured in preterm neonates in several reports. Serum MDA levels were found to be markedly higher in hypoxic preterm neonates in umbilical cord blood as well as blood samples on day 4 of life when compared to hypoxic term neonates [99]. Elevated urinary MDA was reported in infants with bronchopulmonary dysplasia [126]. It has also been observed that the MDA was inversely related to birthweight [126]. Urinary MDA measurements gained legitimacy when it was reported that plasma changes in MDA are followed by changes in urinary MDA 24 h later [127]. Increased levels of MDA have been observed in infants born to mothers who went through labor as opposed to those who had elective caesarean section without labor [128].

Studies measuring nitrotyrosine in hypoxic preterm infants are rare (**Table 3**). Plasma nitrotyrosine was found to be significantly higher in preterm neonates with bronchopulmonary dysplasia [129]. Furthermore, Hoehn *et al.* demonstrated that certain degradation products of nitrotyrosine can also be quantified in the urine of preterm normoxic infants [130]. Thus, it appears that nitrotyrosine can be measured in serum and

urine of preterm neonates. Additional studies are needed to measure nitrotyrosine in hypoxic neonates to determine its utility in this context.

### **Detecting Hypoxia Using The Biochemical Marker Lactate**

Perhaps the most commonly used biochemical marker of hypoxia is lactate. During anaerobic metabolism, glycolysis is sustained by the NADH-dependent reduction of pyruvate to lactate, yielding a net of two molecules of ATP per glucose molecule (**Figure 2**). When the body is reoxygenated, lactate is converted back into pyruvate and eventually glucose in the liver at the expense of ATP (**Figure 2**). The diagnostic value of lactate as a marker of hypoxia is controversial. Studies suggested that lactate is a promising indicator of the severity of hypoxia in high-risk neonates [131] and the neurological outcome and mortality in severely hypoxic neonates [132, 133]. Further investigation into lactate as a marker of hypoxia revealed some inherent flaws. Lactate levels in neonates may be increased by maternal glucose, Plasmalyte A, Ringer's lactate infusion or bronchodilators [134-136]. Growth-retarded infants have elevated lactate levels [137]. Maternal ephedrine administration can increase placental lactate production and alter the fetal plasma lactate level [138]. Owing to such confounding factors lactate may not be the most reliable marker for hypoxia. Nevertheless, clinical lactate determinations are well established, while quantitation of purines is still generally limited to experimental settings. Thus, lactic acid continues to be commonly used, in spite of the ongoing debate.

## Lactate In Animal Models

Owing to evidence of acidosis in cord blood of hypoxic neonates, lactate has been evaluated as a marker of hypoxia and asphyxia in pigs, rats, and fetal lambs (**Table 4**). In the pig model, plasma lactate levels increase linearly with tissue hypoxia, exhibiting high correlation with hypoxanthine during hypoxia as well as recovery [144]. In *ex vivo* experiments using pig coronary arteries, interstitial arterial wall lactate concentrations were observed to increase during hypoxia and fall during reoxygenation [139]. In asphyxiated fetal rat pups, Seidl R *et al.* demonstrated that lactate levels increased in the brain and blood compared to control animals [141]. Subcutaneous and striatal levels of lactate significantly increased in asphyxiated fetal rat pups, with subcutaneous lactate reaching a maximum around 20–21 min of asphyxia. Similarly, striatal lactate levels reached a maximum around 19-20 min of asphyxia [142]. In the adult rat, arterial lactate levels also increased in response to hypoxia [140]. Lastly, in fetal lambs, cerebral lactate does not increase until there is a 30% drop in arterial oxygen saturation [143].

## Lactate Marker In Full-Term Neonates

Lactate was studied in full-term infants as a marker of hypoxia and asphyxia (**Table 5**). A prospective study of 4045 cord samples demonstrated elevated lactate levels in vacuum extraction deliveries and emergency cesareans compared to standard vaginal delivery and elective cesarean [145]. A correlation exists between lactate levels in arterial cord blood, venous cord blood and fetal scalp blood, as well as between cord arterial lactate, pH and base excess in infants that had ominous fetal heart rate patterns[146]. Lactate was evaluated in CSF and blood of neonates with hypoxic-

**Table 4. Values of lactate in animal models.** <sup>1</sup>H-NMR: proton nuclear magnetic resonance; HPLC: high pressure liquid chromatography

Species	Method Of Measurement	Source Of Sample	Condition Of Animal	Recorded Level	Ref.
Pig	Spectrophotometric kinetic enzymatic analyzer	Interstitial	Normoxic	1.07±0.21 mmol/L	[139]
			Hypoxia 30-60 min	2.50±0.240 mmol/L	
Adult Rat	<sup>1</sup> H NMR	Arterial blood	Normoxic	3.6±1.6 mmol/L	[140]
			Hypoxia	13.5±4.3 mmol/L	
			Hypoxia reoxygenation	8.3±3.3 mmol/L	
Fetal Rat	Colorimetric test	Brain	Normoxic	88.55±0.78 µM/g	[141]
			Asphyxia 5min	14.77±2.95. µM/g	
			Asphyxia 10min	19.63±1.36 µM/g	
			Asphyxia 15min	28.26±1.22 µM/g	
			Asphyxia 20min	36.66±3.22 µM/g	
	Enzymatic spectrophotometric end point assay	Blood	Normoxic	8.72±1.97 mM/L	[141]
			Asphyxia 5min	10.35±1.15 mM/L	
			Asphyxia 10min	12.10±1.59 mM/L	
			Asphyxia 15min	14.24±1.30 mM/L	
			Asphyxia 20min	13.34±1.84 mM/L	
HPLC	Subcutaneous	Normoxic	0.7 mM	[142]	
		Asphyxia 2-3min	1.2±0.13 mM		
		Asphyxia 20-21min	2.7±0.36 mM		
		Asphyxia 21-22min	1.5±0.17 mM		
		Normoxic	3.5 µmol/g		
		Asphyxia 5-6min	7±1.3 µmol/g		
		19-20min	16.7±1.9 µmol/g		
Fetal Lamb	Proton magnetic resonance spectroscopy	Cerebral	Normoxic	0.4±0.9 mmol/Kg	[143]
			Hypoxic	6.7±3.6 mmol/Kg	
			Normoxic	88.55±0.78 µM/g	

**Table 5. Values of lactate in full-term neonates.** HIE: Hypoxic-Ischemic Encephalopathy; <sup>1</sup>H-NMR: proton nuclear magnetic resonance; CSF: cerebrospinal fluid

Method Of Measurement	Source Of Sample	Condition Of Patient	Recorded Level	Ref.
<sup>1</sup> H NMR	Urine	Normoxic ≥6hr	0.09±0.02 Lactate/creatinine	[148]
		Normoxic 48-72hr	0.09±0.03 Lactate/creatinine	
		Asphyxia (no HIE) ≥6h	0.19±0.12 Lactate/creatinine	
		Asphyxia (no HIE) 48-72h	0.16±0.17 Lactate/creatinine	
		Asphyxia (HIE) ≥6hr	16.75±27.38 Lactate/creatinine	
		Asphyxia (HIE) 48-72hr	0.92±1.77 Lactate/creatinine	
Electrochemical test strip method	Arterial umbilical blood	Standard vaginal delivery	1.87±0.94 mmol/L	[145]
		Vacuum delivery	2.95±1.20 mmol/L	
		Elective cesarean	1.44±0.10 mmol/L	
		Emergency cesarean	2.44±0.69 mmol/L	
Roche L-640 lactate analyzer [149]	CSF	Normoxic	1.9±0.4 mmol/L	[147]
		Asphyxiated	2.9±1.2 mmol/L	
	Blood	Normoxic	2.2±0.6 mmol/L	
		Asphyxiated	3.9±1.1 mmol/L	

**Table 6. Values of lactate in preterm neonates**

Method Of Measurement	Source Of Sample	Condition Of Patient	Recorded Level	Ref.
ABL Radiometer device	Umbilical artery blood	Normoxic	29.4±2.75 mg/dl	[150]
GM 7 analyzer	Umbilical artery blood	Healthy	2750±470 μmol/L	[131]
		High risk	5100±2700 μmol/L	

ischemic brain insult [147]. The role of lactate as a marker of neonatal stress was further validated when Huang *et al.* reported that the urinary lactate:creatinine ratio, measured soon after birth, may help to identify hypoxic-ischemic encephalopathy in asphyxiated newborns [148].

### Lactate Marker In Preterm Neonates

Despite the common use of lactate as an indicator of hypoxia in the clinical setting, there are few data on the relevance of lactate in preterm neonates (**Table 6**). It was shown that umbilical artery mean lactate levels are higher in preterm infants than in term infants [150]. Chou *et al.* also demonstrated elevated lactate levels in preterm infants compared to full-term ones, but this group further demonstrated elevated lactate levels in high-risk neonates [131]. Lastly, Groenendaal *et al.* have proposed that lactate may be an indicator of adverse outcome. They reported that early arterial lactate levels were associated with a 0.47 positive and a 0.92 negative predictive value for adverse outcomes [151]. Further research needs to be carried out in order to validate lactate as a marker of hypoxia in preterm neonates.

### Conclusions

Quantification of the presence and severity of fetal and neonatal hypoxia remains a challenge. Identification, characterization and validation of biomarkers that swiftly identify neonates at risk of hypoxia-induced damage are needed. It was suggested that the therapeutic window for intervention in hypoxic-ischemic neonates is approximately 6 h following the onset of hypoxia [152]. Since the time allowed for intervention is short, the rapid detection of direct markers of hypoxia are needed. Numerous biochemical

markers are currently being used for the identification of hypoxia [23, 153-156]. Some of the most promising candidates are purine metabolites, lactate, and biochemical markers of ROS or RNS. These markers are primarily measured in body fluids, unlike ATP and inorganic phosphate, which can be measured by tissue MRI. This review highlighted the use of hypoxanthine, xanthine, uric acid, xanthine oxidase, MDA, nitrotyrosine and lactate as biochemical markers of neonatal hypoxia. While the assays for some of these markers are well established, others require further characterization [157, 158]. In addition, several xanthine oxidase inhibitors have been evaluated as potential agents for reduction of hypoxia-induced cellular damage [159-161]. With better diagnostic approaches to hypoxic stress, further improvements in the understanding of affected biochemical processes are expected and more rational interventions can be developed and tested.

### **Future Perspectives**

It is speculated that within the next 5-10 years, several major developments in the field of biochemical markers of hypoxia will occur. First, new biochemical markers of hypoxia will become established. Allantoin, a product of oxidation of uric acid, is currently being investigated as a marker of hypoxia. Allantoin increased in premature infants who subsequently developed chronic lung disease [162], but further research is needed to establish it as a marker of hypoxia. Second, a combination of biochemical markers will improve predictive value for severity of tissue damage and outcome in the clinical setting. For this to occur, some of the current biochemical markers of hypoxia need to be adapted to the clinical setting, and clinical studies using combinations of



markers for higher predictive value will need to be carried out. A candidate combination of markers could include lactate, hypoxanthine, xanthine oxidase, and MDA. This would incorporate circulatory acid/base status, purine metabolism, ROS-generating enzyme level and a product of lipid peroxidation into a combined evaluation of neonatal hypoxia and tissue damage. Lastly, a greater understanding of the biochemical processes of hypoxia will enhance the development of therapeutics. Several compounds are currently being investigated [159, 163].

**Table 7. Executive Summary**

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**EXECUTIVE SUMMARY**

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**Neonatal hypoxia**

- Neonatal hypoxia is a clinical condition with overt biochemical consequences.
- This condition is associated with a high morbidity and mortality rate.
- Hypoxia is associated with the production of reactive oxygen species (ROS), Reactive nitrogen species (RNS), ATP depletion and risk of developmental abnormalities and growth retardation.

**The biochemical processes of hypoxia**

- The biochemical processes of hypoxia are associated with anaerobic metabolism.
- Anaerobic metabolism creates an energy-deprived state, forcing the breakdown of ATP ultimately to uric acid, and driving the formation of lactate to sustain glycolysis.
- Purine metabolism following reperfusion requires the enzyme xanthine oxidase, which generates ROS.
- In the brain, hypoxia triggers a series of events leading to formation of ROS and RNS.

**Clinical evaluation of hypoxia**

- Currently accepted method for diagnosis of fetal/perinatal hypoxia involves a combination of nonspecific approaches: Apgar score, umbilical cord pH, Sarnat staging, and fetal heart rate monitoring
- These methods do not correlate well with the severity of hypoxia, which may be difficult to assess due to its numerous causes
- Several fetal and maternal factors contribute to the onset and severity of hypoxia making it difficult to diagnose with the current nonspecific clinical methods

**Purine biochemical markers of hypoxia**

- Promising candidate markers for quantification of the severity of hypoxia, and prediction of its sequel, are purine degradation products
- Purine biochemical markers include hypoxanthine, xanthine, uric acid, and the enzyme xanthine oxidase
- The value of purines and xanthine oxidase as markers of hypoxia was demonstrated in animals, full-term infants and preterm infants

**ROS and RNS biochemical markers of hypoxia**

- Products of short-lived ROS or RNS, specifically, malondialdehyde (MDA) and nitrotyrosine, respectively, may serve as biochemical markers of hypoxic stress
- MDA measurements are a commonly used indicator of oxygen induced free radical damage.
- Cerebrospinal fluid nitrotyrosine levels were used as a reliable predictor of poor neurological outcome in adults, making it of key interest in correlating severity of hypoxia damage with outcome in neonates.

**Lactate as a marker of hypoxia**

- Lactate is one of the most commonly used biochemical markers of hypoxia and it is formed during anaerobic metabolism to support glycolysis.
- The diagnostic value of lactate as a marker of hypoxia is controversial due to evidence that lactate may also be increased by maternal glucose, Plasmalyte A, Ringer's lactate infusion or bronchodilators.
- Despite the controversy, studies have indicated that lactate is a promising predictor of the severity of hypoxia in high-risk neonates and the neurological outcome and mortality in severely hypoxic neonates.

**Conclusion**

- Identification, characterization and validation of biomarkers that swiftly identify neonates at risk of hypoxia-induced damage are needed.
- Detection of the presence and evaluation of severity of fetal and neonatal hypoxia remains a challenge.
- Better diagnostic approaches to hypoxic stress will further improve the understanding of affected biochemical processes and lead to more rational interventions.

**Future prospective**

- It is speculated that within the next 5-10 years, several major developments with biochemical markers of hypoxia will occur.
  - First, new biochemical markers of hypoxia will be established.
  - Second, a combination of biochemical markers will improve the predictive value for severity of tissue damage and outcome in the clinical setting.
  - Lastly, improved understanding of the biochemical processes of hypoxia will enhance the development of therapeutics.
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CHAPTER THREE  
BIOCHEMICAL MEASUREMENT OF NEONATAL HYPOXIA

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## Abstract

Neonatal hypoxia ischemia is characterized by inadequate blood perfusion of a tissue or a systemic lack of oxygen. This condition is thought to cause/exacerbate well documented neonatal disorders including neurological impairment [1-3]. During hypoxia, aerobic metabolism is switched to anaerobic pathways. Decreased adenosine triphosphate production occurs due to a lack of oxidative phosphorylation. To compensate for this energy deprived state molecules containing high energy phosphate bonds are degraded [2]. This leads to increased levels of adenosine, which is subsequently degraded to inosine, hypoxanthine, xanthine, and finally to uric acid. The final two steps in this degradation process are performed by xanthine oxidoreductase. This enzyme exists in the form of xanthine dehydrogenase under normoxic conditions but is converted to xanthine oxidase (XO) under conditions of hypoxia-reperfusion [4, 5]. Unlike xanthine dehydrogenase, XO generates hydrogen peroxide as a byproduct of purine degradation [4, 6]. Hydrogen peroxide, in combination with other reactive oxygen species (ROS) produced during hypoxia, oxidize uric acid to form allantoin and react with lipid membranes to generate malondialdehyde (MDA) [7-9]. Most mammals, humans exempted, possess the enzyme uricase, which converts uric acid to allantoin. In humans, however, allantoin can only be formed by ROS-mediated oxidation of uric acid. Because of this, allantoin is considered to be a marker of oxidative stress in humans, but not in the mammals that possess uricase.

We describe methods employing high-pressure liquid chromatography (HPLC) and gas chromatography mass spectrometry (GCMS) to measure biochemical markers of neonatal hypoxia ischemia. Human blood is used for most tests. Animal blood may also

be used while recognizing the potential for uricase-generated allantoin. Purine metabolites were linked to hypoxia as early as 1963 and the reliability of hypoxanthine, xanthine, and uric acid as biochemical indicators of neonatal hypoxia was validated by several investigators [10-13]. The HPLC method used for the quantification of purine compounds is fast, reliable, and reproducible. The GCMS method used for the quantification of allantoin, a relatively new marker of oxidative stress, was adapted from Gruber *et al.* [7]. This method avoids certain artifacts and requires low volumes of sample. Methods used for synthesis of MMDA were described elsewhere [14, 15]. GCMS based quantification of MDA was adapted from Paroni *et al.* and Cighetti *et al.* [16, 17]. Xanthine oxidase activity was measured by HPLC by quantifying the conversion of pterin to isoxanthopterin [18]. This approach proved to be sufficiently sensitive and reproducible.

## **Protocol**

### **Sample Collection And Processing**

Collect blood sample in a 6ml K3E EDTA K3 tube which is kept on ice. Within 2 min of collection, centrifuge the sample at 4°C at 1500 g for 10 min. Transfer the supernatant (plasma) to a 1.5ml microcentrifuge tube. Centrifuge at 4°C at 18000 g for 30 min. Remove the supernatant and transfer into separate microcentrifuge tubes for purine (200µl), allantoin (50µl), MDA (100µl), and XO (120µl) analysis. Be careful not to contaminate the samples with red blood cells. You may need to adjust the volumes of plasma for MDA, XO, and purines based upon the total volume of plasma available.

### Preparing Internal Standard, 2-Aminopurine (2-AP), For Purine And XO Analysis

Weigh out 0.01351g 2-AP and add to 8ml of water that has been acidified with 2-5 drops of HCl. If the 2-AP is not dissolving you need to add more acid. Adjust the final volume to 10ml. Use the UV-Vis spectrophotometer to determine the true concentration of your stock 2-AP solution, ( $\lambda_{\max}$  315,  $\epsilon$  4000). Once the concentration of the stock 2-AP solution is determined, calculate the volumes required to contain  $1 \times 10^{-7}$  mol 2-AP internal standard for purine measurements (**Eq. 1**) and  $1 \times 10^{-8}$  mol 2-AP internal standard for XO measurements (**Eq. 2**). Aliquot the calculated volumes into separate microcentrifuge tubes and evaporate to dryness in a SpeedVac.

$$(1 \times 10^{-7} \text{ mol}) \left( \frac{1}{[\text{Stock Solution}]} \right) \left( \frac{1 \times 10^6 \text{ ul}}{1 \text{ l}} \right) = \text{Purine Desired Volume} \quad \text{Eq. 1}$$

$$(1 \times 10^{-8} \text{ mol}) \left( \frac{1}{[\text{Stock Solution}]} \right) \left( \frac{1 \times 10^6 \text{ ul}}{1 \text{ l}} \right) = \text{XO Desired Volume} \quad \text{Eq. 2}$$

### HPLC Measurement Of Purines

Transfer plasma (200 $\mu$ l) to a Microcon YM-10 centrifugal filter device and centrifuge at 4°C at 14000 g for 1.5 hours. Remove the filtrate and transfer to a microcentrifuge tube containing  $1 \times 10^{-7}$  mol 2-AP. Be sure to record the volume of filtrate added to the microcentrifuge tube containing the 2-AP. Vortex the samples for 10-20 sec. Analyze samples with an HPLC. Three 50  $\mu$ l injections are used for each sample. Samples will be injected onto a Supelcosil LC-18-S 15 cm x 4.6 mm, 5  $\mu$ m column equipped with a Supelguard LC-18-S column guard. The gradient conditions

**Table 1: Solvent changes for HPLC measurement of purine compounds.**

	<b>Time</b>	<b>Flow (ml/min)</b>	<b>%A</b>	<b>%B</b>	<b>%C</b>
<b>1</b>		1.00	0.0	0.0	100.0
<b>2</b>	16.00	1.00	0.0	0.0	100.0
<b>3</b>	17.00	1.00	100.0	0.0	0.0
<b>4</b>	22.00	1.00	100.0	0.0	0.0
<b>5</b>	27.00	1.00	0.0	100.0	0.0
<b>6</b>	32.00	1.00	0.0	100.0	0.0
<b>7</b>	33.00	1.00	100.0	0.0	0.0
<b>8</b>	38.00	1.00	100.0	0.0	0.0
<b>9</b>	39.00	1.00	0.0	0.0	100.0
<b>11</b>	45.00	1.00	0.0	0.0	100.0

**Table 2: Typical retention times and  $\lambda_{\max}$  for purines and internal standard.** \*Determined on HPLC using isocratic 50mM ammonium formate buffer (pH 5.5) with a flow rate of 1mL/min. pH is in ().

	<b>Retention Time*</b>	<b>Observed <math>\lambda_{\max}</math>*</b>	<b>Reported <math>\lambda_{\max}</math>[19]</b>	<b>Reported <math>\epsilon_{\max}</math>[19]</b>
<b>Uric Acid</b>	~ 3.5 min	288	283 (2)	11,500 (2)
<b>Hypoxanthine</b>	~7.0 min	248	248 (1)	10,800 (1)
<b>Xanthine</b>	~ 9.5 min	267	267 (2)	10,200 (2)
<b>2-Aminopurine</b>	~ 12.5 min	305	314 (2)	4000 (2)

described in **Table 1** should be used to obtain adequate peak separation: solvent A-water, solvent B- methanol, solvent C- 50mM ammonium formate buffer, pH 5.5. Determine the concentration of purines in the sample. Quantify hypoxanthine, xanthine, and uric acid by obtaining peak areas at the retention times and wavelengths described in **Table 2**. Determine the peak area of 2-AP. Determine the area ratios of hypoxanthine, xanthine, and uric acid to 2-AP and convert the ratios to  $\mu$ molar concentrations using standard curves. Analyze all samples in triplicate, but only include values for later analyses if the coefficient of variation is less than 10%.

#### GCMS Measurement Of Allantoin

Add 50 $\mu$ l of 10 $\mu$ M DL-Allantoin-5-<sup>13</sup>C;1-<sup>15</sup>N (internal standard) to the 50  $\mu$ l plasma set aside for the allantoin assay during sample collection and processing. Add 100  $\mu$ l acetonitrile to this plasma solution. Vortex the mixture for 10-20 sec then centrifuge at 4°C at 20000 g for 10 min. Transfer the supernatant to a GCMS vial and dry under N<sub>2</sub>. After drying, Resuspend in 50  $\mu$ l of derivatizing agent *N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide* (MTBSTFA) in pyridine (1:1 vol/vol), cap the vials and incubate at 50°C for 2 h. This derivatization yields consistently quantifiable m/z peaks of 398.0 and 400.0 for allantoin and DL-Allantoin-5-<sup>13</sup>C;1-<sup>15</sup>N respectively [20]. Analyze samples on a GCMS equipped with an auto sampler.

Perform compound separation on an Agilent 122-5532G capillary column (25.7m length, 0.25mm internal diameter). Use helium as the carrier gas at a flow rate of 1.5 ml/min. Inject derivatized product (1  $\mu$ l) in split mode (split ratio 20:1, split flow 29.4 ml/min, total flow 33.8 ml/min). Set the initial column temperature at 100°C and hold at

that temperature for 2 min before increasing it to 180°C at a rate of 10°C/min. Hold the temperature for 4 min and then increase it to 260°C at a rate of 20°C/min. Maintain this temperature until the end of the run. After each sample, clean the column with 1 injection of acetonitrile and 2 injections of MTBSTFA in pyridine (1:1 vol/vol). Quantify allantoin using select ion monitoring mode while monitoring the 398.00 m/z ion for allantoin and the 400.00m/z for the DL-allantoin-5-<sup>13</sup>C;1-<sup>15</sup>N. Convert the ion abundance ratios of allantoin/(heavy allantoin) to micromolar concentrations of allantoin using a prepared standard curve. All samples are analyzed in triplicate but only values with a coefficient of variation of less than 10% are used in further analyses.

#### Preparing Internal Standard, Methyl Malondialdehyde (MMDA), For MDA Analysis

Add 523µl of 3-ethoxymethacrolein and 1477µl of 7M NaOH to a 100ml round bottom flask. Add a stirbar. Place the flask in a water bath at 45°C and stir until the reaction has gone to completion. This should take roughly 140 min. Monitor the progress of the reaction by removing 10µl of liquid from the flask periodically, around 10 min intervals. Dilute each sample collected to a factor of 10<sup>5</sup> using 50 mM potassium phosphate buffer (pH 7) and measure the absorbance on the UV-Vis. Once the absorbance at 275nm reaches roughly 0.658 the reaction is complete. As the reaction progresses, the solution will turn yellow then orange. Allow the reaction to progress for an additional 15 min. Add 5ml of distilled deionized water to the round bottom flask and transfer the solution to a separatory funnel. Use an additional 5 ml of water to wash the contents of the flask into the funnel. Extract the solution 3 times with 5 ml dichloromethane. After each extraction discard the organic layer. After the third



extraction, transfer the aqueous layer to a round bottom flask and rotovap to dryness. Resuspend the product in 3ml ethanol and transfer to a pre-weighed 15ml conical tube. Rinse the flask with an additional 2ml of ethanol and add the rinse to the conical tube. Add 5ml benzene and incubate the tube in warm water until the product dissolves then immediately place the tube on ice for 10 min. Centrifuge for 5 min at 15000 g and remove the supernatant. Add 5ml ethanol and 5 ml isopropyl ether to the precipitant. Dissolve the precipitant by incubating the tube in warm water and periodically tilting the tube very gently. Once precipitate is dissolved, place the tube on ice for 10 min. Perform this recrystallization 2 more times with ethanol and isopropyl ether. Some insoluble white chunks of product may be formed. After the last recrystallization step remove as much supernatant as possible and dry the resultant product in a Speedvac. Weigh the conical tube with the synthesized product. Add 5ml water to the tube, vortex, and filter out any remaining solids. Use the UV-vis spectrophotometer to determine the final concentration of MMDA. The  $\lambda_{\max}$  for MMDA in 50 mM potassium phosphate buffer (pH 7) is 274 nm with the extinction coefficient of  $29900 \text{ M}^{-1}\text{cm}^{-1}$  [14].

#### GCMS Measurement Of MDA

Prepare a solution of 0.5mM butylated hydroxyl toluene (BHT) in ethanol by adding 0.11 g BHT to 9ml ethanol and adjusting the final volume to 10ml. Then perform a dilution by adding 10 $\mu$ l of this concentrated solution to 990 $\mu$ l ethanol. Prepare a solution of 50mM phenyl hydrazine by adding 4.92 $\mu$ l of phenyl hydrazine to 995 $\mu$ l of water in a dark microcentrifuge tube. Vortex the solution. Add 5 $\mu$ l 10 $\mu$ M MMDA to the 100 $\mu$ l plasma set aside for the MDA assay during sample collection and processing.

Then add 10 $\mu$ l 0.5mM BHT to the MMDA/plasma mixture followed by the addition of 200 $\mu$ l 1M sodium citrate (pH 4). This pH is crucial for correct derivatization of the sample. A higher or lower pH will result in skewed MDA levels. Dilute the final mixture by adding distilled deionized water to a final volume of 480 $\mu$ l. Derivatize the solution by adding 20 $\mu$ l 50mM phenyl hydrazine, capping the vials, and incubating the solution at 25°C for 30 min on an orbital shaker. Add 1ml of hexane, vortex for 1 min, and centrifuge at 3000 rpm for 10 min. Transfer the organic layer to a GCMS vial and concentrate the solution to 100 $\mu$ l by N<sub>2</sub> stream.

Analyze samples on a GCMS using the auto sampler. Perform compound separation on an Agilent 122-5532G capillary column (25.7 m length, 0.25 mm internal diameter). Use helium as the carrier gas at a flow rate of 0.6-0.8 ml/min. Inject derivatized product (2  $\mu$ l) in split mode (split ratio 20:1, split flow 23.0 ml/min, total flow 27.1 ml/min). Set the initial column temperature at 110°C and hold at that temperature for 1 min before increasing it to 250°C at a rate of 40°C/min. Maintain this temperature until the end of the run. After each sample, clean the column with 2 injections of hexane. Quantify MDA using select ion monitoring mode using the 144.00 m/z for MDA and the 158.00 m/z for MMDA. Convert the MDA/MMDA ion abundance ratios to micromolar concentrations using a standard curve. Analyze samples in triplicate and only use values with a coefficient of variation of less than 10%.

#### HPLC Measurement Of Xanthine Oxidase

The detection of xanthine oxidase function is dependent on the ability to sensitively measure the levels of a suitable substrate (pterin) as well as its product

(isoxanthopterin). Plasma is incubated with the substrate either for 0 min (control) or 4 h, and the incubation-dependent substrate conversion is determined. Add 240 $\mu$ l 0.2 M Tris-HCl buffer (pH 9.0) and 4.63 $\mu$ l 7.083 $\times 10^{-3}$  M pterin to each tube (0 and 4h) and preincubate them at 37°C for 5 min. Initiate the enzyme reaction by adding 60 $\mu$ l of plasma to each tube. Immediately add 300 $\mu$ l 4% HClO<sub>4</sub> to the 0 incubation tube but allow the other mixture to incubate for 4 hours before quenching the reaction with 300 $\mu$ l 4% HClO<sub>4</sub>. After the addition of HClO<sub>4</sub>, vigorously shake the tube and then centrifuge at 4°C at 15000 g for 15 min. Remove 500 $\mu$ l of supernatant and neutralize by adding 20 $\mu$ l of 5M K<sub>2</sub>CO<sub>3</sub>. Vigorously shake the tube and then centrifuge at 4°C at 15000 g for 15 min. Add 350 $\mu$ l of the neutralized solution to a microcentrifuge tube containing 1 $\times 10^{-8}$  mol 2-AP.

Analyze samples on an HPLC equipped with a scanning fluorescence detector. Three 50  $\mu$ l injections are used for each sample. Samples will be injected onto a Wacossil 5C-18-200 250 mm x 6.0 mm, 5  $\mu$ m column equipped with a Supelguard LC-18-S column guard. The following isocratic conditions should be used to obtain adequate peak separation and identification: 95% 20mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 2.2) and 5% methanol at a flow rate 1ml/min. Set the excitation wavelength for the fluorescence detector at 345 nm and the emission wavelength at 410 nm. Determine the ratios of pterine and isoxanthopterin to 2-AP by obtaining peak areas from the fluorescence detector spectrum. The first peak in the spectrum, ~ 5 minutes, corresponds to 2-AP. The second and largest peak will be pterine. Isoxanthopterin peak elutes last. Obtain the difference in the isoxanthopterin/2-AP peak area ratios between the 0 and the 4 h incubation time

points. Use this value to calculate the XO activity from standard curves. Analyze samples in triplicates but use only values with a coefficient of variation of less than 10%.

### **Representative Results**

An example of the HPLC quantification of purine compounds is shown in **Figure 1A**. The specific retention times and emission wavelengths of hypoxanthine, xanthine, and uric acid permit the simultaneous quantification of purine compounds (**Table 2**). When the assay is run correctly, the compounds will have adequate separation and the peak shape will be sharp and unimodal. These peaks are then converted into concentrations, ranges shown in **table 3**, through the use of a standard curve. Because sample processing for this assay is minimal, the only sample based problems which may arise would be the lysing of the red blood cells. If the red blood cells lyse before the samples are centrifuged, the plasma will take on an orange/red color and cannot be used for evaluating hypoxic ischemia. The other issues which may arise when measuring purines involves the HPLC system and the column (**Figure 1B**). If there are air bubbles in the HPLC system the retention times will shift and the HPLC pressure will fluctuate dramatically. If the guard cartridge needs to be changed, the pressure will increase and the peaks will widen and become bi or tri-modal.

An example of the GCMS quantification of Allantoin is shown in **Figure 2**. Because the mass of derivatized allantoin and derivatized heavy allantoin is known, select ion mode can be used to identify these compounds on the GCMS. If the assay is done correctly, two peaks will be observed at the same retention time. One corresponding to allantoin (398.00 m/z) and the other to heavy allantoin (400.00 m/z).

These peaks are then converted into concentrations, ranges shown in table 3, through the use of a standard curve. If the assay is run incorrectly, and samples were not derivatized properly, the peaks may not be present or may not be quantitatively representative. Once again, if the red blood cells lysed the plasma cannot be used to evaluate oxidative stress in neonatal hypoxic ischemia. The results for the quantification of MDA are similar to those for allantoin with the exception that the two peaks are observed at different retention times. At ~ 3 minutes retention time, a 144.00 m/z peak for MDA and at ~3.5 minutes retention time, a 158.00 m/z peak for MMDA is observed (**Figure 3**). These peaks are then converted into concentrations, ranges shown in table 3, through the use of a standard curve. If the assay is run incorrectly, or samples are not derivatized properly, no peaks may be observed when selecting for 144.00m/z and 158.00m/z. It should be noted that if there is an excess of lipid in the plasma from bolus oral feedings or intravenous lipid administration, the plasma will take on a milky appearance and cannot be used to evaluate oxidative stress in neonatal hypoxic ischemia.

An example of the HPLC-based quantification of xanthine oxidase function is shown in **Figure 4**. If the assay is run correctly, three peaks should be observed with the fluorescent detector, one for pterin, one for isoxanthopterin, and one for 2-AP. These peaks are then converted into concentrations, ranges shown in table 3, through the use of a standard curve. There should also be a peak corresponding to isoxanthopterin and 2-AP on the spectrum generated from the PDA detector. If the enzyme activity is absent, the peak corresponding to isoxanthopterin will not be seen. Because this assay measures enzyme function, repeated freezing and thawing of the sample may alter this

Figure 1 A

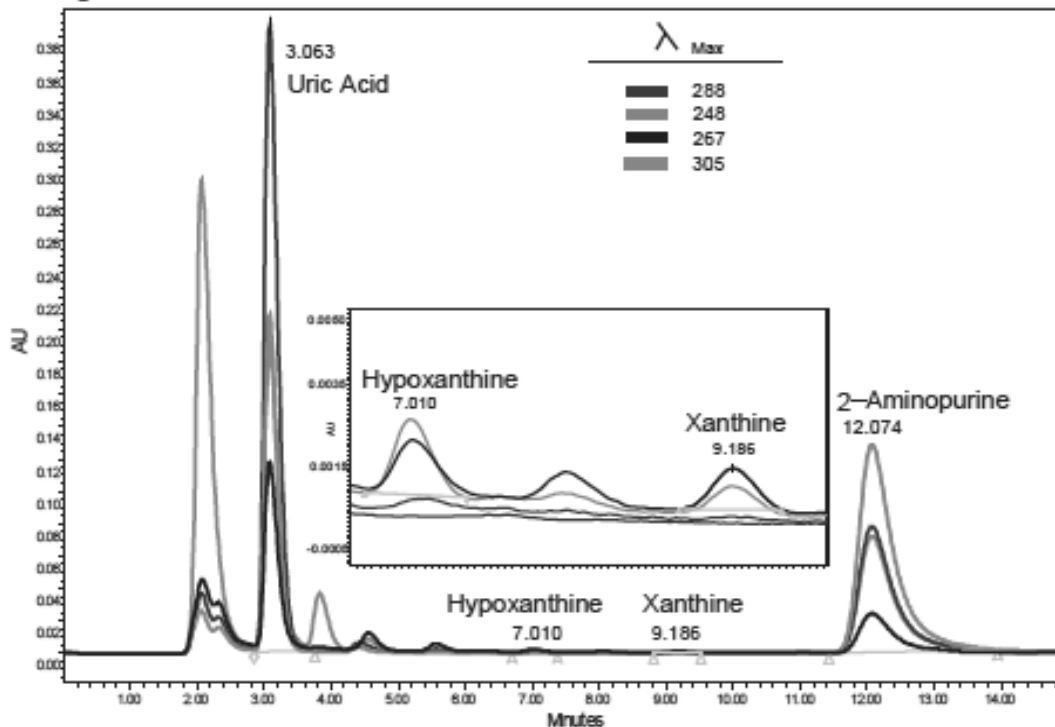
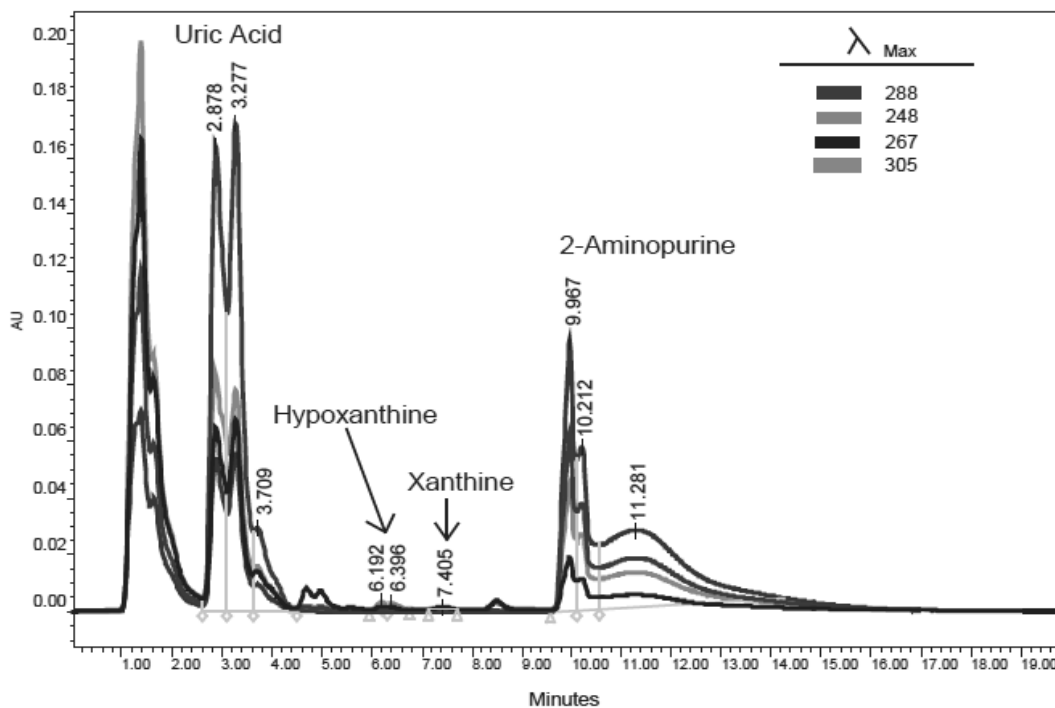


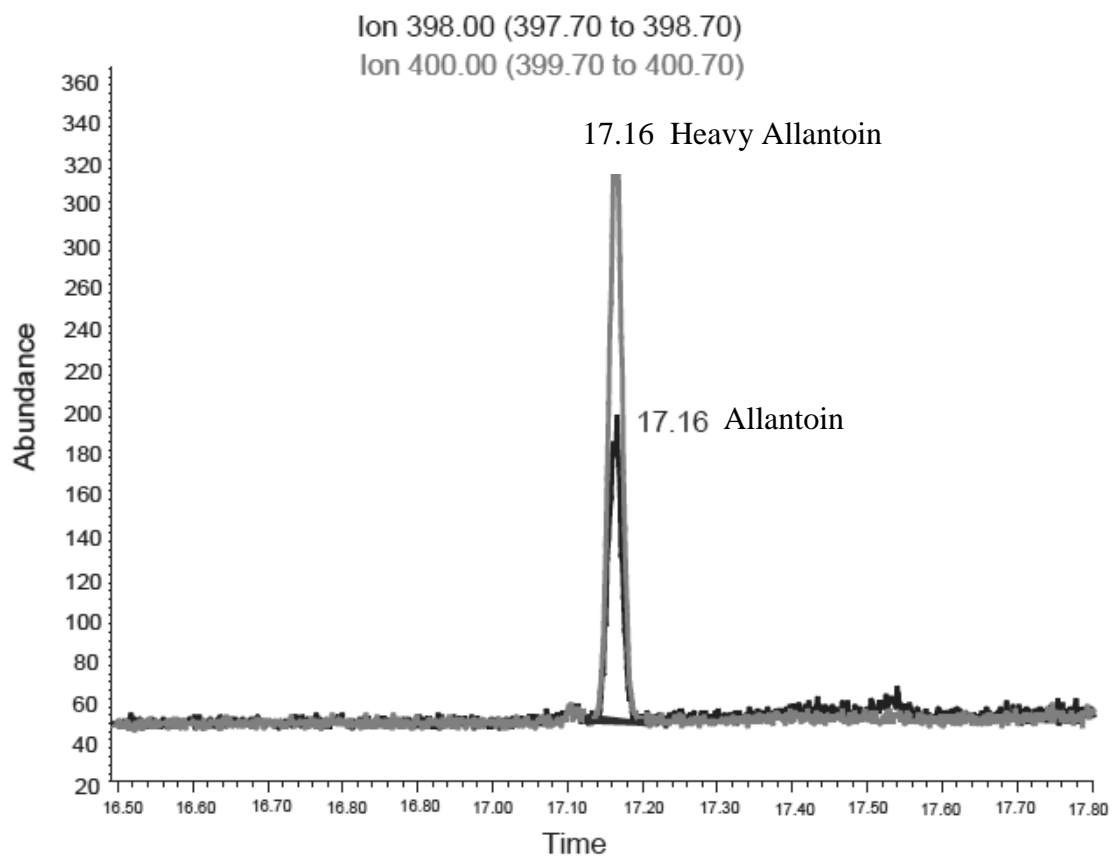
Figure 1 B



**Figure 1: HPLC Chromatogram for the Identification of Purine Compounds.** A). Representative results if the assay was run correctly. B). Representative results if there is a problem with the HPLC, column, or guard cartridge.

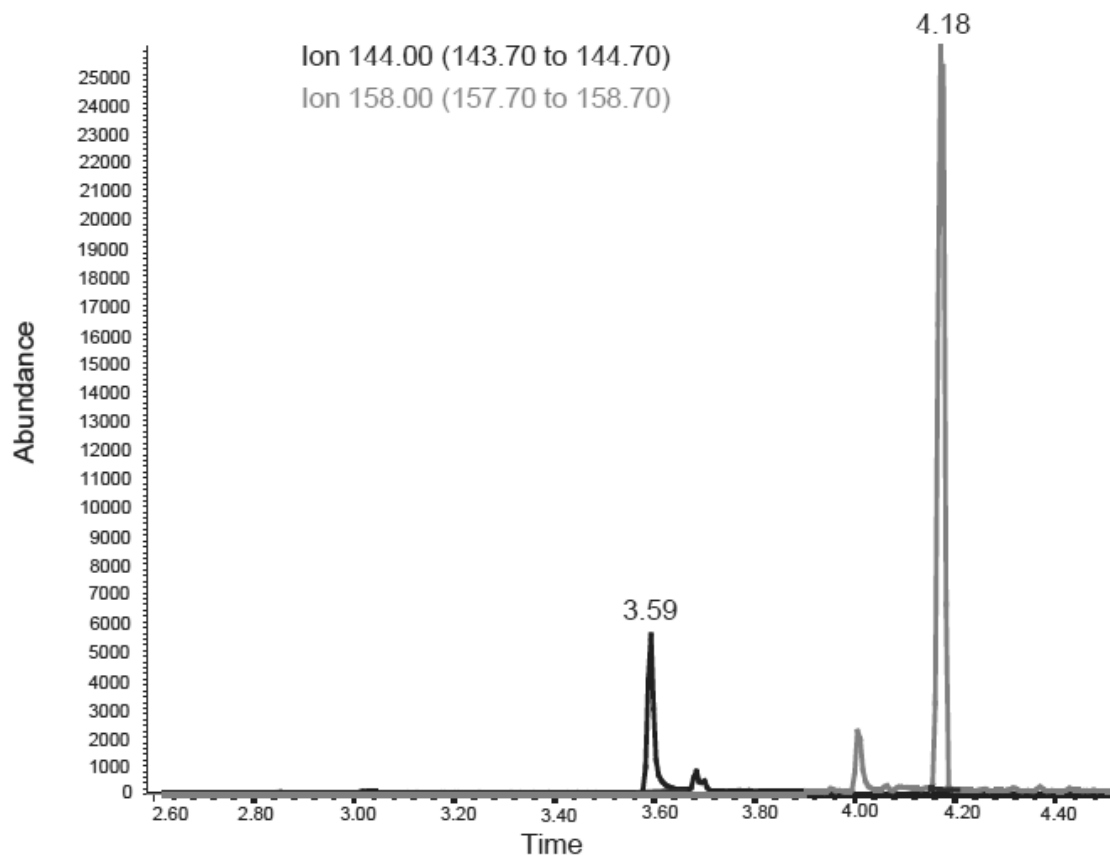
**Table 3: Representative ranges for purines, xanthine oxidase, malondialdehyde, and allantoin. Max-Min (n).**

	Hypoxanthine	Xanthine	Uric Acid	Xanthine Oxidase	MDA	Allantoin
Term Normoxic	1.13-19.3 (n=64)	0.02-3.69 (n=61)	107.20-726.12 (n=63)	2.47x10 <sup>-6</sup> - 3.92x10 <sup>-3</sup> (n=20)	0.44-3.76 (n=53)	NA
Term Respiratory Distress	1.78-12.59 (n=27)	0.07-11.8 (n=24)	225.40-653.32 (n=27)	0 - 1.03x10 <sup>-5</sup> (n=8)	0.82-2.73 (n=24)	NA
Term Hypoxic	0.38-31.80 (n=13)	0.11-2.88 (n=13)	235.65- 1348.13 (n=13)	1.20x10 <sup>-5</sup> - 236.44 (n=9)	0.95-2.15 (n=7)	NA
Preterm Normoxic	1.54-4.39 (n=9)	0.03-1.77 (n=9)	178.92-593.49 (n=9)	2.46x10 <sup>-5</sup> - 5.44x10 <sup>-4</sup> (n=2)	0.95-2.74 (n=8)	2.30-5.26 (n=67)
Preterm Respiratory Distress	3.04-8.04 (n=2)	NA	327.56-365.11 (n=2)	NA	2.40-3.46 (n=3)	NA

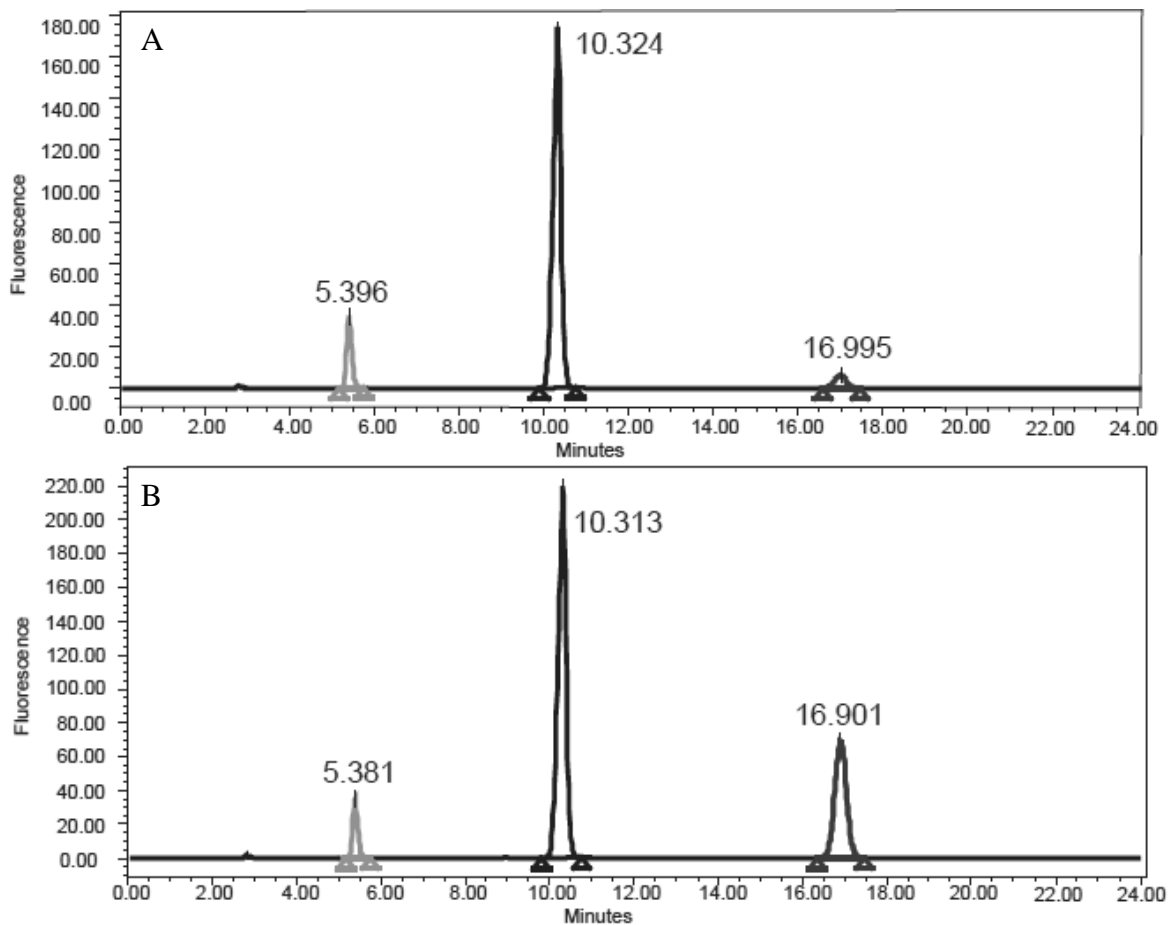


**Figure 2: GCMS Spectrum for the Quantification of Allantoin.**





**Figure 3: GCMS Spectrum for the Quantification of MDA.**



**Figure 4: HPLC spectrum for the measurement of XO activity.** A). Representative results for the 0 min incubation time and B) Representative results for the 4 hour incubation time. Note the higher isoxanthopterin peak for the 4 hour incubation time.

## **Discussion**

The methods described here permit for the evaluation of neonatal hypoxic ischemia. This protocol combines the measurements of markers of energy deprivation, oxidative stress, oxidative damage, and enzyme activity to gain an overall biochemical picture of the presence or even the degree of hypoxic ischemia. Despite the usefulness of this method, there are potential limitations. First, it takes roughly 1-2 ml of blood to collect enough plasma to run all of the assays. This will not be a problem in adults or children, but it poses issues when it comes to term and especially preterm neonates. We work around this issue by prioritizing assays and diluting some of the samples if necessary. Secondly, it can be difficult to synthesize MMDA; however, deuterated MDA can be used as a substitute for MMDA as an internal standard.

Despite these minor limitations, the method described here provides a useful tool in evaluating neonatal hypoxic ischemia as well as other diseases associated with alterations in redox homeostasis. Moreover, all of these markers, with the exception of XO, can be measured in urine, providing a non-invasive means of monitoring neonates.

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## CHAPTER FOUR

### AN ANIMAL MODEL FOR MEASURING THE EFFECT OF COMMON NICU PROCEDURES ON ATP METABOLISM

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**Running Title:** Heel Lance Increases ATP Utilization in Neonatal Rabbit Model

Adapted from Plank, M.S., Boskovic, D.S., Tagge, E., Chrisler, J., Slater, L., Angeles,  
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## Abstract

Infants exposed to common neonatal intensive care unit (NICU) procedures show alterations in heart rate, blood pressure and oxygen saturation; however, it is unclear if these physiologic changes increase adenosine triphosphate (ATP) utilization, thus potentially increasing the risk for tissue hypoxia in medically fragile neonates. Plasma uric acid is a commonly used marker of increased ATP utilization because uric acid levels increase when ATP consumption is enhanced. To examine the effect of a common NICU procedure on plasma uric acid concentration, we developed a model that allows for acute monitoring of this biochemical marker in plasma in 7- to 9-day-old rabbits. In this pilot study, we exposed neonatal rabbits to a single heel lance 2.5 hr after catheter placement. We measured uric acid concentration before and 30 min after the heel lance and compared findings to levels in control animals not exposed to the heel lance. Our pilot data shows a significant difference in uric acid concentration over time between the control and heel lance groups ( $46.2 \pm 7.1 \mu\text{M}$  vs.  $54.7 \pm 5.8 \mu\text{M}$ , respectively,  $P = 0.027$ ). Calculation of percent change from baseline showed uric acid concentration increasing in rabbits exposed to heel lance and decreasing in control rabbits ( $1.5\% \pm 4.7\%$  vs.  $-16.1 \pm 4.2\%$ , respectively,  $P = 0.03$ ). These data suggest that this animal model can be successfully used to examine the biochemical effect of common NICU procedures, such as heel lance, on markers of ATP breakdown and purine metabolism.

## Introduction

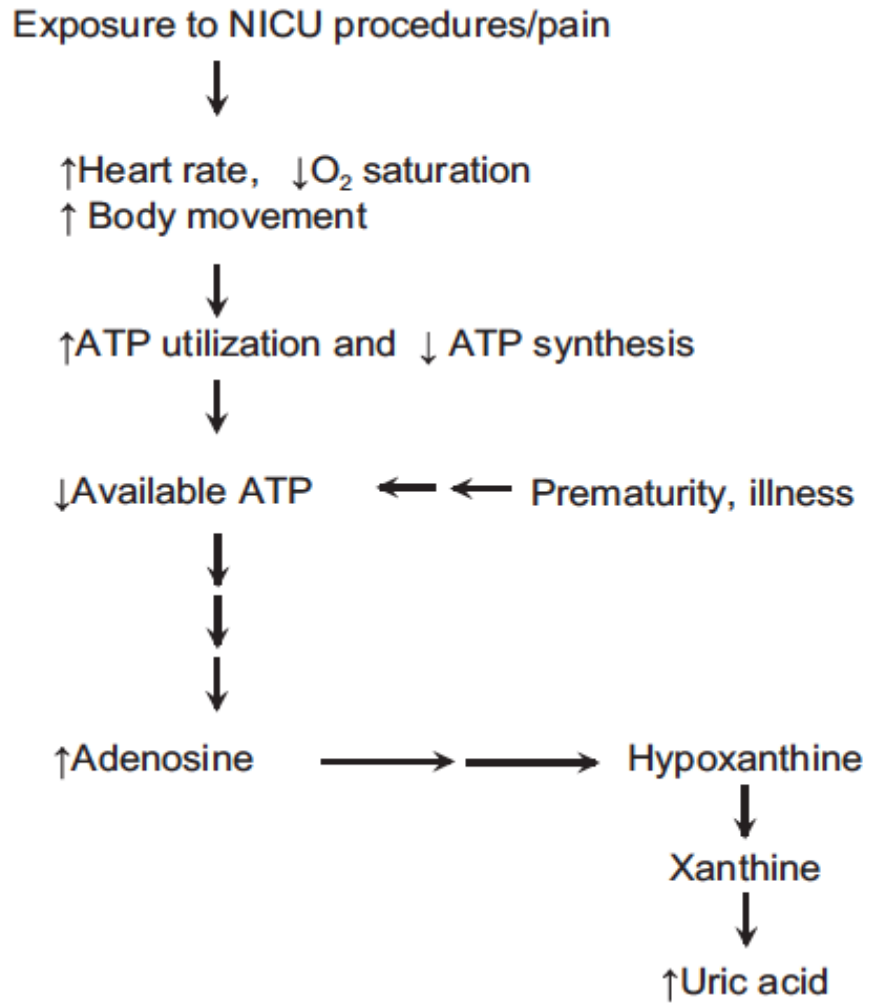
Infants in the neonatal intensive care unit (NICU) routinely undergo painful and invasive procedures. NICU patients are exposed to an average of  $12 \pm 8$  painful procedures per day and  $141 \pm 107$  total procedures over a 14-day period [1]. Invasive and painful NICU procedures are known to alter physiological homeostasis by increasing heart rate and metabolism and decreasing oxygen saturation and transcutaneous PaO<sub>2</sub> (partial pressure of oxygen in arterial blood) [2, 3]. However, it is unclear if painful procedures increase adenosine triphosphate (ATP) utilization in excess of ATP synthesis, increasing the risk for tissue hypoxia. Pilot data from our laboratory demonstrated significant alterations in purine metabolism in the plasma of premature neonates experiencing tape removal, while a recent paper by Bellieni and colleagues showed an increase in markers of oxidative stress in the plasma of premature neonates experiencing heel lance [4, 5]. These data suggest that common NICU procedures can lead to enhanced ATP breakdown and oxidative stress in immature neonates.

To further examine the biochemical effects of commonly performed NICU procedures, our laboratory developed a neonatal rabbit model for acute monitoring of ATP breakdown and purine metabolism. The neonatal rabbit kit is an ideal NICU model for several reasons. First, the neonatal rabbit kit has brain development similar to the premature human neonate, with a high cortical white:gray matter ratio and undeveloped cerebral white matter with undefined masses of neuroblasts [6] These similarities enable evaluation of the white matter injury commonly seen in hypoxic premature neonates. Second, unlike rats or mice, neonatal rabbits are unique in that maternal behavior is restricted and minimal, making it possible to perform procedures and obtain results that



are independent of stresses related to maternal separation, feeding behavior or interference [7]. Lastly, the larger size and higher plasma volume of the postnatal rabbit kit at age 7–9 days when compared to the postnatal rat or mouse of the same age, approximately 15 times bigger than the neonatal rat pup, makes it possible to monitor heart rate, oxygen saturation and blood pressure during procedures as well as collect multiple blood samples from each animal without causing hypovolemia and significantly altering cardiovascular homeostasis.

Based on the above-described characteristics, we examined the possible use of neonatal rabbits as an experimental model to measure the effect of commonly performed NICU procedures on ATP breakdown. It is well documented that when animals and humans are challenged with increased metabolic needs, they will catabolize high-energy phosphate bonds such as ATP [8-13]. The degradation of ATP results in increased levels of free adenosine, which is consequently degraded to inosine and then to hypoxanthine and xanthine and finally to uric acid (**Figure 1**). In humans, an increased level of uric acid is a commonly accepted biochemical marker of hypoxia-ischemia and increased ATP breakdown [8, 11, 13]. If common NICU procedures enhance ATP and purine consumption, we would expect to see a significant increase in the concentration of markers of ATP utilization, specifically uric acid, in rabbit kits exposed to procedures commonly performed in the NICU.



**Figure 1.** Exposure to neonatal intensive care unit (NICU) procedures/pain leads to adenosine triphosphate (ATP) breakdown and uric acid metabolism. O<sub>2</sub> = oxygen.

## Methods

### Animals

We performed all experiments in accordance with approved institutional animal care guidelines. We purchased midgestation New Zealand rabbit dams from a local vendor and allowed them to deliver naturally at term. At postnatal day of life 5, we transported the rabbit dam and kits to the animal care facility, where they were maintained under approved laboratory conditions. Animals were housed in a steel cage with a thermoregulated litter box containing nesting materials for the kits. Food and water were made available ad libitum.

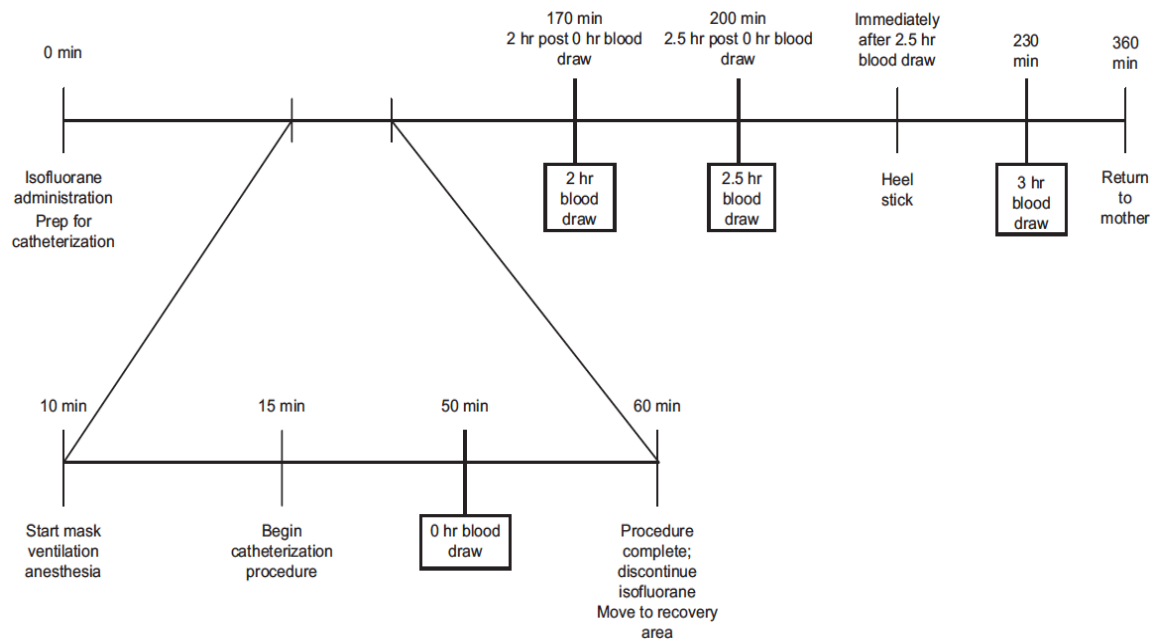
### Central Catheter Placement

We performed catheter placement on a warming pad and facilitated dissection using an OPMI-6 surgical microscope (Karl Zeiss Co., Germany) at approximately 10X magnification. We fashioned the catheters by inserting and gluing PE-10 tubing (Braintree, MA) into PE-50 tubing (Braintree, MA) prior to sterilization. At postnatal day of life 7–9, neonatal rabbit kits were quickly anesthetized with 3%–3.5% isoflurane and then maintained by mask ventilation with 1.5%–2.5% isoflurane to maximize anesthesia while minimizing cardiorespiratory depression. Once the kits were anesthetized, we made an incision in the neck to the right of the ventral midline and isolated the internal jugular vein from surrounding tissue. We made a second skin incision inferior to the left scapula and tunneled a venous catheter subcutaneously from the back incision to the neck incision. We then inserted a 25-gauge butterfly into the free end of the PE-50 tubing and filled the catheter with heparinized saline (20 units/ml). We

ligated the distal internal jugular vein with 8-0 ophthalmic nylon and made a venotomy with a 25-gauge needle. We then passed the catheter antegrade through the venotomy at a predetermined distance to reside at the junction of superior vena cava and right atrium. We secured the catheter to the proximal vein using 6-0 silk after ensuring patency, filled it with heparinized saline, and locked it with gentamicin (120 microgram/ml). We closed subcutaneous tissues in a routine manner with 6-0 vicryl and maintained catheter patency by flushing with heparinized saline (10 units/ml) after each blood draw, as described below. We employed standard aseptic techniques and monitored the rabbit kits continuously for heart rate, respiratory rate and temperature. After completing the catheterization procedures, we discontinued anesthesia. All rabbit kits recovered immediately and were observed to be active and moving freely within 15–30 min. They remained awake (unanesthetized) and active for the duration of the study.

#### Blood Sampling Procedure For Acute Monitoring Of Plasma Uric Acid Concentration

To examine the effectiveness of this animal model in acutely measuring the effects of common NICU procedures on plasma uric acid concentration, we randomized rabbit kits to either a time-control or a heel lance group. We used data gathered from the time-control group to determine the optimal timing of the heel lance procedure in the experimental animals as described below. For all animals, we drew blood from the jugular catheter at specific time points: immediately after catheter insertion (0 hr) and at 2 hr, 2.5 hr and 3 hr after insertion (**Figure 2**). Study conditions (lighting, noise,



**Figure 2.** Time line of experimental procedures.

temperature) were similar during both time-control and heel lance procedures. Animals in the time-control group were allowed to move around the cage freely for the entire study after catheter placement; animals in the heel lance group were allowed to move freely until 2.5 hr after surgery when they were exposed to the heel lance procedure.

#### Heel Lance Procedure.

Based on data gathered in the time-control animals (see “Results,” **Figure 3**), we exposed rabbit kits in the heel lance group to heel lance (Accucheek, Roche Diagnostics Basel, Switzerland) at 2.5 hr after anesthesia and catheterization. We used a lance depth of 2.3 mm for each heel lance procedure due to the presence of fur on the rabbit’s heel and to ensure that the procedure would lead to a visible single drop of blood.

#### Blood Processing And Uric Acid Analysis

Each blood sample was collected into an EDTA tube in the surgical sweet and transported it to a lab in the department, on ice, where lab personnel placed the samples into a Fisher Scientific AccuSpin 1R Centrifuge (Fair Lawn, NJ). Samples were centrifuged for 20 min at 1,500 g and 4°C. The plasma was removed, transferred to separate Eppendorf tubes and centrifuged in a Beckman Microfuge 22R centrifuge (Fullerton, CA) for 30 min at 18000 g, 4°C. Each supernatant, 250µl, was then deproteinated using a Microcon centrifugal filter device (Millipore Corp., Bedford, MA) with a 10K molecular weight cutoff by spinning the loaded device for 90 min at 14000 g and 4°C. Filtrate was removed and 200 µl were transferred to an Eppendorf tube

containing  $1 \times 10^{-7}$  mol of 2-aminopurine as internal standard. Samples were stored at  $-80^{\circ}\text{C}$  until analysis.

For measurement of uric acid, each blood sample was analyzed using high pressure chromatography (HPLC; Waters 600E, Waters 996 PDA, Millipore Corp.) by injecting  $50 \mu\text{l}$  of sample onto a Supelcosil LC-18-S  $15\text{cm} \times 4.6 \text{ mm}$ ,  $5 \mu\text{m}$  column (SGE: Austin, TX) with the following isocratic conditions:  $50\text{mM}$  ammonium formate buffer,  $\text{pH } 5.5$ , flow rate  $1.0 \text{ ml/min}$ . Uric acid and the internal standard, 2-aminopurine, were quantitated by obtaining peak areas at the appropriate retention times ( $\sim 3.0$  and  $\sim 12.0$  min, respectively) and absorbance wavelengths ( $288$  and  $305$ , respectively). The area ratio of uric acid to 2-aminopurine was determined and converted into concentration using standard curves. Samples were analyzed in triplicate and values with a coefficient of variation less than  $10\%$  were included in the final analysis.

### Statistical Analyses

We assessed assumptions of normality and equal variance. We used independent samples t-test to analyze continuous data and reported them as the mean and standard deviation (mean  $\pm$  SD). We assessed repeated measures ANOVA for one between-subject factor (group) and one within-subject factor (time) to evaluate the effect of the two procedures (control vs. heel lance) on plasma uric acid concentration over time using interaction terms in the general linear model. We calculated percent change in uric acid between 0-min (baseline) and 30-min (postprocedure) values as follows (**Eq. 1**):

$$([30 \text{ min}] - [0 \text{ min}]) / [0 \text{ min}] \times 100 \quad \text{Eq. 1}$$

We performed all statistical analyses using SPSS Statistics for Windows Version 19 and considered differences to be significant at  $p < .05$ .

## Results

### Central Catheter Placement

Using the jugular vein, we were successful in obtaining multiple blood samples from a total of 11 out of 15 rabbit kits (73% success rate), 5 of which were in the control group and 6 of which were in the heel lance group. We were not able to obtain multiple blood samples in the other 4 rabbits due to catheter dislodgement or failure of the line to draw, possibly due to kinking or clotting.

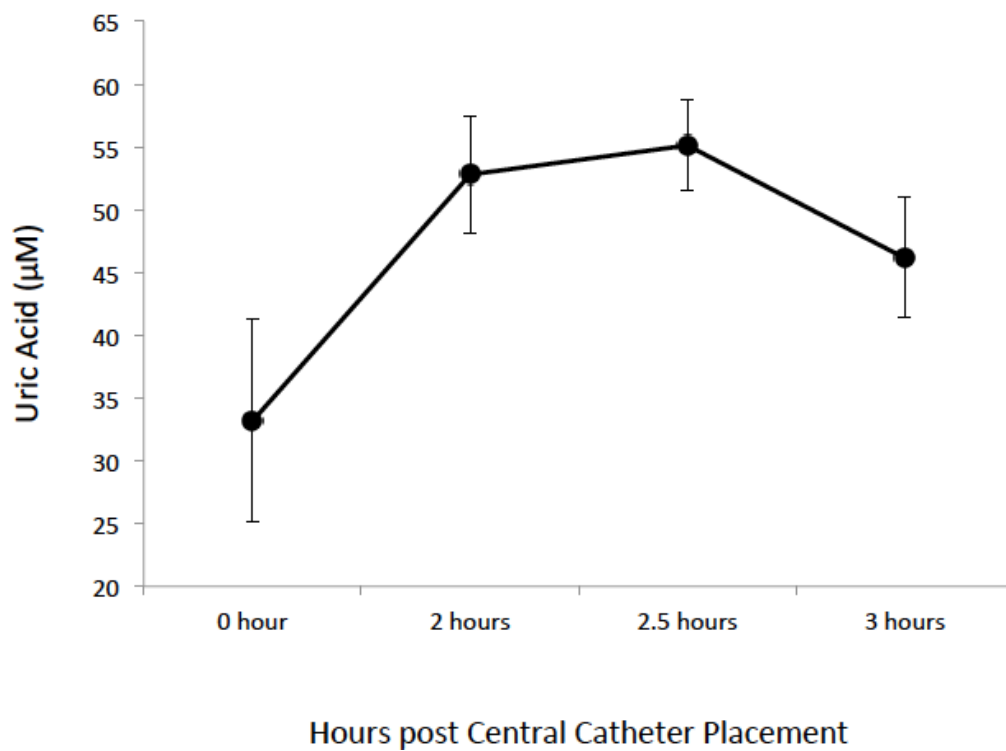
### Stabilization Of Plasma Uric Acid Concentration Post Central Catheter Placement

We measured uric acid concentrations in plasma over multiple time points to determine when this marker stabilized after jugular catheterization. We found in 5 control rabbit kits that uric acid values began to fall at the 2.5- and 3-hr time points, suggesting achievement of steady-state ATP metabolism (**Figure 3**). Thus we determined that the 2.5-hour time point could be used to explore the effect of procedures on biochemical markers of ATP breakdown.

### Effect Of Heel Lance On Uric Acid Levels

We found significant differences in uric acid concentration over a 30 min time period between the control group and the heel lance group ( $46.2 \pm 7.1 \mu\text{M}$  vs.  $54.7 \pm 5.8 \mu\text{M}$ , respectively,  $p = .027$ ; **Figure 4**). Whereas uric acid concentration decreased over





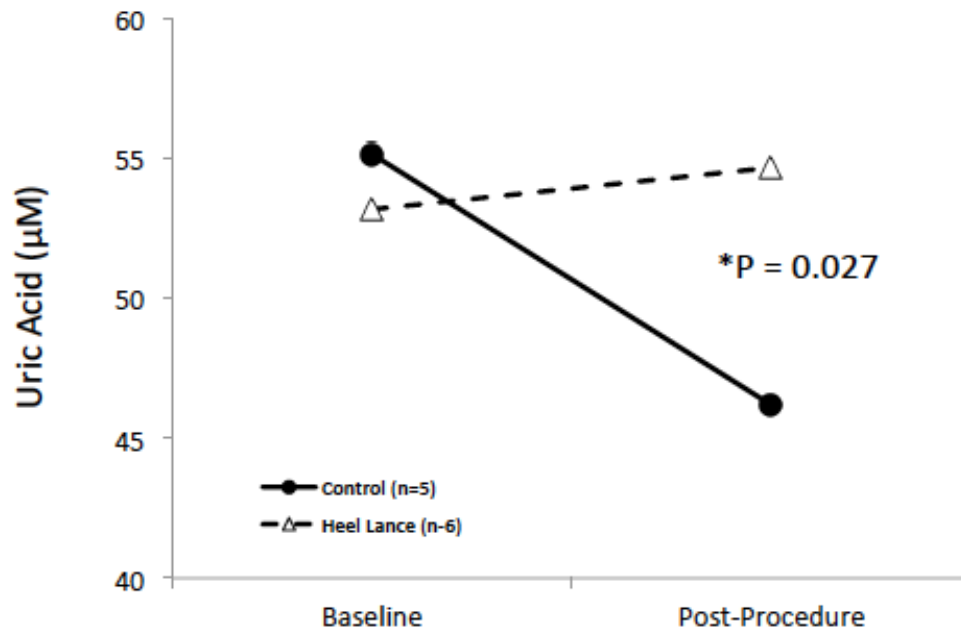
**Figure 3.** Uric acid concentration time course in control rabbit kits ( $n = 5$ ; mean $\pm$ SD).

time in control rabbits, it increased over time in rabbits exposed to heel lance (repeated measures,  $p < .05$ ; **Figure 4**). Calculation of percent change from baseline confirmed that uric acid concentration was increasing in the heel lance group while decreasing in the control group ( $1.5\% \pm 4.7\%$  vs.  $-16.1 \pm 4.2\%$ , respectively,  $p = .03$ ).

## Discussion

Our preliminary work showed that neonatal rabbits can successfully be used as a model for acutely monitoring the effects of common NICU procedures on ATP metabolism and breakdown. After collecting blood at four different time points post catheter placement, we observed that plasma uric acid levels began decreasing at the 2.5- and 3-hr time points. This decrease was most likely due to the enzymatic conversion of uric acid to allantoin or to urinary excretion, which is to be expected in control rabbits. In the absence of enhanced ATP breakdown, plasma uric acid levels will gradually stabilize or decrease [14]. The conversion of uric acid to allantoin occurs through the action of uricase, an enzyme present in rabbits [15, 16].

At the 2.5- and 3-hr mark, we noted an opposite trend in rabbits exposed to a single heel lance (**Figure 4**). This increase in plasma uric acid concentration suggests that this common procedure can increase ATP utilization and breakdown. It is unclear if this increase in energy needs will exacerbate hypoxia in sick premature neonates. Further studies are needed to determine if enhanced ATP utilization due to exposure to common NICU procedures induces a hypoxic response, which may lead to inflammation and organ damage [17, 18].



**Figure 4.** Differences in uric acid concentration over time in rabbits without heel lance (control group,  $n = 5$ ) and rabbits subjected to heel lance (heel lance group,  $n = 6$ ). \*Repeated measures ANOVA.

One comment regarding difficulties we encountered in the use of this animal model might be helpful for future investigators. After evaluating several potential sites for central catheter placement, we found the highest success with the internal jugular vein. Although attempts to insert catheters into the femoral vein were moderately successful, the catheters fell or kinked when the rabbits recovered from surgery and were permitted to move around freely. We disregarded other potential catheter insertion sites, such as the ear vein or the cephalic vein, because of vessel size and high incidence of vessel collapse when a sample is being aspirated. Even with the choice of the internal jugular vein, only 11 of the 15 catheters placed remained patent and allowed for repeated blood draws.

Our pilot study shows preliminary data documenting the use of this animal model to examine the relationship between a single commonly performed NICU procedure, heel lance, and markers of ATP breakdown. The model could also be used to study the biochemical effects of a combination of NICU procedures performed sequentially, leading to an examination of the biochemical effects of repetitive pain- or non-pain-inducing procedures. The rabbit's larger size relative to rats and mice would allow the measurement of other relevant biochemical markers of hypoxia, ATP breakdown or oxidative stress, including plasma concentrations of ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, malondialdehyde or F<sub>2</sub> isoprostane. Although not performed in this current study, future investigators could develop and validate measures of pain specific to neonatal rabbits for use in rabbits exposed to painful procedures, which would allow for the examination of the relationships among biochemical markers of ATP utilization, hypoxia and behavioral markers of pain.

Because the animals survive the catheterization and blood sampling procedure, the model could also be used to investigate the possible link between procedural pain, increased ATP utilization and organ impairment, such as white matter injury, through magnetic resonance imaging and spectroscopy.

### **Relevance To Nursing Practice And Research**

NICU nurses perform many painful and nonpainful procedures on the neonates in their care. Although the effects of these procedures on heart rate and oxygen saturation is clearly visible at the bedside, it is unclear whether these alterations increase ATP utilization and decrease ATP synthesis, potentially increasing the risk of tissue hypoxia in fragile neonates. Researchers have hypothesized that hypoxia from pain and stress during many weeks and months in the NICU may be one contributing factor to white matter injury. However, this link remains speculative because the mechanisms that link pain with organ damage remain elusive. Use of this model may help elucidate the relationship between single, multiple or repetitive NICU procedures and ATP breakdown and hypoxia. Because the animals are allowed to survive after sample collection, the effect of NICU procedures on organ damage could be elucidated through multiple imaging modalities (magnetic resonance imaging and spectroscopy) or tissue assays. Data from these studies could be used to test efficacy of treatment strategies and to develop innovative nursing interventions to prevent and treat potentially adverse consequences of commonly performed NICU procedures in premature neonates [19, 20].

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CHAPTER FIVE  
PROCEDURAL PAIN AND OXIDATIVE STRESS IN  
PREMATURE NEONATES

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**Running Title:** Procedural Pain and Oxidative Stress

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Phillips R, Deming D, Ashwal S, Hougland K, Fayard E, Angeles DA. (2012). J

Pain. 13(6): p. 590-597.



## Abstract

Preterm neonates exposed to painful NICU procedures exhibit increased pain scores and alterations in oxygenation and heart rate. It is unclear whether these physiologic responses increase the risk of oxidative stress. Using a prospective study design, we examined the relationship between a tissue-damaging procedure (TDP, tape removal during discontinuation of an indwelling central arterial or venous catheter) and oxidative stress in 80 preterm neonates. Oxidative stress was quantified by measuring uric acid and malondialdehyde (MDA) concentration in plasma obtained before and after neonates experienced a TDP (n=38) compared to those not experiencing any TDP (control group, n=42). Pain was measured before and during the TDP using the Premature Infant Pain Profile (PIPP). We found that pain scores were higher in the TDP group compared to the control group (median scores: 11 and 5, respectively,  $P < 0.001$ ). Uric acid significantly decreased over time in control neonates but remained stable in TDP neonates ( $132.76\mu\text{M}$  to  $123.23\mu\text{M}$  vs.  $140.50\mu\text{M}$  to  $138.9\mu\text{M}$ ,  $P = 0.002$ ). MDA levels decreased over time in the control group but increased in the TDP group ( $2.07\mu\text{M}$  to  $1.81\mu\text{M}$  vs.  $2.07\mu\text{M}$  to  $2.21\mu\text{M}$ ,  $P = 0.01$ ). We found a significant positive correlation between PIPP scores and MDA. Our data suggest a significant relationship between procedural pain and oxidative stress in premature neonates.

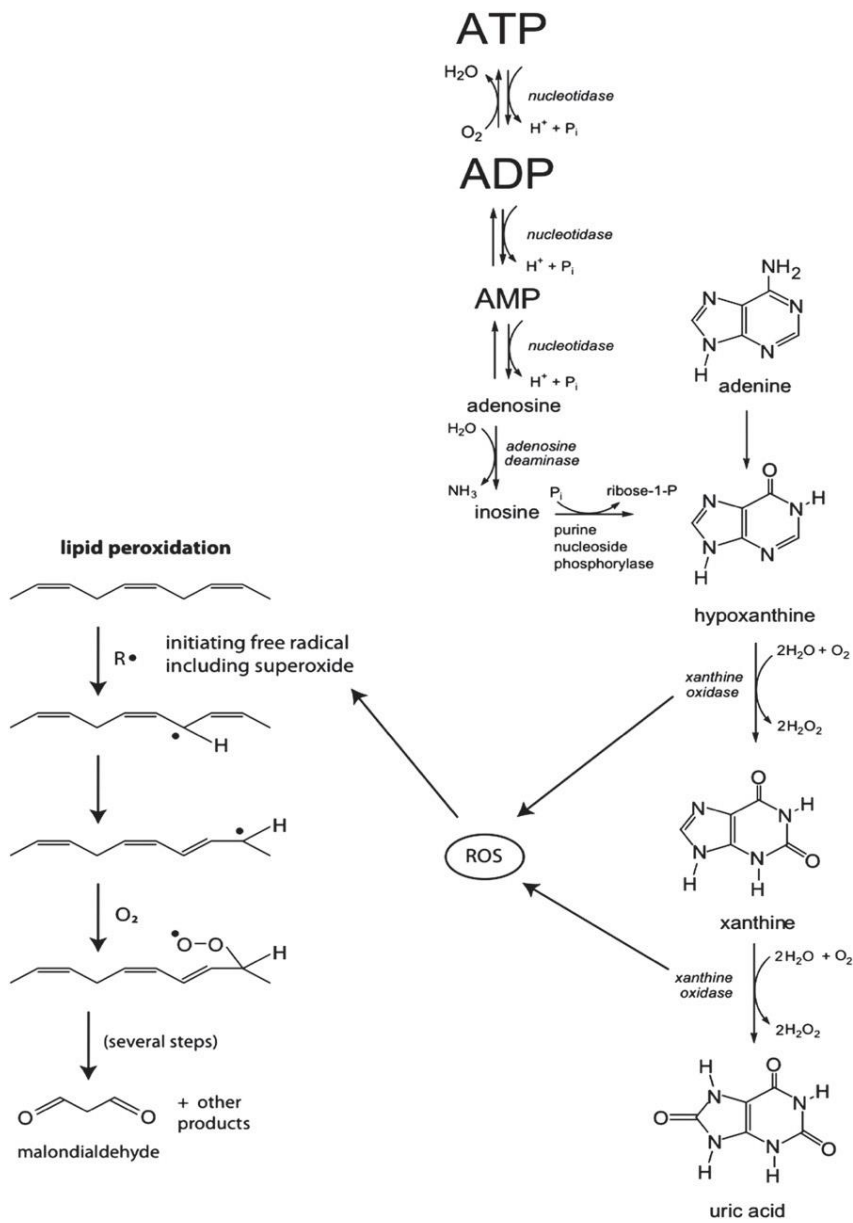
## Perspective

This article presents data describing a significant relationship between physiological markers of neonatal pain and oxidative stress. The method described in this paper can potentially be used to assess the direct cellular effects of procedural pain as well the effectiveness of interventions performed to decrease pain.

## Introduction

During a typical stay in the neonatal intensive care unit (NICU), a newborn often experiences numerous painful procedures in the course of monitoring and treatment. Contrary to previously held convictions, premature neonates are able to perceive pain as demonstrated by several pain scoring methods [1, 2]. Such methods, however, tend to rely on a neonate's alertness and ability to react expressively to the painful experiences. Consequently, caregivers are reluctant to treat pain if there appears to be no clear demonstration that it is occurring or if no immediate untoward effects are observed [3]. An approach that allows the measurement of a systemic reaction to a painful stimulus has the potential benefit of providing objective means of evaluating the pain as well as the effectiveness of its treatment.

It is well documented that exposure to painful procedures often results in tachycardia, which increases energy expenditure and oxygen consumption [4]. However, there are currently few data that quantify the effect of increase oxygen consumption on ATP metabolism. Theoretically, an increase in oxygen consumption should increase the utilization and degradation of ATP to its purine byproducts, specifically uric acid (**Figure 1**). We made this observation in a pilot study from our laboratory in which we reported



**Figure 1:** Pathway from ATP to uric acid and lipid to MDA

increased uric acid concentration in rabbit kits subjected to a single heel lance [5]. Because the process of purine degradation can result in the production of hydrogen peroxide, markers of oxidative stress can theoretically increase. This was observed by Belliene *et al.* who showed increased markers of oxidative stress in the plasma of premature neonates experiencing a single heel lance [6]. However, no studies to date have examined the relationship between behavioral and physiological markers of pain and plasma markers of ATP utilization and oxidative stress. Specifically, do markers of ATP utilization and oxidative stress increase as pain scores increase?

To explore this question, we studied two groups of preterm neonates: one group experiencing a common tissue damaging procedure (TDP group) and a second group not experiencing any TPD (control group). The TDP was tape removal associated with the discontinuation of a central arterial or venous catheter. Pain was measured before and during the TDP using the Premature Infant Pain Profile (PIPP), whose components include physiological (gestational age, heart rate, oxygen saturation) and behavioral (alertness and facial expressions) markers. ATP degradation was quantified by measuring uric acid levels and oxidative stress was quantified by measuring malondialdehyde (MDA) in plasma obtained before and after the TDP. Uric acid is a well-known end product of purine metabolism whose concentration is associated with increased ATP utilization, hypoxia, ischemia or increased ROS (**Figure 1**) [7]. Malondialdehyde is a thiobarbituric acid–reacting substance that is formed by the action of ROS on lipid membranes (**Figure 1**) [8, 9]. Its use as a marker is relevant because the study of oxidative stress and pain in neonates is relatively recent and the association of pain with elevated MDA was reported in adults. Elevated MDA concentrations were

observed in adult patients with vascular related pain [10], young women with dysmenorrheal pain [11, 12], adults with neuropathic pain [13] and adult patients with acute abdominal pain [14]. More recently, MDA and 8-hydroxyguanosine were reported to significantly correlate with pain intensity in patients with temporomandibular joint disease [15].

Evaluation of the effects of pain in premature neonates is challenging. We are presenting biochemical evidence that a single painful procedure is associated with a system wide perturbation in purine metabolism. Furthermore, we show that a single TDP is also associated with elevated MDA levels (correlated with the PIPP score) suggesting distant effects in the form of oxidative damage to lipid membranes.

## **Methods**

### **Subject Enrolment And Sampling Procedure**

We conducted a prospective cohort study at the Loma Linda University Children's Hospital NICU. The Loma Linda University Institutional Review Board approved study protocol and informed consent documents. Families were approached for consent as soon as possible after birth.

Preterm infants less than 37 weeks gestation that met the following inclusion criteria were considered for enrollment: 1) weight of more than 1000 grams at time of enrollment, 2) arterial or central venous catheter in place, 3) no signs or symptoms of hypoglycemia, hypovolemia, hypoperfusion, hyperbilirubinemia, clinical sepsis, pallor or moderate to severe respiratory distress, 4) absence of intraventricular hemorrhage grade 3 or higher and 5) parental consent. Exclusion criteria included 1) multiple congenital

abnormalities 2) facial malformation, 3) complex congenital heart disease 4) receiving analgesia or sedation and 5) endotracheal intubation.

After parental consent was obtained, investigators collaborated with the clinical staff to obtain from a central catheter, approximately 0.8 ml of blood, before and 30 minutes after the TDP to measure levels of uric acid and MDA. In control neonates, similar samples were obtained at 0 and 30 minutes from baseline. The time period of 30 minutes after TDP for blood sample collection was based on previous investigations which showed plasma levels of MDA significantly increasing 15-30 minutes after ischemia-reperfusion [16, 17] and remaining elevated up to 2 hours later [18]. Our pilot study in rabbit kits also showed elevations of uric acid thirty minutes after a single heel lance [5]. To isolate the effects of TDP, subjects were given at least one hour of quiet time in which no TDPs were performed before baseline blood samples were drawn and no additional TDPs were performed during the study period. Samples were centrifuged within five minutes to separate cells from plasma which was then stored at -80°C. All stored plasma samples were analyzed within one week of acquisition.

### Pain Assessment

To assess pain, we used the Premature Infant Pain Profile (PIPP), an instrument that was designed to assess acute pain in preterm neonates [19]. The scoring system includes seven items, each graded from 0 to 3. Two items describe baseline characteristics of the neonate (gestational age and behavioral state), two items are derived from physiologic measurements (heart rate and oxygen saturation), and three items describe facial actions (brow bulge, eye squeeze, and nasolabial furrow). Gestational age,

behavioral state, heart rate and oxygen saturation were assessed and recorded by a trained research nurse (LS) at the bedside. Facial actions were assessed and scored by a neonatologist who was blinded to group assignment (KH), who had undergone training in the use of the PIPP and was experienced in observing and quantifying facial actions. Previous work on validation of the PIPP score showed an ability to differentiate painful from non-painful or baseline events ( $F = 48$ ;  $P = .0001$ ), with interrater reliability coefficients of 0.93 to 0.96 and intrarater reliability coefficients were 0.94 to 0.98 [19-21].

#### Measurement Of MDA

Plasma MDA levels were determined using an adaptation of the selected ion-monitoring gas chromatography-mass spectrometry (GCMS; Agilent, Santa Clara, CA) analysis of phenylhydrazine-derivatized plasma, as described by Cighetti *et al* [22]. Specifically, the sample was prepared by the mixture of 0.1 ml plasma, 0.26 nmol methyl malondialdehyde (MMDA), 5 nmol butylated hydroxytoluene (10  $\mu$ l of 0.5 mM) (Sigma, St. Louis, MO), 0.2 ml citrate buffer (0.4 M, pH 4.0), and deionized water, up to a final volume of 480  $\mu$ l. Then 20 $\mu$ l 50 mM phenylhydrazine (1  $\mu$ mol) (Aldrich, St. Louis, MO) was added as the derivatizing agent. After 30 min incubation at 25°C, the samples were extracted with 1 ml hexane, vortexed for 1 min and centrifuged (3000 rpm, 10 min) at 25°C. The organic phase was removed, concentrated by nitrogen stream to 100  $\mu$ l, and analyzed by GCMS, in selected ion monitoring mode (injection volume of 2  $\mu$ l). Ion 144.00 was monitored for MDA, and ion 158.00 for MMDA. The ion abundance ratios were converted to micromolar concentrations by use of a standard curve. All

measurements were performed in triplicate. Values with coefficients of variation of less than 10% were included in the final analyses.

The internal standard, MMDA, was synthesized using a modified method of Paroni et al [23]. Briefly, 2-methyl-3-ethoxyprop-2-enal (Sigma; St. Louis, MO) was suspended in 7 M NaOH and stirred for 150 min. This was diluted with 5 ml of water and extracted with three 5 ml volumes of CH<sub>2</sub>Cl<sub>2</sub>. After that, the water was evaporated from the aqueous layer. The residue was crystallized once from 5 ml ethanol and 5 ml benzene and then three times from 5 ml ethanol and 5 ml diisopropyl ether. The resulting white powder was diluted in water, filtered, and stored at -80°C.

#### Measurement of Uric Acid

Plasma was transferred to separate Eppendorf tubes, and immediately centrifuged within 5 minutes in Beckman Microfuge 22R (Fullerton, CA) for 30 minutes at 18000 x g, 4°C. The supernatant was immediately transferred to Microcon centrifugal filter devices (Millipore Corp.; Bedford, MA), 200 µl per device, and spun for 90 min at 14000 x g, 4°C. Filtrate was removed, and 150 µl was transferred to an Eppendorf tube containing 1 x 10<sup>-7</sup> mol of 2-aminopurine (Sigma, St. Louis, MO) as the internal standard. Analysis was done the same day using high performance liquid chromatography (HPLC; Waters 996 PDA, 715 Ultra Wisp Sample Processor; Millipore Corp) or the tubes were frozen at -80°C until analysis could be performed. Previous HPLC analysis demonstrated that uric acid values remained stable despite freezing.

Three 45 µl injections were used for each sample. Samples were injected onto a Supelcosil LC-18-S 15 cm x 4.6 mm, 5 µm column (Supelco, Sigma Aldrich, St. Louis,



MO), with the following isocratic conditions: 50 mM ammonium formate buffer, pH 5.5, flow rate 1.0 ml/min. Uric acid concentration was quantitated by first obtaining integrated peak areas for uric acid and for 2-aminopurine at appropriate retention times and wavelengths, as described by our laboratory [24]. The peak area ratios of uric acid to 2-aminopurine were determined and converted to micromolar concentrations using standard curves. Samples were analyzed in triplicates and values with coefficients of variation of less than 10% were included in the final analyses. The limit of detection for uric acid was 5.0  $\mu\text{M}$ .

### Statistics

At the time our study was planned, there were no studies that had examined the relationship between pain and oxidative stress in premature neonates. We based our sample-size calculation on our pilot study that compared MDA and uric acid concentration in neonates born by elective cesarean section to those born by vaginal birth[24]. Based on that calculation, 35 subjects per group were required to demonstrate a difference between groups with 80% power and  $\alpha = .05$  (Sample Power 2.0, SPSS Inc, Chicago, IL). We recruited additional subjects per group to account for data-collection errors. The total sample size was 80 infants (Control  $n = 42$  and TDP  $n = 38$ ).

Assumptions of normality and equal variance were assessed. Demographic data for categorical variables were analyzed using Chi-square test. Repeated measures ANOVA for one between subject factor (group) and one within subject factor (time) were assessed to evaluate the effect of the procedure (control vs. TDP) on plasma uric acid and MDA concentrations over time. Interaction terms in the General Linear Model were used

for this purpose. Percent change in uric acid, as well as MDA, between baseline and thirty-minute values were calculated as follows (**Eq. 1**):

$$(([\text{30min}] - [\text{0min}]) / [\text{0min}]) \times 100 \quad \text{Eq. 1}$$

Correlations between uric acid, MDA and biobehavioral markers (PIPP) were examined using Spearman's rho. All statistical analyses were performed using SPSS Statistics for Windows Version 17. Differences were considered significant at  $P < 0.05$ .

## Results

### General Results

Subject recruitment occurred from July 2007 to August 2009. After obtaining parental consent, samples were obtained from 80 subjects that met study criteria. As described in **Table 1**, no significant differences in demographics were demonstrated between the control and TDP group. The clinical characteristics and the environmental condition of subjects at the time of sampling were also compared (**Table 2**). At the time of sampling, no significant differences were observed in mode of ventilation, baseline oxygen saturation, postnatal age, fraction of inspired oxygen ( $\text{FiO}_2$ ), hemoglobin, acuity (as measured by SNAPPE-II), environmental noise, kidney function (as measured by BUN and creatinine) and number of exposure to TDPs (**Table 2**).

**Table 1. Subject demographics.** TDP: tissue damaging procedure; NS: not significant. Data are Mean  $\pm$  SD. \*Independent samples t-test, +Chi-square

	No Procedures	Single TDP	P Value*
Number of Subjects	42	38	
Birth Weight (g)	1644 $\pm$ 486	1540 $\pm$ 389	NS
Estimated Gestational Age (Weeks)	31.2 $\pm$ 3	30.7 $\pm$ 3.3	NS
Gender (%)	Male 54.1	Male 54.1	NS+
Apgar-1 minute	6 $\pm$ 2	6 $\pm$ 3	NS
Apgar-5 minute	8 $\pm$ 2	7 $\pm$ 3	NS
Race (%)	Caucasian 52.4 Hispanic 23.8 African American 14.3 Asian 7.1 Not Documented 2.4	Caucasian 39.5 Hispanic 44.7 African American 10.5 Asian 5.3 Not Documented 0.0	NS+

**Table 2. Physiological and Environmental Conditions at Time of Sampling.** CPAP: continuous positive airway pressure, NIPPV: noninvasive positive pressure ventilation, NS: not significant. Data are mean±SD, \* Independent samples t-test.

	No Procedures	Single TDP	P Value*
FiO <sub>2</sub>	0.24 ± 0.05	0.24 ± 0.07	NS
Mode of Ventilation	Room air = 24 (57.1%) Nasal cannula = 9 (21.4%) Nasal CPAP = 6 (14.3%) NIPPV = 3 (7.1%)	Room air = 26 (68.4%) Nasal cannula = 4 (10.5%) Nasal CPAP = 5 (13.1%) NIPPV = 3 (7.9%)	NS
Baseline oxygen saturation (%)	97 ± 2.8	97 ± 3.6	NS
Postnatal age (days)	20 ± 12	20 ± 15	NS
Weight (g)	1850 ± 515	1890 ± 568	NS
Hemoglobin (mg/dL)	12.7 ± 2.2	13.2 ± 2.2	NS
SNAPPE-II score	7.9 ± 3.6	7.6 ± 5.5	NS
Blood urea nitrogen (mg/dL)	16.8 ± 10.6	14.8 ± 8.3	NS
Creatinine (mg/dL)	0.47 ± 0.19	0.46 ± 0.18	NS
No. of TDPs from birth to time of sampling	95 ± 69	107 ± 96	NS
Environmental noise (dB)	51 ± 5	53 ± 4	NS

**Table 3. Pain Scores as Measured by PIPP.** NS: not significant, \* Mann-Whitney U test

	No Procedure (N=42)	TDP (N=38)	P value *
Baseline pain score (median)	3 (0-7)	3 (0-8)	NS
Procedural pain score (median)	5 (0-11)	11 (3-19)	< 0.001

### Procedural Pain Score As Measured By PIPP

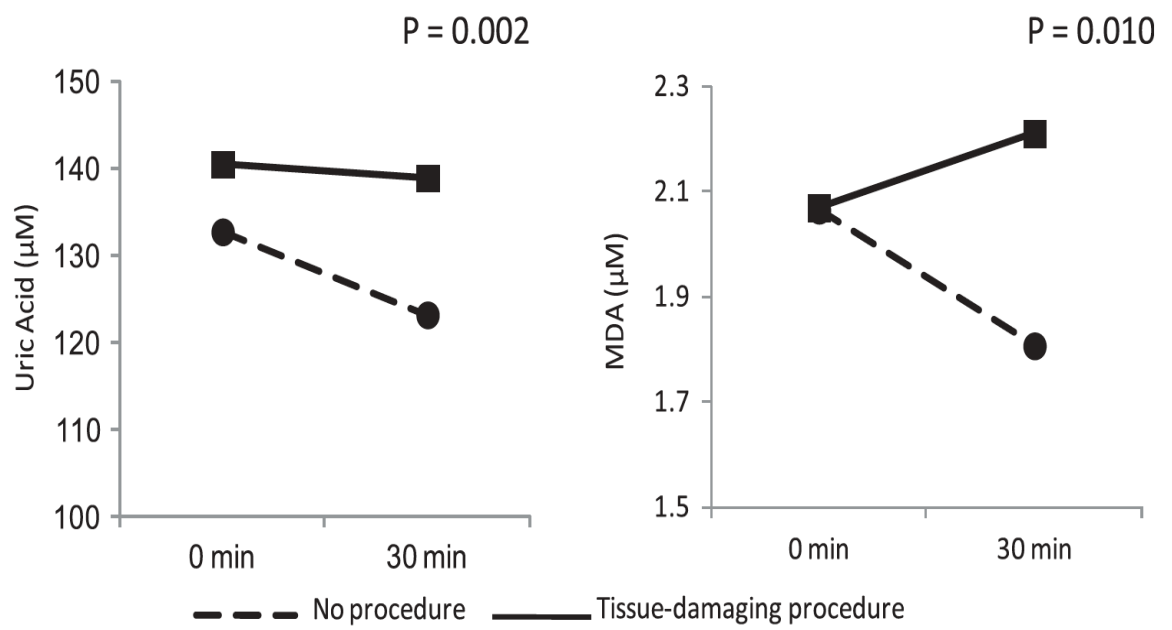
As expected, neonates in the TDP group had significantly higher procedural pain scores compared to the control group (**Table 3**). Median procedural pain scores were as follows: control group: 5 (min-max 0-11) and TDP group: 11 (min-max 3-19),  $P < 0.001$  (**Table 3**).

### Differences Between Baseline And Post-Procedure Uric Acid And MDA Levels In Control Vs. TDP Groups

There were no significant differences in baseline uric acid and MDA levels between control and TDP groups. However, although uric acid levels decreased over time in the control group, uric acid levels remained unchanged in the TDP group (**Figure 2**). More importantly, we saw a significant increase in MDA over time in the TDP group that was not observed in the control group ( $P = 0.02$ ) (**Figure 2**).

### Correlation Between PIPP Pain Score And MDA

We found significant correlations between pain scores and MDA (Pearson correlation, 0.283,  $P = 0.012$ ). As the pain score increased, MDA levels also increased. In addition, we found significant correlations between procedural heart rate and MDA concentration (Correlation Coefficient 0.286,  $P = 0.014$ ). We observed that as heart rate increased, MDA concentration also increased. More importantly, we also found a significant correlation between MDA concentration and oxygen saturation; as oxygen saturation decreased, MDA concentration increased (Correlation coefficient, 0.454,  $P < 0.001$ ). We found no significant correlation between plasma uric acid levels and total PIPP pain scores.



**Figure 2: Plasma [Uric Acid] and [MDA] at baseline and 30 minutes post-TDP.**

## Discussion

Premature neonates in the intensive care unit have reduced endogenous substrate fuel stores and metabolic reserve [25]. Their ability to respond to acute stressors is limited due to their physiologic immaturity, which impedes their ability to mobilize substrate during catabolic metabolism [26]. Their response to routine NICU procedures often include significant alterations in transcutaneous oxygen levels ( $T_cPO_2$ ) and heart rate [27, 28]. Because neonates have fixed stroke volumes, acute changes in heart rate can potentially reduce preload, decrease cardiac output and cause tissue hypoperfusion, increasing the risk of ischemia before compensatory mechanisms take effect. Ischemia combined with decreased arterial oxygen tension can contribute to increased ATP utilization in the face of decreased ATP synthesis. This can lead to enhanced degradation of ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) [29]. Further degradation leads to dramatic increases in adenosine levels. In turn, adenosine is converted to inosine and subsequently to hypoxanthine, xanthine, and uric acid (**Figure 1**). During conditions of decreased ATP supply, the enzyme xanthine oxidase is activated catalyzing the conversion of hypoxanthine to xanthine and xanthine to uric acid, while generating reactive oxygen species [30]. In this context, there has been little research evaluating the link between painful procedures, increased ATP utilization and oxidative stress in premature neonates.

Our data demonstrate a significant relationship between procedural pain and MDA, a well-accepted marker of oxidative stress. Specifically, we observed an increase in MDA in neonates exposed to a single painful procedure compared to those exposed to no such procedure. None of the neonates in this study had conditions that were shown to

increase plasma MDA concentration such as moderate to severe respiratory distress, elevated  $\text{FiO}_2$  requirements, lipid infusions, hyperbilirubinemia [31, 32] or clinical signs of septicemia [33]. Instead, the elevated MDA is consistent with an increase in the production of reactive oxygen species secondary to enhanced ATP degradation (**Figure 1**) in response to increased energy requirements and reduced oxygenation brought about by the painful procedure. Elevations in heart rate and reductions in oxygen saturation significantly correlated to elevated MDA levels. Further studies are required to investigate this mechanism as well as other possible mechanisms that may increase MDA such as pain-related tissue injury, inflammation and cytokine production. To validate the relationship between procedural pain and oxidative stress, it will also be important to measure other oxidative stress markers, such as damage to DNA (8-hydroxy-2'-deoxyguanosine), lipids (4-hydroxy-2-nonenal, isoprostanes, isofurans) and nitration of proteins (nitroalbumin, nitrotyrosine, 3-nitro-4-hydroxyphenylacetic acid (NHPA) and para-hydroxyphenylacetic acid) in plasma and other biological fluids [34-36].

Our findings have important implications for clinical practice. Because neonates are exposed to multiple painful procedures per day [37, 38], previous investigations have focused on examining and decreasing the painful effects of these procedures. A number of interventions, such as the use of sucrose [39-41], pacifiers [41, 42], or morphine [43, 44] have been successful in decreasing signs of pain, but studies investigating the possible biochemical sequelae of painful procedures in neonates are needed. If exposure to multiple painful procedures is shown to contribute to oxidative stress, biochemical markers of such stress might be useful to evaluate mechanism-based interventions that could decrease the adverse effects of exposure to these procedures.



Although our findings are novel in the neonatal population, our study was performed without randomization. The painful procedure (tape removal) was a clinical requirement necessary in the normal course of care in the NICU setting, and no additional painful procedures were employed solely for the benefit of a randomized experimental protocol. However, as shown in **Table 1**, the demographic and clinical characteristics of subjects in both the control and TDP groups were not significantly different. All of the subjects were clinically stable premature neonates, with minimal oxygen requirements and similar clinical acuity status. Randomized trials that examine the biochemical effect of painful procedures in more acutely ill neonates with higher SNAPPE II scores are needed.

### **Conclusion**

Our data demonstrate an important relationship between exposure to a single painful procedure and oxidative stress. Because neonates are exposed to many painful procedures during hospitalization, it is important to further examine the effect of single, multiple and accumulated TDPs in a randomized clinical trial. Mechanistic studies that will determine whether or not procedure-related increases in oxidative stress exacerbate preexisting pathological conditions or trigger development of new abnormalities would provide insight.

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## CHAPTER SIX

### ORAL SUCROSE FOR HEEL LANCE INCREASES ADENOSINE TRIPHOSPHATE USE AND OXIDATIVE STRESS IN PRETERM NEONATES

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## Abstract

### Objective

To examine the effects of sucrose on pain and biochemical markers of adenosine triphosphate (ATP) degradation and oxidative stress in preterm neonates experiencing a clinically required heel lance.

### Study Design

Preterm neonates that met study criteria (n = 131) were randomized into 3 groups: (1) control; (2) heel lance treated with placebo and non-nutritive sucking; and (3) heel lance treated with sucrose and non-nutritive sucking. Plasma markers of ATP degradation (hypoxanthine, xanthine, and uric acid) and oxidative stress (allantoin) were measured before and after the heel lance. Pain was measured with the Premature Infant Pain Profile. Data were analyzed by the use of repeated-measures ANOVA and Spearman rho.

### Results

We found significant increases in plasma hypoxanthine and uric acid over time in neonates who received sucrose. We also found a significant negative correlation between pain scores and plasma allantoin concentration in a subgroup of neonates who received sucrose.

### Conclusion

A single dose of oral sucrose, given before heel lance, significantly increased ATP use and oxidative stress in premature neonates. Because neonates are given multiple doses of sucrose per day, randomized trials are needed to examine the effects of repeated sucrose administration on ATP degradation, oxidative stress, and cell injury.



## Background

Premature neonates experience many painful procedures as part of their standard care in the neonatal intensive care unit [1, 2]. To prevent or treat procedural pain, the use of oral sucrose is recommended by many national and international clinical guidelines on the basis of results from multiple randomized clinical trials in which investigators determined sucrose to be effective in reducing signs of pain [3]. However, there are no studies to date in which investigators examine the effects of sucrose, a disaccharide of fructose and glucose, on neonatal cellular adenosine triphosphate (ATP) metabolism, despite the well-documented relationship between fructose metabolism and reductions in ATP synthesis in adult animals [4], in children ages 11 months to 12 years [5], and in healthy adults [5, 6].

Inhibition of ATP synthesis, as a consequence of sucrose administration, may reduce a premature neonate's already modest ATP stores [7]. In addition, evidence shows that the administration of sucrose does not attenuate the tachycardia that often accompanies painful procedures [8]. In neonatal pigs and newborn lambs, tachycardia significantly increased glucose oxidation and myocardial oxygen requirement [9], which paralleled significant reductions in phosphocreatine/ATP and significant elevations in adenosine diphosphate (ADP) and inorganic phosphate [10]. More importantly, sucrose may not be an effective analgesic as evidenced by its lack of impact on neonatal nociceptive circuits in the brain and spinal cord [11]. Together, these considerations imply that oral sucrose administration may alter ATP metabolism and may have adverse cellular effects in neonates with limited energy stores.

The aim of this study is to examine the effects of a single dose of oral sucrose on

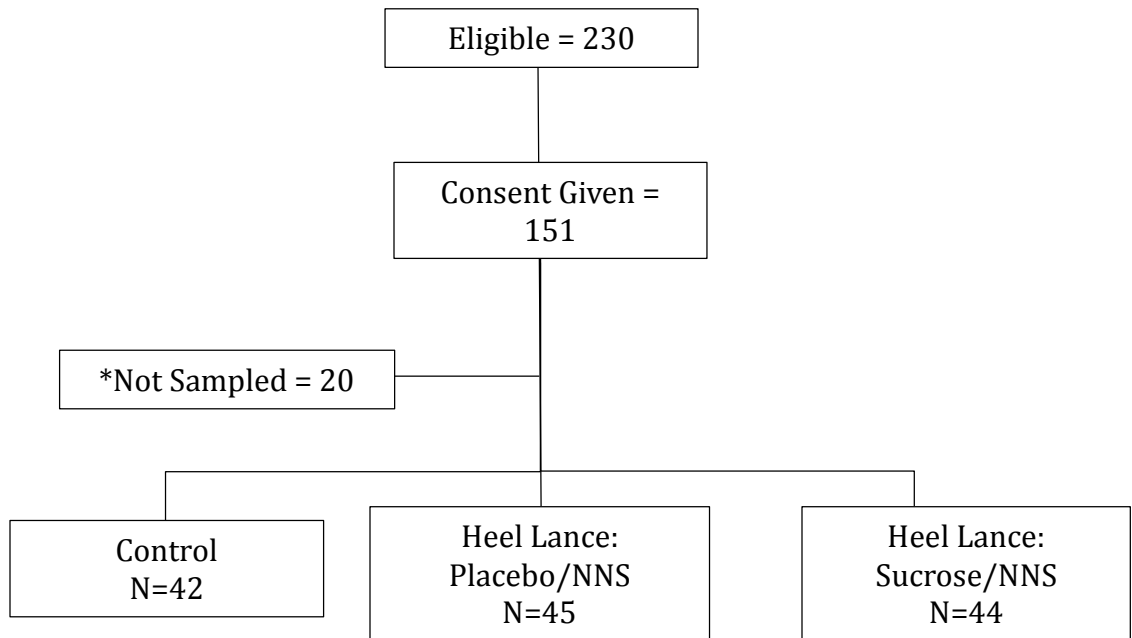
behavioral/physiological markers of pain and biochemical markers of ATP metabolism and oxidative stress in premature neonates experiencing a clinically required heel lance. The heel lance was chosen because it is a frequent painful procedure in the neonatal intensive care unit, as shown in 26 different clinical trials [12]. Pain was quantified using the Premature Infant Pain Profile (PIPP) [13], ATP metabolism was quantified by measuring plasma concentrations of purines (hypoxanthine, xanthine, and uric acid), which are well-documented markers of ATP use and breakdown, and oxidative stress, measured as plasma concentrations of allantoin, a well-accepted in vivo free radical marker [14].

## **Methods**

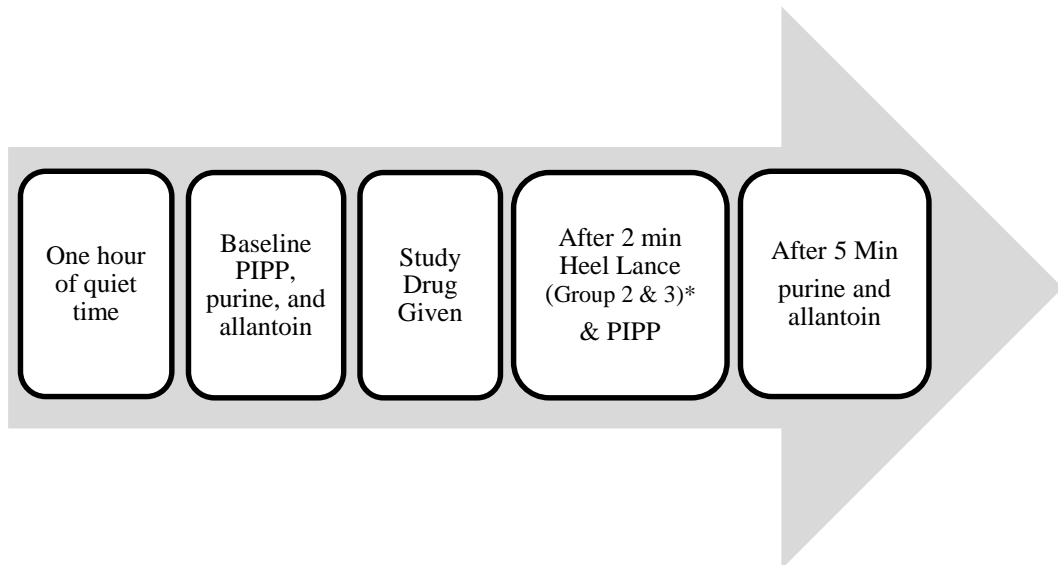
### **Subject Enrolment And Sampling Procedure**

We conducted a prospective double-blind randomized controlled study at Loma Linda University Children's Hospital neonatal intensive care unit. Study protocol and informed consent documents were approved by the Loma Linda University Children's Hospital Institutional Review Board. Subjects included in the study were premature infants  $\leq 36.5$  weeks' gestation who: (1) weighed  $\geq 800$  g; (2) had a central catheter in place; and (3) required a heel lance. Exclusion criteria included neonates: (1) with unstable oxygenation and hemodynamic status; (2) receiving opioids or sedatives or any antiepileptic medications; (3) diagnosed with intraventricular hemorrhage  $\geq$  grade 3; or (4) with facial or multiple congenital anomalies that might alter the pain response. The heel lance was performed for an accurate measurement of blood glucose from neonates receiving glucose-rich total parenteral nutrition through a central catheter. Parents of

premature infants who met study criteria were approached for informed consent as soon after birth as possible. With consent, subjects were randomized into 1 of 3 groups: (1) control; (2) placebo with non-nutritive sucking (NNS, or pacifier); or (3) sucrose (Sweet-Ease; Children's Medical Ventures, Phillips Healthcare, Andover, Massachusetts) with NNS (**Figure 1**). Randomization was performed by a research pharmacist, who used a permuted block randomization table generated by the study statistician. The experimental procedure is described in **Figure 2**. Investigators collaborated with the clinical staff to obtain a sample of approximately 0.8 mL of blood from a central catheter before (“0” minute) and 5 minutes after the heel lance to measure purine and allantoin levels. In control neonates who did not receive a study drug or undergo a heel lance, similar samples were collected at “0” and 5 minutes from baseline. The time period of 5 minutes after heel lance for blood sample collection was based on previous investigations, which showed plasma levels of purines and organic hydroperoxides significantly increasing 5 minutes after conditions such as incomplete ischemia [15]. These data were validated by unpublished preliminary studies in our laboratory, where we found increases in plasma purines compared with baseline five minutes after heel lance, and purine values that were less than baseline, 20-30 minutes after heel lance. Blood samples were centrifuged within 5 minutes to separate the plasma which was then stored at  $-80^{\circ}\text{C}$ . All samples were analyzed within 1 week of acquisition.



**Figure 1. Enrollment Flow Chart.** \*Reasons why subjects were not sampled include: change in acuity or failure to meet study criteria after consent was obtained (n = 9); central line will not draw or lipids were being infused preventing sampling (n = 5); and line was discontinued before sampling could be scheduled (n = 6).



**Figure 2. Study Procedure.** \*Control neonates received no study drug, no heel lance. PIPP and plasma markers are obtained at baseline and five minutes after baseline.

## Heel Lance Procedure And Administration Of Study Drug

The study drug was prepared immediately before the experimental procedure by the research pharmacist and labeled as “study drug” to ensure blinding. The dose of sucrose was based on previously published studies in premature infants [12, 16-18]. Neonates randomized to the sucrose group received a single dose of 24% sucrose in the following volumes: 2 mL for neonates >2 kg, 1.5 mL for neonates 1.5-2 kg, and 0.5 mL for neonates that were <1.5 kg. The study drug was administered slowly via syringe to the anterior tongue along with a pacifier (NNS) 2 minutes before the heel lance. Multiple studies showed that sucrose was most effective when given approximately two minutes before heel lance [8, 18-23]. Neonates randomized to the placebo group received an equal volume of sterile water to the anterior portion of the tongue along with a pacifier. The neonate's face was videotaped by trained research staff to record facial action at “0” minutes, during the heel lance and up to 30 seconds post heel lance.

## Pain Assessment

To assess pain, we used the PIPP, an instrument designed to assess acute pain in preterm neonates [13]. This scoring system includes seven items, each graded from 0 to 3. Two items describe baseline characteristics of the neonate (gestational age and behavioral state), 2 items are derived from physiologic measurements (heart rate and oxygen saturation), and 3 items describe facial actions (brow bulge, eye squeeze, and nasolabial furrow). Baseline pain was scored before the heel lance (0 minute) during a 30-second window. Procedural pain was scored from the time of heel lance to 30 seconds after the lance. Facial actions were recorded with a digital camera with real-time

counter that allowed for intensive slow motion stop frame, videocoding, and playback. Previous work on validation of the PIPP score showed an ability to differentiate painful from non-painful or baseline events [13, 24].

### Measurement Of Purines

Purine metabolites were measured as previously published by our laboratory [25]. Specifically, plasma was removed, transferred to separate Eppendorf tubes, and immediately centrifuged in Eppendorf 5702R (Pittsburgh, Pennsylvania) centrifuge, for 30 minutes at 18000 g. The supernatant was transferred to Microcon centrifugal filter devices (Millipore Corp, Bedford, Massachusetts), 200  $\mu$ L per device, and spun for 90 minutes at 14000 g, 4°C. Filtrate was removed, and 150  $\mu$ L was transferred to an Eppendorf tube containing  $1 \times 10^{-7}$  mol of 2-aminopurine (internal standard). High-performance liquid chromatography (Waters 996 PDA, 715 Ultra Wisp Sample Processor; Millipore Corp) analysis was done in the same day, or the tubes were frozen at  $-80^{\circ}\text{C}$  until analysis. Previous analysis via high-performance liquid chromatography of plasma demonstrated that purines remained stable with freezing.

Samples, 45- $\mu$ L, were injected onto a Supelcosil LC-18-S 15 cm  $\times$  4.6 mm, 5- $\mu$ m column (SGE, Austin, Texas), with the following isocratic conditions: 50 mM ammonium formate buffer, pH 5.5, flow rate 1.0 mL/min. Hypoxanthine, xanthine, and uric acid were quantitated by obtaining peak areas at appropriate retention and wavelengths [26]. Once the peak area of 2-aminopurine at approximately 10.8 minutes and 305 nm was determined, the area ratios of hypoxanthine, xanthine, and uric acid to 2-aminopurine were determined and converted to micromolar concentrations using standard

curves. Samples were analyzed in triplicates and values with a coefficient of variation of less than 10% were included in the final analyses. The limits of detection for the purines are as follows: 1.58  $\mu\text{M}$  hypoxanthine, 1.32  $\mu\text{M}$  xanthine, and 5.0  $\mu\text{M}$  uric acid.

### Measurement of Allantoin

Allantoin was measured in plasma using an adaptation of the method developed by Gruber *et al* [27] and Al-Khalaf and Reaveley [28]. Plasma (50  $\mu\text{L}$ ) was transferred to an Eppendorf tube containing  $5 \times 10^{-10}$  mol internal standard (50  $\mu\text{L}$  10  $\mu\text{M}$  [ $^{15}\text{N}$ ]-labeled allantoin). Spiked samples were simultaneously deproteinized and extracted by the addition of 100  $\mu\text{L}$  of acetonitrile. Samples were then vortexed and centrifuged at 20000 g, 4°C for 5 minutes, and the supernatant was dried under  $\text{N}_2$ . After drying, 50  $\mu\text{L}$  of MTBSTFA (N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide) in pyridine (1:1 vol/vol) was added and the derivatization reaction was facilitated by incubation at 50°C for 2 hours.

Analysis was performed on Agilent 6890N Network GC System connected to an Agilent 5973 Inert Mass Selective Detector (both Agilent Technologies, Inc, Santa Clara, California). Separation was performed using an Agilent 122-5532G capillary column (25.7 m length, 0.25 mm internal diameter). Helium was used as the carrier gas at a flow rate of 1.5 mL/min. Derivatized product (1  $\mu\text{L}$ ) was injected in split mode (split 20:1, split flow 29.4 mL/min, total flow 33.8 mL/min). The initial column temperature was set at 100°C and held at that temperature for 2 minutes, then increased to 180°C at a rate of 10°C/min. The column was held at this temperature for 4 minutes and then increased to 260°C at a rate of 20°C/min. This temperature was maintained until the end of the run.

Allantoin was quantified using selected ion monitoring mode with the 398.00 m/z ion being monitored for allantoin and the 400.00 m/z for DL-allantoin-5-<sup>13</sup>C;1-<sup>15</sup>N. The ion abundance ratios (398.00/400.00) were converted to micromolar concentrations by use of a standard curve.

### Statistical Analyses

A repeated-measures ANOVA with one between factor (type of intervention) and one within factor (time) was used to compute the minimum sample size needed for this study. The sample size was based on the following assumptions: (1) the significance level was set to 0.05; and (2) required power was 80%. After adjusting for a 10% dropout rate, we enrolled 42-45 participants per group for a total of 131 subjects.

To analyze the data, assumptions of normality and equal variance were assessed. Demographic data for categorical variables were analyzed by use of the  $\chi^2$  test. Repeated-measures ANOVA for one between subject factor (group) and one within subject factor (time) were assessed to evaluate the effect of the heel lance on plasma purines and allantoin concentrations over time. Interaction terms in the general linear model were used for this purpose. The interaction terms assess the differences between the groups over time. Correlations between purines, allantoin, and biobehavioral markers (PIPP) were examined with the Spearman rho. All statistical analyses were performed using SPSS Statistics for Windows Version 20 (SPSS Inc, Chicago, Illinois). Differences were considered significant at  $P < .05$ .



## Results

### General Results

Of the 151 subjects who provided consent between the months of July 2009 and February 2012, 131 subjects were randomized into 1 of 3 groups: control (n = 42), heel lance and placebo (n = 45), or heel lance and 24% sucrose (n = 44; **Figure 1**). All subjects randomized to the heel lance groups were given a pacifier (NNS) immediately before, during, and after study drug administration. There were no significant differences between the groups (**Table I**).

### Effects of Oral Sucrose on Behavioral and Physiological Markers of Pain

There were no significant differences in baseline pain score between the 3 groups (Table II). Sucrose significantly attenuated the increase in pain score in response to heel lance, compared with placebo (Table II). The heart rate response to heel lance was greatest in the sucrose group ( $P < .001$ ). Heart rate increased by 11% in the sucrose group, compared with 6% in the placebo group and 0.5% in the control group. We observed no significant changes in mean oxygen saturation in response to heel lance. These data suggest that the lower pain scores in the sucrose group were attributable to significant reductions in the behavioral components of the PIPP scoring tool and not from physiological markers of pain such as heart rate or oxygen saturation.

### Effects of Oral Sucrose on Markers of ATP Metabolism (Purines) and Oxidative Stress (Allantoin)

There were no significant differences in baseline purine and allantoin levels in any of the groups. However, although plasma purine and allantoin concentration

**Table 1. Subject demographics.** EGA, estimated gestational age; NCPAP, nasal continuous positive airway pressure; NIPPV, noninvasive intermittent positive pressure ventilation; RA, room air; SNAPPE-II, Score for Neonatal Acute Physiology–Perinatal Extension-II. \*One-way ANOVA. †  $\chi^2$  test.

	<b>Control (n = 42)</b>	<b>Heel lance, placebo-NNS (n = 45)</b>	<b>Heel lance, Sweet-Ease/NNS (n = 44)</b>	<b>F value</b>	<b>P value</b>
EGA, weeks	30.5 ± 2.6	30.3 ± 3.2	30.1 ± 3.1	0.218*	.804
Birth weight, g	1456.4 ± 502	1498.4 ± 706	1374.1 ± 552	0.499*	.608
Apgar, 1 minute	5 ± 3	5 ± 3	6 ± 2	0.961*	.385
Apgar, 5 minute	7 ± 2	7 ± 2	7 ± 2	1.008*	.368
Sex, n (%)					
Male	25 (60%)	22 (49%)	22 (50%)	1.175†	.556
Female	17 (40%)	23 (51%)	22 (50%)		
Race, n (%)					
White	15 (36%)	14 (31%)	19 (43%)	4.277†	.831
Hispanic	15 (36%)	21 (47%)	15 (34%)		
African American	6 (14%)	7 (16%)	6 (14%)		
Asian	2 (4%)	2 (4%)	2 (4.5%)		
Other	4 (10%)	1 (2%)	2 (4.5%)		
Condition at time of sampling					
F <sub>i</sub> O <sub>2</sub> , %	0.25 ± 0.07	0.26 ± 0.09	0.23 ± 0.03	1.783*	.172
EGA, weeks	32.5 ± 2.3	32.6 ± 2.6	33.1 ± 2.1	0.759*	.470
SNAPPE-II	7.8 ± 10.8	7.9 ± 11.9	8.3 ± 11.6	0.020*	.980
Mode of O <sub>2</sub> delivery					
Spontaneous RA	19	19	24	6.861†	.334
Nasal cannula	6	11	11		
NCPAP	6	6	1		
NIPPV	11	0	8		
Hemoglobin	13.1 ± 2.4	12.7 ± 2.3	12.5 ± 2.2	0.846*	.432
Hematocrit	38.7 ± 6.5	37.6 ± 6.4	36.9 ± 5.7	0.844*	.433

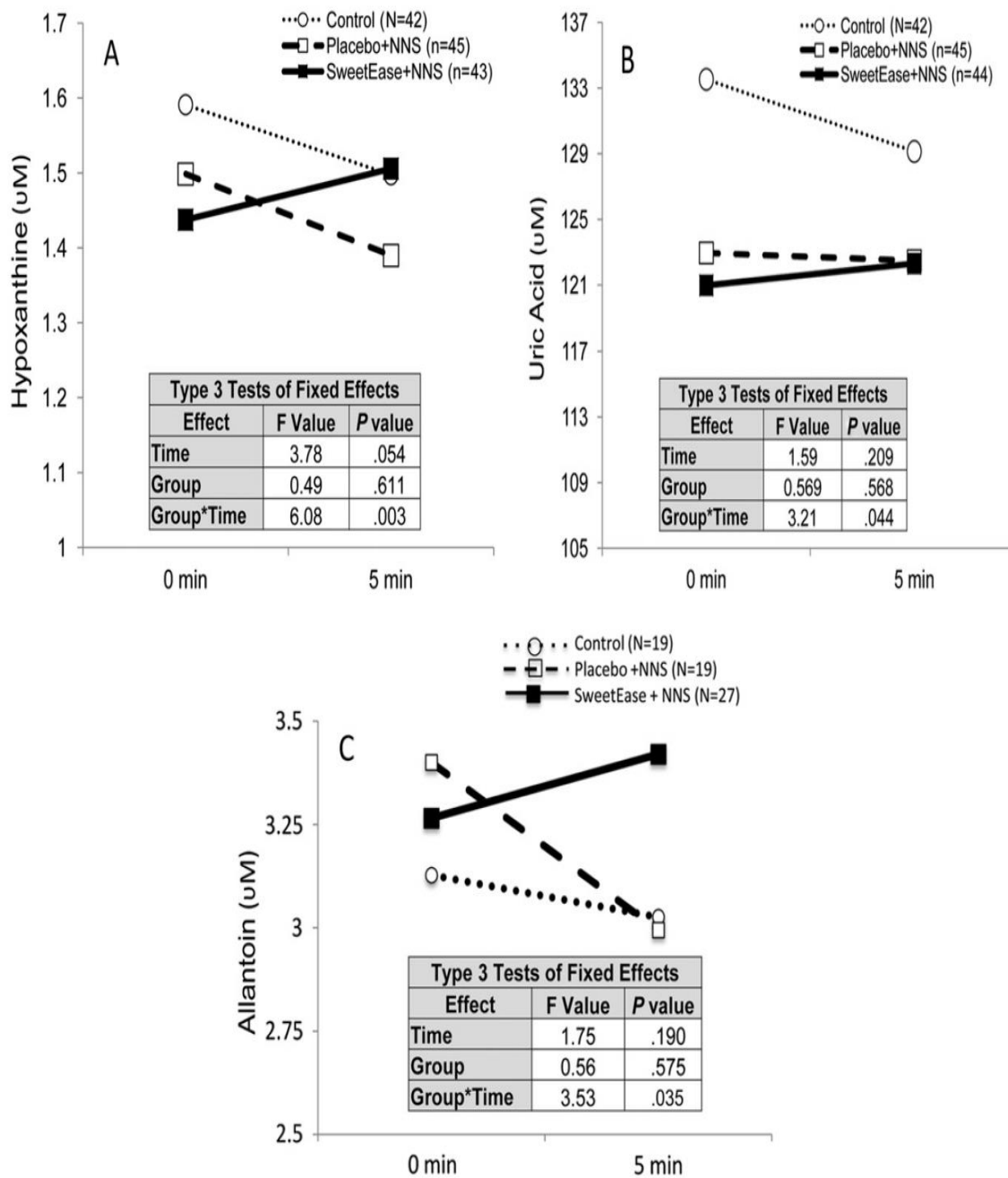
**Table 2. Pain score, heart rate, and oxygen saturation** \*One-way ANOVA. † Sweet-Ease group significantly lower than control or placebo groups. ‡ Control group significantly lower than both heel lance groups.

	Control (n = 42)	Heel lance, placebo-NNS (n = 45)	Heel lance, Sweet-Ease/NNS (n = 44)	F value	P value
Pain score Mean (min-max)					
Baseline	3.9 (1-8)	3.8 (1-8)	3.8 (1-7)	0.233*	.792
Procedural	5.9 (2-15)	6.3 (2-12)	4.6 (2-10)	6.216*	.003†
Heart rate					
Baseline	154.4 (12.8)	155.9 (14.4)	154.1 (13.3)	0.206*	.814
Procedural	154.9 (13.9)	164.9 (14.6)	170.5 (14.7)	14.480*	<.001‡
Oxygen saturation					
Baseline	96.5 (0.5)	96.2 (0.5)	96.2 (0.5)	0.123*	.885
Procedural	96.2 (0.6)	95.8 (0.6)	96.4 (0.6)	0.235*	.791

decreased over time in subjects randomized to the control and placebo groups, we observed a significant increase over time in plasma hypoxanthine and uric acid in neonates who received sucrose before the heel lance (**Figure 3A and B**). This effect persisted even when analysis was limited to subjects <33 weeks' gestation at the time of sampling. Xanthine concentrations remained stable over time in each of the 3 groups. Plasma allantoin concentration increased over time in those who received sucrose; however, this effect was not statistically significant (data not shown).

#### Effects of Oral Sucrose on Plasma Allantoin in Neonates with a Minimal Pain Response to Heel Lance

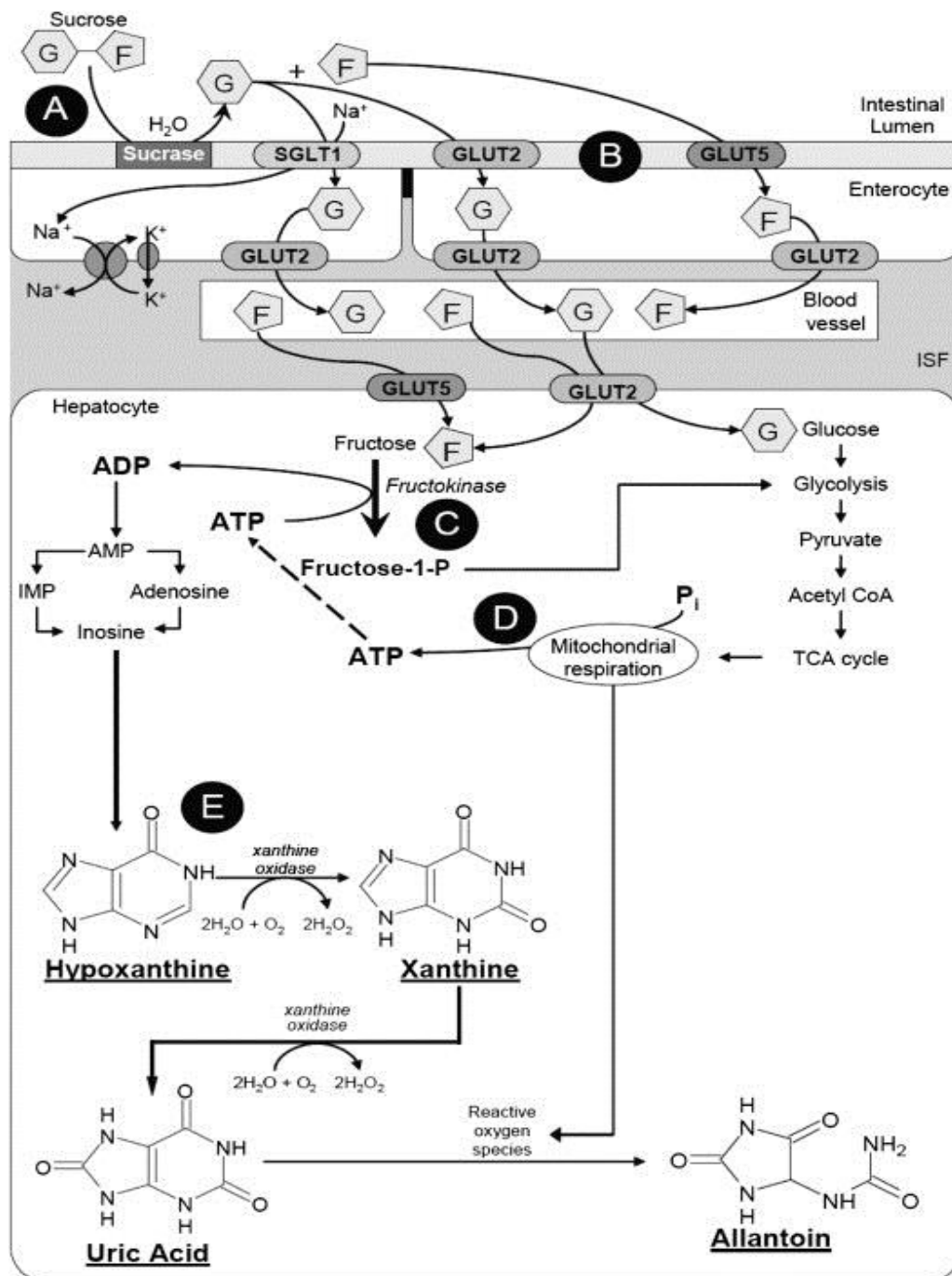
We found that 63% of neonates who received sucrose demonstrated a minimal response to heel lance, defined as an increase in PIPP score of <33%. When we examined the effect of sucrose in this subgroup, we found that plasma allantoin concentration increased significantly over time (**Figure 3C**). When we examined the correlation between the percent change in PIPP pain score over time and the percent change in allantoin concentration over time, we found a significant negative correlation (Spearman rho,  $-0.378$ ,  $P = .014$ ), suggesting that although sucrose significantly decreased the pain scores, it also increased markers of oxidative stress in this subgroup of premature neonates.



**Figure 3. Differences in Purine and Allantoin Concentrations 5 min post TDP.** A and B, Plasma hypoxanthine and uric acid concentration increased over time in preterm neonates who received oral sucrose before a clinically required heel lance. C, In neonates with minimal pain response (<33% increase in PIPP with heel lance), plasma allantoin concentration increased over time.

## Discussion

Although oral sucrose given before a single heel lance significantly decreased behavioral markers of pain, consistent with the findings of numerous clinical investigators [8, 17, 23, 29], it also increased markers of ATP use, as evidenced by significant increases over time in plasma hypoxanthine and uric acid concentrations. The relationship between sucrose, ATP use/depletion, and increased purine production is well documented in adult animal and human literature (**Figure 4**)[6, 30, 31]. Sucrose is a disaccharide of glucose and fructose. It is hydrolyzed by sucrase, an enzyme secreted by epithelial cells of the villi in the small intestine (**Figure 4A**). Both glucose and fructose are rapidly absorbed from the gastrointestinal tract through glucose transporter GLUT5 (fructose) and sodium-glucose cotransporter/GLUT2 (glucose) transporters in the apical membrane and transferred to the portal circulation via GLUT2 transporters in the basolateral membrane of enterocytes (**Figure 4B**). The expression of these GLUTs is up-regulated by exposure of intestinal lumen to fructose solutions [32] or by previous exposure to corticosteroids [33]. Once in circulation, glucose uptake is insulin-dependent while fructose uptake is independent of insulin [4]. Inside the cell, fructose is rapidly phosphorylated to form fructose-1-phosphate by the enzyme fructokinase (**Figure 4C**). The activity of fructokinase is 4-fold greater than glucokinase (the enzyme that phosphorylates glucose). Moreover, fructokinase activity is relatively unregulated, being limited only by fructose concentration [34]. Fructose-1-phosphate is split by aldolase (aldolase B) into glyceraldehyde and dihydroxyacetone phosphate, a member of the glycolysis sequence of intermediates. The third enzyme in the fructose pathway is triokinase, which catalyzes the phosphorylation of glyceraldehyde to glyceraldehyde-3-



**Figure 4. Sucrose metabolism.** A) Sucrose is hydrolyzed into fructose and glucose by the enzyme sucrase. B) Glucose and fructose are absorbed from the gastrointestinal tract, transferred to the portal circulation, and enter hepatocytes. C) Fructose is phosphorylated to form fructose-1-phosphate by the enzyme fructokinase. D) Fructose phosphorylation rapidly depletes the cell of ATP and inorganic phosphate. E) Decreases in ATP production, in addition to increased ATP utilization, results in increased hypoxanthine, xanthine, and uric acid production. If this is combined with increased oxidative stress, uric acid can be oxidized to form allantoin. SGLT, sodium-glucose cotransporter; ISF, interstitial fluid; TCA, tricarboxylic acid cycle; IMP, inosine monophosphate.

phosphate, another intermediate in the glycolytic pathway.

These fructose-related biochemical reactions are significant because each phosphorylation step requires ATP [31]. As ATP is consumed, it is degraded to ADP, leading to an increase in ADP concentration [4]. Simultaneously, inorganic phosphate levels decrease because they are sequestered in fructose-1-phosphate or the mitochondria to generate ATP necessary to maintain fructose phosphorylation (**Figure 4D**) [4]. As inorganic phosphate concentration decreases, oxidative phosphorylation is inhibited, reducing ATP synthesis and rapidly depleting ATP [4, 6, 30, 31]. ATP and ADP catabolism results in increased concentration of purines such as uric acid (**Figure 4E**) [5, 6, 35]. These observations have been documented in adult animals as well as in children ages 11 months to 12 years [5] and in healthy adults [5, 6]. We show similar effects in preterm neonates, in which a single dose of sucrose significantly increased plasma hypoxanthine and uric acid concentrations.

We also found that neonates in the sucrose treatment group had the largest increase in heart rate compared with those in the control or placebo groups, providing additional evidence that sucrose does not attenuate the tachycardia that accompanies painful procedures, but may increase it. This increase in heart rate may be due to the stimulatory effect of sucrose on the sympathetic nervous system, as shown in rats [36, 37] and healthy young adults [38]. Interestingly, in humans, the sympathetic response to sucrose ingestion was enhanced under conditions of acute moderate hypoxia[39]. Together, these data suggest that the apparent analgesic effect of oral sucrose may come at a price, namely tachycardia, which in turn contributes to increased ATP use.

An additional finding of this study is the effect of sucrose administration on



plasma allantoin concentration in neonates with reduced pain responses (PIPP score increased by <33%). We found that plasma allantoin increased over time when compared to control and placebo groups; however, this increase was only significant when neonates within the sucrose group who exhibited a larger pain response (defined as having an increase in PIPP score of  $\geq 34\%$  compared with baseline) were excluded (**Figure 3C**). The demographics of this subgroup of neonates were not significantly different from those with a larger pain response. Although newborns with reduced pain responses had significantly lower baseline heart rates ( $151 \pm 11.6$  beats/min vs  $159 \pm 14.4$  beats/min,  $P = .028$ ), they tended to have a greater percent change in heart rate with the heel lance procedure compared with those with larger pain responses ( $12 \pm 7\%$  vs  $9 \pm 7\%$ ,  $P = .290$ ). These data suggest that changes in heart rate due to sucrose administration may be more predictive of oxidative stress than changes in facial or behavioral activity. Additional studies are required to examine the relationship between sucrose administration, pain reactivity, and oxidative stress.

A limitation of this study is that although statistical power of more than 80% was achieved for hypoxanthine and uric acid, it was not achieved for allantoin. A larger sample size may be required to adequately examine the effect of sucrose treatment on allantoin. It is possible that a significant increase in allantoin may not be always evident five minutes after the heel lance procedure.

In this prospective, randomized, double-blind study, we made the observation that a single dose of oral sucrose, given before a heel lance, significantly increased markers of ATP use and oxidative stress in premature neonates over time. This finding suggests that the apparent analgesic effect of oral sucrose may come at a price, namely increased ATP

use. Because neonates can be exposed to numerous painful procedures per day requiring multiple doses of sucrose, randomized trials should be performed to examine the effects of repeated sucrose administration not only on markers of ATP breakdown and oxidative stress but also on cellular injury. If it is determined that the metabolic risks of using sucrose in neonates is indeed greater than the known benefits of reducing behavioral indices of pain, additional studies need to be performed to identify alternative effective substances or methods to prevent or treat pain in neonates.

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## CHAPTER SEVEN

### THE EFFECT OF GESTATIONAL AGE, WEIGHT FOR GESTATIONAL AGE AND MORBIDITY ON URINARY PURINE METABOLITES AND ALLANTOIN

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## Abstract

Before urine can be used to evaluate the biochemical consequences of pain on adenosine triphosphate (ATP) utilization and oxidative stress, the clinical factors that alter the urinary concentrations of markers of ATP breakdown and oxidative stress need to be determined. Here we report the effects of gestational age, weight for gestational age, and neonatal morbidity on urinary hypoxanthine, xanthine, uric acid, and allantoin.

Urine was collected from 294 premature infants born at <28 weeks gestation (n=47), 28-30<sup>6</sup>/<sub>7</sub> weeks gestation (n=21), 31-33<sup>6</sup>/<sub>7</sub> weeks gestation (n=67), and 34-36<sup>6</sup>/<sub>7</sub> weeks gestation (n=159). Of the infants born between 34-36<sup>6</sup>/<sub>7</sub> weeks gestation, 22 infants were small for gestational age (SGA), 125 infants were appropriate for gestational age (AGA), and 12 infants were large for gestational age (LGA). The effect of neonatal morbidity was also determined in the 34-36<sup>6</sup>/<sub>7</sub> weeks gestation, late preterm, cohort. The most prevalent morbidities within this group were poor nipple (n=11), poor nipple plus hyperbilirubinemia (n=24), poor nipple plus early respiratory disease (n=40), or respiratory disease alone (n=32). HPLC and GCMS were used to evaluate the effects of gestational age, weight for gestational age, and mild morbidity on urinary markers of ATP utilization (hypoxanthine/creatinine, xanthine/creatinine, uric acid/creatinine) and oxidative stress (allantoin/creatinine).

We found that infants born before 28 weeks estimated gestation age (EGA) had significantly higher mean day of life 1-4 urinary uric acid compared to infants born between 28-30<sup>6</sup>/<sub>7</sub> weeks EGA (P<0.001) as well as significantly higher urinary hypoxanthine, xanthine, uric acid and allantoin compared to infants born between 31-33<sup>6</sup>/<sub>7</sub> weeks EGA (P<0.001 for all markers) and infants born between 34-36<sup>6</sup>/<sub>7</sub> weeks



gestation ( $P < 0.001$ ,  $P = 0.002$ ,  $P < 0.001$ , and  $P < 0.001$  respectively). In the late preterm cohort (34-36<sup>6/7</sup> weeks EGA) we found that SGA infants had significantly lower urinary hypoxanthine compared to AGA ( $P = 0.020$ ) and LGA ( $P = 0.026$ ) infants. Also within this population, we found that respiratory morbidity significantly altered urinary purine concentrations. Infants diagnosed with respiratory disease alone had significantly higher mean day of life 1-4 urinary hypoxanthine compared to infants diagnosed with poor nipple alone ( $P = 0.003$ ), poor nipple plus hyperbilirubinemia ( $P < 0.001$ ), or poor nipple plus early respiratory disease ( $P = 0.003$ ). In addition, infants with respiratory disease had significantly higher xanthine compared to infants diagnosed with poor nipple plus hyperbilirubinemia ( $P = 0.001$ ) as well as significantly higher uric acid compared to infants with poor nipple alone ( $P = 0.014$ ). Allantoin, a marker of oxidative stress, was only significantly altered by gestational age.

These data indicate that factors such as gestational age, weight for gestational age and respiratory disease significantly alter markers of ATP breakdown and must be accounted for when using purines or allantoin to evaluate the biochemical consequences of pain.

## **Background**

To examine the relationship between procedural pain and hypoxia in infants, our laboratory measured and reported alterations in plasma markers of adenosine triphosphate (ATP) utilization and oxidative stress in rabbit kits [1] and premature infants [2, 3] exposed to tape removal or heel lance, two painful procedures commonly performed in the neonatal intensive care unit (NICU). These studies required subjects to have a central

arterial or venous catheter in order to obtain blood samples without causing additional pain; however, many infants in the NICU do not have a central line or have their lines discontinued soon after birth. Furthermore, these studies were limited to evaluating a single painful procedure due to the limited blood volume of premature infants and to reduce the risk of infection.

One possible solution to these problems is to utilize urinary biomarkers to identify the effects of procedural pain on oxidative stress and ATP utilization in premature infants. Urine provides a non-invasive means of measuring purines and allantoin; however, these markers are not specific to pain. Purine degradation products are well-established markers of hypoxia [4-9]. When the supply of oxygen cannot meet tissue demand, metabolism switches from aerobic to anaerobic and ATP is broken down to generate energy [10]. The degradation of ATP results in elevated levels of adenosine monophosphate and free adenosine, which are subsequently catabolized into inosine monophosphate and inosine, respectively. Both inosine monophosphate and inosine are then converted to hypoxanthine, then to xanthine, and finally to uric acid. Uric acid has the ability to act as an antioxidant by scavenging reactive oxygen species which converts the compound mainly to allantoin, a marker of oxidative stress in humans [11]. Research indicates that allantoin is also elevated under conditions of ischemia-reperfusion and inflammation [12, 13].

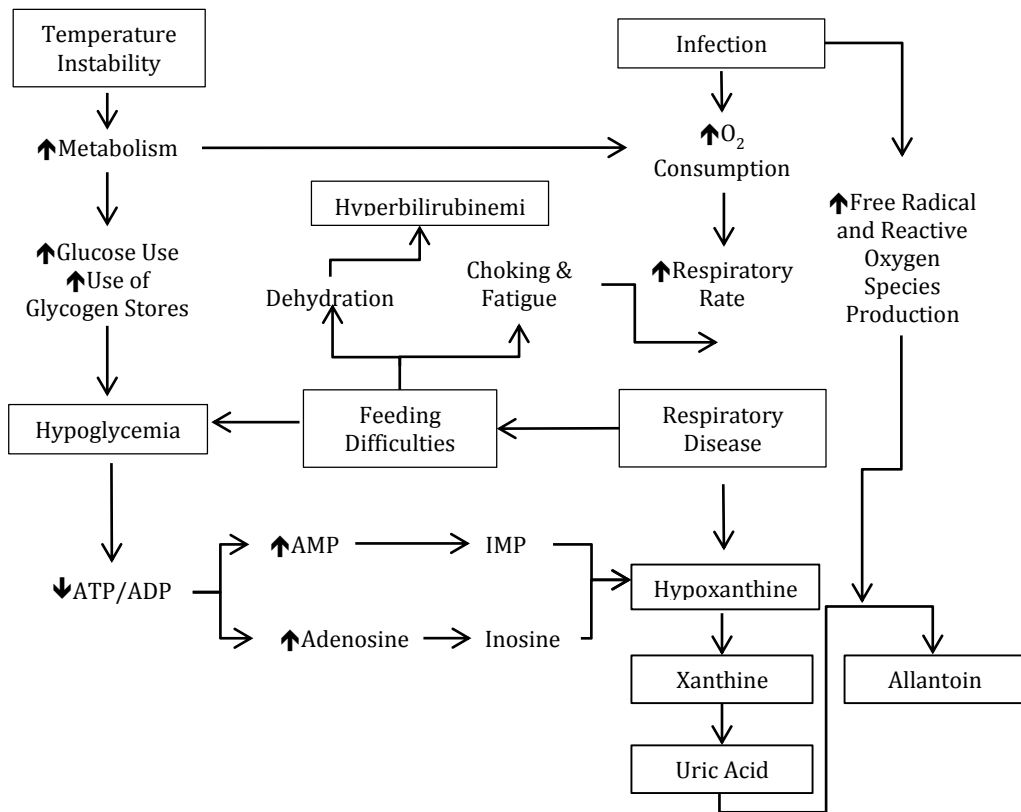
Because of the effects of tissue hypoxia on ATP metabolism, we examined common neonatal conditions that may cause measurable changes in the urinary concentrations of purines and allantoin. Several investigators have reported that both gestational age and weight for gestational age affect neonatal mortality rates [14-16]. It

has further been reported that the that fractional excretion of uric acid is inversely related to gestational age [17]. Pulver *et al.* recently reported that even late preterm infants who are born small for gestational age are 44 times more likely to die in the first month of life and are 21 times more likely to die in the first year of life than appropriate weight for gestational age term infants [14]. To the best of our knowledge, the effects of weight for gestational age on purine and allantoin concentrations have not been reported in infants; however, research indicates that elevated maternal uric acid concentrations is associated with an increase rate of small for gestational age birth [18].

It is also known that neonatal morbidities associated with poor prognosis or major neurological impairment (hypoxia-ischemia, asphyxia, intraventricular hemorrhage, and periventricular leukomalacia) increase ATP breakdown [8, 19, 20]. The effect of more mild morbidities (respiratory disease, hyperbilirubinemia, poor nipping) on oxidative stress and ATP breakdown has not been well studied. Late preterm infants, infants born between 34 and 36<sup>6/7</sup> weeks gestation, provide a unique population for studying the effects of mild to moderate neonatal morbidity on ATP breakdown and oxidative stress. This population has a higher incidence of morbidity than term infants, but have lower incidence of morbidity than more premature infants [21-31]. The morbidities most frequently experienced by late preterm infants tend to be milder and include temperature instability, hypoglycemia, respiratory distress, hyperbilirubinemia, infection and feeding difficulties [22-31]. Many of these morbidities can be linked to increases in oxygen demand, metabolism, energy utilization, and oxidative stress (**Figure 1**). In addition, these infants are less likely to have central lines than more premature infants and may benefit most from urinary analysis of purines and allantoin. The goal of this study was to

determine the effect of gestational age, weight for gestational age, and mild/moderate neonatal morbidity on the urinary concentrations of purines and allantoin in premature infants. Late preterm infants were chosen to evaluate the effects of mild/moderate morbidity for several reasons. First, this population is less likely to have central lines when compared to more premature infants. Second, research has already established the effects of severe morbidity on purine concentrations; however, the effects of clinically mild to moderate morbidities on the urinary concentrations of purines and allantoin is unknown. Third, the late preterm population is understudied and often clinically managed as if they were term. Lastly, because we hypothesized that urinary purine and allantoin excretions will be inversely related to gestational age, we wanted to control for gestation in our morbidity studies. These studies reveal that gestational age, weight for gestational age, and morbidity alter the urinary excretion of purines and allantoin and need to be accounted for if these compounds are to be utilized to evaluate the metabolic consequences of pain in premature infants.

The goal of this study was to determine the effect of gestational age, weight for gestational age, and mild/moderate neonatal morbidity on the urinary concentrations of purines and allantoin in premature infants. Late preterm infants were chosen to evaluate the effects of mild/moderate morbidity for several reasons. First, this population is less likely to have central lines when compared to more premature infants. Second, research has already established the effects of severe morbidity on purine concentrations; however, the effects of clinically mild to moderate morbidities on the urinary concentrations of purines and allantoin is unknown. Third, the late preterm population is understudied and often clinically managed as if they were term. Lastly, because we



**Figure 1.** Pathway depicting the interrelationship between common late preterm morbidities and biochemical markers of ATP breakdown and oxidative stress.

hypothesized that urinary purine and allantoin excretions will be inversely related to gestational age, we wanted to control for gestation in our morbidity studies. These studies reveal that gestational age, weight for gestational age, and morbidity alter the urinary excretion of purines and allantoin and need to be accounted for if these compounds are to be utilized to evaluate the metabolic consequences of pain in premature infants.

## **Methods**

### **Subject Enrolment And Sample Collection**

Premature neonates admitted to Loma Linda University Children's Hospital neonatal intensive care unit were included in this study. The Loma Linda University Institutional Review Board approved study protocol and informed consent documents. No infants were excluded from the study; however, major anomalies, severe morbidity, and comorbidities were noted. Weight for age and morbidity studies were carried out in late preterm infants. Primary and secondary diagnosis was determined by a neonatologist.

After parental consent was obtained, investigators collaborated with the clinical staff to obtain urine samples. Urine was collected in 12-hour aliquots over the first seven days of life by placing cotton balls over the urethral meatus. Urine-soaked cotton balls were removed with every diaper change and stored at 0°C. Validation studies performed in our laboratory showed that purines, creatinine, and allantoin concentrations remained stable under our sampling and processing conditions. Urine was extracted from the cotton using pressure, centrifuged for 10 min at 20000xg and 4°C, filtered through a

Millex syringe driven filter (Low Protein Binding Durapore PVD filter, 0.45µm, 13mm; Millipore Corp.), and stored at -80°C until analysis.

### Subject Classification

Infants with birth weights in the lower 10% or upper 90% of the weight for age growth charts were classified as small and large for gestational age respectively.

Neonates with poor nipple are those with oral motor immaturity without signs and symptoms of feeding intolerance or necrotizing enterocolitis. Neonates with poor nipple plus hyperbilirubinemia are those with oral motor immaturity who received phototherapy at some point over the first six days of life. Neonates with poor nipple plus initial respiratory disease are those with oral motor immaturity and evidence of apnea or ventilatory support for three days or less. Neonates with respiratory disease alone are those with transient tachypnea of the newborn (TTNB) or radiographic evidence of respiratory distress syndrome (RDS) or pneumonia requiring oxygen and/or ventilatory support over at least a successive four-day period in the first six days of life.

### Purine And Creatinine Quantification

Urinary purine and creatinine concentrations were determined using an adaptation of the HPLC method described by George *et al* [32]. Briefly, urine samples were thawed and sonicated before 200 µl was transferred to an Eppendorf tube containing  $1 \times 10^{-7}$  mol of 2-aminopurine (internal standard). The samples were then analyzed on an HPLC (Waters 996 PDA, Waters 600 controller, and 717 plus autosampler; Millipore Corp) by injecting 35 µl onto a Supelcosil LC-18-S 15 cm x 4.6 mm, 5 µm column (SGE; Austin, TX), with the following isocratic conditions: 10 mM potassium dihydrogen phosphate

buffer, pH 4.7, flow rate 1.0 ml/min. Creatinine, hypoxanthine, xanthine and uric acid were quantitated by obtaining peak areas at the appropriate retention times (~3.5, 8, 9.5, and 6.5 min respectively) and wavelengths (230, 248, 267, and 288nm respectively). The area ratios of each compound to 2-aminopurine (13.5 min and 305 nm) were determined and converted into micromolar concentration using standard curves. Samples were analyzed in triplicate and values with a coefficient of variation less than 10% were included in the final analysis. The limits of detection for the purines and creatinine are as follows: 1.58  $\mu\text{M}$  hypoxanthine, 1.32  $\mu\text{M}$  xanthine, 5.0  $\mu\text{M}$  uric acid, and 3.2  $\mu\text{M}$  creatinine.

#### Allantoin Quantification

Urinary allantoin concentrations were determined using an adaptation of the gas chromatography mass spectrometry method described by Gruber *et al*[33]. Briefly, urine samples were thawed and sonicated before 25  $\mu\text{l}$  was transferred to an Eppendorf tube containing  $5 \times 10^{-10}$  mol of internal standard (50  $\mu\text{l}$  of 10  $\mu\text{M}$  solution of  $^{15}\text{N}$ -labeled allantoin). Spiked samples were deproteinated and extracted with 100 ml of acetonitrile. Samples were then vortexed, centrifuged at 4°C for 5 minutes at 20000 x g, and the supernatants were dried under  $\text{N}_2$ . After drying, 50  $\mu\text{l}$  of MTBSTFA in pyridine (1:1 vol/vol) was added and the derivatization reaction was facilitated by incubation at 50°C for 2 h.

Analysis was performed on an Agilent 6890N Network GC System connected to an Agilent 5973 Inert Mass Selective Detector. Separation was performed on an Agilent 122-5532G capillary column (25.7m length, 0.25mm internal diameter) with helium, at a



flow rate of 1.5mL/min, as the carrier gas. Allantoin was quantified using selected ion monitoring mode with the 398.00m/z ion being monitored for allantoin and the 400.00m/z for DL-Allantoin-5-<sup>12</sup>C;1-<sup>15</sup>N. The ion abundance ratios were converted to micromolar concentration units by use of a standard curve. All measurements were performed in triplicate. Values with a coefficient of variation of less than 10% were included in the final analyses.

### Stability Of Purines, Allantoin And Creatinine

To determine the stability of hypoxanthine, xanthine, uric acid, allantoin, and creatinine over time and at varying temperatures, urine samples were collected from volunteers the compounds were measured by HPLC or GCMS. Samples were subjected to the following conditions: direct analysis, immediate cotton extraction and analysis, or incubation at room temperature (3 hours, 24 hours, 3 days, and 1 week), 36°C (3 hours or 6 hours), 0°C (24 hours or 1 week), or -80°C (24 hours or 1 week). Purines and creatinine as well as the ratio of each to creatinine were found to be stable under all conditions tested with coefficient of variation between the mean concentrations for each processing condition being within 10% of fresh urine. Allantoin, on the other hand, significantly increased in concentration when left at room temperature for 24 hours but remained stable when incubated at 36°C for 6 hours or stored at temperatures of 0°C and lower.

### Statistics

To analyze the data, assumptions of normality and equal variance were assessed. Demographic data for categorical variables were analyzed using Chi-square test. One-

way ANOVA was used to evaluate the effects of gestational age, weight for gestational age, and morbidity on mean DOL 1-4 urinary purines and allantoin. All statistical analyses were performed using SPSS Statistics for Mac OS Version 21. Differences were considered significant at  $P < 0.05$ .

## Results

### General Results

Urine was collected from 294 premature infants born at <28weeks gestation (n=47), 28-30<sup>6</sup>/<sub>7</sub> weeks gestation (n=21), 31-33<sup>6</sup>/<sub>7</sub> weeks gestation (n=67), and 34-36<sup>6</sup>/<sub>7</sub> weeks gestation (n=159). As expected, significant differences were observed between gestational age groups for 1-min Apgar, 5-min Apgar, and birth weight (**Table 1**). There was also a trend for more males in each group except for the poor nipple plus hyperbilirubinemia group. Of the infants born between 34-36<sup>6</sup>/<sub>7</sub> weeks gestation, 22 infants were SGA, 125 infants were AGA, and 12 infants were LGA. No significant differences were found between these groups; however, LGA infants tended to have lower Apgar scores and a large majority were born via cesarean section (**Table 2**).

**Table 1: Subject Demographics for Gestational Age Groups.** SVD: Spontaneous vaginal delivery; C/S: Cesarean Section; Data are Mean  $\pm$  SD; \*One-way ANOVA, †Chi-square test ‡<28 and 28-30<sup>6/7</sup> significantly different from 31-33<sup>6/7</sup> and 34-36<sup>6/7</sup>. +<28 and 28-30<sup>6/7</sup> significantly different from all other groups. †All groups are significantly different from one another.

	<28 (n=47)	28-30 <sup>6/7</sup> (n=21)	31-33 <sup>6/7</sup> (n=67)	34-36 <sup>6/7</sup> (n=159)	P value*
Apgar, 1 min	4 $\pm$ 3	5 $\pm$ 2	7 $\pm$ 2	7 $\pm$ 2	0.000‡
Apgar, 5 min	6 $\pm$ 2	7 $\pm$ 2	8 $\pm$ 1	8 $\pm$ 2	0.000+
Birth Weight (g)	829.5 $\pm$ 187.4	1150.6 $\pm$ 275.5	1875.4 $\pm$ 308.1	2325.0 $\pm$ 493.3	0.000‡
Gender, n (%)					
Male	32 (68.1%)	6 (8.6%)	37 (55.2%)	83 (52.2%)	0.024†
Female	15 (31.9%)	15 (71.4%)	30 (44.8%)	76 (47.8%)	
Mode of Birth, n (%)					
SVD	15 (31.9%)	5 (23.8%)	22 (32.8%)	63 (39.6%)	NS (0.409)†
C/S	32 (68.1%)	16 (76.2%)	45 (67.2%)	96 (60.4%)	
Weight for age, n (%)					
AGA	44 (93.6%)	16 (76.2%)	58 (86.6%)	125 (78.6%)	NS (0.153)†
SGA	1 (2.1%)	3 (14.3%)	8 (11.9%)	22 (13.8%)	
LGA	2 (4.3%)	2 (9.5%)	1 (1.5%)	13 (7.5%)	

**Table 2: Subject Demographics for Weight for Gestational Age Groups.** EGA: estimated gestational age; SVD: Spontaneous vaginal delivery; C/S: Cesarean Section; Data are Mean  $\pm$  SD; \*One-way ANOVA, †Chi-square test

	SGA (n=22)	AGA (n=125)	LGA (n=12)	P value*
Apgar, 1 min	7 $\pm$ 2	7 $\pm$ 2	5 $\pm$ 3	NS (0.141)
APGAR, 5 min	8 $\pm$ 1	8 $\pm$ 1	7 $\pm$ 2	NS (0.058)
EGA (weeks)	35 $\pm$ 0.8	35 $\pm$ 0.8	35 $\pm$ 0.8	NS (0.325)
Gender, n (%)				
Male	12 (54.5%)	65 (52.0%)	6 (50.0%)	NS (0.964)†
Female	10 (45.5%)	60 (48.0%)	6 (50.0%)	
Mode of Birth, n (%)				
SVD	8 (36.4%)	54 (43.2%)	1 (8.3%)	NS (0.059)†
C/S	14 (63.6%)	71 (56.8%)	11 (91.7%)	

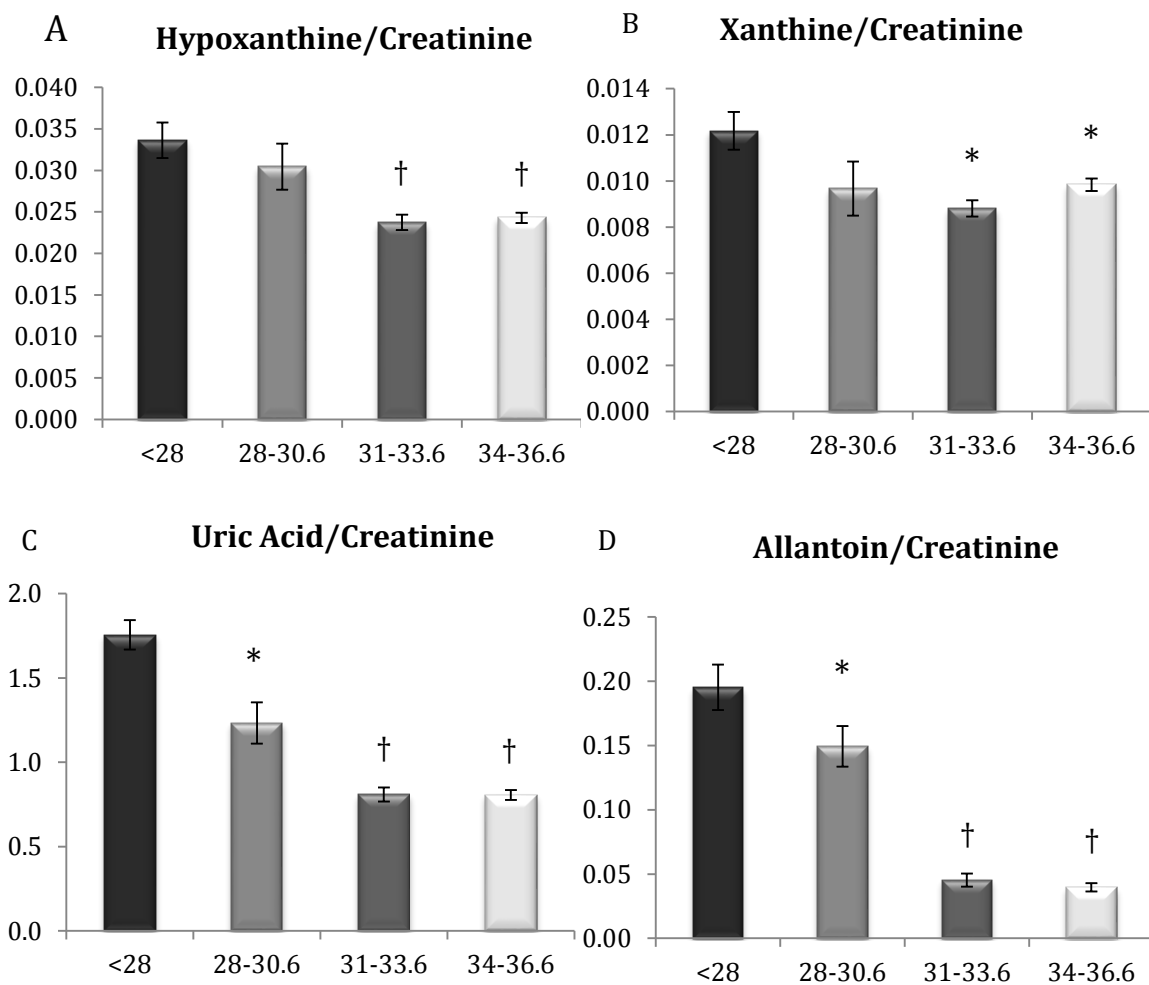
**Table 3: Subject Demographics for Late Preterm Morbidity Groups.** EGA: estimated gestational age; SVD: Spontaneous vaginal delivery; C/S: Cesarean Section; Data are Mean  $\pm$  SD; \*One-way ANOVA; †Chi-square test. ‡All groups significantly different than Respiratory Disease. †Poor nipple plus Hyperbilirubinemia significantly different from Poor nipple plus Mild Respiratory Disease and Respiratory Disease alone. ‡Respiratory disease significantly different from Poor nipple plus Hyperbilirubinemia and Poor nipple plus Mild Respiratory Disease

	Poor Nippling (n=11)	Poor Nippling plus Hyperbilirubinemia (n=24)	Poor Nippling plus Early Respiratory Disease (n=40)	Respiratory Disease (n=32)	P value*
Apgar, 1 min	7 $\pm$ 2	7 $\pm$ 2	7 $\pm$ 2	6 $\pm$ 3	NS (0.393)
Apgar, 5 min	9 $\pm$ 1	9 $\pm$ 21	8 $\pm$ 1	7 $\pm$ 2	0.000‡
EGA (weeks)	35 $\pm$ 0.7	34 $\pm$ 0.4	35 $\pm$ 0.7	35 $\pm$ 0.7	0.001‡
Birth Weight (g)	2251.7 $\pm$ 721.8	2108.8 $\pm$ 455.2	2294.5 $\pm$ 387.9	2593.1 $\pm$ 463.1	0.002 <sup>+</sup>
Gender, n (%)					
Male	8 (72.7%)	14 (58.3%)	20 (50.0%)	15 (46.9%)	NS (0.454)†
Female	3 (27.3%)	10 (41.7%)	20 (50.0%)	17 (53.1%)	
Mode of Birth, n (%)					
SVD	5 (45.5%)	17 (70.8%)	18 (46.2%)	9 (28.1%)	0.018†
C/S	6 (54.5%)	7 (29.2%)	21 (53.8%)	23 (71.9%)	
Weight for age, n (%)					
AGA	8 (72.7%)	19 (79.2%)	33 (82.5%)	24 (75.0%)	NS (0.107)†
SGA	2 (18.2%)	4 (16.7%)	6 (15.0%)	2 (6.3%)	
LGA	1 (9.1%)	1 (4.2%)	1 (2.5%)	6 (18.8%)	

The effect of neonatal morbidity was determined in the late preterm, 34-36<sup>6/7</sup> weeks gestation, cohort. The most prevalent morbidities within this group were poor nipple (n=11), poor nipple plus hyperbilirubinemia (n=24), poor nipple plus early respiratory disease (n=40), or respiratory disease alone (n=32). Significant differences were observed between morbidity groups for 5-min Apgar (P<0.001), gestational age (P=0.001), and birth weight (P=0.002, **Table 3**). We also noted that more infants in the poor nipple plus hyperbilirubinemia group were born via spontaneous vaginal deliveries versus cesarean section.

#### Effect Of Gestational Age On Purine Catabolites And Allantoin

There was a trend for infants of lower gestation to have higher purines and allantoin (**Figure 2**). Specifically, infants born at < 28 weeks gestation had significantly higher mean day of life 1-4 urinary uric acid (P<0.001) and allantoin (P=0.043) compared to infants born between 28-30<sup>6/7</sup> weeks gestation. Similarly, infants born at <28 weeks gestation also had significantly higher hypoxanthine, xanthine, uric acid and allantoin compared to infants born between 31-33<sup>6/7</sup> weeks gestation (P<0.001 for all markers) and infants born between 34-36<sup>6/7</sup> weeks gestation (P<0.001, P=0.002, P<0.001, and P<0.001 respectively). In addition, infants born between 28-30<sup>6/7</sup> weeks gestation had significantly higher hypoxanthine, uric acid, and allantoin compared to infants born between 31-33<sup>6/7</sup> weeks gestation (P=0.030, P=0.001, P<0.001 respectively) and infants born between 34-36<sup>6/7</sup> weeks gestation (P=0.033, P<0.001, and P<0.001 respectively).



**Figure 2. Effect of gestational age on mean day of life 1-4 urinary purines and allantoin.** A) hypoxanthine/creatinine, B) xanthine/creatinine, C) uric acid/creatinine, and D) allantoin/creatinine. Bars are mean  $\pm$  standard error of the mean. \*Significantly different from <28 weeks, †Significantly different from <28 and 28-30<sup>6/7</sup>.

## Effect Of Weight For Gestational Age On Urinary Excretion Of Purines And Allantoin

After controlling for gestational age by selecting for infants between 34-36<sup>6/7</sup> weeks gestation we found that SGA infants had significantly lower hypoxanthine compared to AGA (P=0.020) and LGA (P=0.037) infants (**Figure 3A**). No significant differences were found between weight for gestational groups for xanthine, uric acid, or allantoin (**Figure 3B-D**).

## Effect Of Morbidity On Purines And Allantoin In Late Preterm Infants

Infants diagnosed as having respiratory disease alone had significantly higher hypoxanthine than infants diagnosed with poor nipple alone (P=0.003), poor nipple plus hyperbilirubinemia (P<0.001), or poor nipple plus early respiratory disease (P=0.003, **Figure 4A**). Xanthine was found to be significantly lower in infants diagnosed with poor nipple plus hyperbilirubinemia (P=0.001) compared to infants diagnosed with respiratory disease alone (**Figure 4B**). Infants diagnosed with poor nipple alone had significantly lower uric acid (P=0.014) compared to infants diagnosed with respiratory disease alone (**Figure 4C**). Urinary allantoin was not significantly different between any of the groups (**Figure 4D**).

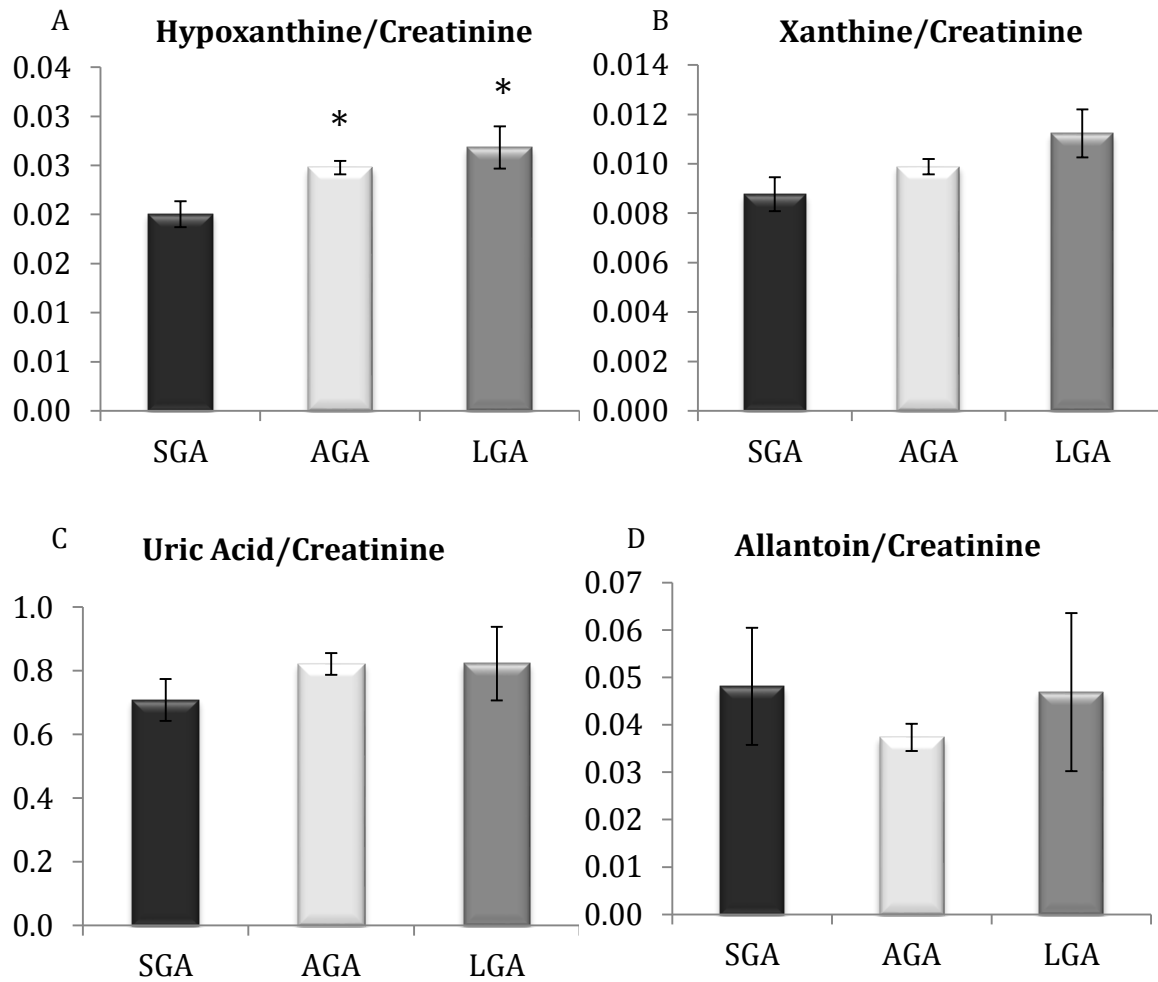
## Discussion

The aim of this study was to examine the effects of gestational age, weight for gestational age, and mild morbidity on the urinary concentration of purines and allantoin. We found that gestational age significantly altered the urinary concentrations of purines and allantoin. Weight for gestational age also altered the urinary concentration of purines and

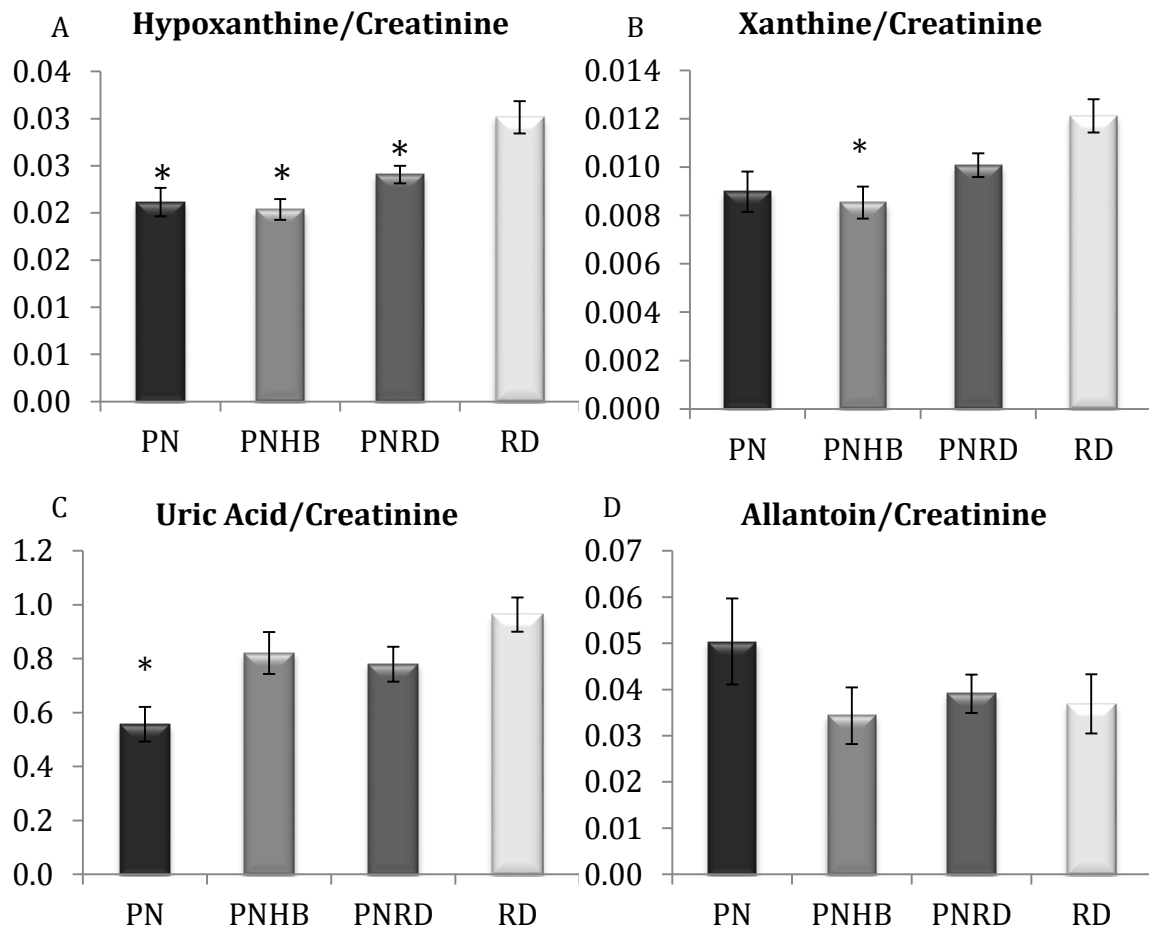


allantoin; however, the changes were only significant for hypoxanthine. In addition, neonatal morbidity significantly changed urinary purines in that infants diagnosed with respiratory disease alone had the highest concentration for all purines.

Previous studies have demonstrated an inverse relationship between gestational age and uric acid [17]. Our data provide additional support for higher urinary purine concentration in infants at the lowest gestational age groups, until roughly 31 weeks gestation. In addition, we also report significantly higher allantoin, a marker of inflammation and oxidative stress, in more premature infants. The observed increase in urinary purine and allantoin concentrations could be caused by increased purine and allantoin production and/or increased renal clearance. It is known that infants excrete substantially greater quantities of uric acid per unit of body weight than adults or older children [17, 34, 35]. Stapleton *et al.* reported that fractional excretion of uric acid was nearly 70% for infants born between 29-31 weeks gestation and decreased to roughly 40% for infants born between 38-40 weeks gestation [17]. Increased urinary purine concentrations were also shown to be a result of clinical conditions associated with hypoxia or perinatal asphyxia [19, 36, 37]. Our data provide additional support that uric acid is easily filtered and excreted and can be altered by prematurity and conditions that increase ATP utilization. The mechanism for urinary excretion of allantoin is not well studied; however, it was demonstrated that elevated urinary allantoin concentration were associated with increased oxidative stress in adults exposed to continuous exercise or therapies that increase synthesis of free radicals [38, 39], establishing this compound as an important free radical marker in urine.



**Figure 3. Effect of weight for age on mean day of life 1-4 urinary purines and allantoin.** A) hypoxanthine/creatinine, B) xanthine/creatinine, C) uric acid/creatinine, and D) allantoin/creatinine. SGA: small for gestational age, AGA: appropriate for gestational age, LGA: large for gestational age. Bars are mean  $\pm$  standard error of the mean. \*Significantly different from SGA.



**Figure 4. Effect of morbidity on urinary purines and allantoin in late preterm infants.** A) hypoxanthine/creatinine, B) xanthine/creatinine, C) uric acid/creatinine, and D) allantoin/creatinine. PN: poor nipple; PNHB: poor nipple plus hyperbilirubinemia; PNRD: poor nipple plus early respiratory disease; RD: respiratory disease. Bars are mean  $\pm$  standard error of the mean. \*Significantly different from respiratory disease.

To the best of our knowledge, we are the first to report the effects of weight for gestational age on urinary purine and allantoin concentrations in late preterm infants. Surprisingly, we found that SGA infants tended to have lower urinary purine concentrations than AGA or LGA infants; with SGA infants having significantly lower hypoxanthine than both AGA and LGA infants. This trend continued, but was not significant, after controlling for neonatal co-morbidities (data not shown). The mechanism for this reduction in urinary purines in SGA infants is unclear but could be a result of increased purine salvage, decreased energy use/purine production, or decreased renal clearance. However, an increase in purine salvage is unlikely because hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity was shown to be depressed in cord blood lymphocyte from SGA infants when compared to term infants [40]. Furthermore, this is not likely a result of an overall reduction in purine metabolism because this same study found similar adenosine deaminase activity between premature SGA infants and term infants. This study, however, compared preterm SGA infants to term infants. The HGPRT activity for SGA infants needs to be compared to age matched AGA infants for purine salvage to be ruled out as an explanation for the decreased urinary purine concentrations observed in our study.

It was further reported that SGA infants have similar metabolic parameters and higher resting energy expenditure than age matched AGA infants in the first week of life; indicating that the observed decrease in urinary purines in SGA infants may be due to decreased excretion rather than decreased production [41]. Wang *et al.* reported higher insulin levels in SGA infants compared to age matched AGA infants at 72 hours of life[42]. Research indicates that hyperinsulinemia decreases uric acid excretion without

significantly altering plasma uric acid concentration in adults [43]. Bauer *et al.*, however, have reported similar blood insulin levels between SGA and AGA infants [41]. Studies investigating the mechanism of decreased urinary purine excretion in SGA infants need to be conducted. Based upon these studies, as well as the data presented in the paper, it is important to control for, or exclude, SGA infants from studies that use urinary purines as markers of ATP breakdown.

We also found that respiratory morbidity within the late preterm population can significantly alter urinary purines, but not allantoin. This was most evident for urinary hypoxanthine. The elevated urinary purine concentrations in infant with respiratory disease could be caused by decreased ATP production or increased ATP utilization. It is well documented that hypoxia and asphyxia resulted in increased purine levels [5-9, 44]. In addition, it has been reported that plasma uric acid positively correlated with fraction of inspired oxygen ( $FiO_2$ ) in intubated premature infants [45]. In this study,  $FiO_2$  was adjusted per patient need. In our study, all of the infants in the respiratory morbidity group were receiving oxygen and/or ventilatory support, indicating a need for supplemental oxygen or assisted breathing and an increased purine production. It has also been reported that respiratory morbidity can increase energy expenditure in premature infants [46, 47]. The increase in purines observed in our study could be a result of increased ATP utilization due to increased energy expenditure. It is well documented that increases in energy expenditure result in increased purine production [48, 49]. Our data indicate that infants with respiratory morbidities need to be grouped separately from infants without respiratory morbidities if urinary purines are to be used to

measure the relationship between procedural pain and hypoxia on ATP breakdown in urine.

These data suggest that gestational age, weight for gestational age, and neonatal morbidity alter the urinary concentration of purine metabolites. Allantoin was also found to be significantly altered by gestational age. Based upon these data, we recommend conducting age-matched as well as weight for age-matched studies when using urinary purines and allantoin to evaluate the relationship between procedural pain and hypoxia on ATP utilization and oxidative stress. Lastly, it is important to stratify subjects based on health status or morbidity, due to the significant effects of neonatal respiratory diseases on ATP use and oxidative stress.

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