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Dietary Intake and Bio-activation of Nitrite and Nitrate in Newborn Infants

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Dietary Intake and Bio-activation of
Nitrite and Nitrate in Newborn Infants

by

Jesica Ann Jones

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

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September 2015

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

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ABBREVIATIONS

NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
eNOS	Endothelial NO synthase
DASH	Dietary Approaches to Stop Hypertension
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
PN	Parenteral nutrition
sPN	Starter parenteral nutrition
C	Celsius
cPTIO	2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
NEM	N-ethylmaleimide
FeCN	Ferricyanide
PO_2	Partial pressure of oxygen
LPO	Lactoperoxidase
2-MMI	2-mercaptio-1-methylimidazole
3-AT	3-amino-1,2,4-triazole
LLUCH	Loma Linda University Children's Hospital
MetHb	Methemoglobin
SNO	S-nitrosothiols
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
BHI	Brain heart infusion

O_2	Oxygen
N_2	Nitrogen
H_2	Hydrogen
DNA	Deoxyribonucleic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCl	Hydrochloric acid
Ct	Cycle threshold
ADMA	Asymmetric dimethylarginine
iNOS	Inducible NOS
EPA	Environmental Protection Agency
WHO	World Health Organization
ADI	Acceptable Daily Intakes
NSAID	Non-steroidal anti-inflammatory drug
ATP	Adenosine triphosphate
GI	Gastrointestinal
L-NAME	NG-nitro-L-arginine methyl ester
FF	Formula-fed
I/R	Ischemia-reperfusion
iNO	inhaled NO
NaCl	Sodium chloride

ABSTRACT OF THE DISSERTATION

Dietary Intake and Bio-activation of Nitrite and Nitrate in Newborn Infants

by

Jesica Ann Jones

Doctor of Philosophy, Graduate Program in Pharmacology
Loma Linda University, September 2015
Dr. Arlin B. Blood, Chairperson

Nitrate and nitrite are commonly thought of as inert end products of nitric oxide (NO) oxidation, possibly carcinogenic food additives, or well-water contaminants. However, recent studies have shown that nitrate and nitrite play an important role in cardiovascular and gastrointestinal homeostasis through conversion back into NO via a physiological system involving enterosalivary recirculation, bacterial nitrate reductases, and enzyme-catalyzed or acidic reduction of nitrite to NO. The diet is a key source of nitrate in adults; however, infants ingest significantly less nitrate due to low concentrations in breast milk. In the mouth, bacteria convert nitrate to nitrite, which has gastro-protective effects. However, these nitrate-reducing bacteria are relatively inactive in infants. Swallowed nitrite is reduced to NO by acid in the stomach, affecting gastric blood flow, mucus production, and the gastric microbiota. These effects are likely attenuated in the less acidic neonatal stomach. Systemically, nitrite acts as a reservoir of NO bioactivity that can protect against ischemic injury, yet plasma nitrite concentrations fall dramatically at birth and remain markedly lower than in adults for the first few weeks of life. The physiological importance of the diminished nitrate→nitrite→NO axis in infants and its implications in the etiology and treatment of newborn diseases such as necrotizing enterocolitis and hypoxic/ischemic injury are yet to be determined.

CHAPTER ONE

INTRODUCTION

This dissertation shows that nitric oxide bioavailability is markedly lower in newborn infants than adults. This is characterized by low dietary nitrate and nitrite ingestion, a lack of oral bacterial nitrate reductase activity, enhanced urinary nitrite excretion, and a rapid fall in plasma nitrite levels at birth. Nitric oxide (NO), a potent vasodilator formed by nitric oxide synthases, is rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) in the body. Although the biological transformation of NO to nitrite and nitrate was previously believed to be unidirectional, recent studies have demonstrated pathways by which nitrate is converted to nitrite and nitrite back into NO via a nitrate $\rightarrow$ nitrite $\rightarrow$ NO axis (1).

The key steps in nitrate and nitrite transport and metabolism are shown in Figure 1 and consist of 1) the introduction of nitrate into the mouth by the diet or active transport from plasma into the saliva by the salivary glands, 2) reduction of nitrate to nitrite by oral bacteria, 3) ingestion of nitrite, which is either converted to NO in the stomach or absorbed into the blood stream, 4) oxidation of nitrite and NO back into nitrate which can then again be secreted into the saliva, and 5) oxidation of NO into nitrite in the plasma and tissues (2).
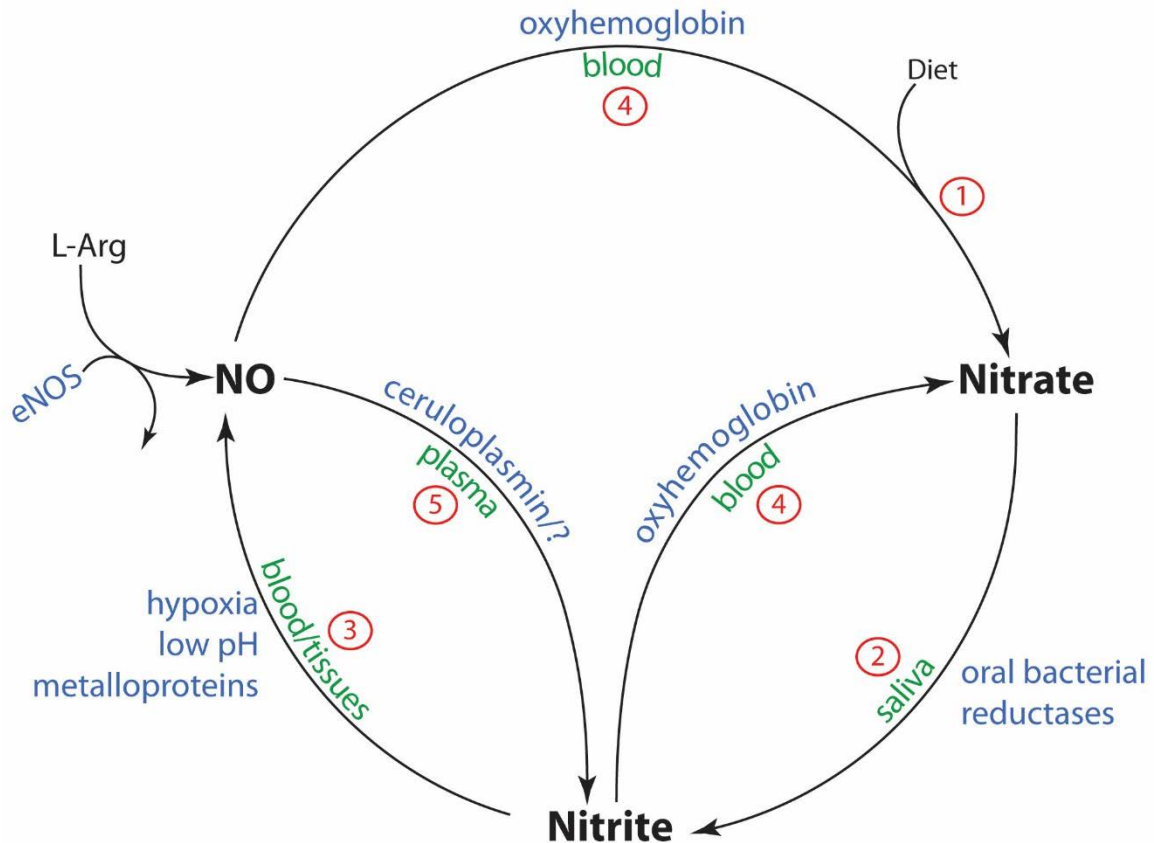
Figure 1. Simplified schema showing the pathways and interconversions of NO, nitrite, and nitrate. 1.) Nitrate enters the mouth from the diet and from the plasma by a concentrating action of the salivary glands. 2.) In the mouth nitrate is converted to nitrite by commensal bacteria on the tongue (3). 3.) In the acid milieu of the stomach nitrite is converted to NO by disproportionation (4) or absorbed into the circulation. Nitrite may also be converted to NO in blood and tissues by the action of metalloproteins (2). NO, deriving from nitrite or from the conversion of L-arginine to L-citrulline by eNOS, can be converted to 4.) nitrate by reaction with oxyhemoglobin (5) or 5.) nitrite by ceruloplasmin (6).

Via this endothelial NO synthase (eNOS)-independent pathway, nitrate and nitrite are gaining recognition as potential reservoirs of NO bioactivity. This has important physiological implications considering NO's wide range of actions throughout the body, including regulation of vascular homeostasis, neurotransmission, host defense, redox signaling, and cellular respiration (1). Under hypoxic and ischemic conditions in the circulation and tissues, nitrite is thought to act as a vasodilator via conversion to NO (7-

10). This is of particular importance in the ischemic heart, for example (11). More than 20 studies now show that increasing circulating nitrite levels, even only two-fold higher than basal levels, protects against ischemic stress in the brain, heart, lungs, liver, and kidney (see review by Dezfulian, 2007 (12)). Clearly, nitrite shows promise as a therapy in a multitude of vascular pathologies. Indeed, preclinical trials are currently testing nitrite as a therapy for pulmonary hypertension (13) and acute myocardial infarction (14). Furthermore, in the gastrointestinal tract, nitrite-derived NO kills pathogenic bacteria, protects against gastric ulcers, and increases gastric mucus production and local intestinal blood flow (15-18). Given these beneficial effects in adults and animals, we asked if nitrite would also be protective in premature infants who are at significant risk of suffering from hypoxic/ischemic injury due to dysregulation of cerebral blood flow (e.g. intraventricular hemorrhage and periventricular leukomalacia) and episodes of inadequate systemic oxygenation. We reasoned that low plasma nitrite levels in preterm infants could potentially predispose them to the severe effects of hypoxia and ischemia. Specifically, we hypothesized that preterm infants would have low plasma nitrite levels because of diminished activity of each step of the nitrate → nitrite → NO axis. To address our questions, we looked at the dietary intake of nitrate and nitrite, the bacterial conversion of salivary nitrate to nitrite, and plasma and urine nitrite levels in preterm and term newborn infants.

Dietary Nitrate and Nitrite

While about 70% of circulating nitrite comes from eNOS-derived NO oxidation (19), dietary intake of nitrate and nitrite also contributes to resting nitrite concentrations.

High levels of nitrate and nitrite are found naturally in green-leafy vegetables like spinach and beetroot and fruits like strawberries. They are also found in high levels in processed meat because they are used as curing agents (20). Consumption of dietary nitrate elevates plasma nitrite, as shown by Lundberg and Govoni who found that adults who consumed the equivalent of 300 g of spinach[1] saw their plasma nitrite levels increase from 123 ± 19 nM to 392 ± 68 nM, a striking 4-fold increase, ninety minutes after nitrate ingestion (21). Two further studies highlight the importance of dietary intake by showing that plasma nitrite and nitrate concentrations drop by nearly 50% in rats given diets low in nitrite and nitrate (22-23). Accumulating evidence now indicates that dietary nitrate ingestion, and subsequent elevation in plasma nitrite, has significant cardiovascular effects. Increasing dietary nitrate, even at relatively low doses [2](24), has consistently been shown to decrease blood pressure in healthy normotensive people (24-26) and now recently in patients with chronic obstructive pulmonary disease (27) and high blood pressure (28). Furthermore, it protects against ischemia-reperfusion-induced endothelial dysfunction, decreases platelet aggregation (25), decreases the oxygen cost of exercise, and improves exercise tolerance (29). It is now proposed that the high nitrate content in vegetables is one reason why the Mediterranean and Dietary Approaches to Stop Hypertension (DASH) diets are thought to be so cardioprotective (20). Considering the important gastro- and cardioprotective effects of nitrate and nitrite ingestion in adults, we saw the importance of measuring the levels of nitrate and nitrite in the diet of newborn infants. Given the close link between plasma nitrite and nitrite/nitrate ingestion, we

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[1] The dose of nitrate used was 10 mg/ml.
[2] Administration of 100 g of beetroot juice produces a significant drop in systolic and diastolic blood pressures.

hypothesized that diminished nitrite/nitrate ingestion in premature infants would lead to low plasma nitrite levels.

The second chapter of this dissertation describes our work of measuring dietary nitrite and nitrate ingestion in newborn infants. To estimate the amount of nitrate and nitrite infants ingest daily, we measured nitrite and nitrate concentrations in breast milk, formula, and parenteral nutrition. In addition, we looked at how clinical practices, such as freezing and thawing breast milk, effect the concentrations of nitrite and nitrate.

Salivary Nitrate and Nitrite

The third chapter of this dissertation describes our studies of the oral component of the nitrate → nitrite → NO axis in infants. In adults, the salivary glands concentrate twenty five percent of circulating nitrate into the saliva, leading to nearly ten-fold higher concentrations in fasting saliva (~200 μM in saliva versus ~20-40 μM in the plasma) (21). Twenty percent of this nitrate, together with nitrate from the diet, is reduced to nitrite by the normal microflora on the tongue. Although nitrate ingestion plays an important role in determining plasma nitrite levels, it is critically dependent on this bacterial reduction of salivary nitrate to nitrite. Adults who consume a high nitrate meal while using antibacterial mouth rinse do not have the corresponding postprandial rise in plasma nitrite (30). Moreover, if subjects refrain from swallowing saliva after a dietary nitrate load or are given antibacterial mouthwash to decrease bacterial nitrate-reducing activity, the hypotensive effects of nitrate are attenuated and there is no inhibition of platelet aggregation (25, 31). Thus, the many beneficial effects of increasing dietary nitrate are only seen when there is bacterial activation of nitrate to nitrite.

Due to the importance of these bacteria in the nitrate → nitrite → NO axis in adults, we sought to measure their activity in newborn infants. Conventional wisdom is that the fetus is sterile[3] and then acquires bacteria during birth and in the following weeks from the mother's skin and breast milk and the surrounding environment (33). However, it was unknown when infants acquire nitrate reducing bacteria and are able to reduce salivary nitrate effectively. Furthermore, it was unknown if premature infants, who often receive broad-spectrum antibiotics that would impact bacterial growth (34), have delayed colonization compared to term infants. Thus, we measured the activity of the specific oral nitrate-reducing bacteria in both term and preterm infants, in outpatient and intensive-care settings. We also address whether infants are able to concentrate nitrate from the blood into the saliva and how this impacts their salivary nitrate and nitrite concentrations and subsequent ingestion. Additionally, we show how a lack of oral nitrate reducing bacteria influences plasma nitrite levels in adults.

Plasma and Urine Nitrite

Plasma nitrite concentrations of healthy term newborns are measured to be 0.18 ± 0.03 μM (35). These are ~30% lower than the concentrations of adults, which typically range from 50 to 300 nM (2). Given that elevated nitrite levels protect against ischemic injury, the reverse is also potentially true: a deficiency in nitrite would enhance risk of injury. Thus, a significant reduction in plasma nitrite may put newborn infants, and

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[3] Intriguing new data suggest that the fetus is not, in fact, sterile and receives bacterial colonization from the placenta (32). Interestingly, the microbiome of the placenta is strikingly similar to that of the mother's oral flora and could potentially include these nitrate-reducing bacteria (32), although this remains to be determined.

particularly those born prematurely, at an even higher risk for diseases that involve ischemic injury such as necrotizing enterocolitis (NEC).

As the most common gastrointestinal disease among premature infants, NEC affects approximately 5-14% of infants born weighing less than 1500 grams (36-38). Despite over thirty years of clinical management, mortality rates in patients with NEC remain as high as 20% and the underlying cause remains largely unknown (36, 39). Premature birth is a prominent risk factor. Consensus is emerging that NEC results from epithelial mucosal injury secondary to prematurity, feeding substrate, weakened immune resistance to bacteria, and impaired response to stressors such as ischemia (40, 41). Considering nitrite's protective effects in the gastrointestinal tract and in animal models of ischemia, we hypothesized that preterm infants who are diagnosed with NEC have lower plasma nitrite levels in the days preceding diagnosis compared to other preterm infants, term infants, and adults. As discussed in chapter four, we tested this hypothesis by collecting plasma from preterm infants at risk for NEC for the first three weeks of life. We also measured the nitrite concentration in urine to determine whether NEC, and any associated changes in plasma nitrite levels, may impact nitrite excretion in these infants.

In addition to measuring plasma nitrite levels in infants at risk for NEC, we also attempted to address whether dietary nitrite supplementation would prevent NEC in a newborn rat pup model of the disease. However, due challenges with this animal model, we were unable to adequately test our hypothesis. A more thorough explanation of these experiments and the challenges we encountered appears in the discussion of this dissertation.

Summary

The studies described herein characterize the nitrate → nitrite → NO axis in newborn infants. Specifically, we measured the levels of nitrate and nitrite in breast milk, formula, and parenteral nutrition (Chapter 2), the oral bacterial nitrate reducing activity (Chapter 3), and the plasma and urinary nitrite levels in preterm infants at risk for NEC (Chapter 4). This work reveals that newborn infants differ greatly from adults due to low nitrate and nitrite ingestion, negligible bacterial nitrate reduction in the oral cavity, high urinary nitrite excretion, and diminished plasma nitrite levels. These findings give us a baseline of nitrate and nitrite bioactivity in newborn infants and help us to begin to address the possibility of using nitrite supplementation as a therapeutic intervention against hypoxic and ischemic pathologies common to newborn infants.

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CHAPTER TWO

NITRITE AND NITRATE CONCENTRATIONS AND METABOLISM IN
BREAST MILK, INFANT FORMULA, AND PARENTERAL NUTRITION

Abstract

Dietary nitrate and nitrite are sources of gastric NO, which modulates blood flow, mucus production, and microbial flora. However, the intake and importance of these anions in infants is largely unknown. Nitrate and nitrite levels were measured in breast milk of mothers of preterm and term infants, infant formulas, and parenteral nutrition. Nitrite metabolism in breast milk was measured after freeze-thawing, at different temperatures, varying oxygen tensions, and after inhibition of potential nitrite-metabolizing enzymes. Nitrite concentrations averaged 0.07 ± 0.01 μM in milk of mothers of preterm infants, less than that of term infants (0.13 ± 0.02 μM) ($P < .01$). Nitrate concentrations averaged 13.6 ± 3.7 μM and 12.7 ± 4.9 μM, respectively. Nitrite and nitrate concentrations in infant formulas varied from undetectable to many-fold more than breast milk. Concentrations in parenteral nutrition were equivalent to or lower than those of breast milk. Freeze-thawing decreased nitrite concentration ~64%, falling with a half-life of 32 minutes at 37 C. The disappearance of nitrite was oxygen-dependent and prevented by ferricyanide and three inhibitors of lactoperoxidase. Nitrite concentrations in breast milk decrease with storage and freeze-thawing, a decline likely mediated by lactoperoxidase. Compared to adults, infants ingest relatively little nitrite and nitrate, which may be of importance in the modulation of blood flow and the bacterial flora of the infant GI tract, especially given the protective effects of swallowed nitrite.

Introduction

Since being established as a potent vasodilator, nitric oxide (NO) has been one of the most intensely studied compounds in biology and is now considered to be an essential signaling molecule in a diverse set of pathways. Endogenous NO is produced predominantly through the conversion of L-arginine to L-citrulline by NO synthase enzymes. Through reactions with metal-containing proteins and oxygen, endogenous NO is rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-). Although these anions were once thought to be inert at physiological concentrations, more recent evidence indicates that nitrite plays a significant role in cardiovascular homeostasis (1) and protects against hypoxic and ischemic stress in the brain, (2,3) heart, (4,5) lungs, (6) and kidney (7). Thus, there is growing interest in factors that contribute to the concentrations of nitrite in the body.

A large portion of plasma nitrite is derived from the oxidation of NO produced by endothelial NO synthases (8). However, plasma nitrite concentrations are also influenced by the ingestion of nitrite and nitrate, the latter being converted to nitrite in the mouth by commensal bacteria present on the dorsal surface of the tongue (9). The oral conversion of nitrate to nitrite is enhanced by active secretion of nitrate from the blood into the saliva. Once swallowed, the salivary nitrite can contribute to plasma nitrite concentrations, (10) or it can be protonated to nitrous acid resulting in a cascade of reactions leading to several bioactive products including nitrotyrosines, nitrosothiols, and nitrated lipids (see review by Rocha et al (11)). Although the chemistry of nitrite in the acidic gastric milieu is not fully characterized, it likely plays a role in the observed effects

of ingested nitrite in the adult rat. These include increased gastric blood flow (12,13) and mucus production (13) and protection against ulcers (12,14).

In the newborn period, breast milk and artificial breast milk substitutes (referred to herein as "infant formulas") are the sole dietary sources of nitrate and nitrite. As will be discussed in chapter three, the nitrate-nitrite-NO pathway of adults does not similarly function in newborn infants due to diminished bacterial conversion of nitrate to nitrite in the mouth, (15) making the diet a particularly important source of nitrite. Indeed, plasma nitrite levels are lower in newborn infants in the neonatal intensive care unit (NICU) than in adults, (16) but the contribution of dietary nitrite and nitrate is not known. Concentrations of nitrate and nitrite in human breast milk have previously been reported in milk from mothers of healthy term infants (17,18). However, the effect of preterm birth and of common manipulations such as freeze-thaw cycles and storage on these concentrations have not been reported.

In this study we hypothesized that the freeze-thaw and storage of breast milk results in significant reductions in the dietary intake of nitrate and nitrite of newborns. We further hypothesized that nitrite and nitrate intake would be significantly reduced in infants receiving infant formula or intravenous parenteral nutrition (PN) compared to infants receiving fresh breast milk. We report that the levels of nitrate and nitrite change in the handling and storage of breast milk and describe potential mechanisms by which these levels change, including an examination of the roles of various key milk proteins that may contribute to nitrite metabolism.

The experimental protocols were approved by the Institutional Review Board of

Loma Linda University. All chemicals were purchased from Sigma Aldrich (St Louis,

MO) unless otherwise specified.

Breast Milk and Formula Collection and Processing

Fresh breast milk was collected from 11 mothers of term infants and 13 mothers

of preterm infants. The demographics of these mothers and their infants are provided in

Table 1.

Table 1. Demographics of breast milk study participants

Characteristic	Term (>36 weeks)	Preterm (>35 weeks)
Number of women, n	11	13
Maternal age, years	28.4 ± 2.4	28.9 ± 2.0
Parity, n	2.0 ± 0.7	3.5 ± 0.7
Times breast pumped before donation, n	4.2 ± 0.6	3.8 ± 0.4
Number of infants, n	11	13
Gestational age, weeks	39.4 ± 0.3	30.8 ± 0.7
Birth weight, grams	3158 ± 323	1390 ± 180
Infant age at time of collection, days	24.3 ± 8.7	18.9 ± 3.9

Feeding, breast pumping, and milk collection routines were not changed by participation

in the study. After milk had been expressed for the first two minutes of pumping, a 3 mL

sample of newly expressed milk was collected in a polyurethane bottle, then divided into

500 µL aliquots and placed on ice. A portion of these aliquots was then immediately

placed in a −20 C freezer, while the remainder was assayed for nitrite and nitrate

concentrations within 30 minutes of collection. To measure the effect of freeze-thawing,

frozen samples were thawed on ice ~48 hours after collection and then assayed for nitrite and nitrate.

Nitrite concentrations of colostrum (milk expressed days 1-3), transition milk (expressed days 4-7), and mature milk (expressed days >7) (18) were measured to follow changes during the first 3 weeks after giving birth. Samples were collected daily from 12 lactating women and immediately stored at −20 C until assay. Nitrite and nitrate concentrations were also analyzed in a number of infant formulas used in the NICU of the Loma Linda University Children's Hospital (LLUCH). These included: Enfamil® Premature Lipil (Mead Johnson Nutritionals, Evansville, IN); Enfamil® Lacto Free Lipil, Enfamil® ProSobee® Lipil, Enfamil® EnfaCare® 22, Enfamil® Premium Infant, Similac® Special Care (Abbott Nutrition, Columbus, OH); Similac NeoSure®, Similac Advance EarlyShield®, Pregestimil® Lipil (Mead Johnson Nutritionals); and Nestlé® Good Start® (Nestlé Infant Nutrition, Florham Park, NJ). Assays were performed in 3 samples from 2 different lots of each formula. The nitrite concentrations were also measured in 5 samples of starter parental nutrition (sPN) and 14 samples of parenteral nutrition (PN) used for infants unable to receive milk or formula feeds.

Nitrite Metabolism in Breast Milk

The metabolism of nitrite in breast milk was assessed in a series of experiments in which nitrite was added to freeze-thawed samples of breast milk to initial concentrations of ~12 μM. The milk was then incubated at 37 C (unless otherwise stated) and changes in nitrite concentrations were measured over a 5-hour time course.

To determine the effect of temperature on the metabolism of nitrite in breast milk, samples were incubated at 0, 10, 25, and 37 C. In a separate study, aliquots of milk were boiled for 5 minutes to denature proteins prior to incubation at 37 C. To test whether nitrite was being oxidized to nitrate, nitrite was added to 6 samples of milk and both nitrite and nitrate concentrations were measured over a 5-hour time course. In a separate experiment, additional nitrite was added to breast milk samples for final concentrations ranging from 10 μM to 160 μM and the increase in nitrate was measured after 5 hours. The increase in the nitrate concentration in a control sample of milk was subtracted from the rise in nitrate measured in the nitrite supplemented samples and plotted versus the initial nitrite concentration.

To assess the role of lipids in the stability of nitrite in breast milk, lipids were removed from 10 samples of milk by either chemical or centrifugation methods. The chemical method utilized an adaptation of a method for the delipidation of plasma, (19) while the centrifugation method involved centrifuging the milk twice at 13,400 rpm for 90 seconds. The lipid content was measured before and after the delipidation procedures using a Calais Human Milk Analyzer, a midrange infrared spectrophotometer (Metron Instruments, Inc, Solon, OH).

To measure whether nitrite is reduced to NO in breast milk, nitrite was added to breast milk and then 100 μL of sample was immediately injected into buffered saline solution (pH = 7.4) in a purge vessel being continuously sparged with argon in line with a chemiluminescence NO detector. The presence of NO formation was detected over a period of 20 minutes (model 280i NO analyzer, Sievers Instruments, Boulder, CO). The lower limit of NO detection by this method was 20 nM. The possibility of a flux of nitrite

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reduction to NO that could subsequently be oxidized back into nitrite was examined by incubating milk samples with the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (final concentration 200 µM).

Additionally, to address the possibility that nitrite was being reduced to NO and then combining with thiols to produce s-nitrosothiols, nitrosothiol concentrations were measured in 6 samples of breast milk over a 3-hour period following the addition of nitrite using a method described previously (20). The possible role of thiols was also assessed by measuring the effect of addition of 2 mM N-ethylmaleimide (NEM) (21) on the rate of nitrite metabolism. To assess whether nitrite metabolism was dependent on enzymes with oxidizable transition metals, experiments were performed after addition of 10 mM ferricyanide (FeCN).

To determine the role of dissolved oxygen in nitrite metabolism in breast milk, 6 samples of milk were equilibrated with gas phases of various mixtures of nitrogen and oxygen. The oxygen tension of the samples were adjusted to approximately <5, 7, 37, 73, or >600 mmHg by measuring the partial pressure of oxygen (PO2) in the sample (Radiometer, model ABL5, Copenhagen, Denmark). The rate of nitrite metabolism was determined by measuring the magnitude of decrease in nitrite concentrations after incubation for 60 minutes at 37 C.

To test the possibility that xanthine oxidase was involved in the metabolism of nitrite, experiments were conducted following the addition of 100 µM of the selective antagonist allopurinol. A possible role for the enzyme lactoperoxidase (LPO) was studied by adding 1 of 3 selective inhibitors of LPO to samples of breast milk. First, to inactivate the heme center of LPO, 10 µM of 2-mercaptio-1-methylimidazole (2-MMI) was added

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to breast milk. As a second test, 4 mM 3-amino-1,2,4-triazole (3-AT), a compound that inhibits LPO by covalent binding to the polypeptide chain of LPO rather than the heme, (22) was added to a separate set of samples. In a third set of samples, the LPO inhibitor dapson was added to a concentration of 0.6 mmol/L. Optimal concentrations for each inhibitor were chosen in accordance with those used in the literature (22-25). To assess whether addition of exogenous LPO would accelerate the loss of nitrite, 10 µL of 1.33 mM LPO from bovine milk was added to 5 mL of breast milk for a final LPO concentration of 500 nM. Nitrite was added to the sample immediately before 1 mM hydrogen peroxide was added to initiate the reaction. Control samples lacked hydrogen peroxide. The samples were incubated at 37 C and the nitrite concentrations were measured after 10 seconds and 1, 2, 3, 30, and 60 minutes.

Nitrite and Nitrate Assays

Nitrite concentrations were measured by triiodide chemiluminescence as described by Pelletier et al, (26) enabling quantification above 10 nM with a precision of ±5 nM. Nitrate concentrations were measured by triiodide chemiluminescence after reduction to nitrite with a nitrate reducing enzyme as previously described (15).

Data Analysis

Data are presented as mean ± standard error. Differences between study groups were detected using a t-test when the tested hypothesis involved only two sample groups and a 1-way ANOVA followed by Bonferroni post hoc analysis when three or more sample groups were involved. One-way ANOVA with repeated measures was used to

detect significant changes from baseline measurements in time-course experiments. Two-way ANOVA followed by Bonferroni post hoc analysis was used to detect significant differences between groups in time-course experiments. The overall rate of nitrite metabolism in breast milk was determined by fitting nitrite disappearance curves to a monoexponential equation. Where indicated, the initial rate of nitrite metabolism was determined as the amount of nitrite consumed during the first 60 minutes following addition of nitrite. Statistical analyses were performed using Prism 5 for Mac OS X (Graphpad Software, Inc, La Jolla, CA).

Results

Nitrite and Nitrate Concentrations in Breast Milk and Formula

The nitrite concentrations of fresh and freeze-thawed breast milk from mothers of term and preterm infants are shown in Figure 2A. Nitrite concentrations in the milk of the mothers of preterm infants were significantly less than in milk from the mothers of term infants (0.07 ± 0.01 µM vs 0.13 ± 0.02 µM, respectively, $P < .01$). After freeze-thawing, nitrite concentrations were significantly decreased in the milk of mothers of both preterm and term infants (0.03 ± 0.01 µM and 0.04 ± 0.01 µM, respectively, $P < .05$ compared to fresh milk).

Nitrate concentrations averaged about 100-fold higher than the nitrite levels in breast milk, as shown in Figure 2B. Nitrate in the milk of the mothers of preterm infants (13.6 ± 3.7 µM) did not differ significantly from nitrate concentrations in the milk from mothers of term infants (12.7 ± 4.9 µM). Nitrate concentrations tended to increase following freeze-thawing, but this change did not reach statistical significance.

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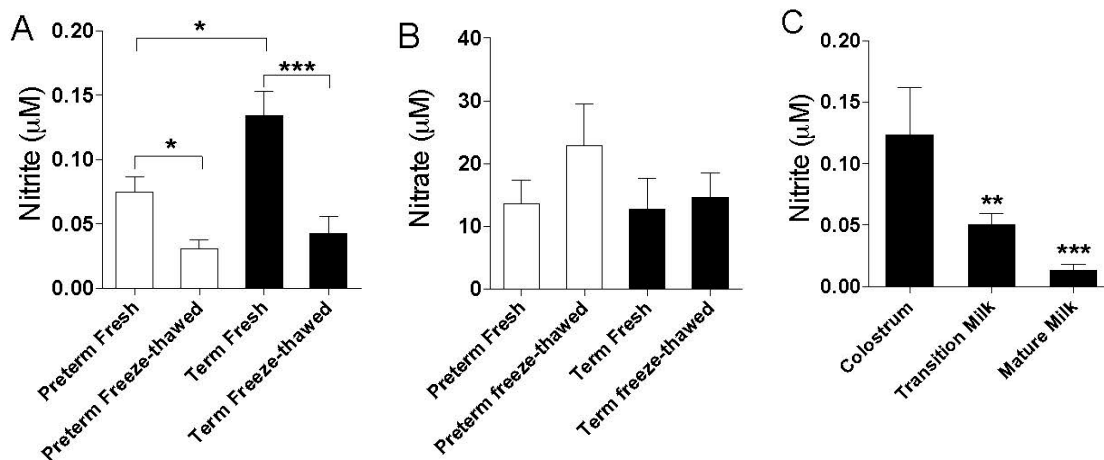
Figure 2. Comparison of nitrite (A) and nitrate (B) concentrations in breast milk of mothers of term and preterm infants and after freeze-thawing. C) Nitrite concentrations are higher in colostrum than transition (**p<0.01) or mature milk (***p<0.001).

To examine changes in nitrite concentrations in milk in the days following birth, samples were collected from 12 lactating mothers during the first 21 days postpartum (Figure 2C). In the first 3 days after birth, the nitrite concentration averaged 0.12 ± 0.03 µM. Nitrite concentrations decreased significantly over time following parturition, falling to 0.05 ± 0.01 µM in days four through seven (1-way ANOVA, $P < .01$) and 0.01 ± 0.005 µM in days eight through twenty one ($P < .001$).

In a convenience sample of commercially available infant formulas used commonly in the Loma Linda University Children's Hospital (LLUCH) NICU, nitrite and nitrate concentrations averaged 0.28 ± 0.1 µM and 43 ± 5.8 µM, respectively (Figures 3A, 3B). The nitrite concentrations were also measured in sPN and PN. Nitrite concentrations averaged 0.02 ± 0.008 µM in sPN and 0.08 ± 0.03 µM in PN. Nitrate concentrations averaged 4.6 ± 0.2 µM in sPN and 9.5 ± 0.8 µM in PN. Figures 4A, 4B

include the nitrite and nitrate concentrations of the PN samples alongside the mean

concentrations in breast milk, colostrum, and formula.
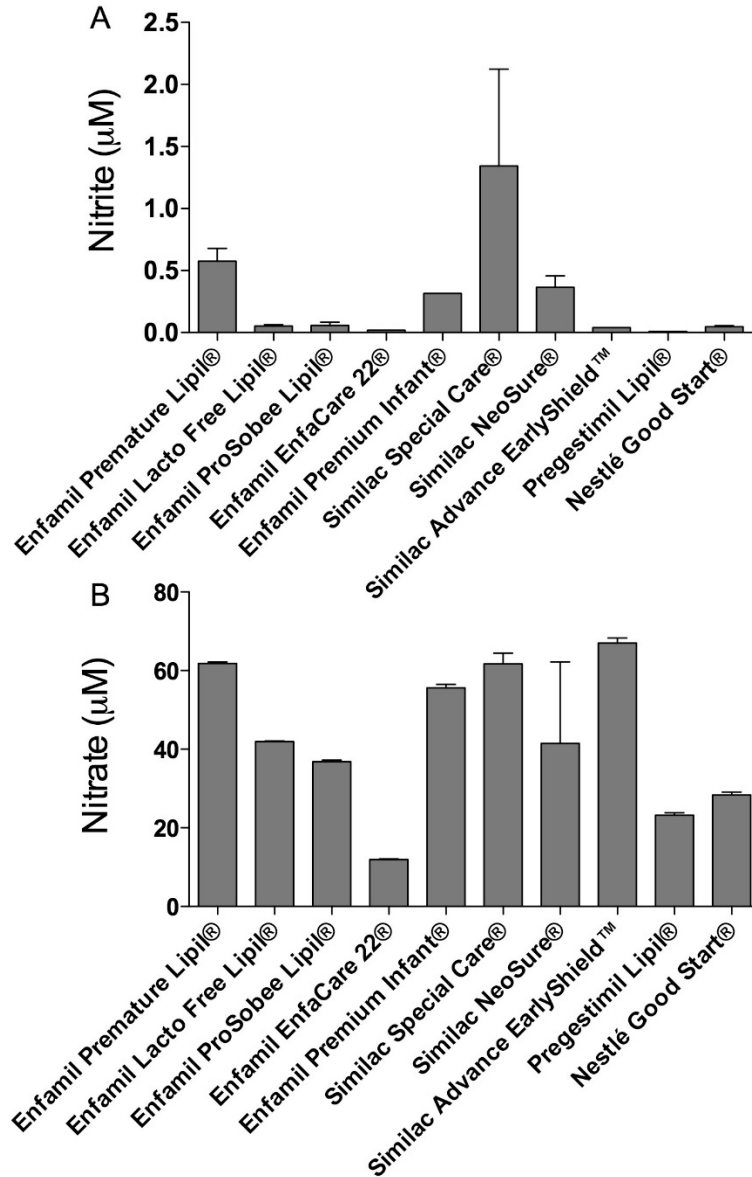
Figure 3. Nitrite concentrations (A) and nitrate (B) concentrations in a variety of formulas used in neonatal intensive care units. Nitrite levels vary widely, ranging from barely detectable to more than 13-fold higher than breast milk.
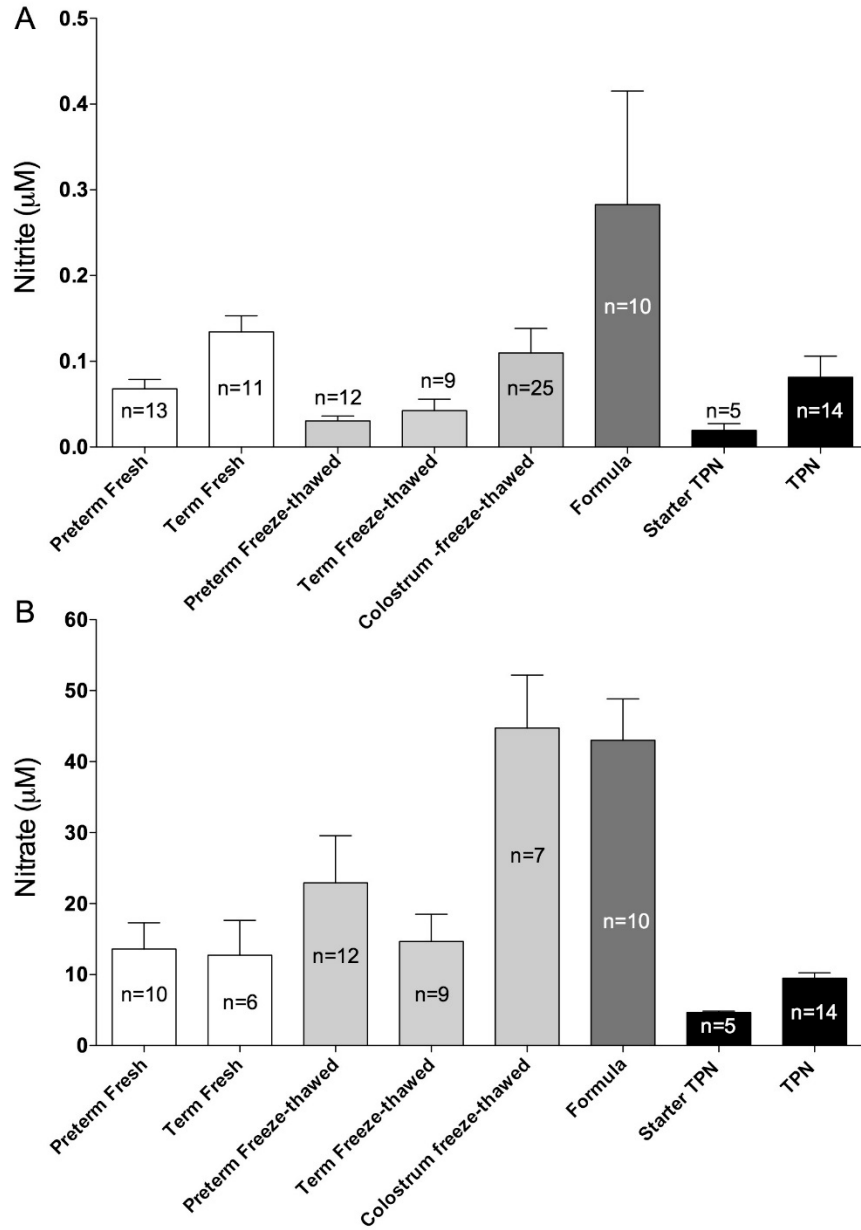
Figure 4. Summary nitrite (A) and nitrate (B) concentrations in all forms of nutrition provided to newborns in an intensive care setting. The nitrite and nitrate concentrations in starter PN and PN samples are similar to those found in breast milk.

Nitrite Metabolism in Breast Milk

The lower concentrations in the freeze-thawed milk compared to fresh samples

indicated a time-dependent metabolism of nitrite. This was confirmed by measuring the

disappearance of 12 µM nitrite added to freeze-thawed milk and incubated at 37 C. Under

these conditions, nitrite concentrations decreased in a manner approximating first-order

kinetics, with a rate constant of 0.020 ± 0.003 min^{-1} and an effective half-life of 32

minutes. The initial rates of nitrite metabolism of breast milk incubated at the different

temperatures are shown in Figure 5A. An Arrhenius plot of the nitrite concentrations and

temperature, shown in Figure 5B, revealed an activation energy of 6551 cal•mol^{-1} and a

Q_{10} of 1.5.
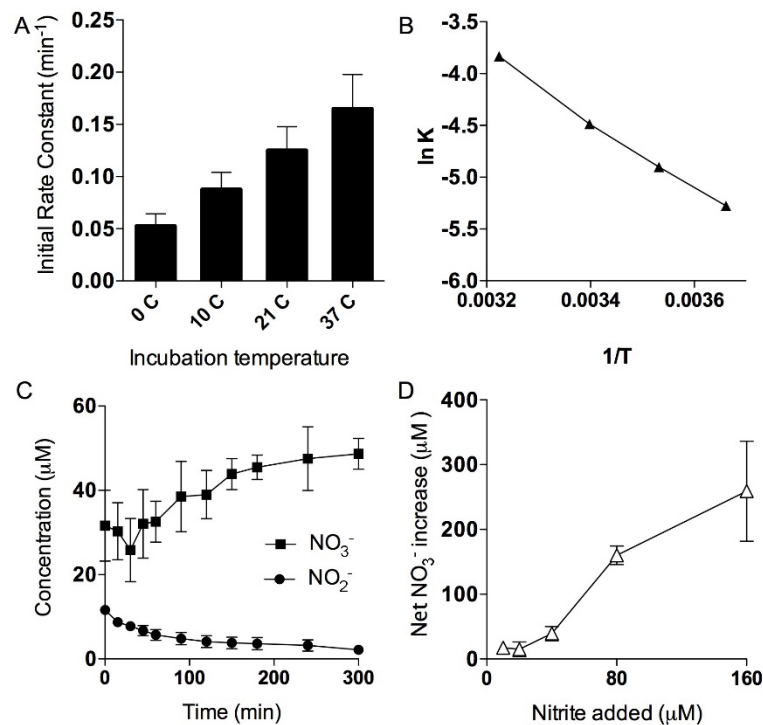
Figure 5. A) Nitrite metabolism rates under various temperature conditions. B) Arrhenius plot of nitrite metabolism and temperature. C) Nitrate and nitrite concentrations in six samples of breast milk incubated over five hours. D) Relationship between initial nitrite concentration and nitrate production.

As shown in Figure 6, this rate of disappearance was temperature dependent, with rate constants of 0.010 ± 0.001 min–1, 0.007 ± 0.001 min–1, and 0.005 ± 0.002 min–1 at 21, 10, and 0 C. When the milk was boiled for 5 minutes to denature proteins, the nitrite concentrations remained stable when incubated at 37 C (data not shown).
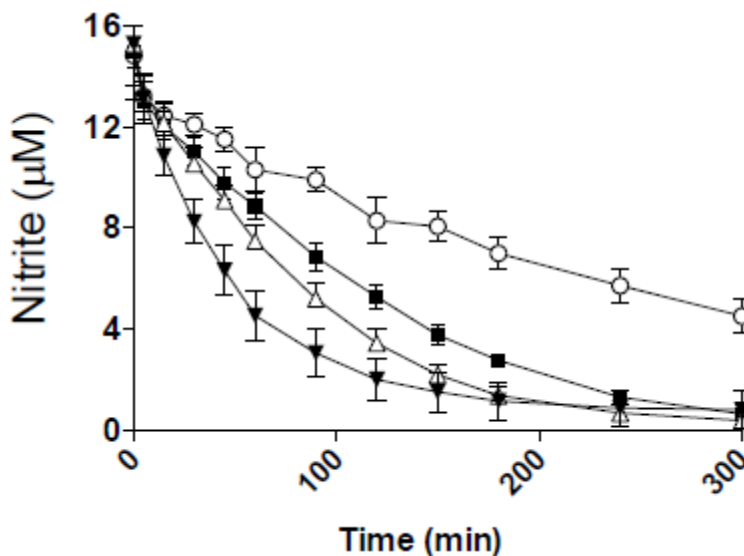
Figure 6. Time-course of nitrite disappearance in samples of milk incubated under different temperatures, 37 C (▼), 21 C (Δ), 10 C (■), or left on ice (○). Nitrite decreased in a mono-exponential fashion with apparent half-lives of ~31, ~54, ~75 min, and ~100 min, respectively.

To examine the possibility that nitrite was being oxidized to nitrate, we made measurements of nitrite and nitrate concentrations following addition of nitrite to an initial concentration of 12.5 µM in breast milk. We observed an increase in nitrate concentrations that was comparable in magnitude to the decrease in nitrite concentrations (Figure 5C), consistent with a pathway of oxidation of nitrite to nitrate. To confirm these results, additional nitrite was added to a second set of breast milk samples for final concentrations ranging from 10 to 160 µM. The increase in the nitrate concentration in a

control sample of milk was subtracted from the rise in nitrate in the nitrite supplemented samples and plotted vs. the initial nitrite concentration (Figure 5D).

We next examined a number of different possible mechanisms for the disappearance of nitrite including its reaction with lipids, oxidation/reduction, and enzymatic catalysis by key milk proteins.

The role of lipids was assessed by measuring nitrite metabolism before and after delipidation of breast milk samples from mothers of term infants. Fat content in these samples averaged 2.6 ± 0.1 g/dl and decreased to 1.1 ± 0.2 g/dl after delipification for samples that were delipified via the chemical process. The extent of delipification was similar for the centrifugation method, with the starting fat content averaging 3.2 ± 0.4 g/dl and the ending amount averaging 0.8 ± 0.1 g/dl after delipification (P = .31). However, lipid removal, by either a combination of chemical delipification and centrifugation or centrifugation only, had no effect on the rate of metabolism of nitrite, as shown in Figure 7A, and thus the role of lipids could be discounted.
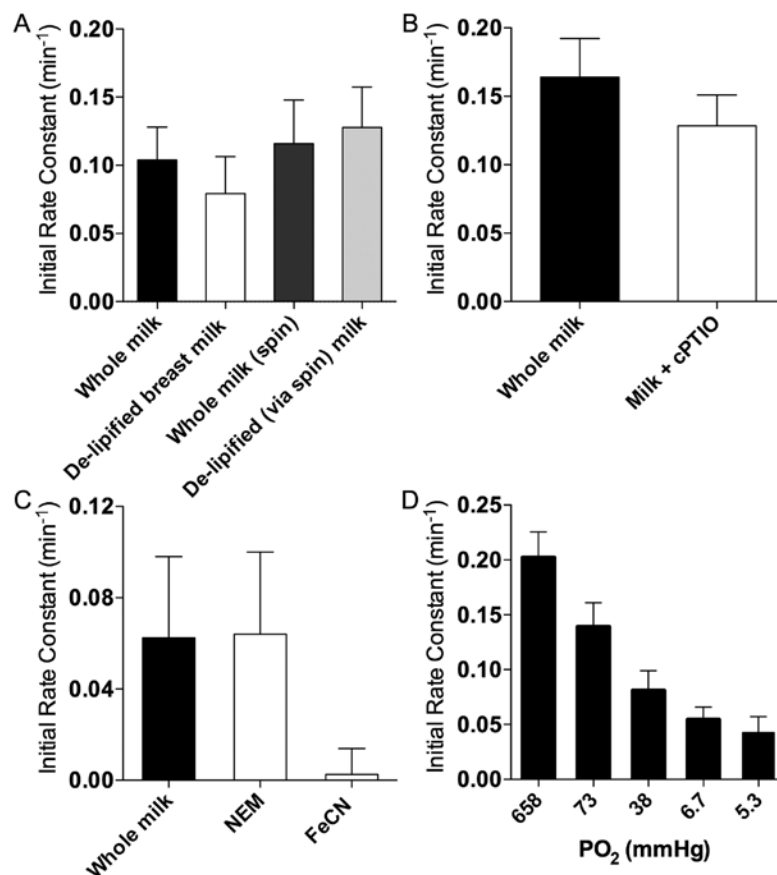
Figure 7. Initial rates of nitrite metabolism in breast milk after removal of lipids (A), treatment with the NO scavenger, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (B), incubation with N-ethylmaleimide or ferricyanide (C), or at different partial pressures of oxygen (D).

We were unable to detect any free NO in breast milk following addition of nitrite, indicating no reduction of nitrite to NO. We also considered the possibility of a rapid flux of nitrite reduction to NO with subsequent oxidation of the NO back into nitrite. If so, the removal of NO being produced would be anticipated to speed the metabolic loss of nitrite by mass action. However, addition of the NO scavenger, cPTIO, did not change the rate of nitrite metabolism (Figure 7B). Alternatively, any NO produced might have immediately reacted with cysteine residues to produce nitroso compounds, including s-nitrosothiols (SNO). However, we did not detect measurable amounts of SNO production

in any of the milk samples. The combination of these results speaks against a reductive

process that might have converted nitrite to NO.
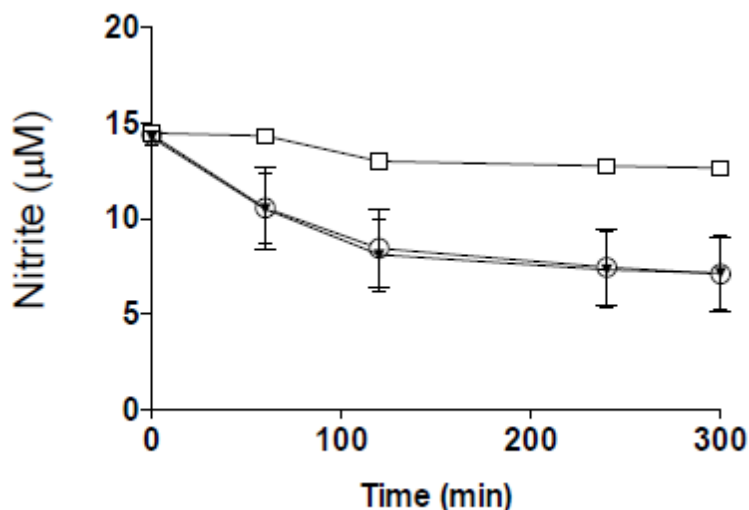
Figure 8. Time course of nitrite disappearance in samples of milk treated with PBS (○), N-ethylmaleimide (NEM) [2mM] (▼), or ferricyanide (FeCN) [10mM] (□). The metabolic loss of nitrite was blocked by incubation with FeCN, suggesting the involvement of an oxidizable transition metal.

We also investigated the potential roles of catalytic proteins in the metabolism of

nitrite. The prevention of nitrite metabolism after boiling milk is consistent with an

enzyme-mediated metabolism of nitrite. The metabolic loss of nitrite was halted by the

addition of 10 mM ferricyanide, suggesting the involvement of a protein containing an

oxidizable transition metal (Figure 7C and Figure 8). Other experiments tested the

possible binding of nitrite or its metabolic byproducts to thiol groups. Again, the rate of

nitrite disappearance was not affected by the presence of 2 mM NEM used to block

available thiol groups (Figure 7C and Figure 8).

The role of oxygen in nitrite metabolism was tested by measuring rates of nitrite disappearance in breast milk samples after equilibration with various concentrations of oxygen. We found the initial rate of nitrite disappearance to be directly related to oxygen tensions (Figure 7D). These results are consistent with an oxygen-dependent oxidation of nitrite to nitrate. In further support of this mechanism, nitrate production was observed to increase in approximate proportion to the magnitude of nitrite disappearance, as shown in Figures 5C, 5D.

The blockade of xanthine oxidase, an enzyme previously reported to metabolize nitrite to NO in breast milk, (27) by addition of allopurinol had no effect on the rate of nitrite disappearance (Figure 9 and Figure 11A).
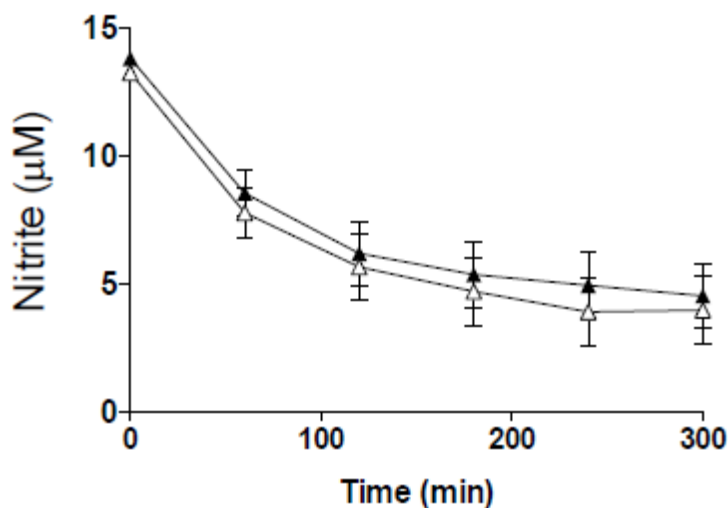
Figure 9. Time course of nitrite in samples of milk treated with either PBS (▲) or with the xanthine oxidase inhibitor allopurinol (100 µM final concentration) (Δ). Xanthine oxidase appears not to be responsible for the nitrite metabolism measured in breast milk.

Lactoperoxidase, an enzyme previously reported to be present in breast milk, (28) has been shown to contribute to oxidative mechanisms in milk, (29) in addition to

catalyzing the oxidation of nitrite in vitro (30). Inhibition of LPO's iodide-binding site

with 2-mercaptio-1-methylimidazole resulted in the blockade of nitrite metabolism.

Similarly, inhibiting the polypeptide chain of LPO with 3-AT resulted in the complete

inactivation of enzymatic activity, corresponding with a lack of nitrite metabolism

(Figure 10 and 11B). Treating milk samples with excess dapson, a potent inhibitor of

LPO activity, also effectively prevented the loss of nitrite over time. The nitrite

metabolism curves for milk treated with the LPO inhibitors are shown in Figure 10. The

addition of exogenous LPO (final concentration, 500 nM) also accelerated the loss of

nitrite, shortening the half-life from ~30 to ~16 min (Figure 11C).
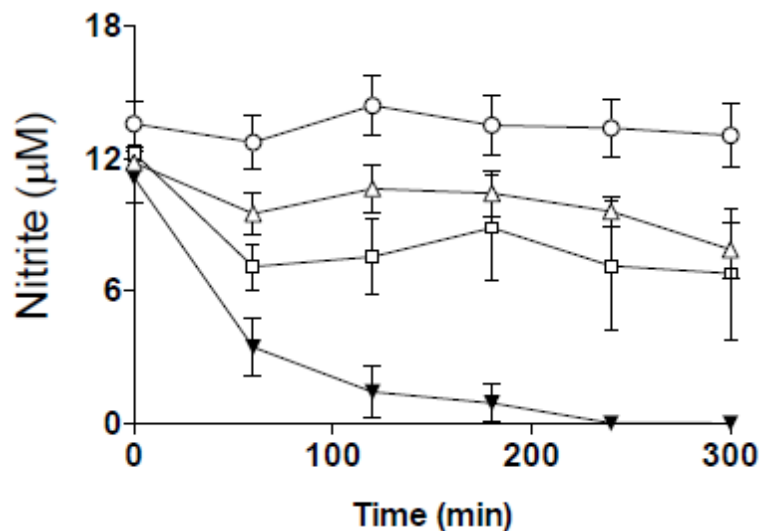
Figure 10. Time course of nitrite in five samples of milk treated with PBS (▼) or one of three inhibitors of lactoperoxidase activity: 2-mercaptio-1-methylimidazole (2-MMI) (□), 3-amino-1,2,4-triazole (3-AT) (Δ), or Dapson (○). Inhibition of lactoperoxidase activity effectively blocked the metabolism of nitrite in breast milk.
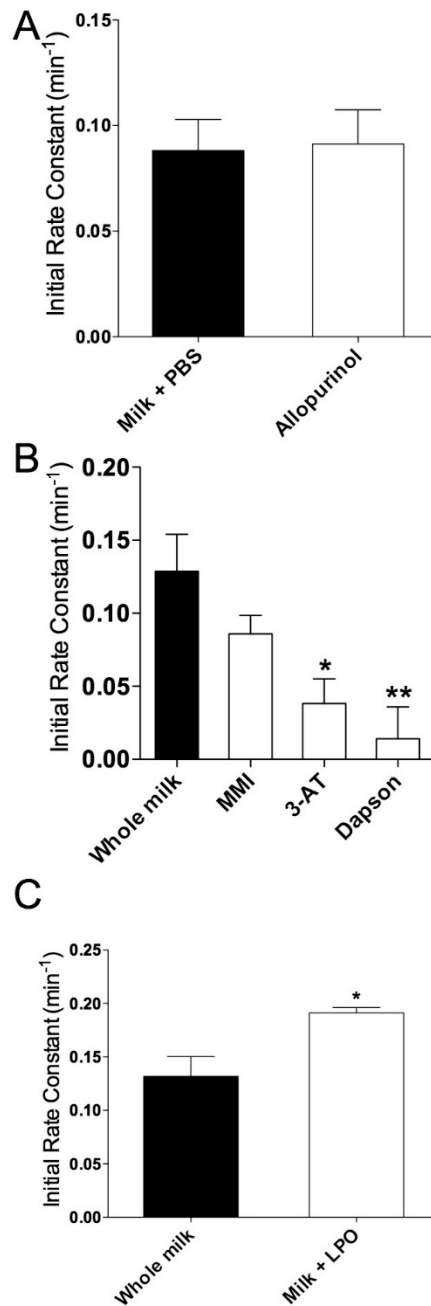
Figure 11. Initial rates of nitrite metabolism in breast milk after treatment with the xanthine oxidase inhibitor allopurinol (A), inhibition of lactoperoxidase by 2-mercaptio-1-methylimidazole, 3-amino-1,2,4-triazole, or Dapson (B), or addition of purified lactoperoxidase enzyme (C).

Discussion

These studies sought to determine whether the dietary intake of nitrite and nitrate is limited for term and preterm infants in the NICU compared to normal infants. We find that the dietary intake of nitrate and nitrite is significantly lower in infants receiving freeze-thawed breast milk or breast milk from mothers of preterm infants or in infants receiving PN. In addition, the intake of nitrate and nitrite of infants receiving infant formulas range from less than to more than that of infants receiving fresh breast milk, depending on the brand of formula. Finally, the findings of the current experiments also add a novel breast milk component to the nitrate-nitrite-NO system, with nitrite being oxidized to nitrate by LPO, as shown in Figure 12.
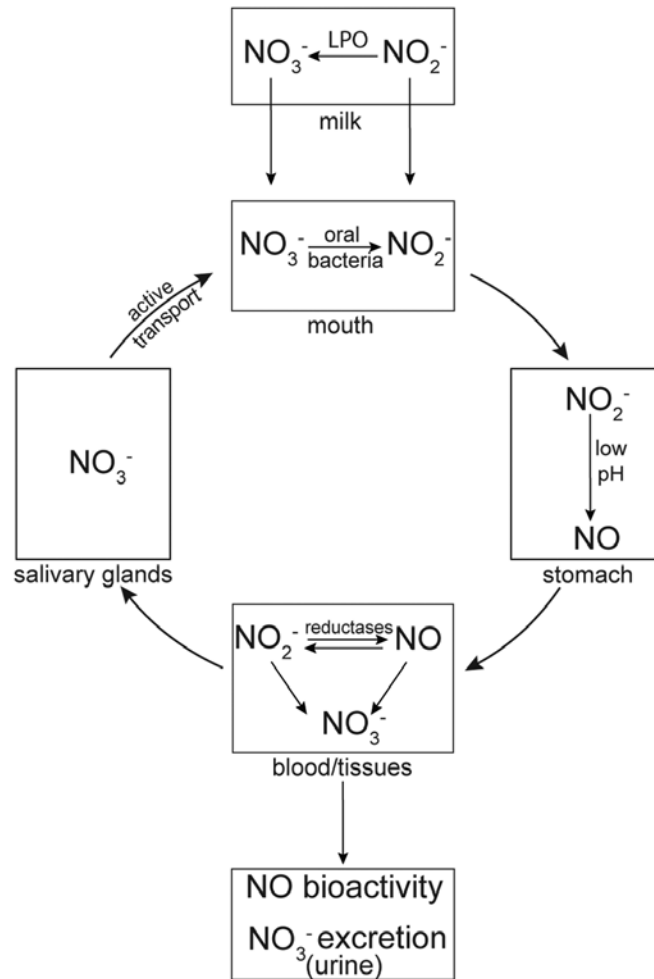
Figure 12. Simplified schema showing the nitrate-nitrite-NO axis.

We find widely variant concentrations of nitrite and nitrate in the diets of newborn infants in the NICU. The lowest levels of nitrite and nitrate measured were those in sPN at nearly undetectable concentrations, whereas standard PN had concentrations comparable to those of breast milk. Infant formulas, however, were found to have levels ranging from undetectable to nearly 100 times those of breast milk. This suggests that the manufacturers of commonly used infant formulas do not tightly control for nitrite and nitrate concentrations, especially as levels were found to vary significantly across different lots of the same formula.

With regard to breast milk, our results show that nitrite intake is limited for preterm infants in the NICU compared to term infants. The milk of the mothers of preterm infants has significantly less nitrite than the milk of the mothers of term infants, a finding in line with studies that have shown that the breast milk of mothers of term and preterm infants differ with respect to a number of components (31-34). Consistent with reports that the composition of milk changes in the days postpartum, (18, 34) we find that nitrite concentrations decrease from relatively high levels in colostrum to lower levels in the days and weeks after birth. The physiological importance of this progression is not known, but it is worth noting that it is not matched in the care of newborns in the NICU where initial feeds may be delayed and concentrations in sPN are well below those of breast milk.

Nitrite Metabolism in Breast Milk

In all samples of breast milk analyzed, nitrite concentrations fell after freeze-thawing. Our experiments indicate that the mechanism for this fall involves the metabolism of nitrite by LPO. In support of this idea, we demonstrate that nitrite metabolism is oxygen-dependent, blocked by the presence of FeCN to oxidize the heme moiety of LPO, and is blocked by 3 different selective antagonists of LPO activity. We also demonstrate that the metabolism of nitrite in breast milk is accelerated by the addition of exogenous LPO. This idea is consistent with previous reports that LPO is known to contribute to oxidative mechanisms in milk (29) and to metabolize nitrite in vitro (30).

In contrast to previous reports that nitrite is reduced to NO by xanthine oxidase in breast milk,(35) we observed no change in the rate of nitrite metabolism following addition of allopurinol to block xanthine oxidase activity. Likewise, addition of the NO scavenger cPTIO also had no effect. Finally, the rate of nitrite metabolism was observed to be directly proportional to oxygen concentrations, in contrast to the inverse relationship that would be expected from the reduction of nitrite by xanthine oxidase (36).

The observation that FeCN prevented nitrite metabolism, together with evidence of the involvement of oxygen, suggested the involvement of an oxidative process involving a heme-containing protein. Lactoperoxidase falls into this category and is known both to contribute to oxidative mechanisms in milk (38) and to metabolize nitrite in vitro (30). Our finding that each of the three LPO antagonists studied abolished nitrite disappearance strongly indicates a role for LPO in the metabolism of nitrite in breast milk. Further supporting the role of LPO in the metabolism of nitrite, we found that adding purified LPO enhanced the conversion of nitrite to nitrate by approximately 2-fold.

Neonatal Nitrite and Nitrate Ingestion

Based on the values reported in this study, we have estimated the daily intake of nitrate in newborns in the NICU and compared this to adults. Assuming a representative milk intake of 150 ml•kg^{-1}•day^{-1} and based on the nitrite and nitrate concentrations measured in our study, infants consuming fresh breast milk would ingest 0.0007 mg•kg^{-1}•day^{-1} of nitrite and 0.12 mg•kg^{-1}•day^{-1} of nitrate. This may be compared to adult

ingestion of nitrite of ~0.109 mg•kg^{-1}•day^{-1} and ~2.65 mg•kg^{-1}•day^{-1} of nitrate, based on

an average of seven studies summarized by Pennington (39). Thus, on a per kg body

weight basis, neonatal intake of nitrite and nitrate are only ~0.6% and ~5% of the adult,

respectively. In addition, nitrite intake would decrease another 50%-75% if the milk is

freeze-thawed prior to ingestion and by another 50% if the milk came from the mother of

a preterm infant. For infants who are fed infant formula instead of breast milk, nitrite and

nitrate intake may range from markedly lower to several-fold above that of breast milk–

fed infants, and all formulas provide less nitrite and nitrate than the average adult diet.

The amount of nitrite and nitrate being administered to infants receiving PN is 50%-80%

less than that ingested by term infants receiving fresh breast milk. To date, we are

unaware of any adult studies examining the effects of chronically low intake of nitrate

and nitrite similar to that of infants. Consequently, the physiological effects of this

deficiency in newborns cannot be compared to similar deficiencies in adults.

Relation to Plasma Nitrite Levels

We have recently shown that plasma levels of nitrite in infants in the NICU

average only 35%-55% of the levels of adults (16). One potential explanation is the

current finding that infants ingest far lower amounts of nitrite and nitrate relative to

adults. Demonstrating this effect, two recent studies showed a nearly 50% reduction in

plasma nitrite and nitrate concentrations in rats given diets low in nitrite and nitrate

(40,41). However, it remains to be determined whether reduced plasma nitrite levels in

newborn infants is beneficial or detrimental and thus optimal levels of nitrate and nitrite

in the diet of infants are as yet unknown.

It is important to consider that until recently, these anions were solely considered to be toxic by-products of NO metabolism because of their potential ability to form carcinogenic N-nitrosamines and methemoglobin (MetHb) (42). Cases of methemoglobinemia have contributed to the idea that nitrate and nitrite ingestion must be carefully regulated for infants less than three months of age. However, even the high nitrate and nitrite levels we measured in infant formulas are well below concentrations known to cause methemoglobinemia, so we can assume that there is a wide range of safe nitrite and nitrate concentrations in the nutritional sources of newborn infants used today. Moreover, infants exposed to nitrate intakes as high as 700 mg/day maintained nontoxic MetHb levels (43) and even when MetHb levels are artificially inflated in newborn cord blood, there is sufficient MetHb reductase activity such that the half-life of MetHb is only ~3.5 hours (44).

Clinical Implications

The current findings inform speculation on the relationship between ingested nitrate and nitrite and the occurrence of newborn necrotizing enterocolitis (NEC), a disease that is characterized by a combination of decreased gastrointestinal blood flow, breakdown of the mucus barrier lining the lumen of the gut, and invasion by pathogenic bacteria (45). Nitric oxide, which can be derived from swallowed nitrite, has been shown to counteract all three of these factors in adult animal models (13,14,46,47). It is clear that the dietary intake of nitrite and nitrate is significantly lower in infants compared to adults, whether an infant is receiving fresh breast milk, freeze-thawed breast milk, infant formula, or PN. We demonstrate in chapter three that the endogenous reduction of nitrate

to nitrite is markedly diminished in the newborn infants due to a lack of oral nitrate-reducing bacteria and significantly reduced saliva production (15). As a result, the impact of adding nitrate to the diet may be limited. Alternatively, addition of nitrite may provide an important source of NO to support the integrity of the gut lining by promoting enteric blood flow and mucus production. Although our results do not support the recent postulate that formula-fed infants are predisposed to NEC because of deficient nitrate or nitrite intake (48) since the concentrations in most formulas for preterm infants we measured were well above those of breast milk, given the protective effects of nitrite in the gastrointestinal tract, it is reasonable to propose that a deficiency of circulating nitrite could potentially predispose infants to NEC. We explore this hypothesis further in our work discussed in chapter four.

One possible consequence of low plasma nitrite concentrations would be a diminished capacity of this anion to function as a reservoir of NO bioactivity, a pathway thought to be important in protecting tissues against ischemic stress (2-7) (also see recent review by Weitzberg and Lundberg (50)). Animal studies from multiple laboratories, species, and disease models now support the idea that increasing plasma nitrite levels, in some cases by as little as 2- to 3-fold, may protect against hypoxic/ischemic stress (see review by Dezfulian et al (49)). Interestingly, plasma nitrite concentrations of healthy term newborns are ~30% lower than those of adults and increase by ~2-fold with no known adverse effects during administration of inhaled NO (20 ppm) to infants with pulmonary hypertension (16). However, there are not yet any clinical reports on the therapeutic effects or safety of exogenous nitrite to treat ischemia/reperfusion injury, and although it may have therapeutic possibilities, there are also inherent risks to consider.

For example, in adults, ingestion of nitrate results in a significant decrease in arterial blood pressure, (10) which would be undesirable in the care of most premature infants. In addition, MetHb production is of particular concern in neonates as they possess low levels of MetHb reductase activity, (44) and compared to adults, fetal hemoglobin is more rapidly oxidized to methemoglobin by reaction with nitrite (50). Thus, the possibility of methemoglobinemia cannot be ignored.

In summary, infants in neonatal intensive care units ingest markedly lower levels of nitrate and nitrite than adults on a per kg basis, possibly contributing to lower circulating concentrations in the plasma. The naturally occurring enzyme lactoperoxidase is likely a primary factor in the oxidation of nitrite to nitrate during the handling and storage of breast milk and is likely responsible for the ~65% fall in nitrite concentrations that occurs through freeze-thawing, potentially exacerbating the effects of the little dietary nitrite made available to infants. Whether this limited ingestion puts the newborn infant population at risk for gastrointestinal and cardiovascular diseases or whether supplementation of these anions in the diet would be beneficial on one hand or safe on the other calls for future study.

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CHAPTER THREE

NITRATE REDUCTASE ACTIVITY OF BACTERIA IN SALIVA OF TERM
AND PRETERM INFANTS

Abstract

The salivary glands of adults concentrate nitrate from plasma into saliva where it is converted to nitrite by bacterial nitrate reductases. Nitrite can play a beneficial role in adult gastrointestinal and cardiovascular physiology. When nitrite is swallowed, some of it is converted to NO in the stomach and may then exert protective effects in the gastrointestinal tract and throughout the body. It has yet to be determined either when newborn infants acquire oral nitrate reducing bacteria or what the effects of antimicrobial therapy or premature birth may be on the bacterial processing of nitrate to nitrite. We measured nitrate and nitrite levels in the saliva of adults and both preterm and term human infants in the early weeks of life. We also measured oral bacterial reductase activity in the saliva of both infants and adults, and characterized the species of nitrate reducing bacteria present. Oral bacterial conversion of nitrate to nitrite in infants was either undetectable or markedly lower than the conversion rates of adults. No measurable reductase activity was found in infants within the first two weeks of life, despite the presence of oral nitrate reducing bacteria such as *Actinomyces odontolyticus*, *Veillonella atypica*, and *Rothia mucilaginosa*. We conclude that relatively little nitrite reaches the infant gastrointestinal tract due to the lack of oral bacterial nitrate reductase activity. Given the importance of the nitrate–nitrite–NO axis in adults, the lack of oral nitrate-

reducing bacteria in infants may be relevant to the vulnerability of newborns to hypoxic stress and gastrointestinal tract pathologies.

Introduction

Until recently nitrite and nitrate were thought to be biologically inert at physiological concentrations. However, evidence now indicates that these anions play a significant role in cardiovascular homeostasis, and in responses to hypoxic and ischemic stress (See review by Lundberg et al. (1)). Nitrite has been found to provide protective effects in various animal models of ischemia–reperfusion (2–5). Clinical trials are currently evaluating the safety and efficacy of nitrite in pulmonary hypertension and myocardial infarction. While the mechanism underlying the protective effects of nitrite is not yet well understood, a common hypothesis posits that nitrite is reduced to NO, a reaction that can either occur spontaneously under acidic conditions such as those found in the stomach (6), or which may be catalyzed by a number of metal-containing proteins (7). Irrespective of the mechanism, there is growing consensus that nitrite is cytoprotective during ischemia–reperfusion insult which has lead to an interest in the factors that determine plasma nitrite concentrations.

Nitrate, which is inert in mammalian tissues, is converted to nitrite by bacteria normally found on the dorsal surface of the adult tongue (1, 8). These microbes act upon nitrate that is concentrated in the saliva by the salivary glands or ingested in the diet. Dietary nitrate primarily derives from vegetables (9) and from breast milk and formula, albeit in significantly lower levels, in the diet of newborns (10). These microbes also act on nitrate that is actively transported from plasma into the saliva by the salivary glands

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(9). The resulting salivary nitrite is swallowed, and can then be converted to NO by nonenzymatic disproportionation in the acidic environment of the stomach (11, 12). It can also enter the circulation where it may serve as a long-term reservoir of NO-bioactivity (1). Increasing dietary nitrate lowers blood pressure (13, 14), improves exercise performance (1, 15, 16), and increases plasma nitrite concentrations (14, 17). Disruption of the salivary nitrate–nitrite–NO pathway in adults by not swallowing saliva prevents the fall in blood pressure associated with ingestion of nitrate (13). Thus, there is strong evidence that nitrate-reducing oral bacteria play an important role in determining plasma nitrite concentrations and the cardiovascular homeostasis of adults.

The bacterial colonization of the mouth and gastrointestinal tract of newborn infants begins at birth and progresses over the first few weeks of life (18). This progression is almost certainly altered in an intensive care setting where sanitary practices and the use of antibiotics may diminish oral bacterial quantity and nitrate reducing capacity. The appearance of nitrate-reducing bacteria in the mouths of newborn infants, whether in outpatient or intensive care settings, had not yet been characterized.

Our previous work has shown that plasma nitrite concentrations are lower in newborn infants in the neonatal intensive care unit compared to adults (19). We postulate that this is in part due to low oral nitrate reductase activity in newborn infants. The current studies were designed to measure the activity of nitrate reducing bacteria in saliva, to characterize the type of oral nitrate reducing bacteria in the mouth of newborn infants, and to compare newborn and adult salivary nitrite and nitrate concentrations. We also tested the hypothesis that elimination of oral nitrate-reducing bacteria leads to decreases in circulating nitrite in adults. Given the importance of the nitrate–nitrite–NO

axis in adults, the appearance and activity of oral nitrate-reducing bacteria in infants may contribute to reduced plasma nitrite levels and may be a significant factor in the course of development.

Methods

All experimental protocols were approved by the Institutional Review Board of Loma Linda University. Newborn study subjects were recruited from the neonatal intensive care unit, well-baby nursery, and pediatric outpatient clinic of Loma Linda University Children's Hospital. Infants were considered to be term if born after 36 weeks gestation, and to be preterm if born before 35 weeks gestation; they were excluded from the study if they were born at 35 or 36 weeks gestation. In addition, infants with congenital malformations were excluded from the study. The infants' gestational age, birth weight, type of feeding, and antibiotic therapy were recorded. Adult subjects were healthy males and females who, ranged from 24 to 72 years of age, had not used antiseptic mouthwash within 24 hours, and had not received antibiotic treatment within two weeks prior to the study. Separate cohorts of adults were studied for the salivary nitrate and nitrite concentrations and nitrate reductase activity portions of the study.

Salivary Nitrite and Nitrate Concentrations

To determine baseline salivary nitrite and nitrate concentrations, samples were collected from infants and adults by gently rotating a sterile cotton swab (Kendall Q-Tips, Tyco Healthcare Group, Mansfield, MA) against the sublingual posterior aspect of the tongue for 90 seconds. Samples were collected at least one hour following meals. The cotton end of the swab was removed and immediately transferred to 500 µL of water. The

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weight of the cotton swab was recorded to the nearest milligram before and after placement in the mouth to determine the amount of saliva collected. The amount of nitrite and nitrate collected in the swab was determined by comparison with standard curves generated from known nitrite or nitrate concentrations in 200 μL of water soaked into swabs and prepared on each day samples were assayed. Both nitrite and nitrate standards resulted in a linear relationship between the nominal and measured concentrations (nitrite $R^2 = 0.98$, nitrate $R^2 = 0.87$). Nitrite levels were consistently found to be below the lower limit of assay detection (10 nM) in samples of dry swabs, demonstrating no background contamination. There was measurable background contamination of nitrate (60.32 ± 11.61 μM) in the dry swabs, which was subtracted from the raw nitrate levels by the use of the standard curve.

Salivary Nitrate Reductase Activity

Salivary samples were collected by rotating a sterile cotton swab in a single 360-degree twisting motion against the dorsum of the tongue. The volume of saliva collected was determined via the weight gain of the swab. The swab tip was immediately transferred to 3.0 ml of sterile anaerobic BHI broth (BD bacto™ brain heart infusion; BD Bacto™ yeast extract, Becton Dickinson, Franklin Lakes, New Jersey; L-cysteine hydrochloride monohydrate, Fisher Scientific, Pittsburg PA; Hemin; Menadione; Sigma, St. Louis MO) and then placed in a water bath held at 37 C. Within ten minutes of placing the swab in broth, a baseline nitrite measurement was recorded and then 10 mM nitrate was added (final concentration 333 μM) to provide substrate for the bacterial conversion to nitrite. Nitrite concentrations were then measured at five-minute intervals

beginning after the first collection at one minute, and continuing for 30 min. The nitrate

reductase activity was calculated from the slope of a linear regression fit to the plot of

increasing nitrite concentrations over time, with the result providing nanomoles of nitrite

production per minute per milligram of saliva collected. We verified this method of

measuring oral nitrate reductase activity by treating four adult subjects with six antiseptic

mouth rinse treatments consisting of 0.12% chlorhexidine solution (Peridex, 3 M ESPE

Dental, St. Paul, MN) over a period of three days (12-hour intervals). Baseline activities

were recorded before the mouth rinse treatment, and immediately following the last

mouth rinse treatment, demonstrating that the subjects no longer had any measurable

activity (Figure 13). Subsequent measurements at 8, 24, and 48 h after the last treatment

indicated a return to baseline levels of nitrate reductase activity within 48 h.
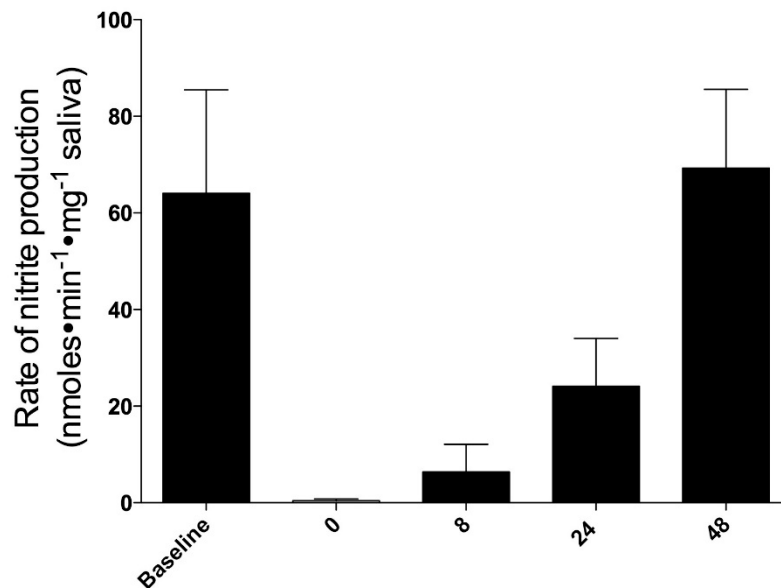
Figure 13. Effect of antiseptic mouthwash on the assay for oral nitrate-reducing bacteria. Following six treatments with antimicrobial mouth rinse at twelve hour intervals, oral nitrate reductase activity in four adult subjects was decreased to unmeasurable levels compared to baseline values. Nitrate reductase activity returned to normal within 48 h following the last antimicrobial mouth rinse treatment.

To verify that nitrate was being metabolized in the broth as nitrite concentrations were increasing, we also measured the rate of nitrate disappearance using swab samples from five adult subjects. Over the course of the 30-min experiment, the mean rate of nitrate disappearance was 103 ± 20 nmol$\cdot$min$^{-1}\cdot$mg^{-1} of saliva, while the mean rate of nitrite production was 47 ± 17 nmol$\cdot$min$^{-1}\cdot$mg^{-1} (Figure 14).
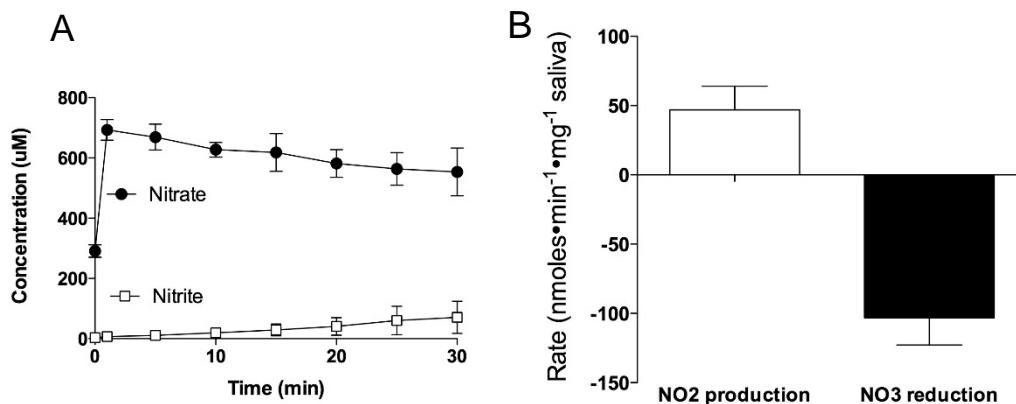
Figure 14. A) Time course of nitrite production and nitrate reduction by nitrate-reducing bacteria cultured from saliva samples of five adults. B) Summary of kinetic results based on average rates of change of nitrite and nitrate concentrations with time.

Effect of Antimicrobial Mouth Rinse on Blood and Saliva Nitrite and Nitrate Concentrations in Adults

Twenty-four normal healthy adults received six mouth rinse treatments over a period of three days (at 12-h intervals) with either 0.12% chlorhexidine solution (n = 12) or saline solution (n = 12) as placebo control. Saliva and venous blood samples were collected just prior to the first mouth rinse, and then again one to two hours following the final mouth rinse. Blood was immediately added to a nitrite preservation solution (4:1 v/v) (20), deproteinized by methanol precipitation (1:1 v/v), and stored at 70 C until

assay. Approximately 5 ml of saliva was collected by expectoration of passively secreted saliva into a 50 ml tube, which was immediately frozen until assay at a later date. Subjects were asked to refrain from eating high-nitrate foods (e.g. beets, radishes, hotdogs, and leafy greens) from 24 h prior to the first sample until after the collection of the final sample.

Salivary Bacterial Analyses

To assess for the presence of bacteria capable of nitrate reductase activity, PCR analysis and real-time PCR was performed on bacterial DNA isolated from saliva collected from healthy infants in the pediatric outpatient clinic, and from preterm infants in the NICU and from healthy adults after six chlorhexidine treatments. We chose to test for the presence of the four most prevalent nitrate reducing bacterial species in the adult mouth (21): *Veillonella atypica, Actinomyces odontolyticus, Rothia mucilaginosa*, and *Staphylococcus epidermidis*. Saliva was collected using a Vacutainer™ anaerobic specimen collection vial (Becton–Dickinson, Sparks, Maryland) by rotating a sterile anaerobic specimen collector swab in one 360-degree rotation against the dorsum of the tongue. Swabs were stored in an anaerobic specimen collector for up to 48 h at room temperature after which they were placed in 5.0 ml of sterile anaerobic brain heart infusion (BHI) broth. The swabs were then incubated overnight at 37 C in an anaerobic chamber (0% O_2, 99.8% N_2, 0.2% H_2), until the broth had reached an optical density of 0.8–1.0 (600 nm). Bacterial DNA was extracted using a Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI) following the manufacture's protocol for isolating genomic DNA from gram positive bacteria. For efficient lysis, 60

µL of 10 mg/ml lysozyme and 60 µL of 10 mg/ml lysostaphin (Sigma–Aldrich, St. Louis, MO) were added to each sample. The DNA was resuspended in 100 µL of rehydration solution, quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE) and then stored at 70 C.

PCR Analysis

Qualitative PCR was performed on the DNA samples initially to confirm the accuracy of the primers used for the four bacterial species of interest, and to confirm the presence of target bacterial genes in the DNA samples. The primers for PCR analysis are listed in Table 1 and were designed using the NCBI primer select program. The reaction mixture (50 µL) contained 1 µg of template DNA in the high fidelity PCR master mix (Invitrogen, Carlsbad, CA). PCR cycling conditions were 1 cycle of 5 min at 94 C followed by 30 cycles of 30 s at 94 C, 30 s at 54 C and 1 min at 68 C with final extension of 5 min at 68 C.

Real-time PCR analysis

Real time PCR was carried out on 1 µg aliquots of purified DNA using a Smart cycler II (Cepheid, Sunnyvale CA). The primers for the real time analysis are listed in Table 2. The amplification efficiency of each primer set was determined empirically by using DNA template dilutions over four orders of magnitude. The amplification efficiency for each primer set varied between 95.1% and 102.5%, showing that the amplicons were generated with comparable efficiency. The specificity of the amplification was ascertained based on the melting peak generated during each run.

Table 2. Primer sequences used for bacterial PCR analysis.

Bacterial species	Target gene primer set
1. *Veillonella atypica*	Forward primer- 5' – GTGCTGCAGAGAGTTTGATCCTGGCTCAG -3' Reverse primer-5'- CACGGATCCTACGGGTACCTTGTTACGACTT -3'
2. *Rothia mucilaginosa*	Forward primer-5'-AGCCTCAGGG ATTGATGGGTTCTT-3' Reverse primer- 5'-TTCTGGTGGTT GTACAGGGCGTTA-3'
3. *Actinomyces odontolyticus*	Forward primer- 5'- GCGGATTAATT CGATGCAACGCGA-3' Reverse primer- 5' CATTGTAGCAT GCGTGAAGCCCAA-3'
4. *Staphylococcus epidermidis*	Forward primer- 5'-CTGCCTTTCAA TGCGAGTTGGCTT-3' Reverse primer-5'-ACAGCTAAACT TGCAGCATGTGGG-3'

The real time-PCR reaction contained 12.5 μL of QuantiTect SYBR Green qPCR master

mix (Qiagen, Valencia, CA), 0.2 μM of each gene-specific primer and 1 μL of DNA

template. The cycling conditions were 50 C for 2 min, 95 C for 2 min, then 40 cycles of

94 C for 15 s, 58 C for 30 s, and 72 C for 30 s. Distilled water was used as a negative

control in each run. All reactions were carried out in triplicate. A standard curve was

generated using dilution of the DNA from each species of bacteria plotted against the

cycle threshold value (Ct). The concentration of DNA was converted to copy number

using the formula $n = N_A – m/M_W – L$ where n = the number of target sequence copies

per microliter, N_A = Avagadro's constant (mol^{-1}), m = mass of the amplicon per

microliter, M_W = mean molecular weight of 1 base pair, and L = amplicon length in base

pairs. The Ct values obtained were plotted against DNA concentration to generate

standard curves from which the cutoff Ct for each bacterial species was determined. The

cutoff Ct value, above which the absence of the bacteria is indicated, was 43 for

Veillonella atypica, 49 for *Rothia mucilaginosa*, 39 for *Actinomyces odontolyticus*, and 47 for *Staphylococcus epidermidis*.

Nitrite and Nitrate Assays

Nitrite concentrations were measured by triiodide chemiluminescence as described by Pelletier et al. (20), enabling quantification above 10 nM with a precision of ±5 nM. Nitrate concentrations were measured by incubation of 205 μL of sample with 5 μL nitrate reductase enzyme (10 U/ml solvent, Roche, Indianapolis IN) in 10 μL of 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer solution (Fisher Scientific, Pittsburgh PA), 10 μL of 0.1 mM flavin adenine dinucleotide disodium salt hydrate (Sigma Aldrich, St. Louis, MO), and 20 μL of 1 mM nicotinamide adenine dinucleotide phosphate-oxidase tetrasodium salt (Roche, Indianapolis, IN) at 37 C for 45 min to convert all the nitrate to nitrite, which was then measured by triiodide chemiluminescence. Whole blood nitrate concentrations were measured by chemiluminescence assay following reduction of nitrate to NO in a purge vessel containing vanadium III and HCl at 90 C.

Data Analysis

Data are presented as means ± standard error. Differences between study groups were detected using Student's t-test for two group comparisons and one-way ANOVA followed by Bonferroni post hoc analysis for comparison of three or more datasets. Oneway ANOVA with repeated measures was also used to detect significant changes from baseline measurements in time-course experiments. Two-way ANOVA followed by

Bonferroni post hoc analysis was used to detect significant differences between groups in time-course experiments. Statistical analysis was performed using Prism 5 for Mac OS X (Graphpad Software, Inc, La Jolla, CA).

Results

Nitrite and Nitrate Concentrations in Saliva

Nitrite and nitrate concentrations were measured in saliva collected from ten infants and twelve healthy adults. The results are shown in Figure 15 and participant demographics are provided in Table 3.

Table 3. Saliva study infant participant demographics.

	Infants
Participants, n	10
Gestational age, weeks	39 ± 0.3
Birth weight, grams	3662 ± 170
Age at time of collection, days	3.1 ± 0.3
Infants receiving antibiotics, n	0
Breast fed, n	7
Formula and breast milk fed, n	3

Nitrite concentrations in infant saliva averaged 8.2 ± 5.6 µM. These concentrations were significantly lower than adult salivary nitrite concentrations which averaged 50 ± 13 µM (p = 0.0501) (Figure 15A). Nitrate concentrations were measured in saliva collected from ten infants and fourteen adults and averaged 284 ± 83 µM in the saliva of infants and were not measurably different than those found in adults (681 ± 184, p = 0.11) (Figure 15B). The newborn salivary nitrate concentrations were approximately ten-fold greater

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than previously reported newborn plasma nitrate levels (19,22,23), demonstrating that

salivary glands are able to concentrate nitrate from the plasma into the saliva of infants,

and to levels comparable to adults. Notably, the amount of saliva obtained during the

timed 90-second collection periods from infants (39 ± 8 µL) was less than the amount

collected from adults (143 ± 15 µL) ($p < 0.001$).
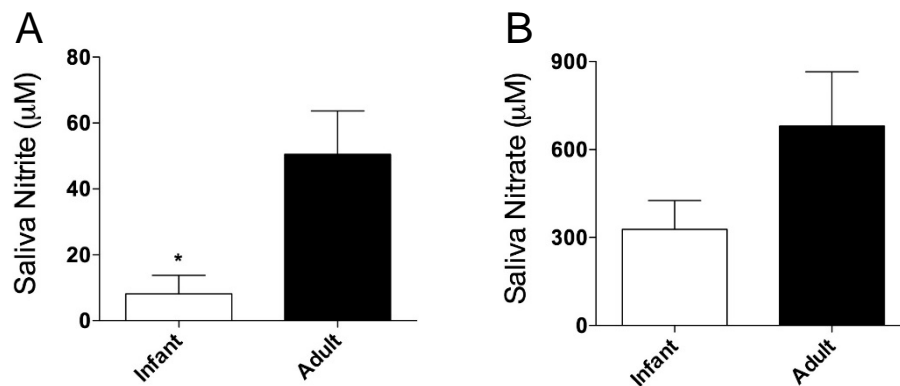
Figure 15. Nitrite and nitrate concentrations in saliva of infants and adults. (A) Nitrite concentrations in the saliva of infants (8 ± 5 µM) were less than adult saliva (55 ± 22 µM, *= $p < 0.01$). (B) Salivary nitrate levels were not significantly lower in the saliva of infants (328 ± 97 µM) than in the saliva of adults (538 ± 125 µM).

Conversion of Nitrate to Nitrite in Saliva

Oral bacterial nitrate-reducing activity was measured in fresh oral swabs

immediately after placing them in culture media. Swabs were obtained from 25 infants

less than five days of age, from 19 infants between 14 and 40 days of age, from 9 infants

approximately two months old, and from 13 healthy adults. The demographics of the

infant subjects are shown in Table 4.

Table 4. Saliva nitrate reductase study infant participant demographics.

	NICU preterm on antibiotics	NICU term on antibiotics	NICU preterm	Outpatient term	NICU term >2 weeks old	NICU preterm > 2 weeks old	Outpatient > 2 weeks old	Outpatient 2 months
Participants, n	7	6	5	7	5	8	6	9
Gestational age, weeks	32.1 ± 0.7	38.6 ± 0.5	32.6 ± 0.7	39.2 ± 0.5	38.4 ± 0.5	30.8 ± 1.1	38.9 ± 0.7	39.5 ± 0.3
Birth weight, grams	1495 ± 201	3322 ± 86	1964 ± 124	3391 ± 140	3350 ± 235	1581 ± 174	3199 ± 133	3425 ± 127
Age at time of collection, days	3 ± 0.4	4.2 ± 0.3	4.6 ± 0.2	3.7 ± 0.4	26.6 ± 5.6	27.3 ± 2.9	17.2 ± 1.7	64.6 ± 1.1
Breast fed, n	0	2	0	3	1	0	6	7
Nipple/gavage breast milk fed, n	3	3	2	2	1	7	0	0
Formula fed only, n	0	0	2	2	3	1	0	2
Intravenous feeding, n	4	1	1	0	0	0	0	0

Swabs collected from infants contained similar amounts of saliva as those collected from adults (17 ± 1 vs. 17 ± 3 mg, respectively, p = 0.9). The rate of nitrite production in the cultures, normalized to the weight of the saliva collected, was linear during the 30 min sampling period, as shown in Figure 16A, with the slope thus providing an index of bacterial nitrate reductase activity. In samples taken from adults nitrite was produced at a rate of 147 ± 36 nmoles•min^{-1}•mg^{-1}. In marked contrast, however, saliva cultures from newborns showed little or no detectable nitrite production.

In the cultures collected from preterm (n = 6) and term (n = 6) infants on antibiotics, the rates of nitrite production were not significantly different than zero (0.05 $\pm$ 0.04 nmoles•min^{-1}•mg^{-1} saliva and 0.04 $\pm$ 0.11 nmoles•min^{-1}•mg^{-1} saliva, respectively). In addition, there was no appreciable nitrite production in the salivary cultures taken from preterm infants in the NICU not receiving antibiotics, or from healthy term infants less than two weeks of age in the well-baby nursery and outpatient clinic (4 $\pm$ 4 nmoles•min^{-1}•mg^{-1} saliva and 5 $\pm$ 3 nmoles•min^{-1}•mg^{-1} saliva, respectively). Nitrite production was detectable in saliva samples collected from healthy infants between 14

and 40 days of age in the outpatient clinic (22 ± 15 nmoles•min^{-1}•mg^{-1}) as well as term

(23 ± 12 nmoles•min^{-1}•mg^{-1}) and preterm infants in the NICU (28 ± 19 nmoles•min^{-1}•mg^{-1}), although this activity was still measurably less than that of the adults ($p < 0.01$).

Even by two months of age, the rate of nitrite production (23 ± 12 nmoles•min^{-1}•mg^{-1})

was significantly lower than that of adults ($p < 0.01$, Figure 16B).
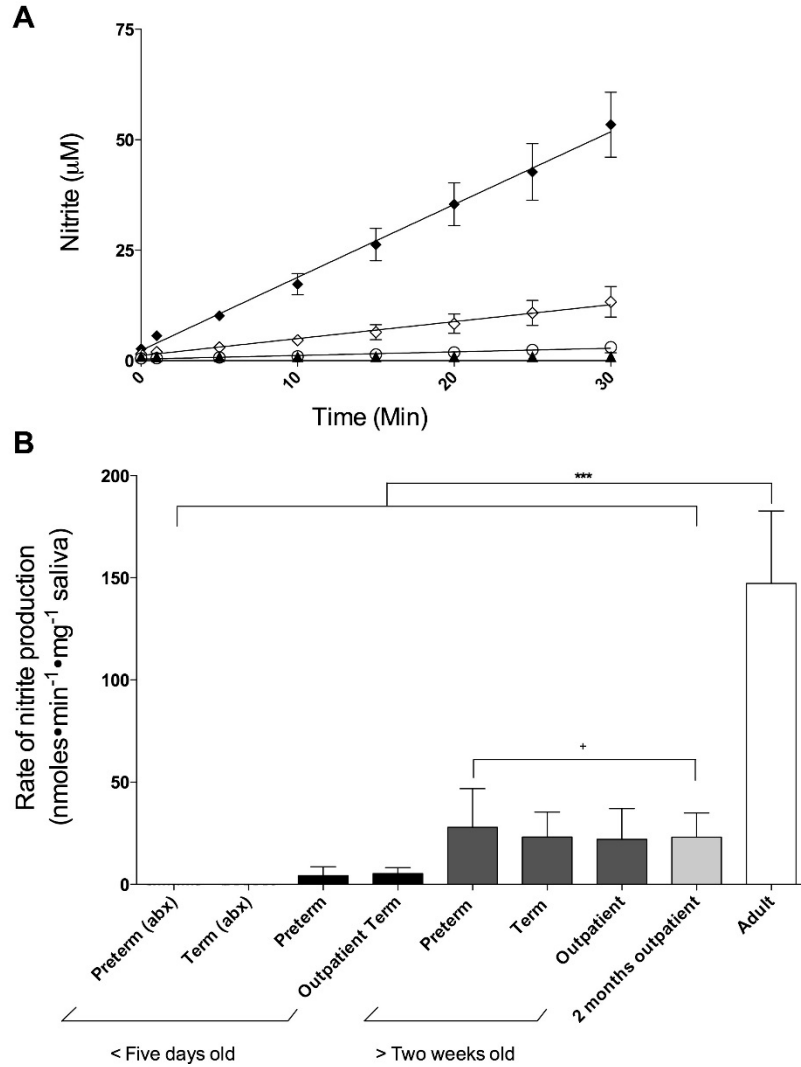
Figure 16. A) Time course of nitrite production by nitrate-reducing bacteria cultured from saliva samples. After introduction of nitrate substrate into cultures, nitrite was measured in samples from adults (♦), infants greater than two weeks of age (◊), newborn infants less than five days old (○), and newborn infants less than five days old receiving antibiotics (▲). B) Summary of kinetic results based on average rates of change of nitrite concentration with time, as shown in A. There was no significant nitrite production in the saliva of infants <5 days old, regardless of whether they were on antibiotics (abx). There was significant nitrite production in the infants older than two weeks (+ = p<0.01), and nitrite production was significantly greater in the adult saliva compared to infants (*** = p<0.001).

PCR Detection of Nitrate Reducing Bacteria

To investigate the possibility that the lack of nitrate reducing activity in the samples collected from infants was due to the absence of nitrate-reducing bacteria, PCR detection was performed on samples collected from five healthy term infants in the outpatient clinic and seven preterm infants in the NICU, who were all less than five days of age. The specificity of the amplification of target genes was confirmed by conventional PCR analysis (data not shown). The quantitative PCR demonstrated the presence of *Veillonella atypica* and *Rothia mucilaginosa* in all the infants studied. *Actinomyces odontolyticus* was present in four of the five term infants and five of the seven preterm infants, and *Staphylococcus epidermidis* was present in two of the five term infants and all of the preterm infants studied (Figure 17). These values suggest that the most abundant oral nitrate reducing bacteria in adults are also present in the mouths of neonates within a few days after birth. Thus, the lack of nitrate reductase activity in infants is more likely to be due to low abundance or activity of these bacteria as opposed to lack of inoculation. To assess this possibility further, PCR was also performed on eight adult subjects after six treatments with chlorhexidine, which lowers oral nitrate reductase activity by our assay to levels comparable to those of infants (see above). We found detectable presence of all four bacterial species in all adults studied, with the exception of one adult in whom *Actinomyces odontolyticus* was not detectable, suggesting that although chlorhexidine significantly decreases overall oral nitrate reducing activity, it does not completely eliminate the presence of nitrate reducing bacteria.
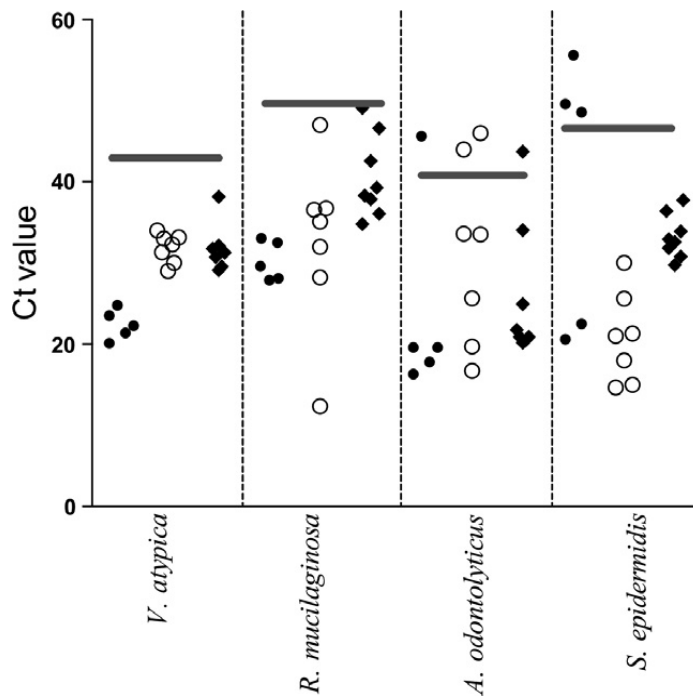
Figure 17. Real time PCR detection of nitrate-reducing bacteria. The presence of *Veillonella atypica, Rothia mucilaginosa, Actinomyces odontolyticus,* and *Staphylococcus epidermidis* in bacterial cultures collected from the mouths of term (●) and preterm (○) infants and adults (◆) was confirmed using primers specific to the respective bacteria. Horizontal gray bars represent the cutoff Ct value, below which the presence of the bacteria is confirmed. The data indicate the presence of most of these nitrate reducing bacteria in the infant mouth within the first few days of life.

Effect of Antimicrobial Mouth Rinse on Blood and Saliva Nitrite and Nitrate

Concentrations in Adults

Changes in blood nitrite were examined in 24 adult subjects after three days of treatment with either anti-microbial mouth rinse or placebo. Mean blood nitrite concentration decreased by approximately 20% from 0.27 ± 0.01 μM to 0.22 ± 0.01 μM following chlorhexidine treatment ($p < 0.05$), but was not affected by the placebo. As shown in Figure 18, blood nitrite concentrations decreased in 9 out of 12 subjects

following chlorhexidine mouth rinse treatments, compared to only 5 out of 12 subjects

with placebo mouth rinses. There were no significant differences in the blood nitrate

concentrations of the study subjects before or after treatment with chlorhexidine (41 ± 8

vs. 48 ± 7 µM) or placebo (35 ± 4 vs. 32 ± 6 µM, data not shown). Saliva nitrite

concentrations fell from 322 ± 70 to 109 ± 35 µM ($p < 0.01$) following three days of

antimicrobial mouthwash, and were unchanged (246 ± 54 vs. 284 ± 45 µM) following

treatment with placebo. Saliva nitrate concentrations increased from 189 ± 39 to $1083 \pm$

268 µM ($p < 0.01$) following the antimicrobial mouthwash, and were again unchanged

(118 ± 40 vs. 141 ± 37 µM) following treatment with placebo.
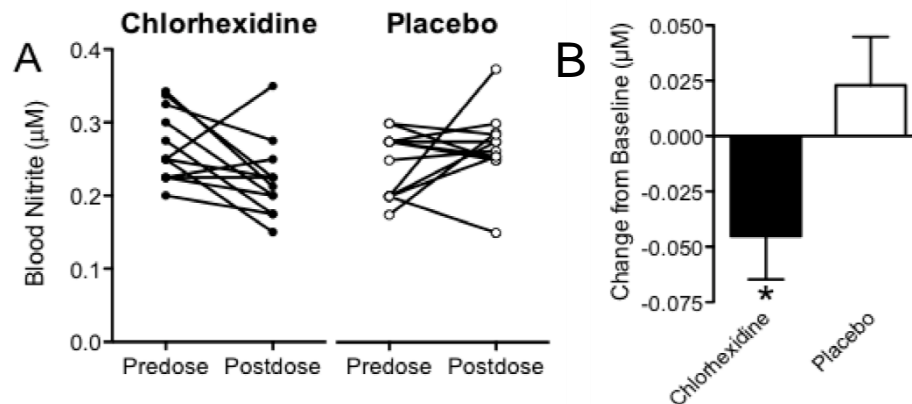
Figure 18. Effect of antiseptic mouth rinse on adult blood nitrite concentrations. Blood nitrite concentrations in healthy adult volunteers were significantly decreased by ~19% compared to baseline levels following six treatments with antiseptic mouth rinse (chlorhexidine) at 12-hour intervals (*=$p < 0.04$). No significant change was observed in a parallel group of placebo (saline) control subjects.

Discussion

The current studies demonstrate that bacterial nitrate reductase activity in the mouth of neonates is minimal compared to that of adults. The low nitrate-reducing activity in the newborn persists for at least the first two months of life, and results in significantly lower salivary nitrite concentrations. In spite of this reduced activity, PCR evidence shows that newborn mouths do contain the major nitrate-reducing bacteria found in adults. This suggests that the diminished nitrate-reducing activity of newborns may be due to lower numbers of these bacteria, as opposed to a lack of inoculation. Thus, this study shows that oral nitrite production, which plays a prominent role in cardiovascular and gastrointestinal homeostasis of adults, has less capacity to function in neonates during the first few weeks of life.

Impact of Oral Bacteria on Nitrite Ingestion

Normally, adults swallow on average 600 ml of saliva per day (24). To our knowledge, the quantification of saliva production or ingestion in newborns has not yet been accomplished due to obvious technical challenges. However, the newborn mouth is relatively dry compared to the adult mouth, as is reflected by significantly lower volumes of saliva collected by the oral swabs in the current study. In adults, even when nitrate-reducing bacteria are present, not swallowing saliva effectively blocks the physiological effects of dietary nitrate (13). Thus, it is reasonable to hypothesize that the relatively low rate of saliva production in newborns, coupled with a low concentrations of nitrite in the

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saliva and diet, has a compounded impact upon overall nitrite ingestion by newborn infants resulting in significantly decreased nitrite intake compared to adults.

Linkage between Oral Bacterial Activity and Plasma Nitrite

We have recently observed that the blood nitrite concentration of newborn infants (1.4 ± 0.5 days of age) is approximately 35–55% lower than that of adults (19). The current studies found that a blockade of oral bacterial nitrate reduction activity in adults resulted in a 20% reduction in blood nitrite concentrations, demonstrating that oral nitrate reductase activity makes a significant contribution to basal plasma nitrite concentrations. This finding is consistent with experiments in which increases in plasma nitrite, following nitrate ingestion, have been shown to require the activity of oral bacteria (13, 25). Notably, plasma nitrite concentrations are also heavily influenced by NO derived from endothelial nitric oxide synthase (eNOS) activity (26, 27). However, the rate of eNOS activity in newborns, relative to adults, has not been determined.

Oral Bacteria

A number of specific nitrate-reducing bacteria, most of them facultative anaerobes, have been identified in the oral cavity of adult humans (21, 28). Previous studies have reported the presence of *Veillonella* and *Actinomyces spp.* (29, 30) in infants. To our knowledge, however, our work is the first to report both the presence of nitrate reducing bacteria and measurement of oral nitrate reducing activity. The significantly lower nitrate reductase activity in newborns, despite evidence of their mouths having been colonized by these bacteria, may be due to relatively low bacterial abundance at this age, a factor not measured in the current study. This idea is supported by our observation

of the presence of these bacteria in the mouths of adults following chlorhexidine treatment, concomitant with a lack of measurable nitrate reductase activity. The significantly lower bacterial nitrate reductase activity observed in infants in both an intensive care setting and the home environment, regardless of whether the infants were receiving antibiotics, serves as further evidence that the low nitrate reducing activity of newborns was not due to a delayed inoculation of these infants with nitrate-reducing bacteria.

Study Limitations

We observed that salivary nitrite concentrations in adult saliva collected with cotton swabs (55 ± 22 μM) were consistently lower than concentrations measured in saliva collected by expectoration (284 ± 63 μM) in addition to being lower than most previously reported salivary nitrite concentrations which range from 90 to >670 μM. Standard curves generated by dipping swabs in small amounts of water with varying concentrations of nitrite were highly linear with significant slope. However, we observed that nitrite concentrations measured in ten expectorated adult saliva samples were significantly higher than concentrations measured from samples collected at the same time using swabs (131 ± 32 vs. 45 ± 14 μM, $p = 0.024$). This suggests that nitrite in saliva may bind to the swab itself or to other components of saliva that bind to the swab, resulting in an artifact of low absolute nitrite concentrations. Nevertheless, the measurement of nitrite in saliva collected in the swabs provides a relative comparison of newborn and adult concentrations where collection of expectorated samples from infants is not possible. Improved methods of neonatal saliva collection in the future may lead to

more precise determinations of absolute concentrations in neonatal saliva. It is also important to note that although our findings indicate that nitrate reducing activity is markedly less in the mouths of infants compared to adults despite the PCR detection of nitrate reducing bacteria, the PCR results cannot be used as a quantifiable comparison of bacterial abundance in infant and adult mouths due to the method of sample collection and overnight culture prior to DNA isolation.

Clinical Perspective

Hypoxia and ischemia play key roles in several diseases of the newborn period. Because nitrite is protective against hypoxic and ischemic insults, questions arise as to the potential role of nitrite supplementation of the newborn infant. Such treatment may be particularly beneficial to premature infants who require prolonged periods of intubation and mechanical ventilation which has been demonstrated in adults to result in significant depletion of intragastric NO, presumably due to marked decreases in saliva production and swallowing (31). Necrotizing enterocolitis, the most common gastrointestinal disorder to affect premature infants, likely results from a combination of decreased gastrointestinal blood flow, breakdown of the mucus barrier lining the lumen of the gut, and invasion by pathogenic bacteria (32). Intragastric nitric oxide derived from non-enzymatic disproportionation of swallowed salivary nitrite counteracts all three of these factors in adult animal models (31, 33, 34). However, the gastric pH of newborn infants is relatively high compared to adults (35, 36), leading to diminished NO production, which may hinder its gastroprotective effects and compound the effects of low levels of nitrite ingestion in newborn infants.

Premature infants are also at significant risk of suffering intraventricular hemorrhage and episodes of inadequate systemic oxygenation. Circulating nitrite provides protection against cerebral vasospasm following subarachnoid hemorrhage in baboons (37), and can also increase cerebral blood flow (38) and decrease oxygen consumption during hypoxic stress (39). During reperfusion, nitrite is found to protect against oxidative stress and to reduce the generation of harmful reactive oxygen species (40). Whether these protective effects of nitrite are diminished in neonates due to the attenuation of enterosalivary nitrate–nitrite–NO metabolism calls for future study.

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CHAPTER FOUR

CHANGES IN PLASMA AND URINARY NITRITE AFTER BIRTH IN PREMATURE INFANTS AT RISK FOR NECROTIZING ENTEROCOLITIS

Abstract

Plasma nitrite serves as a reservoir of NO bioactivity. Because nitrite ingestion is markedly lower in newborns than adults, we hypothesized plasma nitrite levels would be lower in newborns than in adults, and that infants diagnosed with NEC, a disease characterized by ischemia and bacterial invasion of intestinal walls, would have lower levels of circulating nitrite in the days prior to diagnosis. Single blood and urine samples were collected from nine term infants and twelve adults, 72 preterm infants every five days for three weeks, and from 13 lambs before and after cord occlusion. Nitrite fell 50% relative to cord levels in the first day after birth; and within 15 min after cord occlusion in lambs. Urinary nitrite was higher in infants than adults. Plasma and urinary nitrite levels in infants who developed NEC were similar to those of preterm control infants on Days 1 and 5, but significantly elevated at 15 and 20 days after birth. Plasma nitrite falls dramatically at birth while newborn urinary nitrite levels are significantly greater than adults. Abnormally low plasma nitrite levels do not appear to predispose infants to NEC. Yet, active NEC is associated with elevated plasma and urinary nitrite levels.

Introduction

The free radical molecule nitric oxide (NO) plays a key signaling role in a number of physiological processes. NO is produced from L-arginine by the action of NO synthase enzymes found throughout the body. NO can diffuse readily through tissues to distances several cells away from its site of production. However, its half-life in whole blood is <10 ms and thus the range of free NO itself is paracrine in nature (1). As a free radical, NO can be metabolized by a number of different biochemical pathways. Many of these pathways result in nitrogen oxide species that are stable enough to circulate systemically yet retain the bioactivity of NO, such as nitrite and nitrosothiols. Other pathways oxidize free NO to nitrate. While nitrate is inert in mammalian cells, it can be secreted in saliva and converted to nitrite by oral bacteria (2), although this pathway is markedly diminished in the newborn compared to the adult (3). These products of NO metabolism make up a humoral source of NO bioactivity (see review by Lundberg and Weitzberg (4)). Thus, it is now recognized that the overall level of NO bioactivity in tissues is a result of not only local NO synthase activity, but also the concentration of nitrogen oxide species which may be delivered from other organs via the blood.

There is accumulating evidence that nitrite plays a role in the regulation of cardiovascular homeostasis and responses to hypoxic/ischemic stress. Numerous studies have demonstrated the positive effects of increasing dietary nitrate intake and subsequent elevation in plasma nitrite concentrations on exercise performance and lowering blood pressure (5). Modest increases in blood nitrite levels also confer protection in animal models of ischemia/reperfusion injury in the brain, heart, liver, kidney, and lung (6). The bioactivity of nitrite results from its conversion to NO by reaction with a number of

metal-containing proteins with nitrite reductase activity (7), a reaction that is favored in hypoxic/ischemic tissues.

Infants born prematurely are at increased risk of systemic hypoxia and compromised cardiovascular homeostasis during the first few days of life. This dysregulation of tissue oxygen delivery is proposed to contribute to necrotizing enterocolitis (NEC), a gastrointestinal disease that affects 5 to 14% of infants born weighing less than 1500 grams (8). While premature birth is a prominent risk factor, the underlying cause of NEC remains unknown. Although there is little evidence to support the idea that NEC is caused by a single acute perinatal ischemic event (9), poor splanchnic oxygenation and reduced blood flow are associated with increased incidence of NEC (10-12), and histological evidence of ischemia is consistently found (13,14). In the adult rodent gastrointestinal tract, swallowed salivary nitrite, derived from the reduction of salivary nitrate by oral bacteria, confers protection by increasing mucus production, improving local blood flow, killing bacterial pathogens, decreasing inflammation, and improving epithelial injury (5). The role that nitrate and nitrite play in regulating gastrointestinal blood flow in the newborn has not been studied.

We have previously demonstrated that newborn infants have lower blood nitrite concentrations than adults, possibly due to markedly lower levels of nitrate and nitrite ingestion (15). Preterm infants in particular ingest 50% less nitrite than term infants (15), leading us to hypothesize that nitrate and nitrite levels would be decreased in preterm compared to term infants. Furthermore, given animal evidence for the protective effects of nitrite in the gastrointestinal tract, we hypothesized infants diagnosed with NEC would have lower levels of circulating nitrite in the days prior to diagnosis. We tested this

hypothesis by measuring plasma nitrate and nitrite and urine nitrite concentrations in preterm infants over the first three weeks of life and making comparisons between infants who developed NEC and those who did not.

Methods

Human protocols were pre-approved by the Loma Linda University (LLU) Institutional Review Board and written informed consent was obtained from a parent or legal guardian, or from the study subject (adult group). The animal protocol was pre-approved by the LLU Institutional Animal Care and Use Committee.

Human Protocol

The infants studied in this investigation were patients in the neonatal intensive care unit or well baby nursery of LLU Children's Hospital (Loma Linda, California). They fell into two groups: term infants born after 37 weeks and with a birth weight more than 2500 grams, and preterm infants born at a gestational age less than 32 weeks. Preterm infants weighing less than 800 grams were excluded from the study due to limitations in the amount of blood that could be collected. Also excluded were infants with congenital malformations or chromosomal abnormalities, those small for gestational age, intrauterine growth restricted, anemic (Hgb < 11 g/dL) or septic (positive blood, urine, CSF cultures). The infants' gestational age, birth weight, gender, type of feeding (breast milk or formula), antibiotic therapy, whether a PDA ligation had been performed, and whether indomethacin or a blood transfusion had been given were noted.

Blood and urine samples were obtained from the preterm infants between 12 to 24 hours, and 5, 10, 15, and 20 days of extrauterine life. Within this 20-day period, if an infant developed signs and symptoms of NEC (defined as Bell's stage 2A (16)) an additional sample of blood and urine was collected within 24 hours of diagnosis. Samples of placental cord blood were collected from term infants following uncomplicated cesarean section. Blood (1 ml) and urine (1 to 3 ml) samples were also collected from these term infants in conjunction with standard newborn screening tests at approximately 24 hours of life. Single blood and urine samples were also collected from healthy adults (24 to 72 years of age).

Fetal Sheep Protocol

Fetal sheep were delivered via C-section at 124 to 126 days of gestation (term=145) as part of another study protocol (17). Venous blood samples were collected for plasma nitrite measurement before birth and at 15, 60, 120, and 180 min after ligation of the umbilical cord. Samples were immediately centrifuged for 60 seconds at 10,000 rpm and the plasma was decanted, frozen in liquid nitrogen, and stored at -80 C for subsequent measurement of nitrite concentrations.

Sample Collection and Handling

Blood samples (1 ml each) from newborn infants were collected from an indwelling catheter whenever possible; otherwise by heel stick timed so as to coincide with routine clinical care (blood glucose measurements, blood gas measurements, weekly nutrition labs, newborn screen). Blood was collected from adults by venipuncture of the

antecubital vein. The blood was immediately centrifuged for one minute at 10,000g. The plasma was stored at -80 C until assay.

Urine samples (1 to 3 ml each) were collected by placing a cotton ball into the infant's diaper before urination. Sodium hydroxide (0.1 M) was added to the urine sample in a 1:1 ratio v/v to stabilize nitrite, and the sample was then stored at -80 C for subsequent nitrite analysis.

Nitrite and Nitrate Assay

Urine and plasma nitrite concentrations were measured by triiodide chemiluminescence (18), enabling quantification above 10 nM with a precision of ± 5 nM. Nitrate concentrations were measured by enzymatic reduction of nitrate to nitrite as previously described (19).

Statistical Analysis

An *a priori* power analysis was performed using G*Power (Heinrich Heine University, Dusseldorf, Germany), and indicated that with an effect size of 50%, $p < 0.05$, and 80% power, a total of six patients with NEC would be needed to detect a change in plasma and urine nitrite levels over time and compared to preterm controls. Data are presented as mean $\pm$ SEM. Differences between study groups were detected using one-way ANOVA. A two-way ANOVA was used to compare samples from preterm infants with or without NEC. When ANOVA indicated significance, Bonferroni's post hoc analysis was applied to detect differences between sample pairs. A Kruskal-Wallis test was used to compare term, preterm, and adult plasma nitrite and nitrate levels due to a

large variation in the small number of samples in the term and adult groups. Outlying

values, likely due to nitrite or nitrate contamination occurring during sample collection,

were identified and removed using the ROUT method (20) with a Q value of 0.1% (0.1%

chance of excluding a valid value). For comparisons between preterm infants with or

without NEC, two-way ANOVA was applied to the Day 1 and 5 data to test whether

nitrite levels were lower in infants who would go on to develop NEC, and to Days 10, 15,

and 20 to test whether nitrite levels were different in infants with developing or active

NEC. All statistical analyses were performed using Prism 6 for Mac OSX (Graphpad

Software, Inc, La Jolla, CA), except for two-way ANOVA with unequal sample sizes

which was performed with R (R Foundation for Statistical Computing, Vienna, Austria).

Results

Nitrite concentrations were measured in blood and urine collected from 79

preterm infants, nine healthy term infants, twelve adults and twelve cord blood samples.

Of the 79 preterm infants, six infants developed Bell's stage 2A necrotizing enterocolitis.

The time of NEC diagnosis ranged from 9 to 29 days after birth (mean 17 days). Six

preterm infants without NEC developed sepsis and were excluded from analysis because

plasma nitrite and nitrate levels have been shown to be elevated in pediatric and neonatal

patients with sepsis (21-24). One preterm infant died on the 18th day of life with an

isolated ileal perforation and was excluded from the analyses. Thus, 66 preterm infants

without NEC were analyzed. The demographics of the patients are provided in Table 5.

Table 5. Plasma and urine study preterm patient demographics

	Preterm	Preterm with NEC	Term
Participants (*n*)	66	6	9
Gestational age (weeks)	29.6 ± 0.2	28.1 ± 0.9	38.4 ± 0.3
Birth weight (grams)	1349 ± 41	1224 ± 128	2791 ± 282
Male (*n*)	26	5	4
Female (*n*)	31	1	5
Mean day of diagnosis	-	17	-

Nitrite and Nitrate Concentrations in Infant Plasma

Twelve to 24 hours after birth, nitrite concentrations of preterm infants (0.03 ± 0.01 µM) were lower than term infants (0.08 ± 0.01 µM; $p<0.05$), cord blood samples (0.18 ± 0.01 µM, $p<0.001$), and adults (0.16 ± 0.01 µM, $p<0.001$). Plasma nitrite levels in term infants tended to be lower than cord blood and adult levels, but post hoc analysis did not indicate statistical significance. Plasma nitrite concentrations of preterm infants increased significantly during the 20 days after birth ($p<0.001$, Figure 19A).

Plasma nitrate concentrations of term infants (16.4 ± 1.5 µM) 12 to 24 hours after birth were lower than those of preterm infants (25.3 ± 1.7 µM, $p<0.05$), cord blood samples (33.1 ± 3.5 µM, $p<0.01$), and adults (33.4 ± 3.1 µM, $p<0.01$) (Figure 19B). One-way ANOVA indicated that there was significant variation in plasma nitrate concentrations of preterm infants over the first 20 days after birth ($p=0.014$).
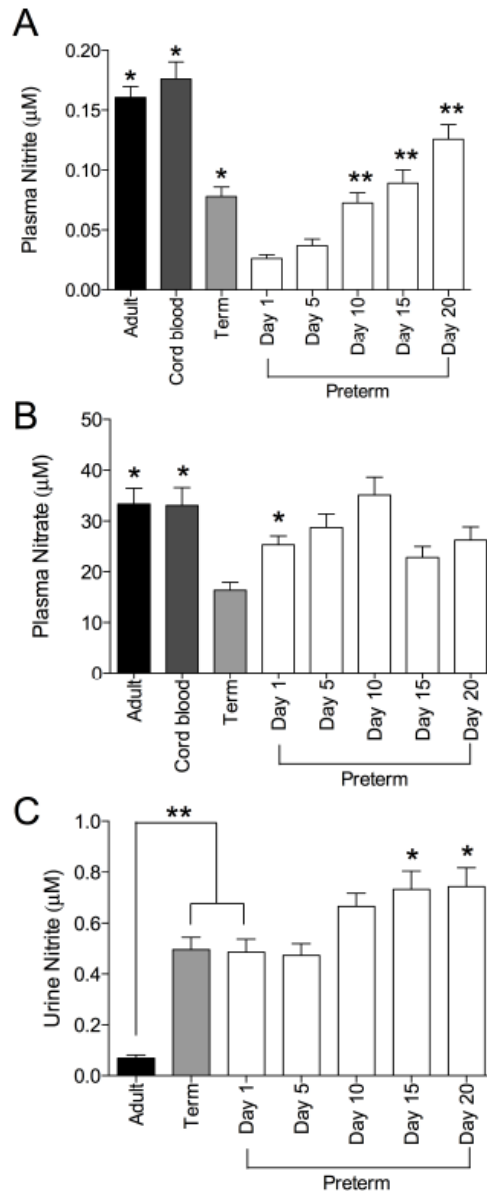
Figure 19. Plasma nitrite and nitrate and urine nitrite concentrations. A) Plasma nitrite concentrations were significantly higher in adults, cord blood, and term infants than in preterm infants on the first day after birth (*=difference from preterm infants on Day 1, $p<0.05$). Nitrite concentrations increased over the first 20 days after birth, with Days 10, 15, and 20 greater than Day 1 (**=$p<0.01$). B) On Day 1, plasma nitrate concentrations in term infants were significantly lower than those of adults, cord blood, and preterm infants (* =difference from term infants, $p<0.05$). The variation in preterm nitrate levels over the first 20 days after birth was also significant ($p<0.01$), although post hoc analysis did not detect a significant difference between Day 1 and any subsequent time point. C) Urine nitrite concentrations in preterm and term infants on Day 1 were significantly higher than those of adults (**=$p<0.001$). Urinary nitrite concentrations of preterm infants increased over the first 20 days of life, with concentrations on Days 15 and 20 being significantly greater than Days 1 and 5 (* =$p<0.05$).

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Nitrite Concentrations in Newborn Lambs

The markedly lower nitrite concentrations on the first day after birth led us to examine the time course of changes in plasma nitrite concentrations in more detail in 13 newborn lambs. Prior to ligation of the cord, plasma nitrite concentration in umbilical arterial blood was 0.16 ± 0.01 μM. Fifteen minutes after birth, nitrite concentrations had fallen to 0.06 ± 0.01 μM (p<0.001), and remained low for at least 180 minutes (Figure 20).
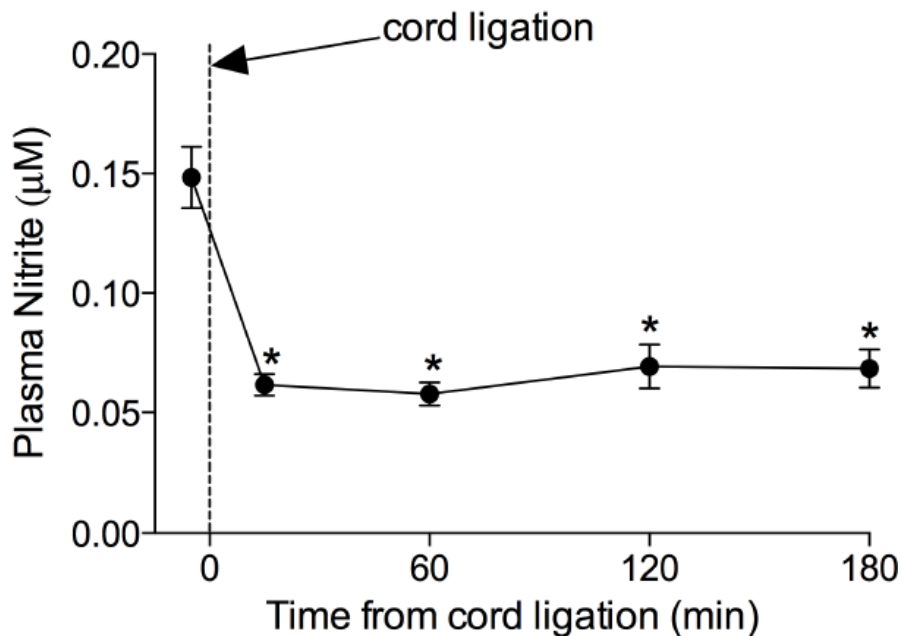
Figure 20. Plasma nitrite concentrations in fetal sheep before and after ligation of the umbilical cord and initiation of ventilation. Within 15 min following birth, nitrite concentrations had fallen by >60% (*=p<0.001 compared to baseline) and remained lower than baseline 60, 120, and 180 minutes after birth.

Nitrite Concentrations in Urine

On the first day after birth, urinary nitrite concentrations averaged 0.49 ± 0.05 μM in preterm infants and 0.50 ± 0.05 μM in term infants, values not significantly different from one another (Figure 19C). The levels in both preterm and term infants on

Day 1 were significantly higher than the levels measured in urine collected from adults (0.07 ± 0.01 µM, p<0.001). In preterm infants, the urine nitrite concentrations increased significantly over the first 20 days after birth (p<0.01, Figure 19C).

Plasma Nitrite and Nitrate Concentrations in Preterm Infants with and without NEC

Plasma nitrite and nitrate concentrations in those preterm infants who developed NEC were not significantly different than those of control preterm infants on Days 1 and 5. On Days 10, 15 and 20, plasma nitrite concentrations were higher in preterm infants who developed NEC than in control preterm infants, with post-hoc analysis indicating significance between the two groups on Days 15 and 20 (p<0.05, Figure 21A and B). Plasma nitrite concentrations of preterm infants with NEC increased over the first 20 days after birth (p<0.01). Although plasma nitrate concentrations tended to increase in infants with NEC, this change did not meet the criteria for significance (p=0.09). Individual and mean plasma nitrite and nitrate concentrations on days relative to diagnosis with NEC are shown in Figure 22 A-D.

Urine Nitrite Concentrations in Preterm Infants with and without NEC

On Days 10, 15, and 20, plasma nitrite concentrations were higher in the preterm infants who developed NEC than in control preterm infants, with post hoc analysis indicating significance on Days 15 and 20 (p<0.01). Urine nitrite concentrations of preterm infants with NEC increased over the first 20 days after birth (p<0.001) (Figure 21 C). Individual and mean urine nitrite concentrations on days relative to diagnosis with NEC are shown in Figure 22 E and F.
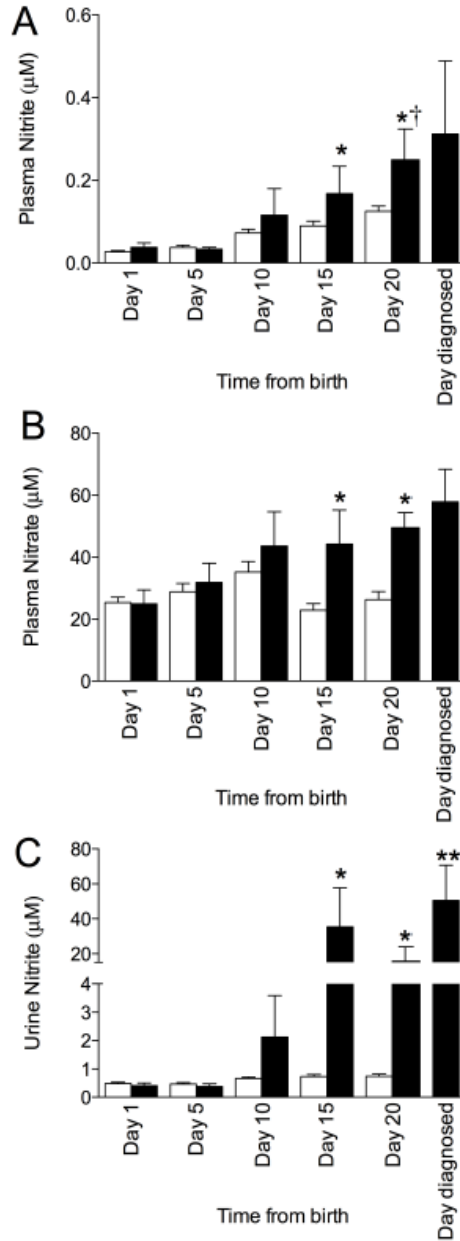
Figure 21. Plasma and urine nitrite concentrations in preterm infants with or without diagnosis of NEC. There were no significant differences between preterm infants with NEC (black bars) or without NEC (white bars) with respect to plasma nitrite (A) or nitrate (B) concentrations on Days 1 and 5. In contrast, on Days 10, 15, and 20, plasma nitrite and nitrate levels were higher in preterm infants diagnosed with NEC compared to those without NEC. C) Fifteen and 20 days after birth, urine nitrite concentrations in preterm infants who developed NEC were significantly higher than those of control preterm infants (*=p<0.001). Nitrite concentrations in urine from the preterm infants with NEC increased over the first 20 days after birth, with levels on the day they were diagnosed with NEC (mean=Day 17) being significantly higher than the levels measured in these infants on Days 1 and 5 (**=p<0.05). (*=difference from non-NEC infants at the same time point, p<0.05; †=difference from Day 1, p<0.05).
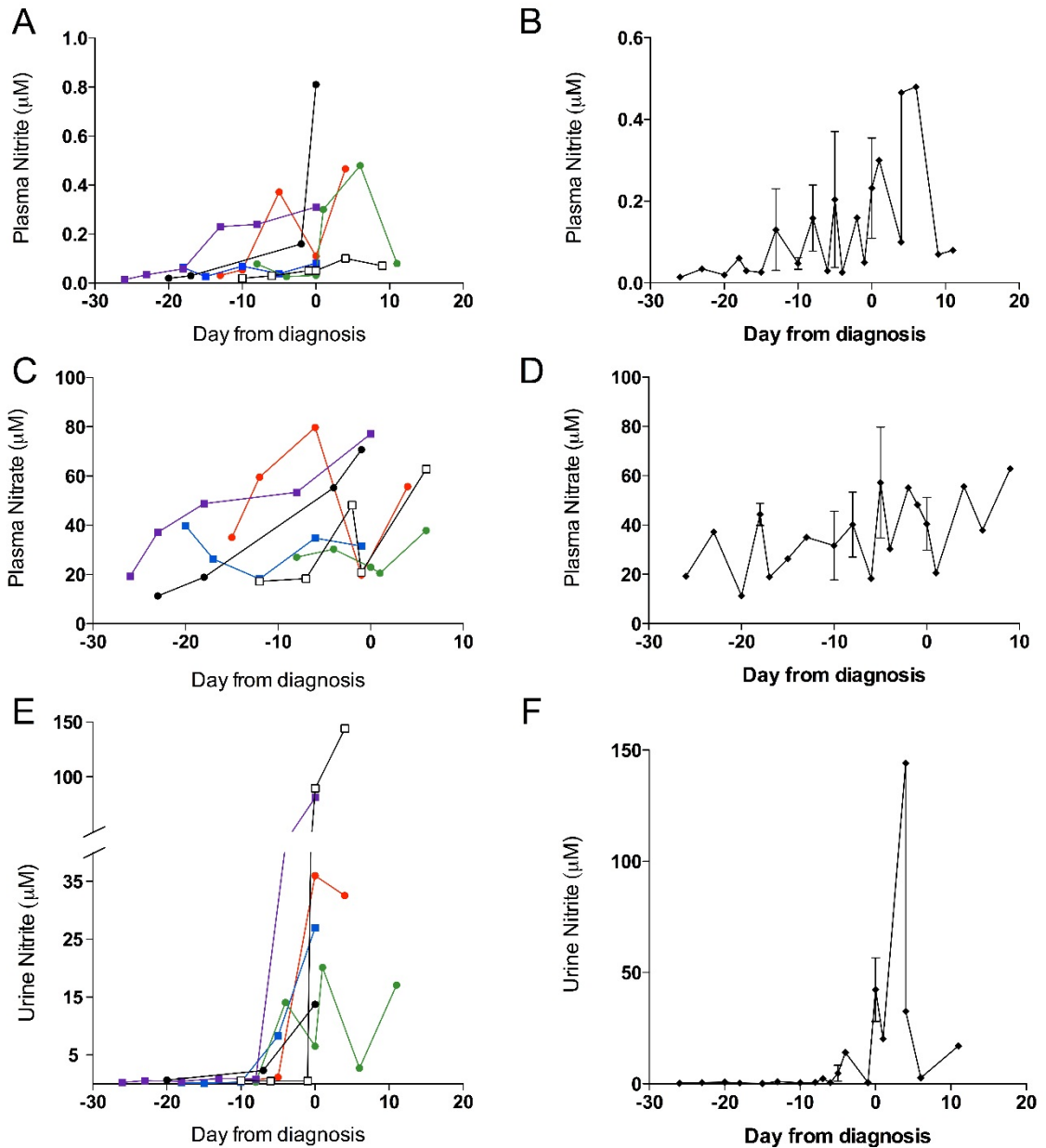
Figure 22. Plasma nitrite and nitrate concentrations and urine nitrite concentrations in preterm infants with a diagnosis of NEC. X-axis shows days relative to the day of diagnosis with Bell's Stage II NEC (Day 0). Each curve represents an individual patient in figures A, C, and E, where NEC 1 (●), NEC 2 (■), NEC 3 (●), NEC 4 (■), NEC 5 (□), and NEC 6 (●). Figures B, D, and F are the mean (± SEM) data from all patients with NEC (◆).

Discussion

The current study tested the hypotheses that plasma nitrite levels fall significantly at birth, that levels in preterm infants fall below those of term infants, and that infants diagnosed with NEC would have lower levels of circulating nitrite in the days prior to diagnosis. We found that the plasma nitrite concentrations of preterm infants on the first day of life were lower than those of term infants, adults and placental cord blood, a difference also observed to appear in newborn lambs within 15 minutes after birth. Contrary to our hypothesis, plasma and urine nitrite levels of preterm infants who developed NEC were similar to those of their non-NEC counterparts on Days 1 and 5 after birth. Once NEC became apparent, plasma and urine nitrite concentrations were measurably higher than those of non-NEC counterparts 15 and 20 days after birth.

Decrease in Plasma Nitrite and Nitrate Concentrations at Birth

Several reasons might account for the marked decrease in plasma nitrite in the minutes and hours after birth. Previous studies have shown that up to 70% of plasma nitrite is derived from NO produced by eNOS (25). Several studies have assessed the effects of development on organ-specific eNOS levels (26, 27), but, to our knowledge none have measured whole body eNOS activity in newborns. Preterm infants are deficient in arginine which could lead to decreased NO synthesis from eNOS (28). Neonates also have two- to threefold higher concentrations of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS, compared to adults (29). Thus, low L-arginine and increased ADMA concentrations may lead to low eNOS activity in newborns, contributing to the fall in plasma nitrite levels after birth.

It is also possible that the mother and placental tissue constitute a significant source of fetal nitrite that is lost at birth. We have previously reported that nitrite concentrations of chronically instrumented fetal sheep are the same as those of the mother under normoxic conditions, but become two-fold higher than the ewe following long term exposure to moderate hypoxia (18). This finding suggests transport of nitrite from the ewe into the fetus, production of nitrite by placental tissue, or an accelerated rate of nitrite production in the fetus in response to hypoxia. These possibilities have not been evaluated experimentally but could influence the changes that occur soon after birth.

Although most plasma nitrite is derived from NO oxidation, this reaction is only one of a number of competing reactions by which NO is consumed. One of the fastest of these competing pathways is the reaction of NO with superoxide to form peroxynitrite. This reaction proceeds at a nearly diffusion-limited rate ($k = 4.3$ to $20 \times 10^9\,M^{-1}\cdot s^{-1}$) (30) and can become a significant scavenger of NO if superoxide concentrations are increased (31). While we are not aware of reported changes in whole body superoxide levels at birth, it is reasonable to hypothesize that the increases in arterial and tissue oxygen tensions that occur in the transition from fetus to newborn increase superoxide production that, in turn, scavenges NO away from nitrite production.

Regardless of the mechanism, the fall in nitrite levels at birth appears to be consistent with other cardiovascular homeostatic events that facilitate the vascular transition from a fetal state characterized by low pressures and high flows to a newborn state with higher overall resistance to blood flow. Factors that contribute to the increased vascular tone of the newborn include decreases in circulating vasodilators such as prostaglandin E2 (32) and adenosine (33), and increases in vasoconstricting

catecholamines (34). Given the vasodilatory and NO-like bioactivity ascribed to nitrite (see review by Lundberg and Weitzberg (4)), the fall in concentrations within minutes of birth raises the possibility that nitrite may also play a role in the transition.

While circulating nitrate concentrations are of cardiovascular relevance to the adult due to its conversion to nitrite (35), the same may not be true for newborns as the activity of oral nitrate-reducing bacteria is markedly reduced (3). Plasma nitrate concentrations tended to decrease at birth, in parallel with nitrite concentrations, although the changes were less pronounced in the preterm infants. This may reflect the contribution of non-NO-related influences, such as denitrifying enzymes in the liver (36) or decreased renal excretion.

Nitrite Concentration in Urine

Although there are numerous reports of combined nitrite/nitrate concentrations in newborn urine (see Honold *et al* for comprehensive measurements (37), we are unaware of previous reports of urine nitrite levels in healthy infants. Urinary nitrite concentrations are typically less than 5% of the total nitrite/nitrate signal, thus the combined nitrite and nitrate level is not a useful indication of nitrite excretion. In fact, in contrast to our finding of higher urinary nitrite levels in infants compared to adults, Honold *et al*. reported higher urinary nitrite/nitrate concentrations in adults compared to preterm and term infants (37). Using the body weights of our infants and estimates of normal urinary output (48 $mL \cdot kg^{-1} \cdot day^{-1}$), one may calculate a urinary nitrite output for preterm infants of 3.6 $\mu g \cdot kg^{-1} \cdot day^{-1}$ and for term infants of 5.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$. A similar calculation for a typical 70 kg adult with a urine output of 12 $mL \cdot kg^{-1} \cdot day^{-1}$ indicates a urinary nitrite

output of only 0.24 μg·kg^{-1}·day^{-1}, about 5-10% of newborn output. This marked

difference between newborns and adults cannot be explained by dietary intake, since

newborns ingest less than 1% as much nitrite as adults when normalized to body weight

(38). Interestingly, for newborns the urinary excretion of nitrite is significantly higher

than their estimated dietary intake (~0.7 μg·kg^{-1}·day^{-1}) (38), whereas excretion by adults

is less than intake (109 μg·kg^{-1}·day^{-1}) (39). This result suggests net endogenous

production of nitrite by newborns and consumption by adults. Nitrite is actively

reabsorbed in the kidney of adults via a pathway dependent upon carbonic anhydrase

activity (40), and a deficiency in this pathway in newborns would also contribute to

higher urinary nitrite levels, although this possibility remains to be studied.

Possible Role for Nitrite in NEC

Various lines of evidence suggest NO and nitrite potentially play a protective role

against NEC. L-arginine supplementation to promote eNOS activity has been shown to

reduce the severity and risk of NEC (41, 42). Nitrite, serving as a source of NO, protects

against ischemia/reperfusion injury in a number of organs and animal models (6). In the

gastrointestinal tract, NO derived from nitrite also protects against bacterial pathogens

and supports thickening of the protective mucosal layer, and thus may support the barrier

function of the gastrointestinal lining (5). Supplemental dietary nitrite is also protective in

a mouse model of NEC (43). Thus, our hypothesis was that preterm infants with lower

plasma nitrite levels during the first few days of life would be more likely to develop

NEC. However, plasma nitrite levels of preterm infants destined to develop NEC were

similar to healthy preterm controls, indicating that NEC is not caused by plasma nitrite

levels that are lower than normal for a preterm infant. Notably, plasma nitrite levels were already markedly lower in preterm infants than in term infants and adults, and whether supplementation of nitrite would be protective requires further study.

In contrast to the proposed beneficial effects of nitrite and NO under physiological conditions, it appears that once NEC has reached an acute inflammatory stage, it has been postulated that excessive NO production from inducible NOS (iNOS) contributes to the disease progression (44). Resected sections of intestine from infants with acute NEC have increased levels of iNOS and iNOS knockout mice appear to be protected against LPS-induced bacterial translocation (45). Overproduction of NO by enhanced iNOS activity leads to toxic levels of reactive nitrogen oxide derivatives, like peroxynitrite, that are thought to contribute to the epithelial injury and disruption of repair mechanisms that lead to intestinal barrier failure characteristic of NEC (46). Consistent with overproduction of NO by iNOS, plasma nitrite and nitrate and urinary nitrite levels in infants developing NEC became significantly elevated fifteen and twenty days after birth (Figure 21), suggesting that urinary nitrite levels could be a useful non-invasive biomarker of NEC. However, more rigorous studies need to be done to validate nitrite as a reliable marker for NEC. While the cause of this increase cannot be determined from the present study, it is worth noting that similar increases in urinary nitrite and/or nitrate are found in infants with systemic inflammatory responses (47), consistent with markedly increased iNOS activity.

Clinical Perspective

The current study finds that a marked fall in circulating nitrite concentrations occurs at birth. Given increasing evidence that nitrite plays a key role as a circulating source of NO, future studies are needed to establish the extent to which changes in nitrite concentrations contribute to the cardiovascular transition at birth, and whether manipulation of these concentrations might improve outcomes in infants exposed to hypoxic/ischemic stress.

Acknowledgements

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CHAPTER FIVE

DISCUSSION

The discovery that nitric oxide (NO) is produced endogenously by NO synthases initiated a paradigm shift from thinking of NO as a toxic gas to the realization that it is a key regulator of vascular homeostasis, amongst many other physiological roles. One of the greatest clinical impacts of this discovery has been the use of inhaled NO gas for the treatment of persistent pulmonary hypertension in newborns where it has significantly reduced the need for extracorporeal oxygenation (1-2). Initially it was thought that the effects of inhaled NO would be confined to the lungs, as free NO gas in blood is scavenged by reactions with hemoglobin in a few milliseconds (3). However, it is now widely reported that inhaled NO has an array of extrapulmonary effects, suggesting that one or more of its metabolites serve as reservoirs of NO bioactivity capable of circulating from the lungs to peripheral organs. The existence of such an endocrine mediator of NO and its effects would be of great physiological relevance and much recent research has focused on identifying metabolites of NO that may serve in that role.

Nitrate (NO_3^-) and nitrite (NO_2^-) are the two major end products of NO metabolism. Historically, these compounds were considered to be relatively inert at physiological concentrations and environmental pollutants that posed potential health risks to humans at high concentrations. However, similar to the turnabout course of our understating of NO in biology, evidence now indicates that while nitrate and nitrite may be toxic at high concentrations, they play an important physiological role as they can be converted back into NO via a system involving enterosalivary recirculation, bacterial nitrate reductases, and enzyme-catalyzed or acidic reduction of nitrite to NO. This

discussion will summarize our current knowledge regarding the bioactivity of nitrate and nitrite in adults and will outline known differences in infants (summarized in Figure 23 and Table 6).
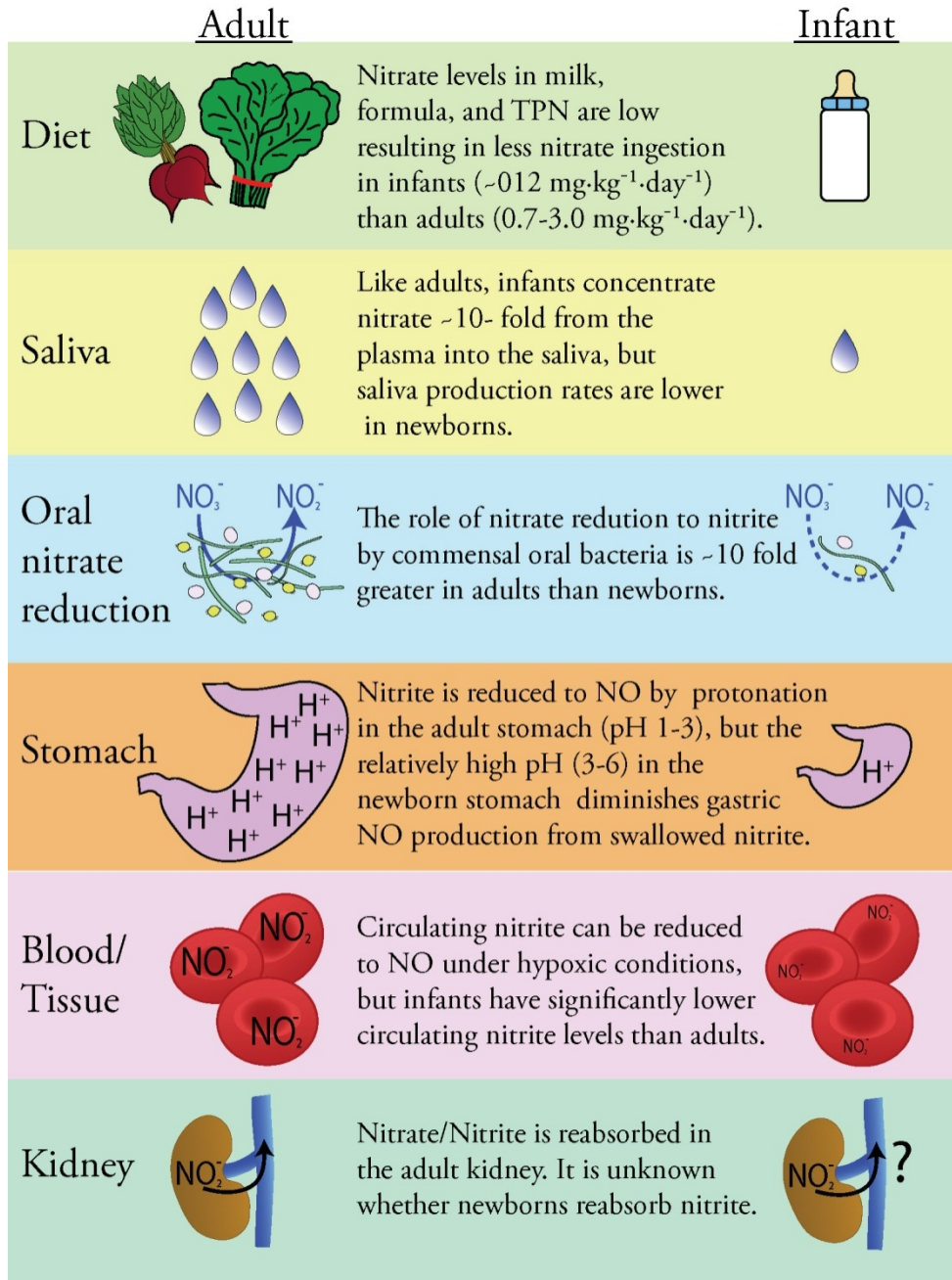
Figure 23. Schematic summary showing major differences in the supply and handling of nitrite and nitrate by the adult and infant. Multiple deficiencies in the infant lead to diminished nitric oxide (NO) bioactivity in the stomach and lower circulating nitrite concentrations in the blood.

Table 6. Summary of the major differences in the supply and handling of nitrite and nitrate in adults and infants

	Adults	Infants	References
Dietary nitrate ingestion	0.88-5.83 mg·kg^{-1}·day^{-1}	0.07-2.065 mg·kg^{-1}·day^{-1}	4-8
Saliva nitrate concentrations	190-1600 µM [a]		9-13
	681 ± 184 µM [b]	284 ± 83 µM [b]	14
Saliva nitrite concentrations	90-670 µM [a]		9-13
	50 ± 13 µM [b]	8.2 ± 5.6 µM [b]	14
Saliva production	43-1728 mL/day	43-58 mL/day	15
Oral bacterial nitrite production	147 ± 36 nmoles·min^{-1}·mg saliva^{-1}	20 ± 8 nmoles·min^{-1}·mg saliva^{-1}	14
Stomach pH	1.5-3.5	3-6	15-18
Gastric NO production	16.4 ± 5.8 ppm [c] 89.4 ± 28.6 ppm [d]		19
		1.53 ± 3.1 ppm	20
Plasma nitrite concentrations	0.27 ± 0.01 µM	0.18 ± 0.01 µM	21
Urinary nitrate/mmol creatinine	71 ± 10/75 ± 11 µmol nitrate/mmol creatinine	150 ± 31/136 ± 28 µmol nitrate/mmol creatinine	22

[a] Saliva collected via expectoration [b] Saliva collected via oral swab [c] Basal [d] After dietary nitrate load

Diet

Dietary Nitrate and Nitrite

Nitrate is the most prevalent nitrogen oxide species in the body. Although some of

it is derived as an end product of the oxidation of endogenous nitric oxide and nitrite,

nitrate concentrations are also heavily influenced by dietary intake. Nitrate itself is inert

in mammalian tissues, but it can be reduced to nitrite by symbiotic bacteria that are part

of the normal flora in the mouth and gastrointestinal tract (discussed below). Thus, by the action of these bacteria, dietary nitrate contributes to the body's pool of nitrite. Vegetables are the most common source of dietary nitrate with particularly high concentrations (>2500 mg/kg) in beets, radishes, celery and green leafy vegetables such as lettuce, kale, and spinach. Although daily nitrate ingestion can vary significantly dependent upon the types and amount of vegetables eaten, it is estimated that a typical adult ingests approximately 0.7 to 3.0 mg/kg body weight of nitrate per day (23).

Compared to nitrate, the amount of nitrite ingested in a normal adult diet is relatively small. In fact, it is likely that more of the nitrite in the body is derived from the bacterial reduction of nitrate and oxidation of endogenously-produced NO than from the diet (24, 25). The biggest source of nitrite in the diet is cured and processed meats, where it is used as an additive to prevent bacterial growth and enhance the color. A typical adult ingests about 0.1 mg/kg body weight of nitrite daily (4).

Although there are some discrepancies in the reported concentrations of nitrate and nitrite in breast milk and artificial milk (perhaps due to differing assay methodologies), we and others have recently shown that newborn infants ingest markedly lower amounts of nitrate and nitrite than adults on a per kg body weight basis. This is true regardless of whether they are receiving breast milk, artificial milk, or parenteral nutrition (7, 26, 27). Based on a breast milk intake of 150 $mg \bullet kg^{-1} \bullet day^{-1}$ and our measurements of nitrate and nitrite concentrations (13 μM and 0.13 μM, respectively), we have estimated that infants ingest approximately 0.12 $mg \bullet kg^{-1} \bullet day^{-1}$ of nitrate and 0.0007 $mg \bullet kg^{-1} \bullet day^{-1}$ of nitrite from fresh breast milk, which equates to only 5% and 0.6% of the nitrate and

nitrite intake of adults (27). A comparison of the average dietary nitrate intake in

newborn infants and adults is shown in Figure 24.
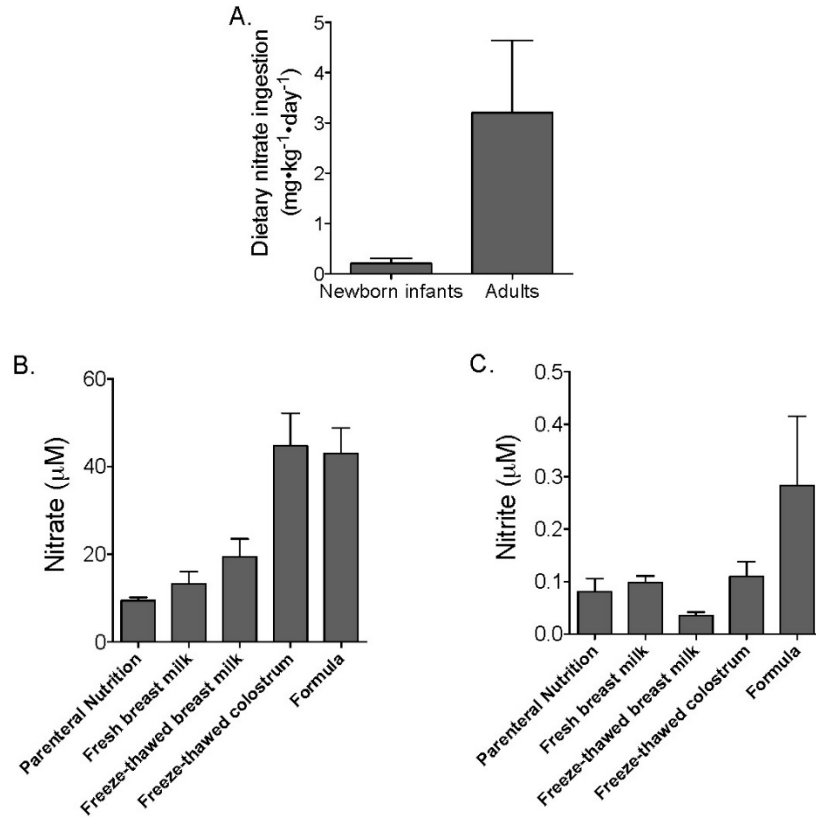
Figure 24. Dietary nitrate and nitrite levels for newborns and adults. A) Daily dietary nitrate ingestion, normalized for body weight, is shown for newborns and adults, based on a mean (±SEM) of reported concentrations in breast milk and formula (for newborns) (7, 26, 27) and a typical adult diet (Mensinga). B,C) Nitrate and nitrite concentrations in total parenteral nutrition (TPN), fresh and freeze-thawed breast milk, freeze-thawed colostrum, and a convenience sample of artificial milk formulas. (Figure adapted from Jones et al., 2014 (27).)

We have also shown that nitrite is oxidized to nitrate in breast milk by an enzyme

normally present in milk, lactoperoxidase, leading to even lower levels in milk that has

been allowed to sit at room temperature or which has been freeze-thawed (27). Breast

milk nitrite concentrations also fall during the first few weeks of life, with the highest

levels found in colostrum and decreasing to nearly undetectable amounts in milk collected after the third week postpartum (26, 27). The levels of nitrate and nitrite in artificial milk vary widely across a range that extends above and below concentrations measured in breast milk, averaging 43 μM and 0.3 μM, respectively (27) (Figure 24). The recently increased use of nutritional additives for caloric and protein enhancement raises the possibility of an additional source of dietary nitrate and nitrite, although to our knowledge concentrations in these additives have not yet been reported. Whether the newborn deficiency of dietary nitrite and nitrate serves an important physiological role, or whether supplementation of breast milk with these anions would be beneficial or problematic remains to be studied.

Until recently, a majority of research related to dietary nitrate and nitrite was in the context of toxicology. It has been known since 1945 that unusually high nitrate concentrations in vegetables and drinking water, often due to contamination with fertilizer, can cause cyanosis due to oxidation of hemoglobin to methemoglobin by nitrite derived from bacterial nitrate reductases, a problem often referred to as "blue baby syndrome." Newborn infants are particularly susceptible to this problem as they have ~25% lower methemoglobin reductase activity than adults (28). It is also proposed that dietary nitrate and nitrite are associated with gastrointestinal cancer due to the formation of carcinogenic N-nitroso compounds. Although a definitive causal link between dietary nitrate and cancer has not been identified (29), the associations between the intake of nitrite-treated meats and gastric cancer are more established (30). This is consistent with evidence that nitrite added to meats as a preservative can be converted to harmful N-nitrosamines in the meat itself before ingestion or in the body after it has been ingested

(31). In an effort to protect against toxicity, the Environmental Protection Agency (EPA) has set limits on inorganic nitrate and nitrite levels in drinking water and the World Health Organization (WHO) has put forward Acceptable Daily Intakes (ADI) for nitrate at 3.7 mg/kg of body weight and for nitrite at 0.06 mg/kg of body weight. These levels are easily exceeded, however, with a high vegetable diet, and some have called for a resetting of these limits based on recent advances in our understanding of the roles of dietary nitrate and nitrite (26). The upper limits of toxicity of dietary nitrate in newborns have been investigated. Phillips, et al. found that up to 21 mg/kg of nitrate per day was well tolerated by seven newborn infants, with six infants showing no increase in methemoglobin and the other one only a slight increase, and not enough to produce detectable cyanosis. Likewise, no symptoms of cyanosis occurred even when 100 mg nitrate $\bullet kg^{-1} \bullet day^{-1}$ was given to an infant for 8 days (32).

In contrast to the evidence of the toxic effects of nitrate and nitrite, the data increasingly indicates that a diet rich in nitrate is beneficial to overall cardiovascular health. In adults, raising dietary nitrate intake has been shown to improve exercise tolerance (30, 33, 34), decrease blood pressure (24, 34), inhibit platelet aggregation (24), decrease risk of cardiovascular disease (35), and improve vascular compliance (36) (see Weitzberg, 2013 for a comprehensive review) (37). In addition, nitrite supplementation ameliorates microvascular inflammation and endothelial dysfunction in mice fed a high-cholesterol diet (38). Dietary nitrate also appears to have beneficial effects in the gastrointestinal tract of adult rats, where it has been shown to protect against non-steroidal anti-inflammatory drug (NSAID)-induced ulcers (39). Weighing the beneficial

effects of increasing dietary nitrate and nitrite (37) against the potential risks of methemoglobinemia and carcinogenicity is the focus of ongoing studies.

Saliva

In addition to dietary intake of nitrate and nitrite, the levels of these anions in swallowed saliva also have a significant impact on the amount of nitrate and nitrite that is ingested. As discussed in this section, this appears to be another point of significant difference between adults and newborns, thus compounding the effects of low dietary nitrate and nitrite ingestion in newborns.

Bacterial Conversion of Salivary Nitrate to Nitrite

Fasting nitrate concentrations average about 200 µM in the saliva of adults but can reach as high as 10 mM after a nitrate-rich meal (12). These concentrations are approximately 10-fold higher than the concentrations measured in plasma due to active transport of nitrate from the blood into the saliva by the salivary glands. The transport of nitrate has been suggested to be mediated by the enzyme sialin via an ATP-dependent electrogenic NO_3^-/H^+ transport mechanism in the salivary acinar cells (40). The nitrate concentration in the saliva of newborns is approximately 200 µM, similar to that of adults (14). As in adults, this concentration is many-fold higher than in blood (16-40 µM). Thus the active transport mechanisms in the salivary glands of newborn infants are present with a concentrating power comparable to that of adults (21, 41). That the body expends energy to actively concentrate nitrate into the saliva suggests that nitrate is not just an inert end product of NO metabolism, but has potential bioactivity in the body.

Although nitrate itself appears to be inert in mammalian tissues, it is made physiologically relevant after reduction to nitrite by bacteria residing in the crypts of the dorsal posterior surface of the tongue. These bacteria utilize nitrate as the terminal electron acceptor in the respiratory chain, rather than oxygen and reduce about 20% of salivary nitrate in adults (30, 42). A true symbiotic relationship between these bacteria and the human host exists as humans lack the requisite enzymes to bring about this conversion independently but provide nitrate to the bacteria that then perform nitrate reduction via respiration. As discussed below, these nitrate-reducing bacteria are critical to the beneficial effects of dietary nitrate.

The primary bacteria that mediate nitrate reduction in the mouth are obligate anaerobes of the *Veillonella* species and facultative anaerobes of the *Actinomyces*, *Rothia*, and *Staphylococcus* species, all of which possess nitrate reductase enzymes that allow them to respire nitrate and rapidly produce nitrite (43). *Veillonella* and *Actinomyces* species have been found in saliva collected from infants in the first two months of life and appear to be some of the first bacteria to colonize the mouths of newborns (12, 44-46). Despite the presence of these bacteria, oral nitrate reductase activity is markedly lower in newborn infants when compared to adults, as shown in Figure 25. It is unknown whether this difference comes from insufficient numbers of bacteria, whether the bacteria do not possess sufficient nitrate-reducing capacity, or whether the mouth of newborns lack some cofactor for nitrate reduction or some other necessary element.
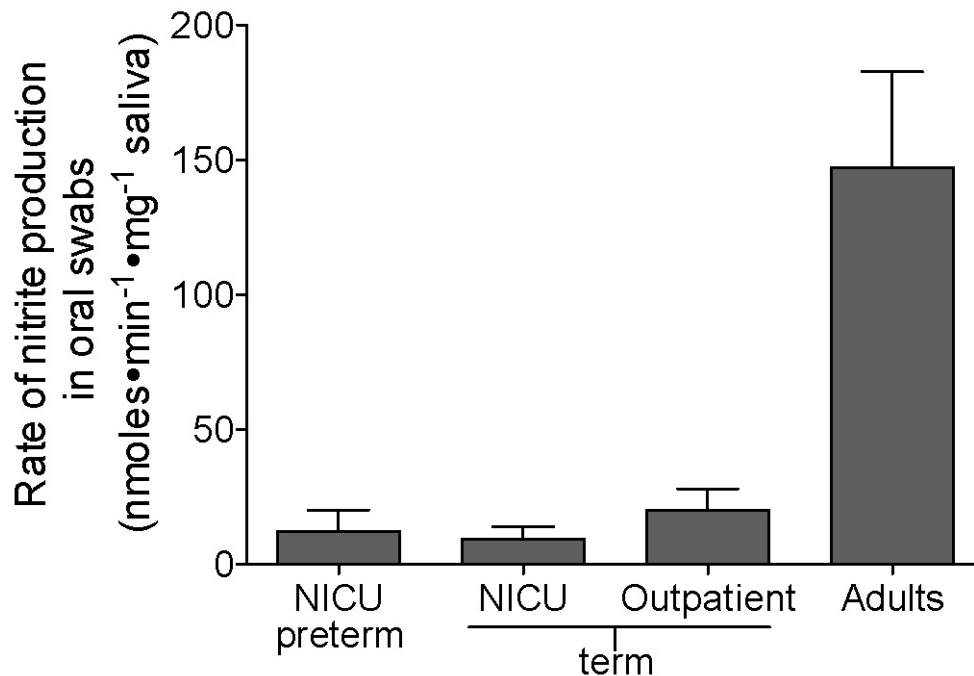
Figure 25. Nitrate-reducing activity, normalized for saliva weight, in swab samples collected from the mouths of preterm (born at <35 wk gestation) and term (born at >36 wk) infants in the neonatal intensive care unit (NICU) or from healthy infants in an outpatient clinic between 2 and 6 wk after birth, or from normal healthy adults. Mammalian cells lack the enzymes required for nitrate reduction, but bacteria dwelling in crypts of the tongue bring about the reaction. Note that the rate in infants is ~10% of that in adults. (Figure adapted from Kanady et al., 2012 (14).)

By measuring the nitrate-reducing capacity of bacteria in oral swabs collected from infants, we have shown that there is essentially no detectable nitrite production from nitrate in the first five days of life. While there is measurable nitrate reducing capacity in swabs collected from infants at two to eight weeks of age, the rate of nitrite production is only ~10% of that of adults (14), as illustrated in Figure 25. Notably, infants also produce relatively small volumes of saliva during the first few weeks of life, which may attenuate bacterial growth and may also result in less swallowed salivary nitrite compared to the adult (14). The developmental time point at which the nitrate reducing capacity of the infant mouth becomes comparable to the adult is unknown.

The diminished bacterial nitrate reducing capacity in infants may be of physiological relevance because salivary nitrite impacts both gastrointestinal and cardiovascular function in adults. Blockade of salivary nitrate secretion by ligation of the submandibular gland duct in rats results in decreased gastric nitrate, nitrite, and NO concentrations and exacerbates stress-induced gastric ulcers (47). The severity of gastric ulcers in these rats is reduced upon supplemental nitrate treatment (47). These gastroprotective effects appear to be mediated through the action of increased salivary nitrite, as nitrite-rich saliva results in increased gastric mucosal blood flow, a thicker mucus layer, and attenuation of the inflammatory response associated with NSAID administration in rats (39, 48-49). These effects of nitrite on the stomach are likely due to its conversion to NO, as discussed below.

In addition to the effects in the gastrointestinal tract, increasing dietary nitrate, and concomitant increases in salivary nitrite, have been shown to decrease arterial blood pressure, protect against ischemia-reperfusion induced endothelial dysfunction, and decrease platelet aggregation (24, 34). The importance of salivary nitrite production by oral bacteria is again highlighted by the finding that if subjects refrain from swallowing saliva after a dietary nitrate-load or are given antibacterial mouthwash to decrease bacterial nitrate-reducing activity, the hypotensive effects of nitrate are attenuated and there is no inhibition of platelet aggregation (24, 50). Increasing dietary nitrate also leads to increased circulating nitrite concentrations (24), which is associated with a host of beneficial effects ranging from improved exercise tolerance to protection against ischemia-reperfusion injury (discussed below).

Thus, considering the beneficial gastrointestinal and cardiovascular effects of dietary nitrate and subsequent salivary nitrite production by oral nitrate reducing bacteria in adults, the lack of the critical bacterial nitrate reduction in infants is noteworthy and deserves investigation. Moreover, the lack of bacterial nitrate reducing activity in infants will compound the already low levels of nitrate and nitrite in their diet, ultimately leading to significantly lower nitrite delivery to the infant stomach. The potential benefit of adding a mother's oral bacteria to an infant's mouth is untested.

Gastrointestinal tract

Intragastric Conversion of Nitrite to NO

In 1994, two independent studies showed that NO was generated from nitrite in the stomach of human adults (51, 52). These studies showed a novel nitric oxide synthase (NOS)-independent in-vivo mechanism by which NO could be generated from nitrite. Since then, there has been great interest in nitrite as not merely an inert NO metabolite but as a physiologically relevant source of NO bioactivity. The chemical reaction by which NO is generated in the acidic stomach of adults involves protonation of nitrite to form nitrous acid (pKa 3.3), which rapidly decomposes to several highly reactive nitrogen oxides, including NO free radical, NO_2, N_2O_3, and peroxynitrite (51, 53). In the stomach, these nitrogen oxides can form new stable products through nitration and nitrosylation of amines, amides, thiols, and fatty acids. These products have wide-ranging bioactivities, which include modulation of inflammatory signaling pathways, inhibition of platelet aggregation, vasodilation, mucus production, and bacterial colonization, among many other functions (53-56).

Via gastric conversion to NO, ingested nitrite has been shown to kill many different enteropathogens including *Salmonella, Shigella, H. pylori, E. coli, Yersinia enerocolitica, C. difficile* and *Candida albicans*, establishing nitrite as a key player in host defense (57-60). In addition to acting as a bactericidal agent, NO plays a key role in host defense in the gastrointestinal tract by stimulating mucus and fluid secretion, regulating the epithelial barrier, mediating vascular smooth muscle tone, diminishing leukocyte adherence to the endothelium, modulating mucosal repair, and influencing the release of inflammatory mediators (57,61). While it is now apparent that nitrite-derived NO plays many protective roles in the stomach and GI tract, it is important to note that nitrite in the stomach (via conversion to nitrous acid and other nitrogen oxides) can also act as a nitrosating agent, converting ingested amines into their carcinogenic N-nitroso derivatives (30). However, while the nitrosating ability of acidified nitrite is clear, there is still no direct evidence that increased ingestion of nitrate, and subsequent conversion to salivary nitrite and gastric NO, causes increased risk of gastric cancer (42).

NO generation from nitrite in the stomach is highly pH dependent and is effectively attenuated with proton pump inhibitors (52). Consequently, the protective effects of swallowed nitrite appear to be highly dependent upon gastric acidity as increasing the pH above a value of 4 effectively prevents nitrite-induced increases in blood flow (48) and reduction in pathogenic bacteria (51, 60), and blocks nitrite's hypotensive effects (62).

The pH dependence of nitrite-derived gastric NO is of particular relevance in the newborn, as the newborn stomach has a relatively high pH compared to the adult stomach (17, 18, 63). Figure 26 illustrates the effect of pH on the rate of NO production from

nitrite using previously calculated rate constants (64). As shown in Figure 26, the less acidic environment of the newborn stomach would attenuate the generation of NO from nitrite delivered to the stomach, compounding the already low nitrite ingestion from the saliva and diet. In fact, non-enzymatic NO production in the stomachs of newborns averages 1.53 ± 3.10 ppm (20), as compared to 16.4 ± 5.8 ppm in fasted adults (19) or 89.4 ± 28.6 ppm after a 2 mmol nitrate load (19). However, reports vary as to the quantitative amount of stomach NO generation in adults, which are also reported as low as 0.6 ± 0.1 ppm in fasted adults and 1.64 ± 0.4 ppm after dietary nitrate intake (52). Interestingly, peak gastric NO generation is shown to be lower in formula-fed infants $(2.24 \pm 15.71$ ppm) versus breastfed infants $(6.03 \pm 5.73$ ppm) (20). Further work should be done to explore these intriguing findings.

High gastric pH in newborns has been associated with an increased risk of necrotizing enterocolitis (NEC) (65, 66), whereas low gastric pH protects against bacterial translocation across the gut wall in neonatal rabbit pups (67). Considering that acidified nitrite kills bacteria, improves mucus secretion and mucosal blood flow, and is protective against ischemia-reperfusion injury (discussed below), it is worth speculating that enhancing NO generation from nitrite would be protective against NEC.
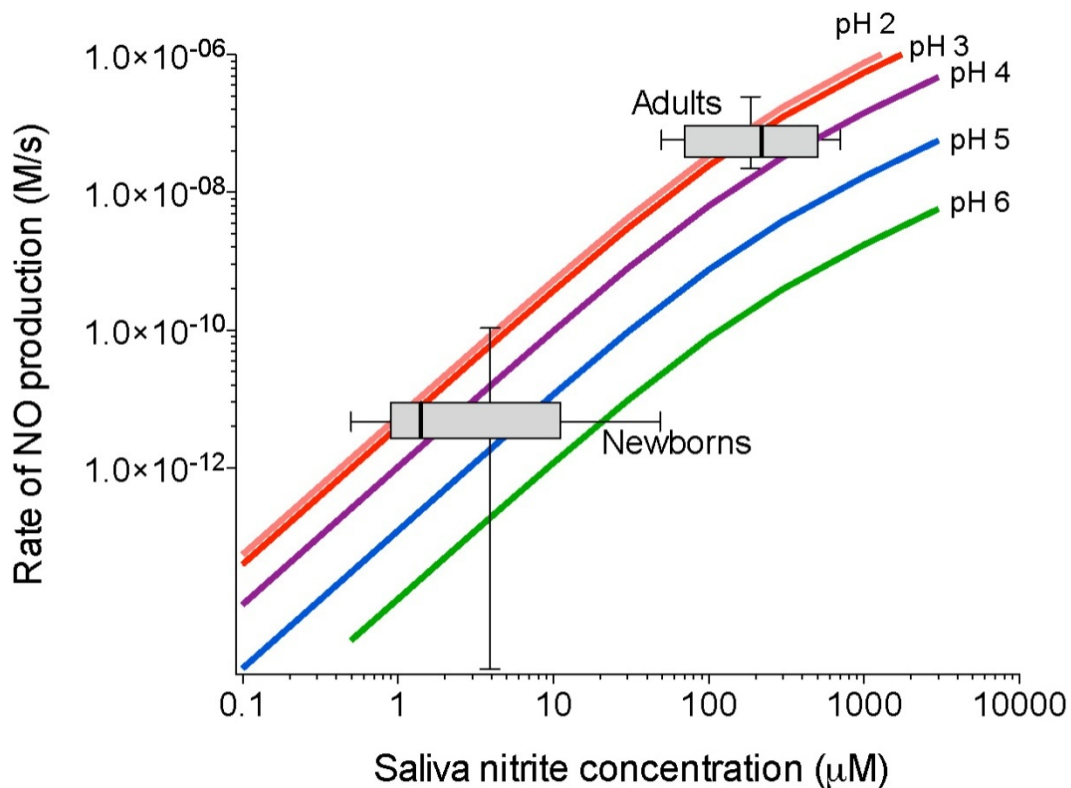
Figure 26. Nomogram showing the rate of nitric oxide (NO) generation in gastric fluid for various pH and nitrite concentrations. The adult and newborn ranges of nitrite concentrations are shown as box and whisker (min to max) plots of saliva nitrite concentrations (14) and placed at reported typical ranges of gastric pH for adults and newborns (18). The infant rate is estimated to be about 100-fold slower than the adult rate due to less acidity in the newborn stomach and lower nitrite levels ingested. Curves were constructed using rate constants and equations given by Zweier et al. (64).

Intestine

In contrast to the NOS-independent generation of NO in the stomach, NO generation in the colon appears to be mediated by NOS-dependent mechanisms, as demonstrated by the finding that rats treated with the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME), have significantly less NO generation in the colon while NO generation in the stomach is unaffected (68). In rats, NO concentrations in the stomach

(>4000 ppb) are orders of magnitude higher than in the small intestine (<20 ppb), cecum (~200 ppb), or colon (<25 ppb) (68). Interestingly, germ-free rats have markedly lower NO generation in all areas of the gastrointestinal (GI) tract, including the stomach, indicating an important role for bacteria in NO production throughout the GI tract. Germ-free rats provide a useful comparison to newborn infants, as diminished gastric NO in germ-free rats is thought to be due to the lack of oral nitrate reducing bacteria since gastric NO production is dependent on substrate (ingested nitrite) availability (68). In the cecum, NO is in part formed via reduction of nitrate and nitrite by strains of *Lactobacilli* and *Bifidobacteria* (69) and stimulation of the mucosal NOS enzymes by GI bacteria (68). NO generation in the intestine, either by *Lactobacilli farciminis* or by administration of an NO-donor, has been demonstrated to have anti-inflammatory effects in an animal model of colitis (70), highlighting the potential protective effects of increased NO generation in the intestine.

Given the beneficial effects of NO derived from ingested nitrite on the gastrointestinal microbiota, blood flow and mucus production (described above), we were interested in nitrite's role in the context of NEC. NEC is the most common gastrointestinal disease to afflict premature infants. It is a disease characterized by intestinal barrier failure (71) most likely subsequent to an ischemic insult. The main factors contributing to the regulation of gastrointestinal blood flow in the preterm infant are poorly defined, and it is not known whether a deficiency in NO contributes to the dysregulation of gastrointestinal blood flow that is thought to precede NEC. Thus, we hypothesized that infants with NEC would have lower circulating nitrite levels (and thus lower NO bioavailability) in the days preceding the onset of NEC. However, we found

that abnormally low plasma nitrite levels do not appear to predispose infants to NEC. Yet, active NEC (Bell's Stage II, diagnosable by radiographic signs (72) is associated with elevated plasma and urinary nitrite levels.

Translocation of bacteria across the compromised gastrointestinal wall leads to activation of an inflammatory response characterized by pronounced up-regulation of NO production by inducible NOS (iNOS). In this inflammatory stage of the disease, overproduction of NO by iNOS results in toxic levels of peroxynitrite, further damaging the integrity of the gut wall by inducing enterocyte apoptosis and necrosis, or by disrupting tight junctions and gap junctions that normally maintain epithelial monolayer integrity (73, 74). Thus, a vicious cycle characteristic of severe NEC is created by bacterial invasion, immune activation, uncontrolled inflammation with production of ROS and nitrogen species, vasoconstriction followed by ischemia-reperfusion injury, gut barrier failure, intestinal necrosis, sepsis and shock (75). Since NEC is predominantly found in preterm infants, and preterm infants have significantly lower plasma nitrite levels than term infants (0.03 ± 0.01 μM vs. 0.08 ± 0.01 μM; $p < 0.05$), it would appear reasonable to hypothesize that NO plays a dichotomous role in NEC, with deficient levels of NO contributing to an increased vascular resistance during the initiating ischemic event, and subsequent overproduction of NO during the inflammatory stage of the disease leading to propagation of the injury. Interestingly, Yazji et al have recently reported that nitrite/nitrate-deficient formula predisposes newborn mice to NEC, and that both the incidence and severity of NEC was ameliorated by nitrite/nitrate supplementation to the formula to achieve levels comparable to that of breast milk (76). However, the clinical relevance of this finding is uncertain given the fact that nitrite and nitrate concentrations

of many commercially available formulas are already higher than those found in breast milk (27). Whether manipulation of the decreased levels of ingested and circulating nitrite in the preterm infant would prevent or alter the course of NEC is an area worthy of study.

Nitrite Supplementation for the Prevention of NEC in Newborn Rat Pups

To test whether nitrite supplementation would be protective against NEC, we utilized a newborn rat pup model of NEC, based on that described by Nadler et al. (77). In this model, neonatal rats are removed from their mothers after Caesarian-section, placed in a humidity and temperature-controlled incubator, gavage-fed formula in lieu of breast milk, and exposed to hypoxia (5% O_2, 95% N_2) twice daily. Littermate controls are placed with a foster dam and allowed to breast feed *ad lib* and are not exposed to hypoxia. Using this model, we sought to test the efficacy of nitrite (0-3mM) in preventing intestinal damage by evaluating the ileum for signs of NEC. Tissue sections were given a score from 0 (healthy) to 4 (severe NEC), with a score of 2 or greater indicating NEC. We also evaluated the tissue for macroscopic evidence of pnuematosis intestinalis and bowel discoloration.

Although this model is used in multiple labs throughout the country and has been helpful in elucidating many of the molecular pathways involved in NEC pathogenesis, its clinical relevance is limited given that NEC typically manifests 2-4 weeks after birth, rather than in the first 72 hours after birth as designed in this experimental model (78). In addition to this inherent problem with the model, we experienced difficulty in being able to reliably reproduce histological NEC in the pups, as determined by two independent

clinical pathologists blinded to the study conditions. Thus, it was impossible to form conclusions about nitrite's efficacy against NEC since NEC could not be shown to be induced.

Through the use of this model, however, we found that the mean histological score of 34 pups that did not receive supplemental NO_2^- (formula-fed (FF) + hypoxia) was significantly worse than that of the healthy control group (0.5 ± 0.08 vs. 0.23 ± 0.03, respectively) ($p<0.01$, ANOVA). The histological scores of the pups that received nitrite were not significantly worse than the healthy controls. However, they were not improved compared to the FF + hypoxia group (Figure 27). Furthermore, nitrite treatment had no effect on the survival rates, weight gain, severity of pneumatosis intestinalis, or intestinal discoloration. Based on the histological scores, nitrite may offer some protection against the early stages of intestinal damage. However, the other points of evaluation suggest the preventative effects of nitrite are minimal. Moreover, the scores for the FF + hypoxia ("NEC" positive control) group did not reach the threshold for NEC and thus, nitrite's efficacy in preventing NEC remains untested.
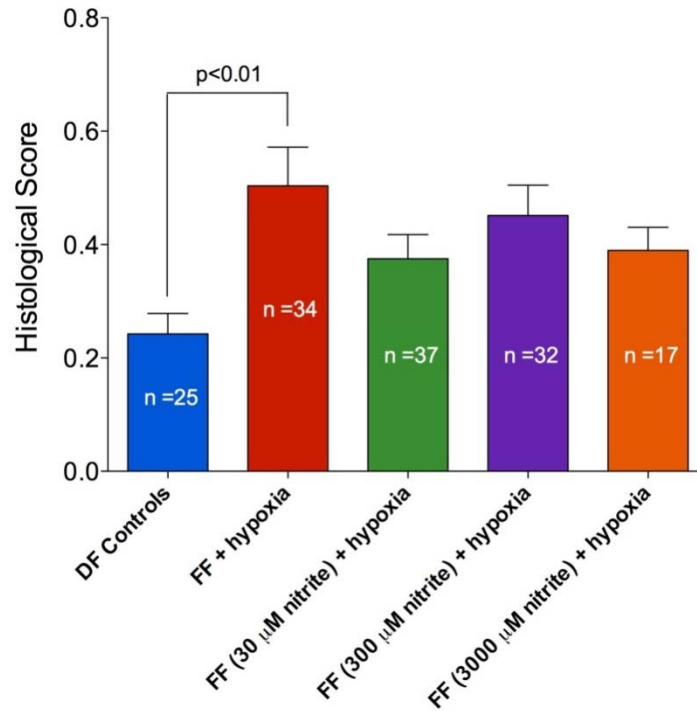
Figure 27. Histological scores of ileum samples collected from neonatal rat pups exposed to formula feeding (FF) with increasing doses of nitrite (0-3 mM) and hypoxia twice daily for 72 hours.

Circulation

Circulating Nitrite

Shortly after the discovery that NO is generated from nitrite in the acidic environment of the stomach, Zweier and colleagues showed that NO could also be generated from nitrite in ischemic heart tissue (79). Nitrite reduction to NO in hypoxic tissues appears to be mediated either by acidic disproportionation (similar to the mechanism in the acidic stomach) or by the activity of metal-containing proteins with nitrite reducing activity. These proteins include the heme-associated globins in their deoxygenated state, such as deoxygenated myoglobin, hemoglobin, cytoglobin and neuroglobin, as well as mitochondrial enzymes like complex III; molybdenum

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metalloenzymes, such as xanthine oxidoreductase (80); cytochrome P450 enzymes; and

endothelial NOS (see the review by Kim-Shapiro and Gladwin) (81). While the initial

report by Zweier suggested that nitrite reduction to NO in acidic tissues exacerbates post-

ischemic injury (79), nitrite has since been consistently shown in experimental animals to

be protective against ischemia-reperfusion (I/R) injury in the heart, brain, liver and

kidney (82). The mechanism by which nitrite confers protection against I/R injury is not

well understood, but is thought to involve reduction to NO which modulates the function

of the mitochondria, leading to more efficient oxygen utilization, decreased reactive

oxygen species formation, and the inhibition of apoptotic signaling (83). The therapeutic

potential of nitrite against I/R injury in newborns has not yet been studied. Considering

that neonates are particularly at risk for hypoxic and ischemic insults, further research is

needed to address the therapeutic and preventative potential for nitrite supplementation in

the neonatal population.

While nitrite supplementation has yet to be studied in neonatal populations, it has

been shown that treatment of persistent pulmonary hypertension of the newborn with

inhaled NO (iNO) increases nitrite levels in the blood at least two-fold (21, 84). Although

the resulting nitrite concentrations reached only ~300 nM after iNO administration,

similar increases in circulating nitrite concentrations have been shown to protect mice

against hepatic infarct (85), increase blood flow in the human forearm (86), and decrease

systolic blood pressure in adults (24, 34, 87). Thus, increases in circulating nitrite levels

resulting from iNO treatment may be enough to cause significant systemic effects (21).

Indeed, there are a number of reports demonstrating protective effects of iNO therapy in a

mouse model of myocardial infarction (88), adult human liver transplant patients (89),

and in children following cardiopulmonary bypass (90). Whether the protective effects of iNO are due to elevations in circulating nitrite remains to be determined.

Given that nitrite could theoretically improve an infant's ability to withstand ischemic stress, it is important to discuss the mounting evidence that normal newborn infants appear to have numerous mechanisms in place that decrease systemic nitrite levels during the first few weeks of life. As shown in Figure 23, these mechanisms include low dietary nitrite and nitrate intake, the lack of bacterial nitrate reduction in the mouth, a relatively high pH in the stomach, enhanced urinary excretion, and a dramatic fall in plasma nitrite concentrations at birth.

We have shown that circulating nitrite concentrations decrease markedly after birth, falling from approximately 0.18 ± 0.02 µM in umbilical cord plasma to 0.08 ± 0.02 µM in plasma collected from term infants on their first day of life. Interestingly, plasma collected from preterm infants has even lower nitrite concentrations (0.03 ± 0.01 µM). Plasma nitrite concentrations are significantly lower in infants than those measured in adults, which averaged 0.17 ± 0.01 µM in our study and typically range from 50 to 300 nM (42). Moreover, plasma nitrite appears to remain lower than adult levels for the first few weeks of life. These findings are consistent with previous reports that adult plasma nitrite levels are significantly higher than those of newborn infants (21), but are similar to those in umbilical cord blood (91). The relevance of the dramatic fall in circulating nitrite levels immediately after birth is uncertain, but may be an important part of the circulatory changes that occur at birth.

There are many factors that contribute to plasma nitrite concentrations. In adults, a majority of plasma nitrite is derived from the oxidation of NO produced by endothelial

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NOS (eNOS) (25). This oxidation depends on enzyme-catalyzed reactions in the plasma (92), which are significantly attenuated in the newborn (93). In addition, eNOS activity may be diminished in newborns due to low levels of L-arginine (94) and increased levels of asymmetric dimethylarginine (ADMA), the endogenous inhibitor of NOS (95-97). Furthermore, the rapid increase in tissue PO_2 that occurs at birth may result in increased superoxide levels, particularly in preterm infants who are likely to have low antioxidant defenses (98-101). This superoxide can rapidly scavenge NO to produce peroxynitrite instead of nitrite. Another potential cause of relatively low plasma nitrite in infants could be the lack of significant oral bacterial nitrate reduction, as discussed above and illustrated in Figure 25. We have shown that adults given antibacterial mouth rinse have significantly reduced plasma nitrite concentrations (14), highlighting the importance of the oral nitrate reducing bacteria to the amount of circulating nitrite. Thus, with the confluence of all of these factors, it appears that nitrite bioavailability is diminished in the newborn by a system of concerted mechanisms, as evidenced by the sharp fall in nitrite concentrations at birth. The physiological relevance of this decreased nitrite in newborns remains to be elucidated and should be more fully understood before efforts are made to study the potentially therapeutic use of nitrite in this patient population.

Summary

Before birth nitrate and nitrite concentrations in fetal blood are similar to those in maternal blood due to rapid passive exchange of the anions across the placenta. Within hours of birth, however, the nitrite concentration in the newborn falls sharply in association with increases in blood pressure, increases in pulmonary blood flow, and

many other adaptations to increasing oxygen tensions. In the early weeks of life nitrate and nitrite levels remain low for several reasons. There is limited ingestion of nitrate and nitrite because their concentrations are low in milk and formula. There is little reduction of nitrate to the physiologically active nitrite by oral bacteria. There is little generation of NO in the newborn stomach because the pH is high. Finally, there is enhanced urinary excretion of nitrite. The net result is that the recirculation of nitrate and nitrite as bioactive sources of NO is markedly lower in the newborn than in the adult.

In recent decades the many serious concerns that nitrite in the diet would cause cancer and methemoglobinemia have lessened and been replaced by new findings of cardiovascular benefits. In the newborn period there arises the prospect of protecting the GI tract from bacterial invasion by supplementation with nitrite, thereby increasing NO bioactivity and its protective actions. However, careful investigation must be done weighing the risks against the benefits before supplementation with nitrite can be undertaken safely in newborn infants.

Future Directions

One of the most exciting things about research is that in trying to answer one question, many more questions arise. The continual pursuit of knowledge is what drives science forward and it is a humbling honor to have been able to contribute my small piece of the puzzle. Our work has raised important questions regarding nitrite and nitrate bioactivity in newborn infants and will hopefully lay the groundwork for future studies to assess the therapeutic potential of nitrite. Indeed, exciting studies are currently underway

in our lab evaluating the protective potential of nitrite against hypoxia in the fetus and newborn.

It is important that future work explore the physiological effects of diminished nitrite and nitrate ingestion in newborn infants. To address the physiological implications of chronic low nitrate ingestion, changes could be observed in adults subjected to a low-nitrate diet. In addition, future work addressing how human milk fortifiers impact the nitrate and nitrite levels in breast milk would have important clinical relevance. It would also be interesting to see whether maternal consumption of a high nitrate diet could raise the nitrate/nitrite content in breast milk and whether this could be beneficial.

Every day we learn more about the importance of the symbiotic relationship we have with bacteria. Given the importance of oral nitrate-reducing bacteria, future studies should identify the age at which oral nitrate reductase activity becomes significant. It would also be fascinating to explore how bacterial colonization differs in preterm infants in the NICU versus healthy term infants and how early use of broad-spectrum antibiotics impact the development of the microbiome. Similarly, studies should be done to investigate whether seeding an infant's mouth with the mother's saliva would be beneficial for normal colonization of flora. In addition, the effects of chronic use of antibacterial mouth rinse, in the context of nitrate and nitrite bioactivity, remain untested. This has important clinical implications as prophylactic chlorhexidine[4] treatment is used to prevent the oral complications of leukemia (102), HIV (103), and prolonged intubation (104).

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[4] Chlorhexidine is the antibacterial mouthwash we used to stop nitrate-reducing activity by oral bacteria in our studies

In addition to decreased dietary intake and bacterial activation of nitrate, decreased gastric NO generation may be another mechanism responsible for the low plasma nitrite levels in newborns and the particularly low levels in preterm infants. Future studies should explore this important component of the nitrate-nitrite-NO axis in newborn infants. As a first step, we collected gastric fluid samples from eleven preterm infants (<32 weeks gestation), thirteen infants born between 32-37 weeks gestation (late preterm), and seven term infants (>37 weeks gestation). As expected for newborns, the average pH of the samples is relatively high (4.4 ± 0.3, preterm; 4.2 ± 0.1, late preterm; and 3.9 ± 0.3, term) and is higher than the pKa for nitrite reduction to nitrous acid, which is ~3.2. Consistent with the other aspects of the nitrate-nitrite-NO axis, we see that the generation of NO from nitrite is greatly diminished due to the high pH of these stomach contents. Figure 28 shows the rate of NO generation in gastric residual samples at various pHs, highlighting the dramatic decrease in NO production at higher pH.
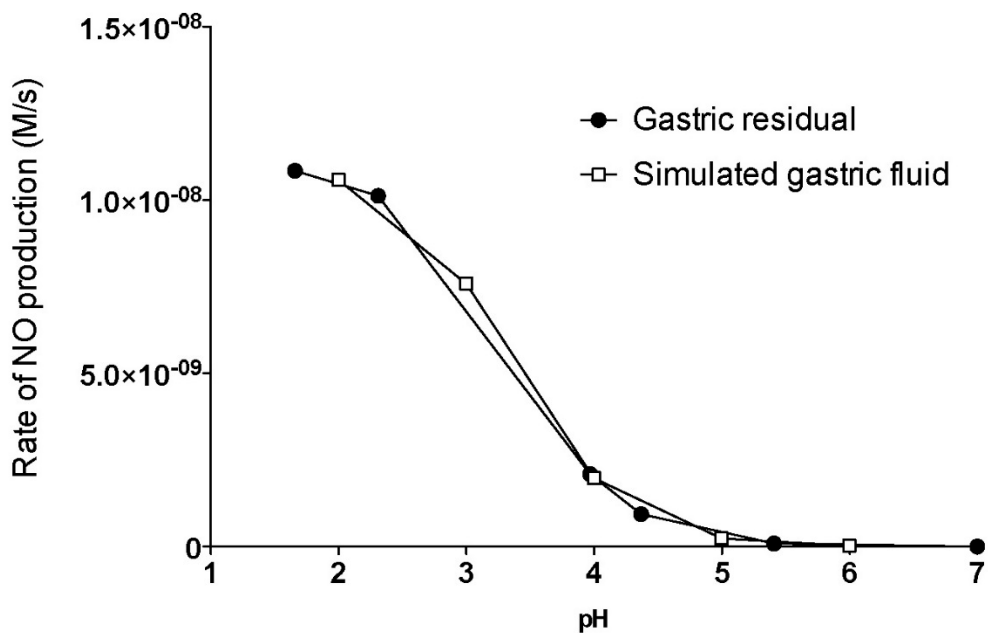

Figure 28. Rates of NO production from 50 μM nitrite added to gastric residual samples (●) and simulated gastric fluid (□) at different pHs.

However, we were surprised to find that the nitrite concentrations in these gastric

residuals are remarkably high in preterm infants (<32 weeks gestation) (10.8 ± 3.6 μM),

infants born at 32-37 weeks gestation (9.7 ± 2.4 μM), and in term infants (4.5 ± 2.0 μM),

as shown in Figure 29.
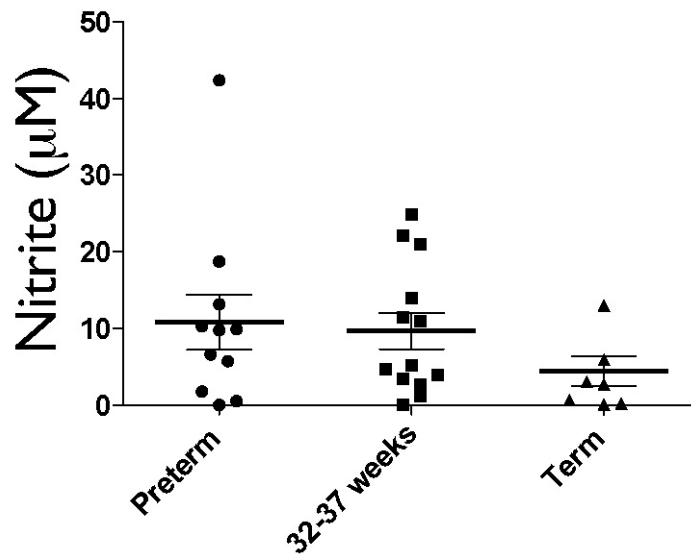
Figure 29. Mean nitrite concentrations measured in gastric residual samples collected from newborn infants during the first three weeks of life. Each data point represents the mean nitrite concentration in all the samples collected from one infant.

These levels are over a 100-fold higher than the plasma and 100-fold higher than

the levels in breast milk, as shown in Figure 30.

One future direction of our work is to explore this exciting finding. We have

generated a few hypotheses regarding the high nitrite levels in these gastric fluids and

herein present preliminary results that will be useful for determining future work.
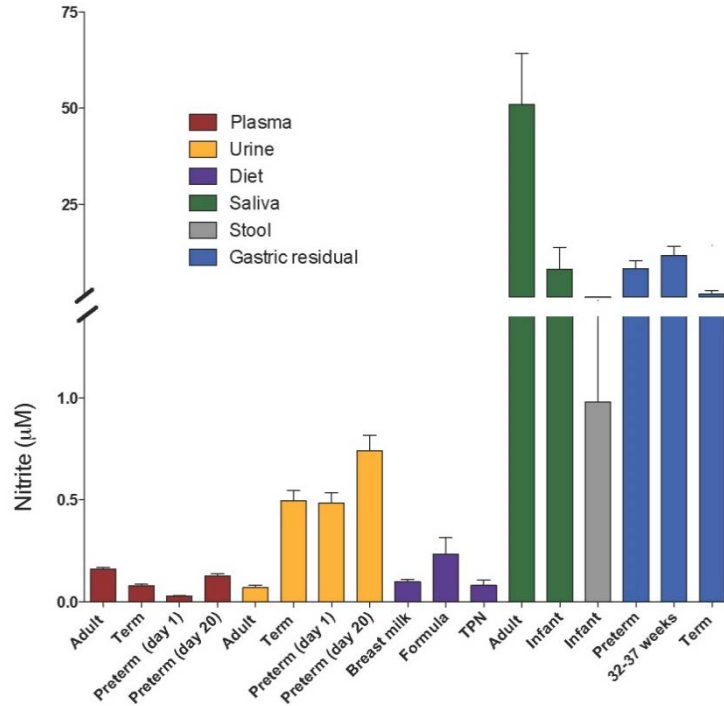
Figure 30. Comparison of the nitrite concentration in the plasma, urine, diet, saliva, stool, and gastric residuals of infants. Adult values were included when available.

Possible reasons for high gastric nitrite in infants include: nitrate reduction to nitrite by bacteria, accumulation of breakdown products of breast milk proteins, active transport across the stomach mucosa, co-transport of nitrite with protons, concentration of nitrite after loss of volume, and diminished reduction of nitrite to nitrous acid and NO because of the high gastric pH.

Nitrate reduction by bacteria seems unlikely because there is no nitrite production after nitrate is added in six of the seven gastric samples measured (Figure 31). Similarly, breakdown of milk proteins in an acid solution does not cause an increase in the nitrite concentration (data not shown).

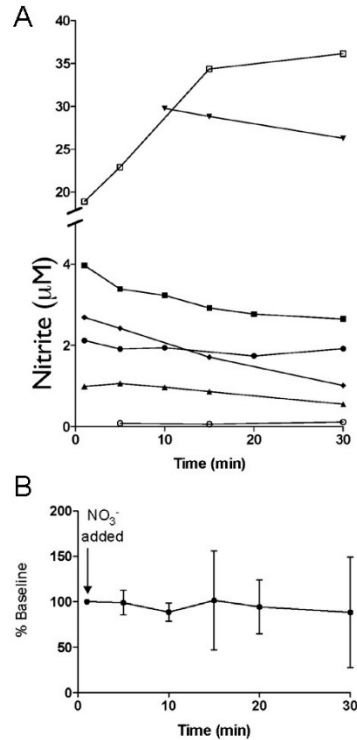*Figure 31*. Nitrate reduction in gastric residual samples. A) After additional nitrate (0.3 mM) was added to seven gastric residuals (represented by individual lines), nitrite concentrations were measured over 30 minutes (similar to the oral bacteria experiments described in Chapter 3). B) Mean data represented as % of baseline.

We have promising data from preliminary studies in adult rats testing the possibility that the high pH in the stomach of newborns is preventing nitrite in the stomach from being converted to nitrous acid and NO. When 4.0 mL of a high pH buffer (10mM bicarbonate buffer, pH 8.4), containing 0.02 mg/ml phenol red (a non-absorbable marker for stomach volume measurements) and 10 μM nitrite, was infused into the rat stomach, the nitrite concentration remained high over the 30 minutes. Conversely, when a low pH buffer (1% pepsin in NaCl and HCl, pH 2.0) was infused into the stomach, the nitrite concentrations fell rapidly during the 30 minutes (Figure 32). In both groups, we did not see an increase in the nitrite concentration over time despite volume loss,

indicating that concentration of nitrite because of loss of water is not a likely explanation for the high nitrite levels in the stomach.
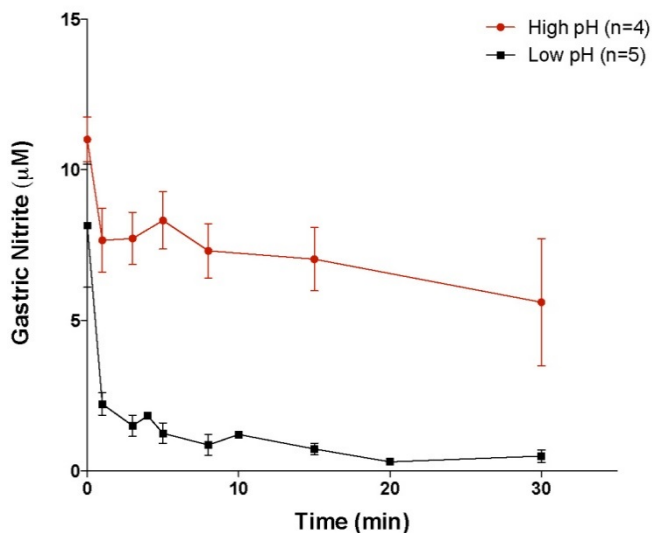

Figure 32. Nitrite concentrations in stomach fluid collected from rats after infusion of a low or high pH buffer into the stomach.

One intriguing hypothesis is that nitrite is actively pumped into the stomach from the plasma. To test this, in our preliminary studies we gave rats intravenous (I.V.) nitrite (0.5 mL of 300 μM) and measured the gastric nitrite levels over 30 minutes. As shown in Figure 33, when the stomach was filled with saline (pH 5.6) or bicarbonate (pH 8.4), it appears that the stomach nitrite concentrations rose as the plasma levels fell. However, when the stomach pH was low (pH 2.0), gastric nitrite levels remained below the level of detection for the entire 30 minutes. Interestingly, the nitrite concentration in the bile significantly increases after a gastric or IV bolus of nitrite, suggesting the liver may play an important role in regulating circulating nitrite levels. These preliminary experiments provide a starting point for future work that will hopefully shed more light into the stomach processing of nitrite when the gastric pH is altered, as it is in newborn infants.
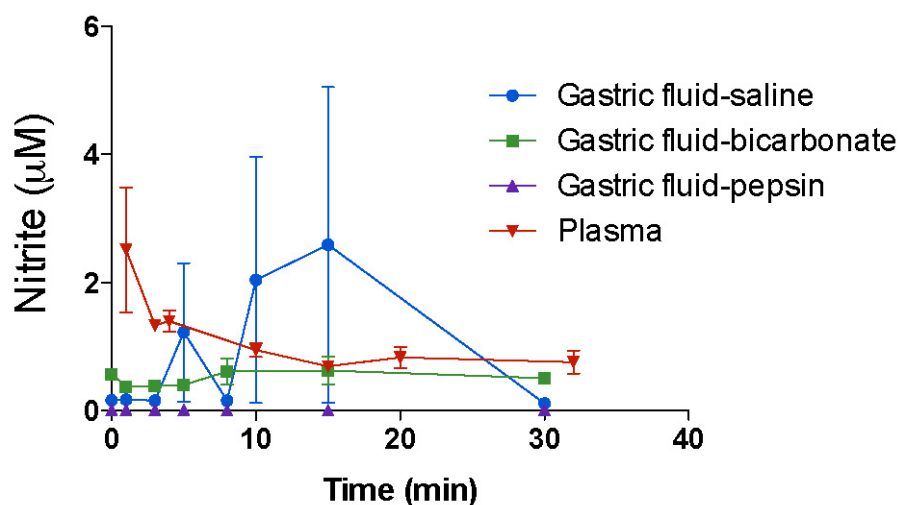
Figure 33. Nitrite concentrations in gastric fluid and plasma after 0.5 mL of 300 μM nitrite was administered IV.

The exciting and intriguing finding that the gastric contents of newborn infants contain high levels of nitrite deserves to be explored further. As a first step, we have ruled out a few hypotheses. It seems unlikely that nitrate reducing bacteria, acid breakdown of breast milk proteins, or simple concentration of nitrite due to water loss from the stomach, are responsible for high stomach nitrite. However, very preliminary work in rats suggest that nitrite may be actively pumped from the plasma into the stomach where, at a high pH, it can accumulate and lead to higher concentrations than can be explained by dietary intake. I urge the future generations of students in our lab and elsewhere to continue to explore this and other potential mechanisms.

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