ABSTRACT

FOGLEMAN, APRIL DANIELLE. Calcium and Phosphorus Supplementation in Human Donor Milk for Premature Infants. (Under the direction of Jonathan C. Allen).

Infants born prematurely are at risk for metabolic bone disease and may need increased minerals for normal bone mineralization. In these situations, supplementation of human milk with calcium and phosphorus is common in the United States; however, the bioavailability of these additives has not been proven. The goal of this research was to study the effect of calcium, phosphorus, and post-discharge formula fortification of donor human milk on the bioaccessibility of calcium, phosphate, and the digestibility of protein and fat. We hypothesized that fortification of donor milk with minerals and premature infant formulas would decrease bioaccessibility of calcium and phosphate as well as the digestibility of fat and protein.

An in-vitro model of the premature infant's gastrointestinal tract was modified from previous studies to simulate digestion and absorption. Calcium and phosphate were measured after in-vitro digestion in donor milk supplemented with Calcium Glubionate, Neutra-Phos (sodium/potassium phosphate), Calcium Glubionate and Neutra-Phos together, Enfamil® Enfacare, Similac® Human Milk Fortifier, and Similac® NeoSure. Additionally, protein, free fatty acids, and ionized calcium were measured before and after in-vitro digestion in milk with and without added Calcium Glubionate and Neutra-Phos.

The percent dialyzable calcium from donor milk was not significantly different from the percent dialyzable calcium in donor milk supplemented with calcium and donor milk supplemented with both calcium and phosphate together, but was significantly higher than percent dialyzable calcium in donor milk supplemented with Enfamil® Enfacare and

Similac® NeoSure. The dialyzable calcium was significantly greater in donor milk supplemented with calcium and donor milk supplemented with calcium and phosphate than in donor milk alone or with added phosphate, Enfamil® Enfacare, and Similac® NeoSure. Dialyzable calcium in donor milk supplemented with premature infant formulas was not significantly different from the dialyzable calcium in donor milk alone. Percent soluble calcium was significantly lower in donor milk supplemented with premature infant formulas than any other treatment.

Calcium and phosphorus supplementation did not negatively impact: total protein, protein breakdown, protein digestibility, or fat breakdown. Supplemental calcium increased ionized calcium, which may replace ionized calcium lost during milk expression, storage, and processing.

Addition of fortifiers to donor milk does not decrease calcium bioaccessibility. Donor milk fortification with post-discharge formulas does not increase calcium bioaccessibility or the amount of bioaccessible calcium. If a premature infant is at risk for bone disease, fortification with Calcium Glubionate and Calcium Glubionate with Neutra-Phos together will provide the most bioaccessible calcium.

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Calcium and Phosphorus Supplementation in Human Donor Milk for Premature Infants

by
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DEDICATION

This document and research is dedicated to the memory of the babies who have passed away and whose tenderhearted mothers continued to express their milk and generously donate it to the Human Milk Banking Association of North America to save the lives of other babies.

I also dedicate this dissertation to my husband, Allen Fogleman.

BIOGRAPHY

April Danielle Fogleman is originally from Georgia and moved to North Carolina in 1999. She has been a student at North Carolina State University since 2002, where she completed her Bachelor of Science degree in Biological Sciences, her Master of Science in Nutrition Science, and her doctoral degree in Nutrition Science. While working on her PhD, she earned the title of International Board Certified Lactation Consultant from the University of North Carolina at Chapel Hill, Gillings School of Global Public Health and expects to receive Registered Dietitian certification through the University of Northern Colorado in 2011. She will be working as Assistant Professor in Nutrition at North Carolina State University beginning in August 2011.

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CHAPTER 1: Literature Review

1.1. Human milk nutrition

Breastfeeding is the natural way of feeding human babies. The World Health Organization recommends exclusive breastfeeding for 6 months of age, with continued breastfeeding along with appropriate complementary foods for two years of age and beyond (23). In 2007 in the United States, 75% of infants were breastfed at least once after delivery; while only 33% were exclusively breastfed at 3 months and 13% at 6 months (1). Although it has long been recognized that breastfed babies somehow have fewer infections than formula-fed babies, we have only recently started to discover why. Human milk supplies infants with nutrition tailored to their specific needs. Human milk is remarkable and life-sustaining; it protects the infant from disease and efficiently transfers nutrients from the mother to the infant (4).

Newberg defines three aspects of lactation efficiency (2), which is responsible for providing the infant nutrition tailored to their specific needs. The first aspect of lactation efficiency is the way in which nutrients in the maternal diet are brought into the milk. Constant milk composition and synthesis are based on the needs of the infant rather than the nutritional status of the mother. De novo milk synthesis is balanced with the availability of preformed precursors of milk components from the mother's diet as well as body reserves. The second aspect of efficiency is that the rate of milk production matches the rate of milk expression, without limiting nutrition for the infant. The third aspect of efficiency is that milk components contribute to nutritional support of the infant as well as to immune system development and support (2).

Human milk provides many methods of protection against pathogens, including support and development of the innate and acquired immune system. Acquired immunity is immunity that develops with exposure to various antigens. Secretory Immunoglobulin A (SIgA), serum antibodies, and leukocytes are part of the acquired immune system and are provided to the infant through human milk. The concentration of these components depends on the mother's prior exposure to their target pathogens. Components of the innate immune system include constitutive components of human milk such as lysozyme, lactoferrin, and NEFA (3). Table 1.1 illustrates the immune benefits of breast milk (4).

Component	*Action*
White Blood Cells	
B lymphocytes	Give rise to antibodies targeted against specific microbes.
Macrophages	Kill microbes outright in the baby's gut, produce lysozyme and activate other components of the immune system.
Neutrophils	May act as phagocytes, ingesting bacteria in baby's digestive system.
T-lymphocytes	Kill infected cells directly or send out chemical messages to mobilize other defenses. They proliferate in the presence of organisms that cause serious illness in infants. They also manufacture compounds that can strengthen a child's own immune response.

Table 1.1 Immune benefits of breast milk at a glance (4)

Table 1.1 continued

Molecules	
Antibodies of secretory IgA class	Bind to microbes in baby's digestive tract preventing them from passing through walls of the gut into body's tissues.
B12 binding protein	Reduces amount of vitamin B_{12}, which bacteria need in order to grow.
Bifidus factor	Promotes growth of *Lactobacillus bifidus*, a harmless bacterium, in baby's gut. Growth helps crowd out dangerous varieties.
Fatty acids	Disrupt membranes surrounding certain viruses and destroy them.
Fibronectin	Increases antimicrobial activity of macrophages; helps repair tissues that have been damaged by immune reactions in baby's gut.
Gamma-interferon	Increases antimicrobial activity of immune cells.
Hormones and growth factors	Stimulate baby's digestive tract to mature more quickly. Once initially "leaky" membranes lining gut mature, infants become less vulnerable to microorganisms.
Lactoferrin	Binds to iron, a mineral many bacteria need to survive. By reducing available iron, lactoferrin thwarts growth of pathogens.
Lysozyme	Kills bacteria and viruses by disrupting their cell walls.
Mucins	Adhere to bacteria and viruses, keeping them from attaching to mucosal surfaces.
Oligosaccharides	Bind to microorganisms and keep them from attaching to mucosal surfaces.

Many studies have shown a protective effect of human milk against pathogens, including *Escherichia coli* (5), *Vibrio cholerae* (6), rotavirus (7), enterotoxigenic *E. coli* (8), *Camphylobacter* (9), and *Guardia duodenalis* (10).

The clinical significance of these immune factors has been demonstrated in reports that compared the morbidity and mortality of breastfed and formula-fed infants (11, 12).

Human milk protects infants from illnesses during and after breastfeeding. According to a meta-analysis by the Agency for Healthcare Research and Quality (13), health outcomes are considerably different for infants who are fed human milk versus formula and for mothers who provide their own milk versus those who do not. For infants, not being breastfed is associated with an increased incidence of infectious diseases, including gastroenteritis, otitis media, pneumonia, upper respiratory tract infections, diarrheal diseases, urinary tract infections, meningitis, and neonatal sepsis (13, 14). Additionally, long-term implications of not breastfeeding include an increased risk of childhood obesity, type I and type II diabetes, leukemia, Crohn's disease, ulcerative colitis, multiple sclerosis, rheumatoid arthritis, hypertension, celiac disease, and sudden infant death syndrome (13, 14). For mothers, not breastfeeding is associated with an increased incidence of premenopausal breast cancer, ovarian cancer, retained gestational weight gain, type II diabetes, and metabolic syndrome (13).

Glycosaminoglycans in human milk reduce the risk of transmission of HIV by preventing the binding of HIV gp120 to the CD4 receptor. Lipids in human milk contribute to innate immunity by providing activity against *Giardia lamblia*, *H. influenzae*, group B streptococci, *S. epidermidis*, respiratory syncytial virus, and herpes simplex virus type 1 (15).

If premature infants do not receive human milk they have an increased risk of necrotizing enterocolitis (NEC) (32). Human milk protects against NEC by decreasing pathogenic bacterial colonization, promoting the growth of non-pathogenic bacteria, promoting maturation of the intestinal barrier, and by ameliorating the pro-inflammatory response (16).

Of particular importance to the premature infant, human milk promotes development of the gastrointestinal tract by quickly reducing intestinal permeability (17) and inducing lactase activity (18), it contains multiple factors to stimulate growth, motility and maturation of the intestine (19), it empties from the stomach faster than formulas (20), and it leads to less residuals and faster realization of full enteral feedings (16).

1.2. Human milk banking

"When mother's own milk is not available, processed human milk from appropriately screened donors contains many of the immunoprotective and bioactive factors absent from commercial formula and is clearly the next best option for feeding both full-term and preterm infants." - Mary Rose Tully, IBCLC

When an infant is born prematurely, which is defined as less than 37 weeks of gestation (21), the mother may be too sick to provide her own milk. Additionally, when infants are born prematurely, feedings should be initiated as soon as possible because infants fed sooner are able to tolerate full oral feeds sooner, have fewer days of feeding intolerance, and have shorter hospital stays (22), but the mother's own milk may not be available in sufficient quantities. The mother's milk supply may be decreased for pre-term infants because their weak or inadequate suckling ability leads to inadequate stimulation for milk letdown and augmenting milk production or if the baby is in the neonatal intensive care unit, separation causes breastfeeding to occur less frequently (23). The World Health Organization recommends donor human milk as the next best option when mother's own milk is not available (24).

Human milk is the preferred source of nutrition for all infants according to the AAP (25) and the WHO (24). While mother's milk is usually the first choice to provide nutrition

to an infant, there are acceptable medical reasons for breast milk substitutes as summarized in Tables 1.2 to 1.6 (26).

Table 1.2 Infants who should not receive breast milk or any other milk except specialized formula
Infants with classic galactosemia: a special galactose-free formula is needed.
Infants with maple syrup urine disease: a special formula free of leucine, isoleucine and valine is needed.
Infants with phenylketonuria: a special phenylalanine-free formula is needed (some breastfeeding is possible, under careful monitoring).

Table 1.3 Infants for whom breast milk remains the best feeding option but who may need other food in addition to breast milk for a limited period
Infants born weighing less than 1500 g (very low birth weight).
Infants born at less than 32 weeks of gestational age (very pre-term).
Newborn infants who are at risk of hypoglycemia by virtue of impaired metabolic adaptation or increased glucose demand (such as those who are preterm, small for gestational age or who have experienced significant intrapartum hypoxic/ischaemic stress, those who are ill and those whose mothers are diabetic) (5) if their blood sugar fails to respond to optimal breastfeeding or breast-milk feeding.

Table 1.4 Maternal conditions that may justify permanent avoidance of breastfeeding
HIV infection: if replacement feeding is acceptable, feasible, affordable, sustainable and safe.

Table 1.5 Maternal conditions that may justify temporary avoidance of breastfeeding
Severe illness that prevents a mother from caring for her infant, for example sepsis.
Herpes simplex virus type 1 (HSV-1): direct contact between lesions on the mother's breasts and the infant's mouth should be avoided until all active lesions have resolved.
Maternal medication: - sedating psychotherapeutic drugs, anti-epileptic drugs and opioids and their combinations may cause side effects such as drowsiness and respiratory depression and are better avoided if a safer alternative is available; - radioactive iodine-131 is better avoided given that safer alternatives are available - a mother can resume breastfeeding about two months after receiving this substance; - excessive use of topical iodine or iodophors (e.g., povidone-iodine), especially on open wounds or mucous membranes, can result in thyroid suppression or electrolyte abnormalities in the breastfed infant and should be avoided; - cytotoxic chemotherapy requires that a mother stops breastfeeding during therapy.

Table 1.6 Maternal conditions during which breastfeeding can still continue, although health problems may be of concern
Breast abscess: breastfeeding should continue on the unaffected breast; feeding from the affected breast can resume once treatment has started
Hepatitis B: infants should be given hepatitis B vaccine, within the first 48 hours or as soon as possible thereafter
Hepatitis C.
Mastitis: if breastfeeding is very painful, milk must be removed by expression to prevent progression of the condition
Tuberculosis: mother and baby should be managed according to national tuberculosis guidelines
Substance use: - maternal use of nicotine, alcohol, ecstasy, amphetamines, cocaine and related stimulants has been demonstrated to have harmful effects on breastfed babies; - alcohol, opioids, benzodiazepines and cannabis can cause sedation in both the mother and the baby. Mothers should be encouraged not to use these substances, and given opportunities and support to abstain.

When breastfeeding or mother's milk feeding is not advised, donor milk feeding for infants is the next recommended option (24). Donor milk is mainly used for feeding preterm or critically ill infants in a hospital setting (27).

The Human Milk Banking Association of North America (HMBANA) is "a multidisciplinary group of health care providers that promotes, protects, and supports donor milk banking" (28). As of 2011, 10 donor milk banks in North America operate under the guidelines of HMBANA. Their role is to set standards and develop guidelines for donor milk banking. They develop protocols for its members, including protocols for donor screening, pasteurization, and post-pasteurization testing. Eleven HMBANA milk banks exist in North America and together they dispensed 409,077 ounces of milk in 2000 and 745,329 ounces in 2005 to hospitals in over 80 cities located in 29 states and 3 Canadian provinces (28). Participation in HMBANA and adherence to the HMBANA guidelines is voluntary. The guidelines, named *Guidelines for the Establishment and Operation of a Donor Human Milk*

Bank (29) were developed through collaboration between the American Academy of Pediatrics (AAP), the Centers for Disease Control and Prevention (CDC), and the Food and Drug Administration (FDA). Similar to the process used by blood banks, mothers interested in donating milk are first screened for unhealthy behaviors and infectious diseases. HMBANA milk banks do not pay donors for the milk. However, a processing fee is charged for each ounce of milk dispensed to cover the costs of screening donors, milk processing, and recordkeeping. Insurance companies may cover the fee if it is approved by the insurance company as a medical necessity. Modern milk banks may be located in large hospitals, while milk donated from the smaller hospitals and voluntary donors often needs to be temporarily stored before being transported to the milk bank (23).

In addition to the health benefits of donor human milk, which are similar to the benefits of human milk and have been discussed previously in this chapter, the use of donor milk results in cost savings to the hospital. Because donor human milk reduces the length of hospital stay compared to infant formula feeding, necrotizing enterocolitis, and sepsis in premature infants, there is a relative savings of about $11.00 - $37.00 to the hospital or health care plan for each $1.00 spent for donor human milk obtained from a HMBANA milk bank (27) or a cost savings of $9,669 for each infant with necrotizing enterocolitis (30). With the establishment of a non-profit milk bank, Torres et al (31) found that the increased availability of donor human milk resulted in a 23% reduction in the number of infants who receive formula at some time during their admission in the neonatal intensive care unit (NICU).

1.3. Nutritional requirements of premature infants

Infants are categorized as premature if they are less than 37 weeks gestation. While many premature infants are at risk for nutritional deficiencies, not all premature infants are the same. Infants born weighing less than 2500 g are referred to as being low birth weight (LBW). An infant weighing less than 1500 g is categorized as very low birth weight (VLBW). An infant weighing less than 1000 g is categorized as extremely low birth weight (ELBW), and the birth of an ELBW infant is a nutritional emergency because weight loss exceeds 10% of their body weight and it takes 10 days or longer to return to birth weight (32).

There are many advantages to feeding human milk to VLBW infants, including the amino acid and fat profile, which provide essential amino acids and fatty acids, cholesterol, and phospholipids, (33) the ease of digestibility of these proteins and fats (34), and the low renal solute load (35). The enzymes in the milk enhance maturation of the infant's immature gut (32). The living cells, immunoglobulins, and antibacterial factors protect the infant from infection and from NEC (32). Additionally, the mother is given the opportunity to participate in an important part of her baby's medical care, which may be good for her psychological well-being (27). An advantage of feeding premature infants milk from mothers who have delivered preterm is that the preterm milk is higher in many nutrients (Table 1.7). The composition is variable depending upon the degree of prematurity (21, 36).

Table 1.7 Nutritional composition of milk of mothers who deliver preterm

Milk of Mothers Who Deliver Preterm	
Levels Increased in Preterm Milk	*Levels Unchanged in Preterm Milk*
Total nitrogen	Volume
Protein nitrogen	Calories
Long-chain fatty acids	Lactose
Medium-chain fatty acids	Fat
Short-chain fatty acids	Linolenic acid
Sodium	Potassium
Chloride	Calcium
Magnesium	Phosphorus
Iron	Copper
	Zinc
	Osmolality
	Vitamin B_{12}

The standards for evaluating the nutritional outcome of a premature infant are poorly defined (32). However, Lawrence and Lawrence (32) outlined three main goals in feeding premature infants. First, help the infant achieve well-defined, standard short-term growth, such as intrauterine growth curves or mimicking body composition of a reference fetus. Second, prevent infant morbidities related to feeding, such as necrotizing enterocolitis and nosocomial infections. Third, optimize the infant's long-term neurodevelopmental and physical growth (32). Although feeding regimens vary, the focus is moving from

intrauterine-based, short-term growth and nutrient retention rates towards a strategy that considers long-term growth and health outcomes (21).

In support of early enteral feeding for premature infant, Lucas said, "It is fundamentally unphysiological to deprive an infant of any gestation of enteral feeding since the deprivation would never normally occur at any stage" (21, 37). Even before birth, infants swallow up to 150 mL/kg/day amniotic fluid from early gestation and it provides up to 3 g/kg of protein each day. Many studies support the practice of early enteral feedings. Schanler (1995) recommended that extremely low-birth-weight (ELBW) infants can be given 10 – 20 mL/kg/day of milk for 3 – 7 days before advancing the feeds (38). Bolus feeding led to shorter time to attain full oral feedings, less feeding intolerance, greater weight gain, and decreased morbidity (39). Meetze et al. (1992) found that infants fed sooner were able to tolerate full oral feeds sooner, had fewer days of feeding intolerance, and had shorter hospital stays (22).

Initiating feedings in the premature infant is a delicate balance between providing too little and too much milk. If the infant is given delayed and insufficient feeds, epithelial cells may atrophy and multi-organ system dysfunction may occur, increasing the risk for injury of the intestinal mucosa, which may lead to invasion of pathogenic bacteria (32). When too little milk is given, gut maturation may not occur, and when too much milk is given the digestive capacity is overwhelmed and injury to the brush border membrane can occur (40).

Estimates of energy requirements for LBW and ELBW infants range from approximately 109 kcal/kg/day (41) to 120 kcal/kg/day (32) in order to achieve optimal growth rates, which many clinicians view as close to the 50^{th} percentile on the growth charts. To achieve a positive protein balance, Brumberg and La Gamma recommend 3.5 – 4.0

g/kg/day of protein because ELBW infants miss the last trimester in utero, when fat and protein are stored, and they lose about 1.1 – 1.5 g/kg of stored protein daily (40). The protein requirements for LBW infants are based on intrauterine accretion rates of 2.5 g/100 kcal or 325 mg/kg body weight/day (35). Protein content of human milk averages 1.09 g/dL and fortified human milk is 2.2 g/dL, although fortified human milk can achieve 3 – 3.5 g/kg/day (32).

The recommendations for fat intake are based on the essential fatty acid proportion as 3% of total caloric intake. The essential fatty acid requirement is met adequately by human milk because nine percent of the lipids are composed of linoleic acid. Infants less than 1500 g are able to absorb 90% of human milk fat and 68% of cow milk fat (42).

1.4. Nutritional adequacy of donor milk and mother's milk for premature infants

As the age of viability decreases with medical advancements, clinicians who care for preterm infants are faced with feeding decisions for which there are no clear guidelines and minimal scientific data to update optimal nutritional strategies (43). Infants born prematurely are often in need of donor milk and there is concern among clinicians that it does not cause as rapid weight gain as does formula. Although an infant fed donor human milk when mother's own milk is not available may not grow as fast as an infant fed formula, they may still be exhibiting a normal, healthy growth trajectory. If the goal of feeding premature infants is to get them to grow at the same rate as term infants, they could experience "catch-up growth," which may set them up for obesity and diabetes later in life (43).

Slow weight gain is not necessarily a problem unless the infant is exhibiting signs of "failure to thrive," which is defined as a rate of weight gain less than the -2 standard

deviation value during an interval of two months or longer for infants less than six months of age, or three months or longer for infants over six months of age, and the weight for length being less than the 5^{th} percentile (44).

The most favorable growth for infants born prematurely is considered to be the growth curve they would have followed had they remained in utero (32). Although formula fed infants grow faster than human milk fed infants, they have higher body fat percentages (45) and an increased risk for type two diabetes and obesity later in life (13, 46).

Although donor human milk provides many nutrients, it would require an excessive and unrealistic volume to achieve adequate amounts of some nutrients. However, growth needs can be met by using donor human milk as a "base" and adding needed nutrients back into the milk through supplementation, while conserving the components of human milk that are essential to normal growth and development, and that are not present in formula, such as immunoglobulins, lactoferrin, lysozyme, growth factors, enzymes, anti-inflammatory factors, cytokines, and oligosaccharides.

Premature infants fed donor human milk may not grow as fast as infants fed formula, but they have higher survival rates and shorter hospital stays. Schanler et al. (1999) found that using fortified human milk was associated with decreased infections and more rapid achievement of full feeds and slower weight gain did not result because feeding tolerance was improved (39). Concerns such as slower growth and loss of important biological components of human milk due to processing were not considered sufficient a reason to deny infants donor human milk when mother's own milk is not available when compared with effectiveness at preventing infections and necrotizing enterocolitis (NEC) and for improving long-term neurological and cardiovascular outcomes (47).

The benefits of human milk compared to formula are widely known and accepted. However, there is more skepticism among health care providers and parents regarding the health benefits of donor human milk. A Cochrane review in 2007 (48) evaluated randomized, controlled trials in preterm and low birth weight infants and found a significantly higher incidence of NEC in formula fed infants compared to donor human milk-fed infants. Boyd et al. (2007) also evaluated randomized, controlled trials that compared donor human milk to formula on incidence of NEC and found that a diet of exclusive donor human milk reduces NEC by 79% (48).

Many health care providers are concerned that feeding donor milk from mothers of full-term infants results in a slower growth rate than does feeding mother's own milk in preterm infants (37, 49). However, there is question as to whether weight gain is the best measure of optimal health outcomes and how much weight gain is optimal. Recently, there has been interest among the scientific community in the concept of "fetal programming,'' in which an insult or stimulus applied at a critical or sensitive period may have long-term or lifetime effects on the structure or function of an organism (37). Singhal et al. provided evidence that diets promoting faster growth in infants increase later cardiovascular disease risk (50-53). Lucas et al. (1992) found that even with slower weight gain, preterm infants fed human milk had significantly higher IQ scores at school age (54).

As previously discussed, human milk protects the infant from pathogens in the environment through specific antibody-targeted mechanisms, such as IgA, IgG, sIgA, and IgM, as well as broad-spectrum mechanisms, such as lactoferrin and lysozyme. The protein κ-casein can act against *Helicobacter pylori* and free fatty acids and monoglycerides formed from the hydrolysis of milk triglycerides have antiviral and antiprotozoan activity (4, 15).

However, in order to prevent the transmission of certain viral pathogens in human milk, the Human Milk Banking Association of North America (29), the United Kingdom Association for Milk Banking (55), and other national milk banking guidelines for donor milk require that Holder pasteurization (62.5°C for 30 minutes) be done on all the donated milk. Holder pasteurization eliminates the threat of viruses and pathogens, such as HIV (56), HTLV-1 (57), and CMV (58, 59), as well as common bacterial contaminants (60); however, it also eliminates the B- and T-cell mediated immunity of milk (61, 62). As seen in Table 1.8 (63), even though some beneficial non-nutritive components of human milk are destroyed along with viruses and pathogens during Holder pasteurization, the nutritive components still remain as do many beneficial non-nutritive components that are not found in infant formulas.

Table 1.8 Changes in selected components of human milk after freezing and pasteurization

Selected Components of Human Milk after Freezing and Pasteurization	
Component of Human Milk	*Percentage Activity*
IgA and sIgA	67 – 100
IgM	0
IgG	66 – 70
Lactoferrin	27 – 43
Lysozyme	75
Lipoprotein lipase	0
Bile salt activated lipase	0
Monoglycerides	100
Free fatty acids	100
Linoleic acid	100
α-linolenic acid	100

1.5. Calcium and phosphorus in human milk

The milk of many species contains high concentrations of calcium and phosphorus (64). Minerals and caseins in milk are in dynamic equilibrium between the soluble and micellar phases, and the partitioning depends upon temperature, minerals, and the pH (65).

When milk pH decreases from 6.7 to 6.0, soluble calcium increases by 20% and soluble phosphorus by 15% (65). The soluble calcium is more bioavailable to the infant (66).

Calcium and phosphate can form many different types of complexes, such as dicalcium phosphate, dicalcium phosphate dihydrate, micellar calcium phosphate, octacalcium phosphate, β-tricalcium phosphate, hydroxyapatite, amorphous calcium phosphate, tricalcium citrate dihydrate, and dimagnesium phosphate (64, 67). They can also exist as amorphous or crystallized. Additionally, calcium binds casein molecules in proportion to the number of phosphoserine residues present on the casein molecule because they are the main cation-binding sites. There is a significant amount of calcium phosphate and calcium citrate bound to casein micelles. Calcium phosphate aids in stabilization of the casein micelle (67, 68).

When the pH of milk is decreased, organic and inorganic phosphate is protonated, calcium phosphate and the citrate and magnesium associated with the casein micelles are dissolved, with the extent depending upon the temperature and pH (65, 67). At a pH of 5.2, one part of calcium and all of the inorganic phosphate are solubilized. Between the pH of 5.2 and 6.7, there is a correlation between solubilized calcium and solubilized inorganic phosphate. At the pH of 3.5, calcium is completely solubilized. The effects of pH on the calcium complexes are irreversible, thus increasing pH will not reconstruct the micellar calcium phosphate, leaving the calcium in a more bioavailable form (67).

Heat treatment of milk at equal to or greater than 90 degrees Celsius for several minutes will result in decreased solubility of calcium and phosphate. However, if heat treatment is less than 90 °C, as is the case in the Holder pasteurization method used to

pasteurize donor milk, the modifications are reversible. When milk is cooled, the solubility of calcium and phosphate increases, but this effect is also reversible (67).

When ionic strength is increased in milk by adding NaCl, there is a slight decrease in pH, which increases the solubility of calcium. These changes are caused by exchanges of divalent cations, such as calcium, bound to the phosphoserine residues of casein with sodium (67, 68). The increase in ionic strength causes a decrease in activity coefficients of the ions, resulting in dissociation of the ion pairs (67).

1.6. Calcium and phosphorus interaction with other nutrients

Calcium, phosphate, and magnesium are partly bound to the casein micelles in milk (67). About one-third of calcium, half of the phosphate, two-thirds of magnesium, and over 90% of citrate are in the aqueous fraction of milk. Some calcium is bound to α-lactalbumin (67).

In the diffusible fraction of milk, interactions between ions depend upon the affinity between the cations and the anions as well as the solubilities (64, 67). Calcium is present as ionized calcium, as a complex with citrate, inorganic phosphate, and chloride. Although calcium may complex with inorganic phosphate, a low quantity of this complex exists due to their low solubilities. Sodium and potassium are usually present as free ions, but some can complex with citrate, inorganic phosphate, and chloride. At a pH of 6.6 – 6.7, calcium and phosphate are more likely to complex with one another (67).

1.7. Development of an in-vitro digestion method

The amount of a nutrient available for absorption is termed bioaccessibility and can be measured using an *in vitro* digestion system followed by analyses of the nutrient of interest. Calcium must be in a soluble form, usually ionized or bound to a soluble organic molecule before it can cross the intestinal wall (69). Although the measurement of bioaccessibility can give an estimate of the amount of a nutrient available for absorption, it does not take into account the absorptive capacity of the intestines, which is affected by physiological factors such as calcium reserves and hormonal regulation.

Previously described methods of *in vitro* digestion (69-71) are not completely representative of the premature infant's gastrointestinal tract because they were either developed to simulate an adult's gastrointestinal tract or did not take into account the increased amount of fat digestion that occurs in the infant's stomach. In order to adapt an *in vitro* digestion to simulate the premature infant's gastrointestinal tract, lipase should be added in the gastric phase because it has been shown that there is a high degree of gastric lipolysis, even in premature infants (72). *Rhizopus niveus* is a lipase with a similar specificity for fatty acids as the gastric lipase, which is responsible for a significant portion of fat digestion in the infant, preferentially hydrolyzing the fatty acids at the Sn-1 and Sn-3 positions of glycerol (73). Additionally, when modeling an infant's gastrointestinal tract, a gastric pH of 5 should be used because gastric contents of gavage-fed premature infants maintain a pH greater than 5 for the entire postprandial period (73).

1.8. Comparison of various methods to estimate bioavailability

Bioavailability of certain nutrients can be measured in-vivo by methods such as classical balance studies, isotope balance methods, urinary excretion of an oral calcium load, measuring isotopes labeled in blood, urine, or bone, long-term evaluation of bone mineralization, and measuring of biological markers in the blood or urine (74). Bioavailability is most accurate when measured using in vivo studies done on humans. However, these studies are labor and time intensive and they are expensive and may yield variable results. Additionally, the use of radioisotopes commonly used in studies to measure bioavailability may be hazardous, especially to infants (71). In vivo studies with laboratory animals are less expensive than in vivo human studies, but they are limited by differences between human and animal metabolism. An alternative to measuring bioavailability by performing human and animal in vivo studies is to measure bioaccessibility through simple and inexpensive *in vitro* methods (75).

Bioaccessibility of certain nutrients can be measured in-vitro by methods such as dialysis, ultrafiltration, and cell-culture models as well as with animal models. Use of an *in vitro* digestion method, with pepsin used in the gastric stage and pancreatin and bile salts used during the intestinal stage, as well as measurement of the nutrient diffusing across a semipermeable membrane during the intestinal stage is used as a measure for the element's availability, yields satisfactory results for bioavailability of the nutrients of interest (76-84). Zemel et al. (78) and Schwartz & Nevins (77) reported that there is not a good correlation between *in vitro* calcium solubility and in vivo calcium bioavailability. Absorption percentages may be higher in vivo due to many physiological factors present which are not

active *in vitro*. Although it is preferable to carry out experiments on animals rather than humans for ethical and financial reasons, the use of animal models is not ideal for measuring bioaccessibility because the main species used, rats, pigs, guinea pigs, and primates, are not completely representative of the premature infant's gastrointestinal tract (74).

1.9. Calcium and phosphate metabolism in the premature infant

Calcium is the most abundant mineral in the human body, with 99% of body calcium located in bone and the remaining 1% located in soft tissues and extracellular fluid. The full-term newborn has about 30 g of body calcium, while a 24-week preterm infant has only 10% to 15% this value, or 3.0 to 4.5 g body calcium (85). From 28 to 40 weeks of gestation, fetal calcium content quadruples due to increased bone mineralization (86). Approximately 85% of body phosphorus is located in bone and 15% in soft tissues and extracellular fluid (87). The term newborn has about 16 g of body phosphorus (87).

Approximately 80% of calcium and phosphorus accretion occurs during the third trimester, between 24 and 40 weeks of gestation (87). Infants born preterm miss this period of calcium and phosphorus accretion, and as a result, failure to meet mineral requirements results in insufficient bone mineralization. Failure to meet the nutritional requirements of the preterm infant may lead to metabolic bone disease, also called rickets or osteopenia of prematurity (87). In order to optimize bone mineralization and prevent metabolic bone disease, the American Academy of Pediatrics recommendations for preterm infant formulas are 140 – 160 mg of calcium per 100 kcal and 95 – 108 mg of phosphate per 100 kcals (88). ESPGAN recommendations for preterm infant formulas are 70 – 140 mg calcium per 100 kcal and 50 – 87 mg phosphate per 100 kcal (88). While these recommendations are meant

to serve as guidelines to ensure adequate calcium and phosphorus intake for the preterm infant, they do not take into account the variable absorption of calcium from various sources, which depend upon many factors other than the amount of calcium provided. For example, these recommendations do not take into account the type of calcium salt, the amount or type of fat in the diet, the type of milk, and the processes used for infant formula manufacturing (89).

Phosphorus is absorbed by passive diffusion in the jejunum and depends upon the amounts of dietary phosphate as well as the relative concentrations of dietary calcium and phosphate and about 80 – 90% of phosphate is absorbed (87). Calcium is absorbed in the intestine by active and passive mechanisms. In children and infants, the majority of calcium is absorbed by passive transport, which is paracellular diffusion of calcium down a chemical gradient. Active transport of calcium is vitamin D-dependent and is not expressed in preterm infants (88).

Parathyroid hormone, vitamin D, and calcitonin are the three main hormones involved in calcium homeostasis. Table 1.9 summarizes the effect of these hormones on calcium homeostasis.

Table 1.9 Hormonal effects on calcium and phosphorus concentrations (88)

Hormone	*Effect on serum calcium*	*Effect on serum phosphorus*
Parathyroid hormone	Increase	Decrease
Vitamin D	Increase	Increase
Calcitonin	Decrease	Decrease

When serum calcium is elevated, parathyroid hormone secretion is inhibited and when serum calcium is decreased, parathyroid hormone secretion is stimulated. Parathyroid

hormone increases serum calcium levels by stimulating osteoblasts, which increases bone resorption. In the kidney, parathyroid hormone aids in calcium resorption and decreases phosphate resorption. It also increases 1-α-hydroxylase activity, which hydroxylates 25-hydroxyvitamin D, converting it to the active 1,25-dihydroxyvitamin D, which aids in intestinal absorption of calcium (88).

The overall action of 1,25-dihydroxyvitamin D is to increase serum calcium and phosphorus concentrations. Vitamin D increases the synthesis of the calcium binding protein, calbindin, which mediates intracellular diffusion of calcium, leading to increased calcium absorption. Vitamin D increases phosphorus absorption. In the bone, 1,25-dihydroxyvitamin D mobilizes calcium and phosphorus by increasing the number of osteoclasts. In the kidney, 1,25-dihydroxyvitamin D increases calcium and phosphorus resorption and provides negative feedback to 1-α-hydroxylase, the enzyme that activates 25-hydroxyvitamin D.

As the preterm infant grows, the calcium and phosphorus requirements remain higher than those of a term infant. These mineral requirements are summarized in Table 1.10.

Table 1.10 Recommended daily enteral intakes for calcium and phosphorus from the American Academy of Pediatrics (88)

	Preterm	*Term*
Calcium	120 – 230 mg/kg/day	210 – 270 mg/day
Phosphorus	60 – 140 mg/kg/day	100 – 275 mg/day
Vitamin D (IU/day)	400	200

When the infant is discharged from the hospital, he or she may need to continue to consume a greater amount of calcium than human milk alone may provide (88). Some authors recommend that infants born prematurely should be fed a calcium-enriched formula

instead of a standard term formula or human milk when discharged from the hospital because it results in increased growth and bone mineralization (88, 90). However, formula-fed infants usually are heavier than breastfed infants. In the DARLING study, anthropometric indexes of infants from 1 to 24 months were compared between matched cohorts of infants either breastfed or formula fed until 12 months of age. Formula fed infants had a significantly greater weight-for-length percentiles and body fat percentages (45). Additionally, human milk contains lactoferrin, which can have powerful anabolic, differentiating, and antiapoptotic effects on osteoblasts, inhibitory effects on the development of osteoclasts, and is an important physiological regulator of bone growth (91).

1.10. Summary

Studies comparing the health outcomes of supplementing human milk with fortifiers are conflicting, but it is widely known that the nutritional needs of premature infants exceed that which is in human milk alone. Fortification of human milk should be done in order to provide nutrients needed by the premature infant, while preserving the beneficial bioactive components in human milk.

In the United States, supplementation of human milk with calcium and phosphorus is common; however, the bioaccessibilities of the wide variety of these additives have not been compared or studied. Calcium and phosphorus are important for bone mineralization (88), but the addition of these minerals to human milk may affect the bioavailability of calcium, phosphate, and other nutrients, such as protein and fat, because they interact with many components in human milk (67). The calcium and phosphorus recommendations do not take

into account the type of calcium salt, fat, milk, or processes used for infant formula manufacturing (89).

Therefore, there is a need to study the effect of common human milk supplements such as calcium, phosphorus, and premature infant formula on nutrients in human milk. As a result, in the present study, bioaccessibility was estimated on the basis of simulated gastrointestinal digestion and calcium solubility and dialysability. We hypothesized that fortification of donor human milk with minerals and premature infant formulas will decrease the bioaccessibility of calcium and phosphate.

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CHAPTER 2: Effect of calcium, phosphorus, and premature infant formula supplementation on calcium and phosphorus bioaccessibility in preterm human donor milk

2.1 Abstract

Background: Infants born prematurely are at risk for metabolic bone disease and may need increased minerals for normal bone mineralization. In these situations, supplementation of human milk with calcium and phosphorus is common in the United States. The bioavailability of these additives has not been proven. The primary goal was to study the effect of calcium, phosphorus, and premature infant formula fortification of donor human milk on the bioaccessibility of calcium and phosphorus by developing an *in vitro* model that simulates digestion of the premature infant. We hypothesized that fortification of donor human milk would decrease the bioaccessibility of calcium and phosphorus.

Methods: We developed a simulated premature infant digestion system to measure the bioaccessibility of calcium and phosphate in donor human milk supplemented with common nutritional fortifiers used in neonatal intensive care units. Calcium and phosphate were measured after in-vitro digestion in donor milk supplemented with Calcium Glubionate, Neutra-Phos (sodium/potassium phosphate), Calcium Glubionate and Neutra-Phos together, Enfamil® Enfacare, Similac® Human Milk Fortifier, and Similac® NeoSure. Additionally, time course experiments of calcium and phosphorus bioaccessibility were performed to determine the rate and kinetics of calcium and phosphate equilibration across the dialysis membrane.

Results: The percent dialyzable calcium from donor milk was not significantly different from the percent dialyzable calcium in any of the treatment groups; however, it was significantly greater in donor milk supplemented with calcium than in donor milk supplemented with premature infant formulas. Dialyzable calcium was significantly greater in donor milk supplemented with calcium and donor milk supplemented with calcium and phosphate than in donor milk alone or with added phosphate, Enfamil® Enfacare, and Similac® NeoSure. Dialyzable calcium in donor milk supplemented with premature infant formulas was not significantly different from the dialyzable calcium in donor milk alone.

Conclusions: Addition of fortifiers to donor milk did not decrease calcium bioaccessibility. Donor milk fortification with premature infant formulas did not increase calcium bioaccessibility. If a premature infant is at risk for bone disease, fortification with Calcium Glubionate and Calcium Glubionate with Neutra-Phos together will provide the most bioaccessible calcium.

Funding Sources: Mothers' Milk Bank, San Jose, CA

2.2. Introduction

Calcium is the most abundant mineral in the human body, with 99% of body calcium located in bone and the remaining 1% located in soft tissues and extracellular fluid. The full-term newborn has about 30 g of body calcium, while a 24-week preterm infant has only 10% to 15% of this value at 3.0 to 4.5 g body calcium (1). From 28 to 40 weeks of gestation, fetal calcium content quadruples due to increased bone mineralization (2). Approximately 85% of body phosphorus is located in bone and 15% in soft tissues and extracellular fluid (3). The term newborn body contains about 16 g of phosphorus (3).

Approximately 80% of calcium and phosphorus accretion occurs during the third trimester, between 24 and 40 weeks of gestation (3). Infants born preterm miss this period of calcium and phosphorus accretion, and as a result, failure to meet mineral requirements results in insufficient bone mineralization. Failure to meet the nutritional requirements of the preterm infant may lead to metabolic bone disease, also called rickets or osteopenia of prematurity (3). In order to optimize bone mineralization and prevent metabolic bone disease, the American Academy of Pediatrics recommendations for preterm infant formulas are 140 to 160 mg of calcium per 100 kcal and 95 to 108 mg of phosphate per 100 kcals (3). The European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGAN) recommendations for preterm infant formulas are 70 to 140 mg calcium per 100 kcal and 50 to 87 mg phosphate per 100 kcal (3). While these recommendations are meant to serve as guidelines to ensure adequate calcium and phosphorus intake for preterm infants, they do not take into account the variable absorption of calcium from different sources, which depend upon many factors other than the amount of calcium provided. For example,

these recommendations do not take into account the type of calcium salt, the amount or type of fat in the diet, the type of milk, and the processes used for infant formula manufacturing (4).

Phosphorus is absorbed by passive diffusion in the jejunum and depends upon the amounts of dietary phosphate as well as the relative concentrations of dietary calcium and phosphate; about 80 – 90% of phosphate is absorbed (3). Calcium is absorbed in the intestine by active and passive mechanisms. In children and infants, the majority of calcium is absorbed by passive transport, which is paracellular diffusion of calcium down a chemical gradient. Active transport of calcium is vitamin D-dependent and is not expressed in preterm infants to a significant extent (5).

As the preterm infant grows, the calcium and phosphorus requirements remain higher than those of a term infant. These mineral requirements are summarized in Table 2.1.

Table 2.1 Recommended daily enteral intakes for calcium and phosphorus from the American Academy of Pediatrics (5)

	Preterm	*Term*
Calcium	120 – 230 mg/kg/day	210 – 270 mg/day
Phosphorus	60 – 140 mg/kg/day	100 – 275 mg/day
Vitamin D (IU/day)	400	200

Following discharge from the hospital, it is recommended that preterm infants continue to consume a greater amount of calcium than human milk alone may provide (5). Some authors recommend that infants born prematurely should be fed a calcium-enriched formula instead of a standard formula or human milk when discharged from the hospital because it results in increased growth and bone mineralization (5, 6). However, when

markers of bone mineralization of premature infants fed human milk (donor milk or mother's milk), pre-term formula, or human milk fortified with calcium, phosphorus, or preterm formula were compared, there were no significant differences between groups (7-9). Faerk et al. (2000) found that infants fed preterm formula had a significantly higher weight at term compared with infants fed only their own mother's milk, but did not differ in length or head circumference (9). Fewtrell et al. (2009) showed that the proportion of human milk in the diet was significantly positively associated with bone mineral content (8). A Cochrane review of human milk fortifiers found that fortification of human milk with multicomponent fortifiers is associated with short-term increases in rates of weight gain, length, and head circumference, but it was not clear if there is an effect on bone mineral content (10).

In the present study, we developed a simulated premature infant digestion system to measure the bioaccessibility of calcium and phosphate in donor human milk supplemented with common nutritional fortifiers used in neonatal intensive care units. Bioaccessibility has been defined as the fraction of a mineral that is soluble in the gastrointestinal environment and available for absorption (11). While bioaccessibility estimates calcium and phosphate that is soluble in gastrointestinal fluids, it may provide a good estimate of the potential bioavailability of the minerals to preterm infants who depend primary on passive calcium absorption through the leaky tight junctions of the intestinal epithelium. We hypothesized that fortification of donor human milk with minerals and premature infant formulas will decrease the bioaccessibility of calcium and phosphate.

Dialyzable calcium, percentage dialyzable calcium, and bioaccessible phosphate were measured in donor milk as well as donor milk supplemented with calcium, phosphate, calcium and phosphate, Similac® Human Milk Fortifier, Similac® Neosure, and Enfamil®

Enfacare to compare the bioaccessibility of calcium and phosphate of the various supplements. With the purpose of determining the time needed for complete dialysis of calcium and phosphate, time course experiments were performed in which the samples were dialyzed and calcium and phosphate bioaccessibility was measured at various time points.

2.3 Materials and methods

2.3.1. Preparation and in-vitro digestion of donor milk samples

Samples

Preterm donor human milk was shipped from the San Jose Mother's Milk Bank (San Jose, CA) to our laboratory. Mothers who donated the milk gave signed consent that their milk may be used for research purposes, as is policy for all donations to milk banks of the Human Milk Banking Association of North America (HMBANA). The study was approved by the Institutional Review Board at North Carolina State University. Five batches of preterm donor milk were used in the study and treated according to standard fortification protocols used in neonatal intensive care units (NICU). It is standard protocol for HMBANA milk banks to pool milk from 4 – 5 mothers in order to reduce variability of the nutritional composition. Therefore, milk from 20 – 25 mothers was included in the analyses. Each sample was treated seven ways: 1) no treatment to serve as a control; 2) addition of 0.15 mL Calcium Glubionate per 1 mL milk; 3) 0.23 mL Neutra-Phos (sodium phosphate and potassium phosphate) per 1 mL milk; 4) 0.15 mL Calcium Glubionate and 0.23 mL Neutra-Phos per 1 mL milk; 5) 0.064 g Enfamil® Enfacare per 1 mL milk; 6) 0.1563 g Similac® Human Milk Fortifier per 1 mL milk; and 7) 0.072 g Similac® NeoSure per 1 mL milk. The

calcium and phosphate content of each treatment is listed in Table 2.1. The quantities of the infant formula supplements were added based on recommendations in NEOFAX (12), the nutritional guide used in neonatal intensive care units. Quantities of Calcium Glubionate and Neutra-Phos were based on the calcium and phosphate fortification protocol used at Lucille Packard Children's Hospital at Stanford University.

Table 2.2 Calcium and phosphate content of fortifiers (12)

Treatment Group	Calcium Added (µg/mL)	Phosphate Added (µg/mL)
Donor Milk	0	0
Donor Milk + Calcium	1130.83	0
Donor Milk + Phosphate	0	725
Donor Milk + Calcium & Phosphate	1130.83	725
Donor Milk + Similac® Human Milk Fortifier	493.33	527.75
Donor Milk + Similac® Neosure	416.66	191
Donor Milk + Enfamil® EnfaCare	394.16	171

In Vitro Digestion Protocol

An *in vitro* digestion model was developed to simulate the gastrointestinal tract of the premature infant. The model was modified from those described previously (13, 14). In the gastric phase, 0.2 g pepsin (Sigma, St. Louis, MO) was dissolved in 5 mL of 0.1 N HCl and 0.25 mL was added to each 4 mL sample of donor milk. Additionally, 1.7 g lipase with

similar specificity as human milk lipase (15) (Sigma, St. Louis, MO) was dissolved into 15 mL 0.1 N HCl and 1.5 mL was added to each donor milk sample. Lipase was added in the gastric phase because it has been shown that there is a high degree of gastric lipolysis, even in premature infants (16). The low pH optimum (2.5 to 6.5), the absence of requirements for cofactors or bile salts, and resistance to pepsin digestion enable lipase to remain active in the infant's stomach and contribute significantly to fat digestion (15, 16). The donor milk samples were adjusted to pH 5.0 by addition of HCl or NaOH and then placed in a shaking water bath at 37˚C for two hours. The donor milk samples were placed on ice for 10 minutes to stop digestion.

In the intestinal phase, 0.05 g pancreatin (Sigma, St. Louis, MO) and 0.3 g bile extract (Sigma, St. Louis, MO) were dissolved in 25 mL of 0.1 M $NaHCO_3$ and 1.25 mL of this solution was added to each donor milk sample. In order to add 17.2 mU of lactase (Sigma, St. Louis, MO) to each donor milk sample, 0.25 g of lactase was dissolved in 200 mL H_2O, and 2 µL was added to each donor milk sample. Donor milk samples were adjusted to a pH of 7.0 by 1 M $NaHCO_3$ and to a final volume of 10 mL by addition of cell culture grade water (Sigma, St. Louis, MO). The donor milk samples were placed in a shaking water bath at 37˚C for two hours. The donor milk samples were placed on ice for 10 minutes to stop digestion and they were adjusted to pH 7.0.

After the gastric and intestinal phases, the samples were centrifuged at 3500 x *g* for 1 hour at 4˚C. Aliquots of the supernatant were transferred to tubes and stored at -20˚C until further analysis, unless samples were analyzed within 24 hours, in which case they were stored at 4˚C.

2.3.2. Dialysis of donor milk digests

Dialysis was completed using Spectra/Por® Float-A-Lyzer® G2 (Model G235067, Spectrum Labs) dialysis tubing with a molecular weight cutoff of 8000 to10000 D. The Spectra/Por® Float-A-Lyzer® was submerged and allowed to soak in deionized water for 15-30 minutes. The hydrated membrane was not allowed to dry out.

Using a pipette, 10 mL of the previously digested sample was added to the inside of the membrane. The cap was replaced and the membrane was placed inside a glass tube that contained 25 mL of either a solution of 0.9% NaCl with 1% albumin or 0.9% NaCl, pH 7. In this experiment, all samples were dialyzed with and without albumin in the buffer in order to determine the effects of having a protein with moderate calcium binding in the dialysate on the final distribution of calcium and phosphate.

The solutions dialyzed for 24 hours, except in the case of the time course experiments, after which time the volumes in the inside and outside of the membranes were measured. The contents on the inside and outside of the dialysis membrane were removed and analyzed for total calcium concentration.

In a separate experiment to determine the rate and kinetics of calcium and phosphate equilibration across the dialysis membrane, total calcium and phosphate concentrations were measured at 1, 4, 8, 12, and 24 hours during dialysis with and without albumin in the buffer. The initial conditions placed the digested milk mixtures inside the dialysis tubing, and either 0.9% NaCl with 1% albumin or 0.9% NaCl as dialysate on the outside. Additionally, total calcium and phosphate concentrations were measured after days 1, 2, 3, 4, and 5 of dialysis with albumin in the buffer to look for long-term changes in the redistribution of calcium and phosphate in the dialysis system.

2.3.3. Biochemical assays

2.3.3.1. Calcium analysis

After dialysis, total bioaccessible calcium was measured by analyzing the calcium content of the dialysate by atomic absorption spectrophotometry (Perkin Elmer Model 3100, Norwalk, CT). Calcium standards were made at calcium concentrations of 0.5 ppm to 10 ppm in buffer containing 0.01N HCl with 0.5% lanthanum oxide. The digested samples were diluted in buffer containing 0.01N HCl with 0.5% lanthanum oxide so they could be measured within the range of the calcium standards.

2.3.3.2. Calcium bioaccessibility

Calcium bioaccessibility is estimated by the equilibrium dialysis of calcium in this system and it is the amount of soluble calcium that disappeared from the dialysis tubing during the 24-hour time period of dialysis. It was determined by adding the calcium content measured inside the dialysis tubing to the calcium content outside of the dialysis tubing after equilibrium.

2.3.3.3. Percent calcium bioaccessibility

Percent calcium bioaccessibility was calculated by dividing the dialyzable calcium by the total calcium content of the sample and multiplying by 100.

2.3.3.4. Total phosphate assay

Phosphate concentration was determined using a phosphate colorimetric assay kit (BioVision K410-500). The assay uses a preparation of malachite green and ammonium

molybdate which forms a chromogenic complex with phosphate ions, resulting in an absorption band around 650 nm. The kit can directly determine phosphate concentrations between 1 μM and 1 mM, with a lower limit of detection of approximately 0.1 nmol.

The assay was performed after *in vitro* digestion on all donor milk samples. A microplate reader (Multiskan EX, Thermo Electron Corp., Vantaa, Finland) was used to measure absorbance at 650 nm. A standard curve was created by plotting absorbance at 650 nm as a function of phosphate concentration. The standard curve was used to determine the phosphate concentration of each unknown sample.

First, 0 to 200 μL of each standard and sample was added to the wells of the 96-well microplate and the volume of each well was adjusted to 200 μL with distilled water. Next, 30 μL of the phosphate reagent was added to all standard and sample wells. The samples were mixed for 30 seconds on a plate shaker and incubated for 30 minutes at room temperature. After the 30-min incubation period, the absorbance of each sample was measured at 650 nm in triplicate.

The equation resulting from the standard curve was used to determine the phosphate concentration of each sample. The equation is as follows:

Phosphate concentration = [(absorbance – intercept) / (slope)] x (DF), where

Absorbance: average of the absorbencies of wells for each sample
Intercept: y-intercept from the standard curve graph
Slope: slope from the standard curve graph
DF: dilution factor used to dilute human milk samples (dilution factor used varied among samples)

2.4. Statistical analysis

Experiments were performed in triplicate for analysis of total calcium and phosphate. Statistical analysis was performed using JMP (SAS, Inc., Cary, NC). One-way analysis of variance (ANOVA) with the Tukey post-hoc test to describe the relation between means was used, and $P < 0.05$ was regarded as statistically significant.

2.5. Results

2.5.1. Percent dialyzable calcium

When albumin was included in the dialysis buffer, the percentage of dialyzable calcium in donor milk without added supplements was not significantly different from percent dialyzable calcium in donor milk supplemented with additional minerals or premature infant formula (Figure 2.1). Percent dialyzable calcium was significantly greater in donor milk supplemented with calcium than in donor milk supplemented with premature infant formulas. Addition of the premature infant formulas, Similac® Human Milk Fortifier, Similac® Neosure, and Enfamil® Enfacare decreased percent dialyzable calcium, although the decrease was not statistically significant.

When albumin was not used in the dialysis buffer, percent dialyzable calcium in donor milk without added supplements was not significantly different from percent dialyzable calcium in donor milk supplemented with calcium, phosphate, calcium and phosphate together, or with Similac® Human Milk Fortifier, but it was significantly greater than percent dialyzable calcium in donor milk supplemented with Similac® Neosure and Enfamil® Enfacare (Figure 2.1).

When the two methods of dialysis are compared, with and without use of albumin in the dialysis buffer, there are no statistically significant differences in percent dialyzable calcium between the two groups (Figure 2.1).

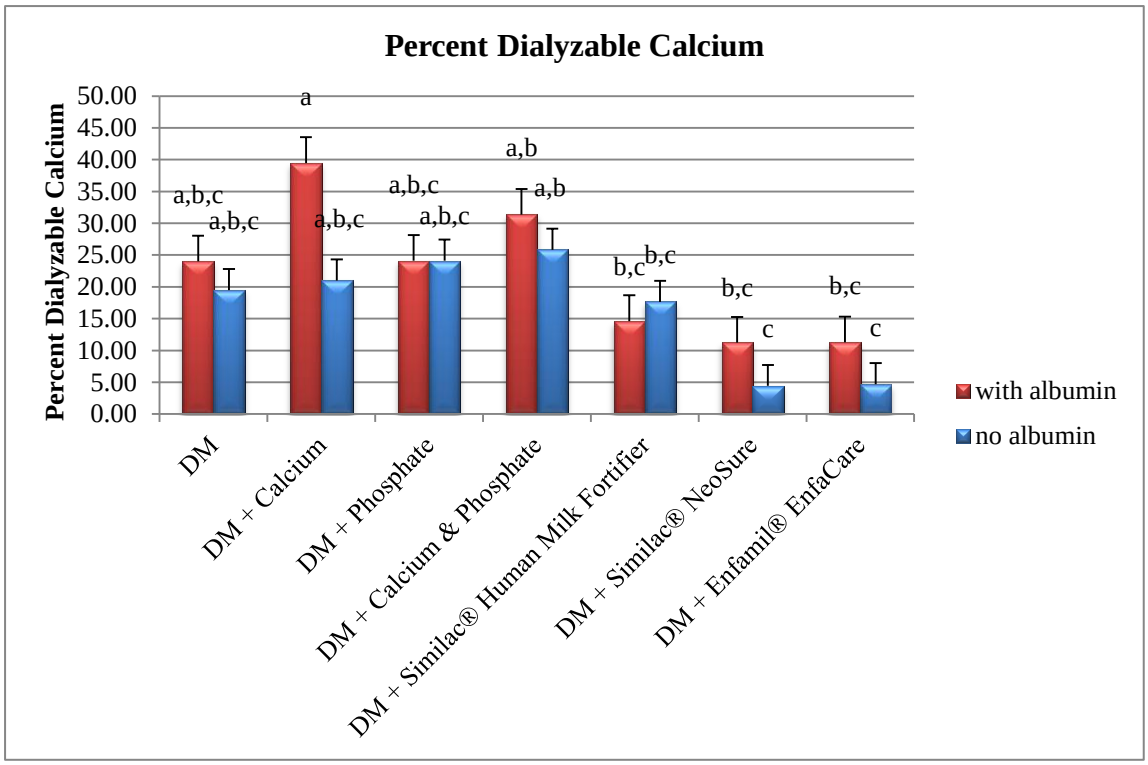

Figure 2.1 Percent bioaccessible calcium with and without albumin in the dialysis buffer. (DM=donor human milk).

2.5.2. Dialyzable calcium

When albumin was used in the dialysis buffer, the dialyzable calcium was significantly greater in donor milk supplemented with calcium and with calcium and phosphate together than in donor milk and donor milk supplemented with phosphate, Similac® Human Milk Fortifier, Similac® Neosure, and Enfamil® Enfacare (Figure 2.2). Addition of the premature infant formulas Similac® Human Milk Fortifier, Similac® Neosure, and Enfamil® Enfacare, did not increase calcium bioaccessibility from donor milk without added supplements.

When albumin was not used in the dialysis buffer (Figure 2.2), the dialyzable calcium was significantly greater in donor milk supplemented with calcium and with calcium and phosphate together than in donor milk and donor milk supplemented with phosphate, Similac® Human Milk Fortifier, Similac® Neosure, and Enfamil® Enfacare. Addition of the premature infant formulas, Similac® Human Milk Fortifier, Similac® Neosure, and Enfamil® Enfacare, did not increase calcium bioaccessibility from donor milk without added supplements.

When the two methods of dialysis are compared, with and without use of albumin in the dialysis buffer, the only milk mixture with a statistically significant difference in dialyzable calcium between the two dialysis buffers was observed in donor milk supplemented with calcium. Dialyzable calcium in donor milk supplemented with calcium was significantly greater when albumin was used in the dialysis buffer than if it was not used.

When albumin was present in the dialysis buffer, it increased the mean bioaccessibility of calcium. When albumin was not present in the dialysis buffer, phosphate increased the bioaccessibility of the calcium added as Calcium Glubionate (but not the native

calcium from the donor milk) perhaps by binding to compounds that would otherwise bind to calcium, rendering it less bioaccessible.

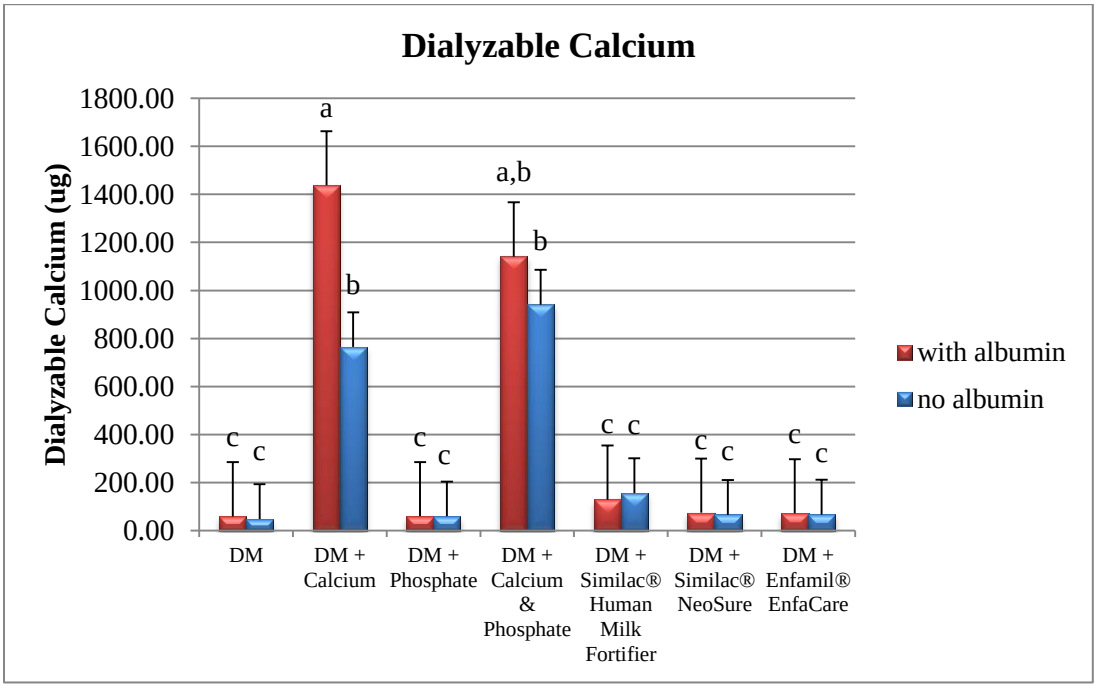

Figure 2.2 Total bioaccessible calcium with and without albumin in the dialysis buffer

2.5.3. Bioaccessible phosphate

When albumin was used in the dialysis buffer, there were no statistically significant differences in bioaccessible phosphate between the treatment groups (Figure 2.3). When albumin is not used in the dialysis buffer, there are no statistically significant differences in bioaccessible phosphate between the treatment groups (Figure 2.3). When the two methods of dialysis were compared (2.3), with and without use of albumin in the dialysis buffer, there were no statistically significant differences in bioaccessible phosphate between the two groups. Although it is not statistically significant, there was a trend for an increase in bioaccessible phosphate when donor milk is supplemented with calcium in the form of Calcium Glubionate or premature infant formula. Two-way ANOVA showed no significant treatment, albumin, or interaction effects.

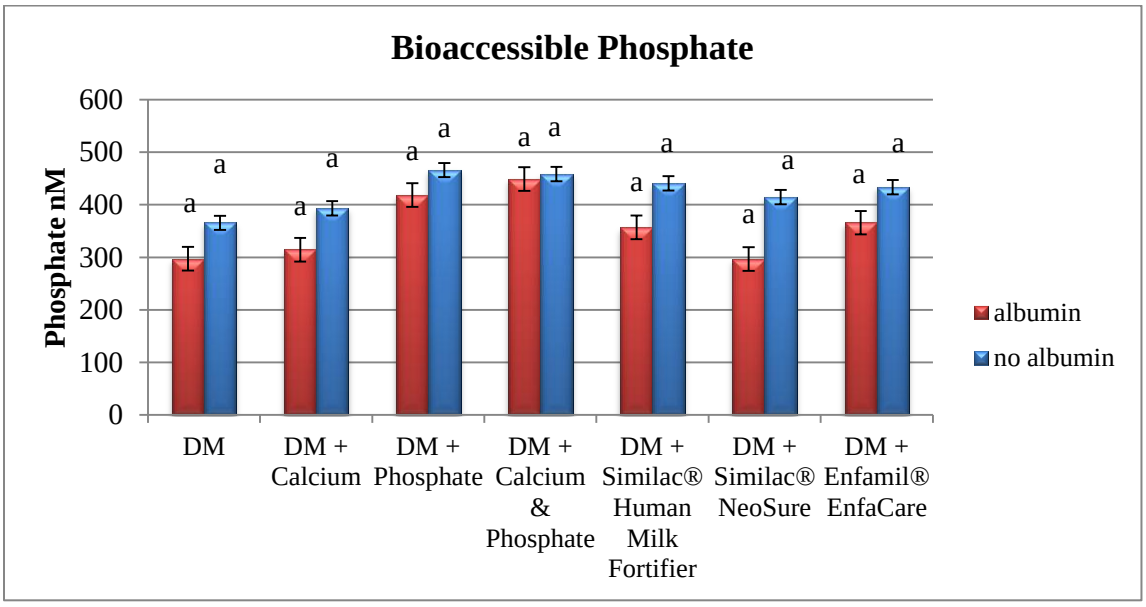

Figure 2.3 Bioaccessible phosphate with and without albumin in the dialysis buffer

2.5.4. Change in bioaccessible calcium concentrations during 24 hours of dialysis

Regardless of whether albumin was used in the dialysis buffer, bioaccessible calcium was greatest in donor milk supplemented with calcium and phosphate (Figures 2.4 and 2.5). When digests of donor milk and donor milk supplemented with Enfamil® Enfacare were dialyzed for 24 hours, with and without albumin in the buffer, bioaccessible calcium concentrations came to equilibrium at approximately 24 hours. Bioaccessible calcium in donor milk supplemented with calcium and phosphate together did not come to equilibrium after 24 hours when albumin was included in the dialysis buffer (Figure 2.4) but it did come to equilibrium when albumin was not included in the dialysis buffer (Figure 2.5).

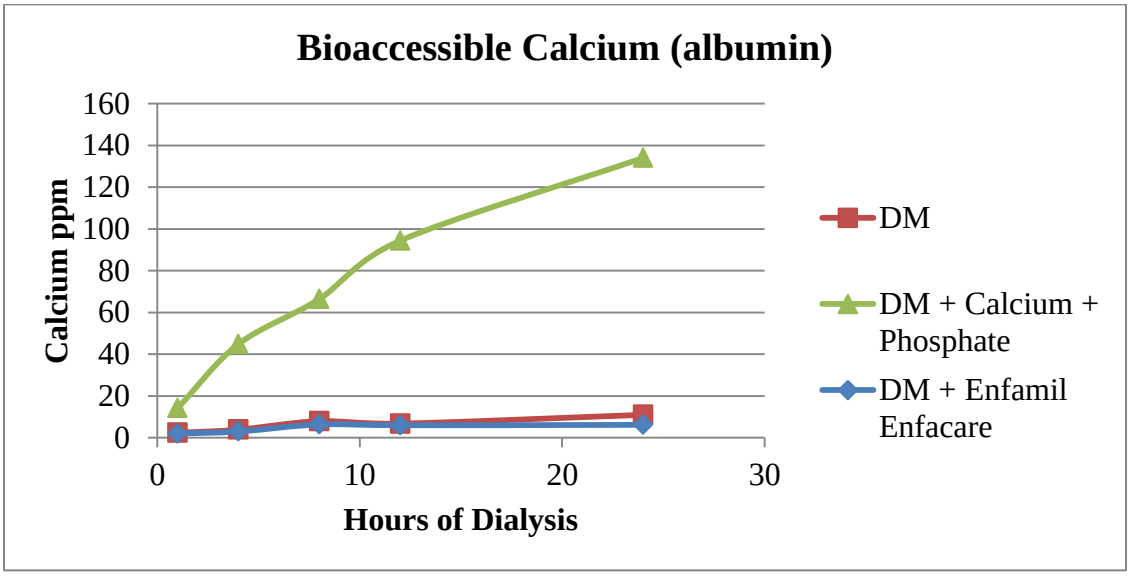

Figure 2.4 Change in bioaccessible calcium during 24 hours of dialysis with albumin in the dialysis buffer

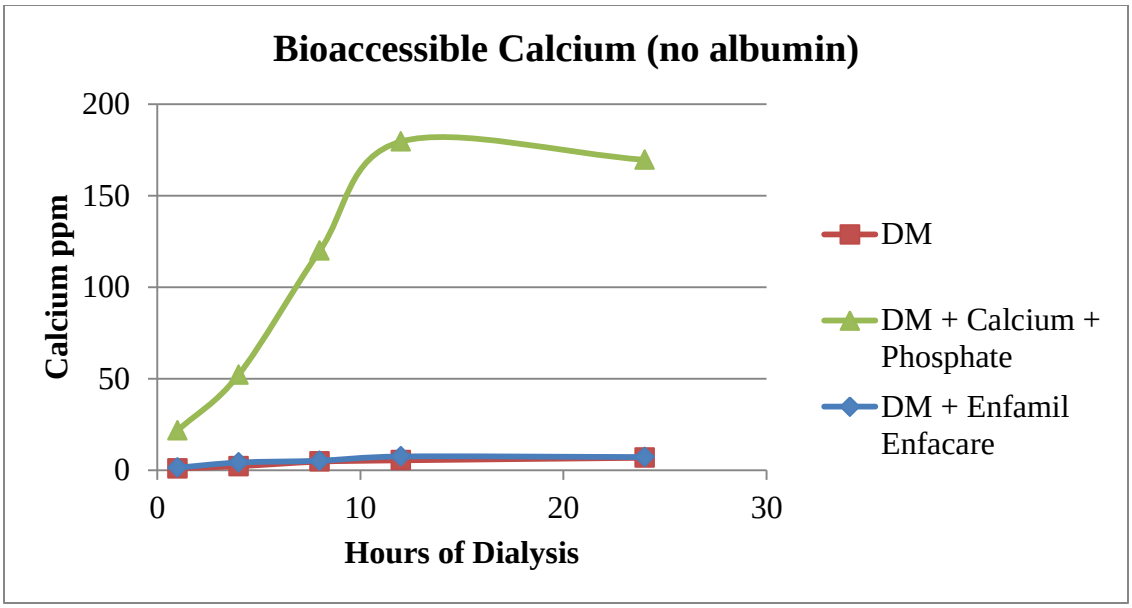

Figure 2.5 Change in bioaccessible calcium during 24 hours of dialysis without albumin in the dialysis buffer

2.5.6. Change in bioaccessible phosphate concentration during 24 hours of dialysis

Regardless of whether albumin was used in the dialysis buffer, bioaccessible phosphate was greatest in donor milk supplemented with calcium and phosphate (Figures 2.6, 2.7). Bioaccessible phosphate plateaued at approximately 10 hours when digests of donor milk, donor milk supplemented with calcium and phosphate, and donor milk supplemented with Enfamil® Enfacare were dialyzed for 24 hours. Bioaccessible phosphate concentrations came to equilibrium at approximately 10 hours.

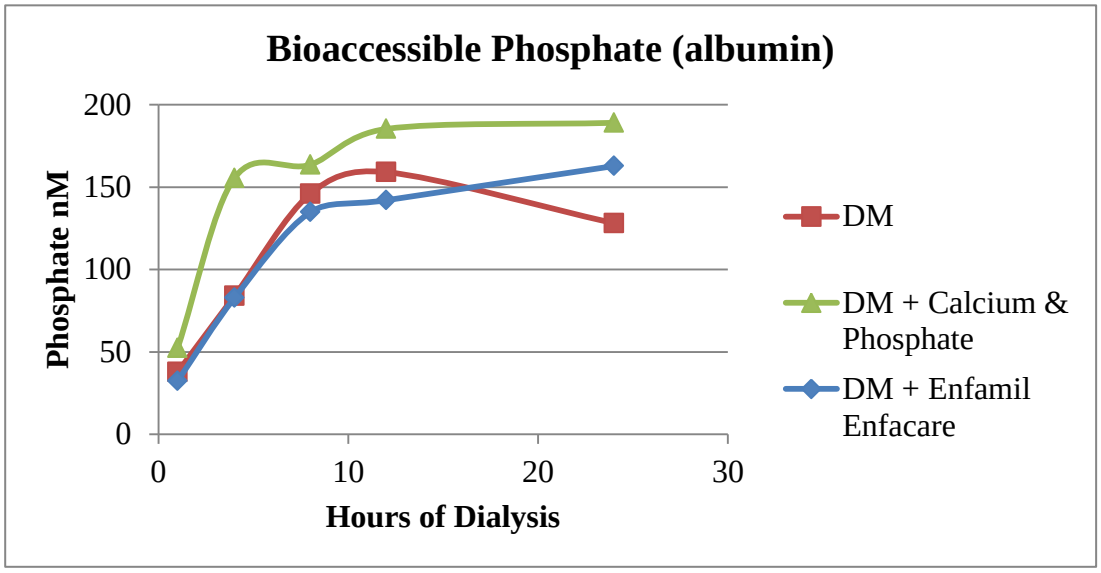

Figure 2.6 Change in bioaccessible phosphate during 24 hours of dialysis with albumin in the dialysis buffer

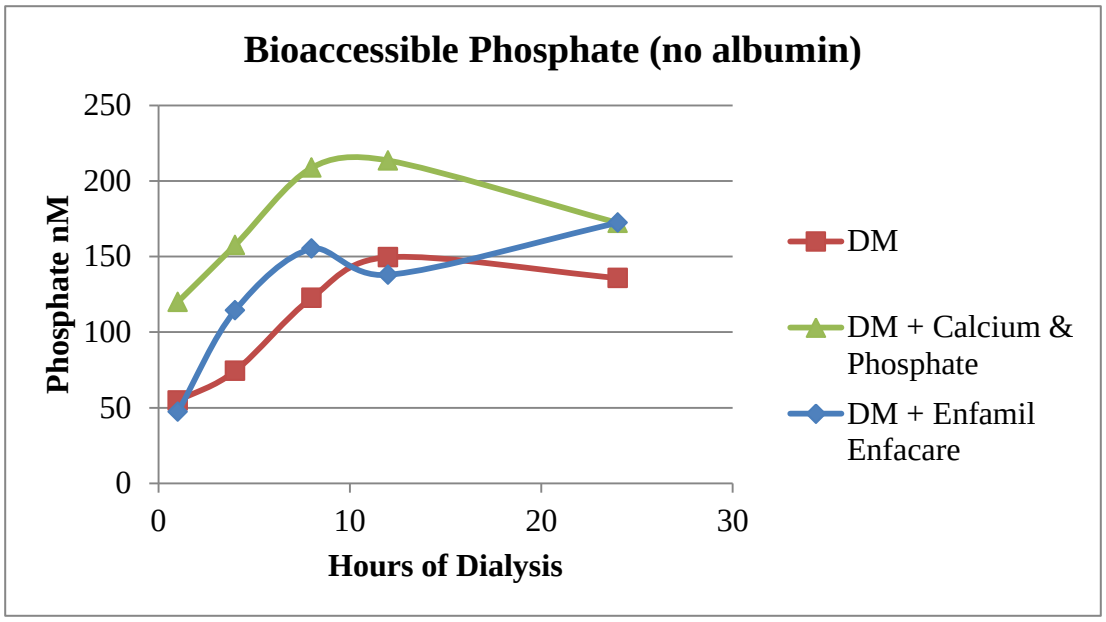

Figure 2.7 Change in bioaccessible phosphate during 24 hours of dialysis without albumin in the dialysis buffer

2.5.6. Quantity of calcium dialyzable during successive 24-hour intervals

The results of the time course data shown in Figure 2.8 indicate that when albumin was used in the dialysis buffer and is replaced every 24 hours, most of the calcium was dialyzed within the 5 days. In donor milk supplemented with calcium or calcium plus phosphate, there was an exponential washout of the calcium over a period of 4 days, but most was removed within the first 24 hours. In all treatment groups, 20 – 40% of the calcium is dialyzed within 24 hours and 40 – 70% within 5 days (Figure 2.9).

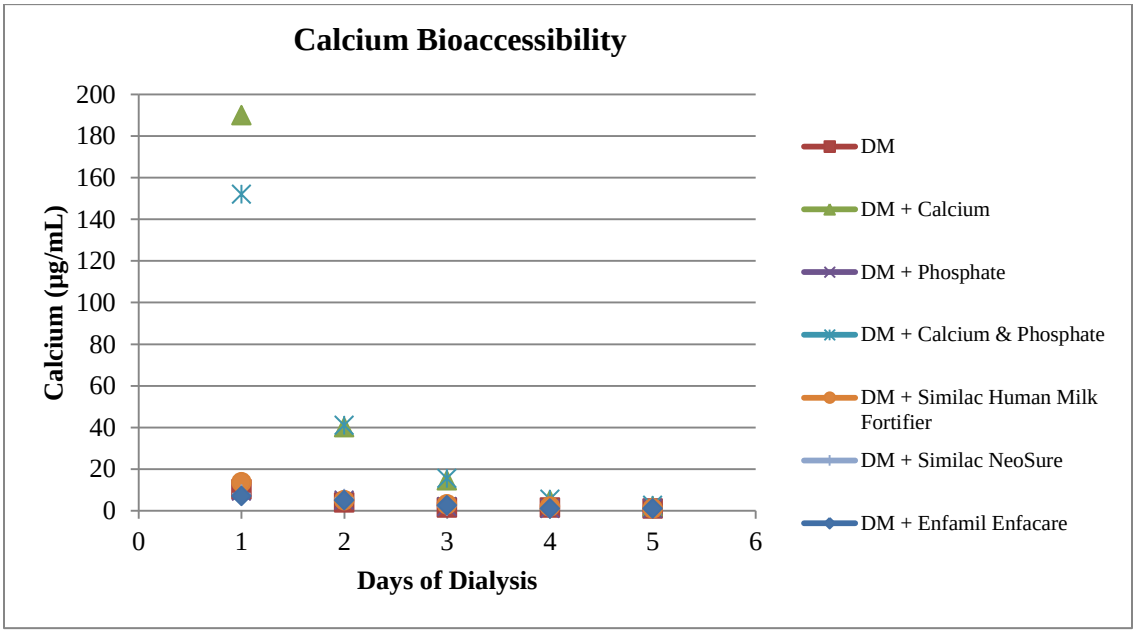

Figure 2.8 Quantity of calcium dialyzable during successive 24-hour interval with albumin in the dialysis buffer

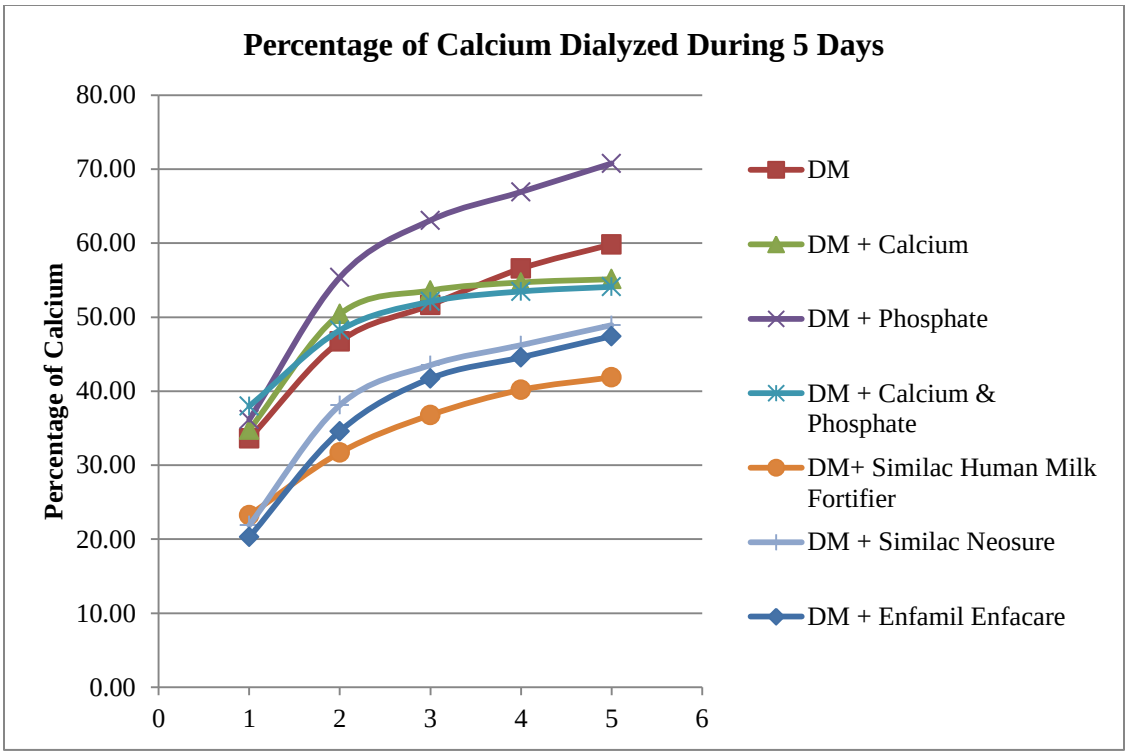

Figure 2.9 Percentage of calcium dialyzable during successive 24-hour interval with albumin in the dialysis buffer

2.5.7. Change in phosphate bioaccessibility during 5 days of dialysis

The results of the time course data shown in Figure 2.8 indicate that when albumin was used in the dialysis buffer and is replaced every 24 hours, most of the phosphate was dialyzed within the 5 days (Figure 2.10). In all treatment groups, approximately 25 – 30% of phosphate was dialyzed within 24 hours and 40 – 50% was dialyzed within 5 days.

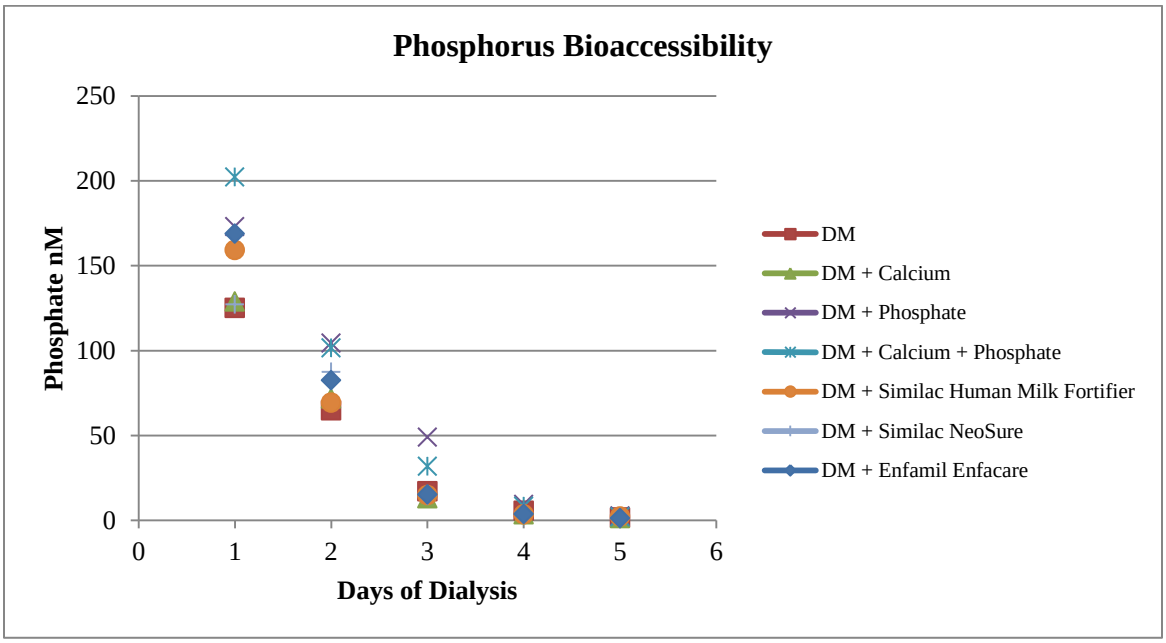

Figure 2.10 Quantity of phosphate dialyzable during successive 24-hour intervals with albumin in the dialysis buffer

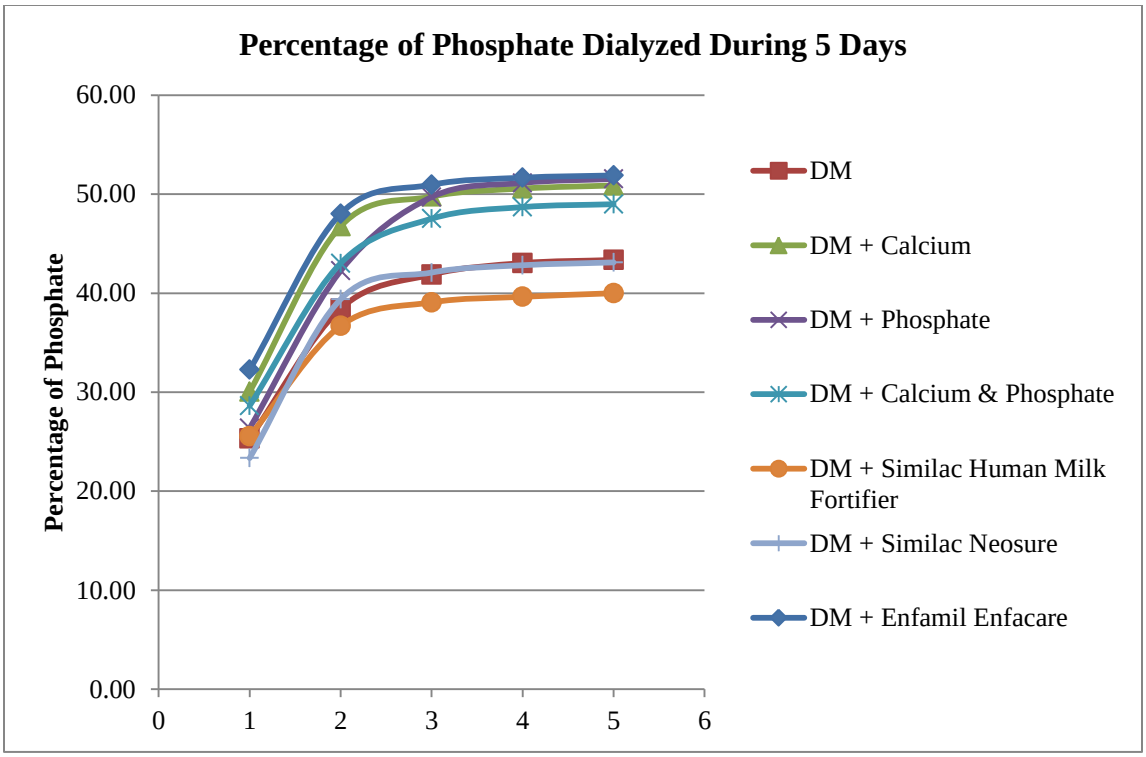

Figure 2.11 Percentage of phosphate dialyzable during successive 24-hour intervals with albumin in the dialysis buffer

2.6. Discussion

To understand the impact of calcium, phosphorus, and premature infant formula supplementation on the bioaccessibility of calcium and phosphorus in donor milk we developed a simulated premature infant digestion system to measure the bioaccessibility of calcium and phosphate in donor human milk supplemented with common nutritional fortifiers used in neonatal intensive care units, including Calcium Glubionate, Neutra-Phos, Calcium Glubionate and Neutra-Phos together, Enfamil® Enfacare, Similac® Human Milk Fortifier, and Similac® NeoSure. Following *in vitro* digestion and absorption, samples were analyzed for bioaccessible calcium and phosphate. A comprehensive literature review was used to design optimal *in vitro* digestion procedures, including the appropriate digestive pH, enzyme types, enzyme levels, and transit time for the gastrointestinal tract of a premature infant.

Modifications to previously described methods of *in vitro* digestion (13, 14) were made. Lipase was added in the gastric phase because it has been shown that there is a high degree of gastric lipolysis, even in premature infants (16). The lipase used was *Rhizopus niveus* because it has a similar specificity for fatty acids as the gastric lipase, preferentially hydrolyzing the fatty acids at the Sn-1 and Sn-3 positions of glycerol, where as human gastric lipase preferentially hydrolyzes at the Sn3-position (15). A gastric pH of 5 was used because gastric contents of gavage-fed premature infants maintain a pH greater than 5 for the entire postprandial period (15).

In our initial design for this *in vitro* experiment, albumin was used in the buffer for dialysis in order to create a physiological simulation of intestinal calcium transport into plasma. As calcium ions leave the gut through the paracellular pathway, they can bind to

plasma albumin and reduce the effect of a calcium ion gradient. In the current experiment, all samples were dialyzed with and without albumin in the buffer in order to compare the albumin effect on the redistribution of calcium and phosphate in the dialysis system. The only statistically significant difference caused by using albumin in the dialysis buffer was in dialyzable calcium in donor milk supplemented with calcium. Additionally, dialysis of calcium did not come to equilibrium over 24 hours of dialysis when albumin was included in the dialysis buffer but it did come to equilibrium when albumin was not present in the dialysis buffer. Dialyzable calcium was significantly greater in donor milk supplemented with calcium when albumin was used in the dialysis buffer, most likely due to the fact that calcium has affinity to albumin, and binding to albumin in the dialysate would reduce the calcium ion gradient and allow more calcium ions to dissociate from larger molecular weight complexes and then diffuse out. However, the same effect was not observed when donor milk was supplemented with calcium and phosphate together, suggesting that calcium preferentially binds to phosphate over albumin, and diffusion out of the dialysis tubing could be in the form of a calcium phosphate complex.

Addition of the premature infant formulas Similac® Human Milk Fortifier, Similac® Neosure, and Enfamil® Enfacare decreased percentage of dialyzable calcium when compared to donor milk without added fortifiers and donor milk supplemented with calcium, phosphate, and calcium and phosphate together. The values measured for percent dialyzable calcium in donor milk without added supplements (24 ± 5%) were higher than those previously reported by Roig et al. (17) and Shen et al. (18), that is, 13.6 ± 0.8% and 19.6 ± 2.1%, respectively (17, 18). It is not possible to compare the values of percentage dialyzable

calcium of the donor milk with added supplements to those in other studies because similar *in vitro* digestion methods, mixtures, and variables have not been studied previously.

Addition of the premature infant formulas Similac® Human Milk Fortifier, Similac® Neosure, and Enfamil® Enfacare, did not impact dialyzable calcium and it significantly decreased percentage dialyzable calcium when added to donor milk. Consistent with the findings in the present study, other authors have found significantly lower values for calcium absorption from infant formulas than from human milk (17, 19-21).

In vivo calcium absorption percentages for human milk measured by various authors are consistently higher than the percentage dialyzable calcium measured in the present study (24 ± 5.25%). Previous in-vivo studies reported values of 65% (22), 67.2 ± 3.6% (19), 76% (21), and 61.3 ± 22.7% (23). Possible reasons for this discrepancy are better mixing in vivo, active transport of Ca^{2+} from the duodenum into a larger blood volume relative to the design of the Float-a-Lyzer™, which would allow for further dissociation of Ca^{2+} from large molecular weight complexes, or direct absorption of larger molecular weight complexes in the lower small intestine in vivo than in the dialysis system.

Calcium, phosphate, and premature infant formula supplementation did not impact phosphorus bioaccessibility in donor milk. As the calcium content of the supplements increased, there was a slight trend for an increase in phosphate bioaccessibility, but the trend is not statistically significant.

When total bioaccessible calcium and phosphate concentrations were measured in digests of donor milk, donor milk supplemented with calcium and phosphate, and donor milk supplemented with Enfamil® Enfacare at 1, 4, 8, 12, and 24 hours during dialysis with and without albumin in the buffer, bioaccessible calcium and phosphate come to equilibrium at

approximately 12 hours in most cases. The exception was the milk sample supplemented with both Calcium Glubionate and Nutra-Phos with albumin in the buffer, which was approaching equilibrium at 24 hr, suggesting slower dissociation of calcium phosphate complexes and binding to albumin than in the case of the other samples. When total bioaccessible calcium and phosphate concentrations were measured with daily dialysate replacement at days 1, 2, 3, 4, and 5 of dialysis with albumin in the buffer, approximately 20 to 25% of the calcium and 25 to 30% of the phosphate were dialyzed within 24 hours. Therefore, 24 hours was determined to be a sufficient time for equilibrium dialysis to occur, and a logical termination for the experiment because anything left in the infant's gut after 24 hours would most likely be excreted in the feces. Additionally, 24 hours should be the maximum amount of time that nutrients would remain in the intestine of premature infants because the appearance of meconium in feces of newborn infants generally begins within 24 hours, suggesting that the rate of passage though the GI tract is within this time frame.

2.7. Conclusion

The amount of calcium dialyzed from donor milk supplemented with Similac® Human Milk Fortifier, Similac Neosure®, and Enfamil EnfaCare® is not significantly different from the amount of calcium dialyzed from milk alone. Supplemental calcium as well as calcium and phosphorus added together increases the total amount of bioaccessible calcium.

Donor milk fortification with premature infant formulas does not have an effect on bioaccessible calcium but fortification with Calcium Glubionate and Calcium Glubionate with Neutra-Phos together increases bioaccessible calcium. When premature infants are at risk for bone disease, fortification with Calcium Glubionate and Calcium Glubionate with Neutra-Phos together is an option that may provide the most bioaccessible calcium.

Premature infants fed enterally retain approximately 70 to 80 mg/kg/day of calcium (19), whereas the intrauterine calcium retention in the last trimester is approximately 140 mg/kg/day (1). Rigo et al. (2007) recommend an intake of 100 to 160 mg/kg/day of highly bioavailable calcium salts, 60 to 90 mg/kg/day of phosphorus, and 800 to 1000 IU of vitamin D/day (24). When infants are able to consume 200 mL/kg/day, mineral fortification is not advised because that volume of milk is sufficient to supply adequate nutrients (9); however, when infants are prescribed a fluid-restricted diet, which is common in neonatal and pediatric intensive care units, fortification of human milk is needed to provide optimal calcium retention.

Studies comparing the health outcomes of fortifiers are conflicting, but it is widely known that the nutritional needs of premature infants exceed that which is in human milk alone. In the United States, supplementation of human milk with calcium and phosphorus is

common, but the bioavailability of these additives has not been proven. This study provides evidence that supplementation of human milk with Calcium Glubionate and Neutra-Phos increases calcium bioaccessibility. However, supplementation with premature infant formula does not increase bioaccessible calcium. Therefore, premature infant formula should be used to increase total calories and protein for the fluid restricted infant, but when the infant is at risk for metabolic bone disease, Calcium Glubionate and Neutra-Phos will provide the greatest amount of bioaccessible calcium of the products tested. Conservative and cautious use of human milk supplementation is advised because adding supplements to the milk displaces the many beneficial non-nutritive factors present in human milk, of which the full benefits are yet to be understood. For example, infants fed 200 ml/kg/day of human milk do not have a higher incidence of bone disease. Human milk contains non-nutritive factors that may positively influence bone mass such as lactoferrin, which has been shown to promote osteoblast growth, reducing bone resorption and increasing bone mass (25). Additionally, prolactin, which is present in human milk, may act together with $1,25(OH)_2D_3$ to increase active intestinal calcium absorption and it may enhance calcium absorption through the paracellular pathway (26)

Our method models calcium digestion in premature infants, for which most calcium absorption is a passive, paracellular transport process (27), whereas Devizia et al. (38) referred to normal infants in which calcium absorption is mostly active transport regulated by vitamin D and other hormones. For adults, vitamin D is important for calcium homeostasis and bone mineralization; however, fetal mineral ion homeostasis appears to be independent of vitamin D (27).

The development of an *in vitro* model to simulate digestion in the preterm infant is a reliable way to compare the relative bioaccessibility of minerals in human milk supplemented with common fortifiers used in neonatal intensive care units. The method is simple and inexpensive compared to methods utilizing animals and cell culture. Future research should focus on improving premature infant feeding protocols to provide optimal growth, bone mineralization, and short-term and long-term health outcomes while still providing and preserving the desirable non-nutritive effects of human milk.

2.8. REFERENCES

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CHAPTER 3: Effect of Preterm Donor Human Milk Fortification on Calcium and Phosphate Bioaccessibility

3.1. Abstract

Background: Infants born prematurely are at risk for metabolic bone disease and may need increased minerals for normal bone mineralization. In these situations, supplementation of human milk with calcium and phosphorus is common in the United States. The bioavailability of these additives has not been proven. The goal was to study the effect of calcium, phosphorus, and premature infant formula fortification of donor human milk on the bioaccessibility of calcium.

Methods: A previously developed model of the premature infant's gastrointestinal tract was used to simulate digestion. Bioaccessible calcium was measured after in-vitro digestion in donor milk supplemented with Calcium Glubionate, Neutra-Phos (sodium/potassium phosphate), Calcium Glubionate and Neutra-Phos together, Enfamil® Enfacare, Similac® Human Milk Fortifier, and Similac® NeoSure.

Results: The percent dialyzable calcium from donor milk was not significantly different from the percent dialyzable calcium in donor milk supplemented with calcium and donor milk supplemented with both calcium and phosphate together, but was significantly higher than percent dialyzable calcium in donor milk supplemented with Enfamil® Enfacare and Similac® NeoSure. The dialyzable calcium was significantly greater in donor milk supplemented with calcium and donor milk supplemented with calcium and phosphate than in donor milk alone or with added phosphate, Enfamil® Enfacare, and Similac® NeoSure. Dialyzable calcium in donor milk supplemented with premature infant formulas was not

significantly different from the dialyzable calcium in donor milk alone. Percent soluble calcium was significantly lower in donor milk supplemented with premature infant formulas than any other treatment.

Conclusions: Addition of fortifiers to donor milk does not impact calcium dialysability but does significantly decrease calcium solubility. If a premature infant is at risk for bone disease, fortification with Calcium Glubionate and Calcium Glubionate with Neutra-Phos together will provide the most bioaccessible calcium.

Funding Sources: Mothers' Milk Bank, San Jose, CA

3.2. Introduction

As the age of viability for premature infants decreases with medical advancements and the survival rates of preterm infants improve, more attention is being given to the providing the best possible quality of life through nutritional interventions. There are no clear guidelines and minimal scientific data to update optimal nutritional strategies (1). The goal of many clinicians is to accelerate the growth of premature infants in order to achieve the intrauterine growth rate and shorten hospital stays (2).

Suitable nutrition is essential for premature infants to prevent extra-uterine growth restriction caused by severe nutritional deficit during the first weeks of life. Improved nutrition in the early postnatal period can improve common morbidities of premature infants (3-6). Additionally, as the rate of weight gain of premature infants increases between the first and fourth quartiles, short-term health and neurodevelopmental outcomes improve significantly (7).

Bone disease of prematurity occurs in approximately 30% of infants who weigh less than 1500 g and 55% of infants who weigh less than 1000 g (8, 9). Infants most at risk of developing bone disease are those less than 28 weeks gestation, weigh less than 1500 g, receive total parenteral nutrition for an extended period of time, and those who receive steroids and diuretics, all of which are common in neonatal intensive care units (10). The current recommended calcium and phosphorus intakes for premature infants are designed to provide postnatal accretion during the period equal to the intrauterine gain of a fetus. For premature infants, the American Academy of Pediatrics (AAP) recommends supplementation of human milk with both calcium and phosphorus or use of a formula with a high mineral

content (11, 12). The European Society for Pediatric Gastroenterology and Nutrition (ESPGAN) recommends routine phosphate supplementation (12).

During the period equal to the third trimester, feeding premature infants 200 mL/kg/day human milk supplies approximately 25% of the amount of calcium and phosphorus needed for normal bone mineralization (13, 14). Meeting the needs for calcium, phosphorus, and other required nutrients would require an excessive and unrealistic volume of human milk. However, needs can be met by using donor milk as a "base" and adding required nutrients back into the milk through supplementation, while conserving the components of human milk that are essential to normal growth and development, and that are not present in formula, such as immunoglobulins, lactoferrin, lysozyme, growth factors, enzymes, anti-inflammatory factors, cytokines, and oligosaccharides (15).

When the clinical outcomes of fortified donor milk feeding are compared to those of infant formula feeding, several studies show that donor milk feeding is associated with significantly slower rate of growth, including weight gain and increase in head circumference, but not in length (14, 16). However, these studies did not address long-term clinical outcomes or body composition, and they were performed in the 1980s when clinical practices may have been different from those in use currently.

Feeding premature infants unfortified human milk during hospitalization leads to decreased growth and bone mineralization during infancy and beyond (17-20). Furthermore, fortification with calcium and phosphorus leads to improved indicators of mineral homeostasis (20-23). Schanler, Shulman, and Lau (1999) found that using fortified human milk is associated with fewer infections and more rapid achievement of full feeds; slower weight gain does not result because feeding tolerance was improved (24).

However, when markers of bone mineralization of premature infants fed human milk (donor milk or mother's milk), pre-term formula, or human milk fortified with calcium, phosphorus, or preterm formula were compared, there were no significant differences between groups (12, 25, 26). Faerk et al. (2000) found that infants fed preterm formula had a significantly higher weight at term compared with infants fed only their own mother's milk, but did not differ in length or head circumference (12). Fewtrell et al. (2009) showed that the proportion of human milk in the diet was significantly positively associated with bone mineral content (26). A Cochrane review of human milk fortifiers found that fortification of human milk with multicomponent fortifiers is associated with short-term increases in rates of weight gain, length, and head circumference, but it was not clear if there is an effect on bone mineral content (27).

Studies comparing the health effects of feeding fortified human milk versus infant formulas to premature infants yield conflicting results because many have enrolled a small number of infants, there were differences in feeding protocols, and few of the participants have been extremely preterm. In addition to the conflicting results of these studies, the bioavailability of fortifiers has not been investigated.

Although studies comparing the health outcomes of supplementing human milk with fortifiers are conflicting, it is widely known that the nutritional needs of premature infants exceed that which is in human milk alone (15). In the United States, supplementation of human milk with calcium and phosphorus is common; however, the bioavailability of these additives has not been measured. The primary goal of this research was to study the effect of calcium, phosphorus, and premature infant formula fortification of donor human milk on the bioaccessibility of calcium and phosphate. Bioaccessibility was estimated on the basis of

simulated gastrointestinal digestion and calcium solubility and dialysability. We hypothesized that fortification of donor human milk with minerals and premature infant formulas will decrease the bioaccessibility of calcium and phosphate.

Dialyzable calcium, percentage dialyzable calcium, and percentage calcium solubility were measured in donor milk as well as donor milk supplemented with calcium, phosphate, calcium and phosphate, Similac® Human Milk Fortifier, Similac® Neosure, and Enfamil® Enfacare to compare the bioaccessibility of calcium of the various supplements.

3.3. Materials and methods

3.3.1. Preparation and in-vitro digestion of donor milk samples

Samples

Preterm human donor milk was shipped from the San Jose Mother's Milk Bank (San Jose, CA) to our laboratory. Mothers who donated the milk gave signed consent that their milk may be used for research purposes, as is policy for all donations to milk banks of the Human Milk Banking Association of North America (HMBANA). The study was approved by the Institutional Review Board at North Carolina State University. Five batches of preterm donor milk were used in the study and treated according to standard fortification protocols used in neonatal intensive care units (NICU). It is standard protocol for HMBANA milk banks to pool milk from 4 to 5 mothers in order to reduce variability of the nutritional composition. Therefore, milk from 20 to 25 mothers was included in the analyses. Each sample was treated seven ways: 1) no treatment to serve as a control; 2) addition of 0.15 mL Calcium Glubionate per 1 mL milk; 3) 0.23 mL Neutra-Phos (sodium phosphate and potassium phosphate) per 1 mL milk; 4) 0.15 mL Calcium Glubionate and 0.23 mL Neutra-Phos per 1 mL milk; 5) 0.064 g Enfamil® Enfacare per 1 mL milk; 6) 0.1563 g Similac® Human Milk Fortifier per 1 mL milk; and 7) 0.072 g Similac® NeoSure per 1 mL milk. Table 3.1 shows the amounts of calcium and phosphate added to each sample. The quantities of the infant formula supplements were added based on recommendations in NEOFAX (28), the nutritional guide used in neonatal intensive care units. Quantities of Calcium Glubionate and Neutra-Phos were based on the calcium and phosphate fortification protocol used at Lucille Packard Children's Hospital at Stanford University.

Table 3.1 Concentrations of calcium and phosphate added to donor milk

Treatment Group	Calcium Added (ppm)	Phosphate Added (ppm)
Donor Milk	0	0
Donor Milk + Calcium	1130.83	0
Donor Milk + Phosphate	0	725
Donor Milk + Calcium & Phosphate	1130.83	725
Donor Milk + Similac® Human Milk Fortifier	493.33	527.75
Donor Milk + Similac® Neosure	416.66	191
Donor Milk + Enfamil® EnfaCare	394.16	171

In Vitro Digestion Protocol

An *in vitro* digestion model was developed to simulate the gastrointestinal tract of the premature infant. The model was modified from those described previously (29, 30). In the gastric phase, 0.2 g pepsin (Sigma, St. Louis, MO) was dissolved in 5 mL of 0.1 N HCl and 0.25 mL was added to each 4 mL sample of donor milk. Additionally, 1.7 g lipase with similar specificity as human milk lipase (31) (Sigma, St. Louis, MO) was dissolved into 15 mL 0.1 N HCl and 1.5 mL was added to each donor milk sample. Lipase was added in the gastric phase because it has been shown that there is a high degree of gastric lipolysis, even in premature infants (32). The low pH optimum (2.5 – 6.5), the absence of requirements for cofactors or bile salts, and resistance to pepsin digestion enable lipase to remain active in the infant's stomach and contribute significantly to fat digestion (31, 32). The donor milk

samples were adjusted to pH 5.0 by addition of HCl or NaOH and then placed in a shaking water bath at 37˚C for two hours. The donor milk samples were placed on ice for 10 minutes to stop digestion.

In the intestinal phase, 0.05 g pancreatin (Sigma, St. Louis, MO) and 0.3 g bile extract (Sigma, St. Louis, MO) were dissolved in 25 mL of 0.1 M $NaHCO_3$ and 1.25 mL of this solution was added to each donor milk sample. In order to add 17.2 mU of lactase (Sigma, St. Louis, MO) to each donor milk sample, 0.25 g of lactase was dissolved in 200 mL H_2O, and 2 µL was added to each donor milk sample. Donor milk samples were adjusted to a pH of 7.0 by 1 M $NaHCO_3$ and to a final volume of 10 mL by addition of cell culture grade water (Sigma, St. Louis, MO). The donor milk samples were placed in a shaking water bath at 37˚C for two hours. The donor milk samples were placed on ice for 10 minutes to stop digestion and they were adjusted to pH 7.0.

After the gastric and intestinal phases, the samples were centrifuged at 3500 x *g* for 1 hour at 4˚C. Aliquots of the supernatant were transferred to tubes and stored at -20˚C until further analysis, unless samples were analyzed within 24 hours, in which case they were stored at 4˚C.

3.3.2. Dialysis of donor milk digests

Dialysis was done using Spectra/Por® Float-A-Lyzer® G2 (Model G235067, Spectrum Labs) dialysis tubing with a molecular weight cutoff of 8000 to10000 D. The Spectra/Por® Float-A-Lyzer® was submerged and allowed to soak in deionized water for 15-30 minutes. The hydrated membrane was not allowed to dry out.

Using a pipette, 10 mL of the previously digested sample was added to the inside of the membrane. The cap was replaced and the membrane was placed inside the container which contained 25 mL of a solution of 0.9% NaCl with 1% albumin. Albumin was used in the buffer for dialysis in order to create a physiological simulation of intestinal absorption.

The solution was allowed to dialyze for 24 hours, after which time the volumes in the inside and outside of the membranes were measured. The contents on the inside and outside of the dialysis membrane were removed and analyzed for total calcium concentration and volume.

3.3.3. Biochemical assays

3.3.3.1. Dialyzable calcium analysis

After dialysis, total bioaccessible calcium was measured by analyzing the calcium content of the dialysate by atomic absorption spectrophotometry (Perkin Elmer Model 3100, Norwalk, CT). Calcium standards were made at calcium concentrations of 0.5 ppm to 10 ppm in buffer containing 0.01N HCl with 0.5% lanthanum oxide. The digested samples were diluted in buffer containing 0.01N HCl with 0.5% lanthanum oxide so they could be measured within the range of the calcium standards.

3.3.3.2. Percent calcium bioaccessibility

Percent calcium bioaccessibility was calculated by dividing the dialyzable calcium by the total calcium content of the sample and multiplying by 100.

2.3.3.3. Percent soluble calcium

Soluble calcium concentrations were determined in the supernatant, dialysis tube contents, and the dialysate. To obtain a value for percent soluble calcium for each sample, these values were added together, divided by the total calcium content of the sample, and multiplied by 100.

2.4. Statistical analysis

Experiments were performed in triplicate for analysis of total calcium. Statistical analysis was performed using JMP (SAS, Inc., Cary, NC). One-way analysis of variance (ANOVA) with the Tukey post-hoc test to describe the relation between means was used, and $P < 0.05$ was regarded as statistically significant.

In order to study the influence of the various fortifiers on calcium dialysability, a regression analysis was applied between calcium dialysability and the nutrient content of each fortifier. The various regression models included the amount of dialyzable calcium as the dependent variable and the nutrient content of each fortifier as the independent variables.

3.5. Results

3.5.1. Percent dialyzable calcium

The percentage of dialyzable calcium in donor milk without added supplements was not significantly different from dialyzable calcium in donor milk supplemented with calcium, donor milk supplemented with phosphate, donor milk supplemented with calcium and phosphate together, or donor milk supplemented with Similac® Human Milk Fortifier (Figure 3.1). Percentage dialyzable calcium was significantly greater in donor milk than in donor milk supplemented with Similac Neosure® or donor milk supplemented with Enfamil EnfaCare®.

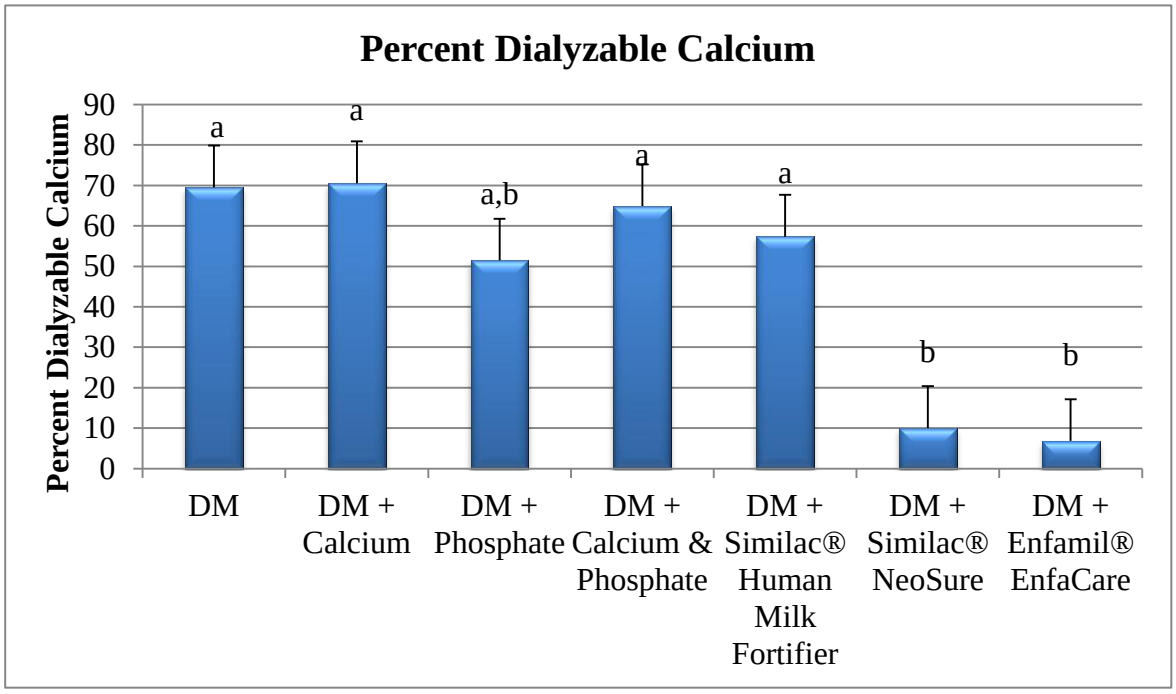

Figure 3.1 Percent bioaccessible calcium

3.5.2. Dialyzable calcium

Dialyzable calcium was significantly greater in the donor milk supplemented with calcium and calcium and phosphorus together than in donor milk, donor milk supplemented with phosphate, donor milk supplemented with Similac Neosure, and donor milk supplemented with Enfamil Enfacare (Figure 3.2). Dialyzable calcium in donor milk supplemented with Similac® Human Milk Fortifier, Similac Neosure®, and Enfamil EnfaCare® was not significantly different from the amount of calcium dialyzed from milk alone.

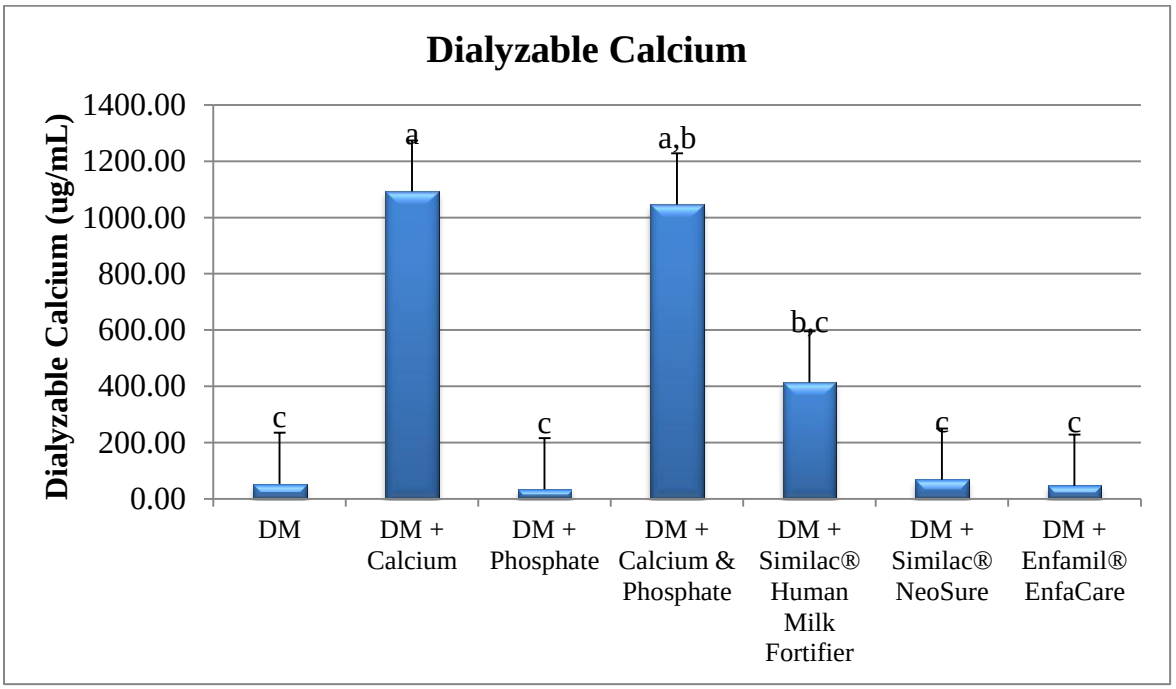

Figure 3.2 Total bioaccessible calcium

3.5.3. Calcium Solubility

As shown in Figure 3.3, the percent calcium solubility was significantly higher in donor milk without added fortifier, donor milk with added phosphate, and donor milk with added calcium and phosphate than in donor milk with supplemental premature infant formula.

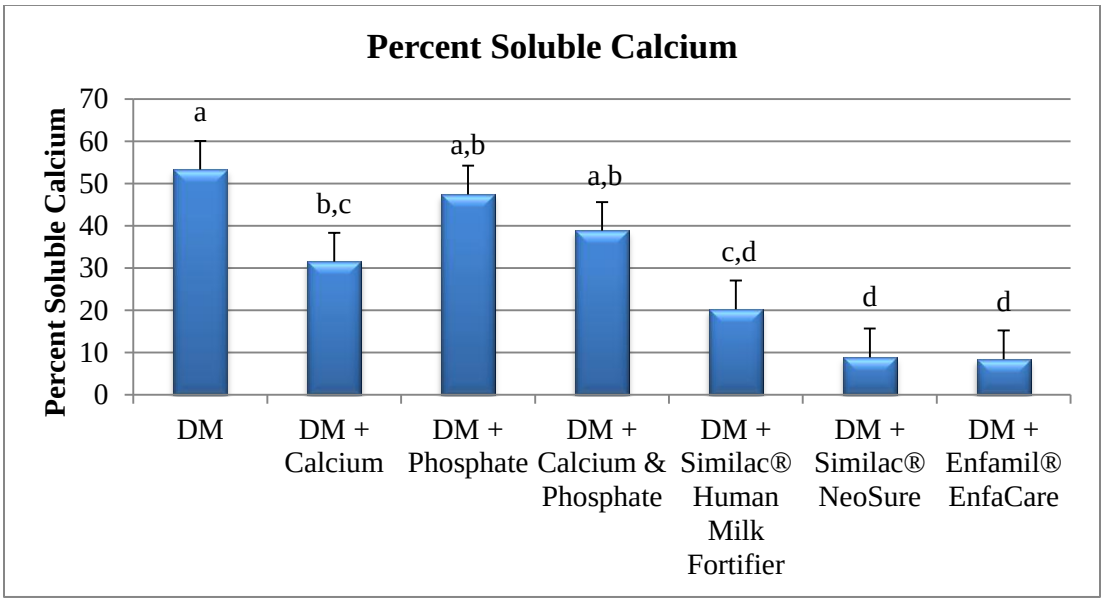

Figure 3.3 Percent soluble calcium

3.5.4. Influence of fortifier on calcium bioaccessibility

Regression models were analyzed in which the dependent variable was the total amount of calcium dialyzed and the independent variables were amounts of nutritional components measured previously in milk (28), including protein, fat, carbohydrate, total energy content, and various minerals such as calcium, phosphorus, magnesium, iron, zinc, manganese, copper, iodine, selenium, sodium, potassium, and chloride. The results displayed a significant, positive correlation between the amount of calcium dialyzed and the calcium content of the sample ($r = 0.73$, $p < 0.0001$). No other nutritional components were correlated with the amount of calcium dialyzed.

Table 3.2 Influence of fortifier on calcium bioaccessibility

Treatment	Bioaccessible Calcium (ppm)	Calcium Added (ppm)
Donor milk, preterm	52 [c]	0 [a]
Donor milk, preterm + Calcium Glubionate	1092 [b]	1130 [a]
Donor milk, preterm + Neutra-Phos	33 [c]	0 [a]
Donor milk, preterm + Calcium Glubionate & Neutra-Phos	1046 [a]	1130 [a]
Donor milk, preterm + Similac Human Milk Fortifier	413 [b,c]	493 [a]
Donor milk, preterm + Similac Neosure	68 [c]	416 [a]
Donor milk, preterm + Enfamil Enfacare	46 [c]	394 [a]

3.6. Discussion

To compare the impact of calcium, phosphorus, and premature infant formula supplementation on the bioaccessibility of calcium in donor milk we used a previously developed simulated premature infant digestion system. We compared the bioaccessibility of calcium in donor milk supplemented with common nutritional fortifiers used in neonatal intensive care units, including Calcium Glubionate, Neutra-Phos, Calcium Glubionate and Neutra-Phos together, Enfamil® Enfacare, Similac® Human Milk Fortifier, and Similac® NeoSure. Following *in vitro* digestion, samples were analyzed for bioaccessible calcium and calcium solubility.

The values measured for percent dialyzable calcium in donor milk in this study (69.5 ± 7.5%) without added supplements were higher than those previously reported by Roig et al. (33) and Shen et al. (34), that is, 13.6 ± 0.8% and 19.6 ± 2.1%, respectively (33, 34). It is not possible to compare the values of percent dialyzable calcium of the donor milk with added supplements to those in other studies because similar *in vitro* digestion methods, mixtures, and variables have not been studied previously. However, Roig et al. (1999) found a percent dialyzable calcium value for bovine milk-based formula to be 8.1 ± 0.9%, which is comparable to the percent dialyzable calcium measured for donor milk supplemented with Similac Neosure and donor milk supplemented with Enfamil Enfacare, both of which are bovine milk-based formulas (33).

Consistent with the findings for percent dialyzable calcium in the present study, other authors have found significantly lower values for calcium absorption from infant formulas than human milk (33, 35-37). In vivo calcium absorption percentages for human milk measured by various authors are similar to the percent dialyzable calcium measured in the

present study and in past studies with values of 65% (38), 67.2 ± 3.6% (35), 76% (37), and 61.3 ± 22.7% (39). A possible explanation for the decreased calcium bioaccessibility of the bovine-based infant formulas used in this study is the protein in these formulas is predominately casein, which contains a substantial portion of the calcium (40). Human milk contains less casein and more whey protein (34).

In a balance study in infants fed formulas containing varying calcium contents, DeVizia et al. (38) reported that the percentage of calcium absorption decreases when the intake of calcium increases. This observation is not consistent with the bioaccessibility results of the present study. A regression model was analyzed in which the dependent variable was the total amount of calcium dialyzed and the independent variable was the calcium content of the sample. The results displayed a significant, positive correlation between the amount of calcium dialyzed and the calcium content of the sample ($r = 0.97$). For adults, vitamin D is important for calcium homeostasis and bone mineralization; however, fetal mineral ion homeostasis appears to be independent of vitamin D (41). Therefore, our method models calcium absorption in premature infants, for which most calcium absorption is a passive, paracellular transport process (41), whereas Devizia et al. (38) referred to normal infants in which calcium absorption is mostly by active transport regulated by vitamin D and other hormones.

The values measured for percent solubility in donor milk without added supplements are slightly lower (53.3 ± 11%) than previously reported by Roig et al. (35), who reported 69.4 ± 2.0%. The use of solubility as an indicator of bioavailability is controversial because solubility is only one of many factors involved in bioavailability, with others being such physiological factors as calcium reserves and hormonal regulation (41).

The differences in the values of percent dialyzable calcium, dialyzable calcium, and percentage soluble calcium between the present study and others may be due to differences in the conditions of the *in vitro* assay and calibration of the atomic absorption spectrophotometer. Each research group performed the *in vitro* digestion experiment slightly differently. Specifically, the present study was the only one to perform the gastric phase of the *in vitro* assay at pH 5 and to include lipase in the gastric phase. Zemel et al. (42) and Schwartz & Nevins (41) reported that there is not a good correlation between *in vitro* calcium solubility and *in vivo* calcium bioavailability, whereas others report a good correlation (33, 42, 43).

3.7. Conclusion

Bioaccessible calcium in donor milk supplemented with Similac® Human Milk Fortifier, Similac Neosure®, and Enfamil EnfaCare® is not significantly different from the bioaccessible calcium in donor milk without added supplements. Supplemental calcium as well as calcium and phosphorus added together increases the total amount of bioaccessible calcium, despite causing an increase in insoluble calcium.

Addition of fortifiers to donor milk does not decrease calcium bioaccessibility, but may increase bioaccessibility when donor milk is supplemented with Calcium Glubionate and Calcium Glubionate and Neutra-Phos together. When premature infants are at risk for bone disease, fortification with Calcium Glubionate and Calcium Glubionate with Neutra-Phos together is an option that may provide the most bioaccessible calcium.

Premature infants fed enterally retain approximately 70 to 80 mg/kg/day of calcium (35), whereas the intrauterine calcium retention in the last trimester is approximately 140 mg/kg/day (2). When infants are able to consume 200 mL/kg/day, mineral fortification is not advised because that volume of milk is sufficient to supply adequate nutrients (12); however, when infants are prescribed a fluid restricted diet, which is common in neonatal and pediatric intensive care units, fortification of human milk is needed to provide optimal calcium retention. Future research should focus on improving premature infant feeding protocols to provide optimal growth, bone mineralization, and short-term and long-term health outcomes while still providing and preserving the desirable non-nutritive effects of human milk.

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CHAPTER 4: Effect of calcium and phosphorus supplementation on the digestibility of protein, fat, and calcium in preterm human donor milk

4.1. Abstract

Background: Infants born prematurely are at risk for metabolic bone disease and may need increased minerals for normal bone mineralization. In these situations, supplementation of human milk with calcium and phosphorus is common in the United States. The interaction of these nutrients and their carriers with other nutrition components of human milk has not been systematically investigated. The primary goal was to study the effect of calcium and phosphorus supplementation on the digestibility of protein, free fatty acids, and calcium in preterm donor human milk.

Methods: An in-vitro model of the premature infant's gastrointestinal tract was modified from previous studies to simulate digestion. Protein, free fatty acids, and ionized calcium were measured before and after in-vitro digestion in milk with and without added Calcium Glubionate and Neutra-Phos (sodium/potassium phosphate).

Results: Calcium and phosphorus supplementation did not negatively impact: total protein, protein breakdown, protein digestibility, or fat breakdown. Supplemental calcium increased ionized calcium, which may replace ionized calcium lost during milk expression, storage, and processing.

Conclusions: Supplemental Calcium Glubionate and Neutra-Phos (sodium/potassium phosphate) does not negatively impact protein or fat breakdown *in vitro*. Donor milk contains less ionized calcium than fresh human milk because it loses CO_2 during expression and processing. Adding supplemental calcium increases ionized calcium in donor milk.

Funding Sources: Mothers' Milk Bank, San Jose, CA

4.2. Introduction

The preterm infant is a nutritional emergency. As the age of viability decreases and the survival rates of preterm infants improve, more attention is being given to the providing the best possible quality of life through nutritional interventions. There are minimal scientific data and conflicting guidelines for feeding premature infant (1). Many clinicians aim to accelerate growth to achieve the intrauterine growth rate and shorten hospital stays (2).

Approximately 30% of infants who weigh less than 1500 g and 55% of infants who weigh less than 1000 g will have bone disease of prematurity (3, 4). Infants less than 28 weeks gestation, weighing less than 1500 g, receiving total parenteral nutrition for an extended period of time, and those who receive steroids and diuretics are most at risk of developing bone disease (5). The current recommended intakes for calcium and phosphate for premature infants are intended to provide postnatal accretion during the period equal to the intrauterine gain of a fetus. For premature infants, the American Academy of Pediatrics (AAP) recommends supplementation of human milk with calcium and phosphorus or a formula with a high mineral content (6, 7), whereas the European Society for Pediatric Gastroenterology and Nutrition (ESPGAN) recommends phosphate supplementation (7).

During the period equal to the third trimester, feeding premature infants 200 mL/kg/day human milk supplies approximately 25% of their calcium and phosphorus needs for normal bone mineralization (8, 9). Meeting the needs for calcium, phosphorus, and other nutrients would require an unrealistic volume of human milk. However, needs can be met by using donor milk as a “base” and adding required nutrients back into the milk through supplementation. This practice attempts to conserve the components of human milk that are

essential to normal growth and development, and that are not present in formula, such as immunoglobulins, lactoferrin, lysozyme, growth factors, enzymes, anti-inflammatory factors, cytokines, and oligosaccharides (10).

Many studies show that feeding premature infants unfortified human milk during hospitalization leads to decreased growth and bone mineralization during and after infancy (11-14). Studies show that fortification with calcium and phosphorus led to improved indicators of mineral homeostasis (14-17). However, other studies show that when markers of bone mineralization of premature infants fed human milk (donor milk or mother's milk), pre-term formula, or human milk fortified with calcium, phosphorus, or preterm formula were compared, there were no significant differences between groups (7, 18, 19). A study by Fewtrell et al. (2009) showed that bone mineral content improved as the amount of human milk in the diet increased (19). According to a Cochrane review of human milk fortifiers, while fortification of human milk with multicomponent fortifiers is associated with short-term increases in rates of gain in weight, length, and head circumference, it was not clear if there is an effect on bone mineral content (20).

Adding calcium and phosphorus to milk may not result in increased intestinal absorption of these nutrients, and could decrease bioavailability of other milk components. When high-calcium, preterm formulas were used, the calcium absorption was low, which resulted in increased fecal calcium excretion, impaired fat absorption, decreased gastrointestinal transit time, and increased stool hardness (4, 21), all of which may increase the risk of necrotizing enterocolitis (4, 21). Supplementation with excessive calcium and phosphorus has led to calcium and phosphate bezoars, nephrocalcinosis, and abdominal distension (21, 22).

The primary goal was to study the effect of calcium and phosphorus supplementation of donor milk on markers for the digestibility of protein, free fatty acids, and calcium in preterm donor human milk. We hypothesized that fortification of donor human milk with minerals and premature infant formulas will decrease the digestibility of calcium, protein, and fat.

4.3. Materials and methods

4.3.1. Preparation and in-vitro digestion of donor milk samples

Samples

Preterm human donor milk was shipped from the San Jose Mother's Milk Bank (San Jose, CA) to our laboratory. Mothers who donated the milk gave signed consent that their milk may be used for research purposes, as is policy for all donations to milk banks of the Human Milk Banking Association of North America (HMBANA). The study was approved by the Institutional Review Board at North Carolina State University. Ten batches of preterm donor milk were used in the study and treated according to standard fortification protocols used in neonatal intensive care units (NICU). It is standard protocol for HMBANA milk banks to pool milk from 4 to 5 mothers in order to reduce variability of the nutritional composition. Therefore, milk from 20 to 25 mothers was included in the analyses. Each milk sample was divided and the aliquots were treated three ways: 1) no treatment to serve as a control; 2) addition of 0.15 mL Calcium Glubionate per 1 mL milk; and 3) 0.23 mL Neutra-Phos (sodium phosphate and potassium phosphate) per 1 mL milk.

In Vitro Digestion Protocol

An *in vitro* digestion model was developed to simulate the gastrointestinal tract of the premature infant. The model was modified from methods of previous investigators (23, 24). In the gastric phase, 0.2 g pepsin (Sigma, St. Louis, MO) was dissolved in 5 mL of 0.1 N HCl and 0.25 mL was added to each 4 mL sample of donor milk. Additionally, 1.7 g lipase with similar specificity as human milk lipase (25) (Sigma, St. Louis, MO) was dissolved into 15 mL 0.1 N HCl and 1.5 mL was added to each donor milk sample. Lipase was added in

the gastric phase because it has been shown that there is a high degree of gastric lipolysis, even in premature infants (26) and one objective was to model lipid digestion. The low pH optimum (2.5 to 6.5), the absence of requirements for cofactors or bile salts, and resistance to pepsin digestion enable gastric lipase to remain active in the infant's stomach and contribute significantly to fat digestion (25, 26). The donor milk samples were adjusted to pH 5.0 by addition of HCl or NaOH and then placed in a shaking water bath at 37˚C for two hours. The donor milk samples were then placed on ice for 10 minutes to stop digestion.

In the intestinal phase, 0.05 g pancreatin (Sigma, St. Louis, MO) and 0.3 g bile extract (Sigma, St. Louis, MO) were dissolved in 25 mL of 0.1 M $NaHCO_3$ and 1.25 mL of this solution was added to each donor milk sample. In order to add 17.2 mU of lactase (Sigma, St. Louis, MO) to each donor milk sample, 0.25 g of lactase was dissolved in 200 mL H_2O, and 2 µL was added to each donor milk sample. Donor milk samples were adjusted to a pH of 7.0 by 1 M $NaHCO_3$ and to a final volume of 10 mL by addition of cell culture grade water (Sigma, St. Louis, MO). The donor milk samples were placed in a shaking water bath at 37˚C for two hours. The donor milk samples were placed on ice for 10 minutes to stop digestion and they were then adjusted to pH 7.0.

After the gastric and intestinal phases, the samples were centrifuged at 3500 x *g* for 1 hour at 4˚C. Aliquots of the supernatant were transferred to tubes and stored at -20˚C until further analysis, unless samples were analyzed within 24 hours, in which case they were stored at 4˚C.

4.3.2. Biochemical assays

4.3.2.1. Total protein

The BCA protein assay (BCA Protein Assay Kit TM #23227, Thermo Fisher Scientific Inc., Rockford, IL) is based on the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium, using bicinchoninic acid for the colorimetric detection and quantification of total protein present in a sample (27). Peptide bonds in protein reduce Cu^{+2} (from cupric sulfate pentahydrate included in the reagent) to Cu^{+1}. The amount of Cu^{+2} reduced to Cu^{+1} is proportional to the amount of protein in solution. Two molecules of bicinchoninic acid chelate with each Cu^{+1} ion and form a purple complex that absorbs light at 562 nm. The amount of protein in solution is then quantified by measuring the absorbance at 562 nm and comparing it with absorbencies of solutions containing known concentrations of protein.

The assay was performed before and after *in vitro* digestion on all donor milk samples. A microplate reader (Multiskan EX, Thermo Electron Corp., Vantaa, Finland) was used to measure absorbance at 620 nm. A standard curve was created by plotting standard bovine serum albumin absorbance at 620 nm against a known concentration (mg/ml). The standard curve was used to determine the protein concentration of each unknown sample.

First, 50 ml reagent A was mixed with 1 ml reagent B to make the working reagent. Donor milk samples were diluted 1:20 so that the absorbencies would fall within the range of the standard curve. Next, 25 µL of each diluted sample or standard was pipetted into the microplate wells. Working reagent (200 µL) was added to each microplate well. The samples were mixed for 30 seconds on a plate shaker and placed in an incubator at 37°C for 30 minutes. After the 30-min incubation period, the absorbance of each sample was measured at 562 nm in triplicate.

The equation resulting from the standard curve was used to determine the protein concentration of each sample. The equation is as follows:

$$\textit{Protein concentration} = [(\textit{absorbance} - \textit{intercept}) / (\textit{slope})] \times (\textit{DF})$$

Absorbance: average of the absorbencies of each sample
Intercept: y-intercept from the standard curve graph
Slope: slope from the standard curve graph
DF: dilution factor used to dilute human milk samples (dilution factor used was 10)

4.3.2.2. Proteolysis

The concentration of free amino ends was measured to determine the extent of proteolysis by using o-phthaldialdehyde (OPA) (28). When hydrolysis of proteins occurs, α-amino groups are released and react with OPA and β-mercaptoethanol to form an adduct that absorbs at 340 nm. The absorptivity is 6,000 M^{-1} and is similar for all α-amino groups.

The o-phthaldialdehyde reagent (Pierce #26025) includes: 100 mM sodium tetraborate, 20% (w/w) sodium dodecyl sulfate, OPA (dissolved in 1 ml methanol), and 2-mercaptoethanol. Sodium dodecyl sulfate terminates proteolysis and insures full exposure and complete reaction of amino groups.

The OPA reagent was stored in the refrigerator, but a given amount was warmed to room temperature before use. Cold reagent will cause condensation on the outside of the microplate wells and lead to an inconsistent and inaccurate absorption reading.

The donor milk samples were diluted with deionized water 1:20 so that the range of absorption was between 0.1 and 1 AU. A microplate reader (Multiskan EX, Thermo Electron Corp., Vantaa, Finland) was used to measure absorbance at 340 nm. OPA reagent (200 μL) was added to 20 μL of each diluted donor milk sample within the wells. The wells

were mixed on a plate mixer for 10 seconds. The absorption at 340 nm was recorded after 2 minutes. Each donor milk sample was analyzed in triplicate.

The following equation was used to determine the number of amino ends in each sample:

$$\textit{Amino ends } (\mu M) = [(Abs / 6{,}000\ M^{-1}) \times (1{,}000{,}000)] / [(\text{Protein concentration } (g/L)) \times (0.025)]$$

Abs: absorption at 340 nm
6,000 M^{-1}: molar protein concentration
Protein concentration: protein concentration (mg/ml)
0.025: 25 µl (0.025 ml) of diluted sample was used

4.3.2.3. Free fatty acids

Microtitration was used to determine the amount of free fatty acids within the breast milk samples. The method was originally used to determine the amount of free fatty acids in lipid extracted from plasma (29).

The Folch technique is commonly used to extract lipid from tissue samples (30). A modified Folch technique was used to extract the lipid from the breast milk in this study by using an extraction mixture consisting of 40 parts isopropyl alcohol, 10 parts heptane, and 1 part 1 N H_2SO_4. The extraction mixture will cause the lipid to be on the top layer of the sample rather than the bottom layer, as is the case with the Folch method. Having the lipid on the top layer allows for easy removal of the lipid layer. The extraction mixture (15 ml) was added to 3 ml of each breast milk sample in a glass-stoppered tube and shaken vigorously twenty times. After standing 10 minutes or longer, the system was divided into two phases by mixing into it an additional 6 ml of heptane and 9 ml of water. The phases separated rapidly without centrifugation and formed a sharp interface. The top layer was

removed using a 10 ml serological pipette. The contents of the pipette were emptied into three test tubes, resulting in 3 ml of the top layer being transferred into each test tube.

The titration mixture (1 ml) was added to each test tube containing the extracted lipid. The titration mixture consisted of 0.01% thymol blue and 90% ethanol in water, made by dilution of a stock 0.1% thymol blue in water with 9 parts redistilled ethanol. The alkali used for the titration consisted of about 0.020 N NaOH (Fisher # 50-440-0364).

Once a 3-mL aliquot of the upper (lipid) phase was transferred to a test tube containing 1 mL of titration mixture, it was then titrated with the alkali using a pipette. Nitrogen was delivered to the bottom of the tube with a fine glass capillary in order to expel carbon dioxide from the sample and to keep the two phases mixed during titration.

As the green-yellow end point was approached, the gas stream was interrupted from time to time for examination of the indicator color in the alcoholic phase. Good lighting was provided by a fluorescent light placed above and in front of the tube.

The percentage of free fatty acids (molar percent) was calculated using the following formula:

% free fatty acids (molar percent) = (mL titrant)(N titrant)(266.16) / (mL sample) x 10

mL titrant: mL NaOH used during titration
N titrant: normality titrant (0.02N)
266.16: average (weighted) molecular weight of NEFA in breast milk
mL sample: mL sample used

4.3.2.4. Ionized calcium

Ionized calcium was measured using calcium electrodes (Fisher Scientific, Catalog Number 13-620-498). The electrodes were calibrated immediately before measuring ionized calcium using calcium standards. Standard solutions for milk contained 1 mmol of Tris HCl/L, 1, 2.5, and 4 mmol of $CaCl_2$/L, and NaCl sufficient to bring the total ionic strength to 28 mmol/L.

4.4. Statistical analysis

Experiments were performed in triplicate for analysis of total protein, free amino ends, NEFA, and ionized calcium. Statistical analysis was performed using JMP (SAS, Inc., Cary, NC). For each biochemical assay, one-way analysis of variance (ANOVA) with the Tukey post-hoc test to describe the relation between means was used, and $P < 0.05$ was regarded as statistically significant.

4.5. Results and discussion

4.5.1. Total protein

Before *in vitro* digestion, the protein content of donor milk supplemented with calcium and donor milk supplemented with phosphate was significantly greater than donor milk without added minerals (Figure 4.1); however, it was only greater by 0.52 to 0.77 mg/mL and this difference could have been due to analytical variation in the protein assay. Another possibility is that the added calcium and phosphate could increase ionic strength or other parameters leading to increased reduction of Cu^{+2} to Cu^{+1} in the BCA assay, causing increased bicinchoninic acid chelation with Cu^{+1} ions, which would increase the intensity of the purple complex that absorbs light at 562 nm.

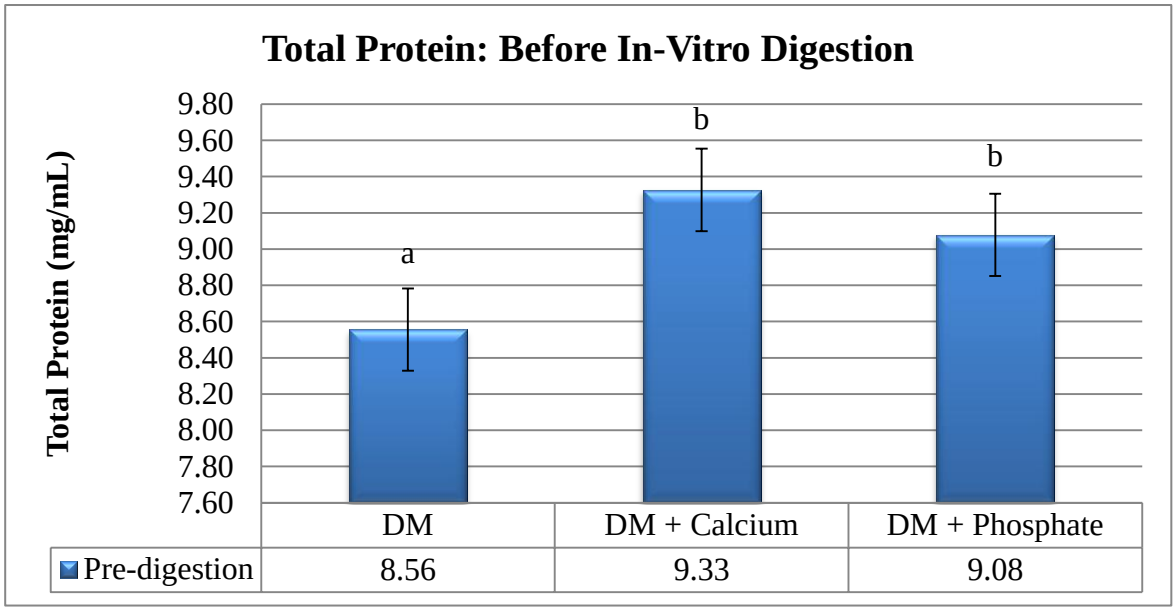

Figure 4.1 Total protein: before in vitro digestion

After *in vitro* digestion, the amount of protein in donor milk supplemented with calcium was not significantly different from the amount of protein in donor milk (Figure 4.2); however, the amount of protein in donor milk supplemented with phosphate was significantly greater than that in either donor milk supplemented with calcium or donor milk. Based on these data, supplementation did not have a negative impact on protein concentration during *in vitro* digestion of donor milk.

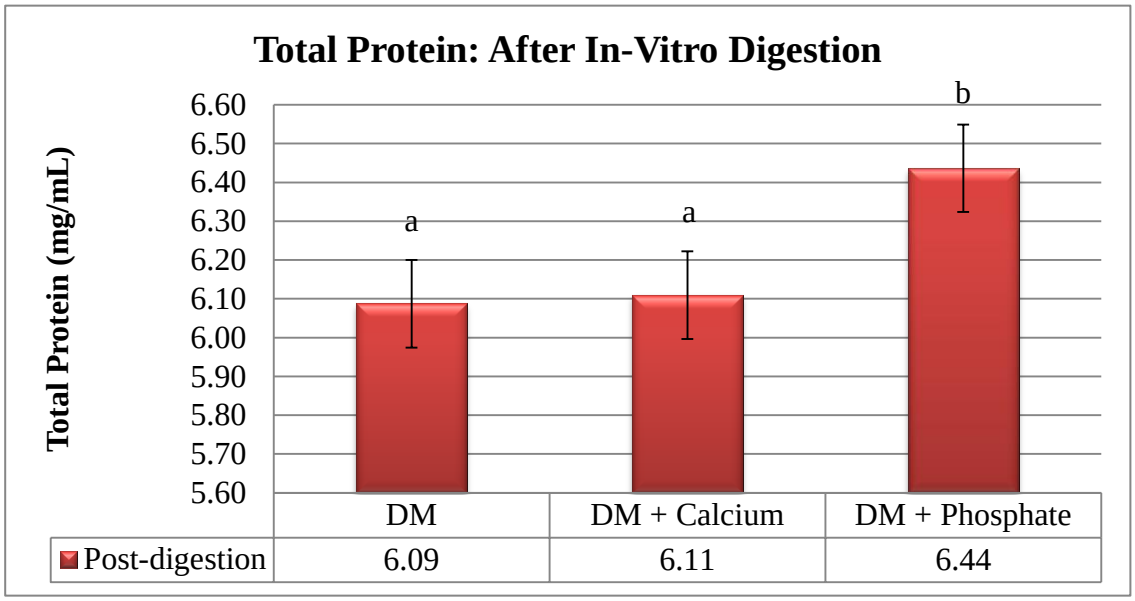

Figure 4.2 Total protein: after in vitro digestion

4.5.2. Proteolysis

Before *in vitro* digestion, there were significantly more free amino ends available to react with the OPA reagent when measured in donor milk than in donor milk supplemented with calcium and donor milk supplemented with phosphate (Figure 4.3). Based on these data, it appears as though calcium and phosphate supplementation decrease protein breakdown; however, a possible explanation is that calcium and phosphorus could bind to the free amino ends so they are not available for detection in the OPA assay.

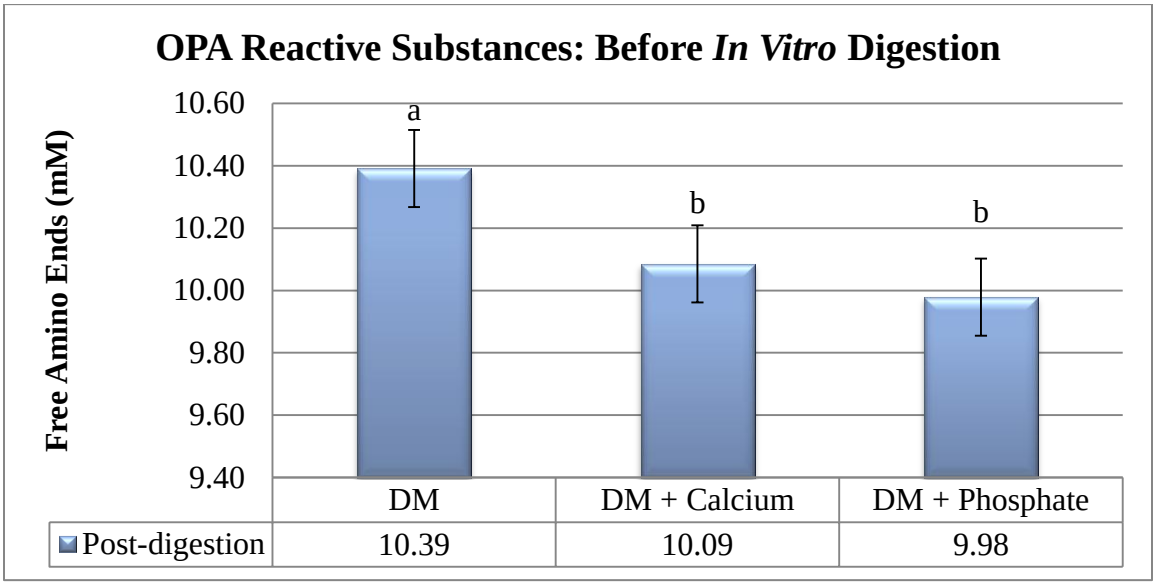

Figure 4.3 OPA reactive substances: before in vitro digestion

After *in vitro* digestion, there were no significant differences between donor milk, donor milk supplemented with calcium, and donor milk supplemented with phosphate (Figure 4.4). Based on these data, it appears as though there were still more free amino ends after digestion in milk with supplemental calcium. Therefore, calcium and phosphorus supplementation did not have a negative impact on protein breakdown in donor milk.

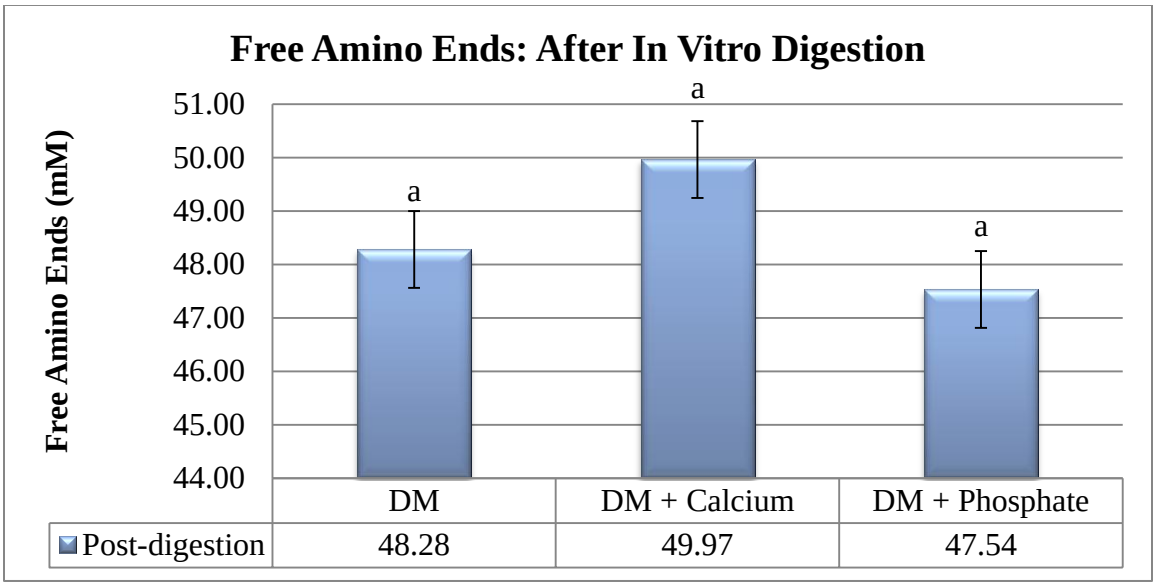

Figure 4.4 Free amino ends: after in vitro digestion

4.5.3. Protein digestibility

Protein digestibility was calculated by dividing the total measured free amino ends by the total measured protein. After *in vitro* digestion, there were no significant differences in protein digestibility between donor milk, donor milk supplemented with calcium, and donor milk supplemented with phosphate (Figure 4.5). Therefore, calcium and phosphorus supplementation did not have a negative impact on protein digestibility in donor milk.

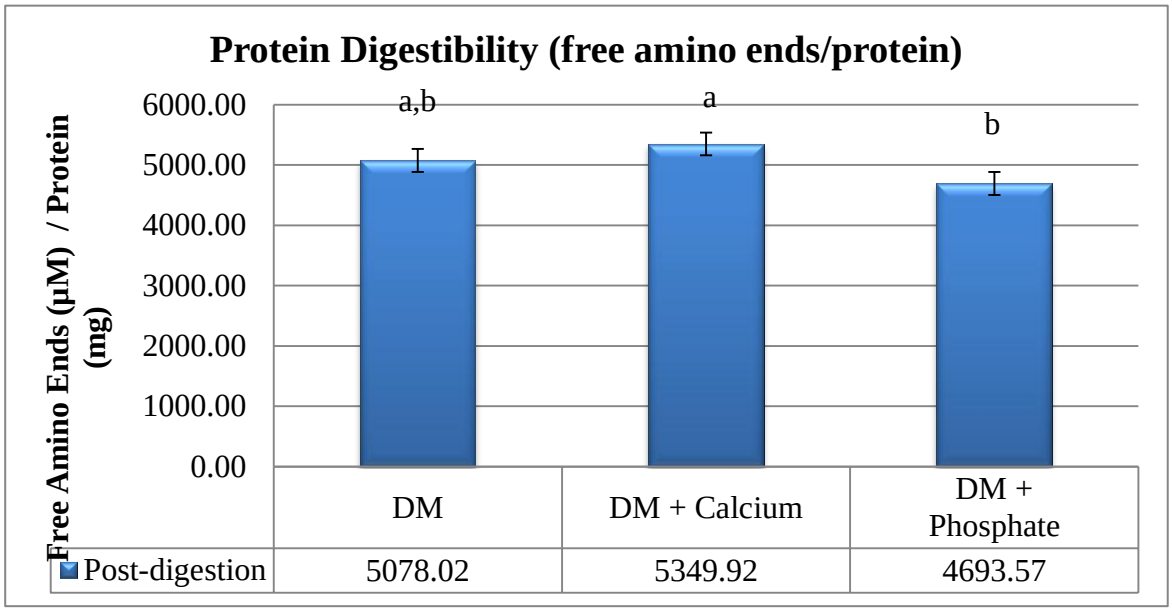

Figure 4.5 Protein digestibility

4.5.4. Free fatty acids

The amount of free fatty acids in donor milk, donor milk supplemented with calcium, and donor milk supplemented with phosphate were not significantly different in the donor milk before or after digestion (Figure 4.6 and 4.7, respectively). The percentage of free fatty acids in donor milk supplemented with calcium was lower than in donor milk or donor milk supplemented with phosphate; however, it was not statistically significant. The data show that supplementation of donor milk with calcium and phosphate did not decrease fat breakdown during *in vitro* digestion of donor milk.

The effects of calcium from dietary supplements or dairy products on quantitative fecal fat excretion in healthy adults were examined in a meta-analysis performed by Christensen et al. (31). They estimated that increasing calcium intake by 1241 mg/day resulted in an increase in fecal fat of 5.2 g/day and concluded that dietary calcium has the potential to increase fecal fat excretion and aid in weight loss (31).

Studies show that calcium supplementation also decreases fat absorption in preterm infants. In metabolic balance studies of 35 healthy premature infants, Chappell et al. (32) found that oral calcium supplements significantly altered the efficiency of lipid absorption in enterally fed preterm infants (32). In metabolic balance studies of six term infants, DeVizia et al. (33) found that absorption of fat was significantly decreased by calcium intake, although the small decrease observed may not be relevant clinically.

It has been proposed that calcium increases fat excretion through an interaction of calcium and fatty acids, which results in the formation of insoluble calcium fatty acid soaps, leading to reduced fat absorption. When analyzing the products formed from a lipase reaction as a function of time, Patton and Carey (34) found that when high levels of calcium

(10 mM) were present in the reaction, the amount of fatty acids ionized were increased and a pellet was formed, whereas at low levels of calcium (1 mM), the amount of fatty acids ionized were decreased and no pellet was formed. Their research supports the observation that calcium promotes the ionization of fatty acids, rendering them unavailable for absorption (34).

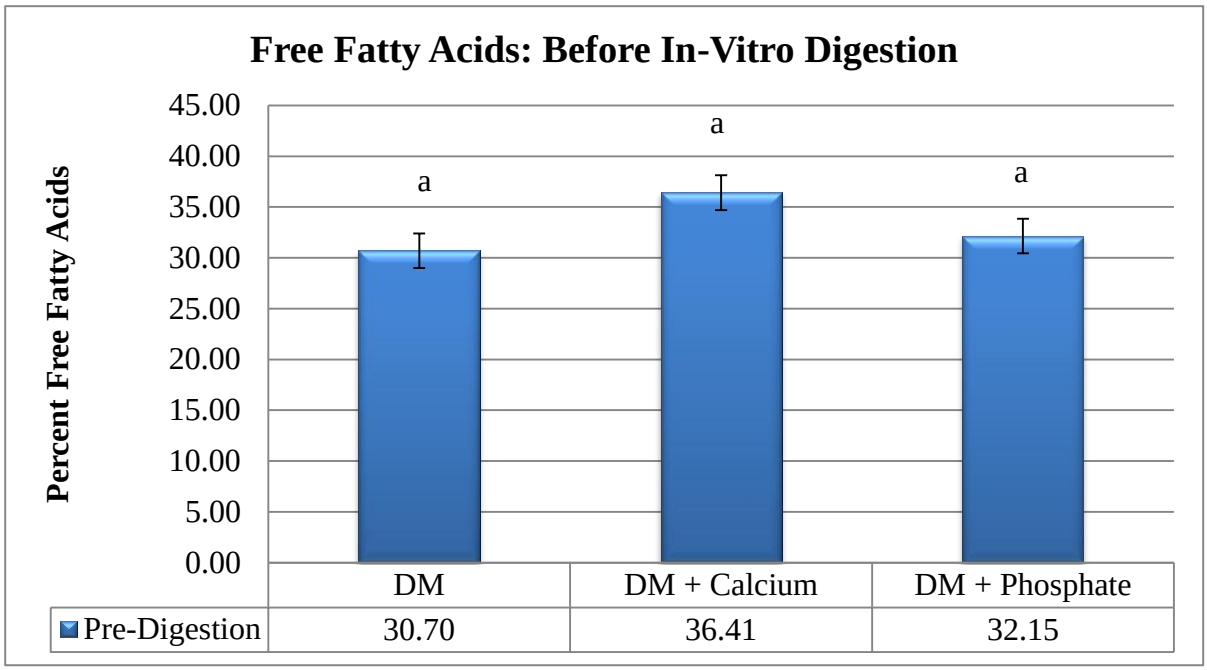

Figure 4.6 Free fatty acids: before in vitro digestion

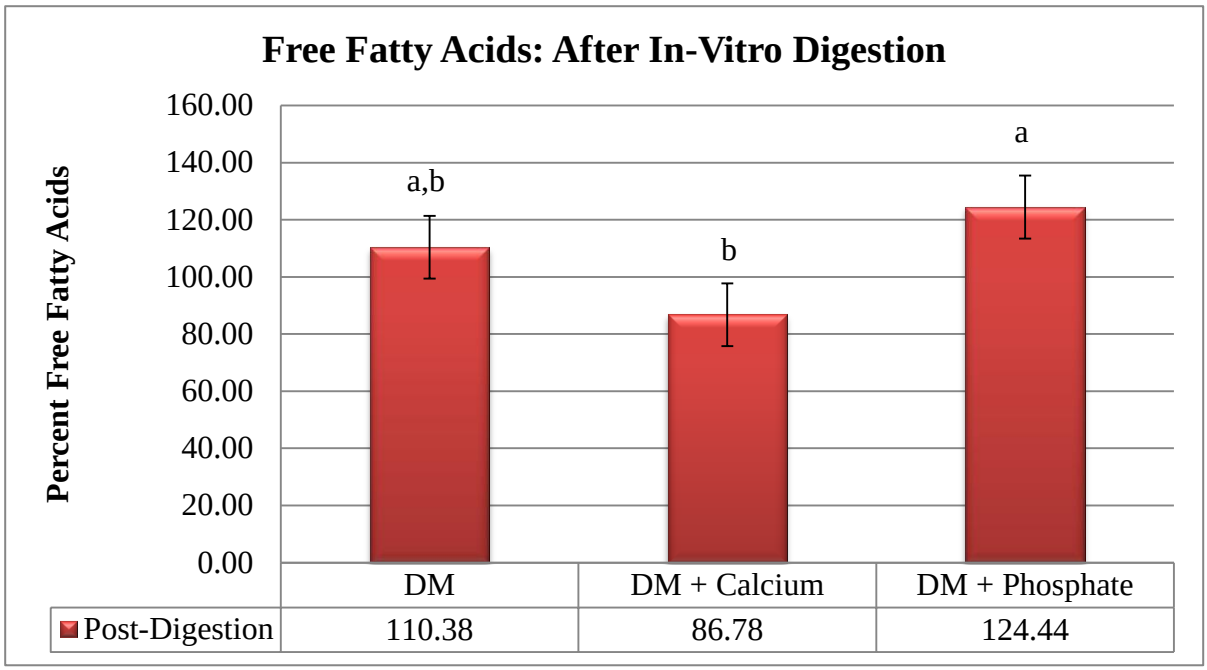

Figure 4.7 Free fatty acids: after in vitro digestion

4.5.5. Ionized calcium

Before digestion, donor milk supplemented with calcium contained significantly more ionized calcium than donor milk or donor milk supplemented with phosphate (Figure 4.8). Based on these data, supplemental calcium increases ionized calcium in donor milk, which may replace ionized calcium lost during milk expression, storage, and processing that occurs during the milk donation process.

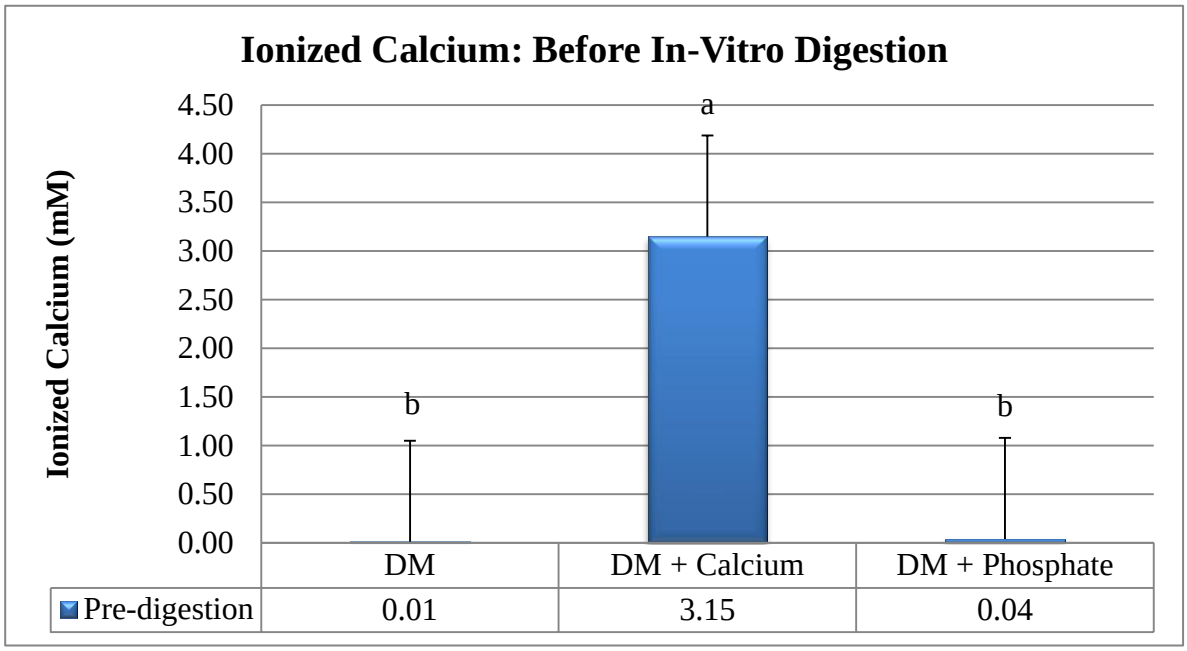

Figure 4.8 Ionized calcium: before in vitro digestion

After digestion, donor milk supplemented with calcium still contained significantly more ionized calcium than donor milk (Figure 4.9), but about 50 fold less than before digestion. Although ionized calcium was greater in donor milk supplemented with calcium than in donor milk supplemented with phosphate after digestion, the difference was not statistically significant. The data still show that both fortification of calcium and phosphate increased ionized calcium in donor milk, which may replace ionized calcium lost during milk expression, storage, and processing that occurs during the milk donation process.

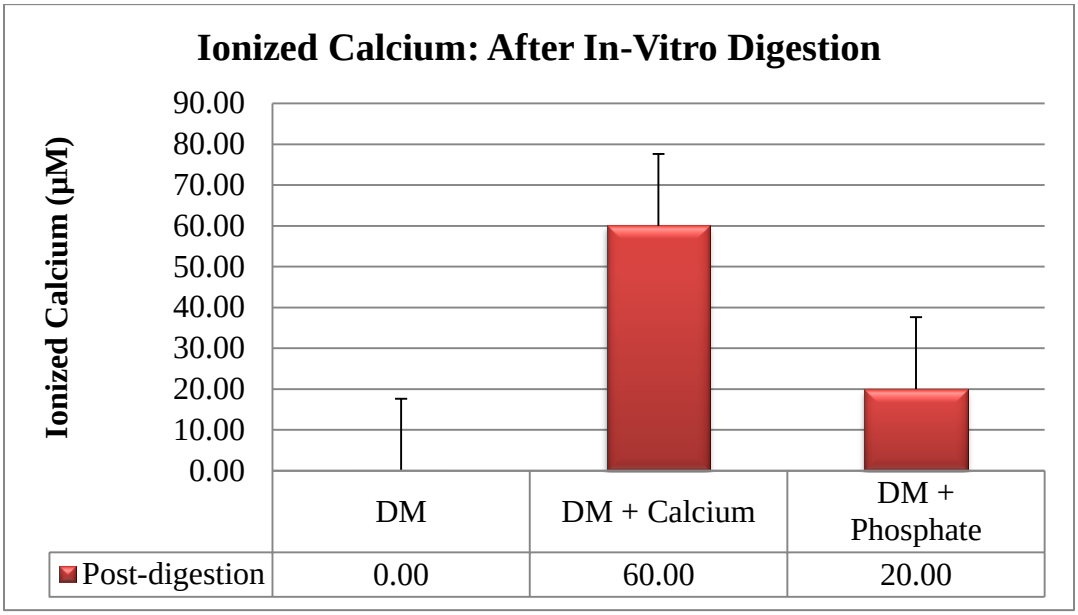

Figure 4.9 Ionized calcium: after in vitro digestion

Table 4.1 compares data from the present study to that of Allen and Neville (35), who measured ionized calcium in human milk by collecting drops of milk on the nipple while the mother hand expressed her milk. The samples were pulled into a plastic 1-mL syringe, with care to minimize contact of the sample with air. Collection of the milk in this manner minimized the loss of ionized calcium, which occurs upon exposure to air when the

bicarbonate, which is naturally present in milk, converts to carbonate and forms a complex with the ionized calcium, rendering it unavailable for absorption (35).

During the processing of donor milk, there are many steps that allow for loss of ionized calcium. The mother expresses her milk and it is exposed to air. She then freezes the milk and it is shipped to the milk bank. The milk bank thaws the milk, pasteurizes it, cultures it for bacterial growth, freezes it again, and ships it to the recipients. As a result of the milk processing, the ionized calcium was negligible in the donor milk without added calcium. However, when Calcium Glubionate was added to the donor milk, the amount of ionized calcium returned to the level that is present in freshly expressed milk collected in a way to minimize loss of ionized calcium. However, in the milk collected by Allen and Neville (35), the ionized calcium to total calcium ratio is 1:2.3. In the donor milk fortified with calcium, the ionized calcium to total calcium ratio is 1:16.2, indicating that calcium must be added in a much greater amount than that naturally present in human milk in order to obtain physiologic levels of ionized calcium.

	Total Calcium (mM)	**Ionized calcium (mM)**	**Non-protein phosphate (mM)**	**Bicarbonate (mM)**	**pH**
Fresh Milk [1]	7.5	3.0	1.8	6.0	6.8
Preterm Donor Milk	5.7	0	1.8	~0	7.5
Preterm Donor Milk + Calcium	50.2	3.1	1.8	~0	7.0
Preterm Donor Milk + Phosphorus	5.3	0.04	22	~0	7.0
[1]Allen et al., (1991) AJCN 54:69-80					

Table 4.1 Total and ionized calcium in fresh, donor, and fortified donor human milk

4.6. Conclusion

To understand the impact of calcium and phosphorus supplementation on the digestibility of protein, free fatty acids, and calcium in donor milk we performed *in vitro* digestion of donor milk, donor milk supplemented with calcium, and donor milk supplemented with phosphate as well as analyses of total protein, free amino ends, free fatty acids, and ionized calcium. Data from the present study indicate that calcium and phosphorus supplementation does not negatively impact total protein, protein breakdown, or protein digestibility. However, added calcium may make fat breakdown more difficult as compared to phosphorus supplementation, but this effect was not statistically significant. Added calcium increases ionized calcium, which may replace ionized calcium lost during milk expression, storage, and processing.

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CHAPTER 5: Conclusion

The quantity of calcium available to be absorbed from donor milk supplemented with Similac® Human Milk Fortifier, Similac Neosure®, and Enfamil EnfaCare® is not significantly different from the quantity of calcium available to be absorbed from milk alone. Supplemental calcium as well as calcium and phosphorus added together increases the total amount of bioaccessible calcium, despite causing an increase in insoluble calcium.

Addition of fortifiers to donor milk does not decrease calcium bioaccessibility. Donor milk fortification with post-discharge formulas does not increase calcium bioaccessibility but fortification with Calcium Glubionate and Calcium Glubionate with Neutra-Phos together increases calcium bioaccessibility. When premature infants are at risk for bone disease, fortification with Calcium Glubionate and Calcium Glubionate with Neutra-Phos together is an option that may provide the most bioaccessible calcium.

Calcium and phosphorus supplementation does not negatively impact total protein, protein breakdown, or protein digestibility. However, added calcium may make fat breakdown more difficult as compared to phosphorus supplementation, but this effect was not statistically significant with the current sample size. A portion of the added calcium increases ionized calcium, which may replace ionized calcium lost during milk expression, storage, and processing.

Premature infants fed enterally retain approximately 70 to 80 mg/kg/day of calcium (1), whereas the intrauterine calcium retention in the last trimester is approximately 140 mg/kg/day (2). When infants are able to consume 200 mL/kg/day, mineral fortification is not advised (3).

However, when infants are prescribed a fluid-restricted diet, which is common in neonatal and pediatric intensive care units, fortification of human milk is needed to provide optimal calcium absorption. Future research should focus on improving premature infant feeding protocols to provide optimal growth, bone mineralization, and short-term and long-term health outcomes while still providing and preserving the desirable non-nutritive effects of human milk. If a premature infant is at risk for bone disease, fortification with Calcium Glubionate and Calcium Glubionate with Neutra-Phos together will provide the most bioaccessible calcium.

5.1. REFERENCES

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APPENDICES

APPENDIX A: Nutritional composition of human milk with added fortifiers

Energy, protein, and fat content of preterm donor milk, fortified and unfortified (1)

Treatment	Energy (kcals/L)	Protein (g/L)	Fat (g/L)
Donor milk, preterm	671	14.09	38.93
Donor milk, preterm + Calcium Glubionate	671	14.09	38.93
Donor milk, preterm + Neutra-Phos	671	14.09	38.93
Donor milk, preterm + Calcium Glubionate & Neutra-Phos	671	14.09	38.93
Donor milk, preterm + Similac Human Milk Fortifier	790	23.46	41.41
Donor milk, preterm + Similac Neosure	760	12.7	43.3
Donor milk, preterm + Enfamil Enfacare	740	13.4	42.4

Carbohydrate, calcium, and phosphorus content of preterm donor milk, fortified and unfortified (1)

Treatment	Carbohydrate (g/L)	Calcium (mg/L)	Phosphorus (mg/L)
Donor milk, preterm	66.4	248	128
Donor milk, preterm + Calcium Glubionate	66.4	3640	128
Donor milk, preterm + Neutra-Phos	66.4	248	725
Donor milk, preterm + Calcium Glubionate & Neutra-Phos	66.4	3640	725
Donor milk, preterm + Similac Human Milk Fortifier	82.2	1381	777
Donor milk, preterm + Similac Neosure	80.1	370	190
Donor milk, preterm + Enfamil Enfacare	83	350	171

Ca:P ratio, magnesium, and iron content of preterm donor milk, fortified and unfortified (1)

Treatment	Ca:P ratio	Magnesium (mg/L)	Iron (mg/L)
Donor milk, preterm	1.94	30.90	1.21
Donor milk, preterm + Calcium Glubionate	28.44	30.90	1.21
Donor milk, preterm + Neutra-Phos	0.34	30.90	1.21
Donor milk, preterm + Calcium Glubionate & Neutra-Phos	5.02	30.90	1.21
Donor milk, preterm + Similac Human Milk Fortifier	1.78	98.20	4.58
Donor milk, preterm + Similac Neosure	1.95	42.00	1.90
Donor milk, preterm + Enfamil Enfacare	2.05	40.00	1.50

Zinc, manganese, and copper content of preterm donor milk, fortified and unfortified (1)

Treatment	Zinc (mg/L)	Manganese (mcg/L)	Copper (mcg/L)
Donor milk, preterm	3.42	6	644
Donor milk, preterm + Calcium Glubionate	3.42	6	644
Donor milk, preterm + Neutra-Phos	3.42	6	644
Donor milk, preterm + Calcium Glubionate & Neutra-Phos	3.42	6	644
Donor milk, preterm + Similac Human Milk Fortifier	13.07	76	2283
Donor milk, preterm + Similac Neosure	2.2	10	350
Donor milk, preterm + Enfamil Enfacare	2.1	18	330

Iodine, selenium, and sodium content of preterm donor milk, fortified and unfortified (1)

Treatment	Iodine (mcg/L)	Selenium (mcg/L)	Sodium (mg/L)
Donor milk, preterm	107	14.8	248
Donor milk, preterm + Calcium Glubionate	107	14.8	248
Donor milk, preterm + Neutra-Phos	107	14.8	248
Donor milk, preterm + Calcium Glubionate & Neutra-Phos	107	14.8	248
Donor milk, preterm + Similac Human Milk Fortifier	105	19.2	388
Donor milk, preterm + Similac Neosure	120	17	210
Donor milk, preterm + Enfamil Enfacare	120	17	175

Potassium and chloride content of preterm donor milk, fortified and unfortified (1)

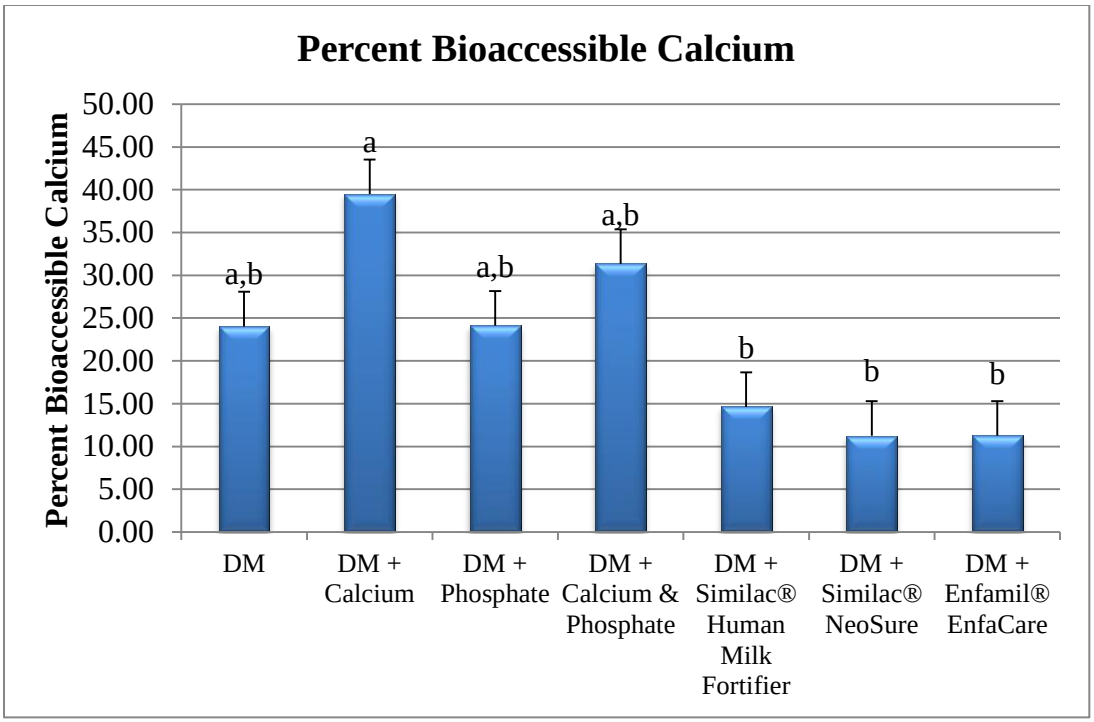
Treatment	Potassium (mg/L)	Chloride (mg/L)
Donor milk, preterm	570	550
Donor milk, preterm + Calcium Glubionate	570	550
Donor milk, preterm + Neutra-Phos	570	550
Donor milk, preterm + Calcium Glubionate & Neutra-Phos	570	550
Donor milk, preterm + Similac Human Milk Fortifier	1169	906
Donor milk, preterm + Similac Neosure	650	480
Donor milk, preterm + Enfamil Enfacare	580	470

APPENDIX B: Chemical forms of calcium in fortifiers for human milk

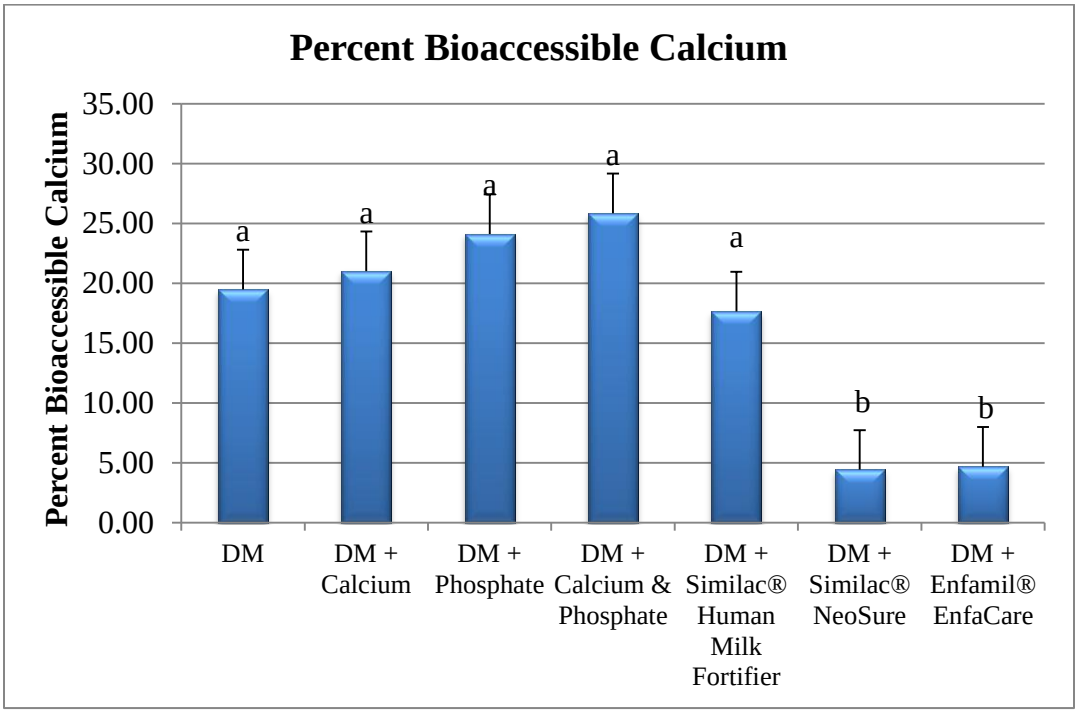
Human Milk Supplement	Form of Calcium
Similac NeoSure	Calcium Phosphate Calcium Carbonate
Similac Human Milk Fortifier	Calcium Phosphate Calcium Carbonate Calcium Pantothenate
Enfamil Enfacare	Calcium Pantothenate Calcium Carbonate Calcium Phosphate
Calcium Glubionate	Calcium d-Gluconate Lactobionate Monohydrate

APPENDIX C: Results from chapter 2

Percent dialyzable calcium

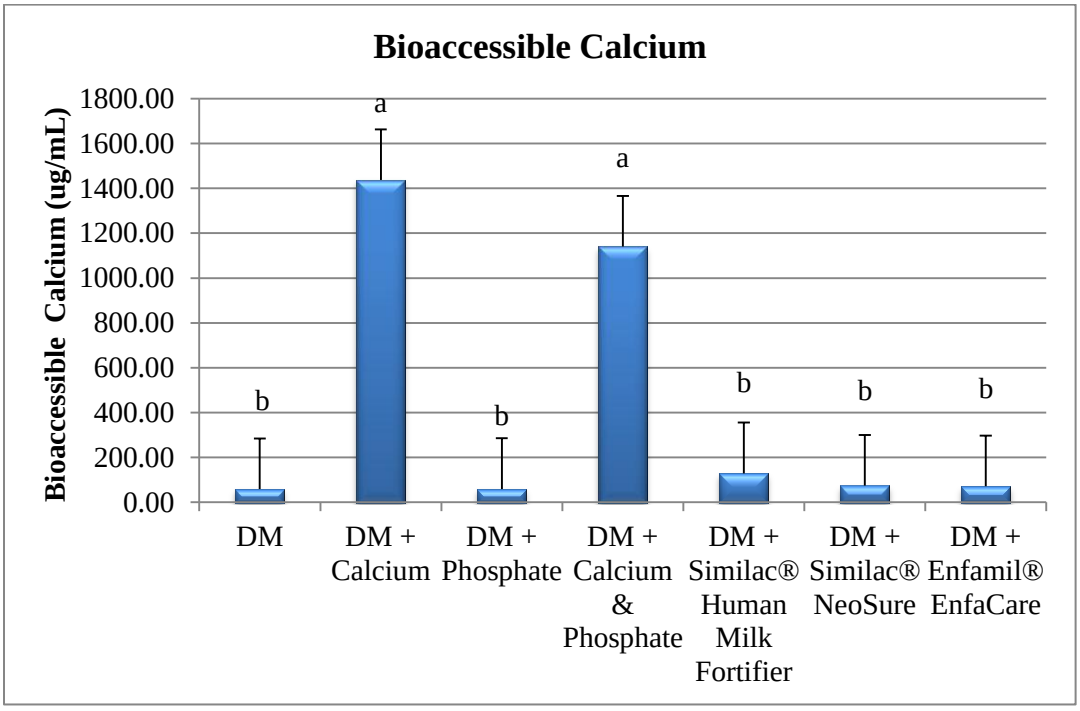


Percent Bioaccessible Calcium with Albumin in the Dialysis Buffer

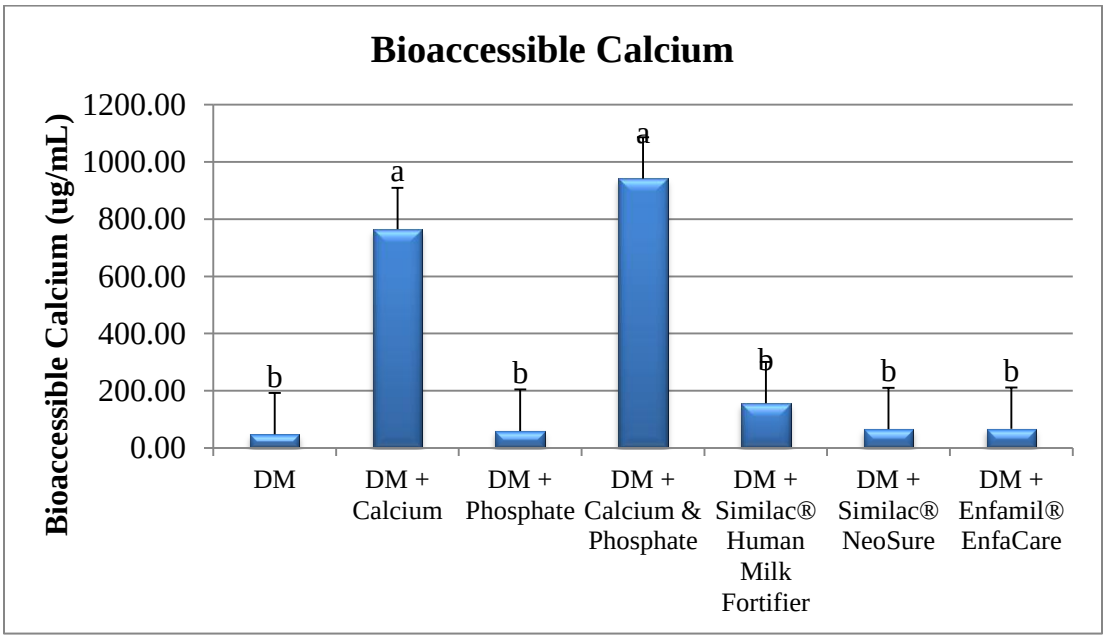


Percent Bioaccessible Calcium without Albumin in the Dialysis Buffer

Dialyzable calcium

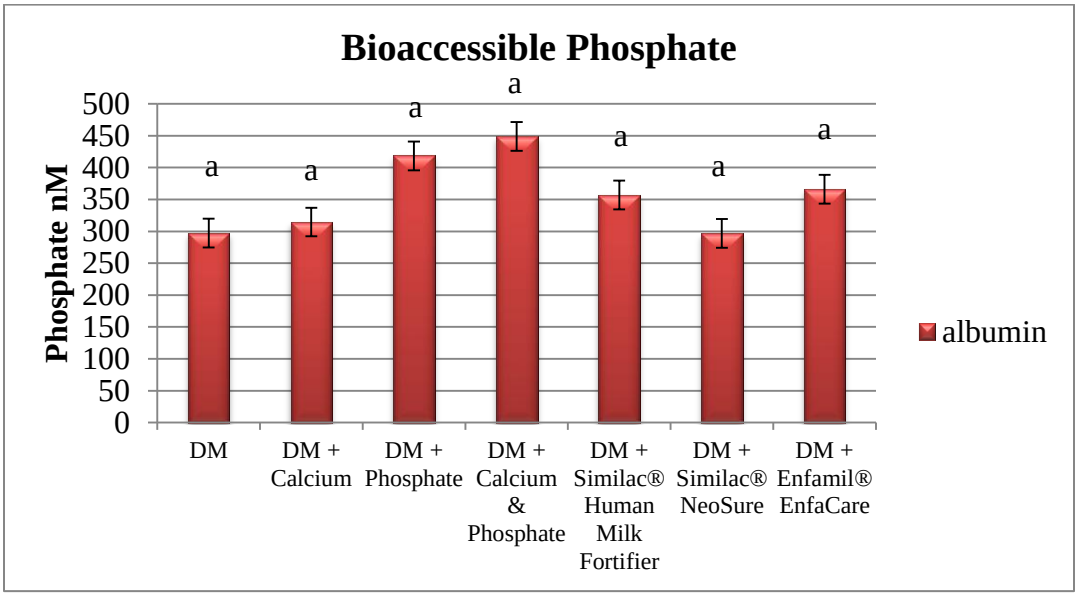


Total Bioaccessible Calcium with Albumin in the Dialysis Buffer

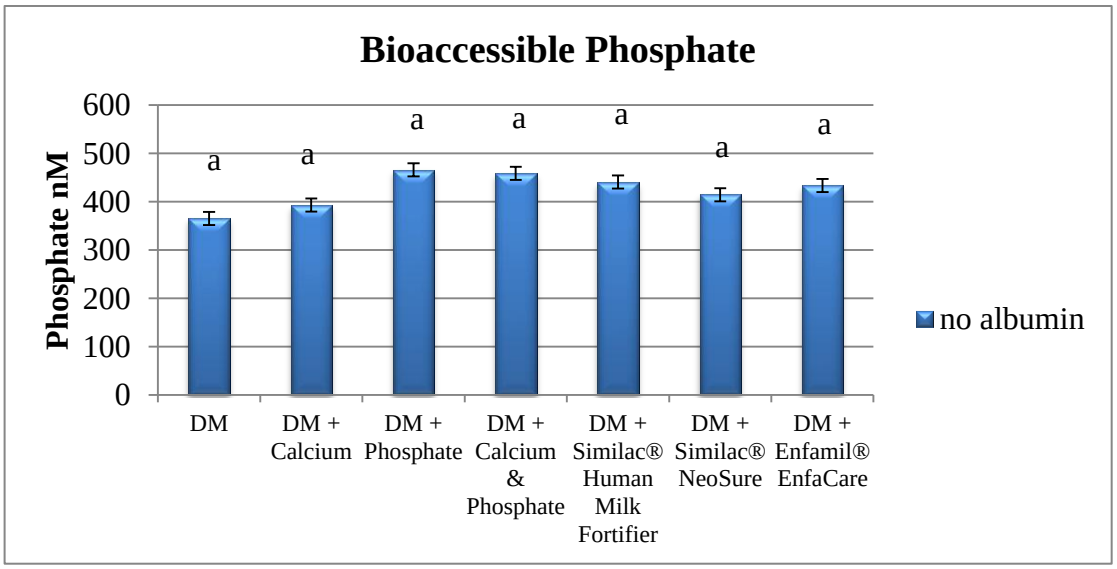


Total Bioaccessible Calcium without Albumin in the Dialysis Buffer

Bioaccessible phosphate



Bioaccessible Phosphate with Albumin in the Dialysis Buffer



Bioaccessible Phosphate without Albumin in the Dialysis Buffer

APPENDIX D: Data from chapter 2

Calcium data from samples dialyzed with albumin in the dialysis buffer, represented as averages of three replicates

Treatment	Total Ca before digestion and centrifugation (µg)	Total in milk before digestion ppm (µg/mL)	Calcium Inside Dialysis Tubing (µg/mL)	Volume Inside of Dialysis Tubing mL	Calcium Content Inside Dialysis Tubing (µg)
Donor milk	2976.00	248.00	38.50	8.50	327.25
DM + Calcium	43686.00	3640.50	979.18	10.00	9791.75
DM + Phosphate	2976.00	248.00	40.75	9.00	366.75
DM + Calcium & Phosphate	43686.00	3640.50	760.55	8.50	6464.68
DM + Similac® Human Milk Fortifier	10642.00	886.83	96.58	9.00	869.25
DM + Similac® NeoSure	13468.67	1122.39	54.67	8.00	437.33
DM + Enfamil® EnfaCare	12848.00	1070.67	50.92	8.00	407.33

Treatment	Calcium Outside Dialysis Tubing (µg/mL)	Volume Outside of Dialysis Tubing	Calcium Content Outside Dialysis Tubing (µg)
DM	21.08	23.00	484.92
DM + Calcium	458.17	23.00	10537.83
DM + Phosphate	19.08	23.00	438.92
DM + Calcium & Phosphate	380.50	23.00	8751.50
DM + Similac® Human Milk Fortifier	33.08	23.00	760.92
DM + Similac® NeoSure	19.75	23.00	454.25
DM + Enfamil® EnfaCare	21.50	23.00	494.50

Treatment	Dialyzable Calcium	% Dialyzable Calcium
DM	59.58	24.03
DM + Calcium	1437.34	39.48
DM + Phosphate	59.83	24.13
DM + Calcium & Phosphate	1141.05	31.34
DM + Similac® Human Milk Fortifier	129.67	14.62
DM + Similac® NeoSure	74.42	11.24
DM + Enfamil® EnfaCare	72.42	11.27

Calcium data from samples dialyzed without albumin in the dialysis buffer, represented as averages of three replicates

Treatment	Total Ca before digestion and centrifugation (µg)	Total in milk before digestion ppm (µg/mL)	Calcium Inside Dialysis Tubing (µg/mL)	Volume Inside of Dialysis Tubing mL	Calcium Content Inside Dialysis Tubing (µg)
DM	2976.00	248.00	35.33	8.50	300.33
DM + Calcium	43686.00	3640.50	438.13	10.00	4381.33
DM + Phosphate	2976.00	248.00	41.00	9.00	369.00
DM + Calcium & Phosphate	43686.00	3640.50	494.23	8.50	4200.91
DM + Similac® Human Milk Fortifier	10642.00	886.83	112.83	9.00	1015.50
DM + Similac® NeoSure	17983.00	1498.58	46.75	8.00	374.00
DM + Enfamil® EnfaCare	17172.00	1431.00	46.33	8.00	370.67

Treatment	Calcium Outside Dialysis Tubing (µg/mL)	Volume Outside of Dialysis Tubing	Calcium Content Outside Dialysis Tubing (µg)
DM	13.00	23.00	299.00
DM + Calcium	326.67	23.00	7513.33
DM + Phosphate	18.83	23.00	433.17
DM + Calcium & Phosphate	447.00	23.00	10281.00
DM + Similac® Human Milk Fortifier	43.67	23.00	1004.33
DM + Similac® NeoSure	19.58	23.00	450.42
DM + Enfamil® EnfaCare	20.75	23.00	477.25

Treatment	Dialyzable Calcium	% Dialyzable Calcium
DM	48.33	19.49
DM + Calcium	764.80	21.01
DM + Phosphate	59.83	24.13
DM + Calcium & Phosphate	941.23	25.85
DM + Similac® Human Milk Fortifier	156.50	17.65
DM + Similac® NeoSure	66.33	4.43
DM + Enfamil® EnfaCare	67.08	4.69

Phosphate data from samples dialyzed with albumin in the dialysis buffer, represented as averages

		With Albumin in the Dialysis Buffer	
Treatment	Total Phosphorus before digestion (nM)	Outside: Phosphate (nM)	Inside: Phosphate (nM)
DM	1474000.00	297.15	946.39
DM + Calcium	1474000.00	314.51	779.29
DM + Phosphate	9108000.00	418.29	1233.59
DM + Calcium & Phosphate	9108000.00	448.78	1178.48
DM + Similac® Human Milk Fortifier	7031000.00	356.98	1125.49
DM + Similac® NeoSure	3485000.00	296.82	1032.93
DM + Enfamil® EnfaCare	3275000.00	365.87	1006.44

Phosphate data from samples dialyzed without albumin in the dialysis buffer, represented as averages

		Without Albumin in the Dialysis Buffer	
Treatment	Total Phosphorus before digestion (nM)	Outside: Phosphate (nM)	Inside: Phosphate (nM)
DM	1474000.00	365.63	1134.32
DM + Calcium	1474000.00	393.26	1100.41
DM + Phosphate	9108000.00	465.98	1672.34
DM + Calcium & Phosphate	9108000.00	458.40	1368.89
DM + Similac® Human Milk Fortifier	7031000.00	440.71	1247.72
DM + Similac® NeoSure	3485000.00	414.54	1103.59
DM + Enfamil® EnfaCare	3275000.00	433.53	1216.98

Reference

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