THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF NUTRITIONAL SCIENCES

MARGINAL ZINC DEFICIENCY DISRUPTS MAMMARY GLAND MORPHOLOGY AND COMPROMISES MILK SECRETION

COLLEEN DEMPSEY

SPRING 2011

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Nutritional Sciences
with honors in Nutritional Sciences

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--ABSTRACT--

Zinc (Zn) is the second most abundant trace element in the body and is present in all cells. As an essential component of over 300 enzymes and proteins, Zn is involved in numerous biological processes at both the physiological and molecular levels. It is estimated that 82% of pregnant and lactating women worldwide are at risk of developing Zn deficiency due to inadequate Zn intake from the diet. Few studies have addressed the maternal factors that may contribute to marginal Zn deficiency in the infant, which may include Zn concentration in the mammary gland and milk, mammary gland structure, and milk secretion. We used a mouse model and fed them a diet adequate in Zn (ZA: 25 mg Zn/kg) or a diet reduced in Zn (ZD: 12 mg Zn/kg) for thirty days prior to conception. Our findings indicate that mammary gland morphology was severely compromised by marginal Zn deficiency which resulted from apoptosis in the mammary glands of ZD mice, despite the fact that total Zn content of the mammary gland was unaffected. A key finding was that milk secretion was reduced by ~75% in marginally ZD mice. By using a modified Bradford assay to measure protein content in milk, we found that ZD mice had significantly higher milk protein concentrations compared with ZA mice. In addition, we found that the amount of caseins and whey acidic protein was significantly lower in the milk of ZD mice by utilizing SDS-PAGE gel electrophoresis and staining with Coomassie Blue. Taken together, our data indicate that marginal Zn deficiency disrupts mammary gland morphology, interferes with milk secretion and alters milk composition. Due to the high prevalence of marginal Zn deficiency worldwide, well-controlled studies in women are needed to determine if marginal Zn intake during lactation has an impact on infant health and development.

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Most importantly, I would like to thank Dr. Shannon Kelleher for the support, guidance and encouragement that she was able to offer me over the course of this project. She has instilled within me a true passion for research for which I will be forever grateful. I would also like to thank the fellow members of the Kelleher Lab: Tom Croxford, Nick McCormick, Young Ah Seo, Vanessa Velasquez, Wynston Morris, Philip Lieberman and Dr. Veronica Lopez, who have assisted me with various techniques and provided me with support and insight throughout the duration of my project.

I would also like to thank Dr. Rebecca Corwin for academic advising, and the Schreyer Honors College for granting me the opportunity to take full advantage of my undergraduate experience at Penn State. Lastly, I would like to thank my mother and father, whose endless support and love has enabled me to be where I am today.

--LITERATURE REVIEW--

I. The Mammary Gland and Milk Secretion

A. Morphology

The mammary gland is comprised of numerous cell types with specialized roles to maintain proper structure and function of the organ. Milk is produced and stored in alveolar units (1). Mammary epithelial cells line the alveolar lumen and are responsible for the synthesis and secretion of milk components (2). Mammary epithelial cells regulate the accumulation, production and secretion of milk components and promote milk secretion when stimulated by prolactin (2). Branching ducts are characterized by an outer myoepithelial and an inner luminal epithelial cell layer (1). Milk is removed from the alveoli through the contraction of the mammary epithelial cells and then moves through the ductal branching to the sub-areolar sinuses and through the nipple for secretion (1). The ductal branching and alveoli are embedded in the stroma which is comprised of fibroblasts, adipocytes, plasma cells and blood vessels (1). Fibroblasts and adipocytes provide the mammary gland with growth factors, like IGF-1 and hepatic growth factor, and produce lipoprotein lipase (1). Plasma cells develop from lymphocytes and produce immunoglobulins that are secreted in milk (1). During lactation, blood flow increases in order to provide higher amounts of substrates required for milk synthesis (1).

B. Major Components of Milk

Breast milk alone provides all essential nutrients for optimal growth and development during the first six months of life (3). The American Academy of Pediatrics (AAP) recommends that infants be exclusively breast fed for the first six months due to the associated benefits of breastfeeding against conditions such as bacterial meningitis, bacteremia, diarrhea, respiratory tract infection, necrotizing enterocolitis, otitis media, urinary tract infection, late-onset sepsis in preterm infants, type 1 and type 2 diabetes, lymphoma, leukemia, and childhood overweight and obesity (4). In terms on macronutrient distribution, mature human milk contains 3-5% fat, 0.8-0.9% protein and 6.9-7.2% carbohydrate (as lactose) (5). Colostrum, the secretory product of the early post-partum period, contains higher amounts of protein and lower amounts of carbohydrates than mature milk, but the fat content remains relatively constant throughout lactation (5).

Human milk is comprised of numerous proteins that can be divided into three main categories: whey, caseins and mucins (6). Mucins are heavily glycosylated proteins involved with lubrication and protection that surround the lipid globules in milk and account for only a small percentage of the proteins in milk (6). Caseins, however, account for about 40% of protein and whey proteins account for approximately 60% of protein in human milk (5). As milk production increases throughout lactation, casein synthesis and secretion increase while whey protein synthesis decreases, which likely reflects the inability of the newborn infant to digest caseins until later in life (6). With the exception of serum albumin, proteins found in milk are synthesized in the mammary gland (6). In contrast, serum albumin originates from maternal circulation and binds 28% of zinc in breast milk (6, 7). Proteins in milk contribute to nutrient absorption, antimicrobial activity, gastrointestinal development and immunocompetence (6). For instance, immunoglobulins, β-casein, lysozyme, lactoferrin, haptocorrin, α-lactalbumin and

lactoperoxidase have critical roles in the digestion and absorption of nutrients in milk due to their resistance against proteolysis in the gastrointestinal tract (6). Many proteins act as defense mechanisms against bacteria and viruses. For instance, lactoferrin inhibits the growth of iron-dependent bacteria in the gastrointestinal tract such as coliforms and yeast (8). Lysozyme promotes the growth of *Lactobacilli* and *Bifidobacteria* to create an acidic gut environment to limit the growth of several pathogens (6, 8). Peptides resulting from β-casein digestion (casein phosphopeptide, CPPs) enhance the absorption of calcium (6). Furthermore, CPPs in milk have been associated with antithrombotic, antihypertensive and opioid activities, which may contribute to sleeping behavior and fluid transport in the small intestine of breastfed infants (6).

During lactation, both lactose and proteins are synthesized in the Golgi apparatus and transported through the secretory compartments for exocytosis into milk (9). Lactose is the primary carbohydrate found in human milk and accounts for approximately 40% of kilocalories provided by milk (5). Lactose is a β-disaccharide generated from a reaction involving glucose and α-lactalbumin in mammary epithelial cells. Lactose promotes the growth of beneficial bacteria in the gastrointestinal tract and assists in the absorption of calcium, phosphorus and magnesium (5, 8). In addition, lactose plays a dominant role in the osmoregulation of milk, accounting for ~50% of the osmotic pressure in milk. The presence of lactose in the Golgi draws in water for the dilution of lactose, which likely induces an electrical potential difference across the apical membrane when fused with secretory vesicles. Approximately 98% of fat in breast milk is in the form of triglycerides, with palmitic and oleic acids as the most abundant fatty acids (5, 8). High amounts of linoleic and linolenic acid are also present in breast milk, along with relatively high proportions of arachidonic acid and docosahexaenoic acid (8). Long chain fatty acids are crucial for brain, retina and nervous system development in early life (5). For transport into the milk, lipids are extruded from the apical membrane as droplets surrounded by the milk fat globule

membrane (9). The lipid components in milk are crucial for the transport and absorption of fat-soluble micronutrients such as vitamin A, D, E and K (8).

Aside from delivering essential macronutrients to infants, breast milk provides infants with substantial amounts of vitamins, minerals, cytokines, antimicrobial factors, anti-inflammatory substances, hormones, growth modulators and digestive enzymes (5, 8). Adequate concentrations of all vitamins, with the exception of vitamins D and K, are thought to be provided in milk (5). Yet, the regulation and transfer of micronutrients into milk is a complex process that is not completely understood.

II. Zinc: An Essential Micronutrient

Zinc (Zn), an essential trace mineral, is vital to human health. Zn is the second most abundant trace element in the body and is present in all cells (10). As an essential component of over 300 enzymes and proteins, Zn is involved in numerous biological processes at both physiological and molecular levels (2, 11). Although an understanding of Zn transport and regulation remains incomplete, research throughout the past decade has confirmed Zn's role in gene expression, cell and tissue growth, immune function, protein synthesis, skin integrity, hemoglobin activity, respiration and antioxidant defense (2, 12).

Physically, Zn exists as a bluish white metallic element and has an atomic weight of 65.4 amu (13). Physiologically, Zn is found in all organs and tissues and the human body contains approximately 1.5 to 2.5 grams of Zn (13). The highest sources of Zn include oysters (513% Daily Value), crabmeat, shrimp, tuna, red meat and poultry. Fortified grains and dairy products are moderate sources of Zn, while fruits, vegetables and refined cereals are considered poor

sources (14). Certain compounds are able to enhance or inhibit the absorption of Zn. Low-molecular weight ligands and chelators can form an absorbable complex with Zn and increase solubility (14). Ligands and chelators include EDTA, amino acids (histidine, methionine, cysteine), and organic acids (citric, picolinic) (14). Also, due to Zn's high binding affinity for sulfur and nitrogen, Zn typically binds to ligands such as amino acids cysteine and histidine, glutathione, and tripeptides. Such compounds enable Zn to maintain solubility throughout the GI tract (15). Zn absorption is also regulated by homeostatic mechanisms requiring the integration of twenty-four different Zn transporting proteins. For example, if an increased amount of Zn is present in a meal, the fractional Zn absorption will decrease (14).

Phytate is a major inhibitor of Zn absorption and is often present in plant foods such as cereals and legumes. The human gastrointestinal tract lacks the enzyme phytase, and therefore cannot eliminate the phosphate groups that form strong and insoluble complexes with cations (14). Rather, the phytate-bound minerals will be excreted in the stool. This mechanism is a critical problem in developing countries, where most of a population's diet consists of foods high in phytate, such as corn, cereals, rice, legumes (13). Several methods have been shown to reduce phytase activity, which include leavening of bread, fermentation, germination, milling, treatment with commercial phytase or addition of phytase to the diet (14). In addition to phytate, oxalate, found in spinach, berries, chocolate, tea, polyphenols and tannins, bind Zn to inhibit absorption (15). Conclusive evidence remains incomplete in regards to the effects of iron, calcium and copper on Zn absorption. While some studies suggest that the divalent cations may compete with Zn for absorption, other studies show no significant inhibitory effects (14). Lastly, toxic levels of cadmium, which are often present in cereals, have been shown to inhibit Zn absorption (14).

Recommended Daily Allowances vary depending on age, gender, pregnancy and lactation (16). The National Institute of Health (16) recommends males and females of 19-13 years to consume 8 mg/day; males 14+ years to consume 11 mg/day; females 14-18 years to consume 9 mg/day; and, females 19+ years to consume 8 mg/day. Recommendations increase with pregnancy and lactation. Females of 14-18 years are recommended to intake 13 mg/day when pregnant and 14 mg/day when lactating, while those of 19+ years should consume 11 mg/day when pregnant and 12 mg/day when lactating. Such increased recommendations are proposed in order to ensure adequate fetal growth during pregnancy and to protect against poor neonatal growth and insufficient immune function during lactation (17). Although the mechanisms relating to Zn transport by the mammary gland remain unclear, studies have shown that milk Zn concentration is maintained throughout various levels of dietary Zn intake, suggesting that the mammary gland is tightly regulated for the sake of the nursing newborn (18, 19).

III. Zinc in the Body

An understanding of Zn regulation throughout the human body at a physiological and cellular level remains incomplete. However, progress has been made over the past ten years pertaining to an understanding of Zn transport. Within the human body, 90% of Zn is found within muscle and bone, while other organs containing high concentrations of Zn include the prostate, liver, gastrointestinal tract, kidney, skin, lung, brain, heart and pancreas (20). After dietary Zn is digested and absorbed through the small intestine, it is distributed through the serum while bound to proteins such as albumin, α macroglobulin and transferrin (21). On a cellular level, the cytosol contains 50% of Zn, the nucleus contains 30-40% of Zn, and the remainder of Zn is associated with membranes (20). Thus far, there are 24 known Zn transporters, which can be divided into

two categories. The SLC39A gene family encodes for Zn importers, Zip 1-14, which transport Zn into the cytosol from extracellular fluid or from inside a vesicle (2). The SLC30A gene family encodes for the expression of Zn exporters, ZnT 1-10, which transport Zn out of the cytosol to carry out various functions (2). Zn transporters are localized to specific cellular compartments with tissue- and cell-specific expression. In regards to the mammary gland, expression of ZnT1, ZnT2, ZnT4, and all Zip proteins aside from Zip4, Zip9, and Zip11 has been detected in mammary tissue or cultured mammary cells (2).

Zn is stored in most tissues by binding to the protein thionein, thereby becoming metallothionein. Metallothionein is found in most tissues and also binds copper, cadmium and mercury. One metallothionein molecule can bind up to seven Zn ions due to the high amounts of cysteine residues that can complex metal ions (20). Additionally, increased gene expression of thionein will result from elevated intracellular Zn concentration (20). Zn will interact with metal transcription factors (MTF) which bind to metal regulatory elements (MRE) located on the promoter region of the thionein gene, in order to induce expression (20). When intestinal cells are sloughed off, metallothionein-bound Zn is lost into the lumen for excretion.

IV. Main Functions of Zinc

As an essential micronutrient, Zn plays both a catalytic and structural role in hundreds of biological processes (2, 11). One principal function of Zn is its involvement in gene expression (2). Specifically, Zn interacts with DNA-binding proteins to promote transcription. These "zinc fingers" permit the binding of about 2000 metal transcription factors to regulatory elements in the promoter region of a gene (15). As mentioned, Zn positively regulates thionein expression (15, 20) through metal regulatory elements. In this manner, Zn also regulates ZnT1 expression; a high

concentration of intracellular Zn results in increased gene expression of ZnT1, and therefore more Zn can be exported from the cell. Such processes are necessary in order to regulate the amount of Zn within a cell and avoid toxicity or deficiency.

Zn also plays a vital role in immune function. Zn is required for efficient immune function and affects both non-specific and specific immune responses (22). The components of innate immunity are compromised in a case of Zn deficiency. For instance, epidermal cells, linings of the GI tract and linings of the respiratory tract can be damaged by marginal Zn deficiency (22). Without adequate Zn, gene expression for these particular cells is not initiated and the barriers remain damaged. Furthermore, specific immunity can also be severely compromised due to Zn's role in both concentration and function of T and B lymphocyte function (23). Studies have shown that mice fed a Zn deficient diet for two weeks had lower numbers of T and B lymphocytes in peripheral blood and spleen tissue, eventually leading to a 50% reduction in blood lymphocyte and macrophage concentration (23). Zn's association with immune function is not limited to one role; rather, there are numerous catalytic and structural effects (23). Specific examples include Zn serving as a co-factor for thymulin, a hormone that promotes T-cell differentiation and cytokine release; Zn influencing lymphocyte response to mitogens and cytokines; and, Zn impacting leukocyte signal transduction (20).

V. Causes of Zinc Deficiency

Because the essential roles of Zn cover an extremely broad array of processes within the body, mutations or interruptions in Zn regulation may have detrimental effects. The first documented case of Zn deficiency was diagnosed from the occurrence of severe growth retardation in Egypt and Iran (25). Due to a diet high in phytic acid, several twenty-year old men were found to have

the stature of six-year old boys and had not gone through puberty. These severe cases of Zn deficiency were rapidly reversed when the patients were given Zn supplements. Typical clinical symptoms of a Zn deficient individual include immune deficiencies, diarrhea, intestinal inflammation, impaired taste, delayed wound healing, dermatitis, alopecia, glossitis, growth retardation, delayed sexual maturation, and, in severe deficiencies, neurological disturbances and death (17, 20, 23). Such Zn deficient symptoms can arise from a numerous defects in Zn metabolism, which include inadequate dietary intake, genetic mutations, increased requirements, malabsorption, increased losses and impaired utilization (15, 20).

Research has also demonstrated the positive association between Zn deficiency and stunted growth (13, 24). Zn's role in cell division and gene expression is necessary for normal growth, especially during the critical periods of infancy and childhood (13). It is also possible that the direct correlation may be due to Zn's impact on hormonal growth mediators, effects on appetite, or effects on immune response (13). A marginal Zn deficiency may also lead to growth retardation in infants and children due to the increased requirements during these stages of growth. Zn has a major impact on protein and energy metabolism and serves as a catalytic and structural cofactor for enzymes involved in protein digestion, including carboxypeptidase A and aminopeptidase (15, 24). Therefore, in cases of protein-energy malnutrition, Zn has been successfully used as a supplement to enhance weight gain (24). Castillo-Duran, et al. found that marasmic infants provided with Zn supplements had a significantly lower incidence of infection compared to the control group, and also experienced greater growth in terms of weight-for-height assessment (24).

A. Inadequate Dietary Intake

Recommended Daily Allowances vary depending on age, gender, pregnancy and lactation (12). It has been estimated that ~82% of pregnant women worldwide are marginally Zn deficient (17). However, despite the amount of Zn present in a certain food item, the bioavailability of the source may inhibit the optimum amount of Zn from being absorbed in the body. Inhibitory sources of Zn include those high in phytic acid, such as corn, cereals, rice, legumes (14). These grain products also happen to be the most affordable and prevalent sources of nourishment for individuals living in developing countries or communities of low socioeconomic level. Recall that Zn deficiency was first documented in Egypt and Iran, where inhabitants commonly consume flat breads without yeast and high in phytic acid (25). When estimating Zn consumption by total daily per-capita in a population's food supply, Hotz, et al. determined that 71.2% of the population in Southeast Asia was at risk of developing Zn deficiency (34). Moreover, studies have concluded that one out of every two pre-school children suffered from Zn deficiency after conducting a community-based study in the North-West Frontier Province in Pakistan (35).

It is clear that dietary content plays a significant role in the development of Zn deficiency. Of course, attempts have been made to reverse the effects of Zn deficiency caused by low bioavailability in food. For instance, Troesch, et al. conducted a study that tested the effects of a micronutrient powder with low doses of iron and Zn (22). The micronutrient powder also contained exogenous phytase that was active at gut pH, and was added to high-phytate maize porridge for South African schoolchildren. Prior to the study, one-half of the subjects were identified as Zn deficient. The effectiveness of the micronutrient powder was significant and reduced the prevalence of Zn deficiency by 66%, which is likely due to the reduction in phytate activity. Additionally, the weight-for-age ratio significantly improved, suggesting that the

micronutrient powder with low doses of iron and Zn may significantly improve growth, iron and Zn status in micronutrient deficient children by being added to cereals and legumes in developing countries (36).

B. Increased Requirements

There is an extensive amount of research that investigates the effects of Zn deficiency in children and infants, especially in developing countries, because it is these individuals whom are at the highest risk. Zn requirements increase during periods of growth, pregnancy, and lactation, and the most significant period of growth throughout the lifecycle occurs during infancy and childhood (16). This being said, it is also crucial for the pregnant and lactating mother to maintain adequate Zn status for the sake of herself and her baby.

The United States Department of Agriculture suggests that pregnant and lactating women increase their Zn intake by 3-5 mg/day compared with the recommendation for non-pregnant, non-lactating women (16). This increased recommendation is due to the transfer of Zn across the placenta and into breast milk. Requirements are higher for lactating women due to the 1-3 mg Zn/day that is transferred from maternal circulation into milk during lactation, which is three times greater than the amount of Zn that is transferred to the placenta during pregnancy (37). Compared with other micronutrients, the concentration of Zn in the milk (~2 mg/L) is ~10 times greater than the concentration of iron or copper in the milk (~0.2 mg/L) (9). The high concentration of Zn in milk suggests that Zn transport in the mammary gland is tightly regulated for the sake of the nursing neonate (18, 19).

The regulation of Zn within the mammary gland and the effect on the infant has not been extensively investigated. However, one study conducted in rural Bangladesh found that normal weight infants breast-fed from 4-6 months had a higher plasma Zn concentration than normal weight infants breast-fed < 4 months (38). The researchers suspect that the association between higher plasma Zn status and a longer duration of breast-feeding might be due to the higher intake of Zn from breast milk than from complementary foods. Nevertheless, iron and Zn deficiencies remain to be a problem in this country regardless, with or without breast feeding, due to the high prevalence of diarrhea, low birth weight infants, and micronutrient disease (38).

C. Genetic Mutations

A genetic mutation has been identified which leads to diminished Zn transfer from the mammary gland into the milk (30). Transient neonatal Zn deficiency is a condition that results from a point mutation in the Zn exporter ZnT2, in which a conserved histidine is substituted with an arginine in the N-terminal domain, leading to decreased Zn secretion from mammary epithelial cells (30). Consequently, the low milk Zn concentration causes an infant to become severely Zn deficient and cannot be corrected by maternal Zn supplementation (3). While lactating mothers experience no physical symptoms, breast-fed infants will develop severe eczema, decreased growth, sparse hair growth and diarrhea (2, 30).

To illustrate the effects of transient neonatal Zn deficiency, Sambasiviah, et al. reported a case study on a nine-month-old female who was experiencing such symptoms (39). Topical and systemic medication did not improve the skin lesions on her face, perineal region, and upper and lower limbs, and no abnormalities were found in hematological and urine laboratory tests, stool examinations, serum creatine, hair examinations, blood urea and liver function tests. However,

the Zn level in the serum was assessed and recorded at 45 µg/dl, while the normal value is typically 70-120 µg/dl. Furthermore, the maternal breast milk contained only 7 µg/L of Zn, for what should be 11-12 µg/L. Gradual weaning from breast feeding and oral Zn supplementation allowed conditions to drastically improve within 48 hours.

An additional genetic mutation can lead to Zn deficiency in a neonate. A hereditary Zn disorder, acrodermatitis enteropathica, is caused by a mutation in the Zip4 gene and consequently impairs the uptake of Zn into the enterocyte and inhibits absorption (40). The inadequate absorption of Zn will impair the proliferation of the intestinal, epidermal and tongue papillae cells first due to their rapid turnover, and symptoms similar to those of transient neonatal Zn deficiency will be exhibited. Fortunately, acrodermatitis enteropathica is known to be reversible with Zn supplementation.

The distinction between these two disorders is crucial, because while an infant diagnosed with acrodermatitis enteropathica must take Zn supplements for life, continuing to supplement a child who had suffered from transient neonatal Zn deficiency could be toxic (2). Unlike acrodermatitis enteropathica, an individual with transient neonatal Zn deficiency is able to sufficiently absorb Zn, so over-supplementation must be avoided in order to inhibit the associated effects, such as copper and iron deficiency (39).

VI. Assessment of Zinc and Future Implications

Although Zn status is often evaluated by plasma or serum Zn measurements, such levels are known to be inaccurate because Zn is so tightly regulated in the body. For instance, plasma Zn can be affected by dietary intake, time of the day, stress, infection and steroid use. Clinical

effects of Zn deficiency can therefore be present without abnormal laboratory results (12). Attempts have also been made to assess Zn by measuring urinary excretion, hair concentration, Zn-dependent enzyme activity, red blood cells, leukocytes and metallothionein, but none have been proven effective (15). It is important to develop an adequate method of Zn assessment, especially given the amount of affected women (~82%) in the world who are likely to have a marginal Zn deficiency without obvious symptoms (17). For instance, a breast-fed infant may develop typical symptoms of Zn deficiency, but the epidemiology of such symptoms would remain unclear and worsen due to the lack of adequate assessment and lack of awareness about the clinical signs of Zn deficiency.

In addition to enhancing Zn assessment techniques, future research should also further investigate the roles of Zn transporters within the mammary gland in order to evaluate the impact of mammary gland Zn metabolism and the effects on nutrient secretion into the milk. For instance, could dysregulation in Zn metabolism lead to insufficient secretion of macronutrients or other micronutrients like copper and iron? Thus far, research has shown that Zn deficiency lowers immune function, inhibits cell growth and expression, and affects the activity of over 300 Zn-dependent enzymes and processes in the body (2). The effects and clinical symptoms may vary in severity depending on the level of Zn deficiency, and attempts must be made to reduce the prevalence of the primary causes. In any case, based on discussed studies above, it is clear that Zn deficiency is prevalent in the world today and can affect the health of the fetus, neonate, child and adult, and research should continue to be conducted in all stages of the human lifecycle.

VII. Zinc in the Mammary Gland

Throughout lactation, the mammary gland must regulate the milk secretion of a complex mixture of nutrients. Breast-fed infants are completely dependent upon the volume of milk and nutrient composition of milk secreted from the mammary gland (25). It is therefore essential to have an elaborate understanding of nutrient metabolism in the mammary gland due to the consequences that compromised nutrition may have on neonates.

During lactation, the mammary gland transfers 1-2 mg of Zn into the milk per day (2). The amount of Zn that is transferred into the mammary gland for secretion is almost twice the amount of Zn that is transferred to the fetus during the third trimester of pregnancy (2). This evidence demonstrates the tight regulation of Zn within the mammary gland. As discussed, recommended Zn intakes increase during periods of growth, pregnancy and lactation. Due to these increased requirements and coupled with inadequate Zn intake, it is estimated that 82% of pregnant and lactating women worldwide are marginally Zn deficient (17). This alarming prevalence is a concern not only for lactating women, but even more so for nursing infants who are completely dependent upon breast milk for their nutrition.

Research has shown that the Zn transporters ZnT1, ZnT2 and ZnT4 are expressed in mammary gland tissue (2). ZnT1 is a ubiquitously expressed Zn exporter, primarily localized at the plasma membrane, and has been shown to increase in mRNA expression upon Zn exposure (27). The role of ZnT1 in the mammary gland is not completely understood. Studies have proposed that ZnT1 is only highly expressed in the placenta, small intestine and kidney (28). However, additional research has shown that ZnT1 is localized to the luminal membrane of the mammary

gland and it has since been suggested that ZnT1 contributes to intracellular Zn accumulation and Zn export from the mammary gland (2).

ZnT2 has been detected in the mammary gland, prostate and pancreas (29). The restricted expression of ZnT2 appears limited to secretory tissues, suggesting that ZnT2 contributes to the secretion of biological fluids such as milk, seminal fluid and pancreatic fluid. Specifically, ZnT2 is localized proximal to the luminal membrane of the mammary epithelial cell (2). Previous studies have identified a mutation associated with dysregulated ZnT2 function in the mammary gland that leads to a ~75% reduction in milk Zn concentration (30). More recently, polymorphisms in ZnT2 have been associated with dysregulated Zn secretion and metabolism in mammary cells (48). The transcription of ZnT2 is regulated by prolactin, causing ZnT2 to play a significant role in the transfer of Zn into the milk (2).

ZnT4 is localized to intracellular vesicles and is expressed ubiquitously, yet abundantly in the mammary gland, brain and kidney (2). Specifically, ZnT4 was found to be most abundant in cells surrounding the alveolar ducts and may co-localize with milk-protein containing vesicles (2, 29). Like many aspects of Zn regulation in the mammary gland, the exact role of ZnT4 in milk secretion has yet to be determined. However, studies have confirmed that a mutation in SLC30A4 in mice, which encodes for ZnT4, leads to a ~35% reduction in milk Zn concentration of mice and produces the“lethal milk” syndrome (31). This evidence suggests that ZnT4 may be a critical component to milk secretion in the mammary gland.

As discussed, the SLC39A gene family encodes for Zn transport into the cytosol from extracellular fluid or a vesicle. The expression of Zip proteins 1-4 has been detected in the mammary gland (2). Despite this documentation, the particular role of each Zip transporter in the

mammary gland has yet to be understood. Most evidence for mammary gland function pertains to Zip1 and Zip3.

Studies have investigated the effects of a *Zip3*-null phenotype on Zn transfer within the mammary gland (33). Observations have indicated that Zip3 may not have a critical role in Zn transfer from maternal circulation to the mammary gland, contrary to previous hypotheses (33). Rather, studies have shown that Zip3 is localized proximal to the luminal membrane of the mammary gland and may mediate Zn reuptake from the alveolar lumen in order to retain Zn from the milk pool (33). Because Zip3 functions in Zn reuptake from the lactating mammary gland, *Zip3*-null mice had less Zn retention in the mammary gland and greater Zn retention in the milk pool compared with wild-type mice. Additionally, the morphology of the mammary gland was compromised in *Zip3*-null mice, which further suggests that Zn regulation in the mammary glands of *Zip3*-null mice was impaired (33).

Although a complete understanding of Zn metabolism in the mammary gland has yet to be established, studies have suggested that Zn dysregulation in the mammary gland may compromise the health and development of a nursing infant. Due to the fact that ~82% of women worldwide are at risk of developing Zn deficiency, it is essential to gain an enhanced understanding of the implications from Zn deficiency on both the mother and nursing neonate. Evidence, though limited, has shown that Zn status may affect the structure and function of the mammary gland throughout lactation, which could consequently cause to infant to be inadequately nourished. Future research is needed to further explore associations between Zn deficiency and the morphology of the mammary gland and nutritional content of breast milk during lactation.

--INTRODUCTION--

Zinc (Zn), an essential trace mineral, is vital to human health. Zn is the second most abundant trace element in the body and is present in all cells (10). As an essential component of over 300 enzymes and proteins, Zn is involved in numerous biological processes at both the physiological and molecular levels (2). Although an understanding of Zn transport and regulation remains incomplete, research throughout the past decade has confirmed Zn's role in cell and tissue growth, immune function, protein synthesis, skin integrity, respiration and antioxidant defense (2, 12). Importantly, recent studies have also revealed the high prevalence of Zn deficiency worldwide (17, 34, 35). One of the principal causes of Zn deficiency has been linked to the action of phytate, a storage form of phosphorus that if often present in corn, cereals, rice and legumes. The human gastrointestinal tract lacks the enzyme phytase, and therefore cannot eliminate phosphate groups from forming strong and insoluble complexes with cations; rather, the phytate-bound minerals will be excreted in the stool (14). This cause of Zn deficiency is most common in developing countries where the population mainly consumes high phytate diets due to availability and affordability. When estimating Zn consumption by total daily per-capita in a population's food supply, Hotz, et al. determined that 71.2% of the population in Southeast Asia was at risk of developing Zn deficiency due to inadequate Zn intake (35).

This issue primarily impacts women and children, with 450,000 children at risk of dying each year from the effects of Zn deficiency due to increased incidence of diarrhea, pneumonia and malaria (11). Zn requirements increase during periods of growth, pregnancy, and lactation, causing pregnant women, lactating women and young children to be the most vulnerable

population groups (41). Zn is essential during rapid periods of growth due to its role in gene expression, cell growth and cell differentiation, explaining why the rapidly growing embryo, fetus, infant and young child are particularly susceptible to faltered growth, delayed neurological development, and an impaired immune response if Zn intake is inadequate (42). During lactation, the mammary gland transfers 1-2 mg of Zn into the milk per day (2). The amount of Zn that is transferred into the mammary gland for secretion is almost twice the amount of Zn that is transferred to the fetus during the third trimester of pregnancy (9). Due to increased requirements coupled with low Zn intake, it is estimated that 82% of pregnant and lactating women worldwide are marginally Zn deficient (17). This alarming prevalence is a concern not only for lactating women, but even more so for nursing infants who are completely dependent upon breast milk for their nutrition.

The concentration of Zn in the milk (~2 mg/L) is ~10 times greater than the concentration of other trace elements such as iron or copper (~0.2 mg/L) (9). Although the concentration of Zn in the milk does decrease throughout lactation, the concentration of Zn in the mammary gland is maintained, suggesting that Zn is tightly regulated and accumulated within the mammary gland (43). Although further research is needed in order to completely understand the mechanisms of Zn accumulation within the mammary gland and secretion into milk, our previous studies have suggested that Zn transfer into milk involves the integration of mammary gland Zn import, Zn sequestration and Zn secretion mechanisms (9, 33). We have also found that the lactogenic hormone prolactin increases Zn uptake into mammary cells, which may explain why Zn accumulates in the mammary gland during lactation (33). Specifically, our studies *in vivo* and *in vitro* have shown that Zn accumulates within the mitochondria and Golgi apparatus of the mammary gland. Once stimulated by prolactin, Zn is redistributed into vesicles for secretion into milk (9).

This evidence demonstrates the essentiality of proper Zn regulation within the mammary gland in order to provide optimal nourishment for the neonate. Errors in mammary gland Zn metabolism may therefore compromise the amount of Zn consumed by the neonate. Transient neonatal Zn deficiency has been documented as a condition that results from a point mutation in the Zn exporter ZnT2, in which a conserved histidine is substituted with an arginine in the N-terminal domain (30). This mutation leads to a diminished transfer of Zn from the mammary gland into milk, and consequently causes an infant to become severely Zn deficient (30). The condition cannot be corrected by maternal Zn supplementation, and while lactating mothers experience no physical symptoms, breast-fed infants will develop severe eczema, decreased growth, alopecia and diarrhea (2, 30, 39).

However, studies suggest that compromised Zn metabolism in the mammary gland may alter mammary gland function and milk composition. Mammary epithelial cells surround the lumen of the alveoli and milk components are accumulated and released into the lumen of mammary gland alveoli when stimulated by prolactin (9). Our previous studies have shown that mammary gland morphology was compromised in Zip3 knockout mice (33). Because Zip3 functions in Zn reuptake from the alveolar lumen in lactating mammary gland, *Zip3*-null mice had less Zn retention in the mammary gland and greater Zn retention in milk compared with wild-type mice, suggesting that the *Zip3*-null mice may have compromised mammary gland Zn metabolism (33). Although alterations in the mammary glands of *Zip3*-null mice were not drastic, the alveoli of *Zip3*-null mice were less dense and ductal branching was less-defined (33). These findings suggest that Zn status may affect the morphology of the mammary gland, and the effects of Zn deficiency on mammary gland structure and function should be further investigated.

Previous studies have also demonstrated that Zn deficiency may compromise the secretion of milk from the mammary gland (26). For instance, rats fed a moderately Zn deficient diet (7 mg Zn/kg) and a marginally Zn deficient diet (10 mg Zn/kg) had lower milk volume during lactation compared with those fed a control diet (26). Due to the high prevalence of marginal Zn deficiency worldwide, this outcome should be further investigated in order to account for compromised milk intake in infants of marginally Zn deficient mothers. Furthermore, the possibility of lower milk secretion raises questions about the nutritional value of the milk. Research investigating the effects of Zn deficiency on milk nutrient composition is limited. Studies have suggested that milk Zn concentration is maintained over a wide range of dietary intake in order to provide optimal Zn to a nursing infant (2, 43). Yet, the total Zn in the mammary gland and milk Zn concentration were significantly compromised by moderate Zn deficiency in rats (26).

In addition to milk Zn concentration, few studies have addressed the association between Zn deficiency and the nutritional composition of milk. During lactation, the mammary gland must regulate the secretion of a complex mixture of nutrients. Specifically, lactose and secreted proteins are synthesized in the Golgi apparatus and transported through the secretory compartment for exocytosis into milk (9). Lipids, on the other hand, are removed from the apical membrane as droplets surrounded by the milk fat globule membrane (9). Although evidence is limited, one study does suggest that milk protein composition may be altered by Zn deficiency (44). Although total protein concentration in milk was not significantly affected by inadequate Zn intake, there was a higher concentration of β-casein in the milk of rats fed low-Zn diets (7 mg Zn/kg) (44). This suggests that Zn intake may alter the protein composition milk. However, the study did not explore the effects of marginal Zn deficiency on protein composition. Future

studies are needed to identify the effects of marginal Zn deficiency on protein concentrations and investigate additional effects on macronutrient composition in milk.

The high prevalence of Zn deficiency throughout the world demonstrates the need to further understand the physiological and molecular consequences of Zn deficiency. Based the role for Zn on the structure and function of the mammary gland, we hypothesized that marginal Zn deficiency would disrupt mammary gland morphology and compromise secretion. Additionally, due to the limited evidence suggesting an association between Zn deficiency and milk nutrient composition, we hypothesized that marginal Zn deficiency would alter Zn and macronutrient composition in milk. Our findings suggest that mammary gland morphology is compromised by marginal Zn deficiency and milk secretion in mice is significantly lower. Although plasma and mammary gland Zn concentrations were not compromised, our results indicated that milk Zn secretion was reduced by marginal Zn deficiency. We found that protein concentration and composition were significantly altered in milk of marginally Zn deficient mice.

--MATERIALS AND METHODS--

I. Candidate's Role

The author of this thesis was primarily responsible for every component of this project, aside from assistance with animal care and dissections. Additionally, Zn measurements (Table 2) were obtained from Nicholas McCormick (Kelleher Lab).

II. Animal Care

Animals - This study was approved by the IACUC Committee at the Pennsylvania State University, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Female C57BL/6 mice were obtained commercially (Charles River; Wilmington, MA) and housed individually in polycarbonate cages. Mice were fed a purified diet based on AIN93 containing 25 mg Zn/kg (ZA) or a diet reduced in Zn (12 mg Zn/kg, ZD) for thirty days prior to conception. Mice were maintained on a 12 h light/dark cycle under controlled temperature and humidity. Mice (n=10) were bred and allowed to deliver naturally. On lactation day (LD) 7-10, dams were removed from pups for 2 h to control for effects of suckling, and milk was collected as previously described (45). Briefly, mice were anesthetized with isofluorane and milk was manually expressed after subcutaneous oxytocin injection (0.2 ml/dam). Mice were subsequently euthanized by CO2 asphyxiation. Blood was drawn by cardiac puncture, collected

into heparinized vials, and centrifuged to measure the Zn concentration of plasma. Mammary glands were removed and snap frozen or fixed in phosphate-buffered paraformaldehyde (4%).

III. Histological Staining

A. Tissue Sections

Fixed mammary glands were embedded in paraffin and sectioned (5 μm) onto glass slides. For each staining procedure, tissues from ZA and ZD mice were used (n=2) with approximately 5-7 sections per slide. Following each of the staining procedures described below, glass cover slips (VMR; Radnor, PA) were mounted onto slides with DePex Mounting Medium (Electron Microscopy Sciences; Hatfield, PA) after counterstaining was complete.

B. Hematoxylin and Eosin Staining

Hematoxylin and Eosin staining was used to assess cell morphology. Harris hematoxylin is a basic dye that stains nuclei blue or purple, while Eosin Y is an acidic dye that stains cytoplasm, collagen, muscle fibers and red blood cells pink. Mammary gland sections from ZA and ZD mice were deparaffinized and dehydrated by immersing slides in xylene, 3 min; xylene, 2 min; 100% ethanol, 2 min; 100% ethanol, 2 min; 95% ethanol, 2 min; 95% ethanol, 2 min; and 70% ethanol, 2 min. Sections were then rinsed with distilled water and stained with Harris hematoxylin solution for 4 min. Sections were again rinsed in distilled water and quickly dipped into 1% acid alcohol (1% HCL acid) until stained lavender. Sections were placed in blueing solution (1% lithium carbonate) for 2 min and visually checked for the blueing of nuclei. After being dipped in distilled water for 4 min, slides were immersed in 80% ethanol for 2 min and

95% ethanol for 2 min. Sections were counterstained with eosin Y solution for 45-60 sec and again dehydrated with ethanol and xylene: 100% ethanol, 2 min; 100% ethanol, 2 min; 100% ethanol, 2 min; xylene, 2 min; and xylene, 2 min.

C. Apoptosis Detection

The TACS-XL Basic In Situ Apoptosis (TUNEL) Detection kit (Trevigen; Gaithersburg, MD) was used to identify apoptotic cells in the mammary gland. All reagents were used from kit unless noted otherwise, and methods were obtained from manual instructions. Mammary gland sections from ZA and ZD mice were deparaffinized and dehydrated with xylene, 3 min; xylene, 2 min; 100% ethanol, 2 min; 100% ethanol, 2 min; 95% ethanol, 2 min; 95% ethanol, 2 min; 70% ethanol, 2 min; and 1X PBS, 5 min. Each section was digested with Proteinase K solution for 30 min at 37°C in a humidity chamber, and then immersed in Quenching Solution for 5 min. Sections were immersed in 1X TdT Labeling Buffer for 5 min, covered with Labeling Reaction Mix for 60 min in a 37°C humidity chamber, and immersed in 1X TdT stop buffer for 5 min. Sections were treated with Antibody Solution (2 µL Anti-BrdU/100 µL strep dilutant) for 30 min in humidity chamber, rinsed in 1X PBS and then covered in 100 µl Strep-HRP Solution and incubated at room temperature for 10 min. DAB Peroxidase Substrate Kit (Vector; Burlingame, CA) was used to detect HRP-conjugated antibody and produce insoluble brown-colored nicked DNA. Sections were counterstained with Toluidine Blue for 20-30 sec and washed in 50% ethanol, 30 sec; 70% ethanol, 30 sec; 70% ethanol, 1 min; 95% ethanol, 2 min; 95% ethanol, 2 min; 100% ethanol, 2 min; 100% ethanol, 2 min; xylene, 2 min; and xylene, 3 min. In order to quantitate data, nicked DNA (represented as brown ‘dots’) was counted twice by investigators blinded to treatment in order to prevent experimental bias.

D. Ki67 Immunohistochemistry

To quantitate effects of ZD on cell proliferation, mammary gland sections were immunostained for Ki67 protein. All reagents were from Vector (Burlingame, CA) unless otherwise noted. Sections were deparaffinized and dehydrated with xylene, 3 min; xylene, 2 min; 100% ethanol, 2 min; 100% ethanol, 2 min; 95% ethanol, 2 min; 95% ethanol, 2 min; 70% ethanol, 2 min; and 1x PBS, 5 min. Slides were immersed in a boiling solution of Antigen Unmasking Solution and water for 10 min, cooled in 1X PBS, immersed into 3% Hydrogen Peroxide for 10 min, and submerged into a Trypsin solution (0.5 mg/ml) for 15 min at 37°C. Sections were then blocked with Avidin (1 drop) and Biotin (1 drop) each for 15 min, respectively, and incubated in Blocking Buffer (10% heat inactivated Goat Serum/1% BSA/0.05% Tween-20/89% PBS) for 60 min. Sections were incubated with Ki67 Antibody (Abcam; Cambridge, MA) for 60 min and Biotinylated Anti-Rabbit Antibody for 2 h. Sections were incubated in ABC reagent (1 mL PBS, 6.25 µl Solution A, 6.25 µl Solution B) for 2 h to detect the biotin and amplify the detection signal obtained from DAB Substrate. Slides were rinsed with 1X PBS between each step. Lastly, sections were counterstained with Toluidine Blue, ~20 sec; 50% ethanol, 30 sec; 70% ethanol, 30 sec; 70% ethanol, 1 min; 95% ethanol, 2 min; 95% ethanol, 2 min; 100% ethanol, 2 min; 100% ethanol, 2 min; xylene, 2 min; and xylene, 3 min.

E. Microscopy

Sections were viewed under Leica DM IL LED microscope and images were obtained by Leica EL600 compact light source (Germany).

IV. Milk Secretion (Weigh-Suckle-Weigh Technique)

The protocol for this study was adapted from a study in mice conducted by McDonald and Nielsen (46). To assess milk secretion, weigh-suckle-weigh was performed on ZA and ZD mice between 7 to 10 days of lactation. Table 1 shows the timeline used for periods of suckling and separation (46). During separation periods, the litters were each placed into a separate clean cage to prevent suckling and were provided with bedding to prevent heat loss. The pups were then placed back into their cages with the dams to conduct a "suckle-emptying" period. This period was used to ensure that all dams had empty mammary glands to normalize milk secretion measurements. After 2 h, the litters were again separated from the dams for 3 h. This period allowed for dams to produce milk and pups to become hungry. Litters were weighed and then placed back into the cage with dams and allowed to suckle for 2 h. Litters were weighed at the end of this suckling period and milk secretion was calculated as the difference between final and initial litter weights.

V. Determining Fat Percentage in Mouse Milk (Creamatocrit)

Frozen milk samples from mice fed ZA and ZD diets were warmed in a 37°C water bath for 10 min to thaw. Samples were mixed by vortexing, approximately 20 µl of milk was drawn into a 60 mm micro-hematocrit tube (VMR; Radnor, PA), and the tube was sealed with Critoseal (Fischer Scientific). Capillary tubes were spun in a hematocrit microcentrifuge for 10 min at 2500 rpm. In order to determine the percentage of fat, the total length of the milk in the capillary tube was measured (cm). The length of the milk fat in the capillary tube (designated as the thicker, creamier fraction) was measured (cm). To calculate the creamatocrit (% fat), the length of milk fat was divided by the total milk length and was expressed as a percentage.

VI. Measuring Protein Concentration in Mouse Milk

To measure the protein concentration in milk from ZA and ZD mice, total protein was first isolated from mouse milk. Each milk sample (50 µl) was diluted in two volumes of buffer (100 µl; sodium phosphate (50 mM), sodium chloride (150 mM), and EDTA (50 mM, pH 7.4)) and was centrifuged at 11,600 g for 15 min at 4°C. The supernatant was retained in a separate eppendorf tube and total milk protein was measured by the Bradford assay to determine protein concentration.

VII. Defatting Mouse Milk

Whole milk samples were defatted prior to measuring lactose concentration and assessing protein composition by Coomassie blue staining following SDS-PAGE gel electrophoresis (see below). Frozen whole milk samples were thawed on ice and centrifuged at 2000 g for 15 min at 4°C. The top of the sample consisted of solidified fat, while the skim milk supernatant lay beneath. The fat was gently scraped aside in order to remove the supernatant and transferred into a new tube.

VIII. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Skimmed milk (0.5 µl protein/well or 50 µg protein/well) from mice fed ZA or ZD diets was separated by SDS-PAGE using 12.5% polyacrylamide gels. Two gels were run, one using a constant sample volume (0.5 µl milk/well) in order to determine differences in milk protein concentrations, and the other using a constant protein amount (50 µg protein/well) in order to determine differences in the relative distribution of proteins. Samples were loaded onto a 12.5%

gel with running buffer (10% 10x Tris Glycine) and electrophoresed at 200 volts for 45 min. Proteins were stained with Coomassie Blue for 30 min followed by destaining in Destaining Solution (50% methanol/10% acetic acid/40% distilled water) for 5-6 h. Gels were visualized by Gel Logic 212 Pro and band densities were analyzed by densitometry in Carestream Molecular Imaging Software (Rochester, NY).

IX. Measuring Lactose Concentration in Milk

A Lactose Assay Kit was used (Abcam; Cambridge, MA) and the protocol was obtained from the kit's instruction manual. A standard curve was prepared in duplicate for a colorimetric assay by diluting the Lactose Standard (1 nmol/µl) with Lactose Assay Buffer (1% standard /99% buffer). Skimmed milk samples from mice fed a ZA and ZD diet were diluted with Lactose Assay Buffer (10% milk/90% buffer). To each well of a 96-well plate, 1 µl of the diluted sample, 2 µl of Lactase, and 50 µl of Lactose Reaction Mix (88% Lactose Assay Buffer/4% Probe/4% Lactose Enzyme Mix/4% HRP) was added. Each sample was prepared in duplicate. Lactase was added in order to convert lactose to galactose. Because free galactose interferes with the assay, controls were also analyzed to account for background by substituting 2 µl of Lactase with 2 µl Lactose Assay Buffer for each sample. The reaction was incubated for 60 min at 37°C and protected from light. Optical density was measured at 570 nm using a microplate spectrophotometer (Epoch BioTek; Winooski, VA). In order to determine the lactose concentration of each sample, the galactose background values of the controls were subtracted from the sample readings. A standard curve was plotted as lactose amount (nmol) vs. absorption readings and the sample readings were applied to the standard curve. To calculate lactose concentration, the galactose amount in the sample wells (nmol) was divided by the sample volume added into the wells (µl) and was converted into µmol/ml.

X. Statistical Analysis

Results are presented as mean ± standard deviation. Statistical comparisons were performed using Student's t-test (Prism Graph Pad, Berkeley, CA) and a significant difference was demonstrated at $p<0.05$.

--RESULTS--

I. Plasma and mammary gland zinc concentrations were not compromised by zinc deficiency.

Although we fed the mice in our study a marginally Zn deficient diet, mammary gland Zn levels, plasma Zn levels and liver Zn levels were not compromised (Table 2). However, milk Zn concentration was significantly lower ($p<0.05$) in ZD mice (20.84 ±2.4 µg Zn/ml) compared with mice fed ZA (24.28 ±2.3 µg Zn/ml). By measuring Zn concentration in the plasma, mammary gland, liver and milk, our results indicated that marginal Zn deficiency did not significantly alter Zn "status" but significantly compromised milk Zn concentration.

II. Mammary gland morphology was compromised by zinc deficiency.

To our knowledge no studies have investigated the effects of Zn deficiency on mammary gland morphology and function. To determine if mammary gland morphology was affected by marginal Zn deficiency, we compared mammary gland sections from ZA and ZD mice using hematoxylin and eosin staining. We found that mammary gland morphology from ZD mice was severely compromised as reflected by distorted mammary gland histology and less dense alveoli compared with ZA mice (Figure 1). Mammary epithelial cells tightly regulate the accumulation, production and secretion of milk components (2). Our results suggest that marginal Zn deficiency severely compromises mammary gland morphology and thus may impair milk secretion due to the reduced number of alveoli.

III. Greater apoptosis was detected in the mammary gland of ZD mice.

Previous studies have shown that Zn deficiency is associated with dysregulation in apoptosis and cell proliferation (20). We hypothesized that marginal Zn deficiency induces apoptosis within the mammary gland. To determine the cause of alterations in mammary gland morphology, the extent of apoptosis and cell proliferation were investigated. As illustrated in Figure 2, TUNEL staining indicated that the occurrence of apoptosis was significantly higher in ZD mammary glands compared with ZA mammary glands ($p<0.05$). We found no significant effects on cell proliferation using Ki67 staining when comparing the mammary gland sections from ZA and ZD mice. These results demonstrate that marginal Zn deficiency induces cell death within the mammary gland.

IV. Milk secretion in mice was significantly reduced by zinc deficiency.

Mammary epithelial cells surround the alveolar lumen and tightly regulate the accumulation, production and secretion of milk components (2). We hypothesized that the compromised morphology and fewer alveoli in the mammary gland from ZD mice may impair milk secretion. To determine if milk secretion was affected, a weigh-suckle-weigh protocol was used over a 2-h suckling period for ZA and ZD mice between 7-10 days of lactation (Figure 3). We found that milk secretion was significantly lower in ZD mice compared with ZA mice ($p<0.05$). Our results indicate that marginal Zn deficiency reduced the amount of milk secreted from the mammary gland during periods of suckling.

V. Milk protein concentration was higher in milk of ZD mice.

Because milk secretion was lowered by Zn deficiency, we hypothesized the macronutrient content of milk from ZD mice would be lower. To determine effects of marginal Zn deficiency on lipid content, the percentage of fat in milk from ZA and ZD mice was determined by creamatocrit. We found no significant differences in lipid content. To determine effects of marginal Zn deficiency on carbohydrate content, lactose concentration was measured in milk of ZA and ZD mice by a lactose assay. We found no significant differences in lactose content. To determine effects of marginal Zn deficiency on protein content, protein concentration in milk from ZA and ZD mice was measured by the modified Bradford assay. We found that ZD mice had significantly higher milk protein concentration compared with ZA mice ($p<0.05$). Our results indicate that marginal Zn deficiency alters the concentration protein in milk, but does not significantly change the lipid or carbohydrate content (Figure 4).

VI. Relative distribution of major milk proteins was compromised in ZD mice.

Marginal Zn deficiency lowers milk secretion in mice. Due to the higher protein concentration observed in milk of ZD mice, we hypothesized that protein composition of milk would be altered due to Zn deficiency. To determine if protein composition was altered, skimmed milk (50 µg/well or 0.5 µl/well) from mice fed a ZA or ZD diet was separated by SDS-PAGE gel electrophoresis and the relative distribution of major milk proteins were assessed by staining with Coomassie Blue (Figure 5). We found that the amount of caseins and whey acidic protein was significantly lower in milk of ZD mice compared with milk of ZA mice ($p<0.05$) by analyzing band densities by densitometry. There were no significant differences in the amount of lactoferrin or serum albumin in milk from ZA and ZD mice. Our results indicate that marginal

Zn deficiency alters milk protein composition by reducing the amount of caseins and whey acidic protein in milk of ZD mice.

--DISCUSSION--

The increased accumulation of Zn in the mammary gland required for secretion into milk during lactation reflects the essentiality of Zn for optimal health and development in the nursing neonate (2). It is estimated that 82% of pregnant and lactating women worldwide are at risk of developing Zn deficiency due to inadequate Zn intake from the diet (17). This alarming prevalence is a concern for both lactating women and their nursing infants who are completely dependent upon breast milk for nutrition. The consequences of severe Zn deficiency on the infant during lactation have been researched thoroughly and have been shown to cause detrimental clinical effects on the infant which include severe eczema, inhibited growth, compromised immune function, alopecia and diarrhea (2, 30, 39). However, studies investigating the association between marginal Zn deficiency during lactation and the effects on the nursing infant are limited. Furthermore, few studies have addressed the maternal factors that may contribute to marginal Zn deficiency in the infant, which may include compromised Zn concentration in the mammary gland and milk, impaired mammary gland structure, and decreased milk secretion. Our findings suggest that mammary gland morphology is severely compromised by marginal Zn deficiency and results from greater apoptosis in the mammary glands of Zn deficient mice despite the fact that total Zn content of the mammary gland was unaffected. A key finding from our study was the reduction in milk secretion from the mammary glands of marginally Zn deficient mice. In addition, we found that milk protein concentration and composition were significantly altered in milk of marginally Zn deficient mice. Based on our results, we suggest that marginal Zn deficiency disrupts mammary gland morphology and interferes with milk secretion. Due to

the high prevalence of marginal Zn deficiency worldwide, these implications are a major cause of concern for compromised maternal and infant health during lactation.

Previous studies have indicated that mammary gland and milk Zn levels are maintained over a wide range of dietary intake (37, 45). Although studies in rats have shown that milk Zn concentration is compromised during severe Zn deficiency, changes in milk Zn concentration have not been observed during marginal Zn deficiency. To explore the effects of marginal Zn deficiency in lactating mice, we fed mice a marginally Zn deficient diet and determined that despite the fact that Zn levels in plasma, liver and mammary gland were not compromised, milk Zn was reduced. These contradicting results may be due to difference between species. Because Zn metabolism in the mammary gland in humans is not understood, more research is needed to determine which species is more characteristic of humans in terms of mammary gland Zn metabolism. Our findings suggest that mammary gland Zn metabolism is tightly regulated at the expense of milk Zn. One possible theory is that in addition to maternal circulation, milk Zn pools are utilized to maintain adequate Zn concentration in the lactating mammary gland. Previous studies have illustrated the reuptake of Zn from the milk into the mammary gland in order to maintain Zn homeostasis (33). For instance, milk Zn concentration was higher in Zip3-null mice compared with wild-type mice, suggesting that Zip3 plays an important role in mammary gland Zn reuptake (33). Our previous studies have demonstrated that Zn is transferred into the mammary gland from two Zn pools, uptake from maternal circulation and reuptake from milk (33). Because milk Zn concentration was reduced by marginal Zn deficiency yet mammary gland Zn concentration was maintained, we questioned whether the secretory functions of the mammary gland were compromised. To determine if mammary gland function was disrupted, we examined mammary gland morphology. To our knowledge, no studies have directly investigated the effects of marginal Zn deficiency on the structure of the mammary gland. However, previous studies in

our lab have found that mammary gland morphology was compromised in Zip3-null mice (33). The alterations in tissue architecture consisted of less-defined ductal branching and less dense alveoli (33). Because we found that Zip3 functions in Zn reuptake from the alveolar lumen, the observations suggested that marginal Zn deficiency may underlie the Zip3-null phenotype (33). To investigate the effects of marginal Zn deficiency on mammary gland morphology, we used hematoxylin and eosin staining to examine differences between the mammary glands of marginally Zn deficient mice and Zn adequate mice. We found that mammary gland morphology from Zn deficient mice was severely compromised as reflected by distorted mammary gland histology and less dense alveoli compared with Zn adequate mice. These data suggest that milk Zn pools may be drawn upon to maintain mammary gland function to a limited extent.

Studies have shown that changes in cellular Zn pools can induce apoptosis and inhibit cellular proliferation (47). Mitochondrial Zn plays a critical role in regulating bioenergetics and apoptosis (48). An accumulation of Zn in the mitochondria activates the cytochrome c caspase leading to apoptosis (48). We detected greater apoptosis in the mammary glands of Zn deficient mice. However, Ki67 (a marker for cell proliferation) was not affected, suggesting that the higher incidence of apoptosis in the mammary glands of Zn deficient mice contributed to the compromised mammary gland morphology and reduced density of alveoli. Although cellular Zn levels in the mammary gland were maintained, the sub-cellular distribution of Zn was not investigated. We speculate that perhaps marginal Zn deficiency dysregulated cellular function by increasing Zn uptake into the mitochondria, thereby activating apoptosis. Although mammary gland weights were not documented in this study, our previous study investigating the role of Zip3 the mammary gland found that reduced mammary gland weight was correlated with greater apoptosis in Zip3-null mice (33). This evidence supports the hypothesis that greater apoptosis in

the mammary gland results from Zn deficiency and compromises the development of morphological components in the mammary gland.

Other factors aside from a greater incidence of apoptosis may contribute to the disrupted structure of the mammary gland. Because Zn is an essential component of over 300 enzymes and proteins (33), Zn deficiency may impair enzyme activity and inhibit vital cellular processes. For instance, Lmo4 is a Zn finger LIM domain protein that is highly expressed in ductal and alveolar luminal cells of the mature mammary gland (49). Sum, EYM, et al. found that reduced expression of Lmo4 results in impaired lobuloalveolar development in the mammary glands of mice (49). These findings suggest that Lmo4 induces alveolar epithelial proliferation and is required for normal development of the mammary gland. In the case of marginal Zn deficiency, perhaps Lmo4 is unable to carry out its role in alveolar epithelial proliferation without its essential Zn fingers, which would result in lower density of alveoli and impaired ductal development. We speculate that while marginal Zn deficiency may cause Zn to accumulate in the mitochondria and signal apoptosis, perhaps marginal Zn deficiency reduces the Zn pool in the nucleus and has an inhibitory effect on Zn-dependent enzymes like Lmo4. Future studies should examine the concentrations of expressed Lmo4 in the mammary glands of Zn deficient mice compared with Zn adequate mice in order to account for differences in mammary gland morphology.

The severely compromised mammary gland morphology raises additional questions concerning cell function, specifically milk secretion. Nursing neonates are completely dependent upon the content of breast milk for health and nutrition. Previous studies have indicated that severe Zn deficiency results in reduced milk secretion from the mammary gland, yet there is limited evidence concerning the effects of a marginal Zn deficiency (26). To further investigate the effects of marginal Zn deficiency on milk secretion, we fed mice a Zn deficient diet and

determined that marginal Zn deficiency compromised milk secretion (79% decrease). During lactation, milk is produced and stored within the alveoli and secreted upon contraction by mammary epithelial cells (40). However, due to the impaired ductal branching and alveoli function of mammary glands from Zn deficient mice, our data suggest that the mammary gland is unable to carry out optimal secretory functions. This effect may have detrimental implications on the health and development of the nursing neonate. In order to further explore this phenomenon, we investigated the nutritional content of secreted milk.

Effects of Zn deficiency on macronutrient content in milk have not been extensively investigated. To our knowledge, no studies have identified associations between Zn deficiency and carbohydrate or lipid content. Zubieta and Lönnerdal found that the total protein concentration was not altered between Zn deficient and Zn adequate mice (44). In contrast to reports from Zubieta and Lönnerdal (44), our results indicated that protein concentration was higher in milk of marginally Zn deficient mice. The mechanism for an increase in total protein concentration is not yet understood. Perhaps the higher protein concentration results from an increased synthesis of protein in the mammary gland in response to Zn deficiency. Alternatively, it is more likely that the increase in protein concentration results from the reduction in milk secretion that was observed in Zn deficient mice. To test this hypothesis, we measured lactose in milk and postulated that lactose concentration would decrease due to its role in regulating osmolality (50). However, we did not detect a significant effect on lactose concentration. Extensive research is needed in order to investigate this mechanism and determine the underlying mechanism for the increased protein concentration in milk.

To further investigate the observed alterations in protein concentration, we analyzed protein composition in milk. The main proteins in mouse milk include α-lactalbumin, lactoferrin, serum albumin, α_{s1}-casein, glycosylated κ-casein, β-casein, γ-casein, ε-casein, κ-casein, and whey

acidic protein (WAP). Previous studies have indicated that the concentration of β-casein in milk was significantly higher in the milk of Zn deficient mice (44). However, our results indicated that the amount of caseins and WAP was significantly lower in milk from Zn deficient mice compared with milk from Zn adequate mice. The mechanisms responsible for the lower concentrations of caseins and WAP are unclear and numerous factors may contribute to this phenomenon. Because Zn plays an essential role in gene expression (2), marginal Zn deficiency may inhibit the expression of caseins and WAP in the mammary gland, thus accounting for the lower concentrations in milk. Or, perhaps mammary gland function in general is compromised due to marginal Zn deficiency. Such inferences should be extensively examined in future studies in order to better understand the altered concentration of protein in milk.

Future investigation concerning the altered protein concentration in milk with reduced Zn levels may have important implications for nursing infants. Due to the similar protein composition in the milk of mice and humans, our results suggest that if the concentration of caseins and WAP are compromised this may contribute to the depressed immune function of Zn deficient infants. Studies have shown that in addition to inhibiting the proliferation of mammary epithelial cells and inhibits tumorigenesis, WAP also has antibacterial activity (53). For example, WAP isolated from rat milk was shown to inhibit the growth of *Staphylococcus aureus*, resulting in shrunken and twisted bacterial cells. WAP, however, is not found in human milk, and a protein equivalent to WAP in humans has not yet been discovered (53). Yet, because WAP does have a similar structure to protease inhibitors which are found in humans (53), examination of WAP in mouse milk may be beneficial to understanding the function of protease inhibitors found in human milk on immune function in neonates. Caseins are highly phosphorylated proteins that are present in human milk (14). β-casein is a key component of breast milk due to its assistance in the digestion and absorption of macronutrients and micronutrients from milk, specifically

calcium (54). Casein phosphopeptides (CPP) are formed during the digestion of casein and keep calcium in a soluble form, contributing to high bioavailability of calcium in milk (14). Furthermore, CPP in milk have been associated with antithrombotic, antihypertensive and opioid activities, which may contribute to sleeping behavior and fluid transport in the small intestine of breastfed infants (54). κ-casein, also found in human milk, has been shown to prevent Helicobacter pylori from attaching to the mucosal lining of the intestine. Caseins may therefore play critical roles in the digestive and immune functions of breastfed infants, and perhaps may account for the lower incidence of bacterial infection and colic in breastfed infants (54). Future studies in our lab will measure the relative protein composition in human milk with low Zn concentrations in order to further investigate whether lower casein concentration can compromise the health of infants.

In summary, results from this study document the compromised mammary gland morphology, milk secretion and protein composition in mice fed a marginally Zn deficient diet. Our results demonstrate that marginal Zn deficiency may have critical effects on the mammary gland structure and function of a lactating woman and may also compromise the health and development of nursing neonate. Current studies are underway to determine whether similar nutrient alterations are found in human milk with low Zn concentration, which could provide important implications considering the high prevalence of women at risk for marginal Zn deficiency worldwide. We hope to enhance our understanding of the implications resulting from Zn deficiency throughout lactation in order to eliminate the detrimental effects and optimize sustainability and development in a nursing infant.

TABLE 1: Timeline for weigh-suckle-weigh protocol [46]

Time (h)	Pups (7-10 days)
0	Separate from dams
1	Return to dams for suckle (emptying) period
2	
3	Separate from dams
4	
5	
6	Weigh litter; Return to dams to suckle
7	
8	Weigh litter

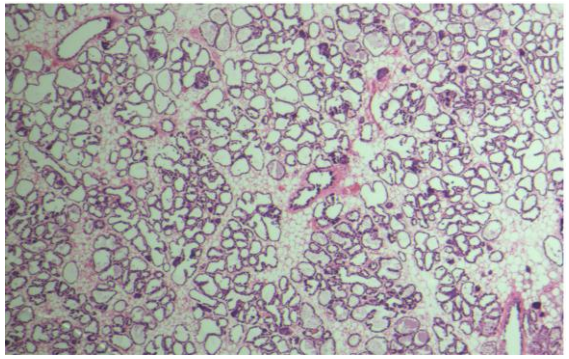
Pups were separated from dams for 1-h. During separation periods, the litters were each placed into a separate clean cage to prevent suckling and were provided with bedding to prevent heat loss. The pups were then placed back into their cages with the dams to conduct a "suckle-emptying" period. After 2-h, the litters were again separated from the dams for 3 h. Litters were weighed then placed back into the cage with dams and allowed to suckle for 2-h. Litters were weighed at the end of this suckling period.

TABLE 2: Plasma and mammary gland zinc concentrations were not compromised by zinc deficiency.

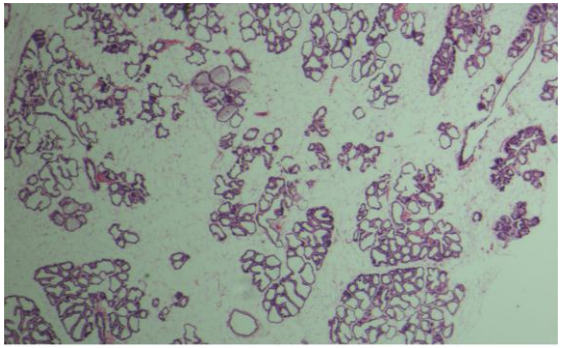
	ZA	ZD	Significance
Plasma (µg/ml)	0.69 ±0.08	0.83 ±0.16	$p<0.05$
Mammary gland (µg/g)	15.41 ±2.96	14.03 ±2.9	NS
Liver (µg/g)	28.59 ±2.43	27.44 ±2.2	NS
Milk (µg/ml)	24.28 ±2.3	20.84 ±2.4	$P<0.05$

Mice were fed a purified diet based on AIN93 containing 25 mg Zn/kg (ZA) or a diet reduced in Zn (12 mg Zn/kg, ZD) for thirty days prior to conception. On lactation day 7-10, dams were removed from pups for 2-h to control for effects of suckling, and milk was collected. Blood was drawn by cardiac puncture, and mammary glands and liver were dissected and snap frozen. By measuring Zn concentration in the plasma, mammary gland, liver and milk, our results indicated that marginal Zn deficiency did not compromise alter Zn "status" but significantly alter milk Zn concentration.

FIGURE 1: Mammary gland morphology was compromised by zinc deficiency.



(a)

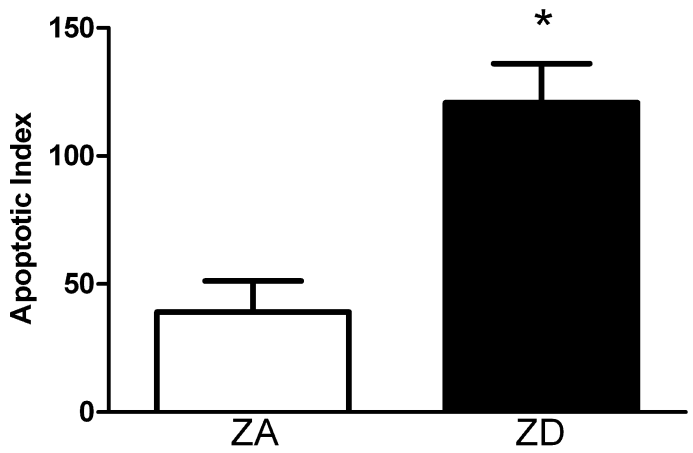


(b)

Mammary gland tissue from mice sectioned (5 µm) and stained with hematoxylin and eosin shows reduction in alveoli. Magnification x10

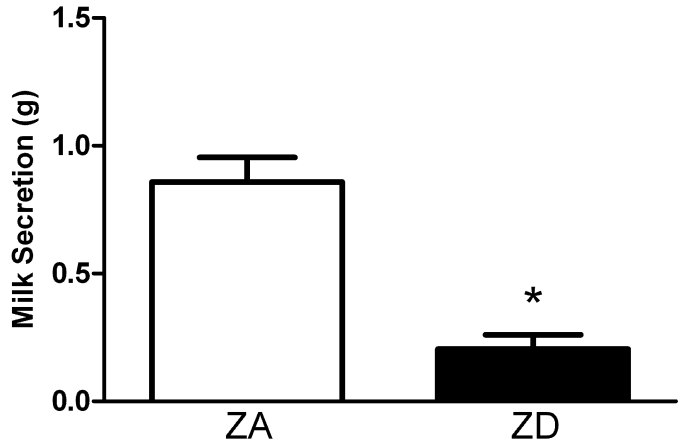
(a) ZA
(b) ZD

FIGURE 2: Greater apoptosis was detected in the mammary gland of ZD mice.



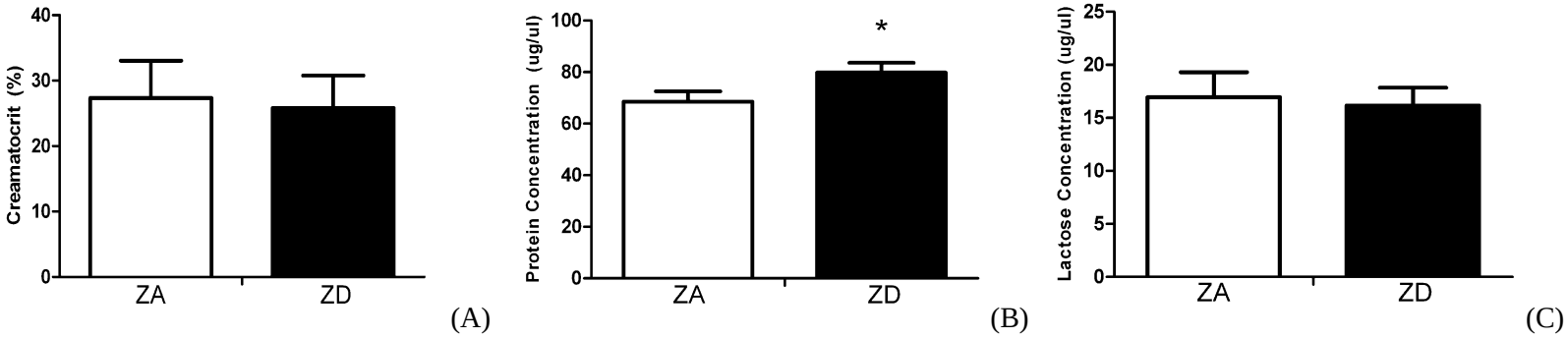
Quantification of TUNEL. Data was also quantified blindly to control for experimental bias. The number of apoptotic cells is significantly higher in ZD mice (n=5) than ZA mice (n=5), $p < 0.05$

FIGURE 3: Milk secretion in mice was significantly reduced by marginal zinc deficiency.



Weigh-suckle-weigh protocol was used to estimate milk secretion over a 2-h suckling period for ZA and ZD mice between 7-10 days of lactation. Milk secretion was significantly lower for ZD (n=5) compared with ZA (n=4) based on changes litter weight, $p < 0.05$.

FIGURE 4: Milk protein concentration was higher in milk of ZD mice.

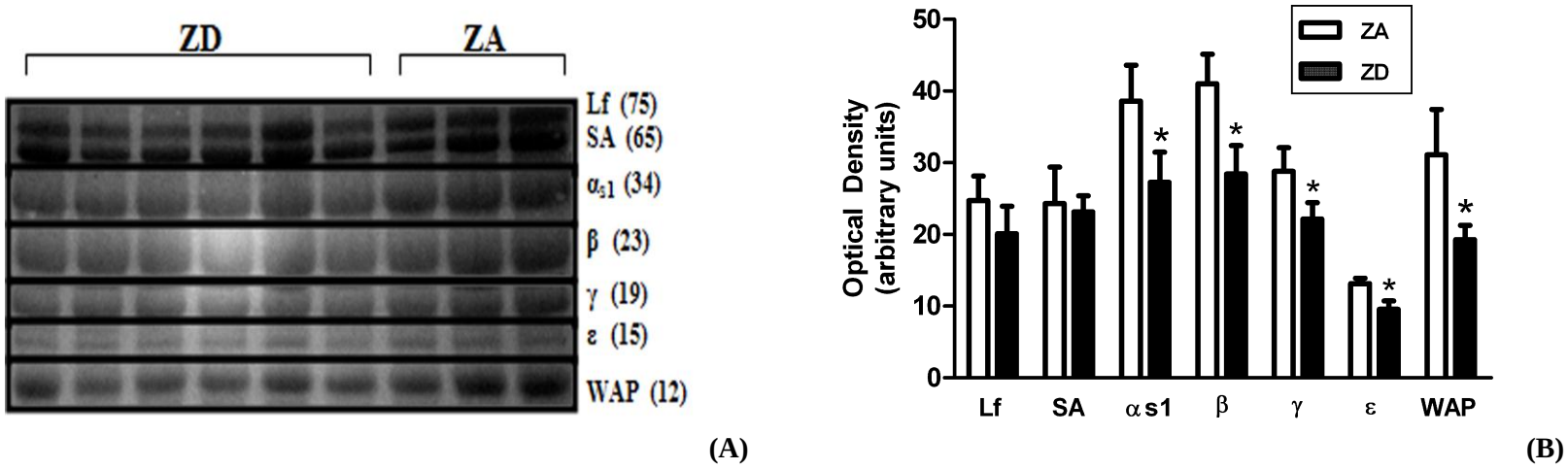


(A) Fat percentage in milk was measured by creamatocrit for ZA (n=4) and ZD (n=6) mice, but results were not significant.

(B) Protein was isolated from skimmed milk and quantified using Bradford Method. ZD mice (n=6) had significantly higher protein concentration than ZA mice (n=3), $p < 0.05$.

(C) Lactose assay was used to measure carbohydrate content in skimmed milk for ZA (n=3) and ZD (n=6) mice, but results were not significant.

FIGURE 5: Relative distribution of major milk proteins was compromised in ZD mice.



(A) SDS-PAGE analysis (12.5% polyacrylamide gel with (50 µg protein/well) and Coomassie staining of skimmed milk after 7-10 days of lactation for ZA (n=3) and ZD (n=6) mice. Protein concentrations determined by Bradford Method. The name of proteins and their corresponding theoretical molecular weight are as indicated: Lf: lactoferrin, SA: serum albumin, α_{s1}:α_{s1} casein, β: β-casein, γ:γ-casein, κ:κ-casein, WAP: whey acidic protein.

(B) Quantification of protein concentrations (50 µg protein/well) obtained by densitometry on Carestream Molecular Imaging Software. Optical density was measured and provided in arbitrary units. The name of proteins are as followed: Lf: lactoferrin, SA: serum albumin, α_{s1}:α_{s1} casein, β: β-casein, γ:γ-casein, κ:κ-casein, WAP: whey acidic protein. Differences in α_{s1}, β, γ, ε and WAP concentrations were significant for ZA and ZD ($p < 0.05$).

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COLLEEN DEMPSEY

EDUCATION

The Pennsylvania State University **University Park, PA**
Schreyer Honors College *September 2007-May 2011*
BS Nutritional Sciences, Basic Science Option
Minor in Spanish

HONORS AND SKILLS

Academic Recognition

- Student scholar of The Schreyer Honors College *2009-2011*
- Schreyer Honors College Travel Grant recipient *2010*
- Dean's List student *2007-2011*
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Language

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EXPERIENCE

Undergraduate Research Assistant **August 2010-May 2011**
Penn State Nutrition Department *Dr. Shannon Kelleher*

- Honors Thesis: "Marginal zinc deficiency disrupts mammary gland morphology and compromises milk secretion"
- Experience with range of techniques including immunoblotting, immunohistochemistry, cell culture, animal care

Research Intern **June 2010-August 2010**
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- Presentation: "Investigating the differences of subunit and splice variant expression of voltage gated calcium channels in arterial smooth muscle cells"
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ACTIVITIES

- Healthworks: Health Advocate & Promoter on campus
- Habitat for Humanity Member: service trip to New Orleans in March 2010
- Student Nutrition Association
- Penn State Dance Marathon for Pediatric Cancer