EFFECTS OF ZINC ON BOVINE MAMMARY EPITHELIA

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Abstract

Two experiments were conducted to explore potential cellular and biochemical mechanisms by which zinc may impact milk production and mammary health of dairy cattle. For the first experiment, twelve lactating Holstein cows were enrolled in a replicated 3 × 3 Latin square, and provided a balanced diet supplemented with either 30 ppm zinc as $ZnSO_4$ (30-ZS), 60 ppm zinc as $ZnSO_4$ (60-ZS), or 60 ppm zinc as a mixture of $ZnSO_4$ and Zn methionine (60-ZM). Blood and milk were collected and analyzed for markers of blood-milk leak and dairy food properties of milk. Milk cells were also isolated and analyzed for RNA abundance of genes related to zinc-mediated apoptosis. Aside from a tendency for 60-ZS to increase feed intake and 60-ZM to increase milk fat compared to 30-ZS, no effects were observed on performance, markers of blood-milk leak, transcript abundance of selected genes, or dairy food properties of milk. A cell culture study was also conducted using immortalized bovine mammary epithelial (MAC-T) cells. Messenger RNA abundance of GPR39—a G protein-coupled receptor shown to regulate cellular activity in response to extracellular zinc concentrations in other epithelial cell lines—was knocked down using RNA interference. Following GPR39 knockdown, cells were treated with 0 or 100 µM zinc. Phosphorylation of the kinases ERK1/2 and AKT was measured in GPR39-knockdown and control cells treated with either 0 or 100 µM zinc. No effect of zinc treatment or GPR39 knockdown was detected on kinase phosphorylation. Interestingly, the 100 µM zinc treatment showed a tendency to increase GPR39 mRNA abundance in control cells. There remain many questions about the cellular mechanisms whereby zinc can impact milk production in dairy cattle.

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Dedication

To S. Who else?

Chapter 1 - Literature Review: Zinc Nutrition in the Dairy Cow

Introduction

The soft, gray element which occupies the topmost position of the Group 12 transition metals may not possess great inherent value. As the 27th-most abundant element in Earth's crust (Vallee and Falchuk, 1993), it is not particularly rare. In industry, it is extensively used as a galvanizing material to protect other metals from rust. However, removal of this common material from the diet of any higher animal quickly demonstrates its paramount importance as a micronutrient.

Many properties of zinc lend themselves toward important and unique biochemical functions. As a Group 12 transition metal with a filled D orbital, zinc has virtually no redox activity in biological systems. As a divalent cation, it possesses a high affinity for specific amino acids—in particular, cysteine, histidine, aspartate, and glutamate. Furthermore, it is highly stable in a wide variety of binding geometries, and easily accepts electron pairs, making it an excellent Lewis acid. Likely because of these properties, zinc is a known component of hundreds of enzymes—by some estimates, over 10% of the human proteome (Andreini et al., 2006). Knowing this, it is no surprise that the biological functions of zinc are numerous and variable, that deficiency of this element can lead to a number of complications, and that supplementation can lead to a number of benefits.

The importance of zinc in nutrition has been known since the early 1930's, when its essentiality was described in rats (Todd et al., 1934). For some twenty-odd years following this discovery, it was widely believed that, while deficiencies could be demonstrated in the laboratory, they simply did not occur in practice. It wasn't until 1955 that a practical example of zinc deficiency was described in farm animals, and zinc supplementation was recognized as a

potent treatment for parakeratosis in swine (Tucker and Salmon, 1955). Soon after, zinc deficiency was also noted in human populations (Halsted et al., 1972) and cattle (Legg and Sears, 1960).

Since then, study of this micronutrient has greatly expanded knowledge of the many ways in which zinc interacts with biological systems. Deficiency leads to gastrointestinal upset, skin disorders, immune suppression, delayed wound healing, growth retardation, and inhibited reproductive development if left uncorrected for a long enough time (reviewed in Machen et al., 1996; Vallee and Falchuk, 1993). Zinc supplementation has been shown to improve performance of beef (Greene et al., 1988) and dairy cattle (Nayeri et al., 2014). Especially impactful for dairy cattle, dietary zinc levels have been shown to impact mammary health (Nayeri et al., 2014). While no definitive explanation has been presented for zinc effects on mammary health in cattle, basic research in mouse models have uncovered a variety of mechanisms in mammary epithelial cells (Hennigar et al., 2015; Dempsey et al., 2012).

Zinc Requirements and Homeostasis

Requirements for the dairy cow

In the NRC model (2001), recommendations for dietary zinc in dairy cattle are determined by a factorial approach. Considerations are made for endogenous fecal and urinary losses, gestation, lactation, and growth, where they apply. The sum of these requirements is considered to be the total requirement for absorbed zinc. Dairy cattle absorb zinc with an efficiency of about 15% (Miller and Cragle, 1965), so the absorbed requirement is divided by 0.15 to determine the dietary requirement. Using this model, the dietary recommendation for a lactating cow (650 kg, producing 40 kg milk/d) is about 63 mg Zn/kg dry matter intake (DMI).

By comparison, a pregnant nonlactating cow (270 d gestation) would require much lower dietary levels (23 mg/kg), despite also having a lower predicted DMI. Even a growing heifer (300 kg, 0.7 kg average daily gain) requires lower dietary levels of zinc (33 mg/kg). These predictions illustrate the relatively high impact of lactation on zinc requirements, even in comparison to growth or gestation, making the management of this essential element particularly important in dairy cattle. Cope et al. (2009) have shown that provision of a moderately zinc-deficient diet (~ 40 mg Zn/kg diet DM) to early lactation Holstein cows, as determined by the NRC (2001) model, results in increased milk somatic cell counts and amyloid A concentrations compared to a zinc-sufficient diet (~60 mg Zn/kg diet dry matter). In this study, milk and component yield was not affected by level of supplemental zinc. These results suggest that feeding dietary zinc levels below NRC recommendations can be done without directly impacting milk production; however, mammary health is likely to be compromised.

Research has also been conducted into supplementing above the NRC recommendations, with limited effects on animal performance or health. In a study by Kincaid et al. (1997), growing heifers receiving a base diet containing 60 ppm zinc were compared to heifers on the same diet plus 150 ppm supplemental zinc provided as a zinc-amino acid chelate (zinc methionine/lysine), 300 ppm zinc as zinc methionine/lysine, or 300 ppm zinc as zinc oxide. In this study, no effects were observed on either immune function or performance of the calves; the only observed result was a higher bioavailability of the amino acid-chelated zinc as judged by increases in tissue zinc concentrations.

A 1998 study by Campbell and Miller reported that provision of 800 mg supplemental zinc to dry cows receiving ~ 1100 mg zinc/d from their base diet reduced days to first observed estrus following calving, but did not affect other health or performance measures. In the context

of this study, zinc was tested as a potential antioxidant to ameliorate negative effects of oxidative stress in the periparturient cow. Interestingly, supplementing with iron, a prooxidant, also reduced days to first estrus as well as days open. In the words of the authors, "it cannot be concluded with certainty that reproductive efficiency was improved by prepartum supplementation with vitamin E or Zn without also acknowledging it was improved by excess Fe."

Responses have been demonstrated in dairy cattle when comparing zinc sources. Amino acid-chelated zinc has been shown to be more bioavailable than other inorganic forms of zinc in chickens (Wedekind et al., 1992), catfish (Paripatananont and Lovell, 1995), lambs, and heifers (Spears, 1989; Kincaid et al., 1997). Nayeri et al. (2014) have further shown that increasing the proportion of supplemental zinc supplied as zinc methionine can increase milk production, reduce services/conception, and improve feed efficiency in Holstein cows receiving a diet containing 111 – 149 ppm zinc. Further, multiparous cows receiving zinc methionine had reduced somatic cell counts in milk and increased concentration of IgG in colostrum. While some question remains about the effect of the organic constituent (e.g. methionine in zinc methionine) in these supplements, their benefit compared to inorganic forms of zinc have been demonstrated.

Absorption

Zinc absorption is controlled by a dynamic, multifactor system in cattle. At various sites along the gastrointestinal tract, zinc can be either absorbed from digesta as a nutrient or secreted as a component of endogenous digestive factors. The efficiency at which dietary zinc is absorbed and retained is impacted by a number of known factors including animal age, feeding rate, zinc

content of the diet, and zinc status of the animal. Mechanisms for absorption of zinc occur in three general stages: cellular uptake, transcellular transport, and transport into the bloodstream.

Zinc is absorbed primarily in the small intestine, though the location of highest absorption may vary across species (Naveh et al., 1988; Lee et al., 1989). In general, zinc secretion is highest in the duodenum, and absorption of dietary and endogenous zinc occurs along the remaining length of small intestine. In cattle, appreciable uptake also occurs in the abomasum (Miller and Cragle, 1965). In addition, some zinc secretion occurs at or prior to the rumenoreticulum (Hiers Jr et al., 1968), possibly as a component of saliva.

Miller et al. (1969) have shown that both zinc-deficient animals and animals fed zinc-deficient diets absorb and retain a larger percentage of dietary zinc. Observations have also shown absorption rates (amount absorbed/total intake) to be higher in calves compared to adult cows (Miller and Cragle, 1965). In a study by Miller et al. (1968), young calves (2.5 mo) retained a higher proportion of dietary zinc than older calves (4.5 mo) when fed a zinc-sufficient diet; however, age did not affect zinc retention rates when a zinc-deficient diet was fed. Growing calves fed at maintenance have also been shown to retain a lower proportion of dietary zinc than calves receiving the same diet at amounts calculated to support normal growth (Miller, 1969). It is likely, then, that observed differences in zinc retention (and, possibly, absorption) at different ages may be a reflection of different growth rates and rates of tissue deposition.

The cellular mechanisms by which zinc is absorbed from the diet are not yet completely elucidated. It has been known for a long time that zinc uptake in the small intestine occurs by both saturable and unsaturable processes, or that zinc both passively diffuses and is actively transported across the apical membrane of intestinal epithelial cells (Steel and Cousins, 1985; Krebs, 2000). The main zinc transporter on the apical membrane of enterocytes is a zinc import

protein encoded by the ZIP4 gene (Wang and Zhou, 2010). Mutations in this gene are responsible for the genetic disorder known as acrodermatitis enteropathica in humans (Wang et al., 2002; Küry et al., 2002) and for bovine hereditary zinc disorder (BHZD or lethal trait A46) in cattle (Yuzbasiyan-Gurkan and Bartlett, 2006). Both of these disorders are characterized by skin disorders and diarrhea, among other signs, and are effectively treated with supplemental zinc in the diet, consistent with the idea of a redundant unsaturable pathway for zinc uptake. On the basolateral side of enterocytes, another zinc transporter, ZnT1, transports zinc into the bloodstream (Wang and Zhou, 2010).

Inside the enterocyte, almost all zinc is bound by proteins, nucleic acids, and various small organic molecules (Eide, 2006), leaving very little free ionized zinc. Transport of zinc across the enterocyte is generally believed to be the product of the interactions of two proteins—CRIP and metallothionein—described quite thoroughly in rodents. Both CRIP (Birkenmeier and Gordon, 1986; Hempe and Cousins, 1991) and metallothionein (Kägi and Schäffer, 1988) bind to zinc and are highly expressed in intestinal tissue. As described by Hempe and Cousins (1991, 1992) in rats, CRIP appears to be responsible for transport of zinc across the cell, while metallothionein binds to and sequesters zinc within the cell, until the cell is sloughed and passed in the feces. While some early research suggested a direct relationship between metallothionein levels and absorption rate of zinc (Starcher et al., 1980), other studies have since shown that increased intestinal metallothionein leads to decreased absorption of zinc. Davis et al. (1998) found that metallothionein-knockout mice had higher serum zinc levels, while metallothionein-transgenic (overexpressing) mice had lower serum zinc than control mice following an oral zinc dose. By conducting the experiment in transgenic animals, they were able to attribute the observed decrease in serum zinc directly to the increased metallothionein expression, an

observation consistent with previous research correlating increases in metallothionein with decreases in zinc absorption (Hoadley et al., 1988; Hempe and Cousins, 1992). In addition, metallothionein expression in many tissues, including intestinal tissue, is induced by zinc exposure (Kägi and Schäffer, 1988), supplying a plausible mechanism by which zinc absorption rates may be regulated by dietary zinc intake. In line with this, Hempe and Cousins (1992) found that rats fed a low zinc diet had lower levels of intestinal metallothionein than high-zinc rats. Low-zinc rats also were found to have higher fractional zinc absorption rates, a lower proportion of a ^{65}Zn dose bound to metallothionein, and a higher proportion of ^{65}Zn bound to CRIP. Further, as luminal zinc levels increased from 5 to 300 µM, the percentage of ^{65}Zn bound to CRIP dropped from 42% to 25% while the moles of ^{65}Zn bound to CRIP remained the same, in line with previous predictions of saturable transport kinetics (Steel and Cousins, 1985).

Distribution

Zinc is ubiquitous in the tissues of ruminants, with generally higher concentrations in muscle and bone. Once absorbed, zinc appears in tissues at variable rates. Metabolically active tissues have very rapid turnover of zinc. In blood plasma and liver, absorbed zinc accumulates very quickly (peaking in 1-3 days), but is retained for a relatively short period (declining rapidly over 3-4 weeks; Miller, 1969). In contrast, bone, hair, muscle, and erythrocytes have a very low turnover, and accumulate dietary zinc over the course of several weeks.

As stated above, the vast majority of intracellular zinc is bound to proteins and various organic molecules. This holds true also in blood serum (Faure et al., 1990). Serum zinc is mostly protein-bound; in humans, the majority of serum zinc has been shown to be bound to albumin and alpha-2-macroblobulin (Foote and Delves, 1984). In portal circulation of rats, a large amount of zinc is also bound to a protein called transferrin (Evans and Winter, 1975). Plasma zinc

concentrations in cattle fall normally in the range of 0.8 – 1.2 ppm, and drop rapidly in the case of deficiency (Mills et al., 1967). While most tissue zinc concentrations do not change appreciably during zinc deficiency, repeated measures below 0.4 ppm of plasma zinc are strong enough indicators of a deficiency to be used diagnostically in cattle (Miller, 1970).

Cow's milk typically contains ~ 4 ppm zinc (Miller, 1970), over 95% of which is associated with casein micelles (Blakeborough et al., 1983). When lactating cows are fed high dietary levels of zinc, zinc levels of milk also rise, though to a lesser degree than plasma zinc (Miller et al., 1965a), suggesting that zinc secretion into milk is regulated within the mammary gland. While knowledge of mammary epithelial cell zinc regulation in the bovine is limited, it has been studied extensively in mice and mouse cell lines (reviewed in McCormick et al., 2014). Pathway analysis combined with cell localization studies indicate that ZIP5, ZIP8, and ZIP10 are likely involved in zinc import to mouse mammary epithelial cells during lactation (Kelleher et al., 2012). On the luminal membrane, ZnT2 and ZnT4 have both been shown to transport zinc from mammary epithelial cells into milk (Michalczyk et al., 2002; Chowanadisai et al., 2006). In addition, a mutation in ZnT4 causes the "lethal milk" phenotype in mice, a disorder leading to low zinc concentrations in milk which causes zinc deficiency and death in nursing pups (Piletz and Ganschow, 1978; Huang and Gitschier, 1997). Zinc can also be resorbed into the cell from the alveolar lumen. Kelleher *et al*. (2009) have shown that the zinc import protein, ZIP3, functions in this manner. Other ZIP proteins also likely function in this way (Kelleher et al., 2012), based on cell localization and expression.

Feces is the main route of zinc excretion. In mature cows, 86% of a daily dose of ^{65}Zn was observed in the feces, while less than 0.2% was observed in the urine (Miller and Cragle, 1965). Alimentary secretions of zinc in cattle are impacted by the both the zinc level of the diet

and the zinc deficiency status of the animal. Fecal endogenous zinc decreases for both deficient diets and deficient animals (Miller, 1969).

Dietary factors

A number of factors can impact the availability of zinc in a diet. Determining those factors, the degree of their impact, and potential interactions between factors has been the subject of much research to date. To illustrate this, studies have been performed determining the zinc requirements of ruminants fed semi-purified diets (Mills et al., 1967). In such diets, zinc requirements for growing calves are very low, 8 – 12 ppm. In contrast, growing calves on late-maturity pastures have been documented to show signs of severe zinc deficiency on diets providing at least 20 ppm Zn (Legg and Sears, 1960; Mayland et al., 1980). This disparity shows that dietary factors exist that drastically impact the efficiency with which cattle utilize dietary zinc.

Cadmium and lead both interfere with zinc absorption (Powell et al., 1967; NRC, 2001). In humans, supplemental iron has also been shown to interfere with zinc absorption. Sandstrom et al. (1985) showed that iron supplements, when taken on an empty stomach, interfered with zinc absorption. The inclusion of histidine or the consumption of a meal along with the supplement reversed this effect. While ruminants are not likely to consume large amounts of iron on an empty stomach, this does provide an interesting observation of improved zinc bioavailability with inclusion of a zinc ligand. In very large amounts, copper can also interfere with zinc absorption, though this is unlikely in cattle, and typically it is excess zinc which interferes with copper absorption (NRC, 2001). In monogastrics, high levels of calcium can also negatively impact zinc absorption (Wood and Zheng, 1997), especially in conjunction with high

levels of dietary phytate (Oberleas et al., 1960), though this effect also has not been observed in ruminants.

Several organic molecules also influence the availability of zinc in the diet. Perhaps the most prominent of these is phytate. In monogastrics, phytate inhibits zinc absorption by binding zinc and preventing its absorption in the intestine (Lonnerdal, 2000). This effect can be observed in preweaned calves receiving soybean protein in milk replacer (Miller, 1967); however, in mature ruminants, most phytate is digested by rumen microbes before it reaches the small intestine, preventing its interference with zinc absorption (NRC, 2001). Small molecules acting as chelating agents may also enhance zinc uptake, by keeping zinc in solution and allowing it access to the brush border membrane. For instance, inclusion of EDTA in the diet of turkey poults was shown to ameliorate the negative effects of phytate on zinc absorption (Kratzer et al., 1959). However, these chelating agents can also interfere with transport of zinc into the blood once it is absorbed (Hill et al., 1987; Hempe and Cousins, 1989). Some amino acids, when complexed with zinc, will also improve zinc bioavailability. Histidine (Schölmerich et al., 1987; Sandström et al., 1985) and methionine (Wedekind et al., 1992) have been shown to improve bioavailability of zinc. In lambs fed a semi-purified diet, zinc methionine had the same absorption rate as zinc oxide, but a higher retention rate (Spears, 1989). Replacing inorganic forms of zinc with zinc methionine can lead to improvements in carcass quality of finishing steers (Greene et al., 1988), and milk production and mammary health of lactating cows (Nayeri et al., 2014). These production and health benefits are well established; however, the specific cellular and molecular mechanisms responsible remain to be determined.

Biological Impacts

Zinc plays important roles in integumentary, immune, neurological, and reproductive systems. Deficiency is characterized by skin lesions, diarrhea, inhibited immune function, and growth retardation (Chai et al., 1999; Yuzbasiyan-Gurkan and Bartlett, 2006). Its importance in the diet of animals has been known at least since the 1930's, when it was shown to be necessary for the growth of rats (Todd et al., 1934). Deficiencies were not described in humans until the 1960's when "nutritional dwarfism" was linked to zinc-poor, phytate-rich diets of the Middle-Eastern lower class (Halsted et al., 1972). About the same time, zinc deficiency was described in cattle grazing late-maturity pastures (Legg and Sears, 1960). Since then, this element has been shown to impact a number of cellular pathways in a variety of tissue types.

Concentrations of intracellular zinc range from 0.1 – 0.5 mM in both prokaryotic and eukaryotic cells (Eide, 2006). The vast majority of this intracellular zinc is bound to proteins, nucleic acids, lipids, and a variety of small organic molecules. Direct measurement of unbound "free" zinc in the cell is difficult; however, binding affinities of many zinc-containing metalloproteins are in the nanomolar to picomolar range. If intracellular free zinc is in this range, it composes at most 0.001% of total intracellular zinc, suggesting tight regulation within the cell.

Zinc and the proteome

Recent estimates predict that approximately 10% of the human proteome is made up of zinc-binding proteins (Andreini et al., 2006) belonging to all classes of enzymes (Vallee and Falchuk, 1993), in which zinc provides both structural and functional benefits.

These zinc metalloproteins can be categorized based on their function and binding geometry (reviewed in McCall et al., 2000). When only one zinc is bound per active site, the zinc atom is said to be catalytic. Multiple zinc atoms bound to a single active site are considered co-

catalytic. Finally, when a single zinc atom is bound to a motif and simply stabilizes the protein's tertiary structure, it is said to be a structural zinc atom. For example, matrix metalloproteases (Eide, 2006), a class of enzymes which are involved in remodeling of extracellular matrix, tissue growth, and angiogenesis, require a catalytic zinc to function. Other metalloenzymes in which zinc performs a catalytic role include carbonic anhydrase and carboxypeptidase, whereas zinc has a co-catalytic role in alkaline phosphatase and phospholipase C (Holt et al., 2012). Protein kinase C is an example of a metalloenzyme containing a structural zinc ion. Alcohol dehydrogenase is a unique zinc metalloenzyme in that it is the only known enzyme to contain both a catalytic and a structural zinc (Vallee and Falchuk, 1993).

One highly important and biologically influential group of zinc-binding proteins are the transcription factors, many of which contain a number of "zinc finger" motifs. These zinc fingers allow the transcription factors to recognize and bind to response elements in DNA to promote or inhibit expression of specific genes. One such transcription factor, MTF-1 actually responds to extracellular zinc concentrations to regulate expression of genes related to zinc homeostasis within cells (Andrews, 2001). Other examples of zinc-containing transcription factors include steroid and retinoid receptors (Hardin et al., 2012), and factors which influence cellular autophagy (Li et al., 2015) and tissue development (Sum et al., 2005).

The metallothioneins are a group of metalloproteins which bind metal ions in a fashion which defies the classification scheme described above. At 61-62 amino acids, these small proteins are named for their high sulfur and metal composition. A highly-conserved sequence of 20 cysteine residues is present in all mammalian metallothioneins, which binds strongly to zinc and other heavy metals at a ratio of 7 mol metal/mol protein (Vallee and Falchuk, 1993). Unlike any other known metalloproteins, metallothioneins bind metal ions in clusters with a unique

binding geometry unlike the stereotypical catalytic, co-catalytic, or structural geometries. Regulation of metallothionein is thought to be a mechanism by which cells can regulate the intracellular concentrations of unbound zinc and other metals (Kägi and Schäffer, 1988).

Zinc transport

Zinc transport in mammalian cells is mediated nearly entirely by transporters from two families: ZIP (SLC39) and ZnT (SLC30). Transporters belonging to these families are believed to be largely responsible for maintaining zinc levels within the cell and intracellular compartments. The two groups have opposite functions—ZIP transporters move zinc into the cytosol of cells, either from the extracellular matrix or from intracellular compartments; ZnT transporters transfer zinc out of the cytosol, into the extracellular matrix or intracellular compartments. Members of both groups can be found in all taxonomic kingdoms (Gaither and Eide, 2001), and in all tissue types in mammals (Lichten and Cousins, 2009; Eide, 2006). ZIP transporters typically have 8 transmembrane domains with N- and C-termini located on the extracytosolic side of a membrane. ZnT transporters have 6 transmembrane domains, with both termini on the cytosolic side of a membrane. Both contain a histidine-rich loop between transmembrane domains 3 and 4, which is thought to be a zinc-binding site. There are 14 known members of the SLC39 (ZIP) family found in mammals, and 10 of the SLC30 (ZnT) family (Lichten and Cousins, 2009).

Transporters from both families are known to perform essential functions in a variety of cell types. The first zinc transporter described in mammals was ZnT1 (Palmiter and Findley, 1995). As discussed above, ZnT1 transports zinc from enterocytes into the blood (Wang and Zhou, 2010), and may perform a similar function in kidney cells (Lichten and Cousins, 2009).

The ZIP1 protein is constitutively expressed in all tissues of mice and humans (Eide, 2006). It is located in the plasma membrane of zinc-deficient cells and the intracellular compartments of zinc-replete cells. As discussed above, ZIP4 is an important protein for dietary zinc uptake. Mutations in the ZIP4 gene lead to zinc deficiency disorders in humans (Wang et al., 2002) and cattle (Yuzbasiyan-Gurkan and Bartlett, 2006). Other ZIP proteins are involved in cellular zinc uptake in other tissues such as liver (Liuzzi et al., 2005), and mammary epithelium (Kelleher and Lönnerdal, 2005).

Apoptosis

The importance of dietary zinc can be inferred from the frequent observations of zinc-containing enzymes, and from the impacts of zinc deficiency *in vivo.* Closer analyses of a more basic nature also reveal a number of cellular pathways which are directly affected by zinc availability. For example, zinc availability has been shown to affect apoptosis rates in a variety of tissues—the trachea (Truong-Tran et al., 2003), intestine (Cohen et al., 2012), mammary gland (Hennigar et al., 2015; Dempsey et al., 2012; Seo et al., 2011), skin, thymus, testes, retina, and pancreas (Truong-Tran et al., 2001), to name a few.

The exact mechanism by which zinc impacts apoptosis varies in these tissues. In the case of airway epithelial cells, zinc protects cells from oxidative damage, and prevents activation of the pro-apoptotic protein, caspase (Truong-Tran et al., 2003). *In vitro*, intestinal epithelial cells are protected from butyrate-induced apoptosis by a zinc-dependent upregulation of the anti-apoptotic protein, clusterin (Cohen et al., 2012). Mammary epithelial cells undergo two forms of cell death during involution: the first a lysosomal-mediated cell death occurring during an early reversible stage of involution, the second being via the intrinsic mitochondrial-mediated apoptotic pathway which occurs during a later irreversible stage of involution (McCormick et al.,

2014). Zinc availability has been shown to impact lysosomal-mediated cell death (Hennigar et al., 2015), and may also play a role in apoptosis via the intrinsic pathway (Seo et al., 2011) in mammary epithelial cells.

Immune effects

Many nutritional deficiencies can cause immune dysregulation. In human medicine, protein-energy malnutrition, as well as a number of micronutrient deficiencies are known to impair immune responses (reviewed in Calder, 2013). Zinc deficiency is classically associated with reductions in thymus weight and circulating lymphocytes, as well as retarded healing and increased risk of secondary infections (reviewed in Fraker and King, 2004).

In cattle, severe zinc deficiency leads to multiple quantifiable impacts on immune function. One convenient model which has been extensively used to study zinc deficiency in cattle is autosomal recessive bovine hereditary zinc disorder (BHZD). As discussed above, BHZD is caused by a mutation in the gene encoding an important zinc transporter for dietary zinc uptake. Calves born with BHZD exhibit signs closely resembling diet-induced zinc deficiency—diarrhea, lethargy, delayed wound healing, etc.—which are reversible with addition of zinc to the diet (Machen et al., 1996). Perryman et al. (1989) observed marked reduction in lymphocyte activity in BHZD calves. Reduced thymus weights are another feature of BHZD (Brummerstedt, 1971). Calves in controlled studies modeling diet-induced zinc deficiency also exhibit changes to the immune system. Miller et al. (1965b) showed that zinc deficient calves experienced delayed and abnormal healing compared to control calves.

Moderate zinc deficiencies leading to negative impacts on production often do not impact immune function. Supplementing zinc to a practical ration for growing heifers did not impact lymphocyte activity (Kincaid et al., 1997). Likewise, lymphocyte activity and swelling response

of grower-finisher steers on a practical diet did not respond to zinc supplementation (Spears and Kegley, 2002).

However, in some scenarios, zinc supplementation to practical diets has led to enhanced immune response compared to unsupplemented animals. Zinc supplements decreased recovery time following an infectious bovine rhinotracheitis challenge in feedlot steers (Chirase et al., 1991). Also, lactating dairy cows receiving an ostensibly more bioavailable source of zinc had lower somatic cell counts in milk and higher IgG levels in colostrum than control cows receiving a zinc sulfate supplement (Nayeri et al., 2014).

Research in rodent models has illuminated numerous cellular and biochemical interactions between zinc status and immunity. Diet-induced zinc deficiency in mice leads to thymic atrophy, as well as decreased numbers of lymphocytes, particularly the immature B cells (King et al., 1995). While total numbers of circulating lymphocytes decline in zinc deficient mice, the functionality of the remaining cells remains the same (Cook-Mills and Fraker, 1993). Concurrent with immune suppression, zinc deficiency coincided with increased levels of circulating glucocorticoids (GC; King et al., 1995), implicating the adrenocortical axis in this process. In an attempt to further explore the role of GC, DePasquale-Jardieu and Fraker (1980) compared adrenalectomized and sham-operated mice with and without zinc deficiency. Interestingly, removal of the adrenal gland (and subsequent drops in circulating corticosterone) completely protected zinc deficient mice from thymic atrophy. Considering the nutritional demands of a normally-functioning immune system, it has been proposed that immune suppression such as that described above may have a nutrient-sparing effect in animals experiencing a variety of deficiencies (reviewed in Fraker et al., 1995).

Integumentary effects

The effects of zinc on the integumentary system are also widespread and profound. Perhaps the most characteristic signs of zinc deficiency are the skin disorders which accompany it. In cattle, hair loss, parakeratosis, and various skin lesions are observed in calves and cows following zinc deficiency (Legg and Sears, 1960). *In vitro*, zinc has been shown to prevent apoptosis and promote proliferation in a variety of epithelial cell lines, including intestinal (Cohen et al., 2012, 2014), airway (Truong-Tran et al., 2003), and mammary (Hennigar et al., 2015) epithelial cells. In a mouse model, moderate zinc deficiency has been shown to dramatically impact apoptosis, lactation rates, and gross morphology of the lactating mammary gland (Dempsey et al., 2012). In cattle, feeding a greater proportion of zinc as an ostensibly more bioavailable amino acid complex results in increased milk production and improved mammary health in multiparous cows (Nayeri et al., 2014). Whether the improved performance is a reflection of enhanced immune response or a direct effect on the milk-secreting epithelial cells is not yet known.

Conclusion

The importance of zinc in the diet of cattle is readily illustrated by the extensive and diverse complications associated with its deficiency. Many benefits of zinc supplementation have been documented in beef and dairy cattle. However, the cellular and biochemical mechanisms remain, for the most part, to be elucidated. Basic research in rodent models provide a number of tantalizing hints about the mechanisms behind production improvements in response to zinc supplementation. As methods for the study of these basic mechanisms become more readily

available, thorough descriptions of the biochemistry of zinc specifically in the bovine may soon become a reality.

References

Andreini, C., L. Banci, I. Bertini, and A. Rosato. 2006. Counting the Zinc-Proteins Encoded in the Human Genome. J. Proteome Res. 5:196–201.

Andrews, G.K. 2001. Cellular zinc sensors: MTF-1 regulation of gene expression. BioMetals. 14:223–237. doi:10.1023/A:1012932712483.

Bicalho, M.L.S., F.S. Lima, E.K. Ganda, C. Foditsch, E.B.S. Meira, V.S. Machado, A.G. V Teixeira, G. Oikonomou, R.O. Gilbert, and R.C. Bicalho. 2014. Effect of trace mineral supplementation on selected minerals, energy metabolites, oxidative stress, and immune parameters and its association with uterine diseases in dairy cattle. J. Dairy Sci. 97:4281–95. doi:10.3168/jds.2013-7832.

Birkenmeier, E.H., and J.I. Gordon. 1986. Developmental regulation of a gene that encodes a cysteine-rich intestinal protein and maps near the murine immunoglobulin heavy chain locus. Proc. Natl. Acad. Sci. USA. 83:2516–2520.

Blakeborough, P., D.N. Salter, and M.I. Gurr. 1983. Zinc binding in cow's milk and human milk. Biochem J. 209:505–512.

Brummerstedt, E. 1971. The effect of zinc on calves with hereditary thymus hypoplasia. Acta pathalogica Microbiol. Scand. 79:686–687. doi:10.1300/J014v21n04.

Calder, P.C. 2013. Feeding the immune system. Proc Nutr Soc. 72:299–309. doi:10.1017/S0029665113001286.

Chai, F., A.Q. Truong-Tran, L.H. Ho, and P.D. Zalewski. 1999. Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: A review. Immunol. Cell Biol. 77:272–278. doi:10.1046/j.1440-1711.1999.00825.x.

Chirase, N.K., D.P. Hutcheson, and G.B. Thompson. 1991. Feed intake, rectal temperature, and serum mineral concentrations of feedlot cattle fed zinc oxide or zinc methionine and challenged with infectious bovine rhinotracheitis virus. J. Anim. Sci. 69:4137–4145.

Chowanadisai, W., B. Lonnerdal, and S.L. Kelleher. 2006. Identification of a Mutation in SLC30A2 (ZnT-2) in Women with Low Milk Zinc Concentration That Results in Transient Neonatal Zinc Deficiency. J. Biol. Chem. 281:39699–39707. doi:10.1074/jbc.M605821200.

Cohen, L., H. Azriel-Tamir, N. Arotsker, I. Sekler, and M. Hershfinkel. 2012. Zinc sensing receptor signaling, mediated by GPR39, reduces butyrate-induced cell death in HT29 colonocytes via upregulation of clusterin. PLoS One. 7:1–13. doi:10.1371/journal.pone.0035482.

Cohen, L., I. Sekler, and M. Hershfinkel. 2014. The zinc sensing receptor, ZnR/GPR39, controls proliferation and differentiation of colonocytes and thereby tight junction formation in the colon. Cell Death Dis. 5:e1307. doi:10.1038/cddis.2014.262.

Cook-Mills, J.M., and P.J. Fraker. 1993. Functional capacity of the residual lymphocytes from zinc-deficient adult mice. Br J Nutr. 69:835–848.

Davis, S.R., R.J. McMahon, and R.J. Cousins. 1998. Metallothionein knockout and transgenic mice exhibit altered intestinal processing of zinc with uniform zinc-dependent zinc transporter-1 expression. J. Nutr. 128:825–31.

Dempsey, C., N.H. McCormick, T.P. Croxford, Y.A. Seo, A. Grider, and S.L. Kelleher. 2012. Marginal Maternal Zinc Deficiency in Lactating Mice Reduces Secretory Capacity and Alters Milk Composition. J. Nutr. 142:655–660. doi:10.3945/jn.111.150623.

DePasquale-Jardieu, P., and P.J. Fraker. 1980. Further characterization of the role of corticosterone in the loss of humoral immunity in zinc-deficient A/J mice as determined by adrenalectomy. J. Immunol. 124:2650–5.

Eide, D.J. 2006. Zinc transporters and the cellular trafficking of zinc. Biochim. Biophys. Acta. 1763:711–722. doi:10.1016/j.bbamcr.2006.03.005.

Evans, G.W., and T.W. Winter. 1975. Zinc transport by transferrin in rat portal blood plasma. Biochem. Biophys. Res. Commun. 66:1218–1224.

Faure, H., A. Favier, M. Tripier, and J. Arnaud. 1990. Determination of the Major Zinc Fractions in Human Serum by Ultrafiltration. Biol. Trace Elem. Res. 24:25–37.

Foote, J.W., and H.T. Delves. 1984. Albumin bound and alpha 2-macroglobulin bound zinc concentrations in the sera of healthy adults. J. Clin. Pathol. 37:1050–1054. doi:10.1136/jcp.37.9.1050.

Fraker, P.J., and L.E. King. 2004. Reprogramming of the Immune System During Zinc Deficiency. Annu. Rev. Nutr. 24:277–98. doi:10.1146/annurev.nutr.24.012003.132454.

Fraker, P.J., F. Osati-Ashtiani, M.A. Wagner, and L.E. King. 1995. Possible roles for glucocorticoids and apoptosis in the suppression of lymphopoiesis during zinc deficiency: a review. J. Am. Coll. Nutr. 14:11–17. doi:10.1080/07315724.1995.10718467.

Gaither, L.A., and D.J. Eide. 2001. Eukaryotic zinc transporters and their regulation. BioMetals. 14:251–270.

Ganda, E.K., R.S. Bisinotto, A.K. Vasquez, A.G.V. Teixeira, V.S. Machado, C. Foditsch, M. Bicalho, F.S. Lima, L. Stephens, M.S. Gomes, J.M. Dias, and R.C. Bicalho. 2016. Effects of injectable trace mineral supplementation in lactating dairy cows with elevated somatic cell counts. J. Dairy Sci. 99:7319–7329. doi:10.3168/jds.2016-10989.

Greene, L.W., D.K. Lunt, F.M. Byers, N.K. Chirase, C.E. Richmond, R.E. Knutson, and G.T. Schelling. 1988. Performance and Carcass Quality of Steers Supplemented With Zinc Oxide or Zinc Methionine. J. Anim. Sci. 66:1818–1823.

Halsted, J.A., H.A. Ronaghy, P. Abadi, M. Haghshenass, G.H. Amirhakemi, R.M. Barakat, and J.G. Reinhold. 1972. Zinc deficiency in man. The Shiraz experiment. Am. J. Med. 53:277–284.

Hardin, J., L.J. Kleinsmith, G. Bertoni, and W.M. Becker. 2012. The regulation of gene expression. In Becker's World of the Cell. Pearson Benjamin Cummings. 710–757.

Hempe, J.M., and R.J. Cousins. 1989. Effect of EDTA and Zinc-Methionine Complex on Zinc Absorption by Rat Intestine. J. Nutr. 119:1179–1187.

Hempe, J.M., and R.J. Cousins. 1991. Cysteine-rich intestinal protein binds zinc during transmucosal zinc transport. Proc. Natl. Acad. Sci. U. S. A. 88:9671–4.

Hempe, J.M., and R.J. Cousins. 1992. Cysteine-rich intestinal protein and intestinal metallothionein: an inverse relationship as a conceptual model for zinc absorption in rats. J. Nutr. 122:89–95.

Hennigar, S.R., Y.A. Seo, S. Sharma, D.I. Soybel, and S.L. Kelleher. 2015. ZnT2 is a critical mediator of lysosomal-mediated cell death during early mammary gland involution. Sci. Rep. 5:8033. doi:10.1038/srep08033.

Hiers Jr, J.M., W.J. Miller, and D.M. Blackmon. 1968. Endogenous secretion and reabsorption of zinc in ruminants as affected by zinc deficiency and feeding of ethylenediaminetetraacetate or cadmium. J. Dairy Sci. 51:730–736. doi:10.3168/jds.S0022-0302(68)87063-8.

Hill, D.A., E.R. Peo, and A.J. Lewis. 1987. Effect of zinc source and picolinic acid on 65Zn uptake in an in vitro continuous-flow perfusion system for pig and poultry intestinal segments. J. Nutr. 117:1704–7.

Hoadley, J.E., a S. Leinart, and R.J. Cousins. 1988. Relationship of 65Zn absorption kinetics to intestinal metallothionein in rats: effects of zinc depletion and fasting. J. Nutr. 118:497–502.

Holt, R.R., J.Y. Uriu-Adams, and C.L. Keen. 2012. Zinc. In Present Knowledge in Nutrition. J.W. Erdman, I.A. Macdonald, and S.H. Zeisel, editors. International Life Sciences Institute. 521–539.

Huang, L., and J. Gitschier. 1997. A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nat. Genet. 17:292–297.

Kägi, J.H., and a Schäffer. 1988. Biochemistry of metallothionein. Biochemistry. 27:8509–8515. doi:10.1021/bi00423a001.

Kelleher, S.L., and B. Lönnerdal. 2005. Zip3 plays a major role in zinc uptake into mammary epithelial cells and is regulated by prolactin. Am. J. Physiol. Cell Physiol. 288:C1042-7. doi:10.1152/ajpcell.00471.2004.

Kelleher, S.L., V. Lopez, B. Lönnerdal, J. Dufner-Beattie, and G.K. Andrews. 2009. Zip3 (Slc39a3) functions in zinc reuptake from the alveolar lumen in lactating mammary gland. Am. J. Physiol. Regul. Integr. Comp. Physiol. 297:R194–R201. doi:10.1152/ajpregu.00162.2009.

Kelleher, S.L., V. Velasquez, T.P. Croxford, N.H. McCormick, V. Lopez, and J. Macdavid. 2012. Mapping the zinc-transporting system in mammary cells: Molecular analysis reveals a phenotype-dependent zinc-transporting network during lactation. J. Cell. Physiol. 227:1761–1770. doi:10.1002/jcp.22900.

Kellogg, D.W., D.J. Tomlinson, M.T. Socha, and A.B. Johnson. 2004. Effects of Zinc Methionine on Milk Production and Somatic Cell Count of Dairy Cows : Twelve-Trial Summary. Prof. Anim. Sci. 20:295–301.

Kincaid, R.L., B.P. Chew, and J.D. Cronrath. 1997. Zinc oxide and amino acids as sources of dietary zinc for calves: effects on uptake and immunity. J. Dairy Sci. 80:1381–8. doi:10.3168/jds.S0022-0302(97)76067-3.

King, L.E., F. Osati-Ashtiani, and P.J. Fraker. 1995. Depletion of cells of the B lineage in the bone marrow of zinc-deficient mice. Immunology. 85:69–73.

Kratzer, F.H., J.B. Allred, P.N. Davis, B.J. Marshal, and P. Vohra. 1959. The effect of autoclaving soybean protein and the addition of ethylenediaminetetraacetic acid on the biological availability of dietary zinc for turkey poults. J. Nutr. 313–322.

Krebs, N.F. 2000. Overview of Zinc Absorption and Excretion in the Human Gastrointestinal Tract 1 , 2. J. Nutr. 130:1374–1377.

Küry, S., B. Dréno, S. Bézieau, S. Giraudet, M. Kharfi, R. Kamoun, and J.-P. Moisan. 2002. Identification of SLC39A4, a gene involved in acrodermatitis enteropathica. Nat. Genet. 31:239–240. doi:10.1038/ng913.

Lee, H.H., a S. Prasad, G.J. Brewer, and C. Owyang. 1989. Zinc absorption in human small intestine. Am. J. Physiol. 256:G87-91.

Legg, S.P., and L. Sears. 1960. Zinc sulphate treatment of parakeratosis in cattle. Nature. 186:1061.

Li, Y., L. Zhang, K. Li, J. Li, R. Xiang, J. Zhang, H. Li, Y. Xu, Y. Wei, J. Gao, P. Lin, and Y. Wei. 2015. ZNF32 inhibits autophagy through the mTOR pathway and protects MCF-7 cells from stimulus-induced cell death. Sci. Rep. 5:9288. doi:10.1038/srep09288.

Lichten, L. a, and R.J. Cousins. 2009. Mammalian zinc transporters: nutritional and physiologic regulation. Annu. Rev. Nutr. 29:153–176. doi:10.1146/annurev-nutr-033009-083312.

Liuzzi, J.P., L. a Lichten, S. Rivera, R.K. Blanchard, T.B. Aydemir, M.D. Knutson, T. Ganz, and R.J. Cousins. 2005. Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc. Natl. Acad. Sci. 102:6843–6848. doi:10.1073/pnas.0502257102.

Lonnerdal, B. 2000. Dietary Factors Influencing Zinc Absorption. J. Nutr. 130:1378–1383.

Machen, M., T. Montgomery, R. Holland, E. Braselton, R. Dunstan, G. Brewer, and V. Yuzbasiyan-Gurkan. 1996. Bovine hereditary zinc deficiency: lethal trait A 46. J. Vet. Diagnostic Investig. 8:219–227.

Mayland, H.F., R.C. Rosenau, and A.R. Florence. 1980. Grazing cow and calf responses to zinc supplementation. J. Anim. Sci. 51:966–974.

McCall, K.A., C. Huang, and C.A. Fierke. 2000. Function and Mechanism of Zinc Metalloenzymes. J. Nutr. 130:1437–1446.

McCormick, N.H., S.R. Hennigar, K. Kiselyov, and S.L. Kelleher. 2014. The biology of zinc transport in mammary epithelial cells: Implications for mammary gland development, lactation, and involution. J. Mammary Gland Biol. Neoplasia. 19:59–71. doi:10.1007/s10911-013-9314-4.

Michalczyk, A.A., J. Allen, R.C. Blomeley, and M.L. Ackland. 2002. Constitutive expression of hZnT4 zinc transporter in human breast epithelial cells. Biochem. J. 364:105–113.

Miller, J.K. 1967. Effect of Protein Source and Feeding Method on Zinc Absorption by Calves. J. Nutr. 93:386–392.

Miller, J.K., and R.G. Cragle. 1965. Gastrointestinal Sites of Absorption and Endogenous Secretion of Zinc in Dairy Cattle. J. Dairy Sci. 48:370–373. doi:10.3168/jds.S0022-0302(65)88231-5.

Miller, W.J. 1969. Absorption , Tissue Distribution, Endogenous Excretion, and Homeostatic Control of Zinc in Ruminants. Am. J. Clin. Nutr. 22:1323–1331.

Miller, W.J. 1970. Zinc Nutrition of Cattle: A Review. J. Dairy Sci. 53:1123–1135. doi:10.3168/jds.S0022-0302(70)86355-X.

Miller, W.J., C.M. Clifton, P.R. Fowler, and H.F. Perkins. 1965a. Influence of High Levels of Dietary Zinc on Zinc in Milk, Performance and Biochemistry of Lactating Cows. J. Dairy Sci. 48:450–453. doi:10.3168/jds.S0022-0302(65)88251-0.

Miller, W.J., Y.G. Martin, R.P. Gentry, and D.M. Blackmon. 1968. Zn and stable zinc absorption, excretion and tissue concentrations as affected by type of diet and level of zinc in normal calves. J. Nutr. 94:391–401.

Miller, W.J., J.D. Morton, W.J. Pitts, and C.M. Clifton. 1965b. Effect of zinc deficiency and restricted feeding on wound healing in the bovine. Proc. Soc. Exp. Biol. Med. 118:427–430.

Mills, C.F., A.C. Dalgarno, R.B. Williams, and J. Quarterman. 1967. Zinc deficiency and the zinc requirements of calves and lambs. Br. J. Nutr. 21:751–768. doi:10.1079/bjn19670076.

Naveh, Y., L. Bentur, and E. Diamond. 1988. Site of Zinc Absorption in Dog Small Intestine. J. Nutr. 118:61–64.

Nayeri, A., N.C. Upah, E. Sucu, M. V Sanz-Fernandez, J.M. DeFrain, P.J. Gorden, and L.H. Baumgard. 2014. Effect of the ratio of zinc amino acid complex to zinc sulfate on the performance of Holstein cows. J. Dairy Sci. 97:4392–4404. doi:10.3168/jds.2013-7541.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev ed. Natl. Acad. Press, Washington DC.

Oberleas, D., M.E. Muhrer, and B.L. O'Dell. 1960. Effects of phytic acid on zinc availability and parakeratosis in swine. J. Anim. Sci. 20:57–61.

Palmiter, R.D., and S.D. Findley. 1995. Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J. 14:639–649.

Paripatananont, T., and R.T. Lovell. 1995. Chelated zinc reduces the dietary zinc requirement of channel catfish, Ictalurus punctatus. Aquaculture. 133:73–82.

Perryman, L.E., D.R. Leach, W.C. Davis, W.D. Mickelsen, S.R. Heller, H.D. Ochs, J.A. Ellis, and E. Brummerstedt. 1989. Lymphocyte alterations in zinc-deficient calves with lethal trait A46. Vet. Immunol. Immunopathol. 21:239–248. doi:10.1016/0165-2427(89)90034-2.

Piletz, J.E., and R.E. Ganschow. 1978. Zinc Deficiency in Murine Milk Underlies Expression of the Lethal Milk (lm) Mutation. Science (80-.). 199:181–183.

Powell, G.W., W.J. Miller, and D.M. Blackmon. 1967. Effects of Dietary EDTA and Cadmium on Absorption, Excretion and Retention of Orally Administered 65Zn in Various Tissues of Zinc-deficient and Normal Goats and Calves. J. Nutr. 93:203–212.

Sandström, B., L. Davidsson, A. Cederblad, and B. Lönnerdal. 1985. Oral iron, dietary ligands and zinc absorption. J. Nutr. 115:411–4.

Schölmerich, J., a Freudemann, E. Kottgen, H. Wietholtz, B. Steiert, E. Lohle, D. Haussinger, and W. Gerok. 1987. Bioavailability of Zinc from Zinc-Histidine Complexes .1. Comparison with Zinc-Sulfate in Healthy-Men. Am. J. Clin. Nutr. 45:1480–1486.

Seo, Y.A., V. Lopez, and S.L. Kelleher. 2011. A histidine-rich motif mediates mitochondrial localization of ZnT2 to modulate mitochondrial function. Am. J. Physiol. Cell Physiol. 300:C1479–C1489. doi:10.1152/ajpcell.00420.2010.

Spears, J.W. 1989. Zinc methionine for ruminants: relative bioavailability of zinc in lambs and effects of growth and performance of growing heifers. J. Anim. Sci. 67:835–843.

Spears, J.W., and E.B. Kegley. 2002. Effect of zinc source (zinc oxide vs zinc proteinate) and level on performance, carcass characteristics, and immune response of growing and finishing steers. J. Anim. Sci. 80:2747–2752.

Starcher, B.C., J.G. Glauber, and J.G. Madaras. 1980. Zinc absorption and its relationship to intestinal metallothionein. J. Nutr. 110:1391–7.

Steel, L., and R.J. Cousins. 1985. Kinetics of zinc absorption by luminally and vascularly perfused rat intestine. Am J Physiol. 248:G46-53.

Sum, E.Y.M., M. Shackleton, K. Hahm, R.M. Thomas, L. a O'Reilly, K.-U. Wagner, G.J. Lindeman, and J.E. Visvader. 2005. Loss of the LIM domain protein Lmo4 in the mammary gland during pregnancy impedes lobuloalveolar development. Oncogene. 24:4820–4828. doi:10.1038/sj.onc.1208638.

Teixeira, A.G. V, F.S. Lima, M.L.S. Bicalho, A. Kussler, S.F. Lima, M.J. Felippe, and R.C. Bicalho. 2014. Effect of an injectable trace mineral supplement containing selenium, copper, zinc, and manganese on immunity, health, and growth of dairy calves. J. Dairy Sci. 97:4216–26. doi:10.3168/jds.2013-7625.

Todd, W.R., C.A. Elvehjem, and E.B. Hart. 1934. Zinc in the nutrition of the rat. Am. J. Physiol. 107:146–156. doi:10.1111/j.1753-4887.1980.tb05879.x.

Truong-Tran, A.Q., J. Carter, R.E. Ruffin, and P.D. Zalewski. 2001. The role of zinc in caspase activation and apoptotic cell death. Biometals. 14:315–30.

Truong-Tran, A.Q., D. Grosser, R.E. Ruffin, C. Murgia, and P.D. Zalewski. 2003. Apoptosis in the normal and inflamed airway epithelium: Role of zinc in epithelial protection and procaspase-3 regulation. Biochem. Pharmacol. 66:1459–1468. doi:10.1016/S0006-2952(03)00498-2.

Tucker, H.F., and W.D. Salmon. 1955. Parakeratosis or zinc deficiency disease in the pig. Proc. Soc. Exp. Biol. Med. 88:613–616. doi:10.3181/00379727-88-21670.

Vallee, B.L., and K.H. Falchuk. 1993. The biochemical basis of zinc physiology. 73. 79-118 pp.

Wang, K., B. Zhou, Y.-M. Kuo, J. Zemansky, and J. Gitschier. 2002. A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. Am. J. Hum. Genet. 71:66–73. doi:10.1086/341125.

Wang, X., and B. Zhou. 2010. Dietary zinc absorption: A play of zips and znts in the gut. IUBMB Life. 62:176–182. doi:10.1002/iub.291.

Wedekind, K.J., A.E. Hortin, and D.H. Baker. 1992. Methodology for assessing zinc bioavailability: efficacy estimates for zinc methionine, zinc sulphate and zinc oxide. J. Anim. Sci. 70:178–187.

Wood, R.J., and J.J. Zheng. 1997. High dietary calcium intakes reduce zinc absorption and balance in humans. Am. J. Clin. Nutr. 65:1803–9.

Yuzbasiyan-Gurkan, V., and E. Bartlett. 2006. Identification of a unique splice site variant in SLC39A4 in bovine hereditary zinc deficiency, lethal trait A46: An animal model of acrodermatitis enteropathica. Genomics. 88:521–526. doi:10.1016/j.ygeno.2006.03.018.

Chapter 2 - Effects of dietary zinc amino acid complex on mammary epithelium and dairy food chemistry

Abstract

Twelve lactating Holstein cows (132 ± 21 DIM) were enrolled in a replicated 3 x 3 Latin square experiment to explore the extent to which the source and amount of supplemental Zn in the diet of lactating cows can impact the structure of mammary epithelial tissue. Cows received either 0.97 g supplemental Zn/d as $ZnSO_4$ (34.5 mg Zn/kg DM; **30-ZS**), 1.64 g supplemental Zn/d as $ZnSO_4$ (56.5 mg Zn/kg DM; **60-ZS**), or 1.68 g supplemental Zn/d as a mixture of $ZnSO_4$ and Zn methionine (58.2 mg Zn/kg DM; **60-ZM**). Treatments lasted for 17 days followed by 4 days of sample collection. Blood and milk were collected and analyzed for markers of blood-milk leak including plasma lactose and α-lactalbumin, and blood and milk electrolytes. Total RNA was also isolated from milk cells, and abundance of ZnT2 and clusterin, genes with potential impact on Zn-dependent apoptosis, were measured. Finally, dairy food properties of milk (heat coagulation time, nonprotein nitrogen, and noncasein nitrogen) were also analyzed. 60-ZS cows tended to have greater feed intake than 30-ZS ($P = 0.06$), and milk fat percentage tended to be greater for 60-ZM compared to 30-ZS ($P = 0.08$). No other effects on milk composition, yield, or production efficiency were observed. No effects were observed on markers of blood-milk leak, RNA abundance of ZnT2 or clusterin, or properties of dairy food chemistry. Concentration and source of dietary Zn did not impact mammary epithelial integrity in lactating cows during late lactation.

Introduction

Dietary zinc is important for immunity, reproduction, hormone activity, and the activity of many enzymes in dairy cattle (NRC, 2001). Zinc availability has been shown to impact apoptosis in different epithelial cell lines and tissue types. Cohen et al. (2014, 2012) described a zinc signaling pathway in colonocytes in which extracellular zinc influences the expression of the antiapoptotic protein, clusterin. Mammary epithelial cells (MECs) may also be sensitive to zinc availability. Dempsey et al. (2012) have shown in a mouse model that moderate zinc deficiency can impact apoptosis rate, gross morphology, and milk secretion in the mammary gland of mice. Hennigar et al. (2015) have further shown that overexpression of the zinc transporter, ZnT2, stimulates lysosomal-mediated cell death in the mouse mammary gland.

Research in applied nutrition has shown that supplementing zinc as a zinc amino acid complex increases milk production in dairy cattle, likely due to an increased bioavailability of the zinc (Kellogg et al., 2004). In addition to production benefits, Nayeri et al. (2014) observed decreased milk somatic cell counts and increased colostrum IgG in multiparous cows fed increased proportions of zinc as a zinc amino acid complex.

Blood lactose and α-lactalbumin, milk albumin, and the ratio of blood electrolytes:milk electrolytes have been used as biomarkers for blood-milk leak in previous research (Stelwagen et al., 1997, 1995, 1994). In this study, we explored the extent to which variable sources and levels of zinc supplementation in practical rations impact MEC structure and apoptosis. We hypothesized that cows fed higher concentrations or more bioavailable zinc would show fewer signs of cellular degeneration and epithelial permeability in the mammary gland.

Methods

Cows and treatments

Twelve multiparous Holstein cows in mid- to late-lactation (132 ± 21 DIM) were enrolled in a replicated 3 × 3 Latin square experiment. Cows were blocked by average milk yield (51 ± 3 kg/d) and randomly assigned to treatment sequence within block. The experiment was divided into 3 periods of 21 days, with 17 days of diet adaptation and 4 days of sample collection in each period. Throughout the course of the study, cows were housed in individual tie stalls equipped with automatic waterers, provided with a balanced basal ration (Table 2.1) fed twice daily at 0700 h and 1600 h to provide 110% of expected intake, and milked 3 times daily at 0700 h, 1600 h, and 2300 h.

Treatments consisted of (1) 0.97 g Zn/d provided as $ZnSO_4$ (34.5 mg Zn/kg diet DM; 30-ZS), (2) 1.64 g Zn/d as $ZnSO_4$ (56.5 mg Zn/kg diet DM; 60-ZS), and (3) 0.55 g Zn/d provided as $ZnSO_4$ plus 1.13 g Zn/d provided as a zinc methionine complex (58.2 mg Zn/kg diet DM; 60-ZM). Treatments were administered once daily at 1700 h as an oral bolus (Torpac, size #10, Valley Vet Supply, Marysville, KS) containing all supplemental trace minerals except for Se, which was included in the grain mix. All trace minerals except for Zn were provided in equal amounts across treatments. Smartamine M (Adisseo Inc., France) was also included in the 30-ZS and 60-ZS boluses to provide metabolizable methionine equivalent to that provided in 60-ZM (Table 2.2).

An additional cow (second lactation, 128 DIM) was kept throughout the first treatment period as a potential replacement, fed the same basal diet, and received the 60-ZS treatment bolus daily. Data from this replacement cow for the first treatment period were included in analysis. During the second treatment period, one cow developed a case of clinical mastitis, was

removed from the study, and period 2 and 3 data this cow were excluded from analysis. Also, during the third treatment period, one cow became lame, suffered a drop in intake, and was removed from the study; period 3 data from this cow were excluded from analysis. In total, 34 cow × period observations were included in the analysis.

Data collection and sampling procedures

Initial body weights were measured on the first day of the study, refusals were weighed daily to monitor feed intake, and water intake was recorded daily using in-line meters on each waterer. During sampling periods, milk samples (~ 25 mL) were collected at each milking for component analysis by DHIA (MQT Labs, Kansas City, MO). Additional milk samples (≥ 20 mL) were collected and stored at -20°C for later analysis. On the last day of each sampling period, 2 additional milk samples were collected at the morning milking (0700 h). One sample (~2 L) was analyzed that day for heat stability and prepared for analysis of protein fractions. The second sample (50 mL) was immediately placed on ice and centrifuged within 30 min of collection (15 min, 3,000 × *g*, 4°C). The pellet was then suspended in TRIzol reagent (Thermo Fisher Scientific, Inc.) and stored at -80°C until later analysis of RNA transcript abundance.

On either the third or fourth day of each sampling period, blood samples were collected in K_3EDTA tubes via coccygeal venipuncture at 0900 h, 1100 h, 1300 h, and 1500 h. Blood samples were centrifuged for 15 min at 1500 × g and plasma was collected and stored at -20°C for later analysis of α-lactalbumin and lactose concentrations. An additional blood sample was collected at 1300 h in Li heparin tubes and whole blood was immediately analyzed for Na, K, and Cl concentrations as well as hematocrit and hemoglobin, using a handheld blood analyzer (VetScan i-STAT 1; E3+ cartridges; Abaxis, Union City, CA).

Milk sample preparation and analyses

Milk samples (n = 12/cow per period) were defatted and composited for each cow within each treatment period. Briefly, sample tubes were brought to 33°C in a water bath, inverted 4 times, and centrifuged for 30 min at 3,000 × g and 4°C. The fat layer was removed and equal proportions of the aqueous phase were combined into a sterile microcentrifuge tube. Composited samples were then centrifuged a second time (60 min, 15,000 × g, 4°C). The translucent supernatant was used for quantification of electrolytes. Sodium and potassium concentrations were determined using atomic absorbance spectrophotometry (model 3110, Perkin Elmer, Waltham, MA) measured at 589 nm and 766.5 nm for Na and K respectively. Attempts were also made to measure bovine albumin concentrations in milk using a commercially available kit (Bethyl Laboratories, Inc.; Montgomery, TX). Inter-assay CV for technical replicates of samples averaged 35% for this assay. Eight ng of bovine albumin was spiked into 100 μL of 4 samples with CV < 15%, but recovery averaged -19% (absorbance decreased relative to the unspiked samples). Because of the poor performance of this assay, albumin concentrations were not considered in our analysis.

Whole milk samples (~2 L) collected on the final day of each treatment period were kept on ice immediately following collection and used the same day for determination of heat coagulation time (HCT) and quantification of nonprotein nitrogen (NPN) and noncasein nitrogen (NCN). Heat coagulation time was performed following the method of Davies and White (1966) with minor modifications. Briefly, 5 subsamples were allowed to equilibrate to room temperature and adjusted to different pH's (pH 6.4, 6.6, 6.8, and 7.0, and native pH) using 0.5 N NaOH or HCl. Three mL of each subsample were collected into a tightly-capped glass test tube and placed on a rocker. The rocker was immersed in an oil bath (140°C) and monitored visually. HCT was

recorded as the time (min) required for the onset of visible clots. NPN and NCN were measured as described by Zhang and Metzger (2011).

Plasma sample preparation and analyses

Plasma samples (n = 4/cow per period) were composited in duplicate for each cow within each treatment period. Composited plasma samples were diluted 1:15 and the concentration of bovine α-lactalbumin was measured by ELISA using a commercially available kit (Bethyl Laboratories, Inc.; Montgomery, TX) according to the manufacturer's protocol. Samples were analyzed in duplicate on a single plate and the mean intra-assay CV was 25%. To test the validity of the assay in our samples, 0.05 ng of bovine α-lactalbumin was spiked into 100 µL of 4 representative samples (5 ng/mL spike). The mean recovery was 75 ± 38%. Because of the variability in results obtained with this kit, analysis of bovine α-lactalbumin should be interpreted with caution.

Composited plasma samples were also deproteinized for measurement of lactose. Briefly, 30 µL of 6 N $HClO_4$ was added to 300 µL of composited plasma. Samples were kept on ice for 20 min and centrifuged for 2 min at 10,000 × g. Two hundred µL of supernatant were collected in a fresh tube containing 18 µL of 6 N KOH. Tubes were left on ice for another 20 min, centrifuged for another 2 min at 10,000 × g, and the supernatant was collected for analysis of lactose concentration. Lactose concentration was measured in the deproteinized plasma using a commercially available kit (Abnova, Taiwan) according to the manufacturer's protocol. Samples were analyzed in duplicate on 2 plates. The mean intra-assay CV was 22%. To test the validity of the assay, 4 nmol lactose was spiked into 20 µL of a representative sample (200 µM spike) and recovery was measured at 118%.

Milk mRNA isolation and abundance

RNA was isolated from whole milk pellets using the RNeasy Mini procedure (Qiagen). Concentration of RNA was measured on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.) and used to determine volumes for reverse transcription. The amount of RNA used for reverse transcription ranged from 78.6 to 431.4 ng.

Reverse transcription was performed on a DCL 25 thermal cycler (Dynalab Corp.; Rochester, NY) using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Inc.; Hercules, CA). The resulting cDNA was amplified with primers for ZnT2, clusterin, occludin, RPS9, and RPS15 on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc.) using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.; Hercules, CA). Normalized transcript abundance was calculated for ZnT2, clusterin, and occludin using the ΔΔCT method with the geometric mean of RPS9 and RPS15 as control genes, and a mix of all samples as a reference sample. Primers were designed using the Primer-BLAST tool on the National Center for Biotechnology Information website (Table 2.3).

Statistical analyses

Measurements were analyzed in JMP v10.0 (SAS Institute, Cary, NC) with a mixed model using fixed effects of treatment and period, and the random effect of cow. Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$. Interactions of treatment and period were also tested and removed from the model if they did not contribute ($P > 0.10$).

Results

Feed intake tended to be greater for 60-ZS cows ($P = 0.06$) compared to 30-ZS, and milk fat percentage tended to be greater for 60-ZM ($P = 0.08$) compared to 30-ZS. No other effects on milk composition, yield, or production efficiency were observed (Table 2.4). This study was not

designed to be sensitive enough to detect modest production effects. However, previous research has shown increases in milk production when feeding higher proportions of Zn as a Zn-amino acid complex (Kellogg et al., 2004).

Plasma electrolyte, lactose and α-lactalbumin concentrations were also unaffected by treatment, as were milk electrolytes (Table 2.5). No effects of treatments were observed on heat coagulation time or the proportion of nonprotein nitrogen or noncasein nitrogen in the milk (Table 2.6).

No treatment effects were observed in transcript abundance of ZnT2 or clusterin in milk cells (Table 2.5). Occludin transcript abundance was too low to reliably measure in our samples.

Discussion

Results from this study indicate that supplemental zinc source and level in practical diets of lactating dairy cattle have little effect on the integrity of the blood-milk barrier of the healthy mammary gland. In previous research, plasma lactose and α-lactalbumin levels have been shown to increase with disruption of the epithelial barrier separating blood from milk in the mammary gland (Stelwagen et al., 1997). The lack of treatment effects observed in this study likely indicates that epithelial integrity was not appreciably affected by our treatments, perhaps in part due to the use of healthy, non-mastitic cows.

Feeding of marginally zinc-deficient diets in early lactation has been shown to increase somatic cell counts in dairy cattle (Cope et al., 2009). Further, multiparous cows supplemented with an increased proportion of zinc as zinc methionine had reduced somatic cell counts and higher colostrum IgG (Nayeri et al., 2014), indicating improved mammary health and possibly enhanced immune function. One possible explanation for this observation is that zinc supplementation leads to less apoptosis in the mammary gland, leading to a tighter blood-milk

barrier. Marginally zinc deficient mice have been shown to experience dramatic increases in apoptosis rates in mammary tissue leading to differences in gross morphology of the mammary gland during lactation (Dempsey et al., 2012). Were this the case for our model, we would likely have observed differences in markers of blood-milk leak.

Another potential explanation for zinc supplementation leading to improved mammary health is a direct impact on immune responses. Dietary zinc levels impact lymphocyte numbers as well as other aspects of the immune response in mice (reviewed in Fraker and King, 2004); however, zinc supplementation in practical diets of cattle does not always impact measures of immune function (Kincaid et al., 1997; Spears and Kegley, 2002). It is possible that the improvements in mammary health and milk production noted in previous studies were the product of improved immune response to mastitis challenge, without directly affecting apoptosis of mammary epithelial cells or integrity of the blood-milk barrier.

Transcript abundance of selected genes was determined as a further exploratory attempt to discover cellular pathways impacted by zinc supplementation in the mammary gland. Collecting RNA from an internal tissue of a living organism is typically a difficult task, requiring a biopsy procedure. In the case of mammary epithelial cells, there are alternatives to biopsy, which in turn present their own challenges. In the present study, we utilized milk somatic cells as a source of mammary tissue. Isolation of RNA from milk somatic cells has been shown to produce RNA sequencing results comparable to other more intensive sampling methods such as biopsies, laser-capture of cells from fixed tissue, and antibody-capture of milk epithelial cells (Canovas et al., 2014). However, in a healthy cow, less than 10% of milk somatic cells are expected to be MEC (Wagner et al., 2009), with the balance largely made up of immune cells. It is possible that transcript abundance of genes expressed preferentially in MEC could be diluted

by the low proportion of MEC in milk somatic cells. Abundance was low for all of our chosen transcripts (Table 2.3), which may partially explain why no differences were detected.

ZnT2 is a zinc transporter which is involved in zinc transport into milk (Chowanadisai et al., 2006), as well as participating in lysosomal-mediated cell death during mammary involution (Hennigar et al., 2015). Dempsey et al. (2012) found that transcript abundance of ZnT2 was higher in mice fed a marginally zinc-deficient diet throughout gestation and lactation. This was coupled with decreased milk secretion and increased numbers of apoptotic cells in the mammary gland. In the present study, all cattle were fed a diet which met NRC (2001) requirements for daily zinc intake, and treatment diets were administered for only a portion of the lactation period, in order to facilitate a Latin square experimental design. Although no effects were detected in the current study, this does not rule out the possibility that a marginally zinc-deficient diet, or provision of a supplement for an extended period of time may elicit a response in ZnT2 expression.

Clusterin is a ubiquitously-expressed protein which is upregulated by cellular stress in a diverse array of tissue types and prevents apoptosis by reducing oxidative stress (reviewed in Park et al., 2014). Further, clusterin is associated with drug resistance in a variety of tumor cells, and is upregulated in prostate, renal, colon, breast, and other cancer cells in humans (reviewed in Djeu and Wei, 2009). Cohen et al. (2012) found that 100 μM zinc caused increased clusterin expression in butyrate-challenged HT29 colonocytes. It is important to note that butyrate treatment was necessary for extracellular zinc to impact clusterin expression, and that in the absence of cellular stress, there was no zinc effect. In the present study, the lack of response in clusterin expression may be due to a lack of cellular stress in the mammary gland. The study was conducted during post-peak lactation, a period where cellular stability is relatively high, in

healthy animals. In mastitic cows, or during involution, clusterin regulation may be more dramatic.

Conclusion

Zinc supplementation of dairy rations at 30 ppm as opposed to 60 ppm did not appear to impact the integrity of the blood-milk barrier or dairy food properties of milk, nor did the inclusion of a greater proportion of the Zn as an amino acid complex. Milk production responses to amino acid-bound zinc sources which have been documented in studies with larger sample sizes are likely due to mechanisms other than a direct impact on mammary epithelial integrity. However, it is possible that these treatments could elicit a response in cohorts with greater mastitis pressure, with more challenges to mammary epithelial integrity.

References

Canovas, A., G. Rincon, C. Bevilacqua, A. Islas-Trejo, P. Brenaut, R.C. Hovey, M. Boutinaud, C. Morgenthaler, M.K. VanKlompenberg, P. Martin, and J.F. Medrano. 2014. Comparison of five different RNA sources to examine the lactating bovine mammary gland transcriptome using RNA-Sequencing. Sci. Rep. 4:1–7. doi:10.1038/srep05297.

Chowanadisai, W., B. Lonnerdal, and S.L. Kelleher. 2006. Identification of a Mutation in SLC30A2 (ZnT-2) in Women with Low Milk Zinc Concentration That Results in Transient Neonatal Zinc Deficiency. J. Biol. Chem. 281:39699–39707. doi:10.1074/jbc.M605821200.

Cohen, L., H. Azriel-Tamir, N. Arotsker, I. Sekler, and M. Hershfinkel. 2012. Zinc sensing receptor signaling, mediated by GPR39, reduces butyrate-induced cell death in HT29 colonocytes via upregulation of clusterin. PLoS One. 7:1–13. doi:10.1371/journal.pone.0035482.

Cohen, L., I. Sekler, and M. Hershfinkel. 2014. The zinc sensing receptor, ZnR/GPR39, controls proliferation and differentiation of colonocytes and thereby tight junction formation in the colon. Cell Death Dis. 5:e1307. doi:10.1038/cddis.2014.262.

Davies, D.T., and J.C.D. White. 1966. The stability of milk protein to heat. 1. Subjective measurement of heat stability of milk. J. Dairy Res. 33:66–81.

Dempsey, C., N.H. McCormick, T.P. Croxford, Y.A. Seo, A. Grider, and S.L. Kelleher. 2012. Marginal Maternal Zinc Deficiency in Lactating Mice Reduces Secretory Capacity and Alters Milk Composition. J. Nutr. 142:655–660. doi:10.3945/jn.111.150623.

Djeu, J.Y., and S. Wei. 2009. Clusterin and chemosresistance. Adv. Cancer Res. 105:77–92. doi:10.1097/MPG.0b013e3181a15ae8.Screening.

Fraker, P.J., and L.E. King. 2004. Reprogramming of the Immune System During Zinc Deficiency. Annu. Rev. Nutr. 24:277–98. doi:10.1146/annurev.nutr.24.012003.132454.

Hennigar, S.R., Y.A. Seo, S. Sharma, D.I. Soybel, and S.L. Kelleher. 2015. ZnT2 is a critical mediator of lysosomal-mediated cell death during early mammary gland involution. Sci. Rep. 5:8033. doi:10.1038/srep08033.

Kellogg, D.W., D.J. Tomlinson, M.T. Socha, and A.B. Johnson. 2004. Effects of Zinc Methionine on Milk Production and Somatic Cell Count of Dairy Cows : Twelve-Trial Summary. Prof. Anim. Sci. 20:295–301.

Kincaid, R.L., B.P. Chew, and J.D. Cronrath. 1997. Zinc oxide and amino acids as sources of dietary zinc for calves: effects on uptake and immunity. J. Dairy Sci. 80:1381–8. doi:10.3168/jds.S0022-0302(97)76067-3.

Nayeri, A., N.C. Upah, E. Sucu, M. V Sanz-Fernandez, J.M. DeFrain, P.J. Gorden, and L.H. Baumgard. 2014. Effect of the ratio of zinc amino acid complex to zinc sulfate on the performance of Holstein cows. J. Dairy Sci. 97:4392–4404. doi:10.3168/jds.2013-7541.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev ed. Natl. Acad. Press, Washington DC.

Park, S., K.W. Mathis, and I.K. Lee. 2014. The physiological roles of apolipoprotein J/clusterin in metabolic and cardiovascular diseases. Rev. Endocr. Metab. Disord. 15:45–53. doi:10.1007/s11154-013-9275-3.

Schukken, Y.H., D.J. Wilson, F. Welcome, L. Garrison-Tikofsky, and R.N. Gonzalez. 2003. Monitoring udder health and milk quality using somatic cell counts. Vet. Res. 34:579–596. doi:10.1051/vetres.

Spears, J.W., and E.B. Kegley. 2002. Effect of zinc source (zinc oxide vs zinc proteinate) and level on performance, carcass characteristics, and immune response of growing and finishing steers. J. Anim. Sci. 80:2747–2752.

Stelwagen, K., S.R. Davis, V.C. Farr, and S.J. Eichler. 1994. Effect of Once Daily Milking and Concurrent Somatotropin on Mammary Tight Junction Permeability and Yield of Cows. J. Dairy Sci. 77:2994–3001. doi:10.3168/jds.S0022-0302(94)77240-4.

Stelwagen, K., V.C. Farr, R. Davis, and C.G. Prosser. 1995. EGTA-induced disruption of epithelial cell tight junctions in the lactating caprine mammary gland. Am. J. Physiol. 269:848–855.

Stelwagen, K., V.C. Farr, H. a McFadden, C.G. Prosser, and S.R. Davis. 1997. Time course of milk accumulation-induced opening of mammary tight junctions, and blood clearance of milk components. Am. J. Physiol. 273:R379–R386.

Wagner, S.A., D.E. Jones, and M.D. Apley. 2009. Effect of endotoxic mastitis on epithelial cell numbers in the milk of dairy cows. Am. J. Vet. Res. 70:796–799.

Zhang, H., and L.E. Metzger. 2011. Noncasein nitrogen analysis of ultrafiltration and microfiltration retentate. J. Dairy Sci. 94:2118–2125. doi:10.3168/jds.2010-3690.

Table 2.1 Formulation and composition of basal diet

Item	Value
Ingredient, % of diet DM	
Alfalfa hay	18.6
Corn silage	18.9
Wet corn gluten feed[1]	24.3
Cotton seed	4.3
Lactation grain mix[2]	33.9
Nutrient, % of diet DM unless specified	
DM, % as-fed	53.4
CP	17.9
ADF	19.3
aNDF	30.4
Lignin (Sulfuric Acid)	4.7
NFC	42.1
Fat (EE)	4.4
Zinc, ppm	58.3
NE_L[3], Mcal/kg	1.6

[1]Sweet Bran (Cargill Inc., Blair, NE)

[2]Lactation grain mix consisted of 66.22% fine rolled corn, 20.2% expeller soybean meal (SoyBest, Grain States Soya, West Point, NE), 4.04% limestone, 0.5% stock salt, 0.5% potassium chloride, 3.53% sodium bicarbonate, 0.81% magnesium oxide, 0.13% selenium premix (0.06% Se from sodium selenite), 0.05% vitamin A premix (30 kIU/g), 0.02% vitamin D premix (30 kIU/g), 0.5% vitamin E premix (44 IU/g), 0.02% Rumensin 90 (Elanco Animal Health, Greenfield, IN), 0.63% XP Yeast (Diamond V, Cedar Rapids, IA), 0.32% Biotin 100 (ADM Alliance Nutrition, Quincy, IL), 2.52% Ca salts of long-chain fatty acids (Megalac R, Arm & Hammer Animal Nutrition, Princeton, NJ)

[3]Estimated according to NRC (2001)

Table 2.2 Treatment bolus composition and nutrient supply

Item[1]	30-ZS	60-ZS	60-ZC
DM, %	97.97	98.28	97.74
Ca, % DM	1.55	1.33	5.23
P, % DM	0.01	0.01	0.01
Mg, % DM	0.13	0.10	0.11
K, % DM	0.23	0.21	0.26
S, % DM	15.42	15.31	10.35
Mn, % DM	12.84	9.95	9.69
Zn, % DM	7.99	11.55	10.37
Cu, % DM	1.89	1.40	1.32
Fe, % DM	0.23	0.26	0.23
Na, % DM	2.61	1.94	2.01
Cl, % DM	2.32	2.42	8.83
DCAD, mEq/100g	-909.72	-935.44	-801.66
Dose[2], grams/day			
Ca	0.19	0.19	0.85
P	0.00	0.00	0.00
Mg	0.02	0.01	0.02
K	0.03	0.03	0.04
S	1.87	2.17	1.68
Mn	1.56	1.41	1.57
Zn	0.97	1.64	1.68
Cu	0.23	0.20	0.21
Fe	0.03	0.04	0.04
Na	0.32	0.28	0.32
Cl	0.28	0.34	1.43
Met[3]	2.71	2.71	2.69
Metabolizable Met[3]	2.19	2.19	2.18

[1]Values are results of analysis of mineral mixes (Dairyland labs, Arcadia, WI).

[2]Doses are calculated using observed average daily intakes.

[3]Methionine supplied from Zinpro 120 (27.3% methionine, 90% bypass, AA digestibility of 90%; Zinpro Corp., Eden Prairie, MN) or Smartamine M (70% methionine. 90% rumen bypass, 90% intestinal digestibility; Adisseo USA Inc., Alpharetta, GA)

Table 2.3 Primers used for RT-qPCR

Gene	Accession number	Forward primer Reverse primer	Region amplified	Reaction Efficiency	Mean CT
ZnT2	NM_001191496.1	AGCTTGCAGTCGGGACTTAC CCAGGACCCTTCTGGGAATG	38 – 176	91%	32.09
Clusterin	XM_005209841.1	GATTGATTGGTGCTGCCTGC CACCTCTCTTGGGTCACTCG	88 – 214	71%	31.15
Occludin	NM_001082433.2	TACCACTCCTCCTCCCTAGC AGATCAGCCAATCTGCGGCT	55 – 180	undetermined	35.34
RPS9	DT860044.1	GAACAAACGTGAGGTCTGGAGG ATTACCTTCGAACAGACGCCG	233 – 344	106%	22.44
RPS15	NM_001024541.2	GGCGGAAGTGGAACAGAAGA GTAGCTGGTCGAGGTCTACG	23 – 96	99%	21.93

Table 2.4 Intake and milk production

	Least Square Means				Prob > F	
Item	30-ZS	60-ZS	60-ZM	SEM	Trt	Period
DMI, kg/d	28.15	29.01	28.88	0.65	0.06	< 0.001
Zn intake, g/d	2.61[a]	3.33[b]	3.37[b]	0.04	< 0.001	< 0.001
Milk, kg/d	47.23	47.34	47.79	1.34	0.74	0.16
ECM, kg/d	48.03	48.88	49.58	1.17	0.36	0.23
Milk/DMI	1.68	1.63	1.66	0.04	0.13	< 0.001
ECM/DMI	1.71	1.69	1.73	0.03	0.42	< 0.001
Milk protein, %	3.04	3.03	3.02	0.06	0.52	< 0.001
Milk protein, kg/d	1.43	1.43	1.44	0.04	0.97	0.25
Milk fat, %	3.56	3.69	3.76	0.13	0.08	0.35
Milk fat, kg/d	1.67	1.74	1.78	0.06	0.15	0.17
Milk lactose, %	4.90	4.94	4.91	0.04	0.21	0.098
Milk lactose, kg/d	2.31	2.33	2.34	0.06	0.71	0.09
SCC LS[1]	1.35	1.18	1.55	0.30	0.30	0.96
MUN, mg/dL	13.15	13.53	13.49	0.50	0.23	0.21

[1]SCC linear score (LS) calculated using the formula, LS = $\log_2$ (SCC/100) + 3, where SCC = number of somatic cells/µL of milk (Schukken et al., 2003).

[a b]Within row, values not sharing common superscript differ ($P < 0.01$).

Table 2.5 Mammary epithelial integrity markers

	Least Square Mean				Prob > F	
Item	30-ZS	60-ZS	60-ZM	SEM	Trt	Period
Blood K, mM	4.35	4.59	4.36	0.09	0.12	0.11
Blood Na, mM	136.86	136.47	136.31	0.38	0.52	0.57
Blood Cl[3], mM	99.52	99.84	99.82	0.52	0.80	0.43
Blood Hb[3], *mM	5.62	5.70	5.67	0.07	0.48	0.04
Blood Hct[3] (% PCU)	26.58	27.00	26.85	0.33	0.48	0.04
Plasma lactose, µM	21.32	20.91	19.14	2.35	0.73	0.53
Milk Na[1], ppm	354	349	369	11	0.15	< 0.001
Milk K, ppm	1516	1492	1527	30	0.55	< 0.01
ZnT2, NTA[2]	0.59	0.39	0.35	0.15	0.25	0.054
Clusterin, NTA	2.27	0.98	1.20	0.72	0.41	< 0.01

[1]Modeled with blood Na as a covariate ($P < 0.01$)

[2]NTA = Normalized transcript abundance

[3]Treatment × period effect significant and included in the model ($P < 0.1$)

Table 2.6 Dairy food chemistry

	Least Square Mean				Prob > F	
Item	30-ZS	60-ZS	60-ZC	SEM	Trt	Period
Milk NPN, ppm	286	306	296	12	0.51	< 0.01
Milk non-casein N[1], ppm	791	816	809	42	0.92	0.052
Native HCT[1], min	11.29	11.35	10.89	1.66	0.78	0.19
6.4 HCT, min	0.90	1.35	2.80	0.27	0.40	0.09
6.6 HCT, min	12.31	12.46	11.20	0.77	0.39	0.87
6.8 HCT, min	12.28	12.98	13.03	1.68	0.89	0.22
7.0 HCT, min	12.12	10.17	10.41	1.68	0.59	0.33

[1]Treatment × period effect significant and included in the model ($P < 0.1$)

Chapter 3 - Effects of extracellular Zn and Zn receptor GPR39 on ERK and AKT phosphorylation in bovine mammary epithelial cells

Abstract

The orphaned G protein-coupled receptor, GPR39, has been shown to regulate cellular proliferation and apoptosis in response to extracellular zinc in a variety of tissues and cell lines. To explore if a similar mechanism exists in bovine mammary epithelia, GPR39 was knocked down at the mRNA level using RNA interference in immortalized bovine mammary epithelial (MAC-T) cells. Twenty-four hours post-transfection, control and siRNA-treated cells were treated with either 0 or 100 μM Zn and harvested for measurement of total and phosphorylated ERK1/2 and AKT. Two siRNA constructs (**siGPR39a** and **siGPR39b**) were tested for knockdown efficiency of GPR39. At the high-Zn level, transcript abundance of GPR39 was reduced 63% by siGPR39a ($P = 0.02$) and 57% by siGPR39b ($P = 0.04$). Interestingly, there was a tendency for Zn to increase abundance of GPR39 in cells not treated with siRNA ($P = 0.096$). Phosphorylation of ERK1/2 and AKT was not affected by knockdown of GPR39 or Zn treatment. Given the response in GPR39 mRNA abundance to extracellular Zn, it is possible that GPR39 possesses Zn-dependent functions in MAC-T cells. However, extracellular Zn and knockdown of GPR39 do not impact phosphorylation of ERK or AKT, key intermediates of common kinase signaling cascades.

Introduction

Zinc has long been recognized as an essential component of the diet of cattle (Legg and Sears, 1960). As such, its supplementation to diets is not only advisable, but occasionally necessary for normal growth, development, and health of the animal. Severe Zn deficiency leads to sudden and drastic consequences on health and production, which are just as quickly reversed by addition of Zn to the diet (reviewed in Miller, 1970).

Improvements in production can also be realized by Zn supplementation to practical diets. For instance, Spears and Kegley (2002) noted improved average daily gain and carcass quality in grower/finisher steers supplemented with 25 mg Zn/kg diet dry matter. Also, Cope et al. (2009) have shown that provision of approximately 1.4 g Zn/cow per day effectively reduced milk somatic cell counts and amyloid A content compared to cattle fed approximately 0.9 g Zn/cow per day.

Further production benefits have been reported by supplementing with ostensibly more bioavailable Zn sources. Greene *et al*. (1988) found that steers supplemented with Zn methionine showed improvements in carcass quality compared to Zn oxide-supplemented controls. More recently, Nayeri *et al*. (2014) observed a marked decrease in milk somatic cells and an increase in colostrum IgG of multiparous cows, as well as increased milk production in all cows receiving a higher proportion of supplemental Zn as an amino acid complex. However, little is known about the molecular mechanisms by which supplemental Zn can support increased milk yield or quality.

One interesting possibility is the potential for Zn to act as a signaling ligand, altering cellular function in various tissues. Currently, the best-characterized example of a mineral which actively participates in cellular signaling is calcium, which regulates a variety of cellular

activities in a number of tissue types in addition to the classical regulation of parathyroid hormone secretion (reviewed in Chattopadhyay, 2000). Recently, another G protein-coupled receptor, GPR39, which responds to Zn as a ligand, has been characterized in intestinal, salivary gland, and keratinocyte cell lines (Hershfinkel et al., 2001; Cohen et al., 2014) as well as mouse neural tissue (Besser et al., 2009). Cohen et al. (2014) have shown that GPR39 mediates a Zn-dependent increase in activity of the MAPK and PI3K signaling cascades in HT29 colonocytes, as well as a Zn-dependent increase in cellular proliferation. In other tissue types, GRP39 has been observed to mediate a Zn-dependent release of intracellular calcium stores (Hershfinkel et al., 2001).

The rapidity with which cattle are affected by Zn deficiency, coupled with the small change in tissue Zn content (reviewed in Miller, 1970, 1969), would fit with the idea of a Zn-sensing receptor, which could impact cellular function without changing the cellular Zn content. The purpose of this study was to determine whether the putative Zn receptor, GPR39, is expressed in the immortalized bovine mammary epithelial cell line, MAC-T, and if so, to explore its role in regulating function of these cells.

Methods

Cell Culture and Transfection

Immortalized bovine mammary epithelial (MAC-T) cells were cultured in DMEM with 10% FBS, 50 µg/mL gentamycin, 20 µg/mL streptomycin, 20 units/mL penicillin, and 5 µg/mL insulin (growth medium) in an incubator at 37°C and 5% CO_2. Cells were cultured in two 12-well plates for 24 hours (~80% confluence) and subsequently transfected using the TransIT X2 Dynamic Delivery System (Mirus Bio) following manufacturer's protocol, with minor modifications. Prior to transfection, siRNA/transfection reagent complexes were prepared in a

reduced serum medium (Opti-MEM, Thermo Fisher Scientific) and allowed to incubate 15 minutes at room temperature. Cells were then washed once with Ca^{++}/Mg^{++}-free PBS, immersed in Opti-MEM (300 µL/well), and transfected with either: a) one of two siRNA constructs designed to knock down GPR39 (Table 3.1), or b) a universal negative control (Sigma Life Science, cat # SIC001). All wells contained a final concentration of 0.27% TransIT X2 and 45 nM siRNA. Following transfection, plates were incubated at 37°C, 5% CO_2 for 4 hours. At that time, 600 µL growth medium was added back to each well, and cells were incubated for another 20 hours (37°C, 5% CO_2).

Kinase Activation

Twenty-four hours after transfection, cells were removed from the incubator and tested for kinase activation following a Zn treatment. Briefly, wells were washed once with Hanks Balanced Salt Solution (HBSS), and 1 mL of either 0 or 100 µM Zn in HBSS was added to each well. Wells were incubated for 10 minutes in the incubator (37°C, 5% CO_2). Following incubation, wells were washed and incubated a second time with only HBSS (10 minutes, 37°C, 5% CO2) to allow completion of phosphorylation. After the second 10-min incubation, cells were washed with cold PBS and suspended in 300 µL Cell Disruption Buffer (Thermo Fisher Scientific) + 0.5% phenylmethane sulfonyl fluoride.

RNA/Protein Isolation and RT-qPCR

Following kinase activation, protein and RNA were isolated from cells using the mirVana PARIS kit (Thermo Fisher Scientific). Concentration of RNA was measured on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and used to determine sample volumes for reverse transcription.

Reverse transcription was performed on a DCL 25 thermal cycler (Dynalab Corp.; Rochester, NY) using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Inc.; Hercules, CA). The resulting cDNA was amplified with primers for GPR39, RPS9, and RPS15 on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc.) using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.; Hercules, CA). Normalized transcript abundance was calculated for GPR39 using the ΔΔCT method with the geometric mean of RPS9 and RPS15 as control genes, and a mix of all samples as a reference sample. Primers for GPR39 were designed using the Primer-BLAST tool on the National Center for Biotechnology Information website (Table 3.2).

Western Blotting

Protein concentration of cell lysates were determined as described by Bradford (1976). Samples were electrophoresed on two 4-12% Tris Glycine gels for 1 hour at 225 V. Loading volumes were calculated to contain 20 µg protein. Following electrophoresis, gels were blotted to nitrocellulose membranes using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific). Membranes were sequentially probed for AKT1/2/3, pAKT1/2/3, ERK1/2, and pERK1/2. Briefly, membranes were blocked with 0.1 M Tris-HCl containing 5% dry milk and 1% BSA. Following blocking, membranes were washed and left overnight with the appropriate primary antibody at 4°C. The next day, membranes were incubated for 1 h with the corresponding secondary antibody-HRP conjugate in 0.1 M Tris-HCl containing 5% dry milk. After this, bands were detected on a photodocumentation system using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Densitometric analysis was performed on the bands using the gel analysis tool in ImageJ. To determine the level of phosphorylation, band intensity for pAKT1/2/3 was normalized against total AKT1/2/3, and pERK1/2 normalized against total ERK1/2.

Between probes, antibodies from the previous probe were stripped from the membrane by incubating in a stripping solution (62.5 mM Tris HCl, 2% SDS, 0.5% β-mercaptoethanol) for 30 minutes at 60°C. Primary antibodies were rabbit polyclonal for AKT1/2/3 (Santa Cruz Biotechnology, Dallas, TX; sc-8312), pAKT1/2/3 (sc-7985), ERK1 (sc-94), and ERK2 (sc-154); and goat polyclonal for pERK1/2 (sc-7976). HRP-conjugated goat anti-rabbit and HRP-conjugated donkey anti-goat were used as secondary antibodies (Santa Cruz Biotechnology). Primary antibody concentrations were 0.8 µg/mL, and secondary antibody concentrations were 0.2 µg/mL.

Statistical Analysis

Data were analyzed using the mixed procedure of SAS with the fixed effects of siRNA treatment, Zn treatment, and their interaction. Results are reported as LS means with SEM. Where main effects were significant, differences among means were investigated by adjusting for multiple comparisons using Tukey's honestly significant difference method. Significance was set at $P < 0.05$ and tendencies at $P < 0.10$.

Results

Preliminary tests showed GPR39 mRNA to be present in MAC-T cells, as well as a variety of bovine tissues including skeletal muscle, adipose, and milk cells (Table 3.3). In an attempt to explore the role of GPR39 in extracellular Zn signaling of bovine MEC's, MAC-T cells were transfected with one of two anti-GPR39 siRNA constructs (siGPR39a or siGPR39b), or a universal negative siRNA construct (siCON), and subsequently treated with 100 µM Zn or 0 µM Zn, harvested, and analyzed for ERK and AKT phosphorylation. Abundance of GPR39 mRNA was significantly impacted by siRNA treatment ($P < 0.01$) and level of Zn ($P = 0.01$;

Figure 3.1). There was no significant interaction between siRNA treatment and Zn level. At the high Zn level, siGPR39a treatment reduced GPR39 by 63% ($P = 0.02$), while siGPR39b reduced it by 57% ($P = 0.04$) compared to the control. At the low Zn level, no difference in GPR39 mRNA was detected between siRNA treatments and control; however, abundance for both siRNA treatments was numerically lower than the control. Also, the low-zinc control tended to have lower GPR39 abundance compared to the high-zinc control ($P = 0.096$).

There was no significant effect of siRNA, Zn, or their interaction on ERK phosphorylation (Figure 3.2). There was a tendency for siRNA treatment to affect AKT phosphorylation ($P = 0.094$, Figure 3.3), but Zn and the interaction of siRNA and Zn had no effect.

Discussion

Analysis by qPCR confirmed the presence of GPR39 mRNA in MAC-T cells. Interestingly, the relative abundance of GPR39 mRNA increased with Zn treatment. Cohen et al. (2014) found that Caco-2 colonocytes increased expression of GPR39 when differentiating. It is possible that Zn treatment caused the cells in this study to start differentiating as well, possibly by supplying a limiting nutrient, stimulating increased GPR39 expression.

Zinc treatment (100 µM) had no distinguishable effect on phosphorylation of ERK or AKT, key intermediates of the MAPk and PI3K pathways. Zinc concentration in cow's milk is approximately 60 µM, and plasma normally ranges from 12 – 24 µM (Miller, 1969, 1970). The concentration of zinc in our growth medium was 5.98 µM, originating entirely from the 10% fetal bovine serum. Our zinc treatment was 100 µM. In previous studies, extracellular Zn treatment of 100 µM was effective in eliciting kinase phosphorylation, calcium release, and/or

elevated cellular proliferation in a variety of cell types including mouse brain cells, HT29 and Caco-2 colonocytes, human salivary gland cells, and keratinocytes (Hershfinkel et al., 2001; Cohen et al., 2014, 2012; Besser et al., 2009). Given the lack of response in phosphorylation of key intermediates of these pathways, it is not likely that extracellular Zn levels regulate MAPk or PI3K pathways in MAC-T cells. However, these results do not preclude the possibility of receptor-mediated Zn signaling in bovine mammary epithelia. Many other Zn responses have been described in other cell types. The most complete characterization is in colonocytes, where extracellular Zn stimulates a variety of GPR39-dependent responses, such as MAPk/PI3K activation; regulation of the anti-apoptotic gene, clusterin; regulation of sodium/hydrogen exchange; intracellular Ca^{++} release; and increased proliferation rates (Cohen et al., 2014, 2012; Hershfinkel et al., 2001). Here, we have investigated only MAPk/PI3K signaling. Other responses are also possible.

Conclusion

Given its expression in MAC-T cells, GPR39 may play a role in regulation of bovine mammary epithelial cells. This role could possibly be affected by extracellular Zn concentration, given the response in GPR39 mRNA abundance to extracellular Zn treatment. Extracellular Zn and GPR39 did not impact phosphorylation of ERK or AKT, key intermediates of the MAPk and PI3K signaling pathways. Further research is necessary to elucidate the function of this receptor in the bovine mammary gland.

References

Besser, L., E. Chorin, I. Sekler, W. Silverman, S. Atkin, J. Russell, and M. Hershfinkel. 2009. Synaptically-released zinc triggers metabotropic signaling via a zinc sensing receptor in the hippocampus. J. Neurosci. 29:997–1003. doi:10.1016/j.biotechadv.2011.08.021.Secreted.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein– dye binding. Anal. Biochem. 72:248–254.

Chattopadhyay, N. 2000. Biochemistry, physiology and pathophysiology of the extracellular calcium-sensing receptor. Int. J. Biochem. cell Biol. 32:789–804. doi:10.1300/J014v21n04.

Cohen, L., H. Azriel-Tamir, N. Arotsker, I. Sekler, and M. Hershfinkel. 2012. Zinc sensing receptor signaling, mediated by GPR39, reduces butyrate-induced cell death in HT29 colonocytes via upregulation of clusterin. PLoS One. 7:1–13. doi:10.1371/journal.pone.0035482.

Cohen, L., I. Sekler, and M. Hershfinkel. 2014. The zinc sensing receptor, ZnR/GPR39, controls proliferation and differentiation of colonocytes and thereby tight junction formation in the colon. Cell Death Dis. 5:e1307. doi:10.1038/cddis.2014.262.

Cope, C.M., A.M. Mackenzie, D. Wilde, and L.A. Sinclair. 2009. Effects of level and form of dietary zinc on dairy cow performance and health. J. Dairy Sci. 92:2128–35. doi:10.3168/jds.2008-1232.

Greene, L.W., D.K. Lunt, F.M. Byers, N.K. Chirase, C.E. Richmond, R.E. Knutson, and G.T. Schelling. 1988. Performance and Carcass Quality of Steers Supplemented With Zinc Oxide or Zinc Methionine. J. Anim. Sci. 66:1818–1823.

Hershfinkel, M., a Moran, N. Grossman, and I. Sekler. 2001. A zinc-sensing receptor triggers the release of intracellular Ca2+ and regulates ion transport. Proc. Natl. Acad. Sci. U. S. A. 98:11749–11754. doi:10.1073/pnas.201193398.

Legg, S.P., and L. Sears. 1960. Zinc sulphate treatment of parakeratosis in cattle. Nature. 186:1061.

Miller, W.J. 1969. Absorption , Tissue Distribution, Endogenous Excretion, and Homeostatic Control of Zinc in Ruminants. Am. J. Clin. Nutr. 22:1323–1331.

Miller, W.J. 1970. Zinc Nutrition of Cattle: A Review. J. Dairy Sci. 53:1123–1135. doi:10.3168/jds.S0022-0302(70)86355-X.

Nayeri, A., N.C. Upah, E. Sucu, M. V Sanz-Fernandez, J.M. DeFrain, P.J. Gorden, and L.H. Baumgard. 2014. Effect of the ratio of zinc amino acid complex to zinc sulfate on the performance of Holstein cows. J. Dairy Sci. 97:4392–4404. doi:10.3168/jds.2013-7541.

Spears, J.W., and E.B. Kegley. 2002. Effect of zinc source (zinc oxide vs zinc proteinate) and level on performance, carcass characteristics, and immune response of growing and finishing steers. J. Anim. Sci. 80:2747–2752.

Table 3.1 Description of siRNA constructs designed for GPR39 knockdown

Construct[1]	Sense strand Antisense strand	Knockdown Efficiency
siGPR39a	CCGACATCCTGGTCTTCCT AGGAAGACCAGGATGTCGG	63%
siGPR39b	CCATGGAGTTCTACAGCAT ATGCTGTAGAACTCCATGG	57%

[1] Both constructs designed to target bovine GPR39, accession number: NM_001143744

Table 3.2 Primers used for RT-qPCR

Gene	Accession number	Forward primer Reverse primer	Region amplified	Reaction Efficiency	Mean CT
GPR39	NM_001143744	TGAGAAGGTCTTTGGACCCG CAATGACCTGGGAGCAGTCC	94 – 183	105%	27.26
RPS9	DT860044.1	GAACAAACGTGAGGTCTGGAGG ATTACCTTCGAACAGACGCCG	233 – 344	87%	18.17
RPS15	NM_001024541.2	GGCGGAAGTGGAACAGAAGA GTAGCTGGTCGAGGTCTACG	23 – 96	92%	15.50

Table 3.3 Average CT values for GPR39 and control genes, β-actin and RPS15, in MAC-T cells and various bovine tissues

	Gene		
Tissue	GPR39	β-actin	RPS15
Adipose	22.3	17.2	24.5
MAC-T	28.2	17.7	18.2
Milk somatic cells	26.3	20.2	23.6
Skeletal muscle	31.2	22.3	22.1

Figure 3.1 GPR39 mRNA abundance is reduced by siRNA constructs, siGPR39a and siGPR39b, at the 100 µM Zn level.

[1,2]Control cells receiving the 0 µM Zn treatment tended to have lesser abundance of GPR39 than those receiving the 100 µM Zn treatment ($P < 0.1$). [a,b]Within the 100 µM Zn series, both siRNA treatments significantly reduced GPR39 abundance ($P < 0.05$). [3]Normalized Transcript Abundance (NTA) = 2 ^ [$(CT_{sample,reference} - CT_{sample,target}) - (CT_{control,reference} - CT_{control,target})$] where CT ≡ average cycles of PCR required for absorbance of a well to pass a specified threshold, sample ≡ a given treatment combination, control ≡ a mixed combination of all samples in equal proportions, reference ≡ the geometric mean of CT values for reference genes RPS9 and RPS15, target ≡ GPR39. Values are reported as a proportion of the highest treatment combination: siCON, 100Zn.

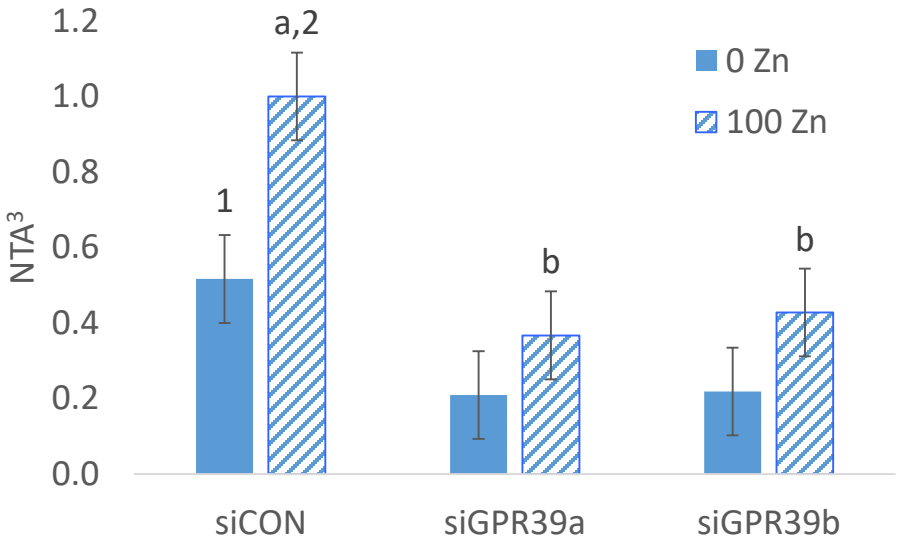

Figure 3.2 Phosphorylation of ERK was unaffected by Zn treatment or silencing of GPR39.

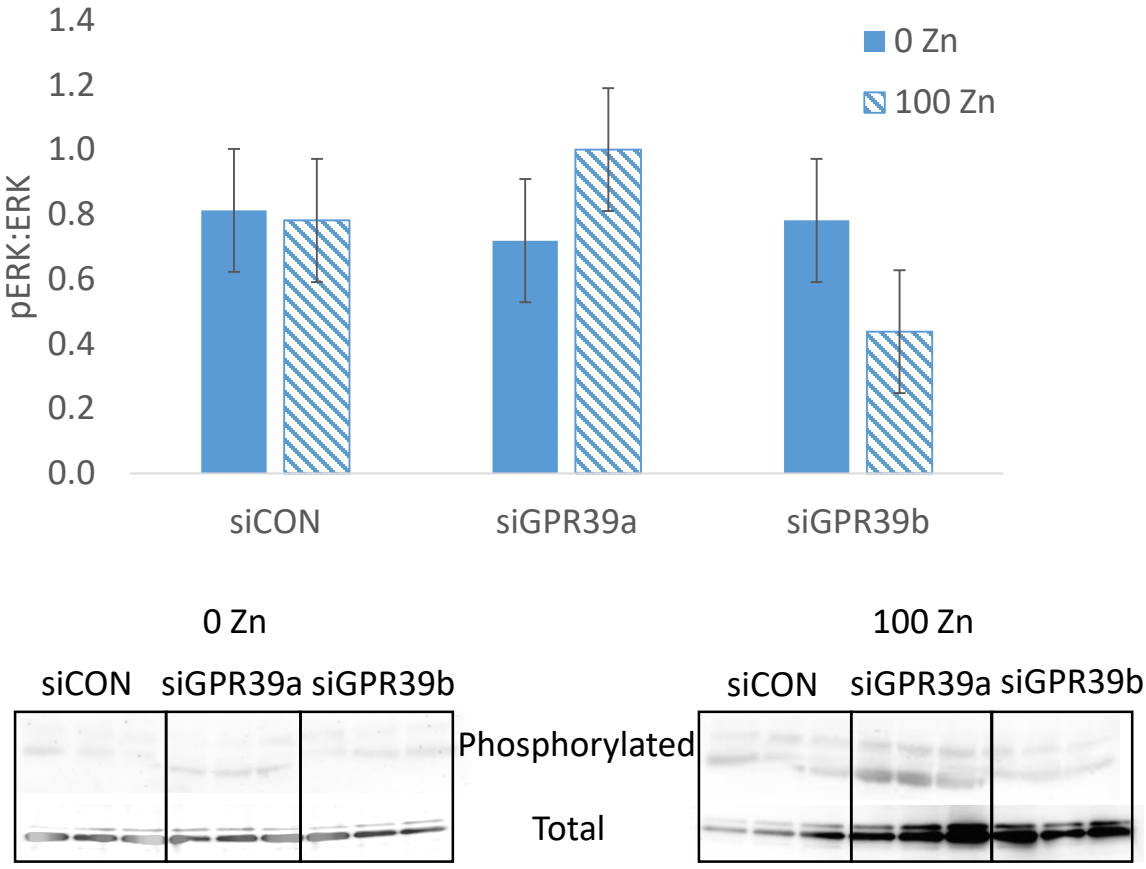

Figure 3.3 Phosphorylation of AKT was unaffected by Zn treatment, and tended to be affected by GPR39 silencing (*P* = 0.094).