ZINC DEFICIENCY AND EXERCISE: EFFECTS ON THE PROTEIN COMPOSITION OF LIPID RAFTS

by

JENNY YING-CHIEH HWANG

(Under the Direction of ARTHUR GRIDER)

ABSTRACT

The present study determined the effects of the lethal acrodermatitis (LAD) mutation, dietary zinc deficiency, and exercise on the protein composition of lipid rafts. The lipid rafts from LAD dog kidney and rat liver were separated using the ice-cold Triton X-100. Two dimensional gel electrophoresis (2DE) was utilized for identifying differential protein expression in these membrane fractions. Rats were fed diets containing 5 and 30 ppm zinc, and were separated into exercise and sedentary groups for 42 days. Differences were assessed using the general linear model (GLM) and the student's unpaired *t* test with repeated measures. One protein was up regulated with 5 ppm zinc. One protein was down regulated during exercise. One protein spot was decreased in the affected LAD kidney lipid rafts. In summary, these results will further our understanding of the LAD mutation and the role of exercise in altering zinc homeostasis.

INDEX WORDS: 16-BAC, Bull Terrier, Exercise, Kidney, Lethal Acrodermatitis, Liver, Zinc, Zinc Deficiency

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JENNY YING-CHIEH HWANG

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JENNY YING-CHIEH HWANG

Major Professor:	Arthur Grider
Committee:	Joan G. Fischer James L. Hargrove

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION

Zinc is the second most abundant trace metal ion in the human body, with iron being the first. More than 300 enzymes and proteins contain zinc as a component. Numerous cellular and biochemical pathways utilize zinc-containing proteins, including many proteins involved in signal transduction and intracellular sorting pathways (Cousins 2003). Zinc also plays an integral role in gene expression through the many zinc-finger DNA binding proteins and transcription factors. These proteins require zinc to stabilize their structural domains (Shu 2008).

The current recommended dietary allowance is 11 mg for males and 8 mg for females (Food and Nutrition Board, Institute of Medicine 2000). There is no tissue storage site for zinc, though bone has the lowest rate of turnover and may reflect long-term zinc status (Rink and Gabriel 2000). Clinical signs of zinc deficiency include growth retardation, hair loss, poor appetite, and various skin lesions. The tolerable upper intake level, which is 40 mg per day (Food and Nutrition Board, Institute of Medicine 2000), was assessed by the hazard identification of a decrease in the levels of erythrocyte superoxide dismutase activity (Food and Nutriton Board, Institute of Medicine 2000).

Two genetic defects result in systemic zinc deficiency, including acrodermatitis enteropathica (AE) in humans and lethal trait A46 in cattle. Defective intestinal zinc absorption is the phenotype in both AE and lethal trait A46, and is caused by mutations in the ZIP4 zinc transporter gene. The phenotype of systemic zinc deficiency is also observed in lethal acrodermatitis (LAD) in bull terrier dogs, though the genetic defect has not been identified (Jezyk et al. 1986). LAD is a fetal disorder that causes several syndromes, such as hair loss, growth retardation, redness and crusted skin around mouth, eyes, and between toes of

experimental dogs (McEwan et. al 2000). Zinc supplementation does not ameliorate the zinc deficiency symptoms of the affected dogs, in comparison to its success with treating AE and lethal trait A46 (Jezyk et al. 1986). A previous proteomic study has shown that the LAD phenotype may be related to defective zinc metabolism and impaired inflammatory response (Grider et al. 2007), although the specific biochemical defect was not determined. The use of proteomic techniques to identify differentially expressed proteins in LAD tissues will provide insight to the effect of this mutation on proteins requiring zinc, or involved in the zinc metabolic pathway.

Stressors such as exercise can increase the requirement for zinc in humans and experimental animals, and cause changes in cellular pathways either requiring zinc or involved in zinc metabolism. Liver proteins from rats consuming either normal or marginal zinc diets have been separated using proteomic techniques to determine the impact of dietary zinc and exercise on protein expression within lipid rafts. The results from this study will increase our understanding of the potential role of lipid rafts in mediating zinc-dependent responses to stress, particularly as related to signal transduction pathways.

Lipid rafts are specialized compartments within the plasma membrane that contain high concentrations of cholesterol and sphinogolipids immersed in a phospholipid rich environment. These plasma membrane compartments have been shown to contain proteins involved with signal transduction such as the Src-family kinases, and glycosylphosphatidylinositol (GPI) – anchored proteins (Simons and Toomre 2000). They are also sites for cholesterol transport (Simons and Ikonen 2000, Mayor 1998). The rafts are thought to provide platforms for activating binding and signaling processes in response to extracellular stimuli. Isolation of lipid rafts occurs based on their insolubility to non-ionic detergents, such as Triton X-100, at 4^{o}C (Simons and Vaz

2004). Caveolae are a subset of lipid rafts that are invaginated on the cell surface. They form using the scaffolding protein caveolin. Caveolin is the hairpin-like palmitoylated integral membrane scaffolding protein for caveolae (Simons and Toomre 2000). The function of caveolae is not clear yet, but some studies have shown that caveolae are involved with endocytosis and transcytosis (Simons and Toomre 2000). Several studies have shown that the structure and function of caveolae are disrupted following removal of their cholesterol (Rothberg et al. 1990). Zinc uptake into MCF10A cells was also shown to be reduced with cholesterol removal, suggesting that the cells' specialized plasma membrane compartments are involved with zinc metabolism (Mouat et al. 2003). Since lipid rafts/caveolae have been linked with signal transduction, and several signal proteins contain zinc-finger motifs (Morita et al. 1996, Glait et al. 2006), acquiring proteomic data on the lipid rafts/caveolae will further our understanding of the role of cellular zinc status on signaling pathways. Further, the results obtained from both of these models will increase our understanding of the role of genetic mutations and stress on zinc metabolism. Finally, the results obtained from the LAD tissues will help to more clearly define this mutation and its effect on zinc-dependent homeostatic pathways.

CHAPTER 2

LITERATURE REVIEW

Zinc

Zinc has been known as an essential mineral for eukaryotes for over a century (Raulin 1869). Zinc is considered the second most abundant trace element in the human body, with an average amount about 2-3 g (Berg and Shi 1996). About 60% of zinc is found in skeletal muscle, 30% in bone, and about 5 % in our skin (Ganapathy and Volpe 1999). Semen (15-30 mg/100 ml) (Marmar et. al. 1975) contains high concentration of zinc as well (King et al. 2000). There are over 3,000 proteins in the human body containing zinc prosthetic groups which include zinc fingers (Hershfinkel et al. 2007). The recommended dietary allowance (RDA) of zinc is shown in Table 1.

Food Containing Zinc

A variety of foods contain zinc (Food and Nutrition Board, Institute of Medicine 2000), especially those enriched with proteins. For example, oysters have a high concentration of zinc compared to other food sources (Bobroff 2001). In the United States, research has shown that Americans consume more red meat and poultry in their diet and both of these contain zinc. Zinc can also be obtained from beans, nuts, mushrooms, spinach, whole grains, fortified breakfast cereals, and some dairy products (Food and Nutrition Board, Institute of Medicine 2000). Zinc from animal protein is more bioavailable than plant protein food sources (Food and Nutrition Board, Institute of Medicine 2000). However, only 20% of zinc consumed will be absorbed by the human body (Bobroff 2001). Several studies have indicated that phytates in whole grain products, cereal, and legumes can reduce zinc absorption, thereby reducing bioavailability (Table

2) (Food and Nutrition Board, Institute of Medicine 2000, Sandstrom 1997, & Prasad 1991). For example, pumpkin seeds are the most concentrated source of zinc in a vegetarian diet; however, phytanic acid in pumpkin seeds forms an insoluble complex with zinc which reduces zinc absorption (Bobroff 2001). Phytic acid can also reduce the zinc absorption by forming an insoluble complex (McClung et al. 2006). Therefore, vegetarians have to consume more foods rich in zinc in order to obtain adequate amounts to meet the RDA. A recent study has suggested that phytases can remove the insoluble complex from phytic acid. This can help to increase the bioavailability of zinc (McClung et al. 2006). Also, a study shows that people with cadmium toxicity may also have reduced zinc absorption because zinc and cadmium compete with each other in the absorption process. Some chemical substances contained in these processed foods will also affect the absorption of zinc, such as EDTA and phosphates (Bobroff 2001).

Zinc Upper Limit

The Food and Nutrition Board, in 2000 set 40 milligrams as the tolerable upper limit (UI) level of daily zinc intake for individuals aged 19 or over (Table 1; Food and Nutrition Board, Institute of Medicine 2000). A metallic or bitter taste may indicate zinc toxicity; symptoms of zinc include stomach pain, cramps, vomiting, nausea, and diarrhea (Fosmire 1990). Studies to determine the toxicity of dietary zinc in animal models and humans consistently show that excess zinc antagonizes copper metabolism and affect erythrocyte superoxide dismutase activity (Kavas et. al. 2002). However, the Food and Nutrition Board used the reduction in erythrocyte superoxide dismutase activity as the biochemical indicator for establishing the tolerable upper intake level (Food and Nutrition Board, Institute of Medicine 2000).

Functions of Zinc

Zinc is an important component of over 300 enzymes (Berg and Shi 1996), and plays three biochemical roles: 1) catalytic, 2) structural, and 3) regulatory (Cousins 1998). For its catalytic function, zinc catalyses RNA polymerases to promote gene expression for cell replication and protein synthesis (Cousins 1998). Consequently, one of the classical features that zinc deficiency causes is growth retardation problems. Another study has shown that LTA_4 hydrolase, a zinc metalloenzyme, has a catalytic role at the final step of conversion to the proinflammatory leukotriene B_4 (Blomster et al. 1995). Leukotriene B_4 is involved in inflammation and leukocyte activation in the human body (Blomster et. al. 1995). In addition, protein farnesyltransferase (FTase) is a zinc metalloenzyme that helps the cysteine residue of the protein to increase its nucleophilicity and activate the diphosphate leaving group (Huang et al. 1997).

Zinc also helps stabilize the structure of proteins via zinc finger motifs. About 1% of the genome code consists of zinc-finger proteins. Zinc finger proteins act like transcription factors that regulate the gene expression. They also relate to cell signaling and hormone release with nerve impulse transmission (Truong-Tran et al. 2000). Zinc-finger proteins are highly stable (on the order of 10^{12} M) due to their tetrahedral structure which consists of four cysteine and/or histidine residues in many different forms. A few studies have suggested that zinc status is related to zinc-finger proteins and gene regulation (Cousins 1998). For example, Zif 268 contains a total three zinc finger proteins; it is also considered as a mammalian transcription factor that is now called Egr1. These zinc finger proteins bind to the major groove of B-DNA and wrap around the double heliex, which creates a great framework to design DNA-binding proteins (Pavletich and Pabo 1991). Botulinum toxins (BoNTs) block acetylcholine release over the

neuromuscular junctions in order to treat some neuromuscular disorders, such as strabismus and blepherospasm (Fu et. al. 1998). Zinc has been suggested to related and bound to its structure. The data has shown that BoNTs can lose its neurotoxin activity by removing the zinc from its structure (Fu et. al. 1998). Therefore, zinc plays a critical role with its functional and biological activities (Fu et. al. 1998). In addition, zinc deficiency can alter cell membrane structure, which will result in oxidative damage and functional impairment (Ho et al. 2003).

Zinc functions in its regulatory role through its control of gene expression. The zinc finger transcription factor GATA4 is required to interact with a cardiac muscle-restricted homeobox protein Csx in order to activate gene expression (Lee et. al. 1998). GATA4 protein has been involved in embryogenesis and in myocardial differentiation and function (Lee et. al. 1998). Another study has shown that the zinc-finger protein A20 has two functions in inhibiting NF-kB activation and suppressing apoptosis (Evans et. al. 2004). NF-kB acts as a transcription factor that is involved in regulation of the immune response for stimuli such as stress and bacterial infection (Lademann et. al. 2001).

Zinc-binding transcription factors control gene expression by binding to metal response elements (MRE) (Cousin 1998). The control of metallothionein expression via its MRE is one of the most studied examples. Metallothionein (MT) can also protect cells from free radicals (Sato and Bremner 1993) and metal toxicity (Murphy et. al 1999). For instance, an antioxidant response element (ARE) works synergistically with MRE to response to free radicals (Davis and Cousins 2000). In addition, the MREs can be activated by both hypoxia and oxidative stress signals, controlled by MTF-1. MTF1 is involved in zinc homeostasis of gene expression and against free radicals from oxidative stress (Andrews 2001).

MTF-1 (metal responsive transcription factor-1) is normally found complexed with MTI (metallothionein transcription inhibitor) (Palmiter 1994). When zinc is present, MTF-1 dissociates from MTI and interacts with the MREs in the MT promoter, which starts the transcription (Palmiter 1994). It has been well-known that RNA polymerases I are zinc metalloenzymes (Auld et. al. 1976) and zinc motif proteins are involved in DNA binding factors during gene transcription (Cousins and Lee-Ambrose 1992). Therefore, zinc deficiency can reduce the activity of RNA polymerase (Terhune and Sandstead 1972).

There are other physiological functions for zinc. Zinc can help to support a healthy immune system (Solomons 1998 and Prasad 1995). An immune system is defined as a collection of biological processes in the human body that guard against diseases or kill pathogens. Also, zinc deficiency can reduce the effectiveness of recruitment chemotaxis of neutrophils (Rink and Gabriel 2000). Another key role of the immune system is the T-cell, which is a type of white blood cell known a lymphocyte. T-cell development and activation have to involve zinc when binding tyrosine kinase to T-cell receptors (Oteiza et. al. 1995). Therefore, zinc deficiency can decrease T and B lymphocyte concentration in the human body, which can reduce immune function (Shankar and Prasad 1998).

Under normal conditions zinc is involved in DNA synthesis, protein synthesis, and cellular proliferation. Zinc deficiency reduces wound healing by reducing fibroblast proliferation and collagen synthesis, ultimately delaying wound healing time. Since zinc deficiency is associated with impaired immune function, the risk of infection also increases (Arnold and Barbul 2006).

The effect of zinc deficiency on growth retardation is well-known (Fons et al. 1992) . Zinc deficiency reduces the level of insulin-like growth factor-1 (s-IGF-1) and insulin (s-insulin)

(Dorup et. al. 1991). In addition, IGF-1 plays a significant role during the childhood growth and anabolic processes in adults.

Zinc Deficiency

Zinc deficiency can result from impaired zinc absorption and inadequate intake during growth in early life (Prasad 1991). RNA is required for protein synthesis and gene expression, and RNA plolymerase used DNA as a template to generate RNA. RNA polymerase declines in activity during zinc deficiency (Terhune and Sandstead 1972).

This can result to decreased gene transcription and translation. Also, zinc deficiency causes a decline in the level of IGF-1, which leads to growth retardation (Dorup et. al. 1991). A decline in insulin is also noted, and may be related to the structural role zinc plays in maintaining insulin protein conformation (Zalewski et al. 1994). In addition, immune function can decline during zinc deficiency because of insufficient development of white blood cells (Oteiza et. al. 1995). Zinc deficiency can induce poor appetite because it may inhibit the Neuropeptide Y (NPY) release from the paraventricular nucleus (PVN) (Jing et. al. 2007). Normally, NPY can be secreted by hypothalamus and helps to increase the food intake. However, the relationship between the level of zinc intake and the effect on NPY still needs further study. Consequently, it is important to have a balanced zinc diet.

Genetic Mutations Causing Zinc Deficiency

Acrodermatitis enteropathica (AE) was first found by Danbolt and Closs in 1942 (Danbolt and Cross 1942). AE is a rare autosomal recessive disorder in the human, initially thought to be caused by impaired zinc intake or increased zinc excretion (Yu 2007, Danbolt and

Cross 1942). Within the past decade mutations in the SLC39A4 gene, which encodes the Zip4 intestinal zinc transporter, was identified as the cause of AE (Maverakis et. al. 2007, Nakano 2003, Wang 2004).

Lethal trait A46 is an autosomal recessive disorder causing hereditary zinc deficiency in Black Pied Friesian cattle (Machen et al. 1996).Affected cattle were first described in 1964 as having hereditary parakeratosis and hereditary thymus hypoplasia. The symptoms can include skin lesions and diarrhea, identical to the clinical symptoms of AE. As with AE, the biochemical defect in lethal trait A46 is the result of mutations in the SLC39A4 gene (Yuzbasiyan-Gurkan and Bartlett 2006).

Lethal acrodermatitis (LAD) is a rare genetic disease that only affects bull terrier dogs (Jezyk et al. 1986). In the 1980s, researchers were first documented this metabolic disease (McEwan et al. 2000). As yet, there is insufficient research regarding this genetic disorder. The clinical symptoms for dermatohistopathology of LAD are similar to AE in humans (McEwan et al. 2000) and lethal trait A46 in Black Pied Friesian cattle (Jezyk et al. 1986). Clinical studies indicate that AE is successfully treated with oral zinc supplements of 35 mg zinc daily (Danbolt 1979) and 14 g zinc acetate every other day for cattle (Machen 1996); however, 100 mg zinc sulfate twice a day was ineffective in treating dogs affected with LAD (Kunkle 1980). Zinc supplementation increased to 880 mg zinc sulfate twice daily could improve, but not fully ameliorate their condition, the dog's clinical condition (Kunkle 1980). LAD symptoms include diarrhea, behavioral abnormality, anorexia, growth retardation, skin lesions, eating difficulties, and bacterial infections (McEwan et al. 2000 & Jezyk et al. 1986). Although LAD exhibits the same phenotype as AE and lethal trait A46, the genotype remains undetermined. Consequently, there is no definitive experimental evidence that this is a zinc deficiency disease.

Zinc, Lipid Rafts, and Detergent Resistance Membrane (DRM) Proteins

Lipid Rafts

The concept of lipid rafts originated in the late 1980s, though their existence is somewhat controversial. Lipid rafts are defined as compartments within the plasma membrane consisting of cholesterol, sphinogolipids, and GPI-anchored proteins (Lucero and Robbins 2004). Previous works had found that polarized epithelial cells have formed into apical and basolateral membranes with totally different proteins and lipid compositions within these membranes. Therefore, the lipid rafts theory was proposed and experiments designed to determine their existence (Hanzal-Bayer and Hancock 2007). One means for separating lipid rafts from other cellular components was the use of different detergents during extraction procedures. For an example, lipid rafts were isolated using non-ionic detergents such as Triton-X100 at 4^{o}C (Simons and Vaz 2004). Other detergents, such as CHAPS, Tween20, Brij 58, Lubrol WX, Brij98, and Triton X-100, were also used (Schuck et. al 2003). Removal of the lipid components of the lipid rafts was another tactic used to define them. Incubation of cells with cyclodextrins such as methyl-β-cyclodextrin (MβCD) or fillipin, deplete the plasma membrane of cholesterol leading to disruption of the lipid rafts (Simons and Vaz 2004). However, some studies were using other newer non-ionic detergents presented results suggesting that lipid rafts do not exist (Shogomori and Brown 2003). The reason was that the detergents used to isolate lipid rafts can be vary in their ability to solubilize cellular components. For example, a study has suggested that Brjj58, Lubrol WX, Triton X-100, and CHAPS detergents poorly solubilized non-raft lipids and non-raft plasma membrane proteins (Schuck et. al. 2003). Results from more recent studies support the lipid raft theory (Lucero and Robbins 2004). A study suggested that rafts appear in the liquid-

ordered (lo) state, containing certain proteins, such as GPI-anchored proteins, Src-family tyrosine kinases, and other signaling receptors (Shogomori and Brown 2003).

Caveolae

Caveolae have been recognized in electron microscopic images as invaginated vesicles (Westermann et al. 1999). Plasmalemmal vesicles were the earlier term used for caveolae (Fujimoto et al. 1995). Caveolae are considered as a subset of lipid rafts. The primary difference is that lipid rafts have a planar membrane structure compared to caveolae's invaginated membrane structure. Also, caveolae consist of caveolins, hairpin-like membrane proteins, which are bound to cholesterol within the membrane (Simons and Toomre 2000). Lipid rafts or caveolae have been described with many cellular functions, such as signal transduction, intracellular trafficking, and secretion and endocytosis (Simons and Toomre 2000). For example, one study has shown that caveolin was presented at the apical membrane of small intestine. The caveolin also has helped the influx of cholesterol molecules from the lumen into the endoplasmic reticulum (Field et. al. 1998). The most important function of lipid rafts and caveolae may be signal transduction. For example, the T-cell antigen receptor (TCR), which was started with Src-like tyrosine kinases, LCK and FYN, which phosphorylate the ITAMs (Immunoreceptor Tyrosine-based Activation Motifs). When ZAP70, a protein-tyrosine kinase family, is bound to phosphorylated ITAMs and is activated. Also, the small GTP-binding protein Ras was activated and finally has activated gene transcription and T-cell growth factor (Janes et. al. 2000).

Zinc and Detergent Resistance Membrane (DRM) Proteins

Zinc homeostasis and uptake may be associated with lipid rafts/caveolae, though this might be dependent on the cell type. Mouat (2003) has suggested that zinc uptake can be

decreased by cholesterol depletion with cyclodextrin in the MCF-10A cells. A study has shown that two human zinc uptake transporters, ZIP1 and 2 have been found to locate within the plasma membrane and inhibit the zinc uptake during the depletion of cholesterol (Gaither and Eide 2000).

Various studies have showed that cholesterol can disrupt the formation of lipid rafts and caveolae. Researchers identified the lipid rafts/caveolae compartment by its resistance to solubilization with detergent resistance membrane proteins. For example, a study tried to use T-cell resistance membrane domains to find out the characteristic of those DRM proteins. The results have shown that those DRM proteins are categorized into two groups: cytoskeletal proteins and proteins involved in signal transduction. These results have indicated that some proteins have been highly associated with lipid rafts (Haller et. al. 2001). Another study has suggested that glycosyl phosphatidylinositol-anchored proteins (GPI-APs) do not seem to be present in lipid rafts (Brown 2006). Raft dynamics in cells are still not fully understood; therefore, it is important to continue further research to define their function in cells.

Exercise

Exercise stress affects zinc metabolism. Serum zinc levels are increased immediately after short-duration, high intensity exercise (Ohno et. al. 1985). Serum zinc levels are reduced by prolonging the duration of exercise, but there was no immediate effect observed on serum zinc levels when people started exercise, such as running (Anderson et. al. 1984). Zinc is required for energy metabolism since exercise, as a stressor, increases nutrient requirements. Therefore, the lack of zinc can adversely affect physical activity and muscle capacity during exercise (Couzy et. al. 1990). Also, zinc protects cellular membranes from free radical oxidation (Zago and Oteiza

2001). For example, zinc deficiency can alter the activity of Cu, Zn superoxide dismutases (SODs) during the increase of oxidative stress level. These enzymes provide a defense against oxidative stress (Liochev and Fridovich 2001).

The purpose of this research is to compare the protein expression of detergent-resistant membranes (DRM) proteins in the lipid rafts from zinc-deficient rats (liver) and bull terriers afflicted with the LAD mutation (kidney). Also, the protein expression of exercised and non-exercised rats will be compared by using the same proteomic analysis.

Hypothesis

It is hypothesized that the LAD mutation, zinc deficiency and exercise will affect the protein composition of plasma membrane lipid rafts. It is expected that the affected proteins will be involved in normal zinc homeostasis and/or zinc-responsive signal transduction pathways.

Tables

TABLE 1

Recommended Dietary Allowances (RDA) for zinc (Bobroff 2001)

Life Stage	Age	Allowance, mg/d	
		Males	Females
Infants	0-6 mo	2	2
	7-12 mo	3	3
Children	1-3 y	3	3
	4-8 y	5	5
	9-13 y	8	8
Adolescents	14-18 y	11	9
	Pregnant, 19 y	NA	12
	Breastfeeding, 19 y	NA	13
Adults	All, 19 y	11	8
	Pregnant, 19 y	NA	11
	Breastfeeding, 19 y	NA	12

NA = not applicable.

TABLE 2

Zinc content of zinc-rich foods (Food and Nutrition Board, Institute of Medicine 2000, Sandstrom 1997, & Prasad 1991)

Food	Serving Size	Zinc Content, mg
Oysters	6 medium, cooked	43.4
Dungeness crab	3 oz., cooked	4.6
Beef	3 oz., cooked	5.8
Turkey, dark meat	3 oz., cooked	3.5
Chicken, dark meat	3 oz., cooked	2.4
Pork	3 oz., cooked	2.2
Cashews	1 oz.	1.6
Baked beans	0.5 cup	1.8
Yogurt, fruit	1 cup (8 oz.)	1.8
Chickpeas (garbanzo beans)	0.5 cup	1.3
Almonds	1 cup (8 oz.)	1.0
Milk	1 cup (8 oz.)	1.0
Cheese cheddar	1 oz.	0.9
Peanuts	1 cup (8 oz.)	0.9

CHAPTER 3

DIETARY ZINC DEFICIENCY AND EXERCISE ALTER PROTEIN EXPRESSION IN DETERGENT RESISTANCE MEMBRANES OF RATS

Introduction

Lipid rafts are special compartments within the plasma membrane and are sites associated with signal transduction pathways (Simons and Toomre 2000). These structures contain high concentrations of cholesterol and sphingolipids, as well as glycosylphospholipid inositol-linked proteins (Simons and Toomre 2000). Caveolae are lipid rafts that contain many hairpin-like palmitoylated integral membrane proteins called caveolins. Caveolins as tightly bound to cholesterol in lipid rafts (Parton 1996). Cholesterol depletion can cause two effects: reduction of invaginated caveolae numbers (Chang et al. 1992) and unclustered folate receptors – GPI-anchored membrane proteins (Rothberg et al. 1990). A study showed that GPI-anchored membrane proteins, which serve as a folate receptor, are highly clustered in caveolae (Ying et al. 1992). These results have suggested cholesterol plays a significant role on caveolae membrane domain and GPI-anchored membrane proteins.

Protein kinase C (PKC) has contained two identical zinc-binding motifs at the N-terminus as its regulatory region (Parker et al. 1986). Also, the translocation of PKC to the cell membrane requires zinc. Activation of the PKC can help to phosphorylate transcription factors (TF) and lead to gene expression (Beyersmann and Haase 2001). A study has shown that PKC was essential for the hematopoietic protein tyrosine phosphatase (HePTP) and it phosphorylated HePTP at Ser-225, which was required for lipid raft translocation (Nika 2006). Another study has shown that the GPI-anchored protein is involved with the translocation of those immune

receptors, such as the high-affinity IgE receptor and the T-cell receptor (Sedwick and Altman, 2002). These signaling processes can help to amplify the T-cell activation events.

Insulin-like growth factor- binding protein 3 (IGFBP-3), has a zinc finger motif, which binds to insulin growth factors (IGFs) to increase the duration of IGF effects in the human body (Singh et al. 2004). Also, IGFs have shown to couple with caveolin which is involved in signal transduction and membrane traffic (Gustavsson et al. 1999, Chamberlain et al. 2002).

Cholesterol depletion has shown to disrupt the stability of lipid rafts/caveolae (Mouat 2003). A study has shown that zinc uptake into MCF-10A cells was inhibited by depleting cholesterol from lipid rafts/caveolae (Mouat 2003). These results have shown the importance of cholesterol in the formation of lipid rafts/caveolae.

Lipid rafts/caveolae are defined as structures apart from the plasma membrane by their resistance to solubility following treatment with ice-cold nonionic detergent, such as Triton X-100 (Ilangumaran et al. 1998, Scheiffele et al. 1997).

Exercise, as a stressor, will alter zinc metabolism. Increased secretion of hormones such as adrenaline, epinephrine, and cytokines stimulate metallothionein synthesis and increase the movement of zinc into the liver (Gleeson 2000, Shinogi et al. 1999). Since zinc uptake was shown to be sensitive to cellular cholesterol depletion (Mouat 2003), which disrupts the structure and function of lipid rafts/caveolae, then exercise may alter the protein composition of these plasma membrane structures to accommodate increased zinc transport. Previous work has shown that soluble soleus muscle proteins were down-regulated in rats consuming marginal zinc deficient diet (5 ppm zinc) compared with the control group consuming a 30 ppm zinc (Grider 2007).

The goal of this research is to test the hypothesis that dietary zinc and exercise will alter the composition of the liver lipid raft compartment in weanling rats. The results will further our understanding of the role these specialized structures in zinc metabolism and the stress response.

Materials and Methods

Animals and Diets

Male Sprague-Dawley rats (n=34; Charles River Laboratories, Wilmington, MA, USA) were housed in 18 X 20-cm stainless steel wire-bottom cages. The temperature of the environment was set at 22 ± 2^{o}C, and the light was controlled between 0700 to 1900 h for a 12-h light-dark cycle. The humidity was maintained between 40-60% in the room. The study was approved by the Institutional Animal Care and Use Committee at the United States Army Research Institute for Environmental Medicine. All the laboratory procedures that used the rats as the experiment followed the "Guide for Care and Use of Laboratory Animals" from the National Research Council. All laboratory rats weighed between 125 to 149g. All rats were fed a stock diet (LM-485, Harlan Teklad, Indianaplois, IN, USA) for the first 14 days and then separated (Grider 2007). Eight rats with the same weight were assigned for each group. Post hoc power analysis of a previous experiment has shown that five to seven rats for each dietary treatment group would be enough to show any significant changes (Chu et al. 2003). All rats were fed sprayed dried egg-whites with fixed 12 % protein content and different levels of Zn carbonate of 5 and 30 ppm. for 42 days. The LM-485 diet (test diet) contained 80g cellulose per kilogram of diet from Research Diets, Inc. (New Brunswick, NJ, USA) (Reeves et al. 1993). This test diet (Meals Ready to Eat) was a modified diet that contained a average 34% of fat and 38% of daily calories.

Animals and Exercise

Rats were arranged in a 3x2 complete factorial design, with half the colony (n=34) being assigned to cages equipped with 345-mm (diameter) running wheels (Mini Mitter Co., Bend, OR, USA). Exercised rats had unrestricted access to the running wheels. Each cage was fitted with a magnetic switch to allow for the counting of wheel revolutions using Vital View 3000 software (Mini Mitter Co.).

Sample Preparation

Tissues from rats in each dietary zinc group (5ppm zinc with exercise, 5 ppm zinc sedentary, 30 ppm zinc with exercise, 30 ppm zinc sedentary) were processed individually. Each liver sample was pulverized with liquid nitrogen. A total of 34 rat samples of dietary zinc (5 or 30 ppm) and exercise treatments (exercise or sedentary) were obtained. Because of the quality of gels, only data of 3 samples for 5ppm Zn/ exercise group, 4 samples for 30ppm Zn/exercise group, 3 samples for 5ppm Zn/ non-exercise group, 3 samples for 30ppm Zn/ non-exercise were collected. All of these rat samples were treated with the ReadyPrep (Signal) Protein Extraction Kit to extract and remove hydrophilic and soluble proteins, leaving those detergent insoluble fractions of signaling proteins out. From the stock of tissue powder 50 mg tissue powder was processed with 1-ml protein lysis buffer containing 10 µl protease inhibitor. The tissue was then sonicated on ice using three to four 30s pulses, with incubation for 1 min. in between each sonication. Afterwards, samples were centrifuged at 15,000Xg for 30 min at 4°C. The supernatant was stored at -80^{o}C, and pellet was extracted again because it contains the signal proteins. This supernatant was stored as the detergent resistant membrane protein fraction at -

80^{o}C. This fraction was measured by the RC/DC protein assay (Bio-Rad Laboratories) to estimate the protein content of the sample (Lowry et al.1951).

Electrophoresis and Sample Analysis

First dimension BAC-PAGE (benzyldimethyl-n-hexadecylammonium chloride (16-BAC)-PAGE) was performed on each of liver samples. Samples were separated towards the cathode in an acidic PAGE system by using the cationic detergent BAC. We usually use a 1.5-mm-thick slab gel measuring about 14 x 16 cm in a vertical slab gel. The composition and preparation of the stacking and running gels as well as the solubilizing and running buffers are given in Table 3. The running gel was prepared and poured at room temperature and overlaid with butanol. After polymerization, the gel is removed from the overlay and poured in the stacking gel solution, then inserted the comb.

After determining protein concentration of our samples, the protein samples (~200 µg in each 1.5-ml tube) were thawed on ice and acetone was addedto precipitate samples (100 µL of water and 400 µL of 100% acetone). The mixtures were left on ice for 20 minutes, and centrifuged at 5000 rpm at 4°C for 10 min. The supernatant was removed and discarded, and the pellet was dried in the air for 5 minutes. To each sample 35 µL of solubilizing buffer was added. The pellet was disrupted with a pipette tip and vortexed for 5 minutes. The samples were heated at 60°C for 5 minutes, then vortex again for 5 minutes. The samples were then centrifuged at 13,000 x g at room temperature for 5 minutes, and incubated at 60°C for 5 minutes again. After centrifuging the samples, they were loaded immediately into the wells of the 16-BAC PAGE gel. The electrodes were reversed at the power supply. Sixty mA of constant current was used for electrophoresis. Following electrophresis, the gel was fixed in 35% isopropanol, 10% acetic acid

for one hour. Afterwards, the gel was stained in Coomassie blue R-250 solution. The lanes were cut from the gel and incubated in 100 mM Tris pH 6.8 buffer for 30 minutes. The strips were then loaded horizontally on SDS-PAGE gels and run at 60 mAmps for 5 hours. Following SDS-PAGE, the gels were fixed in 40% methanol, 10% acetic acid for one hour and washed with water for three times (each time for 30 minutes). The gels were visualized by staining in colloidal Coomassie Blue G-250 (BioSafe stain, Bio-Rad).

The images of stained gels were digitized and the densities were determined by using Phoretix 2D software (Nonlinear Dynamics, New Castle upon Tyne, UK). The density of protein spots whose differences from the controls were determined to be a ≥1.5-fold difference in density were noted and saved for mass spectrometry.

Statistical Analysis

For protein spot intensity data, differences were assessed using general linear models (GLM) due to unbalanced dataset (Some group has 4 samples and some has 3 samples). The comparison between different treatments was analyzed by Student's *t* test. All data were shown as means ± S.E. Significant differences were considered as P less than 0.05.

Results

A total of 50 spots were detected in detergent resistant membranes from the rat samples. A representative 2D gel image of the control group is shown in Fig. 1. Two spots were found to exhibit differential staining intensities between the treatments. Various dietary group gels were magnified to show the variation in protein expression levels, are illustrated in Fig 2.

The level of changes and normalize volumes are listed in Table 4. The normalized volume and background intensity have been carefully considered and calculated for each gel spots. One spot (No.1) was up regulated by moderate zinc deficiency. Exercise reduced the expression of the second spot compared to the sedentary controls.

Discussion

This study was designed to test the effects of dietary zinc levels and exercise on protein expression in rat models. Also, I have hypothesized that the zinc deficiency and exercise will affect the protein composition of plasma membrane lipid rafts. I accepted my hypothesis from my study results. The two-dimensional expression gels indicated a total of 2 protein spots from rat samples. Spot 1 was significantly increased in rat consuming 5ppm zinc (zinc deficiency). Zinc transporters have been involved with protein signaling transduction within the plasma membrane. For example, zinc 1 and zinc 3, as zinc uptake transporter, found in lipid rafts (Wang et al. 2004). In addition, zinc uptake required cholesterol that located with lipid raft or caveolae. Another possibility can be increased the number of zinc transporter because the liver sensed the zinc deficiency and tried to increase the zinc uptake. By extracting out most of the detergent resistant membrane proteins, it helped us understand the relationship between lipid raft and dietary zinc. The result has shown that zinc deficiency can affect the detergent resistant protein expression in lipid raft.

Spot 2 was significantly reduced in exercised rats when compared to non-exercise treatment. It has been shown that exercise can alter zinc status. During the prolonged exercise, plasma or serum zinc levels have been significantly decreased and significant zinc depletion in muscle or other organs has been found (Ganapathy and Volpe 1999). The study also suggested

that exercise during a short period of time can increase blood zinc concentration immediately but decrease after within an hour (Ganapathy and Volpe 1999). These results have shown the exercise acts as a stressor which can increase muscle damage, and cause zinc depletion. Another study has suggested that the adequate zinc level can help the cell against oxidative stress and prevent cell damage (Liochev and Fridovich 2000). Therefore, exercise can possibly cause protein to become deactivated or move to other compartment in lipid rafts.

Among those detected sample spots, we will need the actual identification of these proteins by MALDI-MS. However, we do not have sufficient time to finish identify those protein spots and this protein identify process will be complete in the near future. In addition, we would use our soluble membrane protein fractions to test on this hypothesis in order to obtain more information. Also, there were several reasons that may contribute to the difference between the results. First, the sensitivity of Coomassie blue to the protein gel. Although the Coomassie blue is a great tool to detect protein in polyacrylamide gels, but we needed to neutralize the fix solution (40% methanol, 10% acetic acid) with filtrated water before staining any gels. Without this neutralization procedure, the sensitivity of Coomassie blue was decreased and affected the quality of protein visualization. However, there was no guarantee to remove all of fix solution for the gel. We encountered some visual variations during staining procedures. Second, the samples needed to be extracted and processed continuously soon after they were pulverized to minimize the chance of protein denaturation. Third, the travel distance on the 10% constant SDS-PAGE slab gel for these high molecular weight proteins is relatively longer which maximizes the separation distance of these proteins. There were few limitations in this experiment that required further research, such as variety of animal models and various organs. Currently, we only focused on the insoluble protein fraction of kidney in rat; therefore, we should expand our

research to other organs, such as a small intestine, brain, and muscle. Also, we can try to include the soluble protein fraction from kidney to be able to present a more completely experiment. By expanding our research, we can enhance our understanding the protein interaction between zinc, exercise, and lipid rafts.

Conclusion

This study used proteomic techniques to study the effect of zinc deficiency and exercise factors on the proteins in lipid rafts. The protein expression pattern of these gels suggests that zinc deprivation and exercise can regulate the protein expression. The identity of one protein spot with different zinc dietary levels increased significantly. Only one protein spot was detected to show that it was regulated the protein expression by exercise. Future experiments need to identify those differentiated proteins from this study and expand the sample variety by using different type of organs. The differences of protein expression results between normal/zinc deficiency and exercise/sedentary groups support the hypothesis that the zinc status and exercise can affect the protein composition of lipid rafts.

Tables

TABLE 3

Recipe for running 16-BAC PAGE gels

16-BAC PAGE (14 x 16 cm)

7.5% Mini-16-BAC Running Gel

3.6 g	Urea
5 ml	Acrylamide (Duracryl 30%/0.8%)
5 ml	300 mM potassium phosphate pH 2.1
5 ml	Ultra-pure water
1.0 ml	1.7% bisacrylamide
1.0 ml	80 mM ascorbic acid
32 µL	5 mM ferrous sulfate
200 µL	250 mM 16-BAC
800 µL	1:1200 hydrogen peroxide

4% Stacking Gel

1.0g	Urea
1.33 ml	Acrylamide (Duracryl 30%/0.8%)
2.5 mL	500 mM potassium phosphate pH 4.1
3.0 ml	Ultra-pure water
1.38 ml	1.7% bisacrylamide
500 µL	80 mM ascorbic acid
8.5 µL	5 mM ferrous sulfate
70 µL	250 mM 16-BAC
500 µL	1:750 hydrogen peroxide

Solublizing Buffer

2.25 g	Urea
500 mg	16-BAC
500 µL	Glycerol
250 µL	1.5 M Dithiothreitol

Electrode Buffer

150 mM	Glycine
2.5 mM	16-BAC
50 mM	Phosphoric Acid

TABLE 4

Staining intensity of protein spots with 50% variation from the control group in rats.

RAT	**30ppm (control)**	**5ppm**
Spot 1*	25.8 ± 1.9	71.9 ± 16.9

RAT	**Sedentary (control)**	**Exercise**
Spot 2*	278.0 ± 59.0	134.6 ± 0

* Means ± Standard error

Figures

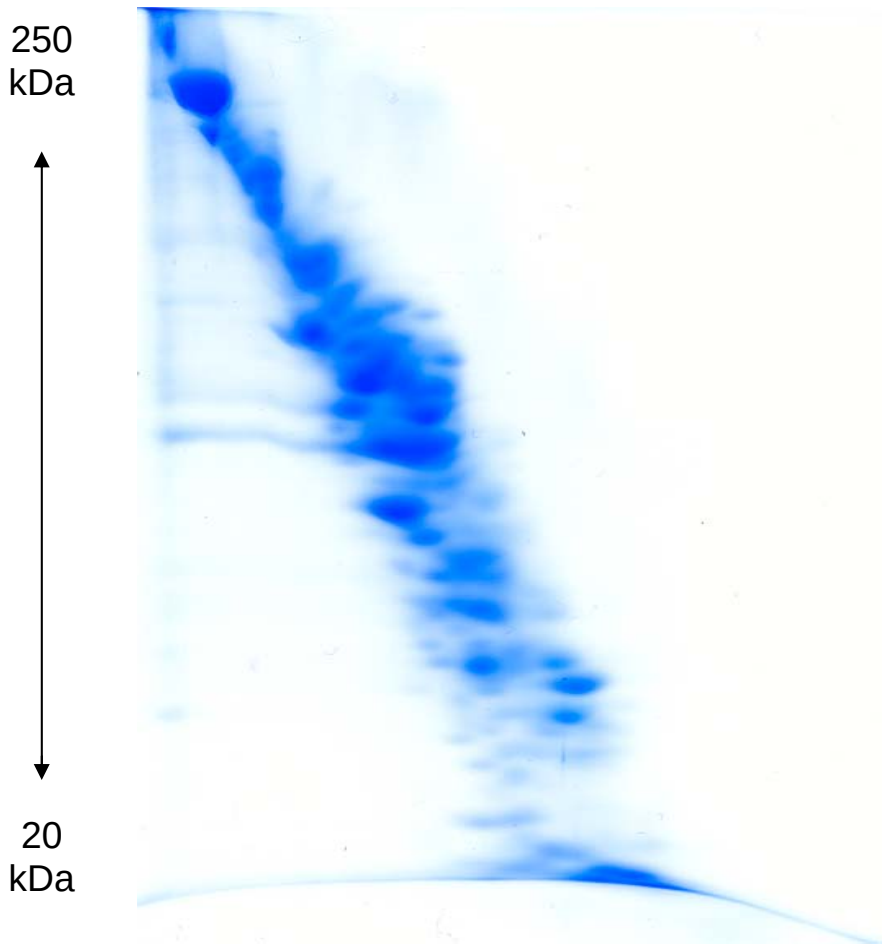
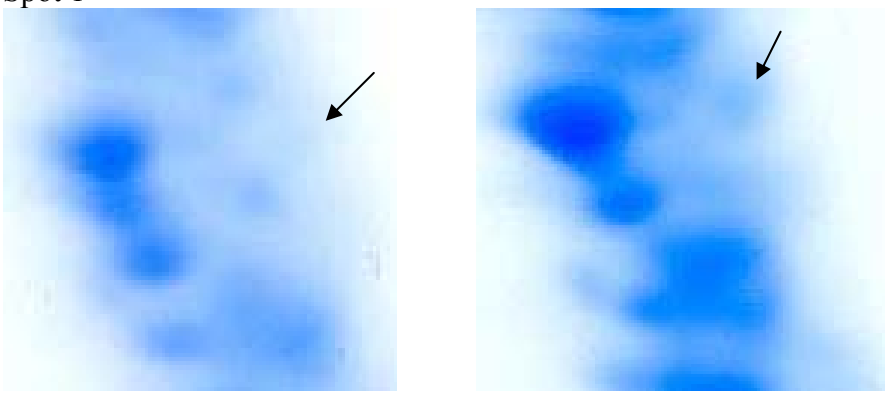


FIGURE 1 Enlarged images of regions of 2D gels showed as an example for each dietary and exercises treatment groups. Protein spots in the control, zinc deficiency or exercise gels with staining intensity greater or less than 50% of normalized values. The levels of change of differential spots are listed in Figure 2.

Control (30ppm) **Zinc Deficiency (5ppm)**

Spot 1



Sedentary (Control) **Exercise**

Spot 2

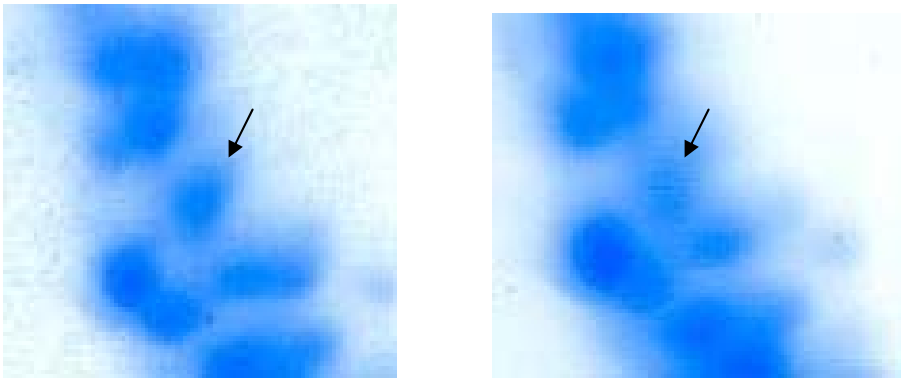


FIGURE 2 Presents images at the lower molecular weight region for spot 1 and the higher molecular weight region for spot 2 in the gel. The gel analysis program indicated few changes in spot intensity in this region.

CHAPTER 4

ANALYSIS OF INSOLUBLE PROTEIN EXPRESSON IN KIDNEYS FROM BULL TERRIERS WITH LETHAL ACRODERMATITIS DISORDER

Introduction

Lethal acrodermatitis (LAD) is an inherited genetic disease, which was first described in 1986 by Jezyk. The etiology of the disease is still unknown, and it has been suggested to relate to the zinc deficiency (Grider et. al. 2007). Uchida and others (1997) found a significant zinc deprivation has occurred in LAD dogs. Also, the human zinc deficiency disease – acrodermatitis enteropathica (AE) and cattle zinc deficiency disease – lethal trait A46 in cattle have similar clinical conditions. Both of the diseases are caused by mutations in SLC39A4. The symptoms of LAD have included growth retardation, acrodermatitis, hypopigmentation of hair coat, and hyperkeratosis (McEwan et. al. 2000). Another study suggested that zinc deficiency is related to increases in keratinolytic activity (Hsu et. al. 1991), which indicated the down regulation of keratin 10 gene in LAD liver can be related to zinc deficiency (Grider et. al. 2007). The purpose of this study was to identify the differences of insoluble protein expression from bull terriers with lethal acrodermatitis disease. Another purpose of this study was to expand our understanding of the disease in order to develop diagnostic criteria to identify affected dogs.

Materials and Methods

Animals and Diets

Bull Terriers' kidney samples were obtained from Medical Genetics at the Veterinary School of the University of Pennsylvania. There were a total of three puppies, one normal and two affected, aged about 8 to 12 weeks. At birth, the affected puppies were described by their

owners as having less hair and smaller size as compared to the normal puppy. At the age of 8 to 10 weeks, these affected puppies had lost more hair and hadbehavior changes (more aggressive). At the age of 14 weeks, all three puppies were euthanized. The affected puppies were found to have skin lesions, thymic hypoplasia, bilateral cataracts and mild hydrocephalus conditions. However, there was no inflammation in the kidney and the kidney was dissected from all three dogs and frozen with liquid nitrogen at -80°C (Grider et al. 2007).

Sample Preparation

One normal puppy and two affected puppies' kidney samples were processed separately. Each kidney sample was pulverized with liquid nitrogen. Approximately 5 g of kidney tissue was pulverized and stored at -80^{o}C until use. All of these 3 dog samples were treated with the ReadyPrep (Signal) Protein Extraction Kit to extract and remove hydrophilic and soluble proteins, leaving those detergent insoluble fractions of signaling proteins out. From the stock of tissue powder 50 mg tissue powder was processed with 1-ml protein lyses buffer containing 10 µl protease inhibitor per gram tissue, and then was sonicated on ice using three to four 30s pulses. Afterwards, samples were centrifuged at 15,000Xg for 30 min at 4°C. The supernatant was stored at -80^{o}C, and pellet was extracted again because it contains the signal proteins. This supernatant was stored as the detergent resistant membrane protein fraction at -80^{o}C. This fraction was measured by the RC/DC protein assay (Bio-Rad Laboratories) to estimate the protein content of the sample (Lowry et al. 1951).

Electrophoresis and sample analysis

First dimension BAC-PAGE (benzyldimethyl-n-hexadecylammonium chloride (16-BAC)-PAGE) was performed on each of kidney samples. Samples were separated towards the cathode in an acidic PAGE system by using the cationic detergent BAC. We usually use a 1.5-mm-thick slab gel measuring about 14 x 16 cm in a vertical slab gel. The composition and preparation of the stacking and running gels as well as the solubilizing and running buffers are given in Table 3. The running gel was prepared and poured at room temperature and overlaid with butanol. After polymerized, the gel is removed from the overlay and poured in the stacking gel solution, then inserted the comb.

After determining protein concentration of our samples, the protein samples (~200 µg in each 1.5-ml tube) were thawed on ice and acetone was added to precipitate samples (100 µL of water and 400 µL of 100% acetone). The mixtures were left on ice for 20 minutes, and centrifuged at 5000 rpm at 4°C for 10 min. The supernatant was removed and discarded, and the pellet was dried in the air for 5 minutes. To each sample 35 µL of solubilizing buffer was added. The pellet was disrupted with a pipette tip and vortexed for 5 minutes. The samples were heated at 60°C for 5 minutes, then vortex again for 5 minutes. The samples were then centrifuged at 13,000 x g at room temperature for 5 minutes, and incubated at 60°C for 5 minutes again. After centrifuging the samples, they were loaded immediately into the wells of the 16-BAC PAGE gel. The electrodes were reversed at the power supply. Sixty mA of constant current was used for electrophoresis. Following electrophresis, the gel was fixed in 35% isopropanol, 10% acetic acid for one hour. Afterwards, the gel was stained in Coomassie blue R-250 solution. The lanes were cut from the gel and incubated in 100 mM Tris pH 6.8 buffer for 30 minutes. The strips were then loaded horizontally on SDS-PAGE gels and run at 60 mAmps for 5 hours. Following SDS-

PAGE, the gels were fixed in 40% methanol, 10% acetic acid for one hour and washed with water for three times (each time for 30 minutes). The gels were visualized by staining in colloidal Coomassie Blue G-250 (BioSafe stain, Bio-Rad).

The images of stained gels were digitized and the densities were determined by using Phoretix 2D software (Nonlinear Dynamics, New Castle upon Tyne, UK). The density of protein spots exhibiting ≥1.5-fold difference in expression were picked to show as the result.

Statistical analysis

For protein spot intensity data, differences were assessed using general linear models (GLM) due to an unbalanced dataset. The comparison between different treatments was analyzed by Student's *t* test. All data were shown as means ± S.E. Significant differences were considered as P less than 0.05.

Results

Normalization has been applied to every gel. Only one spot that expressed significantly differences between the normal and LAD dogs (illustrated in Fig 3). The intensity of the spot in the LAD phenotype gel was down regulated when compared to the normal dog gel. The spot was about 50% down regulated and showed in Table 5.

Discussion

Also, I have hypothesized that lethal acrodermatitis will affect the protein composition of plasma membrane lipid rafts. I accepted my hypothesis from my study results. This study was designed to identify the effect of LAD on differential protein expression. The genotype of this

disorder has still not found, but its phenotype has very similar to zinc deficiency clinical symptoms. Both of Acrodermatitis Enteropathica (AE) and lethal trait A46 in cattle can be treated by supplemental zinc, but is not for the lethal acrodermatitis. Both of AE and lethal trait were related to Zip4 transporter mutation in the small intestine (Machen et al. 1996). Therefore, it suggested that LAD did not involve with Zip4 transporter mutation in the small intestine. Jezyk's (1986) study has injected as high as 880mg of zinc sulfated to those affected dogs, but they don't see a significant improvement for their symptoms. Few investigators have applied biochemical techniques to the study of this mutation. For example, a study has been done with the liver soluble proteins from bull terriers with lethal acrodermatitis. The results have shown that a total of 13 proteins were expressed in the affected dogs (Grider et al. 2007). The results here are consistent in showing that the LAD mutation affects protein expression in affected dogs. The protein spot has been down regulated for the LAD phenotype sample, although the identity of this protein is not known. We will need the actual identification of these proteins by MALDI-MS. The down regulation exhibited with the LAD kidney lipid raft protein may be directly related to the mutation or to secondary to stress or infection in the affected dogs. Obviously, a significant limitation to our interpretation of these results is the small number of animals in the study. Nevertheless, these results indicate that biochemical differences between normal and affected dogs are observable. Future studies will look at the proteomic differences within the soluble fraction of the kidney, which are currently stored at -80^{o}C.

Conclusion

This study used proteomic techniques to study how the differentially expressed proteins that were identified can aid the early diagnostic criteria of lethal acrodermatitis dogs. The protein

expression patterns of these gels suggest that LAD can regulate the protein expression. The identity of the protein spot for LAD samples was significantly differentiated. Indentifying the protein spot would be beneficial in order to understand more about the relationship between protein and LAD. The study results show that LAD can affect the detergent resistant membrane proteins, which suggested there is a possible relationship between lipid rafts and lethal acrodermatitis.

Tables

TABLE 5

Receipt for running 16-BAC PAGE gels

16-BAC PAGE (14 x 16 cm)

7.5% Mini-16-BAC Running Gel

3.6 g	Urea
5 ml	Acrylamide (Duracryl 30%/0.8%)
5 ml	300 mM potassium phosphate pH 2.1
5 ml	Ultra-pure water
1.0 ml	1.7% bisacrylamide
1.0 ml	80 mM ascorbic acid
32 µL	5 mM ferrous sulfate
200 µL	250 mM 16-BAC
800 µL	1:1200 hydrogen peroxide

4% Stacking Gel

1.0g	Urea
1.33 ml	Acrylamide (Duracryl 30%/0.8%)
2.5 mL	500 mM potassium phosphate pH 4.1
3.0 ml	Ultra-pure water
1.38 ml	1.7% bisacrylamide
500 µL	80 mM ascorbic acid
8.5 µL	5 mM ferrous sulfate
70 µL	250 mM 16-BAC
500 µL	1:750 hydrogen peroxide

Solublizing Buffer

2.25 g	Urea
500 mg	16-BAC
500 µL	Glycerol
250 µL	1.5 M Dithiothreitol

Electrode Buffer

150 mM	Glycine
2.5 mM	16-BAC
50 mM	Phosphoric Acid

TABLE 6

Staining intensity of protein spots with 50% variation from the control group in bull terriers.

Bull Terrier	**Normal**	**LAD**
Mean *	204.4 ± 16.3	126.0 ± 20.7

* Means ± Standard error

Figures

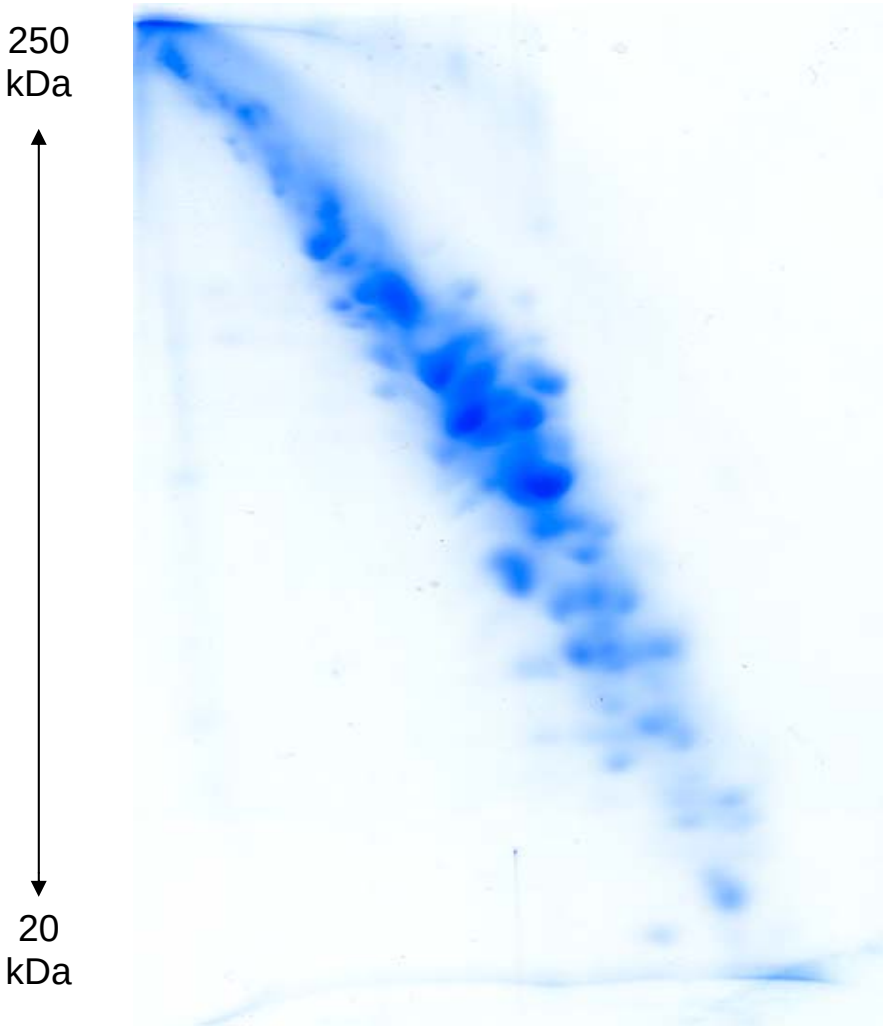
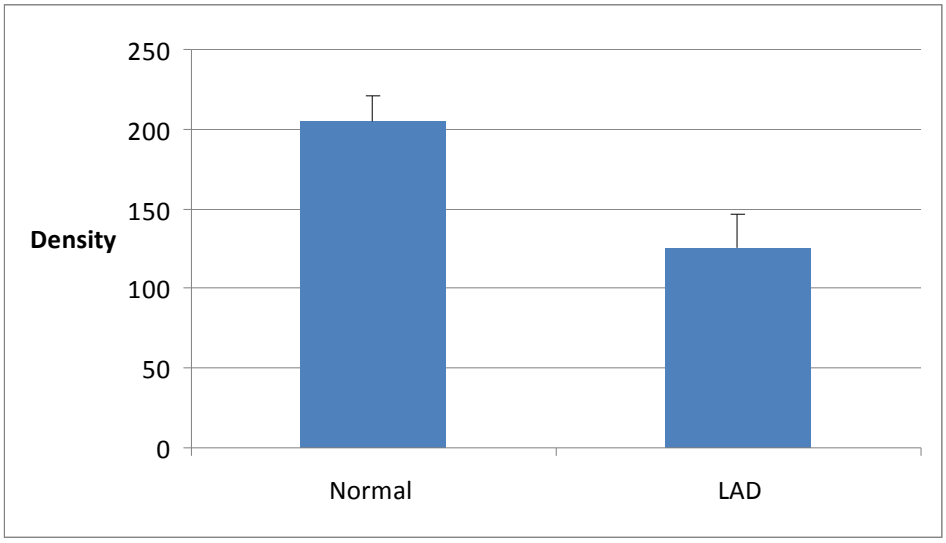


FIGURE 3 Enlarged images of regions of 2D gels showed as an example for one normal and two affected dogs. Protein spots in the normal and LAD dog gels with staining intensity greater or less than 50% of normalized values. The levels of change of differential spots were listed in Figure 4.

FIGURE 4 Presents images at the lower molecular weight region for spot 1 and the higher molecular weight region for spot 2 in the gel. The gel analysis program indicated few changes in spot intensity in this region.



* Means ± Standard error

FIGURE 5 The level of density for the spot in the normal dog (204.4 ± 16.3)* and the average of the two LAD dogs (126.0 ± 20.7)*.

CHAPTER 5

SUMMARY

The hypothesis for this study was to test the lethal acrodermatitis mutation, zinc deficiency, and exercise affect the protein composition of the lipid rafts. We used a total of 13 rat liver samples and 3 dogs liver samples as the subjects. Also, we measured the effect of the protein expression with four different groups: 5ppm exercise, 5ppm sedentary, 30ppm exercise, and 30ppm sedentary groups. We found that one protein spot has up regulated from the zinc deficiency (5ppm) group when compared with the control group (30ppm). In addition, there was a significant difference between sedentary and exercise group: one protein spot has down regulated in exercise group. These results have shown that zinc deficiency and exercise can alter the protein composition of the lipid rafts.

For the lethal acrodermatitis (LAD) study, the results have shown that LAD has down regulated a protein expression. Consequently, the down regulation of this protein suggests that LAD and lipid rafts can be related. One of the limitations is that we do not have enough sample size for this LAD dog samples; therefore, we will try to include more samples, finish analyzing the soluble fraction of samples in the future. Plus, we will need to include other parts of tissues or organs for both rats and bull terriers studies in order to enhance our understanding of the etiology of LAD and relationship between zinc deficiency/exercise with lipid rafts.

REFERENCES

Anderson, R. A. et al. (1984) Strenuous Running: acute effects on chromium, copper, zinc, and selected clinical variables in urine and serum of male runners. *Biological Trace Element Research,* 6: 327–336.

Andrews, G.K. (2001). Cellular Zinc Sensors: MTF-1 regulation of gene expression. *Biometals*, 14: 223-237.

Arnold, M. and Barbul, A. (2006). Nutrition and Wound Healing. *Journal of the American Society of Plastic Surgeons*, 117: 42S-58S.

Auld, D.S. et al. (1976) Yeast RNA Polymerase I: a eukaryotic zinc metalloenzyme. *Biochemical and Biophysical Research Communications*, 69: 548-554.

Berg, M. J. & Shi, Y. (1996).The Galvanization of Biology: a growing appreciation for the role of zinc. *Science*, 271:1081-1085.

Beyersmann, D. and Haase, H. (2001). Function of Zinc in Signaling, Proliferation and Differentiation of Mammalian Cells. *Biometals*, 14: 331-341.

Blomster, M. et al. (1995). Evidence for a Catalytic Role of Tyrosine 383 in the Peptidse Reaction of Leukotriene A_4 Hydrolase. *European Journal of Biochemistry*, 231: 528-534.

Brown, D. A. (2006). Lipid Rafts, Detergent-Resistant Membranes, and Raft Targeting Signals. *Physiology*, 21: 430-439.

Chamberlain, L.H. and Gould, G.W. (2002). The Vesicle- and Target-SNARE Proteins That Mediate Glut4 Vesicle Fusion Are Localized in Detergent-insoluble Lipid Rafts Present on Distinct Intracellular Membranes. *The Journal of Biological Chemistry*, 277: 49750-19754.

Chang, W.J. et al. (1992). Lowering the Cholesterol Content of MA104 Cells Inhibits Receptor-Mediated Transport of Folate. *The Journal of Cell Biology*, 118: 63-69.

Chu, Y. et al. (2003). Water Maze Performance and Changes in Serum Corticosterone Levels in Zinc-Deprived and Pair-Fed Rats. *Physiol Behav*, 78: 569-578.

Cousins, R. J. (1998). A Role of Zinc in the Regulation of Gene Expression. *Proceedings of the. Nutrition Society*, 57: 307-311.

Cousins, R. et al. (2003). Regulation of Zinc Metabolism and Genomic Outcomes. *The Journal of Nutrition*, 133: 1521S-1526S.

Cousins, R., and Lee-Ambrose, L.M. (1992). Nuclear Zinc Uptake and Interactions and Metallothionein Gene Expression are Influenced by Dietary Zinc in Rats. *The Journal of Nutrition*., 122: 56-64.

Couzy, F. et al. (1990). Zinc Metabolism in the Athlete: influence of training nutrition and other factors. *The International Journal of Sports Medicine*, 11: 263-266.

Danbolt, N. (1979). Acrodermatitis Enteropathica. *British Journal of Dermatology***,** 100: 37-40.

Danbolt, N. and Closs, K. (1942). Acrodermatitis Enteropathica. *Acta Dermatol Venereol,* 23: 127-69.

Davis, S.R., and Cousins, R.J. (2000). Metallothionein Expression in Animal: a physiological perspective on function. *The Journal of Nutrition*., 130: 1085-1088.

Dorup, I., et al. (1991). Role of Insulin-Like Growth Factor-1 and Growth Hormone in Growth Inhibition Induced by Magnesium and Zinc Deficiency. *British Journal Nutrition*, 66: 505-521.

Evans, P.C. et al. (2004). Zinc-Finger Protein A20, a Regulator of Inflammation and Cell Survival, has De-ubiquitinating Activity. *Biochemistry Journal*, 378: 727-734.

Field, F.J. et al. (1998). Caveolin is Present in Intestinal Cells: role in cholesterol trafficking? *The Journal of Lipid Research*, 39: 1938-1950.

Fons, C. et al. (1992). Serum Zinc and Somatic Growth in Children with Growth Retardation. *Biological Trace Element*, 32: 399-404.

Fosmire, J.G. (1990). Zinc Toxicity. *American Journal of Clinical Nutrition*, 51: 225-7.

Fu, F.-N. et al. (1998). Role of Zinc in Structure and Toxic Activity of Botulinum Neurotoxin. *Biochemistry*, 37: 5267-5278.

Fujimoto, T. et al. (1995). Inositol 1,4,5-Trisphosphate Receptor-Like Protein in Plasmalemma Caveolae is Linked to Actin Filaments. *Journal of Cell Science*, 108: 7-15.

Gaither, L. A. and Eide, D.J. (2000). Functional Expression of the Human hZIP2 Zinc Transporter. *Journal of Biological Chemistry*, 275: 5560–5564.

Ganapathy, S., and Volpe, S. (1999). Zinc, Exercise, and Thyroid Hormone Function. *Critical Reviews in Food Science and Nutrition*, 39: 369-39.

Glait, C. et al. (2006). Caveolin-1 Up-Regulates IGF-I Receptor Gene Transcription in Breast Cencer Cells via Sp-1 and P53-Dependent Pathways. *Experimental Cell research*, 312: 3899-3908.

Gleeson, M. (2000). Interleukins and Exercise. Journal of Physiology, 529: 1.

Gustavsson, J. et al. (1999). Localization of the Insulin Receptor in Caveolae of Adipocyte Plasma Membrane. *The FASEB Journal*, 13: 1961-1971.

Grider, A. et al. (2007). Analysis of the Liver Soluble Proteome from Bull Terriers Affected with Inherited Lethal Acrodermatitis. *Molecular Genetics and Metabloism*, 92: 249-257.

Haller, P.D. et al. (2001). Mass Spectrometric Characterization of Prtoeins Extracted from Jurkat T Cell Detergent-Resistant Membrane Domains. *Proteomics*, 1: 1010-1021.

Hanzal-Bayer, M.F. and Hancock, J.F. (2007). Lipid Rafts and Membrane Traffic. *FEBS Letters*, 581: 2098-2104.

Hershfinkel, M., Silverman, F.W.,and Sekler, I. (2007). "The Zinc Sensing Receptor, a Link Between Zinc and Cell Signaling". *Mol Med* 13 (7-8): 331-336.

Ho, E. et al. (2003). Zinc Deficiency Induces Oxidative DNA Damage and Increases P53 Expression in Human Lung Fibroblasts. *The Journal of Nutrition*, 133: 2543-2548.

Hsu, D.J. et al. (1991). Effect of Zinc-Deficiency on Keratins in Buccal Epithelium of Rats. *Archives of Oral Biology*, 36: 759-763.

Huang, C.C. et al. (1997). Evidence for a Catalytic Role of Zinc in Protein Farnesyltransferase. *The Journal of Biological Chemistry*, 272: 20-23.

Hug, H., and Sarret, T.F. (1993). Protein Kinase C Isoenzymes: divergence in signal transduction?

Biochemistry Journal, 291: 329-343.

Ilangumaran, S. et al. (1998). Effects of Cholesterol Depletion by Cyclodextrin on the Sphingolipid Microdomaiins of the Plasma Membrane. *Biochemisty Journal*, 335: 433-440.

Janes, P.W. et al. (2000). The Role of Lipid Rafts in T Cell Antigen Receptor (TCR) Signalling. *Immunology*, 12: 23-34.

Jezyk, P.F. et al. (1986). Lethal Acrodermatitis in Bull Terriers, Journal of American Veterinary. Medical Association, 188: 833-839.

Jing, M.Y. et al. (2007). Insights on Zinc Regulation of Food Intake and Macronutrient Selection.

Biological Trace Element Research, 115: 187-194.

Kabouridis, P.S. et al. (2000). Cholesterol Depletion Disrupts Lipid Rafts and Modulates the Activity of Multiple Signaling Pathway in T Lymphocytes. *Eur. J. Immunol*., 30: 954-963.

Kavas, G.O. et al. (2002). Zinc Treatment Affects Superoxide Dismutase Acitvity in Growth Retardation. *Biological Trace Element Research*, 90:39-46.

King, C.J. et al. (2000). Zinc Homeostasis in Humans. *The Journal of Nutrition,*.130: 1360S-1366S.

Lademann, U. et al. (2001). A20 Zinc Finger Protein Inhibits TNF-induced Apoptosis and Stress Response Early in the Signaling Cascades and Independently of Binding to TRAF2 or 14-3-3 Proteins. *Cell Death and Differentiation*, 8: 265-272.

Lee, Y. et al. (1998). The Cardiac Tissue-Restricted Homeobox Protein Csx/Nkx2.5 Physically Associates with the Zinc Finger Protein GATA4 and Cooperatively Activates Atrial Natriuretic Factor Gene Expression. *Molecular and Cellular Biology*, 18: 3120-3129.

Liochev, S.I, and Fridovich, I. (2001). The Oxidation of 3-Hydroxyanthranilic Acid by Cu,Zn Superoxide Dismutase: Mechanism and Possible Consequences. *Archives of Biochemistry and Biophysics*, 388: 281-284.

Lowry, O.H. et al. (1951). Protein Measurement with the Folin Phenol Reagent. The Journal of Biological Chemistry, 193: 265-275.

Lucero, A.H. and Robbins, W.P. (2004). Lipid Rafts – protein association and the regulation of protein activity. *Archives of Biochemistry and Biophysics*, 426: 208-224.

Machen, M. et al. (1996). Bovine Hereditary Zinc Deficiency: lethal trait A 46, The *Journal of*

Veterinary Diagnostic Investigation, 8: 219–227.

Marmar, J.L. et al. (1975). Semen Zinc Levels in Infertile and Postvasectomy Patients and Patients with Prostatitis. *Fertility* and *Sterility*, 26:1057-63.

Maverakis, E. (2007). Acrodermatitis Enteropathica and an Overview of Zinc Metabolism. *Journal of the American of Dermatology*, 56: 116-24.

Mayor, S. et al. (1998). Cholesterol-Dependent Retention of GPI-Anchored Protein in Endosomes. *The EMBO Journal*, 17: 4626-4638.

McClung, J.P. et al. (2006). Effect of Dietary Phytase on Body Weight Gain, Body Composition and Bone Strength in Growing Rats Fed a Low-Zinc Diet. *Journal of Nutritional Biochemistry*, 17; 190-196.

McEwan, N.A. et al. (2000). Diagnostic Features, Confirmation and Disease Progression in 28 Cases of Lethal Acrodermatitis of Bull Terriers. *Journal of Small Animal Practice*, 41: 501-507.

Morita, E.H. et al. (1996). Implications of the Zinc-Finger Motif Found in the DNA-Binding Domain of the Human XPA Protein. *Genes to Cells*, 1: 437-442.

Mouat, M.F. et al. (2003). Zinc Uptake into MCF-10A Cells is Inhibited by Cholesterol Depletion. *Journal of Nutritional Biochemistry*, 14: 74-80.

Murphy, B.J. et al. (1999). Activiation of Metallothionein Gene Expression by Hypoxia Involves Metal Response Elements and Metal Transcription Factor-1. *Cancer Research*, 59: 1315-1322.

Nika, K. et al. (2006). Lipid Raft Targeting of Hematopoietic Protein Tyrosine Phosphatase by Protein Kinase C – Mediated Phosphorylation. *Molecular and Cellular Biology*, 26:

1806-1816.

Nokano, A. et al. (2003). Novel SLC39A4 Mutations in Acrodermatitis Enteropathica. *Journal of Investigative Dermatology*, 120: 963-966.

Ohno, H. et al. (1985). Exericse Induced Changes in Blood Zinc and Related Proteins. *Journal of Applied Physiol*ogy, 58: 1453-1458.

Oteiza, P.I. et al. (1995). Zinc Deficiency Causes Oxidative Damage Proteins, Lipids and DNA in Rat Testes. *The Journal of Nutrition*, 125: 823-829.

Palmiter, R.D. (1994). Regulation of Metallothionein Genes by Heavy Metals Appears to be Mediated by a Zinc-Sensitive Inhibitor that Interacts with a Consitiutively active Transcription factor, MTF-1. *Proceeding of the National Academy of Science*, 91: 1219-1223.

Parton, R.G. (1996). Caveolae and Caveolins. *Current Opinion in Cell Biology*, 8: 542–548.

Pavletich, N.P. and Pabo, C.O. (1991). Zinc Finger-DNA Recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. *Science*, 252: 809-817.

Parker, P. et al. (1986). The Complete Primary Structure of Protein Kinase C – the major phorbol ester receptor. *Science*, 233: 853–859.

Parton, R. G. (1996). Caveolae and Caveolins. *Current Opinion in Cell Biology*, 8: 542-548.

Prasad, A.S. (1991). Discovery of Human Zinc Deficiency and Studies in an Experimental Human Model. *American Journal of Clinical Nutrition*, 53: 403–12.

Prasad, A.S. (1995). Zinc: an overview. *Nutrition*, 11: 93-99.

Rink, L., and Gabriel, P. (2000). Zinc and the Immune System. *Proceedings of the Nutrition Society*, 59: 541–552.

Rothberg, K.G.. et al. (1990). Cholesterol Controls the Clustering of the Glycophospholipid-Anchored Membrane Receptor for 5-Methyl-Tetrahydrofolate. *Journal of Cell Biology*, 111: 2931-2938.

Raulin, J. (1869). Etudes Cliniques Sur La Vegetation. *Annual Science Nature Botanical Biolological Vegetable*, 11: 93.

Reeves, P.G. et al. (1993). AIN-93 Purified Diets for Laboratory Rodents — final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. *Journal of Nutrition*, 123: 1939-1951.

Rink, L. and Gabriel, P. (2000). Zinc and the Immune System. *Proceedings of the Nutrition Society*, 59: 541-52.

Sandstrom, B. (1997). Bioavailability of Zinc. *European Journal of Clinical Nutrition,* 51: 17-19.

Sato, M., and Bremner, I. (1993). Oxygen free radicals and Metallothionein. *Free Radical Biology & Medicine*, 14: 325-337.

Scheiffele, P. et al. (1997). Interaction of Influenza Virus Haemagglutinin with sphingolipid-cholesterol Membrane Domains via Its Transmembrane Domain. EMBO Journal, 16: 5501-8.

Schuck, S. et al. (2003). Resistance of Cell Membranes to Different Detergents. *Proceeding of the National Academy of Science*, 100: 5795-5800.

Sedwick, C. E. and Altman, A. (2002). Ordered Just So: lipid rafts and lymphocyte function. *Science STKE,* RE2.

Shankar, H. A., and Prasad, S. A. (1998). Zinc and Immune Function: the biological basis of altered resistance to infection. *American Journal of Clinical Nutrition*, 68: 447S–63S.

Shinogi, M. et al. (1999). Metallothionein Induction in Rat Liver by Deitary Restriction or Exercise and Reduction of Exercise-Induced Hepatic Lipid Peroxidation. *Biological & Pharmaceutical Bulletin*, 22: 132-136.

Shogomori, H., and Brown, D.A. (2003). Use of Detergents to Study Membrane Rafts: the good, the bad, and the ugly. *The Journal of Biological Chemistry*, 384: 1259-1263.

Shu, N. et al. (2008). Prediction of Zinc-Binging Sites in Proteins from Sequence. *Bioinformatics*, 24: 775-782.

Simons, K. and Toomre, D. (2000). Lipid Rafts and Signal Transduction. *Nature Reviews Molecular Cell Biology*, 1: 31-39.

Simons, K. and Ikonen, E. (2000). How Cell Handle Cholesterol. *Science*, 290: 1721-1726.

Simons, K and Vaz, L.C. W. (2004). Model Systems, Lipid Rafts, and Cell Membranes. *Annual Review of Biophysics and Biomoecular. Structure,* 33:269-295.

Solomons, N.W. (1998). Mild Human Zinc Deficiency Procedures an Imbalance between Cell-Mediated and Humoral Immunity. *Natural Review*, 56: 27-28.

Terhune, M.W. and Sandstead, H.H. (1972). Decrease RNA Polymerase Activity in Mammalian Zinc Deficiency. *Science*, 177: 68-69.

Truong-Tran, A.Q. et al. (2000). Cellular Zinc Fluxes and the Regulation of Apoptosis/Gene-Directed Cell Death. The Journal of Nutrition, 130: 1459S-2466S.

Uchida, Y. et al. (1997). Serum Concentrations of Zinc and Copper in Bull Terriers with Lethalacrodermatitis and Tail-Chasing Behavior. *American Journal of Veterinary*

Research, 58: 808–810.

Wang, F. et al. (2004). Acrodermatitis Enteropathica Mutations Affect Transport Activity, Localization, and Zinc-Responsive Trafficking of the Mouse ZIP4 Zinc Transporter. *Human Molecular Genetics*, 13: 563-571.

Wang, F. et al. (2004). Zinc-Stimulated Endocytosis Controls Activity of the Mouse ZIP1 and ZIP 3 Zinc Uptake Transporters. *The Journal of Biological Chemistry*, 279: 24631-24639.

Westermann, M. et al. (1999). Membrane Structure of Caveolae and Isolated Caveolin-Rich Vesicles. *Histochemistry and Cell Biology*, 111: 71-81.

Yuzbasiyan-Gurkan, V., and Bartlett, E. (2006). Identification of a Unique Solice Site Variant in SLC39A4 in Bovine Hereditary Zinc Deficiency, Lethal Trait A 46: an animal model of acrodermatitis enteropathica. *Genomics*, 88: 521-526.

Ying, Y.S. et al. (1992). Each Caveola Contains Multiple Glycosyl-Phosphatidylinositol-Anchored Membrane Proteins. *Cold Spring Harbor Symposia on Quantitative Biology*, 57: 593-604.

Yu, H.H. et al. (2007). Zinc Deficiency with Acrodermatitis Enteropathica-Like Eruption After Pancreaticoduodenectomy. *Journal of the Formosan Medical Association*, 106: 864-868.

Zago, M.P., and Oteiza, P.I. (2001). The Antioxidant Properties of Zinc: interactions with iron and antioxidants. *Free Radical Biology & Medicine*, 31: 266-274.

Zaichick, V.Y. et al. (1997). Zinc in the Human Prostate Gland: Normal, Hyperplastic and Cancerous. *International Urology and Nephrology*, 29: 565-574.

Zalewaki, P.D. et al. (1994). Video Image Analysis of Labile Zinc in Viable Pancreatic Islet Cells Using a Soecific Fluorescent Probe for Zinc. *The Journal of Histochemistry and Cytochemistry*, 42: 877-884.