

Zinc Deficiency Contributes to Chronic Inflammation in Obesity

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for Graduation “with Honors Research Distinction” in the Undergraduate Colleges of The Ohio State University

by
Dara Burris

The Ohio State University
June 2011

Project Advisor: Professor Daren Knoell, College of Pharmacy

Introduction

Obesity is recognized as a world-wide public health problem that has approached epidemic proportions in the United States. Obesity increases the risk of developing insulin resistance, diabetes, cardiovascular disease, stroke, lung disease, and cancer. The exact cause for increased morbidity resulting from obesity is unclear. Recent evidence has revealed that obesity is associated with a state of low-grade systemic inflammation that further promotes the risk of developing cardiovascular and pulmonary diseases, such as atherosclerosis and asthma [1-3]. In obese subjects, adipose tissue secretes specific adipokines leading to infiltration by macrophages that further promote the production of inflammatory mediators in concert with adipocytes [4]. The amount of visceral adipose tissue in obese subjects directly correlates with elevated circulating cytokine levels including interleukin (IL-6), tumor necrosis factor- α (TNF- α), and C-reactive protein (CRP) [5, 6]. Leptin, an adipokine exclusively manufactured by adipocytes, was first identified as a satiety factor that induces appetite suppression [7]. Most recently, leptin has been identified as a pro-inflammatory adipokine whose up-regulation in the setting of obesity leads to the development of a chronic inflammatory state that contributes to inflammatory-mediated chronic disease [8].

Consequent to abnormal metabolism, micronutrient deficiencies are common in obese individuals across all age groups [9]. In particular, obese subjects have an increased incidence of developing zinc deficiency [10-13]. Interestingly, weight loss has been shown to significantly increase circulating zinc levels while decreasing the extent of obese-driven morbidity [14]. With regard to obesity-derived inflammation, obese subjects that have lower dietary zinc intake exhibit more systemic inflammation in parallel with altered lipid profiles when compared to similar subjects with normal zinc intake [15]. Further, in a recent randomized, placebo-

controlled, cross-over study conducted in 60 obese children, zinc supplementation significantly attenuated plasma levels of glucose and insulin [16].

Zinc deficiency perturbs innate immune function and enhances inflammation. Among children less than 5 years old, zinc deficiency is one of the leading causes of newly acquired respiratory and gastrointestinal infections [17]. Our group was the first to report that zinc deficiency causes increased systemic inflammation and mortality in response to serious infection [18]. Specifically, we have identified that zinc deficiency results in over-activation of the NF- κ B pathway, a central signal transduction pathway that is responsible for immune activation and inflammation. Further, we have shown that zinc supplementation restores normal pathway function [19]. Based on the above observations, we postulate that obesity combined with zinc deficiency will further promote the development of a chronic, low-grade systemic inflammatory state. To counterbalance this pathogenic cycle we predict that zinc supplementation will benefit the host by suppressing inflammation.

The purpose of this study was to determine whether zinc deficiency contributes to increased inflammation in the setting of obesity. Since inflammation is a key driver of obesity related diseases and zinc modulates inflammation, a lack of zinc may contribute to increased inflammation and susceptibility to related diseases. Diet induced models of obesity and zinc deficiency were used to examine differences in inflammation and zinc metabolism between zinc normal and deficient obese mice.

Methods

Animal Model of Obesity and Zinc Deficiency

Four-week old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) were randomly assigned to receive either a 60% high fat diet (HFD) (70 parts per million (ppm) Zn; TD.10873; Harlan Tekland) or normal matched control diet (ND) (50 ppm Zn; TD. 85420; Harlan Tekland with zinc content adjusted to account for a lower caloric density. Body weights were recorded every week, and within six weeks mice fed the HFD experienced, on average, a 29% weight gain with altered glucose metabolism, thereby establishing a valid model of obesity. After six weeks, half of the animals from each group were randomly placed on a zinc deficient diet (Zn-), either 60% high fat (1 ppm Zn; TD.10872; Harlan Tekland) or normal (1 ppm Zn; TD.85419; Harlan Tekland), respective to the original diet, for an additional three weeks. A zinc-free environment was carefully maintained during the course of the entire study using deionized water in zinc-free containers and stainless steel cages. As previously reported, zinc deficient diets consistently result in a ~2.5 fold decrease in plasma zinc levels [19]. Animal studies were conducted in accordance with prior approval by The Ohio State University Institutional Animal Care and Use Committee.

Magnetic Resonance Imaging

Adipose tissue was analyzed by *in vivo* Magnetic Resonance Imaging (MRI) using a 1.5 T Siemens scanner. Kidneys were used as anatomical landmarks and were depicted using a coronal gradient echo, T1-weighted localizing sequence (TR/TE 800/1.00). Thirty, axial slices spanning the iliac bifurcation to the superior pole of the topmost kidney were obtained using a T1-weighted gradient echo turbo FLASH protocol (FOV 288/384; TR/TE 230/5.6; NEX=3; BW

100MHz; time of acquisition \approx 11 minutes). Adipose tissue area was analyzed using Image J software (NIH, Bethesda, Maryland).

RNA Isolation and Quantitative RT-PCR

Following the dietary regimen, livers and adipose tissue (inguinal and epididymal) from animals were flash frozen for later analysis. Liver RNA was isolated by the TRIZol method (Invitrogen Carlsbad, California). Adipose tissue RNA isolation was performed using QIAzol and the RNeasy kit (Qiagen, Valencia, California). cDNA synthesis was performed using ThermoScript RT-PCR System (Invitrogen). Real-time PCR was performed with the 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, California) using SYBR Green reagents. All analyses were normalized against the average cycle threshold number of mouse GAPDH genes. The sequences of all the PCR primers are available on request.

Glucose Tolerance Testing

Animals were fasted overnight in a clean cage for sixteen hours. Mice were injected with 2 mg glucose per gram body weight intraperitoneally. Blood glucose was measured with a Contour blood glucose meter (Bayer HealthCare, Mishawaka, Indiana) at 30 minute intervals from 0 to 120 minutes. Glucose tolerance was evaluated by measuring the area under the curve (AUC) of the blood glucose concentration over time.

Diabetes and Obesity Marker Levels in Plasma

Multiplex quantification of factors associated with diabetes and obesity was evaluated in mouse plasma samples. The concentration of each marker was analyzed using the Bio-Plex Diabetes assay that includes ghrelin, resistin, glucagon-like peptide- 1 (GLP-1), glucagon, leptin,

plasminogen activator inhibitor-1 (PAI-1), gastric inhibitory polypeptide (GIP), and insulin using the Bio-Plex 200 Analysis System (Bio-Rad, Hercules, CA).

Data Analysis

All data are expressed as mean \pm SD. Statistical comparisons among different groups were performed using ANOVA. Significance was assumed at a p value of <0.05 .

Results

Mice maintained on the 60% high fat diet during the course of this study (9 weeks) experienced, on average, a 42% increase in body weight regardless of zinc intakes compared to mice maintained on the normal diets (Figure 1a).

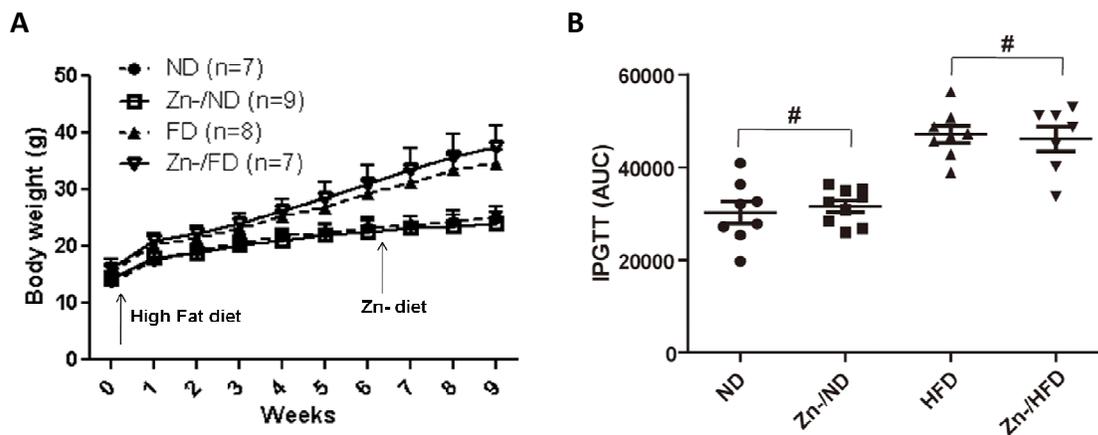


Figure 1. A) High fat intake resulted in an expected increase in total body weight (42 % increase by 9 weeks); however, zinc deficiency did not further increase body weight. B) Glucose tolerance was evaluated for two hours following a glucose challenge (2 mg glucose/gram body weight). The area under the curve (AUC) of the blood glucose concentrations over time demonstrates an expected decrease in glucose tolerance in mice receiving a high fat diet. Glucose tolerance was not further affected by zinc deficiency as determined by a one-way ANOVA with Tukey's Multiple Comparison Test, # not significantly different.

Mice maintained on HFDs also experienced an expected decrease in glucose tolerance within six weeks following intraperitoneal glucose injection after overnight fasting. The difference in plasma glucose levels was further exacerbated after nine weeks, but there was no difference between mice that received the zinc deficient and zinc sufficient diets (Figure 1b). Consistent

with these observations, qualitative and quantitative MRI analysis of fat tissue showed an increase in the total amount of fat in HFD mice, but again there was no difference between animals maintained on different zinc intakes (Figure 2b). Measurement of inguinal and epididymal fat weight demonstrated that HFD mice had a significant increase in fat content compared to ND mice (Figure 2a).

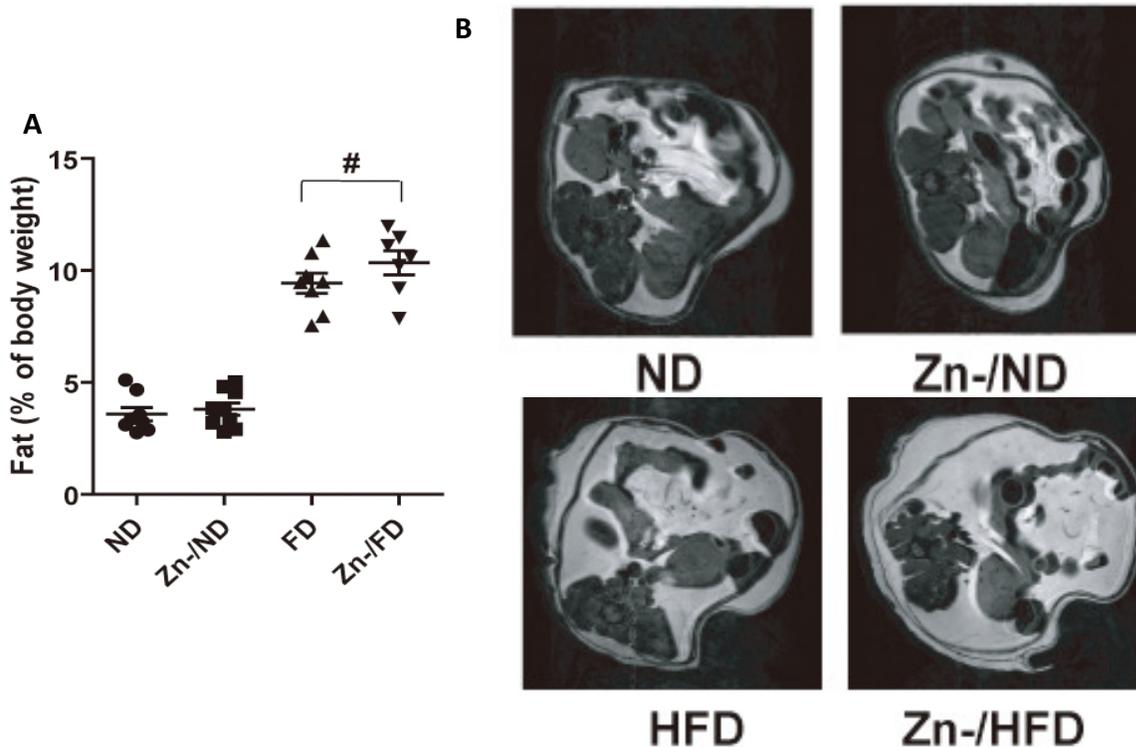


Figure 2. A) Combined epididymal and inguinal fat weights were recorded following nine weeks of high fat intake. Animals in the HFD and combined HFD/Zn deficient groups experienced a similar increase in fat content as determined by a one-way ANOVA with Tukey's Multiple Comparison Test, # not significantly different. B) Magnetic resonance imaging also showed an increase in fat accumulation in the high fat diet groups.

Next we determined whether obesity combined with zinc deficiency altered the circulating levels of factors associated with obesity-driven inflammation. Out of the eight factors evaluated, only serum leptin levels were changed. In particular, mice maintained on the combined HFD zinc deficient diet had the most significant increase in circulating leptin levels. An expected increase in mice maintained only on HFDs was also observed (Figure 3). There were no differences between treatment groups in circulating levels of ghrelin, resistin, glucagon-like peptide-1 (GLP-1), glucagon, plasminogen activator inhibitor-1 (PAI-1), gastric inhibitory polypeptide (GIP), and insulin. Serum levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF α) were also analyzed but could not be detected in any of the four treatment groups (data not shown).

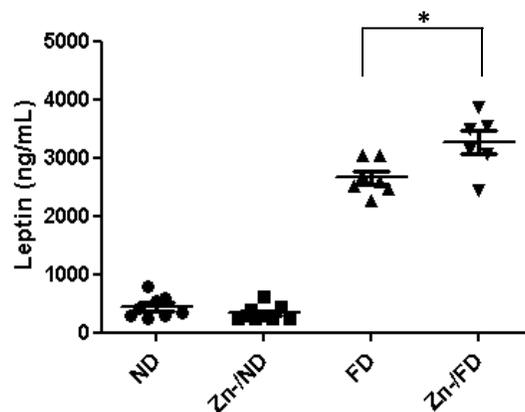


Figure 3. Serum leptin levels were evaluated at the end of the dietary regimens (Bio-Plex[®] Pro Diabetes Assay). Circulating leptin levels were elevated in obese animals and further significantly increased in mice that were also subject to a zinc deficient diet as evaluated by a one-way ANOVA with Tukey's Multiple Comparison Test, * $p < 0.05$. Other markers evaluated (but not shown) were unaffected including ghrelin, resistin, GLP-1, glucagon, GIP, insulin, and PAI-1.

Knowing that maintaining mice on a zinc restricted diet lowers plasma zinc levels on average 2.5-fold, we wanted to determine whether changes in zinc metabolism occurred. To do this we measured mRNA levels of Zip14, a zinc transporter, and metallothionein, an intracellular zinc binding protein, in liver tissue. We observed a consistent but insignificant decrease in Zip14 levels in both of the Zn- diet groups. Metallothionein-1 (MT-1) mRNA levels were significantly lower in HFD/Zn- liver tissue when compared to HFD liver tissue (Figure 4). Using the same assay, we next analyzed RNA extracted from adipose tissue to determine whether mRNA levels unique to genes expressed by macrophages were present within this tissue. Analysis of the macrophage markers MAC-1, CD68, and ADAM8 in adipose tissue revealed a significant increase, suggestive of an increased presence of macrophages, in the HFD/Zn- mice when compared to the HFD mice (Figure 5).

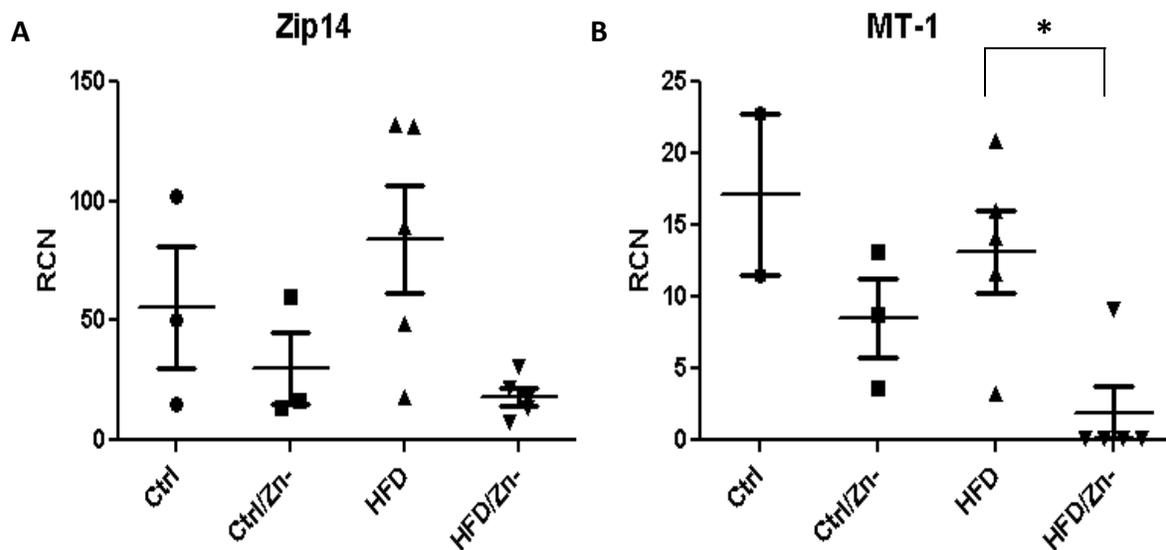


Figure 4. Intracellular zinc metabolism is regulated by zinc transporters and metallothionein. As an exploratory component to our study we evaluated the mRNA expression profile of two of these factors within liver tissue following different dietary exposures. RNA levels were quantitatively measured using real-time

PCR. A) Zinc deficiency resulted in decreased metallothionein-1 (MT-1) RNA levels in the livers of the high fat diet groups as evaluated by a one-way ANOVA with Tukey's Multiple Comparison Test, * $p < 0.05$. B) Zinc deficiency also resulted in decreased expression of Zip14, a zinc transporter, in liver tissue, but did not reach statistical significance. These findings suggest that zinc metabolism may be significantly altered by zinc deficiency in the setting of obesity. The consequences of which remain to be determined.

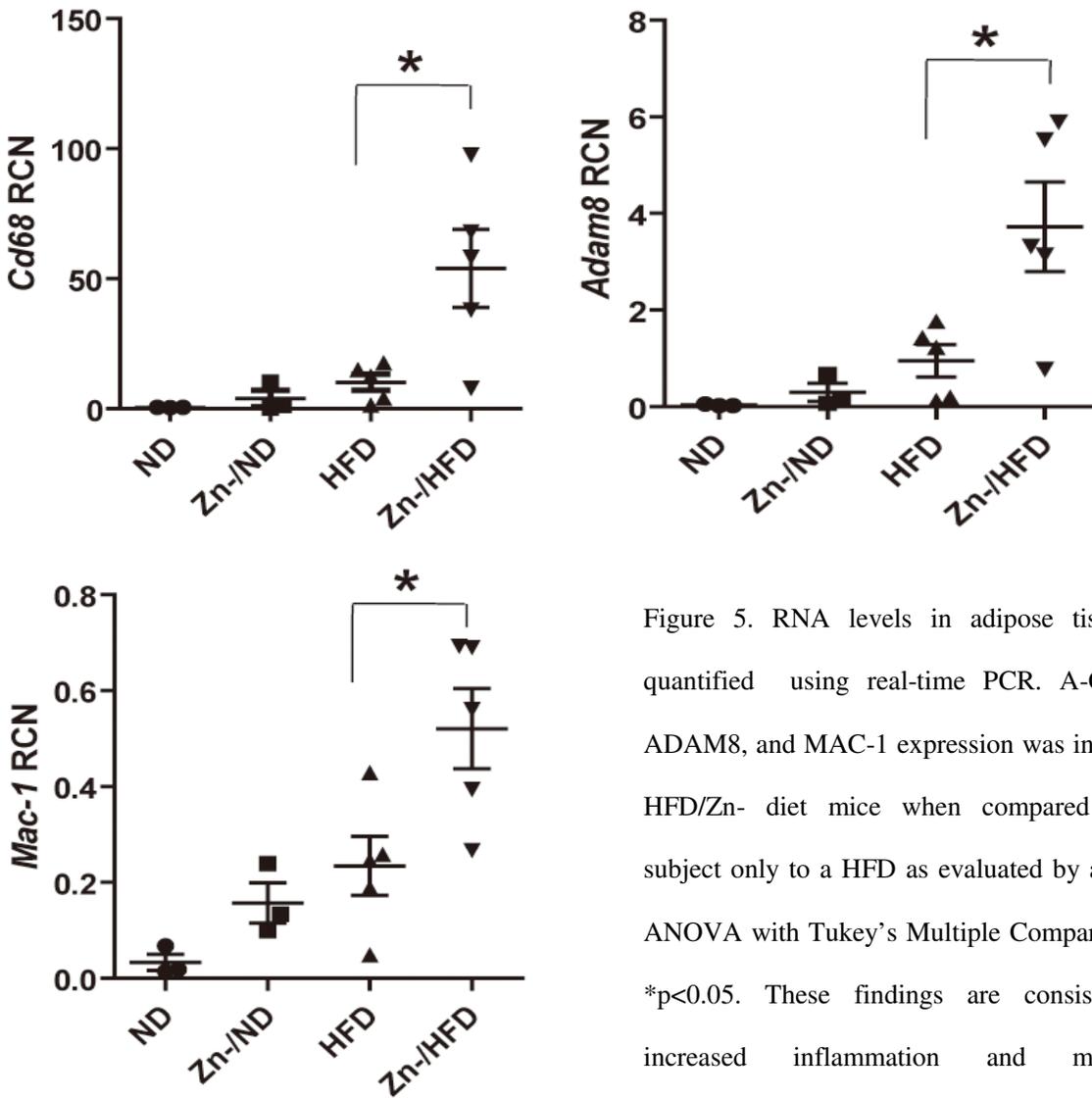


Figure 5. RNA levels in adipose tissue were quantified using real-time PCR. A-C) CD68, ADAM8, and MAC-1 expression was increased in HFD/Zn- diet mice when compared to mice subject only to a HFD as evaluated by a one-way ANOVA with Tukey's Multiple Comparison Test, * $p < 0.05$. These findings are consistent with increased inflammation and macrophage infiltration, previously identified to be a hallmark of obesity-driven systemic inflammation.

Discussion

In our investigation, differences between treatment groups and particularly, obese, zinc deficient mice were primarily observed at the molecular level. Interestingly, changes in body weight, adipose tissue mass, and glucose tolerance in obese mice were not significantly affected by zinc deficiency. We predict that this may be in part due to the relatively short dietary regimen that clearly did produce obese mice, but may not have been sufficient time to induce a chronic, systemic inflammatory state. In future studies, we will consider implementing a longer duration of the combined high fat and zinc restricted diet to induce more obesity and inflammation. This would be relevant in the context of human disease since obesity and the development of a chronic inflammatory state typically take years to manifest.

Most striking, differences in serum leptin levels were observed within nine weeks of high fat intake which was further significantly increased in obese mice in just three weeks of zinc deficient intake. Leptin is a regulator of metabolism and inflammation and its production is restricted to adipocytes. Specifically, leptin production is induced by cytokines including TNF α , IL-1, and IL-6 [20]. Leptin also plays a role in promoting an inflammatory response by inducing the production of cytokines in peripheral blood mononuclear cells and macrophages and by enhancing macrophage accumulation within adipose tissue, a hallmark of obesity-mediated systemic inflammation, by increasing the release of monocyte chemoattractant protein-1 (MCP-1) [20]. We observed that zinc deficiency led to an increase in serum levels of leptin in obese mice, so we speculate that zinc may play a role in directly controlling leptin production, or, in support of our hypothesis, zinc deficiency itself may increase inflammation in adipose tissue that in turn stimulates leptin production. Further studies will be required to answer these important questions.

Based upon our preliminary studies which were restricted to mRNA analysis of liver tissue, we contend that zinc metabolism is altered in zinc deficient, obese mice. The decrease observed in Zip14 levels suggests that less zinc is taken into the cells, which is also consistent with the observation that zinc deficiency caused lower MT-1 levels in the liver. Clearly, more extensive analysis will need to be conducted so that we can better understand whether changes in zinc metabolism directly or indirectly affect development of an inflammatory state in the setting of obesity.

A hallmark characteristic during the development of chronic systemic inflammation in obesity is the accumulation of macrophages in adipose tissue. Consistent with this characteristic, obese, zinc deficient animals had an increase in RNA levels of macrophage-specific markers that were significantly higher than all other treatment groups, including HFD mice. This suggests that although zinc deficient, obese mice did not accumulate more adipose tissue and did not have elevated levels of cytokines when compared to obese, zinc sufficient mice, their adipose tissue had a higher level of inflammation. We believe this suggests that zinc deficiency within only three weeks resulted in an increase in inflammation within adipose tissue. Given more time, we predict that changes in adipose function may lead to increased systemic inflammation. Consistent with our findings, obese individuals with the same body mass index exhibit an increase in the number of macrophages in adipose tissue which is associated with a higher risk of cardiovascular disease [21]. Taken together, these findings indicate that the quality, and not just the quantity, of adipose tissue is an important factor in the risk for obesity-related disease.

We predict that zinc deficiency can contribute to the morbidity observed within the obese population in that a lack of zinc has the potential to increase inflammation which then may increase the risk of developing inflammation-mediated diseases. If correct, zinc supplementation

has the potential to lower the risk of the progression of obesity to cardiovascular diseases. The NF- κ B pathway, which has been previously shown by our group to be zinc responsive [19], may be involved in the regulation of the innate immune response in obesity; however, further studies are required to determine whether this signaling pathway is actually involved. Based on our findings, it appears that leptin production in adipose tissue may serve as sensitive biomarker of zinc status in obese subjects. Further, this raises many questions regarding whether leptin expression is directly regulated by zinc and whether leptin, or other yet to be identified factors, induce macrophage recruitment in the setting of combined zinc deficiency and obesity. Finally, if zinc directly regulates the adipose response, then further studies that evaluate zinc metabolism within adipose tissue will be required.

Acknowledgements

The contributions of Mingjie Liu, Shengying Bao, Eric Bolin, Qinghua Sun, Martha Belury, Mark Failla, Xiaohua Xu, and Daren Knoell to this work are gratefully acknowledged. This work is supported by RO1 HL086981-01 (DLK) and the Ohio State University Food Innovation Center (DLK and M-JL).

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