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INVESTIGATING THE EFFECTS OF SUBCHRONIC DIETARY ZINC
SUPPLEMENTATION ON DIET-INDUCED METABOLIC DYSFUNCTION-ASSOCIATED
STEATOTIC LIVER DISEASE

By

Oluwanifemi Esther Bolatimi

B.A., Lewis & Clark College, 2019

A Thesis

Submitted to the Faculty of the
School of Medicine of the University of Louisville

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for the Degree of

Master of Science

In Pharmacology and Toxicology

Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

December 2023

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ABSTRACT

INVESTIGATING THE EFFECTS OF SUBCHRONIC DIETARY ZINC SUPPLEMENTATION ON DIET INDUCED METABOLIC DYSFUNCTION-ASSOCIATED STEATOTIC LIVER DISEASE

By

Oluwanifemi Esther Bolatimi

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Zinc deficiency is associated with metabolic dysfunction-associated steatotic liver disease (MASLD). Previous studies show zinc supplementation improves steatosis, but its therapeutic effects in established MASLD remain unclear. We developed a model to characterize the effects of zinc supplementation on high-fat diet (HFD) induced MASLD and hypothesized established MASLD would be attenuated. Mice were fed control diet or HFD for 12 weeks and then grouped into normal or zinc-supplemented diets for 8 additional weeks. At euthanasia, plasma and liver tissues were collected for phenotypic analysis. Twelve weeks of HFD altered glucose clearance and body composition. Eight weeks of subsequent zinc supplementation did not change glucose handling, steatosis, or liver injury. Results from our model suggest 8-week zinc supplementation cannot reverse established MASLD. The HFD may have caused disease progression beyond rescue by the 8-week supplementation. Future studies will address these limitations, providing insights into zinc as a therapy for established MASLD.

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CHAPTER 1

INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease

The global prevalence of obesity is paralleled by a corresponding increase in the severity and prevalence of various metabolic diseases, including metabolic dysfunction-associated steatotic liver disease (MASLD, formerly termed non-alcoholic fatty liver disease (NAFLD)). MASLD represents a spectrum of liver diseases ranging from simple hepatic steatosis or lipid accumulation to more severe steatohepatitis in the absence of alcohol consumption [1]. Presently, MASLD has surpassed alcohol-associated liver disease as the most common chronic liver disease, affecting up to 25% of the global adult population [2]. By the year 2030, the MASLD population is projected to increase to 100.9 million cases, a 21% increase since 2015 [3]. With a wide range of etiologies, MASLD is commonly attributed to excessive consumption of a high-calorie diet with poor nutritional content [2]. MASLD, characterized by the accumulation of lipid in the liver, is regarded as the hepatic manifestation of an array of multifactorial diseases, such as metabolic syndrome, obesity, and diabetes [1, 4-6].

Metabolic dysfunction-associated steatotic liver, the leading contributor to liver morbidity and mortality, has become a major health concern as there are no overt symptoms of the disease, and left untreated it can progress into steatohepatitis (MASH), cirrhosis, or hepatocellular carcinoma [7]. The latter stages of disease progression may necessitate liver transplantation or result in abrupt and severe liver failure culminating in death [8]. Beyond hepatic outcomes, MASLD has implications for non-hepatic health consequences such as cardiovascular disease [3] and impaired

kidney function [9]. Upon the reduction of steatosis, liver injury is reversible as hepatic metabolic function improves. The current management of MASLD consists of lifestyle adjustments encompassing diet restriction and increased physical activity, but there is still a lack of effective therapeutics approved by the Food and Drug Administration for the treatment of MASLD[3]. This necessitates a critical search for efficacious and appropriate therapeutic agents for the treatment of MASLD.

Zinc Deficiency

The underlying pathogenesis of MASLD still remains unclear but is associated with mechanisms encompassing oxidative stress, insulin resistance, dyslipidemia, and deficiencies in micronutrients [10, 11]. Deficiency in the micronutrient zinc, which affects 17.3% of the global population due to inadequate zinc intake [12], is implicated in playing a crucial role in the pathogenesis of MASLD. Zinc is the second most concentrated trace mineral in the body [13]. This vital trace element contributes to various biological processes serving structural, catalytic, signaling, and regulatory functions. Some of these include a role in energy metabolism, anti-inflammation, and antioxidative pathways [11, 14]. Disruption in these processes constitute a risk factor and/or may increase susceptibility to the development of MASLD. Zinc deficiency is most prevalent in developing and socioeconomically disadvantaged areas, but it is also increased in vegan and vegetarian populations, as well as in the elderly and people with disorders associated with reduced zinc absorption [15-17]. Clinical studies have demonstrated low serum levels of zinc in patients with chronic hepatitis, cirrhosis, and liver cancer. Zinc levels are also correlated with disease severity and prognosis. In particular, low levels of serum zinc acts as an independent risk factor for significant hepatic fibrosis in MASLD patients [18].

Physiological and Hepatic Role of Zinc

Zinc affects the activity of enzymes required for normal hepatic identity, function, and lipid metabolism [19]. Zinc has additionally been shown to reduce hepatic lipid accumulation by preventing lipogenesis and stimulating lipolysis through autophagy-mediated lipophagy [20]. Insufficient hepatic zinc leads to disruptions in liver function and energy metabolic profiles, promoting hepatic lipid accumulation [21] and the development of MASLD. Studies investigating zinc supplementation consumed concurrently with a high-fat diet (HFD) show reduced hepatic steatosis and improved glucose metabolism [22]. Furthermore, zinc supplementation has been shown to be protective in lowering serum glucose in rodent models [10]. While studies addressing the role of zinc supplementation prior to the development of MASLD have been conducted, the therapeutic effects of dietary zinc supplementation following MASLD onset remain to be determined. Finding from this study will have valuable implications in broadening patient treatment approaches and potential MASLD outcomes.

Study Objective

Hence, the primary objective of this study was to develop an in vivo model exploring dietary zinc supplementation as a potential therapeutic agent after the establishment of high-fat diet-induced MASLD. We explored the effects of zinc supplementation on glucose metabolism, hepatic lipid accumulation, hepatic injury, and gene expression of hepatic enzymes, of which zinc is a major structural component.

CHAPTER 2

METHODS AND MATERIALS

2.1. Animals Model and Diets

Animal use, protocols, and procedures were approved by the University of Louisville Institutional Animal Care and Use Committee (OLAW/PHS Assurance No. A3586-01; USDA Registration No. 61-R-001-01). The University of Louisville is an Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) accredited institution. Male C57BL/6J mice (8-weeks-old; n = 60) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in a temperature- and light-controlled room with a 12 h light/dark cycle. Mice were allowed to acclimate after the facility transfer for one week and were fed autoclaved standard laboratory rodent chow. After acclimation, mice were switched to purified diets to minimize the influence of metal contamination found in standard chow on experimental outcomes [23, 24]. Specifically, mice (n = 30) were either fed a control diet (CD, 10% kcal fat; Research Diets D14020202, New Brunswick, NJ, USA) or a HFD (60% kcal fat; Research Diets D14020205, New Brunswick, NJ, USA) for 12 weeks. After 12 weeks, mice were further grouped (n = 15) into diets containing either 30 mg or 90 mg zinc/4057 kcal, representing the normal zinc (NZ) and added zinc (ZS) diets, respectively (CD + Zn—Research Diets D14020203; HFD + Zn—Research Diets D14020206, New Brunswick, NJ, USA) for an additional 8 weeks [Figure 1]. Food and water were provided ad libitum. Body weights and food consumption were recorded weekly. Intraperitoneal glucose tolerance tests (IPGTT) were performed at weeks 12 and 19. Prior to euthanasia, the fat and lean tissue mass of the animals were determined by quantitative magnetic resonance imaging using an EchoMRI-500 Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA).

At the end of 20 weeks, mice were anesthetized (ketamine/xylazine, 120/16 mg/kg body weight) via intraperitoneal (i.p.) injection, followed by blood collection from the inferior vena cava and subsequent euthanasia via exsanguination. Blood samples were centrifuged at 2000 rcf for 10 minutes at 4°C, and plasma was stored at –80°C for further biochemical analysis. Liver weight was recorded for each mouse and was used to calculate organ to body weight ratios. Portions of liver tissue were snap-frozen in liquid nitrogen, processed for RNA isolation, fixed in 10% neutral buffered formalin for histology, and used for metal analysis. The liver weight to tibia length ratio (liver weight in grams divided by tibia length in millimeters) was used as an index of liver size changes. Mice were fasted overnight prior to sacrifice.

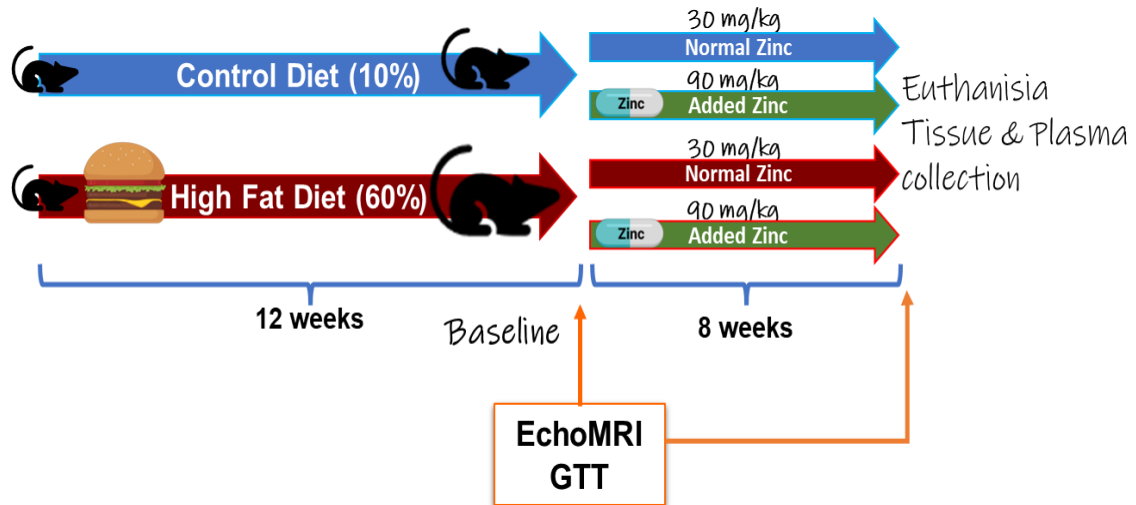


Figure 1. Exposure paradigm. 9-week-old male C57BL/6J mice were fed control (n = 30, 10% fat-kcal) or HFD (n = 30, 60% fat-kcal) for 12 weeks, after which they were further subdivided into normal zinc (n = 15, 30 mg zinc/4057 kcal) or zinc supplemented (n = 15, 90 mg zinc/4057 kcal) diet groups for an additional 8 weeks. Prior to zinc supplementation and animal euthanasia (use “euthanasia”), IPGTTs were conducted, and body composition determined by Echo magnetic resonance imaging (MRI). IPGTT = intraperitoneal glucose tolerance test.

2.2. Glucose Tolerance Test

IPGTT was performed at week 12, before the start of zinc supplementation, and at week 19, one week prior to the end of the study. Mice were fasted for 6 h, weighed, and assessed for baseline blood glucose. Mice were then injected with glucose (2 mg glucose/g body weight, sterile saline, i.p.) [25, 26]. Blood glucose levels were measured at +15, +30, +60, and +120 minutes post-injection with a hand-held glucometer (Contour Next EZ, Parsippany, NJ, USA) using blood from tail prick. A time course of absolute blood glucose measurement and the area under the curve (AUC) calculated using the trapezoid rule were determined for each animal.

2.3. Blood Chemistry Analysis

Plasma alanine transaminase (ALT), aspartate transaminase (AST), triglycerides, cholesterol, glucose, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) levels were measured using Lipid Panel Plus diskettes (catalog:400-0030; Abaxis Inc.; Union City, CA, USA) with the Abaxis Piccolo Xpress Chemistry Analyzer (Abbott; Abbott Park, IL, USA).

2.4. Measurement of Hepatic Triglyceride and Cholesterol Content

Liver tissues were washed in 50 mM NaCl and homogenized in 1 mL 50 mM NaCl with 0.5 mm glass silica beads and beaded for 30 seconds. Hepatic lipids were extracted using an aqueous solution of chloroform and methanol in a 2:1 ratio based on the Bligh and Dyer method (Bligh and Dyer, 1959). Triglyceride and cholesterol standards (catalog: T7531-STD, C7509-STD; Point Scientific; Canton, MI) were utilized to generate a standard curve to quantify the extracted lipids. Extracted triglycerides and cholesterol were colorimetrically quantified with absorbance at 500 nm using a microplate absorbance reader (BioTek Gen 5; Winooski, VT, USA). Reagents used in the assay: Infinity Triglycerides Reagent (TR22421, ThermoFisher Scientific, Middletown, VA, USA) and Infinity Cholesterol Reagent (TR13421, ThermoFisher Scientific, Middletown, VA, USA).

2.5. Liver Histology

Liver tissues were fixed in 10% neutral buffered formalin for at least 24 h and moved to 75% ethanol until tissue processing prior to being embedded in paraffin. Paraffin embedded tissues were sectioned at 5 μ m with Leica Biosystem's Histocore Autocut Automated Rotary Microtome (Leica Biosystem; Deer Park, IL, USA). Tissue sections were deparaffinized with a citrisolv hybrid, rehydrated with graded ethanol washes, and stained with either hematoxylin and eosin (H&E) to assess the overall hepatic structure or Masson Trichrome to assess the collagen deposition as an indicator for fibrosis using the EpreDia™ Gemini™ AS Automated Slide Stainer (Fisher Scientific, Pittsburgh, PA, USA). Steatosis was scored as a percent of liver cells in a 10 \times field containing fat (<25% = 1+; <50% = 2+; <75 = 3+; >75% = 4+) [27]. For each animal, ten 10 \times fields were scored. 10 \times and 20 \times field Masson Trichrome images were taken, and five 10 \times and five 20 \times images were quantified for collagen deposition using ImageJ (v1.53k) software (National Institute of Health; Bethesda, MD, USA). Images were captured on cellSens Standard XV image processing software using the Olympus DP74 digital camera and Olympus BX43 microscope (Olympus America, Breinigsville, PA, USA).

2.6. Real-Time qPCR

Liver tissues were homogenized using 1 mL of chilled RNA-STAT 60™ (catalog: CS502; Tel-test Inc.; Friendswood, TX) per 50–100 mg of tissue in a 2 mL screwcap tube containing 0.5 mm glass silica beads (Biospec Products, Bartlesville, OK, USA), followed by homogenization for 30 seconds using a mini-beadbeater (Sigma Aldrich, St. Louis, MO, USA). Total RNA was then extracted from clear homogenate by addition of chloroform and precipitated with isopropanol. RNA pellet was washed with 75% ethanol, evaporated until dry and resolubilized in RNA free diethyl pyrocarbonate (DEPC) treated water (Invitrogen, catalog: AM9906, Life Technologies Corp, Austin, TX, USA) following the RNA-STAT 60 reagent protocol for the isolation of total RNA, DNA, and protein by AMSBIO. RNA quantity and purity were assessed with Nanodrop

OneC (Thermo Scientific, catalog: 701-058112; Madison, MI, USA). cDNA was reverse transcribed from 3 µg total RNA to yield 60 µL using the single step cDNA synthesis reagent QScript (Quantabio; catalog: 95048-500), following the manufacturer's protocol. qRT-PCR was performed on a CFX384 Touch Real-Time PCR Thermal Cycler (Bio-Rad, Hercules, CA, USA) using iTaq Universal Probe Supermix (catalog:1725134; Biorad, Hercules, CA, USA) according to the manufacturer's protocol. Primer sequences from TaqMan Gene Expression Assays (ThermoFisher Scientific) were as follows: hepatocyte nuclear factor 4 alpha (*Hnf4a*); (Mm01247712_m1), tumor necrosis factor alpha (*Tnfa*); (Mm00443258_m1), peroxisome proliferator-activated receptor alpha (*Ppara*); (Mm00440939_m1), adiponectin (*Adipoq*); (Mm04933656_m1) and apolipoprotein B (*Apob*); (Mm01545150_m1). Levels of mRNA expression were calculated using the 2- $\Delta\Delta C_t$ method. Fold induction was calculated and normalized relative to the amount of Glyceraldehyde-3-Phosphate Dehydrogenase (*Gapdh*) mRNA (catalog:4352339E, ThermoFisher Scientific; Madison, MI, USA), and expression levels of mice fed the control diet and normal zinc, which were set to 1.

2.7. Metal Analysis

Each liver sample was weighed and digested in 600 µL of 70% concentrated trace metal grade nitric acid (Fisher Scientific) in a 65°C shaking incubator for 4 h. After digestion, samples were cooled to room temperature and filtered using a 100 µm filter to remove undigested debris. Furthermore, 8.2 mL of Milli-Q deionized water was added to each sample, bringing the final concentration of nitric acid to 5%. Metal levels were assessed using an Agilent 7800 inductively coupled plasma mass spectrometry (ICP-MS) equipped with an Agilent SPS 4 autosampler (Agilent Technologies, Inc., Santa Clara, CA, USA) for sample injection. During sample injection, internal standards including Bi, In, Li, Sc, Tb, and Y (Inorganic Ventures) were mixed with each sample for drift correction and accuracy improvement. Each sample was analyzed three times, and metal

levels were calculated and presented as $\mu\text{g/g}$ wet tissue. Anything less than the intercept concentration was considered non-detectable.

2.8. Statistics

Statistical analyses were conducted using GraphPad Prism (version 9.2.0) for Windows (GraphPad Software Inc.; La Jolla, CA, USA). Data are expressed as mean \pm SD. Two group comparisons were performed using an unpaired t-test. Multiple group data was compared using the two-way Analysis of Variance (ANOVA) followed by Bonferroni's post-hoc test to correct for multiple hypothesis testing. Statistical significance was set at an alpha level of 0.05 ($p \leq 0.05$).

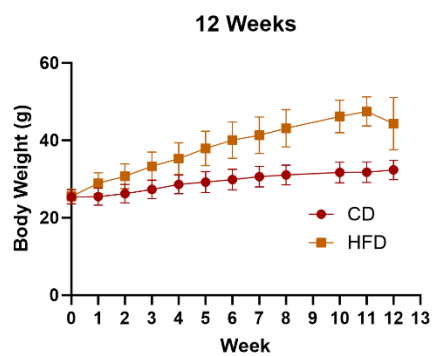
CHAPTER 3

RESULTS

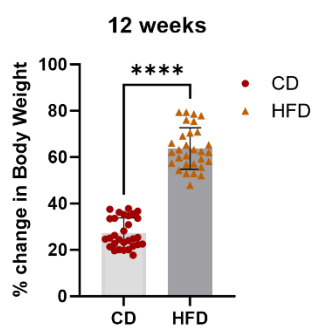
3.1. Body Weight and Composition

Body weights were recorded weekly during the 20-week study period. All groups experienced weight gain, with HFD-fed mice gaining significantly more weight compared with CD-fed mice ($p < 0.0001$) (Figure 2A, B, E, F). Zinc supplementation had no significant effect on the mean percent body weight gain in either the HFD or CD zinc-supplemented mice (Figure 2F). As expected, HFD-fed mice experienced a significant increase in percent body fat ($p < 0.0001$) and a contrasting decrease in percent lean tissue ($p < 0.0001$) compared with CD-fed mice (Figure 2C, D, G, H). Similar to the effects on body weight, zinc-supplemented mice did not show significant differences in percent body fat or percent lean tissue compared with normal zinc-fed mice. The observed data suggest the HFD was successful in inducing obesity in HFD-fed mice.

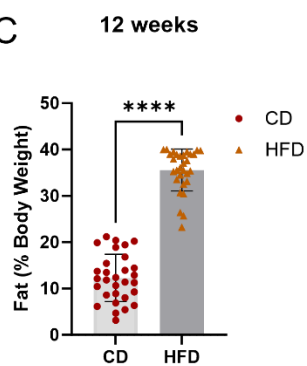
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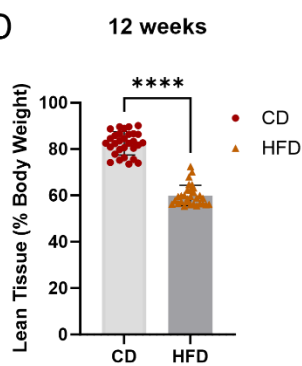
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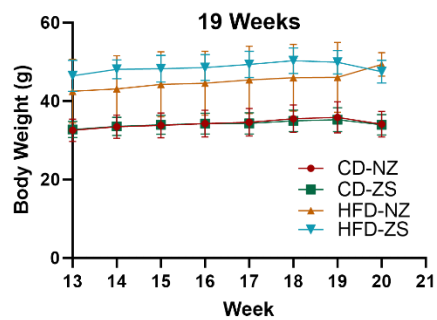
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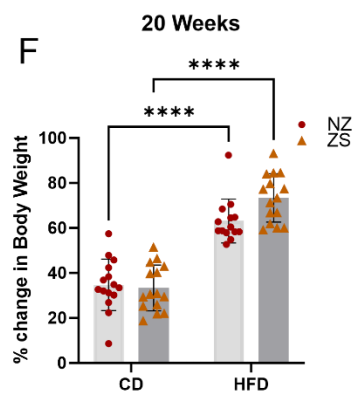
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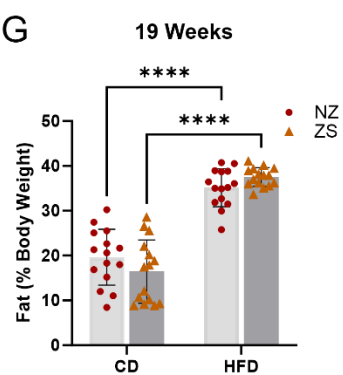
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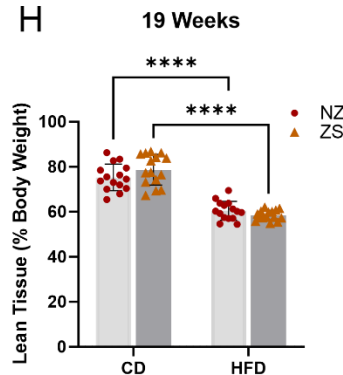


Figure 2. Effects of HFD and zinc supplementation on body weight and composition. Twelve weeks of HFD resulted in significant body weight gain and altered body composition which zinc supplementation could not ameliorate. Body weights were measured weekly for the duration of the study and body composition determined by Echo MRI scan at weeks 12 and 19. Panels A–D show outcomes for weeks 1–12 (+/– HFD). Panels E–H show outcomes for weeks 13–20 (+/– HFD and +/- zinc supplementation). Data are reported as mean \pm SD (n = 30 mice/group, A–D; n = 14–15 mice/group, E–H) with significance set to 0.05. **** p < 0.0001. CD = control diet, HFD = high-fat diet, NZ = normal zinc, ZS = zinc supplement.

3.2. Dietary and Hepatic Zinc Levels

Mice were administered zinc in their food; therefore, we measured zinc levels in the diet and liver tissues. As expected, diets supplemented with zinc had three times the amount of zinc compared with levels in the normal zinc diets. Mice fed CD with and without zinc supplement had 90.3 μg zinc/g of diet and 30.3 μg zinc/g of diet, respectively, while the HFD with and without zinc supplement had 124.0 μg zinc/g of diet and 40.0 μg zinc/g of diet, respectively. No significant differences were observed in hepatic zinc levels across groups (Figure 3). Zinc supplementation did not alter zinc accumulation in HFD nor CD zinc supplemented mice compared with normal zinc diet fed mice.

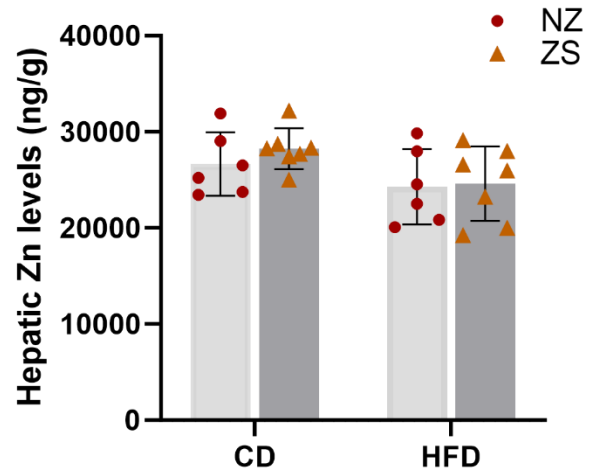


Figure 3. Hepatic zinc levels. Hepatic zinc content was measured by ICP-MS. Data are reported as mean \pm SD (n = 6 NZ, n = 7 ZS) significance set to 0.05. NZ = normal zinc, ZS = zinc supplement.

3.3. IPGTT

To assess glucose intolerance, often associated with MASLD, glucose tolerance tests were performed at weeks 12 and 19. At week 12, HFD-fed mice had significantly increased blood glucose and AUC compared with CD-fed mice (Figure 4A, C) ($p < 0.0001$). After 7 weeks of subsequent zinc supplementation, HFD-fed mice still showed reduced glucose clearance and increased glucose AUC (Figure 4B, D). However, zinc supplementation did not result in a significant difference in blood glucose and AUC between the supplemented and normal zinc groups. Similar observations were made in CD-fed mice. In summary, HFD was associated with reduced glucose clearance, of which zinc supplementation had no impact.

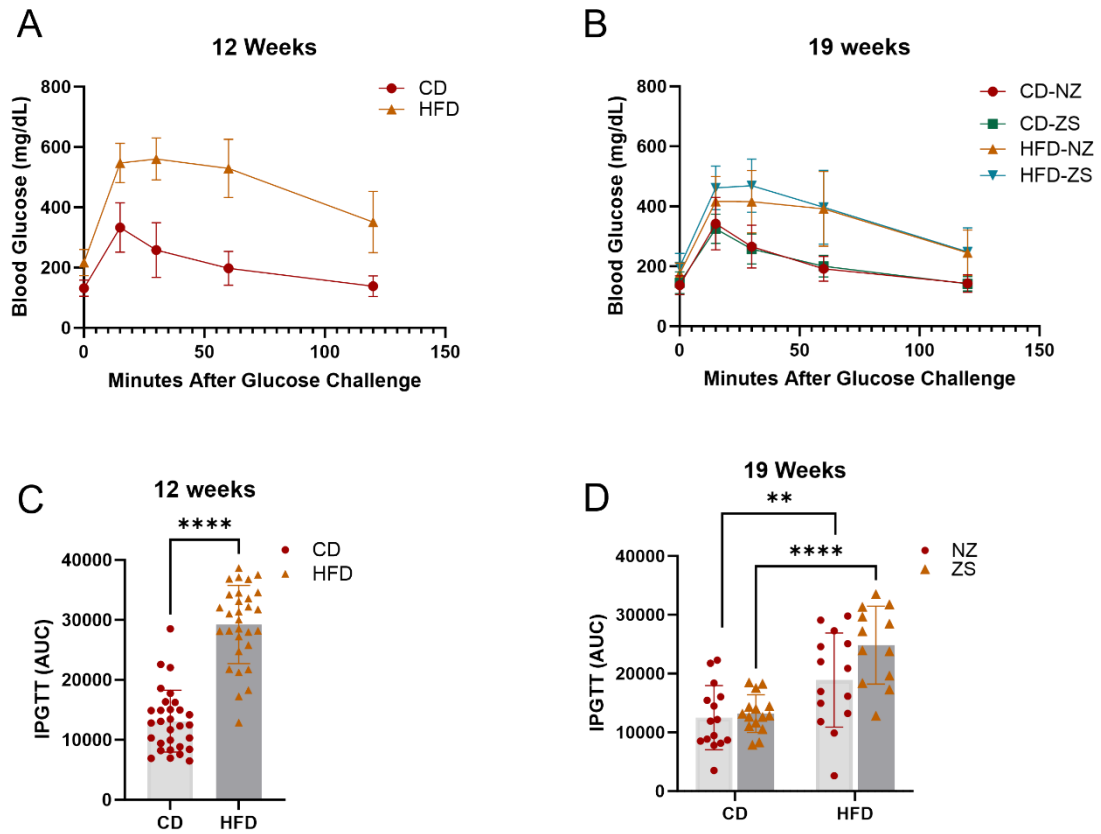


Figure 4. Effects of HFD and zinc supplementation on glucose tolerance. HFD-induced increase in glucose intolerance was unaffected by zinc supplementation. Blood glucose levels in CD and HFD groups after IPGTT, performed (A) before zinc supplementation and (B) one week prior to sacrifice. (C, D) Integrated AUC was calculated showing higher changes in blood glucose levels in HFD groups. Data are reported as mean \pm SD ($n = 30$ mice/group, A, C; $n = 14$ – 15 mice/group, B, D) with significance set to 0.05. ** $p < 0.05$, *** $p < 0.0004$, **** $p < 0.0001$. CD = control diet, HFD = high-fat diet, NZ = normal zinc, ZS = zinc supplement, IPGTT = intraperitoneal glucose tolerance test.

3.4. Plasma Lipids

Plasma total cholesterol, triglycerides, HDL, LDL, and vLDL levels were measured in all animal groups (Table 1). Overall, plasma cholesterol and HDL levels were increased with HFD feeding, while triglycerides and vLDL levels were decreased. Mean lipid levels were not significantly different between HFD plus zinc supplement and normal zinc-fed mice. Similarly, no significant differences were observed in CD zinc-supplemented compared with normal zinc-fed mice

	CD+NZ (n)	CD+ZS (n)	HFD+NZ (n)	HFD+ZS (n)
Cholesterol	109.8 ± 28.1 (14)	110.7 ± 17.3 (15)	178.4 ± 38.8 (14) #	163.5 ± 35.4 (14) #
Triglycerides	73.2 ± 20.8 (14)	74.7 ± 19.5 (15)	58.4 ± 19.9 (14)	54.1 ± 15.5 (14) #
HDL	84.4 ± 23.2 (14)	85.3 ± 12.3 (15)	96.9 ± 9.4 (14)	97.1 ± 7.5 (14)
LDL	8.8 ± 4.3 (12)	8.3 ± 5.5 (13)	14.0 ± 14.1 (2)	7.0 ± 1.4 (2)
vLDL	14.7 ± 4.2 (14)	15.1 ± 4.0 (15)	11.6 ± 4.0 (14)	10.8 ± 3.1 (14) #

Table 1. Plasma levels of total cholesterol, triglycerides, HDLs, LDLs, vLDLs. Data reported as mean ±SD (mg/dL), with significance set at 0.05. # p < 0.05 compared with mice fed normal zinc or zinc supplemented control diet. CD = control diet, HFD = high-fat diet, NZ = normal zinc, ZS = zinc supplement.

3.5. Hepatic Injury and MASLD

Liver-to-tibia and liver-to-body weight ratios were calculated as markers for changes in liver size. HFD feeding alone resulted in enlarged livers compared with CD (Figure 5A, B). No significant differences in either liver-to-tibia or liver-to-body weight ratios between zinc-supplemented and normal zinc-fed mice were observed. Plasma aminotransferase activity was used to evaluate hepatic injury. The mean plasma levels of AST and ALT were significantly elevated in HFD-fed mice compared with CD-fed mice. In HFD-fed mice, zinc supplementation resulted in reductions in AST and ALT levels compared with mice fed normal zinc, although the decrease was not statistically significant. In CD-fed mice, AST and ALT levels were unaffected by dietary zinc supplementation. (Figure 5C, D). Ultimately, the increases in liver injury markers induced by HFD feeding were not ameliorated by zinc supplementation.

Histological analysis showed significant increases in macrovesicular steatosis in the HFD-fed mice compared with the CD-fed mice, as seen in representative photomicrographs of H&E (Figure 6A) as well as in the quantification of steatosis in the H&E sections (Figure 6C). Furthermore, the biochemical analysis of liver tissue revealed mice fed HFD had greater total cholesterol and hepatic triglyceride levels compared with mice fed CD (Figure 6D, E). Dietary zinc supplementation did not significantly alter the histological or biochemical parameters of MASLD in either diet group. No qualitative differences in collagen deposition, as seen in representative photomicrographs of Masson Trichrome stain, were observed between any of the experimental groups (Figure 6B), and this was confirmed quantitatively (data not shown). Taken together, the data showed HFD was able to induce liver injury, of which zinc supplementation was unable to attenuate.

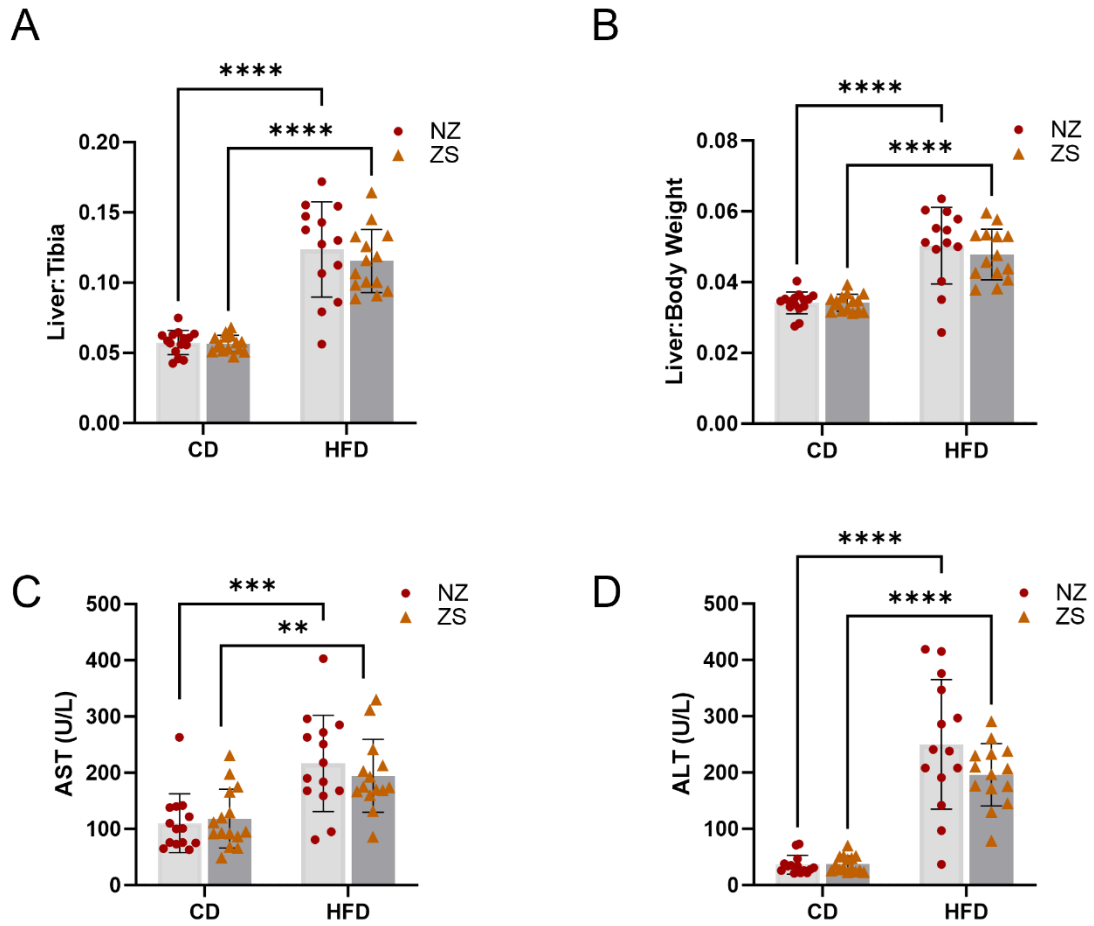
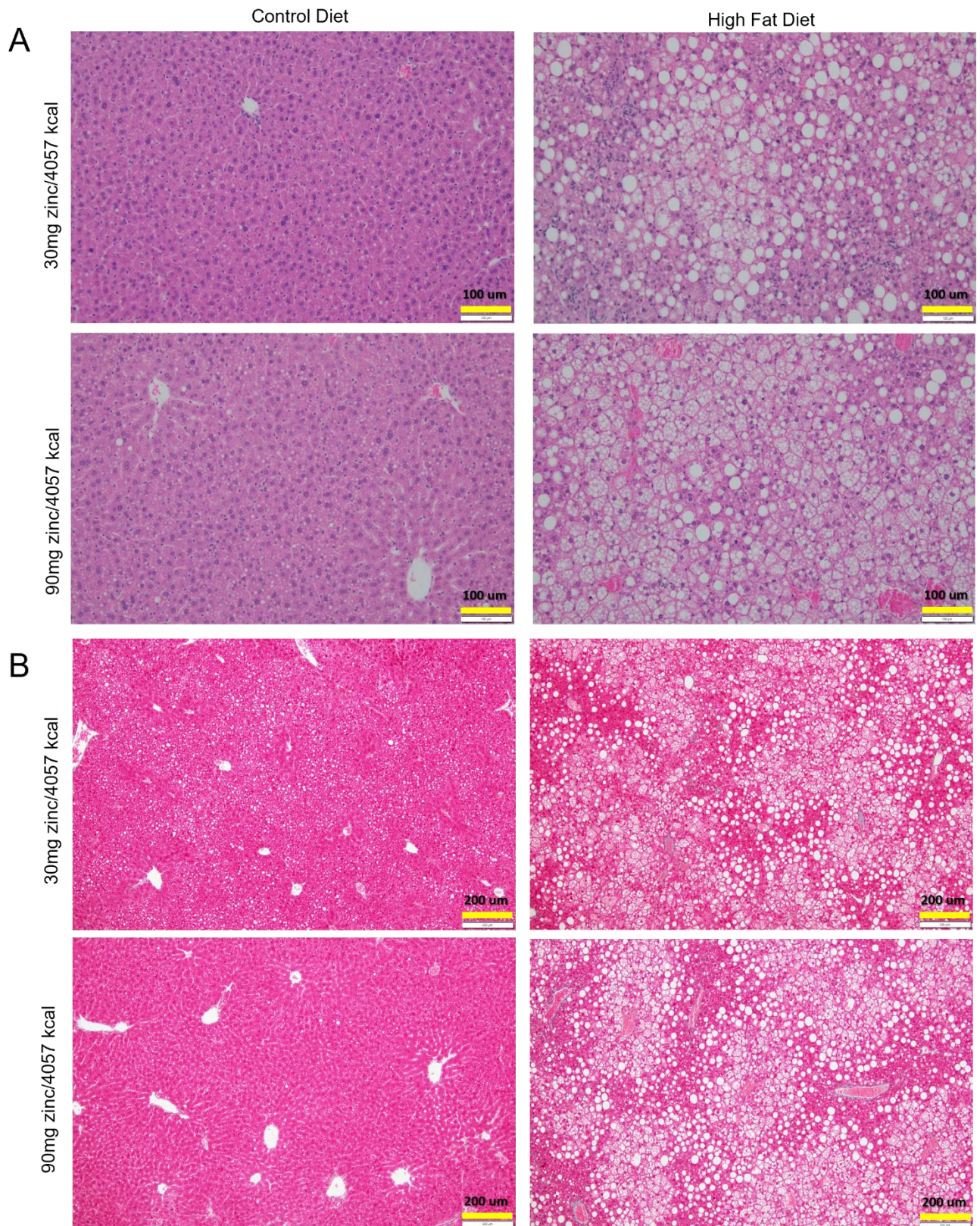


Figure 5. Effects of HFD and zinc supplementation on liver injury. HFD-induced liver injury was not reduced by zinc supplement. (A, B) Ratio of liver to tibia and body weight, indices of altered liver sizes. (C) Plasma aspartate aminotransferase (AST) and (D) alanine aminotransferase (ALT) activities were increased in HFD fed groups. Data are reported as mean \pm SD (n = 14–15 mice/group) with significance set to 0.05. ** p < 0.05, *** p < 0.005, **** p < 0.0001. CD = control diet, HFD = high-fat diet, NZ = normal zinc, ZS = zinc supplement.



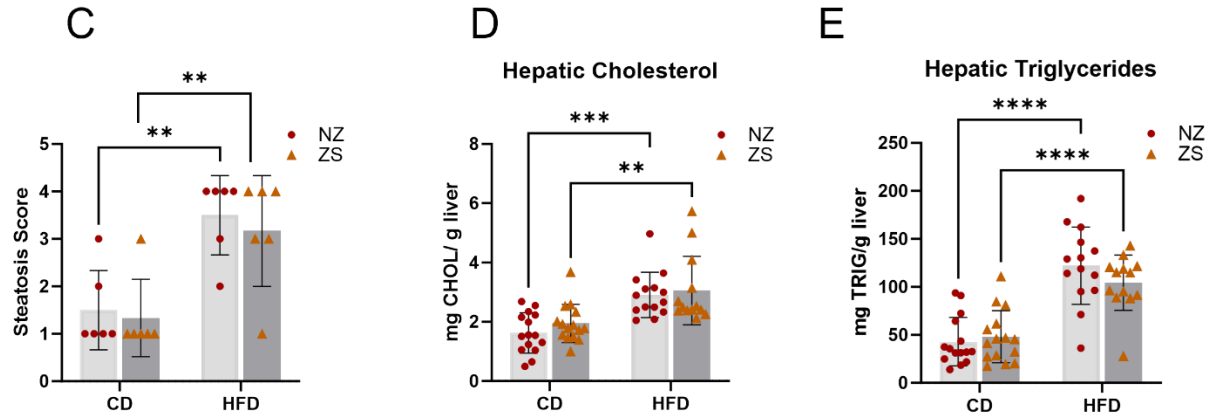


Figure 6. Effects of HFD and zinc supplementation on hepatic steatosis. Zinc supplementation did not attenuate HFD-induced steatosis nor increase hepatic triglycerides or cholesterol. 5 μ m liver tissue sections stained by (A) H&E (20 \times) and (B) Masson Trichrome (10 \times) showed hepatic morphology and macrovascular steatosis or collagen deposition, respectively. (C) Steatosis was scored as percentage of liver cells in 10, 10 \times fields per liver containing fat. (D) Hepatic levels of cholesterol (CHOL) and (E) triglycerides (TRIG). Data are reported as mean \pm SD (n = 6 mice/group, C; n = 14–15 mice/group, D, E) with significance set to 0.05. ** p < 0.05, *** p < 0.005, **** p < 0.0001. CD = control diet, HFD = high fat diet, NZ = normal zinc, ZS = zinc supplement.

3.6. Altered Gene Expression

Key regulators involved in hepatic function, fatty acid transport, and metabolism were examined by qRT-PCR for changes in gene expression. Expression of *Hnf4a*, a gene responsible for the transcriptional regulation of hepatic progenitor genes, was reduced in HFD-fed mice compared with mice fed CD (Figure 7A). Independent of fat content, there was a trend toward increased *Hnf4a* expression in zinc-supplemented mice, although they were not significantly different from mice fed normal zinc diets. Similarly, the expression of *Ppara*, a gene involved in the transcriptional regulation of hepatic lipid metabolism, was reduced in HFD-fed mice compared with CD-fed mice (Figure 7B). In CD-fed mice, a non-significant trend of increased *Ppara* expression was observed in the mice supplemented with zinc compared with mice fed a normal zinc diet. In HFD-fed mice, zinc supplementation did not significantly alter *Ppara* expression. Expression of *Apob*, a gene involved in lipid transport, was reduced in zinc-supplemented CD-fed, normal zinc HFD, and zinc-supplemented HFD-fed mice compared with normal zinc CD mice, although there was no statistical difference between the groups (Figure 7C). Increased expression of *Tnfa*, a proinflammatory gene, was observed in HFD-fed mice compared with CD-fed mice (Figure 7D). However, zinc supplementation did not change expression levels of *Tnfa* in either CD- or HFD-fed mice. Overall, zinc supplementation did not alter the expression levels of these selected genes.

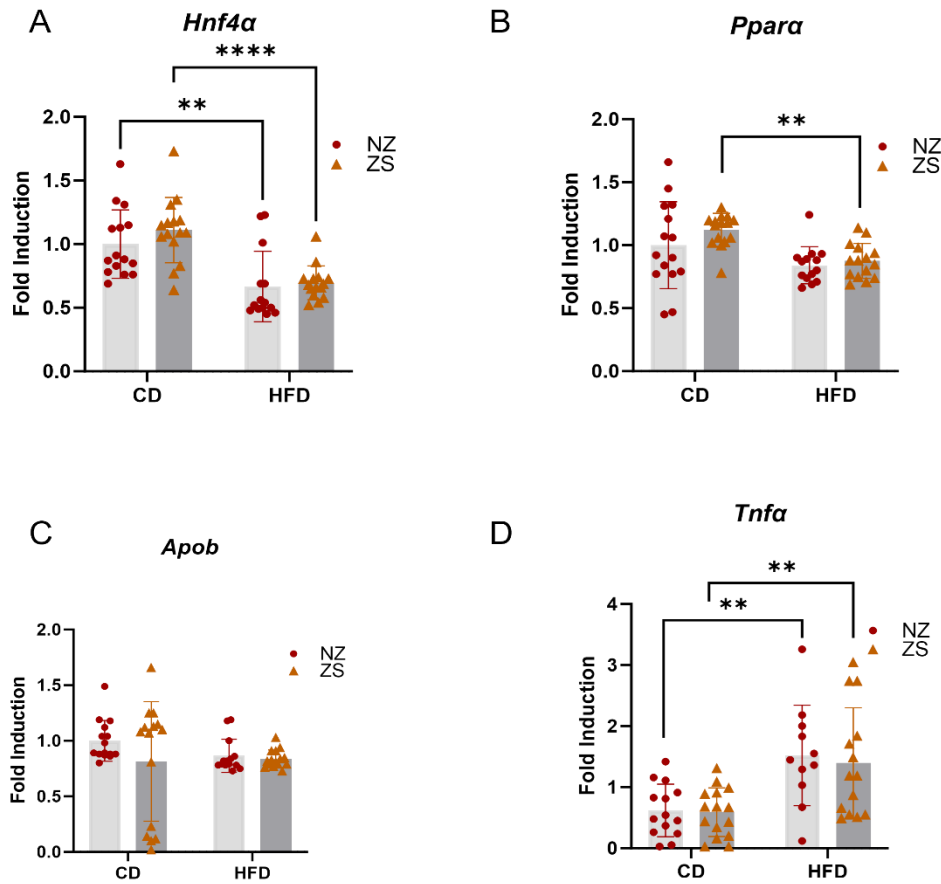


Figure 7. Effects of zinc on hepatic gene expression. Zinc supplementation did not affect HFD-induced dysregulated gene expression of transcription factors involved in hepatic function and lipid metabolism. Hepatic (A) *Hnf4α* (B) *Ppara* (C) *Apob* and (D) *Tnfa* mRNA expression. Data are reported as mean \pm SD (n = 14–15 mice/group) with significance set to 0.05. ** p < 0.05, **** p < 0.0001. CD = control diet, HFD = high fat diet, NZ = normal zinc, ZS = zinc supplement. *Hnf4α*, Hepatocyte nuclear factor 4 alpha; *Ppara*, Peroxisome proliferator-activated receptor alpha; *Apob*, apolipoprotein b; *Tnfa*, Tumor necrosis factor.

CHAPTER 4

DISCUSSION AND SUMMARY

Treatment options for MASLD are limited to dietary and lifestyle changes focused on weight loss and the reversal of syndrome factors. These lifestyle interventions are at times conducted with pharmacological therapies; however, there remains a need for an approved therapeutic agent for the treatment or prevention of MASLD. Previous studies have shown zinc deficiency is associated with increased metabolic disorders, dyslipidemia, oxidative stress, and inflammation [11, 19]. Using zinc as a preventive therapy, dietary zinc supplementation has been shown to diminish alcohol-induced steatosis [21] and improve liver function [28]. In this present study, we developed an in vivo model to test the hypothesis that dietary zinc supplementation can act as a treatment therapy to attenuate established HFD-induced MASLD. To our knowledge, this is the first animal study investigating the effects of zinc supplementation after MASLD progression. We successfully induced marked hepatic lipid accumulation by feeding mice a HFD (60% fat-kcal). The results obtained from this study demonstrated that 8-week dietary zinc supplementation after the establishment of steatotic liver disease in mice did not significantly lessen or rescue HFD-induced MASLD.

While zinc supplementation has been shown to improve lipid and glucose metabolism, which is dysregulated in steatotic liver disease progression [21], dietary zinc supplementation from our study was unable to alleviate the disrupted metabolic endpoints associated with HFD-induced MASLD. Our data is contrary to other studies [10, 22] that reported zinc supplementation improved HFD-induced liver injury and decreased hepatic lipid accumulation and serum lipids. In the animal study by Shidfar et al. (2018) [10], rats were fed a HFD for 28 weeks, with zinc and

selenium supplementation introduced in the last 8 weeks of the study. Consistent with our results, total cholesterol, HDL, ALT, AST, and glucose, as well as increased levels of steatosis, were elevated in the HFD-fed group. In contrast to our findings, serum triglycerides, cholesterol, ALT, AST, and hepatic steatosis were decreased, while hepatic levels of zinc were increased in zinc-supplemented rats. These differences may be attributed to their use of a rat animal model as opposed to the murine model used in our study. Additionally, the supplementation after disease progression was composed of both zinc and selenium, versus our investigation of the effects of zinc supplementation alone. Their results indicate the potential necessity of an additional micronutrient in addition to zinc to provide synergistic therapeutic effects on adverse phenotypes of MASLD, or the positive effects of the combined supplementation may be limited to the rat animal model.

More comparable to our study is the murine HFD model study by Qi et al. (2020) [22]. Results from their study correlated with our observations of increased obesity, hepatic lipid accumulation, and liver injury in HFD-fed mice compared with control diet mice. However, while dietary zinc supplementation did not alter metabolic endpoints in our study, Qi et al. (2020) reported improvements in fat and lean mass, glucose tolerance, hepatic injury, and lipid deposition in zinc-supplemented HFD-fed mice [22]. Possible explanations for these paradoxical differences in results may be that, whereas in our study zinc supplementation was introduced in the last 8 weeks after disease progression, Qi et al. (2020) began administering zinc supplementation concurrently with HFD for a total period of 14 weeks [22]. This suggests there may be a certain threshold of MASLD progression beyond which zinc supplementation is unable to have a therapeutic effect. Supplementation may be required well in advance of the establishment of MASLD to have the desired restorative benefits. The implication of this is that patients begin taking recommended dietary zinc supplementation either ahead of or with the consumption of HFD, of which the likelihood may be rather low.

Ppara and *Hnf4a* are zinc finger transcription factors that play key regulatory roles in hepatic gene expression, lipid homeostasis, and vLDL secretion [29, 30]. *Hnf4a* affects hepatic fat storage through the induction of lipophagy, while *Ppara*, a nutritional sensor, is essential to lipid transport and β -oxidative pathways. Our data showed decreased mRNA expression of *Ppara* and *Hnf4a* in HFD-fed mice. This is consistent with literature showing *Hnf4a* activity is decreased by fatty acids and decreased *Ppara* results in enhanced steatosis. In the alcohol-induced model of steatosis described by Kang et al. (2009)[21], zinc supplementation was reported to significantly increase the expression levels of these genes, which were unaffected by alcohol. Our results similarly showed slight increases in *Ppara* and *Hnf4a* expression due to zinc supplementation.

While our data did not confirm our hypothesis of zinc supplementation attenuating established MASLD, we have not looked at other parameters that may have contributed to the null findings. Zinc is primarily absorbed into circulation through the gut [13], and gut permeability and the microbiome are altered in MASLD [31]. How HFD may have altered gut permeability before and after zinc supplementation in this model is yet to be investigated. Studies on this may elucidate changes to zinc transporters in the gut that may have resulted in unchanged hepatic zinc levels and liver injury phenotypes between zinc-supplemented and normal zinc mice.

Potential limitations of this study may include the fat percentage of the diet administered to the HFD group. The 60% fat diet is known to cause rapid obesity in mice but presents a much higher distortion of the fat content compared with the normal rodent chow, resulting in exaggerated metabolic responses [32]. The rapid induction of obesity may have also accelerated the development of MASLD, exceeding attenuation by zinc supplementation. While a 60% fat-kcal HFD was used in the study by Qi et al. (2020), it should again be noted that the introduction of the diet and zinc supplementation began at the same time [22]. In future studies, it may be more beneficial to utilize the 45% fat rodent diet, as obesity in mice can be achieved with the 45% fat diet, albeit more slowly, and this may be more relevant to human physiology than the 60% fat diet.

Additionally, the period of zinc supplementation may have been too brief for a striking phenotypic difference to be detected between normal zinc and zinc-supplemented mice. Qi et al. (2020) provided zinc supplementation for a total duration of 14 weeks [22]. The longer treatment period may be a necessary requirement for the desired therapeutic effects of zinc on the liver. Lastly, zinc deficiency plays a vital role not only in disease progression but also in the efficacy of zinc supplementation in the reversal of excess hepatic lipids. In alcohol-associated liver disease, micronutrient depletion is commonly noted and rectified by replenishing essential micronutrients. Particularly, clinical studies have established significantly low serum and liver zinc concentrations in patients with alcohol-induced steatosis, hepatitis, and cirrhosis [33]. Our study did not assess zinc status prior to introducing zinc supplementation, which would impact the effectiveness of the supplemented zinc. On the one hand, if our HFD-fed mice were zinc deficient after 12 weeks, improved hepatic lipids and glucose metabolism may have been observed after zinc supplementation. On the other hand, if our HFD-fed mice were not zinc deficient after 12 weeks, the additional zinc may have been simply excreted out of the body, explaining why there were no observed differences in hepatic zinc levels. These limitations will be addressed in our future studies. Taken together, our data demonstrate the use of zinc supplementation as an effective therapeutic for the treatment of established MASLD requires a sensitive consideration of the initiation and duration of administration for developed MASLD. Further research insights are required to elucidate and ascertain underlying mechanisms to provide desirable outcomes of zinc supplementation for MASLD.

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APPENDIX

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3. University of Louisville Graduate Student Travel Award- AASLD annual The Liver Meeting, November 10-14, 2023, Boston, MA, USA
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Presentations

1. **Bolatimi, OE**; Hua, Y; Luo, J; Gripshover, TC; Watson, WH; Wahlang, B. Investigating the sex-specific effects of environmental toxicant mixtures on steatotic liver disease.

- American Association of the Study of Liver Disease. The Liver Meeting 2023. Boston, MA, USA. Poster Presentation.
2. **Bolatimi, OE**; Hua, Y; Luo, J; Gripshover, TC; Watson, WH; Wahlang, B. Investigating the sex-specific effects of environmental toxicant mixtures on steatotic liver disease. Center for Integrative Environmental Health Science Symposium, 2023. Louisville, KY, USA. Poster Presentation.
 3. **Bolatimi, OE**; Hua, Y; Luo, J; Gripshover, TC; Watson, WH; Wahlang, B. Investigating the sex-specific effects of environmental toxicant mixtures on steatotic liver disease. Ohio Valley Society of Toxicology, 2023. Indianapolis, IN, USA. Platform Presentation.
 4. **Bolatimi, OE**; Hua, Y; Luo, J; Gripshover, TC; Watson, WH; Wahlang, B. Investigating the sex-specific effects of environmental toxicant mixtures on steatotic liver disease. Research! Louisville, 2023. Louisville, KY, USA. Poster Presentation.
 5. **Bolatimi, OE**; Hua, Y; Luo, J; Wahlang, B. The sex-specific effects of dioxin and dioxin-like chemicals on hepatic receptor activation. Summer Ohio Vally Society of Toxicology, 2023. Virtual. 3 Min Presentation.
 6. **Bolatimi, OE**; Young, JL; Wahlang, B; Luo, J; Head, KZ; Gripshover, TC; Lin, Q; White, C; Adiele, NV; Watson, WH; Wilkerson, C; Cai, L; Cave, MC. Effects of subchronic zinc supplementation on high fat diet-induced non-alcoholic fatty liver disease. American Association of the Study of Liver Disease. The Liver Meeting 2022. Washington DC, USA. Poster Presentation.
 7. **Bolatimi, OE**; Young, JL; Wahlang, B; Luo, J; Head, KZ; Gripshover, TC; Lin, Q; White, C; Adiele, NV; Watson, WH; Wilkerson, C; Cai, L; Cave, MC. Effects of subchronic zinc supplementation on high fat diet-induced non-alcoholic fatty liver disease. Ohio Valley Society of Toxicology, 2022. Louisville, KY, USA. Platform Presentation.
 8. **Bolatimi, OE**; Young, JL; Wahlang, B; Luo, J; Head, KZ; Gripshover, TC; Lin, Q; White, C; Adiele, NV; Watson, WH; Wilkerson, C; Cai, L; Cave, MC. Effects of subchronic zinc

supplementation on high fat diet-induced non-alcoholic fatty liver disease. Research! Louisville, 2022. Louisville, KY, USA. 2nd Place, Master's Basic Science Poster Competition.

9. **Bolatimi, OE**; Young, JL; Wahlang, B; Luo, J; Head, KZ; Gripshover, TC; Lin, Q; White, C; Adiele, NV; Watson, WH; Wilkerson, C; Cai, L; Cave, MC. Effects of subchronic zinc supplementation on high fat diet-induced non-alcoholic fatty liver disease. Summer Ohio Vally Society of Toxicology, 2022. Virtual. 3 Min Presentation.

Peer-Reviewed Publications

1. **Bolatimi OE**, Head KZ, Luo J, Gripshover TC, Lin Q, Adiele NV, Watson WH, Wilkerson C, Cai L, Cave MC, Young JL. Can Zinc Supplementation Attenuate High Fat Diet-Induced Non-Alcoholic Fatty Liver Disease? *Int J Mol Sci.* 2023 Jan 16;24(2):1763. doi: 10.3390/ijms24021763. PMID: 36675277; PMCID: PMC9864360.
2. Head KZ, **Bolatimi OE**, Gripshover TC, Tan M, Li Y, Audam TN, Jones SP, Klinge CM, Cave MC, Wahlang B. Investigating the effects of long-term Aroclor 1260 exposure on fatty liver disease in a diet-induced obesity mouse model. *Frontiers in Gastroenterology (Lausanne).* 2023;2:1180712. doi: 10.3389/fgstr.2023.1180712. PMID: 37426695; PMCID: PMC10327714.