

**EFFECTS OF DIETARY ZINC MANIPULATION
ON INSULIN ACTION IN TYPE 2 DIABETES MELLITUS:
A STUDY IN THE db/db MOUSE**

By Sharon Simon

**A thesis submitted to the Department of Foods and Nutrition
in partial fulfillment of the requirements
for the degree of Master of Science**

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ABSTRACT

EFFECTS OF DIETARY ZINC MANIPULATION ON INSULIN ACTION IN TYPE 2 DIABETES MELLITUS: A STUDY IN THE db/db MOUSE

S.F. Simon, MSc. Thesis, Department of Foods and Nutrition

Type 2 diabetes mellitus (DM-2) is a chronic disease characterized by disturbances in insulin action. Recent evidence suggests that environmental factors, such as nutrition, can play a profound role in modulating both the initiation and progression of the disease. One of the possible nutritional factors that may influence diabetic pathogenesis is zinc. Zinc is crucial to pancreatic insulin storage and animals deficient in zinc exhibit a higher degree of insulin resistance, impaired glucose tolerance, and reduced insulin secretion. Diabetic animal models and humans also display an excessive urinary zinc excretion (hyperzincuria), with no compensatory increases in intestinal absorption of zinc. The hyperzincuria may induce a zinc deficiency, as diabetic patients have depressions in serum and pancreatic zinc concentrations and diabetic animal models have whole body maldistributions of zinc.

The objective of this thesis was to investigate the effects of dietary zinc deficiency and zinc supplementation on diabetic parameters in db/db mice, a genetic model for DM-2. Weanling db/db mice and their lean littermate controls (db/m) were fed zinc deficient (dbZD or lnZD), zinc adequate control (dbC or lnC), or zinc supplemented (dbZS or lnZS) diets (3, 30, 300 ppm zinc, respectively) for 6 weeks. At 10 weeks of age, mice were assessed for zinc status (serum, femur, pancreas, kidney), serum (glucose, insulin) and urinary (glucose, protein, zinc) indices of diabetes, as well as skeletal muscle (gastrocnemius) insulin receptor concentrations and tyrosine kinase activity.

After the 6 week feeding trial, the db/db genotype exhibited significantly higher body weight, glycemia, insulinemia, and glycosuria compared to db/m mice. The db/db group also had a significantly lower pancreatic zinc concentration and a higher urinary zinc concentration. Dietary treatment influenced DM-2 status as zinc supplemented diabetics (dbZS) maintained a significantly lower serum glucose concentration than the dbZD group (498.1 ± 74.3 vs. 344.5 ± 51.0 mg/dl, respectively). In contrast, serum glucose concentration was elevated in the dbZD compared to dbC groups (498.1 ± 74.3 versus 424.9 ± 62.5 mg/dl, respectively). These serum glucose concentrations were clearly reflective of body zinc status, as there was a significant negative correlation ($r = -0.37770$; $P = 0.0149$) between femur zinc and serum glucose concentrations. The dbZS group also had a significantly lower body weight and plasma insulin concentration, as well as a repletion of pancreatic zinc concentration to lean control values. These findings suggest that addition of zinc to the diet may restore peripheral glucose disposal and/or lessen excessive insulin secretion from the pancreas. However, insulin receptor concentration and tyrosine kinase activity were unaltered in db/db and db/m groups. The results of this study reveal that zinc influences the serum glucose concentration of genetically diabetic mice and that supplementation may offer a role in ameliorating diabetic signs.

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I. Literature Review

Introduction

Diabetes mellitus (DM) is a chronic disease in which excessive quantities of glucose are retained in the bloodstream (Kahn, 1994). The hormone insulin, through its synthesis and secretion in the pancreas, normally operates to regulate fluctuating levels of blood glucose. In diabetes, this homeostatic mechanism does not operate properly. High blood glucose concentrations in diabetes result either from an insufficiency of insulin (DM-1) or from a cellular resistance to the actions of insulin (DM-2) (Kahn, 1994). DM-2, the focus of the present study, is a common and chronic adult-onset form of diabetes that is initially characterized by hyperglycemia and compensatory hyperinsulinemia. Without proper management, DM-2 often leads to serious complications such as coronary heart disease, renal failure, blindness, limb amputations, neurological complications and premature death (Knowler et al., 1995). Behavioural and dietary modifications are increasingly being recognized as crucial tools for prevention. Dietary modifications generally focus on the macronutrients, however, trace mineral nutrition may play an important role.

Despite current knowledge about potential interrelationships between zinc, diabetes and insulin action, there is still little information available from the literature as to the potential benefits of zinc supplementation in diabetes. Thus, it is the purpose of this chapter and of this thesis to explore potential relationships between diabetes and zinc metabolism, as well as to investigate the influences of both zinc deficiency and zinc supplementation in an animal model of DM-2.

DM-2: Epidemiological Aspects

DM-2 is a disease which is encountered in virtually every part of the world. In industrialized countries, DM-2 is virtually absent in the young, but increases progressively in both sexes to peak at 10% of the population by 70 years of age (Laakso & Pyörälä, 1985).

DM-2 appears to have both genetic and environmental determinants. Its genetic component can be seen by its higher incidence in specific ethnic groupings, such as in Native American and Polynesian populations (Ekoe, 1988). Another commonality amongst these groups is the relationship between DM-2 and obesity. Obesity is very common in DM-2 patients and the incidence of DM-2 has been shown to increase strongly with increasing weight or body mass index (BMI) (Knowler et al., 1990). Most populations with a high prevalence ratio for DM-2 also have a high prevalence ratio for obesity (i.e. Pima Indian populations of Arizona; Mexican Americans) (Knowler et al., 1990).

While DM-2 clearly displays a genetic component, diet and lifestyle can still have significant impact. Marshall et al. (1994) found that the limitation of dietary fat may decrease the risk of progression toward DM-2. This emphasizes that diet and lifestyle may play an important role in the initiation and progression of DM-2.

Insulin Action and DM-2

DM-2 is a chronic disorder of insulin action. Insulin accelerates glucose transport in muscle and adipose tissue, regulates activities of intracellular enzymes and regulates the transcription of selected genes (Taylor et al., 1994). Disturbances in insulin action can influence the disposal of glucose and proteins into tissues (Taylor et al, 1994), as well as produce abnormalities in fat metabolism (Groop et al., 1989). For the purposes of this literature review, the primary focus will be the effects of DM-2 on glucose metabolism.

Glucose metabolism is tightly regulated by a number of interrelated mechanisms. Glucose homeostasis is dependent on three processes that must occur in a coordinated fashion in the body.

i) Insulin Secretion

Insulin is synthesized and stored in the secretory granules of the pancreatic β -cells, from where it is continually secreted into the portal circulation. Insulin secretion is controlled by multiple signals which include substrate availability, hormone concentrations, and autonomic nervous system activity (Taylor et al., 1994). Extracellular glucose and amino acids are the principle regulators of insulin secretion (Pfeifer et al., 1981). In the early stages of DM-2, insulin is secreted in excess to compensate for high concentrations of circulating glucose (DeFronzo, 1992). Over time, hyperinsulinemia may subside as pancreatic insulin responses to glucose diminish. The result is often a need for exogenous insulin administration to maintain normoglycemia (Taylor et al., 1994).

ii) Glucose Uptake or Disposal

Peripheral tissues (muscle and adipose) contain insulin receptors that ultimately signal the carrier-mediated uptake of glucose into the cell from the bloodstream. Glucose uptake is dependent on a number of mechanisms including hormone-receptor concentrations, hormone-receptor affinity, and glucose transporter concentration and function (Crettaz & Jeanrenaud, 1980). In DM-2, an inadequate or impaired cellular response to insulin is termed insulin resistance. Resistance to insulin is considered one of the primary precursor disturbances evident before the clinical diagnoses of DM-2 (Taylor et al., 1994).

iii) Suppression of Hepatic Glucose Production

The third mechanism in which insulin participates in glucose homeostasis is in the suppression of hepatic glycogenolysis and gluconeogenesis (Consoli et al., 1989). In DM-2, there is a resistance to hepatic glucose suppression. Hepatic resistance to insulin contributes to an excessive basal output of glucose by the liver, therefore exacerbating hyperglycemia (DeFronzo, 1987). However, excess hepatic glucose released accounts for only a small percentage of the defect in whole body glucose metabolism. Impaired disposal of glucose into peripheral tissues is considered primary (DeFronzo, 1987).

The Progression Towards DM-2

The defects in endocrine function that cause or precede DM-2 are thought to be insulin resistance and insulin deficiency. It is clear that DM-2 patients are resistant to the actions of insulin. Patients show a diminished response to both endogenous and exogenous insulin in all target tissues (DeFronzo, 1992). However, it has been proposed that insulin deficiency (due to decreased pancreatic insulin secretion) may exacerbate underlying insulin resistance. This is known because aggressive insulin replacement therapy can markedly increase insulin action in vivo (Garvey et al., 1985).

The initiating events in the progression towards DM-2 can be described to occur in distinct phases (see Figure 1). As obesity is a commonly associated risk factor for DM-2, obese subjects are presented against those of normal weight for each phase. The initial stage leading to DM-2, the obese NGT (normal glucose tolerance) state is that of an essentially normal glycemic response to insulin secretion. Obese subjects show a slightly elevated insulin secretion to maintain normoglycemia. In Obese IGT (impaired glucose tolerance), obese subjects exert a very elevated insulin secretory response to maintain near normal glycemic concentrations. Presumably as insulin resistance increases, subjects progress from impaired glucose tolerance to clinical DM-2. This, in initial stages, is defined as hyperglycemia with hyperinsulinemia. This increase in insulin resistance, which is discussed more fully in following sections, results from various possible changes in insulin, insulin receptor binding and/or alterations in the insulin signal transduction pathway. These changes that cause insulin resistance may be fully due to genetics, or diet, or both. In the last phase of DM-2, subjects can no longer

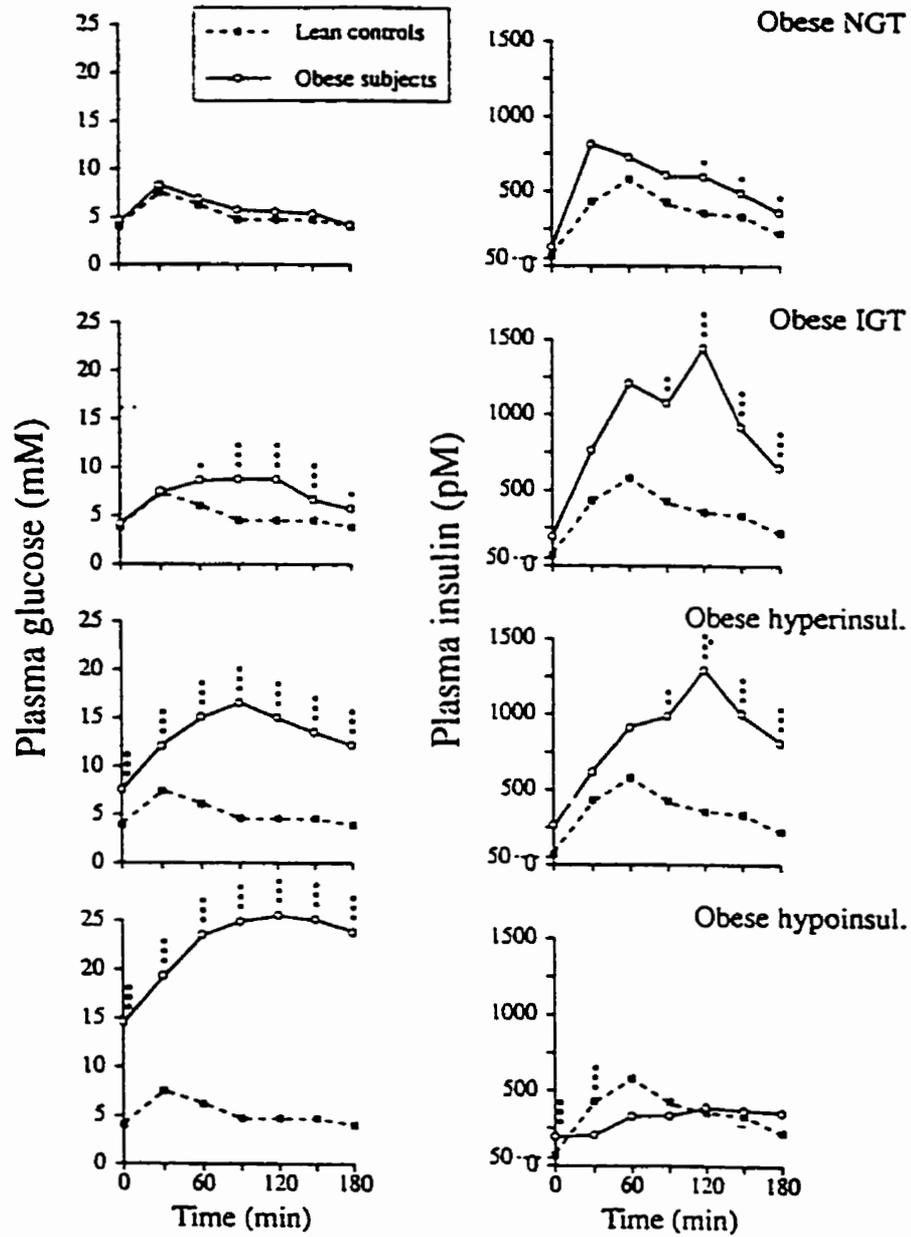


Figure 1. Phase Events in the Progression Towards DM-2

Adapted from: Felber et al. (1981)

* = $p < 0.05$ ** = $p < 0.02$ *** = $p < 0.001$

maintain hyperstimulation of the pancreatic islet cells, and insulin secretion drops dramatically. This phase, often termed β -cell exhaustion or glucose toxicity, requires exogenous insulin administration to maintain normoglycemic concentrations (Taylor et al., 1994).

Molecular Mechanisms in Insulin Action

Insulin resistance is clearly one of the primary events leading to clinical DM-2. At the cellular level, there are three distinct processes that influence or interfere with insulin action. Insulin receptor binding, signal transduction events, and the biological signal responses all determine the magnitude of insulin action.

i) Insulin Binding to the Insulin Membrane Receptor

The insulin receptor is a heterotetrameric transmembrane glycoprotein composed of 2 α and 2 β -subunit structures (Myers and White, 1993). The extracellular α -subunits contain the insulin binding domain, while the β -subunits span the extracellular, membrane and intracellular domains (see Figure 2).

The adequate binding of insulin to its receptor is influenced by the concentration of circulating insulin, the concentration of receptors and the affinity of the receptor for insulin (Kahn,1994). In DM-2 and other insulin resistant states such as obesity, higher concentrations of circulating insulin will decrease insulin receptor concentration in a dose-dependent manner in a process known as 'down-regulation' (Gavin et al 1974). The concept that insulin directly influences the concentration of its own receptors has been

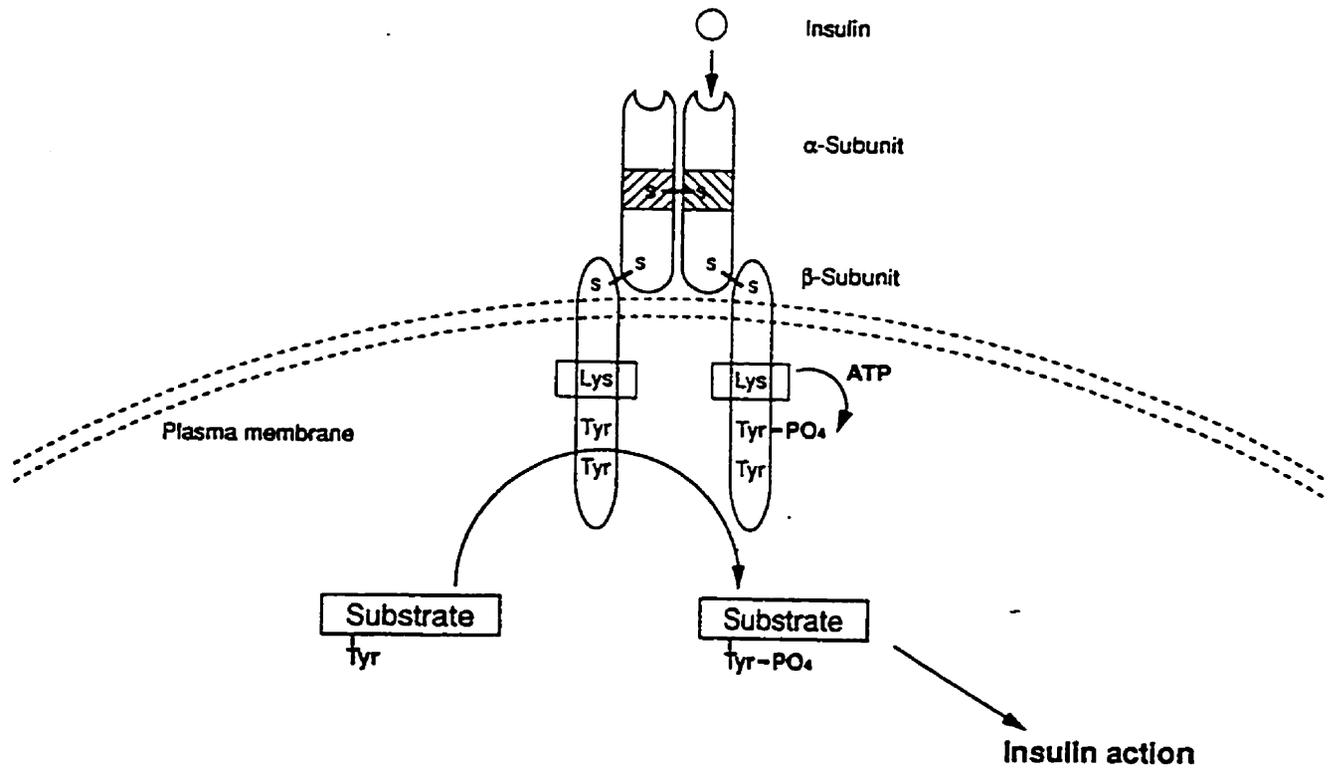


Figure 2: Insulin Binding to the Transmembrane Insulin Receptor

Adapted from: Kahn and White (1988)

confirmed both *in vitro* and *in vivo* (Gavin et al., 1974; Kobayashi et al., 1978). This cycle of lower receptor concentration with higher insulin concentrations can be exacerbated by a decrease in affinity of the insulin hormone for its receptor (Kahn, 1994). However, indications are that these states may be transient and can revert to normal when initial conditions are restored. In a study by Bathena et al. (1986), rats fed a high-fat diet developed insulin resistance due to lower insulin receptor numbers and binding affinity, which were then restored to normal with a standard diet.

ii) Transformation of Binding to Signal Transduction

Post-receptor binding events may be another source of insulin resistance in DM-2. When insulin binds to the α -subunit of its receptor, a conformational change in the receptor results in the autophosphorylation of tyrosine residues on the β -subunits and activation of tyrosine kinases (see Figure 2). This leads to the transfer of phosphate groups from ATP to multiple tyrosine, serine and threonine residues on the insulin receptor itself, as well as phosphorylation of intracellular substrate proteins (Rosen, 1987; Myers & White, 1993).

Considerable evidence suggests that the tyrosine kinase activity of the insulin receptor is crucial for proper insulin signalling. Naturally occurring mutations of the insulin receptor that inhibit kinase activity and block autophosphorylation are associated with severe insulin resistance (Odawara et al., 1989). In cultured cells, insulin receptors with point mutations that destroy ATP binding and thus kinase activity also show no signalling activity (McClain et al., 1987). Decreases in insulin receptor autophosphorylation and

receptor tyrosine kinase activity have also been reported in DM-2 patients (Freidenberg et al., 1987). Thus, initial insulin stimulated phosphorylation events are crucial for the activation of the downstream insulin cascade.

iii) Biological Effectors in the Insulin Cascade

Insulin stimulates the transport of sugars, amino acids, and ions across the plasma membrane of certain target tissues such as muscle and adipose (Pfeifer et al., 1981). One of the major actions of insulin in insulin sensitive tissues is the stimulation of glucose transport across the plasma membrane. The hydrophilic glucose molecule uses carrier-mediated passive transport proteins to gain entry into cells (Barnard & Youngren, 1992). The transport proteins have a succession of hydrophilic and hydrophobic domains which insert into membranes to create a pore through which glucose can be transported. Stimulation by insulin translocates the glucose transporters from a microsomal pool to the plasma membrane (Barnard & Youngren, 1992). In a state of insulin resistance and DM-2, this microsomal pool of transporters appears to be unaltered (Scheck et al., 1991). It appears that defects responsible for insulin resistance in DM-2 are a result of upstream signal events.

The Role of Zinc in Insulin Action

It is evident from the literature that dietary intervention can play a role in modulating insulin resistance in DM-2 (Taylor et al., 1994). The trace element zinc, with its possible

participation in insulin action, may prove to be another example. Zinc is an essential micronutrient that has both catalytic and structural functions in the body (Bettger & O'Dell, 1993). In its catalytic role, zinc acts as a co-factor for zinc metalloenzymes, such as in transferases, hydrolases, isomerases and oxidoreductases (Bettger & O'Dell, 1993). Structurally, zinc participates in the conformation of metalloproteins in cysteine and histidine-rich domains. Zinc also acts as a structural component in peptide hormone storage, release and metabolism (Bettger & O'Dell, 1993; Vallee & Falchuk, 1993).

The complex and diverse biochemical functions of zinc also include its effects on plasma membrane function. Bettger and O'Dell (1993) propose that zinc is crucial to plasma membrane function in that it provides the conformational stability required to permit normal signal transduction. Dietary deprivation of zinc would then alter receptor binding and membrane channels, eventually leading to the cell's desensitization to external messengers. If this is the case for the insulin receptor, then dietary zinc deficiency would generate an increase in resistance to the actions of insulin. This would ultimately result in a reduced rate of cellular growth, differentiation and proliferation, which are characteristic signs of dietary zinc deficiency (Bettger & O'Dell, 1993).

To explore this potential mechanism further, the role of zinc in pancreatic insulin function and peripheral tissue receptor binding will be discussed.

i) Pancreatic Insulin Storage and Release

Zinc is a structural component of insulin stored in the pancreas. Scott and Fisher (1938) were the first to observe that the human pancreas contains zinc in its crystalline

structure and that the human diabetic pancreas contains 50% less zinc. In most species, insulin is stored in the β -cells of the pancreas as a hexameric crystal containing a variable number of zinc molecules (Dodson et al., 1978). Alterations in this zinc-insulin crystal formation have been shown to alter the antigenic determinants of insulin in solution (Arquilla et al., 1978b). Zinc ions also enhance the effectiveness of insulin in vitro (Couldston & Dandona, 1980; May & Contoreggi, 1982). Mice pretreated with zinc injections show a two-fold increase in insulin binding to liver plasma membranes compared to mice injected with a sodium control (Arquilla et al., 1978a).

In a state of dietary zinc deficiency, the pancreas is one of the first tissues to lose appreciable amounts of zinc (Roth & Kirchgessner, 1981). In 1969, Boquest & Lernmark were the first to observe a decreased granulation of the pancreatic β -cells from zinc deficient Chinese hamsters. Since then, it has been suspected that lower pancreatic zinc concentrations could significantly alter insulin's synthesis, storage, release or structural integrity in circulation (Roth & Kirchgessner, 1981). Zinc deficient animals often exhibit reduced circulating plasma insulin concentrations. Huber & Gershoff (1973) found serum insulin, measured by an in vitro adipose tissue assay, to be significantly reduced in zinc deficient rodents when compared to *ad libitum* and pair-fed controls. Insulin secretion from the pancreas, measured by incubation of the pancreas with glucose, was also significantly reduced in zinc deficient rats compared to *ad libitum* and pair-fed controls. In a study by Droke et al. (1993), serum insulin concentrations in zinc deficient lambs were found to be lower 1 hour after feeding when compared to lambs fed marginal (5 ppm Zn) or zinc adequate (40 ppm Zn) diets. In addition, a maternal zinc deficiency

can also reduce the content of insulin in the pancreas of the fetus (Robinson & Hurley, 1981).

Studies of insulin metabolism in zinc deficient animals have the potential to be confounded by the fact that zinc deficient animals often exhibit significant anorexia (Prasad, 1983). Reduced food intake itself would increase insulin sensitivity and reduce circulating plasma insulin concentrations (Droke et al., 1993). More research with pair-fed (caloric reduced) controls, such as the studies cited above, is required to better discriminate findings in studies utilizing severe zinc deficiencies.

ii) Insulin Receptor Binding

Evidence for the structural role of zinc in peripheral tissue hormone/receptor binding is just beginning to be generated in the literature. Binding studies of the insulin receptor have confirmed that small regions in cysteine-rich domains of the insulin receptor are responsible for hormone binding specificity (Gustafson & Rutter, 1990). These cysteine-rich regions often contain zinc-binding sites in conformations such as zinc fingers, clusters or twists that act to enhance structural integrity and function (Vallee & Falchuk, 1993). Sunderman & Barber (1988) recently reported the discovery of zinc finger loop domains in the human insulin receptor. Arquilla et al. (1978a) found that administration of zinc *in vitro* enhances the binding of insulin in liver plasma membranes. Furthermore, Gomot et al. (1992) observed that a zinc deficient diet (< 0.2 ppm zinc for 4 weeks) produced a significantly lower insulin receptor binding in rat adipocytes when compared to *ad libitum* controls. However, when the zinc deficient group was compared to pair-fed

controls, there were no significant differences in insulin receptor binding. The authors suggest that the lower insulin receptor binding in both the zinc deficient and the pair-fed control groups could be the result of caloric restriction, impaired receptor synthesis and/or decreased cell membrane fluidity and receptor translocation into the cells.

Tyrosine kinase phosphorylation activity has been found to be elevated with the addition of zinc in vitro. Findik and Presek (1988) observed that Zn^{2+} significantly enhanced tyrosine kinase phosphorylation activity compared to other cations (Mg^{2+} , Mn^{2+}) in human platelet membranes. Ezaki (1989) found that Zn^{2+} ions also stimulated glucose transport activity in rat adipocytes when compared to buffer alone. Further development of techniques and study are needed to define the specific role of zinc ions in the conformation and binding of insulin to its receptor.

Zinc Status in Type 2 Diabetes Mellitus

Given the role of zinc in pancreatic and cell surface receptor function, it becomes important to determine the zinc status of DM-2 patients and diabetic animal models. Significantly, one of the clinical manifestations of DM-2 is an excessive excretion of zinc in the urine (hyperzincuria), with no compensatory increases in intestinal absorption of zinc (Kinlaw et al., 1983). This hyperzincuria is commonly observed in a number of hypercatabolic states such as surgery, burns, injuries, protein deprivation and starvation (Prasad, 1983). Patients with cirrhosis of the liver, nephrotic syndrome, and sickle cell disease also present with higher zinc excretion (Prasad, 1983). In diabetes, the degree of

hyperzincuria evidently appears to be proportional to the severity of the diabetic state (Pidduck et al., 1970; Walter et al., 1991).

The effects of hyperzincuria on tissue zinc status have been investigated in human DM-2 patients and significant alterations in zinc metabolism have been observed. Blostein-Fujii et al. (1997) observed that plasma zinc concentrations and plasma activity of 5'-nucleosidase (a zinc-containing enzyme) were significantly lower in DM-2 women. Winterberg et al. (1989) found serum zinc concentrations to be negatively correlated with glycosylated hemoglobin (a measure of longstanding glucose status) as well as serum glucose concentrations. Kinlaw et al. (1983) found lower (10%) serum zinc concentrations in 25% of their DM-2 patients as well as significantly depressed serum zinc concentrations after an oral zinc tolerance test. This may suggest a malabsorption of zinc by diabetics as well as an excessive excretion. Sjögren et al. (1988) also observed significant depressions in plasma zinc (11%) as well as a two fold elevated urinary zinc excretion in 18 DM-2 patients. In a similar study by Walter et al. (1991), significant depressions in serum zinc (16%) were associated with a sixfold increase in urinary zinc excretion.

Various animal models of obesity and DM-2 are also used to assess physiological events in DM-2 in a controlled setting. The most common are the diabetes (db/db) and the obese (ob/ob) mice. In the ob/ob mouse, Bégin-Heick et al. (1985) reported higher zinc concentrations in muscle, liver and brown adipose tissue, while concentrations of zinc were lower in the pancreas and femur. In the Israeli diabetic sand rat, hyperinsulinemia and hyperglycemia were associated with significant reductions in

hepatic, renal and muscle zinc concentrations, but elevated concentrations of zinc in the femur (Raz et al., 1988). In the db/db mouse, depressions in plasma, femur and pancreatic zinc concentrations were found in association with significant urinary zinc excretion and elevated serum glucose concentrations (Levine et al., 1983; Southon et al., 1988). The db/db mouse also exhibits impairments in cell-mediated immunity, gonadal function and wound healing, all of which may be indicative of a deficiency in zinc (Coleman, 1982). Given this evidence of altered zinc metabolism in animals and humans, it has been proposed that a subset of DM-2 patients may suffer a zinc deficiency, thus possibly exacerbating their diabetic condition.

Zinc Supplementation and Diabetes

The effects of a zinc repletion or supplementation on tissue zinc status have had only limited examination in the literature. In human studies, Winterberg et al. (1989) found that DM-1 patients supplemented with zinc (50 mg zinc oxide/day for 3 weeks) showed a significantly higher serum zinc concentration as well as a significantly lower serum glucose concentration. In DM-2 women, Blostein-Fujii (1997) also found a significantly higher serum zinc concentration with dietary zinc supplementation (30 mg/day as amino acid chelate for 3 weeks). It did not, however increase 5'-nucleotidase activity to the degree observed in non-diabetic controls. In animal model studies, Bégin-Heick et al. (1985) observed that zinc supplementation (1000 ppm Zn) to 9-12 week old ob/ob mice for 4 weeks lowered fasting plasma glucose and insulin concentrations during glucose

tolerance testing. Dietary zinc also diminished abnormally elevated insulin secretory responses to glucose in pancreatic islets. In the db/db mouse, Levine et al. (1983) determined that 30 ppm zinc as zinc acetate in drinking water slightly repleted serum and femur concentrations of zinc. However, their study is inconclusive regarding diabetic parameters in that they failed to test serum glucose or insulin concentrations. In an examination of the current literature, there no known studies examining the effects of zinc supplementation on diabetic parameters in a DM-2 animal model.

An Animal Model of DM-2: The Genetically Diabetic Mouse (db/db)

Several different rodent models are available for the study of diabetes. The best studied models develop diabetes from a single gene mutation, ie. obese (ob), diabetes (db), fat (fa), tubby and yellow (Coleman, 1982). The single gene mutation allows for accurate breeding, as well as identification of new mutations.

The diabetes mouse (db), the mouse model used in this investigation, is an inbred strain of C57BL/KsJ (BL/Ks) mice with an inherited autosomal mutation for diabetes on chromosome 4 (Coleman, 1982). This single gene mutation is also closely linked to the coat colour gene 'misty' (m), which facilitates identification of the heterozygous lean black (db/m) breeding stocks from the grey homozygous recessive leans (m/m). The db/db black homozygous diabetic mouse is characterized by marked obesity, hyperphagia and hyperglycemia (Coleman, 1978) (Table 1). It exhibits increases in plasma insulin concentration by 10 days of age, peaking at 6-10 fold normal at 2-3 months, and then drops off to near normal concentrations. This decline in insulin function is precipitated

by increasing pancreatic pathology, with the pancreatic islets undergoing significant atrophy. During the early stages of the disease, pancreatic cells become increasingly hyperplastic and hypertrophic in an attempt to manage rising glycemia (Coleman, 1978; Shafrir, 1992).

i) Origins of Diabetes in the db/db Mouse

The causative factors responsible for diabetes in the db/db mouse are still under investigation. However, much information has also been gained from parabiotic experiments of db and normal mice. In parabiotic experiments, Coleman and Hummel (1969) found that when normal and diabetic (db/db) mice were paired, it resulted in death by starvation of the normal mouse, usually within 3–4 weeks. In their experiments, they determined that the diabetic db/db mouse secreted, but did not respond to, a satiety factor that prevents overeating. The lean mouse in the parabiotic experiment received this satiety factor from db, resulting in a diminished eating drive and eventual starvation. The db mouse in turn, did not respond to the secreted satiety signal and exhibited hyperphagia.

This faulty satiety signal response in the db/db was suspected to be a result of defects in the hypothalamus (Coleman, 1978). It is now suspected that this satiety factor that the db/db mouse secreted, but did not respond to was the hormone leptin (Caro et al., 1996). Leptin is considered integral to the regulation of energy balance, appetite and degree of adipose tissue storage in humans and animal models (Caro et al., 1996). In the ob/ob mouse, the obese gene has been found to contain a mutation that results in a complete leptin deficiency (Zhang et al., 1994). In experiments by Halaas et al. (1995), exogenous

Table 1. The Life Cycle of the db/db Mouse

Life Cycle of the db/db Mouse ¹	
Weeks of Age	
1-2	-rising plasma insulin to maintain normoglycemia
2-4	-significant deposition of fat in axillary and inguinal regions -higher plasma glucose concentrations -lower number of β -cell granules -significant insulin resistance -no response to exogenous insulin administration
6-7	-significant polyuria, polydipsia, polyphagia, and glycosuria -hyperactive lipogenesis -marked weight gains
8-12	-hyperglycemia peaks at 400-600 mg/dl -6-10 fold increase in insulinemia
16-24	-hyperinsulinemia recedes - β -cells have significant degranulation -decrease in body weight -dilation of pancreatic ducts -animals become ketotic
24-40	-renal, microvascular and neuropathic complications from diabetes -survival is rare beyond 40 weeks of age

¹ Data compiled from Hummel et al., 1966; Coleman, 1978 and 1982; Leiter et al., 1981 & Shafir, 1992.

leptin administered to the ob/ob mouse resulted in a 40% decrease in body weight within 33 days. In the db/db mouse, there was no response to exogenous leptin administration, either in body weight or food intake (Halaas et al., 1995). It has been determined that the db/db mouse secretes leptin, but exhibits a mutation of the leptin receptor that renders the mouse resistant to the actions of this hormone (Chen et al., 1996; Lee et al., 1996).

II. Study Rationale

The interrelationships between zinc and DM-2 are multi-fold. The micronutrient zinc plays an important role in glucose homeostasis as exemplified by increases in serum glucose with zinc deficiency. Zinc is present in the conformational structure of insulin and may be important for insulin storage and release from the pancreas. Zinc may also play an important role in peripheral insulin receptor binding, as putative zinc fingers have been reported in the human insulin receptor. In DM-2, there is also an excess excretion of zinc in the urine, thus providing for the possibility of an altered zinc metabolism or zinc deficiency.

It is the hypothesis of this study that dietary zinc may influence the development of insulin resistance and that supplementation of dietary zinc will improve diabetic parameters in the db/db mouse model of DM-2. The objective of this experiment was to investigate the effects of dietary zinc deficiency and supplementation on diabetic parameters in the DM-2 mouse model db/db. The effects of dietary zinc manipulation on tissue zinc status were determined by zinc concentrations in the femur, serum, pancreas and kidney. An assessment of diabetic progression involved analyses at both the molecular and physiological levels. At the whole body level, serum glucose and insulin, liver lipid, and urine glucose, protein, zinc and creatinine concentrations were measured. At the molecular level, the concentration of insulin receptors as well as tyrosine kinase activity were assessed. These indices allowed for an examination of any alterations in the underlying molecular mechanisms of insulin action. These molecular analyses also

allowed us to investigate a possible location for the insulin receptor defect, be it at the cell membrane surface or an intracellular signal kinase alteration.

This study is unique in its scope as no other investigations in the db/db mouse have involved a dietary supplementation of zinc as well as a zinc deficiency. While the literature contains some evidence for relationships between zinc status and diabetes, most studies do not attempt to manipulate the dietary intakes of zinc. A comprehensive assessment of the role of zinc supplementation in diabetic animal models in the literature is lacking. The one report of zinc supplementation in the db/db mouse involved no analysis of diabetic parameters (Levine et al., 1983). Since diabetic animals often have low zinc status and zinc is known to influence glucose concentrations in non-diabetic rodents, it would seem logical to investigate the potential benefits of zinc supplementation in diabetic animals. Furthermore, there has been no attempt to link gross physiological findings to molecular biological events at the receptor level. The development of new molecular biological techniques and previous investigations into cell signal events permits new questions on hormone-receptor interactions to be addressed.

In regards to the experimental design, other important considerations were the length of experimental/dietary treatment, the age of db/db mice at trial initiation, the severity of the zinc deficiency, and the amount of zinc supplementation. Considerations of the length of experimental treatment and the age of db/db at baseline were interrelated in this study. With an examination of Table 1, it can be seen that diabetic symptomology starts very early, peaks at 3 months of age, then declines as pancreatic pathologies set in. Other investigations with db/db and ob/ob mice often initiate the dietary treatment at 9 weeks of

age, coinciding with the peak of diabetes, and then terminate at 12 weeks. We chose not to follow others in this respect. Since it was our intention to test the hypothesis of a diminution of diabetic symptoms with zinc supplementation, we felt that a preventative dietary approach should be initiated at the weanling age. This could potentially allow the dietary excess of zinc to offset any natural alterations in zinc status that may set in due to the diabetic state. We felt that significant and potentially irreversible pathologies in tissue zinc status and clinical signs of diabetes could be in place by 9 weeks of age. In addition, higher serum zinc concentrations may be beneficial to peripheral insulin sensitivity. Therefore, to start at weanling age (4 weeks) and to terminate the mice at the height of insulin secretion, the length of trial was set from 4 weeks to 10 weeks of age.

The severity of the dietary zinc manipulations was based on the age of the mice and the overall objectives of the experiment. Most studies of zinc deficient rodents initiate a severe zinc deficiency by feeding a 1 ppm zinc diet to young growing rodents. Our goal was to initiate a marginal zinc deficiency in weanling mice that have elevated requirements due to growth. Since Southon et al. (1988) found no evidence of anorexia or skin lesioning in db/db mice fed a 1 ppm diet for 4 weeks, we felt that a 3 ppm zinc diet would not severely compromise growth requirements.

The decision to initiate a supplementation of 300 ppm zinc was based on a balance between establishing excess zinc in the diet based and not severely compromising the metabolism of other trace minerals. In the ob/ob mouse, Bégin-Heick et al. (1985) supplemented dietary zinc at a concentration of 1000 ppm. We felt, however that this concentration of dietary zinc was excessive as copper absorption is known to be inhibited

by excessive quantities dietary zinc (Sandstead, 1995). L'Abbé and Fischer (1984) found that a diet of 240 ppm zinc compromised copper metabolism in rodents, as serum copper concentrations were significantly lower than in rats fed the control diet of 30 ppm zinc. While we felt this was significant, it is important to note that the magnitude of body weight gains are far more pronounced in the growing rat than in the mouse. Furthermore, since they noted that the serum copper concentration resulting from the zinc supplemented diet was sufficient to meet normal growth requirements for 6 weeks of study in the rat, we felt that a 300 ppm concentration of dietary zinc was acceptable for this study.

III. Materials and Methods

Animals and Diet

Female weanling C57BL/KsJ (BL/Ks) homozygous diabetic (db/db) and heterozygous lean (db/m) control mice were purchased the Jackson Laboratory (Bar Harbour, Maine). Mice were segregated at the Jackson Laboratory before shipment. Homozygous diabetic (db/db) were recognized by their relative obesity and characteristic body shape. Upon arrival, the 42 mice were initially housed in groups of 7 and held on a nutritionally complete standard chow diet for a 5 day adaptive period. The diabetic and lean mice were then randomly assigned to six treatment groups (see Table 2). The animals were fed *ad libitum* diets low (3 ppm), adequate (30 ppm) or high (300 ppm) in zinc, based on the AIN-93G formulation (Reeves et al., 1993), for 6 weeks (see diet formulations in Table 3). During this period, the mice were maintained in a controlled environment of 21-23° C, 55% humidity, and a 14 hour light, 10 hour dark cycle. During the feeding trial, the mice were housed separately in stainless steel hanging cages and fresh zinc-free double deionized water was available in polypropylene bottles with stainless steel sipper tubes. At weeks 0, 2, 4, and 6 of trial, mice were weighed and housed overnight (12 hours) in polycarbonate metabolic cages (Nalgene, Fisher Scientific) for the purpose of urine collection. Animals were provided water *ad libitum* (but not feed) for the 12 hours to ensure urine samples were not contaminated by zinc from the diets. Animals were terminated at week 6 after an overnight fast in the metabolic cages.

Table 2 Experimental Groups ¹

Diet	Diabetic (db) Mice (db/db)	Lean (ln) Control Mice (db/m)
Zinc Deficient (3 ppm)	7 dbZD	6 ^a lnZD
Control Diet (30 ppm)	7 dbZC	7 lnC
Zinc Supplemented (300 ppm)	8 ^b dbZS	6 ^b lnZS
TOTALS	22	19

¹ Discrepancies in subject numbers due to ^a death of a zinc deficient lean, and ^b misassignment of db/db at weanling by Jackson Laboratory.

Table 3. Diet Formulations¹

Ingredient	Zn Deficient (3.0 ppm)	Control (30 ppm)	Zn Supplement (300 ppm)
Dextrose²	2655.1	2618.4	2258.4
Egg White	850	850	850
Soybean Oil³	280	280	280
Mineral Mix (AIN-93; zinc-free)	140	140	140
Potassium PO₄	21.6	21.6	21.6
Vitamin Mix (AIN-93)	40	40	40
Choline bitartrate	10	10	10
Zinc Premix⁴	3.3	40	400

¹ Diet ingredients were purchased from Harlan Teklad (Madison, WI) except dextrose and soybean oil

² Dextrose was purchased from Moonshiners (Winnipeg, MB)

³ Soybean Oil was purchased from Vita Health (Winnipeg, MB)

⁴ Zinc Premix (11.55g Zn Carbonate/1000 g dextrose)

Tissue Collection

After an overnight fast at weeks 0, 2, 4, and 6, urine samples from the mice were collected in 1.5 ml microcentrifuge tubes from the metabolic cages and weighed to determine volume. Due to the small size of the weanling mice at weeks 0 and 2, insufficient quantities of urine were collected. For samples obtained at weeks 4 and 6, they were stored at -80° C until assayed for glucose, zinc, protein and creatinine content.

Animals were terminated at week 6 by CO₂ asphyxiation and cervical dislocation following Canadian Council on Animal Care Guidelines. Trunk blood was collected after decapitation and samples were stored on ice until centrifuged (1290 x g for 15 minutes, Beckman TJ-6 centrifuge) to obtain serum. The liver, pancreas, kidneys, axillary and inguinal region fat pads, gastrocnemius muscles and hind quarters were excised and immediately frozen in liquid nitrogen. All tissue samples and serum were stored at -80°C until required for analysis.

Glucose Assay

Glucose in the serum and urine was assessed using an enzymatic colorimetric kit developed for the quantitative determination of glucose in biological fluids (Procedure #315, Sigma Chem.Co., St. Louis, MO). The method is based on the work of Trinder (1969).

The glucose assay involves the conversion of a quantity of glucose to a quinoneimine dye. The quinoneimine dye has an absorbance maximum of 505 nm and the intensity of

colour is directly proportional to the concentration of glucose in the sample. The assay is linear to a glucose concentration of 750 mg/dl.

Reagents:

Trinder reagent:

4-Aminoantipyrine	0.5 mmol/L
p-Hydroxybenzene Sulfonate	20 mmol/L
Glucose Oxidase (<i>Aspergillus niger</i>)	15 000 U/L
Peroxidase (Horseradish)	10 000 U/L
Buffer	pH 7.0 ± 0.1
Stabilizers and fillers	

Urine Control, Level 2 (Sigma Chem. Co.)

Glucose Standard (300 mg/dl)

Assay Procedure:

Urine samples were initially diluted 0-25 times (depending on sample concentration) with isotonic saline (0.85 % NaCl). Plasma samples required no dilution. Once samples were prepared, 1.0 ml of Trinder reagent was pipetted into 10 mm disposable cuvetts and allowed to warm to room temperature. Then, at 20 second intervals, duplicate 5 µl volumes of distilled water (assay blank), glucose standard, urine control, or plasma or urine samples were pipetted into each cuvet. Cuvets were mixed immediately by gentle inversion 3 times and allowed to incubate at room temperature for 18 minutes. At 18

minutes, each sample was read at 20 second intervals at 505 nm with a Milton Roy Spectronic 3000 spectrophotometer (Fisher Scientific, Nepean, ON). A set of glucose standards (50-750 mg/dl) was used to verify linearity of the procedure. A 1.0 ml volume of distilled water was used as a reference.

Calculation:

$$\text{Glucose (mg/dl)} = \frac{([A] \text{ sample} - [A] \text{ blank}) \times \text{dilution factor}}{[A] \text{ standard} - [A] \text{ blank}} \times \text{Conc. of Standard}$$

Insulin Assay

Insulin in the serum was assayed using a sensitive rat insulin radioimmunoassay kit (# SRI-13K, Linco Research Inc., St. Charles, MO). The procedure follows the basic principle of radioimmunoassay where there is a competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. The amount of ¹²⁵I-insulin bound is inversely proportional to the concentration of unlabelled insulin present.

Reagents:

Assay Buffer:

0.05 M Phosphosaline (pH 7.4) containing:

0.025 M EDTA

0.1% Sodium Azide

1% RIA grade Bovine Serum Albumin

Sensitive Rat Insulin Antibody

¹²⁵I-Insulin label (<3 μCi) and label hydrating buffer

Insulin standards (0.02-1.0 ng/ml) & Quality controls

Precipitating reagent

Procedure:

On day 1, serum insulin samples were diluted in Assay Buffer by 40 fold for db/db samples and by 2.5 fold for db/m to be within range of the standard curve. Assay buffer was then pipetted into Borosilicate glass (12 x 75 mm) tubes: 300 μl into 2 non-specific binding tubes, 200 μl each into 2 total binding reference tubes, and 100 μl into tubes for standards, controls and unknowns. One hundred μl of standards, quality controls, or diluted sample were then pipetted into duplicate into appropriate tubes. One hundred μl of rat insulin antibody was pipetted to all tubes except total count and non-specific binding tubes. Tubes were vortexed, covered with parafilm, stored in a styrofoam test tube rack in a sealed plastic container, and incubated overnight at 4°C. The following day, 100 μl of ¹²⁵I-insulin was pipetted into all tubes. Tubes were vortexed, covered with parafilm, stored in a styrofoam test tube rack in a sealed plastic container, and incubated overnight at 4°C. On the third day, 1.0 ml of precipitating reagent was added to all tubes except total count tubes. Tubes with the precipitating reagent were vortexed, incubated for 20 minutes at 4°C, and centrifuged for 30 minutes at ~2000 x g to achieve a firm pellet. The supernatant fractions was decanted and tubes were held inverted for 1 minute for complete blotting of all liquid. Remaining pellets were counted for ¹²⁵I in a gamma counter (Beckman Gamma 8000, Scientific Instruments, Irvine, CA).

Calculations:**Definitions:**

Total count = total ^{125}I alone.

Non-specific binding = tube with no antibody reagents except antibody and sample or standard

Total binding reference tube= all reagents except sample or standard.

1. Non-specific binding count was subtracted from all tubes except total counts.
2. Percentage of maximum binding ($\%B/B_0$) = $\frac{\text{sample or standard cpm}}{\text{total binding reference cpm}} \times 100$

A log-log plot was constructed for $\%B/B_0$, with standard cpm on the y-axis versus the known concentration of that standard on the x-axis. A reference curve was created by manually joining points for smooth curve. Insulin concentrations (ng/ml) for unknown samples and quality controls were then determined by interpolation on this standard reference curve.

Insulin Receptor Concentration / Tyrosine Kinase Phosphorylation

Insulin receptor concentrations and tyrosine kinase activity were assessed in the gastrocnemius muscle, a primary site for glucose disposal. The procedure initially involves the isolation and immobilization of insulin receptors from total muscle homogenate by means of an anti-insulin receptor antibody that was bound to 96-well plates. Tyrosine kinase phosphorylation activity of the isolated receptors is tested by initiating phosphorylation of an exogenous protein substrate with labelled ^{32}P -ATP. The

tyrosine kinase activity was determined under both basal and insulin-stimulated conditions. The isolated receptors were also used to determine the insulin receptor concentration per mg protein of muscle homogenate by the degree of binding of 125 I-insulin. The amount of 125 I-insulin bound is directly proportional to the concentration of receptors in the sample. These methods are adapted from the publications of Klein et al. (1995 & 1993), Hamann et al. (1995), and Suárez et al. (1995).

Buffers:*

Buffer A:

1% Triton X-100

2.5 mmol/L PMSF

800 trypsin U/ml aprotinin

8 mmol/L EDTA

2.5 mg/ml benzamidine

2.5 µg/ml pepstatin

2.5 µg/ml leupeptin

160 mmol/L NaF

10 mmol/L Na Pyrophosphate

0.2 mmol/L Na vanadate

2 mmol/L dichloroacetic acid

20 mmol/L HEPES

Buffer B:

0.05% Triton X-100

50 mmol/L NaCl

1.2 mmol/L KCl

0.5 mmol/L $\text{CaCl}_2 \cdot \text{H}_2\text{O}$

10 mmol/L HEPES

10% glycerol

10% Na azide

0.5% bovine serum albumin

* All components in this assay were of reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Procedure:

In preparation for insulin receptor immobilization, 96-well plates were coated with goat anti-mouse IgG (Oncogene Science, Uniondale, NY). An assessment of insulin receptor concentrations determined that the appropriate dilution for the IgG was 2 $\mu\text{g}/\text{ml}$. A 0.05 M phosphate buffer (13.4 g/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 6.9 g/L NaH_2PO_4 , pH 7.4) was used for all antibody dilutions.

Fifty μl of IgG (2 $\mu\text{g}/\text{ml}$) was pipetted onto 96-well easy-wash ELISA grade polystyrene plates (#25805-96, Corning Glass Works, Corning, NY). Plates were covered and allowed to incubate at 37°C for 2 hours to ensure adherence. Plates were subsequently washed 3 times with deionized water, inverted to blot dry, and incubated with 50 μl of anti-Insulin Receptor antibody (AB-3, Oncogene Science, Uniondale, NY) previously diluted to 1 $\mu\text{g}/\text{ml}$ in 0.05 M phosphate buffer. Plates were covered with parafilm and allowed to incubate overnight at room temperature.

On Day 2, gastrocnemius muscles were removed from the freezer and set on ice to maintain a frozen state. Muscle samples, with the addition of ice cold Buffer A (8 μ l/mg muscle tissue) were homogenized (Potter-Elvehjem Homogenizer, Wiarnton, ON) on ice for one minute on setting 10 using a 20 ml homogenizing tube and pestle. After 30 minutes at 4^oC, samples were centrifuged at 15 000g (Brinkmann-Eppendorf 5414 centrifuge, Brinkmann Instruments Inc., Mississauga, ON) for 20 minutes at 4^oC to remove insoluble materials. In preparation for plating tissue lysate, the 96-well plates were washed 3 times with deionized water to remove excess antibody. Plates for the tyrosine kinase assay and the insulin binding assay were inverted to blot dry, and 40 μ l aliquots of muscle lysate were pipetted into wells at 4^oC. Insulin, (final concentration of 10⁻⁷ mol/L) was also pipetted into appropriate wells for tests of insulin-stimulated tyrosine kinase phosphorylation activity. Plates were again covered with parafilm and incubated overnight at 4^oC.

On Day 3, the reaction buffer (Buffer B with the addition of 5 mmol/L MnCl₂, 10 mmol/L MgCl₂, 0.5 μ mol/L ATP and γ -³²P ATP [3000 Ci/mmol, (New England Nuclear, Dorval, PQ); 1 000 000 cpm/ 20 μ l Buffer B] was prepared. Just before initiating the tyrosine kinase procedure, wells containing lysate were washed 5 times with ice cold buffer B (no additions) and inverted to blot dry. The exogenous substrate, 20 μ l of poly GLUTYR [poly(glutamate:tyrosine 4:1) 4 mg/ml in water], was added to the washed plates with a repeat pipetter. The reaction was initiated with the addition of the γ -³²P ATP containing buffer B (20 μ l/well). After 15 minutes, the reaction was terminated by spotting 35 μ l per well onto prelabelled 2 x 2 cm squares of Whatman 3MM filter papers.

Filter papers were allowed to dry for 5 minutes, then washed in 10% trichloroacetic acid and 10 mmol/L Na Pyrophosphate 3 times, for 15 minutes each time. Filter papers were subsequently immersed for 5 minutes in 100% ethanol, allowed to dry, and placed into prelabelled disposable polyethylene scintillation vials (Fisher Scientific, Pittsburgh, PA) with 10 ml scintillation fluid (ScintiSafe™ Econo 1, Fisher Scientific, Pittsburgh, PA) per vial. Total counts, blanks and samples were counted for one minute each in a ³²P scintillation counter (Beckman LS 6000TA, Beckman Instruments, Mississauga, ON).

For the insulin binding assay, plates previously prepared with 40 µl aliquots of muscle lysate with were washed 5 times in ice cold buffer B. In preparation for the insulin binding assay, 40 µl/well Buffer B [with the addition of 30 000 cpm ¹²⁵ I-insulin/well, (New England Nuclear, Dorval, PQ)] was added and allowed to incubate overnight at 4°C.

On Day 4, wells containing ¹²⁵ I-insulin were washed 5 times with Buffer B to remove unbound insulin. The ¹²⁵ I-insulin that had bound to receptors was collected by incubating the wells twice with 100 µl of a 10% solution of sodium dodecyl sulfate (SDS), for 15 minutes each time. After each incubation, 95 µl of the solution was pipetted into prelabelled borosilicate test tubes. Tubes were covered with parafilm and total counts, blanks and samples were quantified by gamma counting (Beckman Gamma 8000 Scientific Instruments, Irvine, CA).

Calculations:**Insulin receptor numbers:**

Protein content (mg/well) = protein conc. from BCA assay (mg/ml) X 0.04 ml/well

Insulin receptor number (cpm/mg protein) = $\frac{\text{cpm/well}}{\text{mg protein/well}}$

Tyrosine Kinase Phosphorylation:

ATP conc = 0.02 ml (well size) x 5000 nmol/1000 ml = 0.1 nmoles

Specific Activity = R = cpm per 20 μ l ATP/0.1 nmol per 20 μ l = cpm/nmol

T = total phosphate transferred

T = (sample cpm x 40 μ l/35 μ l) - blanks cpm

P = pmol phosphate transferred per minute

15 minutes = incubation time

$P = (T * 1000) / (15 * R)$

Zinc, Calcium and Phosphorus Determinations

Femur, pancreas, kidney, serum, urine and treatment diets were analyzed for zinc concentration by atomic absorption spectrophotometry. Serum and urine samples (100 μ l) were subjected to a direct analysis at a 10 fold dilution in deionized water, while tissues, bone and diet required acid digestion.

Procedure:

The following technique of digesting tissue is based on the method of Clegg et al. (1981). In preparation for digestion, femurs were first thawed and carefully scraped of all musculature using a scalpel blade. Wet weights were obtained for femurs, pancreas, and

half of 1 kidney. Tissues were dried for 48 hours at 85°C. After drying, all tissues were immediately weighed to determine dry weights. Dried tissue samples plus 1 ml of zinc-free 70% nitric acid (VWR Canlab, Mississauga, ON) were put into previously acid-washed (20% nitric acid) zinc-free pyrex test tubes. For diet samples, 1 g of diet plus 2 ml of a 70% nitric acid solution was used. A bovine liver reference (0.1 g/10 ml; ref.#1577b, U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) was also digested as a quality control. All test tubes were covered with acid-washed glass marbles, allowed to digest at room temperature for 24 hours, and then heated for 48 hours at 85°C using a dry bath heater. Tissue samples were diluted with double deionized water to 5 ml in a volumetric flask. Diet samples were diluted to 10 ml in a volumetric flask. Samples were analyzed using an atomic absorption spectrophotometer (Varian Spectra AA-30 Spectrophotometer, Georgetown, ON) and zinc standards (0.1-1ppm) were prepared from a zinc atomic absorption standard (1000 ppm, #H595-01 Mallinckrodt, Paris, Kentucky).

Calculations:

For femur, kidney, pancreas, & diet:

$$\text{Zinc } (\mu\text{g/g dry weight}) = \frac{\text{Sample zinc concentration} \times \text{dilution factor}}{\text{Dry weight of sample}}$$

For serum & urine samples:

$$\text{Zinc } (\mu\text{g/ml}) = \text{Sample zinc concentration} \times \text{dilution factor}$$

Mineral content of the femur was further analyzed for calcium and phosphorus concentrations. Following a further 50 fold dilution of femur digests (total dilution of 250), samples were analysed in triplicate using an Emission Spectrometer (Varian Liberty 200 ICP, Varian Canada, Georgetown, ON).

Calculations:

$$\begin{array}{l} \text{Femur Calcium} = \frac{\text{Sample Ca or P concentration} \times \text{dilution factor}}{\text{Dry weight of sample}} \\ \text{or Phosphorus} \\ (\mu\text{g/g dry weight}) \end{array}$$

Protein Assay

Gastrocnemius muscle and urine were analyzed for protein concentration using the bicinchoninic acid (BCA) protein assay method (Pierce, Rockford IL). This method employs the biuret reaction (Lowry, 1951) where protein reduces Cu^{2+} to Cu^{1+} in an alkaline environment. The result is a purple coloured reaction product with 2 molecules of BCA plus the reduced cuprous ion (Cu^{1+}). The concentration of colour is directly proportional to the reduction of copper by the protein in a sample.

Reagents:

Reagent A:

In 1000 ml 0.2 N NaOH:

sodium bicarbonate

sodium carbonate

BCA detection reagent

sodium tartrate

Reagent B:

25 ml of 4% copper sulfate solution

Procedure:

The method for muscle homogenization prior to protein assessment is described under the method for Insulin Receptor Concentration/Tyrosine Kinase Phosphorylation. Muscle samples were diluted ten-fold with muscle homogenizing buffer prior to assay. Urine samples were first precipitated in cold 10% trichloroacetic acid and centrifuged for 5 minutes at 15 000g (Brinkmann-Eppendorf 5414 centrifuge, Brinkmann Instruments Inc., Mississauga, ON). Supernatant fractions were removed, and the protein pellet was resuspended in phosphate buffered saline (pH 7.4) solution in preparation for the protein assay.

Albumin protein standards (0.05-2.0 mg/dl) were used for the standard curve. Samples, standards and buffer blanks of 10 μ l were pipetted in triplicate into 96-well polystyrene microtiter plate wells (#25880-96, Corning Glass Works, Corning, NY). Two hundred μ l of BCA reagent was then added to the wells using a multi-channel pipet. Samples on the microplate were shaken for 5 seconds by the SpectroMax Microtiter Plate Reader (Molecular Devices Corporation, Sunnyvale, CA), and allowed to incubate for 30 minutes at 37°C. Absorbances were read at 562 nm. Unknown sample concentrations (mg/ml) were calculated by the Soft Max Pro software.

Manual Calculation:

$$\text{Protein Concentration (mg/ml)} = \frac{\text{Concentration of standard} \times [\text{A}] \text{ sample}}{[\text{A}] \text{ standard}}$$

Creatinine Assay

Creatinine is an urinary excretion product that is proportional to body muscle mass (Bowers and Wong, 1980). Since creatinine excretion from the body is relatively constant, it was used in this study as a basis for calculating urinary concentration in samples analyzed for glucose, protein and zinc determinations.

The colorimetric creatinine assay (Procedure #555, Sigma Chem.Co., St. Louis, MO) is based on the method of Heinegard and Tiderstrom (1973). Upon contact with the alkaline picrate solution, a sample will form an orange/yellow colour. This colour, derived solely from creatinine, is destroyed by the addition of an acidic pH solution. Therefore the difference in colour intensity, as measured before and after acidification, is proportional to creatinine concentration.

Reagents:

Sodium Hydroxide Solution: 1.0 N

Acid Reagent: A mixture of sulfuric and acetic acid

Creatinine Colour Reagent: 0.6% Picric acid, sodium borate and surfactant

Creatinine Standards: 3.0 or 15.0 mg/dl creatinine in 0.02 N hydrochloric acid

Urine Control, Level 2 (Sigma)

Alkaline Picrate Solution: 5 parts creatinine colour reagent to 1 part NaOH (1.0 N)

Assay Procedure:

Urine samples were diluted 5-10x with distilled water. Duplicate samples of 100 μ l of distilled water (blank), 3 mg/dl creatinine standard, urine control or sample were added to 10 mm disposable cuvetts. To all cuvetts, a 1.0 ml volume of alkaline picrate solution was pipetted, gently mixed by inversion 3 times and allowed to stand at room temperature for 10 minutes. After 10 minutes, all cuvetts were read and recorded at 500 nm using a Milton Roy Spectronic 3000 Spectrophotometer (Fisher Scientific, Nepean, ON). This reading is the INITIAL [A]. Once read, 33.3 μ l of acid reagent was pipetted into all cuvetts, immediately mixed by inversion 3 times and allowed to stand at room temperature for 5 minutes. At 5 minutes, all cuvetts were read at 500 nm and recorded. This reading is the FINAL [A]. A 1.0 ml volume of distilled water in the same cuvet type was used as a reference for the spectrophotometer during this assay. A calibration curve of 0-10 mg/dl was prepared to establish the linearity of the assay.

Creatinine Calculation:

BLANK [A] was subtracted from all unknown and standard samples, and creatinine was calculated as follows:

$$\text{Creatinine (mg/dl)} = \frac{(\text{INITIAL [A]}_{\text{test}} - \text{FINAL [A]}_{\text{test}}) \times \text{dilution factor}}{[\text{A}]_{\text{standard}} - [\text{A}]_{\text{blank}}} \times \text{Conc. of Standard}$$

Liver Lipid

The method of Folch (1956) was used to quantify total lipid content in the liver. The method utilizes specific ratios of chloroform, methanol and water to extract the lipid fraction in a two day procedure.

Procedure:

Livers were thawed, cut into 1.0 g portions and placed in 22 ml of a 2:1 by volume chloroform/methanol solution. Each liver was homogenized twice at 20 second intervals using setting #4 on a Polytron Homogenizer (model #PT 1020 3500, 115 volt, Brickmann Instruments, Rexdale, ON). The homogenate was filtered (#1 Whatman filter) and the volume of eluate collected in a 25 ml graduated cylinder was recorded. Twenty percent of this volume was added as water, shaken gently by inversion with venting, covered and allowed to separate overnight.

The following day, the top methanol layer and a protein disc layer were removed using an aspirator. Ten mls of the bottom chloroform layer were carefully removed and placed in a 25 ml glass vial that was previously desiccator dried and weighed. The chloroform was evaporated from the vial using a heated water bath (OA-SYS heating system, Organomation Associates, Berlin, MA) with a stream of nitrogen air (0.7 Kg/cm^2) for one hour. The lipid-containing vials were allowed to re-dry in the desiccator for a minimum of one hour. They were then weighed for lipid content.

Liver Lipid Calculation:

liver lipid (g) =

$$\frac{\text{dried vial \& lipid} - \text{dried vial alone}}{\text{chloroform volume removed (10 ml)}} \times \frac{\text{volume of initial chloroform layer}}{\text{amount of liver tissue used (1g)}}$$

Statistical Analysis

Differences between dietary treatment groups were analysed using ANOVA (SAS 6.04, SAS Institute, Cary, NC). Main effects were diet and genotype and the interaction was of diet x genotype. Significant differences between means were determined by Duncan's multiple range test. Correlational analysis was performed using the Spearman's Correlation Coefficient. Differences were accepted as significant at $P < 0.05$.

IV. Results

The findings reported in this study can stem from 2 major areas in the investigation. First are the genotypic effects. These refer to significant differences in the animals that are due strictly to the genetic differences between the diabetics (db/db) and their lean littermates (db/m). Second are the diet effects. These effects are a result of the dietary manipulations of zinc and diet effects can only properly be compared within animals of the same genotype. Both genotypic and dietary findings will be reported in the results. For data in Figures, means and standard errors of the mean are also presented in tabular form in Appendixes A and B. Main effects of diet, genotype and a diet x genotype interaction are reported in Appendixes C and D.

Body Weight

The effects of genotype and dietary treatment on body weight is presented as weekly means across the course of treatment (Figure 3). At week 0 of treatment, the 4 week old mice assessed as lean or diabetic by Jackson Laboratory were clearly discernable by weight alone. Diabetic mice had a significantly higher body weight (21.6 ± 0.5 g) than lean control mice (15.9 ± 0.4 g). By week 2, all groups had experienced slight weight gains, but dbC (25.8 ± 1.2 g) could now be differentiated by weight from other diabetic mice (21.9 ± 1.5 g and 22.5 ± 1.4 g for dbZD and dbZS, respectively). This trend was maintained until termination with dbC (33.8 ± 1.0) weighing significantly more than dbZD

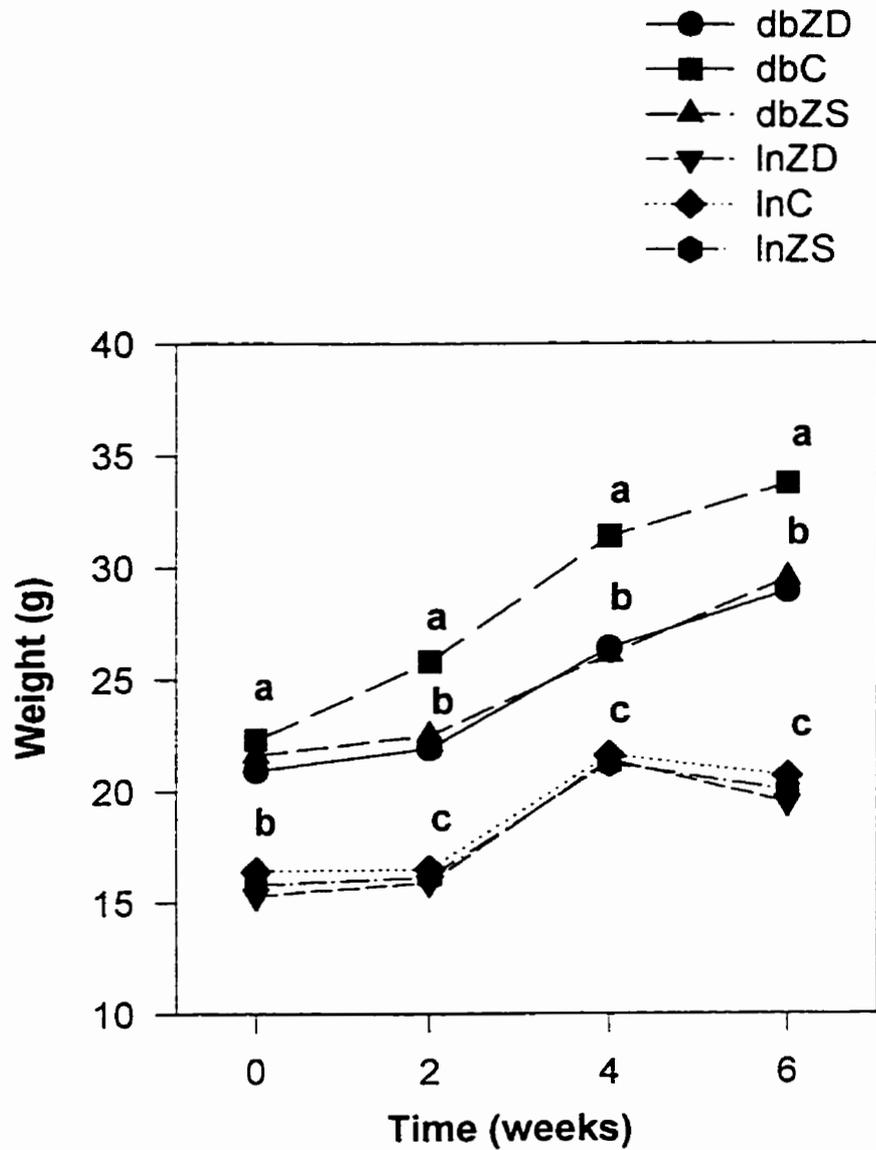


Figure 3. The effect of dietary zinc on body weight (g) at weeks 0, 2, 4 and 6. Treatment groups were dbZD = db/db zinc deficient, dbC = db/db control, dbZS = db/db zinc supplement, lnZD = db/m zinc deficient, lnC = db/m control, and lnZS = db/m zinc supplement. Data points represent means \pm SEM. $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, $n = 6$, $n = 6$, respectively. Different lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

and dbZS ($29.0 \text{ g} \pm 1.9$ and $29.5 \pm 1.0 \text{ g}$, respectively at week 6). All lean mice were significantly different in weight from diabetics ($20.1 \pm 1.5 \text{ g}$ versus $30.7 \pm 4.0 \text{ g}$ respectively), but there were no significant differences in body weight among lean mice due to dietary treatment during the course of the study.

Mineral Status (Zn, Ca, P)

Zinc status in this study was assessed in the femur as an indicator of longer term zinc body pools. The dbZS group had a higher femur zinc concentration compared to both dbZD and dbC (311.4 vs. 224.0 and $242.9 \text{ } \mu\text{g/g}$ dry weight, respectively) (Figure 4). This trend of higher femur zinc with higher concentrations of zinc in the diet was also observed in the lean mice, with lnZS having the highest zinc concentration in the femur and lnZD the lowest (303.5 vs. $219.32 \text{ } \mu\text{g/g}$ dry weight, respectively). In terms of genotype, no significant differences in femur zinc concentration were observed in diabetic versus lean mice (258.6 vs $266.2 \text{ } \mu\text{g/g}$ dry weight, respectively). In a further assessment of bone mineral content, calcium and phosphorus concentrations were measured (Table 4). Calcium and phosphorus concentrations exhibited a 2:1 ratio in the femur (Table 4) and were significantly different in the diabetic versus lean mouse genotype (220.9 vs. 240.7 mg/g dry weight, respectively, for calcium; 111.5 vs. 126.7 mg/g dry weight, respectively, for phosphorus). Both calcium and phosphorus femur concentrations were lowest in the dbZD groups (Ca = 210.45 and P = $107.66 \text{ } \mu\text{g/g}$ dry weight, respectively) and were both

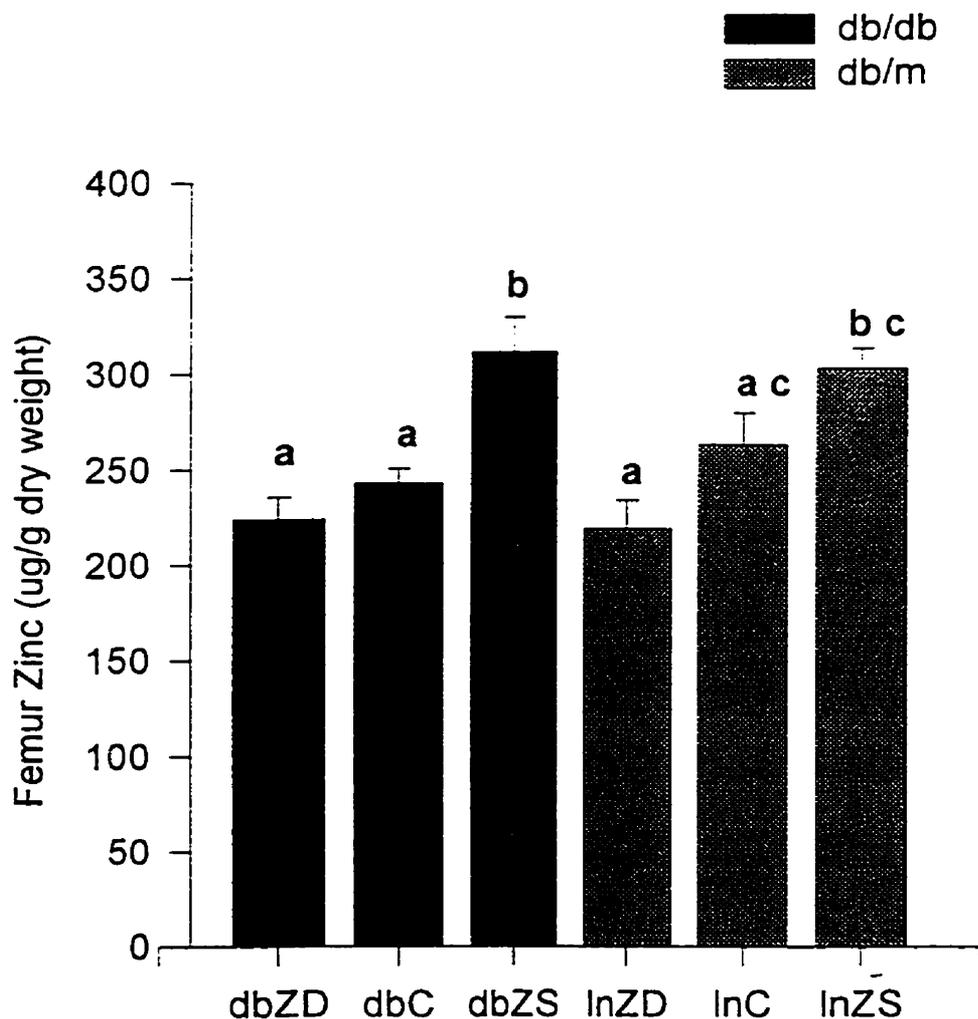


Figure 4. The effect of dietary zinc on femur zinc concentration. Treatment groups were dbZD = db/db zinc deficient, dbC = db/db control, dbZS = db/db zinc supplement, lnZD = db/m zinc deficient, lnC = db/m control, and lnZS = db/m zinc supplement. Columns represent mean \pm SEM. $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, $n = 6$, $n = 6$, respectively. Different lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

Table 4. Effects of dietary zinc on femur calcium and phosphorus, serum and kidney zinc, serum insulin, and urine indices of diabetes after 6 weeks of dietary treatment¹

	Treatment Groups ²					
	dbZD	dbC	dbZS	lnZD	lnC	lnZS
Femur Ca ($\mu\text{g/g}$ dry wt.)	210.5 ^B ± 1.4	223.5 ^{A,B} ± 10.4	228.8 ^{A,B} ± 16.0	251.1 ^A ± 8.8	237.8 ^{A,B} ± 2.5	233.2 ^{A,B} ± 2.6
Femur P ($\mu\text{g/g}$ dry wt.)	107.7 ^A ± 4.5	113.0 ^{A,B} ± 5.1	113.9 ^{A,B} ± 8.4	132.3 ^C ± 4.7	125.1 ^{B,C} ± 1.2	122.7 ^{A,B,C} ± 1.4
Femur Ca:P ratio	2.0:1.0	2.0:1.0	2.0:1.0	1.9:1.0	1.9:1.0	1.9:1.0
Serum Zn ($\mu\text{g/ml}$)	1.29 ^{A,B} ± 0.06 n = 5	1.30 ^{A,B} ± 0.08 n = 6	1.84 ^A ± 0.16 n = 8	1.54 ^{A,B} ± 0.35 n = 3	1.27 ^{A,B} ± 0.32 n = 3	1.09 ^B ± 0.18 n = 5
Kidney Zn ($\mu\text{g/g}$ dry wt.)	73.7 ^A ± 8.9	127.1 ^B ± 24.5	90.0 ^{A,B} ± 11.9	87.0 ^A ± 4.1	81.8 ^A ± 6.5	76.3 ^A ± 3.0
Serum Insulin (ng/ml)	12.3 ^A ± 4.9 n = 7	33.4 ^B ± 3.3 n = 6	13.4 ^A ± 3.9 n = 7	0.1 ^C ± 0.0 n = 5	0.1 ^C ± 0.0 n = 7	0.1 ^C ± 0.0 n = 6
Urine glucose (mg/12 hours)	5.4 ^{A,B} ± 1.7 n = 7	25.6 ^C ± 11.1 n = 7	18.9 ^{B,C} ± 3.9 n = 8	0.1 ^A ± 0.0 n = 6	0.1 ^A ± 0.0 n = 7	0.1 ^A ± 0.0 n = 5
Urine protein (mg/12 hours)	1.63 ^A ± 0.31 n = 6	3.28 ^{A,B} ± 1.32 n = 5	1.79 ^A ± 0.43 n = 7	3.51 ^{A,B} ± 1.11 n = 6	4.69 ^B ± 0.83 n = 7	3.17 ^{A,B} ± 1.06 n = 5
Urine Creatinine (mg/12 hours)	0.0725 ^{A,B} ± 0.0121 n = 6	0.0401 ^A ± 0.0116 n = 6	0.0769 ^{A,B} ± 0.0103 n = 4	0.124 ^B ± 0.0274 n = 6	0.115 ^B ± 0.0151 n = 7	0.0776 ^{A,B} ± 0.0186 n = 6

¹ Values are means \pm standard error of the mean. Superscript letters indicate significant differences between means as determined by Duncan's multiple range test. n = 7 except for dbZS, lnZD, and lnZS where n = 8, n = 6, n = 6, respectively. Due to sample pooling, n values are listed separately in the table where applicable.

² dbZD = db/db zinc deficient, dbC = db/db control, dbZS = db/db zinc supplement, lnZD = db/m zinc deficient, lnC = db/m control, lnZS = db/m zinc supplement

highest in the lnZD group (Ca = 251.09 and P = 132.31 $\mu\text{g/g}$ dry weight, respectively). However, there were no significant diet effects within either the db/db or db/m genotypes.

Changes in serum zinc concentration in diabetic and lean genotypes were not significant in this study (1.52 vs. 1.26 $\mu\text{g/ml}$, respectively). In the diabetic group, there was a trend toward higher serum zinc concentration in the dbZS group versus dbZD and dbC (1.84 vs. 1.29 and 1.30 $\mu\text{g/ml}$, respectively), but it did not reach statistical significance (Table 4). There was, however, a significant diet x genotype interaction.

Zinc in the pancreas and kidneys was assessed as a measure of zinc distribution/maldistribution in the body. Pancreatic zinc content in this study showed both genotypic and dietary treatment differences (Figure 5). In terms of genotype, the mean pancreatic zinc concentration in the diabetic mice was significantly lower than the lean mice (98.2 vs. 118.9 $\mu\text{g/g}$ dry weight, respectively). The diabetic pancreas is often reported to be depleted of zinc, and this study confirmed these findings as dbC was significantly lower than lnC (81.9 vs. 109.3 $\mu\text{g/g}$ dry weight). The zinc deficient diet in the db/db mice, however, failed to further decrease pancreatic zinc concentrations compared to the dbC group (87.8 vs. 81.9 $\mu\text{g/g}$ dry weight, respectively). The dietary zinc supplementation in the diabetics, however, restored pancreatic zinc concentrations to lean values. The dbZS had a mean pancreatic zinc concentration of 117.7 $\mu\text{g/g}$ dry weight, which was not significantly different from lean controls, but significantly different from the other diabetic groups, dbZD and dbC. This higher concentration of pancreatic zinc in the dbZS group was the result of a higher zinc content in the pancreas, as pancreatic dry

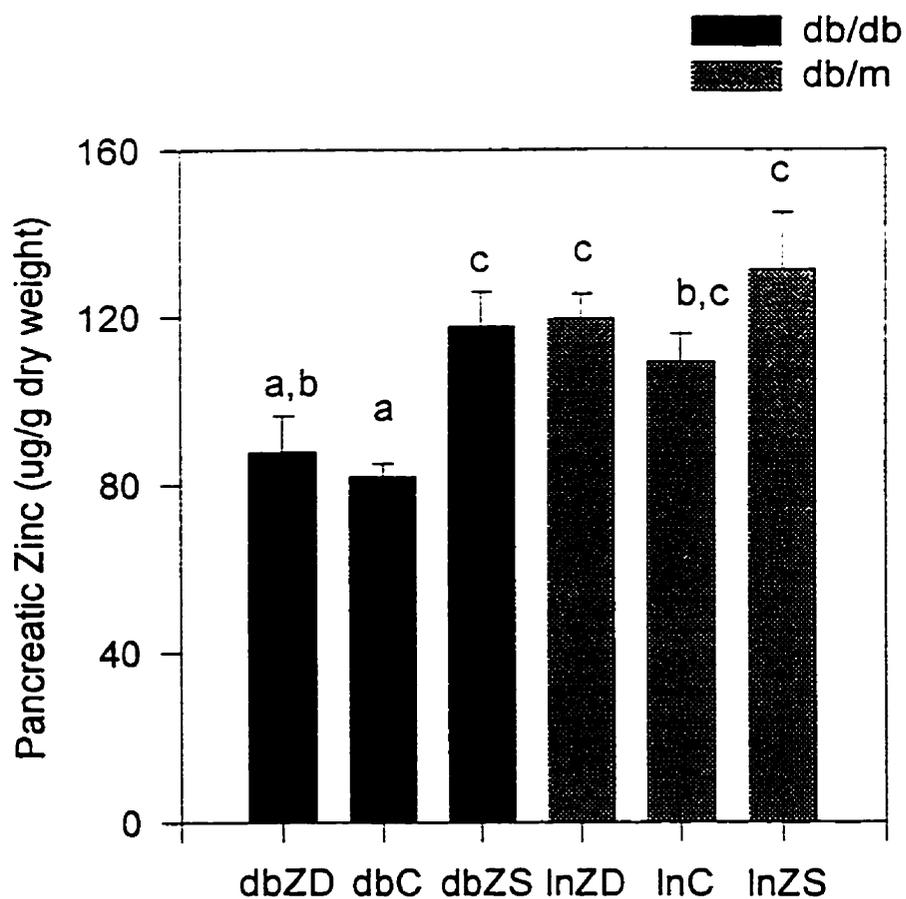


Figure 5. The effect of dietary zinc on pancreatic zinc concentration. Treatment groups were dbZD = db/db zinc deficient, dbC = db/db control, dbZS = db/db zinc supplement, lnZD = db/m zinc deficient, lnC = db/m control, and lnZS = db/m zinc supplement. Columns represent mean \pm SEM, $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, $n = 6$, $n = 6$, respectively. Different lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

weight did not significantly differ in the dbC versus the dbZS group (53.9 vs. 52.3 mg dry weight, respectively) (Appendix A). Kidney zinc, another measure of tissue zinc distribution, showed no significant differences due to genotype (97.4 vs. 81.2 $\mu\text{g/g}$ dry weight in diabetics vs. lean mice, respectively). Kidney zinc concentration was highest in the dbC group (127.1 $\mu\text{g/g}$ dry weight), and significantly different from all other groups except dbZS (90.0 $\mu\text{g/g}$ dry weight) (Table 4). It appears that the pancreas and kidney respond differently to both the diabetic state and dietary zinc administration in terms of zinc retention.

Serum and Urine Indices of Diabetes

This study examined the effects of diet and genotype on common diabetic indices, including serum concentrations of glucose and insulin, and urinary glucose, zinc, protein and creatinine excretion. The effects of both diet and genotype on serum glucose concentrations were significant (Figure 6). The diabetic group exhibited significantly higher serum glucose concentrations compared to lean groups (419.5 vs. 172.0 mg/dl, respectively). While this would be expected in diabetics, there was an interesting trend with respect to dietary zinc intake. Zinc supplemented diabetics (dbZS) had a significantly lower serum glucose concentration than the dbZD group (498.1 vs. 344.5 mg/dl, respectively). The dbC group had an intermediate glucose concentration of 424.9 mg/dl. This trend of lower serum glucose concentration with higher zinc in the diet was also observed in the lean mice, but did not reach statistical significance. To further

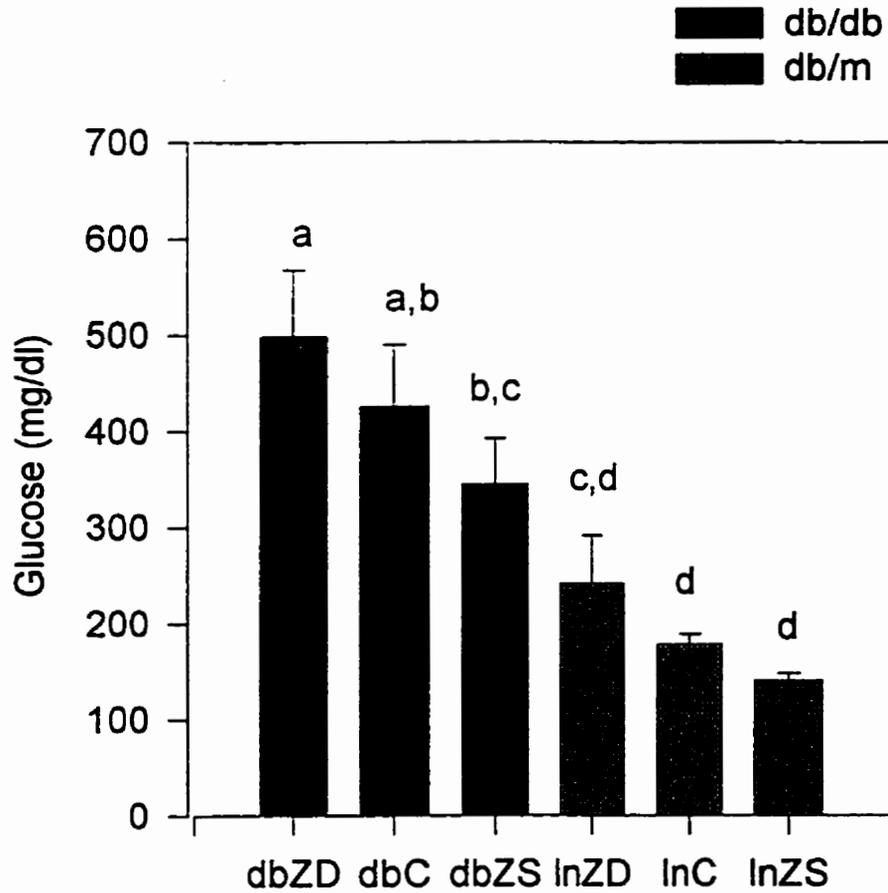


Figure 6. The effect of dietary zinc on serum glucose concentration. Treatment groups were dbZD = db/db zinc deficient, dbC = db/db control, dbZS = db/db zinc supplement, lnZD = db/m zinc deficient, lnC = db/m control, and lnZS = db/m zinc supplement. Columns represent mean \pm SEM, $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, $n = 6$, $n = 6$, respectively. Different lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

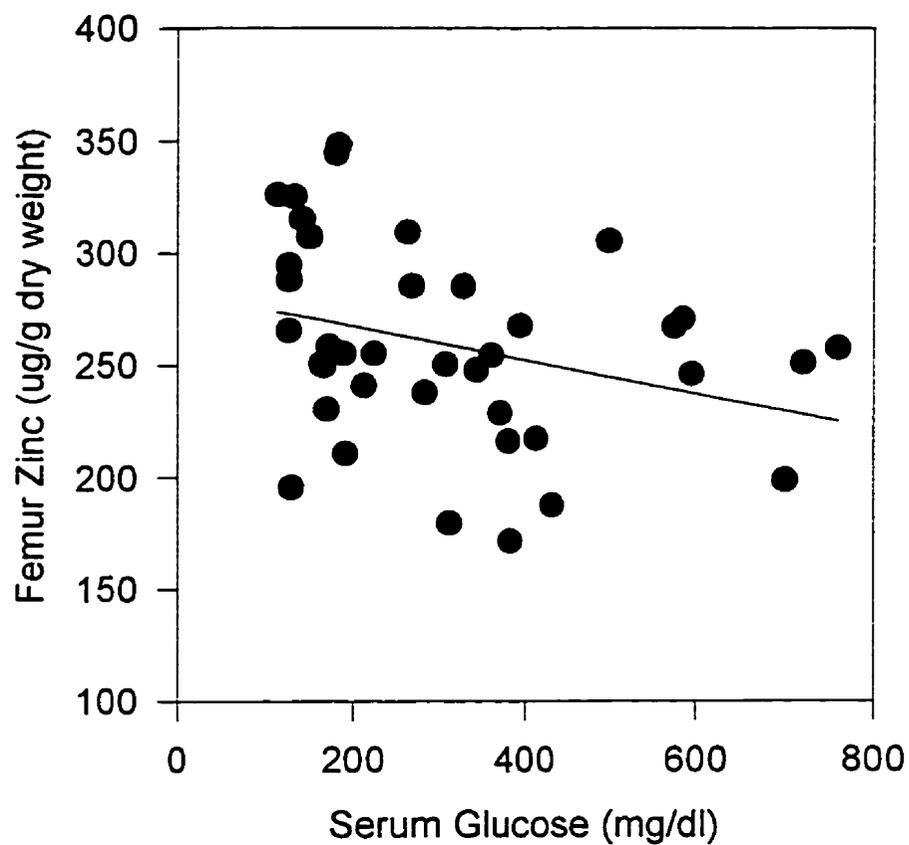


Figure 7. Scatter plot of femur zinc concentration vs. serum glucose concentration. Data points are values obtained from individual mice, $n = 38$. Analysis by Spearman's correlation coefficient (r) revealed a significant negative correlation ($r = -0.38$, $p = 0.015$).

confirm an association between zinc status in the body and serum glucose concentrations, a Spearman correlation was performed. There was a significant negative correlation ($r = -0.38$, $p = 0.015$) between serum glucose and femur zinc concentrations (Figure 7).

Serum insulin concentrations were highly significant in terms of genotype and diet. Insulin concentrations were 100-275 fold higher in diabetic versus lean mice. The mean insulin concentration in the diabetic group was 19.02 ng/ml versus a mean of 0.12 ng/ml in the lean genotype. This is to be expected because at the age of termination (10 weeks of age), db/db mice would be at their peak of hyperinsulinemia. Effects of dietary treatment were present only within the diabetic group. Serum insulin concentration in diabetic controls (dbC) was 2 fold higher than either dbZD or dbZS (33.39 vs. 12.26 and 13.46 ng/ml, respectively) (Table 4). The lower insulin concentration in dbZS corresponds with the lower serum glucose concentration, suggesting that these diabetic parameters improved with zinc supplementation. However, in the dbZD group, the lower insulin concentration was associated with the highest serum glucose concentration of all groups in the study.

Urinary zinc excretion was measured to evaluate the degree of hyperzincuria in diabetic mice. Urinary zinc excretion in this study was influenced by genotype, with dietary zinc intake having a nonsignificant impact. The diabetic groups excreted approximately twice as much zinc as the lean groups over a 12 hour period (0.77 vs. 0.37 $\mu\text{g Zn}/12$ hours, respectively) (Figure 8). This confirms the higher urinary excretion by diabetic animals.

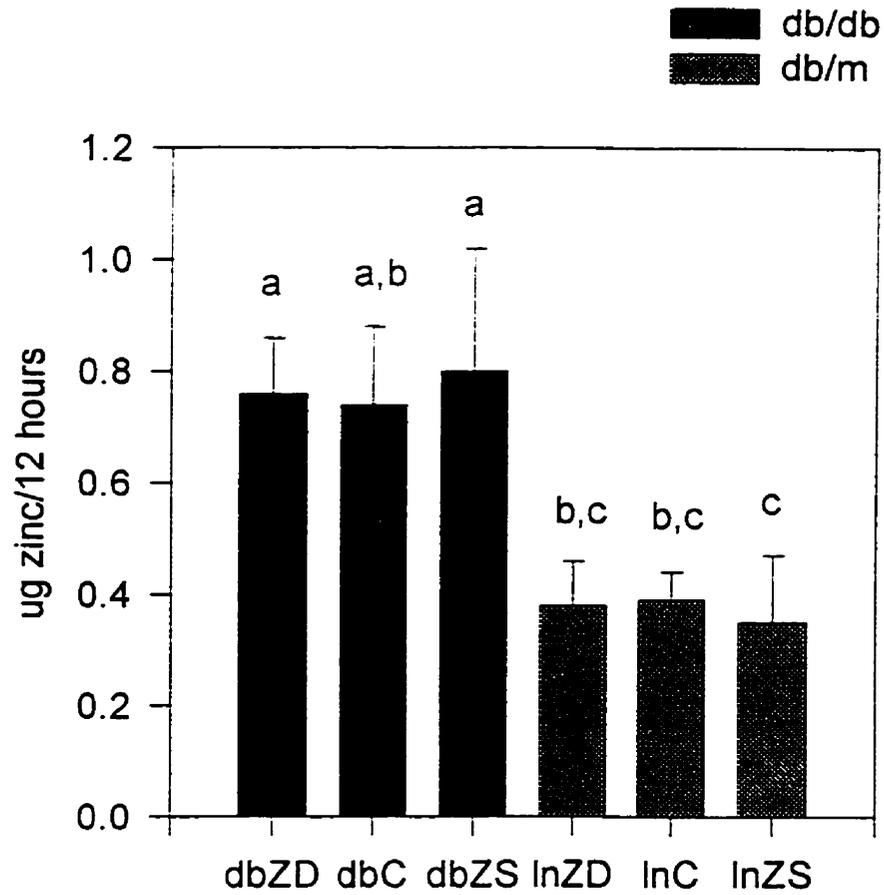


Figure 8. The effect of dietary zinc on urine zinc concentration. Treatment groups were dbZD = db/db zinc deficient, dbC = db/db control, dbZS = db/db zinc supplement, lnZD = db/m zinc deficient, lnC = db/m control, and lnZS = db/m zinc supplement. Columns represent mean \pm SEM, $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, $n = 6$, $n = 6$, respectively. Different lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

Urine parameters are presented in Table 4. Urinary glucose excretion was 50-200 fold higher in diabetic versus lean groups. Glucose excretion in the dbZD group was significantly lower than dbC (5.4 vs. 25.6 mg/12 hours, respectively). This was in contrast to the high serum glucose concentrations in dbZD. The dbC and dbZS groups were not significantly different. Urinary protein excretion, an index of renal damage, was not significantly different between lnC and dbC groups. Urinary protein excretion in the dbZD and dbZS groups was lower (50% and 45%, respectively) compared to the dbC group, however, these differences were not statistically significant.. Urinary creatinine excretion was originally employed in this study to act as a stable measurement against which urine glucose and protein could be quantified. However, it was revealed that creatinine values demonstrate significant genotypic variability, with lower creatinine excretion evident in the db/db mice compared to lean control mice (0.061 vs. 0.106 mg/12 hours, respectively). Creatinine excretion was significantly higher in lnC versus dbC, with lnC being over twice as high as the dbC group (0.11 vs 0.04 mg/12 hours, respectively).

Liver Lipid and Liver Weight

Liver lipid concentration and weight of the liver were both genotypically different in this study. Diabetic mice had significantly higher liver weights compared to the lean mice (1.36 vs. 0.94 g wet weight at termination, respectively) (Table 5). In terms of diet, the dbC had a significantly higher liver weight than either dbZD or dbZS groups (1.49 vs. 1.28 and 1.32 g, respectively). Liver weights were proportional to body weight, as the

ratio of liver weight to body weight was approximately 0.045 in all treatment groups (Table 5). Higher liver weights in the diabetic genotype did not translate into higher liver lipid concentration, as dbC and dbZS had less lipid per gram liver than the lean groups (96.5 and 98.6 mg lipid/g liver vs. 137.4-142.4 mg lipid/ g liver, respectively). Interestingly, the dbZD group had a liver lipid concentration not significantly different from the lean groups. However, when total lipid content of the liver was analysed, there were no significant differences with either genotype or diet.

Fat Pad Weight

Fat pad weights were measured as an indication of obesity in the db/db mice. Fat pad weight was significantly influenced by genotype, as fat content of the body was significantly higher in the diabetic versus the lean genotype (5.0 vs. 1.1 g, respectively) (Table 5). In terms of diet, the dbC group was found to have a significantly higher body fat content than the dbZD group (5.99 vs. 4.64, respectively). This higher fat content of the body of dbC did not translate to a higher percent body fat, as treatment groups within the diabetic or lean genotype did not significantly differ.

Insulin Receptor Concentration and Tyrosine Kinase Phosphorylation

Insulin receptor concentration was measured in this study to assess any dietary or genotypic effects as well as any relationships between receptor concentrations and serum glucose disposal and insulin status. Receptor concentrations in this study were not

Table 5. Effects of dietary zinc on liver lipid and liver weights, fat pad weights, insulin receptor concentration, basal and insulin-stimulated tyrosine kinase activity ¹

	Treatment Groups ²					
	dbZD	dbC	dbZS	lnZD	lnC	lnZS
Liver lipid concentration (mg/g liver)	113.4 ^{AB} ±16.5	96.5 ^B ±10.2	98.6 ^B ±9.7	139.1 ^A ±0.9	137.4 ^A ±1.0	142.4 ^A ±0.0
Liver lipid content (mg/ liver)	141.3 ^A ±15.8	146.5 ^A ±21.8	130.2 ^A ±13.2	125.4 ^A ±15.5	133.1 ^A ±7.6	136.8 ^A ±14.5
Liver weight (g)	1.28 ^A ±0.07	1.49 ^B ±0.07	1.32 ^A ±0.04	0.90 ^C ±0.04	0.97 ^C ±0.02	0.96 ^C ±0.07
Liver weight / body weight (g) %	4.5 ^A ±0.2	4.4 ^A ±0.3	4.5 ^A ±0.2	4.6 ^A ±0.1	4.7 ^A ±0.1	4.8 ^A ±0.2
Fat Pad Weight (g)	4.64 ^A ± 0.52	5.99 ^B ± 0.42	5.01 ^{AB} ± 0.35	1.01 ^C ± 0.33	0.98 ^C ± 0.12	1.16 ^C ± 0.21
Fat pad weight/ body weight (g) %	15.8 ^A ±1.1	17.7 ^A ±1.1	16.9 ^A ±0.8	4.9 ^B ±1.6	4.7 ^B ±0.6	5.7 ^B ±1.0
Insulin Receptor Concentration (cpm/mg protein)	673.6 ^{AB} ±89.2	746.2 ^{AB} ±104.2	744.2 ^{AB} ±80.2	847.6 ^A ±105.8	666.6 ^{AB} ±128.6	457.3 ^B ±117.4
Tyrosine Kinase Phosphorylation (basal) (pmol/min/mg phosphate)	0.000911 ^A ± 0.000260	0.000884 ^A ± 0.000211	0.00141 ^A ± 0.00025	0.00143 ^A ± 0.00041	0.00133 ^A ± 0.00027	0.00109 ^A ± 0.00027
Tyrosine Kinase Phosphorylation (+ insulin) (pmol/min/mg phosphate)	0.00254 ^{AB} ± 0.00029	0.00306 ^A ± 0.00050	0.00232 ^{AB} ± 0.00031	0.00266 ^{AB} ± 0.00038	0.00200 ^{AB} ± 0.00035	0.00185 ^B ± 0.00036

¹ Values are means ± standard error of the mean. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test. n = 7 except for dbZS, lnZD, and lnZS where n = 8, n = 6, n = 6, respectively.

² dbZD = db/db zinc deficient, dbC = db/db control, dbZS = db/db zinc supplement, lnZD = db/m zinc deficient, lnC = db/m control, lnZS = db/m zinc supplement

significantly different in diabetic and lean genotypes (740.9 vs. 665.9 cpm/mg protein, respectively) (Table 5). The lnZD had a significantly higher receptor concentration when compared to lnZS (847.6 vs. 457.3 cpm/mg protein, respectively). In the diabetic group, there were no significant differences in insulin receptor concentration.

Tyrosine kinase phosphorylation was measured to determine if there were any alterations in signal transduction activity in gastrocnemius muscle. Measurements were performed at basal activity levels as well as in an insulin stimulated state. Since phosphorylation activity is higher in the insulin stimulated state, any differences between groups would be more easily discriminated. Results in this study indicated that there was little difference in tyrosine kinase activity in terms of diet. Activity was similar in all groups in the basal state (Table 5). In the insulin-stimulated state, overall activity was elevated and there was a significant effect of genotype. The dbC showed a trend toward higher values and significance was reached when comparing dbC versus lnZS (0.0031 vs. 0.0019 pmol/min/mg phosphate, respectively).

V. Discussion

The db/db mouse was used as model for DM-2 to investigate the effects of dietary zinc intake on the progression of diabetes. The diets were initiated at an early age, ie. weanling, before significant clinical signs were present to investigate the possibility of delaying diabetic progression in the zinc supplemented db/db and exacerbating clinical signs with marginal zinc deficiency in db/db. The dbZS had a significantly lower serum glucose (Figure 6) and insulin concentrations (Table 4), a significantly lower body weight (Figure 3), as well as pancreatic zinc concentrations equivalent to lean control values (Figure 5). Also interesting was the finding of a significant negative correlation between femur zinc and serum glucose concentrations (Figure 7). Serum glucose values clearly showed a trend to lower concentrations at higher concentrations of dietary zinc. Each of these results will be discussed in detail in the following sections. Together, these findings further confirm the relationship between zinc status and glucose metabolism.

Body Weights

Both diet and genotype had a significant effect on body weight (Figure 3). Genotypic differences in weight were significant and evidenced by lower weights in lean mice starting from week 0 of treatment. From 6 weeks of age and only 2 weeks into dietary treatment, the dbC group showed a significantly greater propensity towards obesity than either dbZD or dbZS groups. The lower weights in the dbZD and dbZS groups may have resulted from an interaction of the dietary treatment and the db/db

genotype, as manipulation of dietary zinc did not significantly change body weights in the lean genotype. No significant change in body weight in the lean mice also confirms that the dietary zinc supplementation was not toxic at 300 ppm zinc.

The differences due to genotype in weight gains and total weight are well documented in the db/db mouse. Hummel et al (1966) first chronicled the significant weekly weight gains of the homozygous db/db compared to their heterozygous lean littermates, and this study is clearly consistent with their findings. Other more recent reports by Orland & Permutt (1987) and Donaldson et al. (1986) also support the trend towards obesity in the db/db genotype.

The differences in body weight due to diet in the db/db mice in this study are not supported by existing literature. In the one available zinc study, Donaldson et al. (1986) reported no differences in the body weight of 17-18 week old control db/db mice compared to db/db mice fed a 2 ppm zinc diet for 12 weeks. Zinc deficiencies are characteristically known to initiate anorexia and retard growth in animal and human models (Prasad, 1983; Reeves & O'Dell, 1983). It is plausible that our study initiated the zinc deficient diet at a more crucial point in growth and development than the study by Donaldson et al. (1986), and for that reason, the dbZD mice weighed 14.2% less than the dbC mice at week 6 of treatment.

In a review of the existing literature, there is no data on the effects of dietary zinc supplementation on growth parameters in the db/db mouse. Therefore, one can only speculate as to why the dbZS group in our study also weighed less than the dbC group. The lower body weight in dbZS mice may be the result of a reduced hyperphasia due to

improvements in insulin resistance and glucose disposal. Consequently, the result of a lowered body weight in dbZD and dbZS may be the same, but the physiological mechanisms behind it may differ. It would be interesting to compare the degree of insulin resistance in a glucose tolerance test to further differentiate these dietary groups.

Mineral Status (Zn, Ca, P) and Urine Indices of Diabetes

Femur and serum zinc status are often employed to assess overall zinc status in the body, with femur zinc being a stable reflection of long-standing zinc deposition in the body. Significantly lower femur zinc concentrations have been reported in diabetic mouse models compared to their lean counterparts. Donaldson et al. (1986) reported 10% lower femur zinc concentrations in 18 week old db/db mice, while Begin-Heick et al. (1985) reported a 22% lower femur zinc concentrations in 12-16 week old ob/ob mice compared to lean counterparts. This indicates potential presence of zinc deficiency in diabetic mouse models. We, however, did not observe a significant depletion of femur zinc concentration in the diabetic mice compared to lean groups. Significant differences in this study were the result of dietary zinc manipulations, with higher bone zinc concentrations evident with high dietary zinc intake (Figure 4). The finding of no significant genotypic differences in femur zinc concentrations was unexpected, but it is plausible that there is an age related effect that could be responsible for the discrepancy. Studies by Donaldson et al. (1986) suggest that there is a marked decrease in bone mineral accretion in the db/db compared to their lean controls from age 6 weeks on. In addition, they found a marked increase in lipid vacuoles in the db/db bone marrow

starting at this age. This increase in bone lipid would artificially increase femur dry weight and dilute the zinc concentration. This perhaps explains significant differences in bone zinc between db/db and db/m mice terminated at ~16-18 weeks of age (Donaldson et al., 1986 & 1988; Levine et al., 1983). In this study, mice terminated at 10 weeks of age may not exhibit these age related genotypic differences.

There was a significant effect of dietary zinc supplementation on femur zinc concentrations. Our study demonstrated higher femur zinc concentrations with higher dietary zinc intake in both the db/db and db/m mice. In other studies, the supplementation of zinc in the drinking water of db/db mice and in the diet of ob/ob mice failed to affect femur zinc concentrations (Levine et al., 1983; Begin-Heick et al., 1985, respectively). Again, the ability to modulate femur zinc with dietary zinc may be due to an age effect. Our supplementation began at weanling rather than in adult diabetic mice, when depletions of bone mineral may not be recoverable.

In a further assessment of bone mineral content, calcium and phosphorus concentrations were measured (Table 4). Results showed a proportionally similar relationship between the two minerals as the calcium to phosphorus ratio was ~2:1. We observed a significant effect of genotype, with lower concentrations of both minerals in the db/db groups. In an assessment of femur mineral status in the db/db strain without dietary zinc manipulation, Donaldson et al. (1988) also found that femur calcium concentrations were significantly lower in the diabetic genotype when compared to lean control mice at 18 week of age. The data on calcium and phosphorus supports the concept of lower bone mineral accretion in the db/db mouse.

As in the case of femur zinc, serum zinc concentrations in this study were not found to differ significantly between db/db and db/m genotypes (Table 4). This is similar to Donaldson et al. (1986), who found serum zinc to be the same in db/db and lean controls at 18 weeks of age. These findings in the db/db mouse are in direct contrast with many human studies of non-insulin dependent diabetics, all of which report significantly lower serum zinc concentrations ($< 70 \mu\text{g/dl}$) in all or a subset of DM-2 patients when compared to healthy controls (Canfield et al., 1984; Kinlaw et al., 1983; Niewoehner et al., 1986; Sjogren et al., 1988; Winterberg et al., 1989). Thus, it may be that the zinc status of the diabetic mouse reveals a physiological difference in the etiology of the diabetic state in humans versus mice.

While serum zinc showed no significant changes due to diabetes, the effect of genotype on urinary zinc excretion was significant in our study. The zinc content in the urine was almost double in db/db mice compared to lean controls during a 12 hour collection period (Figure 8). This higher urinary zinc excretion (hyperzincuria) is a common occurrence in diabetes and other hypercatabolic states such as injury or starvation (Prasad, 1983). Excessive losses of zinc in the urine have also been reported by Levine et al. (1983) in the db/db mouse as well as in DM-2 diabetic patients (Pidduck et al., 1970; Kinlaw et al., 1983). The explanation for high urinary zinc excretion is obscure. Kinlaw et al. (1983) have suggested that the high excretion of zinc may be caused by hyperglycemia and/or a renal abnormality. The excretion of zinc in the urine in our study was not influenced by dietary zinc intake.

The excretion of glucose in the urine of the db/db mice was significantly higher than in the db/m control mice. Urinary glucose excretion was 50-200 fold higher in db/db mice versus lean controls (Table 4). Differences due to dietary treatment in diabetic groups revealed that glucose excretion by dbZD was significantly lower than either dbC or dbZS groups. This was in contrast to the high serum glucose concentrations in dbZD. Thus, it seems that the glucose is retained and not excreted as extensively in the dbZD group. Urinary protein excretion, a measure of the severity of diabetes, was not significantly different between dbC and lnC groups (Table 4). There was a trend for lower protein excretion in dbZD and dbZS groups compared to dbC mice, however, these differences failed to reach statistical significance. Urinary creatinine excretion was originally employed in this study to act as a stable measurement against which urine glucose and protein could be quantified. However, it was revealed that creatinine values show significant genotypic variability. Creatinine excretion, which is proportional to body muscle mass, was significantly lower in the diabetic genotype compared to the lean controls (Table 4). It appears that despite a significantly higher body weight (Figure 3), the db/db genotype may have significantly lower muscle mass.

Higher concentrations of glucose and protein in the urine are a common condition in diabetes. However, it is not a variable that is often measured in animal models. There are no known studies in the current literature that identify the glucose, protein or creatinine excretion rates of diabetic animals, with or without dietary zinc manipulation.

Pancreatic and Kidney Zinc Concentrations

Tissue zinc status is known to be altered in many diabetic animal models. In this study, there were significant differences in pancreas and kidney zinc concentrations in db/db versus lean groups. The pancreatic zinc concentrations of dbZD and dbC were significantly lower than lean mice (Figure 5). This finding was expected as the zinc concentration in the human diabetic pancreas has been reported to be ~50% less than non-diabetics (Scott & Fisher, 1938). More recently, significantly lower pancreatic zinc concentrations have been reported in db/db and ob/ob mice (Southon et al., 1988 in db/db; Begin-Heick, 1985, Kennedy & Failla, 1987 in ob/ob). In our study, zinc supplementation of db/db mice (dbZS group) restored the pancreatic zinc concentration to that observed in lean controls. This higher concentration of pancreatic zinc in the dbZS group was the result of a higher zinc content in the pancreas, as pancreatic dry weight did not significantly differ in the dbC versus the dbZS group (Appendix A). This repletion of zinc in the pancreas has not been previously reported in the literature, and may be physiologically significant for diabetics. Since zinc is involved in insulin crystal structure and pancreatic insulin storage, this increased availability of zinc to the β -cells may assist in insulin granule storage and thus, insulin secretion (Roth & Kirchgessner, 1981). Since Boquist & Lernmark (1969) reported a decreased granulation of the β -cells in zinc deficient animals, it would be interesting to investigate these new found effects of zinc supplementation on β -cell pathology and pancreatic function.

The renal zinc concentration in the diabetic mice fed the control diet (dbC) was found to be significantly (1.4-1.7 fold) higher than all other treatment groups except

dbZS (Table 4). This finding is in contrast to Donaldson et al. (1988) who observed a slightly lower renal zinc concentration in db/db mice compared to lean controls. Levine et al. (1983) found no alterations in kidney zinc concentration of the db/db mice, while Kennedy et al. (1986) noted slight, but significant decreases in renal zinc concentration of ob/ob mice. In contrast, streptozotocin-induced diabetic mice (a model for DM-1) have significantly higher kidney zinc concentrations, but this can be normalized with daily injections of insulin (Failla & Kiser, 1981; Failla & Gardell, 1985; Yang and Cherian, 1994). The observation that dbC mice had elevated renal zinc concentrations compared to dbZD and dbZS groups may be a reflection of the severity of their diabetes as indicated by serum and urinary glucose concentrations. Donaldson et al. (1988) noted that the greatest increases in renal zinc were in the mice identified as being the most diabetic. Since Kinlaw et al. (1983) suggested that urinary zinc excretion also may be due to renal abnormalities, investigations into the associations between zinc status, renal function and diabetic severity seem warranted.

Serum Glucose and Insulin

Fasting serum glucose and insulin concentrations both aid in determining the degree of insulin resistance and insulin sufficiency in diabetes. The db/db mice as a group had significantly higher serum glucose concentrations (Figure 6). This finding of higher serum glucose in the db/db was expected and follows the general observations of many others (Coleman, 1978; Hummel et al., 1966; Levine et al., 1983; Roesler &

Khandelws, 1985; Orland & Permutt, 1987). Serum glucose concentrations were modulated with diet, as serum glucose concentrations were lower with higher dietary zinc intake. This trend was observed in both the diabetic and lean genotypes. Since this study was the first to assess glucose status in both the zinc deficient and zinc supplemented diabetic animal model, there is no published literature to confirm our finding of lower serum glucose concentration in the supplemented state. Many papers, however, do agree with our finding of a higher serum glucose concentration in the zinc deficient groups. Southon et al.(1988) found that a low zinc diet (1 ppm zinc) fed for 4 weeks resulted in significantly higher fasting serum glucose concentrations in 8-9 week old db/db mice. As well, Reeves and O'Dell (1983) observed higher serum glucose concentrations in zinc deficient, non-diabetic rats. Others have reported impaired oral glucose tolerances with zinc deficiency (Boquist & Lernmark, 1969; Hendricks & Mahoney, 1972; Park et al., 1986). Given the evidence from other studies as well as our own, it appears that zinc clearly influences tissue glucose uptake in the diabetic as well as in non-diabetic state. It will take further investigation to clearly identify the role of a zinc supplementation in diabetic glycemic management.

Given the findings of lower serum glucose concentrations with higher dietary zinc intake, it was expected that serum insulin concentrations would also reflect a similar relationship. Such was not the case, as the serum insulin concentration was highest in dbC, and similar lower concentrations were evident in both the zinc deficient and zinc supplemented db/db groups (Table 4). It would seem that any dietary shift in zinc, be it deficient or excessive, lowers serum insulin concentrations. The mechanism involved,

however, may differ. The lower serum insulin concentration in the dbZS group in this study is similar to the results of Bégin-Heick et al. (1985). They observed that 9-12 week old ob/ob mice fed a 1000 ppm zinc supplemented diet for 4 weeks had lower fasting plasma insulin concentrations during glucose tolerance testing. It may be that the lower serum insulin concentration in dbZS compared to dbC in our study was the result of an improved glucose uptake by peripheral tissues.

The lower serum insulin concentration in the dbZD group compared to the dbC group has also been confirmed by the literature. Zinc deficient rats have significantly lower concentrations of circulating insulin in the face of high serum glucose concentrations (Huber & Gershoff, 1973). In that study, Huber and Gershoff (1973) determined that pancreatic insulin synthesis was normal. Thus, it appears that in the zinc deficiency, there may be an impaired insulin release and/or an elevated degradation of circulating insulin.

Liver Lipid

It has been proposed that elevated liver lipid concentration in the diabetic state is the result of hyperinsulinemia (Zucker and Zucker, 1962). Liver lipid concentration and weight of the liver were both genotypically significant in this study. Diabetic mice had a significantly higher liver weight compared to the lean groups (Table 5). The liver weight of the dbC group was significantly higher than either dbZD or dbZS mice. Liver weights, however, were found to be proportional to body weight, as the ratio of liver weight to

body weight was approximately 0.045 in all treatment groups (Table 5). This higher liver weight in the diabetic genotype did not translate into higher liver lipid content, as dbC and dbZS had less lipid per gram liver than the lean groups. Interestingly, the dbZD group had a liver lipid concentration not significantly different from the lean groups. However, when total lipid content of the liver was analysed, there were no significant differences with either genotype or diet. This is opposed to the finding of Koe et al. (1985), who observed a higher liver lipid content in diabetic Zucker rats.

Fat Pad Weight

Fat pad weights were measured as an indication of the degree of obesity in the db/db mice. Fat pad weight was significantly influenced by genotype, as fat content of the body was significantly higher in the diabetic versus the lean genotype (Table 5). In terms of dietary treatment, the dbC group had a significantly higher body fat content than the dbZD group. This higher fat content of the body of dbC did not translate to a higher percent body fat, as treatment groups within the diabetic or lean genotype did not significantly differ. These findings are difficult to confirm with the current literature, as fat pad weights have not previously been reported in a zinc manipulated db/db mouse.

Insulin Receptor Concentration and Tyrosine Kinase Phosphorylation

In a state of insulin resistance, one of the possible cellular defects could be reduced binding of insulin to its cell surface receptor. Alternatively, depressed insulin

action could be the result of an altered or deficient intracellular transduction signal (Saltiel, 1990; Crettaz & Jearrenaud, 1980).

Receptor concentrations in this study were not significantly different in diabetic and lean genotypes. In terms of diet, the lnZD group had a significantly lower receptor concentration when compared to lnZS mice (Table 5).

There was little difference in tyrosine kinase activity in terms of genotype or diet. Activity was similar in all groups in the basal state, although there was a trend toward lower values in the dbZD and dbC groups (Table 5). In the insulin-stimulated state, overall activity was elevated and there was a significant effect of genotype. There were no significant differences due to diet, but the dbC group showed a trend towards higher values.

The results of our study are difficult to confirm with the current literature. Firstly, the method for isolating the insulin receptors employed in this study is relatively new (Klein et al., 1995). The use of antibodies to adhere the receptors is preferable to the alternative of partial purification on lectin columns of wheat germ agglutinin agarose (Klein et al., 1995). Isolation of receptors via an anti-insulin receptor antibody is reported to lower background activity in kinase determinations and lower non-specific binding for receptor binding measurements (Klein et al., 1995). It is also reported to reduce the bound insulin receptor to IGF-1 receptor ratio from 2:1 as in the wheat germ agglutinin agarose method to a < 10:1 ratio (Klein et al., 1995). Klein et al. (1995) also note that the procedure avoids significant losses of receptors and does not select receptors according to their glycosylation as the lectin procedures does.

This antibody procedure has not previously been utilized for study in the db/db mouse. Based on the wheat germ agarose method, there were no differences in binding or kinase activity in the hind limb muscle of 24 week old db/db compared to lean controls (Vicario et al., 1987). Their rationale for the finding of no change was that the db/db exhibits a decrease in insulinemia over time. This lowering of insulin concentration would reduce the down-regulation of the insulin receptors, thus restoring normal binding. This, however, does not seem likely as our study also failed to find a decrease in insulin receptor concentrations or tyrosine kinase activity, despite very high concentrations of circulating insulin at the age of 10 weeks. The highest serum insulin concentrations were in the dbC group and the lowest were in the non-diabetic leans. Yet, the concentration of receptors or kinase activity in dbC mice was not significantly lower than that of any lean group.

In human studies, Comi et al. (1987) reported that diabetic patients exhibited a significantly lower insulin receptor tyrosine kinase activity. They note that this lower activity is above that which may be the result of lower insulin receptor concentrations. It appears that alterations at the receptor level reveal differences in the etiology of DM-2 in animal models versus humans. Since these animals are clearly insulin resistant, further investigation into signal events in the insulin receptor transduction pathway will be required to reveal the causative factors for their pathology. The db/db mouse model also offers an excellent means to investigate the interaction of nutrition and genetics.

VI. Summary and Conclusions

The objective of the current experiment was to investigate the effects of dietary zinc manipulation on zinc status and diabetic parameters in the diabetic mouse (db/db), a model for DM-2. The goal was to determine the role of zinc in insulin action and to elucidate the potential benefits of a zinc supplementation to diabetic pathology.

The use of the db/db mouse as a model for DM-2 was successful. By the age of 10 weeks, db/db mice exhibited significant increases in body weight, glycemia, insulinemia and glycosuria compared to db/m littermates. The zinc status of the db/db mice was also influenced by the diabetic state as indicated by alterations in pancreatic and urine zinc concentrations in db/db compared to db/m control mice. The use of db/m as the lean control also documented the effects of zinc in a non-diabetic mouse.

Zinc supplementation in the db/db mouse was investigated to ascertain its beneficial effects on diabetic parameters. The dbZS group had a significantly lower serum glucose concentration compared to dbZD and also exhibited a significantly lower concentration of serum insulin than dbC. These findings suggest that dietary zinc supplementation may restore peripheral glucose disposal and lessen excessive insulin secretion from the pancreas. The dbZS had the lowest serum glucose and the highest pancreatic zinc concentration amongst the db/db groups. These results further confirm the requirement for adequate zinc concentrations in the pancreas. Since the trend was for glucose concentrations to be lower with higher concentrations of dietary zinc in both the

diabetic and lean genotypes, it appears that zinc supplementation may have a beneficial effect for diabetic management. Further investigations into optimal dose requirements and the effects of zinc supplementation on glycemic control in humans with DM-2 appears to be indicated.

The effects of the zinc deficiency in db/db mice were also pronounced. The dbZD exhibited a significantly lower body weight by week 6 compared to dbC, the highest serum glucose concentrations of all treatment groups, and lower pancreatic zinc concentrations compared to dbZS and all db/m groups. These findings would be indicative of reduced glucose utilization and disposal. The dbZD group also had a significantly lower concentration of circulating serum insulin compared to dbC. These results were unexpected given the high serum glucose values, but the lower concentrations of serum insulin may be an indication that pancreatic secretion is altered or limited in marginally zinc deficient db/db mice. Alternatively, there may be a higher degradation of circulating insulin compared to dbC. Further research into pancreatic islet function as well as time-charted oral glucose tolerance tests in the zinc deficient db/db mouse appear warranted.

The effect of genotype on tissue zinc status was determined to assess the degree of zinc maldistribution in the diabetic state. The db/db mouse, as evidenced by findings in the db/db genotype compared to the db/m genotype, exhibited a significantly lower pancreatic zinc concentration and significant hyperzincuria. The db/db mouse did not, however, exhibit significant depletions of serum or femur zinc concentration. This was unexpected, but reveals that the db/db mouse may be more resistant to the actions of

marginal zinc deficiency. This is in contrast to many reports of a lower serum zinc concentration in DM-2 patients (Canfield et al., 1984; Kinlaw et al., 1983; Niewoehner et al., 1986; Sjogren et al., 1988; Winterberg et al., 1989). It appears that zinc status reveals some physiological variations in DM-2 as evidenced by animal models.

The final goal of this study was to relate the effects of the zinc diets to events at the molecular level. The findings in this study revealed no significant effects due to diet on insulin receptor concentration or tyrosine kinase activity. In addition, the diabetic groups did not exhibit lower insulin receptor numbers or tyrosine kinase activity. This was unexpected and reveals that the underlying causes for insulin resistance in the db/db mouse remain to be elucidated. Study into the basic mechanisms and pathways in insulin signal transduction will further the effort in establishing the molecular basis for altered insulin action in DM-2.

In conclusion, the results of this study reveal that zinc status is inversely correlated with serum glucose concentrations in genetically diabetic mice. Dietary zinc supplementation also assisted in ameliorating high serum glucose concentrations in both db/db and db/m mice. Further inquiry into the role of zinc supplementation for DM-2 patients is clearly warranted.

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Effects of dietary zinc on body weights (0-6 weeks), and femur and pancreatic zinc, serum glucose and urine zinc (at 6 weeks of dietary treatment).¹

	Treatment Groups ²					
	dbZD	dbC	dbZS	lnZD	lnC	lnZS
Body Weight week 0 (g)	20.9 ^A ±0.8	22.3 ^A ±0.5	21.6 ^A ±1.0	15.3 ^B ±0.6	16.4 ^B ±0.4	15.8 ^B ±1.1
Body Weight week 2 (g)	21.9 ^A ±1.5	25.8 ^B ±1.2	22.5 ^A ±1.4	15.9 ^C ±0.4	16.5 ^C ±0.7	16.1 ^C ±1.2
Body Weight week 4 (g)	26.4 ^A ±2.2	31.4 ^B ±1.7	26.1 ^A ±1.8	21.4 ^C ±0.8	21.6 ^C ±0.9	21.2 ^C ±0.7
Body Weight week 6 (g)	29.0 ^A ±1.9	33.8 ^B ±1.0	29.5 ^A ±1.0	19.5 ^C ±0.7	20.7 ^C ±0.4	20.0 ^C ±0.7
Femur Zn (µg/g dry wt.)	224.0 ^A ±11.6	242.9 ^A ±7.5	311.4 ^B ±18.5	219.3 ^A ±14.8	263.2 ^{A,C} ±16.7	303.5 ^{B,C} ±10.7
Pancreatic Zn (µg/g dry wt.)	87.8 ^{A,B} ±8.6	81.9 ^A ±3.0	117.7 ^C ±8.2	119.5 ^C ±5.9	109.3 ^{B,C} ±6.6	131.1 ^C ±13.8
Pancreas Wt. (mg dry wt.)	64.2 ^A ±3.5	53.9 ^{A,B} ±5.9	52.3 ^{A,B} ±5.9	35.2 ^C ±4.0	41.5 ^{B,C} ±6.1	53.7 ^{A,B} ±7.0
Serum Glucose (mg/dl)	498.1 ^A ±68.8	424.9 ^{A,B} ±65.2	344.5 ^{B,C} ±47.7	241.2 ^{C,D} ±49.7	177.5 ^D ±11.1	139.8 ^D ±8.2
Urine Zn (µg/12 hrs.)	0.76 ^A ±0.10	0.74 ^{A,B} ±0.14	0.80 ^A ±0.22	0.38 ^{B,C} ±0.08	0.39 ^{B,C} ±0.05	0.35 ^C ±0.12

¹ Values are means ± standard error of the mean. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test. n = 7 except for dbZS, lnZD, and lnZS where n = 8, n = 6, n = 6, respectively.

² dbZD = db/db zinc deficient, dbC = db/db control, dbZS = db/db zinc supplement, lnZD = db/m zinc deficient, lnC = db/m control, lnZS = db/m zinc supplement

Effects of dietary zinc on urine indices of diabetes at week 4 of dietary treatment ¹

	Treatment Groups ²					
	dbZD	dbC	dbZS	lnZD	lnC	lnZS
Urine Zn ($\mu\text{g}/12$ hours)	0.38 ^A ± 0.07 n = 7	0.58 ^{A,B} ± 0.14 n = 7	1.32 ^C ± 0.23 n = 6	0.35 ^A ± 0.08 n = 6	0.51 ^{A,B} ± 0.06 n = 7	0.59 ^{A,B} ± 0.15 n = 6
Urine glucose (mg/12 hours)	4.7 ^A ± 3.5 n = 7	15.1 ^B ± 3.0 n = 7	4.2 ^A ± 2.2 n = 7	0.1 ^C ± 0.0 n = 5	0.3 ^C ± 0.1 n = 7	0.3 ^C ± 0.01 n = 6
Urine protein (mg/12 hours)	0.65 ^A ± 0.23 n = 6	0.83 ^{A,B} ± 0.37 n = 7	0.70 ^A ± 0.17 n = 7	0.87 ^{A,B} ± 0.47 n = 6	1.62 ^C ± 0.35 n = 7	1.82 ^C ± 0.70 n = 6
Urine Creatinine (mg/12 hours)	0.044 ^A ± 0.008 n = 6	0.058 ^{A,B} ± 0.014 n = 4	0.046 ^A ± 0.009 n = 4	0.083 ^B ± 0.025 n = 4	0.113 ^C ± 0.016 n = 6	0.107 ^C ± 0.032 n = 4

¹ Values are means \pm standard error of the mean. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test. Due to sample shortage and variability, n values are listed separately in the table.

² dbZD = db/db zinc deficient, dbC = db/db control, dbZS = db/db zinc supplement, lnZD = db/m zinc deficient, lnC = db/m control, lnZS = db/m zinc supplement

Main effects of genotype, diet, and genotype x diet interaction on body and fat pad weights, trace mineral status (Zn, Ca, P), serum glucose and insulin after 6 weeks of dietary treatment.

Main Effects ¹			
	Genotype	Diet	Genotype X Diet
Body Weight (g)	0.0001 *	0.0332 *	0.133
Fat Pad Weight (g)	0.0001 *	0.171	0.0879
Femur Zinc ($\mu\text{g/g}$ dry weight)	0.604	0.0001 *	0.613
Femur Calcium ($\mu\text{g/g}$ dry weight)	0.0387 *	0.966	0.345
Femur Phosphorus ($\mu\text{g/g}$ dry weight)	0.0014 *	0.849	0.569
Serum Zinc ($\mu\text{g}/12\text{hrs}$)	0.260	0.601	0.0242 *
Pancreatic Zinc ($\mu\text{g/g}$ dry weight)	0.0025 *	0.0087 *	0.480
Kidney Zinc ($\mu\text{g/g}$ dry weight)	0.137	0.152	0.127
Serum Glucose (mg/dl)	0.001 *	0.117	0.706
Serum Insulin (ng/ml)	0.0001 *	0.0027 *	0.0027 *

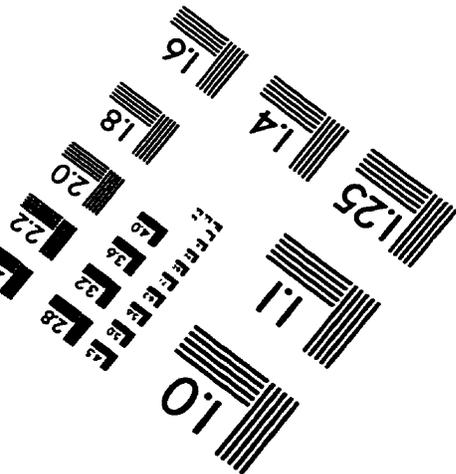
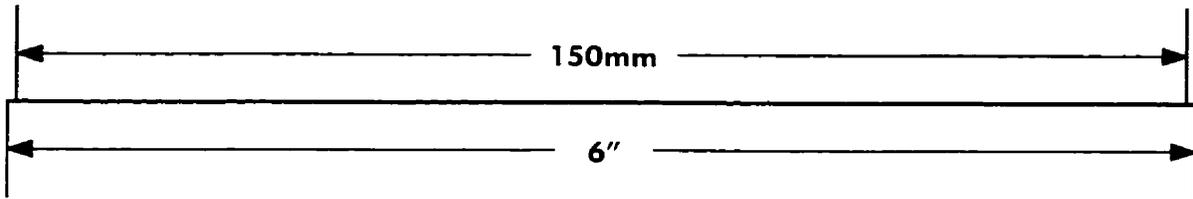
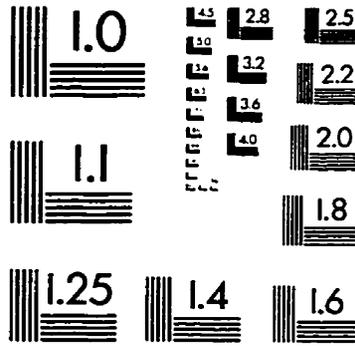
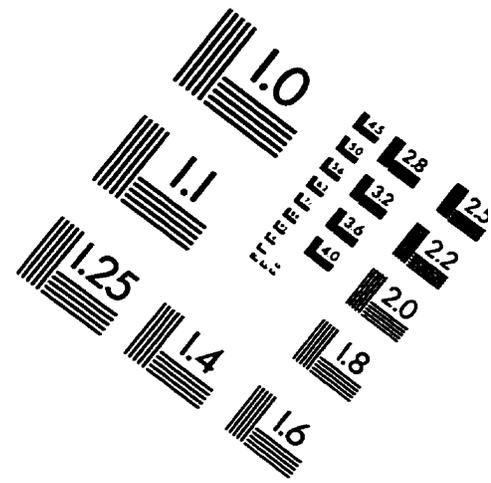
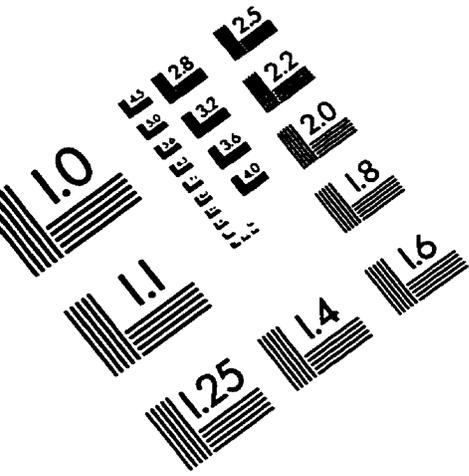
¹ Values are $Pr > F$ as assessed by ANOVA. * = significant main effect at $\alpha \leq 0.05$.

Main effects of genotype, diet, and genotype x diet interaction on urine and tissue indices of diabetes after 6 weeks of dietary treatment.

Main Effects ¹			
	Genotype	Diet	Genotype X Diet
Urine Zinc ($\mu\text{g}/12$ hrs)	0.0003 *	0.956	0.919
Urine Glucose (mg/12 hrs)	0.0007 *	0.151	0.152
Urine Protein (mg/12 hrs)	0.0544	0.202	0.993
Urine Creatinine (mg/12 hrs)	0.0174 *	0.302	0.120
Liver Lipid (mg/g liver)	0.0007 *	0.682	0.508
Liver Weight (g)	0.0001 *	0.0287 *	0.225
Insulin Receptor Concentration (cpm/mg protein)	0.305	0.608	0.515
Tyrosine Kinase Phosphorylation (basal)(pmol/min/mg phosphate)	0.145	0.810	0.967
Tyrosine Kinase Phosphorylation (+insulin)(pmol/min/mg phosphate)	0.0491 *	0.305	0.414

¹ Values are $Pr > F$ as assessed by ANOVA. * = significant main effect at $\alpha \leq 0.05$.

IMAGE EVALUATION TEST TARGET (QA-3)



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