# THE EFFECT OF CHROMIUM DEPLETION AND STREPTOZOTOCIN-INDUCED DIABETES IN PREGNANCY

By

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in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1996

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*Dedicated to and in memory of Bernardo F. Caluya*

## ACKNOWLEDGEMENTS

First, I would like to thank my family: my husband, Leon; my children, Anna, Michael and Melissa; my mother, Virginia Caluya Torres; my father, Carlos C. Torres; my uncle, Bernardo F. Caluya; my grandfather, Pio Q. Caluya, and the rest of the clan for their endless support and patience, and, for believing in me. Most especially, I would like to thank my major adviser and committee chairwoman, Dr. Barbara J. Stoecker, for her constructive guidance, her calm responses, her valuable suggestions, her support which came in many forms and many ways, and, for her endless patience and steady keel. Thank you, Dr. Stoecker.

I am grateful to the members of my committee: Dr. Andrea Arquitt, Dr. Lea Ebro and Dr. Robert P. Wettemann for their input towards this dissertation and this graduate degree. Thank you, All, for sticking with me.

I wish to express my sincere gratitude to all those that have helped with this project, most especially: Bernice Adeleye, Swarna Mandali, Tracey Chen, Emilia Achmadi, Shannon Dougherty, Melissa Davis Whitenack, Beth Keefer, Pat Bowen, Pam Ventris, Kerri Shirley, Jarod King, Wen Wang and Terra Smith. Thank you.

Finally, I would like to acknowledge the physical and logistic support of the Department of Nutritional Sciences, the College of Human Environmental Sciences, the Animal Science Department, the College of Veterinary Medicine Lab Animal Resources, Bruce Nance and Robin Guinn, Dr. Joseph McCann and Oklahoma State University. All the labs including the Math Science computer lab were invaluable.

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## CHAPTER V

## LIST OF ABBREVIATIONS

| | |
|---|---|
| Cr | chromium |
| $CrCl_3$ | chromium chloride |
| CrPic | chromium tripicolinate |
| Db | diabetes |
| ESADDI | Estimated Safe and Adequate Daily Dietary Intake |
| GDM | gestational diabetes mellitus |
| GLM | general linear models |
| GTF | glucose tolerance factor |
| GTT | glucose tolerance test |
| HDL | high density lipoproteins |
| IGF | insulin-like growth factors |
| IGFBP | IGF binding proteins |
| kD | kilo Dalton |
| LDL | low density lipoproteins |
| LPL | lipoprotein lipase |
| LiOH | lithium hydroxide |
| RDA | Recommended Dietary Allowances |
| SAS | Statistical Analysis System |
| SD | Sprague-Dawley |
| SGA | small for gestational age |
| STZ | streptozotocin |
| TPN | total parenteral nutrition |
| VLDL | very low density lipoproteins |

# CHAPTER I

# INTRODUCTION

## Research Problem

Numerous metabolic changes related to nutrition occur in normal uncomplicated pregnancy. Insulin resistance increases and glucose tolerance decreases. This is manifested by elevated blood insulin concentrations which maintain blood glucose within normal limits (1). As pregnancy progresses, lipid metabolism is altered such that lipogenesis is favored early in pregnancy but not in late pregnancy. In late gestation, decreased lipoprotein lipase activity (LPL) in adipose tissue and increased triglyceride production by the liver result in hypertriglyceridemia. Before parturition, LPL activity in the mammary glands increases utilizing circulating triglycerides for milk production (2).

Gestational diabetes mellitus (GDM) is impaired glucose intolerance of varying severity first diagnosed during pregnancy (3). High perinatal risks such as premature birth, infant morbidity and mortality, macrosomia, congenital abnormalities and obstetric complications such as urinary tract infections, polyhydramnios, pre-eclampsia and toxemia result from uncontrolled GDM as well as from poorly managed diabetes in pregnancy (4,5). In the early 1980's, GDM was a global problem that developed in 2-3% of all pregnancies (6). In 1988, 4% of all pregnancies that resulted in live birth in the United

States were complicated with diabetes. Of these 154,000 pregnancies, 88% were complicated by gestational diabetes mellitus, 8% by pre-gestational non-insulin dependent diabetes and 4% by pre-gestational insulin dependent diabetes (7). Congenital malformations, stillbirth and neonatal death still occur despite more intensive prenatal care for the diabetic pregnant woman (8).

Maternal nutrition is a significant contributor to the outcome of pregnancy. Most mothers with GDM can achieve good metabolic control by only modifying their diets. Some require supplemental insulin to help control glucose concentrations in the blood. Insulin dependent diabetic mothers may have a more difficult time with glucose control resulting in large fluctuations in blood glucose and unpredictable reactions to insulin especially in adolescent pregnancy. Adequate chromium in the diet may potentiate the action of insulin especially in those individuals who have suboptimal intakes of the trace mineral. The analysis of freely chosen and institutional composite diets showed that even well planned diets contain less than the recommended estimated safe and adequate daily dietary intake of 50 μg chromium per day (9). Dietary chromium supplementation of patients, subjects and experimental animals presumed to be deficient in chromium has been shown to improve glucose tolerance and decrease the need for exogenous insulin (10 - 14). The effect of supplemental dietary chromium in promoting better glucose control and minimizing the need for exogenous insulin in pregnant women that have difficulty achieving normal glucose homeostasis has not been studied (15).

In murine pregnancy, the insulin-like growth factors (IGF's) are necessary for embryogenesis and fetal growth (16 - 19). Streptozotocin induced diabetic pregnancies in rats resulted in impaired fetal growth (20 - 22). Chromium deficient mature rats are

growth retarded compared to rats fed chromium (23). Growth retarded fetal rats had decreased IGF-I and -II gene expression, decreased fetal serum IGF-I and increased IGFBP-1 gene expression (24 - 26). Decreased serum IGF-I, differential gene expression of IGF-I and IGFBP-I as well as discordant organ specific regulation of IGF-I mRNA have been observed in streptozotocin induced diabetic male rats and non-pregnant female rats (27 - 29). In adult streptozotocin diabetic male Sprague Dawley rats, renal IGF-I accumulation occurred despite a reduction in plasma IGF-I concentrations (30). The effect of streptozotocin diabetes as well as dietary chromium on the IGF system has not been studied in pregnant rats.

Diabetes mellitus results in the alteration of micronutrient status (31). Abnormal trace element metabolism occurs in diabetic rat dams and their fetuses (20 - 33). Manganese, copper and zinc accumulated in the liver and kidneys of diabetic dams. Compared with normal controls, fetuses of diabetic dams have low levels of liver zinc and elevated levels of liver manganese. In the placenta, copper, magnesium and calcium are reduced. The effect of chromium on macro- and micromineral distribution in the tissues and its interactions with other microminerals has not been studied in the STZ-induced diabetic pregnant rat model.

## Objectives and Hypotheses

The first objective of this study was to determine the effect of chromium depletion and diabetes induced during pregnancy on glucose tolerance, and on circulating total cholesterol, total triglycerides, non-esterified fatty acids and β-hydroxybutyrate concentrations.

Null Hypothesis: Chromium depletion and diabetes during pregnancy do not affect maternal glucose tolerance or total plasma cholesterol, triglycerides, non-esterified fatty acids and β-hydroxybutyrate concentrations.

The second objective of this study was to determine the effect of chromium depletion and diabetes induced during pregnancy on plasma insulin, glucose, IGF-I, IGF-II, maternal weight gain and pregnancy outcome.

Null Hypothesis: Chromium depletion and diabetes during pregnancy do not affect maternal plasma insulin, glucose, IGF-I, IGF-II, maternal weight gain or pregnancy outcome.

The third objective of this study was to determine the effect of chromium depletion and diabetes during pregnancy on fetal plasma insulin, glucose, IGF-I, IGF-II, fetal weight, fetal protein and hydroxyproline concentrations.

Null Hypotheses: Chromium depletion and diabetes during pregnancy do not affect fetal plasma insulin, glucose, IGF-I, IGF-II, fetal weight, fetal protein or hydroxyproline concentration.

The fourth objective of this study was to determine the effect of chromium depletion and diabetes during pregnancy on macromineral and trace mineral concentrations in the liver, kidney, spleen, heart, placenta and fetuses of the experimental animals.

Null Hypotheses: Chromium depletion and diabetes during pregnancy do not affect macromineral and trace mineral concentrations in the liver, kidney, spleen, heart, placenta and fetuses of the experimental animals.

## Format of Dissertation

This dissertation will contain six chapters: the introduction, literature review, methodology, two journal articles and a conclusion. All chapters will be formatted according to the recommended manuscript preparation style required by Diabetes, the journal of the American Diabetes Association.

# CHAPTER II

# REVIEW OF THE LITERATURE

## Chromium

The trace mineral, chromium, has effects on both glucose and lipid metabolism. Chromium deficiency in man and animals is associated with impaired glucose tolerance and a syndrome simulating diabetes mellitus (23,34). Fasting hyperglycemia and glycosuria have been observed in rats with an intake of 0.17 μg chromium per gram diet (34). Severe chromium deficiency in rats leads to retarded growth, decreased glycogen reserves, increased incidence of aortic lesions and disturbances in amino acid utilization for protein synthesis (23). In patients receiving total parenteral nutrition, symptoms of insulin resistance, hyperglycemia and diabetic neuropathy were alleviated by chromium supplementation (10, 11, 35).

Two considerations need to be made in evaluating chromium studies: accuracy and experimental protocols utilizing different dietary sources for chromium. Through the years, analytical methods and handling of samples for chromium analysis have improved. Due to these improvements, reported values for chromium concentrations in tissues, urine and blood have decreased to trace or micro concentrations. Contamination is a problem in chromium analysis and has to be considered throughout this review. It is assumed that the

studies mentioned herein had good controls and that all samples within each study were handled in a consistent manner. Mertz recommends that analytical values for chromium reported before 1980 should not be accepted (15) because values reported then were too high compared with values that have been confirmed more recently with improved analytical knowledge and capabilities (36). The other consideration in evaluating chromium studies is the different dietary sources of chromium which have been used in different experimental protocols. A preliminary study by Ward *et al* (37) has determined that there were no significant differences in the effects of chromium chloride ($CrCl_3$), chromium acetate, chromium oxalate, chromium nicotinate, chromium picolinate (CrPic) or chromium nicotinate-glycine-cysteine-glutamate on growth, carcass characteristics, plasma metabolites or hormones in growing finishing pigs. The following section will discuss common thoughts and observations that have arisen regarding chromium's physiological effects despite differences in dietary sources of chromium, time and experimental protocols.

Effects of chromium on glucose tolerance

Glucose tolerance may be defined as the body's ability to maintain normal blood glucose concentrations in the post-absorptive state as well as postprandially or after a glucose load. Three case reports document the positive effect of chromium supplementation on glucose tolerance in patients receiving total parenteral nutrition (TPN) that were observed to have low levels of plasma chromium. In one case (10), a patient who had been on a TPN regimen for 3 1/2 years without any problem suddenly lost weight and exhibited symptoms of peripheral neuropathy and glucose intolerance. Weight loss

and neuropathy persisted despite the addition of insulin to her treatment. The addition of 250 μg chromium to the TPN solution daily for two weeks abolished the need for insulin. Thereafter, the patient was maintained on 20 μg chromium per day and insulin was no longer needed. Freund and colleagues (11) reported a similar case with a patient that had a complete bowel resection and had been on TPN for 5 months. The patient developed severe glucose intolerance, weight loss and metabolic encephalopathy. Chromium supplementation improved glucose tolerance, reduced insulin requirements and resulted in weight gain. The third case report involved a patient with short bowel syndrome following a resection who required TPN. The patient presented with similar symptoms as the previous cases and was given additional chromium in the TPN solution. Ten days after initiating chromium supplementation, the patient no longer required insulin and was maintained on 26 μg added chromium per day (35).

In 1966, Glinsman and Mertz (38) reported that 3 out of 6 subjects with maturity onset diabetes responded to 180 μg chromium per day from $CrCl_3$ supplementation with improved glucose tolerance. Supplementation with 150 - 1000 μg chromium per day of 10 non-diabetic subjects with normal glucose tolerance tests did not alter their responses except for one person with a positive family history of diabetes who had reactive hypoglycemia. In this one case, chromium supplementation was associated with an improved glucose tolerance curve.

Chromium supplementation with 200 μg chromium from $CrCl_3$ improved glucose tolerance only in patients with marginal hyperglycemia and reactive hypoglycemia but not in normal healthy individuals. Anderson *et al* (39) observed that chromium supplementation of normal, free living subjects significantly decreased fasting and 90

minute glucose concentrations in response to a 1 g/kg glucose challenge in those subjects whose serum glucose concentrations were equal to or greater than 100 mg/dL 90 minutes after the glucose challenge. Subjects whose 90 minute concentrations were at or below their fasting levels did not show a similar response to chromium supplementation. In a later study (13), subjects who consumed controlled low chromium diets were treated with chromium supplementation or placebo in a double blind crossover design. Chromium supplementation did not change glucose tolerance or insulin responses of normal, healthy subjects while the same parameters were improved in hyperglycemic subjects whose diets were supplemented with 200 μg chromium from $CrCl_3$ per day. The Estimated Safe and Adequate Daily Dietary Intake (ESADDI) for chromium set by the National Research Council is 50 - 200 μg/day (40).

In pigs, dietary chromium supplementation from CrPic in conjunction with pituitary porcine growth hormone reduced the increase in blood glucose and insulin concentrations induced by growth hormone treatment (41). In another study (42), barrows fed 200 μg Cr/kg diet as CrPic had reduced fasting plasma insulin compared with controls. In response to intravenous glucose tolerance tests, the chromium fed barrows had a greater glucose clearance rate and a shorter serum glucose half-life. In response to an insulin challenge, glucose clearance rates were greater in the chromium fed group. In pregnant sows, insulin response to feeding was significantly less in animals fed diets supplemented with 200 μg Cr/kg diet from CrPic compared with unsupplemented pregnant sows (43).

Urinary chromium excretion

Morris and colleagues (44, 45) studied the effect of 75 g oral glucose on plasma chromium, glucose, insulin and urinary chromium excretion in normal, healthy adults. They observed that along with the normal glucose and insulin response to the glucose challenge, plasma chromium had a significant inverse relationship with plasma insulin and that urinary chromium concentrations increased between 30 - 60 minutes after the challenge. The inverse relationship between plasma insulin and plasma chromium was also noted when diurnal measurements were plotted. These patterns of urinary chromium excretion are similar to those observed in earlier studies by Gurson and Saner (46, 47). In normal adults, they noted a significant increase in urinary chromium excretion at the end of the 75 g oral glucose tolerance test compared with concentrations before the test (46). Contrary to these observations, Anderson and coworkers (48) did not find any significant difference between urinary chromium excretion before and after glucose loading in placebo or chromium supplemented subjects. However, they did observe a significant difference in urinary chromium excretion between the groups at fasting at the beginning of the study.

Possible mechanisms for chomium's insulin potentiating effect

Natural or synthetic glucose tolerance factor (GTF) has been shown to potentiate the action of insulin (49). Insulin potentiation is expressed as the ratio of radioactive $CO_2$ produced from fat cell oxidation of radiolabeled glucose at a fixed concentration of insulin with added GTF to that produced without the addition of GTF. The addition of increments of GTF alone to basal concentrations of insulin (i.e., no insulin added to the

media) increased $CO_2$ production and achieved maximum response (Vmax) without the addition of insulin. Dietary chromium supplementation by reactive hypoglycemic subjects increased insulin binding and receptor number but not affinity (50) while chromium supplementation by normal control subjects did not show any alterations in receptor binding, receptor number or affinity.

CrPic increased insulin receptor complex internalization rate accompanied by a marked increase in glucose and leucine uptake (51). Insulin receptor internalization was determined by a modified ($^{125}$I)insulin receptor assay utilizing trypsin to release internalized radioactive insulin. Data were not corrected for non-specific binding but the results indicated that both the quantity of initially bound insulin receptors and insulin internalization were significantly greater in muscle cells precultured with CrPic.

Mouse myotubes cultured in low chromium media had a decreased glucose uptake response to insulin (52). As demonstrated by dose response curves, these cells had greater sensitivity to insulin in the presence of chromium. Glucose uptake was measured by monitoring 2-deoxy-{1-$H^3$}glucose. In vitro studies show that $^{51}CrCl_3$ uptake by muscle and adipose tissue was glucose dependent and was blocked by the glucose transport inhibitors, phloretin, phloridzin and cytochalasin b (53).

The effect of diabetes on chromium status and metabolism

Gurson and Saner (46) studied the effect of a glucose challenge on the urinary excretion of chromium by diabetic subjects and individuals with a family history of diabetes. In 8 out of 10 normal adults, urinary chromium excretion increased significantly after an oral glucose tolerance test but only 3 out of 8 diabetic subjects and 5 of 15

subjects with a family history of diabetes had a similar response. In diabetic subjects, Morris and colleagues (44, 54) observed that plasma chromium concentrations were significantly lower than in normal healthy individuals and that there was a significant positive correlation between urinary chromium excretion and urinary glucose as well as the duration of diabetes. Twenty-four hour urinary chromium excretion of the diabetic subjects was three times greater than that of the normal controls (44). Doisy and colleagues (55) observed that $^{51}$chromium absorption was three times greater in diabetics than in normal controls. This suggests that diabetic individuals may have an increased requirement for chromium due to a negative chromium status.

Chromium status in pregnancy and gestational diabetes mellitus

Data are limited and archaic on the chromium status of normal pregnant and GDM women. Plasma chromium was significantly lower in pregnant women compared with non-pregnant women (56). Multiparous women had lower hair chromium content compared with nulliparous women (57 - 59). Hair chromium content decreased as gestation progressed (59, 60) while urinary chromium excretion increased concommitantly (61). In response to i.v. as well as oral glucose challenges, plasma chromium decreased in normal non-pregnant women but not in pregnant women (56). In rats, the fetoplacental uptake of $^{51}$chromium accounts for 25 to 30% of the $^{51}$chromium retained by the mother (62). These observations suggest that chromium status is greatly compromised during and by gestation.

Chromium status may influence insulin sensitivity during pregnancy as well. Gestational diabetic women had significantly greater hair chromium content than normal

pregnant women (60). It was suggested that gestational diabetic women had a "chromium resistance" that resulted in higher hair chromium concentrations but plasma chromium was not measured in the subjects to confirm this theory. It is more conceivable, based on the research by Morris and colleagues (53), that chromium excretion and hair chromium content would be greater in glucose intolerant subjects due to decreased glucose dependent chromium uptake by insulin dependent tissues.

Studies on the effects of chromium supplementatioin on reproductive performance in pigs are inconclusive. In one study, Lindemann *et al* (43) observed significantly larger litter sizes in chromium supplemented sows. But in a following study (63), litter size was not significantly different between the supplemented and unsupplemented group. Although it was not statistically significant, the researchers did point out that there was an average of one more live pig per litter in the supplemented group.

Effect of chromium on lipid metabolism

The effect of chromium on plasma lipids are not in agreement but there are more studies that demonstrate a lipid lowering effect. In 1965, Schroeder (34) observed a lower incidence of aortic plaques (2%) in chromium fed rats than in chromium deficient rats (19%). In hypercholesterolemic rats, Staub *et al* (64) noted a significant decrease in serum cholesterol after the addition of chromium acetate to the drinking water of the experimental group. Both synthetic and naturally occurring GTF lowered plasma triglycerides in normal and genetically diabetic mice (65). In chromium deficient rats, supplementation with high chromium baker's yeast lowered both serum cholesterol and triglyceride concentrations while supplementation with $CrCl_3$ decreased only serum

triglycerides (66).

Elderly subjects whose diets were supplemented with chromium-rich yeast had decreased serum cholesterol and total lipids compared with those that received low-chromium yeast supplemetation (67). In a subsequent study by the same research group (68), however, no significant effects of chromium supplementation on blood lipids were observed.

Chromium treated atherosclerotic rabbits had a significant reduction in aortic plaque and aortic cholesterol content compared with controls (69). HDL cholesterol increased and serum triglycerides decreased in human subjects with established atherosclerotic disease treated daily with 5 μmols (260 μg) chromium from $CrCl_3$ (70). In adult men, HDL cholesterol increased when they received 4 μmols (200 μg) chromium per day from $CrCl_3$ supplementation (71). Responses were more pronounced in the insulin resistant subjects. Both total cholesterol and the total cholesterol:HDL cholesterol ratio decreased significantly in male athletes given 4 or 8 μmols (200 or 400 μg) chromium daily from a chromium-nicotinic acid complex (72).

Hermann and colleagues observed that in elderly subjects with total cholesterol concentrations greater than 6.21 mM (240 mg/dL), supplemental $CrCl_3$ (4 μmols daily) decreased LDL cholesterol, total cholesterol and apoprotein B concentrations (73). Chromium supplementation did not affect HDL cholesterol, serum triglycerides or glucose concentrations.

## Metabolic Characteristics of Pregnancy and Gestational Diabetes Mellitus

A shift in energy substrate metabolism related to hormonal and other physiological

changes normally occurs during pregnancy. In normal uncomplicated pregnancies, insulin resistance characterized by an elevated insulin response to a glucose challenge which maintains blood glucose concentrations within accepted normal limits develops in the later half of gestation. In gestational diabetes, individuals are unable to respond as well to a glucose challenge resulting in elevated blood glucose concentrations despite the elevated insulin response (1). There is a normal increase in blood lipids especially after mid-gestation. Serum triglycerides increase two to threefold reaching an average peak in the range of 2.26 - 3.39 mmol/L (200 to 300 mg/dL) at term while total cholesterol concentrations increase by an average of 50% reaching an average peak in the range of 5.17 - 6.46 mmol/L (200 to 250 mg/dL) at term (74). Significant positive predictors of birth size include: maternal age, prepregnancy weight, pregnancy weight gain, plasma glucose, plasma insulin, human placental lactogen, and estriol (75). Serum creatinine on the other hand is a negative predictor of birth weight. As serum creatinine concentration increases birthweight decreases.

Gestational diabetes mellitus is associated with a 35 - 38% rate of Cesarean sections, 17 - 33% rate of macrosomia, a 7 - 13% incidence of shoulder dystocia and a 28 - 30% incidence of the requirement for exogenous insulin (76). The American Diabetes Association mandates that all pregnant women be screened for gestational diabetes mellitus (3). Pre-conception planning and care are strongly recommended for pre-gestational diabetic women. Gestational diabetes mellitus is diagnosed when two or more blood glucose values during a glucose tolerance test are above the following parameters: 5.6 mM (105 mg/dL) fasting, 10.6 mM (190 mg/dL) 1 hour after a 100 gram glucose load, 9.2 mM (165 mg/dL) 2 hours postload and 8.1 mM (140 mg/dL) 3 hours post load

(3).

The following section will discuss the insulin resistance of normal pregnancy, the metabolic characteristics of normal pregnant women and contrast these normal characteristics to those of gestational diabetic pregnant women.

Insulin resistance in normal pregnancy and in gestational diabetes

In normal pregnant women, fasting plasma insulin concentration increases gradually as gestation progresses (77). At term, these levels are almost double pre-gravid, post partum or non-pregnant concentrations (78 - 80). In normal pregnant women, insulin resistance is manifested by blood glucose concentrations which are maintained within normal limits by elevated insulin concentrations (1). The insulin resistance or decreased insulin sensitivity of pregnancy is further demonstrated by a magnified insulin response to a glucose load such that the area under the insulin response curve is much greater in pregnancy than in the non-pregnant state (77,80 - 82). The insulin to glucose ratio is also much greater in pregnancy than in the non-pregnant state (83).

Euglycemic hyperinsulinemic clamp studies in normal pregnant women show that there is a 27 - 56% decrease in insulin sensitivity at 36 weeks gestation compared with the non-pregnant state (83,84). Clamped at 100 μU/mL of circulating insulin, women in late gestation required a glucose infusion rate of 4.6 ± 1.3 mg glucose/kg fat-free body mass/min to maintain a blood glucose concentration of 5.0 mmol/L (90 mg/dL) while their pregravid infusion rate was 10.5 ± 2.7 mg/kg fat-free mass/min (83). Non-pregnant non-diabetic controls in another study clamped at an infusion rate of 240 $mU/m^2/min$ insulin required a glucose infusion rate of 372 ± 11 $mg/m^2/min$ to maintain a blood glucose

concentration of 4.2 mmol/L (75 mg/dL) while pregnant non-diabetic women required 270 ± 31 mg/m$^2$/min to maintain the same blood glucose concentration (84), a 27% decrease in glucose infusion rate.

In contrast, gestational diabetic women respond to a glucose challenge with a higher response curve resulting in a larger area under the curve (AUC) compared with normal pregnant women in late gestation (81). Under fasting conditions, gestational diabetic women have higher circulating concentrations of blood glucose, insulin, free fatty acids and β-hydroxybutyrate compared with normal pregnant women (85). Diurnal glucose concentrations were higher in GDM women than in normal pregnant women (86) and were consistently above normal pregnant values.

The mean insulin response to a glucose challenge was significantly lower in gestational diabetic women compared with normal pregnant controls. In a study by Buchanan and colleagues (79), first phase insulin responses by 80% of the GDM women in their study were below the 95% CI of the mean response of normal pregnant women. Under euglycemic hyperinsulinemic conditions, GDM women require an even lower glucose infusion rate (157 ± 26 mg/m$^2$/min) than normal pregnant women (270 ± 31 mg/m$^2$/min) representing a 42% decrease in glucose infusion rate compared with normal pregnant women and a 58% decrease in glucose infusion rate compared with normal non-pregnant women demonstrating a more profound insulin resistance or an impaired responsiveness to the action of insulin in gestational diabetes mellitus (84).

Hyperlipidemia of normal pregnancy

In addition to insulin resistance, hyperlipidemia is another normal characteristic of late pregnancy. Total plasma triglycerides increased two to threefold of prepregnancy values while total plasma cholesterol increased by 50 - 60% of prepregnancy values (74,87,88). In a survey of 553 women, the mean total plasma triglyceride concentration at 36 weeks gestation was 2.6 ± 0.9 mmol/L (227 ± 84 mg/dL) compared with a mean of 0.7 ± 0.3 mmol/L (64 ± 27.6 mg/dL) in 100 non-pregnant non-hormone users (89). Mean total plasma cholesterol for the pregnant women was 6.4 ± 1.1 mmol/L (247 ± 42 mg/dL) compared with a mean of 4.4 ± 0.7 mmol/L (169 ± 28.8 mg/dL) in the nonpregnant women. Very low density lipoprotein triglyceride concentrations were three times greater and LDL triglycerides four times greater in the pregnant women than in the nonpregnant women while VLDL cholesterol was double and LDL cholesterol 50% more in the pregnant women compared with the nonpregnant women. Similar concentrations for lipoprotein lipid fractions have been observed by Montelongo and colleagues (90).

Fahraeus *et al* observed that total plasma cholesterol increased gradually through pregnancy up to 54% above early pregnancy values (91). LDL cholesterol followed a similar pattern increasing to 56% above early pregnancy values. HDL cholesterol peaked at 28% above early pregnancy values at 28 weeks gestation. VLDL cholesterol rapidly increased at 20 weeks gestation and peaked at 36 weeks, increasing to almost 200% above week 20 values. All these lipid parameters declined after delivery. Similar patterns have been noted by Potter *et al* (87) and Desoye *et al* (92).

Desoye and coworkers found a significant positive correlation between estradiol,

progesterone and human placental lactogen, and total cholesterol, free cholesterol, phospholipids and total triglycerides during pregnancy in women (92). Total triglyceride concentrations were correlated positively with insulin while Apo-B was positively correlated with estradiol. The strongest correlation was between total triglycerides and progesterone. Knopp and colleagues found a positive correlation between VLDL triglycerides and insulin, total triglycerides and estriol and HDL cholesterol with estradiol and progesterone (93). In another study, a positive linear semilogarithmic relationship was observed between plasma lipoprotein triglycerides and estradiol, progesterone and prolactin in pregnancy (90).

Diurnal plasma triglyceride concentrations were consistently greater in GDM women than in normal pregnant women during late gestation (86). Throughout gestation, GDM women and pre-gestational diabetic women had higher plasma free fatty acids compared with normal pregnant controls (90). Total plasma triglycerides and cholesterol concentrations were not different between normal and diabetic groups while concentrations of LDL triglycerides, LDL cholesterol, LDL phospholipid and HDL phospholipid were consistently less in GDM women (90).

## The Pregnant Rat Model and STZ Diabetes in Pregnancy

### The normal pregnant rat model

Reduced insulin sensitivity similar to that in humans is also evident in the pregnant rat (94). In the fed state, plasma glucose of the pregnant rats was lower than virgin controls while insulin was greater in the pregnant rats than in the virgin controls (95, 96).

Insulin to glucose ratios in the pregnant rat were greater than virgin controls as well. Insulin secretion in response to intravenous glucose in late gestation (19-21 days in the rat) was significantly greater than in early pregnancy (97). Glucose infusion rates under hyperinsulinemic euglycemic clamp experiments were lower in pregnant rats than in virgin controls to maintain euglycemia under hyperinsulinemic conditions. Insulin secretion in response to intravenous glucose in the 19 and 21 day pregnant rat was significantly increased compared with virgin controls (98). Given a low dose of insulin (50 - 150 mU/kg body weight), a smaller hypoglycemic response was observed compared with virgin controls while a large dose of insulin (4 U/kg body weight) produced a normal hypoglycemic response in the late pregnant rat.

Plasma triglycerides increased two to threefold during pregnancy in rats (99,100). The disappearance of labeled chylomicron triglycerides was slower in late pregnancy than during the first half of gestation (99). In addition to elevated total plasma triglycerides, total cholesterol, chylomicron triglycerides, and VLDL triglycerides and cholesterol were greater while LDL triglycerides and cholesterol were less in late pregnant rats compared with non-pregnant controls. Concentrations of adipose tissue free fatty acids were greater in pregnant rats compared with nonpregnant controls (101). Maternal lipid storage declined in late gestation (102). Maternal carcass fat content increased until day 19 of pregnancy then decreased significantly until term (103).

The STZ-induced diabetic pregnant rat model

Scientists have chemically induced diabetes in pregnant rats with STZ since 1969. Streptozotocin was an antibiotic isolated from Streptomyces achromogens which was

found to be diabetogenic in rats by the action of selectively destroying the β-cells of the pancreas. It was found to be less fatal and toxic than alloxan (132).

Table II.1 summarizes different protocols that have been used for the induction of diabetes in pregnancy using STZ. Despite these differences in methods, several common observations can be made. Diabetic pregnant rats gained less weight, were hypo-insulinemic and hyperglycemic compared with non-diabetic pregnant controls. Their insulin to glucose ratios were lower than normal pregnant rats (21,104). Only a few studies showed no difference in litter size between diabetic and normal controls (21,105) while the others reported a significantly smaller number of fetuses per litter. STZ diabetic dams had smaller fetuses compared with non-diabetic dams and had a greater incidence of malformations. Streptozotocin diabetes in pregnancy induced a significant increase in total plasma triglycerides (21,93,106) and plasma β-hydroxybutyrate (106) and a decrease in plasma free fatty acids (106). Streptozotocin diabetic dams as well as their fetuses had a lower concentration of circulating total amino acids (107). Furthermore, the rate of protein synthesis in diabetic dams and their fetuses was lower than non-diabetic controls while maternal liver RNA concentration was decreased and fetal liver RNA content increased (108).

Streptozotocin diabetic dams had larger livers, kidneys and placentas compared with non-diabetic controls (20). Liver and kidney zinc and copper were elevated in diabetic dams compared with non-diabetic dams (20,32,33,109). In addition, maternal and fetal liver manganese and fetal liver iron increased while fetal liver zinc, placental magnesium and calcium and maternal kidney calcium decreased (20).

Table II.1

Studies of STZ-induced diabetes in pregnant rats.

| Authors | Dose/Vehicle | Site/ When given | Strain/ Weight or Age | n | Scope of Study |
|---|---|---|---|---|---|
| Sybulksi (1971) (105) | 50mg/kg BW in citrate buffer | Tail vein/ 6 - 9 days gestation | Wistar/ 225 grams | 93 | Compared with alloxan treatment |
| Golob (1971) (133) | 30 - 50 mg/kg BW in citrate buffer | Tail vein/ 5 days gestation | SD*/ 160 - 190 grams | 141 | Effect on fasting vs non-fasting blood glucose concentration |
| Eriksson (1980) (104) | 20 - 50 mg/kg BW in citrate buffer | I.V./ 2 weeks before breeding | SD/ 250 grams | 173 | Effect on fetal growth |
| Triadou (1982) (134) | 100 μg/g BW in citrate buffer | Saphenous vein/ at birth | Wistar bred at 2 months | 18 | Effect on glucose tolerance |
| Eriksson (1984) (33) | 45 - 50 mg/kg BW in citrate buffer | I.V./ 2 weeks before breeding | SD/ 250 grams | 50 | Effect on fetal outcome and maternal and fetal liver zinc, copper and manganese |
| Uriu-Hare (1985) (20) | 40 - 45 mg/kg BW in citrate buffer | Tail vein/ 2 weeks before breeding | SD vs Wistar/ 250 -275 grams | 60 | Effect on tissue distribution of mineral |
| Aerts (1989) (107) | 22.5 & 30 mg/kg BW | Tail vein/ Day 1 gestation | Wistar/ 200 grams | ? | Effect on amino acids |
| Martin (1991) (106) | 45 mg/kg BW in citrate buffer | I.P./ 1 week before breeding | Wistar/ 160 - 180 grams | ? | Effect on blood lipids |
| Uriu-Hare (1992) (109) | 40 mg/kg citrate buffer | Subcutaneous/1 - 2 weeks before breeding | 210 - 220 grams | 38 | Effect on zinc and copper |
| Martin (1995) (108) | 40 mg/kg BW in citrate buffer | I.P./ 1 week before breeding | Wistar/ 160 - 180 grams | 500 | Polysomal aggregations and rate of protein synthesis |

* SD = Sprague-Dawley

## The Insulin-like Growth Factors (IGF's) and Their Binding Proteins (IGFBP's)

Insulin-like growth factors for embryo development and fetal growth

The uterine environment for the pre-implantation rodent embryo is rich in both IGF's and IGFBP's (16). Diverse expression of the IGF-I and IGF-II gene and the IGF binding proteins occurs throughout murine embryonic development (110, 111). The mouse embryo expresses IGF-II receptor transcripts as early as the 2 cell stage, and IGF-I and insulin receptor transcripts at the 8 cell stage (18, 19). IGF-II is highly abundant in trophoblast derived cells of the rat placenta throughout gestation except at the junctional zone immediately before term (112). In the 20 day old fetus, serum IGF-II concentrations are 6 -7 times greater than IGF-I (24, 113). Marked fetal growth retardation occurred in mice with a hemizygous disruption of the IGF-II gene (114) indicating that IGF-II had a more important role than IGF-I in fetal growth. In situ hybridization studies showed that gene coding for IGF-II was predominant throughout fetal development of the rat compared to IGF-I (115, 116).

The IGF system impacts on numerous tissues of the fetus. For example, IGF-I and II stimulated growth, development and differentiation of embryonic rat brainstem and skeletal muscle cocultures organotypic of the fetal tongue (117). IGF-I induced a more pronounced muscle tissue differentiation while IGF-II induced a more complete neural differentiation. IGF-I and II increased {$^3$H}proline incorporation into type I collagen in rat calvarial cultures and decreased collagen degradation as well (118, 119). IGF-II and IGFBP-2 mRNA were associated with the proliferation of fetal epiphyseal chondrocytes in vitro (120). In rat lung development, IGF-II was prominent in late fetal life while IGF-I

was more prominent postnatally (121). Fetal rat islet cells produced IGF-I and II in vitro (122) while exogenous IGF-I and II increased {$^3$H}thymidine incorporation in fetal rat islets. IGFBP-1 and 2 and IGF-I and II were synergistic in that IGFBP-1 and 2 potentiated the effect of IGF-I and II on increasing DNA synthesis of fetal rat islets.

The relationship among the IGF's and their binding proteins in the mother and fetus

In normal rat pregnancies, maternal IGF-I decreased as gestation progressed (26, 123) while IGFBP-3 was absent by late gestation. The smaller binding proteins 1 and 2 persisted during late pregnancy (26, 124). Labeled IGF-I was cleared from the serum of pregnant rats approximately five times faster than postpartum rates (125). The rapid clearance of IGF-I from maternal circulation was associated with the reduction of IGF binding proteins especially IGFBP-3. The decrease in IGFBP-3 was associated with an increase in IGFBP-3 protease activity which probably promoted the availability of IGF-I to rapidly growing and differentiating tissues (126).

Fetal plasma from starved rat dams was less potent than fetal plasma from fed rat dams in promoting brain cell growth in vitro (127). Analysis of the fetal plasma revealed that fetal plasma from starved dams had reduced IGF-I, elevated IGFBP-1 and unchanged IGF-II concentrations. The addition of IGF-I to the starved fetal plasma stimulated brain cell growth in vitro while the addition of IGFBP-1 to fed fetal plasma inhibited brain cell growth. However, correcting concentrations of IGF-I or IGFBP-1 in starved fetal plasma did not completely restore cell growth. Perhaps, reduced glucose concentrations in the starved fetal plasma could have contributed to the less than optimal growth response. IGF-I concentrations were significantly reduced in small for gestational age (SGA) fetal

rats while IGF-II remained unchanged (24, 128). Davenport observed that in the fetus, IGF-II and IGF-II mRNA were not affected by maternal fasting (113). Intrauterine growth retardation due to maternal fasting was related to decreased IGF-I gene expression in the maternal liver and lungs (25, 113) as well as decreased fetal hepatic IGF-I mRNA, decreased fetal serum IGF-I concentrations and increased fetal hepatic IGFBP-1 mRNA (25). In SGA fetal rats induced by short term (24 hours) uterine artery ligation, fetal serum IGF-I was positively correlated with fetal serum insulin, fetal body weight, fetal liver weight and fetal brain weight while fetal serum insulin was positively correlated to fetal body weight and fetal liver weight but not brain weight (24). Upon further analysis, the relationship between IGF-I and fetal body weight, liver weight and brain weight remained significant even when the effect of insulin was controlled for. These data indicate that fetal growth retardation due to maternal fasting and uterine arterial ligation is related to a decrease in IGF-I gene expression and an increase in IGFBP-1 gene expression which may be related to the metabolic state associated with insulin concentrations. It appears that intrauterine growth retardation due to short term nutrient restriction is not related to IGF-II gene regulation.

Effect of diabetes on the IGF's and their binding proteins

Streptozotocin induced diabetes in nonpregnant rats resulted in discordant organ specific regulation of IGF-I expression (29). Within the kidney itself, there was a varied expression of IGFBP's as a result of STZ diabetes (129). Kidney weight increased dramatically in pre- and postpubertal male SD diabetic rats compared to nondiabetic controls (130). The diabetic postpubertal rats had a larger and more significant increase in

kidney weight than diabetic prepubertal rats. Plasma IGF-I concentrations decreased with diabetes in both age groups. Renal IGF-I concentrations increased in both diabetic and nondiabetic postpubertal rats but not in the prepubertal rats. Phillip and colleagues also observed elevated renal IGF-I concentrations in 3 month old male SD rats treated with STZ despite a decrease in plasma IGF-I and growth hormone concentrations within 30 hours of STZ administration (30). In addition, liver and kidney IGFBP-1 mRNA concentrations were elevated. In young female SD rats, Luo and Murphy also noted a significant increase in renal and hepatic IGFBP-1 mRNA after STZ treatments well as an increase in renal and hepatic IGFBP-1 (28).

In mature male SD rats given varying doses of STZ, hepatic IGF-I mRNA decreased in direct proportion to STZ dose while renal IGF-I mRNA content increased inversely proportional to the dose of STZ (29). Bornfeldt *et al* (131) observed a decrease in liver IGF-I mRNA 7 days after STZ treatment in 6 - 7 week old male SD rats. IGF-I mRNA also decreased in the heart, aorta, kidney and diaphragm compared to non-diabetic controls. Growth hormone receptor mRNA concentrations were significantly lower in the heart and diaphragm but not the liver or kidney compared to non-diabetic controls. Despite diabetes, tibial epiphyseal width and longitudinal tibial bone growth were strongly correlated with IGF-I serum concentrations in young (115 - 125 gram) male Zur:SIV rats (27).

Unterman and colleagues demonstrated that the regulation of the 32kD IGFBP in the serum of mature diabetic female SD rats was regulated by insulin (132). This species of IGFBP increased in uncontrolled diabetes and decreased with insulin administration. When insulin was withdrawn, the concentrations of the binding protein rose. They

concluded that IGF-I binding activity was markedly increased in diabetes due to changes in the 32kD IGFBP which in turn was regulated by insulin or the metabolic status associated with the presence of absence of insulin.

In summary, STZ-induced diabetes resulted the differential expression of the IGF and IGFBP genes in different tissues. Diabetes decreased hepatic IGF-I gene expression and plasma IGF-I concentrations. In contrast, diabetes increased renal and hepatic IGFBP-1 mRNA and the 32kD IGFBP in serum as well as IGFBP-1 concentrations in the tissues and blood. The effect of diabetes on IGF-II per se in non-pregnant animals or the IGF system in pregnant diabetic rats has not been elucidated and remains to be determined.

# CHAPTER III

# METHODOLOGY

## Experimental Design

To study the effect of chromium and diabetes during pregnancy, this experiment utilized a 2x2 factorial arrangement of treatments. The two factors were dietary chromium content at low and high levels, and diabetes, induced with an injection of STZ in citrate buffer as opposed to an injection with citrate buffer without STZ. Thus, the four combinations randomly assigned and applied after pregnancy was confirmed were:

1) low chromium diet and citrate buffer injection

2) low chromium diet and STZ in citrate buffer injection

3) supplemented chromium diet and citrate buffer injection

4) supplemented chromium diet and STZ in citrate buffer injection.

Low chromium diets contained 40 μg chromium per kilogram diet (-Cr). Supplemented chromium diets contained 2 mg chromium per kilogram diet (+Cr). Diabetes was induced with a dose of a 30 milligram per kilogram body weight streptozotocin solubilized in citrate buffer (pH 4.5) obtained from Sigma Chemical Co. (St. Louis, MO). The following procedures were approved by the Oklahoma State University Animal Care and Use Committee.

## Animals and Diets

Forty female weanling Sprague Dawley rats obtained from Sasco Inc. (Omaha, NE) were randomly assigned to one of the four treatments. The rats were individually caged in Plexiglas cages with plastic grating for flooring. They were fed a semi-purified adaptation diet which contained an average of 70 μg chromium/kg diet (Appendix A). The rats had free access to the diet and distilled deionized water for drinking. Ceramic bowls were used to hold food and water.

All diet ingredients were analyzed and selected for chromium content before use. Ingredients lowest in chromium were used for the treatment diets (Appendix A). The mineral mix for the low chromium diet followed AIN-76 recommendations replacing iron citrate with iron chloride, using sodium selenate instead of sodium selenite and did not include the addition of chromium potassium sulfate. The mineral mix for the adequate chromium diet was made similarly to the low chromium mineral mix with the addition of chromium potassium sulfate (Appendix B). Ingredients for the mineral mix were weighed then combined and mixed in a burundum-fortified porcelain jar in a roller type mill for 24 hours. Adaptation (pre-pregnancy) and treatment (pregnancy) diet compositions are outlined in Appendix A. The low chromium diets contained 39.7 ± 7.7 μg chromium/kg diet while the adequate chromium diet contained 2.0 ± 0.3 mg chromium/kg diet.

Diet ingredients were weighed in advance. Vitamin mix was protected from light to prevent the breakdown of light sensitive vitamins. In a small mixing bowl (mixture 1), dextrose, sucrose, mineral mix, L-cystine and choline bitartrate were blended together gently by hand. The oil was added and the mixture mixed in an electric mixer at very low

speed for 3 minutes then set aside (mixture 1). In a large institutional size plastic mixing bowl, the casein and celufil were gently blended together with a plastic spatula after which the vitamin mix was quickly added and blended in. Cornstarch was then added to the mixture. The first mixture (mixture 1) in the small mixing bowl was then added very carefully to the cornstarch-vitamin mix-celufil-casein mixture to avoid dust formation. After making sure that all 10 components of the diet were in the large bowl, the diet was then mixed with an institutional size Kitchen Aid mixer for 15 minutes after which the sides and bottom of the bowl were scraped to ensure that all of the mixture was mixed together well. This was followed by 15 more minutes of machine mixing after which the diets were bagged in plastic bags, marked and stored in the freezer.

## Experimental Protocol

The rats were bred when they were older than 56 days and weighed more than 150 grams. Mean breeding age was 126 ± 3 (mean ± SEM) days. Mean breeding weight was 267 ± 8 (mean ± SEM) grams. The heaviest rats were used first. Rats were individually penned with one male for breeding. Breeding pairs were checked daily for vaginal plugs which confirmed pregnancy. When pregnancy was confirmed, the time was labeled day 1 of pregnancy.

On day 1 of pregnancy, the pregnant rat received an injection containing a dose of 30 mg STZ/kg body weight solubilized in 0.1M citrate buffer (pH4.5) or an injection containing buffer alone via tail vein. From day 1 on, the rat received a low chromium diet or an adequate chromium diet.

A glucose tolerance test was administered on day 18. On day 20, the dams were

anaesthetized with 130 mg ketamine/kg body weight and 5 mg xylazine/kg body weight. The fetuses and placentas were removed by Cesarean-section after which the dams were exsanguinated by cardiac puncture. Maternal heart, liver, spleen and kidneys were removed and weighed. Fetuses and placentas were counted and individually weighed.. Fetuses were quickly decapitated and blood collected with heparinized capillary tubes. The fetal blood was pooled in heparinized microcentrifuge tubes. Heparinized blood samples were kept on ice until they could be centrifuged, separated and stored at -20°C.

Maternal plasma was analyzed for glucose, insulin, non-esterified free fatty acids, cholesterol, triglycerides, β-hydroxybutyrate, IGF-I and IGF-II concentrations and IGF binding proteins. Fetal plasma was analyzed for glucose, insulin, IGF-I and IGF-II concentrations. Proximate analysis was done on maternal liver and fetuses. Fetuses and placenta were analyzed for hydroxyproline content. All the tissues collected were analyzed for calcium, magnesium, iron, copper and zinc. Chromium and manganese concentrations were measured in all the tissues except the spleen because of the large amount of concentrated acid needed to solubilize the ashed spleen samples.

## Statistical Analysis

The GLM procedure of SAS which utilizes the method of least squares to fit general linear models was utilized to determine if chromium, diabetes or their interaction had a significant effect on the dependent variables analyzed in this study (135). Log transformations were performed on variables that were not normally distributed as determined by the UNIVARIATE procedure of SAS and are noted in the tables when used in the analysis.

## Analytical Methods

Glucose: Plasma glucose was analyzed by the glucose oxidase method using Trinder reagent from Ciba Corning. This spectrophotometric method is based on the methodology developed by Trinder (136). The reagent contains glucose oxidase, peroxidase, 4-aminoantipyrene and 2,4-dichlorophenol. Glucose oxidase catalyses the oxidation of D-glucose in water and in the presence of oxygen to D-gluconic acid and peroxide. Peroxidase hydrolyses the peroxide and catalyses the formation of quinonemine dye producing a change in color. Color is measured at 505 nm and is directly proportional to glucose concentration.

Insulin: Plasma insulin was determined using the rat radioimmunoassay kit from Linco (St. Louis, MO). Following the usual RIA protocol, standards and samples are mixed with $^{125}$I-insulin and insulin antibody then incubated overnight. The next day, a precipitating reagent is added. After a short incubation, the tubes are centrifuged then decanted. The pellet was counted for radioactivity in a gamma counter and compared with standards to determine concentration. In principle, insulin in the sample and the radioactive insulin compete for the antibody. The pellet contains bound radioactive insulin. Thus, there is an inverse relationship between insulin concentration and radioactive binding.

Blood Lipids: Non-esterified free fatty acid concentrations were determined by the method of McCutcheon and Bauman (137) using the NEFA-C kit from WAKO Chemicals USA Inc. (Dallas, TX). Based on the Trinder principle, this assay measures the color change from the formation of quinonemine dye from the reaction of peroxide which is

formed from the oxidation of acyl CoA from the fatty acids, 4-aminoantipyrene, 3-methyl-N-ethyl-N-β-hydroxyethylaniline and peroxidase. Measured at 550nm on the Beckman DU-64 spectrophotometer, the absorbance is directly proportional to the concentration of the non-esterified fatty acids.

Sigma diagnostic kits and reagents (Sigma Chemical Co., St. Louis, MO) were used to determine total cholesterol (Catalog No. 352-20), triglyceride (Catalog No. 339-20) and β-hydroxybutyrate (Catalog No. 310-A) concentrations. The COBAS FARA II automatic analyzer (Roche Diagnostic Systems, NJ) was used to determine β-hydroxybutyrate concentrations while cholesterol and triglyceride analysis were performed manually. Both cholesterol and triglycerides analytical methods are Trinder based methodologies measuring color change from the formation of quinonemine dye as discussed in the analysis of glucose and non-esterified free fatty acids. Peroxide is formed from the oxidation of cholesterol by cholesterol oxidase and from triglycerides by the oxidation of glycerol-1-phosphate from triglycerides by glycerol phosphate oxidase.

The analysis of β-hydroxybutyrate depends on the oxidation of the ketone by 3-hydroxybutyrate dehydrogenase in the presence of NAD forming acetoacetate, NADH and $H^+$. NADH absorbs light at 340nm so that the increase in absorbance due to the reaction is directly proportional to the β-hydroxybutyrate concentrations in the sample.

IGF-I and IGF-II: IGF-I and -II were extracted from plasma using the formic acid-acetone method developed by Bowsher et al (138). IGF-I and IGF-II were determined by the RIA method described by Spicer *et al* (139) and Echternkamp *et al* (140).

IGF binding proteins: Western ligand blotting was carried out according to the method described by Echternkamp and colleagues (141). Briefly, 4μL of plasma and 26μL of denaturing buffer were heated then were separated by one dimensional SDS PAGE. The binding proteins on the gel were electrophoretically transferred to nitrocellulose paper which were then incubated with $^{125}$I-IGF-I overnight, washed with buffer and exposed to X-ray film for 2 weeks. Band intensities on the radiographs were quantified with a PDI Model DNA 35 scanner and the Quantity One (Version 2.4) software program for scanning densitometry.

Proximate analysis: For moisture and fat analysis, the method described by Firth *et al* (142) was utilized. Frozen maternal liver samples were ground and aliquots weighed into dry, labeled, ashless filter paper and clipped. Because of their watery consistency, ground fetal aliquots were weighed in special glass tubes with two open ends stopped with cotton wool to allow the flow of ether through the sample for fat extraction. The maternal liver samples were freeze dried to prevent the evaporation of volatile fatty acids and weighed again for moisture determination. Fetal samples were desiccated in a conventional drying oven. Fat was extracted from the dried samples in ether extraction Soxhlets, dried and re-weighed for the determination of total extractable fat (142).

Crude protein was determined with a LECO FP-428 (Leco Corp., St. Joseph, MI) which is a microprocessor based, software controlled instrument that measures thermal conductivity of nitrogen produced by the combustion of the samples (143). Thus, thermal conductivity is directly proportional to the amount of nitrogen produced.

Hydroxyproline: Fetal and placental hydroxyproline were determined using a modification of the methods of Bergman and Loxley(144, 145). Tissue samples were

weighed in dried polypropylene tubes of known weight and dried. Dry weights were recorded to determine sample dry weights. Two mL of concentrated hydrochloric acid was added to the dry samples and incubated for at least 16 hours at 105°C. The hydrolysates were then neutralized with concentrated LiCl solution using phenolphthalein and pH paper to approximate pH. A pH between 4 and 6 was achieved for best results. The slightly acid hydrolysates were then brought to a known volume (10 mL for placenta and 15 mL for fetus samples). Buffer (Appendix C) and standards of 25, 50 and 100 μmol/L hydroxyproline were prepared.

One-half mL of the samples and standards were pipetted into 12x75mm glass test tubes after which 1 mL of 2-isopropanol was added. Oxidant solution and Ehrlich's reagent were prepared immediately before addition to the samples (Appendix C). One-half mL of oxidant solution was added to each of the tubes and vortexed. After 4 ± 1 minutes, 1 mL of Ehrlich's reagent was added and vortexed before incubation in a waterbath set at 60°C for 21 minutes. The tubes were then left at room temperature for 1 hour before spectrophotometric determination was performed at 562nm. This method relies on the formation of pyrrole upon oxidation of the hydroxyproline. The pyrrole then forms a chromophore with the ρ-dimethylaminobenzaldehyde in Ehrlich's reagent (146).

Mineral Analysis: All necessary precautions were taken to minimize the contamination of tissue samples for mineral analysis. Gloves, hair restraints, plastic utensils, distilled deionized water and acid washed glassware were used at all times. When possible, work was done under a clean air hood.

Tissue samples were prepared for mineral analysis by a modified procedure

following the method of Hill *et al* (147). Samples were weighed into tubes and placed in glass beakers covered with Petri dishes and dried for 3 days. After dry tissue weights were recorded the samples in their original containers were ashed in a muffle furnace ramped at 0.5°C per minute to the ashing temperature of 375°C for a duration of 24 hours. The tubes containing the ashed samples were placed in heating blocks and wet ashed at 100°C with 50-100μL each of deionized distilled water, double distilled nitric acid (G. F. Smith Chemicals, Powell, OH) and 30% hydrogen peroxide (J. T. Baker, Phillipsburg, NJ). Hydrogen peroxide was added repeatedly until a clear solution was obtained. The tubes were dried in the heating blocks and dry ashed once more in the muffle furnace. This cycle of wet ashing and dry ashing was repeated until a soluble, colorless and clear solution was obtained. The samples were then analyzed using a Perkin Elmer 5100PC atomic absorption spectrophotometer equipped with an air-acetylene flame and a graphite furnace with Zeeman background correction for trace mineral analysis.

# CHAPTER IV

# THE EFFECT OF CHROMIUM AND STREPTOZOTOCIN-INDUCED DIABETES ON REPRODUCTIVE PERFORMANCE, CARBOHYDRATE AND LIPID METABOLISM AND TRACE ELEMENT INTERACTIONS IN PREGNANCY

## Abstract

This study was designed to determine the effect of chromium (Cr) depletion and diabetes on pregnancy outcome, maternal carbohydrate and lipid metabolism, and the distriibution of minerals in maternal tissues, placenta and fetus. Forty weanling female Sprague-Dawley rats were randomly assigned to four treatment groups in a 2 x 2 factorial design, the factors being the presence or absence of diabetes during pregnancy and 40 μg or 2 mg Cr/kg diet given during pregnancy. Treatments were initiated on day 1 of pregnancy. Glucose tolerance tests were given on day 18 of pregnancy. All rats were necroposied on day 20 for tissue and blood collection. Adequate chromium decreased litter size ($p<0.05$), increased placental weight ($p<0.05$), and increased concentrations of kidney chromium ($p<0.0001$), heart copper ($p<0.05$), and kidney zinc ($p<0.005$). Diabetes decreased plasma insulin concentrations ($p<0.001$), increased plasma glucose ($p<0.01$) and fructosamine ($p<0.05$) concentrations, lowered insulin to glucose ratios

($p<0.001$), increased blood glucose response to the glucose tolerance test ($p<0.05$) and total plasma triglycerides ($p<0.01$), enlarged maternal kidneys ($p<0.05$) and spleens ($p<0.005$), increased fetal resorptions ($p<0.01$), decreased maternal weight gain ($p<0.05$), litter size ($p<0.01$) and fetal weight ($p<0.05$), increased maternal liver and kidney copper ($p<0.05$) and zinc ($p<0.01$). Significant interaction effects on non-esterified fatty acids ($p<0.05$), maternal liver protein concentrations ($p<0.05$) and fetal manganese concentrations ($p<0.05$) were observed. Results of the present study confirm that chromium and diabetes have an effect on pregnancy outcome and mineral distribution in maternal tissues which may be associated with the negative outcomes of pregnancy.

## Introduction

Four percent of all pregnancies that resulted in live births in 1988 were complicated by diabetes (1). Of these 154,000 pregnancies, 88% were complicated by gestational diabetes, 8% by non-insulin dependent diabetes and 4% by insulin dependent diabetes. Gestational diabetes is diabetes mellitus first diagnosed during pregnancy. Pre-gestational diabetes is diabetes mellitus that precludes pregnancy. Uncontrolled gestational diabetes and pre-gestational diabetes are associated with increased prenatal risks such as premature birth, infant morbidity and mortality, congenital abnormalities and obstetric complications such as urinary tract infections, polyhydramnios, pre-eclampsia and toxemia (2 - 5). Congenital malformations, stillbirth and neonatal death still occur despite intensive prenatal care in diabetes (6).

In late gestation, GDM women have responded to oral glucose challenges with higher blood glucose response curves resulting in larger areas under the curve compared

to normal pregnant women (7). According to the American Diabetes Association (8), definitive diagnosis of GDM requires that two or more venous plasma concentrations, including fasting values, during a 100 gram oral glucose tolerance test meet or exceed the following parameters: fasting, 5.8 mM (105 mg/dL); 1 hour, 10.6 mM (190 mg/dL); 2 hour, 9.2 mM (165 mg/dL); 3 hour, 8.1 mM (145 ng/dL).

Maternal nutrition is a significant contributor to the outcome of pregnancy (9). Some gestational diabetic women and pre-gestational non-insulin dependent diabetic women whose blood sugar concentrations are not controlled by diet therapy alone may require supplemental insulin in addition to medical nutrition therapy (9).

Chromium, which is best known for its insulin potentiating effect and as a component of glucose tolerance factor (12 - 14), may be inadequate in the diets for pregnant women. In a preliminary study, chromium supplementation for 8 weeks with chromium from chromium picolinate decreased glycosylated hemoglobin, glucose and insulin concentrations and improved glucose tolerance in gestational diabetic women (15). The analysis of the chromium content of freely chosen and composite institutional diets revealed that daily intakes of chromium may be less than the recommended minimum estimated safe and adequate daily dietary intake of 50 μg chromium per day (16, 17).

Maternal chromium status is greatly compromised by the fetus during gestation. Aharoni (10) observed a significant decrease in hair chromium between 20 and 32 weeks of gestation in normal pregnant, gestational and pre-gestational diabetic women. Wallach and Verch (11) reported that in rats, the fetoplacental uptake of $^{51}$chromium accounted for 25 to 30% of the $^{51}$chromium retained by the mother.

Pregnant rats exhibited similar metabolic characteristics as pregnant human beings as evidenced by reduced insulin sensitivity in the pregnant rat (18). In the fed state, plasma glucose concentrations of pregnant rats were lower than those of virgin controls while insulin concentrations as well as insulin to glucose ratios were higher in pregnant rats than in virgin controls (19, 20). Plasma triglycerides increased two to threefold during pregnancy in women (21, 22) as well as in pregnant rats (23).

Streptozotocin-induced diabetic pregnant rats gained less weight, were hypoinsulinemic, hyperglycemic and had lower insulin to glucose ratios compared to non-diabetic pregnant controls (24, 25). In women with gestational diabetes mellitus (GDM), diurnal blood glucose concentrations were above the concentrations observed in normal pregnant women (26). GDM women had higher blood glucose, insulin, free fatty acid and β-hydroxybutyrate concentrations than normal pregnant women as well (26).

Streptozotocin-induced diabetic rat dams had larger placentas and smaller fetuses compared to non-diabetic controls (27) as well as a greater incidence of malformations (28). In rats, many investigators have reported a significantly smaller number of fetuses per litter (27, 29, 30 - 31). Streptozotocin-induced diabetes in pregnancy produced a significant increase in total plasma triglycerides (32, 33) and plasma β-hydroxybutyrate (33, 34) and a decrease in plasma free fatty acids (33). The rate of protein synthesis in diabetic dams and their fetuses was lower than non-diabetic controls while maternal liver RNA concentrations decreased and fetal liver RNA concentration increased in the diabetic dams at 20 days gestation (35).

In sows, supplemental chromium from chromium picolinate increased gestational

weight gain as well as litter size (36). Furthermore, during gestation, insulin response to feeding in chromium-supplemented sows is significantly lower compared to the response by unsupplemented sows. A follow-up study by the same group (37), however, did not find a significant effect by chromium on litter size but the chromium-treated group did have an average of one more live birth than the controls. The effect of supplemental chromium on litter size in rodents has not been evaluated.

Diabetes mellitus in pregnancy resulted in abnormal micronutrient status (27, 34, 38 - 40). Gestational diabetic women had lower erythrocyte intracellular free magnesium compared to normal pregnant controls and non-pregnant women (41). Gestational diabetic women had lower hair chromium content compared to normal pregnant women (10). Diabetic pregnant rats had higher kidney and liver manganese, zinc and copper while their fetuses had low concentrations of liver zinc and high concentrations of liver manganese (27, 28). Placental calcium, magnesium and iron are reduced in diabetic pregnant rats in these same studies compared to the fetuses of non-diabetic dams (27). It is not known whether dietary chromium depletion exacerbates glucose intolerance in diabetic pregnancies and whether this affects the distribution of minerals in maternal tissues or the fetus. The objective of this study was to determine the effects of chromium depletion and STZ-induced diabetes on maternal carbohydrate and lipid metabolism, pregnancy outcome, organ hypertrophy and the distribution of minerals in the tissues of rats during pregnancy.

## Research Design and Methods

Forty female weanling Sprague Dawley rats, obtained from Sasco Inc. (Omaha,

NE), were randomly assigned to one of the four treatments in a 2 x 2 factorial design. The four treatments were: 1) low chromium diet (40 μg Cr/kg diet), citrate buffer injection, 2) low chromium diet, STZ (30 mg STZ/kg body weight) in citrate buffer injection, 3) adequate chromium diet (2 mg Cr/kg diet), citrate buffer injection, and 4) adequate chromium diet, STZ in citrate buffer injection. The rats were individually caged in Plexiglas cages with plastic grating for flooring. The rats were fed an adaptation diet which contained an average of 70 μg Cr/kg diet. The rats had free access to the diet and distilled deionized drinking water. Ceramic bowls were used to hold food and water. Adaptation and treatment diet compositions are outlined in Table IV.1. The low chromium diets contained 39.7 ± 7.7 μg chromium/kg diet while the adequate chromium diet contained 2.0 ± 0.3 mg Cr/kg diet.

Mean breeding age was 126 ± 3 (mean ± SEM) days. Mean breeding weight was 267 ± 8 (mean ± SEM) grams. The heaviest rats were used first. Rats were individually penned with one male for breeding. Breeding pairs were checked daily for vaginal plugs. For each rat, the day that a vaginal plug was found was labeled day 1 of pregnancy.

On day 1 of pregnancy, each pregnant rat received an injection via tail vein containing either a dose of 30 mg STZ/kg body weight solubilized in 0.1M citrate buffer (pH 4.5) or citrate buffer alone. The STZ and citrate buffer were mixed immediately before the injection. From day 1 on, each rat received a low chromium diet (40 μg/kg diet) or an adequate chromium diet (2 mg/kg diet) until termination of the experiment.

A glucose tolerance test (1 g glucose/kg body weight) was administered on day 18. On day 20, the dams were anesthetized with 130 mg ketamine/kg body weight and 5

mg xylazine/kg body weight. The fetuses and placentas were removed by Cesarean section after which the dams were exsanguinated by cardiac puncture. Fetuses and placentas were counted and individually weighed. Heparinized blood samples were kept on ice untill thery were centrifuged, and plasma separated and stored at -20°C.

Maternal plasma was analyzed for glucose, fructosamine, insulin, non-esterified free fatty acids, cholesterol, triglycerides and β-hydroxybutyrate in day 20 samples. Plasma glucose was analyzed by the glucose oxidase method using Trinder reagent from Ciba Corning (Oberlin, OH). Plasma fructosamine was determined by the ROTAG method with the COBAS FARA II clinical analyzer (Roche Diagnostic Systems, Branchburg, NJ). Plasma insulin was determined using the radioimmunoassay kit from Linco (St. Louis, MO). Non-esterified free fatty acid concentrations were determined by the method of McCutcheon and Bauman (42) using the NEFA-C kit from WAKO Chemicals USA Inc. (Dallas, TX). Sigma diagnostic kits (Sigma Chemical Co., St. Louis, MO) and reagents were used to determine total cholesterol (Catalog No. 352-20), triglycerides (Catalog No. 339-20) and β-hydroxybutyrate (Catalog No. 310-A) concentrations. Samples for β-hydroxybutyrate analysis were deproteinized by the method of Mellanby and Williamson (43) and analyzed with a COBAS FARA II clinical analyzer (Roche Diagnostic Systems, Branchburg, NJ) using the protocol provided by Sigma.

To evaluate glucose tolerance, the area under the glucose tolerance curve (AUC) was calculated by adding the areas under each pair of consecutive blood glucose observations using the formula:

$$AUC = \frac{1}{2}\Sigma (t_{i+1}-t_i, y_i+y_{i+1}) \quad (44)$$

where t = time in minutes and y = blood glucose concentration expressed as mmol/L. The area under the curve will be expressed as GTTAUC units in the text and table.

Maternal liver moisture and fat content were determined following the procedure described by Firth and colleagues (45) utilizing the ether soxhlet extraction method of freeze dried animal tissue. Protein was analyzed using a LECO FP-428 which measures the thermal conductivity of nitrogen gas produced upon combustion of the sample (46).

Tissue samples were weighed into acid-washed borosilicate tubes and dried at 100°C for 24 hours. Dry weights were recorded after which the samples were ashed in a muffle furnace (Lindberg, Watertown, WI). The tubes were then placed in heating blocks which were enclosed in a well ventilated hood and the samples wet-ashed using concentrated nitric acid and 30% hydrogen peroxide as previously described by Hill *et al* (47). Samples were dried on the heating blocks after which they were ashed in the muffle furnace. The wet ashing-dry ashing procedure was repeated until the remaining sample was soluble and clear in solution. The samples were analyzed for calcium, magnesium, copper, iron, zinc, chromium and manganese using a Perkin Elmer Model 5100PC atomic absorption spectrophotometer with an acetylene flame and a graphite furnace with Zeeman background correction (Perkin Elmer, Norwalk, CT).

Statistical analysis was performed using the GLM procedure of SAS (1985) to determine factorial effects and their interactions. Log transformations were used to normalize variables exhibiting heterogenous variance which was determined by the Shapiro-Wilk test for normality utilized by the SAS procedure UNIVARIATE (48).

Analyses of variance using transformed variables have been indicated in the tables. Data in this study are presented as mean ± standard error of the mean.

## Results

Carbohydrate metabolism. Plasma insulin was lower ($p<0.001$) in the diabetic (0.10 ± 0.02 pmol/L, Table IV.2) than in the non-diabetic group (0.40 ± 0.08 pmol/L). In contrast, glucose concentrations were elevated ($p<0.01$) in the diabetic group (27.42 ± 3.01 mmol/L) compared to the non-diabetic group (17.96 ± 0.93 mmol/L). Serum fructosamine concentrations which were significantly greater ($p<0.05$) in the diabetic group confirm that the diabetic group was hyperglycemic during the last half of pregnancy. Mean plasma fructosamine for the diabetic group was 388.1 ± 35.2 μmol/L while the mean for the non-diabetic group was 262.9 ± 39.3 μmol/L. The insulin to glucose ratio was lower ($p<0.0001$) in the diabetic group compared to the non-diabetic group. Glucose tolerance curves are illlustrated in Fig. IV.1. Glucose concentrations were greater ($p<0.05$) in the diabetic group than in the non-diabetic group before glucose loading as well as after glucose was given. The area under the curve (TableIV.2) was significantly greater ($p<0.05$) in the diabetic group (3.31 ± 0.84 GTTAUC units) than in the non-diabetic group (1.04 ± 0.03 GTTAUC units). Chromium did not have an effect on plasma glucose, fructosamine, insulin, insulin to glucose ratio or glucose tolerance.

Blood lipids and lipid metabolites. Plasma cholesterol and β-hydroxybutyrate concentrations were similar among the groups. Mean total plasma cholesterol was 2.45 ± 0.31 mmol/L and mean β-hydroxybutyrate concentration was 307 ± 95 μmol/L. Plasma total triglyceride concentrations were higher in the diabetic group ($p<0.01$) than the non-

diabetic group. Mean plasma total triglyceride concentration for the diabetic group was 6.17 ± 0.97 mmol/L while the non-diabetic group had a mean concentration of 3.02 ± 0.38 mmol/L. A significant interaction effect on plasma non-esterified fatty acids ($p<0.05$) was observed such that plasma concentration within the diabetic group increased with adequate chromium diets.

Outcome of pregnancy. Body weights at breeding and at day 20 of pregnancy as well as the number of ovulations were not different among treatment groups (Table IV.4). However, diabetes decreased ($p<0.05$) maternal weight gain and chromium had a tendency ($p<0.1$) to decrease weight gain as well. Mean weight gain for the diabetic group was 71 ± 11 grams compared to 101 ± 6 grams for the non-diabetic group. Mean weight gain for the adequate chromium group was 76 ± 8 grams and 96 ± 8 grams for the chromium depleted group.

Both chromium and diabetes had significant negative effects on litter size. The average number of viable fetuses per dam in the adequate chromium group was 9 ± 1 fetuses per litter compared to 11 ± 1 fetuses per litter in the chromium depleted group ($p<0.05$). Mean litter size for the diabetic group was 8 ± 1 fetuses per litter compared to 12 ± 1 fetuses per litter in the non-diabetic group ($p<0.01$). In addition, the diabetic group had a larger number of resorptions (7 ± 1) than the non-diabetic group (3 ± 1, $p<0.01$). Diabetes also had a negative effect ($p<0.05$) on mean fetal weight. Average fetal weight at day 20 for the diabetic group was 3.5 ± 0.2 grams in contrast to an average of 4.1 ± 0.1 grams per fetus in the non-diabetic group.

Tissue weights. The diabetic dams had larger kidneys ($p<0.05$) than the non-

diabetic dams (Table IV.5). Mean kidney weight of the diabetic dams was 2.33 ± 0.18 grams compared to 1.82 ± 0.10 grams for the non-diabetic dams. When expressed as percent bodyweight, spleens of the diabetic dams were larger ($p<0.005$) than the non-diabetic dams. Average spleen weight in the diabetic group was 0.71 ± 0.02 grams (0.22 ± 0.01 % body weight) compared to 0.64 ± 0.03 grams (0.18 ± 0.01 % body weight) in the non-diabetic group. Liver weights and lung weights expressed as percent body weight had a tendency to be larger in the diabetic dams ($p<0.1$). Placentas, when expressed as percent body weight, from the adequate chromium dams were larger (0.15 ± 0.01 %, $p<0.05$) than those from the chromium depleted dams (0.13 ± 0.01%).

Liver proximate analysis. Liver moisture and fat content were not significantly different among treatment groups. Mean moisture content was 70 ± 2 % and mean extractable fat was 5 ± 2 %. A significant interaction effect on liver protein content was observed such that within the chromium depleted group diabetic rats had greater liver protein concentrations than the non-diabetic rats.

Chromium (Cr). The adequate chromium group had greater ($p<0.0001$) chromium concentrations in the kidneys than the chromium depleted group (Table IV.7). Mean kidney chromium concentration for the adequate chromium group was 1.53 ± 0.23 μmol Cr/kg dry tissue compared to a mean of 0.35 ± 0.16 μmol Cr/kg dry tissue in the chromium depleted group. A significant interaction effect on concentrations of chromium in the heart was observed such that within the adequate chromium group, diabetes decreased ($p<0.05$) heart chromium concentration but did not exhibit the same effect in the chromium depleted group.

Manganese (Mn). Mean kidney manganese concentration was greater ($p<0.05$) in the chromium depleted group (76.38 ± 2.34 μmol Mn/kg tissue) than in the adequate chromium group (70.69 ± 1.75 μmol Mn/kg tissue, Table IV.8). A significant interaction effect was observed such that within the diabetic group, rats fed adequate chromium diets had lower ($p<0.05$) fetal manganese concentrations than the chromium depleted group while in the non-diabetic group, chromium did not exhibit the same effect.

Copper (Cu). The diabetic dams had elevated liver ($p<0.05$) and kidney ($p<0.0005$) copper concentrations compared to the non-diabetic dams (Table IV.9). Mean liver copper for the non-diabetic group was 0.168 ± 0.005 mmol Cu/kg tissue while mean concentration for the diabetic group was 0.224 ± 0.022 mmol Cu/kg tissue. Mean kidney copper concentration for the non-diabetic group was 0.35 ± 0.03 mmol Cu/kg tissue in contrast to 1.41 ± 0.25 mmol Cu/kg tissue. Mean heart copper concentration for the adequate chromium group was greater ($p<0.05$) in the adequate chromium group (0.325 ± 0.004 mmol Cu/kg tissue) than in the chromium depleted group (0.308 ± 0.007 mmol Cu/kg tissue). There was a tendency ($p<0.1$) for diabetes to increase heart copper concentrations and for a treatment interaction effect on heart copper concentrations.

Zinc (Zn). Mean liver zinc concentration was greater ($p<0.01$) in the diabetic group (1.77 ± 0.11 mmol Zn/kg tissue) than in the non-diabetic group (1.44 ± 0.03 mmol Zn/kg tissue). Mean kidney zinc concentration was also higher ($p<0.005$) in the diabetic group (1.87 ± 0.07 mmol Zn/kg tissue) than in the non-diabetic group (1.62 ± 0.05 mmol Zn/kg tissue). Kidney zinc concentrations were also greater ($p<0.05$) in the adequate chromium group (1.82 ± 0.06 mmol Zn/kg tissue) than in the chromium depleted group

(1.67 ± 0.08 mmol Zn/kg tissue).

Iron. Tissue iron concentrations were not significantly different among groups but there was a tendency for spleen iron concentrations to be lower and fetal iron concentrations to be elevated in the diabetic group (Table IV.11).

Calcium and magnesium. There were no significant differences in tissue calcium and magnesium concentrations among the groups but there was tendency for spleen calcium and liver magnesium to be greater in the adequate chromium group (Tables IV.12 and IV.13).

## Discussion

In agreement with the literature (24, 27 - 28), we observed that diabetic dams had decreased plasma insulin concentrations and increased plasma glucose concentrations resulting in lower insulin to glucose ratios. We also observed that diabetic dams responded to the oral glucose tolerance test with higher plasma glucose concentrations resulting in a larger area under the glucose response curve which indicates severe glucose intolerance.

Evidently uncontrolled insulin dependent diabetes as seen in our model has adverse effects on the outcome of pregnancy. In the present study, the diabetic dams gained less weight, had a greater number of fetal resorptions, smaller fetuses and a smaller litter size. Other investigators have reported similar findings (27, 30-34).

The diabetic dams in the present study had elevated plasma triglyceride concentrations but not elevated β-hydroxybutyrate concentrations as has been observed previously (32, 33). Beta-hydroxybutyrate is one of the end products of the β-oxidation

of fatty acids in the liver. Diabetic keto-acidosis occurs when ketone bodies such as β-hydroxybutyrate accumulate in the body.

Insulin is a potent inhibitor of adipose tissue lipolysis by hormone sensitive lipase (50, 51) and, consequently, the release of NEFA's into the circulation. Thus, elevated concentrations of NEFA's are expected with low concentrations of insulin (50). However, decreased concentrations of plasma NEFA's have been observed in diabetic pregnant rats not treated with insulin (33). In this study, diabetes increased NEFA concentrations in the group of rats receiving adequate chromium in their diet but did not have the same effect in the chromium depleted group. The interaction of dietary chromium and diabetes in this respect suggests that dietary chromium may increase tissue lipolysis in uncontrolled diabetes when insulin is inadequate perhaps by increasing hormone sensitive lipase activity. Chromium depletion on the other hand may decrease hormone sensitive lipase activity. Further investigation is warranted to determine if chromium modulates hormone sensitive lipase activity in adipose tissue and whether this response is different between pregnant or non-pregnant animals with or without diabetes.

Chromium in the present study had a negative effect on litter size and did not affect maternal glucose tolerance. There are several possible reasons our results differ from Lindemann's sow study (37). Aside from species differences, the sows were fed chromium supplemented diets during growth and throughout pregnancy and lactation at a level of 200 μg added Cr per day from CrPic. Total dietary chromium concentrations were not specified. In the present study, the virgin rats were maintained on the adaptation diet containing 70 μg Cr/ kg diet during their growth phase and until pregnancy was

confirmed. This was followed by diets containing 2 mg Cr/ kg diet from chromium potassium sulfate over the 20 day gestation period. Thus, the timing and amount of chromium in the diet may account for the differences in our results. The dietary source of chromium may have affected outcomes as well. Although, different sources of chromium in the diet have been shown to affect the incorporation of chromium into various tissues in weanling Wistar rats (53), results from a preliminary study by Ward and colleagues (52) indicated that different sources of dietary chromium did not differ in their effect on growth, plasma metabolites, insulin and growth hormone in growing, finishing swine. In retrospect, it is possible that the chromium depleted rats ate more to compensate for the inadequate chromium in the diet resulting in increased maternal weight gain and increased litter size. Unfortunately, food intake in this study was not measured.

Interaction effects on maternal liver protein concentrations were such that diabetes increased maternal liver protein concentrations in the chromium depleted rats but not in the diabetic rats receiving adequate chromium in their diet. In contrast, Martin and coworkers (35) noted a decrease in maternal liver RNA and rate of protein synthesis in diabetic versus non-diabetic control rats at 20 days of gestation. It seems that the presence of chromium in the diet of the diabetic dams prevented the negative effect of diabetes on liver protein concentrations. Chromium's effect on liver function and morphology may be worthy of further study to determine if adequate chromium in the diet during diabetes maintains liver integrity.

Previously reported effects of diabetes on renal hypertrophy and the accumulation of zinc and copper in maternal kidneys and liver were confirmed in the present study.

Similar outcomes occurred in pregnant and non-pregnant spontaneously diabetic BB Wistar rats (34, 54). Hyperzincuria has been observed in diabetic human subjects (55) accompanied by low levels of plasma zinc indicative of compromised zinc status in diabetes. Maternal zinc deficiency is associated with a greater incidence of fetal malformations in STZ-induced diabetic rats (39). Perhaps, abnormal zinc status contributed to the adverse outcome of pregnancy in diabetes.

In the present study, chromium depletion increased kidney manganese while diabetes had no effect. Some studies demonstrated that diabetes increased kidney manganese concentrations in pregnancy (27 - 28) while one study (39) reported no differences in kidney manganese concentrations between diabetic and non-diabetic pregnant rats.

Copper, zinc and manganese are components of and associated with the antioxidant property of superoxide dismutase (SOD) (56). In previous studies, manganese superoxide dismutase (MnSOD) and ZnCuSOD activities in the kidneys decreased with manganese deficiency and were exacerbated by diabetes (57, 58). Whether renal superoxide dismutase activity is affected by chromium depletion and diabetes requires further investigation. In addition, the effect of chromium depletion on kidney function and histology should be elucidated.

Fetal liver manganese has been observed to increase with diabetes (27 - 28). In the present study, an interaction effect on total fetal manganese was observed. Total fetal manganese concentrations increased with diabetes in the chromium depleted group while in the adequate chromium group, diabetes caused a slight decrease in fetal manganese

concentrations. If the increase in fetal manganese concentrations as a result of diabetes is associated with the adverse outcome of diabetic rat pregnancies, then the interaction of chromium and diabetes on fetal manganese seems to suggest that chromium opposes the effect of diabetes on the fetus. This warrants further investigation.

In conclusion, in addition to the adverse effects of diabetes on maternal metabolism and the outcome of pregnancy, diabetes also had an effect on tissue mineral distribution, particularly zinc and copper which have antioxidant properties in conjunction with superoxide dismutase. Chromium deficiency also had an effect on the distribution of minerals in the tissues as well as an interactive effect with diabetes on fetal manganese concentrations which may have been related to litter size. Whether this response is the organism's way of coping with the oxidative stress of diabetes and with chromium deficiency during pregnancy needs to be determined. Further research is needed to determine if physiological functions are affected by the redistribution of zinc and copper in the liver and kidneys as well as manganese in the maternal kidneys and fetuses under the stress of diabetes or dietary chromium manipulation associated with negative outcomes and to determine the mechanisms by which these responses occur during pregnancy in the rat.

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Table IV.1
Composition of the adaptation and treatment diets used for this study

| Components: | Adaptation diet: (g/kg diet) | Treatment diet: (g/kg diet) |
|---|---|---|
| Cornstarch* | 430 | 150 |
| Casein* | 200 | 200 |
| Dextrose* | 100 | 0 |
| Sucrose | 100 | 500 |
| Soybean oil | 70 | 50 |
| Celufil* | 50 | 50 |
| AIN-76 mineral mix† | 35 | 35 |
| AIN76A vitamin mix‡ | 10 | 10 |
| L-Cystine | 3 | 3 |
| Choline bitartrate | 2 | 2 |

* Harlan Teklad (Madison, WI)
† Prepared in our laboratory. Mineral mix for the treatment diets contained iron chloride instead of iron citrate. The mineral mix for the low chromium diet did not include the addition of chromium potassium sulfate.
‡ USB (Cleveland, OH)

Table IV.2
Plasma glucose, insulin, insulin:glucose ratio and glucose tolerance test area under the curve (GTTAUC) of normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*.

| Group | Insulin pmol/L | Glucose mmol/L | Insulin: glucose ratio | Fructosamine μmol/L | GTTAUC units |
|---|---|---|---|---|---|
| -Cr-STZ | 0.54 ± 0.15 n=8 | 19 ± 1 n=9 | 0.030 ± 0.009 n=8 | 267.8±55.6 n=6 | 1.12 ± 0.03 n=3 |
| -Cr+STZ | 0.12 ± 0.03 n=9 | 26 ± 3 n=10 | 0.005 ± 0.002 n=9 | 379.6±48.2 n=8 | 3.81 ± 1.30 n=5 |
| +Cr-STZ | 0.30 ± 0.08 n=10 | 17 ± 1 n=10 | 0.020 ± 0.005 n=10 | 258.0±55.6 n=6 | 0.99 ± 0.03 n=5 |
| +Cr+STZ | 0.07 ± 0.02 n=8 | 29 ± 5 n=9 | 0.004 ± 0.001 n=8 | 397.9±51.5 n=7 | 2.89 ± 1.15 n=6 |

Analysis of variance

| Source of variation | P Value | | | | |
|---|---|---|---|---|---|
| Cr | 0.1147 | 0.9093 | 0.1589 | 0.9373 | 0.6276 |
| STZ | 0.0009 | 0.0059 | 0.0006 | 0.0259 | 0.0476 |
| Cr x STZ | 0.2347 | 0.4827 | 0.3160 | 0.7929 | 0.7159 |

* Mean ± SEM

Table IV.3
Total plasma cholesterol, triglycerides, non-esterified free fatty acid (NEFA) and β-hydroxybutyrate concentrations of normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Group | Cholesterol mmol/L | Triglycerides mmol/L | NEFA mmol/L | β-OH butyrate μmol/L |
|---|---|---|---|---|
| -Cr-STZ | 2.19 ± 0.18 n=8 | 3.49 ± 0.65 n=9 | 1.50 ± 0.18 n=9 | 344 ± 54 n=5 |
| -Cr+STZ | 2.88 ± 0.49 n=10 | 5.62 ± 1.48 n=7 | 1.34 ± 0.23 n=8 | 282 ± 30 n=8 |
| +Cr-STZ | 2.24 ± 0.18 n=9 | 2.55 ± 0.36 n=9 | 1.13 ± 0.02 n=7 | 315 ± 30 n=8 |
| +Cr+STZ | 2.41 ± 0.13 n=8 | 6.60 ± 1.35 n=9 | 1.82 ± 0.20 n=8 | 295 ± 45 n=6 |

Analysis of variance

| Source of variation | P Value | | | |
|---|---|---|---|---|
| Cr | 0.5101 | 0.9852 | 0.7803 | 0.8257 |
| STZ | 0.1842 | 0.0050 | 0.1571 | 0.2949 |
| Cr x STZ | 0.4182 | 0.3564 | 0.0279 | 0.5919 |

* Mean ± SEM

Table IV.4

Pregnancy outcome of normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Outcome parameters: | Experimental Groups | | | | P value | | |
|---|---|---|---|---|---|---|---|
| | -Cr-STZ | -Cr+STZ | +Cr-STZ | +Cr+STZ | Cr | STZ | Cr x STZ |
| Weight at breeding (g) | 271 ± 14 n=10 | 279 ± 17 n=10 | 253 ± 19 n=10 | 267 ± 18 n=10 | 0.3820 | 0.5237 | 0.8404 |
| Weight at day 20 (g) | 382 ± 17 n=10 | 338 ± 21 n=7 | 342 ± 28 n=10 | 313 ± 25 n=8 | 0.1839 | 0.1351 | 0.7623 |
| Weight gain (g)† | 111 ± 5 n=10 | 80 ± 15 n=7 | 90 ± 10 n=10 | 61 ± 16 n=8 | 0.0946 | 0.0161 | 0.9202 |
| Ovulations (n) | 16 ± 1 n=10 | 16 ± 1 n=10 | 14 ± 1 n=9 | 16 ± 1 n=10 | 0.2069 | 0.5953 | 0.1599 |
| Viable fetuses/litter (n) | 13 ± 1 n=10 | 9 ± 2 n=10 | 10 ± 1 n=10 | 7 ± 1 n=10 | 0.0290 | 0.0067 | 0.5239 |
| Resorptions (n) | 3 ± 1 n=10 | 6 ± 2 n=10 | 4 ± 1 n=9 | 8 ± 1 n=10 | 0.3924 | 0.0091 | 0.5851 |
| Mean pup weight (g) | 4.2 ± 0.2 n=10 | 3.3 ± 0.4 n=10 | 4.0 ± 0.1 n=10 | 3.6 ± 0.2 n=10 | 0.8328 | 0.0145 | 0.3562 |

* Mean ± SEM

† Weight gain between breeding and day 20 of gestation

Table IV.5
Weights and percent bodyweight of various tissues from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Tissue weight (g) | Treatment Groups | | | | P value | | |
|---|---|---|---|---|---|---|---|
| (% bodyweight) | -Cr-STZ | -Cr+STZ | +Cr-STZ | +Cr+STZ | Cr | STZ | Cr x STZ |
| Liver | 13.5 ± 1.2 | 12.9 ± 0.66 | 12.4 ± 1.2 | 13.4± 1.0 | 0.7927 | 0.8603 | 0.4730 |
| | (3.5 ± 0.2) | (3.8 ± 0.12) | (3.6 ± 0.1) | (4.1 ± 0.2) | (0.2828) | (0.0641) | (0.5501) |
| | n=10 | n=10 | n=10 | n=10 | | | |
| Kidney | 1.9 ± 0.1 | 2.3 ± 0.25 | 1.7 ± 0.2 | 2.3 ± 0.3 | 0.6979 | 0.0183 | 0.6560 |
| | (0.50 ± 0.01) | (0.60 ± 0.06) | (0.50 ± 0.02) | (0.73 ± 0.1) | (0.2984) | (0.0193) | (0.3476) |
| | n=10 | n=10 | n=10 | n=10 | | | |
| Spleen | 0.64 ± 0.04 | 0.72 ± 0.03 | 0.64 ± 0.04 | 0.70 ± 0.03 | 0.9017 | 0.0627 | 0.7944 |
| | (0.17 ± 0.01) | (0.22 ± 0.01) | (0.19 ± 0.01) | (0.22 ± 0.02) | (0.2080) | (0.0011) | (0.4200) |
| | n=10 | n=10 | n=10 | n=10 | | | |
| Heart | 0.86 ± 0.04 | 0.86 ± 0.04 | 0.82 ± 0.05 | 0.84 ± 0.06 | 0.5514 | 0.8854 | 0.8369 |
| | (0.23 ± 0.01) | (0.25 ± 0.01) | (0.24 ± 0.01) | (0.26 ± 0.01) | (0.2017) | (0.1671) | (0.8454) |
| | n=10 | n=10 | n=10 | n=10 | | | |
| Lung | 1.23± 0.06 | 1.23 ± 0.08 | 1.16 ± 0.05 | 1.23 ± 0.05 | 0.6063 | 0.6063 | 0.5629 |
| | (0.33 ± 0.02) | (0.37 ± 0.03) | (0.35 ± 0.01) | (0.40 ± 0.03) | (0.2811) | (0.0552) | (0.9281) |
| | n=10 | n=10 | n=10 | n=10 | | | |
| Placenta | 0.45 ± 0.01 | 0.51 ± 0.03 | 0.48 ± 0.02 | 0.51 ± 0.03 | 0.6034 | 0.1267 | 0.5315 |
| | (0.12 ± 0.005) | (0.15 ± 0.01) | (0.15 ± 0.01) | (0.16 ± 0.01) | (0.0422) | (0.0812) | (0.5482) |
| | n=10 | n=10 | n=10 | n=10 | | | |

* Mean ± SEM

Table IV.6
Liver moisture, fat and protein concentration of normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Group | Moisture (%) | Fat (%) | Protein (%) |
|---|---|---|---|
| -Cr-STZ | 70 ± 2 | 5 ± 2 | 18.5 ± 1.7 |
| -Cr+STZ | 69 ± 2 | 5 ± 2 | 20.6 ± 1.3 |
| +Cr-STZ | 70 ± 2 | 5 ± 2 | 19.7 ± 1.3 |
| +Cr+STZ | 70 ± 3 | 6 ± 4 | 19.4 ± 1.1 |

Analysis of variance

| Source of variation | P Value | | |
|---|---|---|---|
| Cr | 0.6083 | 0.7841 | 0.9810 |
| STZ | 0.6353 | 0.7709 | 0.0872 |
| Cr x STZ | 0.4699 | 0.8468 | 0.0313 |

* Mean ± SEM

Table IV.7
Chromium concentration (μmol/kg dry weight) of various tissues from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Tissue: | Treatment Groups | | | | P value | | |
|---|---|---|---|---|---|---|---|
| | -Cr-STZ | -Cr+STZ | +Cr-STZ | +Cr+STZ | Cr | STZ | Cr x STZ |
| Kidney | 0.0 ± 0<br>n=7 | 0.59 ± 0.24<br>n=10 | 1.67 ± 0.28<br>n=6 | 1.44 ± 0.33<br>n=10 | 0.0001 | 0.5071 | 0.1501 |
| Heart | 0.22 ± 0.05<br>n=10 | 0.30 ± 0.08<br>n=8 | 0.57 ± 0.14<br>n=9 | 0.21 ± 0.06<br>n=9 | 0.1477 | 0.1151 | 0.0198 |
| Fetus | 0.13± 0.05<br>n=9 | 0.03 ± 0.01<br>n=8 | 0.12 ± 0.05<br>n=9 | 0.07 ± 0.03<br>n=8 | 0.7211 | 0.0977 | 0.5518 |
| Placenta | 0.49 ± 0.12<br>n=9 | 0.53 ± 0.18<br>n=6 | 0.56 ± 0.16<br>n=7 | 0.62 ± 0.21<br>n=8 | 0.6559 | 0.7610 | 0.9599 |

* Mean ± SEM
†Results from analysis of log transformed data

Table IV.8

Manganese concentration (μmol/kg dry weight) of various tissues from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Tissue: | Experimental Groups | | | | P value | | |
|---|---|---|---|---|---|---|---|
| | -Cr-STZ | -Cr+STZ | +Cr-STZ | +Cr+STZ | Cr | STZ | Cr x STZ |
| Liver | 54.25 ± 7.17 n=9 | 42.66 ± 1.53 n=10 | 41.10 ± 1.57 n=9 | 44.86 ± 4.00 n=9 | 0.1960 | 0.3522 | 0.0736 |
| Kidney | 77.04 ± 4.51 n=8 | 75.80 ± 2.19 n=9 | 73.40 ± 2.12 n=10 | 65.26 ± 0.94 n=5 | 0.0263 | 0.1320 | 0.2631 |
| Heart | 40.01 ± 2.12 n=8 | 33.34 ± 3.46 n=9 | 34.46 ± 3.86 n=10 | 34.33 ± 2.71 n=9 | 0.4849 | 0.3000 | 0.3187 |
| Fetus | 34.29 ± 0.77 n=10 | 42.05 ± 4.04 n=8 | 36.57 ± 1.49 n=9 | 32.73 ± 1.39 n=7 | 0.1194 | 0.3786 | 0.0129 |
| Placenta | 15.52 ± 1.60 n=10 | 12.99 ± 1.93 n=6 | 14.68 ± 2.71 n=7 | 13.75 ± 1.14 n=8 | 0.9840 | 0.3692 | 0.6781 |

* Mean ± SEM

Table IV.9
Copper concentration (mmol/kg dry weight) of various tissues from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Tissue: | Treatment Groups | | | | P value | | |
|---|---|---|---|---|---|---|---|
| | -Cr-STZ | -Cr+STZ | +Cr-STZ | +Cr+STZ | Cr | STZ | Cr x STZ |
| Liver | 0.16 ± 0.01 n=9 | 0.24 ± 0.03 n=10 | 0.17 ± 0.01 n=10 | 0.21 ± 0.03 n=9 | 0.6990 | 0.0183 | 0.3546 |
| Kidney | 0.33 ± 0.05 n=9 | 1.10 ± 0.22 n=7 | 0.39 ± 0.02 n=7 | 1.72 ± 0.44 n=7 | 0.1537 | 0.0001 | 0.2389 |
| Spleen | 0.11 ± 0.01 n=6 | 0.11 ± 0.01 n=6 | 0.11 ± 0.01 n=10 | 0.10 ± 0.01 n=5 | 0.7327 | 0.2645 | 0.1847 |
| Heart | 0.29 ± 0.01 n=10 | 0.32 ± 0.01 n=9 | 0.324 ± 0.003 n=10 | 0.32 ± 0.01 n=10 | 0.0411 | 0.0622 | 0.0990 |
| Fetus | 0.21 ± 0.01 n=10 | 0.21 ± 0.02 n=8 | 0.22 ± 0.01 n=9 | 0.18 ± 0.02 n=8 | 0.3405 | 0.3003 | 0.1348 |
| Placenta | 0.27 ± 0.03 n=10 | 0.23 ± 0.03 n=6 | 0.24 ± 0.01 n=7 | 0.20 ± 0.03 n=8 | 0.2737 | 0.1960 | 0.9991 |

*Mean ± SEM

Table IV.10
Zinc concentration (mmol/kg dry weight) of various tissues from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Tissue: | Experimental Groups | | | | P value | | |
|---|---|---|---|---|---|---|---|
| | -Cr-STZ | -Cr+STZ | +Cr-STZ | +Cr+STZ | Cr | STZ | Cr x STZ |
| Liver | 1.41 ± 0.06 n=9 | 1.63 ± 0.11 n=10 | 1.48 ± 0.04 n=10 | 1.90 ± 0.18 n=10 | 0.1515 | 0.0076 | 0.3808 |
| Kidney | 1.48 ± 0.05 n=7 | 1.81 ± 0.11 n=10 | 1.71 ± 0.06 n=10 | 1.93 ± 0.10 n=10 | 0.0342† | 0.0032† | 0.4135† |
| Spleen | 1.36 ± 0.08 n=6 | 1.25 ± 0.02 n=6 | 1.37 ± 0.07 n=10 | 1.27 ± 0.07 n=5 | 0.7996 | 0.1721 | 0.9459 |
| Heart | 0.89 ± 0.03 n=10 | 0.90 ± 0.02 n=9 | 0.95 ± 0.03 n=10 | 0.92 ± 0.02 n=10 | 0.1227 | 0.7555 | 0.3465 |
| Fetus | 1.96 ± 0.03 n=9 | 2.00 ± 0.03 n=8 | 1.99 ± 0.07 n=9 | 1.96 ± 0.08 n=8 | 0.9170 | 0.8828 | 0.5140 |
| Placenta | 1.08 ± 0.04 n=10 | 1.04 ± 0.03 n=6 | 1.05 ± 0.03 n=7 | 1.03 ± 0.02 n=8 | 0.5268 | 0.3692 | 0.8496 |

*Mean ± SEM
†Results from analysis of log transformed data

Table IV.11
Iron concentration (mmol/kg dry weight) of various tissues from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Tissue: | Treatment Groups | | | | P value | | |
|---|---|---|---|---|---|---|---|
| | -Cr-STZ | -Cr+STZ | +Cr-STZ | +Cr+STZ | Cr | STZ | Cr x STZ |
| Liver | 7.72 ± 1.64 n=9 | 7.84 ± 1.17 n=10 | 7.73 ± 0.96 n=10 | 8.33 ± 1.47 n=10 | 0.8501 | 0.7857 | 0.8567 |
| Kidney | 4.52 ± 0.44 n=9 | 4.33 ± 0.48 n=10 | 4.42 ± 0.24 n=7 | 4.11 ± 0.36 n=8 | 0.7021 | 0.5623 | 0.8869 |
| Spleen | 59.78 ± 19.00 n=10 | 36.40 ± 5.92 n=6 | 59.09 ± 9.86 n=10 | 38.48 ± 11.87 n=5 | 0.9567 | 0.0968 | 0.9143 |
| Heart | 3.26 ± 0.05 n=10 | 3.19 ± 0.12 n=9 | 3.19 ± 0.05 n=9 | 3.30 ± 0.09 n=10 | 0.8538 | 0.8129 | 0.2991 |
| Fetus | 3.40 ± 0.26 n=10 | 4.46 ± 0.52 n=8 | 3.66 ± 0.26 n=9 | 3.86 ± 0.27 n=8 | 0.6173 | 0.0703 | 0.2129 |
| Placenta | 9.97 ± 0.36 n=9 | 10.17 ± 0.80 n=5 | 10.01 ± 0.91 n=6 | 10.72 ± 0.99 n= | 0.6958 | 0.5489 | 0.7358 |

*Mean ± SEM

Table IV.12
Calcium concentration (mmol/kg dry weight) of various tissues from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Tissue: | Treatment Groups | | | | P value | | |
|---|---|---|---|---|---|---|---|
| | -Cr-STZ | -Cr+STZ | +Cr-STZ | +Cr+STZ | Cr | STZ | Cr x STZ |
| Liver | 1.80 ± 0.06 n=9 | 1.93 ± 0.11 n=10 | 1.92 ± 0.10 n=9 | 1.96 ± 0.06 n=9 | 0.3950 | 0.3750 | 0.6029 |
| Kidney | 274.97 ± 80.72 n=7 | 317.13 ± 88.22 n=7 | 174.32 ± 45.51 n=10 | 300.76 ± 50.74 n=8 | 0.3768 | 0.2063 | 0.5231 |
| Spleen | 3.14 ± 0.21 n=6 | 2.69 ± 0.06 n=6 | 3.32 ± 0.19 n=10 | 3.21 ± 0.18 n=5 | 0.0774 | 0.1509 | 0.3796 |
| Heart | 3.14 ± 0.12 n=10 | 3.46 ± 0.19 n=9 | 3.45 ± 0.16 n=9 | 3.27 ± 0.11 n=10 | 0.6929 | 0.6519 | 0.0970 |
| Fetus | 390.59 ± 11.49 n=9 | 379.86 ± 17.58 n=8 | 391.83 ± 6.85 n=8 | 362.96 ± 12.72 n=8 | 0.5424 | 0.1299 | 0.4813 |
| Placenta | 13.25 ± 0.65 n=9 | 15.41 ± 1.61 n=6 | 16.11 ± 1.62 n=7 | 15.92 ± 1.20 n=8 | 0.1898 | 0.4377 | 0.3547 |

*Mean ± SEM

Table IV.13
Magnesium concentration (mmol/kg dry weight) of various tissues from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Tissue: | Treatment Groups | | | | P value | | |
|---|---|---|---|---|---|---|---|
| | -Cr-STZ | -Cr+STZ | +Cr-STZ | +Cr+STZ | Cr | STZ | Cr x STZ |
| Liver | 23.93 ± 2.78 n=9 | 25.58 ± 1.41 n=10 | 27.33 ± 1.30 n=10 | 28.37 ± 0.87 n=9 | 0.0788 | 0.4360 | 0.8597 |
| Kidney | 34.27 ± 4.51 n=9 | 31.47 ± 2.13 n=9 | 28.73 ± 1.61 n=10 | 28.70 ± 1.41 n=10 | 0.1219 | 0.5931 | 0.6001 |
| Spleen | 35.46 ± 2.79 n=6 | 30.99 ± 1.60 n=6 | 35.73 ± 1.98 n=10 | 35.29 ± 2.82 n=5 | 0.3462 | 0.3130 | 0.4061 |
| Heart | 24.83 ± 1.90 n=10 | 26.19 ± 1.35 n=9 | 25.84 ± 1.18 n=8 | 25.84 ± 0.77 n=9 | 0.8162 | 0.6332 | 0.6329 |
| Fetus | 63.09 ± 1.01 n=10 | 62.45 ± 1.74 n=8 | 61.51 ± 1.42 n=8 | 59.72 ± 2.32 n=7 | 0.1878 | 0.4518 | 0.7211 |
| Placenta | 23.60 ± 1.15 n=10 | 23.52 ± 1.37 n=6 | 24.10 ± 1.18 n=7 | 22.32 ± 1.11 n=8 | 0.7732 | 0.4555 | 0.4945 |

*Mean ± SEM

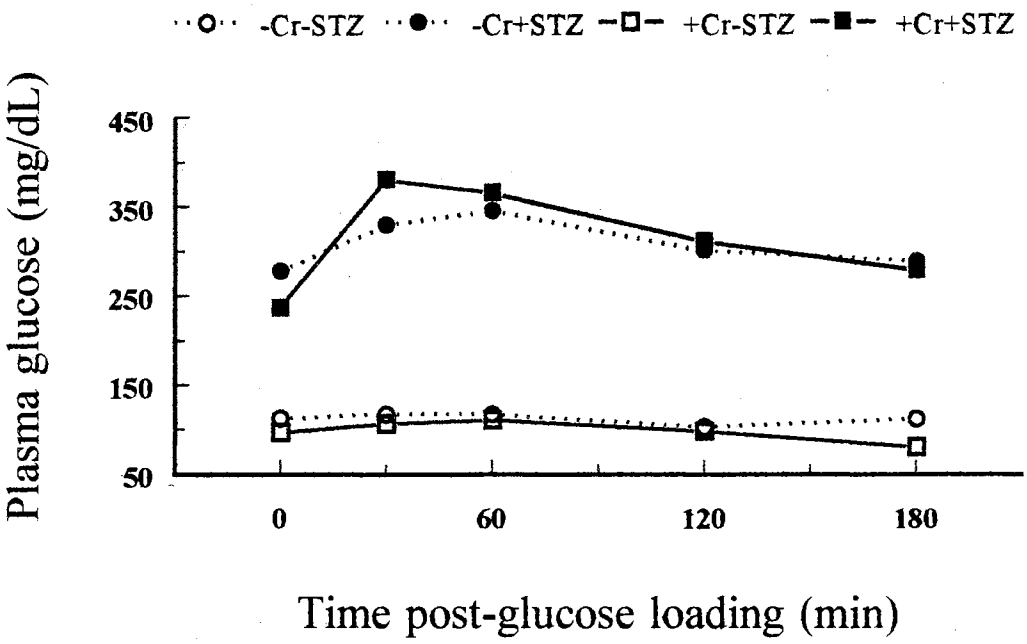


Figure IV.1
Glucose tolerance curves of normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)

# CHAPTER V

# EFFECT OF CHROMIUM DEPLETION AND DIABETES ON MATERNAL AND FETAL INSULIN-LIKE GROWTH FACTORS (IGF) AND FETAL GROWTH DURING PREGNANCY

## Abstract

The hypothesis of this study was that the depletion of the trace mineral chromium (Cr), which potentiates the action of insulin in glucose tolerance, exacerbates hyperglycemia and the negative outcomes of pregnancy in the streptozotocin (STZ) diabetic pregnant rat model perhaps through the regulation of the insulin-like growth factor (IGF) system. To determine the effect of Cr depletion and STZ-induced diabetes on the IGF system and fetal growth, 40 female Sprague-Dawley rats were randomly assigned one of four treatments in a 2X2 factorial design. The four treatments were: 1) very low chromium diet (40 μg Cr/kg diet) + citrate buffer injection, 2) very low chromium diet + STZ (30 mg STZ/kg body weight) in citrate buffer injection, 3) adequate chromium diet (2 mg Cr/kg diet) + citrate buffer injection, and 4) adequate chromium diet + STZ in citrate buffer injection. At weaning, the rats were housed in Plexiglas cages with plastic grate flooring and had free access to their diets and distilled deionized water. Pre-pregnancy diets contained 70 μg Cr/kg diet. Treatments were applied on day 1 of pregnancy. Dams were sacrificed on

day 20 of pregnancy. Day 20 maternal and fetal blood glucose, insulin, IGF-I, IGF-II and the IGF binding proteins (IGFBP's) were measured. Placenta and fetuses were weighed, counted and analyzed for protein and hydroxyproline content. Maternal Cr depletion decreased fetal IGF-II concentrations ($p<0.05$), % protein per fetus ($p<0.05$) and fetal IGF-I concentrations ($p<0.10$). STZ-induced diabetes decreased maternal weight gain ($p<0.02$), number of pups per litter ($p<0.01$), mean pup weight ($p<0.02$) and maternal insulin concentration ($p<0.001$) and increased maternal blood glucose ($p<0.01$) and IGF-I concentrations ($p<0.05$) but did not affect fetal hormones or glucose concentrations. Placental and fetal hydroxyproline concentrations were not affected by treatments. In summary, maternal Cr depletion may negatively affect fetal protein content by decreasing fetal IGF-II concentrations while diabetes may negatively affect fetal growth through its effect on maternal glucose, insulin and IGF-I.

## Introduction

Chromium deficiency in man and animals is associated with impaired glucose tolerance and a diabetic-like state (1, 2). In humans, chromium supplementation has been shown to improve glucose tolerance, lower fasting blood glucose concentrations, potentiate the action of insulin and increase HDL cholesterol (1). Chromium deficiency in rats leads to retarded growth, decreased glycogen reserves, increased incidence of aortic lesions and disturbances in amino acid utilization for protein synthesis (3). However, the effect of chromium deficiency on the growth regulating IGF system is unknown.

Chromium is best known for its insulin potentiating effect. It reduced growth hormone-induced insulin resistance and the resultant rise in blood glucose in pigs (4). Pigs fed chromium from chromium picolinate had faster glucose disappearance rates in

response to I.V. glucose tolerance tests and shorter glucose half-life in response to I.V. insulin challenge tests when compared to controls (5). In growing-finishing pigs, chromium picolinate increased daily gain and percentage of muscling while decreasing tenth rib fat and serum cholesterol (6). A recent preliminary study by Ward and colleagues (7) indicated that different sources of chromium such as chromium chloride, chromium acetate, chromium oxalate, chromium picolinate, chromium nicotinate and chromium nicotinate-glycine-cysteine-glutamate did not differ in their effect on growth, carcass characteristics, plasma metabolites, insulin or growth hormone concentrations in growing, finishing swine.

In sows, supplemental chromium from chromium picolinate increased gestational weight gain as well as litter size (8). Furthermore, during gestation, insulin response to feeding supplemental chromium in sows was significantly less compared to the response by unsupplemented sows. A follow-up study by the same group (9), however, did not find a significant effect of chromium on litter size but they did observe that the chromium-treated group had an average of one more live birth per litter than the controls. The effect of supplemental chromium on pregnancy outcome in rats or humans is unknown.

Although STZ-induced diabetic pregnancies result in impaired fetal growth (10 - 13), little is known about the effect of STZ on the IGF system in pregnancy. The insulin-like growth factors (IGF's) and their binding proteins (IGFBP's) are necessary for murine embryogenesis and fetal growth (14 - 17). Marked fetal growth retardation occurred in mice with a hemizygous disruption of the IGF-II gene (18). Both IGF-I and II increased incorporation of {$^3$H}thymidine in fetal rat islets (19) and {$^3$H}proline in fetal rat type I collagen (20) as well as induced neuronal and muscular differentiation in cocultures of

embryonic rat brainstem slices and skeletal muscle fibers (21). Growth-retarded fetal rats have decreased IGF-I and -II gene expression, decreased fetal serum IGF-I and increased IGFBP-1 gene expression (22, 23).

Decreased serum IGF-I, differential gene expression of IGF-I and IGFBP-1 as well as discordant organ specific regulation of IGF-I mRNA have been observed in diabetic male and non-pregnant female rats (24, 25, 26). Diminished IGF-II expression and retarded development were evident in six-day old conceptus of diabetic dams (27). In normal rat pregnancies, maternal plasma IGF-I concentrations decrease in conjunction with the disappearance of IGFBP-3 (28). This is associated with an increase in IGFBP-3 protease activity which promotes the availability of IGF-I to rapidly growing and differentiating tissues (29). Changes in IGF-I, IGF-II and their binding proteins during normal and diabetic pregnancy might be related to an altered metabolic state and the supply of glucose and insulin in both the mother and fetus which contributes to changes in fetal growth. Thus, the objective of this study was to determine the effect of dietary chromium depletion and STZ-induced diabetes on maternal and fetal insulin, glucose, IGF-I, and IGF-II concentrations, IGF binding protein activity, pregnancy outcome and fetal and placental protein and hydroxyproline content.

## Research Design and Methods

Forty weanling female Sprague Dawley rats were obtained from Sasco Inc. (Omaha, NE). The rats were assigned to one of four treatments in a 2 X 2 factorial design. The treatments were: 1) very low chromium diet (40 μg chromium/kg diet), citrate buffer injection, 2) very low chromium diet, STZ - citrate buffer injection (30 mg/kg body weight), 3) adequate chromium diet (2 mg chromium/kg diet), citrate buffer

injection, and, 4) adequate chromium diet, STZ - citrate buffer injection. All the rats were raised in a light and temperature controlled environment in Plexiglas cages with plastic grate flooring. The rats were fed semi-purified diets which contained 70 μg chromium/kg diet before they were bred. Distilled deionized water was used for drinking water throughout the study. Pre-pregnancy and pregnancy diet compositions are outlined in Table V.1. The rats were allowed free access to the diets and water which were provided in ceramic dishes. Treatments were applied on day 1 of pregnancy which was confirmed by the presence of a vaginal plug. The rats were bred at a mean weight of 267 ± 8 (mean ± SEM) grams and a mean age of 126 ± 3 (mean ± SEM) days. The STZ was obtained from Sigma Chemical Co. (St. Louis, MO), solubilized and delivered in 1 mL of citrate buffer (pH 4.5) and injected into the tail vein.

The rats were not fasted before necropsy on day 20 of gestation. They were anesthetized with a combination of ketamine hydrochloride and xylazine. Fetuses were removed by C-section and decapitated. Fetal blood was collected with heparinized capillary tubes and pooled in heparinized microcentrifuge tubes. Maternal blood was collected by cardiac puncture and mixed gently with 100 units of heparin. The maternal blood and tissues were harvested after all the fetuses were collected. Maternal and fetal plasma was separated and frozen. Embryo loss was determined by subtracting the number of viable fetuses per litter from the total number of corpora lutea observed on the ovaries.

Analytical methods. Dietary chromium analysis was carried out according to the method described by Hill *et al* (30). Insulin was measured with a commercial rat insulin radioimmunoassay kit from Linco Research, Inc. (St. Louis, MO) which, according to the manufacturer, has no detectable cross-reactivity with IGF-I or IGF-II. Blood glucose was

determined by the glucose oxidase method using Trinder reagent from Ciba Corning Diagnostic Corp. (Oberlin,OH). Maternal and fetal IGF was extracted from plasma by the formic acid-acetone method of Bowsher and colleagues (31) and concentrations determined by a radioimmunoassay previously described by Echternkamp *et al* (32) and Spicer *et al* (33). Cross-reactivities for IGF-I in the IGF-II assay and for IGF-II in the IGF-I assay were 5.0% and 0.2% respectively. Cross-reactivities for insulin in the IGF-I and IGF-II assay were <0.001% and <0.06% respectively. IGF binding proteins were analyzed by the Western ligand blot method described by Echternkamp et al (34). Fetal protein content was determined with a direct combustion unit (LECO FP428, Leco Corp., St. Joseph, MI) and fetal hydroxyproline determined by the method of Bergman and Loxley (35). Statistical analysis was performed using the SAS GLM procedure. Variables such as maternal IGF-I and -II that exhibited heterogeneous variance (determined by SAS UNIVARIATE procedure) were log transformed for the analysis of variance. Values reported herein are expressed as mean ± SEM.

## Results

Pregnancy outcome. Adequate chromium diets had a negative effect on litter size ($p<0.05$) and a tendency to decrease maternal weight gain ($p<0.1$, Table V.2). Diabetes has a significant negative effect on maternal weight gain ($p<0.05$), embryo loss ($p<0.01$), litter size ($p<0.01$) and average pup weight ($p<0.05$). Expressed as percent maternal body weight, placentas were significantly larger in the adequate chromium group. (Data presented in Table IV.5).

Maternal insulin, glucose, IGF-I and IGF-II concentrations are listed in Table V.3. Chromium did not significantly affect maternal insulin or glucose concentrations. In

contrast, diabetes significantly decreased ($p<0.001$) maternal plasma insulin such that mean plasma insulin concentration for the diabetic group was $0.10 \pm 0.02$ pmol/L ($0.61 \pm 0.47$ ng/mL) compared with $0.40 \pm 0.08$ pmol/L ($2.35 \pm 2.05$ ng/mL) in the non-diabetic group. As a result, there was a significant elevation of plasma glucose ($p<0.01$); mean plasma glucose for the diabetic group was $27 \pm 3$ mmol/L ($494 \pm 54$ mg/dL) compared with $18 \pm 1$ mmol/L ($324 \pm 17$ mg/dL) in the non-diabetic group.

Dietary chromium did not have an effect ($p>0.1$) on maternal plasma IGF-I or IGF-II concentrations. Diabetes had a significant effect on maternal plasma IGF-I concentration ($p<0.05$) such that mean IGF-I concentration for the diabetic dams was $167 \pm 66$ ng/mL compared with a mean of $129 \pm 54$ ng/mL in the non-diabetic group. Maternal plasma concentrations of IGF-II were not significantly different among groups but there was a tendency for factor interaction to occur ($p<0.1$). Within the chromium depleted group, diabetes had a tendency to decrease maternal plasma IGF-II concentrations but did not have the same effect in the adequate chromium group.

Figure V.1 is a representative autoradiograph from Western ligand blotting of maternal and fetal IGF binding proteins. Maternal plasma IGF binding proteins of the dams from all treatments were almost undetectable regardless of treatment. No significant treatment effect on maternal plasma IGF binding proteins was observed (Table V.4). The most prominent IGFBP detected in fetal plasma was a 32 kD species and was not affected by treatments. The 45 kD IGFBP, which is IGFBP-3, was present in low amounts in the fetal plasma from the diabetic group but not detectable in the non-diabetic group ($p<0.05$, Table V.5). IGFBP activity of the 22 kD species was not consistently observed in all the fetal plasma samples and there was no significant difference observed among the treatment

groups.

Fetal insulin, glucose, IGF-I, IGF-II and fetal protein content are listed in Table V.6. Fetal insulin and glucose concentrations were not significantly different among groups. There was a tendency for IGF-I concentrations to be lower ($p<0.1$) in the fetuses from the chromium depleted dams. Chromium had a significant positive effect on IGF-II concentrations ($p<0.05$) and on fetal protein content ($p<0.05$). Mean IGF-II concentration for the adequate chromium pups was 779 ± 92 ng/mL compared to 697 ± 85 ng/mL in the chromium depleted pups. Mean protein content for the adequate chromium pups was 9.25 ± 0.68 % compared to 8.56 ± 0.76 % in the chromium depleted pups. Fetal hydroxyproline concentrations were not significantly affected by chromium or STZ (data not shown).

## Discussion

We observed that dietary chromium decreased the number of pups per litter and had a tendency to decrease maternal weight gain but did not affect mean fetal weights. It is possible that intrauterine competition per pup due to increased placental size as a result of increased chromium in the diet resulted in decreased litter size. This is in contrast to previous studies where a positive effect of chromium on maternal weight gain and litter size in sows was observed (8, 9). The sows were supplemented with 200 μg chromium from chromium picolinate/kg feed but the total chromium concentration of the feed was not specified. In this experiment, we utilized the AIN-76 recommendation of 0.55 g chromium potassium sulfate/kg mineral mix resulting in a diet containing 2 mg chromium/kg diet, a tenfold difference from the sow studies. In view of the preliminary studies by Ward and colleagues (7) it is unlikely that the dietary source of chromium may

have influenced the animals' reproductive performance. Further studies are required to determine the precise amount of total dietary chromium needed to optimize reproductive performance.

In this study, fetal IGF-II was greater in the chromium supplemented group as was fetal protein content in spite of reduced litter size. It is most likely that chromium increased fetal protein through its effect on IGF-II as it has been observed that the hemizygous disruption of the IGF-II gene in fetal mice resulted in marked growth retardation (24) and that IGF-II stimulated DNA and protein synthesis in numerous fetal rat tissues in vitro (25, 26, 27). It is possible that the increase in placental weight and subsequent increase in maternal nutrient transfer may have contributed to the increase in fetal IGF-II and protein content. Furthermore, we observed an increase in urinary hydroxyproline excretion in the dams fed the chromium depletion diet compared to the dams fed adequate chromium (37) which is a reflection of increased protein breakdown in the rats depleted of chromium.

In agreement with the literature, the toxic effect of STZ on the pancreas and the subsequent development of diabetes was demonstrated by the reduced production of insulin and the resultant elevation of maternal glucose concentrations. Diabetes also had a negative effect on reproductive performance and fetal growth in the present study. As has been observed by other researchers (18, 19), diabetic dams gained less weight during pregnancy, had smaller fetuses, had a greater number of resorptions and a smaller number of viable fetuses per litter. Maternal plasma IGF-I concentrations were higher in the diabetic group when compared to the non-diabetic group in the present study. These hormonal observations have not been documented in diabetic pregnant rats. Hormonal

differences were not explained by IGFBP activity in the plasma. These differences may be explained by organ specific effects of streptozotocin diabetes on IGF production.

Streptozotocin-induced diabetes is associated with increased kidney weight, IGF-I mRNA (29) and IGF-I concentrations (39, 40). In the present study, we observed an increase in kidney weight and a tendency for increased liver weight with diabetes (data presented in Chapter IV). It is possible that increased production of kidney IGF-I contributed to the increase in plasma IGF-I concentrations in the present study. Measurement of kidney and liver IGF-I mRNA is strongly recommended to determine if these contribute to elevated plasma concentrations of IGF-I during pregnancy complicated by STZ-induced diabetes in the rat.

In this study, diabetes did not affect maternal IGF-II, fetal insulin, fetal glucose, fetal IGF-I or IGF-II concentrations but increased IGFBP-3 activity in fetal plasma. This is the first demonstration that the fetal IGF system responds to the mother's diabetic condition by increasing IGFBP-3 activity. Previously, hepatic IGFBP mRNA concentrations have been reported to increase in adult diabetic rats (41, 42). In addition, 14-day old fetuses of diabetic dams appear to have greater IGFBP-1 mRNA expression than fetuses of non-diabetic dams (43). Collectively, our results and the observations from previous studies support the hypothesis that increased fetal IGFBP secretion may contribute to the growth retardation of fetuses from diabetic dams.

In conclusion, this study demonstrates that although adequate dietary chromium has a negative effect on litter size, it does have a positive effect on fetal protein content which is associated with its positive effect on fetal IGF-II concentrations. The present study also demonstrated that streptozotocin-induced diabetes during pregnancy has

profound effects on reproductive performance and fetal growth through its effect on maternal plasma insulin, glucose and IGF-I.

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Table V.1
Diet compositions

| Components: | Adaptation diet (g/kg diet) | Treatment diet: (g/kg diet) |
|---|---|---|
| Cornstarch* | 430 | 150 |
| Casein* | 200 | 200 |
| Dextrose* | 100 | 0 |
| Sucrose | 100 | 500 |
| Soybean oil | 70 | 50 |
| Celufil* | 50 | 50 |
| AIN-76 mineral mix† | 35 | 35 |
| AIN-76 vitamin mix‡ | 10 | 10 |
| L-Cystine | 3 | 3 |
| Choline bitartrate | 2 | 2 |

* Harlan Teklad (Madison, WI)
† Prepared in our laboratory. Mineral mix for the treatment diets contained iron chloride instead of iron citrate. Mineral mix for the chromium depletion diet did not include the addition of chromium potassium sulfate.
‡ USB (Cleveland, OH)

Table V.2
Pregnancy outcome of normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Group | Maternal weight gain (g) | Embryo loss (n) | Pups per litter (n) | Mean pup weight (g) |
|---|---|---|---|---|
| -Cr-STZ | 111±17 | 3±2 | 13±2 | 4.2±0.7 |
| -Cr+STZ | 80±41 | 6±7 | 9±5 | 3.3±1.2 |
| +Cr-STZ | 90±32 | 4±2 | 10±4 | 4.0±0.4 |
| +Cr+STZ | 61±45 | 8±4 | 7±3 | 3.6±0.7 |
| | | Analysis of variance | | |
| Source of variation | ----------P Value---------- | | | |
| Cr | 0.0946 | 0.3924 | 0.0290 | 0.8328 |
| STZ | 0.0161 | 0.0091 | 0.0067 | 0.0145 |
| Cr x STZ | 0.9202 | 0.5851 | 0.5239 | 0.3562 |

*Mean ± SEM

Table V.3
Plasma insulin, glucose, IGF-I & IGF-II from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Group | Insulin pmol/L | Glucose mmol/L | IGF-I ng/dL | IGF-II ng/dL |
|---|---|---|---|---|
| -Cr-STZ | 0.54 ± 0.09 | 19.0 ± 3.3 | 129.0±79.9 | 213.9±65.4 |
| -Cr+STZ | 0.12 ± 0.08 | 26.1 ± 3.2 | 170.6±84.8 | 179.1±28.7 |
| +Cr-STZ | 0.30 ± 0.08 | 17.0 ± 3.2 | 128.6±15.7 | 188.1±20.3 |
| +Cr+STZ | 0.09 ± 0.09 | 28.8 ± 3.3 | 163.7±42.8 | 198.1±20.0 |

Analysis of variance

| Source of variation | P Value | | | |
|---|---|---|---|---|
| Cr | 0.1147 | 0.9093 | 0.5236† | 0.7899 |
| STZ | 0.0009 | 0.0059 | 0.0425† | 0.3376 |
| Cr x STZ | 0.2347 | 0.4827 | 0.6074† | 0.0878 |

* Mean ± SEM
† Result from AOV of log transformed values.

Table V.4
IGF binding protein activity from plasma of normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Group | 45 kD (ADU†) | 32 kD (ADU) | 22 kD (ADU) |
|---|---|---|---|
| -Cr-STZ | 0.329 ± 0.930 | 2.210 ± 0.795 | 0.520 ± 1.002 |
| -Cr+STZ | 0.000 ± 0.000 | 2.381 ± 0.931 | 0.721 ± 0.951 |
| +Cr-STZ | 0.000 ± 0.000 | 2.140 ± 0.618 | 0.185 ± 0.523 |
| +Cr+STZ | 0.376 ± 0.994 | 2.209 ± 0.774 | 0.191 ± 0.506 |

Analysis of variance

| Source of variation | P Value | | |
|---|---|---|---|
| Cr | 0.9686 | 0.6886 | 0.1192 |
| STZ | 0.9764 | 0.6653 | 0.5695 |
| Cr x STZ | 0.1289 | 0.8517 | 0.6487 |

*Mean ± SEM
†ADU = arbitrary densitometric units

Table V.5
IGF binding protein activity from plasma of fetuses from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Group | 45 kD (ADU†) | 32 kD (ADU) | 22 kD (ADU) |
|---|---|---|---|
| -Cr-STZ | 0.000 ± 0.000 | 7.266 ± 3.925 | 1.183 ± 0.921 |
| -Cr+STZ | 0.983 ± 1.822 | 6.249 ± 2.038 | 1.183 ± 0.752 |
| +Cr-STZ | 0.000 ± 0.000 | 7.431 ± 2.096 | 1.157 ± 1.005 |
| +Cr+STZ | 0.446 ± 1.262 | 6.666 ± 1.194 | 1.082 ± 0.934 |

Analysis of variance

| Source of variation | P Value | | |
|---|---|---|---|
| Cr | 0.4041 | 0.6676 | 0.9333 |
| STZ | 0.0483 | 0.2877 | 0.8594 |
| Cr x STZ | 0.4152 | 0.8283 | 0.9770 |

* Mean ± SEM
† ADU = arbitrary densitometric units

TABLE V.6

Plasma insulin, glucose, IGF-I & IGF-II concentrations of fetuses from normal and STZ-induced diabetic dams fed diets with or without added chromium (Cr)*

| Group | Insulin ng/mL (pmol/L) | Glucose mg/dL (mmol/L) | IGF-I ng/mL | IGF-II ng/mL | Protein content % |
|---|---|---|---|---|---|
| -Cr-STZ | 0.91 ± 0.15 | 7.7 ± 1.8 | 48.8±14.5 | 714.9±93.4 | 0.085±0.0009 |
| -Cr+STZ | 0.76 ± 0.14 | 6.8 ± 1.6 | 50.1±8.7 | 664.7±62.7 | 0.086±0.004 |
| +Cr-STZ | 1.06 ± 0.13 | 4.7 ± 1.5 | 59.6±9.1 | 799.3±74.2 | 0.092±0.007 |
| +Cr+STZ | 0.77 ± 0.14 | 8.4 ± 1.5 | 55.2±13.3 | 742.3±121.3 | 0.093±0.007 |

Analysis of variance

| Source of variation | P Value | | | | |
|---|---|---|---|---|---|
| Cr | 0.5825 | 0.6756 | 0.0757 | 0.0397 | 0.0192 |
| STZ | 0.1371 | 0.3878 | 0.7222 | 0.1613 | 0.6971 |
| Cr x STZ | 0.6257 | 0.1758 | 0.5211 | 0.9273 | 0.8966 |

* Mean ± SEM

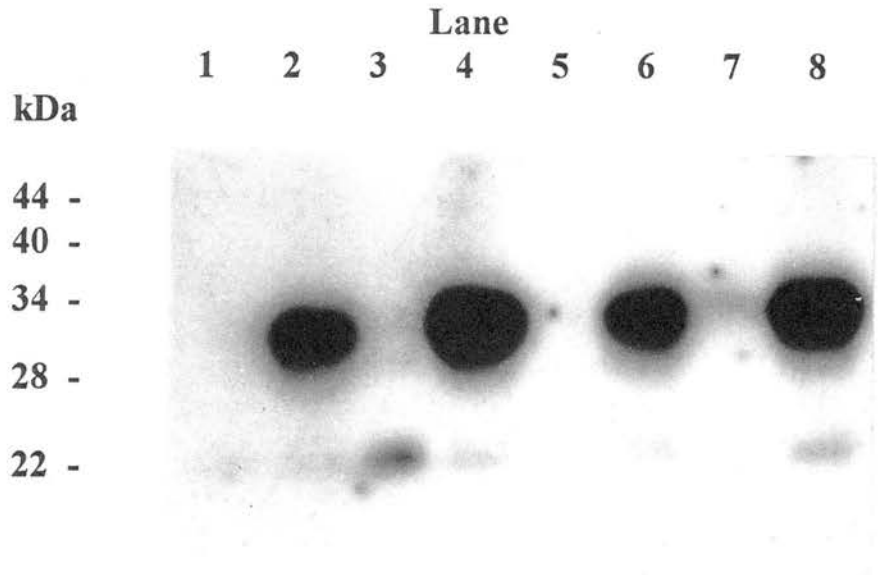


Figure V.1
A representative ligand blot analysis of IGFBP activity in maternal and fetal plasma collected on day 20 of pregnancy. Lane 1, plasma of a dam from the -Cr-STZ group; lane 2, pooled fetal plasma from pups of the dam in lane 1; lane 3, plasma from a dam from -Cr+STZ group; lane 4, pooled fetal plasma from pups of the dam in lane 3; lane 5, plasma from a dam from the +Cr-STZ group; lane 6, pooled fetal plasma from pups of the dam in lane 5; lane 7, plasma from a dam from the +Cr+STZ group; lane 8, pooled fetal plasma from pups of the dam in lane 7.

# CHAPTER VI

# SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

## Summary

This study was designed to determine the effect of chromium depletion and streptozotocin (STZ) - induced diabetes on pregnancy outcome, on maternal carbohydrate and lipid metabolism, on the distribution of minerals in maternal tissues, on the placenta and fetus, and on the IGF system. Forty weanling female rats were assigned to 4 treatment groups in a 2 x 2 factorial design. The treatments in this study were: 1) chromium content of the diet at 40 μg or 2 mg Cr/kg diet given during pregnancy, and 2) diabetes induced or not induced at the onset of pregnancy by injection of streptozotocin (30 mg STZ/kg body weight) in 0.1 M citrate buffer (pH4.5) vs. citrate buffer alone. All rats were fed moderately low chromium diets (70 μg Cr/kg diet) before breeding, and were bred at 126 ± 3 days (mean ± SEM) of age. The average breeding weight was 267 ± 8 grams (mean ± SEM). Treatments were initiated on day 1 of pregnancy when a vaginal plug was observed. Glucose tolerance tests were given on day 18 of pregnancy. All rats were necropsied on day 20 for tissue and blood collection. Maternal and fetal plasma were analyzed for glucose, insulin, total triglycerides, cholesterol, non-esterified free fatty acids, β-hydroxybutyrate, IGF-I, IGF-II concentrations and the IGF binding protein

activity. Maternal kidney, liver, spleen, heart, and the placenta and fetuses were weighed and analyzed for calcium, magnesium, iron, copper, zinc, chromium and manganese concentrations.

Maternal plasma insulin, glucose, fructosamine, glucose tolerance, and other metabolites such as triglycerides, cholesterol, free fatty acids and β-hydroxybutyrate were not significantly affected by chromium depletion. Maternal chromium depletion decreased placenta size when expressed as percent maternal body weight, had a tendency to increase maternal weight gain, significantly increased litter size but did not affect mean pup weights. Chromium depletion decreased concentrations of chromium and zinc while increasing manganese concentration in the kidneys. Maternal chromium depletion also decreased copper concentrations of the heart. Maternal chromium depletion decreased fetal IGF-II concentrations, percent protein per fetus and had a tendency to decrease fetal IGF-I concentrations.

Diabetes decreased maternal weight gain, enlarged maternal kidneys and spleens, and increased fetal loss and therefore decreased litter size, and, decreased fetal weight. Diabetes significantly decreased plasma insulin concentrations increasing plasma glucose and triglycerides. Diabetes thus resulted in severe glucose intolerance as demonstrated by elevated tolerance curves, larger areas under these curves and lower insulin to glucose ratios. Diabetes increased liver and kidney concentrations of copper and zinc and had a tendency to decrease fetal chromium concentrations. Streptozotocin induced diabetes increased maternal IGF-I concentrations but did not affect fetal hormones or glucose concentrations. Maternal IGFBP activity was almost nil while only fetuses from diabetic

dams had the 45kD IGFBP. Placental and fetal hydroxyproline content were not affected by treatments.

Several significant interaction effects between chromium and diabetes were observed. Within the adequate chromium group, diabetes increased plasma non-esterified free fatty acid concentrations but not in the chromium depleted group. Maternal liver protein was increased by diabetes in the chromium depleted group but not in the adequate chromium group. Within the chromium depleted group, diabetes increased fetal manganese concentrations but within the adequate chromium group, diabetes decreased fetal manganese concentrations.

## Conclusions

The first objective of this study was to determine the effect of chromium depletion and diabetes induced during pregnancy on glucose tolerance, and on circulating total cholesterol, total triglycerides, non-esterified fatty acids and β-hydroxybutyrate concentrations. Based on the null hypothesis that chromium depletion and diabetes during pregnancy would not affect maternal glucose tolerance or total plasma cholesterol, triglycerides, non-esterified fatty acids and β-hydroxybutyrate concentrations, the following conclusions were drawn. Chromium depletion had no effect on glucose tolerance, total plasma triglycerides, cholesterol or β-hydroxybutyrate. Diabetes had a detrimental effect on maternal glucose tolerance, and increased total plasma triglycerides but not cholesterol or β-hydroxybutyrate. Diabetes increased non-esterified fatty acid concentrations only in dams receiving an adequate chromium diet but not in those receiving a chromium depletion diet.

The second objective of this study was to determine the effect of chromium depletion and diabetes induced during pregnancy on plasma insulin, glucose, IGF-I, IGF-II, IGF binding protein activity, maternal weight gain and pregnancy outcome. Based on the null hypothesis that chromium depletion and diabetes during pregnancy would not affect maternal plasma insulin, glucose, IGF-I, IGF-II, IGF binding protein activity, maternal weight gain or pregnancy outcome, the following conclusions were drawn. Chromium depletion had a positive effect on litter size and a tendency to increase gestational weight gain but did not affect maternal plasma, insulin, IGF-I, IGF-II, IGF binding protein activity or fetal weight. Diabetes had an adverse effect on maternal insulin, glucose, increased plasma IGF-I, decreased gestational weight gain, litter size and fetal weight but did not affect IGF-II concentrations or IGF binding protein activity. Chromium depletion and diabetes had an interactive effect on maternal liver protein concentration such that within the chromium depleted group, diabetes increased maternal liver protein concentration but did not have the same effect in the adequate chromium group.

The third objective of this study was to determine the effect of chromium depletion and diabetes during pregnancy on fetal plasma insulin, glucose, IGF-I, IGF-II, IGF binding proteins, fetal weight, fetal protein and hydroxyproline concentrations. Based on the null hypotheses that chromium depletion and diabetes during pregnancy would not affect fetal plasma insulin, glucose, IGF-I, IGF-II, IGF binding protein activity, fetal weight, fetal protein or hydroxyproline concentration, the following conclusions were drawn. Chromium depletion decreased fetal plasma IGF-II and fetal protein concentration and had

a tendency to decrease fetal IGF-I concentrations but not plasma insulin, glucose, IGF binding protein activity, fetal weight or hydroxyproline concentrations. Maternal diabetes decreased fetal weight and promoted IGFBP-3 activity in fetal plasma but it did not affect fetal plasma insulin, glucose, IGF-I, IGF-II, fetal protein or hydroxyproline concentrations.

The fourth objective of this study was to determine the effect of chromium depletion and diabetes during pregnancy on macromineral and trace mineral concentrations in the liver, kidney, spleen, heart, placenta and fetuses of the experimental animals. Based on the null hypotheses that chromium depletion and diabetes during pregnancy would not affect macromineral and trace mineral concentrations in the liver, kidney, spleen, heart, placenta and fetuses of the experimental animals, it was concluded that chromium depletion decreased kidney chromium and zinc, decreased heart copper and increased kidney manganese concentrations but did not affect the distribution of calcium or magnesium in the tissues. Diabetes on the other hand increased kidney and liver copper and zinc but did not affect the distribution of calcium, magnesium, iron or chromium in the tissues. Chromium depletion and diabetes had interactive effects on the distribution of chromium in the maternal heart and manganese in the fetus. The adequate chromium non-diabetic group had a significantly greater concentration of chromium in the heart compared to all the other groups. Within the chromium depleted group, diabetes increased fetal manganese concentrations while within the adequate chromium group, diabetes decreased fetal manganese concentrations.

## Recommendations

Further study is recommended to determine the effects of dietary chromium on glucose tolerance and insulin sensitivity in pregnancy. Perhaps, a different animal model such as the spontaneously diabetic BB/Wistar rat, the BHE rat or second generation rats from STZ treated dams might be more suitable than the STZ-induced diabetic dam in that there would be no artificial or chemical destruction of β-cell function. As we have seen in this study, streptozotocin is very toxic to the pancreas so that insulin production is markedly decreased. Another alternative would be to give endogenous insulin during diabetes in pregnancy and base conclusions on the effect of the treatments on insulin requirement to maintain normal glucose control.

Diet composition was also different before and after pregnancy was induced. It is possible that the chromium depleted rats had larger litter size because they were subjected to the least change and may have eaten more to compensate for the lack of chromium in the diet. Therefore, food intake measurements may be a valuable piece of data to consider in similar experiments. Furthermore, the diets changed from 10% sucrose - 43% cornstarch - 10% dextrose before pregnancy to 50% sucrose - 15% cornstarch after pregnancy was induced. This drastic change in simple and complex carbohydrate concentrations may have affected outcomes. Unfortunately, until low chromium complex carbohydrates are made available, the formulation for low chromium diets will remain a problem.

The interaction effects that surfaced in this study warrant further investigation.

The changes in the distribution of manganese concentrations in the fetus and chromium in the maternal heart need to be explored to determine the extent of these changes and how chromium in the diet and diabetes affect each other in this respect. Histological studies as well as organ function studies and DNA analysis are recommended.

## Implications

It was difficult to assess our results adequately because similar types of data were either limited or not available for comparison. Nevertheless, practical implications can be garnered from this study. It is clear that different levels of chromium in the diet can affect fetal physiology as well as mineral distribution in various tissues during pregnancy in the rat. Together with the presence of diabetes, these possibly adverse conditions could be exacerbated. Based on our results, chromium supplementation of the diet for individuals with impaired glucose tolerance or for pre-gestational or gestational diabetic women may not be a safe. Further research is necessary to determine the safety and efficacy of chromium supplementation especially during pregnancy. Chromium supplements such as chromium picolinate which are categorized as a food supplements are readily available in grocery stores, pharmacies and health food stores. Because of the lack of government regulation, quality, purity and safety requirements and standards such as those followed by the Food and Drug Administration for the regulation of drugs, are not required for food supplements. Thus, chromium and other mineral supplements and plant extracts with pharmacological properties and effects are readily available. It is necessary to promote caution in the self-prescribed use not only of chromium supplements but also of other mineral supplements as well until more definitive results are observed.

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# APPENDICES

# Appendix A

## Diet Compositions

| Components: | Adaptation diet (g/kg diet) | Treatment diet: (g/kg diet) |
|---|---|---|
| Cornstarch | 430 | 150 |
| Casein | 200 | 200 |
| Dextrose | 100 | 0 |
| Sucrose | 100 | 500 |
| Soybean oil | 70 | 50 |
| Celufil | 50 | 50 |
| Mineral mix* | 35 | 35 |
| AIN-76 vitamin mix | 10 | 10 |
| L-Cystine | 3 | 3 |
| Choline bitartrate | 2 | 2 |

*AIN-76 mineral mix. Low chromium diets contained mineral mix without added chromium.

Appendix B

Mineral Mix Compositions

| Components: | - Cr diet (g/kg mix) | + Cr diet: (g/kg mix) |
|---|---|---|
| Calcium phosphate, dibasic | 500.0 | 500.0 |
| Sodium chloride | 74.0 | 74.0 |
| Potassium citrate, monohydrate | 220.0 | 220.0 |
| Potassium sulfate | 52.0 | 52.0 |
| Magnesium oxide | 24.0 | 24.0 |
| Manganous carbonate | 0.65 | 0.65 |
| Ferrous chloride | 3.56 | 3.56 |
| Zinc carbonate | 1.60 | 1.60 |
| Cupric carbonate | 0.30 | 0.30 |
| Potassium iodate | 0.01 | 0.01 |
| Sodium selenate | 0.01 | 0.01 |
| Chromium potassium sulfate | 0.00 | 0.55 |
| Sucrose | 121.19 | 120.64 |

# Appendix C

## Reagents for Hydroxyproline Analysis

a. Oxidant solution buffer:

| | |
|---|---|
| Sodium acetate ($3H_2O$) | 57 grams |
| Trisodium citrate ($2H_2O$) | 37.5 grams |
| Citric acid ($H_2O$) | 5.5 grams |
| 2-Isopropanol | 385 mL |
| Distilled deionized water | Add to make 1L total volume. |

Weigh and pour dry ingredients into a 1 L volumetric flask. Add the isopropanol followed by water to volume. Place mixing bar in the flask and mix on low heat until all components are in solution. When cooled, store buffer in a clean opaque plastic bottle.

b. Oxidant solution: After all samples and standards have been aliquoted and the Ehrlich's reagent prepared, weigh 0.7 g chloramine-T and add water to make 10 mL. Add 40 mL buffer solution as described above. Mix gently and cover with foil.

c. Ehrlich's reagent: To make 100 mL of reagent, weigh 17.6 grams para-dimethylaminobenzaldehyde (PDAB). Dissolve PDAB in 40.8 grams of 60% perchloric acid. Add isopropanol to make 100 mL. Cover with foil.

# VITA

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Doctor of Philosophy

Thesis: THE EFFECTS OF CHROMIUM DEPLETION AND STREPTOZOTOCIN (STZ) - INDUCED DIABETES DURING PREGNANCY

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