# THE EFFECT OF CHROMIUM DEPLETION ON BONE AND URINARY HYDROXYPROLINE CONCENTRATION IN STREPTOZOTOCIN INDUCED GESTATIONAL DIABETIC RATS

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# NOMENCLATURE

| | |
|---|---|
| Cr | Chromium |
| GDM | Gestational diabetes mellitus |
| GTF | Glucose tolerance factor |
| Hyp | Hydroxyproline |
| IGF-I | Insulin-like growth factor-I |
| N | Number |
| NGT | Normal glucose tolerance |
| STZ | Streptozotocin |

| | |
|---|---|
| C | centigrade |
| g | gram |
| mg | milligram |
| ml | milliliter |
| mm | millimeter |
| µg | microgram |

# CHAPTER I

# INTRODUCTION

## Research background

Gestational diabetes mellitus (GDM) is a heterogeneous disorder that complicates 2-3% of all pregnancies in the world (Sepe et al., 1985; Gabbe, 1986). It is defined as glucose intolerance with first onset during pregnancy, and it presents significant perinatal risks such as premature birth, congenital abnormalities, fetal morbidity and mortality, and obstetric complications. Gestational diabetes also increases maternal risk for later development of manifest diabetes (Gabbe, 1986; Anonymous, 1988). During normal pregnancy, a gradual deterioration of glucose tolerance can take place so that plasma glucose concentrations following a glucose load or a meal are higher than non-pregnancy (Kuhl, 1975; Kuhl et al., 1977). However, in most pregnant women, plasma glucose stays within normal ranges and deteriorates in only 2-3% of pregnancies sufficiently for the development of diabetes (Kuhl et al., 1984).

Although glucose intolerance in GDM is usually mild,

the higher incidence of maternal complications and fetal perinatal risks make it important to recognize and treat this condition. As a result, the pathophysiologic background of GDM must be known. The reason pregnancy is capable of inducing a temporary diabetic state is still unclear (Kuhl and Anderson, 1988). One of several explanations is that nutritional factors may play a role in the development of GDM (Aharoni et al., 1992).

Chromium is an essential trace mineral for humans and animals. It functions in maintaining normal glucose tolerance by increasing insulin efficiency, therefore reducing the amount of insulin required to control blood glucose concentration and related processes (Anderson, 1992). Marginal states of chromium nutriture may contribute to progressive glucose tolerance impairment and increase the risk of diabetes (Mertz, 1993). There is strong evidence that glucose intolerance induced by insufficient dietary chromium could be alleviated by supplemental chromium (Anderson, 1992). However, not all diabetic patients can benefit from dietary chromium supplementation (Anderson, 1992). Response to chromium is related to the degree of glucose intolerance (Anderson, 1989).

Studies also reported decreased hair chromium concentration in the last stage of pregnancy in GDM (Aharoni et al., 1992), and increased urinary chromium excretion with advancing pregnancy (Saner, 1981). These findings observed

in GDM indicate that chromium deficiency may be a possible factor in the etiology of GDM.

Because diabetes is associated with generalized defects in connective tissue metabolism, and collagen is the major protein component of connective tissue (Bornstein and Traub, 1979), abnormalities in collagen metabolism may play a role in connective tissue alterations in GDM (Spanheimer et al., 1988). An observed decrease in collagen mass in diabetes may result from decreased collagen synthesis, increased collagen degradation, or a combination of these two processes (Lien et al., 1992).

Hydroxyproline is considered to be a good indicator of collagen degradation because it is present almost exclusively in collagen and not reutilized in the synthesis of collagen (Prockop and Kivirikko, 1967). Of the total amount of hydroxyproline produced in the body by collagen catabolism, 90-95% is further broken down, while 5-10% appears in urine (Prockop, 1964). Because collagen is turned over continuously, a relatively constant amount of hydroxyproline is excreted in the urine every day (Berg and Kerr, 1992). This makes it possible to measure 24 or 12 hour urinary hydroxyproline excretion as an index of collagen breakdown.

Moreover, because more than 50% of the total collagen is present in bone tissue (Klein and Curtiss, 1964) and bone collagen has a higher turnover rate than collagen elsewhere in the body, the skeleton is estimated to be the source of

approximately 70-80% of urinary hydroxyproline (Birkenhager, 1970). Significant relationships have been found between diabetes and bone hydroxyproline concentration (Locatto et al., 1990; Ishida et al., 1988). Therefore, in this study we measured bone hydroxyproline as another important indicator to evaluate collagen metabolism in GDM. Furthermore, bone calcium was also measured to evaluate the effect of GDM on bone composition. In streptozotocin (STZ)-induced diabetic rats, researchers found a significant reduction in bone calcium concentration as bone mass was decreased in diabetes (Locatto et al., 1990).

## Significance of the research

A few studies investigating the relationships between chromium nutrition and GDM have been conducted, but they were mostly conducted on human subjects and were hard to control. Likewise, a great deal of research has been performed to evaluate the altered collagen degradation in diabetes using hydroxyproline as an indicator, but these studies were conducted using non-pregnant diabetic models.

Currently, there is no research evaluating the relationship between chromium depletion and the hydroxyproline concentration in GDM using a rat model. Our study was designed to measure bone and urinary hydroxyproline concentration to reflect the effect of

chromium depletion on collagen metabolism in STZ-induced gestational diabetic rats.

## Objectives

The following research objectives were developed:

1. To determine if chromium depletion would alter urinary hydroxyproline excretion in STZ-induced diabetic pregnant rats and control rats.

2. To determine if chromium depletion would alter bone hydroxyproline concentration in STZ-induced diabetic pregnant rats and control rats.

3. To determine if chromium depletion would alter bone calcium concentration in STZ-induced diabetic pregnant rats and control rats.

## Hypotheses

The following research hypotheses were developed:

1. There will be no statistically significant effects of chromium depletion or of STZ-induced diabetes on urinary hydroxyproline excretion in pregnant rats.

2. Chromium depletion or STZ-induced diabetes will not statistically alter bone hydroxyproline concentration in pregnant rats.

3. Bone calcium concentration will not be statistically altered by chromium depletion or STZ-induced diabetes in pregnant rats.

## Limitations

This study was designed to evaluate the effect of chromium depletion on GDM using a rat model, so the data obtained can not be extrapolated directly to humans. In addition, the sample size in this study was small and may not accurately reflect alterations in hydroxyproline concentration in GDM. However, our findings about the relationship between chromium and hydroxyproline concentration in GDM utilizing an animal model may help direct human research. Further studies are suggested in this area using larger numbers of animals.

# CHAPTER II

# LITERATURE REVIEW

## Etiology of gestational diabetes mellitus

Gestational diabetes mellitus (GDM) presents significant risks for both the fetus and the mother (Gabbe, 1986; Anonymous, 1988). Maternal complications of GDM include pregnancy-induced or chronic hypertension, pyelonephritis, preterm labor, hydramnios, abnormalities of labor, birth trauma, and increased risk of later development of manifest diabetes (Goldman et al., 1991). Perinatal risks include higher incidence of macrosomia, hypoglycemia, hyperbilirubinemia, hypocalcemia, polycythemia, and congenital abnormalities (Schwartz, 1990). Hypoglycemia, which may cause neurological damage, and polycythemia, which may cause both neurological damage and renal vein thrombosis, present the greatest danger. Hypoglycemia and polycythemia can be promptly diagnosed and corrected; however, macrosomia can not be reversed once established. Macrosomia may cause delayed chronic morbidity to the offspring by inducing obesity and diabetes

in later life (Hod et al., 1991). As a result, it is very important to recognize the etiology of GDM and to be able to treat and prevent this condition.

Why pregnancy is capable of inducing GDM is still unknown (Kuhl and Anderson, 1988). Glucose tolerance deteriorates during pregnancy in spite of steadily increasing plasma insulin concentration (Kuhl et al., 1985). This indicates that pregnancy may induce insulin resistance. Women with GDM who received an oral glucose load had significantly increased insulin concentration during pregnancy versus postpartum (Kuhl and Holst, 1976; Hornnes et al., 1981). A similar response was observed in pregnant women with normal glucose tolerance (NGT) (Kuhl, 1975). However, in women with GDM, peak plasma insulin concentrations occurred later than in pregnant women with NGT (Kuhl and Holst, 1976). Moreover, the first-phase insulin response and the insulin secretory capacity in women with GDM were significantly reduced compared to that of pregnant women with NGT (Kuhl, 1991). All these data support the hypothesis that pregnancy-induced insulin resistance may be responsible for the development of GDM.

However, the cause of insulin resistance in pregnancy is still unclear. It may be induced by changes in insulin-receptor binding, postreceptor changes (Pedersen, 1984), or cellular effects of high plasma pregnancy-associated hormones or free cortisol (Kuhl, 1991). The effects are a reduction in whole-body insulin sensitivity to about one-

third of non-pregnant state. Most pregnant women are able to have a compensatory increase in both basal and nutrient-stimulated insulin secretions to counteract pregnancy-induced insulin resistance, but approimately 2-3% may not possess the capability to produce a sufficiently large increase in insulin secretion, resulting in glucose intolerance (Kuhl, 1991). These are probably the women who develop GDM.

## The role of chromium nutrition in the etiology of GDM

One assumption for pregnancy-induced insulin resistance is that nutritional factors such as chromium deficiency, may play a role in its development (Aharoni et al., 1992). Chromium is an essential trace mineral in humans and animals, which has a role in glucose homeostasis (Morris et al., 1993). Chromium has been proposed to function in maintaining normal glucose tolerance by forming a glucose-tolerance factor (GTF), which acts as a cofactor with insulin in all insulin-dependent systems, and thus increases insulin efficiency (Mertz, 1976; Hambidge, 1974). As a result, with sufficient chromium, lower insulin is required to control blood glucose and related processes (Anderson, 1992). On the other hand, with inadequate chromium, glucose tolerance is impaired, increasing the risk of diabetes (Mertz, 1969).

There is strong evidence that glucose intolerance induced by insufficient dietary chromium can be alleviated with supplemental chromium (Anderson, 1992). Morris et al. (1985) found plasma chromium concentrations were significantly lower in diabetes (60% of normal) and chromium excretion was elevated (nearly three times) compared to normal healthy subjects. Fasting glucose, circulating insulin, insulin binding, circulating glucagon, and β-cell sensitivity were improved with increased chromium status (Anderson, 1989). Hypoglycemic symptoms including glucose values, insulin binding, and insulin receptor numbers were also improved in hypoglycemic patients following chromium supplementation (Anderson et al., 1987).

However, not all diabetic patients demonstrated beneficial effects of supplemental chromium. One explanation is that response to supplemental chromium may be related to diabetic stages, form and amount of supplemental chromium, duration of supplementation, and chromium status (Anderson, 1992). For example, some diabetic patients may lose the ability to convert inorganic chromium to a physiologically active form and therefore are dependent upon active chromium complexes from foods (Toepfer et al., 1973). When sufficient chromium was consumed in a usable form, insulin requirement was low due to the insulin-potentiating activity of biologically active chromium complexes (Anderson 1992). In addition, studies have found that diabetic mice also lost the ability to convert inorganic chromium to a usable form.

Diabetic mice supplemented with inorganic chromium showed no effect, while biologically active chromium supplementation reduced glucose to normal concentrations (Tuman et al., 1978).

Chromium defeciency is one of the causes of glucose intolerance in diabetes. Because chromium is not a therapeutic agent, only patients whose glucose intolerance is related to insufficient dietary chromium could benefit from improved chromium nutrition (Anderson, 1992).

Pregnancy may lead to chromium deficiency and thus increase the risk of developing GDM (Saner, 1981). Hair chromium concentration of women with GDM significantly decreased in the later stage of pregnancy; this decrease was not observed in non-diabetic pregnant women (Aharoni et al., 1992). Moreover, another study found urinary chromium excretion increased significantly with advancing pregnancy (Saner, 1981). The decreased hair chromium concentration and the increased urinary chromium excretion during later pregnancy, as well as the evidence of impairment of glucose tolerance during pregnancy, suggest that chromium deficiency might be an etiological factor in GDM.

## The abnormalities of collagen metabolism in diabetes

### The nature of connective tissue defect in diabetes

Diabetes is associated with generalized defects in

connective tissue metabolism, such as poor wound healing (Goodson and Hunt, 1979), decreased bone mass and formation rate (Goodman and Hori, 1984), and vascular basement membrane thickening (Raskin et al., 1975). Because collagen is the most abundant protein in vertebrate animals and the major protein component of connective tissues (Bornstein and Traub, 1979), abnormalities in collagen metabolism may play a role in the widespread nature of connective tissue alterations in GDM.

Collagen mass is decreased in diabetes. A net loss of skin and wound healing collagen mass (Goodson and Hunt, 1977) as well as reduced bone mass and decreased bone formation rate have been observed in diabetic patients (Goodson and Hunt, 1979). Loss of interstitial collagen in connective tissues may result from decreased collagen synthesis (Spanheimer et al., 1988), enhanced catabolism of newly synthesized collagen (Schneir et al., 1982), or a combination of these two processes.

## The decrease of collagen synthesis

Collagen synthesis may be decreased in diabetes. Spanheimer and colleagues (1988) reported that within two weeks after induction of diabetes with streptozotocin (STZ), collagen production was significantly reduced 52% and 51% in bone and cartilage of diabetic rats compared to control rats.

The mechanism for this decrease is still unclear. There is evidence that insulin can increase collagen synthesis by promoting intracellular accumulation of certain amino acids, such as L-proline, which is associated with accelerated collagen synthesis (Wettenhall et al., 1969). One hypothesis is that insulin insufficiency in diabetes contributes to decreased collagen synthesis.

The increase of collagen degradation

Diabetes also can increase collagen catabolism. Schneir and coworkers (1982) found the collagen catabolic process was enhanced in the skin of STZ-induced diabetic rats by quantitating the amount of dialyzable [$^3$H]- hydroxyproline in skin homogenates after short-term [$^3$H]-proline injection. Twenty-four hours after [$^3$H]-proline injection, 4.5% of radiolabeled collagen mass in diabetic rats' dried skin existed as low molecular weight [$^3$H]-hydroxyproline-containing material, in contrast to 1.1% in control rats' skin. When wet skin was analyzed, 30-40% of the radiolabeled collagen mass appeared as [$^3$H]-hydroxyproline-containing material in diabetic rats, and only 10-20% in control rats. Thus they concluded that intracellular collagen degradation was accelerated by experimental diabetes.

The mechanism for effects of diabetes on collagen degradation is not clear. In vitro studies demonstrated that glucose inhibited fibril formation and subsequent collagen

cross-linking. Thus, collagen synthesized in a high glucose environment appears to be less cross-linked and thereby more susceptible to collagenase digestion (Lien et al., 1992), which might explain increased collagen degradation in diabetes.

## Hydroxyproline as an indicator of collagen degradation

### Hydroxyproline is related to collagen metabolism

Hydroxyproline is a good indicator of collagen degradation based on the nature of collagen metabolism. Collagen is secreted by fibroblasts and osteoblasts (Birkenhager, 1970). There are three stages in collagen development (Schneir et al., 1982): (1) intracellular procollagen from proline (2) extracellular tropocollagen (recently synthesized collagen) and (3) mature collagen fibers (soluble or insoluble). Collagen contains 33% glycine, 13% proline, and 9% hydroxyproline (Mavrikakis et al., 1993). Certain proline residues in collagen are converted into hydroxyproline by prolyl hydroxylase (Kivirikko et al., 1989). This hydroxyproline in the collagen peptide linkages plays a crucial role in collagen folding, and it can only be hydroxylated from proline, whereas exogenous hydroxyproline does not appear in collagen at all. Collagen catabolism is catalyzed by collagenase, thereafter hydroxyproline is released as a degradative

product. Of the total amount of hydroxyproline produced in the body by collagen degradation, 90-95% is further broken down to carbon dioxide and urea (Birkenhager, 1970), while 5-10% appears in the urine (Prockop, 1964). Urinary hydroxyproline is derived from soluble as well as insoluble collagen. Because of the unique character of hydroxyproline, which is present almost exclusively in collagen and not reutilized in the synthesis of collagen, the production of hydroxyproline has been assumed to reflect collagen catabolism (Prockop and Kivirikko, 1967).

Diabetes may affect collagen catabolism in all three stages of collagen development (Schneir et al., 1982). The catabolism of at least the first two stages which are procollagen and tropocollagen can be quantitated by measuring hydroxyproline-containing products (Bienkowski et al., 1978). In this study we used hydroxyproline as an indicator to evaluate altered collagen metabolism in diabetic rats.

## Urinary hydroxyproline excretion

Since collagen is turned over continuously, a relatively constant amount of collagenous peptides containing hydroxyproline is excreted in the urine every day (Berg and Kerr, 1992). In addition, further study found the degradation of newly synthesized collagen was very rapid (Bienkowski et al., 1978). Approximately 30% of collagen

synthesized by rabbit lung parenchyma explants was degraded within 8 minutes after [$^{14}$C]-proline was added to the incubation. This was within the time required for synthesis and less than that required for secretion (Vuust and Piez, 1970; Lane et al., 1971; Dehm and Prockop, 1972). One explanation for this rapid degradation is that it regulates the quality of proteins that are synthesized, so that abnormal molecules are recognized and destroyed (Bradley and Schimke, 1976; Schimke and Bradley, 1975; Goldberg and Dice, 1974; Goldberg and St.John, 1976). Collagen is a large protein that is subject to many post-translational modifications (Bienkowski et al., 1978), as a result, errors in collagen synthesis can be destructed quickly. This rapid collagen degradation makes it possible to use 24 or 12 hour urinary hydroxyproline excretion as an index of collagen degradation.

There is evidence that urinary hydroxyproline excretion is increased in diabetes. Mani and Mani (1986) found higher 24 hour hydroxyproline excretion in diabetic patients particularly in insulin-dependent diabetes. They reported mean hydroxyproline excretion, expressed on a creatinine basis, was 34.9 µg/mg creatinine for insulin-dependent diabetes, and 16.1 µg/mg creatinine for normals. In another study using the STZ-induced diabetic rat model (Mavrikakis et al., 1993), a significant increase in urinary hydroxyproline excretion was also found after diabetes was induced as compared to baseline concentrations. Mean 24 hour

hydroxyproline concentrations before diabetes induction was 33.5±1.6 μg/mg creatinine, and from the 4th to 87th day after induction, the values were increased from 83.0±4.9 μg/mg creatinine to 115.0±8.2 μg/mg creatinine. This increase may be due to an acceleration in collagen breakdown rate in diabetes.

Bone hydroxyproline and calcium concentration

Because more than 50% of total collagen store is present in bone tissue (Klein and Curtiss, 1964) and bone collagen has on the average a higher turnover rate than the collagen elsewhere in the body, such as skin and tendon, the skeleton is the source of about 70-80% of the urinary hydroxyproline (Birkenhager, 1970). Additionally, because collagen mass decreases in diabetes, hydroxyproline concentration, which is considered as an index of collagen mass, also is thought to decrease in the bones of diabetics. Ishida and coworkers (1988) and Takeshita and colleagues (1993) reported that hydroxyproline per femur was significantly reduced in STZ-induced diabetic rats. Values obtained in the first study were 10.5±0.3 mg/femur in diabetic rats compared to 14.4±0.3 mg/femur in controls. In addition, Locatto et al.(1990) found femur hydroxyproline concentration was directly correlated to plasma insulin concentration, since insulin insufficiency was observed to cause decreased bone hydroxyproline concentration. Therefore, in this study we

measured bone hydroxyproline to evaluate abnormal collagen metabolism in GDM.

Bone calcium content can reflect the influence of GDM on bone composition. Studies found a significant reduction of bone calcium mass in STZ-induced diabetic rats (Verhaeghe et al., 1990). Calcium was decreased by 22% and 17% in the tibia and femur, and by 24% in the four lumbar vertebrae. Bone strength was also decreased in diabetic rats. This is probably because of the effect of insulin insufficiency on bone mass in diabetes. Insulin is important for bone cell function, since insulin receptors were found to be present in osteoblastic cells (Levy et al., 1986). Also, insulin promoted growth in an osteoblast-like cell line (Hickman and McElduff, 1989). Thus insulin insufficiency in diabetes resulted in decreased osteoblast number. This osteoblast dysfunction remained evident and decreased bone mineral apposition and bone strength (Verhaeghe et al., 1990).

Another suggestion is that there is a deficiency of insulin-like growth factor-I (IGF-I) in diabetes. IGF-I stimulates osteoblast replication and bone matrix synthesis (Canalis, 1980; Hock et al., 1988). Receptors for IGF-I were also found in osteoblasts (Bennett et al., 1984). The deficiency of this growth factor in diabetes was considered partly responsible for decreased osteoblast recruitment and function, and may thereby contribute to reduced bone mass, bone mineral apposition, and bone strength in diabetes (Verhaeghe et al., 1990).

As a result, altered collagen metabolism in GDM can be assessed by measuring urinary and bone hydroxyproline concentrations to evaluate the effect of chromium depletion on GDM. In this study, we used STZ-induced diabetic pregnant rats as an animal model of GDM, which could develop chronic connective tissue defects similar to those in human GDM.

# CHAPTER III

# MATERIALS AND METHODS

## Animals

Forty-eight weanling female Sprague Dawley rats were used in this study. The study was a 2x2 factorial design with STZ and dietary chromium as variables (see appendix A). Initially, all rats were maintained on low chromium diet (see appendix B) before they became pregnant. Rats were weighed weekly until they were between 200 to 250 g and were between 56-72 days old. Then each female rat was housed with a male rat. Vaginal plugs were checked every day after breeding, as a pregnancy indicator. If no vaginal plug was observed on the fourth day, we assumed that the rat was pregnant. On the day when a vaginal plug was observed or on the fourth day, the rat was treated with STZ or placebo, and the +Cr or -Cr diet was started. STZ was injected intravenously to the rats in STZ treatment groups via the tail vein. The dose of STZ was 35 mg/kg body weight for the first five rats in each group. Afterwards, it was changed to 25 mg/kg body weight due to an observed tendency for abortion to occur in pregnant rats treated with 35 mg STZ/kg

body weight. Because urine collection began after the STZ dose was changed, we considered the urinary hydroxyproline concentration before breeding would not be altered by the different STZ dosage. Citrate buffer was injected to the rats in placebo groups.

Twenty days later, the animals were necropsied. Animals were not fasted before autopsy. Blood samples were collected by cardiac puncture, placed on ice, and centrifuged. Plasma was separated immediately and stored at -20°C until assayed. Blood glucose was measured by the glucose oxidase method and insulin was determined by radioimmunoassay. Insulin and glucose assays were performed by Maria Spicer.

Because urine collection began about one month after the beginning of the experiment, some of the rats had already been bred or necropsied. Fifteen animals were available for analyzing urine hydroxyproline before mating and 31 after STZ or placebo treatment. Forty animals were available for bone and blood analysis. Non-pregnant rats and those who delivered or died before necropsy were eliminated from this study.

## Determination of urinary hydroxyproline concentration

Urine samples were collected on the day before mating, and 17 days after STZ or placebo (-STZ) treatment. Rats were kept in metabolic cages designed for separation of urine and feces at room temperature from 9 PM to 9 AM. They were on a

12 hour light and dark cycle from 7 AM to 7 PM. Animals were fed water and food during the 12 hour collection period. Urine was collected in a container which was kept cool in an ice holder. Urine samples were stored in polypropylene tubes at -20°C until hydroxyproline was assayed about three months later.

Urinary hydroxyproline concentration was determined using the Bergman and Loxley method (1970). This method has been confirmed successful to measure hydroxyproline in urine hydrolysates in spite of the presence of interfering materials, because internal standards are used (see appendix C). After urine was hydrolyzed, a lithium hydroxide solution was used to neutralize the hydrolysates in order to eliminate possible salt precipitation. Then oxidant solution and Ehrlich's reagent were added. Oxidant solution was added after Ehrlich's reagent in the blank. Chloramine-T in the oxidant solution was inactivated by the perchloric acid which was in the Ehrlich's reagent, thus any color development was due to materials other than hydroxyproline. As a result, a desirable blank for urine could be obtained. The absorbance was measured at 562 nm in a 1x1 cm cuvette.

## Determination of bone hydroxyproline concentration

Bone samples were collected on the day rats were necropsied. Femur and tibia were dissected from adhering connective tissue and muscle, and then stored at -20°C in

plastic bags for about five months. On the day of analyses, the length and weight of the right femur bones were measured. Then a 2 mm thick cross-section was cut from the mid-diaphysis of the bones and weighed (Shaw et al., 1987). These cross-sections were broken into small pieces and placed into polypropylene tubes with 0.5 ml of 6 M hydrochloric acid added. The tubes were then sealed and put in an oven at 105°C for 24 hours. After acid hydrolysis, they were cooled to room temperature and dried in a vacuum. Acid residues were redissolved in distilled water for hydroxyproline assay. The Bergman and Loxley method (1970) was also used for bone hydroxyproline determination.

## Determination of bone calcium concentration

Bone calcium concentration was measured using the method developed by Hill et al.(1986), which includes a combination of wet and dry ashing. Tibial bones were used for this assay. After bone wet weights were measured, the bones were dried for 48 hours at 100°C to obtain dry weights. Bones were ashed in a muffle furnace at 375°C for 48 hours. After cooling, deionized water, nitric acid (concentrated, double distilled, GFS Chemicals, Inc. Columbus, OH), and Ultrex hydrogen peroxide (30%, ultrapure, Baker, Inc. Phillipsburg, NJ) were added to each tube in a heating block. Hydrogen peroxide was added every two hours, four times each day. After several days of wet ashing, samples were again dry

ashed and wet ashed several times until all black carbon particles were digested and bone powder became completely white. Concentrated nitric acid and deionized water were added again to dissolve the bone powder. Samples were vortexed every hour until all residue was dissolved. Then 0.1% lanthenum chloride in 0.5% nitric acid was added to prevent the interference of some elements such as aluminum, beryllium, phosphorus, silicon, titanium, and zirconium, which can give rise to stable oxysalts, and thereby reduce calcium sensitivity. Bone calcium concentration was determined by flame atomic absorption spectrometry at 422.7 nm.

## Statistical analysis

All data were expressed as means ± SEM. Statistical analysis of the data was performed on SAS using analysis of variance and correlation analysis. Log transformations were performed on plasma insulin and glucose to correct for non-homogeneity of variance. Results were considered significant at $p \leq 0.05$.

# CHAPTER IV

## RESULTS AND DISCUSSION

### The effect of chromium and diabetic state on urinary hydroxyproline excretion in pregnant rats

Urine volume and urinary hydroxyproline before and after treatment were available from 15 rats. The total number of urine samples obtained after treatment was 31. Plasma insulin, glucose, bone hydroxyproline and calcium concentrations were available from 40 rats.

On the day before breeding, there were no differences in urine volume (Table 1) among groups. In addition, there were no significant differences in the urinary hydroxyproline excretion per unit body weight among the groups. Because these data were obtained before any treatment was administrated, the results were desired to avoid experimental error.

No significant differences were observed between urine volume measured the day before breeding and 17 days after treatment within the same rats among groups (Table 1). This was probably due to the small sample size, as there were only three to five rats which had paired urine samples in

TABLE I

URINARY HYDROXYPROLINE EXCRETION AND URINE VOLUME IN PREGNANT RATS WITH STZ OR PLACEBO TREATMENT AND WITH CHROMIUM ADEQUATE OR DEPLETION DIETS†

| Trt | N | Urine Vol (ml/12hr) | | | Hyp (µg/g body Wt) | | |
|---|---|---|---|---|---|---|---|
| | | I[1] | II[2] | Δ[3] | I[1] | II[2] | Δ[3] |
| -STZ-CR | 3 | 8±1 | 22±17 | 13±17 | 0.44±0.04 | 0.49±0.09 | 0.06±0.06 |
| -STZ+CR | 5 | 7±1 | 11±14 | 3±14 | 0.43±0.03 | 0.37±0.08 | -0.06±0.06 |
| +STZ-CR | 3 | 7±1 | 20±17 | 14±17 | 0.45±0.04 | 0.96±0.11 | 0.47±0.08 |
| +STZ+CR | 4 | 8±1 | 44±15 | 36±15 | 0.47±0.04 | 0.82±0.08 | 0.35±0.06 |
| | | | | P Values | | | |
| STZ | | 0.71 | 0.34 | 0.33 | 0.52 | 0.0005 | 0.0001 |
| CR | | 0.80 | 0.69 | 0.71 | 0.82 | 0.16 | 0.11 |
| STZ*CR | | 0.36 | 0.30 | 0.34 | 0.78 | 0.94 | 0.99 |

† Values are means ± SEM.
[1] Data were obtained on the day before breeding.
[2] Data were obtained 17 days after treatment.
[3] Values are the differences between I and II.

each treatment group.

Within the same animals, differences between urinary hydroxyproline excretion per unit body weight before and after streptozotocin (STZ) or placebo treatment were greater in GDM rats than control rats ($p=0.0001$; Table 1). This finding indicated that after the induction of diabetes by STZ, hydroxyproline excretion in urine was significantly increased compared with that before STZ administration. However, differences in hydroxyproline concentrations before and after STZ or placebo treatment were not significantly affected ($p=0.11$) by dietary chromium.

Twenty days after treatment, plasma insulin concentration decreased in gestational diabetes mellitus (GDM) rats compared to control rats ($p=0.001$; Table 2), but no difference was observed between the chromium adequate (+Cr) diet groups and chromium depletion (-Cr) diet groups. Moreover, plasma glucose concentrations measured on the same day increased in GDM rats compared to control rats ($p=0.003$), but differences between +Cr and -Cr diet groups were not significant. These observations indicated strong evidence of the induction of diabetes in pregnant rats after STZ treatment. However, an effect of chromium depletion was not found.

Urine volume measured 17 days after treatment was increased in GDM rats compared to control rats ($p=0.03$; Table 2). This finding could be explained by the physiological changes and clinical symptoms observed in

TABLE II

URINARY HYDROXYPROLINE CONCENTRATION AND PLASMA INSULIN AND GLUCOSE IN PREGNANT RATS WITH STZ OR PLACEBO TREATMENT AND WITH CHROMIUM ADEQUATE OR DEPLETION DIETS[1]

| Trt | Urine Vol[2] (ml/12hr) N=31 | Hyp[2] (µg/g body Wt) N=31 | Insulin[3] (ng/ml) N=40 | Glucose[3] (mg/dl) N=40 |
|---|---|---|---|---|
| -STZ-CR | 19±10 | 0.46±0.07 | 4.32±0.77 | 341.8±59.0 |
| -STZ+CR | 10± 9 | 0.38±0.07 | 2.19±0.73 | 292.0±56.0 |
| +STZ-CR | 34±12 | 1.22±0.10 | 0.70±0.73 | 471.2±56.0 |
| +STZ+CR | 43±11 | 0.88±0.07 | 0.84±0.77 | 519.6±59.0 |
| | | P Values | | |
| STZ | 0.03 | 0.0001 | 0.0001 | 0.003 |
| CR | 0.99 | 0.01 | 0.32 | 0.56 |
| STZ*CR | 0.38 | 0.12 | 0.33 | 0.46 |

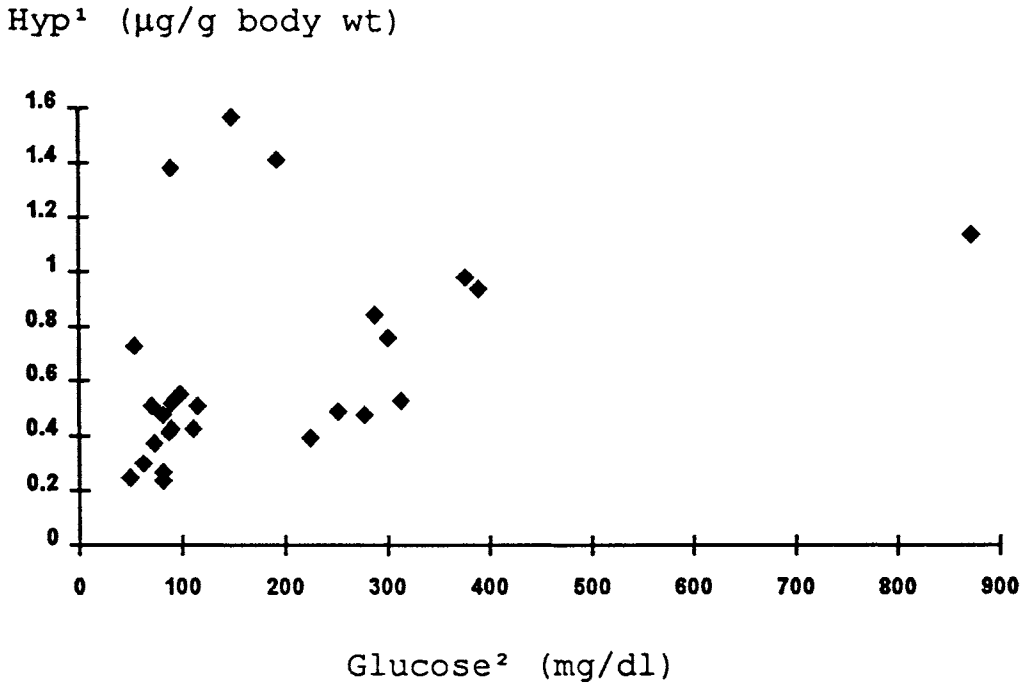
[1] Values are means ± SEM.
[2] Data were obtained 17 days after treatment.
[3] Data were obtained 20 days after treatment.

diabetes. However, an effect of chromium on urine volume was not observed.

Urinary hydroxyproline excretion per unit body weight after STZ or placebo treatment was increased in GDM rats compared to placebo-treated pregnant rats ($p=0.0001$; Table 2). In addition, there was an increase in urinary hydroxyproline excretion in the rats fed a chromium depletion diet compared with those fed a chromium adequate diet ($p=0.01$). The urinary hydroxyproline concentration was increased from 0.88±0.07 µg/12hr/g body weight to 1.22±0.10 µg/12hr/g body weight in GDM rats fed the chromium depletion diet, and it was increased from 0.38±0.07 µg/12hr/g body weight to 0.46±0.07 µg/12hr/g body weight in control rats fed the chromium depletion diet.

Urinary hydroxyproline concentration measured 17 days after treatment was positively correlated with the glucose concentration measured observed 20 days after treatment in pregnant rats ($r=0.66$; Figure 1). This also indicated that diabetes was related to increased urinary hydroxyproline excretion in pregnant rats.

Hydroxyproline is a good indicator of collagen breakdown and is thereby considered to reflect the connective tissue alterations in diabetes state. In this study, 12 hour urinary hydroxyproline excretion per unit body weight was significantly higher in the pregnant rats after STZ treatment compared to placebo treatment. This is probably because of increased collagen breakdown in STZ-induced




¹ Values were obtained 17 days after treatment.
² Values were obtained 20 days after treatment.

Figure 1. Correlation between Urinary Hydroxyproline and Plasma Glucose in Pregnant Rats

diabetic pregnant rats. In diabetes, collagen tends to be less cross-linked and more susceptible to collagenase digestion because high glucose environment can inhibit fibril formation and subsequent collagen cross-linking. Thereafter, urinary hydroxyproline excretion was increased as a product of collagen breakdown (Mavrikakis et al., 1993). Mavrikakis and coworkers reported 24 hour urinary hydroxyproline concentration in rats was 33.5±1.59 μg/mg creatinine before STZ treatment, and increased from 83.0±4.9 μg/mg creatinine to 115.0±8.2 μg/mg creatinine from the 4th day to 87th day after the induction of diabetes by STZ. Our findings were in agreement with their report. The correlation between urinary hydroxyproline concentrations and plasma glucose concentration observed in this study also supports this hypothesis.

A function of chromium is maintaining normal glucose tolerance by increasing insulin efficiency; thus, chromium deficiency is considered a possible etiological factor of GDM. Dietary chromium supplementation may have beneficial effects on GDM. We assumed urinary hydroxyproline excretion would be increased in the rats fed a chromium depletion diet. In this study, urinary hydroxyproline excretion per unit body weight was increased in GDM rats and control rats fed a chromium depletion diet compared with those fed a chromium adequate diet. One explanation is that chromium depletion leads to glucose intolerance, and collagen synthesized in a high glucose environment tends to be more

easily catabolized, resulting in increased urinary hydroxyproline excretion. Dietary chromium supplementation may alleviate some symptoms associated with decreased collagen quality in GDM.

However, the difference in hydroxyproline concentration before and after treatment between +Cr diet groups and -Cr diet groups was not significant. This may be due to our small sample size, as the number of the rats who had paired urine samples was only three to five in each group.

We also found a significant difference in urinary hydroxyproline concentration between GDM rats fed an adequate chromium diet and placebo-treated rats fed a chromium depletion diet ($p=0.002$). This indicated that chromium depletion in pregnant rats did not develop as severe diabetes as that in GDM rats fed a chromium adequate diet.

## The effect of chromium and diabetic state on bone hydroxyproline and calcium concentration in pregnant rats

Body weight obtained on the day of autopsy was not significantly different between GDM rats and control rats (Table 3), nor between the rats fed chromium adequate or chromium depletion diets. When weight gain from the day before breeding to the day of autopsy was compared, GDM rats gained less weight than control rats ($p=0.02$). A significant

TABLE III

BODY WEIGHT AND BONE LENGTH AND WEIGHT IN PREGNANT RATS WITH STZ OR PLACEBO TREATMENT AND WITH CHROMIUM ADEQUATE OR DEPLETION DIETS†

| Trt | N | Body Wt (g) | | | Femur Length (mm) | Femur Wt wet(g) |
|---|---|---|---|---|---|---|
| | | I[1] | II[2] | Δ[3] | | |
| -STZ-CR | 10 | 271±17 | 382±22 | 111±11 | 39.3±0.6 | 0.63±0.03 |
| -STZ+CR | 10 | 253±17 | 343±22 | 90±11 | 39.7±0.6 | 0.58±0.03 |
| +STZ-CR | 10 | 279±17 | 338±27* | 80±13 | 39.9±0.7 | 0.60±0.04 |
| +STZ+CR | 10 | 267±17 | 313±25* | 61±12 | 39.8±0.6 | 0.62±0.03 |
| | | | P Values | | | |
| STZ | | 0.52 | 0.14 | 0.02 | 0.53 | 0.88 |
| CR | | 0.38 | 0.18 | 0.09 | 0.86 | 0.64 |
| STZ*CR | | 0.84 | 0.76 | 0.92 | 0.74 | 0.42 |

† Values are means ± SEM.
[1] Data were obtained on the day before breeding.
[2] Data were obtained 20 days after treatment.
[3] Values are the differences between I and II.
* Two or three rats' data were missing.

effect on weight gain due to chromium depletion was not observed ($p=0.09$).

The weight and length of femoral bones were not different between STZ and placebo treatment groups (Table 3). Chromium depletion also had no effect on bone weight or length.

Bone hydroxyproline concentration per milligram of femur was decreased in GDM rats compared to control rats ($p=0.02$; Table 4), but no difference was found between +Cr and -Cr diet groups. Total hydroxyproline per femur was not affected by any treatment.

Calcium concentration of tibial bones was not different among groups (Table 4). Likewise, the moisture of tibial bones in GDM rats and control rats was not different, nor did chromium status affect tibial moisture.

As shown in Figue 2, bone hydroxyproline concentration (μg/mg femur) was positively correlated with plasma insulin concentration measured 20 days after treatment ($r=0.55$). This indicates an adverse effect of diabetes on bone hydroxyproline concentration in pregnant rats.

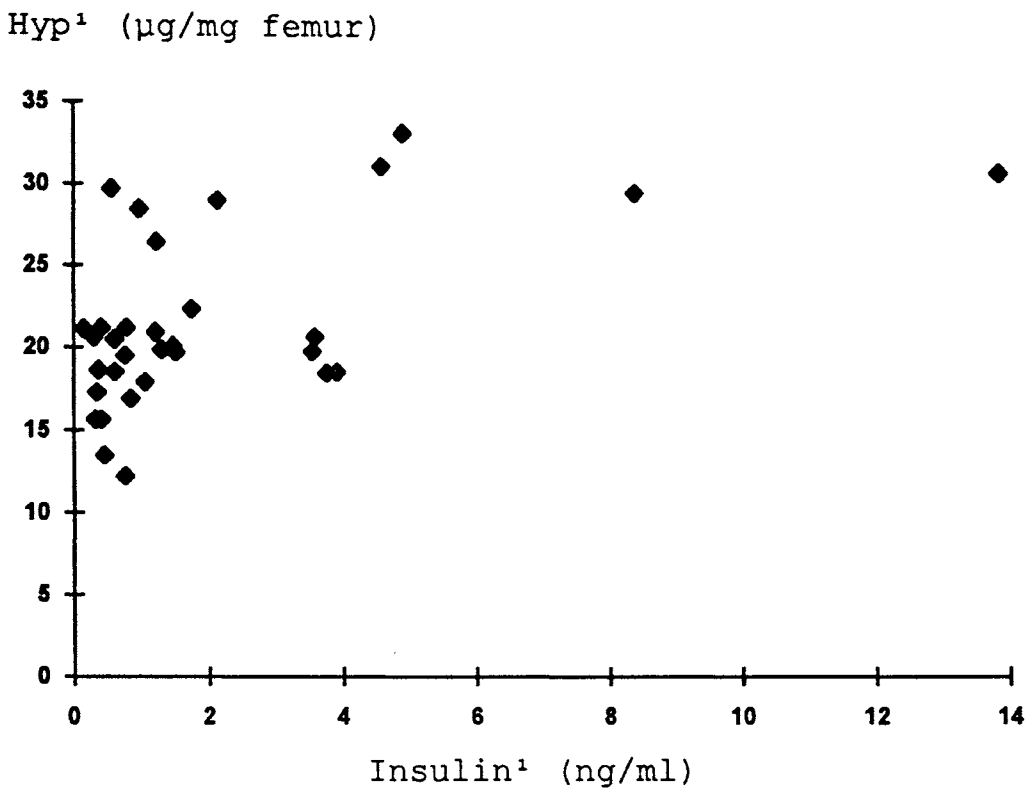
Our finding agreed with the study by Ishida and colleagues (1988). They found bone hydroxyproline per femur was 10.5±0.3 mg in diabetic rats and 14.4±0.3 mg in control rats. Locatto and coworkers (1992) also concluded that insulin deficiency in diabetes was correlated to the decrease of bone hydroxyproline content in alloxan-treated diabetic rats.

TABLE IV

BONE HYDROXYPROLINE AND CALCIUM CONCENTRATIONS IN PREGNANT RATS WITH STZ OR PLACEBO TREATMENT AND WITH CHROMIUM ADEQUATE OR DEPLETION DIETS[1]

| Trt | N | Hyp[2] | | Moisture[3] | Ca[3] |
|---|---|---|---|---|---|
| | | µg/mg femur | mg/femur | % | mg/g |
| -STZ-CR | 10 | 24.32±1.70 | 14.67±1.48 | 28.59±0.86 | 230.5±1.9 |
| -STZ+CR | 10 | 23.33±1.70 | 13.52±1.40 | 27.32±0.86 | 230.7±1.9 |
| +STZ-CR | 10 | 19.58±1.80 | 12.44±1.71 | 28.58±0.86 | 231.4±1.9 |
| +STZ+CR | 10 | 19.19±1.70 | 11.76±1.48 | 27.72±0.86 | 228.6±1.9 |
| | | P Values | | | |
| STZ | | 0.02 | 0.20 | 0.82 | 0.75 |
| CR | | 0.69 | 0.55 | 0.23 | 0.50 |
| STZ*CR | | 0.86 | 0.88 | 0.81 | 0.44 |

[1] Values are means ± SEM.
[2] Femoral bone.
[3] Tibial bone.




[1] Values were obtained 20 days after treatment.

Figure 2. Correlation between Bone Hydroxyproline and Plasma Insulin in Pregnant Rats

Bone hydroxyproline concentration per milligram of femur was decreased in GDM rats compared to control rats. As discussed, collagen mass is reduced in diabetes because of enhanced collagen degradation or decreased collagen synthesis, thus hydroxyproline concentration in collagen is reduced consequently. Because bone contains more than 50% of total body collagen stores, bone hydroxyproline is considered to be decreased in diabetes (Locatto et al., 1992). The positive correlation between bone hydroxyproline concentration and plasma insulin concentration in this study supports this hypothesis. However, bone calcium concentration was not affected by diabetes in this study.

Chromium depletion can lead to glucose intolerance and is speculated to be a possible etiological factor of GDM (Aharoni et al., 1992). When comparing bone hydroxyproline concentrations in GDM and control rats with chromium adequate or depletion diets, we did not find any difference due to dietary chromium. Bone calcium concentration was not affected by chromium depletion either. This may be because of the small sample size, the short duration of this study (20 days), or experimental error. Nevertheless, the effect of chromium depletion on bone hydroxyproline or calcium concentration in GDM can not be clarified in this study.

# CHAPTER V

# SUMMARY, HYPOTHESIS TESTING AND RECOMMENDATIONS

## Summary

The purpose of this study was to evaluate the effect of chromium depletion on collagen metabolism in gestational diabetes mellitus (GDM) by measuring bone and urinary hydroxyproline concentration in streptozotocin (STZ)-induced diabetic pregnant rats.

Chromium functions to maintain normal glucose tolerance by increasing insulin efficiency. In chromium deficiency, glucose tolerance is impaired, increasing the risk of developing diabetes. Therefore, chromium depletion is speculated to be a possible etiological factor in the development of GDM.

Because diabetes is associated with generalized defects in connective tissue metabolism, and collagen is the major protein component of connective tissues, collagen metabolism may be altered in diabetes (Bornstein and Traub, 1979). Hydroxyproline is a degradative product of collagen and is a good indicator of collagen breakdown. In this study, urinary excretion of hydroxyproline was measured as an indicator of

collagen degradation in GDM. Furthermore, bone hydroxyproline and bone calcium concentrations reflect reduced collagen mass in diabetes. These parameters were also used as indicators to evaluate the effect of chromium depletion on GDM.

Plasma insulin was significantly decreased in GDM rats compared to control rats, while plasma glucose was significantly increased in GDM rats compared to control rats. However, chromium depletion did not affect plasma insulin or glucose concentrations in GDM or control rats.

Urinary excretion of hydroxyproline per unit body weight was increased in GDM rats compared with placebo-treated pregnant rats. In addition, hydroxyproline concentration was increased in rats fed a chromium depletion diet compared with those fed a chromium adequate diet in GDM and control rats.

Bone hydroxyproline concentration per milligram of femur was decreased in GDM rats compared to control rats. No difference in bone hydroxyproline concentration between +Cr and -Cr diet groups was observed in this study. Bone calcium concentration was not different among STZ and placebo treatment groups or +CR and -CR diet groups.

Urinary hydroxyproline concentrations measured 17 days after treatment were positively correlated with the plasma glucose concentrations measured 20 days after treatment in the pregnant rats. A positive correlation was also found between bone hydroxyproline concentrations and plasma

insulin concentrations measured 20 days after treatment in the pregnant rats. These findings indicated that diabetic state was related to increased urinary hydroxyproline concentration, and had an adverse effect on the bone hydroxyproline concentration in pregnant rats.

Overall, we found a significant increase in urinary hydroxyproline excretion and a decrease in bone hydroxyproline concentration in STZ-induced diabetic pregnant rats. This is probably because of the reduced bone collagen mass as well as the increased collagen breakdown in GDM rats. Urinary hydroxyproline excretion was elevated as a result of increased collagen breakdown.

Moreover, we found a significant increase in urinary hydroxyproline concentration in rats fed a chromium depletion diet. Because chromium functions to maintain normal glucose tolerance, chromium depletion may lead to glucose intolerance and increased collagen breakdown in pregnant rats. However, no difference was found in the bone hydroxyproline concentrations with chromium depletion or adequate diet. Bone calcium concentration was not affected either. This may be due to the limited sample size or to the short duration of this experiment.

## Hypothesis testing

Based on the data obtained, our hypotheses were tested:

1. Urinary hydroxyproline concentrations were increased in STZ-induced diabetic pregnant rats compared to control rats with chromium adequate or depletion diets. Chromium depletion increased urinary hydroxyproline concentrations in pregnant rats. As a result, hypothesis one was rejected.

2. Bone hydroxyproline concentrations were decreased in STZ-induced diabetic pregnant rats compared to control rats with chromium adequate or depletion diet, but chromium depletion did not have an effect on bone hydroxyproline concentrations. As a result, hypothesis two was partially rejected.

3. Bone calcium concentration was not affected by diabetes or chromium depletion in pregnant rats. As a result, hypothesis three was not rejected.

## Recommendations

To confirm the effect of chromium depletion on the altered hydroxyproline concentration and abnormal collagen metabolism in GDM, further studies are suggested. In our study, the sample size in each treatment group was not large, especially for urine analyses. Because we started collecting urine samples one month after the begining of the experiment, some of the rats were no longer available, especially for initial urine collection. In addition, the duration of diabetes induced by STZ in this experiment may not be long enough to reflect the effect of chromium

depletion on bone hydroxyproline concentrations. Future study in this area with a larger sample size is highly recommended.

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# APPENDIX A

# APPENDIX A

## THE RATS[1] IN STZ OR PLACEBO TREATMENT GROUPS WITH CHROMIUM ADEQUATE OR DEPLETION DIETS

| | -STZ | +STZ |
|---|---|---|
| -CR | 10 | 10 |
| +CR | 10 | 10 |

[1] The numbers of rats were with the elimination of those who were not pregnant, delivered or died before autopsy.

# APPENDIX B

# APPENDIX B

## FORMULATION OF LOW CHROMIUM DIET

| Component | g/kg body weight |
|---|---|
| Cornstarch | 397.5 |
| Casein | 200.0 |
| Dextrose | 132 |
| Sucrose | 100.0 |
| Celufil | 50.0 |
| Vegetable oil | 70.0 |
| Mineral mix, AIN-76 | 35.0 |
| Vitamin mix, AIN-76A | 10.0 |
| L-cystine | 3.0 |
| Choline bitartrate | 2.5 |

# APPENDIX C

# APPENDIX C

## THE DETERMINATION OF HYDROXYPROLINE IN URINE

### Reagents

#### Oxidant solution

Buffer - 57 g of sodium acetate, 37.5 g of trisodium citrate, 5.5 g of citric acid and 385 ml of isopropanol are made up to 1 litre with distilled water. It is stable indefinitely.

7% aqueous chloramine-T - 0.7 g chloramine-T are added with distilled water to 10 ml solution. This is made up daily.

Mix one part of 7% aqueous chloramine-T and four parts of buffer to oxidant solution just before each set of determination.

#### Ehrlich's reagent

17.6 g of p-dimethylaminobenzaldehyde are dissolved in 40.8 g of 60% perchloric acid on the day of use and are made up to 100 ml with isopropanol just before use.

Standard solutions

One mg of hydroxyproline is mixed with 100 ml isopropanol to solution. Then it is diluted to different concentrations.

Standard I - 0.01 µg/ml

Standard II - 0.5 µg/ml

Standard III - 1.0 µg/ml

## Procedures

1. On the first day, mix 2.5 ml of urine and 2.5 ml of 12 N hydrochloric acid in the marked polypropylene tubes.
2. Put two tubes per beaker in an oven. Heat overnight at 100-105°C (at least 16 hours).
3. On the next day, preheat waterbath first. Then turn off oven and let the tubes cool down to about 40°C.
4. Weigh out 0.5 g amount of lithium hydroxide for each tube.
5. Put one drop phenolphthalien solution and add weighed lithium hydroxide to each sample. Cover and mix until solids are in solution.
6. If solution is still clear, add saturated lithium hydroxide drop by drop while shaking tube. Stop when color starts to appear. Then add dilute hydrochloric acid so that solution is clear again.
7. Add distilled water to solution to make it 7.5 ml.

Centrifuge for 10 minutes at medium speed.

8. Transfer 0.5 ml of the neutralized hydrolysates into 6 test tubes, which are marked as a, b (for duplicate samples), 1, 2, 3 (for standards), B (for blank).
9. Add 1 ml isopropanol to tube a, b, and B.
10. Add 1 ml standard I to tube 1, 1 ml standard II to tube 2, and 1 ml standard III to tube 3.
11. Add 0.5 ml oxidant solution to tube a, b, 1, 2, 3, but not to blank. Vortex for 3 seconds each, then wait for 4 minutes (±1 minute).
12. Add 1 ml Ehrlich's reagent to all tubes (a, b, 1, 2, 3, and B).
13. Add 0.5 ml oxidant solution to blank. Vortex for 10 seconds each.
14. Set the tubes in 60°C waterbath for 21 minutes. Then let them sit for one hour at room temprature.
15. Warm up spectrophotometry 15 minutes before the end of the hour, set at 562 nm.
16. Read all tubes (a, b, 1, 2, 3, and B) at one time.

# VITA

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Master of Science

Thesis: THE EFFECT OF CHROMIUM DEPLETION ON BONE AND URINARY HYDROXYPROLINE CONCENTRATION IN STREPTOZOTOCIN INDUCED GESTATIONAL DIABETIC RATS

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