DISPROVING A FIFTY-FIVE YEAR OLD MYTH: CHROMIUM THE ESSENTIAL ELEMENT

by

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ABSTRACT

Over fifty years ago chromium was proposed to have an essential biochemical role in the metabolism of carbohydrates and lipids, and subsequently its status as an essential element was widely accepted. Unfortunately, these studies confused the pharmacological effects of large, supra-nutritional doses of Cr for nutritional effects. Recent research has firmly established that chromium is not an essential or conditionally essential element for mammals but has effects on insulin sensitivity and cholesterol levels only at pharmacologically relevant doses. However, the mechanism of these effects in rodent models of obesity-associated insulin resistance and type 1 and type 2 diabetes at a molecular level has not been elucidated, although a direct effect on insulin signaling cascade is suggested by current data. This research addresses recent studies that demonstrated that chromium is not essential but pharmacologically active through the use of purified rat diets with varying concentrations of chromium. Urinary chromium excretion in response to an insulin challenge is not a biomarker for Cr status, the effect Cr supplementation on tissue metal concentrations and that Cr is not a conditionally essential element for diabetics as increased Cr urinary excretion in diabetics reflects an increase in absorption using Cr^{51} tracer studies.

DEDICATION

To my dad, W. Thomas Love for supporting me

To my mom Aileen Love for loving me

To my sisters Tabia, Freddie, Nishia, and Jamila for listening to me

To my brothers Deon, Tay, JT, and Bakari for protecting me

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Living forever in my heart

LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
x	Times
ºC	Degree Celsius
±	plus or minus
Ag	Silver
AI	Adequate Intake
ANOVA	Analysis of Variance
As	Arsenic
Au	Gold
AUC	Area under the curve
B	Boron
Ba	Barium
Be	Beryllium
Bi	Bismuth
Br	Bromine
Cd	Cadmium
Ce	Cerium
Ci	Curie
Cl	Chloride
cm	Centimeter

Co	Cobalt
CO_2	Carbon dioxide
Cr	Chromium
Cr(III)	Chromium ion in a compound
Cr3	$Cr_3O(propionate)_6(H_2O)_3]^+$
Cr^{3+}	Trivalent Cr ion
$CrCl_3$	Chromium Chloride
$^{51}CrCl_3$	Chromium 51 labelled Chromium Chloride
Cr-Transferrin	Transferrin with Cr bound
Cs	Cesium
Cu	Copper
F	Fluorine
Fe	Iron
g	gram
Ge	Germanium
GTF	Glucose Tolerance Factor
h	Hour
Hg	Mercury
H_2O_2	Hydrogen Peroxide
I	Insulin
I	Iodine
Ir	Iridium
^{125}IRIA	Iodine 125 labelled Radio immuno assay

kg	kilogram
L	Liter
La	Lanthanum
Li	Lithium
LMWCr	Low Molecular Weight Chromium binding substance
LSD	Least Significant Difference
mg	milligram
mL	Milliliter
mM	Millimolar
mm	Millimeter
Mn	Manganese
Mo	Molybdinum
Ni	Nickel
NIST	National Institute of Standards and Technology
nm	Nanometer
no.	Number
Os	Osmium
Pd	Palladium
pic	Picolinate
ppm	parts per million
r^2	coefficient of determination
Rb	Rubidium
Rh	Rhodium

Ru	Ruthenium
s	second
Sb	Antimony
Se	Selenium
SEM	Standard error of the mean
Sn	Tin
Sr	Strontium
STZ	Streptozotocin
Ta	Tantalum
Th	Thorium
$T_{1/2}$	The time it takes half of substance clears an exchange pool
Tf	Transferrin
Ti	Titanium
Tl	Thallium
TPN	Total parenteral nutrition
U	Uranium
V	Vanadium
W	Tungsten
Y	Yttrium
ZDF	Zucker diabetic fatty rat
Zn	Zinc
Zr	Zirconium
µg	microgram

µL	microliter
µM	Micromolar

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Chapter 1: Introduction: Disproving the myth of Cr essentiality

1.1 Glucose Tolerance Factor and the flawed assumptions that led to its beginnings

Over 50 years ago, the field of chromium biochemistry began with an experiment by Mertz and Schwarz. An experiment on rats fed *Torula* yeast-based diets, thought to be deficient in chromium, lead to apparently impaired glucose tolerance in response to an intravenous glucose load and to the rats developing necrotic liver degeneration.[1] Selenium was discovered to reverse the liver disorder (leading to the demonstration that selenium was an essential trace element in the mammalian diet) but not the glucose intolerance; thus, the authors proposed a new dietary requirement was absent from the *Torula* yeast-based diet and responsible for the glucose intolerance.[2] Li, Be, B, F, Si, Ti, V, Mn, Co, Ni, Cu, Zn, Ge, As, Se, Br, Rb, Rh, Pd, Ag, Cd, Sn, Sb, I, Cs, Ba, La, Ce, Ta, W, Os, Ir, Au, Hg, Tl, Bi, Th, and U were screened for glucose tolerance factor activity. The experiments were carried out by repeatedly testing mixtures of 5-10 compounds and assaying subgroups of animals. The dose was 20-50 μg per 100 g of body mass. Upon supplementation of the diets with chromium(III) salts, the diabetes-like symptoms appeared to be reversed.[3] This lead to the coining of "Glucose Tolerance Factor (GTF)," the dietary factor proposed to be essential for proper glucose uptake and missing in the *Torula* yeast diet.

Brewer's yeast and acid-hydrolyzed porcine kidney powder were identified as natural sources of the missing dietary component and were found to contain appreciable quantities of chromium.[3] When given by stomach tube (500-1000 μg/kg body mass), the Brewer's yeast and

porcine kidney powder and concentrates made from them restored proper glucose metabolism in rats on the *Torula* yeast-based diet.[3] The researchers thus proposed the active ingredient of GTF was Cr^{3+}, making chromium an essential trace element.[3]

The diets used in this experiment were not assayed for chromium content; thus, the rats were not shown to actually receive a diet lacking in chromium – only that adding chromium to the diet could lead to potential effects on apparent glucose intolerance. Subsequently, the Cr contents of *Torula* yeasts have been determined but range significantly in value;[4,5] the content probably varies based on the growth conditions. The content of the original diet simply cannot be established. Therefore, the actual Cr intake of the rats in these studies is impossible to gauge; thus, no deficiency was established. Additionally, the animals were housed in wire mesh cages. Rats are known to gnaw on cage components, and by housing the animals in wire mesh cages the animals were potentially exposed to stainless steel, which is between ~10-40% chromium (the metal composition of the wire was not reported.)

The belief that a deficiency in the Cr containing glucose tolerance factor would lead to diabetes like symptoms was born, although the experiments used to justify and isolate GTF were methodologically flawed. One can find in textbooks a figure of the proposed structure of glucose tolerance factor (GTF), the "biologically active form of chromium." This three-dimensional structure displays two *trans* N-bound nicotinic acid ligands with amino acids occupying the remaining four sites of an octahedral around a Cr^{3+} ion.[6] This stems from the report of Mertz and coworkers of the isolation of Brewer's yeast GTF in 1977.[7] (After this paper appeared, the term GTF was generally used for the product of this isolation, not for Cr^{3+}.) However, a careful examination of the isolation procedures and characterization of "GTF" reveals that a naturally occurring form of chromium was neither isolated nor characterized in term of its chemical

composition. The isolation procedure involved rupturing the yeast and binding its contents on activated charcoal, removing the adhered material using a 50:50 mixture of concentrated ammonia and diethyl ether, and refluxing the product of the extraction in 5 M HCl for 18 hours.[7] Any proteins, complex carbohydrates, or nucleic acids would have been hydrolyzed. Thus, the chances that the form of Cr recovered after the treatment resembles the form in the yeast are remote, although it should be noted that the authors were primarily looking for what they believed would be a heat stable, acid stable vitamin or analogue. Nicotinic acid was apparently sublimed from the material and identified (although no data or experimental details were presented for the mass spectral, sublimation, or extraction studies).[7] Amino acid analyses indicated the presence of glycine, glutamic acid, and cysteine as well as other amino acids, although the relative amounts were not reported. The results were interpreted to indicate that GTF was a complex of Cr, nicotinate, glycine, cysteine, and glutamate.

In paper chromatography experiments, the material on which the studies above were performed, gave several chromium-containing spots, only one of which was active in bioassays.[7] Thus, the isolated species used for the composition studies was not pure. (The bioassays measured the ability of a material to activate the metabolism of glucose by adipose tissue from rats on the *Torula* yeast diet in the presence of insulin.) In fact, the Cr in the active band represented only 6% of the total Cr.[7] No data were generated on the more purified chromium-containing component; thus, even if chromium was essential so that GTF existed and was not an artifact generated during the harsh isolation conditions, this study would offer no information on its composition. This is not the end of the story as Cr^{3+} has been demonstrated repeatedly to be separable from agents in yeast responsible for *in vitro* stimulation of glucose metabolism in adipocytes.[8,9,10,11,12,13,14] Thus, the component of yeast that is active in the bioassays does not

contain Cr. Additionally, how such artifacts could potentially be formed in the other suggested source of GTF, acid-hydrolyzed porcine kidney powder, using the harsh isolation conditions has been shown.[15] In 2013, a paper was published analyzing Cr substances from high chromium yeast. The paper contained a substance that was separated using size exclusion chromatography and that led to what is probably an impure substrate called high molecular weight chromium binding substance that was tested on rat adipose cells. The experiment attempted to isolate a biologically active form of Cr, but the isolated material does not appear to be pure.[16,17,18]

A variation on the composition and structure of "GTF" has been proposed. GTF has been suggested to be a Cr(III)-glutathione-nicotinate complex as glutathione is a tripeptide of glutamate, glycine, and cysteine. Although synthetic complexes made from the combination of Cr^{3+}, nicotinate, and glutathione have been reported to have similar biological activity to Brewer's yeast "GTF" and bind tightly to insulin,[19] these suggestions are also unsupported by data on any natural system.

Despite the numerous publications refuting the GTF studies, these studies unfortunately continue to be cited as evidence of the essential role of Cr, pulling attention of researchers from new directions in Cr biochemistry. Studies like this are not helping to advance the field of Cr biochemistry.

1.2 The flawed evidence currently used to prove Cr essentiality

Many studies have attempted to prove the essentiality of chromium, and to date four lines of evidence for the essentiality of chromium have been postulated. 1) Carefully controlled studies with rats have induced apparent Cr deficiency manifested by insulin insensitivity in glucose tolerance tests.[20,21,22] 2) In humans, Cr deficiency has apparently been observed for five

patients on total parenteral nutrition (TPN), where it resulted in symptoms similar to those of type 2 diabetes, which were reversed upon Cr supplementation of the TPN.[23,24,25,26] 3) In humans, Cr absorption is inversely proportional to intake.[27] 4) Changes in plasma insulin levels result in changes in urinary Cr excretion. All four lines are suggestive and not definitive, and more evidence is needed to prove the essentiality of chromium (if it is essential).

Rats were provided a high sugar or high fat diet (supposedly a "low-Cr" diet with ~30 µg Cr/kg diet) with additional mineral stresses for 24 weeks, resulting in compromised lipid and carbohydrate metabolism. The addition of 5 ppm Cr to the drinking water of rats on the stressed diets led to lower plasma insulin levels in intravenous glucose tolerance tests after 24 weeks on the diet.[20] An analysis of the actual Cr content of the diet is in order. A 100 g rat eats about 15 g of food a day.[28] Fifteen grams of food containing 33 µg Cr/kg food provides approximately 0.5 µg Cr. Thus, 0.5 µg Cr per day for a 0.100 kg rat is 5 µg Cr/kg body mass per day, ten times higher than human intakes. Thus, the "low-Cr" diet was not deficient unless rats require more than ten times the Cr that humans do; alternative adjustments for differences between rats and humans using surface area or metabolic rate rather than body mass provide similar results within an order of magnitude. The lowering of plasma insulin levels by addition of Cr can only be considered a pharmacological effect, and will be discussed further in Chapter 4.

Rats on the *Torula* yeast-based diet supplemented with Cr compounds received 400 times this quantity, a supra-nutritional dose. (These comparisons, of course, make the assumption that the biochemistry of Cr is similar in rodents and primates). Statistical concerns have also been raised about these studies;[29] however, many studies in the 1960's apparently observed altered carbohydrate metabolism in rats on diets based on *Torula* yeast.[30]

A similar complication arises for studies on patients on total parenteral nutrition (TPN) that have been used as evidence for Cr being an essential trace element in humans. In five cases, patients on TPN have developed impaired glucose utilization[23] or glucose intolerance and neuropathy or encephalopathy[24,25,26] that could be reversed by inclusion of Cr in the TPN. In the cases where the concentration was reported, the TPN solutions initially provided 2-6 µg Cr per day. Because TPN is an intravenous diet, all the Cr in the TPN is introduced into the bloodstream, while only ~0.5% of Cr in a regular human diet is absorbed into the bloodstream. Thus, the 30 µg of Cr in a typical daily diet presents only ~0.15 µg Cr to the bloodstream. Thus, the TPN solutions cannot be considered Cr deficient. Subjects were treated with 125-250 µg Cr per day added to the TPN solution,[24,25,26] an ~1000-fold increase, to alleviate their conditions. This was clearly a pharmacological dose. This provides no evidence for Cr being an essential element but suggests that large doses of chromium may have pharmacological effects in humans with altered glucose and carbohydrate metabolism, not just rodents with these conditions.

Cr absorption is purportedly inversely proportional to intake in humans. Anderson and Kozlovsky[27] have shown that absorption varies inversely in female subjects, but not male. This inverse relationship may not be real, because the female data requires a full statistical treatment. Cr intake of 10 µg Cr lead to 2% absorption, while 40 µg intake had a decreased intake of 0.5%.[27] However in a study by Kottwitz[31] et al. showed that Cr absorption of $CrCl_3$ in rats was independent over 0.01-20 µg Cr. This suggests that Cr absorption is not inversely proportional in all mammals. Diabetes is known to lead to increased Cr excretion. Increased Cr excretion was hypothesized to lead to conditionally induced Cr deficiency. A study of Cr absorption and its relationship to excretion in Zucker lean, Zucker obese and Zucker diabetic obese animals is discussed in Chapter 2.

Changes in plasma insulin levels result in changes in urinary Cr excretion. This line of evidence is only suggestive. These studies are reviewed in Chapter 3. The demonstration that Cr could potentially be an essential element will probably require the isolation of a biomolecule that is essential to some critical biological process and requires Cr to perform its essential function (assuming such actually exists).

1.3 The chemistry behind the chromium supplements

Several Cr nutitional supplements have been studied. The first were the brewer's yeast and porcine kidney powder as discussed previously. $CrCl_3$, chromium picolinate (or $[Cr(pic)_3]$), $[Cr_3O(propionate)_6(H_2O)_3]^+$(or Cr3), Cr histidine, Cr oligomannutoate, Cr nicotinate and Cr(D-phenylalanine)$_3$ are all Cr compounds that have been studied as a Cr source.[32] In this dissertation, only $[Cr(pic)_3]$, Cr3 and $CrCl_3$ were used and will be thoroughly discussed.

Cell culture studies suggest that Cr supplementation, particularly in the form of chromium picolinate, may have toxic, particularly genotoxic effects.[33] However, in a recent study male and females rats and mice were fed varying amounts of chromium picolinate (up to 5% of the diet by mass) for two years; while no beneficial effects were observed, neither were any consistent deleterious effects.[34] This can readily be explained by recent studies that show that chromium picolinate is not absorbed intact; less than 1% of chromium picolinate from the supplement is in the bloodstream intact.[35] Thus, the complex appears to breakdown in the stomach, serving only a source of Cr(III) similar to other commercial forms of Cr supplements. Both the United States Food and Drug Administration[36] and the European Food Safety Authority[37,38] have determined that nutritional levels of chromium supplementation are safe.

$[Cr(pic)_3]$ has a solubility of only 600 μM at near neutral pH, which drops as the pH is

lowered until the complex hydrolyzes to release picolinic acid.[39] Despite numerous claims that [$Cr(pic)_3$] is absorbed better than inorganic forms of Cr (e.g., $CrCl_3$) used to model dietary Cr, [$Cr(pic)_3$], Cr nicotinate (the second most popular form of Cr sold as a nutritional supplement), and $CrCl_3$ are absorbed to a similar degree in rats when studies examine the fate of ^{51}Cr from labeled compounds in tissues and body fluids;[40,41] given that the picolinate complex is made from a Cr(III) salt, use of $CrCl_3$ should be more cost effective. [$Cr(pic)_3$] is quite stable in water,[42] such that it should not break down appreciably when dissolved or slurried in buffered water. Gammalgaard and coworkers have reported the compound is stable in artificial gastric juice;[43] however, the method used only demonstrates that the chromium remains in a molecule with similar molecular weight to [$Cr(pic)_3$] over time. Lay and coworkers using X-ray absorption studies have found that the compound was stable in simulated gastric juice for 24 hours and simulated intestinal fluid for 24 h.[44] Only about one- fourth and one-half of the compound degraded in artificial gastric juice with added components of a semi-synthetic meal over 1 h and 3 h, respectively,[44] appreciably greater degradation than in actual rat stomach contents.[39] By HPLC using ^{14}C-labelled picolinate, the compound has been shown to be stable for 2 h when added to excised stomach contents of rats and for 4 h when added to excised small intestine contents.[39] Only 1 % of absorbed Cr from the supplement (as followed by HPLC using ^{14}C-labelled picolinate) is found in the bloodstream as [$Cr(pic)_3$], suggesting that little of the intact molecule is absorbed.[39] Double labeling experiments with ^{51}Cr- and ^{3}H-labelled [$Cr(pic)_3$] have shown that gavaged [$Cr(pic)_3$] breaks down rapidly in the gastrointestinal tract, probably primarily in the stomach, as the fate of the labels diverges rapidly in the gastrointestinal tract and body of rats (Gullick and Vincent, unpublished results). Thus, when ingested, the complex probably hydrolyzes near the stomach lining, releasing the Cr and picolinic acid, which are

subsequently absorbed independently examining the greatly different extent of absorbance of chromium and picolinic acid (~90% for the acid, Gullick and Vincent, unpublished results).

The compound appears to be less stable in rat blood serum than in buffer with about 50%, 70%, and 85% degrading in 1, 6, and 24 h, respectively.[44] Intravenously administered [$Cr(pic)_3$] clears the blood stream of rats and enters the tissues and urine rapidly; a large percentage of the [$Cr(pic)_3$] enters the urine intact (>90 % is cleared in 30 minutes). Thirty min after injection greater than 75% of the Cr in the blood stream is present as [$Cr(pic)_3$];[45] this appears to be consistent with the time line for the compound added to rat serum.[44] In tissues the compound breaks down extremely rapidly as all detectable Cr in the cytosolic fraction of hepatocytes was not present as [$Cr(pic)_3$].[45] Hepatocytic microsomes have been shown to degrade [$Cr(pic)_3$] *in vitro*;[46] however, the major product derived from the picolinate ligand is different than that when [$Cr(pic)_3$] is administered by gavage to rats.[39]

A note of caution is in order**.** Comparing the fate of chromium compounds that are administered orally compared to those that are administered intravenously or added to whole blood or blood plasma or serum needs to be made cautiously, if they can be made at all. For example, oral administration of inorganic chromium compounds results in over 90 % of chromium in the blood bound to transferrin,[47] while nearly all the remainder is bound to a low-molecular-weight species[48] (probably low-molecular-weight chromium-binding substance (*vide infra*)). Addition of inorganic compounds to blood plasma or intravenous administration results in chromium binding to albumin and other species in addition to transferrin.[49]

Now the results in the various studies with [$Cr(pic)_3$] can readily be reconciled.[50] When [$Cr(pic)_3$] is intact in the presence of appropriate redox active species, oxidative damage is expected. Thus, [$Cr(pic)_3$] dissolved in water or buffer in a test tube is susceptible to this type of

chemistry. The compound does not cause damage to bacterial cells in Ames assays because it probably cannot diffuse into the cells. Mammalian cells with less robust outer membranes allow the diffusion of $[Cr(pic)_3]$ into the cells; thus, the mammalian cell culture studies almost uniformly observe oxidative damage or DNA damage. Similarly, if $[Cr(pic)_3]$ is administered intravenously, the intact complex has the ability briefly to diffuse into cells and generate damage. In contrast, when given orally to rodents and humans, $[Cr(pic)_3]$ readily breaks down in the gastrointestinal tract so that only ~1% of the small quantity of absorbed Cr exists as intact $[Cr(pic)_3]$. Thus, when administered orally, the animal is fortuitously not exposed to the form capable of entering into the redox chemistry leading to oxidative damage and DNA cleavage. In the very different digestive system of fruit flies, the complex, when absorbed intact, is a potent clastogen, mutagen, and developmental toxin.

Studies on the potential use of $[Cr_3O(propionate)_6(H_2O)_3]^+$, or Cr3, also point out the importance of properly placing chemical and nutritional studies in perspective in a complementary fashion. Cr3 is sold commercially for cattle and swine feed as Kemtrace.[51] Cr3 has an oral LD_{50} greater than 2 g/kg body mass in male and female rats, measured using the Organization for Economic Cooperation and Development (OECD) procedure.[33] In female Wistar rats receiving 100 mg Cr/kg body mass as Cr3 in the diet for 4 weeks, no effects in body mass, organ mass, or feeding efficiency were observed, while Comet assays of lymphocyte DNA revealed no damage.[52] When given orally in doses up to 10 mg Cr/kg body mass to male Sprague-Dawley rats daily for ten weeks, no deleterious effects were observed.[53] Developmental toxicology studies in both rats[34] and mice[35] observed no deleterious effects. Similarly, in contrast to $[Cr(pic)_3]$ and picolinic acid, no developmental delays or changes in hatching or eclosion success have been observed in *Drosophila* with Cr3 or corresponding concentrations of

propionate.[54] Because the amount of propionate delivered by administering the supplement at nutritional or pharmacological doses is small compared to amount of propionate generated by bacterial fermentation of fiber in the gastrointestinal tract, beneficial or deleterious effects from propionate should not be anticipated. An attempt to observe effects in rats from propionate at a dose equivalent to a human taking 1000 mg Cr/kg body mass as Cr3 daily for 24 weeks observed no effects.[36] Lay and coworkers have shown that Cr3 can react in the absence of reducing agents with hydrogen peroxide (0.1 to 5 mM) to form high valent chromium species.[38,37] However, compared to [$Cr(pic)_3$], Cr3 is poor at cleaving DNA *in vitro*.[55] DNA cleavage by the cation required 120 µM Cr3 in the presence of 215 µM hydrogen peroxide. Cr3 could not cleave DNA in the presence of a physiological concentration of ascorbic acid (5 mM). Thus, at anything approaching physiological conditions, Cr3 did not produce sufficient quantities of either high valent chromium or reactive oxygen species (ROS) to cleave detectable quantities of DNA. Thus, in contrast to [$Cr(pic)_3$], studies with Cr3 are uniform in suggesting the complex is not toxic at reasonable doses.

Some discrepancy in the fate of Cr3 has appeared in the literature but this can readily be reconciled by comparing the *in vivo* fate of the compound with the design of *in vitro* studies. In stark contrast to [$Cr(pic)_3$], an oral dose of Cr3 (2 µg Cr) to non-fasted male rats results in over 90% of the dose clearing the stomach in 30 minutes (the earliest time point examined) and over 50% of the dose is absorbed at this time.[56] Thus, Cr3 appears to be absorbed intact; if it decomposed and served only as a source of Cr (as do $CrCl_3$ or [$Cr(pic)_3$]), it should have an absorption of about 1%. Total absorption when corrected for Cr in the bile re-entering gastrointestinal tract appears to approach 100%.[56] When Cr3 is labeled with ^{51}Cr and ^{14}C, the labels co-migrate the first hour after absorption after which their fate significantly diverge

(Gullick and Vincent, unpublished results), supporting that Cr3 is absorbed intact from the stomach and enters the bloodstream intact. However, the compound clearly degrades after this time.

Cr3 can be recrystallized from dilute mineral acids[57,58] so stability in the stomach is not surprising. X-ray absorption studies indicate that it is stable for 24 hours in simulated gastric juice (pH ~2) for 24 hours and artificial gastric juice with a semisynthetic meal added for 24 hours (no change in spectra within error).[44] The compound appears to be surprisingly stable in simulated intestinal fluid for 24 h (80%).[44] When a sample was maintained in artificial gastric juice with a semisynthetic meal for 1 h and then had its environment changed to model the intestinal environment for 2 hours, only ~20% of the Cr3 appeared to stay intact. However, as Cr3 rapidly is absorbed from the stomach, the relevance of the data is questionable.

The fate of ^{51}Cr-labelled Cr3 when administered intravenously has also been examined.[59,60] Unlike [Cr(pic)$_3$], this data should be of relevance as the Cr3 enters the bloodstream intact. In 30 minutes, ~85% of the Cr disappears from the bloodstream as the compound very rapidly enters the tissues. The quantity of Cr from Cr3 in the tissues is maximal 30 to 60 minutes after administration and decreases rapidly thereafter; little Cr corresponding enters the urine the first hr after injection but then the amount lost in the urine increases rapidly, probably as low-molecular-weight chromium-binding substance (LMWCr or chromodulin) (*vide infra*). This unique degree of tissue incorporation suggests that the compound might remain intact for the first hour after injection. In the liver, about 75% of the radiolabel is in the microsomal fraction. Size exclusion chromatography (G-15) of the microsomal liver fraction reveals that the ^{51}Cr elutes as a tight band; the elution profile of Cr3 on the same column is essentially superimposable with that of the major microsomal Cr species.[59] Thus, the data

suggest that Cr3 is incorporated into tissues intact within 30 minutes (earliest time point) of administration after which it is has a short lifetime of 2 hr or less before rapidly decomposing. A similar argument for the unique stability of Cr3 *in vivo* can be made based on tissue accumulation of Cr. Daily oral administration of $CrCl_3$ or $[Cr(pic)_3]$ (~15 mg/kg body mass) results in a dose dependent increase in Cr retention in the kidneys and liver.[28] Contrastingly, daily administration of Cr3 does not lead to significant accumulations at doses up to 1 mg Cr/kg;[36] however, accumulation is observed at a dose of 10 mg Cr/kg.[53] X-ray absorption studies have shown that Cr3 decomposes in 24 hours in rat blood serum or rat blood (~16 and 0% intact, respectively.[44] The results are not physiologically relevant given the long time period utilized. Similarly, the compound decomposes in cell culture medium with 2% fetal bovine serum in 20 hr (and in this media containing L6 rat myotubes in 20 h);[44] again because of the length of time of the incubation, the results are not physiologically relevant. A related study in which Cr3 was added to albumin-depleted bovine blood serum for 2 h at 37 ^{o}C, followed by addition of sample buffer and SDS-PAGE electrophoresis and the Cr contents of the gel examined by X-ray fluorescence revealed that Cr was abound to high molecular weight proteins;[61] again the physiological relevance, given the time interval and the unknown stability of Cr3 in sample buffer and under SDS-PAGE conditions, is uncertain at best.

Commercially available Cr chloride, "$CrCl_3$", is actually the salt *trans*-$[CrCl_2(H_2O)_4]Cl$. In aqueous solution, the compound is susceptible to hydrolysis and oligomerization, especially at basic pH's, leading to the formation of numerous multinuclear hydroxo-bridged chromic species. Thus, Cr in the complex upon exchange of the aquo ligands can bind to biomolecules and be carried by large biomolecules through the gastrointestinal tract or can undergo oligomerization and polymerization to form species of limited solubility, especially at the alkaline pH of the

intestines. Chromium nicotinate is poorly characterized and has limited solubility; the nicotinate ligands are relatively labile, generating forms of Cr susceptible to bind to biomolecules and olation.[62] [$Cr(pic)_3$] has very limited solubility in water (0.6 mM)[63] and in other common solvents and is not particularly lipophilic.[42] In contrast, the molecular cation Cr3 is extremely soluble in water (contra Cr nicotinate and picolinate) and appears to be able to maintain its integrity *in vivo*,[64] not breaking down in the gastrointestinal tract (contra $CrCl_3$ and Cr nicotinate). Depending on dose, Cr3 is absorbed with at least 40-60% efficiency.[56] The effects of the Cr compounds [$Cr(pic)_3$], $CrCl_3$, and Cr3 as well as a vanadium compound, on tissue metal concentrations will be discussed in Chapter 5.

1.4 Understanding Cr transport

Orally administered chromium is absorbed by passive diffusion. This has been convincingly demonstrated by a double perfusion technique using segments of the small intestine of rats that over a 10^2-fold range of Cr^{3+} concentrations (10-1000 ppb) Cr absorption was a nonsaturable process.[65] In studies with rats gavaged with a dose of $CrCl_3$, rats absorbed ~0.2% of the Cr over a 2000-fold range of doses (0.01-20 µg Cr).[31] Another interesting conclusion that can be drawn from the intestinal perfusate studies is that chromium appears to be actively transported out of the intestinal cells, as ~94% of the chromium entering the cells was cleared from the cells (leaving only ~6% behind to be stored). However, no transporter is known for Cr^{3+}. This suggests the possibility is that Cr^{3+} bound to some chelating ligand and is actively transported in this form; this is an area requiring research. Changes in diet could affect the amount of Cr absorption and potentially affect the mechanism, although changes in mechanism have not been demonstrated. For example, the presence of added amino acids, phyate (high levels), ascorbic acid, and oxalate, but not low levels of phyate, in the diet reportedly alter the

extent of Cr uptake,[30] although the changes (while statistically significant in some cases) are relatively small changes in a small percentage absorption.

Once in the bloodstream, Cr(III) binds almost exclusively to the iron-transport protein transferrin. The association of transferrin and chromium has been reviewed.[49] Cr-loaded transferrin has been demonstrated to transport chromium *in vivo*.[66,65] Injection of ^{51}Cr-transferrin into rats results in incorporation of ^{51}Cr into tissues. Transport of iron into tissue by endocytosis of transferrin is insulin sensitive as is the transport of chromium; injection of labeled transferrin and insulin results in a several fold increase in urinary chromium.[65] Thus, transferrin, in an insulin-dependent fashion, can transfer Cr to tissues from which it is excreted in the urine. The binding of chromium to transferrin is quite tight, although apparent the binding constants for the two metal binding sites differ by about 10^5; [67] the *in vitro* binding of Cr^{3+} from inorganic salts is quite slow.[67] This also suggests that Cr may be carried to transferrin as a chelate complex. Once Cr is brought into the cell by endocytosis, it must leave the endosome to enter the cell cytosol. As Cr^{3+} is not readily reduced by any biological reducing agents so that it can be transported by divalent metal ion transporters (in a fashion similar to iron), it must be transported by another mechanism; this is another area requiring further research. Absorbed Cr is found in three pools. A three-compartment model has been proposed to examine the kinetics of chromium tissue exchange and distribution for studies with rats and humans.[68,69,70] Plasma chromium is in equilibrium with the three pools: a small pool with rapid exchange ($T_{1/2}$<1 day), a medium pool with a medium rate of exchange (days), and a large, slowly-exchanging pool (months).

One other molecule is known to bind chromium *in vivo*: low-molecular-weight chromium-binding substance (LMWCr or chromodulin). This molecule occurs in the tissue, the

bloodstream and the urine and appears to bind chromium in the tissues for its elimination from the body via the urine. The history of studies of this molecule has been exhaustively reviewed.[71] The inability of the organic portion of this Cr-peptide complex to be characterized generated significant controversy, as the situation bore similarity to the previous inability to characterize the organic component of glucose tolerance factor.[72] Another concern has been that a Cr-loading procedure is necessary in the purification of LMWCr so that the peptide could be followed (by its Cr content) through the isolation procedure; thus, the animal providing the tissue or body fluid is usually administered a Cr(III) or Cr(VI) source or such a source is added to the tissue homogenate or fluid.[73] Rupture of chromate-treated mammalian cultured cells results in Cr being bound to a low-molecular-weight species with spectroscopic properties similar to LMWCr.[73] This was interpreted in terms of LMWCr being an artifact generated during isolation; however, the unnatural method of presenting chromate in high concentration to cultured cells also suffers from the types of problems discussed above when using cultured cells. Thus, this study only shows that apoLMWCr can potentially bind chromium in a cell extract and potentially binds Cr tight enough to remove it from other biomolecules, consistent with the results of the isolation procedures of LMWCr described above. The chromium environment of LMWCr has been characterized by a variety of techniques including paramagnetic NMR, EPR, X-ray absorbance, and variable temperature magnetic susceptibility.[74,75] The peptide component has recently been sequenced by mass spectrometry;[76] the sequence begins with four glutamate residues whose cyclizing blocked attempts at Edman degradation sequencing. The peptide binds four chromic ions with identical binding constants and cooperativity as apoLMWCr (within experimental error).[76] LMWCr has been shown to stimulate insulin dependent glucose incorporation and

metabolism in isolated rat adipocytes[77,73] and *in vitro* to stimulate (or perhaps retard the deactivation of) the kinase activity of insulin-activated insulin receptor.[78,79]

A mechanism for LMWCr in amplifying insulin signaling has been proposed.[80,81] This proposal was put forward when Cr was thought to be essential; the mechanism needs to be altered so that it would be in vogue under conditions of chromium supplementation so that abnormally high concentrations of holoLMWCr are generated. In this mechanism, apoLMWCr is stored in insulin-sensitive cells. Increases in blood insulin concentrations, result in activation of the insulin signaling cascade: insulin binds to its receptor bringing about a conformational change that results in the autophosphorylation of tyrosine residues on the internal side of the receptor, transforming the receptor into an active tyrosine kinase and transmitting the signal from insulin into the cell. In response to this signaling, transferrin moves from the bloodstream into cells, carrying in part Cr^{3+} into the cells. The chromium flux results in loading of LMWCr with chromium. The holoLMWCr then binds to insulin receptor, presumably assisting to maintain the receptor in its active conformation and amplifying insulin signaling. This mechanism requires demonstration that it can (or cannot be) active *in vivo* to verify (or refute); clear demonstration that insulin receptor is directly involved in increasing insulin sensitivity by chromium (*vide infra*) would support this mechanism. If Cr is not an essential element while it normally has a role in binding Cr, LMWCr could be part of a Cr detoxification system as suggested by Wada and coworkers;[82] Cr supplementation, which leads to increased Cr concentrations in the body, could lead to increased concentrations of holoLMWCr, capable in turn of affecting insulin signaling.

Studies need to determine the origin of LMWCr, i.e. what protein is it made from and what enzymes are involved? Is the holoLMWCr biologically active at physiological levels

(suggesting a potential biological role for Cr) or is it only significantly active only when Cr concentrations are high? Does LMWCr interact with insulin receptor in vivo, or does it manifest its effects elsewhere?

1.5 Cr supplementation appears to have pharmacological effects.

Cr supplementation may result in beneficial responses in mammals with demonstrated glucose intolerance, including type 2 diabetes, cardiovascular disease, and related conditions. Studies with diabetic model rodents using pharmacological doses of chromium have nearly uniformly observed beneficial effects in insulin sensitivity and plasma insulin, cholesterol, and/or triglycerides levels.[54] However, studies in humans using considerably lower doses (in terms of μg Cr/kg body mass) tend to be negative or at best ambiguous.[83] The human studies tend to have small subject pools and not be well designed. One study has dominated attention; the study used 185 adult-onset diabetic Chinese patients in which decreases in the concentration of fasting serum glucose, insulin, hemoglobin A_{1C} and total cholesterol and decreased glucose and insulin levels in response to glucose challenges were observed as a result of Cr supplementation in a dose responsive manner.[84] Unfortunately, attempts to reproduce these results on other populations, including Western populations, have been unsuccessful.[85,86] Consequently, the American Diabetes Association's (ADA) position has been that "Benefit from chromium supplementation in people with diabetes or obesity has not been conclusively demonstrated and, therefore, cannot be recommended."[87] In 2011, chromium was dropped from the position statements of the ADA.[88]

One difference between rodent model studies and the human studies is the relative dose of chromium used. Is the dose crucially important, or does another difference exist in the

bioavailability, distribution, etc. of Cr between humans and the rodent models that gives rise to these differences? Human studies have used doses as high as 1 mg Cr/day. However, rodent studies have utilized 80 to 10,000 µg Cr/kg body mass daily, corresponding to approximately 10 to 1,300 mg daily for a human (based on body mass). If the data are additionally adjusted for the increased metabolic rates of rats, this still corresponds to approximately 2-260 mg daily for humans. Based on available safety recommendations from the Expert Group on Vitamins and Minerals of the Food Standards Agency (United Kingdom) that indicate that up to 10 mg Cr/person daily would be expected to be without adverse health effects,[89] human studies with sufficient power are needed using 5-7 mg Cr(III) daily for 4 to 6 months or longer with careful monitoring for any deleterious effects. Effects on healthy and subjects with compromised insulin sensitivity (and related conditions) should be compared. The use of different supplements and doses should be minimized. The use of $CrCl_3$ or Cr3 is suggested as these are free of potential ligand effects (as with $[Cr(pic)_3]$ or chromium nicotinate);[89] the greater absorption efficiency of Cr3 would seem to make it the preferred supplement. Potential toxic effects, particularly at the higher doses, will need to be monitored carefully. The nutritional status of subjects, including iron metabolism (transferrin levels and saturation) will need to be carefully monitored.

If Cr(III) complexes are found to have beneficial effects on subjects with altered glucose and lipid metabolism, the effects would still be expected to be small compared to treatment with insulin or current diabetes medications. Cr supplementation potentially then may have a role as an inexpensive treatment to allow the use of lower doses of current medications (which have potentially severe side effects) or to treat subjects at the early stages of type 2 diabetes to potentially delay the continued onset of the disease. However, to clearly understand what is

happening, these studies need to be combined with studies examining the mode of action of chromium in enhancing insulin sensitivity at a molecular level and the fate of chromium in the body at a molecular level.

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Chapter 2:Urinary chromium loss associated with diabetes is offset by increases in absorption

2.1 Introduction

Claims of Cr essentiality are rooted in a study by Mertz and Schwarz, where rats fed a diet thought to be Cr deficient developed an inability to efficiently respond to increases in blood glucose.[1, 2] Porcine kidney powder and Brewer's yeast were found to reverse the condition. The porcine kidney powder and Brewer's yeast were found to be rich in Cr. The supplementation of various Cr^{3+} salts to the diet also reversed the glucose tolerance. The dietary factor necessary to prevent impaired glucose tolerance was named glucose tolerance factor. This study was methodologically flawed (as reviewed in Chapter 1). Currently four suggestive and not definitive lines of evidence are used to suggest the nutritional relevance of Cr^{3+} (as reviewed in Chapter 1), but a major question in chromium nutritional and biochemical research is whether individuals who possess conditions that result in or are related to improper carbohydrate and lipid metabolism such as type 2 diabetes can benefit from nutritional Cr supplementation. This question is particularly important for individuals under stresses that result in the abnormally high loss of Cr via the urine. Currently no method for assessing the relationship between stress and effects on humans or other mammals potentially related to changes in Cr nutritional status has been identified. Several attempts to develop assessment protocols have been unsuccessful (as well as an attempt in Chapter 3 in this dissertation), because, for example, urinary and plasma Cr concentrations do not correlate with tissue Cr or plasma glucose, insulin, or lipid levels.

Type 2 diabetic patients have been found to possess ~33% lower plasma Cr and almost 100% higher urine Cr than healthy individuals.[3] Patients diagnosed with diabetes for several years have urinary Cr losses less than those of their corresponding control group. The study suggests that insulin resistance (and the associated increased non-fasting plasma insulin and glucose levels) could possibly result in increased mobilization of Cr from the blood with corresponding increased urinary Cr loss until Cr stores are depleted, resulting in decreased mobilization and urinary Cr loss.[3] Rat models of diabetes excrete greater amounts of Cr than their healthy counterparts.[4] This has been used to support postulates that Cr deficiency exacerbates the symptoms of diabetes and that Cr supplementation can thus reverse the symptoms to a degree.

In this study, Zucker obese and Zucker diabetic fatty rats were used to establish whether rats under stress have altered levels of Cr absorption compared to Zucker lean rats. If these conditions lead to increases in Cr absorption, then increased urinary Cr losses resulting from the conditions are unlikely to lead to Cr deficiency. In contrast, if absorption of Cr is unaltered or decreased, then increased urinary Cr loss from these and related conditions should eventually lead to Cr deficiency, which could be treated by Cr supplements at nutritional levels.

2.2 Materials and Methods

2.2.1. Materials and Instrumentation.

$^{51}CrCl_3$ was obtained from MP Biomedicals, Inc. and diluted with 100 mL of doubly-deionized water. Gamma-counting of collected samples in gamma tubes was performed on a Packard Cobra II auto-gamma counter.

2.2.2. Animals.

The University of Alabama Institutional Animal Care and Use Committee approved all procedures involving the use of rats. Twenty-one male rats (six weeks of age) of each Zucker lean, Zucker obese (an insulin-resistant model of obesity and early stage type 2 diabetes), and Zucker diabetic fatty, ZDF, (a type 2 diabetes model) were obtained from Charles River Laboratory and acclimated to their cages for two weeks (2 rats per cage). Zucker obese rats have a mutation in the gene for the receptor for the hormone leptin; as a result, the rats become obese, have elevated plasma cholesterol and triglycerides levels, and have high normal plasma glucose and insulin concentrations; the ZDF rats have an additional (yet to be identified) mutation which results in their developing the full range of symptoms associated with type 2 diabetes.[5] The rats were maintained on a 12-h light/dark cycle. The rats were housed for 16 weeks, to ensure the ZDF rats developed diabetes prior to the absorption experiments. The Zucker lean and obese rats were fed a standard commercial rat diet (Harlan Teklad LM-485 Mouse/ Rat Sterilizable Diet). The ZDF rats were fed a commercial high fat diet (Formulab Diet 5008), previously shown to assist in the development of diabetic symptoms. Both diets have previously been shown to be chromium sufficient.[6,7] The rats were allowed to feed and drink ad libitum. After the 16 week period, experiments were initiated by gavaging the rats with an appropriate volume of an aqueous $^{51}CrCl_3$ solution (3 μg Cr/kg body mass) by Nicholas Rhodes, after which they were placed in metabolic cages for selected time intervals for the purpose of feces and urine collection. The time intervals were 30, 60, 120, 360, 720, 1440, and 2880 min. The rats were allowed to feed and drink *ad libitum* after Cr administration. Before administration of Cr, the blood glucose levels of the ZDF rats were measured to ascertain if all had developed diabetes; blood glucose measurements were made with a One Touch Ultra from tail slits.

2.2.3 Sample Collection.

At the end of each time interval, the rats were sacrificed by CO_2 asphyxiation. Blood and tissue samples were harvested and placed into pre-weighed 50 mL disposable centrifuge tubes. The stomach, small intestine, large intestine, heart, liver, spleen, testes, kidneys, epididymal fat, right femur, pancreas, and muscle (muscolus triceps surae) from right hind leg were collected and weighed; urine and feces were also collected and weighed. Blood and muscle were assumed to comprise 6% and 30% of the total body mass, respectively, for calculations.[8] For the Zucker obese rats with their large fat content, muscle was assumed to compromise 16% of the total body mass. Studies of the muscle content of the hind legs[9] and carcass (body minus tail, internal organs, and gastrointestinal tract) [10] both reveal about a 53% percent reduction in the % muscle composition of Zucker obese rats versus their normal counterparts.

2.2.4 Statistical Analysis.

Each data point in the figures represents the average value for three rats, except for the 30 min ZDF rats as one rat died of apparent heart complications (common for the disease model) shortly before the Cr administration was to begin. Error bars in the figures denote standard deviation. The data from each replicate was calculated independently, tested for homogeneity of variance by the Levine statistic using SPSS (SPSS Inc. Chicago, Il), and pooled and analyzed to give the reported results. Data was analyzed by repeated measures ANOVA and MANOVA. Specific differences ($p \leq 0.05$) were determined by a Bonferroni post-hoc test. Statistical analysis was performed by Kristin R Di Bona.

2.3 Results

Male Zucker lean, Zucker obese, and ZDF rats were gavaged with a solution of $^{51}Cr^{3+}$ (3 μg/ kg body mass) as ^{51}Cr chloride in water. Assuming a human has an average body mass of 65

kg, the dose corresponds roughly to a human receiving 200 μg Cr daily. Commercial Cr-containing nutritional supplements generally contain 200 to 600 μg Cr; thus, the dose is equivalent to that of a human taking a nutritional supplement.

With time, the ^{51}Cr can readily be followed through the gastrointestinal tract (Fig. 1). At least 90% of the administered dose clears the stomach in 60-120 minutes for the lean and ZDF rats, while only ~80% of the dose passes through the stomach after 2 h for the obese rats. The content of the label in the small intestine of all the groups reaches a maximum in 1 h, although for the ZDF rats the amount in the intestine is statistically equivalent between 1 and 2 h. The maximum content of the label in the large intestine is reached at 6 h, although for the lean rats the amounts are equivalent at 6 and 12 h. This passage through the gastrointestinal tract is accompanied by increased loss of the label in the feces with time (Fig. 1). Nearly 100% of the administered label is lost in the feces within 48 hours of treatment for all three groups.

^{51}Cr appeared rapidly in bloodstream, reaching a maximum for each group 30 min to 1 hr after administration (Fig. 2). After one hour the amount of Cr in the bloodstream dropped quickly. The level returned near the baseline after 2 h for the lean and ZDF rats and by 6 h for the obese rats (Fig. 2). The appearance of Cr in the tissues closely mirrored the appearance in the bloodstream as a function of time, reaching a maximum 30-60 min after administration with a subsequent rapid loss of the radiolabel (Figs. 3 and 4). Thus, the Cr, which is rapidly absorbed from the stomach and/or small intestine (*vide infra*), rapidly passes through the bloodstream to the tissues and then to the bloodstream for elimination. Of the tissues examined, by far the most Cr is found in the skeletal muscle. The maximum retained dosage in the muscle was ~0.8% for the lean rats, ~0.4% for the obese rats, and ~0.6% for the ZDF rats. For the lean and obese rats, the percentage of absorbed dose in any other tissue never exceeds ~0.06% of the applied dose,

while over 0.15% of the applied ^{51}Cr appears in the liver of the ZDF rats. Besides the liver, skeletal muscle, and gastrointestinal tract, only the kidneys of the harvested tissues possess a notable amount of Cr, at maximum 0.01-0.02% of the applied dose for the lean and obese rats and ~0.04% for the ZDF rats.

The disappearance of chromium from the blood and the tissues after approximately the first hour after administration of the radiolabel is accompanied by the rapid appearance of chromium in the urine. For the lean and obese rats, the amount of Cr in the urine rises to approximately 0.3-0.6% of the applied dose. In contrast, the amount of the radiolabel in the urine of the ZDF rats rises to about 1% of the applied dose. The Cr content of the ZDF urine is statistically greater than that of the obese and healthy rats at most of the time points during the study.

2.4 Discussion

Anderson and Polansky[11] have examined the fate of $CrCl_3$ given by gavage administration to rats; in 30 minutes, approximately 90% of the Cr had passed through the stomach and the first 15 cm of the small intestine. After 1 h, almost 100% of the Cr was in the lower portion of the small intestine and the large intestine. After 24 h, about 55% of the label was still in the lower portion of the small intestine and the large intestine[11]; the quantity of Cr in the feces was not reported.

In previous studies examining gavage administered Cr chloride and Cr nicotinate, muscle contained the greatest quantity of the administered dose at all time points up to 24 h after administration, followed by the liver.[8] The situation for Cr picolinate is similar except that the kidneys contain more Cr than the liver for the first 3 h after administration.[8] One to two days

after Cr administration, the amount of Cr in the liver of all three groups of rats increases. This has been observed previously in kidney and/or liver with other forms of Cr [12] [J.B. Vincent, unpublished results], where it has been attributed to movement of Cr into a slowly exchanging pool of Cr in this tissue. Cr concentrations in the liver and kidneys in male Zucker animals are also discussed in chapter 5.

^{51}Cr in tracer studies accumulates in the bone, kidney, spleen, and liver.[13,14,15,16,17,18,19, 20] A three-compartment model has been proposed to examine the kinetics of chromium tissue exchange and distribution for studies with rats and humans.[13,16,17] Plasma chromium is in equilibrium with the three pools: a small pool with rapid exchange ($T_{1/2}$<1 day), a medium pool with a medium rate of exchange (days), and a large, slowly-exchanging pool (months). The increase in Cr in the liver between 1 and 2 days after administration may reflect Cr moving into the medium exchange (and possibly also slow exchange) pool.

In theory adding together the Cr content of the gastrointestinal tract and feces would allow the percentage of Cr absorbed to be estimated; however, the extent of absorption is considerably smaller than the error associated with this calculation. However, the extent of absorption can be estimated by totaling the Cr content of the blood, urine, muscle, and liver as the content of the other tissues examined are basically negligible. For the lean rats, ~1.1% of the applied dose is retained after 30 minutes and 1 h. For the Zucker obese rats, ~0.7% of the applied dose is retained after 30 minutes, and ~1.1% of the applied dose is retained after 1 h. Thus, other than the apparently slower absorption process in the Zucker obese rats, the retention of chromium is similar between the Zucker lean and Zucker obese rats. In contrast, the ZDF rats retain greater quantities of Cr. Approximately 1.3% of the applied dose is retained after 30 minutes, and ~1.8% of the applied dose is retained after 1 h. Thus, the ZDF rats appear to absorb

approximately twice as much Cr as their lean and obese counterparts. The ZDF rats have more ^{51}Cr appear initially in the tissue and bloodstream, followed by a greater quantity of Cr appearing in the urine.

The magnitude of the absorption of the Cr from $CrCl_3$ in the Zucker lean and Zucker obese rats is comparable to that in other studies. In humans, dietary Cr is absorbed with an efficiency of ~0.5-2%.[21] In rats, Olin et al.[8] and Anderson et al.[22] have shown that Cr chloride, Cr nicotinate, and Cr picolinate (popular forms of Cr(III) in nutritional supplements) are absorbed to similar extents (0.5-1.3% of the gavaged dose of 0.14 or 0.15 μg Cr, respectively, after 24 h). Using whole body counting, Kottwitz, et al. found that rats given doses of Cr as $CrCl_3$ between 0.02 μg and 50 μg had ~0.10-0.20% of the dose retained.[23] The poor absorption can readily be understood based on the chemistry of these compounds as reviewed in Chapter 1.

Absorption occurs primarily during the first 60 minutes, as reflected by maximum retention of Cr in the body during this time. This suggests that absorption of Cr from the administered Cr is rapid from the stomach and/or small intestine. The appearance of Cr in the blood an tissues as a function of time closely mirrors the percentage of applied Cr in the small intestines, suggesting that the small intestines may be the more important player in absorption. This is in contrast to Cr3, which is absorbed rapidly in the stomach before entering the intestines, although the complex appears to be absorbed by the small intestine as well.[12] Absorption appears to be essentially complete after two hours. Thus, Cr absorption appears to stop as Cr enters the large intestine. Consequently, Cr absorption is essentially limited to the stomach and small

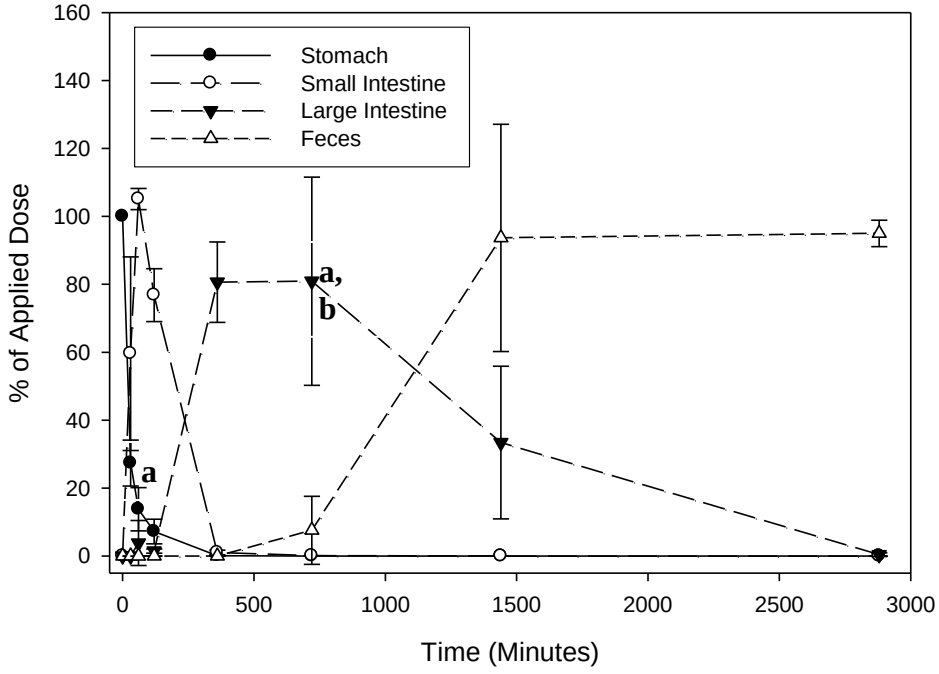

Figure 1A

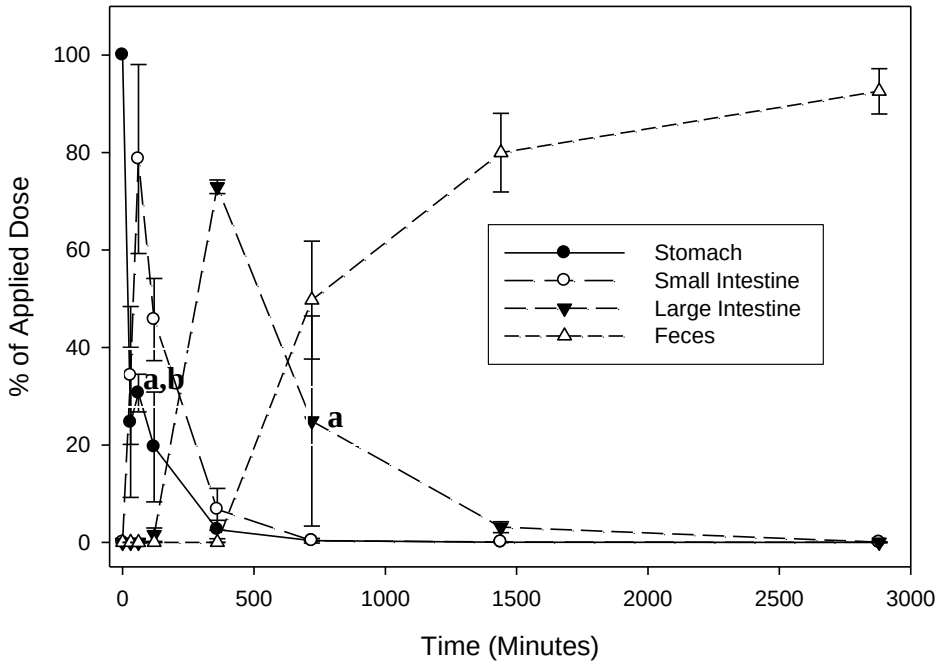

Figure 1B

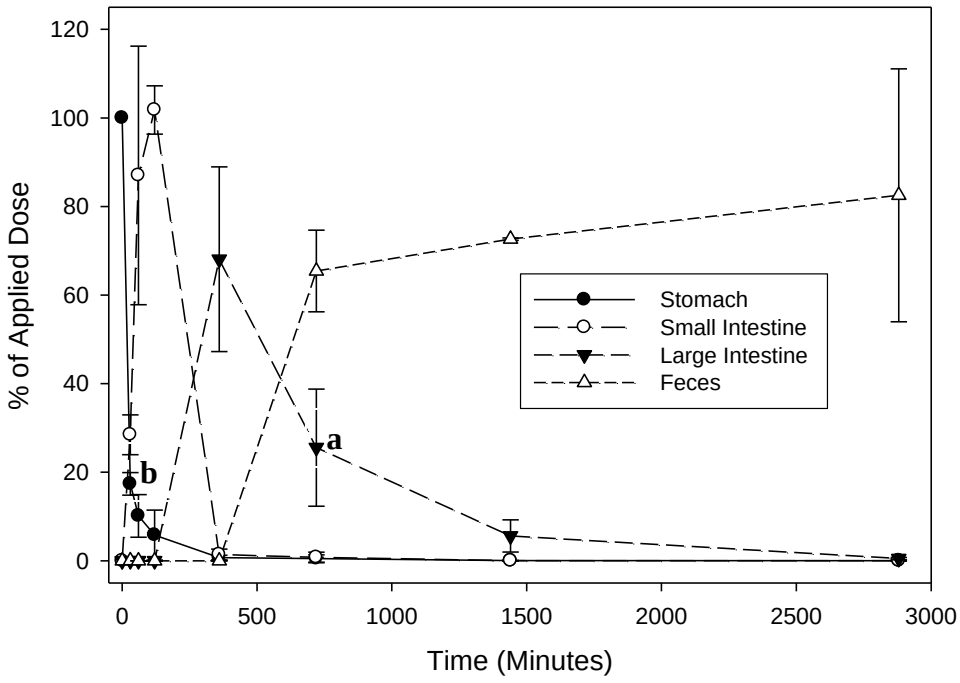

Figure 1C

Figure 1. Distribution of ^{51}Cr from $^{51}CrCl_3$ in the gastrointestinal tract and feces as a function of time: A) Zucker lean rats, a-significantly different from Zucker obese rats ($P<0.05$), b-significantly different from ZDF rats ($P<0.05$); B) Zucker obese rats, a-significantly different from Zucker lean rats ($P<0.05$), b-significantly different from ZDF rats ($P<0.05$); and C) ZDF rats, a-significantly different from Zucker lean rats ($P<0.05$), significantly different from Zucker obese rats ($P<0.05$).

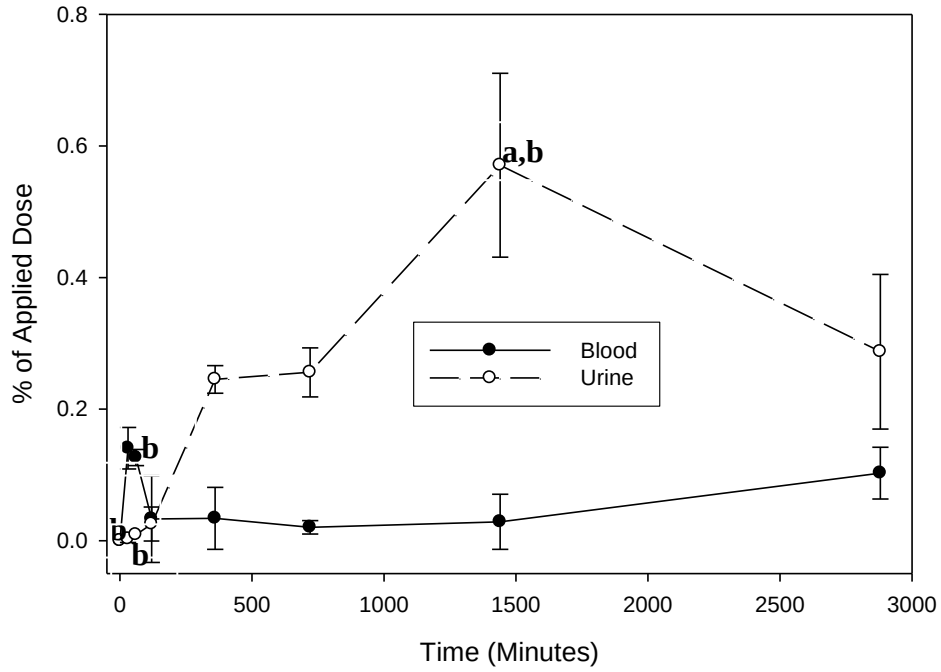

Figure 2A

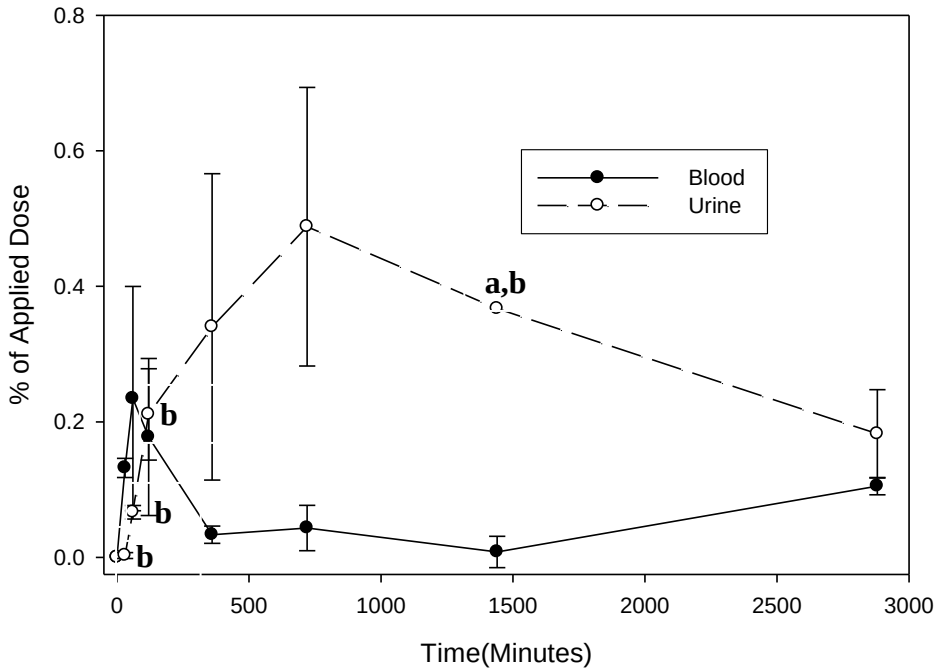

Figure 2B

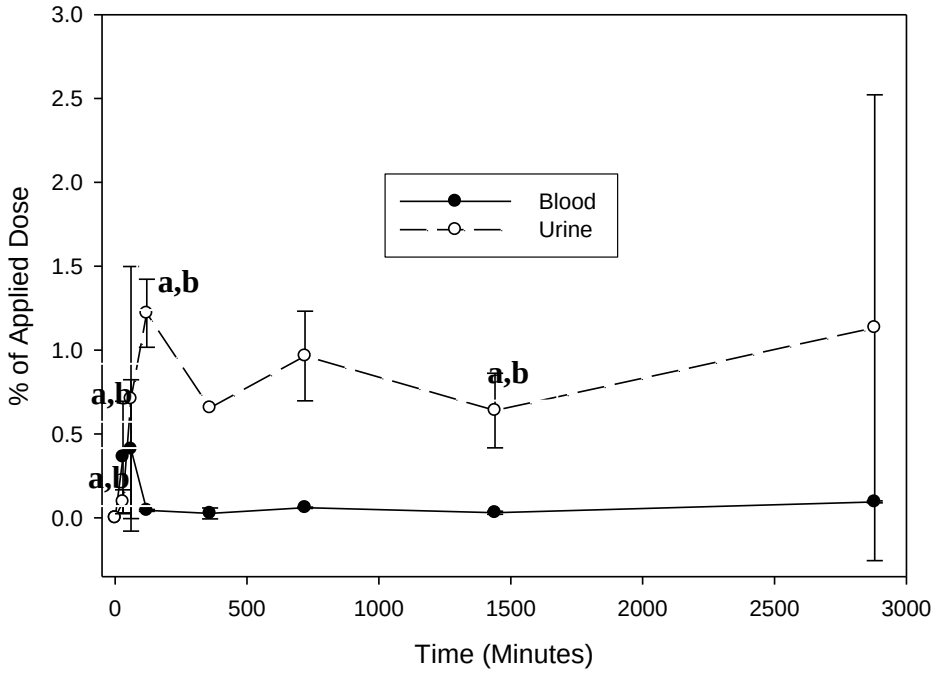

Figure 2C

Figure 2. Distribution of ^{51}Cr from $^{51}CrCl_3$ in the blood and urine as a function of time: A) Zucker lean rats, a-significantly different from Zucker obese rats ($P<0.05$), b-significantly different from ZDF rats ($P<0.05$); B) Zucker obese rats, a-significantly different from Zucker lean rats ($P<0.05$), b-significantly different from ZDF rats; and C) ZDF rats, a-significantly different from Zucker lean rats ($P<0.05$), b- significantly different from Zucker obese rats ($P<0.05$).

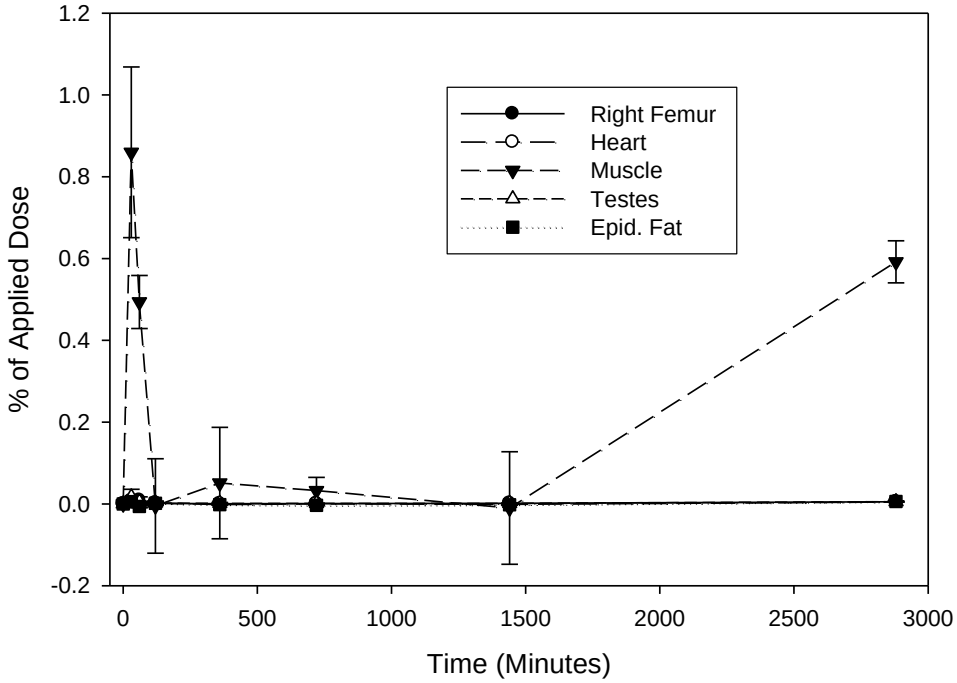

Figure 3A

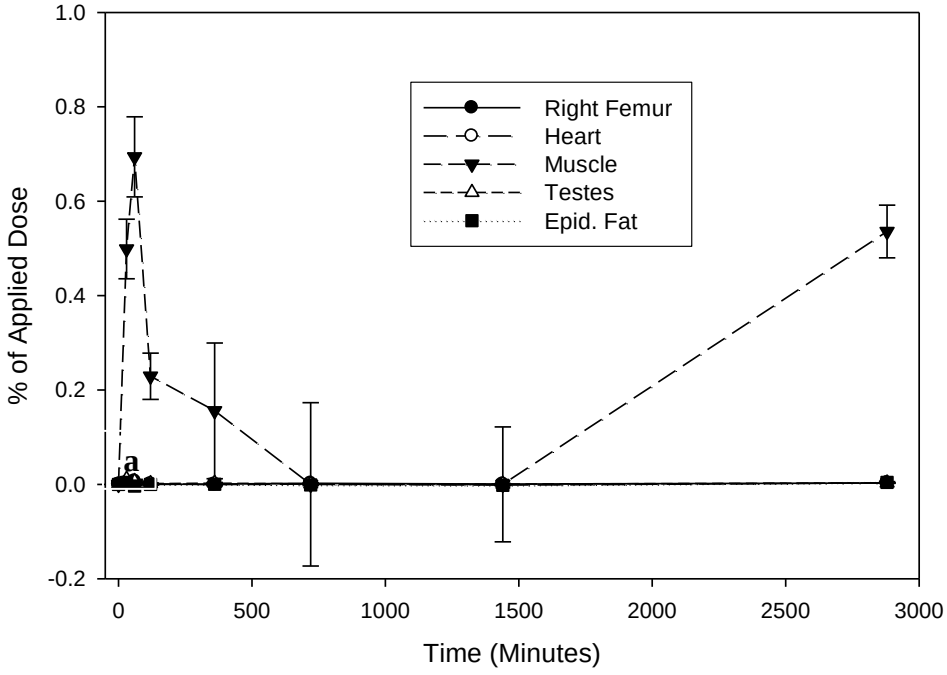

Figure 3B

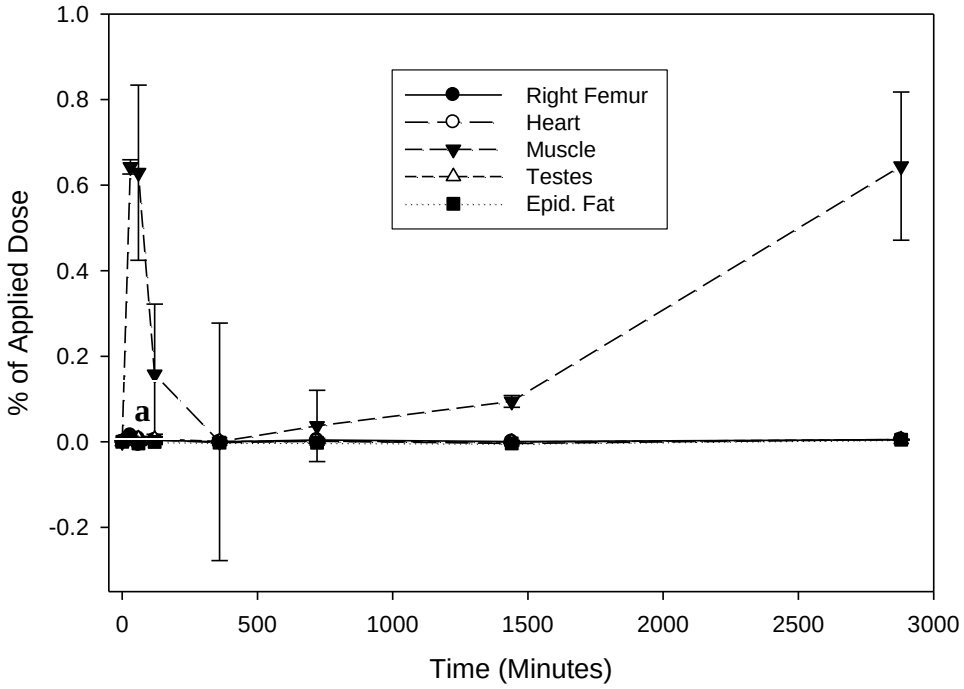

Figure 3C

Figure 3. Distribution of ^{51}Cr from $^{51}CrCl_3$ in the right femur, heart, skeletal muscle, testes, and epididymal fat as a function of time: A) Zucker lean rats; B) Zucker obese rats, a-significantly different from ZDF rats ($P<0.05$); and C) ZDF rats, a-significantly different from Zucker obese rats ($P<0.05$).

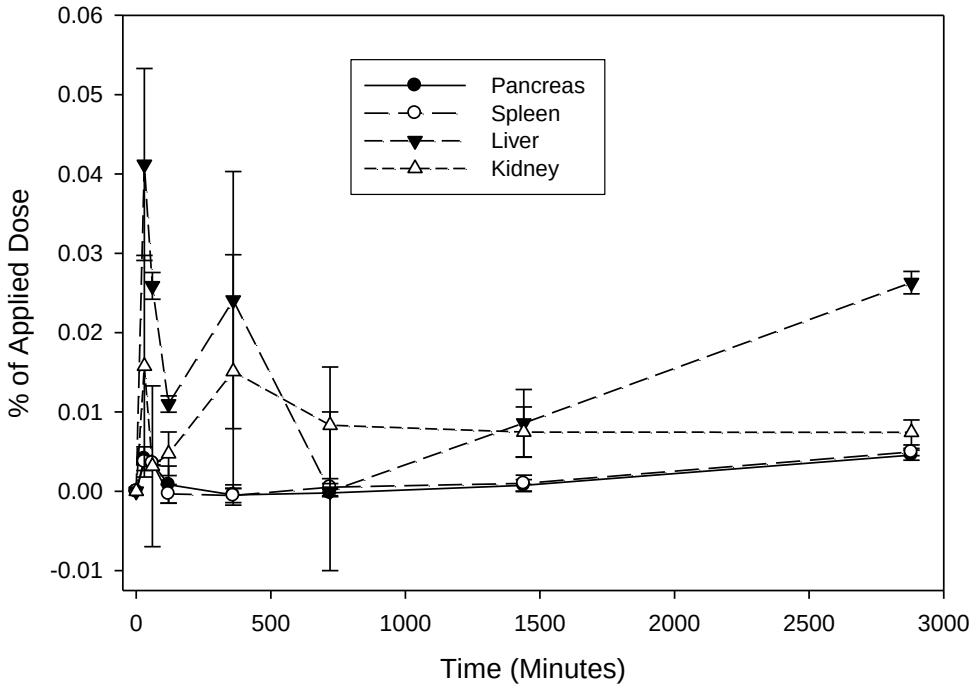

Figure 4A

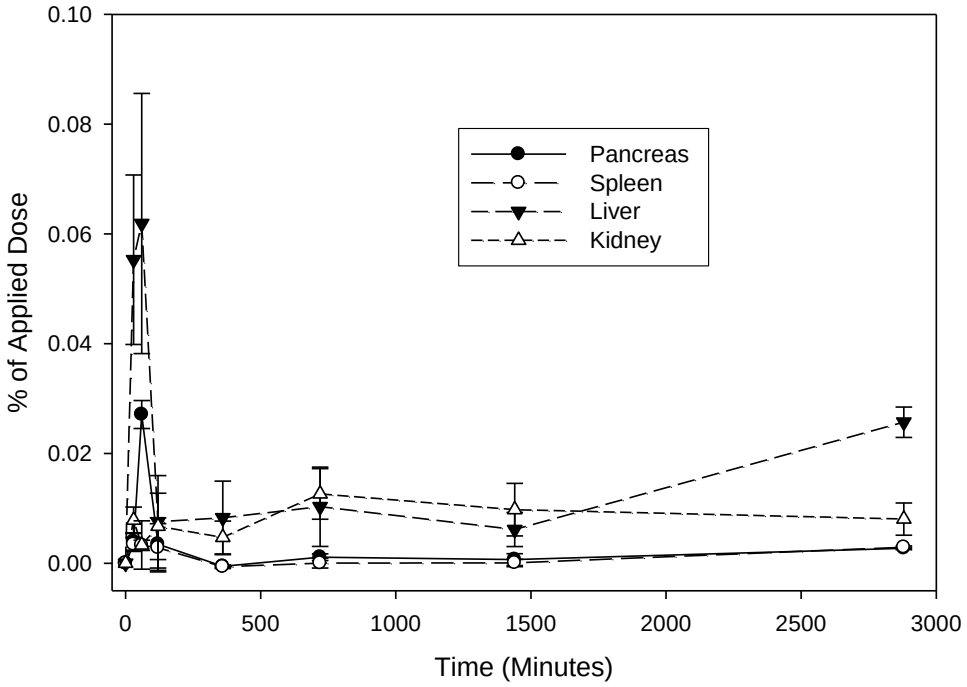

Figure 4B

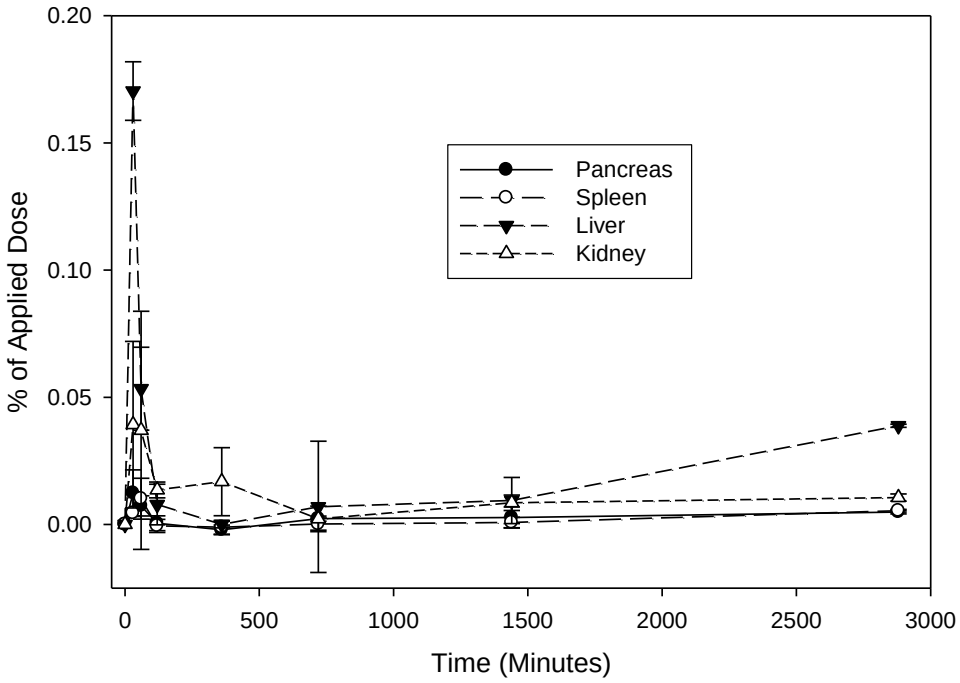

Figure 4C

Figure 4. Distribution of ^{51}Cr from $^{51}CrCl_3$ in the pancreas, spleen, liver, and kidney as a function of time: A) Zucker lean rats; B) Zucker obese rats; and C) ZDF rats.

intestine under these conditions.

Comparison data exists, although it is extremely limited. In 1979, Kraszeski and coworkers examined the blood serum and tissue Cr concentrations of healthy and streptozotocin (STZ)-induced diabetic rats one day and three days after an intravenous injection of ^{51}Cr; Cr was given in the form of $CrCl_3$ mixed with heparinized plasma.[19] Serum Cr levels were higher in the diabetic rats, but the levels returned to normal when the diabetic rats received daily insulin injections (2 insulin units per 100 g body mass) for a week before Cr administration (the last insulin injection was 18 hours before Cr administration). Diabetic rats had less retention of Cr (indicating they had greater urinary losses), and the effect could be partially reversed by insulin. The STZ-treated diabetic rats generally had increased Cr content in the liver and in some experiments had increased content in the pancreas and heart; the increased liver Cr content was reversed by insulin treatment.[19] The average liver Cr concentration of STZ-treated rats two hours after Cr administration was determined in a related study by Clodfelder, et al.[4] and was significantly larger than that of healthy rats, but the levels become equivalent after insulin treatment. Cr(III) was introduced as $Cr(III)_2$-transferrin; Cr is stored in the blood plasma as its transferrin complex. These workers also observed increased urinary Cr loss and increased tissue Cr levels that were reversed upon insulin treatment; thus observations are consistent between these two studies. Clodfelder and coworkers also examined Zucker obese rats in these studies.[4] The obese rats had greater urinary Cr loss than the healthy rat, although the Cr loss was reduced by insulin treatment to near levels of the healthy rats. Levels of Cr in the bloodstream with and without insulin treatment were similar in the obese and healthy rats. Cr content in the liver was only slightly higher in the obese rats, while insulin treatment led to a greater increase in liver Cr for the healthy rats than the obese rats. Cr content in the muscle was greater in the obese rats,

both before and after insulin treatment. The results of this study allow for some comparison with the present study. However, Anderson and Polansky have reported no effects from insulin and several other hormones on Cr retention in healthy and STZ-induced diabetic rats.[11] While no data were presented, 0.6-500 milliunits of insulin were stated to have had no effect on the fasted 200-250 g rats 1, 4, or 24 h after injection of $^{51}CrCl_3$. The form of insulin was not stated, and the time intervals between administration of insulin to the rats and the chromium administration are unclear as the "hormones were administered at times ranging from simultaneous injection of hormone to 24 hours before gavage of labeled Cr".[8] Mathur and Doisy[24] compared the results of the intravenous administration of $^{51}CrCl_3$ to healthy and STZ-treated rats. Cr contents of several tissues and the distribution of Cr in hepatocytes were examined. Unfortunately, the Cr content and distribution were examined 4 days after injection for the healthy rats and 5 days after for the diabetic models. Thus, comparison between groups is problematic, and the results are not comparable to other studies examining the rapid stages of Cr transport. Because the Cr was introduced intravenously in all these studies, they provide no information on whether absorption of chromium is affected by the diabetic conditions; however, the studies of Clodfelder, et al.[4] do demonstrate that the kinetics of the movement of Cr from the bloodstream to tissues and ultimately to the urine is altered in Zucker obese rats compared to healthy rats.

Feng and coworkers have examined the distribution of Cr in healthy and alloxan-induced diabetic rats.[25] The form of chromium administered was not determined, but given the preparation procedure, the form was probably $[Cr(H_2O)_6]^{3+}$. The Cr was administered intragastrically to fasted rats; thus, tissue and fluid Cr levels were influenced by absorption rates. Cr content in the stomach, small and large intestines, feces, urine, blood, liver, kidneys, muscle, femur, testes, heart, spleen, lung, pancreas, and brain were measured 1, 2, 4, 8, 24, 48, 96, and

168 h after administration. Although the rats in the present work were not fasted, the results of Feng, et al. do allow for comparison of the results with the type 2 diabetes models to those of the alloxan-induced, type 1 diabetes model. The distribution of the label with time in the gastrointestinal tract of the alloxan-treated rats is very similar to the three groups of rats in the current study. More than 90% of the Cr passed through the stomach in one hour, when over 80% of the label was located in the small intestine; the maximum level in the stomach (80-90% of the applied dose) was reached between 2-8 hours after application in the healthy rats and 4 hours after dosing in the alloxan-treated rats. The levels of Cr in the blood and liver were maximal 1 h after administration and similar in diabetic and control rats. In contrast, Cr levels in the muscle were maximal 4 h after administration and almost four times higher than the level in controls (which were maximal at 1 h). For the diabetic rats, levels of Cr in the kidney, liver, and femur rose from 8-24 h after administration, again potentially representing Cr movement into the medium-exchanging pool. After the Cr content of the blood and tissues had risen initially and as the Cr content began to drop, the Cr content of the urine increased, with the Cr content of the urine being two times higher for the alloxan-treated diabetic rats after 48 h and over four times higher after 168 h. The alloxan-treated diabetic rats had greater Cr absorption and greater urinary Cr excretion than controls.[25]

Thus, the results of the current study, in addition to those of Feng and coworkers, indicate that type 2 diabetic rats and type 1 diabetic rats have increased urinary Cr loss as a result of their diabetes; however, this increased urinary Cr loss is offset by increased absorption of Cr. Insulin resistant, obese rats have alterations in the rates of Cr transport and distribution compared to lean rats but have similar urinary Cr loss and Cr absorption. Thus, any increases in urinary Cr loss associated with insulin resistance or diabetes are offset by increased absorption. Given that

dietary Cr is normally absorbed with only ~1% efficiency, suitable Cr exists in the diet such that a standard diet possess sufficient chromium to allow for the increases in absorption associated with diabetes. Consequently, supplementing the diet with nutritionally relevant quantities of Cr is not anticipated to have any beneficial effects. Similarly, beneficial effects on plasma variables such as cholesterol, triglycerides, and insulin concentration, from supra-nutritional doses of Cr(III) complexes should not arise from alleviation of chromium deficiency. These beneficial effects must arise from pharmacological effects of high dose Cr(III).

Translating these results is difficult as studies of Cr absorption in humans are limited. [26, 27, 28,29] These studies tend to look at urinary Cr loss at one time period after Cr supplementation[27 ,28] or several time intervals over a period of days. [29] However, the extent of absorption is in the same range of ~2% or less for chromium picolinate and other commercial Cr supplements. One reported difference is that dietary Cr absorption has been reported to vary from ~0.5 to ~2% as a function of intake for humans,[21] while being independent of intake for rats.[23] While the rat study could be performed under very controlled conditions, the human study utilized self-selected diets that were analyzed for Cr content.[21] Fitting a plot of average Cr intake per day over a 7-day period vs. % absorption based on urinary Cr output generated a line with a slight negative slope indicating an inverse relationship between intake and absorption. However, no error analysis was reported. This study, upon which many determinations about the necessity of Cr have been based (*vide infra*), needs to be reproduced with statistical analysis.

In Chapter 4 the essentiality of Cr is discussed. Nielsen proposed that Cr should be considered a "nutritionally or pharmacologically beneficial element" in that in supra-nutritional amounts may have positive effects when an animal is under a stress.[30] Part of the difficulty in being more precise about a role of Cr under these stressors is often determining cause and effect.

For example, recent studies have found a relationship between Cr status and cardiovascular disease.[31, 32] Does the disease or stress result in a mechanism by which the distribution of Cr is altered resulting in the loss of Cr from the body or are low Cr levels in part responsible for the symptoms of the disease? These results clearly indicate that for diabetic rats Cr balance could be maintained and that Cr loss should not be responsible for the symptoms associated with these types of glucose intolerance or insulin resistance. That Cr balance appears to be maintained could be suggested as evidence that Cr is essential and that losses are specifically compensated. However, the increased urinary output of the diabetic rats depletes the body of several chemicals, not just Cr; the increased absorption of several chemicals, including other minerals, could result in the increase in Cr, such that the increase is actually not specific. For example, the absorption, transport, and distribution of Cr utilizes the iron transport protein transferrin.[33] Similarly, increases in plasma insulin concentrations result in increased movement of both iron and Cr from the bloodstream to the tissues via the protein transferrin. Increased iron absorption could potentially readily result in an accompanying increase in Cr absorption. Increased iron stores are associated with type 2 diabetes, [34] while subjects with type 2 diabetes also have increases in urinary iron output.[35] Further study of the relationship between insulin-dependent iron and Cr absorption and transport are necessary before additional conclusions can be drawn.

2.5 Conclusion

Thus, the results described herein in addition to those of Feng and coworkers[25] indicate that type 2 diabetic rats and type 1 diabetic rats have increased urinary Cr loss as a result of their diabetes; however, this increased urinary Cr loss is offset by increased absorption of Cr. Insulin resistant, obese rats have alterations in the rates of Cr transport and distribution compared to lean

rats but have similar urinary Cr loss and Cr absorption. Thus, any increases in urinary Cr loss associated with insulin resistance or diabetes are offset by increased absorption. Given that dietary chromium is normally absorbed with only ~1 % efficiency, suitable Cr exists in the diet so that a standard diet possess sufficient chromium to allow for the increases in absorption associated with diabetes. Consequently, supplementing the diet with nutritionally relevant quantities of Cr is not anticipated to have any beneficial effects. The increased loss of Cr in the urine associated with diabetes cannot be taken as evidence for the essentiality of Cr, especially as supra-nutritional doses of Cr are required for any beneficial effects to be observed. These effects cannot be derived from the restoration of Cr sufficiency from a Cr deficient state but must come from a pharmacological mechanism.

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Chapter 3: Urinary chromium excretion in response to an insulin challenge is not a biomarker for chromium status

3.1 Introduction

Cr has been believed to be an essential element for over 50 years, but as seen in Chapter 2 it is not conditionally essential, due to the increases in absorbance which offset increases in excretion in Zucker diabetic fatty rats. Currently, no known biomarker for Cr status exists, but Cr is known to be excreted in the urine in response to insulin.[1] If the level of increased Cr excretion can be correlated to insulin sensitivity (as determined from glucose or insulin tolerance tests), then it could be used as a possible biomarker for Cr status.

One of the current problems in the field of Cr biochemistry is a lack of a biomarker for chromium status. Studies on supplementation in humans have led to variable results, because of the variation of Cr statuses prior to the start of the studies.[2] Cr status refers to the pool of utilizable Cr, not simply total Cr in the blood or tissues. Urinary and plasma Cr do not correlate with tissue Cr concentrations nor with plasma glucose, insulin, or lipid levels.[3] However, previous studies have not examined mobilizable Cr to determine Cr status as a viable option for determining Cr status.

Rats increase urinary Cr excretion in response to an insulin or glucose challenge.[4] Providing animals a diet with as little Cr as reasonably possible and not seeing a significant difference in urinary Cr excretion would suggest that the diet cannot induce a Cr deficiency. In contrast, if they are truly Cr deficient, then animals on the low Cr diet should have a greatly reduced urinary chromium response to a glucose or insulin challenge, and also the rats with the

lowest level of Cr in their diet should show decreased insulin sensitivity in comparison to the Cr sufficient animals if insulin sensitivity is directly related to Cr status. These experiments should also allow for the determination of whether Cr status (insufficient or sufficient) correlates with insulin-sensitive urinary Cr output.

3.2 Methods

3.2.1 Chemicals and blood variable assays

Glucose and insulin (bovine, zinc) were obtained from Sigma–Aldrich. The final concentrations of glucose and insulin were prepared using doubly deionized water. Plasma insulin was measured using an ^{125}I RIA kit from MP Biomedicals. Gamma counting was performed using a Packard Cobra II auto-gamma counter. Blood glucose levels were measured using a One-Touch glucose meter. Iron content was determined using a modified colorimetric method for determining non-heme iron concentration in biological samples.[5] Iron content assay was performed by Kristin R. Di Bona.

3.2.2 Animals

Thirty-two male Zucker lean rats were obtained from Charles River Breeding Laboratories International at 6 weeks of age. (Male rats were chosen for consistency with previous studies, while the use of Zucker lean rats would allow for the effects of health condition to subsequently be examined by comparison of results with those of Zucker obese and Zucker diabetic fatty rats if urinary Cr loss would prove to be a potential biomarker for Cr status.) Rats were maintained at 22 ± 2 ^{o}C and 40–60% humidity with a 12-h photoperiod and were acclimated for 2 weeks prior to treatment. They were housed individually in specially constructed metal-free housing (*vide infra*) to prevent the introduction of additional chromium into their diets. Rats were fed specific diets and distilled water ad libitum for a 29-week period

prior to glucose and insulin challenges. Rats were weighed, and food consumption was measured twice weekly. All procedures involving these animals were reviewed and approved by The University of Alabama's Institutional Animal Care and Use Committee.

3.2.3 Treatment

Male Zucker lean rats were randomly separated into four treatment groups, each containing eight rats as follows: (1) rats on a purified AIN-93G chromium-sufficient diet [Cr as $KCr(SO_4)_2 \cdot 12H_2O$, the Cr source designated for the AIN-93G diet], (2) rats on the AIN-93G diet, with chromium not included in the mineral mix, (3) rats on the AIN-93G chromium-sufficient diet with an additional 200 µg Cr/kg [Cr as $KCr(SO_4)_2 \cdot 12H_2O$], and (4) rats on the AIN-93G chromium-sufficient diet with an additional 1,000 µg Cr/kg [Cr as $KCr(SO_4)_2 \cdot 12H_2O$]. Purified AIN-93G rodent diets and modified AIN-93G diets were obtained from Dyets (Bethlehem, PA, USA). Diets were received in powder form.

3.2.4 Housing

Housing in metal-free caging was performed. Iris Buckle Up boxes were obtained from Target; the boxes were approximately 18 cm high, 45 cm wide, and 28 cm long. These boxes are made of clear plastic with a removable lid that attaches with latches on both 28-cm sides of the boxes. Holes (4 mm in diameter) were drilled with an electric hand drill in all five sides of the box and in the lid using a square grid pattern with approximately 5 cm between holes. Holes (4 mm in diameter) were also drilled in the corners of the bottom of each box to facilitate urine drainage. Shavings of plastic were removed from the holes, and any rough spots were smoothed using fine sandpaper. An additional hole was drilled in the lid with an appropriate diameter to accommodate the tube of the water bottles, and another hole was drilled in the lip of the box to accommodate a hanging cage card holder. Tube tread no. 116 wet area antifatigue mats were

purchased from General Mat Company. The matting is made of vinyl with a tensile strength of 139 kg/cm and is flexible from -10 to 100 ^{o}C. The matting was cut with a knife to fit inside the base of the boxes. Both the boxes and the matting could pass through multiple cycles of a cage washing machine without noticeable damage. As the boxes are similar in size to shoebox-type housing, they were kept on a standard rack for animal cages. The cages were placed on absorbent bench paper or newspaper. The rear of the cage was elevated approximately 1 cm using scrap pieces of the matting material placed under the rear of the cage to ensure drainage of urine.

3.2.5 Food and water

Wheaton clear straight-sided, wide-mouth glass jars (about 9 cm in diameter, 9.5 cm in height, 473 mL) and plastic lids (89–400-mm screw cap size) were obtained from Fisher Scientific and were used to hold food. A 5-cm-diameter circular opening was cut in the polyvinyl-lined plastic lids to allow the animals access to food. To prevent the rats from dumping the powdered food from the jars, a 2-cm-thick Plexiglas disk (about 7 cm in diameter) was placed on the food. The disk had a 14-mm-diameter circle cut out in the center, with six other 14-mm-diameter circles cut in a hexagonal pattern around the center circle; the disks were prepared by The University of Alabama College of Arts and Sciences machine shop. To provide water, the stainless steel tubes were removed from the water bottles and replaced with glass tubes. The University of Alabama glass shop cut and bent glass tubing of the appropriate diameter to match the length and shape of the stainless steel tubes. To prevent potential injury, the end of the tubing exposed to the rats was fire-polished.

3.2.6 Chromium in diet concentration determinations

Samples of each powdered diet (200 mg) were digested with a 30:1 mixture of ultra high purity concentrated HNO_3 (99.99 % trace element free) and ultra high purity concentrated H_2SO_4

(99.99 % trace element free). The digestion was continued with controlled heating (sub-boiling) until heated to dryness. Then, the residue was diluted to 10 mL with doubly-deionized water (Milli-Q Millipore). All glassware was acid washed. Blank digestions were carried out in the same fashion. Chromium concentrations were determined utilizing a Perkin Elmer Analyst 400 atomic absorption spectrometer equipped with HGA-900 graphite furnace and an AS-800 autosampler using a Cr hollow cathode lamp operating at 10 mA; a spectral bandwidth of 0.8 nm was selected to isolate the light at 353.7 nm. Operating conditions (temperature (^{o}C), ramp time (s), hold time (s)): drying 1 (100, 5, 20), drying 2 (140, 15, 15), ashing (1600, 10, 20), atomization (2500, 0, 5), and cleaning (2600, 1, 3). Other instrumental parameters include curvette: pyrolytic, carrier gas: argon (flow 250 mL/min), sample volume: 20 μL, and measurement mode: peak area. The digestion and atomic absorption methods were verified by analysis of a certified reference material, 1573a Tomato Leaves (NIST). The values were AIN-93G, 1,135 μg Cr/kg; AIN-93G without mineral mix, 16 μg Cr/kg; AIN-93G + 200 μg Cr/kg, 1,331 μg Cr/kg; and AIN-93G + 1,000 μg Cr/kg, 2,080 μg Cr/kg. All values were close to anticipated values. Analysis was performed by Sarmistha Halder Sinha.

3.2.7 Urine Collection

After 21 weeks on the diets, the rats were placed in metabolic cages for 6 hours prior to and removed 12 hours after an intravenous glucose challenge (1.25 mg glucose/kg body mass). Urine was collected prior to injection and two hours, six hours, and twelve hours post injection. The first eight hours of the urine collection occurred during the dark period with the remainder occurring during the photoperiod. The urine was transferred to pre-weighed disposable centrifuge tubes and stored at -20 °C. After continuing on the diet another two weeks, the rats were placed in metabolic cages for 6 hours prior to and removed 12 hours after an intravenous

insulin (5 insulin units (bovine, zinc)/kg body mass) challenge. Urine was collected as described for the glucose challenge. Rats had unrestricted access to food and water during the urine collection period.

3.2.8 Chromium concentration in urine

Each urine sample was digested with a mixture of ultra high purity concentrated HNO_3 (99.99 % trace element free) and 30% H_2O_2. The digestion was continued with controlled heating (sub-boiling) for 15 hours. All glassware was acid washed. Blank digestions were carried out in the same fashion. Chromium concentrations were determined utilizing a Perkin Elmer Analyst 400 atomic absorption spectrometer equipped with HGA-900 graphite furnace and an AS-800 autosampler using a Cr hollow cathode lamp operating at 8 mA; a spectral bandwidth of 0.8 nm was selected to isolate the light at 353.7 nm. Operating conditions (temperature (^oC), ramp time (s), hold time (s)): drying 1 (90, 45, 20), drying 2 (140, 20, 20), ashing (800, 15, 15), atomization (2500, 0, 5), and cleaning (2700, 1, 5). Other instrumental parameters include cuvette: pyrolytic, carrier gas: argon (flow 250 mL/min), sample volume: 20 µL, and measurement mode: peak area. Urine chromium concentrations were calculated using the method of standard additions with samples spiked with 10, 20, 30 and 50 µg/L of PerkinElmer Pure Atomic Spectroscopy Standard 1000 µg Cr/mL in HNO_3. Fits of the standard addition lines had r^2 values >0.98, while each triplicate point generally had standard deviations less than 2%.

To test whether urine could be contaminated by feces in the metabolic cages, ^{51}Cr-containing rat feces (available from previous work) [6] were used to line the urine and feces collection component of the metabolic cage; a rat was housed in the cage (with food and water)

and urine was collected. ^{51}Cr content of the urine was then determined by gamma counting. Contamination of the urine with Cr from the feces was insignificant.

3.2.9 Statistical analysis

Statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA). Data are represented graphically as average values with standard error (SE) bars. Data were calculated independently, tested for homogeneity of variance with Levene's test, and analyzed using univariate analysis of variance and descriptive statistics. For eight animals per group, an expected difference between two means would be significant at $\alpha = 0.05$ level and $1-\beta = 0.01$ if the difference between the means is twice the standard deviation; these values are reasonable based on the effects of insulin on urinary Cr in Sprague Dawley and Zucker obese rats. [7] Blood insulin and blood glucose tolerance tests were further analyzed for the area under each curve. Post hoc least significant difference analyses were used to indicate significant differences at a 95% confidence level ($p \leq 0.05$). Area under the curve was calculated using the trapezoid rule.

3.3 Results

3.3.1 Metal-free Caging

Dietary studies examining the roles of trace metals in the diet require animals be in an environment free from access to extraneous metal, including all cage components and bedding. A majority of all small animal housing uses stainless steel, which, in addition to being comprised of iron, has significant quantities of other metals, most notably chromium. Traditional bedding is made of natural materials that contain a variety of metal ions and is unsuitable for use in many such studies. The removal of metal housing components and bedding can be challenging as replacement components must be stable under the experimental conditions (including those used to wash the housing) and not be hazardous to the animals or susceptible to be damaged by the

animals (for example, by gnawing). Developing suitable, metal-free housing that should require less labor and construction was possible.

Conventional shoebox-type caging utilizes a plastic container for the base and walls of the housing with a stainless steel metal mesh lid. The lid allows for plenty of circulation of air in the cage; the metal mesh is the only surface the rats can chew. The metal housing component must be replaced while maintaining adequate ventilation and without introducing plastic material for the rats to chew. Thus, for example, a simple plastic mesh lid would not suffice. As an alternative, the use of plastic storage boxes was examined. The boxes utilized were Iris Buckle Up boxes with a volume of approximately 22 L (Figure 1). The similar size to shoebox-type housing allows these cages to be placed on regular racks for shoebox-type housing. Latches on two sides of the lid allowed the box to be securely closed so that animals could not escape. The only required modification of the boxes was the drilling of numerous small holes that could be performed with a standard electric hand drill; this greatly simplifies construction compared to previous reported metal-free housing. The pattern of 4-mm holes drilled on all sides of the box and the lid provided ventilation, while the holes on the floor allowed urine to flow out of the box onto absorbent paper. This pattern of holes with the 5-cm spacing does not compromise the integrity of the sides of the boxes, so that the boxes are sturdy and do not break easily. One additional hole was required to accommodate the tube from the water bottle. As The University of Alabama Animal Care Facility normally utilizes water bottles with stainless steel tubes for rodents, replacement of the tubes was readily accomplished by using glass tubing of the same diameter cut to the same length and bent to the same shape as the original steel tube. The end of the glass tubing utilized by the rats was fire-polished.

To prevent rats from walking in fluids draining from the bottom of the cage, a piece of vinyl anti-fatigue matting was placed in the bottom each cage. The matting comes in rolls from which the desired size pieces can readily be cut. The rats had surprisingly little desire to chew on the matting. A rat placed in the box without the matting would rapidly climb on top of its food container to avoid the liquid on the bottom of the cage; the addition of the mat immediately eliminated this problem. The back of the cage bottom was elevated by ~1 cm to facilitate drainage of liquid from the box.

The metal-free housing required cleaning every two days as hair and dander accumulated in the absence of bedding. The cages could be readily cleaned using a standard mechanical cage washing system. The high temperature and pressure water had no observable effects on the plastic boxes or mats, even after 6 months of use. In some areas of the animal care facility rooms where the metal-free cages were kept, moisture accumulated near the top of the housing initially, indicating that sufficient circulation of air to allow the metal-free cages to vent moisture from the rats was not present in all parts of the room. This was readily fixed by placing a household circulating fan in each room. The cages only have sufficient ventilation to house one rat per cage. Measurement of the temperature in the cages indicated that the temperature in a shoebox type cage with bedding stayed one to two degrees warmer than the temperature in the metal-free housing.

Zucker lean rats with the same birth date and shipping date were maintained in either regular shoebox type housing with conventional bedding or in metal free housing. The rats kept in the shoebox type housing were obtained for a different study but provided data useful for this comparison. Over the course of three months, both groups had identical body masses (data not shown) and were identical in appearance. No differences were observed in the rate or type of

health issues between rats in conventional shoebox housing and the metal-free housing; no behavioral differences were noted as well.

3.3.2 Testing Urinary Cr Status as a biomarker

To better determine the urinary Cr status in response to glucose or insulin challenges, controlled diets, as well as a controlled environment, were utilized. To avoid added dietary stresses, the animals were fed a standard USDA diet with only the amounts of added Cr altered. All diet groups averaged approximately 225 g in body mass at the start of the study; the average body masses of the groups at the end of the study were all between 400 and 500 g and statistically equivalent. Diet did not affect body mass. The amounts of Cr in each diet were measured using graphite furnace atomic absorption. If the amounts of Cr excreted are to be used as a biomarker; then, as the amounts of Cr in the diet increase, the amounts should of urinary chromium mobilized should increase and be comparable, while a saturation point for excretion should also be observed. If Cr is essential, the Cr sufficient animal should mobilize Cr more efficiently than the low Cr diet animal because the sufficient animal should be able to maintain the mobilizable pool of Cr more effectively. This method of determining Cr status has not been examined previously.

Samples of urine from each animal in the study were maintained and measured for Cr concentration, which is reflected in the rate of ng Cr/h where each sample is normalized to the amount of time elapsed between sample collections. The trend of increasing Cr excretion can be observed in both Figures 3 and 4. Each point in Figures 3 and 4 are representative of 120 points in a standard addition plot.

Figure 1

Figure 1. The metal-free housing unit with the anti-fatigue matting inside the cage.

In response to glucose, the rate of urinary Cr excretion increases for the animals on AIN-93 diet with no added Cr, with the sufficient amount of supplemented Cr and with an additional 200 μg Cr/kg (Figure 3). The urinary Cr excretion for these animals returns to baseline by 12 hours after the glucose challenge. The animals on the highest supplemented diet, 1000 μg Cr/kg, have no response to the glucose challenge. This situation appears to be reminiscent of the study of Anderson and coworkers[8] where an increase in urinary Cr loss was observed in individuals on a self-selected diet but not for individuals supplemented with 200 μg Cr daily. Thus, a Cr intake apparently can be achieved in humans and rats where the Cr transport system is overwhelmed so that an insulin or glucose challenge results in no apparent increase in urinary Cr loss. Over the course of 12 hours from the challenge, the rate of urinary Cr loss is identical for all groups, as shown from identical areas under the curve (AUC) (Figure 5).

The response to insulin is nearly identical to the response to glucose. The responses are slightly larger, but follow a similar trend. In response to insulin, urinary Cr loss rates tend to increase for rats on the AIN-93G diet with no added Cr, the diet supplemented with the recommended amount of Cr, and the diet supplemented with an additional 200 μg Cr/kg diet (Figure 4). This again is the anticipated result based on the previous studies with humans and rats described above. The urinary Cr rates actually tend to dip below baselines from 2 to 6 hours after to the challenge before tending to return to baseline values by 12 hours after the challenge. However, the insulin challenge again had no response on the rates of urinary Cr loss in the rats receiving the largest amount of Cr, the AIN-93G diet with an additional 1 mg Cr/kg diet (Figure 4). Over the course of 12 hours from the challenge, the rate of urinary Cr loss is identical for all groups, as shown from identical areas under the curve (AUC) (Figure 6).

The Cr content of the diets needs to be put in perspective. For the diet with the lowest Cr content, 16 µg Cr/kg diet corresponds to about 2.4 µg Cr/kg body mass/day (assuming that a 100 g rat eats 15 g of food a day[9]). Assuming an average body mass for a human of 65 kg, this corresponds to approximately 1.6 x 10^2 µg Cr/day. Thus, the diet would be Cr sufficient for humans, based on the AI of 30 µg/day.[10] The diet supplemented with 2 mg Cr/kg corresponds to about 20 mg Cr per day for a 65 kg human in this simple analysis (without potential corrections for surface area, metabolism, etc.). Curiously, humans supplementing their diet with 200 µg Cr/daily appear to have the Cr transport system saturated with Cr so that no increase in urinary Cr loss occurs with a glucose challenge while the transport system in rats is overwhelmed by a proportionally smaller Cr intake.[8]

The standard errors in Figures 3 and 4 are surprisingly large. However, the magnitude of the error does not arise from the determination of the Cr concentrations but rather from the range of individual response to the challenges. (One can readily note in Figure 3 the change in the standard deviation from the baseline values to values after the challenges.) Figure 7 displays the effects of the insulin challenge on the eight individual rats on the AIN-93G diet containing the standard quantity of Cr in the mineral mix. Note the wide variation in responses between rats. This wide range in individual response mirrors that observed for humans on self-selected diets and self-selected diets supplemented with Cr reported by Anderson and coworkers;[11] subjects on supplemented diets displayed a much greater range of urinary Cr loss so that "urinary Cr excretion after a glucose challenge was not predictable."[8] Humans eating self-selected diets experience an increase of urinary Cr excretion in response to an increased level of insulin, which suggested that Cr was mobilized in direct response to insulin rather than glucose. The movement of Cr was found to be variable between individuals, with some individuals having no

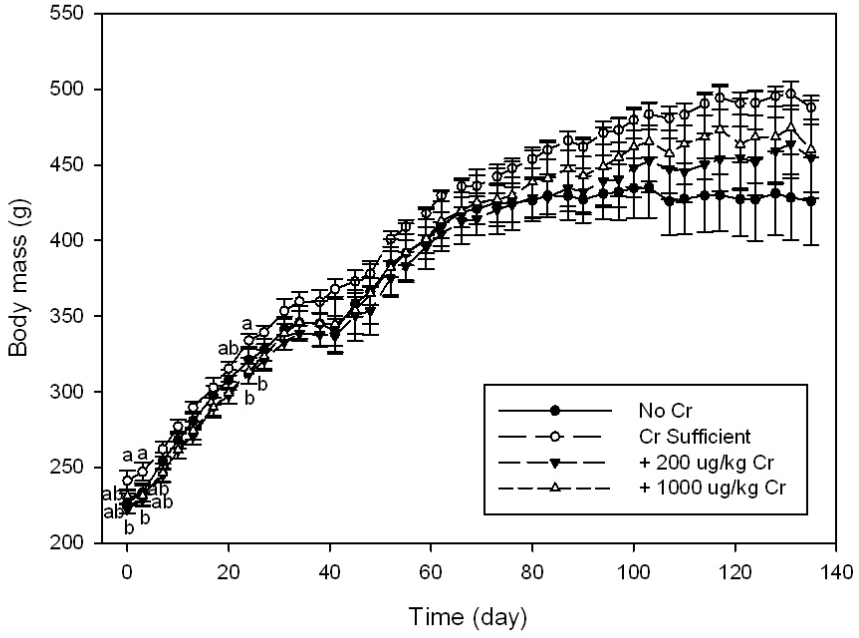

Figure 2. Body mass of Zucker lean rats on the AIN93-G diets. Different letters indicate significant differences between groups. No Cr - rats on the AIN93-G diet, with Cr not included in the mineral mix; Cr Sufficient - rats on a purified AIN-93G Cr sufficient diet (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); + 200 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 200 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); +1000 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 1000 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$).

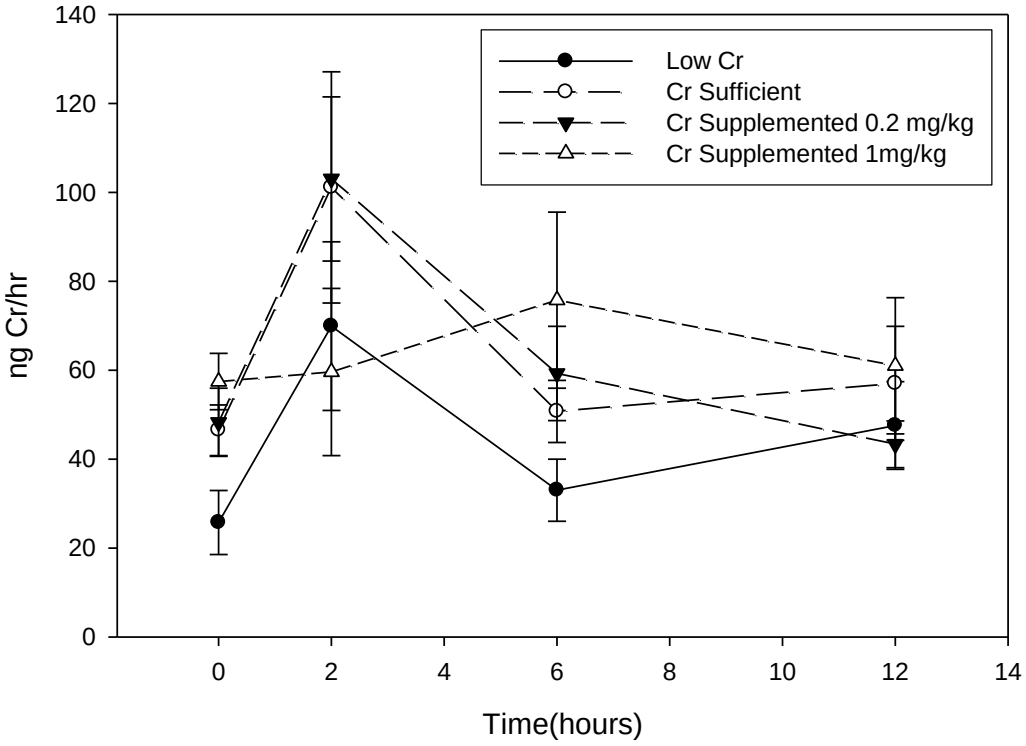

Figure 3. Rate of urinary Cr loss (ng Cr/h) for Zucker lean rats on purified diets over the six hours before an insulin challenge (5 insulin units (bovine, zinc)/kg body mass), over the two hours after a glucose challenge, between two and six hours after a glucose challenge, and between six and twelve hours after a glucose challenge.

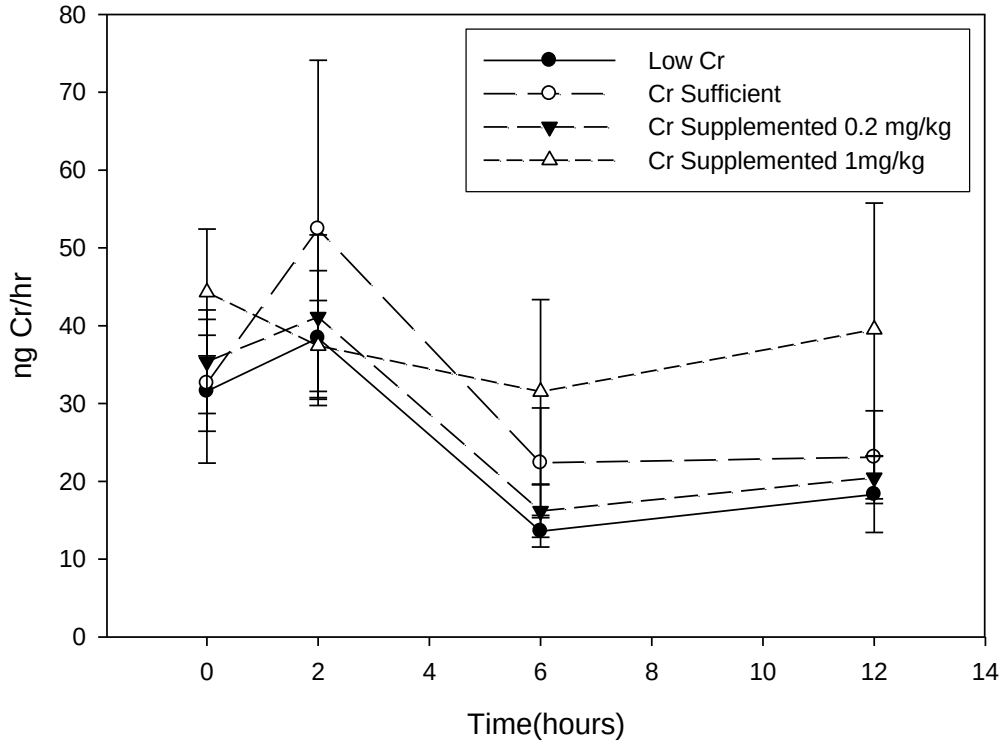

Figure 4. Rate of urinary Cr loss (ng Cr/h) for Zucker lean rats on purified diets over the six hours before a glucose challenge (1.25 mg glucose/kg body mass), over the two hours after a glucose challenge, between two and six hours after a glucose challenge, and between six and twelve hours after a glucose challenge.

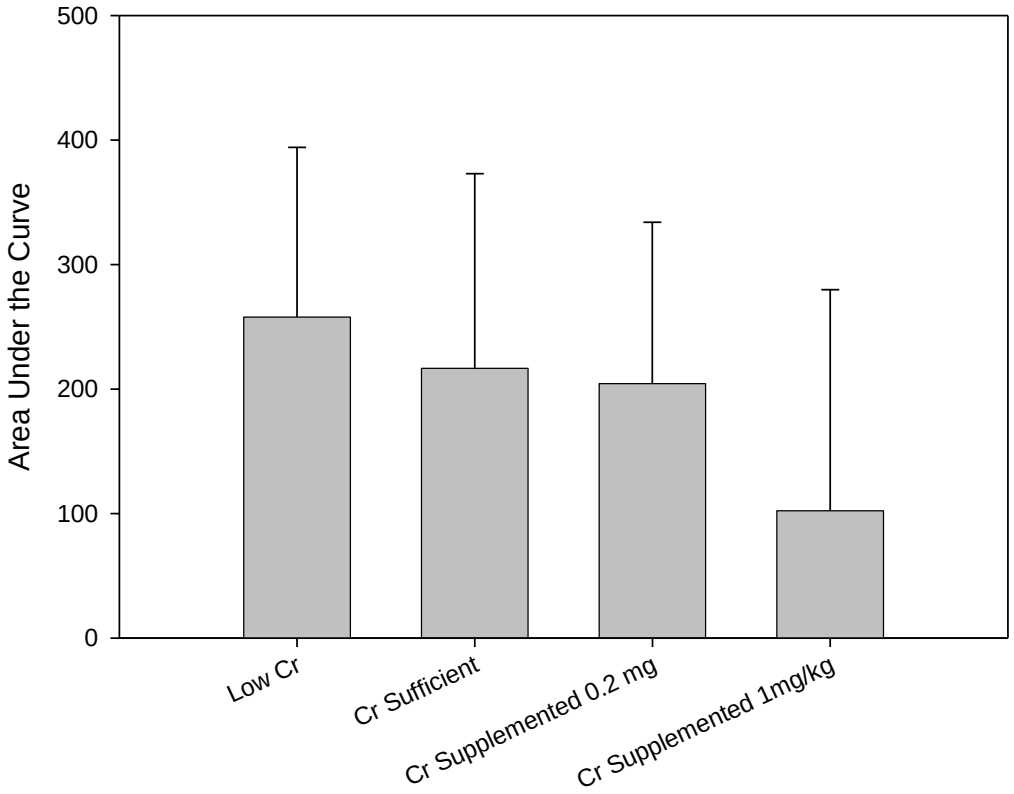

Figure 5. Area under the curve (AUC) for the rate of urinary Cr loss in the insulin tolerance tests. The area is the total area minus the area where no change occurred from baseline values.

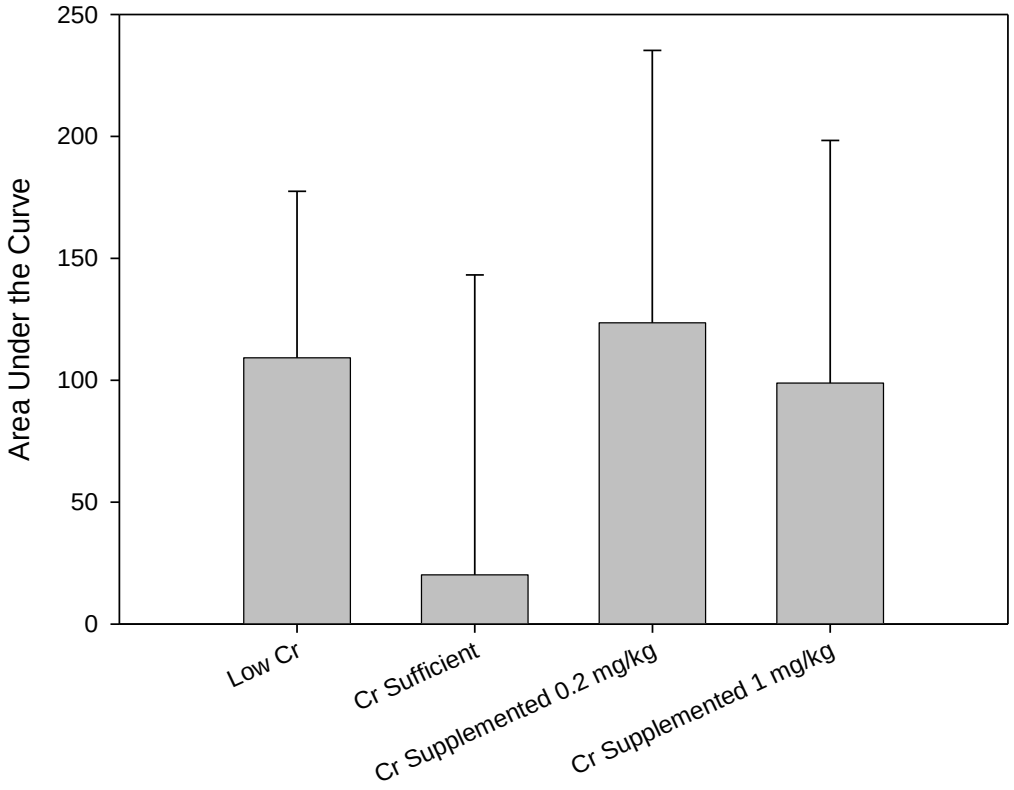

Figure 6. Area under the curve (AUC) for the rate of urinary Cr loss in the glucose tolerance tests. The area is the total area minus the area where no change occurred from baseline values.

change in movement of Cr in response to an increased level of insulin. This variability was attributed to decreased glucose tolerance.[8]

Human urine and serum Cr levels have been shown to reflect Cr intake. Serum Cr levels ninety minutes after a glucose challenge correlate with levels prior to the glucose challenge. Human urine Cr concentrations however do no correlate with serum glucose, insulin, lipid parameters, age or body mass.[2]

3.3.3 Effect of supplemental chromium on blood plasma iron concentrations

Cr (III) is known to compete with ferric iron for the metal binding sites of the iron transport protein transferring in the blood. Kristin R. Di Bona analyzed the non-heme iron concentrations in the blood. The concentration of iron was not significantly altered by the Cr supplementation in the diets as shown in Figure 8. The AIN-93 diet provides 35 mg of Fe per kg diet from its mineral mix[12] (in addition to the Fe contents of the other components) while the diet with the most Cr in this study contained ~2 mg Cr/kg diet, a significant effect on plasma iron would not be expected.

3.4 Discussion

To test the ability of urinary Cr excreted as a biomarker, the conditions in which the animals were studied under had to be carefully controlled. The animals were fed purified diets with no added dietary stresses (e.g., inclusion of high fat or high sugar content). The animals lived in metal free environments. Plastic shoe box type cages with no metal mesh cage tops and with the metal tube of the water bottles replaced with a glass tube were utilized. The Cr content

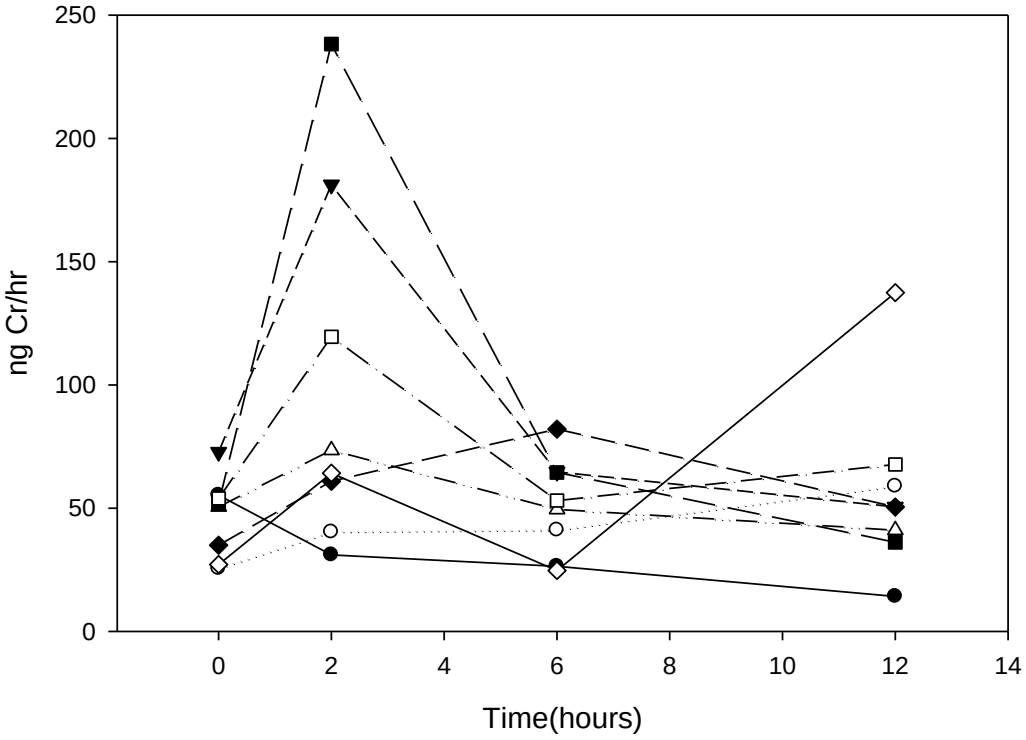

Figure 7. Rate of urinary Cr loss (ng Cr/h) for individual Zucker lean rats on AIN-93G diet containing the standard quantity of Cr in the mineral mix over the two hours before an insulin challenge (5 insulin units (bovine, zinc)/kg body mass), over the two hours after a glucose challenge, between two and six hours after a glucose challenge, and between six and twelve hours after a glucose challenge.

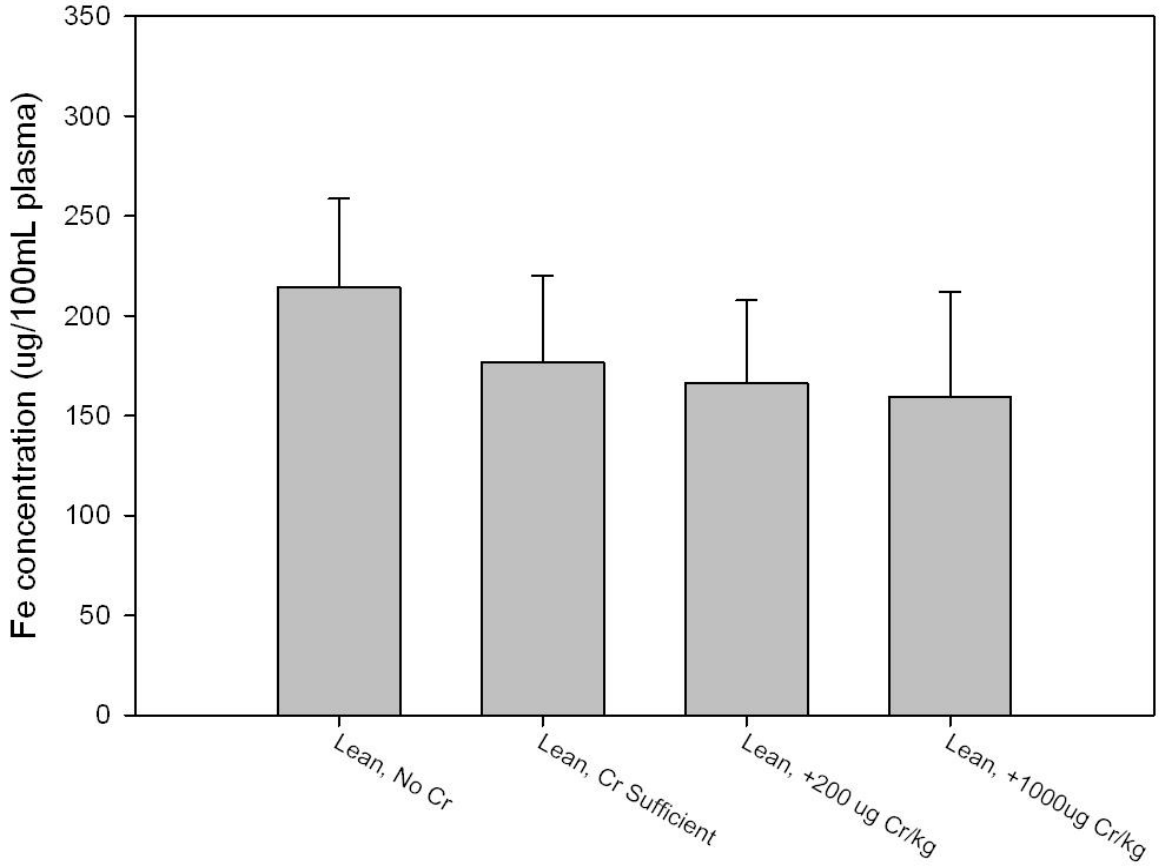

Figure 8. Blood plasma iron concentrations. All values are statistically equivalent.

of the diet without added Cr was measured as a baseline value of Cr. Finally, the animals were provided doubly deionized water to remove the possibility of Cr contamination from the water.

To test for signs of dietary stress, food intake and body mass were monitored. Figure 1 displays the body mass of the rats over time in the study. The diets had no significant effect on body mass or food intake (data not shown) throughout the course of the study. Significant differences for the masses of the various rat groups were only observed on three days.

As shown in Figures 3 and 4, the rate of excretion of chromium after the rats were injected with insulin or glucose increased in all groups except the rats on the diet supplemented with 1 mg Cr /kg. The glucose challenge had a smaller effect on the increase in rate of chromium excretion. None of the points are significantly different, perhaps due to the large standard error of each point. The large standard errors are not to be attributed to issues with sample analysis; but, as seen in Figure 7, the responses of individual rats ranged greatly.

The amount of Cr released in direct response to an insulin or glucose challenge should theoretically reflect whether an adequate amount of Cr is available for insulin potentiation. This in turn should reflect Cr status, assuming Cr is an essential element. In humans, urine Cr levels have been shown to reflect Cr intake.[8, 2, 11] Human serum Cr levels reflect Cr intake.[13] Serum levels of Cr 90 min after a glucose challenge correlate with Cr levels before the challenge, [13] but human urine Cr concentrations do not correlate with serum glucose, insulin, or lipid parameters or with age or body mass.[11] An increase in urinary Cr excretion has been reported for human subjects on self-selected diets in response to a glucose challenge, while no effect was observed for individuals taking a Cr supplement (200 µg Cr as $CrCl_3$ for 3 months).[8] The conclusion drawn from this paper was urinary Cr loss after a glucose challenge was found not to be predictable and suggested to not reflect Cr status.[8] Yet, the extent of movement of chromium to

the urine in response to a glucose challenge did change, from an increase at normal Cr intake to no increase when supplemented with Cr (the inverse of the expected observation). Also in this study, the Cr intake of the individuals in the study was not established. Notably, self-selected American diets have been shown to provide on average about 30 μg Cr daily, a quantity that has been deemed an adequate intake (AI) so that >98% of individuals receiving this quantity cannot be considered Cr deficient.[10] Also, noting that these studies were performed after 1978 when the techniques utilizing graphite furnace atomic absorption for determining Cr concentration in tissue and body fluids were improved and demonstrated the previous values published by others were and order of magnitude too high is important.[3]

The results from humans on self-selected diets are consistent with studies of urinary Cr loss in subjects on diets supplemented with a variety of varying carbohydrates.[14] Cr appears to be mobilized in response to insulin, rather than directly to glucose or other carbohydrates, as determined in this study by the link between increases in circulating insulin as compared to urinary chromium excretion. The greater the increase in the amount of insulin in the blood in response to the various carbohydrates, the more Cr was lost in the urine.[14] A range of responses to the carbohydrates was noted. Subjects receiving glucose or glucose and fructose as the carbohydrate sources showed the highest circulating insulin values and urinary Cr losses. Some of the subjects who in response to the diets had the highest circulating blood insulin levels had decreased abilities to mobilize Cr for excretion in the urine (within 90 min); thus, a group of subjects with decreased carbohydrate tolerance appeared to have decreased urinary Cr loss.[14] The Cr content of the self-selected diets of individuals in the study was not determined, and the subjects do not appear to have been questioned about whether they were consuming any Cr-containing supplements.[14, 15]

Control men or hyperinsulinemic men on diets with differing high amylase cornstarch contents did not have differing urinary Cr excretion after a glucose tolerance test.[15] Eight of 10 healthy individuals have been found to have increased urinary Cr loss (ng Cr/min) for 4 hours after an oral glucose tolerance test compared to the 4 hours before the test such that the mean Cr loss was significantly greater after the test than before, while no mean effect was observed for 8 diabetic subjects or 13 subjects with a diabetic family history.[16] Finally, Morris and coworkers conducting hyperinsulinemic euglycemic clamp studies have shown that increases in blood insulin levels, not specifically blood glucose levels, are responsible for a decrease in plasma Cr and an accompanying increases in urinary Cr loss,[1] consistent with their earlier studies demonstrating increased urinary Cr loss after an oral glucose challenge.[17] Thus, humans appear to increase urinary Cr loss in response to an increase in blood insulin concentrations (whether from a carbohydrate or insulin challenge). The magnitude of the change appears to be variable, including some individuals who may not respond potentially as a result of decreased glucose tolerance. Cr supplementation may eliminate the effect.)

Rats have been conclusively shown to increase Cr excretion in response to an insulin or glucose challenge despite one claim (where no data was presented).[17] Nearly all Cr(III) in the bloodstream is in the form of Cr-transferrin.[18] Using radiolabeled ^{51}Cr-transferrin, Cr has been shown to be transferred from the bloodstream to the tissues to the bloodstream (presumably bound to the peptide low-molecular-weight chromium-binding substance (LMWCr)), and then lost in the urine, again presumably as LMWCr.[7,19,20] This movement of Cr(III) from the bloodstream to the urine is enhanced by insulin.[7,19,20] Insulin does not appear to change the mechanism of chromium transfer or rate of transfer but changes the amount of chromium mobilized from the bloodstream.[7]

The effects of these diets on the insulin sensitivity of Zucker lean rats has been examined and will be further discussed in Chapter 4. Rats on the three AIN-93G diets with added Cr had greater insulin sensitivity than rats on the AIN-93G diet without any added Cr; insulin sensitivity increased as the amount of Cr in the diet increased (see Chapter 4). Given the large quantities of Cr involved, this was clearly a pharmacological effect. Notably, the results of this study cannot be correlated with the insulin sensitivity of the rats. Rats with the greatest insulin sensitivity (AIN-93G diet + 1 mg Cr/kg diet) had no tendency toward increased urinary Cr loss in response to an insulin or glucose challenge. In contrast, these rats appear to be receiving a quantity of chromium that overwhelms the system responsible for increasing urinary Cr loss in response to either challenge. This is reminiscent to the self-selected humans supplemented with 200 µg Cr as $CrCl_3$ after two to three months who were found to be unresponsive to glucose challenge.[8]

The AIN-93G diet is a purified diet that was designed to replace the AIN-76 diet. It is a standard USDA diet that helped to resolve some of the nutritional and technical problems that were being caused by the AIN-76 diet.[21] Several components were substituted to better suit the development and overall health of the animals. The use of this standard diet would help to remove dietary stresses that could be caused by the purified diets in previous studies.[21] The four versions of the AIN-93G diet were assayed for Cr content, and the diet with no Cr added to the mineral mix was found to have 16 µg/kg Cr, which is the lowest amount of Cr in a diet published to date. The diet will be discussed further in Chapter 4.

The blood plasma non-heme iron levels were assayed to determine if the large dose of Cr was detrimental to iron status, and no statistical difference in the samples across the diets was observed.[19] The mineral mix in the diet supplies 6.06 g/kg Fe as ferric citrate, so no iron deficiency was expected.

3.5 Conclusion

The urinary Cr loss in response to glucose or insulin is not decreased in the AIN-93G diet with no added Cr compared to the diet with the added Cr; thus, this study provides no evidence that using a diet with as low a Cr content as reasonably possible generates Cr deficiency. This is consistent with the results discussed in Chapter 4 that demonstrated that this diet provided no observable ill health effects. Therefore, as no diet has been found that can actually generate Cr deficiency in rats (or in humans), [22, 23] no nutritional evidence exists that Cr is an essential element. No biomolecules that bind chromium have been shown to perform an essential function in a biological system. Chromium cannot be considered an essential trace element for mammals.[22,23] Even if evidence existed that Cr were an essential element, the range of individual responses to insulin and glucose challenges in rats (this study) and humans[8] would preclude the use of urinary Cr loss as a biomarker for chromium status. Consequently, the establishment of a biomarker for Cr status is probably impossible as the element cannot be shown to be essential for health.

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Chapter 4: Chromium is not an essential trace element for mammals: Effects of a "low-chromium" diet

4.1 Introduction

In 1959, Mertz and Schwarz performed studies on rats fed a *Torula* yeast based diet that led to an apparent glucose intolerance in response to an intravenous glucose load.[1] The rats on the *Torula* yeast based diet had glucose clearance rates of 2.8%, while the animals on the basal diet had a rate of 4.1%.[1] The glucose tolerance was initially thought to be a symptom of liver disease (from lack of selenium in the diet). The liver disease was reversed by the addition of selenium, but the glucose intolerance was not. The diet was said to be missing "glucose tolerance factor." The active ingredient of glucose tolerance factor was proposed to be Cr^{3+}. The researchers supplemented the diet with numerous inorganic compounds (200-500 µg/kg body mass). Those compounds could not restore glucose tolerance, whereas several chromium(III) complexes (200 µg Cr/kg body mass) did. This experiment was deeply flawed. The *Torula* yeast based diet was not assayed for Cr content, and *Torula* yeast-based diets have subsequently been found to possess variable amounts of Cr. The animals were also housed in cages with metal components. Stainless steel is known to be ~10-40% Cr. Based in part on this study, the estimated safe and adequate daily dietary intake of 50-200 µg of Cr was established by the National Academy of Sciences (USA).

Four lines of evidence support the essentiality of Cr and, similar to the initial study by Mertz and Schwarz, they are riddled with issues. One of the four lines is studies using a "low"-Cr diet. Studies by Striffler et al. with rats housed in metal free cages and low Cr diets are probably the best performed, but due to dietary stresses are not an accurate gauge of the effect of

Cr deficiency.[2, 3] In two papers published by Striffler et al., male Wistar rats were housed in environmental conditions that minimized external Cr contamination. In the first study animals were given a basal low Cr diet made of 55% sucrose, 15% lard, and 25% casein by weight (high sugar diet). In the second study animals were given a basal low Cr diet of 30% sucrose, 40% lard, and 25% casein by weight (high fat diet). Both diets contained 33±14 ng Cr/g diet with or without 5ppm Cr supplementation in the water. The amount of water consumption was not reported. The animals received reduced copper in the diet for the initial six weeks to compromise the functioning of the pancreas.

The high sugar and high fat diets were intended to induce chromium deficiency. In the study involving the high sugar diet, at twelve weeks, the "Cr deficient" rats had lower fasting insulin concentrations and similar fasting glucose concentrations, yet no differences were observed after 24 weeks. An intravenous glucose tolerance test after 24 weeks showed plasma insulin levels to be higher in "Cr-deficient" rats, while the rates of excess glucose were statistically equivalent. Glucose area above basal was reported as significantly higher, but this is probably a consequence of an error in calculating the area because none of the glucose concentrations at individual time points were significantly different. A diet high in sucrose combined with dietary stresses can potentially lead to hyperinsulinemia.

After 16 weeks on the high fat diet, rats had higher fasting plasma insulin levels, but not glucose levels as compared to rats that were receiving supplemented drinking water. Similar results were observed when fasting insulin and glucose levels were compared to rats on a normal chow diet, and insulin and glucose areas after a glucose challenge were equivalent. Thus, the high fat diet with the additional stresses appears to induce increased fasting insulin levels which appear to be corrected with Cr administration.

The "deficient" diets need to be put into perspective. Humans lack signs of Cr deficiency with a daily intake of 30 µg Cr. If an average human has a body mass of 65 kg, 30 µg/day corresponds to 0.46 µg Cr/kg body mass per day. The average 100 g rat eats about 15 g of food per day, which if the rat was receiving 33 µg Cr/kg food would provide approximately 0.5 µg Cr. 0.5 µg Cr per day for a 0.100-kg rat is 5.0 µg Cr/kg body mass per day which would be 10 times what humans take in per kilogram body mass. This negates the claim that the "Cr deficient" diets in the Striffler et al. papers are deficient or even low in chromium unless rats require 10 times more Cr than humans do on a per kilogram body mass basis. Correcting the intake for metabolic rate would still not lead to these diets potentially being Cr deficient. Consequently, the effects of the high fat and high sugar diets cannot be attributed to Cr deficiency, but the supranutritional doses that the supplemented animals should be considered as having a pharmacological effect on the rats, whose physical condition was impaired by the stresses of the diet.

Thus to establish whether or not a standard diet low in Cr can have deleterious effects that can be prevented by the supplementation of Cr or to provide evidence for the essentiality of Cr in mammalian health, rats were maintained in metal free cages and provided standard purified diets supplemented with a variety of Cr concentrations. The effects of diet on food intake body mass, and parameters associated with glucose metabolism and insulin sensitivity were determined.

4.2 Materials and methods

4.2.1 Chemicals, assays, and instrumentation

Glucose and insulin (bovine, zinc) were obtained from Sigma-Aldrich, Inc. Final concentrations of glucose and insulin were prepared using doubly-deionized water. Plasma

insulin was measured using a ^{125}I RIA kit from MP Biomedicals, Inc. Gamma counting was performed using a Packard Cobra II auto-gamma counter. Blood glucose levels were measured using a One-Touch glucose meter; the author was assisted in this by Kristin Di Bona and DeAna McAdory.

4.2.2 Animals

Thirty-two Male Zucker Lean rats were obtained from Charles River Breeding Laboratories International, Inc. at six weeks of age. Rats were maintained at 22 ± 2 °C and 40-60% humidity with a twelve hour photoperiod and acclimated for two weeks prior to treatment. They were housed individually in specially constructed metal-free housing (*vide infra*) to prevent the introduction of additional chromium into their diets. Rats were fed specific diets and distilled water *ad libitum* for a 23 week period prior to glucose and insulin challenges. All procedures involving these animals were reviewed and approved by The University of Alabama's Institutional Animal Care and Use Committee.

4.2.2 Treatment

Male Zucker Lean rats were separated into four treatment groups, each containing eight rats as follows: (1) rats on a purified AIN-93G Cr sufficient diet (Cr as $KCr(SO_4)_2 \cdot 12H_2O$, the Cr source designated for the AIN-93G diet), (2) rats on the AIN93-G diet, with Cr not included in the mineral mix, (3) rats on the AIN-93G Cr sufficient diet with an additional 200 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$), (4) rats on the AIN-93G Cr sufficient diet with an additional 1000 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$). AIN-93G purified rodent diets and modified AIN93-G diets were obtained from Dyets, Inc., Bethlehem, PA. Diets were received in powder form. Chromium content of the diets was determined by graphite furnace atomic absorption spectroscopy utilizing a PerkinElmer Analyst 400 atomic absorption spectrometer equipped with

an HGA-900 graphite furnace and an AS-800 autosampler using a Cr hollow cathode as previously described in Chapter 3. The values were AIN-93G, 1,135 μg Cr/kg; AIN-93G without mineral mix, 16 μg Cr/kg; AIN-93G + 200 μg Cr/kg, 1,331 μg Cr/kg; and AIN-93G + 1,000 μg Cr/kg, 2,080 μg Cr/kg. All values were close to anticipated values.

4.2.3 Housing

Iris Buckle Up boxes were obtained from Target; the boxes were approximately 18 cm in height, 45 cm wide, and 28 cm in depth. These boxes are made of clear plastic with a removable lid that attaches with latches on both 28 cm-sides of the boxes. Holes (4 mm in diameter) were drilled with an electric hand drill in all five sides of the box and in the lid using a square grid pattern with approximately 5 cm between holes. Holes (4 mm in diameter) were also drilled in the corners of the bottom of each box to facilitate urine drainage. Shavings of plastic were removed from the holes, and any rough spots were smoothed using fine sandpaper. An additional hole was drilled in the lid with an appropriate diameter to accommodate the tube of the water bottles, while another hole was drilled in the lip of the box to accommodate a hanging cage card holder. Tube Tread No. 116 Wet Area Anti-fatigue mats were purchased from General Mat Company. The matting is made of vinyl with a tensile strength of 139 kg/cm and is flexible from -10 ^{o}C to 100 ^{o}C. The matting was cut with a knife to fit inside the base of the boxes. Both the boxes and the matting could pass through multiple cycles of a cage washing machine without noticeable damage. As the boxes are similar in size to shoebox-type housing, they were kept on a standard rack for animal cages. The cages were placed on absorbent bench paper or newspaper. The rear of the cage was elevated approximately 1 cm using scrap pieces of the matting material placed under rear of the cage to ensure draining of urine.

4.2.4 Food and water

Wheaton clear straight-sided, wide-mouth glass jars (~9 cm in diameter, 9.5 cm in height, 473 mL) and plastic lids (89 – 400 mm screw cap size) were obtained from Fisher Scientific and were used to hold food. A 5 cm diameter circular opening was cut in the polyvinyl-lined plastic lids to allow the animals access to food. To prevent the rats from dumping the powdered food from the jars, a 2 cm-thick Plexiglas disk (~ 7 cm diameter) was placed on the food. The disk had a 14 mm diameter circle cut out in the center with six other 14 mm diameter circles cut in a hexagonal pattern around the center circle; the disks were prepared by The University of Alabama College of Arts and Sciences machine shop.

To provide water, the stainless steel tubes were removed from water bottles and replaced with glass tubes. The University of Alabama glass shop took glass tubing of the appropriate diameter and cut and bent the tubing to match the length and shape of the stainless steel tubes. To prevent potential injury, the end of the tubing exposed to the rats was fire polished.

4.2.5 Data collection

Animals were weighed, and food consumption was measured twice weekly. At 23 and 25 weeks, respectively, rats were fasted between 10-12 hours then given an intravenous glucose challenge (1.25 mg glucose/kg body mass) or insulin (5 insulin units/kg body mass) challenge. Blood was collected in EDTA lined capillary tubes by a tail vein prick. Blood was collected before intravenous challenges and 30, 60, 90, and 120 minutes after the challenge injections. Area under the curve was calculated using the trapezoid rule, and the calculations were performed by Kristin R Di Bona.

4.2.6 Statistical analysis

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL). Data are represented graphically as average values with standard error bars. Data were calculated

independently, tested for homogeneity of variance with Levene's test, and analyzed using univariate analysis of variance (ANOVA) and descriptive statistics. Blood insulin and blood glucose tolerance tests were further analyzed for the area under each curve. Post hoc Least Significant Difference (LSD) analyses were used to indicate significant differences at a 95% confidence level ($p \leq 0.05$). Statistical analysis was performed by Kristin R Di Bona.

4.3 Results

The AIN-93 diet is a purified diet for experimental rodents reported by the American Institute of Nutrition.[4] Two forms of the diet were designed: the AIN-93G diet, designed for early phase growth and reproduction, and the AIN-93M diet, designed for animal maintenance.[5] The AIN-93G standard purified diet was chosen for use in this study because of the young rats utilized in this study. When chromium is omitted from the mineral mix, the diet is low in Cr compared to standard chow diets. Analysis of the diet revealed only 16 µg Cr/kg diet; this is as low a Cr content as can reasonably be provided to rodents. The only way to obtain a diet lower in chromium would be to have a completely synthetic diet. This diet contains about half the Cr concentration of that in the purified diets used by Anderson and coworkers, [2,3,6] this concentration is actually within error equivalent to that of the other purified diets. It is necessary to note (as described above) that despite the low concentration of chromium that this diet should therefore be considered chromium sufficient based on comparisons with the human AI. Consequently, if no adverse effects from this diet are observed in the rats, then producing a diet that is "deficient" in Cr but sufficient in other dietary requirements is probably not possible. Thus, indicating that no nutritional methodology could possibly demonstrate that Cr is an essential trace element for mammals. The diets that were supplemented with Cr were also

analyzed for their Cr content by graphite furnace atomic absorption spectrometry: AIN-93G, 1135 µg Cr/kg; AIN-93G + 200 µg Cr/kg, 1331 µg Cr/kg; AIN-93G + 1000 µg Cr/kg, 2080 µg Cr/kg. All values were close to anticipated values.

Mammals with Cr deficiency reportedly respond less efficiently to insulin and glucose challenges in terms of maintaining and restoring normal blood plasma glucose and insulin levels. Cr deficiency has been reported to lead to alterations in glucose metabolism and insulin insensitivity.[7,8] Thus, after 23 and 25 weeks, respectively, the rats were fasted for 10-12 hours and given an intravenous glucose (1.25 mg glucose/kg body mass) or insulin challenge (5 units insulin (bovine, zinc) per kg body mass). Blood samples were collected immediately before the challenges and 30, 60, 90, and 120 minutes after the challenges. In the glucose challenge, as shown in Figure 1, the plasma glucose levels of the rats of the various diets were equivalent at all the time points except 60 min after the challenge; at this time, the plasma glucose levels of the rats on the AIN-93G diet without Cr and the AIN-93G diets supplemented with 200 or 1000 µg Cr/kg were statistically equivalent. The glucose concentration for the rats on the AIN-93G diet were statistically higher than the rats on the diet without added Cr and the AIN-93G diet supplemented with 1000 µg Cr/kg. Thus, only the rats on the sufficient AIN-93G appeared to have elevated glucose levels. This is also reflected in the areas under curves (AUC) for the glucose tolerance tests (Figure 2). The AUC for the rats on the diet without added Cr is statistically equivalent to the AUC's for all the diets with added Cr. As the glucose concentrations are statistically equivalent for the rats on the diet without added Cr and the diets supplemented with 200 and 1000 µg Cr/kg at all time points after the challenge, the rates of glucose clearance for these rats are by necessity statistically equivalent. The glucose clearance (K_G) [1,2] was calculated for rats on each diet; K_G is equal the slope of the best fit line of a plot of

ln(% of baseline glucose) vs. time x 100% where % of baseline glucose = (glucose concentration at a given time/glucose concentration at time zero) x 100 %.[9] K_G was 1.07 %/min for the rats without Cr in the mineral mix of their diet, 0.600%/min for the rats on the AIN-93G diet with Cr in the mineral mix, 0.473%/min for the rats on the AIN-93G with 200 µg Cr/kg added, and 0.696%/min for the rats on the AIN-93G with 1000 µg Cr/kg added. As the K_G for the rats on the standard AIN-93G diet is within the range of the other three K_G values, all the K_G values are statistically equivalent. Thus, the glucose tolerance tests indicate that rats on the diet without added Cr could handle increases in blood glucose concentration equally efficiently as rats on Cr sufficient or Cr supplemented diets, providing no evidence that Cr is an essential dietary component.

In insulin tolerance tests, the plasma glucose concentrations of the rats on all the various diets are statistically equivalent; however, prior to the insulin challenge, the rats on the AIN-93G diet supplemented with 200 µg Cr/kg have statistically lower plasma glucose concentrations than the rats on the diet without added Cr (Figure 3). All glucose levels of the diets on the other diets were all statistically equivalent prior to the challenge. Notably, this difference was not observed prior to the glucose challenge. Not surprisingly, this results in the AUC's in the insulin tolerance tests for the rats on all the various diets being statistically equivalent (Figure 4). Thus, the rats

Figure 1

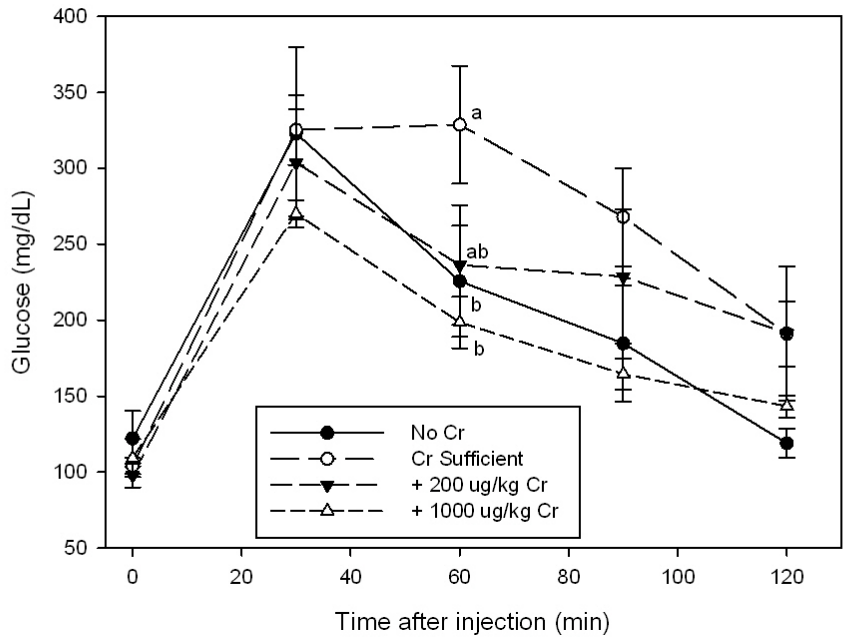

Figure 1. Plasma glucose levels in glucose tolerance tests for Zucker lean rats on the AIN93-G diets. Different letters indicate significant differences between groups. No Cr - rats on the AIN93-G diet, with Cr not included in the mineral mix; Cr Sufficient - rats on a purified AIN-93G Cr sufficient diet (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); + 200 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 200 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); +1000 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 1000 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$).

Figure 2

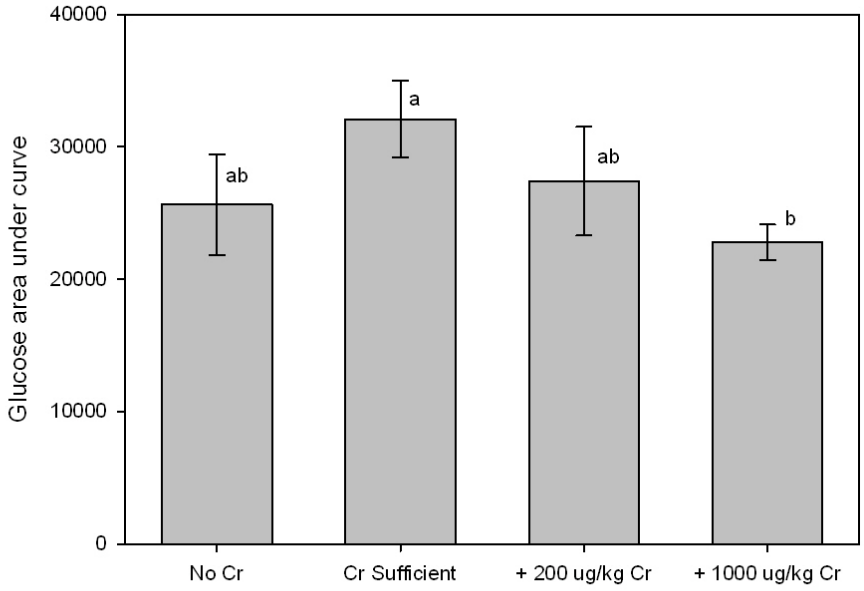

Figure 2. Area under the curve (AUC) for plasma glucose concentrations in glucose tolerance tests for Zucker lean rats on the AIN93-G diets. Different letters indicate significant differences between groups. No Cr - rats on the AIN93-G diet, with Cr not included in the mineral mix; Cr Sufficient - rats on a purified AIN-93G Cr sufficient diet (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); + 200 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 200 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); +1000 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 1000 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$).

on the diet without added Cr could manage their blood glucose after an insulin challenge equally well as rats on Cr sufficient or Cr supplemented diets, providing no evidence that Cr is an essential dietary component.

The effects of the glucose challenge on plasma insulin levels were also examined (Figure 5). Prior to the glucose challenge, a distinct trend is observed in the blood plasma insulin levels as the insulin concentrations drops as the amount of chromium in the diet increases. The difference is only statistically significant between the rats on the diet without added Cr and the rats on the diet with the most added Cr (AIN-93G + 1000 µg Cr/kg). As a consequence, 30 minutes after the challenge, the blood plasma insulin concentration of the rats on the diet without added Cr is statistically greater than all the other diets or the diet with the most added Cr. (No increase in plasma insulin concentration in response to the glucose challenge is observed in Figure 5 as the levels of insulin return to baseline in approximately thirty minutes, the time of the first data point after glucose administration). Sixty minutes after the challenge, the plasma insulin concentration of the rats on the diet without added Cr was greater than that of the rats on the diet with the greatest quantity of added Cr. In other words, increasing the dietary Cr intake of the rats by approximately 100-fold appears to result in a lowering of fasting plasma insulin levels. Thus, high doses of Cr appear to have a pharmacological effect on rats. The effects are best observed in the AUC's for the insulin concentration in the glucose challenge (Figure 6). The AUC's for the rats on the diet without added Cr and on the AIN-93G with Cr are statistically equivalent, indicating no effect from the inclusion of Cr in the diet's mineral mix. However, supplementing the AIN-93G with 200 or 1000 µg Cr/kg results in statistically lower AUC's, while the rats receiving the AIN-93G diet with 1000 µg Cr/kg had areas statistically lower than those of the rats on the AIN-93G diet with the Cr in the mineral mix. Consequently, nutritionally

Figure 3

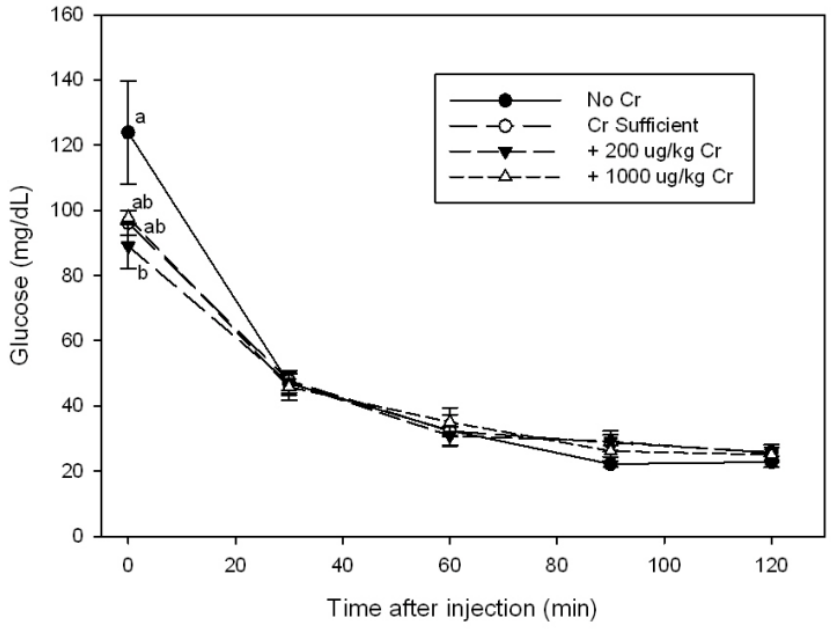

Figure 3. Plasma glucose levels in insulin tolerance tests for Zucker lean rats on the AIN93-G diets. Different letters indicate significant differences between groups. No Cr - rats on the AIN93-G diet, with Cr not included in the mineral mix; Cr Sufficient - rats on a purified AIN-93G Cr sufficient diet (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); + 200 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 200 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); +1000 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 1000 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$).

Figure 4

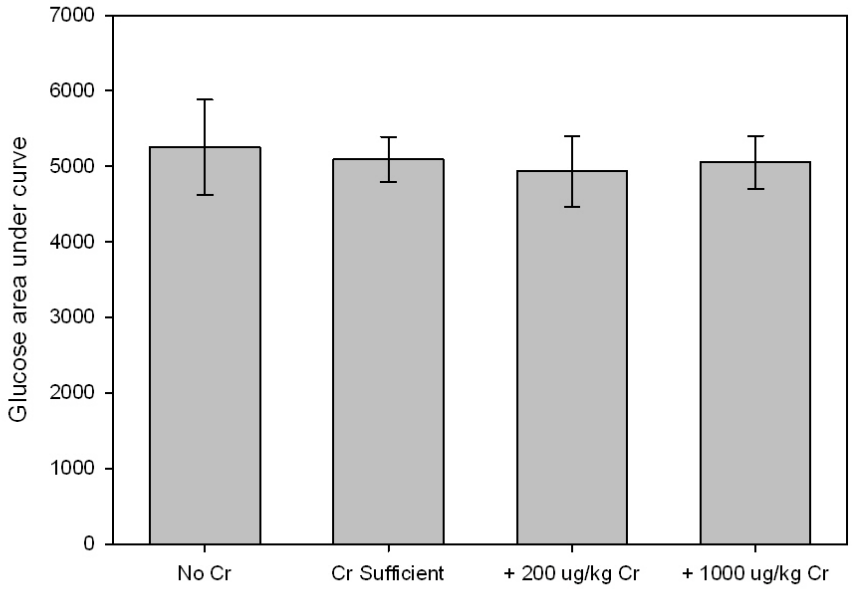

Figure 4. Area under the curve (AUC) for plasma glucose concentrations in insulin tolerance tests for Zucker lean rats on the AIN93-G diets. No significant differences between groups were observed. No Cr - rats on the AIN93-G diet, with Cr not included in the mineral mix; Cr Sufficient - rats on a purified AIN-93G Cr sufficient diet (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); + 200 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 200 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); +1000 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 1000 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$).

relevant amounts of Cr in the diet seem to have no effect on plasma insulin levels in response to a glucose challenge; however, supra-nutritional levels of Cr lead to lower concentrations of insulin being required to restore normal glucose levels in a timely fashion. Thus, supra nutritional levels of Cr appear to increase insulin sensitivity in the healthy Zucker lean rats. This laboratory has previously observed that daily supplementation of high doses of Cr(III) for 24 weeks results in lower fasting plasma insulin concentrations and lower insulin concentrations after a glucose challenge.[8] Insulin levels after the insulin challenge were also measured. The insulin levels were statistically equivalent at all time points for rats on all the various diets (data not shown).

4.5 Discussion

As shown in Chapter 3, the Cr content of the diets had no effect on the body mass of the rats throughout the course of the study. On only three days during the course of the study were the body masses of any of the groups of rats statistically different from the others. This is not unexpected as chromium supplementation has been shown numerous times to not influence body mass.[10, 11] The Cr content also had no effect on food intake (data not shown). Similarly, the rats of the different diets were identical in appearance. No differences were observed in the rate or type of health issues between rats on the various diets.

Four types of studies are generally cited as evidence that Cr is an essential element: 1) studies of rats provided "Cr-deficient" diets,[2,3] 2) studies examining the absorption of Cr as a function of intake,[12,13] 3) studies of patients on total parenteral nutrition (TPN),[14] and 4) studies of the association between insulin response and Cr transport.[7, 8] All are problematic.

Figure 5

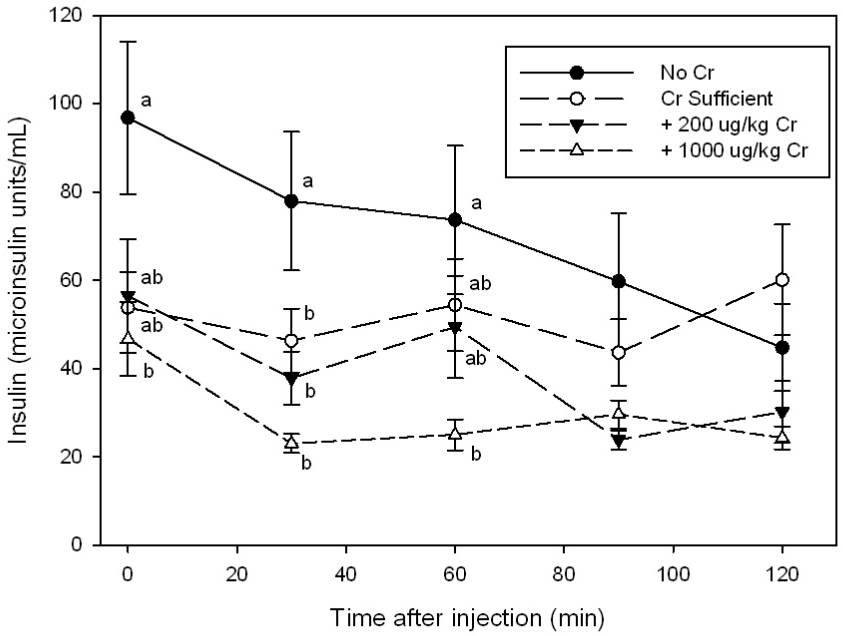

Figure 5. Plasma insulin levels in glucose tolerance tests for Zucker lean rats on the AIN93-G diets. Different letters indicate significant differences between groups. No Cr - rats on the AIN93-G diet, with Cr not included in the mineral mix; Cr Sufficient - rats on a purified AIN-93G Cr sufficient diet (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); + 200 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 200 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); +1000 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 1000 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$).

Figure 6

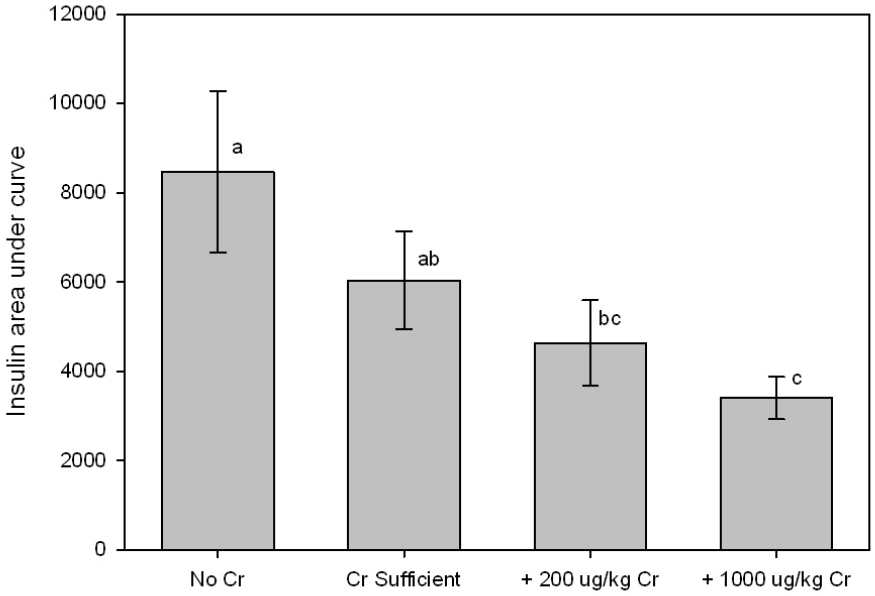

Figure 6. Area under the curve (AUC) for plasma insulin concentrations in glucose tolerance tests for Zucker lean rats on the AIN93-G diets. Different letters indicate significant differences between groups. No Cr - rats on the AIN93-G diet, with Cr not included in the mineral mix; Cr Sufficient - rats on a purified AIN-93G Cr sufficient diet (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); + 200 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 200 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$); +1000 μg/kg Cr - rats on the AIN-93G Cr sufficient diet with an additional 1000 μg Cr/kg (Cr as $KCr(SO_4)_2 \cdot 12H_2O$).

For example, one study has reported that the absorption of Cr in humans is inversely proportional to intake.[12] These results were previously discussed in Chapter 2. A limited number of patients on TPN and demonstrating a variety of symptoms similar to those of type 2 diabetes have had their symptoms improve after addition of Cr to the TPN solution[14]; however, the doses of Cr utilized were pharmacological, not nutritionally relevant. Studies on the relationship between chromium mobilization in the body and insulin action require additional studies to examine what is happening at a molecular level. Finally, as described above, studies with rats on "chromium-deficient diets" have been reported.

A closer analysis of these studies reveals a number of flaws such as failure to determine the Cr content of the diet, the use of metal components in caging that could provide a source of Cr for gnawing rats, and the use of additional stresses other than limiting the Cr content of the diet. Unfortunately, for example, in the original studies by Mertz and Schwarz[1] the Cr content of the diet was not reported (although the experimental procedures at the time would not have likely produced the correct value). Additionally, the rats were maintained in wire mesh cages, possibly with stainless steel components, allowing the rats to obtain chromium by chewing on these components. Consequently, the actual Cr intake of the rats in these studies is impossible to gauge, putting into great question the suggestion that the rats were Cr deficient. The use of the large amounts of the metal ions is also of concern. Supra-nutritional doses of Cr^{3+} have pharmacological effects on rodent models of altered carbohydrate and lipid metabolism including type 2 diabetes.[7]

Woolliscroft and Barbosa have examined the effects of a normal and a *Torula* yeast diet in intravenous glucose tolerance tests in rats.[9] They reproduced the results of Mertz and Schwarz; yet, observation of a significant difference in glucose metabolism between the two

groups of rats depended on the method used to present the data, i.e. using measured plasma glucose concentrations versus using "excess" plasma glucose concentrations. The effect was only statistically significant when "excess" plasma glucose was used. As calculating the "excess" plasma introduces error, use of actual measured plasma glucose is the accepted practice. Thus, these studies do not provide evidence of chromium being an essential trace element. Subsequent studies on healthy rodents in the 1960s, 1970s, and 1980s suffer from similar methodological complications.[7]

The use of these other stresses, such as diets with high sugar or fat content, can lead to alterations in carbohydrate and lipid metabolism. The diets were low in Cu for the first six weeks to impair endocrine pancreas function and also high in Fe to compete with Cr absorption and binding. The low Cr diet in the current study was a standard USDA diet, AIN-93G, that was composed of a more complex list of carbohydrate, fat and protein sources to improve the performance of the animals that consume the diet.[4] The diet contained starch and sucrose because standard diets high in sucrose have been shown to cause complications in rodents. The diet also contains a fat source, soybean oil, which provides appropriate amounts of linoleic and linolenic acid as well as antioxidants to protect the polyunsaturated fatty acids.[5] The diets in the Striffler et al. studies could have led to the effects believed to be observed by Cr deficiency. The effects of the addition of high concentrations of Cr to these diets is better explained by pharmacological effects of Cr, rather than a nutritional effect.

4.6 Conclusion

The current study demonstrates that low Cr diets do not lead to observable deleterious effects and do not provide evidence that Cr is an essential trace element. In fact, no unequivocal

data exists, as seen in Chapters 2 and 3, supporting an essential role for chromium. Given that currently no data confirm that Cr is an essential element, Cr should simply no longer be considered an essential element.

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Chapter 5:Comparison of Tissue Metal Concentrations in Zucker Lean, Zucker Obese, and Zucker Diabetic Fatty Rats and the Effects of Chromium Supplementation on Tissue Metal Concentrations

5.1 Introduction

The concentration of metal ions in tissues has been examined in some rat diabetes or insulin-resistance models including rats on a high-fructose diet,[1,2] streptozotocin-induced diabetic rats,[3] and high-fat fed streptozotocin-induced diabetic rats.[4] The varying models have differing tissue metal concentrations compared to healthy controls, due to different coexisting factors affecting bioavailability, metabolism, and excretion, whose mechanisms are not fully understood.

For example, a high-fructose diet lowers liver Cu concentrations but has little if any effect on kidney copper or kidney or liver Fe, Zn, or Cr concentrations.[1,2] Streptozotocin-treated rats have increased Fe and Cu concentrations but lower Zn and Mg concentrations in the liver and kidney.[3] The administration to rats of both a high-fat diet and streptozotocin results in increases in Fe concentration in the liver and kidney, and increases in Cu concentration in the kidney,[4,5] while other effects are not consistent between studies. Zucker obese rats and Zucker diabetic fatty (ZDF) rats are common models of insulin resistance and type 2 diabetes, respectively.[6] Zucker obese rats have a mutation in the leptin receptor that blocks signaling from the hormone leptin. The Zucker diabetic fatty rats developed from Zucker obese rats and possess another unknown mutation giving rise to the development of type 2 diabetes. A limited number of studies have examined tissue metal ion concentrations in Zucker obese rats[7, 8, 9] while no systematic studies were identified for ZDF rats. Zucker obese rats have been reported to have

lower tissue Cu concentrations that Zucker lean rats, although these differences disappear when the rats are fed a cafeteria-style diet; however, whole rat homogenates, rather than individual tissues were examined in this study.[7] In contrast another lab has reported that the obese rats have higher Cu concentrations than lean rats in the kidney per gram protein at 5 weeks of age and in the kidney and liver at 12 weeks, while no differences per gram protein were observed for Fe and Zn concentrations in liver and kidney.[8] Unfortunately, the use of mg metal per mg protein and mg metal per organ in this work does not allow for direct comparisons with other studies using mg metal per unit dry tissue mass. Another laboratory reported that Zucker obese rats have lower liver Zn and Cu concentrations than Zucker lean rats.[9] The lower concentrations of Zn and Cu per gram dry mass for the obese rats were attributed to the increased fat content of the liver and kidney; when corrected for neutral fat content, the liver Zn and Cu content was equivalent to those of the lean rats. Streptozotocin-treated lean and obese rats resulted in increased liver and renal Cu and Zn concentrations per gram dry mass.[9] Thus, more data on tissue metal concentrations for Zucker lean, Zucker obese, and ZDF rats are needed. Hence, in this study, the hypothesis that tissue trace metal concentrations will vary between the pre-diabetic and diabetic animal models (i.e., rat models) and the healthy controls was tested.

Zucker obese rats and Zucker diabetic fatty (ZDF) rats are common models of insulin resistance and type 2 diabetes, respectively.[6] They serve as a genetic model of insulin resistance and type 2 diabetes in contrast to the chemically-induced diabetic Wistar rats, which model type 1 diabetes. A limited number of studies have examined tissue metal ion concentrations in Zucker obese rats,[7,8,9] while no systematic studies were identified for ZDF rats.

Cr as the trivalent chromic ion is a therapeutic agent capable of improving insulin sensitivity in rodent models of insulin resistance and diabetes (as discussed in Chapters 1 and 4).

Additionally, the ion is also able to improve lipid parameters in some of these models; and some Cr(III) complexes, such as Cr3, $[Cr_3O(propionate)_6(H_2O)_3]^+$, have been reported to improve insulin sensitivity in healthy rats at high doses.[10]

In this study, the effects of chromium picolinate, $[Cr(pic)_3]$ (the most popular commercial chromium nutritional supplement), $CrCl_3$,and Cr3, as well as vanadyl (as a model of chromate [discussed later]) on the metal concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats were examined to test the hypothesis that supplemental Cr could alter concentrations in tissues.

5.2 Methods

5.2.1 Rats

One hundred forty-four male rats, 48 Zucker lean, 48 Zucker obese, and 48 Zucker diabetic fatty (ZDF), approximately 6 weeks old were obtained from Charles River Breeding Laboratories, Zucker obese rats are an insulin resistant model of obesity and early stage Type 2 diabetes and ZDF rats are a Type 2 diabetes model. Rats were maintained in an AAALAC approved animal care facility in rooms with 22±2 °C, 40-60% humidity, and a 12-hour photoperiod. Animals were housed 2 rats/cage containing hardwood bedding and were provided *ad libitium* access to Harlan Teklad rodent chow and water. Following a one week acclimation period, rats were assigned to the following treatment groups with treatments administered by gavage daily at circa 9 am for 12 weeks: (groups 1-3) 8 Zucker lean, 8 Zucker obese, and 8 ZDF as control vehicles receiving distilled water; (groups 4-6) 8 Zucker lean, 8 Zucker obese, and 8 ZDF receiving 1 mg Cr per kg body mass per day as $CrCl_3$; (groups 7-9) 8 Zucker lean, 8 Zucker obese, and 8 ZDF receiving 33 μg Cr per kg body mass per day as Cr3; (groups 10-12) 8 Zucker lean, 8 Zucker obese, and 8 ZDF receiving 1 mg Cr per kg body mass per day as Cr3; (groups 13-15) 8 Zucker

lean, 8 Zucker obese, and 8 ZDF receiving 1 mg Cr per kg body mass per day as [$Cr(pic)_3$]; and (groups16-18) 8 Zucker lean, 8 Zucker obese, and 8 ZDF receiving 2 mg/kg vanadyl sulfate (a source of vanadate *in vivo*) per day. Animals were weighed twice a week.

After the 12-weeks treatment period, rats were anesthetized using isofluorane. A bundle of vastus lateralis muscle fibers and the end of one segment of epididymal fat were dissected from the right side of the body for studies beyond the scope of this report. Rats were then treated intravenously with 5 units insulin (bovine Zn)/kg body mass; after 30 minutes, left muscle and fat samples were collected for other studies. The rats were then sacrificed by carbon dioxide asphyxiation, and the liver, heart, spleen, and kidneys were harvested and weighed by Kristin Di Bona and undergraduates from the Rasco lab. Tissues were transferred directly to plastic weigh boats for weighing and then to disposable plastic centrifuge tubes (capable of withstanding at temperature of 105 ^{o}C). The heart, spleen, kidneys, and a weighed aliquot of liver from each rat were then dried to a constant mass in a vacuum oven at 105 ^{o}C by Nicholas Rhodes, Ge Deng, Liu Ben, and me.

All procedures with the rats were approved by The University of Alabama Institutional Animal Use and Care Committee.

5.2.2 Atomic Absorption Spectrometry

For metal analyses, samples were digested with concentrated 65% spectra pure HNO_3 (Merck) in a Microwave Digestion System (MARS-5, CEM). The concentration of Cu, Zn, Fe, Mg, and Ca was determined by flame atomic absorption spectrometry method (F-AAS) (Zeiss AA-3, with background correction). The concentration of Cr was measured using a graphite furnace atomic absorption spectrometer (AA EA 5 with background correction, Jenoptic). The accuracy of the determination of Cu and Zn was assured by simultaneous analysis of the certified

reference material bovine liver BCR®-185R (IRMM), while analysis of Fe, Mg, and Ca was controlled using the certified reference materials Virginia tobacco leaves CTA-VTL-2 (Poland). Analysis of Cr was assured using the certified reference material mussel tissue ERM®-CE278 (ERM). The recovery for Cu, Zn, Fe, Mg, Ca, and Cr (expressed of the percentage of the mean certified values were 103%, 101%, 97%, 104%, 103%, and 102%, respectively. Atomic absorption spectrometry was performed by Halina Staniek.

5.2.4 Chromium compounds

Chromium picolinate[11] and Cr3[12] were prepared as described previously. $CrCl_3 \cdot 6H_2O$ (actually *trans*-$[Cr(H_2O)_4Cl_2]Cl \cdot 2H_2O$) and vanadyl sulfate were used as received.

5.2.5 Statistics

Each data point in the figures represents the average value for eight rats. Error bars in the figures denote standard deviation. Data were tested for homogeneity of variance by means of the Levine statistic and were analyzed by repeated measures ANOVA using SPSS (SPSS, Inc.). Specific differences ($p \leq 0.05$) were determined by LSD and a Bonferroni post-hoc test. For eight animals per group, an expected difference between two means would be significant at the 0.05 level if the difference between the means is twice the standard deviation. Statistical analysis was performed by Kristin Di Bona

5.3 Results

5.3.1 Rat strains

This research reported herein is one part of a two part study. One part of this study was designed to test the effects of supplementation of a variety of Cr(III) compounds and vanadyl sulfate on the insulin signaling cascade in control rat and rat models of insulin-resistance and type 2 diabetes. This part of the study required the surgeries and insulin treatment. However, the

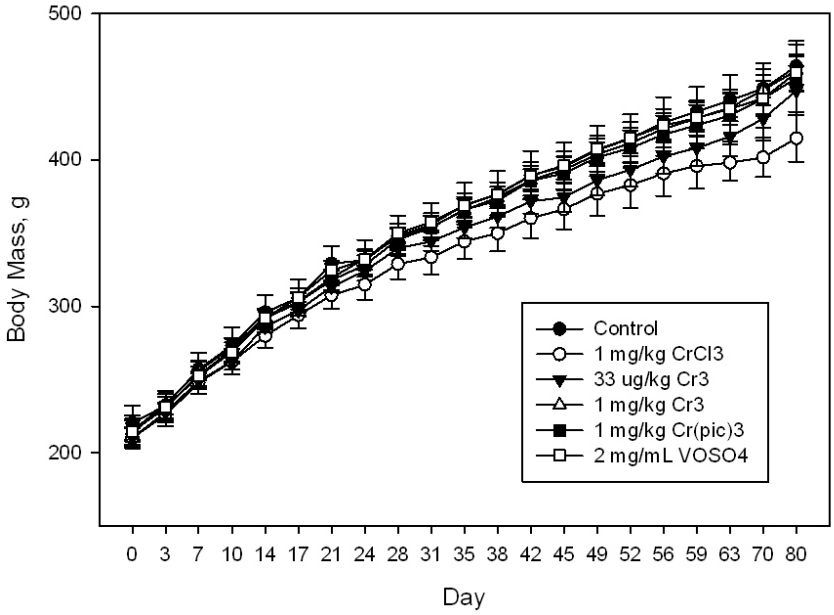
rat carcasses remaining at the end of the study provided a unique opportunity to also (in the portion of the study reported herein) to compare the mineral distribution in tissues of Zucker lean,

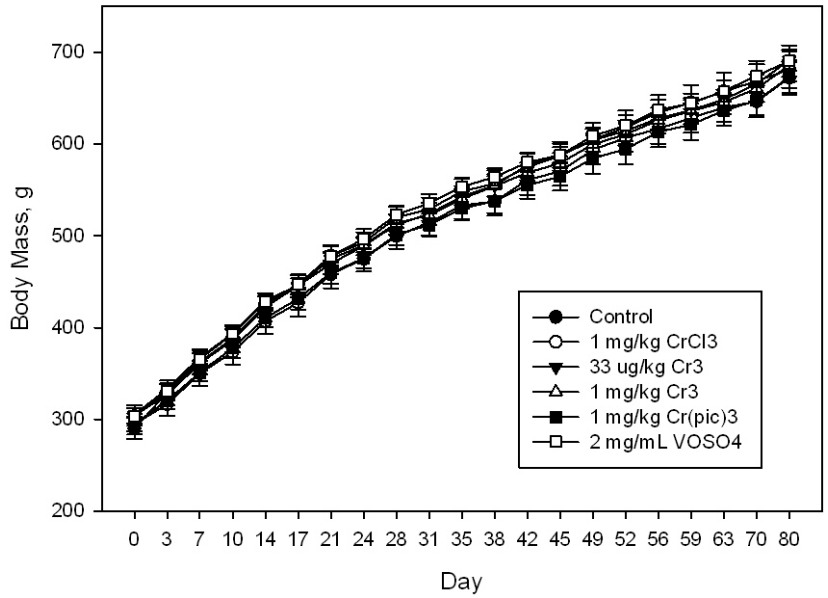
Zucker obese, and Zucker diabetic fatty rats and to study the effects of the Cr(III) compounds and vanadyl sulfate on the mineral distribution of tissues of these animals. Blood mineral concentrations were not determined because of the known effect of insulin to result in movement of various ions to or from the bloodstream. For example, a pool of transferrin is mobilized from the pool to the tissues in response to tissue, resulting in the transport of Cr(III) and Fe(III) from the blood to the tissue.[13,14] However, for many metal ions, multiple storage pools exists. The blood usually represents a small, but rapidly mobilizable pool of the metal ion. In contrast, tissues, such as the liver, represent a large storage pool of the metal, which exchanges only very slowly with the pool of metal in the bloodstream. For example with Cr, most of the metal in the body resides in a large pool in the tissues that very slowly exchanges over a period of months with the pool of Cr ion the bloodstream.[15,16,17] Consequently, the insulin treatment (followed by a 30 minute wait and the time required for the surgery and other operations) is anticipated to have little if any effect on the metal concentrations in the selected tissues. This is indeed borne out in the control Zucker lean rats where the metal concentrations examined are in the normal ranges.

No effects on body mass were observed as a function of diet for the Zucker lean, Zucker obese, or ZDF rats (Figure 1). Tissue levels of Cr, Cu, Zn, Fe, Mg, and Ca in the Zucker lean, Zucker obese, and ZDF rats were generally similar, although some statistically significant differences were identified (Figures 2-7). ZDF rats possessed an increased concentration of Cu in the kidneys (Figure 3B) compared to the Zucker lean and Zucker obese rats, while no other

differences were observed. This is similar to rats with streptozotocin-induced or streptozotocin- and high fat diet-induced diabetes.[3,4,5] Effects on metal concentrations were more common in the Zucker obese rats. Zucker obese rats had a reduction in the concentrations of Cu and Zn compared to that of ZDF rats, a reduction in the concentration of Fe in the liver compared to those of the Zucker lean and ZDF rats, and a reduction in the concentration of Mg in the liver compared to that of the Zucker lean rats. Spleen Fe concentrations were decreased in Zucker obese rats compared to the lean rats. However, Ca concentration was increased in the kidneys of the obese rats compared to those of the Zucker lean and ZDF rats.

The administration of the Cr(III) compounds and the vanadyl sulfate had few effects on tissue metal concentrations (Figure 8). Cr levels were elevated in the kidneys of lean rats and kidneys of obese rats receiving either 1 mg/kg Cr as Cr3 or $CrCl_3$ but not [$Cr(pic)_3$] or the smaller amount of Cr3 (Figure 8A and 8B). No statistically significant effects on the Cr concentration of the liver or kidneys of the other rats were observed. The increased Cu content of kidneys of the ZDF rats was reduced by administration of 1 mg/kg Cr as Cr3 and $CrCl_3$ (Figure 8D). $VOSO_4$ had no significant effects.


Body Mass, g
500
400
300
200
0 3 7 10 14 17 21 24 28 31 35 38 42 45 49 52 56 59 63 70 80
Day
Control
1 mg/kg CrCl3
33 ug/kg Cr3
1 mg/kg Cr3
1 mg/kg Cr(pic)3
2 mg/mL VOSO4


Body Mass, g
700
600
500
400
300
200
0 3 7 10 14 17 21 24 28 31 35 38 42 45 49 52 56 59 63 70 80
Day
Control
1 mg/kg CrCl3
33 ug/kg Cr3
1 mg/kg Cr3
1 mg/kg Cr(pic)3
2 mg/mL VOSO4

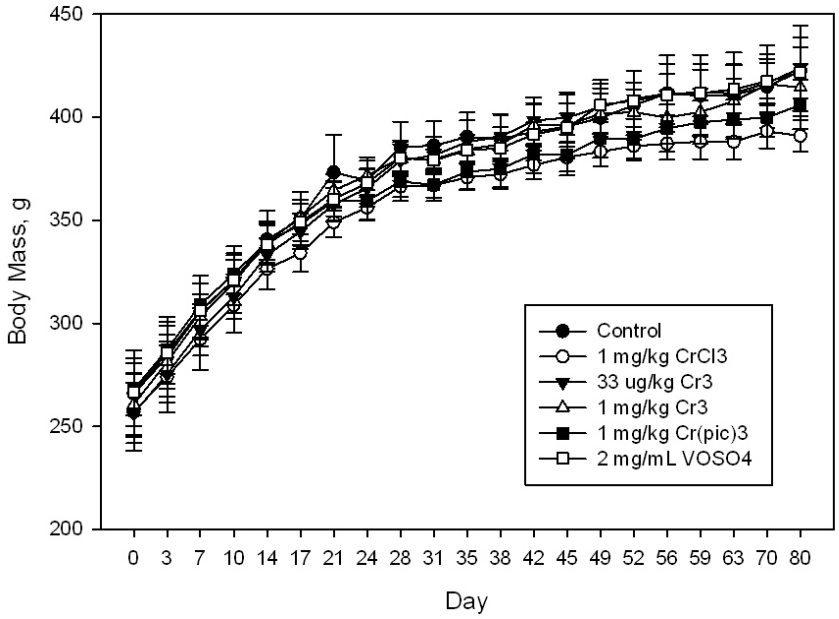


Figure 1. Effects of diets on body in mass. top) Zucker lean rats, middle) Zucker obese rats, bottom) ZDF rats. No statistically significant effects on body mass were found.

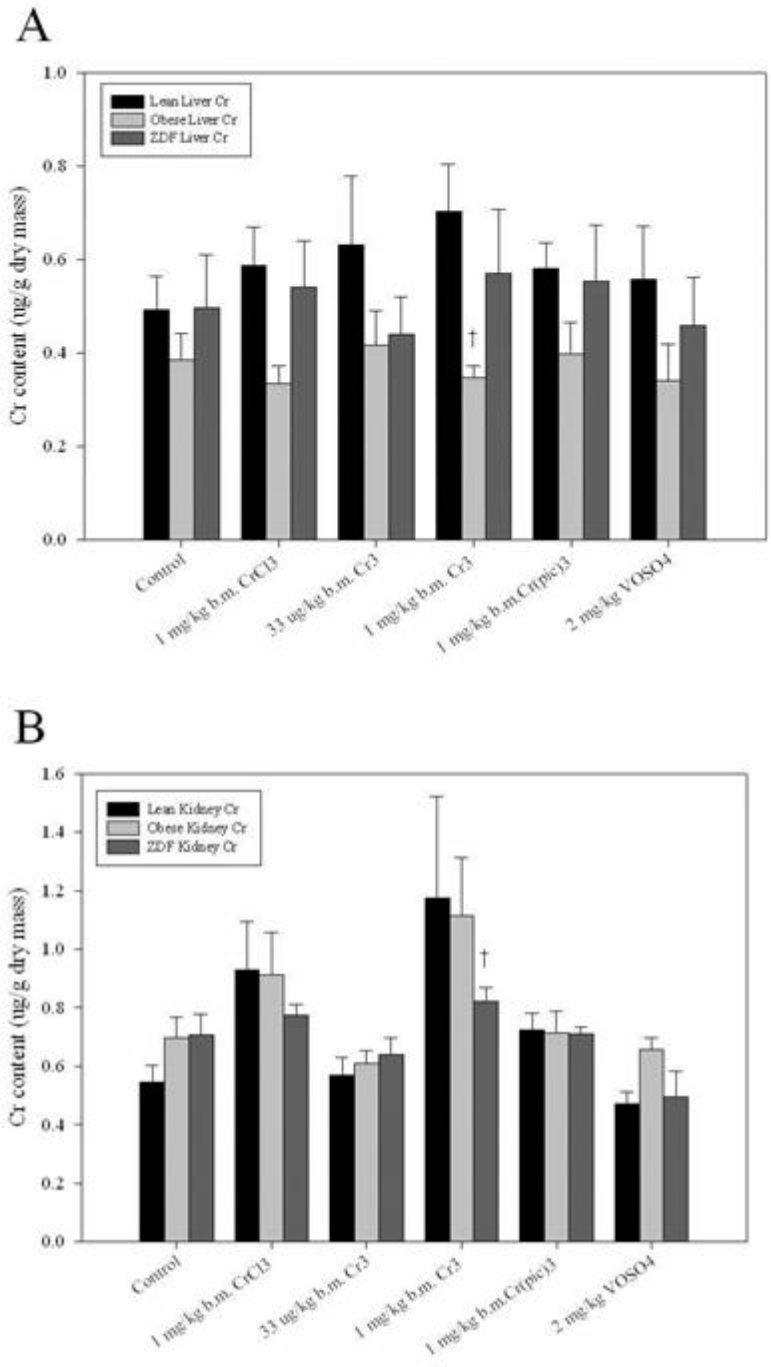
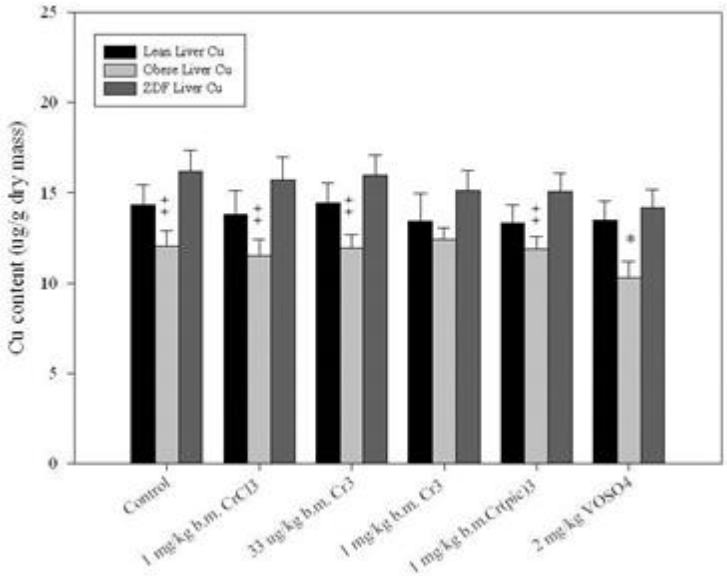
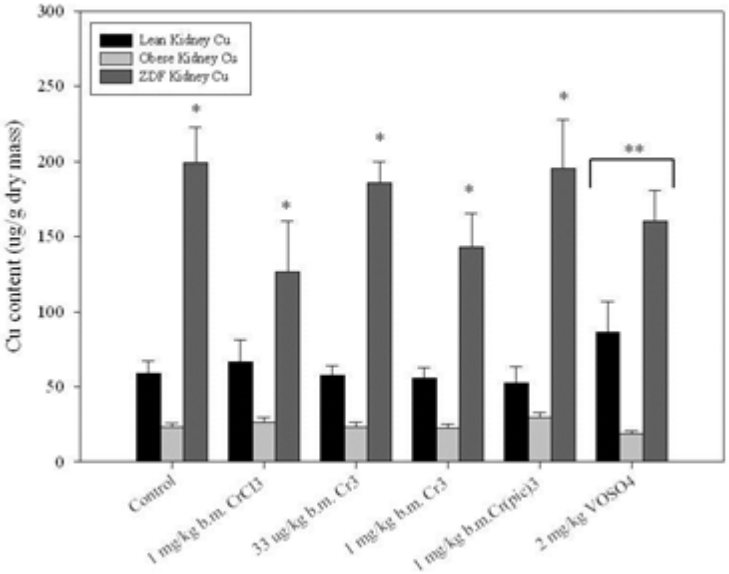


Figure 2. Chromium concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A) liver and B) kidney. † – Difference in concentration from that of Zucker lean rat ($p \leq 0.05$).

A


Lean Liver Cu
Obese Liver Cu
ZDF Liver Cu
Cu content (ug/g dry mass)
25
20
15
10
5
0
++
++
++
*
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Cr(pic)3
2 mg/kg VOSO4

B


Lean Kidney Cu
Obese Kidney Cu
ZDF Kidney Cu
Cu content (ug/g dry mass)
300
250
200
150
100
50
0
*
*
*
*
*
**
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Cr(pic)3
2 mg/kg VOSO4

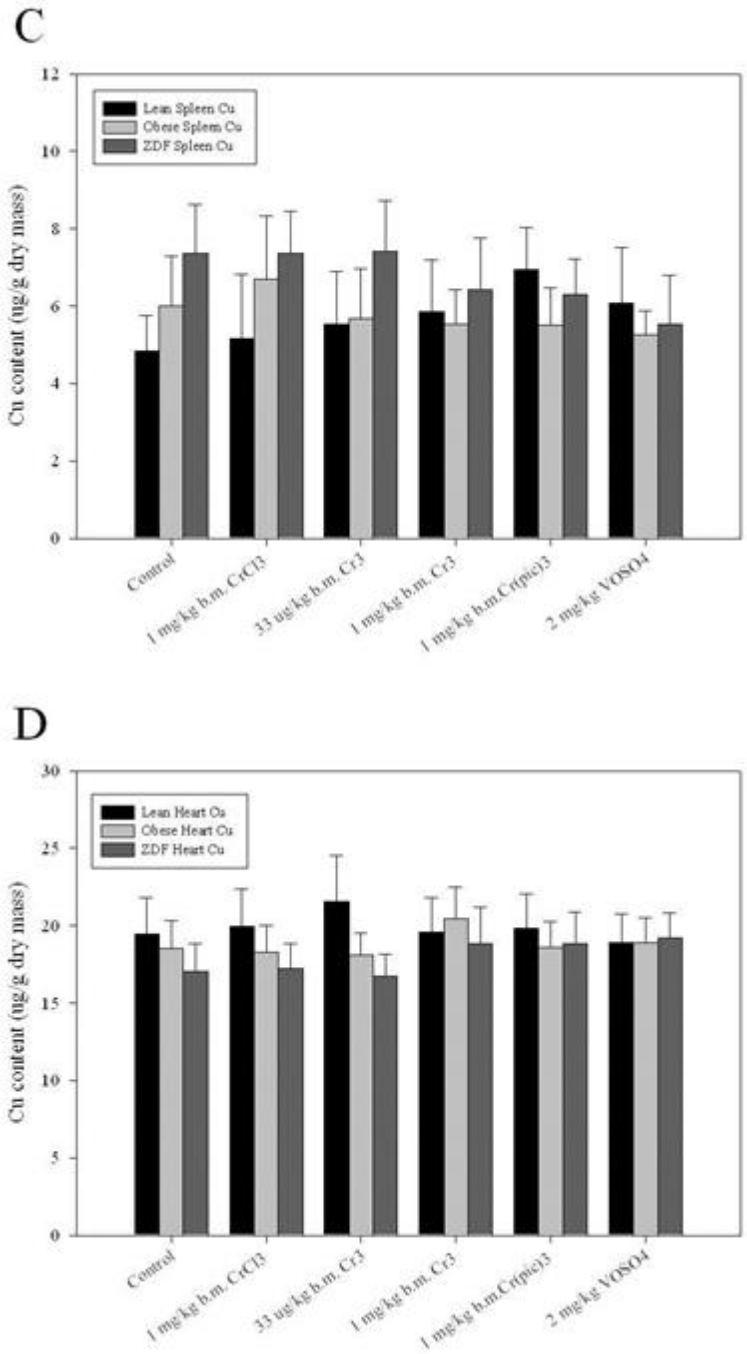
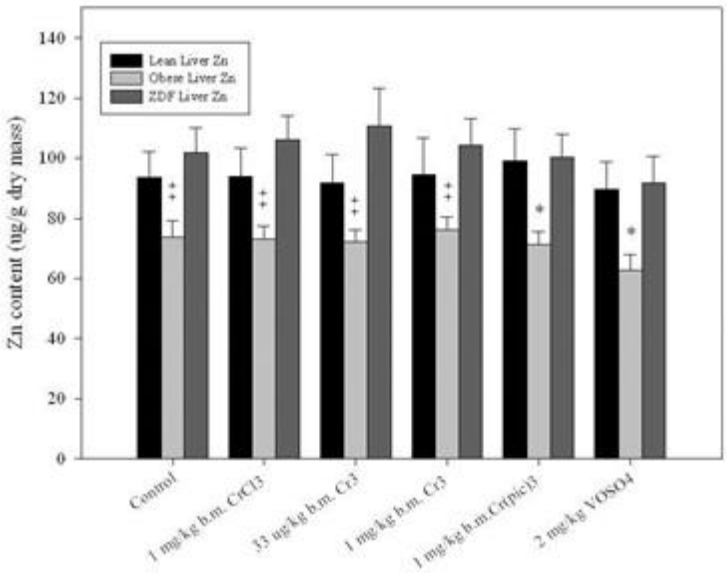
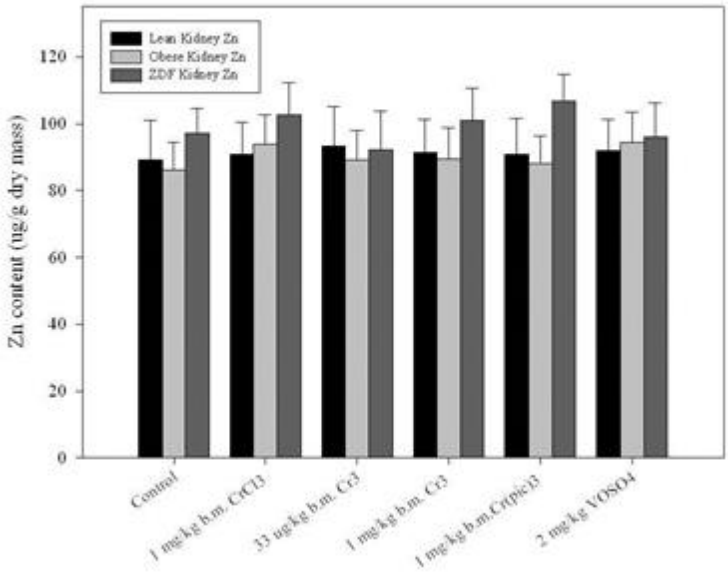


Figure 3. Copper concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A) liver, B) kidney, C) spleen, and D) heart. * - Difference in concentration from those of other two rat strains ($p \leq 0.05$). ** - All strains are significantly different from each other ($p \leq 0.05$). ‡ – Difference in concentration from that of ZDF rat ($p \leq 0.05$).

A


Lean Liver Zn
Obese Liver Zn
ZDF Liver Zn
Zn content (ug/g dry mass)
0
20
40
60
80
100
120
140
++
++
++
++
*
*
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Crpic)3
2 mg/kg VOSO4

B


Lean Kidney Zn
Obese Kidney Zn
ZDF Kidney Zn
Zn content (ug/g dry mass)
0
20
40
60
80
100
120
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Crpic)3
2 mg/kg VOSO4

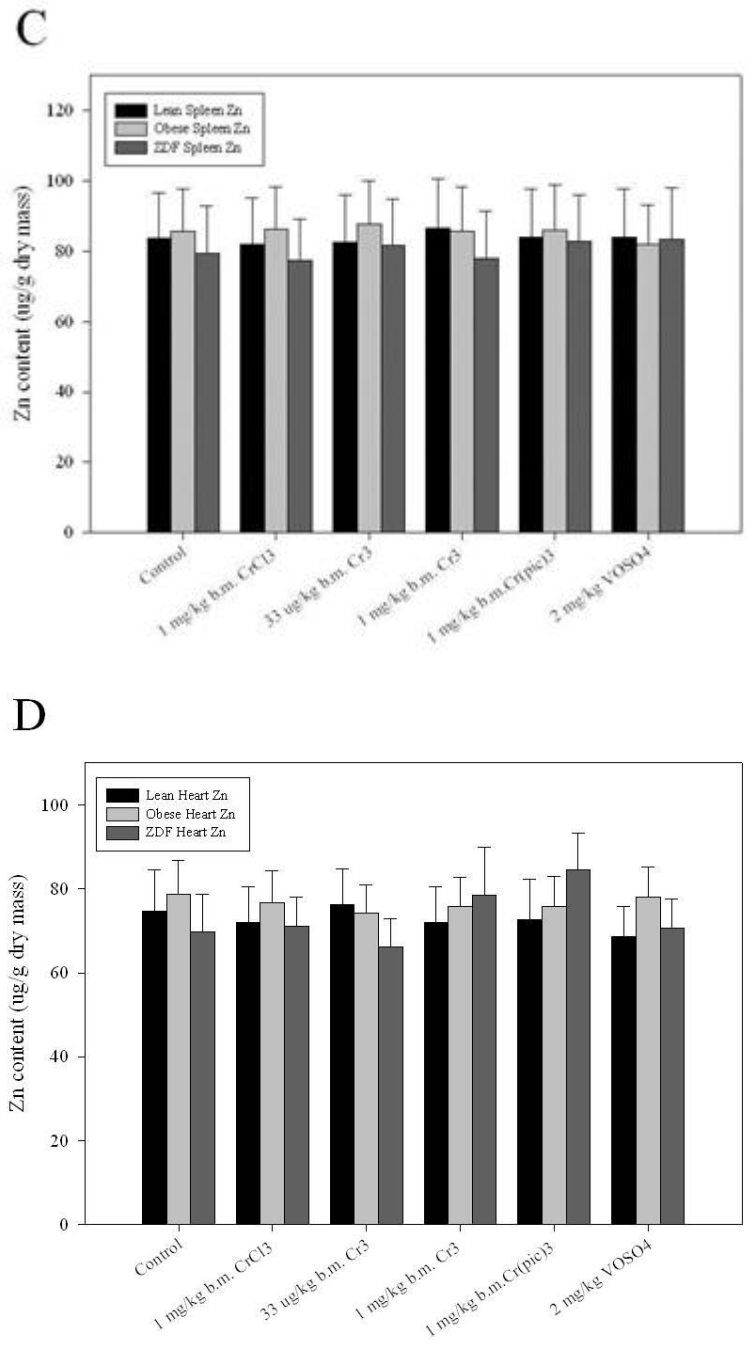
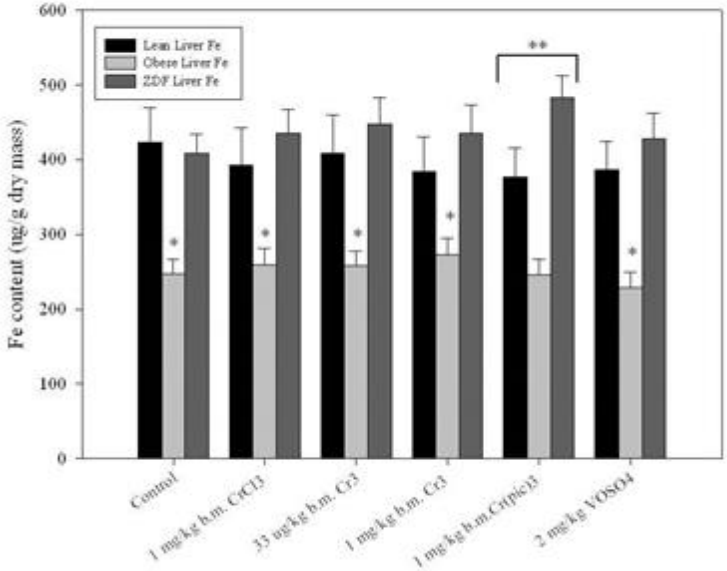
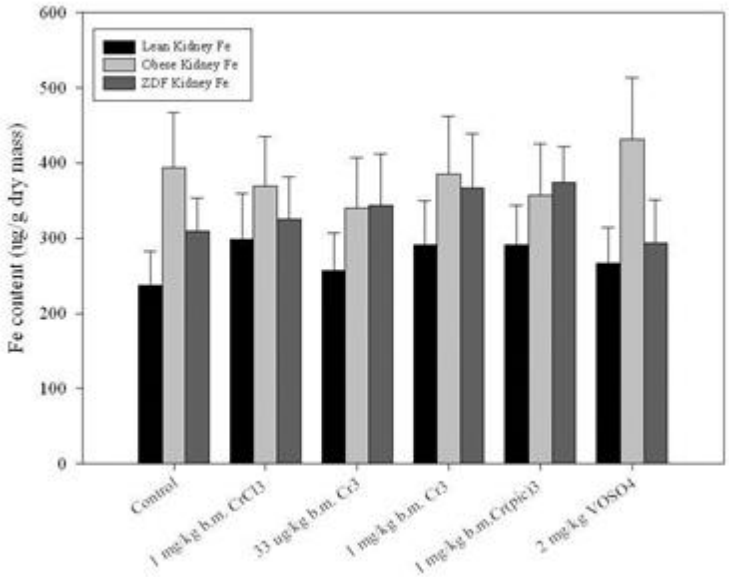


Figure 4. Zinc concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A) liver, B) kidney, C) spleen, and D) heart. * - Difference in concentration from those of other two rat strains ($p \leq 0.05$). ** - Difference in concentration among all three rat strains ($p \leq 0.05$). ‡ – Difference in concentration from that of ZDF rat ($p \leq 0.05$).

A


Lean Liver Fe
Obese Liver Fe
ZDF Liver Fe
Fe content (ug/g dry mass)
600
500
400
300
200
100
0
**
*
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Crpic)3
2 mg/kg VOSO4

B


Lean Kidney Fe
Obese Kidney Fe
ZDF Kidney Fe
Fe content (ug/g dry mass)
600
500
400
300
200
100
0
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Crpic)3
2 mg/kg VOSO4

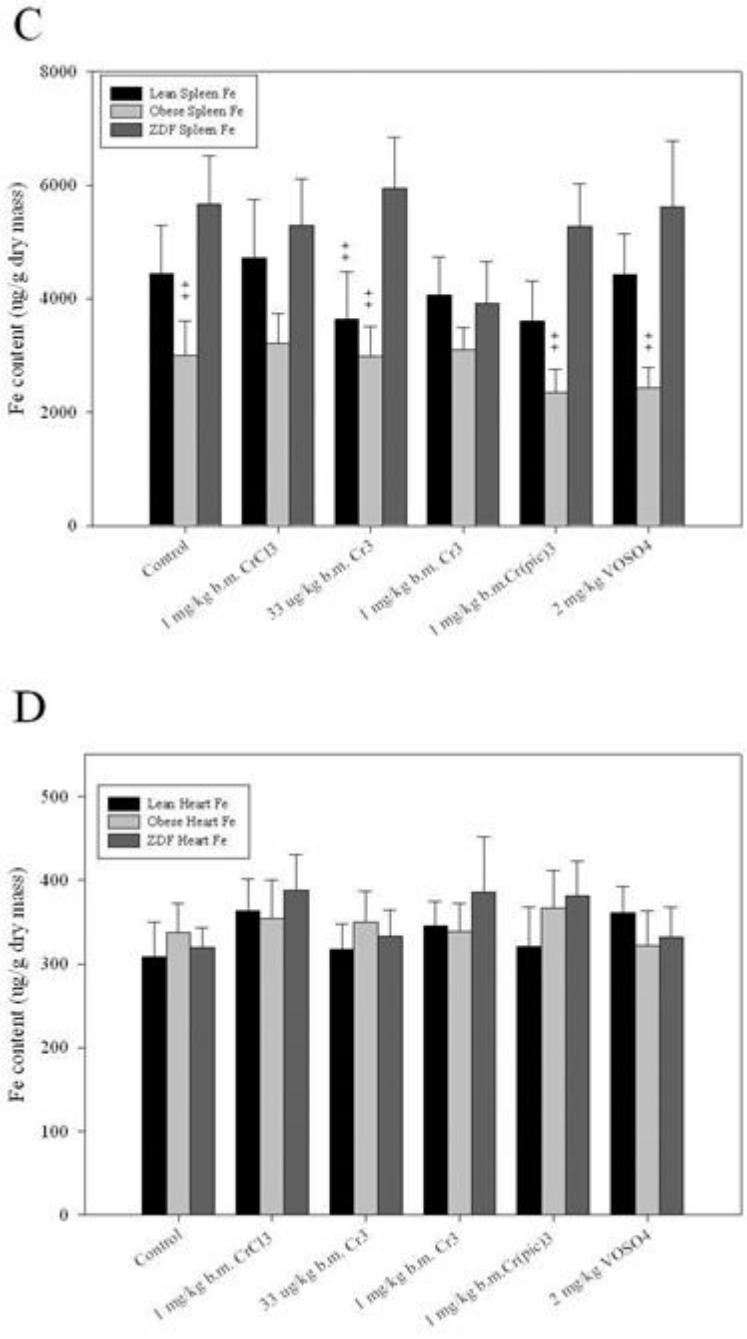
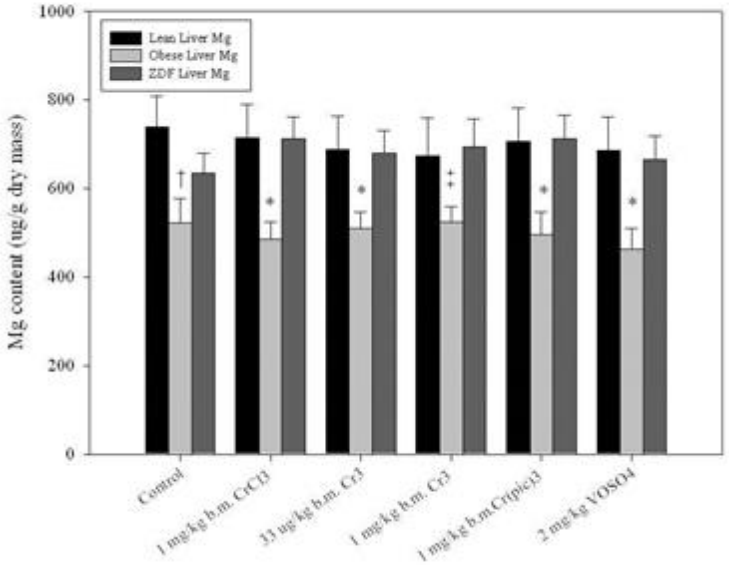
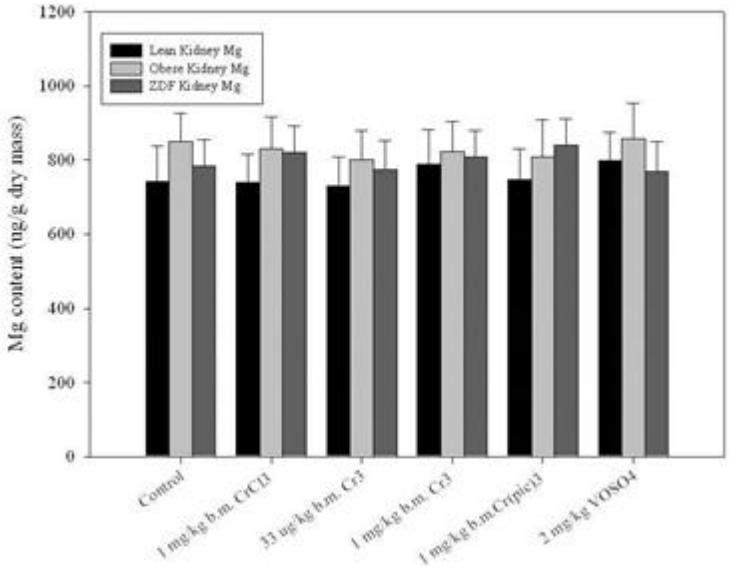


Figure 5. Iron concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A) liver, B) kidney, C) spleen, and D) heart. * - Difference in concentration from those of other two rat strains ($p \leq 0.05$). ‡ – Difference in concentration from that of ZDF rat ($p \leq 0.05$).

A


Lean Liver Mg
Obese Liver Mg
ZDF Liver Mg
Mg content (ug/g dry mass)
1000
800
600
400
200
0
†
*
*
++
*
*
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Cripic)3
2 mg/kg VOSO4

B


Lean Kidney Mg
Obese Kidney Mg
ZDF Kidney Mg
Mg content (ug/g dry mass)
1200
1000
800
600
400
200
0
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Cripic)3
2 mg/kg VOSO4

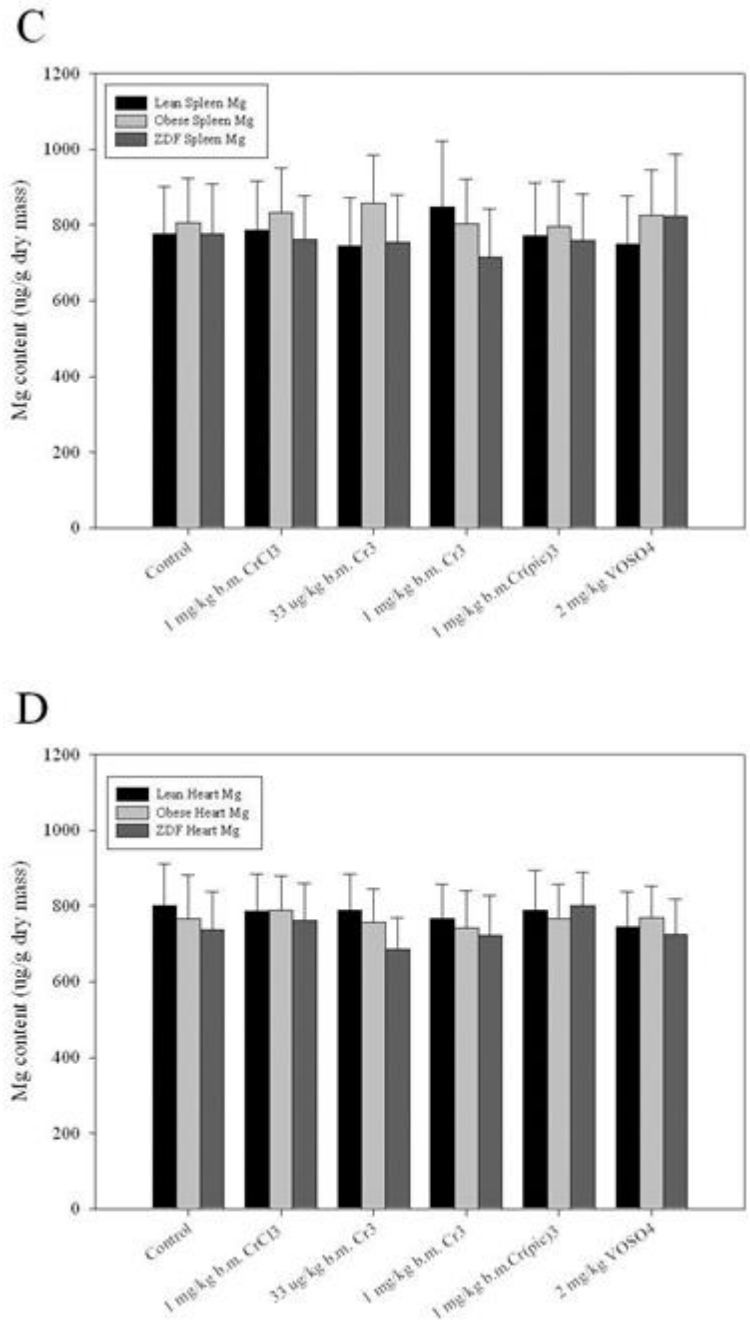
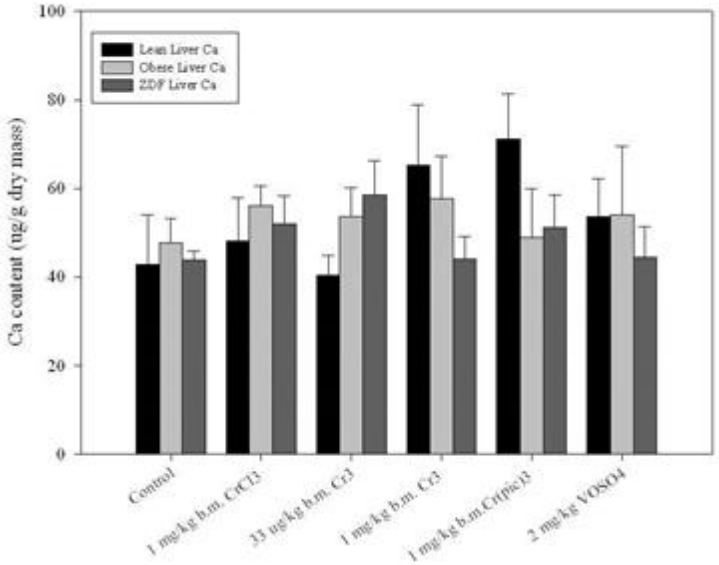
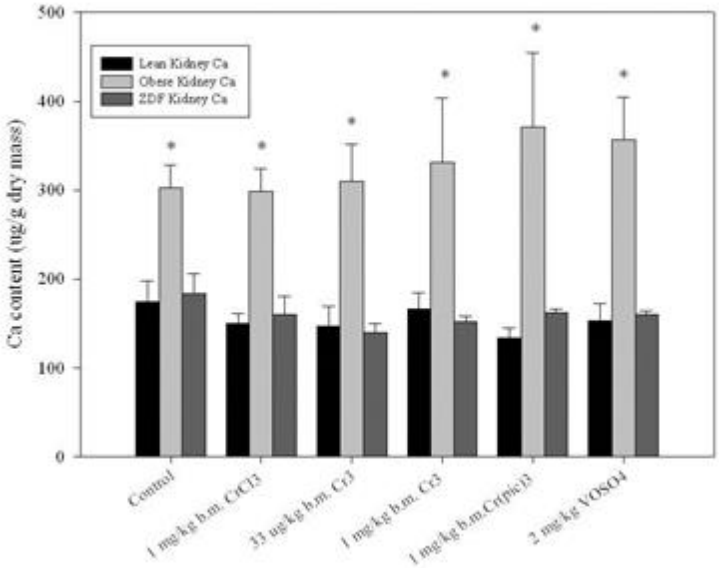


Figure 6. Magnesium concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A) liver, B) kidney, C) spleen, and D) heart. †– Difference in concentration from that of Zucker lean rat ($p \leq 0.05$). * - Difference in concentration from those of other two rat strains ($p \leq 0.05$). ‡ – Difference in concentration from that of ZDF rat ($p \leq 0.05$).

A


Lean Liver Ca
Obese Liver Ca
ZDF Liver Ca
Ca content (ug/g dry mass)
100
80
60
40
20
0
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Cr(pic)3
2 mg/kg VOSO4

B


Lean Kidney Ca
Obese Kidney Ca
ZDF Kidney Ca
Ca content (ug/g dry mass)
500
400
300
200
100
0
*
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Cr(pic)3
2 mg/kg VOSO4

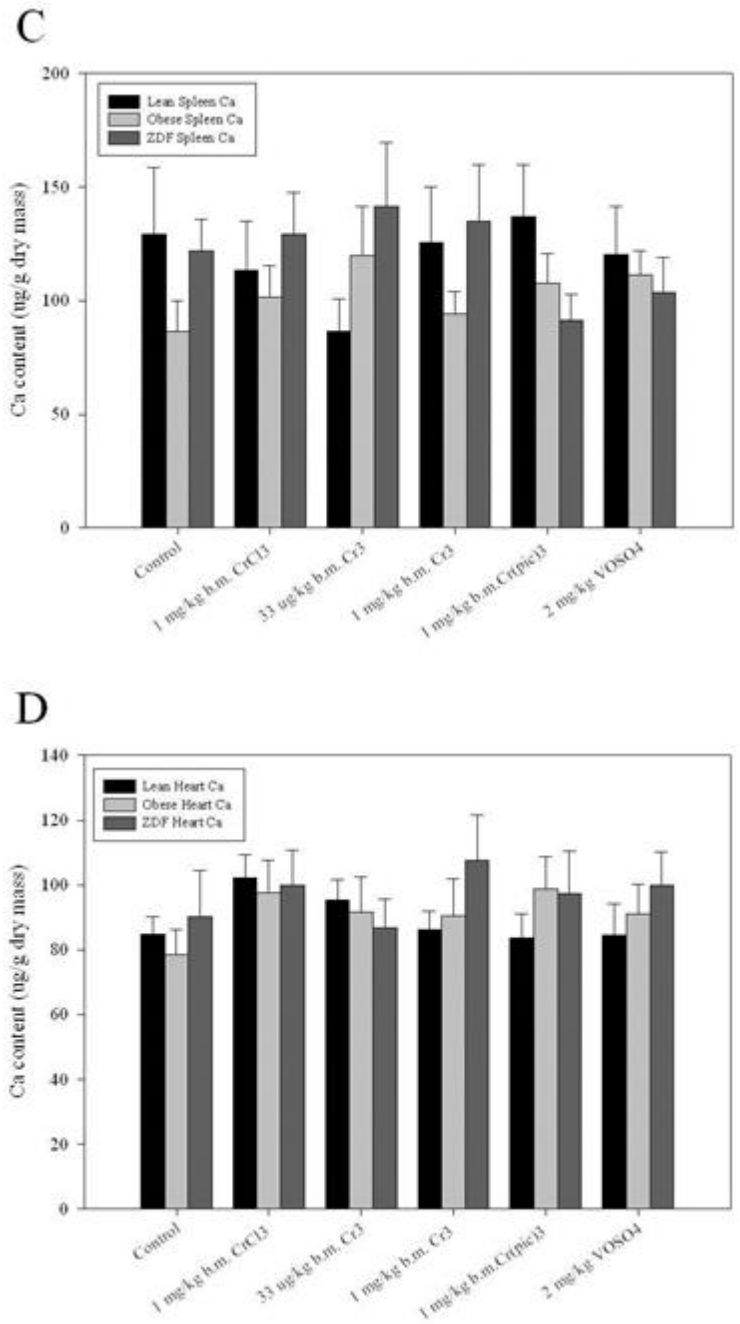
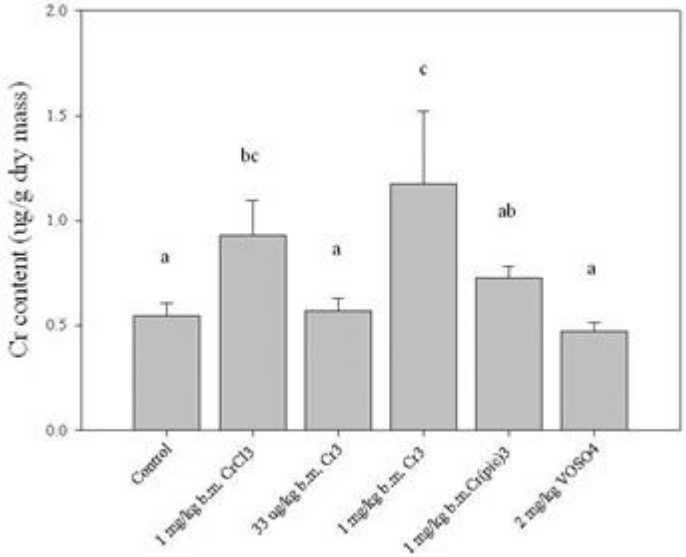
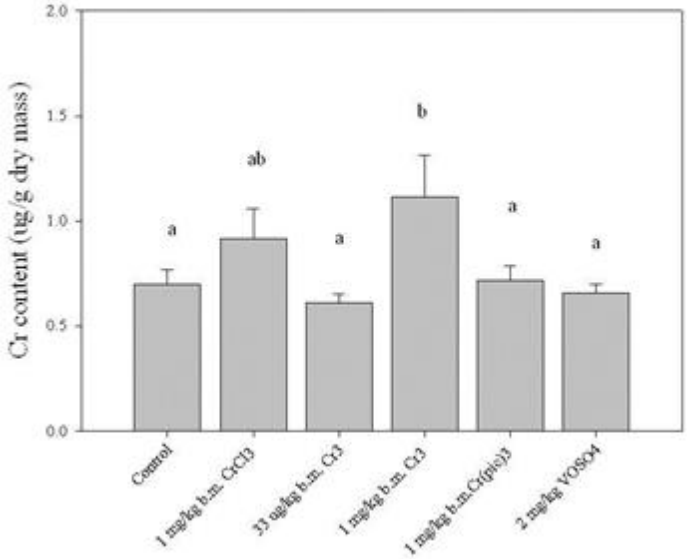


Figure 7. Calcium concentrations of tissues in Zucker lean, Zucker obese, and ZDF rats on the various diets. A) liver, B) kidney, C) spleen, and D) heart. * - Difference in concentration from those of other two rat strains ($p \leq 0.05$).

A


Cr content (ug/g dry mass)
2.0
1.5
1.0
0.5
0.0
a
bc
a
c
ab
a
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Cr(pic)3
2 mg/kg VOSO4

B


Cr content (ug/g dry mass)
2.0
1.5
1.0
0.5
0.0
a
ab
a
b
a
a
Control
1 mg/kg b.m. CrCl3
33 ug/kg b.m. Cr3
1 mg/kg b.m. Cr3
1 mg/kg b.m.Cr(pic)3
2 mg/kg VOSO4

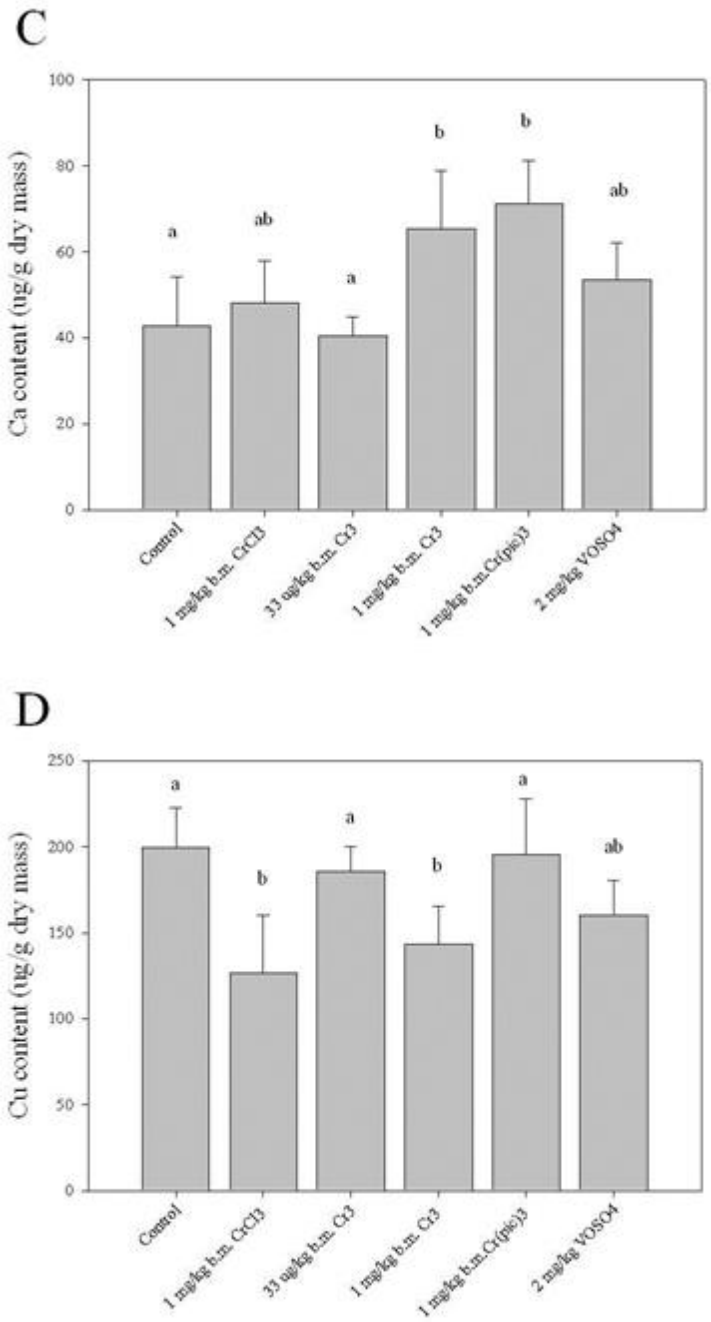


Figure 8. Differences in metal concentration as a function of diet. A) kidney Cr concentration of Zucker lean rats, B) kidney Cr concentration of Zucker obese rats, C) liver Ca concentration of Zucker lean rats, and D) kidney Cu concentration of ZDF rats. Different letters indicate significant difference between treatment groups ($p \leq 0.05$).

5.4. Discussion

5.4.1 Rat strains

Cr complexes have previously been shown to have no effect on body mass of Zucker lean and ZDF rats.[18] Cr3 at an oral dose of 1 mg Cr/kg body mass has previously been found to result in an increase in body mass of Zucker obese rats[10] in contrast to the results of the current study, although an intravenous dose of Cr3 at 20 μg/kg had no effect on body mass.[19]

Although ZDF rats possessed increased kidney copper compared to healthy rats, Zucker obese rats had more differences in metal concentration compared to control rats. Lower Cu and Zn concentrations in the liver of Zucker obese rats have been reported previously.[8] These changes presumably arise as a result of the increased fat content of the liver of the obese rats. Increased kidney Ca concentrations in Zucker obese rats compared to those of lean rats has been observed previously,[20] where the increased Ca concentration was attributed to a specific impairment in Ca-ATPase activity in the obese rats.

A high-fructose diet fed Wistar rat has little if any effect on kidney copper or kidney or liver Fe, Zn, or Cr concentrations but lowers liver Cu concentrations.[1,2] Streptozotocin-treated Wistar rats have lower Zn and Mg concentrations in the liver and kidney but increased Fe and Cu concentrations.[3] The administration to rats of both a high-fat diet and streptozotocin results in increases in Fe concentration in the liver and kidney and increases in Cu concentration in the kidney,[4,5] while other effects are not consistent between studies.

5.4.2 Chromium and vanadium supplementation

Cr(III) complexes have been studied as potential nutrition supplements and therapeutic agents for over 50 years; the complexes have been touted as weight loss agents and muscle development agents, although studies have conclusively shown that the complexes have no such effects.[18] Yet, supranutritional doses of Cr(III) complexes have been shown to have pharmacological effects in rodent models of insulin-insensitivity and diabetes.[18] The current study is designed to test whether high doses of Cr(III) complexes could lead to alterations in metal levels of healthy; obese, insulin-resistant; and diabetic rats. Three Cr compounds were chosen: $CrCl_3$, $[Cr(pic)_3]$, and Cr3. $CrCl_3$ and $[Cr(pic)_3]$ are the most studied forms of Cr(III) as nutritional supplements or pharmacological agents,[18] while Cr3 has also been extensively studied and possesses some unique characteristics such as a high degree of absorption.[18] Most notably Cr3 has previously been studied in Zucker lean, Zucker obese, and ZDF rats.[10,19]

Lay and coworkers have proposed that the pharmacological effects of Cr(III) are actually toxic effects arising from the generation of chromate from Cr(III) in the body; the chromate, as a structural analogue of phosphate, is then proposed to inhibit phosphatase enzymes.[21] Chromate, CrO_4^{2-}, was not used in this study as chromate is readily reduced to Cr^{3+} in the gastrointestinal tract and would primarily (if not solely) serve as another source of Cr^{3+}. To model the potential effects from the generation of chromate, a vanadium compound was utilized. Administration of a variety of vanadium compounds have been shown to have beneficial effects in diabetic rodent models.[22] The vanadium source appears to be converted into vanadate, VO_4^{3-}, a phosphate analogue that inhibits phosphatase enzymes including PTP1B, that deactivates phosphorylated insulin receptor. Unfortunately, the inhibition of a variety of phosphatases appears to result in harmful side effects.[22] Vanadyl sulfate at 2 mg/kg was used as the source of vanadate *in vivo* as

this is the source and dose most commonly used in studies of the effects of vanadyl in rodent models.[24]

The doses of the Cr(III) complexes were carefully chosen. The dose of 1 mg Cr/kg body mass used for $CrCl_3$, $[Cr(pic)_3]$, and Cr3 was chosen as this dose for Cr3 has been observed to result in increased insulin sensitivity and improved cholesterol levels in Zucker lean, Zucker obese, and ZDF rats.[10] Also some data exists on the accumulation of Cr in the kidney and liver of rats given this dose of $CrCl_3$, $[Cr(pic)_3]$, and Cr3.[10,24] The smaller dose of Cr3 was used to account for the difference in absorption (< 2% for $CrCl_3$ and $[Cr(pic)_3]$ [25,26,27] compared to 40-60% for Cr3),[28] while the two different concentrations of Cr3 allowed for concentration dependent effects to be examined. For comparison, the Harland Teklad diet contains ~0.4 mg Cr/kg food.[10] Given that a 100 g rat eats about 15 g food daily,[16] this amounts corresponds to the rats receiving approximately 60 μg Cr/kg body mass from the diet. Thus, the 33 mg Cr/kg body mass as Cr3 represents an approximately 50% increase in oral daily Cr. The 1 mg Cr/kg body mass doses of the Cr compounds represent an approximately 17-fold increase in oral daily Cr, clearly a pharmacologically relevant dose.

The time interval for administration of the Cr and V compounds was chosen so as to guarantee that the model rats fully developed their insulin insensitivity and diabetes and to allow for the potential effects of Cr treatment to be manifested. The rats were approximately 6 weeks old at the initiation of the study, while treatment proceeded for 12 weeks. In previous experiments with Cr3, differences in insulin sensitivity and triglyceride and cholesterol levels were significant after only 4 weeks of treatment.[10] ZDF rats start to show signs of the manifestation of diabetes at week 7 or 8 of age;[6] for males, plasma glucose levels rapidly

increase until approximately 16 weeks of age. Thus, ZDF rats at 18 weeks of age (6 weeks old at start plus 12 weeks of treatment) were ideal for these experiments.

5.4.3 Effects on tissue metal concentrations

In previous research by Krol et al.[1] for Wistar rats on a high fructose diet supplemented daily with 1 mg Cr/kg body mass or 5 mg Cr/kg as Cr3, the liver and kidney levels of Cr of the rats receiving 1 mg Cr/kg were not increased compared to those of rats on a normal diet or on an unsupplemented high-fat diet, while the Cr contents of the organs for the rats on the 5 mg Cr/kg diet were increased.[1] However, a different study from the same laboratory found a significant increase in kidney but not liver Cr concentration in Wistar rats fed a high fructose diet supplemented daily with 1 mg Cr/kg as Cr3.[29] Another study of the effects of Cr3 on streptozotocin-treated Wistar rats on a high-fat diet found that supplementing the diet daily with 1 mg Cr/kg or 5 mg Cr/kg as Cr3 had no effect on liver Cr while the kidney Cr concentrations were raised by both levels of supplementation in a dose dependent fashion.[4] Perhaps, the daily 1 mg Cr/kg dose of Cr3 is near the borderline for where rats can effectively remove the supplemental Cr compared to accumulating the metal in the kidneys and liver.

As seen in Figure 8d, $CrCl_3$ and Cr3 at doses of 1 mgCr/kg, but not [$Cr(pic)_3$], had a restorative effect on elevated Cu levels in the ZDF rats. Only one other effect was identified. The liver Ca concentration in the lean rats were significantly increased by Cr3 and [$Cr(pic)_3$] when administered at the 1 mg Cr/kg level (Figure 8C). Levels of Ca tended to increase for all treatments except the lower dose of Cr3, although the effects were not statistically significant for $CrCl_3$ and vanadyl sulfate. The significance of this observation is uncertain at present.

The ability of Cr3 to have a beneficial effect on a diabetic symptom of the ZDF rats is consistent with effects of Cr3 supplementation on the ZDF rats. In fact, Cr3 has been reported to

have beneficial effects on several rat models of diabetes and insulin resistance by Vincent and coworkers. Healthy Sprague Dawley rats treated daily with 20 μg Cr/kg body mass as Cr3 intravenously for 12 weeks had lower blood plasma insulin, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides, but not glucose, levels.[30] When given intravenously at 20 μg Cr/kg body mass, Cr3 lowered blood plasma insulin, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides, but not glucose, levels in healthy Sprague Dawley rats after 4, 8, 12, 16, 20 and 24 weeks; also 2-h plasma glucose and insulin levels after a glucose challenge were lowered.[19] In rats with streptozotocin-induced diabetes, this treatment had no consistent statistically significant effects although plasma insulin, total cholesterol, and triglycerides tended to be lower;[19] the streptozotocin treatment appeared to have increased the spread of the value of the measured variables resulting in a loss of sufficient power to resolve any effects. Finally, similar intravenous treatment of Zucker obese rats resulted in lower blood plasma insulin, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides, but not glucose, levels; also 2-h plasma insulin, but not glucose, levels after a glucose challenge were lowered.[19] In contrast, Cr3 only lowered plasma insulin levels in Zucker lean rats, suggesting a difference between effects in healthy strains; however, total cholesterol and triglycerides tended to be lower in these animals.[19]

The effects of gavage administration of Cr3 have been examined.[10] At levels of 250, 500, or 1000 μg Cr/kg body mass, the treatment at all concentrations lowered fasting plasma insulin, triglycerides, total cholesterol and LDL cholesterol levels of healthy Sprague Dawley rats while having no effect on plasma glucose or HDL cholesterol. These levels were lower after 4 weeks of treatment and remained lower for the next 20 weeks of treatment. The maintenance of glucose levels with less insulin indicates increased insulin

sensitivity. Both plasma glucose and insulin levels were lowered in 2-h glucose tolerance tests. In Zucker obese rats receiving 1000 μg Cr/kg body mass, the results were similar to those from intravenous administration. The effects of Cr3 on Zucker Diabetic Fatty (ZDF) rats were also examined using 1000 μg Cr/kg body mass. Again, fasting plasma insulin, triglycerides, total cholesterol and LDL cholesterol levels were all lower while glucose concentrations were consistently but not statistically lower. HDL levels were lowered from their very high levels; 2-h plasma insulin levels were also lowered. Plasma glycated hemoglobin levels, a measure of longer term blood glucose status, were examined in the healthy, Zucker obese and ZDF rats after 4, 12 and 24 weeks of treatment. No effect was seen for the healthy rats; however, significant effects were noted for the diabetic models. For the ZDF rats, glycated hemoglobin was lower after 12 and 24 weeks of treatment, reaching almost a 22% drop compared with ZDF controls by week 24; for the Zucker obese rats, glycated hemoglobin was 27% lower at week 24.[10]

The effects of the cation, Cr^{3+}, on healthy and model diabetic rats have also been examined by Krejpcio and co-workers. Male Wistar rats were provided a control diet or a diet containing 5 mg Cr per kg diet as Cr3 for 10 weeks.[31,4] Blood plasma insulin levels were lowered 15.6% by the Cr-containing diet, while glucose transport by red blood cells was increased 9.6%. In another study, Krejpcio and co-workers utilized male Wistar rats with streptozotocin-induced diabetes. Using similar diets for 5 weeks, the rats that had the Cr diet had lower blood serum glucose levels (26%) and increased HDL levels (14%).[31] Cr3 supplementation of the diet (AIN-93M or high-fructose diet) of male Wistar rats for 8 weeks (0, 1, and 5 mg Cr per kg body mass daily) has been shown to result in increased insulin sensitivity without affecting blood plasma glucose or lipid levels; no effects were observed on body mass as an effect of supplemental

chromium.[19] Thus, the results of the short term study with male Wistar rats are very similar to those of the current study. In another study, male Wistar rats were fed a control (AIN-93M) diet or high-fat diet with or without chromium supplementation as Cr3 (0, 1, or 5 mg Cr/kg body mass) for 5 weeks; rats were subsequently injected with streptozotocin before being fed the same diets for another week.[4] Cr3 increased insulin sensitivity and lowered serum total and LDL cholesterol and triglycerides levels but had no effect on blood glucose levels. No effects on body mass were observed except the group receiving high-fat diet containing the highest dose of Cr3 had lower body mass weeks three through five than rats on the high-fat diet not receiving any supplemental chromium; the effect disappeared after streptozotocin treatment.

The accumulation of Cr in the kidney and liver of rats receiving supplemental chromium has been observed previously, although the results are dependent on the form of Cr and dose. A detailed study of chromium accumulation as a function of dose has been reported for $CrCl_3$ and $[Cr(pic)_3]$.[24] Cr content of kidney and livers of Sprague Dawley rats increased in a linear fashion when the rats received daily doses of $CrCl_3$ or $[Cr(pic)_3]$ for 24 weeks ranging from 750 μg Cr/kg body mass to 15 mg Cr/kg.[24] In contrast to the current work, the tissue concentration was greater for $[Cr(pic)_3]$ than $CrCl_3$. The results are consistent with a recent study on absorption of $CrCl_3$ and $[Cr(pic)_3]$ that suggested $CrCl_3$ was better absorbed by rats that $[Cr(pic)_3]$ when tissue Cr concentration were considered in addition to urinary output of Cr.[28]

Previously, no change in kidney or liver Cr concentrations of Zucker obese or ZDF rats receiving 1 mg Cr/kg body mass as Cr3 orally daily for six months has been reported;[10] this was accompanied by no changes in liver Fe concentration but by a small, but statistically significant drop in kidney Fe concentration in the Zucker obese rats but not the ZDF rats.[10] The lack of Cr accumulation from Cr3 in the Zuckler obese and ZDF rats is consistent with the current study.

However, healthy Sprague Dawley rats receiving Cr3 orally daily for 6 months at doses of 250, 500 or 1000 μg Cr/kg also displayed no accumulation in liver or kidney Cr,[10] in contrast to the observed statistically significant increase in liver Cr for the Zucker lean rats in the current study. Whether this difference results from the use of different strains of rats will require more study.

5.5 Conclusion

Cu, Zn, Fe, Mg, and Ca concentrations were compared and contrasted for the first time in the liver kidney heart and spleen and Cr concentration in the liver and kidney of Zucker lean, Zucker obese and ZDF rats. ZDF rats possessed increased concentrations of kidney Cu compared to the lean rats while kidney Ca concentrations were increased in the Zucker obese rats. Zucker obese rats displayed a reduction in the concentration of Cu, Zn, Fe, and Mg in the liver compared to ZDF and/ Zucker lean rats, presumably because of increased fat content in the liver of Zucker obese animals. Spleen Fe concentrations were decreased in Zucker obese animals in comparison to the lean rats. The supplementation with Cr(III) complexes resulted in few tissue metal changes. Treatment with $CrCl_3$ and Cr3 but not $[Cr(pic)_3]$ at 1 mg Cr/kg body mass resulted in the accumulation of Cr in the kidney of lean and obese but not ZDF rats. The lowering of elevated kidney Cu in ZDF rats suggests a beneficial effect on this symptom of type 2 diabetes. $VOSO_4$ had no significant effects.

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Conclusion and future results

Four lines of evidence have been used to suggest that chromium was essential: 1) Carefully controlled studies with rats have induced apparent Cr deficiency manifested by insulin insensitivity in glucose tolerance tests.[1,2,3] 2) In humans, Cr deficiency has apparently been observed for five patients on total parenteral nutrition (TPN), where it resulted in symptoms similar to those of type 2 diabetes, which were reversed upon Cr supplementation of the TPN.[4,5,6,7] 3) In female humans, Cr absorption is inversely proportional to intake.[8] 4) Changes in plasma insulin levels result in changes in urinary Cr excretion.[9]

Cr deficiency was believed to be a byproduct of diabetes, because diabetic mammals excrete more chromium in their urine than healthy mammals. This deficiency could theoretically make Cr conditionally essential for diabetic mammals. The study in Chapter 2 shows that Cr is not conditionally essentially because Zucker diabetic rats appeared to not only excrete more Cr but also absorb more Cr.

Currently no measure of Cr status exists. Cr currently has no biomarker nor is bound to any known biomolecule used in an essential process. Cr is absorbed by passive diffusion, but Cr excretion is known to be insulin stimulated.[9] In Chapter 3 an attempt to use insulin stimulated urinary chromium excretion as a biomarker was made. The results showed that within the groups of animals, variation of responses existed, which made this method of Cr status detection unreliable. Thus, still no biomarker for Cr status exists.

In the mid 1990s Striffler et al. fed rats either a high sugar or high fat "low Cr"diet with a Cu deficiency to manifest the symptoms of "Cr deficiency."[1, 2, 3] The diets contained 33 µg of Cr/kg. After 16 weeks, some animals received Cr supplemented waters and others received water with no added Cr. The "Cr deficient" animals were said to have developed hyperinsulinemia but had statistically equivalent rates of excess glucose. Chapter 4 uses animals on carefully controlled diets with the lowest published dose of 16 µg/kg Cr that does not produce an apparent deficiency or inability to handle an insulin or glucose challenge. This result suggests that the hyperinuslinemia that was reported in the Striffler experiments was possibly a diet induced. In Chapter 4, animals receiving pharmacological doses of Cr were shown to have increased insulin sensitivity. Until further evidence for the essentiality of Cr is given, Cr can no longer be considered an essential element. The effects of Cr supplementation appear to only be pharmacologically relevant and not nutritionally relevant, because Cr deficiency cannot be induced.

Cr should no longer be deemed a nutritionally relevant trace element based on the previous accepted lines of evidence, however a need still exists to determine a pharmacological mechanism for Cr. In Chapter 5, tissue metal concentrations of $CrCl_3$, Cr3 (in two doses), $[Cr(pic)_3]$ or vanadyl sulfate dosed Zucker lean, obese and diabetic animals were compared and contrasted to test the hypothesis that Cr supplementation could alter metal concentrations. $CrCl_3$ and Cr3 at 1mg Cr/kg body mass had a restorative effect on the elevated Cu levels in the ZDF rats but resulted in the accumulation of kidney Cr in the lean and obese rats. Zucker obese rats displayed reduced Cu, Zn, Fe, and Mg in the liver in comparison to ZDF and Zucker lean animals, which could be due to the increased fat content of the liver. Spleen Fe concentrations were decreased in Zucker obese animals in comparison the lean rats.

Understanding the pharmacological mechanism of Cr could potentially lead to the understanding of the mixed results seen in patients supplemented with Cr. Future works should include studying the effect Cr has on the expression of insulin signaling cascade proteins as well as the effect (whether amplifying or disrupting the phosphorylation) on insulin signaling cascade proteins. This could lead to the understanding of the mechanism of action. Understanding the cause of kidney Cu accumulation in diabetic patients and how Cr has a restorative effect could possibly help to understand the role chromium is playing in relieving diabetic symptoms. Performing follow up studies on patients who respond to Cr treatment to probe for similarities could also possibly suggest a reason for the variation in responses from person to person.

The field of Cr biochemistry is not new, but researchers will have to explore new ways of thinking in order to move the field forward. Research as recent as 2013 in still attempting to manipulate high Cr yeast to explore the possibility of a Cr binding substance that will be beneficial in the treatment of disease.[10] More research should be done on understanding the mechanism of Cr and isolating the biologically active species *in vivo*. More research should be done on how Cr enters the cells from endosomes and how it binds low molecular weight binding protein. Research must combine pharmacological research with traditional biochemistry to scientifically understand what is happening in experiments.

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