EFFECTS OF CHROMIUM AND COPPER

DEPLETION OR SUPPLEMENTATION

ON IMMUNITY IN BHE/cdb RATS OR

IN HYPERCHOLESTEROLEMIC

POSTMENOPAUSAL

WOMEN

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ACKNOWLDEGEMENTS

I would like to appreciate to Dr. Barbara Stoecker for her sincere guidance, professional suggestions, support, and encouragement for study, research, and completion of this manuscript. Also, I thank to my committee members Drs. Janice Hermann, Andrea Arquitt, and Kim Burnham for their time, effort, and guidance.

I would like to acknowledge Dr. C. D. Berdanier for supply of BHE/cdb rats for this study, Dr. Tim Kramer for his generous sharing and guidance of whole blood cell proliferation method, Dr. Julie Wallace for helping development of SOD activity measurement method, Dr. Lochmiller for letting me use instruments in his lab, and Dr. Elizabeth Droke for her time and effort for answering my numerous questions.

I would like also thank to individuals helping completion of the research: Dr. Edralin Lucas, Dr. Bernice Adeleye, Jarrod King, Rachel Belvin, Ruth Ann Givens, Bo Burns, Dan Ratford, Mohamad El-Osta, and Jeanette Kniess. I also would like to thank to coffee club members for joyful chatting, attending aerobic classes together, and encouragement. I thank to the Department of Nutritional Sciences at Oklahoma State University for financial support.

I would like to appreciate my family for their support, prayer, and loyalty to the completion of the study. I really appreciate to my loving husband for his love, support, and help throughout the study. I would like to share the Ph.D. degree with him in my heart.

Finally, I would like to thank God for his love and endless grace in my life.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
BHE	bureau of home economics
BMI	body mass index
Ca	calcium
ConA	concanavalin A
Cr	chromium
$CrCl_3$	chromium chloride
Cu	copper
ESADDI	estimated safe and adequate daily dietary intake
Fe	iron
GTF	glucose tolerance factor
GTT	glucose tolerance test
HDL	high density lipoproteins
IL-2	interleukin 2
IL-2R	interleukin 2 receptor
LDL	low density lipoproteins
LPS	*Escherichia coli* lipopolysaccharide
MLI	mixed lymphocyte interaction
Mg	magnesium
Mn	manganese
MNC	mononuclear cells
OGTT	oral glucose tolerance test
PBMNC	peripheral blood mononuclear cells
PHA	phytohemagglutinin
PMN	polymononuclear cells
PWM	pokeweed mitogens
RBC	red blood cell
TH	T helper cell
VLDL	very low density lipoproteins
Zn	zinc

CHAPTER I

INTRODUCTION

Research Problem

Environmental factors including nutrition and stress influence the immune response. The importance of trace elements, including copper, manganese, magnesium, and chromium, in immune function has been studied. Also effects of stress on the immune response, for example, macrophage phagocytosis, mitogenic lymphocyte proliferation, serum levels of specific antibodies, and interleukin 2 production, have been investigated (Lukasewycz and Prohaska, 1983, Good and Lorenz, 1992, Fleshner et al., 1995, Failla and Hopkins, 1998). Well balanced nutritional intake has very important roles in immune function in all age groups of the population. Nutrition has significant roles in immunocompetence in elderly subjects because their sometimes poor dietary intakes lead to impaired immunocompetence. Almost one third of the elderly population may have vitamin and trace mineral deficiencies (Chandra, 1997b). The host defense system of humans is decreased with aging. Also, the number of lymphocytes, mature T cells, and helper T cells is decreased with aging (Kawakami et al., 1999). Decreased nutritional intake detrimentally affects the immune system in elderly and improvement in

nutritional intake would increase immune function (Hulsewe et al., 1999, Kawakami et al., 1999).

Chromium (Cr) is known as a regulator of normal glucose tolerance in experimental animals and in humans because chromium is involved in carbohydrate and lipid metabolism; chromium supplementation potentiates the action of insulin, reduces serum triglycerides, and increases serum high-density lipoprotein cholesterol (Mertz et al., 1974, Anderson et al., 1983, 1992). Cr deficiency produced impaired glucose tolerance in humans and in animals, and Cr supplementation lowered blood glucose in patients with impaired glucose tolerance by improving insulin receptor number, affinity, or both (Anderson et al., 1987, Anderson, 1993). Moreover, Cr supplementation increased serum IgM and total immunoglobulins and decreased serum cortisol in stressed calves. Increased serum immunoglobulins and reduced serum cortisol may improve immune function (Chang and Mowat, 1992, Moonsie-Shageer and Mowat, 1993). Cr supplementation in stressed feeder calves increased primary antibody response to human erythrocytes and levels of IgG_1 on day 14 of treatment, but levels of IgM and IgG_2 were not affected by Cr supplementation (Chang and Mowat, 1992, Moonsie-Shageer and Mowat, 1993). In addition, cell-mediated immune responses to chromium supplementation were studied in stressed dairy cows. The chromium supplemented group (0.5 ppm amino acid-chelated Cr in the diet as Metalosate, 2.68% Cr) increased mitogen-stimulated blastogenic responses of peripheral blood mononuclear cells (PBMNC) compared with unsupplemented controls (Burton et al., 1993).

Copper (Cu) is an essential trace element in the host defense system of humans and animals. Copper deficiency increased susceptibility to pathogens, reduced T-

lymphocyte activities, reduced number of splenic antibody-producing cells, and reduced interleukin production (Mulhern and Koller, 1988, Kramer et al., 1988, Davis et al., 1987).

In spleen and peripheral blood lymphocytes, a metabolic stressor caused reduction of mitogenic proliferation of T lymphocytes but lymph node lymphocytes were not reduced (Fleshner et al., 1995). T cell mitogen or antigen-stimulated human or rat lymphocytes produce T cell growth factor (Interleukin-2). Continuous growth of mitogen-stimulated splenocytes caused depletion of T cell growth factor that resulted in decreased cell proliferation or cell death (Gills et al., 1978).

In this study, effects of chromium and copper on the immune function in animals prone to diabetes mellitus and in hypercholesterolemic postmenopausal women were investigated. Diabetes mellitus prone rats were fed chromium and/or copper depleted or adequate diets for 21 weeks. Hypercholesterolemic postmenopausal women were supplemented with chromium, copper, or chromium and copper combined capsules for 12 weeks.

Objectives

The following research objectives were developed for this study:

1. to determine effects of Cr and/or Cu depletion on T and/or B cell proliferation in BHE/cdb rats.

2. to determine effects of Cr and/or Cu depletion on the number of lymphocytes or neutrophils in BHE/cdb rats.

3. to determine effects of Cr and/or Cu depletion on superoxide dismutase activity in BHE/cdb rats.

4. to determine effects of Cr and/or Cu depletion on serum glucose, insulin, or corticosterone in BHE/cdb rats.

5. to determine effects of Cr and/or Cu supplementation on T cell proliferation in hypercholesterolemic postmenopausal women.

6. to determine effects of Cr and/or Cu supplementation on the number of lymphocytes, neutrophils, monocytes, eosinophils, or basophils in hypercholesterolemic postmenopausal women.

7. to determine effects of Cr and/or Cu supplementation on ceruloplasmin or superoxide dismutase activity in hypercholesterolemic postmenopausal women.

8. to determine effects of Cr and/or Cu supplementation on interleukin 2 production from mitogen stimulated blood cell cultures or serum cortisol in hypercholesterolemic postmenopausal women.

Hypotheses

The following hypotheses were developed for this study:

1. There will be no statistically significant effects of Cr and/or Cu depletion on T and/or B cell proliferation in BHE/cdb rats.

2. There will be no statistically significant effects of Cr and/or Cu depletion on the number of lymphocytes or neutrophils in BHE/cdb rats.

3. There will be no statistically significant effects of Cr and/or Cu depletion on superoxide dismutase activity in BHE/cdb rats.

4. There will be no statistically significant effects of Cr and/or Cu depletion on serum glucose, insulin, or corticosterone in BHE/cdb rats.

5. There will be no statistically significant effects of Cr and/or Cu supplementation on T cell proliferation in hypercholesterolemic postmenopausal women.

6. There will be no statistically significant effects of Cr and/or Cu supplementation on the number of lymphocytes, neutrophils, monocytes, eosinophils, or basophils in hypercholesterolemic postmenopausal women.

7. There will be no statistically significant effects of Cr and/or Cu supplementation on ceruloplasmin or superoxide dismutase activity in hypercholesterolemic postmenopausal women.

8. There will be no statistically significant effects of Cr and/or Cu supplementation on interleukin 2 production from mitogen stimulated blood cell cultures or serum cortisol in hypercholesterolemic postmenopausal women.

Limitations

The following limitations were present in this study:

Both diabetes mellitus prone BHE/cdb rats and hypercholesterolemic postmenopausal women were studied. Results from rats can not be extended to human subjects. The BHE/cdb rats did not develop diabetes mellitus throughout the study, so results can not be applied to diabetic animals or to diabetic human subjects. These animals were fed high fat diets after reaching the growth plateau, so results can not be

extended to animals fed low fat diets.

Results from these human subjects can not be extended to other groups of people. The primary investigator instructed all subjects, and it was assumed that all subjects would take supplemental capsules two times a day with breakfast and dinner for twelve weeks. The supplemental capsules were counted at the end of study to determine the compliance. A subject with leftover capsules of 60 (36% of the total supplements) was considered as a poorly compliant subject and was excluded from the data analysis. The sample size in the human study was small because it was hard to recruit hypercholesterolemic postmenopausal women not on estrogen replacement therapy or cholesterol lowering medication, so results can not be applied to the whole population. Also, there was no monitoring of subjects' fasting before the blood sampling. We requested that they fast for 12 hours prior to each blood collection. Subjects were requested not to change medication intake or eating patterns, but there were no controls on medication intake or on alteration of eating patterns throughout the study, so we can only assume there was no influence of medication or dietary intake on the results of supplementation. Dietary chromium and/or copper intake might affect supplemental chromium and/or copper absorption and excretion. Other nutrients such as zinc or iron also might affect chromium and/or copper absorption or metabolism.

CHAPTER II

REVIEW OF THE LITERATURE

Nutrition and Immunity

Environment as well as nutrition influences the generation and maintenance of the immune response. A proper immune response requires adequate trace metal nutriture (Chandra and Newberne, 1977). The effects of a general nutritional deficiency state or effects of a single nutrient deficiency over a certain time period have been investigated related to immune function in humans and animals. The effects of zinc, copper, selenium, iron, vitamins C, B_6, A, E, and folic acid, and the polyunsaturated fatty acids are most studied related to immune function (Harbige, 1996). Knowledge about nutritional intake, metabolism, concurrent illness, exposure to infectious agents, duration of the deficiency, and genetic factors must be considered in the evaluation of nutritional status related to immunological competence (Cunningham-Rundles, 1982).

Immune responses are affected in the early stage of micronutrient deficiency. The severity of nutrient deficiency, nutrient interactions, and the presence of infection can all impair immune responses (Chandra, 1997a). Also, the specific biochemical and molecular roles of the essential nutrients in the host defense system including

development, activation, differentiation, and effector functions of the diverse cells are hard to define (Failla and Hopkins, 1997).

To analyze the effect of dietary and environmental factors on immunocompetence in vitro, lymphocyte proliferation is most commonly used due to its reliability and technical simplicity (Failla and Hopkins, 1997). The lymphocyte proliferation requires the activation of unprimed T-lymphocytes by antigen-presenting cells and plant lectins such as phytohemagglutinin (PHA) and concanavalin A (ConA) stimulation (Failla and Hopkins, 1997). Also, the microbicidal activity of neutrophils as well as surface marker identification of lymphocyte subpopulations, dermal erythema, and measurement of cytotoxic effector cells can be useful for in vitro immune function analyses related to nutrient deficiency (Cunningham-Rundles, 1982). For humoral immunity evaluation, the antibody response to immunization and measurements of serum immunogloblins and secretory IgA are also useful methods (Cunningham-Rundles, 1982). Well standardized assays and parallel studies with control groups are required to assess immune function in nutritional deficiency (Cunningham-Rundles, 1982).

In this study, effects of chromium and/or copper depletion or supplementation on immune functions in BHE/cdb rats or in hypercholesterolemic postmenopausal women were measured by mitogen stimulated lymphocyte proliferation assays. Also, differential cell profiles including lymphocytes, neutrophils, monocytes, eosinophils, and basophils and stress hormones such as corticosterone (rats) or cortisol (humans) were measured to evaluate chromium and/or copper depletion or supplementation effects on immune function.

BHE /cdb Rats

The Bureau of Home Economics (BHE) rat strain was developed in 1942 by crossing Albino (Yale strain obtained from Columbia University) and black and white hooded rats (Pennsylvania State College strain). The BHE/cdb rat is a substrain of the BHE rat maintained at the University of Georgia. The BHE/cdb rat develops impaired glucose tolerance at midlife (about 300 days old) and type II diabetes mellitus as they age (Adams, 1964, Berdanier, 1991, Mathews et al., 1995).

The BHE/cdb rat has increased lipogenic and gluconeogenic activity and decreased pancreatic endocrine response to a glucose challenge. This strain has 50% of the average life span of a normal rat due mainly to renal disease. All these characteristics are similar to type II diabetes mellitus (Adams, 1964, Berdanier, 1991, Mathews et al., 1995).

Female BHE/cdb rats fed high fat (22%) diets developed abnormal glucose tolerance at an age of 200 days whereas female BHE/cdb rats fed stock diets developed impaired glucose tolerance at an age of 400 days (Mathews et al., 1995). In pregnant female BHE/cdb rats briefly exposed (3 weeks before and during gestation) to the high fat diet, glucose tolerance degenerated by 19 days of gestation (Bue et al., 1989).

Elderly and Immunity

The elderly population is growing fast in the United States (Wood et al., 1995). Nutritional intake of most elderly people is less than recommended dietary allowances

(RDA) and almost one third of the elderly population has vitamin and trace mineral deficiencies (Mowe et al., 1994, Chandra, 1997b). Elderly people seem to be vulnerable to several nutrient deficiencies such as iron, zinc, vitamin C, and vitamin E due to improper dietary intake. These nutrient deficiencies consequently cause depressed immune function in the elderly (Chandra, 1989, Buzina-Suboticanec et al., 1998). In addition, the occurrence of diabetes mellitus is increased with aging (Morely et al., 1987). Impaired immune function leads to increased infections in older diabetic patients and this results in increased morbidity and mortality (Kajanachumpol et al., 1995).

With age, the clonal proliferation of stem cells, the generation of B cells, and homing of precursor cells into the thymus are decreased. These decreased functions of stem cells induce increased response to stress, such as infection (Chandra, 1997a). With age, the lymphocyte proliferation to PHA and ConA and the natural killer cell activity are decreased in humans and animals. For example, in a vitamin E supplementation study, thirty-two men and women (60 years old and older) were assigned to placebo or vitamin E (400 IU dl-α-tocopheryl acetate in soybean oil) supplemented groups for 30 days. The lymphocyte proliferation with ConA stimulation was significantly increased in the treatment group compared to the placebo group. However, there was no significant difference in the lymphocyte proliferation with PHA stimulation between placebo and treatment groups. Moreover, the interleukin 2 (IL-2) production with ConA stimulation was significantly increased in the treatment group compared to the placebo group (Meydani et al., 1990). The number of suppressor T cells and natural killer cells was increased and the number of mature T cells and helper T cells was decreased with aging. Moreover, a mitogenic response to PHA and ConA was decreased in elderly subjects with

malnutrition (Kawakami et al., 1999). In Mazari and Lesourd's study (1998), elderly subjects (80±5 years) had decreased CD3+ and CD8+ T cells compared to healthy young subjects (25±5 years). Elderly subjects with poor nutritional status (low serum folate or low serum albumin) had significant decreases in the number of CD4+ cells and in T cell functions compared to elderly subjects with adequate serum folate (Mazari and Lesourd, 1998).

Postmenopausal Women and Immunity

There are no studies about effects of mineral supplementation on postmenopausal women without estrogen replacement therapy. Most studies investigated the effects of estrogen therapy or different types of estrogen therapy on immune function in postmenopausal women.

In Helgason and Schoultz' study (1981), the mixed lymphocyte reaction of sera from postmenopausal women with estrogen therapy (ethinyl estradiol, 0.05 mg/d for one month or estrone sulfate, 2.5 mg/d for 6 months) was significantly decreased compared to before the estrogen treatment. However, blastogenesis of PHA or ConA stimulated lymphocytes was significantly increased with estrogen therapy compared to postmenopausal women without estrogen therapy (Malarkey et al., 1997). Estrogen increases B-cell mediated immunity or suppresses T-cell mediated immune function (Vollenhoven and McGuire, 1994), so effects of estrogen therapy on immune function in postmenopausal women need further investigation.

Chromium

Chromium potentiates insulin activity and efficiency via increased insulin receptor number, increased insulin binding, and increased insulin receptor phosphorylation and chromium decreases insulin requirements (Anderson, 1986, 1998, Mertz, 1998). The site of chromium action is known as the insulin-sensitive cell membrane (Mertz, 1998). Chromium is transferred from plasma to tissues, and it functions in maintaining glucose levels with insulin as a cofactor (Earle et al., 1989). Moreover, chromium has functions in the prevention of type II diabetes mellitus rather than as a cure for type II diabetes mellitus (Anderson, 1989). Glucose intolerance and diabetes increase with age and the level of chromium decreases with age (Harris, 1990, Harris et al., 1998, Davies et al., 1997).

Also, subjects given chromium-rich brewer's yeast showed improvements in serum cholesterol, total lipids, and glucose tolerance and these suggest that chromium has functions in glucose and lipid metabolism (Offenbacher and Pi-Sunyer, 1980).

The estimated safe and adequate daily dietary intake (ESADDI) of chromium is 50-200 μg/d (National Research Council, 1989), but most people consume close to 25 μg/d which is less than the recommended level. However, most people in the U.S. do not have elevated fasting glucose with less chromium intake than the recommended level. The ESADDI for chromium may be too high because this estimation is based on the early data from chromium studies. Early chromium studies were not able to correct for background in the chromium measurement or to adequately control contamination, so early data were higher than data from recent chromium studies. Therefore, the range of

ESADDI for chromium should be lower than the current range (Hunt and Stoecker, 1996). Also, a supplemental chromium intake above 200 μg/d in normal people without Cr deficiency does not have beneficial effects on glucose tolerance, plasma insulin, cholesterol, or triglycerides. It is hard to reach the upper range of the current ESADDI via dietary chromium intake. Dietary intake of 25-35 μg/d of chromium may be sufficient to maintain normal chromium status (Nielsen, 1997). The chromium concentration of foods is variable depending on growing, processing, preparation, fortification, and handling conditions (Anderson et al., 1992). Approximately 15 μg of chromium is contained per 1000 Kcal of the diet. Turkey ham, grain products, barbecue sauce, and black pepper are good sources of chromium. (Anderson et al., 1992).

Chromium is an essential element in human and animal nutrition because it has functions in carbohydrate and lipid metabolism and immune function. Therefore in this section of the dissertation, the following topics from the literature are reviewed: functions of chromium in carbohydrate and lipid metabolism, absorption and excretion of chromium, and chromium and immune function.

Chromium and Carbohydrate Metabolism

Chromium has a role in maintaining glucose tolerance, so effects of chromium supplementation or depletion on glucose tolerance have been studied. In an animal study, there were severe glucose intolerance symptoms including fasting hyperglycemia and glycosuria in rats fed a diet for 10 months containing rye seed, dried skim milk, and corn oil which contained 170 μg of chromium per kilogram of diet (Donaldson et al., 1985). However, plasma glucose at the age of 12 months was not different at fasting or after

intubation of glucose (250 mg of glucose per 100 g of body weight) between rats fed low chromium or chromium supplemented diets (Donaldson et al., 1985).

A single injection of one of two synthetic complexes of chromium-nicotinic acid-amino acid to normal mice (C57BL/KsJ) reduced the nonfasting plasma glucose either 15% or 20%. However, an injection of a brewer's yeast glucose tolerance factor (GTF), as a biologically active form of chromium, reduced nonfasting blood glucose by 36% in normal mice. In addition, an injection of a synthetic complex or a brewer's yeast GTF to genetically diabetic mice (db/db) reduced elevated blood glucose 727 ± 36 mg/dl (mean±SEM) to 596 ± 21 mg/dl (18% reduction) or 518 ± 40 mg/dl (29% reduction), respectively (Tuman et al., 1978). This study showed that the biologically active form of GTF had better effects on blood glucose reduction than the synthetic complexes.

In Striffler and colleagues' study (1998), a total of fourteen weanling Wistar male rats were fed a Cr depleted or a Cr supplemented high fat diet (40% lard, 30% sucrose, and 25% casein by weight with no Cr added or 5 ppm Cr added as chromium chloride, $CrCl_3$) for 16 weeks to measure insulin resistance. Overnight fasted animals were injected intravenously with glucose (1.25 g/kg body weight) and glucose was measured 40 minutes after the glucose injection. Fasting insulin was increased in the rats fed with a Cr deficient diet (65±10 μU/ml) compared to rats in the Cr supplemented group (31±4 μU/ml). In addition, the glucose clearance rate was slower in the Cr deficient group (1.74±0.22 %/min) compared to the adequate Cr group (2.39±0.11 %/min).

In human studies, chromium supplementation with 200 μg $CrCl_3$/d for 12 weeks in elderly subjects improved glucose tolerance and β-cell sensitivity (Potter et al., 1985). Anderson and coworkers (1983) studied effects of $CrCl_3$ supplementation on glucose.

Subjects with serum glucose greater than or equal to 100 mg/dl at 90 minutes following a glucose load showed a significant decrease in blood glucose with 200 μg/d of Cr supplementation. The mean blood glucose was decreased from 135±9 mg/dl to 116±9 mg/dl and 115±11 mg/dl with 2 months and 3 months of Cr supplementation, respectively. Moreover, subjects with serum glucose less than or equal to their fasting levels at 90 minutes following a glucose load showed a significant increase in serum glucose with Cr supplementation. The mean blood glucose at 90 minutes was increased from 71±2 mg/dl to 77±5 mg/dl and 84±5 mg/dl with 2 months and 3 months of Cr supplementation, respectively. Therefore, Cr supplementation significantly decreased blood glucose in subjects with hyperglycemia and significantly increased blood glucose in subjects with hypoglycemia.

In Ravina and colleagues' study (1999), three corticosteroid-induced diabetic patients were supplemented with 600 μg Cr/d as chromium picolinate. The mean fasting blood glucose was decreased from >250 mg/dl to < 150 mg/dl. This result suggests that serum glucose concentration in patients with corticosteroid-induced diabetes might be improved with chromium supplementation.

Also, effects of a chromium-rich brewer's yeast on glucose tolerance were investigated (Offenbacher and Pi-Sunyer, 1980). Elderly subjects aged 63 years and older received either 9 g of chromium-rich brewer's yeast or 9 g of chromium-poor torula yeast for 8 weeks. The brewer's yeast contained 10.8 μg Cr and the torula yeast contained less than 0.45 μg Cr per 9 g of daily supplement. Eight nondiabetic subjects and four diabetic subjects were included in each group. There was significant improvement in glucose tolerance only in diabetic subjects with chromium-rich brewer's yeast supplementation.

At 90 minutes following a glucose load, the decrease in blood glucose was significant in diabetic and nondiabetic groups with brewer's yeast supplementation and it was the greatest in the diabetic subject group with brewer's yeast supplementation. However, there were no significant changes in serum glucose in the control group with torula yeast supplementation.

Earle and colleagues (1989) compared plasma and urine concentrations of chromium between lean (BMI < 30 kg/m^2) and obese (BMI > 30 kg/m^2) diabetic subjects (type I and type II) following an oral glucose tolerance test (75 g glucose). The study showed that in nondiabetic subjects plasma Cr concentration was higher in obese (22.31±5.00 nmol/l) than lean subjects (12.69±6.73 nmol/l). However, there were no significant differences between obese and lean diabetic subjects. These results are hard to interpret as relevant data, because the values for Cr concentration are several times higher than current reported values. This might be due to uncorrected background values for Cr measurement or contamination during sample collection or analysis. The authors described subjects with BMI <30 kg/m^2 as lean, but this standard for BMI is too high to be defined as lean. A BMI >25 kg/m^2 is considered as grade I obesity and > 30 kg/m^2 is grade II obesity in adults (Lee and Nieman, 1996).

Clausen (1988) studied the effects of chromium supplementation on hypoglycemia. One hundred twenty five μg of chromium as bichrome (yeast chromium, Pharma-Nord, Denmark) was supplemented to twenty subjects for three months and an oral glucose tolerance test was performed three times; prior to supplementation, one month after the beginning of supplementation, and one month after the termination of supplementation. The glucose curve was divided into positive and negative phases based

on the fasting glucose level. If serum glucose was higher than the fasting glucose, it was considered as a positive phase. And if serum glucose was lower than the fasting glucose, it was considered as a negative or hypoglycemic phase. During the chromium supplementation, hypoglycemia was improved (decreased negative phase on the glucose curve). When comparing serum glucose one month after termination of chromium supplementation with glucose during the supplementation, ten subjects showed improved hypoglycemia (decreased negative area on the glucose curve after termination of Cr supplementation). Improved hypoglycemia one month after termination of chromium supplementation might indicate that supplementation of chromium allowed storage of an adequate amount of chromium in the body and affected blood glucose. Moreover, when comparing pre-supplementation values with post-supplementation values, eleven subjects had a decreased negative phase of the glucose curve as a response to supplementation. However, serum glucose was not measured at the end of the supplementation in this study, so it was not possible to compare serum glucose at the beginning and at the end of the study.

In addition to effects of chromium on glucose tolerance, effects of different sources of chromium such as chromium rich brewer's yeast or chromium chloride on carbohydrate metabolism have been studied. Offenbacher and coworkers (1985) studied effects of brewer's yeast, chromic chloride, or placebo on the pre and post supplementation levels of plasma chromium in free-living elderly subjects. Twenty-three free-living elderly subjects (mean age of 73 years old) received one of the treatments daily for ten weeks: brewer's yeast tablets (500 mg, containing five μg of chromium), chromic chloride capsules (containing 200 μg chromium in 200 mg of lactose), and

placebo (200 mg of lactose). Glucose tolerance was not significantly changed after supplementation in any of the three groups. The plasma glucose (mean±SD) at 90 minutes following a 75 g glucose challenge was 156±50 mg/dl and 129±52 mg/dl, before and after brewer's yeast supplementation respectively. The chromic chloride supplemented group had plasma glucose at 90 minutes following a glucose load of 146±41 mg/dl (before supplementation) and 152±52 mg/dl (after supplementation). In the placebo group, means for blood glucose before and after supplementation were 155±56 mg/dl and 129±26 mg/dl at 90 minutes following the oral glucose test. Also, there were no significant differences in the ratio of insulin to glucose after supplementation in any group. Plasma insulin (mean±SD) at 90 minutes following a 75 g glucose load was 117±57 mg/dl and 108±38 mg/dl for pre- and post-supplementation respectively in the brewer's yeast supplemented group, 142±46 mg/dl and 147±73 mg/dl in the chromic chloride supplemented group, and 101±42 mg/dl and 102±30 mg/dl in the placebo group. In addition, there were no correlations between plasma chromium and glucose or insulin. These results might be due to the dietary chromium intake of the subjects. These free-living subjects were well nourished and may have had good chromium status at the beginning of the study that influenced effects of chromium supplementation (Offenbacher et al., 1985).

Anderson and coworkers (1987) studied the effects of $CrCl_3$ supplementation on hypoglycemia. Eight female subjects aged between 33 to 69 years with hypoglycemia were included in the study. Subjects received either placebo or Cr (200 μg/d as $CrCl_3$) for 12 weeks and then supplements were interchanged without having a washout period between supplements. Serum Cr was significantly increased after both 6 and 12 weeks of

Cr supplementation. During the placebo period, serum Cr at 60 and 90 minutes following a glucose challenge was significantly greater than fasting. Glucose tolerance, insulin receptor number, and insulin affinity were not changed with Cr supplementation in normal control subjects. However, hypoglycemic symptoms, hypoglycemic glucose values, the number of insulin receptors, and serum Cr were improved with Cr supplementation in hypoglycemic subjects. The mean hypoglycemic area was significantly lower at six weeks and tended to be lower at 12 weeks in Cr supplemented groups (369±82 at 6 weeks and 448±115 with 12 weeks of supplementation) than in the placebo group (531±108). The insulin binding was significantly or tended to be higher in Cr supplemented groups (12.3±2.1 %/10^9 red blood cells at 6 weeks and 10.2±1.0 %/10^9 red blood cells with 12 weeks of supplementation) compared to the placebo group (8.7±1.0 %/10^9 red blood cells). The number of insulin receptors was significantly or tended to be higher in Cr supplemented groups (1226±207 receptors/cell after 6 weeks Cr supplementation and 623±113 receptors/cell with 12 weeks Cr supplementation) compared to the placebo group (344±100 receptors/cell). The receptor affinity (ng of insulin required to displace 50% of bound tracer) was not significantly changed with either 6 weeks (1.11±0.30) or 12 weeks of Cr supplementation (0.70±0.11) compared with the placebo (1.04±0.12). However, for the placebo group, it was not clear from the paper when the hypoglycemic area, the insulin binding, the number of insulin receptors, or the receptor affinity measurements were made.

Effects of chromium–rich yeast on serum glucose were determined in another study. Twenty-four subjects, between 65-74 years old, were assigned to Cr (160 μg Cr/d

as Cr-rich yeast) or placebo groups for 6 months. There were no significant effects of Cr on the fasting or oral glucose tolerance values for blood glucose and insulin (Uusitupa et al., 1992).

Moreover, Nath and colleagues (1979) studied the effect of inorganic chromium supplementation on glucose and insulin of 12 type II diabetic subjects. Blood glucose and insulin were significantly decreased following supplementation of 500 μg inorganic chromium per day for 60 days.

Chromium plays a role in glucose metabolism by stimulating insulin activity or efficiency, and Cr depletion generally results in impaired glucose tolerance in animals or in humans. Chromium supplementation increases serum glucose in hypoglycemia or improves glucose intolerance in some animals and in some humans.

Chromium and Lipids

Chromium is also involved in lipid metabolism. However, effects of chromium on blood lipid profiles are not consistent among studies; some studies showed improvement in lipids, but some showed no changes in lipid parameters.

In an animal study, there was no effect of dietary chromium on major lipid parameters such as cholesterol or triglycerides in rats fed a chromium supplemented diet for 10 months (Donaldson et al., 1985). In Stoecker and coworkers' study (1996, 1997) eighty-four C57BL/6J lean mice and 84 C57BL/6J-OB obese mice received one of the fifteen diet treatments based on aspirin (0 to 1500 mg/kg diet), Cr (20 to 1000 μg/kg diet, as $CrCl_3$), and Fe (20 to 200 μg/kg diet, as $FeCl_2$) for 24 days. Dietary treatments did not affect the serum cholesterol or triglycerides in either lean or obese mice.

In Anderson and colleagues' study (1983), there were no significant effects of supplementation with 200 μg of Cr as $CrCl_3$ for 12 weeks on serum lipid parameters in normal free-living subjects. In Abraham and colleagues' study (1992), total cholesterol was not significantly changed by 7 to 16 months of chromium (250 μg Cr as $CrCl_3$ in 5 ml of syrup) supplementation. However, serum triglycerides were decreased from 1.84±0.15 mmol/l (mean±SEM, initial visit) to 1.76±0.16 mmol/l (after 3 months supplementation) and to 1.68±0.11 mmol/l (last visit, a mean of 11.1 months) with chromium supplementation and these were significantly different between groups after 3 months of supplementation. However, there were no significant differences in serum triglycerides within the Cr supplemented groups. In addition, the mean HDL cholesterol level was significantly increased by 21% (from 0.94±0.05 mmol/l at initial visit to 1.14±0.07 mmol/l at last visit) and by 25% (from 0.91±0.04 mmol/l after 3 months of supplementation to 1.14±0.07 mmol/l at last visit) in the chromium supplemented subjects. However, serum HDL cholesterol was not significantly different between placebo and chromium supplemented groups. There was no change in the LDL cholesterol, but the VLDL cholesterol was significantly different between placebo and Cr supplemented groups at 3 months of supplementation (1.00±0.07 mmol/l and 0.81±0.08 mmol/l, respectively) and at their last visit (0.95±0.07 mmol/l and 0.77±0.05 mmol/l, respectively). On the contrary, there were no significant differences in serum VLDL cholesterol within chromium supplemented groups.

In addition, supplementation of brewer's yeast or inorganic chromium did not alter plasma cholesterol or triglycerides in elderly subjects (Offenbacher et al., 1985). However, another study by the same authors (Offenbacher and Pi-Sunyer, 1980) using

chromium-rich brewer's yeast or chromium-poor torula yeast showed contrasting effects on plasma cholesterol. Elderly subjects aged 63 years and older received either 9 g of chromium-rich brewer's yeast or 9 g of chromium-poor torula yeast for 8 weeks. Eight nondiabetic subjects and four diabetic subjects were included in each group. Cholesterol was significantly decreased in both diabetic and nondiabetic experimental groups and in the nondiabetic control group. The authors suggested that there might be a beneficial effect of yeast beyond beneficial effects of chromium supplementation, but this effect needs more investigation. However, there was no significant effect on serum triglycerides in either experimental or control groups.

In Potter and colleagues' study (1985), serum triglycerides were increased from 112±17 mg/dl (at presupplementation, mean±SEM) to 126±28 mg/dl (at postsupplementation), but HDL cholesterol (from 51±7 mg/dl at presupplementation to 46±5 mg/dl at postsupplementation), LDL cholesterol (from 147±7 mg/dl at presupplementation to 140±7 mg/dl at postsupplementation), and total cholesterol (from 221±2 mg/dl at presupplementation to 211±9 mg/dl at postsupplementation) were decreased with Cr supplementation (200 μg/d as $CrCl_3$ for 12 weeks) in elderly subjects. However, there were no significant differences in these lipid parameters between pre and post Cr supplementation. There was no placebo group to compare with experimental groups in this study.

In another study, a female patient received 250 μg Cr/d via TPN solution for two weeks and then remained on 20 μg Cr/d in the TPN for 18 months. Plasma free fatty acid concentrations during intravenous glucose tolerance tests (0.5 g glucose/kg body weight as a bolus infusion of 50% glucose solution for two to four min.) were significantly

decreased after seven days of Cr infusion (Jeejebhoy et al., 1977).

Chromium supplementation might have lowering effects on blood cholesterol or triglycerides or it might not have any beneficial effects on blood lipids. The effects of Cr supplementation on blood lipids are not consistent among studies, so further investigations are needed to clarify inconsistent results from existing studies. To investigate chromium effects on lipid metabolism, controlled human studies are required to minimize effects of inconsistent lipid and carbohydrate status of human subjects as well as side effects of dietary intake (Mertz, 1993).

Chromium Absorption and Excretion

Chromium supplementation affects chromium absorption and urinary chromium excretion. Seaborn and Stoecker (1992) found that the dietary intake of chromium influences chromium retention. Chromium deprivation for 23 weeks caused increased uptake of ^{51}Cr following $^{51}CrCl_3$ intubation in weanling male Hartley guinea pigs.

Chromium supplementation also affects tissue chromium concentration of animals. Tissue chromium concentrations in the livers of weanling male Sprague-Dawley rats fed a low-chromium diet were slightly less than those of rats fed a chromium-supplemented diet, but differences were not significant (Donaldson et al., 1985). Jain and coworkers (1981) also found higher chromium concentrations in the liver and kidney of rats provided 5 ppm of chromium in the drinking water compared to those of rats fed a low-chromium diet. Moreover, Donaldson and colleagues (1985) found significant differences in the concentration of kidney chromium between rats fed a low-chromium diet and a chromium supplemented diet. The measurement of tissue Cr concentration

might be important in animal studies, because tissue chromium concentration might represent Cr depletion or retention in animals being Cr depleted or supplemented.

In Anderson and Kozlovsky's study (1985), daily Cr intake was measured for thirty-two subjects aged 25-65 years. Cr content of most self-selected daily diets was 10 to 40 μg of Cr. Duplicate portions of all foods were prepared for seven days, one portion of foods was collected and analyzed for Cr content. When comparing male subjects with female subjects, male subjects consumed higher Cr/d than female subjects. Male subjects consumed a mean±SEM of 33±3 μg Cr per day and female subjects consumed a mean±SEM of 25±1 μg Cr per day. This indicates that people consume about half of the current lower range of the ESADDI for Cr from self-selected diets. Chromium concentrations were high in foods that were high in potassium, fat, saturated fat, and sodium. Also, chromium intake was correlated with oleic acid, phosphorous, vitamin B_6, copper, protein, and total carbohydrate consumption. However, chromium intake was not correlated with zinc and iron consumption.

The amount of chromium absorption has been estimated using urinary Cr excretion because absorbed Cr is mainly excreted in the urine and only small amounts lost in hair, perspiration, and bile (Doisy et al., 1971, Hopkins, 1965). Urinary Cr excretion was significantly increased with 200 μg $CrCl_3$ supplementation for 3 months. Moreover, daily dietary Cr intake and urinary Cr excretion were related inversely. Approximately 0.2 μg of Cr was absorbed and excreted in the urine when daily dietary Cr intake was less than 40 μg. The percent absorption of Cr was 2% (0.20 μg) with dietary Cr intake of 10 μg and 0.4-0.5% (0.16-0.20 μg) with intake of 40 μg. Thus, when dietary

Cr intake is low, absorption is increased (Anderson and Kozlovsky, 1985, Anderson et al., 1982).

The tissue concentration of chromium is decreased with age. The concentration of chromium in hair, sweat, and serum samples from 40,872 ambulatory outpatients between January 1985 and April 1996 was significantly decreased by 49%, 47%, and 42% respectively with age 75 years old or over compared with age 1-4 years old. There were no diet restrictions or timing of sample collection as well as no discriminations based on ethnic origin, place of residence, occupation, clinical symptoms, signs, diagnosis, medications, nutritional supplementation, or dietary or life-style habits throughout the study (Davies et al., 1997).

Measurement of chromium in urine samples can be complicated by several problems such as matrix effects or contamination. The mean basal urinary chromium excretion was 0.20 ng/ml$\pm$0.01 with a range of 0.05 ng/ml to 0.58 ng/ml in free-living subjects. The mean urinary Cr excretion was 1.02$\pm$0.12 ng/ml with a range of 0.05-6.4 ng/ml after 2 months of 200 μg Cr supplementation. The mean urinary Cr excretion was significantly different between Cr supplemented and nonsupplemented groups ($p < 0.05$) (Anderson et al., 1982).

Most studies reported increased urinary excretion of chromium after a glucose load (Schroeder, 1968, Doisy et al., 1971, Gurson and Saner, 1978), however, one study reported decreased urinary chromium excretion (Davidson et al., 1974). Fourteen days after 100 μCi ^{51}Cr administration, urinary ^{51}Cr excretion was significantly higher at 24-48 hours (0.316$\pm$0.080% of original dose) and 48-72 hours (0.210$\pm$0.063% of original dose) following a glucose load (100 g) in the group of type I diabetic subjects compared to the

control and type II diabetic subjects groups. Fourteen days after 100 μCi ^{51}Cr administration, plasma ^{51}Cr was significantly increased in type I diabetes (0.389±0.16% of original dose after 1 hour and 0.398±0.183% of original dose after 2 hours) compared to other groups (0.099±0.031% and 0.082±0.021% of original dose in normal subjects age 21-69; 0.025±0.009% and 0.060±0.010% of original dose in normal subjects age 70 and over) after 1 hour and 2 hours, respectively. Plasma ^{51}Cr was 0.038±0.009% of original dose in type II diabetes after 2 hours (Doisy et al., 1971). However, plasma ^{51}Cr in type II diabetes 1 hour after ^{51}Cr administration was not available in the original article.

Also, in Gurson and Saner's study (1978), the mean urinary Cr excretion was significantly increased from 6.06±0.46 ng/min. to 9.21±0.99 ng/min. following an oral glucose tolerance test (75 g glucose) in healthy subjects. However, there was no significant difference in the mean urinary Cr excretion before and after the glucose tolerance test in individuals from diabetic families. It is hard to interpret the Cr values in this study because more recent studies, using instruments with appropriate background correction, reported much lower Cr values.

In another study (Anderson et al., 1982), effects of chromium supplementation and a glucose load on urinary chromium excretion were determined. Two hundred micrograms of chromic chloride was supplemented to 76 free-living subjects (21 to 69 years old). Subjects were divided into two groups; one group was supplemented with chromium chloride during the first 3 months of the study and the other group was supplemented with chromium chloride during the second 3 months of the study. The mean urinary chromium excretion was 0.20±0.01 ng/ml at baseline and there was no difference between male and female subjects. The urinary Cr excretion was significantly

increased after chromium supplementation (200 μg Cr/d) and after two months of supplementation the value of urinary chromium excretion was greater than 1.02±0.12 ng/ml (with ranges of 0.05-6.4 ng/ml, $p<0.05$). The urinary chromium excretion was not significantly higher after 3 months of chromium chloride supplementation (1.13±0.08 ng/ml with ranges of 0.05-3.1 ng/ml) than that of two months supplementation (Anderson et al., 1982). After two months supplementation with chromium chloride, the urinary chromium excretion was increased fourfold following a glucose load test. Recent chromium intake can be measured by the urinary chromium excretion. However, the urinary chromium excretion fluctuates following a glucose load, so it is not a good indicator of chromium status. However, most subjects showed higher urinary chromium excretion 90 minutes after a glucose load than before the glucose load test (Anderson et al., 1982, Mertz, 1993). Moreover, Davidson et al. (1974) found that the mean ratio of urinary Cr to creatinine excretion was significantly decreased following a glucose load (75 g) from 391±32 ng to 272±46 ng in healthy subjects. However, these values are much higher than current Cr excretion values (0.20±0.01 ng/ml) (Anderson et al., 1982).

Obesity, presence of diabetes, and type of diabetes have effects on glucose tolerance, therefore, the plasma chromium concentration and the urinary chromium excretion have been measured in diabetic and obese subjects. Plasma Cr was significantly higher in lean type II diabetic subjects, especially in subjects using oral hypoglycemic agents, than in the lean control subjects. Moreover, lean type I diabetic subjects (3.17±0.66 nmol Cr/mmol creatinine and 5.77±1.02 nmol Cr/mmol creatinine at baseline and at 3 hours following a 75 g glucose load, respectively) showed significantly higher urinary Cr excretion compared with lean (1.07±0.19 nmol Cr/mmol creatinine and

1.08±0.23 nmol Cr/mmol creatinine at baseline and at 3 hours following a 75 g glucose load, respectively) and obese (1.25±0.34 nmol Cr/mmol creatinine and 1.50±0.28 nmol Cr/mmol creatinine at baseline and at 3 hours following a 75 g glucose load, respectively) nondiabetic subjects (Earle et al., 1989).

Several studies have been conducted about absorption and urinary excretion of chromium related to several factors such as chromium intake or nutrient interactions. In Uusitupa and colleagues' study (1992), the urinary Cr excretion was increased 3-4 fold with Cr supplementation (160 μg Cr/d as four pills of Cr-rich yeast/d) in elderly subjects aged 65-74 years with persistent impaired glucose tolerance, while the urinary Cr excretion of the well matched placebo group was stable during the study. Also, plasma insulin and glucose concentrations affect the plasma Cr concentration. In Morris and coworkers' study (1992), nine healthy people aged 22-41 years participated to investigate the relationship of plasma insulin and Cr concentration. Plasma Cr decreased rapidly from the fasting level following a 75 g oral glucose load (from 6.5±0.8 nmol/l to 3.6±1.1 nmol/l, mean±SE) and there was a significant inverse relationship between plasma insulin and Cr concentration. Also, urinary Cr excretion was increased following a glucose load. In Anderson and colleagues' study (1991) described earlier, urinary Cr excretion was significantly increased with Cr supplementation (1.10±0.29 nmol/90 min. and 0.93±0.20 nmol/90 min. in control and hyperglycemic subjects, respectively) compared to placebo supplementation (0.30±0.05 nmol/90 min. and 0.24±0.06 nmol/90 min. in control and hyperglycemic groups, respectively).

In the subjects with slightly increased blood glucose, insulin level was decreased (from 1787±257 pmol/l to 1320±244 pmol/l for the sum of 0-90 min. and from 2285±425

pmol/l to 1703±274 pmol/l for the sum of 0-240 min. following a glucose load) after Cr supplementation. This decrease between placebo and Cr supplemented groups was significant ($p < 0.05$). However, insulin level was similar in the control between placebo (from 868±194 pmol/l to 883±203 pmol/l for the sum of 0-90 min. and from 1027±211 pmol/l to 1099±231 pmol/l for the sum of 0-240 min. following a glucose load) and Cr supplementation periods.

In Potter and colleagues' study (1985), five elderly subjects received 200 μg Cr as $CrCl_3$ for 12 weeks. Oral glucose tolerance (40 g/m^2 surface area) tests and hyperglycemic clamp tests were performed before and after the supplementation period. The urinary Cr excretion was increased with Cr supplementation (not measured in response to a glucose load). However, the plasma glucose and insulin concentrations following Cr supplementation were not significantly changed.

Seventy-six patients with previous myocardial infarction and/or moderate to severe intermittent claudication were involved in Abraham and coworkers' study (1992). Among these patients, 25 people were type II diabetic patients and the rest of the subjects were the control group. Each group was divided into treatment or placebo subgroups. The treatment group received 250 μg chromium ($CrCl_3(H_2O)_6$) in 5 ml of syrup. The serum Cr concentration was increased about fivefold by 1 month of supplementation, remained for 3 months, and decreased by 7 to 16 months of supplementation in both diabetic and non-diabetic treatment groups. The authors did not explain about or comment on why serum Cr went down by 7 to 16 months of supplementation. However, serum Cr concentration was higher after 7 to 16 months of supplementation compared to initial Cr concentration. Also, fasting serum glucose in the diabetic treatment group was

increased a small amount, but the change was not significant.

The source of Cr, such as inorganic Cr, Cr-rich brewer's yeast, or Cr-poor torula yeast, affects Cr absorption and urinary Cr excretion following a glucose tolerance test. Also, Cr retention or depletion status in the body might affect Cr absorption or urinary excretion as well as the results of Cr supplementation or depletion studies.

Chromium and Immunity

Increased circulating stress hormones influence the retention of absorbed chromium (Seaborn and Stoecker, 1992). Physiological stresses such as trauma, infection, surgery, and intense heat or cold affect chromium metabolism. These stresses cause alteration in hormone secretion and glucose metabolism and lead to changes in chromium metabolism (Nielsen, 1988). Borel et al. (1984) studied effects of trauma on urinary chromium excretion. Seven male trauma patients were involved in the study. Urine samples were collected every 4 hours for the first 24 hours after patients were admitted to a critical care recovery unit or an intensive care unit. The mean±SEM urinary chromium excretion of the first 4 hr was 10.3±2.5 ng/ml. Urinary chromium excretion was decreased gradually and the mean±SEM urinary chromium excretion was 2.0±0.6 ng/ml by 42 hours after admittance and remained at this level at 72 hours.

Stress also affects T helper cell 1 (TH1) and T helper cell 2 (TH2) subsets of T helper cells (TH) in some animals; increased blood cortisol levels decreased the activity of TH2 cells. The TH2 lymphocytes induced cell-mediated immune responses and TH1 lymphocytes induced a specific humoral immune response (Burton et al., 1994).

In Chang and Mowat's study (1992), newly received feedlot calves showed improved performance and decreased morbidity after chromium supplementation (4 mg Cr/d as high-Cr yeast). Also, anti-bovine infectious rhinotracheitis virus (anti-IBR) antibody titers after vaccination were increased with chromium supplementation in steers (Burton et al., 1994).

Wright and coworkers (1995) investigated the effect of low levels of chromium supplementation on alteration in the concentration of hemolytic complement and haptoglobin of newly arrived feeder calves. Seventy-two Charolais-crossed steer calves with an average weight of 250±30 kg were divided into four treatment groups. These four treatment groups were 1) control, 2) four ml intramuscular (IM) vaccination against bovine infectious rhinotracheitis virus (IBR), parainfluenza-3 (PI-3), bovine viral diarrhea (BVD), and bovine respiratory syncytial virus (BRSV) plus two ml IM vaccination against P. *haemolytica,* 3) 0.14 ppm supplemental Cr in the diet, and 4) vaccination plus 0.14 ppm supplemental Cr. Chromium was supplied as amino acid-chelated Cr, containing 3.52% Cr. The concentration of haptoglobin was decreased on the seventh day in the chromium supplemented group compared to nonsupplemented controls. However, there were no significant differences among groups supplemented with nothing or with vaccines, chromium, or chromium plus vaccines.

In another study, the effects of chromium on antibody responses of feedlot calves to IBR and PI-3 antigens were investigated by Burton and colleagues (1994). Thirty-one of a large rotational cross (LR; Simmental X Charolais X Maine Anjou) and 24 of a small rotational cross (SR; Herefored X Angus X Salers) with an average weight of 233 kg were randomly assigned into control (0 ppm supplemental chromium) or experimental

(supplemented with 0.5 ppm chromium per day as Metalosate, 2.68% chromium) groups for 30 days. The IBR seroconversion was significantly higher with chromium supplementation than that of the control group. Overall chromium supplemented calves showed higher anti-IBR antibody titers compared to control calves. Also, LR calves showed higher anti-IBR antibody titers compared to SR calves. However, anti-PI-3 antibody titers were not significantly affected by the treatment. The Metalosate used in this study contained 2.68% of chromium, but what else is in Metalosate was not mentioned in the original article.

In Burton and coworkers' study (1993), twenty Holsteins were randomly assigned into two groups and fed a diet supplemented with 0.5 ppm of chelated Cr per day (Metalosate, 2.68% Cr) or an unsupplemented diet from six weeks before their expected calving date. Also, cows in the Cr supplemented group received 200 g/d of ground corn top dressing containing additional Cr (205 mg Metalosate, 2.68% Cr; equivalent to 5.5 mg Cr) once a day until calving. After parturition, cows in the Cr supplemented group received double the Cr supplement with ground corn throughout lactation (through the 16^{th} week). Chromium supplemented cows had significantly increased anti-ovalbumin and ConA stimulated responses. However, there were no effects on antibody responses to human erythrocytes.

In another study, the immunomodulatory activity of mitogen stimulated PBMC was investigated in chromium supplemented and nonsupplemented periparturient cows. Nine cows received 0.5 ppm of chelated chromium (as Metalosate, 2.68% Cr) per day for 10 weeks and received additional Cr in a top dressing containing 205 mg of Metalosate in 200 g of ground corn once a day until calving. After parturition through the 16^{th} week,

cows received Cr supplemented ground corn two times a day and additional Cr was supplemented (185 mg of Metalosate; equivalent to 5.0 mg of Cr) in 100 g of ground corn at each time of feeding. Cows received total 780 mg of Metalosate (equivalent to 20.9 mg of Cr) a day after parturition throughout the 16^{th} week. The control group (n=10) received diet without added chromium for 10 weeks (Burton et al., 1995). The ConA-induced PBMC blastogenesis in the sera from periparturient cows supplemented with chromium was not suppressed after parturition. However, in the sera from the control cows, PBMC blastogenesis was decreased (Burton et al., 1995). Moreover, there were no differences in the concentration of serum insulin, growth hormone, IGF-1, or TNF-α between Cr supplemented and unsupplemented control groups (Burton et al., 1995). Also, in another study, supplemental chromium improved anti-red blood cell antibody responses of transit stressed feedlot calves, however, there was no effect on in vivo dinitrochlorobenzene-induced cutaneous sensitivity of transit stressed feedlot calves (Moonsie-Shageer and Mowat, 1993).

Chromium supplementation plays roles in immune function by increasing mitogen stimulated immune response or antibody titers. The cell sensitivity to insulin or IGF-1 might be increased with Cr supplementation, which may lead to increased DNA synthesis in cell cultures. Increased DNA synthesis in cell cultures may lead to increased cell proliferation with mitogen stimulation in cell cultures.

Copper

Copper is an important trace mineral in human and animal metabolism because copper has functions in several enzyme activities. Copper containing enzymes include cytochrome C oxidase, superoxide dismutase, tyrosinase, dopamine-β-hydroxylase, lysyl oxidase, and ceruloplasmin (Olivares and Uauy, 1996a, Danks, 1988). Copper also has functions in development and maintenance of the immune response (Percival, 1998). Some examples of copper deficiency symptoms are anemia, neutropenia, and bone abnormalities. Copper deficiency anemia is due to reduced ceruloplasmin activity. Ceruloplasmin plays a role in transformation of Fe^{2+} to Fe^{3+}, therefore iron can not be transferred and bound to transferrin for mobilization in copper deficiency. In addition, bone abnormalities caused by copper deficiency are due to reduced lysyl oxidase activity, because lysyl oxidase has functions in cross-linkage of collagen (Olivares and Uauy, 1996a, Danks, 1988). Furthermore, a reduced superoxide dismutase activity due to copper deficiency causes changes in glucose and cholesterol metabolism (Olivares and Uauy, 1996a).

Copper status can be estimated by measuring serum copper and ceruloplasmin concentrations. Nutrient interactions, infection, inflammation, pregnancy, and amount of copper in the diet influence the serum copper and ceruloplasmin concentrations (Lonnerdal, 1996). The absorption of copper in the body is related to copper concentrations in the diet. When the amount of copper in the diet was high (1.68 mg/d), 36.3% of copper was absorbed in the body. On the other hand, when the dietary copper

intake was low (0.78 mg/d), the copper absorption was 55.6% in young men (Turnlund et al., 1989).

In Milne's study (1997), people consuming less than 1.2 mg Cu/d showed biochemical and physiological changes due to copper deprivation. Dietary intakes of fructose, zinc, and sulfur amino acids affect copper status. Also age, gender, length of copper deficiency, and amount of copper in the diet affect copper depletion.

The estimated safe and adequate daily dietary intake (ESADDI) for copper is 1.5-3.0 mg/d (Olivares and Uauy, 1996b). The average dietary copper intake in the U. S. is 1.24 mg/d in men and 0.93 mg/d in women based on data from the Total Diet Study (Pennington and Young, 1991). Dietary copper sources include liver, shellfish, potatoes, fruit, bread, meat, fish, and legumes (Danks, 1988). However, in spite of low dietary intake of Cu, clinical or biochemical evidence of Cu deficiency problems in the public has not been confirmed (Pennington and Young, 1991).

Copper has important roles in maintaining enzyme activities and in proper nutrient metabolism in humans and animals. Therefore in the following sections, the literature about functions of copper in carbohydrate metabolism, immunity, interleukin 2 secretion, mitogen response, and ceruloplasmin and superoxide dismutase activities are reviewed.

Copper and Carbohydrate Metabolism

Studies of the function of copper in glucose tolerance or carbohydrate metabolism are rarely conducted. Cohen and coworkers (1982) studied the effect of copper on carbohydrate metabolism in rats. Sixty-eight Albino rats of the Hebrew University strain,

5 weeks old, were divided into two groups and each group received either copper deficient high-sucrose diet or copper supplemented synthetic diet for 4 months. In addition to this, six male rats received copper deficient high-starch diet and five male rats received copper supplemented starch diet. At the end of the 4 months, rats were given 350 mg glucose/100 g body weight as an oral glucose tolerance test. Copper deficient rats fed either starch or sucrose had significantly increased fasting plasma glucose at 30 and 60 minutes after the oral glucose load compared to copper supplemented rats. Also, rats fed a copper deficient diet had decreased plasma insulin at 30 and 60 minutes of the test. The difference in decreased plasma insulin with Cu supplementation was significant in the starch fed rats. Rats fed either copper deficient or supplemented diets based on sucrose tended to have higher plasma glucose concentrations following a glucose challenge compared to those in rats fed starch–based diets. However, the increase in plasma glucose of rats fed sucrose was not significant compared to the rats fed starch.

In other studies related to copper and carbohydrate metabolism, inorganic copper (100 μg/ml in Krebs-Ringer bicarbonate buffer, pH 7.4 as $CuCl_2$) stimulated incorporation of glucose into glycogen in diaphragm muscle (Cohen et al., 1982). Also, a higher concentration of plasma insulin was secreted by pancreas from perfused male Albino rats (Hebrew University strain) supplemented with inorganic copper (6.7 ppm as $CuCl_2(H_20)_2$) than from pancreas of rats fed copper deficient diet (1.2 ppm Cu) (Cohen and Miller, 1986).

Copper and Immunity

Lymphocyte and macrophage functions are affected by minerals, which are cofactors for enzymes. For example, copper deficiency caused a significant decrease in phagocytosis of latex beads by peripheral mononuclear cells (PMNs) and this defect was recovered by copper supplementation (Kubena and McMurray, 1996). Moreover, in the offspring of C58 mice fed copper-deficient diets, the number of antibody producing cells following a sheep erythrocyte injection was significantly decreased. Also, reactivity to both T cell and B cell mitogens and mixed lymphocyte reactions was abnormal in the copper deficient offspring of C58 mice (Prohaska and Lukasewycz, 1981, Lukasewycz et al., 1985, 1987).

However, there were no differences in the mitogen reactivity and the antibody production to sheep erythrocytes in copper-deficient NZB mice (Mulhern et al., 1987). Also there were no alterations in the mitogen reactivity of splenocytes in copper deficient Swiss mice, but the number of antibody-producing cells to sheep erythrocytes was altered in these mice compared to copper repleted Swiss mice. Total IgG levels were more influenced than total IgM levels by copper deficiency in these mice (Blakley and Hamilton, 1987).

Decreased antibody-mediated immunity is one of the characteristics of copper deficient mice and rats (Prohaska and Lukasewycz, 1989). The number of antibody producing spleen cells in mice injected with sheep erythrocytes was reduced with copper deficiency.

Copper deficiency leads to suppression of the response of mononuclear cells (MNC) to T cell mitogens as well as to the suppression induced decreased relative

numbers of mature T cells. These results suggested one or more defects in the activity of Cu deficient cells (Bala and Failla, 1992). Rats with copper deficiency showed similar impaired immune systems as mice. Both cytotoxicity of killer cells and the mitogen stimulated proliferation of splenocytes were reduced and antibody titers were decreased in copper deficient rats (Kramer et al., 1988). The percentages and absolute numbers of total T lymphocytes and of the CD4+ (helper) and CD8+ (cytotoxic) subpopulations were decreased in copper deficiency (O'Dell, 1993).

Davis and colleagues (1987) studied the relationship between copper deficiency and lymphoid cell function using weanling male Lewis (LEW/Cr1BR) rats. Rats were fed either copper deficient diet containing 0.6 μg Cu/g diet or Cu adequate diet containing 5.6 μg Cu/g diet. Copper deficient rats showed significantly decreased phagocytic activities of spleen lymphoid cells (SLC) collected on density gradients compared to copper adequate rats.

Also, in animals, thymic hormone activity was decreased in copper deficient rats. A consequence of decreased thymic factor activity is impaired humoral response to dependent antigens because thymic factor activity is related to T cell maturation (Chandra, 1979).

In humans, serum immunoglobulin levels were normal in subjects with copper deficiency or in subjects with genetic copper deficiency (Heresi et al., 1985, Sullivan and Ochs, 1978). In addition, T and B lymphocytes response to mitogen stimulation was normal in subjects with genetic copper deficiency (Sullivan and Ochs, 1978, Smith et al., 1994).

Copper and Interleukin 2

Since secretion of IL-2 is necessary to activate unprimed T-lymphocytes, several researchers have investigated the effect of copper deficiency on IL-2 production. To activate unprimed T cells, several signals are required. Antigen presenting cells (APC) provide the first signal to activate T cells and APC also secrete interleukin-1 (IL-1). IL-1 provides the second signal for T cell activation. T helper cells (TH) then respond to these signals and secrete IL-2 (O'Dell, 1993). To activate proliferation and differentiation of IL-2, IL-2 needs to bind to its receptor (IL-2R) (Bala and Failla, 1992).

In Bala and Failla's study (1992), IL-2 restored the PHA reactivity of splenic MNCs from rats fed Cu deficient diets. Cultures of the splenic MNC from Cu deficient rats showed decreased incorporation of ^{3}H-thymidine into DNA with PHA stimulation. The blastogenic activity of PHA-treated MNCs from Cu deficient and control rats was increased by 5.9- and 1.2-fold, respectively, when rat IL-2 was added to cultures at the initiation of the incubation. Therefore with addition of IL-2 to cell cultures, the incorporation of ^{3}H-thymidine by cells from Cu deficient rats and control rats was similar.

Likewise, the incorporation of ^{3}H-thymidine into DNA in PHA treated cultures of splenic MNC from Cu-deficient rats was significantly increased by addition of 3-6 μM Cu. Moreover, the addition of 12 μM Cu increased the incorporation of ^{3}H-thymidine into DNA to control levels. The addition of 24-96 μM Cu showed gradual increases in the blastogenic activity. However, the supplementation of 1.5-100 μM Cu in the medium without PHA did not change the incorporation of ^{3}H-thymidine into DNA. Incorporation of ^{3}H-thymidine into DNA also was not stimulated with the supplementation of

1.5-100 μM Zn or Mn to cultures of splenic MNCs from Cu-deficient and control rats. These data suggest that Cu had a specific ability to restore the mitogenic reactivity of splenic MNCs, but Cu itself was not mitogenic.

The T cell proliferation and the clonal expansion required binding of IL-2, which is the ultimate mitogen, to its receptor. In Bala and colleagues' study (1991), ConA stimulated splenic MNCs from Cu-deficient and control rats showed similar increases in the expression of IL-2 receptors. This result suggests that the trace mineral deficiency did not compromise the early events of T-cell activation.

Bala and Failla (1992) also investigated the relationship of dietary Cu deficiency and decreased IL-2 activity in cultures of stimulated rat splenic MNCs. The activity of IL-2 in the cultures of PHA-treated splenic MNCs from Cu deficient rats was increased by only 40-50% that of control cultures.

Copper and Stress Hormone

Hormones such as corticosteriods and adrenocorticotropic hormone (ACTH) can influence copper status. These hormones decrease plasma copper concentrations (Solomons, 1979). Nockels and coworkers (1993) measured effects of stress on copper balance using eight Charolais crossbred steer calves. Calves were fed millet hay supplemented with Zn methionine (ZnMet, 36.6 ppm) + Cu lysine (CuLys, 10.5 ppm) or Zn sulfate ($ZnSO_4$, 36.6 ppm) + Cu sulfate ($CuSO_4$, 9.2 ppm) for 18 days. An injection of ACTH (80 IU, same amount to all calves) was given every 8 hours for 3 days to induce stress effects. Food and water were withheld for the first 36 hours of the treatment and for the next 36 hours calves were fed 0.5% of body weight of hay and 4 liters of water

was provided every 12 hours. There was a significant difference in copper absorption between CuLys (prestress 19.1% and poststress 20.1%) and $CuSO_4$ (prestress 16.6% and poststress 13.1%). During stress, the copper excretion in feces was significantly decreased from 36.95 mg/d (at baseline, day 5) to 20.41 mg/d (at the first day of stress, day 9), 7.79 mg/d (at the second day of stress, day 10), and 8.39 mg/d (at the third day of stress, day 11). Urinary copper excretion was also significantly decreased from baseline of 2.95 mg/d to 1.85 mg/d and 0.59 mg/d at the first and second day of stress, respectively. However, during the third day of stress, urinary copper excretion (2.54 mg/d) was similar to the baseline. The Cu retention was also significantly decreased from 5.03 mg/d to -18.89 mg/d, -7.19 mg/d, and -8.54 mg/d during the 3 days of stress treatment, respectively. Fecal and total Cu excretion were significantly different between pre and post stress periods ($P < 0.001$). Decreased fecal and urinary Cu excretion as well as decreased Cu retention might be due to decreased food intake during stress. Decreased urinary Cu excretion also might be due to decreased urinary volume during stress.

Copper and Organ Size

The sizes of organs were changed with chronic dietary copper deficiency. The changes included smaller thymus, enlarged spleen, enlarged heart, and heavier liver (Prohaska, 1983, Prohaska et al., 1983, Prohaska and Lukasewycz, 1989, Mulhern and Koller, 1988).

In Mulhern and Koller's study (1988), the offspring of C57BL/6J mice were assigned to one of four diet treatment groups: 1) diet containing 0.5 mg Cu/kg diet, 2) diet containing 1.0 mg Cu/kg diet, 3) diet containing 2.0 mg Cu/kg diet, and 4) diet containing

6.0 mg Cu/kg diet. The sizes of spleen, thymus, and heart were dependent on the concentration of copper in the diet. Mice receiving 0.5 mg Cu/kg diet or 1.0 mg Cu/kg diet had larger hearts and spleens as percentage of body weight and smaller thymuses as percentage of body weight than mice receiving 2 mg Cu/kg diet or 6 mg Cu/kg diet. However, organ sizes of mice fed 2 mg Cu/kg diet were not significantly different from mice fed 6 mg Cu/kg diet.

In Babu and Failla's study (1990a, 1990b), male Lewis rats, weighing 40-50 g, were divided into copper deficient (0.6-0.7 mg Cu/kg diet) and copper adequate diet (6.7-7.0 mg Cu/kg diet) groups. Copper deficient rats showed enlarged hearts and livers compared to copper adequate rats.

Kishimoto and coworkers (1993) also studied the effect of copper deficiency on the organ size of the animal. Male Fischer 344 rats, weighing 80-90 g, were fed a copper depleted AIN-76 diet supplemented with 0.6% triethylenetetramine tetrahydrochloride as a copper chelator. Control rats were fed a copper supplemented diet without a copper chelator. The body weight of rats in the control group increased rapidly. The weight of rats in the copper deficient group was increased until the 3rd week of the treatment, but plateaued after the 3rd week. Some copper deficient (copper depleted AIN - 76 diet) rats were killed at several intervals during the treatment period and weights of pancreas were measured. Copper deficient rats showed increased pancreas weights until the 2nd week of treatment, but the pancreas weights decreased thereafter.

In Koller and colleagues' (1987) study, the pups of Harlan Sprague-Dawley rats were fed a copper depleted diet (0 ppm Cu added), a marginally deficient diet (2 ppm), or an adequate copper diet (6 ppm) for 8 weeks. The rats fed with marginal copper deficient

or depleted diets had enlarged livers and hearts. However, the absolute spleen weights were significantly decreased only in the female rats fed the copper depleted diet (0 ppm added).

In Windhauser and colleagues' study (1991), fifty male Sprague-Dawley rats were divided into adequate Cu (5.0 μg Cu/g diet), mildly deficient (3.5 μg Cu/g diet), moderately deficient (2.0 μg Cu/g diet), or severely deficient (0.5 μg Cu/g diet) diet groups. After 8 weeks of feeding experimental diets, rats were regrouped based on plasma Cu concentrations to measure effects of Cu status on immune function. Severely copper deficient rats (< 0.14 μg Cu/ml plasma) had significantly larger livers and smaller thymuses compared to other groups. Also, the moderately copper deficient rats (0.14-0.66 μg Cu/ml plasma) had larger livers compared to mildly deficient rats (0.67-1.07 μg Cu/ml plasma) or adequate rats (> 1.07 μg Cu/ml plasma) after 8 weeks of supplementation. However, spleen weights were not affected by the dietary treatment. Copper deficiency caused myocardial vacuoles in the heart, alterations in the liver enzyme activity, and abnormalities in either medulla or cortical regions of the thymus and led to changes in organ sizes (Mulhern and Koller, 1988).

Copper deficiency changed organ sizes including heart, liver, spleen, and thymus in animals. Altered organ sizes might affect the activity, content, or amount of enzymes or lymphocyte cells in tissues. Changes in the activity, amount or content of enzymes or lymphocyte cells in tissues may cause decreased immune responses in animals.

Copper and Mitogen Reactivity

Effects of copper depletion or copper supplementation on T cell or B cell immune response can be evaluated by the mitogen response. In Hopkins and Failla's study (1995), male rats fed a low copper diet had decreased ConA and PHA stimulated mitogen activity of splenocytes compared to rats fed an adequate copper diet.

In another study, weaned Lewis rats were used to investigate effects of dietary copper deficiency on the phenotypic and mitogen reactivity of rat splenocytes. Rats were divided into control (6 μg Cu/g diet) and copper deficient (0.6 μg Cu/g diet) groups. Copper deficient rats showed decreased numbers of T lymphocytes and CD4 and CD8 cells compared to copper adequate rats. However, copper deficient rats showed increased relative percentages of splenic B cells and macrophages compared to copper adequate rats. In vitro the blastogenesis of splenocytes by PHA, ConA, or *Escherichia coli* lipopolysaccharide (LPS) stimulation was lower in the copper deficient rat group than in the control group (Bala et al., 1991).

Windhauser and colleagues (1991) studied effects of suboptimal levels of dietary copper on the immune response of rats. Fifty male Sprague-Dawley rats, initial weight of 100 g, were divided into the following four treatment groups: an adequate copper (5.0 μg Cu/g diet), a mild copper deficiency (3.5 μg Cu/g diet), a moderate copper deficiency (2.0 μg Cu/g diet), and a severe copper deficiency (0.5 μg Cu/g diet). Six days prior to necropsy, 0.5% sheep red blood cell suspension was injected into rats. Copper deficiency did not affect the percentages of T and B cells in the peripheral blood. There were no significant differences among means of lymphocyte stimulation indices because lymphocyte-stimulation data was highly variable within each week. However, severely

deficient rats showed lower mean PHA and ConA lymphocyte stimulation indices than other groups. Also, PHA stimulation indices for severely and marginally deficient rats were not increased with 8 weeks of treatment, but those for adequate control and pair-fed rats were increased by the 8th week. Moreover, PHA stimulation indices and T cell numbers showed significant negative correlations for all rats, whereas PHA stimulation indices and ceruloplasmin were significantly correlated in all groups at the 8th week (Windhauser et al., 1991).

In Lukasewycz and Prohaska's (1983) study, 92 offspring of inbred C58 mice were fed a purified diet containing 0.6 mg copper per kilogram diet. Half of the dams had received supplemental copper (20 μg/ml as $CuSO_4$) in drinking water and the other half of the dams received deionized water. The ConA, PHA, LPS, or pokeweed mitogen (PWM) stimulated splenocyte proliferation of Cu deprived animals was less than one-half of those values of the copper adequate mice.

Kramer and coworkers (1988) also investigated effects of copper deficiency on immunological changes. Male and female weanling Lewis (LEW/Crl BR) rats were divided into four treatment groups in a 2 x 2 factorial experiment: 1) copper deficient and iron adequate containing 0.6 μg Cu/g diet and 50 μg Fe/g diet, 2) copper adequate and iron adequate containing 5.6 μg Cu/g diet and 50 μg Fe/g diet, 3) copper deficient and iron excess containing 0.6 μg Cu/g diet and 300 μg Fe/g diet, 4) copper adequate and iron excess containing 5.6 μg Cu/g diet and 300 μg Fe/g diet. The diet treatment lasted for 42 days. Copper deficient male rats showed decreased hemoglobins and hematocrits regardless of dietary iron. Copper deficient female rats had higher hemoglobin than male rats. Also, copper concentrations in the liver and kidney and iron concentrations in the

kidney were lower in copper deficient male and female rats than in the copper adequate groups. Copper deficient male rats had higher proliferation of unstimulated splenocytes than copper-adequate male rats. However, copper deficient male rats had similar proliferation of unstimulated splenocytes to that in either copper deficient or copper adequate female rats. Also, copper deficient female and male rats had lower peak proliferation of ConA stimulated splenocytes than copper adequate rats.

In Lukasewycz and colleagues' study (1985), offspring of C58 mice were divided into two groups; a copper supplemented group in drinking water (20 μg/ml as $CuSO_4$) and a control group provided deionized water. The mitogen stimulated proliferation was increased in the experimental group in terms of total counts per minute and in terms of stimulation index.

Lukasewycz and colleagues (1987) studied the relative reactivity of - Cu and + Cu splenocytes and the relative capacity of - Cu and + Cu splenocytes to act as stimulator cells in the mitogen lymphocyte reaction. The offspring of inbred BALB/c, C57BL and C58 mice were divided into two groups. One group received supplemental copper (20 μg Cu/ml as $CuSO_4$) in their drinking water with semipurified diet containing 0.6 mg copper per kilogram diet. The other group received the same diet with deionized water to drink. Splenocytes of copper deficient mice showed 49% of the mean stimulation index response of the copper adequate C58 mice. Moreover, mitogen responses of splenocytes from copper deficient mice were decreased and also mixed-lymphocyte reactions were decreased (Lucasewycz et al., 1987).

In human studies, twelve 21 to 23 years old healthy nonsmoking men lived in the metabolic suite of the Western Human Nutrition Research Center for 90 days. The

treatment period was divided into three phases according to the concentration of copper in the diet; metabolic period 1 (days 1-24, 0.66 mg Cu/d), metabolic period 2 (days 25-66, 0.38 mg Cu/d), and metabolic period 3 (days 67-90, 2.49 mg Cu/d). A three-day rotation menu was supplemented with vitamin capsules and liquid formulas to meet other nutrient requirements except copper. Four age-matched men were selected as a control group and they maintained their usual diet and activities throughout the study. Fasting blood samples were collected at days of 1, 24, 59, 66, 83, and 90. The concentrations of 20 mg/L PHA, 20 mg/L ConA, and 2 mg/L PWM were used to stimulate PBMNC proliferation. The PBMNC proliferation by all three mitogens was lower with the low copper diet (metabolic period 2) compared to metabolic period 1 or metabolic period 3 (Kelley et al., 1995).

The secretion of IL-2 receptor in the ConA stimulated PBMNC medium was decreased with the low copper diet (0.38 mg/d, metabolic period 2). However, differential blood cell profiles such as concentrations of leukocytes, monocytes, neutrophils, or lymphocytes were not affected significantly with the different levels of copper intakes (0.66, 0.38, or 2.38 mg/d). Likewise, the number of lymphocyte subsets such as helper T cells, suppressor T cells, or total T cells was not affected by the concentration of copper in the diet. The percentage of B cells and the number of B cell subsets were significantly increased in metabolic period 2 compared with metabolic period 1. However, the clinical significance of the effects of different levels of copper intakes on the changes in the percentage or the number of B cell subsets is not known (Kelley et al., 1995).

In another study, eleven men aged 21 to 32 years received a marginal copper diet (0.66 mg Cu/d) for 24 days, a depletion diet (0.38 mg Cu/d) for 24 days, and a repletion diet (2.48 mg Cu/d) for 24 days to measure effects on copper status. During depletion and at the end of depletion, polymorphonuclear leukocyte (PMN) copper concentration was significantly decreased (91±42 nmol Cu/g protein and 54±27 nmol Cu/g protein, respectively, mean±SD) compared to the beginning of the study (125±61 nmol Cu/g protein, mean±SD). And the mean PMN copper concentration tended to be higher at the end of repletion (79±35 nmol Cu/g protein, mean±SD) than at the end of depletion (54±27 nmol Cu/g protein, mean±SD), but it was not significant (Turnlund et al., 1997).

Copper deficiency and supplementation have effects on carbohydrate metabolism, IL-2 production, lymphocyte response to mitogen stimulation, stress hormones, or organ sizes in animals or in humans. Maintaining appropriate dietary copper intake or copper status is important in preventing altered carbohydrate metabolism or immune responses in animals and in humans.

Copper and Ceruloplasmin and Superoxide Dismutase

Copper status can be evaluated by measuring serum copper and ceruloplasmin concentrations (Milne, 1994, Solomons, 1979). Copper deficiency causes anemia due to reduced ceruloplasmin activity that results in immobilization of iron (Danks, 1988). Ceruloplasmin functions in transformation of $Fe^{2+}$to Fe^{3+} (Turnlund, 1994, Linder, 1996) and this step is required in the binding of iron to circulating transferrin. Therefore, a reduced ceruloplasmin level results in immobilization of iron in the reticuloendothelial system making it unavailable for erythropoiesis (Olivares and Uauy, 1996a).

Copper status also affects the Cu, Zn superoxide dismutase (SOD) activity in the body. The erythrocyte and/or leukocyte Cu, Zn SOD activity is increased under oxidative stress with low dietary copper intake, therefore, this might not be a good indicator of copper status (Milne, 1994). In DiSilvestro and Marten's study (1990), male Sprague-Dawley rats weighing 50-75 g were fed Cu deficient (0.5 mg Cu/kg), marginal Cu (2.5 mg Cu/kg), or adequate Cu (6.0 mg Cu/kg) diets. One to three days before killing, rats were injected with 0.1 ml of turpentine intramuscularly to induce inflammation. The plasma ceruloplasmin activities were increased and Cu, Zn SOD activities in the liver were decreased with inflammation in all rats. However, copper deficient rats did not show a significant increase in plasma ceruloplasmin activity. The level of copper without inflammation affected the concentration of erythrocyte Cu, Zn SOD activity in a proportional way which resulted in decreased Cu, Zn SOD activity with copper deficiency (15.8±0.9 U/mg Hgb with the adequate Cu diet, 7.7±1.1 U/mg Hgb with the marginal Cu diet, and 5.8±0.8 U/mg Hgb with the Cu deficient diet).

Babu and Failla (1990a, 1990b) found tissue copper, Cu, Zn SOD activity, and hematological indices were similar in rats fed adequate (7 mg/kg diet) or low Cu (0.7 mg/kg diet) diets for 5 weeks. However, neutrophils from rats fed a low copper diet had less Cu, Zn SOD activity than those from rats fed an adequate diet.

In copper depleted male and female Harlan Sprague-Dawley rats fed low Cu diet (2.8 mg/kg) for 6 months, tissue such as liver, kidney, brain, and bone copper concentrations of female rats were significantly higher than those of male rats (Hopkins and Failla, 1995). In Paynter and colleagues' study (1979), male Wistar rats were divided into three treatment groups to measure SOD activity of different tissues: basal (0.8 mg

Cu/kg diet), basal+4.0 mg Cu/kg diet (copper as $CuSO_4(H_20)_5$), and basal+24 mg Cu/kg diet. The plasma ceruloplasmin activities were significantly lower in rats fed a basal diet (<0.1 mU/ml) compared to Cu supplemented groups (55.8±22.5 mU/ml with basal+4.0 mg Cu/kg diet and 84.6±2.3 mU/ml with basal+24.0 mg Cu/kg diet). Moreover, SOD activities in red blood cell (RBC) and all tissues except brain and muscle were significantly decreased in rats fed a basal diet for 6 weeks. The liver had the greatest decrease in SOD activity with Cu deficiency.

In offspring of Harlan Sprague-Dawley rats fed a copper depleted diet for 8 weeks, body copper levels were significantly decreased. This indicated that 8 weeks of copper depletion is enough to decrease stored copper in the liver and available copper as ceruloplasmin in the blood (Koller et al., 1987). Also, plasma ceruloplasmin (2.79±1.9 U/l) activities of copper deficient mice were lower than those of copper adequate mice (13.2±1.9 U/l) (Lukasewycz and Prohaska, 1983).

In two adults with anemia and neutropenia, plasma copper concentration, plasma ceruloplasmin activity, and erythrocyte superoxide dismutase activity were low with spontaneous copper deficiency (Smith et al., 1994). The plasma copper, ceruloplasmin, and superoxide dismutase were reduced in young men who consumed low Cu diets (containing 0.4 mg Cu/d) for 90 days, but intakes of 0.8 to 7.5 mg Cu/d did not affect these parameters (Turnlund et al., 1990, 1994).

As reported in the literature, Cr and Cu have functions in glucose and insulin metabolism as well as in immune response in animals and in humans. Increased insulin activity and efficiency with Cr supplementation result in improved glucose tolerance in some people. Improper nutritional intake may cause the risk of nutritional deficiencies

including vitamin as well as trace mineral deficiencies. Glucose intolerance is also increased with aging and glucose intolerance might cause a decrease in immune response. Moreover, nutrient deficiencies lead to lower immune responses in animals and humans. Therefore, effects of Cr and/or Cu depletion or supplementation on carbohydrate metabolism as well as immune functions including measurements of stress hormones, cytokine production, and lymphocyte proliferation were determined in this study.

CHAPTER III

METHODLOGY

MATERIALS AND METHODS

Animal Study

Research Design

A 2 X 2 factorial design was applied for this study using dietary chromium and copper as variables. Animals were randomly divided into four treatment groups.

Animals

Forty diabetes mellitus prone inbred BHE/cdb weanling male rats were obtained from University of Georgia, Athens, GA. The rats were housed individually in polystyrene cages with stainless-steel covers and acrylic grating floors. The rats had *ad libitum* access to experimental diets and deionized water throughout the study. The deionized water was changed three times a week. Acrylic grating floors were changed twice a week and polystyrene cages were changed once a week. The diets were provided in ceramic food bowls and water was provided in a glass bottle with a rubber stopper and

a stainless steel sipper tube. The temperature and humidity of the room were controlled and checked by personnel from Laboratory Animal Resources in the College of Veterinary Medicine at Oklahoma State University. The light cycle was controlled to maintain 12 hours of day and night time. The animal protocol was approved by the Institutional Animal Care and Use Committee.

Treatment

Four different diet treatments were applied in a 2 X 2 factorial design. The four dietary treatments were:

1) chromium and copper depletion (-Cr and -Cu)
2) adequate chromium and copper depletion (+Cr and -Cu)
3) chromium depletion and adequate copper (-Cr and +Cu)
4) adequate chromium and adequate copper (+Cr and +Cu).

Growth Diet

A semi-purified diet modified from the American Institute of Nutrition (AIN-93) diet was used during the rapid growth phase (Appendix A, Reeves et al., 1993). For the copper depletion treatment, 10% of the AIN-93 copper recommendation (0.6 ppm in the diet, as $CH_2Cu_2O_5$) was added to the diet and for the chromium depletion treatment, no chromium was added to the diet (0 ppm in the diet). For the adequate chromium treatment, 0.1466 g chromium chloride/kg mineral mix (1 ppm in the diet, as $CrCl_3$ $(H_2O)_6$) was added to the diet and for the adequate copper treatment, 0.30 g cupric carbonate/kg mineral mix was added to the diet (6 ppm in the diet, as$CH_2Cu_2O_5$). In

addition, vitamin E (0.6 g/kg diet) was added to all diets to protect rats from oxidative damage (Kullen and Berdanier, 1992).

Maintenance Diet

After reaching a growth plateau at the 16th week of age, rats were fed high-fat diets (220 g soybean oil/kg diet, Appendix A) modified from the AIN-93 diet (Reeves et al., 1993). For the copper depletion treatment, 10% of the AIN-93 copper recommendation (0.6 ppm in the diet, as $CH_2Cu_2O_5$) was added to the diet and for the chromium depletion treatment, no chromium was added to the diet (0 ppm added to the diet). For the adequate chromium treatment, 0.1466 g chromium chloride/kg mineral mix (1 ppm in the diet , as $CrCl_3\,(H_2O)_6$) was added to the diet and for the adequate copper treatment, 0.30 g cupric carbonate/kg mineral mix was added (6 ppm in the diet, as $CH_2Cu_2O_5$). In addition, vitamin E (0.6 g/kg diet) was added to all diets to protect rats from oxidative damage (Kullen and Berdanier, 1992). To increase fat content in the diet, partial casein (decreased from 20% to 14% of total diet) and dextrose (decreased from 37.95% to 28.95% of total diet) were replaced with soybean oil (increased from 7% to 22% of total diet).

Preparation of the Diet

Semi-purified diets and mineral mixes were prepared in our laboratory (Appendix C). All diet components were analyzed for trace mineral content and the lot numbers with minimum trace mineral concentrations were selected. Reagent grade or ultrapure chemicals were used for mineral mix preparation. Vitamin mix was protected from the

light to avoid breakdown of light sensitive vitamins. All ingredients for diets and mineral mixes were weighed in advance. All components of mineral mixes were combined and mixed in a burundum-fortified porcelain jar on a roller type mill for 15 hours. All diet ingredients were combined and mixed in an electric mixer for 40 minutes. Diets were bagged in plastic bags, labeled, and kept in the refrigerator at 4 °C until used.

Experimental Protocol

Three-week-old weanling male rats were received and divided into four treatment groups. Experimental diets were fed on the first day rats were received in the facility. Initial body weights were measured on the first day of feeding the experimental diets and weekly throughout the study.

At three, ten, and sixteen weeks of age, urinary glucose was tested in a subset of the rats. Six rats from each group were randomly selected and were caged in metabolic cages for a 6 hour urine collection. Food was withheld but water was available. Urine samples were collected into a cup that was kept on ice below the metabolic case. Urinary glucose was tested using glucose test strips (Clinistix, Bayer Corporation, Diagnostics Division, Elkhart, IN).

At twelve, eighteen, and twenty-one weeks of the age, oral glucose tolerance was tested in randomly selected rats. One gram of glucose per kg body weight (as a 50% solution) was dosed after a 12 hour fast. Blood was collected from the tail prior to the dosing as a baseline and 2 hours after the glucose load. Blood glucose was measured by a β-glucose analyzer (HemoCue Inc., Mission Viejo, CA) to test for impaired glucose tolerance. The changes in glucose in response to the oral glucose load were determined

by the difference between the baseline-glucose and the two hour-glucose.

After twenty-one weeks of feeding the experimental diets, four animals per day were necropsied for a total of 10 days. One rat from each treatment group was randomly selected and caged in the metabolic cage overnight for 12 hours for collection of urine samples. Rats were maintained without food, but water was provided. At the end of the collection, rats were injected with anesthesia (ketamine HCl 60 mg/kg body weight and xylazine 6 mg/kg body weight, Mallinckrodt Veterinary Inc., Mundelein, IL and Bayer Co., Shawnee Mission, KS, respectively) and were scanned using the EmScan SA-2 small animal body composition analyzer to determine lean body mass (Appendix D). Then rats were exsanguinated by cardiac puncture. Blood was collected for serum and plasma samples using different syringes. Blood samples for serum were kept on ice for 30 minutes to clot and then separated by centrifugation for 20 minutes at 2000 x g. Sodium citrate was used as an anticoagulant to collect blood for plasma samples. After collecting blood samples, plasma was separated by centrifugation for 20 minutes at 2000 x g. Serum and plasma samples were stored in the freezer at –20 °C for further assays. Tissues were collected in the following order: liver, spleen, heart, kidneys, pancreas, thymus, and bones. Spleen samples were collected aseptically to minimize contamination for further immune function assays. All tissues (except spleen) were trimmed, weighed, bagged in plastic bags, and stored in the freezer at –20 °C until used. The spleen was divided into half. One piece of the fresh spleen was used for immune function assays and the other half was stored in the freezer at –20 °C for trace mineral analysis.

Analytical Methods

Glucose

Glucose for BHE/cdb rats was measured using a Roche kit (number 47382, Roche Diagnostics Systems, Inc., Branchburg, NJ) and a COBAS FARA autoanalyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). The principle of the glucose analysis assay is based on glucose phosphorylation by hexokinase. Hexokinase phosphorylates glucose into glucose-6-phosphate (G-6-P) and adenosine diphosphate. Adenosine triphosphate and magnesium ions are required in this procedure. Glucose-6-phosphate is oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. In this step nicotinamide adenine dinucleotide (NAD) is reduced to the reduced form of nicotinamide adenine dinucleotide (NADH). One micromole of glucose oxidation produces one micromole of NADH. The absorbance of NADH can be detected spectrophotometrically at 340 nm.

Insulin

Insulin for BHE/cdb rats was determined using a radioimmunoassay kit from Linco (RI-13K, St. Charles, MO). The principle of the insulin assay is based on the ability of insulin in the sample to bind a radiolabeled antigen. Duplicates of samples, controls, standards, total counts, and nonspecific binding (NSB) were used to measure insulin for BHE/cdb rats. Two hundred microliters and 100 µl of assay buffer were added into NSB tubes and the rest of the tubes, respectively. Fifty microliters of standards, controls, or samples were added into tubes. One hundred microliters of ^{125}I insulin was pipetted to all tubes and rat insulin antibody was pipetted to all tubes except total counts and NSB tubes. Tubes were vortexed and incubated for 18 hours at 4 °C. After the

incubation, 1.0 ml of precipitating reagent was pipetted to all tubes except total count tubes. Tubes were then vortexed and incubated for 20 minutes at 4 °C. After the incubation, samples were centrifuged for 15 minutes at 2000 x g. Finally, supernatants were decanted and drained for 1 minute. Excess liquids were blotted on a paper towel and then pellets were counted for 1 minute on the Cobra II automatic gamma counter (Packard Instrument Co., Meriden, CT).

Cell proliferation

Mitogen stimulated T and B cell proliferation using rat splenocytes was measured using a modified method of Lukasewycz and Prohaska (1983). PHA-L, ConA, and LPS were purchased from Sigma Chemical Company (St. Louis, MO). The cell proliferation assay measures in vitro mitogenic responses of spleen lymphocytes to PHA-L, ConA, or LPS. The spleens were processed, splenocytes were collected by centrifugation, and were counted using a Tripan blue solution on the hemacytometer under the microscope. The splenocytes and mitogen were pipetted into a 96-well microtiter plate and incubated for 72 hours. Four hours before the end of the incubation, 1.0 μCi of ^{3}H-thymidine was added into each well. The ^{3}H-thymidine uptake by lymphocytes was counted on the 1600 TR liquid scintillation analyzer (Packard Instrument Co., Dowers Grove, IL). The stimulation index was calculated by a following formula: cpm of stimulated cell cultures/cpm of unstimulated cell cultures.

Differential cell profiles

Different cell profiles for BHE/cdb rats were measured using a Wright stain (Leukostain, Fisher Diagnostic, Kennesaw, GA). The distribution of lymphocytes and neutrophils was measured.

Stress hormone

Corticosterone for BHE/cdb rats was measured using a radioimmunoassay kit from Diagnostic Products Corporation (Los Angeles, CA). Duplicates of samples, controls, total counts of ^{125}I corticosterone, and nonspecific binding were measured. Twenty five microliters of calibrators, controls, and samples were pipetted into antibody-coated tubes and the same quantities of zero calibrator were pipetted into plain tubes. One ml of ^{125}I corticosterone was added into all tubes. Tubes were vortexed for 10 seconds and then were incubated for 45 minutes at room temperature. After finishing the incubation, all tubes except the total count tubes were decanted and drained for 1 minute. Excess liquids were blotted on a paper towel and then counted for 1 minute on the Cobra II automatic gamma counter (Packard Instrument Co., Meriden, CT).

Copper, zinc superoxide dismutase

Liver Cu, Zn superoxide dismutase for BHE/cdb rats was measured using a RANSOD kit (Randox Laboratories Ltd., Co. Antrim, United Kingdom) with a COBAS FARA autoanalyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). The toxic superoxide radical (O_2) is produced under the oxidative energy processes and it is dismutased by superoxide dismutase (SOD) to hydrogen peroxide and oxygen. In this

method, xanthine and xanthine oxidase (XOD) produce superoxide radicals and they react with 2-(4-iodophenyl)-2-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The degree of inhibition of this reaction is measured spectrophotometrically at 500 nm as the superoxide dismutase activity.

Tissue and diet mineral concentrations

Chromium, copper, iron, zinc, magnesium, and calcium concentrations of tissues including kidney, liver, spleen, and tibia of BHE/cdb rats and diets for rats were measured using a wet and dry ashing method of Hill et al. (1986) by flame or graphite furnace atomic absorption spectrometry. Samples were weighed into acid washed tubes, placed in acid washed beakers, and covered with acid washed Petri dishes. Samples were dried at 100 °C for three days and dried samples were weighed. Dried samples were placed in the heating blocks (Fisher Scientific, Pittsburgh, PA) and wet ashed at 100 °C by adding 50-100 µl each of deionized distilled water, double distilled nitric acid (Ultrapure, G. F. Smith Chemicals, Powell, OH), and 30% hydrogen peroxide (Ultrapure, J. T. Baker, Phillipsburg, NJ). Hydrogen peroxide was added repeatedly until a clear solution was obtained and samples were dried in the heating block. Then samples were dry ashed at 375 °C in the muffle furnace. The cycle of wet and dry ashing was repeated until samples became white. The ashed samples were analyzed for trace mineral concentration using an air-acetylene flame or a Zeeman background corrected graphite furnace atomic absorption spectrophotometer (Perkin Elmer 5100PC, Norwalk, CT).

Statistical Analysis

The general linear model (GLM) was utilized to analyze data using the Statistical Analysis System (version 6.11, SAS Institute Inc., Cary, NC, 1989). Groups were analyzed as a 2 x 2 factorial experiment. All data are presented as mean$\pm$SEM and differences at the level of $p<0.05$ are considered significant.

Human Study

The protocol of this study was approved by the Institutional Review Board (IRB) for human subjects at Oklahoma State University (Appendix I). All personnel including the primary investigator and research assistants had training sessions for biohazard safety and radioactive material safety at Oklahoma State University.

Research Design

A 2 X 2 factorial design was applied in this study. Ten subjects were randomly assigned into each treatment group.

Subjects

Forty subjects were recruited from around Stillwater through the Stillwater News Press, hospitals, local department stores, and several organizations such as the senior citizens center, a senior citizen exercise group, and church women's groups. Postmenopausal women with high blood cholesterol concentration (> 200 mg/dl) not taking lipid lowering medications or hormone replacement therapy were qualified as subjects for the study. The primary investigator explained the purpose and treatments of the study, and subjects signed the informed consent (Appendix K) form voluntarily. Subjects were reimbursed $50 at the end of the study.

Treatments

The treatment groups were as listed below:

1) placebo (-Cr-Cu)
2) chromium supplemented (+Cr-Cu)
3) copper supplemented (-Cr+Cu)
4) chromium and copper supplemented (+Cr+Cu) groups.

Subjects took one capsule at breakfast and one capsule at dinner for 12 weeks. Each month, a new bottle of supplements was delivered to subjects and the leftovers from the previous bottle were collected to check the compliance.

Preparation of Supplements

For the placebo supplementation (-Cr-Cu), #2 gelatin capsules (Apothecary Products, Inc., Minneapolis, MN) were filled with 0.1979 g of U.S.P. grade lactose monohydrate, $C_{12}H_{22}O_{11}H_2O$ (Spectrum Quality Products, Inc., Gardena, CA). For the chromium supplementation (+Cr-Cu), 2.653 g of U.S.P. grade $CrCl_3\,(H_2O)_6$ (Professional Compounding Centers of America, Inc., Houston, TX) and 0.1970 g of U.S.P. $C_{12}H_{22}O_{11}H_2O$ were mixed in a burundum-fortified porcelain jar on a roller type mill for 8 hours. Then, #2 gelatin capsules were filled with 0.1970 g of chromium and lactose mixture to contain 100 μg chromium as $CrCl_3\,(H_2O)_6$ per capsule. For the copper supplementation (-Cr+Cu), 29.47 g of U.S.P. grade $CuSO_4\,(H_2O)_5$ (Spectrum Quality Products, Inc., Gardena, CA) and 940.53 g of U.S.P. grade $C_{12}H_{22}O_{11}H_2O$ were mixed in a burundum-fortified porcelain jar on a roller type mill for 8 hours. Then, #2 gelatin capsules were filled with 0.194 g of copper and lactose mixture to contain 1.5 mg copper

as $CuSO_4 (H_2O)_5$ per capsule. For the chromium and copper supplementation (+Cr+Cu), 2.561 g of U.S.P. grade $CrCl_3 (H_2O)_6$, 29.47 g of U.S.P. grade $CuSO_4 (H_2O)_5$, and 1116.97 g of U.S.P. grade $C_{12}H_{22}O_{11}H_2O$ were mixed in a burundum-fortified porcelain jar on a roller type mill for 8 hours. Then, #2 gelatin capsules were filled with 0.230 g of chromium, copper, and lactose mixture to contain 100 μg of chromium as $CrCl_3 (H_2O)_6$ and 1.5 mg of copper as $CuSO_4 (H_2O)_5$ per capsule.

Before starting the supplementation, chromium and copper contents of sample capsule fillings were analyzed using a wet and dry ashing method of Hill et al. (1986) by flame or furnace atomic absorption spectrometry. The average chromium and copper content of placebo was 2.1 μg Cr/g supplement mix and 0 μg Cu/g supplement mix. The average chromium content of the chromium supplement was 288.72 μg Cr/g supplement mix, the copper content of the copper supplement was 7004 μg Cu/g supplement mix, and the chromium and copper content of the chromium and copper combined supplement was 378.4 μg Cr/g supplement mix and 5895.4 μg Cu/g supplement mix. Therefore, the weight of filling for each capsule was adjusted based on mineral content analysis. Final weights of chromium supplement capsule was 0.346 g, of copper supplement capsule was 0.214 g, and of chromium and copper combined supplement capsule was 0.264 g. The final concentration of chromium was 100 μg Cr/capsule, of copper was 1.5 mg Cu/capsule, and of chromium and copper combined supplement was 100 μg Cr and 1.5 mg Cu/capsule.

Blood Collection

Subjects were screened before starting the study. For the screening blood sample, subjects did not fast. Fasting blood subsequently was collected two more times for the beginning (pre-supplementation) and the end (post-supplementation) of the study. Subjects were told not to eat or drink beverages except water for 12 hours. Blood was collected between 7:30 a.m. and 9:00 a.m. After blood collection, a light breakfast was provided. Blood was collected in five syringes for different assays: in two neutral syringes for the separation and collection of serum samples for clinical assays, in an EDTA coated syringe for hematological analyses, in a heparin coated syringe for cell proliferation assays, and in a sodium citrate (300 μg/ml) coated syringe for trace mineral analyses. Siliconized butterfly type needles were used for blood collection to minimize trace mineral contamination. After the blood collection, blood samples were kept on ice for 30 minutes before centrifugation and were centrifuged for 30 minutes to separate plasma or serum samples for further assays. After plasma separation, red blood cells were washed four times with saline (0.9% NaCl solution) for further assays. Serum and plasma samples were labeled and stored at –20 °C until used. Serum and red blood cell samples for enzyme assays were labeled and stored at –70 °C until used. However, blood samples for the cell proliferation assay were kept at room temperature and whole blood cell proliferation assays were initiated within 2 hours of the first blood draw.

Anthropometric Measures

Each subject's height without shoes was measured (in cm) during the screening test. Their weight in light clothing was measured (in kg) at the screening, beginning, and end of the study.

Health Background

Subjects completed the health questionnaire (Appendix L) at the screening, beginning, and end of the study. Indicators of health status such as presence of illness, medication intake, and vitamin and mineral supplement intake were asked on the health questionnaire.

Dietary Intake Records

Subjects were taught about completion of the food frequency questionnaire (Appendix M) using food models by the primary investigator. Subjects filled out the food frequency questionnaires for 7 days before the baseline and final (after 12 weeks of supplementation) blood collection. Completed food frequency questionnaires were collected at each blood collection time and analyzed for 7 days of dietary intake using the Food Processor software (version 7.0, ESHA Research, Salem, OR, 1997).

Analytical Methods

Glucose

Glucose for human subjects was measured using a Roche kit (number 47382, Roche Diagnostics Systems, Inc., Branchburg, NJ) and a COBAS FARA autoanalyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). The principle of the glucose analysis assay is based on glucose phosphorylation by hexokinase. Hexokinase phosphorylates glucose into glucose-6-phosphate (G-6-P) and adenosine diphosphate. Adenosine triphosphate and magnesium ions are required in this procedure. Glucose-6-phosphate is oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. In this step nicotinamide adenine dinucleotide (NAD) is reduced to the reduced form of nicotinamide adenine dinucleotide (NADH). One micromole of glucose oxidation produces one micromole of NADH. The absorbance of NADH can be detected spectrophotometrically at 340 nm.

Insulin

Insulin for human subjects was measured using a radioimmunoassay kit from Diagnostic Products Corporation (Los Angeles, CA). The principle of the insulin assay is based on the ability of insulin in the sample to bind a radiolabeled antigen. Two hundred microliters of calibrators, controls, and samples were pipetted in duplicate into antibody-coated tubes. The same amount of zero calibrator was pipetted into plain tubes to measure non-specific binding. One ml of ^{125}I insulin was added into all tubes. Tubes were vortexed for 10 seconds and incubated for 18 hours at room temperature. After

finishing the incubation, all tubes except the total count tubes were decanted and drained for 1 minute. Excess liquids were blotted on the paper towel and then counted for 1 minute on the Cobra II automatic gamma counter (Packard Instrument Co., Meriden, CT).

Cell proliferation

Mitogen stimulated T cell proliferation using human whole blood cell cultures was measured following a Kramer and Burri's (1997) method. PHA-L and ConA were purchased from Sigma Chemical Company (St. Louis, MO). The cell proliferation assay measures in vitro mitogenic responses of blood to PHA-L or ConA. Whole blood cells and mitogen were pipetted into a 96-well microtiter plate and incubated for 96 hours. Eighteen hours before the end of the incubation, 1.0 μCi of ^{3}H-thymidine was added into each well. The ^{3}H-thymidine uptake by lymphocytes was counted on the 1600 TR liquid scintillation analyzer (Packard Instrument Co., Dowers Grove, IL). The stimulation index was calculated by a following formula: cpm of stimulated cell cultures/cpm of unstimulated cell cultures.

Differential cell profiles

Different cell profiles for human subjects were measured using a VEGA hematology autoanalyzer (ABX Montpellier, Cedex, France). The distribution of lymphocytes, monocytes, neutrophils, eosinophils, and basophils was measured.

Stress hormone

Cortisol for human subjects was measured using a radioimmunoassay kit from Diagnostic Products Corporation (Los Angeles, CA). Duplicates of samples, controls, total counts of ^{125}I cortisol, and nonspecific binding were measured. Twenty five microliters of calibrators, controls, and samples were pipetted into antibody-coated tubes and the same quantities of zero calibrator were pipetted into plain tubes. One ml of ^{125}I cortisol was added into all tubes. Tubes were vortexed for 10 seconds and then were incubated for 45 minutes at room temperature. After finishing the incubation, all tubes except the total count tubes were decanted and drained for one minute. Excess liquids were blotted on a paper towel and then counted for one minute on the Cobra II automatic gamma counter (Packard Instrument Co., Meriden, CT).

Interleukin 2

The production of IL-2 by whole blood cell cultures of human subjects was measured using an ELISA assay kit from BioSource Europe S. A. (Fleurus, Belgium). This assay measures the direct binding of a blend of monoclonal antibodies (Mabs) to distinct epitopes of IL-2. First, IL-2 containing samples or standards and then monoclonal antibody labeled horseradish peroxidase (Mab 2-HRP) were pipetted into a monoclonal antibody (Mab 1) coated microtiter plate. A sandwich of coated Mab 1-IL-2-Mab2-HRP was formed during the two hours of incubation at room temperature and unbound enzyme labeled antibodies were removed by washing steps. A chromogenic solution was pipetted into each well and incubated for 15 minutes at room temperature to measure bound enzyme labeled antibodies. The stop solution was pipetted and the plate

was read at 450 nm on the plate reader (ELX 808 Ultra Microplate Reader, Bio-Tek Instruments Inc., Winooski, VT).

Copper, zinc superoxide dismutase

Red blood cell Cu, Zn superoxide dismutase for human subjects was measured using a RANSOD kit (Randox Laboratories Ltd., Co. Antrim, United Kingdom) with a COBAS FARA autoanalyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). The toxic superoxide radical (O_2) is produced under the oxidative energy processes and it is dismutased by superoxide dismutase (SOD) to hydrogen peroxide and oxygen. In this method, xanthine and xanthine oxidase (XOD) produce superoxide radicals and they react with 2-(4-iodophenyl)-2-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The degree of inhibition of this reaction is measured spectrophotometrically at 500 nm as the superoxide dismutase activity.

Ceruloplasmin

Serum ceruloplasmin for human subjects was measured using a COBAS FARA autoanalyzer (Roche Diagnostic Systems, Inc., Montclair, NJ) following a method of Henry et al. (1974). The oxidation rate of p-phenylenediamine at 37 °C and pH 6.0 is measured for determination of ceruloplasmin concentration. Oxidation products produce purple color and this color can be measured spectrophotometrically at 520-530 nm.

Statistical Analysis

The general linear model (GLM) was utilized to analyze data using the Statistical Analysis System (version 6.11, SAS Institute Inc., Cary, NC, 1989). The effects of chromium, copper, and chromium and copper combined supplementation on dependent variables of the study were determined. Groups were analyzed as a 2 X 2 factorial experiment. The differences between baseline and final (after 12 weeks of supplementation) variables were analyzed using GLM. Also, the differences in dietary intake among groups at baseline and final (after 12 weeks of supplementation) were analyzed. All data are presented as mean$\pm$SEM and p values at the level of $p<0.05$ are considered significant.

CHAPTER IV

RESULT AND DISCUSSION

Animal Study

Result

One of the rats was severely sick at necropsy, so it was excluded from the data analysis. Data from a total of 39 rats were analyzed in the present animal study.

Weight gain. The initial weight was similar among the four experimental groups. There were no significant effects of depletion of Cr, Cu, or a combined depletion of Cr and Cu on the weight gain of the animals throughout the experiment (Table 1).

Tissue weight. Liver, kidney, spleen, heart, thymus, pancreas, and testes were weighed at the end of the experiment (after 21 weeks of treatment). Tissue weights were similar among different diet groups (Table 2). However, when tissue weights were expressed as percentages of body weight, Cr depletion caused significant increases in liver ($p<0.02$), kidney ($p<0.03$), and spleen ($p<0.03$) (Table 2).

Carbohydrate metabolism. Fasting serum glucose and insulin were measured as indicators of carbohydrate metabolism. There were no significant effects of depletion of Cr, Cu, or a combined depletion of Cr and Cu on the serum glucose or insulin (Table 3).

Cu, Zn Superoxide Dismutase. The liver Cu, Zn SOD activity was measured as an indicator of the copper status. There were no significant effects of depletion of Cr, Cu, or a combined depletion of Cr and Cu on the liver Cu, Zn SOD (Table 4).

Corticosterone. Serum corticosterone was measured as an indicator of stress hormone level. There were no significant effects of depletion of Cr, Cu, or a combined depletion of Cr and Cu on serum corticosterone (Table 5).

Differential cell profiles. Lymphocytes and neutrophils in blood were counted. The mean values of these parameters were similar among different diet groups (Table 6).

Lymphocyte proliferation. Proliferation of splenocytes was expressed as a mitogen stimulation index according to different mitogens. The concentrations of 10 μg/ml and 25μg/ml of PHA-L for T cell proliferation, 10 μg/ml of ConA for T cell proliferation, and 10 μg/ml of LPS for B cell proliferation were used as mitogens. The mitogen stimulation indices were similar among different diet groups and mitogen treatments. However, there was a significant interactive effect of a combined depletion of Cr and Cu on T lymphocyte proliferation using 25 μg/ml of PHA as a mitogen ($P < 0.006$, Table 7). In the Cr depleted and Cu adequate group, lymphocyte proliferation was significantly decreased with a PHA-L 25 μg/ml stimulation compared to the -Cr-Cu group (Table 7). In the adequate Cr and adequate Cu group, stimulation index with PHA-L 25 μg/ml was significantly increased compared to the +Cr-Cu group or the –Cr+Cu group (Table 7).

Tissue Mineral Concentrations. Cr, Cu, Fe, Zn, Mg, and Ca concentrations of kidney, spleen, liver, and tibia were analyzed (Tables 8-12). Cr concentrations of kidney, spleen, and liver samples and Ca concentration of liver were not detectable. Non-detectable Cr or Ca concentrations might be due to increased sample dilution or contamination of the

deionized distilled water. Cr concentrations of tibia samples could not be measured without having interference from Ca in the tibia.

Cu concentration of kidney was significantly decreased ($p<0.0001$) in Cu depletion groups compared to adequate Cu groups (Table 8). Cr or Cu depletion significantly affected different mineral concentrations in liver. Cu concentration of liver was significantly decreased in Cu depletion groups ($p<0.02$, Table 8). Iron concentration of the liver was significantly increased in Cu depletion groups ($p<0.02$). Zinc concentration of liver was significantly increased in Cr depletion groups ($p<0.03$) compared to adequate Cr groups (Table 10). Magnesium concentration of liver was significantly increased in Cu depletion groups compared to adequate Cu groups ($p<0.01$, Table 11). Also, a significant interactive effect of Cr and Cu depletion on the magnesium concentration of tibia was observed ($p<0.03$, Table 11). In adequate Cu groups, Mg concentration of tibia was significantly increased with Cr depletion in the diet.

Table 1
Initial and final weights of BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu[1, 2]

Group	Initial weight (g)	Final weight (g)	Weight Gain (g)
-Cr-Cu	59±3	523±13	464±12
+Cr-Cu	55±4	552±19	497±17
-Cr+Cu	52±3	540±23	488±21
+Cr+Cu	55±3	558±18	503±18
-Cr	56±2	531±13	476±12
+Cr	55±2	555±13	500±12
-Cu	57±2	537±12	480±11
+Cu	53±2	548±14	495±14
Factors		P Values	
Cr	0.77	0.22	0.16
Cu	0.27	0.54	0.38
Cr x Cu	0.31	0.77	0.61

[1]Values are mean±SEM, n=9-10
[2]-Cr-Cu: chromium and copper depletion; +Cr-Cu: adequate chromium and copper depletion;
-Cr+Cu: chromium depletion and adequate copper; +Cr+Cu: adequate chromium and adequate copper

Table 2

Tissue weights and percentage of body weights of various tissues from BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Tissue Weight (g) (% body weight)						
	Liver	Kidney	Spleen	Heart	Thymus	Pancreas	Testes
-Cr-Cu	14.09±0.42 (2.70±0.07)	3.38±0.10 (0.65±0.02)	1.21±0.05 (0.23±0.01)	1.26±0.04 (0.24±0.01)	0.22±0.03 (0.04±0.01)	0.30±0.07 (0.06±0.01)	3.37±0.11 (0.65±0.02)
+Cr-Cu	13.80±0.74 (2.49±0.07)	3.15±0.08 (0.58±0.02)	1.10±0.06 (0.20±0.01)	1.29±0.03 (0.24±0.01)	0.27±0.04 (0.05±0.01)	0.35±0.04 (0.06±0.01)	3.39±0.08 (0.62±0.02)
-Cr+Cu	13.90±0.58 (2.58±0.05)	3.45±0.23 (0.64±0.04)	1.14±0.04 (0.21±0.01)	1.30±0.06 (0.24±0.01)	0.27±0.05 (0.05±0.01)	0.36±0.07 (0.07±0.01)	3.33±0.07 (0.62±0.02)
+Cr+Cu	14.00±0.41 (2.51±0.04)	3.41±0.09 (0.61±0.01)	1.11±0.05 (0.20±0.01)	1.28±0.04 (0.23±0.01)	0.25±0.04 (0.05±0.01)	0.26±0.06 (0.05±0.01)	3.40±0.09 (0.61±0.02)
-Cr	13.99±0.35 (2.64±0.04)	3.42±0.12 (0.64±0.02)	1.17±0.03 (0.22±0.01)	1.28±0.04 (0.24±0.004)	0.24±0.03 (0.05±0.004)	0.33±0.05 (0.06±0.01)	3.35±0.06 (0.63±0.01)
+Cr	13.89±0.42 (2.50±0.04)	3.27±0.06 (0.59±0.01)	1.10±0.04 (0.20±0.01)	1.28±0.03 (0.23±0.004)	0.26±0.03 (0.05±0.01)	0.31±0.03 (0.05±0.01)	3.39±0.06 (0.62±0.01)
-Cu	13.94±0.41 (2.59±0.05)	3.27±0.07 (0.61±0.01)	1.15±0.04 (0.22±0.01)	1.27±0.03 (0.24±0.004)	0.24±0.02 (0.05±0.004)	0.32±0.04 (0.06±0.01)	3.38±0.06 (0.63±0.01)
+Cu	13.95±0.35 (2.55±0.03)	3.43±0.13 (0.63±0.02)	1.12±0.03 (0.21±0.01)	1.29±0.04 (0.24±0.01)	0.26±0.03 (0.05±0.01)	0.32±0.05 (0.06±0.01)	3.36±0.05 (0.62±0.01)
Factors	P Values						
Cr	0.87 (<0.02)	0.33 (<0.03)	0.19 (<0.03)	0.91 (0.17)	0.62 (0.93)	0.66 (0.46)	0.58 (0.33)
Cu	0.99 (0.43)	0.25 (0.49)	0.56 (0.38)	0.75 (0.70)	0.62 (0.65)	0.82 (0.72)	0.86 (0.44)
Cr x Cu	0.73 (0.24)	0.50 (0.34)	0.39 (0.33)	0.67 (0.75)	0.32 (0.35)	0.26 (0.35)	0.80 (0.71)

[1]Values are mean±SEM, n=7-10

[2]See Table 1 for description of groups

Table 3

Fasting serum glucose and insulin of BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Glucose (mg/dl)	Insulin (ng/ml)
-Cr-Cu	296±14	0.62±0.04
+Cr-Cu	295±13	0.77±0.08
-Cr+Cu	302±15	0.69±0.08
+Cr+Cu	320±16	0.80±0.09
-Cr	299±10	0.65±0.04
+Cr	307±10	0.79±0.06
-Cu	296±9	0.69±0.04
+Cu	311±11	0.74±0.06
Factors	P Values	
Cr	0.55	0.07
Cu	0.29	0.47
Cr x Cu	0.48	0.81

[1]Values are mean±SEM, n=9-10

[2]See Table 1 for description of groups

Table 4

Liver Cu, Zn superoxide dismutase of BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Cu, Zn SOD (U/mg liver)
-Cr-Cu	4.1±0.6
+Cr-Cu	3.3±0.5
-Cr+Cu	4.0±0.5
+Cr+Cu	3.6±0.6
-Cr	4.1±0.4
+Cr	3.4±0.4
-Cu	3.7±0.4
+Cu	3.8±0.4
Factors	P Values
Cr	0.25
Cu	0.93
Cr x Cu	0.71

[1]Values are mean±SEM, n=9-10

[2]See Table 1 for description of groups

Table 5

Serum corticosterone of BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Corticosterone (ng/ml)
-Cr-Cu	256±22
+Cr-Cu	272±17
-Cr+Cu	258±32
+Cr+Cu	259±16
-Cr	257±19
+Cr	266±12
-Cu	264±14
+Cu	259±18
Factors	P Values
Cr	0.72
Cu	0.83
Cr x Cu	0.74

[1]Values are mean±SEM, n=9-10

[2]See Table 1 for description of groups

Table 6

The distribution of lymphocytes and neutrophils in blood of BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Lymphocytes (%)	Neutrophils (%)
-Cr-Cu	85±3	11±3
+Cr-Cu	87±5	14±5
-Cr+Cu	87±3	15±2
+Cr+Cu	80±5	18±5
-Cr	86±2	13±2
+Cr	84±4	16±3
-Cu	86±3	13±3
+Cu	84±3	17±3
Factors	P Values	
Cr	0.61	0.41
Cu	0.52	0.32
Cr x Cu	0.33	0.90

[1]Values are mean±SEM, n=6-7

[2]See Table 1 for description of groups

Table 7

Stimulation indices with mitogen stimulation of BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	PHA-L (10 μg/ml)	PHA-L (25 μg/ml)	ConA (10 μg/ml)	LPS (10 μg/ml)
-Cr-Cu	1.13 ±0.11	3.69±0.69	25.60±10.82	13.34±1.91
+Cr-Cu	1.25±0.31	2.33±0.56	26.29±9.67	11.61±2.09
-Cr+Cu	1.03±0.18	1.80±0.37	17.23±5.72	12.23±3.05
+Cr+Cu	1.08±0.18	4.22±0.78	16.20±5.18	11.82±2.10
-Cr	1.08±0.10	2.74±0.47	21.42±5.97	12.79±1.75
+Cr	1.17±0.18	3.12±0.52	21.72±5.74	11.70±1.43
-Cu	1.19±0.16	2.96±0.46	25.95±6.92	12.48±1.39
+Cu	1.05±0.12	2.90±0.54	16.78±3.72	12.03±1.83
Factors		P Values		
Cr	0.69	0.39	0.99	0.65
Cu	0.52	1.00	0.29	0.85
Cr x Cu	0.88	<0.006	0.92	0.78

[1]Values are mean±SEM, n=5-9

[2]See Table 1 for description of groups

Table 8
Copper concentrations of various tissues from BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Kidney (μmol/g dry weight)	Spleen (μmol/g dry weight)	Liver (μmol/g dry weight)	Tibia (μmol/g dry weight)
-Cr-Cu	0.188±0.004	0.059±0.008	0.118±0.009	0.042±0.001
+Cr-Cu	0.174±0.006	0.051±0.006	0.104±0.006	0.043±0.001
-Cr+Cu	0.264±0.015	0.064±0.006	0.136±0.009	0.046±0.001
+Cr+Cu	0.264±0.013	0.064±0.006	0.128±0.011	0.043±0.002
-Cr	0.226±0.011	0.061±0.005	0.127±0.006	0.044±0.001
+Cr	0.216±0.013	0.057±0.004	0.115±0.007	0.043±0.001
-Cu	0.181±0.004	0.055±0.005	0.111±0.006	0.042±0.001
+Cu	0.264±0.010	0.064±0.004	0.132±0.007	0.044±0.001
Factors		P Values		
Cr	0.50	0.57	0.23	0.40
Cu	<0.0001	0.19	<0.02	0.10
Cr x Cu	0.49	0.57	0.70	0.10

[1]Values are mean±SEM, n=9-10
[2]See Table 1 for description of groups

Table 9
Iron concentrations of various tissues from BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Kidney (μmol/g dry weight)	Spleen (μmol/g dry weight)	Liver (μmol/g dry weight)	Tibia (μmol/g dry weight)
-Cr-Cu	3.92±0.23	40.94±4.55	5.20±0.28	1.28±0.06
+Cr-Cu	4.08±0.24	49.67±4.03	5.08±0.20	1.29±0.06
-Cr+Cu	3.83±0.17	55.27±6.99	4.87±0.25	1.41±0.07
+Cr+Cu	3.66±0.18	53.34±6.62	4.03±0.43	1.23±0.14
-Cr	3.87±0.14	48.10±4.38	5.04±0.19	1.33±0.05
+Cr	3.88±0.15	51.41±3.70	4.61±0.25	1.26±0.07
-Cu	4.00±0.16	45.31±3.12	5.14±0.17	1.29±0.04
+Cu	3.75±0.12	54.36±4.70	4.50±0.25	1.32±0.08
Factors		P Values		
Cr	0.98	0.55	0.11	0.32
Cu	0.23	0.12	<0.02	0.72
Cr x Cu	0.43	0.35	0.23	0.30

[1]Values are mean±SEM, n=9-10
[2]See Table 1 for description of groups

Table 10

Zinc concentrations of various tissues from BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Kidney (μmol/g dry weight)	Spleen (μmol/g dry weight)	Liver (μmol/g dry weight)	Tibia (μmol/g dry weight)
-Cr-Cu	1.01±0.04	0.92±0.04	1.36±0.04	3.94±0.14
+Cr-Cu	1.02±0.05	0.95±0.04	1.27±0.02	3.98±0.11
-Cr+Cu	1.03±0.05	0.87±0.02	1.31±0.04	4.12±0.15
+Cr+Cu	1.07±0.06	0.92±0.04	1.22±0.06	3.80±0.18
-Cr	1.02±0.03	0.89±0.02	1.33±0.03	4.01±0.10
+Cr	1.04±0.04	0.94±0.03	1.25±0.03	3.89±0.10
-Cu	1.01±0.03	0.93±0.03	1.32±0.02	3.96±0.08
+Cu	1.05±0.04	0.90±0.02	1.27±0.03	3.98±0.12
Factors		P Values		
Cr	0.62	0.27	<0.03	0.34
Cu	0.50	0.31	0.18	0.99
Cr x Cu	0.69	0.75	0.93	0.23

[1]Values are mean±SEM, n=9-10

[2]See Table 1 for description of groups

Table 11
Magnesium concentrations of various tissues from BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Kidney (μmol/g dry weight)	Spleen (μmol/g dry weight)	Liver (μmol/g dry weight)	Tibia (μmol/g dry weight)
-Cr-Cu	11.24±1.05	22.60±1.01	21.05±0.62	137.2±4.8
+Cr-Cu	11.53±1.50	23.36±1.11	21.78±0.57	138.7±3.3
-Cr+Cu	11.38±0.10	21.42±0.99	20.39±0.82	147.5±2.5
+Cr+Cu	10.58±0.89	22.46±0.86	18.36±1.04	129.8±5.2
-Cr	11.31±0.70	22.01±0.70	20.72±0.51	142.1±3.0
+Cr	11.08±0.88	22.93±0.70	20.26±0.68	134.5±3.1
-Cu	11.39±0.89	22.98±0.74	21.42±0.42	138.0±2.8
+Cu	11.00±0.66	21.91±0.65	19.49±0.68	138.6±3.5
Factors		P Values		
Cr	0.83	0.38	0.40	0.06
Cu	0.72	0.31	<0.01	0.87
Cr x Cu	0.64	0.89	0.08	<0.03

[1]Values are mean±SEM, n=9-10
[2]See Table 1 for description of groups

Table 12

Calcium concentrations of various tissues from BHE/cdb rats depleted of Cr, Cu, or both Cr and Cu for 21 weeks[1, 2]

Group	Kidney (μmol/g dry weight)	Spleen (μmol/g dry weight)	Tibia (mmol/g dry weight)
-Cr-Cu	2.88±0.17	2.78±0.28	4.65±0.36
+Cr-Cu	2.81±0.18	2.90±0.13	4.64±0.49
-Cr+Cu	2.70±0.15	2.72±0.17	4.84±0.51
+Cr+Cu	2.78±0.14	3.06±0.18	4.04±0.60
-Cr	2.79±0.11	2.75±0.16	4.97±0.22
+Cr	2.80±0.11	2.98±0.11	4.36±0.38
-Cu	2.84±0.12	2.84±0.15	4.65±0.30
+Cu	2.74±0.10	2.88±0.13	4.46±0.39
Factors		P Values	
Cr	0.99	0.25	0.42
Cu	0.53	0.80	0.68
Cr x Cu	0.65	0.59	0.43

[1]Values are mean±SEM, n=9-10

[2]See Table 1 for description of groups

Human Study

Results

A total of eleven subjects were excluded from the data analysis: five subjects were diabetic, three subjects dropped out themselves after the baseline sample collection, one subject was on estrogen, one subject stopped taking supplement capsules towards the end of the study (60 capsules leftover, 36% of total capsules), and one subject was not able to have blood drawn at the baseline due to dehydration. Data from a total of 29 subjects were analyzed and included in the present human study. Data are discussed based on differences in values between baseline and after 12 weeks of supplementation.

Age. The mean age of subjects was 59 years with a range of 38-81 years. There were no significant age differences at baseline among supplement groups (Table 13).

Weight and Height. There were no significant differences in body weights at baseline or after 12 weeks of supplementation among supplement groups. The average weight of subjects was 82 kg at baseline and after 12 weeks of supplementation (Table 13). The average height of subjects was 163 cm and was not significantly different by supplement groups (Table 13).

Body Mass Index. There were no significant differences among supplement groups in BMI at baseline or after 12 weeks of supplementation. The average BMI of subjects was 31 kg/m^2 at baseline and after 12 weeks of supplementation (Table 13).

Dietary Intake. There were no significant differences in carbohydrate, protein, or fat intake at baseline among supplement groups. However, Kcal intake was significantly

high in the Cr and Cu supplementation group compared to the Cr supplementation (+Cr-Cu) or Cu supplementation (–Cr+Cu) group at baseline ($p<0.02$, Table 14). The average percentage of Kcal provided by carbohydrate, protein, and fat in baseline diet was 48%, 17%, and 33%, respectively. After 12 weeks of Cr and/or Cu supplementation, there were no differences in Kcal intake among groups, but in Cu supplemented groups, percentage of carbohydrate in the diet was significantly decreased ($P<0.02$) and percentage of fat in the diet was significantly increased ($P<0.02$) after 12 weeks of supplementation (Table 14).

Carbohydrate Metabolism. Serum glucose and insulin were measured as indicators of carbohydrate metabolism. There were significant differences in serum glucose ($p<0.02$) and insulin ($p<0.006$) at baseline in Cu supplementation groups (Table 15). There were significant effects of 12 weeks of Cu supplementation on glucose and insulin. Serum glucose ($P<0.04$) and insulin ($P<0.004$) were significantly decreased with 12 weeks of Cu supplementation (Table 15).

Superoxide Dismutase and Ceruloplasmin. Red blood cell Cu, Zn SOD activity and serum ceruloplasmin were measured as indicators of Cu status. There were no significant differences in RBC Cu, Zn SOD activity and in serum ceruloplasmin at baseline among supplement groups. The change in RBC Cu, Zn SOD activity was not significantly affected by 12 weeks of supplementation (Table 16). Also, non-significant differences among supplement groups in serum ceruloplasmin after 12 weeks of supplementation were observed (Table 16).

Cortisol. Serum cortisol was measured as an indicator of stress hormone level. There were significant differences in serum cortisol at baseline in Cu supplementation groups

($p<0.02$). However, serum cortisol was not significantly affected by 12 weeks of treatments (Table 17).

Interleukin 2. The production of IL-2 was measured as an indicator of immune function. There was a significant difference in IL-2 production at baseline among supplement groups ($p<0.04$, Table 18). IL-2 production was significantly high in the Cr and Cu combined supplementation group compared to the Cr supplementation group (+Cr-Cu) at baseline. However, the production of IL-2 was not significantly affected by 12 weeks of Cr and/or Cu supplementation (Table 18).

Differential cell profiles. Differential cell profiles were measured as indicators of health status. However, the distribution of neutrophils was significantly different in Cr ($p<0.05$) or Cu ($p<0.01$) supplementation groups at baseline (Table 19). The changes in lymphocytes, monocytes, neutrophils, and eosinophils after 12 weeks of supplementation were not significantly different among supplement groups. Basophils were significantly increased with 12 weeks of copper supplementation ($p<0.003$) compared to groups not supplemented with copper (Table 19).

Lymphocyte proliferation. Lymphocyte proliferation was measured as an indicator of immune function. There were significant differences in stimulation index at baseline in Cr supplementation groups with PHA-L 40 μg/ml ($p<0.03$) and PHA-L 80 μg/ml ($p<0.04$) stimulation (Table 20). However, changes in stimulation indices were not affected by 12 weeks of supplementation with any of the concentrations of PHA-L used (Table 20).

Likewise, there were significant differences in stimulation index at baseline in Cr supplementation groups with ConA 25 μg/ml ($p<0.04$) and ConA 50 μg/ml ($p<0.01$)

stimulation. A significant interactive effect of chromium and copper was observed on lymphocyte proliferation with ConA 50 μg/ml stimulation after 12 weeks of supplementation ($P<0.05$, Table 21). In chromium supplemented groups, when copper was supplemented, the stimulation index was decreased. When copper was not supplemented, chromium supplementation increased the stimulation index.

Moreover, the stimulation index was significantly decreased ($P<0.02$) with ConA 100 μg/ml stimulation with 12 weeks of Cu supplementation (Table 21). On the contrary, there were no significant differences in the stimulation index among supplement groups after 12 weeks of supplementation with ConA 12.5 μg/ml and ConA 25 μg/ml stimulation (Table 21). In addition, chromium supplementation had the highest stimulation index with either PHA-L or ConA stimulation among supplement groups (Tables 20 and 21).

Table 13
Age, height, weight, and body mass index at baseline and after 12 weeks of supplementation with Cr, Cu, or both Cr and Cu[1, 2]

Group	Age (year)	Height (cm)	Weight (kg)			BMI (kg/m^2)		
			Baseline	12 weeks	Change[3]	Baseline	12 weeks	Change[3]
-Cr-Cu	57±4	166±1	77.4±3.7	79.2±3.5	1.8±0.8	28.0±1.5	28.7±1.4	0.6±0.3
+Cr-Cu	59±5	163±1	85.3±6.5	80.9±1.5	-4.3±6.5	32.0±2.4	30.4±1.0	-1.6±2.4
-Cr+Cu	59±2	162±2	81.6±5.2	82.2±5.2	0.5±0.5	31.6±2.6	31.8±2.6	0.2±0.2
+Cr+Cu	58±5	162±3	80.7±3.3	81.4±3.1	0.7±0.6	30.9±1.6	31.1±1.5	0.3±0.3
-Cr	58±2	164±1	79.6±3.1	80.7±3.0	1.1±0.5	29.8±1.5	30.2±1.5	0.4±0.2
+Cr	58±3	163±2	83.1±3.7	81.1±1.6	-2.0±3.5	31.5±1.5	30.7±0.8	-0.7±1.3
-Cu	58±3	165±1	81.1±3.7	80.0±2.0	-1.1±3.1	30.0±1.4	29.5±0.9	-0.4±1.1
+Cu	59±3	162±2	81.2±3.2	81.8±3.2	0.6±0.4	31.3±1.6	31.5±1.6	0.2±0.1
Factors					P Values			
Cr	0.94	0.47	0.49	0.91	0.37	0.46	0.77	0.37
Cu	0.93	0.10	0.97	0.66	0.56	0.58	0.30	0.56
Cr x Cu	0.75	0.36	0.38	0.75	0.34	0.30	0.53	0.34

[1]Values are mean±SEM, n=7-8

[2]-Cr-Cu: placebo group; +Cr-Cu: chromium supplemented group; -Cr+Cu: copper supplemented group; +Cr+Cu: chromium and copper supplemented group

[3]Change: difference in values between baseline and the end of the study (after 12 weeks of supplementation)

Table 14

Kcal, carbohydrate, protein, and fat intake at baseline and after 12 weeks of supplementation with Cr, Cu, or both Cr and Cu[1, 2]

Group	Kcal intake			Carbohydrate intake (% Kcal)			Protein intake (% Kcal)			Fat intake (% Kcal)		
	Baseline	12 weeks	Change[3]	Baseline	12weeks	Change[3]	Baseline	12 weeks	Change[3]	Baseline	12 weeks	Change[3]
-Cr-Cu	1859±224	1653±234	-206±183	48±2	51±2	3±1	17±1	17±1	0±1	31±4	31±2	0±2
+Cr-Cu	1574±135	1633±207	59±175	47±3	48±2	1±3	17±2	18±1	1±1	36±2	34±2	-2±2
-Cr+Cu	1632±169	1651±64	19±197	49±2	47±3	-2±4	16±1	16±1	0±1	32±5	38±3	6±5
+Cr+Cu	2327±254	2102±162	-225±212	51±4	42±2	-9±4	16±1	15±1	-1±2	33±3	43±3	10±4
-Cr	1746±139	1652±117	-94±133	49±2	49±2	0±2	16±1	16±1	0±1	31±3	35±2	4±3
+Cr	1922±171	1849±145	-72±136	49±2	45±2	-4±3	17±1	17±1	0±1	35±2	38±2	3±3
-Cu	1726±136	1644±152	-82±128	48±2	50±2	-2±1	17±1	17±1	0±1	33±2	33±2	0±2
+Cu	1930±169	1844±97	-86±143	50±2	45±2	-5±3	16±2	15±1	-1±1	32±3	40±2	8±3
Factors	P Values											
Cr	0.31	0.25	0.96	0.84	0.10	0.17	0.93	0.77	0.75	0.41	0.12	0.83
Cu	0.20	0.21	0.88	0.37	<0.02	<0.02	0.24	0.06	0.50	0.73	<0.004	<0.02
Cr x Cu	<0.02	0.21	0.20	0.66	0.74	0.52	0.96	0.54	0.58	0.65	0.75	0.51

[1]Values are mean±SEM, n=7-8

[2]See Table 13 for description of groups

[3]Change: difference in values between baseline and the end of the study (after 12 weeks of supplementation)

Table 15
Fasting serum glucose and insulin concentrations at baseline and after 12 weeks of supplementation with Cr, Cu, or both Cr and Cu[1, 2]

Group	Glucose (mg/dl)			Insulin (μIU/ml)		
	Baseline	12 weeks	Change[3]	Baseline	12 weeks	Change[3]
-Cr-Cu	106±1	111±4	5±5	12.4±1.2	14.2±2.8	1.8±2.8
+Cr-Cu	109±3	119±8	10±7	12.1±1.2	14.4±2.2	2.3±2.4
-Cr+Cu	112±4	113±4	1±2	20.0±4.1	9.9±1.3	-11.0±3.8
+Cr+Cu	124±8	115±6	-9±8	22.0±3.6	16.7±2.8	-5.3±3.3
-Cr	109±2	112±3	3±3	16.2±2.3	12.2±1.7	-4.0±2.8
+Cr	116±4	117±5	1±6	16.7±2.2	15.4±1.7	-1.2±2.2
-Cu	107±2	115±4	8±4	12.3±0.9	14.3±1.8	2.0±1.8
+Cu	117±4	114±3	-3±4	20.9±2.8	13.0±1.7	-8.2±2.6
Factors			P Values			
Cr	0.10	0.41	0.63	0.78	0.16	0.36
Cu	<0.02	0.82	<0.04	<0.006	0.68	<0.004
Cr x Cu	0.30	0.55	0.16	0.69	0.18	0.45

[1]Values are mean±SEM, n=7-8
[2]See Table 13 for description of groups
[3]Change: difference in values between baseline and the end of the study (after 12 weeks of supplementation)

Table 16
Red blood cell Cu, Zn superoxide dismutase and ceruloplasmin activities at baseline and after 12 weeks of supplementation with Cr, Cu, or both Cr and Cu[1, 2]

Group	RBC Cu, Zn SOD (U/g Hgb)			Ceruloplasmin (mg/dl)		
	Baseline	12weeks	Change[3]	Baseline	12 weeks	Change[3]
-Cr-Cu	1003±55	812±40	-191±43	57.3±4.0	20.4±3.5	-37.0±4.9
+Cr-Cu	866±115	856±41	-10±80	56.6±3.4	18.8±1.8	-38.0±2.0
-Cr+Cu	1118±103	839±60	-279±111	56.6±4.4	22.3±4.9	-34.3±6.6
+Cr+Cu	1001±101	841±55	-160±140	54.5±4.7	17.5±2.6	-37.0±6.1
-Cr	1065±60	826±36	-238±62	57.0±2.9	21.3±2.9	-35.7±4.0
+Cr	929±77	849±33	-80±78	55.6±2.7	18.2±1.5	-37.4±2.9
-Cu	935±64	834±28	-101±50	57.0±2.6	19.6±2.0	-37.3±2.7
+Cu	1068±72	840±40	-228±85	55.7±3.1	20.3±3.0	-35.5±4.4
Factors			P Values			
Cr	0.20	0.66	0.14	0.73	0.39	0.75
Cu	0.21	0.90	0.24	0.75	0.93	0.75
Cr x Cu	0.92	0.68	0.76	0.87	0.66	0.86

[1]Values are mean±SEM, n=7-8
[2]See Table 13 for description of groups
[3]Change: difference in values between baseline and the end of the study (after 12 weeks of supplementation)

Table 17

Serum cortisol concentrations at baseline and after 12 weeks of supplementation with Cr, Cu, or both Cr and Cu[1, 2]

Group	Cortisol (nmol/l)		
	Baseline	12 weeks	Change[3]
-Cr-Cu	392±51	375±61	-17±53
+Cr-Cu	335±15	375±32	39±42
-Cr+Cu	246±29	328±59	82±61
+Cr+Cu	279±52	281±33	2±47
-Cr	319±34	351±42	32±41
+Cr	309±26	331±26	22±31
-Cu	366±28	375±35	9±34
+Cu	260±27	307±36	47±40
Factors		P Values	
Cr	0.77	0.65	0.82
Cu	<0.02	0.18	0.57
Cr x Cu	0.27	0.66	0.21

[1]Values are mean±SEM, n=7-8

[2]See Table 13 for description of groups

[3]Change: difference in values between baseline and the end of the study (after 12 weeks of supplementation)

Table 18

Interleukin 2 production from ConA (100 μg/ml) stimulated whole blood cell cultures at baseline and after 12 weeks of supplementation with Cr, Cu, or both Cr and Cu[1, 2]

Group	IL-2 (U/ml)		
	Baseline	12 weeks	Change[3]
-Cr-Cu	0.30±0.06	0.64±0.09	0.34±0.05
+Cr-Cu	0.19±0.01	0.44±0.02	0.25±0.02
-Cr+Cu	0.27±0.04	0.45±0.05	0.18±0.03
+Cr+Cu	0.41±0.09	0.62±0.12	0.20±0.09
-Cr	0.28±0.03	0.54±0.05	0.26±0.03
+Cr	0.29±0.05	0.52±0.06	0.22±0.04
-Cu	0.24±0.03	0.53±0.05	0.29±0.03
+Cu	0.33±0.05	0.52±0.06	0.20±0.04
Factors		P Values	
Cr	0.78	0.77	0.48
Cu	0.10	0.94	0.07
Cr x Cu	<0.04	<0.02	0.22

[1]Values are mean±SEM, n=7-8

[2]See Table 13 for description of groups

[3]Change: difference in values between baseline and the end of the study (after 12 weeks of supplementation)

Table 19

The distribution of lymphocytes, neutrophils, monocytes, eosinophils, and basophils in blood at baseline and after 12 weeks of supplementation with Cr, Cu, or both Cr and Cu[1, 2]

Group	Lymphocytes (%)			Monocytes (%)			Neutrophils (%)			Eosinophils (%)			Basophils (%)		
	Baseline	12 wks	Change[3]	Baseline	12 wks	Change[3]	Baseline	12 wks	Change[3]	Baseline	12 wks	Change[3]	Baseline	12 wks	Change[3]
-Cr-Cu	30.2±1.7	30.5±1.8	0.3±1.9	6.7±0.4	8.3±0.5	1.6±0.5	59.0±1.4	57.4±1.9	-1.6±1.9	3.0±0.4	3.0±0.4	0.0±0.1	1.1±0.1	0.9±0.1	-0.2±0.1
+Cr-Cu	28.7±2.2	29.0±2.0	0.2±3.1	6.9±0.3	7.8±0.4	0.9±0.3	60.5±2.3	59.5±2.5	-1.0±0.5	2.9±0.4	2.9±0.6	0.0±0.5	1.0±0.1	0.9±0.1	-0.1±0.1
-Cr+Cu	36.7±2.3	33.6±2.9	-2.5±1.2	7.2±0.4	8.7±0.8	1.4±0.6	51.3±1.7	52.6±2.0	1.0±1.0	4.0±0.8	4.0±0.6	0.0±0.5	0.8±0.2	1.1±0.1	0.2±0.1
+Cr+Cu	30.1±1.9	27.5±2.1	-2.6±2.1	7.6±0.9	9.4±1.0	1.8±0.5	57.7±2.1	59.0±1.9	1.3±2.7	3.7±0.7	3.0±0.5	-0.7±0.3	1.0±0.1	1.1±0.2	0.1±0.2
-Cr	33.7±1.7	32.0±1.7	-1.1±1.2	7.0±0.3	8.5±0.4	1.5±0.4	54.9±1.5	54.9±1.5	0.0±1.1	3.5±0.4	3.4±0.4	-0.1±0.3	0.9±0.1	1.0±0.1	0.1±0.1
+Cr	29.4±1.4	28.3±1.4	-1.1±1.9	7.2±0.5	8.5±0.5	1.3±0.3	59.2±1.6	59.2±1.5	0.0±2.3	3.3±0.4	2.9±0.4	-0.4±0.3	1.0±0.1	1.0±0.1	0.0±0.1
-Cu	29.5±1.4	29.7±1.5	0.3±1.8	6.8±0.3	8.1±0.3	1.3±0.3	59.8±1.3	58.4±1.6	-1.4±2.0	3.0±0.3	3.0±0.4	0.0±0.3	1.0±0.1	0.9±0.1	-0.1±0.1
+Cu	33.9±1.7	30.8±2.0	-2.6±1.1	7.3±0.4	9.0±0.6	1.6±0.4	54.0±1.5	55.6±1.6	1.2±1.3	3.9±0.5	3.6±0.4	-0.3±0.3	0.9±0.1	1.1±0.1	0.2±0.1
Factors								P Values							
Cr	0.07	0.11	0.98	0.64	0.88	0.87	<0.05	<0.05	0.85	0.81	0.32	0.43	0.74	1.00	0.92
Cu	0.07	0.72	0.21	0.28	0.17	0.50	<0.01	0.23	0.33	0.15	0.29	0.41	0.23	0.10	<0.003
Cr x Cu	0.23	0.32	0.99	0.81	0.36	0.26	0.21	0.32	0.94	0.84	0.39	0.51	0.34	1.00	0.45

[1]Values are mean±SEM, n=6-8

[2]See Table 13 for description of groups

[3]Change: difference in values between baseline and the end of the study (after 12 weeks of supplementation)

Wks: weeks

Table 20

Lymphocyte proliferation with PHA-L stimulation from whole blood cell cultures at baseline and after 12 weeks of supplementation with Cr, Cu, or both Cr and Cu[1, 2]

	PHA-L 10 µg/ml			PHA-L 20 µg/ml			PHA-L 40 µg/ml			PHA-L 80 µg/ml		
Group	Baseline	12 weeks	Change[3]	Baseline	12 weeks	Change[3]	Baseline	12 weeks	Change[3]	Baseline	12 weeks	Change[3]
-Cr-Cu	81.5±24.7	124.9±44.9	43.3±43.3	130.5±29.4	237.2±86.4	106.7±73.9	211.7±38.1	303.5±101.3	91.9±86.1	233.2±41.4	335.4±107.6	102.2±88.0
+Cr-Cu	41.4±18.4	155.3±42.9	113.9±48.3	75.1±21.8	256.9±63.9	181.8±72.4	92.6±22.4	319.3±73.3	226.7±85.5	108.7±31.8	321.6±74.9	203.2±94.4
-Cr+Cu	85.1±15.0	132.8±36.0	47.7±40.4	151.1±24.9	180.2±41.1	29.1±54.6	184.5±32.2	213.2±49.4	28.7±66.4	211.1±37.9	216.5±35.4	5.5±57.5
+Cr+Cu	86.2±25.2	90.3±25.0	4.1±31.0	131.4±31.3	136.0±46.3	4.6±50.0	149.9±37.4	174.1±59.9	24.2±73.8	165.0±42.9	199.9±56.8	34.9±67.1
-Cr	83.4±13.5	129.1±27.4	45.7±28.4	141.5±18.6	206.8±44.7	65.3±44.7	197.2±24.1	255.3±53.4	58.2±52.2	221.4±27.1	272.0±53.9	50.6±51.0
+Cr	62.1±15.9	128.2±27.8	62.7±35.0	101.1±19.5	206.5±44.1	100.6±55.2	119.1±21.7	258.8±52.1	133.9±65.2	134.7±26.3	266.3±49.8	114.7±64.7
-Cu	61.5±15.8	140.1±30.1	78.6±32.7	102.8±19.2	247.0±51.7	144.2±50.8	152.1±26.9	311.4±60.1	159.3±61.2	171.0±30.4	329.0±64.8	148.8±63.3
+Cu	85.6±13.2	116.5±24.1	25.9±27.8	142.6±18.9	163.2±30.3	12.9±39.5	169.7±23.9	198.1±37.0	19.2±47.9	191.3±28.0	210.1±29.5	6.7±42.0
Factors						P Values						
Cr	0.36	0.88	0.88	0.17	0.85	0.81	<0.03	0.88	0.50	<0.04	0.84	0.52
Cu	0.25	0.49	0.19	0.17	0.18	0.06	0.65	0.13	0.09	0.66	0.13	0.08
Cr x Cu	0.33	0.38	0.16	0.51	0.62	0.39	0.21	0.72	0.33	0.32	0.99	0.54

[1]Values are mean±SEM, n=7-8

[2]See Table 13 for description of groups

[3]Change: difference in values between baseline and the end of the study (after 12 weeks of supplementation)

Table 21

Lymphocyte proliferation with ConA stimulation from whole blood cell cultures at baseline and after 12 weeks of supplementation with Cr, Cu, or both Cr and Cu[1, 2]

	ConA 12.5 µg/ml			ConA 25 µg/ml			ConA 50 µg/ml			ConA 100 µg/ml		
Group	Baseline	12 weeks	Change[3]	Baseline	12 weeks	Change[3]	Baseline	12 weeks	Change[3]	Baseline	12 weeks	Change[3]
-Cr-Cu	77.5±14.5	78.5±16.4	1.0±25.2	149.0±14.3	147.6±37.4	-1.3±39.0	167.1±12.2	167.9±39.1	0.8±39.4	180.3±18.2	211.2±49.0	30.8±51.3
+Cr-Cu	40.5±9.9	121.6±16.7	81.1±16.8	84.9±22.7	190.3±27.8	105.5±37.8	93.1±20.0	246.5±32.5	153.4±40.7	105.8±24.4	279.5±35.5	173.7±47.4
-Cr+Cu	66.0±9.6	92.4±29.6	26.4±26.5	122.5±19.7	166.6±53.7	44.1±57.0	153.1±21.1	175.9±36.9	22.9±41.8	178.8±21.3	166.5±35.3	-12.3±40.4
+Cr+Cu	61.3±23.3	73.1±23.0	11.8±25.3	94.2±30.1	108.8±28.2	14.6±32.4	110.7±27.8	119.1±23.4	8.4±33.7	150.9±39.6	134.6±30.5	-16.3±49.8
-Cr	71.4±8.3	85.9±17.1	14.5±18.1	134.9±12.5	157.7±32.5	22.9±34.7	159.6±12.3	172.2±25.9	12.6±28.0	179.5±13.7	188.8±29.7	7.1±32.0
+Cr	50.1±11.8	99.2±15.0	49.1±17.3	89.2±17.7	152.7±22.3	63.5±27.5	101.2±16.2	187.7±27.0	86.5±33.2	126.6±22.4	212.7±30.9	86.0±42.8
-Cu	59.0±9.9	100.0±12.7	41.0±18.3	116.9±15.7	169.0±23.1	52.1±30.0	130.1±15.2	207.2±26.8	77.1±34.5	143.1±17.9	245.4±30.6	102.3±39.0
+Cu	64.0±10.9	84.1±19.1	20.1±18.1	110.4±16.8	141.8±32.9	31.4±34.5	134.9±17.3	151.6±23.9	16.7±27.0	166.9±20.3	151.8±23.1	-16.5±30.3
Factors						P Values						
Cr	0.16	0.61	0.19	<0.04	0.85	0.40	<0.01	0.76	0.10	0.06	0.64	0.14
Cu	0.75	0.46	0.37	0.70	0.45	0.62	0.93	0.10	0.14	0.41	<0.02	<0.02
Cr x Cu	0.27	0.19	0.06	0.42	0.23	0.14	0.45	0.06	<0.05	0.38	0.21	0.15

[1]Values are mean±SEM, n=7-8

[2]See Table 13 for description of groups

[3]Change: difference in values between baseline and the end of the study (after 12 weeks of supplementation)

Discussion

Body and Tissue Weight of BHE/cdb Rats

Initial body weights were measured before feeding experimental diets and rats were randomly assigned into four treatment groups. Body weight changes and lean body mass were not affected significantly by treatments (Appendix D). The liver, kidney, and spleen as percent of body weight were significantly increased in Cr depletion groups. Liver of all rats seemed to be enlarged and have high fat composition at necropsy. This increased percentage of body weight of liver might be due to either effects of Cr depletion or high fat composition in the liver. The percentage of moisture loss of liver was not significantly affected by treatments (Appendix D). The average percentage moisture loss of liver was 60% which suggests that these rats had fatty livers. In Spicer's study (1996), the average percentage moisture loss of liver was 70% in both diabetic and control rats.

Tissue weights in this study did not increase with Cu deficiency which contradicts results of some other Cu deficiency studies. Cu deficient animals had enlarged liver and enlarged spleen compared to copper adequate animals in studies by other investigators (Prohaska, 1983, Prohaska et al., 1983, Prohaska and Lukasewycz, 1989, Mulhern and Koller, 1988). However, Cu depletion did not affect the percentage of weight of liver or spleen in the present study. The results from this animal study suggest that Cr depletion had more effects than Cu depletion on weight of liver, spleen, and kidney as percentage of body weight. However, the severity of Cr or Cu depletion could not be determined because there are no standard reference values for Cr or Cu concentrations in tissues to compare to Cr and Cu concentrations in tissues of the BHE/cdb rats in the present study.

Body Mass Index and Dietary Intake in Human Subjects

The average BMI of human subjects was 31 kg/m^2 which should classify the subjects in this study as obese. A BMI >25 kg/m^2 is considered as obesity (Lee and Nieman, 1996). The average Kcal intake of human subjects was 1824 Kcal, less than the recommended energy allowances. The recommended energy allowances for females over 51 years are 1900 Kcal (National Research Council, 1989). The average percentage of Kcal provided by carbohydrate, protein, and fat was 48%, 17%, and 33% in these subjects. The percentage of carbohydrate intake is lower than U. S. dietary goals (58%) and of protein and fat is higher than U. S. dietary goals (12% protein and 30% fat, Lee and Nieman, 1996). The average dietary Cu intake was 1.0 mg Cu/d in these human subjects. It is lower than the ESADDI for Cu (1.5-3.0 mg/d) and higher than the average intake of females (0.93 mg/d) in the U. S. (Olivares and Uauy, 1996b, Pennington and Young, 1991). The significant changes in percentage of Kcal from fat and carbohydrate intake in Cu supplemented groups were largely due to one subject. One subject's Kcal and fat intake was increased by 42% and 26%, respectively and carbohydrate intake was decreased by 20% at the end of the study compared to the baseline. However, other subjects' Kcal intake after 12 weeks of supplementation remained similar to the baseline.

In spite of less Kcal intake, these subjects were classified as obese. This might indicate that there were possibilities of errors in height and weight measurements. These errors might be from a researcher, an uncalibrated scale, or a measuring ruler. Also, errors might have occurred in the food intake recall estimates. Subjects were taught about completion of food frequency forms using food models. These subjects might have

reported less Kcal intake than their actual Kcal intake because subjects had to depend on their memories for completion of food frequency forms for seven days.

It is common that obese people underestimate their intake when self-reporting their dietary intake. People report about 50% of their actual Kcal intake and they report foods what should have consumed instead of actual foods they consumed (Schoeller, 1995, Fricker et al., 1992). These factors might have created more errors in dietary intake assessment.

Carbohydrate Metabolism

Chromium deficiency influences the action of insulin by increasing circulating insulin concentrations before the development of glucose intolerance (Mertz, 1979, 1993). Also, the response of insulin-sensitive tissue to insulin is decreased in chromium deficiency and tissue-insulin sensitivity is improved by trivalent chromium (Mertz, 1976, Campbell and Mertz, 1963). Dietary chromium increases pancreatic β-cell sensitivity to glucose and increases insulin sensitivity and thus results in normalization of insulin responses to glucose (Mertz, 1979, 1998, Potter et al., 1985, Striffler et al., 1995).

Fasting serum glucose of BHE/cdb rats was not affected by depletion of Cr, Cu, or both Cr and Cu in the present study. These results are different from some of the other Cr and/or Cu depletion or supplementation studies. In Tuman's study (1978), an injection of a synthetic complex or a brewer's yeast GTF to genetically diabetic mice (db/db) reduced elevated blood glucose. In Cohen and coworkers' study (1982), Cu deficient rats had a significantly increased plasma glucose at 30 and 60 minutes after the oral glucose load compared to Cu supplemented rats.

BHE/cdb rats are prone to type II diabetes as they age (Berdanier, 1991, Mathews et al., 1995). However, BHE/cdb rats did not develop or have glucose intolerance or diabetes in the present study. Normal glucose tolerance might not have been affected by depletion of Cr, Cu, or both Cr and Cu in BHE/cdb rats. Glucose tolerance tests were conducted at the 12^{th}, 18^{th}, and 21^{st} weeks of the study (Appendix E). However, fasting serum glucose was within the normal glucose range of rats and there were no signs of impaired glucose tolerance from these glucose tolerance tests. When comparing blood glucose among the three tests, overall blood glucose was the highest at the first test compared to other two tests. Fasting serum glucose at necropsy was higher than normal fasting serum glucose of rats. Normal fasting glucose of rats is 50-135 mg/dl (Mitruka and Rawnsley, 1981), but it was 235-401 mg/dl in BHE/cdb rats in the present study. The increased serum glucose at necropsy might be due to the stress from the anesthesia. In addition, normal glucose tolerance tests in unanesthetized animals supported the idea that the high glucose at necropsy might be due to the stress from the anesthesia. Fructosamine indicates glucose control over the last one to three weeks, and this assay may be a more accurate assessment of glucose status because glucose may be affected by anesthesia. Fructosamine was not significantly affected by treatment (Appendix E), but there was a trend for Cr and Cu to have an interactive effect on fructosamine (Givens, 1997). Fructosamine tended to be lower in the –Cr+Cu group compared to the +Cr+Cu group. BHE/cdb rats might not have had Cr depletion by feeding Cr depleted diets throughout the study. Thus, serum glucose was not affected by Cr depletion or adequate Cr in the diets. However, the tissue Cr concentration was not detectable, so the tissue Cr depletion could not be determined in the present study. Non-detectable Cr concentrations

might be due to increased sample dilution or contamination of the deionized distilled water. Kidney, spleen, and liver samples were hard to dissolve into clear solutions, so the volume of the acids and deionized distilled water was increased to obtain clear solutions. Also, deionized distilled water from our laboratory was found to be Cr contaminated, so Cr concentration of the sample blank was too high, and resulted in non-detectable Cr concentrations.

In Bue and colleagues' study (1989), BHE/cdb female rats were fed 22% fat diets (21% menhaden oil and 1% corn oil) containing 25% sucrose from weaning. These rats showed impaired glucose tolerance at an age of 200 days. In the present study, BHE/cdb male rats were fed 22% fat diets (22% soybean oil) containing 10% sucrose after reaching the growth plateau (the 16^{th} week of treatment). The menhaden oil contains higher saturated fatty acids and omega-3 fatty acids (33.6% saturated fatty acids and 21.7% omega-3 fatty acids) compared to soybean oil (14.4% saturated fatty acids and 6.8% omega-3 fatty acids) (Mahan and Escott-Stump, 1996). The different source of fat and lower sucrose contents in the diets, shorter periods of feeding (about 5 weeks) high fat diets, and different stage of life for introduction of high fat diets might have caused a failure in developing impaired glucose tolerance in the present study.

Serum insulin was not significantly influenced by Cr, Cu, or both Cr and Cu depletion in BHE/cdb rats. However, serum insulin of BHE/cdb rats at necropsy was within or close to a normal range. The normal insulin value for rats is 0.5-2.0 ng/ml (Mitruka and Rawnsley, 1981) and it was 0.2 to 1.2 ng/ml in BHE/cdb rats. The normal or close to normal serum insulin supports that these rats were not Cr depleted in this study.

In the present human study, fasting serum glucose ($p<0.04$) and insulin ($p<0.004$) were significantly decreased in the Cu supplemented groups compared to non-supplemented groups after 12 weeks of supplementation. However, the significantly decreased fasting serum glucose and insulin might also be affected by significantly higher values for these variables in Cu supplementation groups at baseline ($p<0.02$ for glucose and $p<0.006$ for insulin).

In the present study, there was no effect of Cr on serum glucose and insulin. In Anderson and colleagues' study (1983), people with impaired glucose tolerance responded to 200 μg Cr supplementation. The degree of glucose intolerance affects the response to Cr. In Anderson and colleagues' study (1991), control subjects with normal glucose tolerance and without Cr deficiency did not respond to Cr supplementation.

Fasting serum glucose indicated that about 17% of subjects had impaired fasting glucose (based on >110 mg/dl but <126 mg/dl, American Diabetes Association, 1998). The number of subjects with impaired glucose tolerance was not large enough to evaluate effects on serum glucose or insulin due to Cr supplementation in these subjects alone.

In Abraham and colleagues' study (1992), glucose was not affected by 7-16 months of 250 μg $CrCl_3$ supplementation in elderly subjects. However, in Potter and colleagues' study (1985), blood glucose at 60 minutes following an OGTT (40 g/m^2 surface area) was significantly decreased with 12 weeks of 200 μg $CrCl_3$ supplementation. In Mertz's review (1993), most of the controlled Cr supplementation studies had significant effects of Cr on glucose tolerance in subjects with glucose intolerance. Mertz concluded that Cr deficiency might cause insulin resistance and Cr supplementation might improve insulin resistance. In addition, Cr supplementation

studies have several problems in Cr analysis, interaction of Cr with other dietary factors, and measurement of Cr status. As an approach to solve these problems, "well standardized urinary Cr excretion measurement following a glucose load might be necessary in Cr supplementation research" (Mertz, 1998). The urinary excretion of Cr reflects the absorption of Cr. Urinary Cr excretion would be increased with increased dietary Cr intake or Cr supplementation and also with a glucose load (Anderson et al., 1982). Increased urinary Cr excretion following a glucose load is due to insulinogenic characteristics of glucose. Increased blood glucose following a glucose load leads to an increase in insulin in the blood and increases in urinary Cr excretion (Anderson et al., 1991, Mertz, 1998). Acute increase in Cr in the blood following a glucose load is controversial and it needs further investigation (Mertz, 1993).

Corticosterone in Rats or Cortisol in Human

The secretion of ACTH and cortisol is increased in response to stress. Increased cortisol reacts as an immunosuppressor and increases the risk of infection (Moonsie-Shageer and Mowat, 1993). In the present study, serum corticosterone was not affected by depletion of Cr, Cu, or both Cr and Cu in BHE/cdb rats. However, these results contradict a number of other studies. In Seaborn and Stoecker's study (1992), a stress state was created in guinea pigs by depletion of ascorbic acid. Plasma cortisol tended to be increased with ascorbic acid depletion in these guinea pigs. Urinary ^{51}Cr excretion was significantly increased in ascorbic acid depletion groups and it might be due to increased stress hormone by ascorbic acid depletion. The serum cortisol of newly arrived

feeder calves was decreased with chromium supplementation (Moonsie-Shageer and Mowat, 1993).

In Nockels and coworker's study (1993), the copper excretion in feces and in urine and Cu retention were decreased during stress. The decreased fecal and urinary Cu excretion and decreased retention of Cu during stress might be due to the decreased food intake and volume of urine excretion. On the other hand, in Turnlund and colleagues' study (1979), plasma Cu concentration was not changed with prednisolone (1 mg/kg body weight) treatment for up to 24 weeks in adult female guinea pigs. However, plasma Cu concentration was increased with a high dose of prednisolone (100 mg/kg body weight) in these guinea pigs.

In the present study, BHE/cdb rats may not have been metabolically stressed. In other words, Cr and/or Cu depletion and high fat diets may not have been severe enough to cause significant metabolic stress effects. Or the short feeding period of the high fat diets may not have caused significant metabolic stress effects. Serum corticosterone of BHE/cdb rats was within a normal range. The reference value for serum corticosterone from a radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA) was 328 ng/ml in 16 male Wistar rats. Mean serum corticosterone in the experimental groups was 255.5±22.4 to 272.0±14.5 ng/ml in these BHE/cdb rats. To investigate Cr and/or Cu depletion effects on serum corticosterone, experimental animals need to be in a stressd status. It would be hard to determine Cr and/or Cu effects on stress hormones without creating or having a stress state in experimental animals. In the present study, chromium and/or Cu depleted high fat diets were fed to BHE/cdb rats to produce the metabolic stress. However, Cr and/or Cu depletion as well as high fat contents in the diets did not

create metabolic stress in the present animal study. These rats were fed high fat diets after reaching the growth plateau, so if these rats were fed high fat diets from weaning, it might have created metabolic stress in these BHE/cdb rats.

In the present human study, serum cortisol was significantly lower in Cu supplementation groups compared to non-Cu supplementation groups at baseline ($p<0.02$). However, serum cortisol was not significantly influenced by 12 weeks of Cr or Cu supplementation or their interaction in the present human subjects. Non-significant changes in serum cortisol after 12 weeks of Cr and/or Cu supplementation in the human subjects might indicate that these human subjects did not have Cr and/or Cu deficiency in the beginning and throughout the study. Thus, adequate Cr and/or Cu status in the body was not affected by Cr and/or Cu supplementation and led to non-significant effects on serum cortisol. If subjects had a Cr and/or Cu deficiency in the beginning and throughout the study, the Cr and/or Cu deficiency might have created a stressed state and might have been affected by Cr and/or Cu supplementation. However, serum cortisol of these subjects was within the normal range or close to the normal range at baseline (115-600 nmol/l) and 12 weeks after supplementation (122-689 nmol/l). The normal reference range for human cortisol is 138-690 nmol/l for the radioimmunoassay kits from Diagnostic Products Corporation (Los Angeles, CA). As mentioned above, to measure the effects of Cr and/or Cu supplementation on the stress hormones, the subjects must be under metabolic stress.

Immune Response

In Burton and coworkers' study (1993), chromium supplemented cows had increased anti-ovalbumin and ConA stimulated responses. The ConA-induced PBMNC blastogenesis was not suppressed in the sera after parturition from periparturient cows supplemented with chromium. However, in the sera from the control (unsupplemented) cows, PBMNC blastogenesis was decreased.

Bala et al. (1991) found in vitro that blastogenesis of splenocytes in response to PHA, ConA, or LPS was lower in cultures from copper deficient rats than from the control group. Severely Cu deficient rats showed lower mean PHA and ConA lymphocyte stimulation indices than other groups (Windhauser et al., 1991). In Lukasewycz and Prohaska's (1983) and Lukasewycz and colleagues' (1987) studies, ConA, PHA, and LPS stimulated splenocyte proliferation was less than one-half of those values of the copper adequate mice. Also, in Kramer and coworkers' (1988) study, spleens from copper deficient male rats had higher proliferation of unstimulated splenocytes than spleens from copper adequate male rats. Prohaska and Lukasewycz (1989) found alterations in the overall incorporation of thymidine into DNA following stimulation by ConA, PHA, or LPS in C58 mice fed a copper-deficient diet. Other researchers found decreased mitogen reaction to LPS in vivo in C57BL mice fed a copper deficient diet from birth (Mulhern and Koller, 1988). However, there were no changes in lymphocyte blastogenesis in post-weaning copper deficient mice (Blakley and Hamilton, 1987, Prohaska and Lukasewycz, 1989). Differences in animal strains may contribute to these discrepancies among studies (Lukasewycz and Prohaska, 1983, Lukasewycz et al., 1985).

Bala and Failla (1992, 1993) found impaired T-cell proliferation in copper-depleted rats and restored normal T-cell function by repletion of copper. Lower splenocyte proliferation in response to ConA was found in copper-deficient Lewis rats (Davis et al., 1987, Kramer et al., 1988). In most studies, lymphocyte proliferation was decreased with Cu deficiency.

In the present study, splenocyte proliferation from BHE/cdb rats with PHA-L (10 μg/ml), ConA, or LPS stimulation was not influenced by Cr, Cu, or the combined Cr and Cu depletion. However, there was a significant interactive effect of Cr and Cu depletion on splenocyte proliferation with PHA-L 25 μg/ml stimulation in BHE/cdb rats. When either Cr or Cu was adequate in the diets, there was a beneficial effect of Cu or Cr supplementation. However, when Cr was depleted in the diet, there was a suppressive effect of Cu supplementation on lymphocyte proliferation.

These results are different from most studies of effects of copper deficiency on mitogen responses in animals. These differences in results from other studies might be due to the species of animals. The BHE/cdb rats are diabetes or glucose intolerance prone animals. These rats are reported to develop glucose intolerance or diabetes genetically as they age (about 300 days old) due to genetic abnormalities in the liver (Berdanier, 1991). Genetic abnormalities in the liver might cause significant decreases in available ATP in the liver and these lead to increased phosphorylation in the liver. Increased cytosolic phosphorylation leads to increased fatty acid synthesis and storage in the liver. Increased fatty cell sizes in the liver cause insulin resistance. Also, in response to increased fatty acid synthesis in the liver, gluconeogenesis is increased and causes increased glucose in the blood (Berdanier, 1991). However, the effects of these genetic

abnormalities in the liver on the mitogen response could not be clearly explained in the present study.

In the current human study, subjects with postmenopausal women with high cholesterol, not taking lipid lowering medication or estrogen replacement therapy were included. Other researchers from this study investigated effects of Cr and/or Cu supplementation on lipid parameters in these subjects.

There was no literature to review in this area. This study did not measure effects of estrogen on the study parameters, so results from other studies about effects of estrogen therapy on immune function can not be applied to this study.

In the current human study, whole blood cell cultures were used for the lymphocyte proliferation measurement. Whole blood cell cultures have beneficial effects on lymphocyte proliferation compared to lymphocyte cultures separated from whole blood by a density gradient. Whole blood cell cultures prevent changes in leukocyte populations and other mediators in cell cultures (Bloemena et al., 1989, Leroux et al., 1985, Bocchieri et al., 1995). These changes in cell profiles affect further immune response (Petrovsky et al., 1994). Moreover, the lymphocyte proliferation assay using whole blood cell cultures is simpler and easier compared to using separated lymphocyte cultures. Whole blood cell cultures respond better to PHA or ConA stimulation compared to the separated lymphocyte cultures (Bloemena et al., 1989, Leroux et al., 1985, Bocchieri et al., 1995).

In the present study, significant interactive effects of Cr and Cu on lymphocyte proliferation with ConA 50 μg/ml stimulation were observed. Comparing groups supplemented with Cr and Cu or Cu alone, supplementation of Cr enhanced lymphocyte

proliferation with ConA 50 μg/ml stimulation. Also, the same trend ($p<0.06$) of beneficial effect of Cr supplementation on lymphocyte proliferation was observed with ConA 12.5 μg/ml stimulation. A significantly decreased lymphocyte proliferation with ConA 100 μg/ml stimulation after 12 weeks of Cu supplementation might indicate that this dose of Cu (3.0 mg/d) suppressed immune function in these human subjects. Moreover, there was a trend for a suppressive effect of Cu supplementation on the lymphocyte proliferation with PHA-L stimulation at various doses.

The non-significant effects of Cr and/or Cu supplementation on mitogen response with PHA-L stimulation might be due to excessive variability among cell cultures. Lymphocyte proliferation was measured on a daily basis, so the daily measurement of lymphocyte proliferation might have caused high variabilities in lymphocyte proliferation with PHA-L stimulation. These results with PHA-L stimulation are in agreement with Meydani and colleagues' study (1990). The lymphocyte proliferation with PHA stimulation was similar to control, but it was significantly increased with ConA stimulation compared to control in the vitamin E supplemented group of elderly subjects. However, the PBMNC proliferation with PHA, ConA, or PWM was lower with the low copper diet compared to the adequate copper diet or the restored copper diet in young men (Kelley et al., 1995). These different results between PHA-L and ConA stimulated mitogen responses might be due to the stimulation of different subsets of T cells as well as high variabilities within cell cultures with PHA-L stimulation. Thus mitogen responses with PHA or ConA stimulation are not always consistent among studies.

Cr and Cu might have interactive functions in lymphocyte proliferation. A beneficial effect of Cr or Cu supplementation on lymphocyte proliferation was observed

in BHE/cdb rats. Also, a beneficial effect of Cr supplementation on lymphocyte proliferation was observed in human subjects. Cu supplementation blocked enhancing effcts of Cr on lymphocyte proliferation in the current human study. This might suggest that Cu supplementation (3.0 mg/d) was greater than the optimal concentration for promoting immune response. However, the interactive effects of Cr and Cu need further investigation.

Differential Cell Profiles

Neutrophils have phagocytic functions and the number of neutrophils is rapidly increased in response to bacterial stimuli or foreign cells. Eosinophils and basophils are rapidly increased during allergic conditions and parasitic infection. Eosinophils and basophils also have phagocytic functions, so these cells engulf and remove foreign cells or allergic mediators.

The percentage of lymphocytes or neutrophils was within a normal range in BHE/cdb rats. The normal range of lymphocytes is 50.2-84.5% and of neutrophils is 4.40-49.2% in rats (Mitruka and Rawnsley, 1981). The distribution of lymphocytes or neutrophils was not significantly different among treatment groups with depletion of Cr, Cu, or Cr and Cu combined in BHE/cdb rats. Moreover, there were no known allergic reactions or infections in BHE/cdb rats throughout the study, so lymphocytes or neutrophils presumably would not be changed for extraneous reasons.

The results from the animal study suggest that the level of Cr and/or Cu depletion in the diet may not have been low enough to make alterations in the number of lymphocytes or neutrophils. Or the distribution of lymphocyte or neutrophils might not

be influenced by Cr and/or Cu depletion. The average Cu concentration was 0.9 mg Cu/kg diet and 5.0 mg Cu/kg diet in Cu depleted and adequate Cu diets, respectively. The average Cr concentration was 9.1 μg Cr/kg diet and 1007.2 μg Cr/kg diet in Cr depleted and adequate Cr diets, respectively.

In the present human study, the percentage of basophils was significantly increased with 12 weeks of Cu supplementation. However, the numbers of lymphocytes, neutrophils, or eosinophils were not significantly affected by 12 weeks of Cr and/or Cu supplementation. Similarly, the number of leukocytes, monocytes, neutrophils, or lymphocytes were not significantly affected by different levels of copper intake, 0.38, 0.66, or 2.38 mg/d, in young men (Kelley et al., 1995).

Presumably there were no infections among human subjects throughout the study because the health status of human subjects was checked at the screening test, baseline, and end of the study using health questionnaires. There were no subjects who reported having infectious disease or taking antibiotics throughout the study. About 20% of the subjects reported that they had allergies at some time before or during the study. However, the number of subjects with allergies was not high enough to make changes in the distribution of the eosinophils among subjects. In addition, the percentages of eosinophils in subjects with allergies were similar to those in subjects without allergies.

Moreover, there were three subjects with anemia, two subjects with hypertension, two subjects with heart diseases, and two subjects who had cancers in the past. These subjects were not excluded from the data analysis. It was assumed that there were no effects of these diseases on the measurement variables throughout the study. In addition, there were no subjects who reported taking additional Cr or Cu supplementation (not

supplemented by researchers) during the study. There were two subjects taking potassium, one subject taking vitamin E and Ca, one subject taking niacin, and one subject taking vitamin B_{12} and folic acid during the study. These subjects were from all treatment groups and were not excluded from the data analysis. The effects of these vitamin or mineral supplements on Cr and/or Cu supplementation as well as on analysis variables were not determined. However, a significant increase in basophils might support that there were unknown infections or allergic reactions in Cu supplemented groups. It was not clear that the increased basophils might be due to allergic conditions, infectious conditions, or 12 weeks of copper supplementation. However, the number of basophils at baseline (0.9%) and after 12 weeks of supplementation (1.1%) was within a normal or close to a normal range. Normal basophils are <1% in the blood, so this significant change in the basophils may not have any clinical importance.

The results from the human study indicate that the distribution of cells including lymphocytes, monocytes, neutrophils, and eosinophils might not be sensitive to Cr and/or Cu supplementation in hypercholesterolemic postmenopausal women. Or subjects may not have had a Cr or Cu deficiency before participating in the study, so the distribution of cells in the blood might not have been affected.

Interleukin 2

Reports from the literature indicate that production of IL-2 was decreased with Cu deficiency in experimental animals. Also, the addition of copper to PHA-treated splenocyte cultures from copper deficient rats increased IL-2 activity and DNA synthesis to control levels. The inadequate availability of copper within lymphocytes caused

decreased IL-2 secretion and resulted in impaired T-lymphocyte response to mitogen stimulation in the copper deficient rats (Failla and Hopkins, 1997, Bala and Failla, 1992).

The non-significant effect on IL-2 production with 12 weeks of Cu supplementation might be due to the Cu status of human subjects in the present study. The average dietary Cu intake of human subjects was 1.0 mg Cu/d in this study. This dietary Cu intake was higher than those in average American women aged 60-65 years (0.86 mg/d, Pennington and Young, 1991), so it was assumed that these subjects did not have Cu deficiency. The production of IL-2 was significantly increased with Cu supplementation in Cu deficient animals, so if subjects in this study were Cu deficient, IL-2 production would have been significantly increased with Cu supplementation.

The concentration of ceruloplasmin in serum and RBC Cu, Zn SOD activity were measured to determine Cu status in these subjects. There were non-significant differences in these parameters among supplementation groups. However, ceruloplasmin was higher than the normal value at baseline, but it was decreased to about 50% of the baseline after 12 weeks of supplementation in all groups. These decreases in serum ceruloplasmin might be due to analytical errors rather than due to the effect of supplementation. The ceruloplasmin assay measures the *p*-phenlyenediamine oxidation rate. The oxidation rate is measured spectrophotometrically as the rate of purple color development. The ceruloplasmin assay needs to be done in the dark because the light increases *p*-phenylenediamine oxidation rate (Henry et al., 1974). The ceruloplasmin assay in the current study was conducted in the light, so the light might have increased *p*-phenylenediamine oxidation rate. The increased *p*-phenylenediamine oxidation rate might have contributed to analytical errors in the current study. RBC Cu, Zn SOD

activity was lower than the normal value in these subjects at baseline and it was decreased after 12 weeks of supplementation. Also, these decreases in RBC Cu, Zn SOD activity might be due to analytical errors during analysis rather than the effects of supplementation. The procedure from the RANSOD kit recommended to use fresh red blood cell lysates for RBC Cu, Zn SOD measurement. However, RBC lysates were kept frozen for a month at –70 °C, so it might have caused low SOD activity in the current study. Therefore, Cu status could not be determined by ceruloplasmin and RBC Cu, Zn SOD activity in these subjects.

Superoxide Dismutase and Ceruloplasmin

In the liver Cu, Zn SOD assay, manganese containing SOD was inactivated with chloroform and ethanol treatment of tissue homogenate without affecting copper containing SOD according to the DiSilvestro and Marten's method (1990).

In a study by DiSilvestro and Marten (1990), the liver Cu, Zn SOD activity was decreased with copper deficiency (4.1±0.6 U/mg liver, <0.5 mg Cu/kg diet) compared to liver Cu, Zn SOD activity from an adequate Cu (15.5±1.1 U/mg liver, 6 mg Cu/kg diet) or a marginal Cu (10.0±0.5 U/mg liver, 2.5 mg Cu/kg diet) diet group. However, in the current animal study, there was no change in liver SOD activity (3.7±0.4 U/mg liver from both Cu depletion and adequate Cu diet groups) in BHE/cdb rats. The BHE/cdb rats did show Cu depletion with the low copper in the diet. The Cu concentration of liver and kidney was significantly lower in rats fed a Cu depletion diet. The average Cu concentration was 0.9 mg Cu/kg diet and 5.0 mg Cu/kg diet in Cu depletion and adequate Cu diets, respectively.

Liver Cu, Zn SOD activity in both groups in this study was similar to a copper deficient group studied by DiSilvestro and Marten (1990). Perhaps this value was not high enough to allow changes in activity based on adequate Cu or depleted Cu treatments. In DiSilvestro and Marten's study (1990), liver samples were kept at -10 °C up to 2 weeks after sample collection, then liver samples were analyzed for SOD activity. However, in the present study, liver samples were kept at –20 °C for 2 years before measuring SOD activity. These might have caused lower Cu, Zn SOD activity in the current animal study.

In the human study, serum ceruloplasmin or RBC Cu, Zn SOD activity were not significantly affected by 12 weeks of Cr and/or Cu supplementation. Serum ceruloplasmin values or RBC Cu, Zn SOD activity were not in agreement with those obtained by Smith and colleagues' study (1994) or Turnlund and colleagues' studies (1990, 1994). The plasma copper concentration, plasma ceruloplasmin activity, and RBC superoxide dismutase activity were low in copper deficient adults (Smith et al., 1994). The plasma copper, ceruloplasmin, and superoxide dismutase activity were reduced in young men with 0.4 mg Cu/d diets, but intakes of 0.8, 3.0, or 7.5 mg Cu/d did not affect these measures of copper status (Turnlund et al., 1990, 1994). RBC Cu, Zn SOD activity was higher in young men (Turnlund et al., 1990) with 0.4 mg Cu/d diets and supplementation of 0.8 mg Cu/d (2600±200 U/g Hgb, mean±pooled SEM) or of 7.5 mg Cu/d (2500±200 U/g Hgb, mean±pooled SEM) compared to hypercholesterolemic postmenopausal women (831±30 U/g Hgb without Cu supplementation and 851±46 U/g Hgb with Cu supplementation) in this study. Also, in a study of Milne and Jonson (1993), female subjects age 50-59 years had higher RBC Cu, Zn SOD activity (2600-3500 U/g Hgb) compared to those in the current study. In addition, it is known that there are

no sex differences in RBC Cu, Zn SOD activities. In Milne and Jonson's study (1993), RBC Cu, Zn SOD was not significantly different between male and female subjects. There were no premenopausal control subjects, so reasons for lower RBC Cu, Zn SOD activity in subjects in the present study compared to young men in Turnlund and colleagues' study (1990) could not be determined.

The average dietary Cu intake of our subjects was 1.0 mg/d and the Cu supplemented group received an additional Cu supplement of 3.0 mg/d. These subjects did not have any signs of Cu deficiency initially and the dietary Cu intake was higher than average dietary Cu intake of females in the U. S. (Pennington and Young, 1991). It was presumed that serum ceruloplasmin or RBC Cu, Zn SOD activity was not significantly affected by Cr and/or Cu supplementation due to high dietary Cu intake of the subjects. To investigate Cr and/or Cu supplementation effects on serum ceruloplasmin or RBC SOD activity, a Cu deficiency may need to be induced to observe significant effects.

The RBC Cu, Zn SOD activity was decreased compared to baseline by 12 weeks of supplementation with Cr, Cu, or both Cr and Cu. However, RBC Cu, Zn SOD activity was lower than a normal reference range from the RANSOD kit (Randox Laboratories Ltd., Co. Antrim, United Kingdom). The normal reference range of RBC Cu, Zn SOD is 1102-1601 U/g Hgb, but it was 486-1643 U/g Hgb at baseline and was 565-1145 U/g Hgb after 12 weeks of supplementation in the present study. On the other hand, serum ceruloplasmin was higher than normal range at baseline and it was within a normal range at the end of the study. The normal range of serum ceruloplasmin is 20-40 mg/dl (Sunderman and Nomoto, 1970). As mentioned above in the IL-2 discussion section, decreased serum ceruloplasmin or RBC Cu, Zn SOD after 12 weeks of supplementation

compared to baseline might be due to experimental errors in the assays. The ceruloplasmin measurement assay should have conducted in the dark to prevent increase in oxidation rate of *p*-phenylenediamine. However, ceruloplasmin assays both at baseline and after 12 weeks of supplementation were conducted in the light, so decreased ceruloplasmin values after 12 weeks of supplementation (50% of the baseline) in all groups can not be explained completely in the current study. The RBC Cu, Zn SOD activity measurement assay should have carried out using unfrozen RBC samples instead of frozen ones. The measurement of ceruloplasmin in the dark and use of unfrozen RBC samples would have minimized analytical errors in the present study.

Tissue Mineral Concentration

Alterations in trace mineral metabolism are one of the characteristics of diabetic animals. Streptozotocin induced or spontaneously diabetic rats had accumulations of Cu and Zn in the liver and kidney (Failla and Kiser, 1981, 1983, Failla and Gardell, 1985, Raz and Havivi, 1988). BHE/cdb rats are diabetes prone animals, so it was assumed that the concentration of Cu in the liver and kidney would be increased. The range of mean Cu concentration in the kidney was 0.174±0.004 to 0.264±0.015 μmol/g dry weight and in the liver was 0.104±0.006 to 0.136±0.008 μmol/g dry weight in the BHE/cdb rats. These values are lower than those values in other diabetic animals. In Failla and Kiser's study (1981), Cu concentration of liver and kidney was 0.56±0.06 and 2.22±0.12 μmol/g dry weight, respectively in streptozotocin induced diabetic male Sprague-Dawley rats fed purified diets containing 15.6 μg Cu/g for seven days. The Cu concentration in the diet is about three times higher than that in BHE/cdb rat diet (0.9 mg Cu/kg diet in Cu depletion

diets and 5.0 mg Cu/kg diet in adequate Cu diets) in the present study. This high concentration of Cu in the diet may have caused higher Cu concentration in the liver and kidney in Failla and Kiser's study (1981) compared to those in BHE/cdb rats in the present study. Also, Cu concentration in the liver in BHE/cdb rats was lower than that in diabetic BB Wistar rats (Failla and Gardell, 1985). Cu concentration of the liver in BB Wistar rats was 0.25±0.01 μmol/g dry weight and this value was higher than nondiabetic control rats (0.22±0.01 μmol/g). The Cu concentrations in the diet and duration of feeding were not explained in the original article (Failla and Gardell, 1985). In addition, Cu concentrations of liver and kidney in the BHE/cdb rats were lower than those concentrations in Raz and Havivi's study (1988). Male Sabra rats from the Hebrew University Breeding colony were fed diets containing 7.9 mg Cu/kg diet for 180 days. Cu concentrations of liver in the streptozotocin induced diabetic rats (0.25±0.04 μmol Cu/g dry weight) were significantly increased compared to control rats (0.16±0.05 μmol Cu/g dry weight). Cu concentrations of kidney in the streptozotocin induced diabetic rats (0.79±0.20 μmol Cu/g dry weight) were similar to control rats (0.71±0.23 μmol Cu/g dry weight). However, in a Raz and Havivi's study (1988), Cu concentration in the diet was higher than that in the present animal study (0.9 mg Cu/kg diet in Cu depletion diets and 5.0 mg Cu/kg diet in adequate Cu diets). This higher concentration of Cu in the diet might have caused higher Cu concentration of kidney.

Because Cr was not detectable, Cr depletion status of rats due to feeding low Cr diets was not measurable in the present animal study. Cr concentrations in the liver, kidney, and spleen were not detectable because samples were diluted too much. These samples were very hard to dissolve into clear solutions prior to the analysis, so the

volume of the acids and deionized distilled water was increased to obtain clear solutions. This increased sample dilution might have caused undetectable Cr concentrations in liver, kidney, and spleen samples. In addition, deionized distilled water from our laboratory was found to be Cr contaminated, and resulted in Cr contamination of the sample blank. Cr concentration in the tibia was not measurable due to the interference of Ca in tibia samples.

CHAPTER V

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

These studies were conducted to measure effects of Cr, Cu, or combined Cr and Cu depletion or supplementation on immune function in diabetes prone BHE/cdb rats or hypercholesterolemic postmenopausal women, respectively. A 2 X 2 factorial experimental design was used for both studies. Forty BHE/cdb rats were fed with a Cr and/or Cu depleted (9.1 μg Cr/kg diet Cr and/or 0.9 mg Cu/kg diet) and/or an adequate Cr and/or Cu (1007.2 μg Cr/kg diet and/or 5.0 mg Cu/kg diet) diet for 21 weeks. There were no significant differences in weight gain, tissue weight, fasting serum glucose, insulin, liver Cu, Zn SOD activity, corticosterone, the number of lymphocytes and neutrophils, or lymphocyte proliferation with PHA-L 10 μg/ml, ConA 25 μg/ml, or LPS 10 μg/ml stimulation among treatment groups in BHE/cdb rats. However, when tissue weights were expressed as percent of body weight, liver, kidney, and spleen were significantly increased with Cr depletion diets in BHE/cdb rats. Moreover, a significant interactive effect of Cr and Cu was observed in lymphocyte proliferation with PHA-L 25 μg/ml stimulation ($p<0.01$). Cr depletion in the diet significantly affected Cu concentration in the kidney. Cu depletion in the diet significantly affected Cu, Fe, Zn, and Mg

concentrations in the liver. Also, Cr and Cu depletion in the diet significantly affected Mg concentration in the tibia.

Forty human subjects received 0.394 g lactose, 200 μg Cr, 3.0 mg Cu, or 200 μg Cr and 3.0 mg Cu/d for 12 weeks. Twelve weeks of Cr, Cu, or Cr and Cu combined supplementation did not significantly affect subjects' BMI, cortisol, IL-2 production, RBC Cu, Zn SOD activity, ceruloplasmin, the number of lymphocytes, monocytes, neutrophils, and eosinophils, or lymphocyte proliferation with PHA-L stimulation. However, 12 weeks of Cu supplementation caused a significant increase in the number of basophils and significant decreases in fasting serum glucose and insulin in human subjects. Lymphocyte proliferation in response to ConA 50 μg/ml stimulation was significantly increased with Cr supplementation in Cr supplemented groups, when Cu was not supplemented. On the other hand, lymphocyte proliferation was significantly decreased with Cu supplementation in the combined Cr and Cu supplementation group or in the Cu supplemented group with ConA 50 μg/ml or ConA 100 μg/ml stimulation. These results support potential beneficial effects of diets adequate in chromium and potential suppressive effects of diets high in copper on immune function in hypercholesterolemic postmenopausal women.

CONCULSIONS

Eight hypotheses were developed for this study. The first hypothesis was that there will be no statistically significant effects of Cr and/or Cu depletion on T and/or B cell proliferation in BHE/cdb rats. The first hypothesis was rejected because T lymphocyte proliferation was significantly increased with Cr or Cu supplementation in BHE/cdb rats.

The second hypothesis was that there will be no statistically significant effects of Cr and/or Cu depletion on the number of lymphocytes or neutrophils in BHE/cdb rats. The second hypothesis was accepted because the number of lymphocytes or neutrophils was similar among treatment groups in BHE/cdb rats.

The third hypothesis was that there will be no statistically significant effects of Cr and/or Cu depletion on superoxide dismutase activity in BHE/cdb rats. The third hypothesis was accepted because the activity of superoxide dismutase was similar among treatment groups in BHE/cdb rats.

The fourth hypothesis was that there will be no statistically significant effects of Cr and/or Cu depletion on serum glucose, insulin, or corticosterone in BHE/cdb rats. The fourth hypothesis was accepted because serum glucose, insulin, or corticosterone was not significantly affected by Cr and/or Cu depletion in BHE/cdb rats.

The fifth hypothesis was that there will be no statistically significant effects of Cr and/or Cu supplementation on T cell proliferation in hypercholesterolemic postmenopausal women. The fifth hypothesis was rejected because a significant interactive effect of Cr and Cu on T cell proliferation and also a significant decrease in T

cell proliferation with 12 weeks of Cu supplementation were observed in human subjects.

The sixth hypothesis was that there will be no statistically significant effects of Cr and/or Cu supplementation on the number of lymphocytes, neutrophils, monocytes, eosinophils, or basophils in hypercholesterolemic postmenopausal women. The sixth hypothesis was rejected because the number of basophils was significantly increased with 12 weeks of Cu supplementation in human subjects.

The seventh hypothesis was that there will be no statistically significant effects of Cr and/or Cu supplementation on ceruloplasmin or superoxide dismutase activity in hypercholesterolemic postmenopausal women. The seventh hypothesis was accepted because there were no significant differences in ceruloplasmin or SOD activity with Cr and/or Cu supplementation in human subjects. However, this hypothesis needs to be qualified because of possible problems in the SOD and ceruloplasmin measurement methods.

The eighth hypothesis was that there will be no statistically significant effects of Cr and/or Cu supplementation on IL-2 production from mitogen stimulated blood cell cultures or serum cortisol in hypercholesterolemic postmenopausal women. The eighth hypothesis was accepted because the production of IL-2 from mitogen stimulated blood cell cultures or serum cortisol was similar among treatment groups in hypercholesterolemic postmenopausal women.

RECOMMENDATIONS

In the present animal study, Cr, Cu, or Cr and Cu combined depletion diets were based on the AIN-93 diet. Chromium was not added to the diet for Cr depletion and 10% of the AIN-93 Cu level was added to the diet for Cu depletion. BHE/cdb rats are prone to develop glucose intolerance or diabetes when fed high fat diets. However, the BHE/cdb rats did not develop glucose intolerance or diabetes throughout the study. The hypotheses of this study involved measuring effects of Cr, Cu, or Cr and Cu combined depletion on immune function as well as carbohydrate metabolism in diabetic or glucose intolerant animals. Since animals did not develop glucose intolerance, changes in immune function including lymphocyte proliferation or differential cell profiles with Cr, Cu, or Cr and Cu combined depletion were not observed. To induce glucose intolerance, BHE/cdb rats need to be fed with high fat diets. In the current study, these rats were fed high fat diets only after reaching the growth plateau. It would be appropriate to feed BHE/cdb rats with high fat diets from the weanling age to induce glucose intolerance. Soybean oil was provided as a fat source in the diet. Soybean oil (14.4%) has a lower content of saturated fatty acids compared to menhaden oil (33.6%). If saturated fatty acid content was high in the diet, it might have affected the development of diabetes in BHE/cdb rats. Or if streptozotocin induced diabetic animals or other diabetes prone animal models, for example, BB rats or db/db rats, were used in this study, significant effects of Cr, Cu, or Cr and Cu combined depletion on immune function as well as carbohydrate metabolism might have been observed.

Trace mineral concentrations of tissues were measured using wet and dry ashed liver, spleen, kidney, and tibia samples. Tissues were supposed to be dissolved into clear

solutions for the mineral analysis. Liver and tibia samples were dissolved into clear solutions prior to mineral analysis. However, kidney and spleen samples were not completely dissolved into clear solutions prior to mineral analysis. Tissue samples were kept frozen for two years after collection due to laboratory renovation. However, the length of sample storage might not have caused problems in dissolving kidney and spleen samples. The length of sample ashing might have affected the solubilization of tissues. The average cycle of wet and dry ashing was three to four cycles for tibia and liver samples and was six to seven cycles for kidney and spleen samples. Moreover, the size of samples (~0.2-0.5 g) might have affected the length of ashing as well as solubilization. The big sample size might have caused samples to bake in the bottom of the ashing tubes and to be harder to ash and dissolve into solutions. Therefore, less than 0.1 g of wet sample size would be appropriate for mineral analysis.

In the present human study, the supplementation level of Cr, Cu, or combined Cr and Cu was based on ESADDIs. Dietary intake of trace minerals is low in the elderly population. Thus, it was hypothesized that the supplementation of Cr, Cu, or combined Cr and Cu at the upper level of the ESADDIs would affect immune responses. However, there was not much effect of either Cr or Cu supplementation on the immune function in hypercholesterolemic postmenopausal women. Subjects with diabetes or glucose intolerance or poor compliance (60 capsules, or 36% of the total leftover during the 12 weeks) were excluded from the data analysis. Subjects in the current study were well nourished and may not have been Cr or Cu depleted before entering the study. Subjects with a Cr or Cu deficiency might have better responses to Cr or Cu supplementation.

Five diabetic subjects were excluded from the data analysis in the present human study. To avoid exclusion of subjects from the data analysis, more detailed subject inclusion criteria and health background checks would be necessary at the beginning of the study. Also, it was very hard to recruit subjects for this study because most postmenopausal women identified as postential subjects in Stillwater were already on estrogen replacement therapy. Appropriate knowledge about demographic characteristics of the town or surrounding areas before starting the study would be helpful for easier or better recruitment of the subjects.

There were large standard errors in some of the variables from the data analysis. These might be due to experimental assay errors by the researchers or to problems in the methods. For future studies, a proper storage of samples according to assay methods would reduce or minimize the experimental errors and would result in smaller standard errors. In addition, all persons involved with sample collection, storage, and assay methods need to practice the assays before starting the study.

Predominate findings of these studies were the significant interactive effects of Cr and Cu on immune response. Cr had a beneficial effect while Cu had a suppressive effect on immune response in hypercholesterolemic postmenopausal women. However, interactive effects of Cr and Cu on immune function need to be further investigated.

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APPENDICES

APPENDIX A

DIET COMPOSITION

Components	Growth diet		Maintenance diet	
	g/kg diet	Percentage	g/kg diet	Percentage
Casein	200	20	140	14
Cornstarch	150	15	150	15
Celufil	50	5	50	5
Dextrose	379.48	37.95	289.5	28.95
Sucrose	100	10	100	10
Vitamin mix*	10	1	10	1
Soybean oil	70	7	220	22
Choline	2.5	0.25	2.5	0.25
L-cystine	3	0.3	3	0.3
Mineral mix**	35	3.5	35	3.5
Vitamin E	0.6	0.06	0.6	0.06

*Vitamin mix formulated to meet the AIN-93G recommendations for growing rats except for a five fold increase in Vitamin E, or 0.6 g/kg diet mix.

**Mineral mix formulated to meet the AIN-93G recommendations for growing rats except for inadequate levels of chromium (0 g/kg added to mineral mix) and copper (0.03 g/kg added to mineral mix) as specified by the experimental design.

APPENDIX B

MINERAL CONCENTRATIONS OF THE DIET[1]

Group	Cr (μg/kg)	Cu (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Ca (mg/kg)	Mg (mg/kg)
Growth Diet						
-Cr-Cu	8.2	1.3	42	56	4765	558
+Cr-Cu	1160.4	1.2	44	55	4643	545
-Cr+Cu	0.0	6.4	48	62	4717	540
+Cr+Cu	906.6	6.4	46	62	4711	548
Maintenance Diet						
-Cr-Cu	11.6	1.1	46	53	4782	525
+Cr-Cu	882.0	0.0	46	48	4116	479
-Cr+Cu	16.4	3.5	45	50	4606	491
+Cr+Cu	1079.8	3.6	49	56	4579	526

[1]-Cr-Cu: chromium and copper depletion; +Cr-Cu: adequate chromium and copper depletion;-Cr+Cu: chromium depletion and adequate copper; +Cr+Cu: adequate chromium and adequate copper

APPENDIX C

DIET PREPARATION

Instructions for mixing:

Step 1: Put on clean mineral free gloves.
Step 2: In the small mixing bowl, combine the following ingredients one at a time:
1) Vitamin mix
2) L-Cysteine
3) Choline
4) Casein (~ 1 cup)
5) Dextrose (~ 3 cups)
6) Sucrose

Step 3: Mix each ingredient thoroughly as they are added using your hand with mineral free gloves (make sure all small clumps are broken).
Step 4: Use the large mixing bowl to combine the following ingredients. Again, add each ingredient one at a time and use your hands to combine and break up any clumps.
1) Remainder of Dextrose
2) Remainder of Casein
3) Celufil
4) Cornstarch

Step 5: Add around 2 Tbsp. of oil and mix at a setting of "1" on the large mixer for 10-15 minutes (or until well mixed). Add the remainder of the oil and continue mixing.
Step 6: When the six ingredients in the small mixing bowl have been thoroughly combined by hand, add about ½ Tbsp. of oil and mix at low speed (setting of "1") for 5-10 minutes or until well mixed. Next, add the Mineral Mix and stir with a plastic spoon. Continue mixing at a setting of "1" until the ingredients are completely mixed.
Step 7: When the ingredients in each mixer appear to be ready, combine all ingredients into the large mixing bowl and set speed at "1.5" and mix for approximately 20 minutes.
Step 8: Place diet in a plastic bag, label it accordingly, and store it in the refrigerator.

APPENDIX D

LEAN BODY MASS (LBM) AND MOISTURE LOSS OF LIVER AND TIBIA[1,2]

Group	LBM		Liver moisture loss		Tibia moisture loss	
	(g)	(% Final wt.)	(g)	(% loss)	(g)	(% loss)
-Cr-Cu	310±8	63±1	0.10±0.01	58.3±1.2	0.26±0.02	29.3±0.7
+Cr-Cu	388±11	64±2	0.13±0.02	56.4±3.7	0.26±0.01	29.6±0.8
-Cr+Cu	330±13	66±1	0.13±0.01	61.1±0.3	0.26±0.02	29.4±1.3
+Cr+Cu	341±11	64±2	0.12±0.01	63.8±3.9	0.25±0.02	28.9±1.2
-Cr	321±8	64±1	0.11±0.01	59.7±0.7	0.26±0.01	29.3±0.7
+Cr	340±7	64±1	0.12±0.01	59.9±2.8	0.25±0.01	29.2±0.7
-Cu	324±8	63±1	0.12±0.01	57.4±1.9	0.26±0.01	29.5±0.5
+Cu	335±8	65±1	0.12±0.01	62.4±1.8	0.25±0.01	29.2±0.9
Factors			P Values			
Cr	0.09	0.85	0.24	0.89	0.62	0.94
Cu	0.30	0.36	0.48	0.07	0.64	0.77
Cr x Cu	0.46	0.44	0.06	0.41	0.59	0.67

[1]Values are mean±SEM, LBM n=6-7, moisture loss n=9-10

[2]-Cr-Cu: chromium and copper depletion; +Cr-Cu: adequate chromium and copper depletion;-Cr+Cu: chromium depletion and adequate copper; +Cr+Cu: adequate chromium and adequate copper

APPENDIX E

GLUCOSE TOLERANCE TESTS AND FRUCTOSAMINE[1,2]

Group	Test 1 Glucose 0 hr (mg/dl)	Test 1 Glucose 2 hr (mg/dl)	Test 1 Change[3] (mg/dl)	Test 2 Glucose 2 hr (mg/dl)	Test 3 Glucose 2 hr (mg/dl)	Fructosamine (µmol/l)
-Cr-Cu	72±2	128±13	56±12	121±9	115±4	128±4
+Cr-Cu	78±2	137±13	59±12	132±5	119±3	123±4
-Cr+Cu	82±3	133±12	51±14	113±6	120±7	117±5
+Cr+Cu	77±3	136±10	58±11	119±7	128±7	129±4
-Cr	77±2	131±9	54±9	117±5	117±4	123±3
+Cr	78±2	137±8	59±8	126±4	123±4	126±3
-Cu	75±2	133±9	58±8	126±5	117±2	126±3
+Cu	80±2	134±8	55±9	116±5	124±5	123±4
Factors						
Cr	0.78	0.62	0.67	0.25	0.24	0.40
Cu	0.08	0.91	0.80	0.18	0.21	0.58
Cr x Cu	<0.05	0.81	0.85	0.74	0.73	0.06

[1]Values are mean±SEM, test 1 (at 12th week) n=9-10, test 2 (at 18th week), and test 3 (at 21st week) n=5-6

[2]-Cr-Cu: chromium and copper depletion; +Cr-Cu: adequate chromium and copper depletion; -Cr+Cu: chromium depletion and adequate copper; +Cr+Cu: adequate chromium and adequate copper

[3]Values are the difference between glucose 2 hour and 0 hour for test 1

APPENDIX F

SPLENOCYTE PROLIFERATION ASSAY

Complete RPMI-1640 (CRPMI) Culture Medium:
Add 1.0 mM/ml nonessential amino acid, 1.0 mM/ml sodium pyruvate, 100 u/ml penicillin-streptomycin solution, 10 % heat inactivated fetal bovine serum, 200 mM/ml L-glutamine, and 5 $x10^{-5}$M/ml 2-mercaptoethanol.

PHA-L:
Final concentrations of 10 µg/ml and 25 µg/ml PHA-L were used.

LPS:
Final concentration of 10 µg/ml LPS and dextran sulfate mixture was used.

ConA:
Final concentration of 10 µg/ml ConA was used.

Tris-Ammonium chloride:
Final concentration of 0.9 x 0.16 M, pH 7.2 was used.

Cell Suspension:
1. Mince spleen with scissors to release cells.
2. Pipette the cell suspension into clean tubes without clumps.
3. Centrifuge the cell suspension for 10 minutes at 2000 rpm (1400 x g) at 4°C.
4. After centrifugation, pour off supernatant, and add 5 ml of tris buffered ammonium chloride solution per spleen to lyse red blood cells.
5. Incubate about 5 minutes at room temperature.
6. After incubation, wash cells once in CRPMI medium, and resuspend cells in CRPMI medium.

Cell count:
1. Count cells using a Trypan Blue solution under the microscope.
2. Make the final cell suspension of $2x10^6$ lymphocyte/ml by adding CRPMI media.

Procedure:
1. Add 100 µl cell mixture, 100 µl CRPMI alone (unstimulated), and 100 µl PHA-L, 200 µl ConA, or 100 µl LPS and dextran sulfate mixture to each set of triplicate culture wells in 96-well tissue culture plates.
2. Incubate cell cultures in a 5% CO_2 incubator at 37°C for 72 hours.
3. At 4 hours before the termination of incubation, add 1.0 µCi methyl-^{3}H-thymidine to each culture well.
4. After the termination of incubation, harvest cell cultures onto fiberglass filters.
5. Count on a liquid scintillation counter using a single-label count per minute (cpm) program.

Stimulation index: cpm of stimulated cell cultures/cpm of unstimulated cell cultures.

APPENDIX G

WHOLE BLOOD CELL CULTURE PROLIFERATION ASSAY

Complete RPMI-1640 (CRPMI) culture medium:
Add 2.0 mmol/l L-glutamine, 100,000 U/l penicillin, and 100 mg/l streptomycin to RPMI-1640 culture medium.

PHA-L:
Final concentrations of 10 μg/ml, 20 μg/ml, 40 μg/ml, and 80 μg/ml PHA-L were used.

ConA:
Final concentrations of 12.5 μg/ml, 25 μg/ml, 50 μg/ml, and 100 μg/ml ConA were used.

Procedure:

1. Collect fasting blood in a heparin coated syringe, keep blood samples at room temperature, and process within 2 hours of the first blood draw.
2. Dilute 400 μl heparinized blood with 1200 μl CRPMI in 4.0 ml polystyrene tubes.
3. Add 50 μl diluted blood, 50 μl CRPMI alone (unstimulated), and 50 μl PHA-L or 50 μl ConA to each set of triplicate culture wells in 96-well tissue culture plates. Then add 100 μl CRPMI-1640 to each culture well.
4. Incubate cell cultures in a 5% CO_2 incubator at 37°C for 96 hours.
5. At 18 hours before the termination of incubation, add 1.0 μCi methyl-^{3}H-thymidine to each culture well.
6. After the termination of incubation, harvest cell cultures onto fiberglass filters
7. Count on a liquid scintillation counter using a single-label count per minute (cpm) program.

Stimulation index: cpm of stimulated cell cultures/cpm of unstimulated cell cultures.

APPENDIX H

CERULOPLASMIN ASSAY

Use serum samples.

Acetate Buffer, 0.1 M/L, pH 5.45:
1. 0.2 M/L acetic acid: dilute 0.57 ml glacial acid with distilled water to 50 ml.
2. 0.2 M/L sodium acetate: 2.72 g $CH_3COONa3H_2O$ dilute to 100 ml with distilled water.
3. Add 86.0 ml of sodium acetate solution 14.0 ml of acetic acid solution to 80.0 ml of distilled water, then warm to 37°C in water bath.
4. Adjust pH to 5.45 with sodium acetate or acetic acid.
5. Dilute to 200 ml with distilled water.

Sodium Azide, 1.5 M/L:
Dissolve 2.43 g of Sodium Azide in 25 ml of distilled water and store in the refrigerator.

1 N NaOH;
Dissolve 20 g of NaOH in 500 ml distilled water.

Buffered *p*-phenylenediamine(PPD) solution, 27.6 mM/L:
1. Dissolve 0.25 mg PPD in 37.5 ml acetate buffer (warmed at 37°C in water bath).
2. Adjust the pH to 5.45 by adding 1 N NaOH dropwise (approximately 0.1 ml required).
3. Add acetate buffer to final volume of 50 ml.
4. Prepare this reagent 2 hours before use and keep it in the dark.

Procedure:
Read absorbance of blank (at 10 min.) and of test (at 30 min.) at 530 nm using COBAS FARA autoanalyzer.

Calculation:
Ceruloplasmin = 87.5 (Abs test-Abs blank)

APPENDIX I

OKLAHOMA STATE UNIVERSITY
INSTITUTIONAL REVIEW BOARD
HUMAN SUBJECTS REVIEW

Date: 08-28-96 **IRB#:** HE-97-001

Proposal Title: EFFECTS AND INTERACTIONS OF CHROMIUM AND COPPER ON LIPID METABOLISM IN HYPERCHOLESTEROLEMIC POST MENOPAUSAL WOMEN

Principal Investigator(s): Janice R. Hermann, Andrea B. Arquitt, Barbara J. Stoecker

Reviewed and Processed as: Expedited

Approval Status Recommended by Reviewer(s): Approved

ALL APPROVALS MAY BE SUBJECT TO REVIEW BY FULL INSTITUTIONAL REVIEW BOARD AT NEXT MEETING.
APPROVAL STATUS PERIOD VALID FOR ONE CALENDAR YEAR AFTER WHICH A CONTINUATION OR RENEWAL REQUEST IS REQUIRED TO BE SUBMITTED FOR BOARD APPROVAL.
ANY MODIFICATIONS TO APPROVED PROJECT MUST ALSO BE SUBMITTED FOR APPROVAL.

Comments, Modifications/Conditions for Approval or Reasons for Deferral or Disapproval are as follows:

Signature: _____ Date: August 28, 1996
Chair of Institutional Review Board

APPENDIX J

HIGH BLOOD CHOLESTEROL???

Female Participants Wanted

Did you know that the risk of heart disease increases for women after menopause?

Would you like to know if adequate chromium and copper lower blood cholesterol and heart disease risk among women after menopause?

Volunteers will receive $ 50 for participating in this study.

We have an opportunity for women if you are:

- Past menopause
- Have high blood cholesterol, over 200 mg/dl
- Not using estrogen replacement therapy
- Not using medication to lower blood cholesterol

This study will investigate the effects and interactions of chromium and copper supplementation on the risk of cardiovascular disease in postmenopausal women. The aims of this study are to determine if twelve weeks supplementation with chromium, copper or a combination of chromium and copper lowers blood cholesterol and other indicators of heart disease risk in post-menopausal women.

Volunteers will be given either a placebo, chromium, copper, or a combination of chromium and copper. Volunteers will be asked to take one supplement each morning and one each evening with meals for twelve weeks. The total daily intake is 3 mg copper and 200 μg/day chromium, which are the upper level of the safe and adequate intake range for each nutrient, as set by the National Research Council, Food and Nutrition Board.

Volunteers will participate in fasting blood and early morning urine collections. A light breakfast will be served after the fasting blood collection. Volunteers will also participate in routine measurements of wrist, waist and hip circumferences, skin fold, height and weight. Volunteers will be asked to keep diet records before blood collections.

This study has been approved by the Institutional Review Board for Protection of Human Subjects at Oklahoma State University and is funded by the Oklahoma Center for the Advancement of Science and Technology.

Sound Like Fun??? If you are interested or for further information please contact:

Janice R. Hermann, Ph.D, RD/LD
Andrea B. Arquitt, Ph.D, RD/LD
Department of Nutritional Sciences
Oklahoma State University
Stillwater, Oklahoma 74078
(405) 744-6824

APPENDIX K

Individual Consent to Participate in Research

Women's Cholesterol Study: Effect and interactions of chromium and copper on lipid metabolism in hypercholesterolemic post menopausal women

I,_____, voluntarily agree to participate in the above titled research which is sponsored by Agriculture Experiment Station, College of Human Environmental Sciences at Oklahoma State University.

I understand that:

(1) the purpose of the study is to measure the effects of mineral supplementation on plasma lipids and trace mineral status in post menopausal women;

(2) I will receive supplement containing ONE of the following:

(a) 0.25 mg lactose as a placebo
(b) 1.5 mg copper
(c) 100 μg chromium
(d) 1.5 mg copper plus 100 μg chromium

(3) I will take one supplement with each morning and evening meal for 12 weeks;

(4) I will not take any new vitamin or mineral supplement other than those that are part of the study;

(5) a phlebotomist will draw fasting blood samples of 30 ml (about 6 teaspoons) by venipuncture at the beginning and the end of the study, and that slight bruising or discomfort may result from the venipuncture;

(6) my blood will only be used for the study protocol, and any remaining blood tissue will be discarded and no further tests will be run;

(7) a urine sample will be collected at the beginning and the end of the study;

(8) routine data will be collected or measured for age, height, body weight, wrist, waist and hip circumferences at the beginning and end of the study;

(9) I will complete a Health Questionnaire concerning health conditions, medication use, vitamin and mineral supplement use and exercise practices at the beginning of the study; and a follow-up health and exercise questionnaire after each month of supplementation;

(10) I will complete a 7 day food frequency questionnaire at the beginning and end of supplementation;

(11) as a reward for participation and as an incentive to complete the study, I will receive $25 at both the beginning and end blood collections;

(12) all records are confidential and that my name will not be associated with any reports or data records at the end of the study;

(13) participation is voluntary and that I have the right to withdraw from the study at any time by contacting the principal investigators;

(14) I will withdraw from the project if a need to begin taking medication for my health during the study;

(15) this research is beneficial to the public in that the risk of cardiovascular disease increase among women after menopause;

(16) I may contact Dr. Janice Hermann, Dr. Andrea Arquitt, or Dr. Barbara Stoecker at (405) 744-5040 should I wish further information. I may also contact the office of University Research Services, 001 Life Sciences East, Oklahoma State University, Stillwater, OK 74078 telephone (405) 744-5700.

I have read and fully understand the consent form. I sign it freely and voluntarily. A copy has been given to me.

Date_____ Time_____

I certify that I have personally explained all elements of this form to the subject before requesting the subject to sign it.

Signed_____
(project director or her authorized representative)

APPENDIX L

Health Questionnaire

1. Subject Number_____

2. Date of Birth_____

3. Do you have or have you had any of the following conditions?

Conditions	NO	YES	Specify
Allergies			
Anemia			
Sickle Cell Anemia			
Blood Clotting Disease			
Cancer			
Diabetes			
Heart Disease			
Intestinal Disorder			
Liver Disease			
Osteoporosis			

4. Do you Currently take any medications on a regular basis? No_____ Yes_____

Specify all medications taken on a regular basis:

Name_____ How often_____ per day Or_____ per week
Name_____ How often_____ per day Or_____ per week
Name_____ How often_____ per day Or_____ per week
Name_____ How often_____ per day Or_____ per week
Name_____ How often_____ per day Or_____ per week
Name_____ How often_____ per day Or_____ per week
Name_____ How often_____ per day Or_____ per week

5. What types of exercise do you do on a regular basis?

How many times a week do you exercise?_____
How many minutes would you estimate your exercise in a week?_____

6. Height_____cm Weight_____

APPENDIX M

FOOD FREQUENCY FORM

Code Number_____ Date_____

Vitamin and Mineral Supplement

1. Do you take any vitamin or mineral supplement(s)? Yes ____ No _____
2. If **Yes**, please, list all **names** of vitamin or mineral supplements, and **how often** do you take the supplement(s)?

Name _____ How often ____ per day Or ____ per week
Name _____ How often ____ per day Or ____ per week
Name _____ How often ____ per day Or ____ per week
Name _____ How often ____ per day Or ____ per week
Name _____ How often ____ per day Or ____ per week

Seven Day Food Frequency Questionnaire

This questionnaire asks you about your consumption of foods and beverages over the past week. The "How Often" columns are for day, week, or rarely/never. We want you to think back over the past week and tell us how many times (per day or per week) you consumed each item. A medium serving is in parentheses.

EXAMPLES:

Ate ½ grapefruit about twice last week.

Ate 1 large hamburger four times last week.

Drank 2 cups of whole milk each day.

Type of Food (Medium Serving)	**How Often**			**Size**		
	Day	**Week**	**Rarely/ Never**	**S**	**M**	**L**
Grapefruit (1/2)		2			X	
Hamburger, regular (1 patty, 3 oz)		4				X
Whole milk (1 cup, 8 oz)	2				X	

Type of Food (Medium Serving)	How Often			Size		
	Day	Week	Rarely/ Never	S	M	L
DAIRY FOODS						
Whole milk (1 cup, 8 oz)						
2% milk (1 cup, 8 oz)						
Skim milk (1 cup, 8 oz)						
Cream, whipped (1 Tbsp)						
Sour cream (1 Tbsp)						
Coffee cream (1 Tbsp)						
Ice cream (1/2 cup)						
Low fat ice cream (1/2 cup)						
Frozen yogurt (1/2 cup)						
Yogurt (1 cup)						
Low fat yogurt (1 cup)						
Cottage cheese (1/2 cup)						
Cream cheese (1 oz)						
Low fat cream cheese (1 oz)						
Other cheese (1 slice or 1 oz)						
Low fat cheese (1 slice or 1 oz)						
Margarine (1 tsp)						
Butter (1 tsp)						
Reduced fat margarine (1 tsp)						

Type of Food (Medium Serving)	How Often			Size		
	Day	Week	Rarely/ Never	S	M	L
FRUITS, FRUIT JUICES						
Raisins (1 oz or 1 sm box)						
Grapes (20)						
Prunes (½ cup)						
Bananas						
Cantaloupe (¼ melon)						
Watermelon (1 slice)						
Apples, apple sauce or pears (1 fresh, ½ cup)						
Apple juice (½ cup)						
Oranges						
Orange juice (½ cup)						
Grapefruit (½ cup)						
Grapefruit juice (½ cup)						
Other fruit juices (½ cup)						
Strawberries—fresh, frozen, or canned (½ cup)						
Blueberries—fresh, frozen, or canned (½ cup)						
Peaches (1 fresh, ½ cup canned)						
Apricots (1 fresh, ½ cup canned)						
Plums (1 fresh, ½ cup canned)						
Honeydew melon (¼ melon)						

Type of Food (Medium Serving)	How Often			Size		
	Day	Week	Rarely/ Never	S	M	L
VEGETABLES, VEGETABLE JUICE						
Tomatoes (1)						
Tomato juice (½ cup)						
Tomato sauce (½ cup)						
Spaghetti sauce (½ cup)						
Red chili sauce, taco sauce, or salsa (1 Tbsp)						
Tofu or soybeans (3-4 oz)						
String beans, green beans (½ cup)						
Broccoli (½ cup)						
Cabbage (½ cup)						
Cole slaw (½ cup)						
Cauliflower (½ cup)						
Brusssels sprouts (½ cup)						
Carrots, raw (½ carrot or 2-4 sticks)						
Carrots, cooked (½ cup)						
Corn (1 ear or ½ cup frozen or canned)						
Peas (½ cup fresh, frozen or canned)						
Lima beans (½ cup frozen, or canned)						
Mixed vegetables (½ cup)						
Beans or lentils, baked or dried (½ cup)						
Summer or yellow squash (½ cup)						
Winter squash (½ cup)						
Zucchini (½ cup)						
Yam or sweet potato (½ cup)						
Spinach (cooked ½ cup, raw 1 cup)						
Iceberg lettuce, romaine or leaf (1 cup)						
Celery (4" stick)						
Beets (½ cup)						
Alfalfa sprouts (½ cup)						
Kale, mustard, or chard greens (½ cup)						
Vegetable, vegetable beef, minestrone or tomato soup (1 cup)						

Type of Food (Medium Serving)	How Often			Size		
	Day	Week	Rarely/ Never	S	M	L
EGGS, MEAT, ECT.						
Eggs (2)						
Chicken or turkey, roasted or broiled with skin (3-4 oz)						
Chicken or turkey, roasted or broiled skinless (3-4 oz)						
Chicken, fried with skin (3-4 oz)						
Bacon (2 slices)						
Hot dogs (2)						
Low fat hot dogs (2)						
Sausage (2 patties or 2 links)						
Bologna (1 slice)						
Other processed luncheon meats (1 slice)						
Liver, chicken or beef (3-4 oz)						
Hamburger, regular (1 patty, 3-4 oz)						
Hamburger, lean(1 patty, 3-4 oz)						
Meat loaf (3-4 oz)						
Pork, chops, roasts (3-4 oz)						
Lamb (4-6 oz)						
Beef, roast, steak (3-4 oz)						
Beef stew with vegetables (1 cup)						
Ham (3-4 oz)						
Tuna fish (3-4 oz)						
Tuna salad (1/2 cup)						
Fish, baked or broiled (3-4 oz)						
Fish, fried or fish sandwich (3-4 oz)						
Shrimp, Lobster, Scallops						
Pizza (2 slices)						
Mixed dishes with cheese (1 cup)						
Lasagna or meat pasta dishes (1 cup)						

Type of Food (Medium Serving)	How Often			Size		
	Day	Week	Rarely/ Never	S	M	L
BREADS, CEREALS, STARCHES						
Cold breakfast cereal (1 cup)						
Cold breakfast cereal-fortified (1 cup)						
Cooked oatmeal (1 cup)						
Other cooked breakfast cereal (1 cup)						
White bread (1 slice)						
Pita bread (1 piece)						
Dark bread (1 slice)						
English muffin (1)						
Bagel (1)						
Dinner roll (1)						
Hamburger or hotdog bun (1)						
Muffin (1)						
Biscuit (1)						
Corn bread, corn muffin (1)						
Brown rice (1 cup)						
White rice (1 cup)						
Spaghetti noodles (1 cup)						
Macaroni noodles (1 cup)						
Other pasta noodles (1 cup)						
Bulgar, kasha, couscous (1 cup)						
Pancakes or waffles (2)						
Potatoes, french fries or fried (1/2 cup)						
Potatoes, baked or boiled (1)						
Mashed potatoes (1 cup)						
Potato chips or corn chips (small bag or 1 oz)						
Saltine crackers (5)						
Saltine crackers, low sodium (5)						
Saltine crackers, fat free (5)						
Other cracker (5)						
Other crackers, low fat (5)						

Type of Food (Medium Serving)	How Often			Size		
	Day	Week	Rarely/ Never	S	M	L
BEVERAGES						
Regular soft drink (1)						
Diet soft drink (1)						
Caffeine free soft drink (1)						
Caffeine free, Diet soft drink (1)						
Lemonade or other non-carbonated drink (1 glass, bottle, or can)						
Coffee (1 cup)						
Decaffeinated coffee (1 cup)						
Tea (1 cup)						
Herbal tea (1 cup)						
Beer (1 glass, bottle, or can)						
Red wine (4 oz glass)						
White wine (4 oz glass)						
Whiskey, gin, or other liquor (1 drink or shot)						

Type of Food (Medium Serving)	How Often			Size		
	Day	Week	Rarely/ Never	S	M	L
SWEETS, BAKED GOODS, MISC.						
Chocolate (1 small bars or 1 oz)						
Candy bar (1 small bar)						
Candy without chocolate (1 oz)						
Cookies, home baked (2)						
Cookies, ready made (2)						
Brownies (2)						
Doughnuts (2)						
Cake, home baked (1 slice)						
Cake, ready made (slice)						
Sweet roll, coffee cake, or other pastry ready made (1 serving)						
Sweet roll, coffee cake, or other pastry home baked (1 serving)						
Pie, homemade (1 slice)						
Pie, ready made (slice)						
Jam, Jelly, Preserves, Syrup, or Honey (1 Tbsp)						
Peanut butter (1 Tbsp)						
Popcorn (1 cup)						
Popcorn, air popped (1 cup)						
Nuts (small packet or 1 oz)						
Bran, added to food (1 Tbsp)						
Wheat germ (1 Tbsp)						
Chowder or cram soup (1 cup)						
Oil and vinegar dressing (1 Tbsp)						
Mayonnaise or other creamy salad dressing (1Tbsp)						
Mustard, dry or prepared (1 tsp)						
Salt (1 shake)						
Pepper (1 shake)						

VITA

Yeong S. Rhee

Candidate for Degree of

Doctor of Philosophy

Thesis: EFFECTS OF CHROMIUM AND COPPER DEPLETION OR SUPPLEMENTATION ON IMMUNITY IN BHE/cdb RATS OR IN HYPERCHOLESTEROLEMIC POSTMENOPAUSAL WOMEN

Major Field: Human Environmental Sciences

Area of Specialization: Human Nutrition

Biographical:

Personal Data: Born in Cheonan, Chungnam, South Korea, August 15, 1968, the daughter of Nam Soo Rhee and Kyoung Ja Cha.

Education: Graduated from Bokja Girl's High School, Cheonan, Chungnam, in February 1987; received Bachelor of Science degree in Home Economics from Sejong University, Seoul, Korea in February, 1991; Licensed for teaching in field of Home Economics, Seoul, Korea in February, 1991; received Master of Science degree, Sejong University in February, 1993; completed the requirements for the Doctor of Philosophy degree at Oklahoma State University in December 1999.

Professional Experience: Assistant, Sejong University, Seoul, Korea, 1992-1993; Research Assistant for the Department of Nutritional Sciences, College of Human Environmental Sciences, Oklahoma State University, Stillwater, OK, 1994-1999, Dietetic Intern, Oklahoma State University Dietetic Internship, 1998-1999.

Professional Organizations: American Dietetic Association