EFFECTS OF CHROMIUM AND A CHINESE HERB ON GLUCOSE, CHOLESTEROL AND NUCLEIC ACIDS

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CHAPTER I

INTRODUCTION

Chromium (III) is an essential nutrient required for normal glucose, lipid and nucleic acid metabolism. In early experiments, Schwarz and Mertz discovered that trivalent chromium is an essential trace element in preventing and reversing glucose intolerance (Schwarz and Mertz 1957, 1959). Chromium supplementation of patients on total parenteral nutrition (TPN) showed a significant improvement in several diabetic-like symptoms which were not improved by conventional treatments, such as use of exogenous insulin (Jeejeebhoy et al. 1977, Freund et al. 1979, Mossop 1983). Chromium supplementation affected children with protein calorie malnutrition (Hopkins et al. 1968, Gurson & Saner 1971), diabetics (Glinsmann & Mertz 1966, Nath et al. 1979, Mossop 1983), elderly (Levine et al. 1968), individuals of varying ages with marginally impaired glucose tolerance (Anderson et al. 1983) and hypoglycemic patients (Anderson et al. 1984).

In lipids, chromium affected serum cholesterol (Schroeder & Balassa 1965, Abraham et al. 1982), the ratio of cholesterol:HDL-cholesterol (Riales & Albrink 1981), arterial plaque size (Abraham et al. 1980), and serum

insulin (Nath et al. 1979, Mossop 1983, Anderson et al. 1984). All of these factors affected the development of atherosclerosis.

Glucose tolerance factor (GTF) was considered to be a trivalent chromium compound (Toepfer et al. 1977) first found in brewer's yeast. Studies showed the improvement of impaired intravenous glucose tolerance (Schwarz and Mertz 1959, Tuman et al. 1978) and the enhancement of glucose metabolism in rat epididymal fat pad (Mertz et al. 1961) by GTF compound. However the chemical structure of GTF is not yet known.

The interaction between chromium and nucleic acids was first demonstrated by Herrmann and Speck (1954). Later Wacker and Vallee (1954) observed that chromium content was high in nucleic acids and the bond between chromium and nucleic acids was strong. Chromium enhanced RNA synthesis both in vitro (Okada et al. 1982) and in vivo (Okada et al. 1983). Chromium accumulated in the nucleotide may participate in gene expression (Okada et al. 1984). Cr(III) possibly stimulated in vitro RNA synthesis by binding to DNA (Okada et al. 1982).

The relationship between copper and lipids was first found by Klevay (1973). His study showed that copper deficiency increases serum cholesterol in rats and dietary Zn:Cu ratio influences the animal model of copper deficiency. Other studies have shown hypercholesterolemia in copper deficiency (Allen and Klevay 1978a, 1978b, 1978c, Lei

1978). Copper also is an essential component of superoxide dismutase (SOD) which protects lipid cell membrane structures from oxidation by free oxygen radicals (McCord and Fridovich 1969). Animal studies have shown that SOD activity in chicks (Bettger et al. 1979), pigs (Williams et al. 1976), and rats (Paynter et al. 1979) is related to copper nutrition. Therefore SOD activity can be considered as an potential index of copper nutritional status.

CH is a traditional Chinese herb which has been used in hyperlipidemia and diabetes in China for centuries. But little is known about it's physiological and chemical properties. In recent years its hypolipidemic effects have been investigated in pigeons (Guo and Song 1986), rats (Niou et al 1988), Japanese quail (Wang and Jin 1984), and mice (Cheng et al. 1988, Cheng and Stoecker 1990). All the results showed a significant hypocholesterolemic effect.

Purpose and Objectives

The purposes of this study are:

(1) to test the hypoglycemic and hypolipidemic effects of chromium in mice.
(2) to select a Chinese herb with high IPA (insulin potentiating activity).
(3) to test the hypoglycemic and hypolipidemic functions of a Chinese herb with high insulin potentiating activity (IPA) in mice.

(4) to investigate the effects of Cr on nucleic acids in rats and guinea pigs.

Hypotheses of Research

H1. There will be no significant differences in blood glucose or cholesterol between Cr-supplementation and Cr-depletion groups in mice.

H2. There will be no significant differences in blood glucose or cholesterol due to CH extract-supplementation.

H3. There will be no significant differences in blood glucose or cholesterol due to CH extract- or Cu-supplementation in mice fed a hypercholesterolemic diet.

H4. There will be no significant differences in the blood glucose or cholesterol of mice fed a hypercholesterolemic diet and supplemented with single CH fractions.

H5. There will be no significant differences in the blood glucose or cholesterol supplemented by the combination of CH fractions in mice fed a hypercholesterolemic diet.

H6. There will be no significant differences in the blood glucose and cholesterol by the supplementation of fraction 1 of CH in the obese mice.

H7. There will be no significant differences in hepatic RNA and DNA between Cr-supplementation and Cr-depletion groups of rats.

H8. There will be no significant differences in hepatic RNA and DNA between Cr-supplementation and Cr-depletion groups of guinea pigs.

Limitations

In this study, we will investigate the metabolism of glucose, lipids and nucleic acids in Cr-depleted and Cr-supplemented animals. Making the Cr-depleted animal model is one of the limitations in this study because of the possibility of external chromium contamination. Another limitation is the unavoidable differences in IPA and concentrations of Cr among different batches of the Chinese herbs during the entire experiment.

Definition of Terms

1. Chromium (III) - Trivalent chromium is the most stable valence state of chromium. It has a strong tendency to form coordination compounds, complexes, and chelates. Chromium (III) is an essential elements for animal and humans.
2. Glucose intolerance - Glucose intolerance is one of the first measurable signs of chromium deficiency and diabetes. Glucose intolerance may be seen in persons with close relatives having diabetes, the obese, the elderly, pregnant females, and patients with arteriosclerosis, heart disease, and hypertension. The oral glucose tolerance test (OGTT) is the method used to determine the blood glucose levels after an overnight fast and at specified times following an oral glucose load.

3. GTF (Glucose tolerance factor) - GTF has been defined as a dietary component required for the maintainance of normal glucose tolerance. Trivalent chromium is considered as its active ingredient.
4. Hypoglycemia - Low blood sugar; below normal levels (normal whole blood concentration = 70-90 mg/dl).
5. Hyperglycemia - Elevated blood sugar; above normal level (normal whole blood concentration = 70-90 mg/dl).
6. Hypocholesterolemia - Low serum cholesterol; below normal level (normal serum concentration = 100-230 mg/dl).
7. Hypercholesterolemia - Elevated serum cholesterol level; above normal level (normal serum concentration = 100-230 mg/dl).

CHAPTER II

REVIEW OF LITERATURE

Chromium (Cr) can occur in every one of the oxidation states from -2 to +6, but only the ground state 0, +2, +3, and +6 are common. The trivalent is the most stable valence state of chromium. It has a strong tendency to form coordination compounds, complexes, and chelates. Chromium complexes have a slow rate of ligand exchange.

Chromium is an essential nutrient that functions in carbohydrate, lipid and nucleic acid metabolism. Insufficient dietary Cr intake leads to signs and symptoms similar to those associated with maturity-onset diabetes and cardiovascular disease (Borel and Anderson 1984).

Chromium and Glucose

In 1957, Schwarz and Mertz (1957) observed impaired glucose tolerance in rats fed certain diets and postulated that the condition was due to a deficiency of a new dietary agent, designated the glucose tolerance factor (GTF). Chromium affected glucose tolerance by potentiating insulin activity. Much higher amounts of insulin were required when Cr levels were very low both in vivo and in vitro. Overall glucose breakdown can be increased by increasing insulin

concentration but also by adding biologically active Cr and keeping the total amount of insulin constant (Anderson et al. 1978). Because high levels of insulin were associated with cardiovascular disease as secondary symptoms in diabetes (Stout 1977), keeping circulating insulin normal is a good way to reduce cardiovascular damage in diabetes. Chromium potentiated the insulin activity with less insulin requirement.

The clinical reports (Jeejeebhoy et al. 1977, Freund et al. 1979, Brown et al. 1986) support the effect of chromium on insulin potentiation. A female patient had been on TPN for three years and had severe diabetic-like symptoms including glucose intolerance, inability to utilize glucose for energy, neuropathy, high fatty acid levels, low respiratory quotient and abnormalities of nitrogen metabolism. Supplementation of 250 ug daily of Cr as chromium chloride changed intravenous glucose tolerance and respiratory quotient to normal. All the symptoms disappeared after a daily maintenance dose of 20 ug (Jeejeebhoy et al. 1977). Similar results were observed in a second patient receiving total parenteral nutrition (Freund et al. 1979). In a third case, a sixty-three year old female developed unexplained hyperglycemia and glycosuria during total parenteral nutrition thearapy. After fourteen days of supplemental intravenous chromium chloride (200 ug/day), the patient had no more hyperglycemia and glycosuria and did not require exogenous insulin (Brown et al. 1986).

Chromium supplementation affected not only TPN patients but also others. Children with protein-calorie malnutrition, the elderly, insulin and non-insulin dependent diabetics, hypoglycemic subjects and subjects of varying ages with marginally impaired glucose tolerance have all responded to chromium supplementation.

Malnourished children with low-chromium diets responded to chromium supplementation. The glucose removal rates of Jordanian infants were improved from 0.6 to 2.9%/min and those of the Nigerian infants were improved from 1.2 to 2.9 %/min following Cr supplementation. The infants from each area without Cr supplementation had no significant changes in glucose removal rates (Hopkins et al. 1968).

However, the glucose tolerance of malnourished children from Egypt was not improved following Cr supplementation. The dietary Cr intake of those children was higher than that of the children whose glucose tolerance improved following supplemental Cr (Carter et al. 1968). This suggests that chromium supplementation only benefited the subjects who had chromium deficiency.

Glucose removal rates of 9 of 14 Turkish children with marasmic protein-calorie malnutrition also improved within 18 h of oral administration of 250 ug of Cr as $CrCl_3.6H_2O$. There was no change in the control group (5 children) (Gurson and Saner 1971). There was a significant increase in growth in those children who responded to Cr supplementation.

Many studies have reported beneficial effects of Cr on subjects with various degrees of glucose intolerance (Glinsmann and Mertz 1966, Levine et al. 1968, Hopkins et al. 1968, Gurson and Saner 1971, Nath et al. 1979; Anderson et al. 1983, 1984, Mossop 1983). Supplemental Cr also improved the glucose tolerance of elderly subjects, maturity-onset diabetics and insulin-requiring diabetics. Glinsmann and Mertz (1966) found that 150-1000 ug of inorganic Cr/day for 2-17 wk improved oral glucose tolerance in 3 of 6 noninsulin-dependent diabetics (Glinsmann and Mertz 1966). Levine et al. (1968) reported that 4 of 10 elderly subjects with abnormal glucose tolerance normalized after treatment with 150 ug Cr/day for 3-4 months. Nath et al. (1979) and Mossop (Mossop 1983) also studied the effects of supplemental Cr in maturity-onset diabetics. Both studies reported beneficial effects of supplemental Cr on glucose and insulin parameters. The study by Mossop (1983) was a double-blind design. Twenty-six diabetics treated with either oral therapy or insulin were divided into two groups. Thirteen subjects received 600 ug chromium chloride daily and 13 received placebo for 2-4 months. Mean fasting blood glucose of diabetics receiving Cr decreased from 259 to 119 mg/dl and level of oral therapy or insulin treatment decreased in 5 of the 13 subjects. Mean fasting glucose levels of the 13 diabetics on placebo reduced slightly from 259 to 221 mg/dl but this decrease was probably due to the fact that treatment for diabetes was increased in 4 of the

13 patients. High-density lipoprotein cholesterol (HDL) increased nearly 17 mg/dl in the group receiving Cr and decreased more than 6 mg/dl in the placebo group (Mossop 1983).

Anderson et al. (1983) reported that 76 normal subjects were given Cr (200 ug Cr/day in the form of chromic chloride) or placebo in a double-blind crossover study with 3 month experimental periods. The serum glucose of the subjects with marginally impaired glucose tolerance was decreased significantly following Cr supplementation. In China, 63 noninsulin-dependent diabetic patients were given high-Cr yeast (100 ug Cr/day), low-Cr yeast, or placebo (Cheng et al. 1987). After two months, the fasting blood glucose was decreased and glucose tolerance was improved significantly in the high-Cr yeast group but not in the low-Cr yeast or placebo groups (Cheng et al. 1987).

Anderson and co-workers (1991) investigated glucose tolerance of 11 female and 6 male subjects whose low-chromium diets contained chromium in the lowest quartile of normal intake in a 14 wk study. Subjects were divided into hyperglycemic and control group with a double-blind study design. The results showed that glucose tolerance and circulating insulin and glucagon of the hyperglycemic group all improved during chromium supplementation (200 ug/d) whereas those of the control group were unchanged. Glucose and insulin concentrations 60 min after the oral glucose challenge and the sum of the 0-90 min and 0-240 min glucose

values were all significantly lower after chromium supplementation in the hyperglycemic group. The study demonstrated that consumption of diets in the lowest 25% of normal chromium intake led to detrimental effects on glucose tolerance, insulin, and glucagon in subjects with mildly impaired glucose tolerance (Anderson et al. 1991).

Chromium also showed effects on hypoglycemia. Eight female patients with symptoms of hypoglycemia were supplemented with 200 ug of Cr as chromic chloride for three months in a double-blind crossover experimental design study (Anderson et al. 1987). Chromium supplementation improved hypoglycemic symptoms and increased the minimum serum glucose values significantly two to four hours following a glucose load. Insulin binding to red blood cells and insulin receptor number also improved significantly during Cr supplementation. One study showed that chromium has growth stimulating effects on the endocrine pancreas (Hubner et al. 1988). Wistar rats were given Cr (III) orally as chromium chloride for 8 weeks (15 ppm or 3 ppm/day) while consuming high (HFD) or low fat diets (LFD). In HFD animals (n = 12), chromium supplementation increased volume of islets (41%) compared to HFD controls. Many small islets were found which may be newly formed. In general polynesia and hyperplasia of islets were observed. In LFD animals (n = 12), morphometric changes were not found but macronesia, micronesia and polynesia of islets were observed in the chromium supplemented group compared with the control group.

Some studies have reported no beneficial effects with Cr. Sherman and co-workers (1968) reported no effect of Cr supplementation (150 ug of trivalent chromium per day for 16 weeks) in four normal subjects and ten diabetics. Uusitupa et al. supplemented 10 noninsulin-dependent diabetic patients with 200 ug trivalent chromium per day. There were no significant differences in the serum glucose and lipids, except for a decrease in serum insulin (Uusitupa et al. 1983). Rabinowitz et al. reported no significant change in glucose values of 43 diabetics following supplements of inorganic chromium and yeast for 12 months (Rabinowitz et al. 1983).

Obviously, chromium deficiency was only one of the reasons for glucose intolerance. No matter what the results of supplemental Cr on glucose tolerance were, one thing to note is that chromium supplementation only benefits the subjects who were marginally or clearly deficient in Cr. Chromium should be considered as a nutrient not as a therapeutic agent.

Glucose Tolerance Factor (GTF)

In 1957, Schwarz and Mertz proposed that glucose tolerance factor (GTF) was required for the maintenance of normal glucose tolerance in rats (Schwarz and Mertz 1957). GTF was extracted and concentrated from brewer's yeast and pork kidney powder and contained chromium as its active ingredient. Although the chemical structure of the

compound(s) with GTF activity is not known, some of the biological properties have been detected (Mertz and Thurman 1968). Mertz proposed that the structure responsible for the activity was a nicotinic acid-chromium-nicotinic acid axis with ligands of glutamic acid, glycine and cysteine (Mertz 1969, Mertz et al. 1974, Mertz 1974). The functions of GTF included improvement of impaired intravenous glucose tolerance in vivo (Schwarz and Mertz 1959), enhancement of glucose metabolism in yeast (Mirsky et al. 1980) and a potentiating effect on the action of insulin on glucose metabolism in rat fat pads (Mertz et al. 1961). GTF potentiated the effects of insulin on glucose metabolism in epididymal fat tissue more than did simple chromium complexes. In vivo the impaired glucose tolerance of chromium-deficient rats can be improved by small amounts of chromium in the GTF form. So GTF, an organic chromium complex, has strong effects on glucose metabolism of epididymal fat tissue quantitatively.

GTF-like substances have been found in foods (Toepfer et al. 1973, Haylock et al. 1983), and animal tissues (Schwarz and Mertz 1959, Haylock et al. 1983). Yamamoto and his co-workers reported a biologically active, low-molecular-weight, chromium-binding substance from milk (M-LMCr) and rabbit liver which appeared to have GTF properties similar to yeast and the low-molecular-weight, chromium-binding substance present in mammalian liver

(Yamamoto et al. 1981, 1984, 1988). However, the chemical nature of GTF has not yet been established.

Urberg and Zemel have studied the synergism between chromium and nicotinic acid in glucose metabolism (Urberg and Zemel 1987). Sixteen healthy elderly volunteers were divided into three groups and given 200 ug Cr, 100 mg nicotinic acid, or 200 ug Cr $\pm$ 100 mg nicotinic acid daily for 28 days. The results showed that fasting glucose and glucose tolerance were affected neither by chromium nor by nicotinic acid alone. The combination Cr-nicotinic acid supplement caused a 15% decrease in a glucose area integrated total ($P < 0.025$) and a 7% decrease in fasting glucose.

Fischer and co-workers reported that cationic samples with low molecular weight were obtained by partial purification from yeast extract. These samples increased the rate of glucose transport in isolated cardiomyocytes 2.0 - 2.5 fold. A further purification by gel filtration suggested that L-alanine might be involved in the regulation of glucose transport in cardiomyocytes (Fischer et al. 1992).

Khan and co-workers found an unidentified factor that potentiates the function of insulin in selected foods and spices. They also found that insulin potentiating activity of these foods and spices did not correlate with total chromium (Khan et al. 1990).

Simonoff and co-workers reported that individual fractions with GTF activity did not differ between Cr-rich

and Cr-deficient yeast, and there was also no relationship between Cr concentration and GTF activity. This study does not support the hypothesis that chromium is an obligatory constituent of GTF, assuming that GTF is a unique substance (Simonoff et al. 1992). Further study of organic Cr complexes is needed to resolve these inconsistencies.

Chromium and Lipid

Marginal Cr intake has been related to abnormal lipid metabolism and ultimately atherosclerosis in both animal and human studies. Rats fed a low Cr diet increased serum cholesterol, aortic lipids and plaque formation with increasing age (Schroeder and Balassa 1965). In rabbits atherosclerotic plaques were induced by feeding a high-cholesterol diet (standard diet with 1.5% cholesterol added). After intraperitoneal administration of Cr (20 ug potassium chromate per day) for 60 days, the size of aortic plaques and aortic cholesterol concentration were decreased (Abraham et al. 1980). Eight rabbits fed a 1% cholesterol diet for 30 days were injected daily with potasium chromate for 60 days. A 50% reduction in aortic intimal plaque area and in aortic total cholesterol concentration was observed. Six rabbits were injected with potassium chromate and fed a 1% cholesterol diet for 12 weeks. Mean aortic cholesterol concentration was significantly lower than the control group and the area of aortic intima covered by macroscopic plaques was smaller than the control group (Abraham et al. 1982). A

recent study from Abraham and co-workers investigated the effects of Cr on atherosclerosis in rabbits again (Abraham et al. 1991). Thirty-three rabbits fed a hypercholesterolemic diet were randomized into 6 groups and treated with daily injection of either water, 10 or 20 ug of potassium chromate or 1, 5, 10 or 20 ug of chromium chloride, respectively for 135 days. The percentage of aortic intimal surface covered by plaque was reduced in the treated animals. Rabbits treated with 20 ug of chromium chloride showed a better response than those treated with either 10 or 20 ug of potassium chromate.

An early study reported that individuals who died of coronary artery disease had lower Cr concentration in aortic tissue compared to subjects dying from accidents, even through the Cr concentration of other tissues analyzed was similar (Schroeder et al. 1970). The concentration of serum chromium in the subjects with coronary artery disease was lower than in the subjects without symptoms of disease (Newman et al. 1978). In that study low serum Cr and high serum triglyceride were highly correlated ($p < 0.01$ and $p < 0.05$ respectively) with the appearance of coronary artery disease whereas there was no correlation between other risk factors.

Chromium was given to 12 adult men (200 ug Cr as chromium chloride daily). After 12 weeks. serum triglyceride concentrations decreased significantly and the concentrations of high-density lipoprotein cholesterol

increased significantly compared to the subjects given placebo (Riales and Albrink 1981). Chromium supplementation (600 ug Cr as chromium chloride daily) of diabetic patients significantly increased the high density lipoprotein cholesterol and increased the total cholesterol (Mossop 1983}. In China, high-chromium yeast was supplemented to nineteen hypertriglyceridemic subjects (200 ug Cr/day). After two months the concentrations of serum triglyceride were decreased significantly in the Cr-supplemented group, but there was no change in the control group (Jiang et al. 1990). Also many studies have reported beneficial effects on serum lipids following supplementation with brewer's yeast that was high in Cr (Borel and Anderson 1984).

Twenty-eight subjects were given either chromium tripicolinate (200 ug Cr) or a placebo daily for 42 days in a double-blind crossover study. The results showed that levels of total cholesterol, LDL, and apolipoprotein B (the principal protein of the LDL fraction) were decreased significantly in the chromium picolinate supplemented group. The concentration of apolipoprotein A-1 (the principal protein of the HDL fraction) was increased substantially during chromium picolinate supplementation. These results showed that chromium picolinate has hypolipidemic effects in humans (Press et al. 1990).

Chromium and Nucleic Acids

Before the interaction of chromium with insulin was identified, the relationship between chromium and nucleic acids was suggested by the observation that treatment of tissues with chromates and dichromates reduced the amount of nucleic acid that could be extracted with trichloroacetic acid (Herrmann and Speck 1954). The effects were presented by reduction of the hexavalent to the trivalent form of Cr and formation of a Cr(III) complex with nucleic acids. Later, Wacker and Vallee (1959) investigated the concentrations and potential functions of several transition metals in nucleic acid preparations. They found that chromium bound to nucleic acids more tightly than other metals and was not removed by repeated treatments with ethylenediamine tetraacetic acid (EDTA). The concentrations of chromium were between 260 and 1080 ug/g in a beef liver nucleoprotein fraction. More pure preparations of RNA and DNA were found to contain from 18 to 400 ug Cr/g, depending on source and the mode of extraction. They also found that chromium stabilized the tertiary structures of nucleic acids by demonstrating a reduction of heat-induced changes of optical density in vitro. We have checked the chromium concentration of the hepatic nucleic acids in rats (5 ± 1 ug/g dry weight) and found them lower than those reported earlier (Cheng et al. 1990a) .

Recently, more studies have been done because the interaction of chromates with nucleic acids was related to carcinogenicity in industrially exposed workers. The role of trivalent chromium as a physiological component in the metabolism of nucleic acids and in protein synthesis is also being studied. Tamino et al. have reported that Cr(III) binds to mammalian DNA and RNA in vitro (Tamino et al. 1981). Okada and collaborators reported that in vitro RNA synthesis directed by free DNA was significantly enhanced when Cr(III) was bound to the template (Okada et al. 1981a). The increase of RNA synthesis with Cr(III)-bound DNA is proportional to the molar binding ratio of Cr(III) to DNA. This enhancement possibly was caused by an increase in the number of initiation sites on DNA (Okada et al. 1982). In an in vitro system, Cr(III) bound to DNA preferentially in chromatin and increased the number of initiation sites (Ohba et al. 1986) which enhanced RNA synthesis. Okada et al. also reported that chromium was present in regenerating rat liver in vivo in the form of a chromium-protein of approximately 70,000 daltons containing between 4 and 5 atoms of chromium, corresponding to approximately 3,600 ug chromium/g protein (Okada et al. 1984). In our study hepatic DNA of the Cr-depleted groups was significantly lower than the Cr-supplemented animals and hepatic RNA was similarly reduced by Cr depletion (Cheng et al. 1990b).

Wolf et al. examined the interaction of chromium (III) and chromium (VI) with the phosphate groups of di- and

triphosphate nucleotides by ^{31}P-NMR spectroscopy. The formation of Cr-nucleotide complexes could only be detected with Cr(III) through the chemical shifts of the phosphate groups (Wolf et al. 1989).

Chromium has not been studied enough in this expanding field of nucleic acid metabolism and the regulation of protein synthesis. This area should be considered one of the most promising for further research on the biological role of chromium.

Copper and Lipid

Copper, like chromium, is also involved in lipid metabolism. Klevay (1973) first found that copper deficiency increases serum cholesterol in rats which relates to Zn:Cu ratio. Because a high level of dietary zinc increases copper deficiency, dietary zinc:copper ratio is considered as an important factor in hypercholesterolemia. Lei (1978) also showed hypercholesterolemia in copper deficient rats. Allen and Klevay (1978a) investigated the relationship between liver cholesterol and plasma chlesterol by injecting ^{3}H-mevalonate into copper-deficient and control rats. The results suggested that copper deficiency causes rapid cholesterol release from the liver to plasma. The hyperlipidemic results were observed in rats with varied copper intake and feeding period (Petering et al. 1977, Allen and Klevay 1978a, 1978b, 1978c, Lau and Klevay 1981, 1982, Katya-Katya et al. 1984, Looney and Lei 1978). Copper

also is an essential component of several enzymes including the Cu-Zn form of the antioxidant enzyme superoxidase (Fridovich 1975). Superoxide dismutase (SOD) is an important copper metalloenzyme that protects lipid cell membrane structures from oxidation by free oxygen radicals (McCord and Fridovich 1969, Johnston and De Chatelet 1974). Animal studies have shown that SOD activity in chicks (Bettger et al. 1979), pigs (Williams et al. 1976), and rats (Paynter et al. 1979) is related to copper nutrition. Therefore SOD activity can be considered as a potential index of copper nutritional status.

The Hypocholesterolemic Effect of a Chinese Herb (CH) in Animals

A Chinese herb (CH) has been used for centuries as an important medicine and nutrient in the attempt to prevent aging in China. There have been several studies of CH in animals, but still very little is known of its physiological and chemical properties.

The relationship between cholesterol and CH was investigated by Guo and Song in 1986. Eighty pigeons including males and females, mean body weight 300 g, were fed a hypercholesterolemic diet and were divided into three groups: CH supplemented (2g/day) group (n = 20), nicotinic acid supplemented (100 mg/day) group (n = 20), and the control group (n = 40). After 2 months, all pigeons were killed except 24 of the control group. These 24 pigeons were

divided into two groups again as CH supplemented and control groups for one more month. The results showed that mean serum cholesterol of both CH and nicotinic acid supplemented groups were significantly decreased ($P < 0.001$) compared with the control group. In the continued study of the 24 pigeons, serum cholesterol was significantly decreased ($P < 0.025$) by CH supplementation compared to the control group. This study showed the hypocholesterolemic effect of CH supplementation in pigeons fed a hypercholesterolemic diet (Guo and Song 1986).

There was another study of effects of CH on cholesterol, related enzymes, and pathological observations in rats (Niou et al. 1988). Thirty-nine male rats, 135-145 g, were divided into four groups and fed the following diets: normal diet ($n = 9$), hypercholesterolemic diet (2% cholesterol and 0.5% cholic acid) ($n = 9$), hypercholesterolemic diet with an extract from CH ($n = 10$), and hypercholesterolemic diet with CH powder ($n = 11$). After 17 days, serum cholesterol levels in both the CH extract and the CH powder group were decreased significantly ($P < 0.05$) compared to the group fed the hypercholesterolemic diet alone. The hepatic ATPase and SDH (succinate dehydrogenase) in both CH supplemented groups were lower than in the control group. The hepatic G-6-P (glucose-6-phosphate) in the CH powder supplemented group was lower than the other groups. There were many neutral fat drops in the lobules of the liver in the group fed the hypercholesterolemic diet.

Both CH supplemented groups had very few fat drops and were almost like the normal diet group. The results suggested that CH can be considered as a hypolipidemic agent.

More lipidemic indices and atherosclerotic changes have been investigated in the Japanese quail (Wang and Jin 1984). Forty-six Japanese quail were divided into four groups. These groups were supplemented with water (control) or with small (0.084 g/kg/day) (n = 10), medium (0.84 g/kg/day), or large doses (8.4 g/kg/day) (10) of CH extract with hypercholesterolemic diets (1% cholesterol). The results showed that plasma HDL-cholesterol levels of the three CH-supplemented groups were increased compared to the control group. The HDL-cholesterol/total cholesterol ratios in the three CH-supplemented groups were increased after 2-5 weeks. Plasma cholesterol and cholesterol esters in the three CH-supplemented groups were decreased compared to the control group after 6 weeks. Plasma triglyceride levels were decreased compared to the control group. Atherosclerotic changes in the aorta with the three levels of CH supplementation were less than in the control group, especially in the supplementation group with the large dose of CH. This study showed that CH had hypolipidemic effects and reduced atherosclerosis in Japanese quail.

In another study, the conversion of cholesterol and cholic acid in vitro was observed in liver with

(7-^{3}H)-cholesterol and three levels of CH. The result showed that the lowest concentration was the best for stimulating conversion of cholesterol to cholic acid (Xu and Li 1987).

CHAPTER III

METHODOLOGY

Study 1 (Chapter 4): The Effects of Chromium and a Chinese Herb on Serum Glucose and Cholesterol in Mice

Assay of Insulin Potentiating Activity (IPA) of 24 Chinese herbs:

Twenty-four Chinese herbs were separately stirred with a 20-fold excess (w/v) of 0.1 N NH_4OH for 2 hours and then centrifuged at 1,000 x g for 20 minutes. Insulin potentiating activity of supernatant in each Chinese herb was evaluated using isolated adipocytes from epididymal fat tissue (Anderson et al. 1978) (see Appendix A).

Animals and diets:

Forty-two male weanling mice were randomly assigned to four groups: Cr-depletion group; Cr-depletion and Chinese herb-supplementation group; Cr-supplementation group (2 ppm Cr as $CrCl_3$); and Cr- and Chinese herb-supplementation group.

The diet contained: 20% Casein (U. S. Biochemical); 0.3% DL Methionine (U. S. Biochemical); 65.0% Sucrose (Food Club); 5.0% Cellulose (Celufil); 5.0% corn oil (Wesson);

3.5% AIN mineral mix without Cr; 1.0% AIN vitamin mix; 0.2% choline bitartrate.

Chinese herb (#13, i.e. CH)) was stirred with a 20-fold excess (w/v) of 0.1 N NH_4OH for 2 hours and then centrifuged at 1,000 x g for 20 minutes. Then the supernatant was adjusted to pH 7. Each mouse was given a daily oral dose of the CH or water as a placebo by micropipette. During the first 10 days, 25 ul per day was given; during the next 10 days, 50 uL per day; then 100 uL per day was given until the end of the experiment.

The mice were maintained on a 12-hr light dark cycle and had access to diet and deionized water ad libitum. Trace metal contamination was reduced by use of plastic housing and feeding equipment.

After 6 weeks, mice were fasted 14 hr and then were given an oral glucose load (1 mg glucose /g body weight of a 50% solution) 90 min before decapitation and blood collection. Tissues were weighed and serum was prepared and frozen.

Serum glucose was analyzed by the glucose oxidase method using a glucose analyzer (Beckman Instruments, Inc., Fullerton, CA). Serum insulin was determined by radioimmunoassay (Pharmacia Diagnostics, Piscataway, NJ) and serum cholesterol was determined enzymatically (Sigma Chemical Co., St. Louis, MO).

The experimental design was a 2 x 2 factorial with chromium and CH as factors. Data were evaluated by analysis

of variance.

Study 2 (Chapter 5): The Effects of a Chinese Herb and Copper on Plasma Lipids in Mice Fed a Hypercholesterolemic Diet

Extraction of Chinese herb (CH)):

CH was stirred with a 20-fold excess (w/v) of 0.1 N NH_4OH for 2 hours and then centrifuged at 1,000 g for 20 minutes. Then the supernatant was adjusted to pH 7.

Animals:

Thirty male weanling mice were randomly assigned to four groups. The four groups are: Low-Cu diet, Chinese herb supplemented group (LowCu+CH), Low-Cu diet without Chinese herb group (LowCu-CH), Adequate-Cu diet, Chinese herb supplemented group (AdeqCu+CH), and Adequate-Cu without Chinese herb (AdeqCu-CH).

The mice were maintained on a 12-h light-dark cycle and had access to diet and deionized water ad libitum. Trace metal contamination was reduced by use of plastic housing and feeding equipment.

Diets:

The diet contained 20% casein, 0.3% DL-methionine, 48.5% sucrose, 5% cellulose (Celufil), 5% corn oil (Mazola), 3.5% AIN mineral mix or 3.5% modified AIN mineral mix, 1.0% AIN vitamin mix, 0.2% choline bitartrate, 1.0% cholesterol, and 0.5% cholic acid. Using the AIN mineral mix added 7 mg Cu as cupric carbonate/kg diet while the modified mineral

mix added 0.7 mg Cu/kg diet. Each mouse was given 100 uL oral dose of Chinese herb or water as a placebo by micropipette daily.

After 12 weeks, mice were anesthetized and exsanguinated by heart puncture. Tissues were weighed and plasma was prepared and frozen. Plasma glucose and cholesterol were analyzed using enzymatic methods. Triglycerides also were determined enzymatically. Red cell superoxide dismutase (SOD) was analyzed using the method of Roth and Gilbert (Roth and Gilber 1984).

Study 3 (Chapter 6): The Effects of Fractions from a Chinese Herb on Plasma Glucose and Cholesterol in Mice Fed a Hypercholesterolemic Diet

Fractionation of Chinese herb:

CH was extracted with 0.1 N NH_4OH (1:20) and centrifuged (1,000 x g). The supernatant was applied to a Sephadex G-25 column (50-150 u of particle size, 60 x 2.6 cm). Three fractions were collected and evaporated at room temperature to 1/3 of their original volume for experiment 1 and to 1/10 of their original volume for experiment 2. Each combination of fractions was mixed from the specified fractions and equal concentrations were made. Each mouse was given 100 ul of Chinese herb or water orally by micropipette daily.

Animals:

Experiment 1: Thirty-nine male weanling mice were randomly assigned to four groups. The four groups were: fraction 1, fraction 2, fraction 3, or control group.

Experiment 2: Sixty-one male weanling mice were randomly assigned to six groups: fraction 1 & 2, fraction 1 & 3, fraction 2 & 3, fraction 1 & 2 & 3, crude extraction, or control.

Diets and housing:

The diet contained 20% casein, 0.3% DL-methionine, 48.5% sucrose, 5% cellulose (Celufil), 5% corn oil (Mazola), 3.5% AIN mineral mix or 3.5% modified AIN mineral mix, 1.0% AIN vitamin mix, 0.2% choline bitartrate, 1.0% cholesterol, and 0.5% cholic acid.

All the mice were maintained on a 12-h light-dark cycle and had access to diet and deionized water ad libitum. Trace metal contamination was reduced by using of plastic housing and feeding equipment.

Experiment 1 was finished after 10 weeks. Experiment 2 was terminated after 9 weeks. Mice were anesthetized and exsanguinated by heart puncture. Tissues were weighed and plasma was prepared and frozen. Plasma glucose and cholesterol were analyzed using enzymatic methods. Insulin was analyzed by radioimmunoassay.

Study 4 (Chapter 7): The Effects of Fraction 1 from a Chinese Herb on Plasma Glucose and Cholesterol in Obese Mice Fed a Hypercholesterolemic Diet

Fractionation of Chinese herb (CH):

CH was extracted with 0.1 N NH_4OH (1:20) and centrifuged (1,000 x g). The supernatant was applied to a Sephadex G-25 column (50-150 u of particle size). Three fractions were separated. The first fraction was collected and evaporated to 1/3 of its original volume at room temperature.

Animals and diets.

Nineteen male obese mice (51 days old) were randomly assigned to two groups: fraction 1 and control. The diet contained 20% casein, 0.3% DL-methionine, 48.5% sucrose, 5% cellulose (Celufil), 5% corn oil (Mazola), 3.5% AIN mineral mix or 3.5% modified AIN mineral mix, 1.0% AIN vitamin mix, 0.2% choline bitartrate, 1.0% cholesterol, and 0.5% cholic acid. Each mouse was given 100 ul of fraction 1 of CH or water orally by micropipette daily. The mice were maintained on a 12-h light-dark cycle and had access to diet and deionized water ad libitum. Trace metal contamination was reduced by using plastic housing and feeding equipment. After 7 weeks, mice were fasted 14 hr and then were given an oral glucose load (1 mg/g body weight in a 50% solution) 60 minutes before sacrifice. Mice were anesthetized and

exsanguinated by heart puncture. Tissues were weighed and plasma was prepared and frozen.

Analysis:

Plasma glucose and cholesterol were analyzed using enzymatic methods. Insulin was analyzed by radioimmunoassay.

Study 5 (Chapter 8): Chromium Interaction with Nucleic Acids in Rat Liver

Animals and materials:

Male Sprague Dawley rats, 45-50 g, were divided into three groups and feed either a low Cr diet, a low Cr diet plus 1 ug Cr as chromium chloride per g of diet or a low Cr diet plus 100 ug of Cr per g of diet. Chromium concentration of the low Cr diet was approximately 30 ng/g. After eight to ten weeks, rats were sacrificed and livers were removed, rinsed, and stored on ice or frozen at -20^{o}C. Cold liver samples were homogenized with an equal weight of water and centrifuged (1000 x g). The supernatant was applied to a Sephadex G-15 column. RNA was determined by an orcinal method (Keleti and Lederer 1974), DNA by diphenylamine procedure (Keleti and Lederer 1974) and protein by the Biorad method (Bio-Rad Protein Assay Kit). Chromium was determined by atomic absorption spectroscopy as described previously (Anderson and Kozlovsky 1985).

Study 6 (Chapter 9): The Effects of Chromium Depletion on Hepatic Nucleic Acids in Guinea Pigs

Animals and diets:

Thirty-six guinea pigs (mean weight = 179 g) were divided into four groups: Cr and ascorbate-depletion group; Cr-depletion and ascorbate-supplementation group; Cr-supplementation and ascorbate-depletion group; and Cr- and ascorbate-supplementation group.

The diet contained (g/kg): 370 dextrose, 300 casein, 30 L-arginine HCl, 150 Celufil, 70 corn oil, 50 mineral mix, 35 potassium acetate and 22 vitamin mix without ascorbate. The mineral mix contained (g/kg): 600 $CaHPO_4$, 80 NaCl, 100 MgO, 0.90 $ZnCO_3$, 1.8 $MnCO_3$, 0.25 $CuCO_3$, 3.5 KIO_3, 0.67 $NaSeO_3$, 1.42 $FeCl_2$ and a quantity of sucrose sufficient to make 1 kg. Diets contained < 60 ng/g chromium (-Cr) or were supplemented with 2 ug/g Cr as $CrCl_3$ (+Cr). Animals were dosed orally with 1 mg ascorbate/day (-Cr) or with 10 mg/day (+Cr).

Animals had diet and deionized water ad libitum. They were housed in plastic cages to reduce trace mineral contamination.

Guinea pigs were weighed weekly. After 20 weeks, guinea pigs were anesthetized and exsanguinated by heart puncture. Liver was frozen immediately. Hepatic RNA and DNA were separated (Keleti and Lederer 1974)(see Appendix B).

Analysis:

The experimental design was a 2 x 2 factorial with chromium and ascorbate as factors. Data were evaluated by analysis of variance.

CHAPTER IV

THE EFFECTS OF CHROMIUM AND A CHINESE HERB (CH) ON SERUM GLUCOSE AND CHOLESTEROL IN MICE

Abstract

A Chinese herb (CH), which had high IPA, was selected to feed mice. Forty-two male weanling mice were randomly grouped in a 2 x 2 factorial design. Groups were: -Cr+CH, -Cr-CH, +Cr+CH, +Cr-CH. CH was extracted with 0.1 N NH_4OH (1:20). Each mouse was given an oral dose of CH or water by micropipette. After 6 weeks, neither CH nor Cr affected body weight gain. Chromium supplementation decreased liver weight ($P < 0.02$) and CH decreased spleen weight ($P < 0.02$). Serum glucose was not altered by treatment. Serum cholesterol concentrations were significantly decreased ($P < 0.02$) with CH supplementation. These data demonstrated that the Chinese herb, CH, had significant insulin-potentiating activity in vitro and reduced serum cholesterol in mice.

INDEXING KEY WORDS:

. chromium . insulin potentiating activity
. Chinese herb . chromium chloride . cholesterol

Glucose intolerance is usually one of the first measurable signs of Cr deficiency and also of diabetes. The early experiments of Schwarz and Mertz (Schwarz and Mertz 1957, 1959) led to the discovery of Cr as an essential nutrient in preventing and reversing glucose intolerance. In these studies, supplementing the apparently low Cr diet of rats with Cr led to rapid improvements in glucose tolerance. Chromium supplementation of patients on total parenteral nutrition also led to significant improvements in several diabetic-like symptoms including glucose intolerance (Jeejeebhoy et al. 1977). Chromium also affects lipid metabolism, such as serum cholesterol (Schroeder and Balassa, 1965, Abraham et al. 1982), and serum insulin (Nath et al. 1979, Mossop 1983, Anderson et al. 1984). Chromium functions in glucose tolerance primarily through its insulin potentiation (Anderson et al. 1978).

There are several traditional medicines which have been used for diabetic-like symptoms in China for centuries, but their insulin-potentiating activity (IPA) has not been evaluated. In this study several of these Chinese herbs were assayed for IPA. A Chinese herb (CH) which had high IPA was selected to use in an animal study. The purpose of this study was to investigate the effects of chromium and CH on serum glucose and cholesterol in mice.

Materials and Methods

Extraction of Chinese herbs and Assay of IPA. Chinese herbs were stirred with a 20-fold excess (w/v) of 0.1 N NH_4OH for 2 hr and then centrifuged at 1,000 x g for 20 min. The supernatant of each Chinese herb was assayed for insulin potentiating activity (IPA) in the rat epididymal fat cell bioassay (Anderson et al. 1978) (see Appendix A).

Animals and diets. Forty-two male weanling mice were randomly assigned to four groups: Cr-depletion group; Cr-depletion and CH-supplementation group; Cr-supplementation group (2 ppm Cr as $CrCl_3$); Cr- and CH-supplementation group.

The diet contained: 20% casein (U. S. Biochemical); 0.3% DL methionine (U. S. Biochemical); 65.0% sucrose (Food Club); 5.0% cellulose (Celufil); 5.0% corn oil (Wesson); 3.5% AIN mineral mix without Cr; 1.0% AIN vitamin mix; 0.2% choline bitartrate.

Each mouse was given a daily oral dose of CH or water as a placebo by micropipette. During the first 10 days, 25 ul per day was fed. During the next 10 days, 50 uL per day was given. Then 100 uL per day of CH was given until the end of the experiment.

The mice were maintained on a 12-hr light dark cycle and had access to diet and deionized water ad libitum. Trace metal contamination was reduced by use of plastic housing and feeding equipment. After 6 weeks, mice were fasted 14 hr

and then were given an oral glucose load (1 mg glucose/g body weight in a 50% solution) 90 min before decapitation and blood collection. Tissues were weighed and serum was prepared and frozen.

Analysis. Serum glucose was analyzed by the glucose oxidase method using a glucose analyzer (Kadish et al. 1968). Serum insulin was determined by radioimmunoassay (Crowley & Garbien 1974) and serum cholesterol was determined enzymatically (Allain et al. 1974).

Statistics. The experimental design was a 2 x 2 factorial with chromium and CH as factors. Data were evaluated by analysis of variance.

Results and Discussion

The insulin potentiating activity of Chinese herb #13 (CH) was higher than all other Chinese herbs (Table 1). Thus CH might enhance glucose utilization.

Neither CH nor Cr significantly affected body weight gain (Table 2). Chromium supplementation decreased liver weight significantly in this study ($P < 0.02$) (Table 2). Chromium functions by increasing the activity of insulin (Jeejeebhoy 1977, Freund 1979). Insulin plays the role of stimulation of lipogenesis and inhibition of lipolysis which might be an explaination for the increasing liver weight in the chromium-depletion animals. CH also changed spleen weight in this study. We really don't know the mechanism for these results. Further study needs to be done.

Chromium supplementation did not affect blood glucose, insulin and cholesterol compared to the chromium depletion group (Table 3). Flatt and his co-workers reported there were no effects on plasma glucose and insulin between Cr(III) supplemented mice (1 mg Cr/kg diet) and Cr (III) deficient mice (0.03 mg/kg) after 32 days (Flatt et al. 1989). Donaldson and coworkers reported that there were no significant differences in plasma glucose, cholesterol and triglyceride in rats fed either a low-chromium (60 to 100 ug/kg of diet) or chromium- supplemented (5 mg/kg of diets) hyperglycemic and hyperlipidemic diets (Donaldson et al. 1985).

Some human studies also showed no effect of chromium supplementation. Sherman and coworkers (Sherman et al. 1968) reported no effect on glucose and lipids with chromium supplementation (150 ug of chromium as $CrCl_3$/day for 16 weeks) in four normal subjects and ten diabetics. Uusitupa et al. 1983) supplemented chromium (200 ug/day as $CrCl_3$) in 10 noninsulin-dependent diabetic patients. There were no significant changes in serum glucose and lipids. Rabinowitz et al. also reported no effect on glucose of forty-three diabetics following supplements of inorganic chromium and Cr-yeast for 12 months (Rabinowitz et al. 1983).

Chromium deficiency is considered as one of the reasons for glucose intolerance. But chromium supplementation only can benefit the subjects who have chromium deficient status. In an animal studies, making the Cr-depleted animal model is

the most important step to get effective results from Cr-supplementation. From the results of this study, the mice might not have become Cr-depleted for some reasons, such as time, on the diet, or chromium content of the diet (Unfortunately we did not analyze the chromium content in this diet).

With the high IPA in the crude extract of CH, an hypoglycemic effect was expected instead of a hypocholesterolemic effect. In this study, serum cholesterol concentrations were significantly decreased ($P < 0.02$) with CH supplementation. The same results were reported in pigeons, rats and Japanese quail (Guo and Song 1986, Niou et al. 1988, Wang 1984). Our study confirmed the hypocholesterolemic effect of CH. But little is known about their biological and chemical properties. So further study is needed. These data demonstrate that the Chinese herb (CH) has high insulin-potentiating activity in vitro and a hypocholesterolemic effect in mice.

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TABLE 1

INSULIN POTENTIATION OF CHINESE HERBS

Samples	Insulin Potentiation
Standard Yeast #1	2.9
Standard Yeast #2	1.7
No.1	0.9
No.2	1.1
No.3	0.6
No.4	0.5
No.5	1.5
No.6	1.1
No.7	1.0
No.8	1.1
No.9	1.3
No.10	0.8
No.11	1.2
No.12	2.1
No.13 (CH)	4.1
No.14	0.9
No.15	1.2
No.16	1.3
No.17	1.2
No.18	1.2
No.19	1.2
No.20	1.2
No.21	1.3
No.22	1.0
No.23	1.1
No.24	1.1

TABLE 2

WEIGHT GAIN AND TISSUE WEIGHTS OF MICE FED EXPERIMENTAL DIETS[1]

Group	Wt Gain (g)	Liver (g)	Spleen (g)
-Cr + CH	14.2 ± 0.3	1.04 ± 0.03	0.06 ± 0.002
-Cr - CH	14.9 ± 0.5	1.09 ± 0.03	0.07 ± 0.004
+Cr + CH	14.1 ± 0.5	1.00 ± 0.03	0.06 ± 0.002
+Cr - CH	13.8 ± 0.5	1.01 ± 0.02	0.07 ± 0.004
Source of Variation	Analysis of Variance P Value		
Cr	0.18	0.02[2]	0.68
CH	0.67	0.19	0.02[2]
Cr*CH	0.29	0.55	0.21

1. Mean ± SEM, n=9-11.

2. Significant difference.

TABLE 3

SERUM CONCENTRATION OF INSULIN, GLUCOSE AND CHOLESTEROL[1]

Group	Insulin (uU/ml)	Glucose (mg/dl)	Cholesterol (mg/dl)
-Cr + CH	8.0 ± 0.6	147 ± 9.8	94 ± 5
-Cr - CH	9.0 ± 1.0	153 ± 7.7	116 ± 7
+Cr + CH	7.7 ± 0.7	140 ± 8.0	112 ± 6
+Cr - CH	7.1 ± 0.5	156 ± 10.7	124 ± 8
	Analysis of Variance		
Source of Variation	P Value		
Cr	0.13	0.84	0.07
CH	0.77	0.22	0.02[2]
Cr*CH	0.31	0.63	0.52

1. Mean ± SEM, n=7-11.

2. Significant difference.

CHAPTER V

THE EFFECTS OF A CHINESE HERB AND COPPER ON PLASMA LIPID IN MICE FED A HYPERCHOLESTEROLEMIC DIET

Abstract

The purpose of this study was to investigate the effects of a Chinese herb (CH) with high insulin potentiating activity and two levels of dietary copper in mice fed a casein-based hypercholesterolemic diet containing 1.0% cholesterol and 0.5% cholic acid. Diets were supplemented with 0.7 (LowCu) or 7.0 (AdeqCu) ug/g copper as cupric carbonate. Thirty male weanling mice were grouped in a 2 x 2 factorial design. Groups were: LowCu-CH, AdeqCu-CH, LowCu+CH, and AdeqCu+CH. The Chinese herb was extracted with 0.1 N NH_4OH (1:20) and then adjusted to pH 7.0. Mice were fed daily with 100 uL of CH extract by micropipette. After 12 wk of treatment, body and tissue weights, plasma glucose and triglycerides, and erythrocyte superoxide dismutase (SOD) activity were not significantly affected by either CH or Cu. Mean plasma cholesterol was 191 mg/dl for the -CH groups and 171 mg/dL for +CH groups ($p < 0.04$). These

results demonstrated the hypocholesterolemic effect of CH in mice fed a hypercholesterolemic diet.

INDEXING KEY WORDS:

.chromium .Chinese herb .glucose .cholesterol
.insulin potentiating activity .superoxide dismutase (SOD)

Various traditional Chinese herbs have been used for treatment of diabetes and hyperlipidemia in China, but little is known about their biological and physiological functions. One Chinese herb (CH) consistently had high insulin potentiating activity and had a hypocholesterolemic effect in our preliminary animal study (Cheng et al. 1988).

In an initial experiment, high ratios of zinc to copper produced hypercholesterolemia in rats (Klevay 1973). Other experiments have shown hypercholesterolemia in copper deficiency (Allen and Klevay 1978a, 1978b, 1978c, Lei 1978). Different copper intakes and feeding periods influenced the hyperlipidemic result in rats (Petering et al. 1977, Allen and Klevay 1978a, 1978b, 1978c, Lau and Klevay 1981, 1982, Katya-Katya et al. 1984, Looney and Lei 1981). Copper also is an essential component of several enzymes (Fridovich 1975) including superoxide dismutase (SOD). According to animal studies (Bettger et al. 1979, Williams et al. 1976, Paynter et al. 1979), SOD activity can be considered as a potential index of copper nutritional status.

Because copper has been implicated as a micronutrient affecting cholesterol metabolism, both copper and Chinese herbs were selected as variables in a 2 X 2 factorial experiment. The purpose of this study was to investigate the effect of the Chinese herb and two levels of copper in mice fed a hypercholesterolemic diet.

Materials and Methods

Animals. Thirty male weanling mice were randomly assigned to four groups. The four groups were: Low Cu diet supplemented with Chinese herb (LowCu+CH), Low Cu diet without Chinese herb (LowCu-CH), Adequate-Cu diet supplemented with Chinese herb (AdeqCu+CH), and Adequate Cu without Chinese herb (AdqeqCu-CH).

The mice were maintained on a 12-h light-dark cycle and had access to diet and deionized water ad libitum. Trace metal contamination was reduced by use of plastic housing and feeding equipment.

Diets. The diet contained 20% casein, 0.3% DL-methionine, 48.5% sucrose, 5% cellulose (Celufil), 5% corn oil (Mazola), 3.5% AIN mineral mix or 3.5% modified AIN mineral mix, 1.0% AIN vitamin mix, 0.2% choline bitartrate, 1.0% cholesterol, and 0.5% cholic acid. Using the AIN mineral mix added 7 mg Cu as cupric carbonate/kg diet while the modified mineral mix added 0.7 mg Cu/kg diet. CH was extracted with 0.1 N NH_4OH (1:20) and then adjusted to pH 7.0. Each mouse was given a 100 uL oral dose of CH or water

as a placebo by micropipette daily. After 12 weeks, mice were anesthetized and exsanguinated by heart puncture. Tissues were weighed and plasma was prepared and frozen.

Analysis. Plasma glucose and cholesterol were analyzed using enzymatic methods (Method #510 and Method #352, Sigma Chemical Co., St Louis, MO). Triglycerides also were determined enzymatically (Seragen Diagnostics, Indianapolis, IN). Erythrocyte SOD was analyzed using the method of Roth and Gilbert (Roth and Gilbert 1984).

Statistics. The experimental design was a 2 X 2 factorial with CH and Cu as factors. Data were evaluated by analysis of variance.

Results and Discussion

After 12 weeks, body and tissue weights (Table 1), plasma glucose and triglycerides, and erythrocyte superoxide dismutase (SOD) activity (Table 2) were not significantly different among groups. The plasma cholesterol concentration (Table 2) was significantly reduced by CH supplementation ($P < 0.04$). The reduction of plasma cholesterol by CH in this study confirmed the hypocholesterolemic effect of this Chinese herb in different animals (Guo and Song 1986, Niou et al 1988, Wang and Jin 1984).

In this study, the copper concentration in diet did not affect the plasma cholesterol (Table 2). Several studies (Fischer et al. 1980, Caster & Doster 1979, Geiger et al. 1984, Hunsaker et al. 1984, Lin & Lei 1981, Sherman 1981)

showed no effect on cholesterol at various copper intakes and with different Zn/Cu ratios. Dietary interactions may influence the copper effect, such as source of protein (Cohn et al. 1984, Nagata et al. 1981, Terpstra et al. 1983, Truswell 1978), amino acid composition of the protein (Sugano et al. 1982, Huff & Carroll 1980, Kritchevsky 1979), mineral interactions (Lee et al. 1983, Van Der Meer 1983, Samman & Roberts 1984), and the type of carbohydrate (Carroll and Hamilton 1975). Lactose compared to sucrose may further decrease copper status (Carville and Strain 1989). Dietary fructose can increase the signs of Cu deficiency (Lewis et al. 1990). In our study, sucrose, which is the least effective carbohydrate for creating copper deficiency was used instead of lactose or fructose. Different species also can influence the hypercholesterolemic effect in copper study. Rats are the most common animal model used to study effects of copper intake on lipids (all of the above studies). In the pig, Bruch and co-workers (1975) showed a fall in plasma cholesterol level at a constant Cu intake (1.8 ug/g) as the dietary Zn/Cu ratio decreased from 67:1 to 4:1. Eisemann and co-workers (1979) showed that there was no effect on plasma cholesterol and none of the animals became hypercholesterolemia by using Zn/Cu ratios of 0.5:1 to 73:1. The lowest level of dietary copper (8 ug/g) was four times higher than that required to create hypercholesterolemia in rats. There was no effect on plasma cholesterol of diets containing from 6 to 60 ug Cu/g and Zn/Cu ratios from 1 to

20:1 in chickens (Helwig et al. 1978). There were not many mice studies in copper and lipid research. Klevay had a study of mice fed diets containing 0.4 - 10 ug Cu/mL, plasma cholesterol did not change (Klevay 1985). In our mouse study, there was no significant difference in the lipids due to copper which might be explained if mice are an unsuitable animal model. No differences in SOD values among groups suggested that we might have failed to produce copper deficiency in this study. These results demonstrated the hypocholesterolemic effect of the Chinese herb in mice fed a hypercholesterolemic diet.

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TABLE 1

BODY, LIVER, SPLEEN, AND KIDNEY WEIGHTS
OF MICE FED HYPERCHOLESTEROLEMIC DIET[1]

Group	Body (g)	Liver (g)	Spleen (g)	Kidney (g)
-Cu-CH (8)	31.6±1.3	3.1±0.20	0.17±0.01	0.42±0.02
-Cu+CH (6)	34.1±1.5	2.5±0.18	0.12±0.02	0.39±0.02
+Cu-CH (8)	30.8±1.3	2.9±0.16	0.14±0.01	0.14±0.01
+Cu+CH (8)	33.5±1.3	2.9±0.16	0.14±0.01	0.38±0.02
Source of Variation	Analysis of Variance P Value			
Cu	0.60	0.62	0.64	0.19
CH	0.07	0.13	0.06	0.81
Cu*CH	0.97	0.16	0.11	0.32

1. Mean±SEM, N = 6-8.

TABLE 2

PLASMA GLUCOSE, CHOLESTEROL, TRIGLYCERIDE, AND ERYTHROCYTE SOD OF MICE FED HYPERCHOLESTEROLEMIC DIET[1]

Group	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	SOD (U/g Hb)
-Cu-CH (8)	289.0±16.5	182.0±8.7	69.9±5.2	881±38
-Cu+CH (6)	346.5±19.0	174.8±10.0	50.5±6.0	890±49
+Cu-CH (8)	323.6±16.5	200.0± 8.7	53.6±5.2	856±38
+Cu+CH (8)	327.3±16.5	167.5± 8.9	54.1±5.2	889±41
Source of Variation	Analysis of Variance P Value			
Cu	0.66	0.56	0.26	0.76
CH	0.09	0.04[2]	0.09	0.62
Cu*CH	0.13	0.17	0.08	0.78

1. Mean±SEM, N = 6-8.

2. Significant difference.

CHAPTER VI

THE EFFECTS OF FRACTIONS FROM A CHINESE HERB ON PLASMA GLUCOSE AND CHOLESTEROL IN MICE FED A HYPERCHOLESTEROLEMIC DIET

Abstract

High insulin potentiating activity (IPA) was found in a Chinese herb (CH). Three fractions were collected after an extract of CH was applied to G-25 Sephadex. Based on the results of our previous studies, the crude extract from CH had hypoglycemic effects. Therefore the purpose of this study was to test the effects of fractions and the combinations of fractions from CH on the metabolism of glucose and cholesterol. Two experiments were performed. In experiment 1, thirty-nine weanling male mice were randomly assigned to four groups: fraction 1, 2, or 3 supplemented or control. In experiment 2, sixty-one weanling male mice were randomly divided into six groups: fraction 1 & 2, 1 & 3, 2 & 3, 1 & 2 & 3, crude extract, or the control. All mice were fed a hypercholesterolemic diet with 1.0% cholesterol and 0.5% cholic acid. In experiment 1, low chromium diets were fed. Each mouse was given 100 ul of fraction(s) or water orally by micropipette daily. Experiment 1 and 2 were

terminated after 10 and 9 weeks, respectively. The results showed that body weight was significantly decreased by fraction 1. The tissue weights were not affected. The mean plasma glucose was decreased significantly by fraction 1, 1 & 2, and 1 & 3. Mean plasma insulin was significantly decreased by fraction 1 & 2, 1 & 3, 2 & 3, and 1 & 2 & 3. The glucose/insulin ratio was increased significantly by fraction 1 and 1 & 3. No fraction(s) had hypocholesterolemic effects. Fraction 1 alone, 1 & 2, and 1 & 3 had a hypoglycemic effect.

INDEXING KEY WORDS:

. Chinese herb . insulin potentiating activity
. glucose . cholesterol . glucose/insulin ratio

Insulin potentiating activity is high in a Chinese herb (CH) which has been used as a hypocholesterolemic medicine in China. In animal studies, CH showed hypocholesterolemic effects in pigeons, rats, and mice (Guo and Song 1986, Niou et al. 1988, Cheng et al. 1988, Cheng and Stoecker 1990). Mean serum cholesterol decreased significantly after 2 months of supplementations with CH or nicotinic acid compared with the control group ($P < 0.001$) in a study of eighty pigeons fed a hypercholesterolemic diet (Guo and Song 1986). In a continuing study in pigeons, CH supplementation again decreased serum cholesterol significantly ($P < 0.025$) compared with the control group (Guo and Song 1986). CH in

the form of extract or powder also was supplemented to rats fed hypercholesterolemic diets (2% cholesterol, 0.5% cholic acid) (Niou et al. 1988). After 17 days, mean serum cholesterol in rats fed either extract or powder of CH was decreased significantly ($P < 0.05$) compared with the control group (Niou et al. 1988). In a study of mice fed normal diets, mean serum cholesterol was decreased significantly by CH extract
($P < 0.02$) after 6 weeks (Cheng et al. 1988). In another study in which mice were fed a hypercholesterolemic diet for 12 weeks, mean plasma cholesterol was again significantly ($P < 0.04$) decreased (Cheng and Stoecker 1990).

Because of the hypocholesterolemic effect of CH, further study is needed to investigate the components of CH. The purpose of this study was to test the effects of different fractions of CH separated on Sephadex G-25 on glucose and cholesterol in mice fed hypercholesterolemic diets.

Materials and Methods

Animals.

Experiment 1: Thirty-nine male weanling mice were randomly assigned to four groups: fraction 1, 2, 3, or the control.

Experiment 2: Sixty-one male weanling mice were randomly assigned to six groups: fraction 1 and 2, fraction

1 and 3, fraction 2 and 3, fraction 1 & 2 & 3, crude extract, or control.

Diet and housing. The diet contained 20% casein, 0.3% DL-methionine, 48.5% sucrose, 5% cellulose (Celufil), 5% corn oil (Mazola), 3.5% modified AIN mineral mix. 1.0% AIN vitamin mix, and 0.2% choline bitartrate, 1.0% cholesterol, and 0.5% cholic acid. In experiment 1 chromium was omitted from the hypercholesterolemic diet. In experiment 2 the AIN mineral mix containing chromium was used to formulate the hypercholesterolemic diet. Each mouse was given 100 ul of a fraction of CH, or the combination of fractions, or water orally by micropipette daily.

The mice were maintained on a 12-h light-dark cycle and had access to diet and deionized water ad libitum. Trace metal contamination was reduced by using of plastic housing and feeding equipment.

Experiment 1 was finished after 10 weeks. Experiment 2 was terminated after 9 weeks. Mice were anesthetized and exsanguinated by heart puncture. Tissues were weighed and plasma (syringes were rinsed by 10,000 units/ml EDTA solution) was prepared and frozen.

Extraction. Chinese herb (CH) was stirred with an excess 20:1 of 0.1 N NH4OH for 2 hr (w/v) and then centrifuged at 1,000 g for 20 min. The supernatant was applied to a Sephadex G-25 column (50-150 u particle size, 60 x 2.6 cm). Three fractions were collected and evaporated at room temperature to 1/3 of their original volume for

experiment 1 and to 1/10 of their original volume for experiment 2. Each combination of fractions was prepared by mixing the appropriate fractions and equal concentrations were maintained.

Analysis. Plasma glucose (Kadish et al. 1968) and cholesterol (Allain et al. 1974) were analyzed using enzymatic methods. Insulin was analyzed by radioimmunoassay (Crowley and Garbien 1974).

Results

The results showed that in experiment 1 body weight was significantly decreased by fraction 1 compared with the control group ($P < 0.01$) (Table 1). In experiment 2 none of the combinations of fractions affected body weight (Table 3). In experiment 1 kidney weight was decreased by fraction 3 alone compared to the control group ($P < 0.04$) (Table 1). Spleen and liver weights were not affected by single fraction supplementation (Table 1). The weights of liver, kidney, and spleen were not affected by the combinations of fractions (Table 3).

The mean plasma glucose in the fraction 1 supplemented group (245 mg/dl) was significantly decreased compared with the control group (294 mg/dl) ($P < 0.04$) (Table 2). The mean plasma glucose in mice supplemented with combinations of fraction 1 & 2 (195 mg/dl) or fraction 1 & 3 (188 mg/dl) was significantly decreased ($P < 0.04$ and $P < 0.02$, repectively) compared to the control group (Table 4).

The mean plasma insulin concentrations in groups supplemented with combinations of fraction 1 & 2, fraction 1 & 3, fraction 2 & 3, or fraction 1 & 2 & 3 were all significantly lower than the control group (Table 4). The plasma glucose/insulin (G/I) ratio of the fraction 1 group (32.9) was higher than the control (20.1) group ($P < 0.05$) (Table 3). The plasma glucose/insulin ratio of fraction 1 & 3 group (226.5) was much higher than the control group (7.5) ($P < 0.005$) (Table 4). Five animals in this study had plasma insulin concentrations that were not distinguishable from zero. Three of these animals were in the group supplemented with the combination of fractions 1 & 3. Thus, we are not certain if the high G/I ratio in this group is a real effect of fraction 1 & 3 or an artifact.

No fraction alone nor the combinations of fractions decreased plasma cholesterol. The mean plasma cholesterol concentration in mice fed fraction 2 & 3 (240 mg/dl) was significantly ($P < 0.01$) higher than the control group (194 mg/dl) (Table 4).

Discussion

In contrast to the crude extract from CH, none of the fractions or the fraction combinations showed a hypocholesterolemic effect. The combination of fraction 2 and 3 even increased plasma cholesterol. The hypocholesterolemic effect from CH does not occur with supplementation of any of the single fractions or

combinations of fractions. The active hypocholesterolemic substance might become inactive through the purification procedure. Further study is needed to try to purify the hypocholesterolemic substance through the proper column, buffer, and pH. This study showed the combination of fraction 2 and 3 had a negative impact and actually raised plasma cholesterol.

In this study, a significant hypoglycemic effect was found when fraction 1 was supplemented alone. The combination of fraction 1 and 2 or fraction 1 and 3 also lowered plasma glucose significantly. This might be explained by insulin potentiation. Because CH had high insulin potentiating activity (IPA) in vitro, we expected a hypoglycemic effect in the animal study. But the crude extract from CH had a hypocholesterolemic effect (Cheng et al. 1988, Cheng and Stoecker 1990) instead of a hypoglycemic effect. In this study, fraction 1, fraction 1 and 2, fraction 1 and 3 again had a hypoglycemic effect instead of a hypocholesterolemic effect. The mechanism is not clear; perhaps the crude extract was not pure enough to be able to strongly affect glucose metabolism producing a hypoglycemic effect in animals. Fraction 1 alone and the combination of fraction 1 & 3 had the highest G/I ratio. This suggests that fraction 1 or the combined form of fraction 1 and 3 potentiate insulin sensitivity.

Fraction 1 also had lowest weight gain compared with the control group. To know the mechanism, further study is needed.

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TABLE 1

WEIGHT GAIN AND TISSUE WEIGHTS IN MICE SUPPLEMENTED WITH FRACTION 1, 2, OR 3 OF CH (EXPERIMENT 1)[1]

Group	Wt gain (g)	Liver wt (g)	Kidney wt (g)	Spleen wt (g)
Frct 1	9.8±0.7	2.06±0.11	0.30±0.02	0.13±0.01
Frct 2	11.4±0.7	2.11±0.11	0.30±0.02	0.14±0.01
Frct 3	11.6±0.7	2.17±0.11	0.28±0.02	0.13±0.01
Control	12.8±0.8	2.32±0.12	0.33±0.02	0.14±0.01
Sources of variation	Analysis of variance P value			
F1 vs Ct	0.01[2]	0.12	0.22	0.36
F2 vs Ct	0.20	0.20	0.19	0.55
F3 vs Ct	0.29	0.36	0.04[2]	0.25

1. Values are means ± SEM; n = 9-10.

2. Significant difference from control group.

TABLE 2

PLASMA GLUCOSE, INSULIN, G/I RATIO AND CHOLESTEROL IN MICE SUPPLEMENTED WITH FRACTION 1, 2, OR 3 OF CH (EXPERIMENT 1)[1]

Group	Glucose (mg/dl)	Insulin (uU/ml)	G/I (ratio)	Cholesterol (mg/dl)
Fract 1	244.8±16.2	8.5±5.0	32.9±4.1	243.6±15.2
Fract 2	257.0±18.0	14.9±5.5	24.0±4.6	261.7±16.0
Fract 3	249.4±16.2	30.4±4.9	18.4±4.1	238.1±15.2
Control	293.9±17.0	16.5±5.5	20.1±4.6	247.5±16.0

Source of variation	Analysis of Variance P Value			
F1 vs Ct	0.04[2]	0.29	0.05[2]	0.86
F2 vs Ct	0.15	0.83	0.55	0.53
F3 vs Ct	0.07	0.07	0.78	0.67

1. Values are means ± SEM; n = 9-10.

2. Significant difference from control group.

TABLE 3

MEAN BODY WEIGHT AND TISSUES WEIGHTS OF MICE SUPPLEMENTED WITH THE COMBINATIONS OF FRACTIONS OF CH (EXPERIMENT 2)[1]

Group	Weight Gain (g)	Liver (g)	Kidney (g)	Spleen (g)
Fra 1&2	7.9±0.8	2.4200±0.0905	0.3163±0.0253	0.1301±0.2785
Fra 1&3	7.6±0.8	2.4749±0.0905	0.3247±0.0253	0.1263±0.2785
Fra 2&3	6.0±0.8	2.5293±0.0905	0.3239±0.0253	0.1515±0.2785
Fra 1&2&3	7.8±0.8	2.4599±0.0905	0.3371±0.0253	0.1279±0.2785
Crude extr	7.4±0.7	2.4175±0.0863	0.3153±0.0253	0.1292±0.2655
Contr	7.6±0.7	2.3379±0.0863	0.3557±0.0241	0.8073±0.2785

1. Values are means ± SEM, n = 10-11.

TABLE 4

MEAN PLASMA GLUCOSE, INSULIN, G/I RATIO, AND CHOLESTEROL IN MICE SUPPLEMENTED WITH COMBINATIONS OF FRACTIONS FROM CH (EXPERIMENT 2)[1,2]

Group	Glucose (mg/dl)	Insulin (uU/ml)	G/I (ratio)	Cholesterol (mg/dl)
F1&2	194.6±10.8[a]	13.9±2.6[e]	98.7±53.2	209.7±13.2
F1&3	188.3±11.4[b]	11.2±2.7[e]	226.5±56.1[d]	225.1±14.0
F2&3	197.6±10.8	15.1±2.6[e]	56.6±53.2	240.0±13.2[c]
F1&2&3	211.8±10.8	21.8±2.6[e]	12.3±53.2	197.4±13.2
Extr	214.7±10.3	24.9±2.4	9.1±50.7	196.4±12.6
Cont	225.2±10.3	31.4±2.4	7.5±50.7	193.6±12.6

1. Values are means ± SEM; n = 10-11.

2. The significant differences from control (a: $P < 0.05$; b: $P < 0.02$; c: $P < 0.01$; d: $P < 0.005$; e: $P < 0.001$).

CHAPTER VII

THE EFFECTS OF A FRACTION FROM CHINESE HERB ON PLASMA GLUCOSE AND CHOLESTEROL IN OBESE MICE FED A HYPERCHOLESTEROLEMIC DIET

Abstract

Three fractions were separated through a medium G-25 Sephadex column from CH, a Chinese herb which has high insulin potentiating activity (IPA). The purpose of this study was to test the effects of fraction 1, which had the highest IPA, on glucose and cholesterol metabolism in obese mice. Nineteen male obese weanling mice were randomly assigned to two groups: fraction 1 supplemented group and control group. Mice were fed a casein-based hypercholesterolemic diet containing 1.0% cholesterol and 0.5% cholic acid. Each mouse was given 100 ul of fraction 1 or water orally by micropipette daily. After 7 weeks, neither body weights nor liver weights were affected by fraction 1. Both kidney and spleen weights were increased significantly by fraction 1 supplementation. The mean values of plasma glucose, insulin and glucose/insulin (G/I) ratio were not affected by fraction 1. However, the mean plasma cholesterol in the fraction 1 supplemented group (169 $\pm$ 39

mg/dl) was significantly lower ($P < 0.003$) than the control group (237 ± 47 mg/dl).

INDEXING KEY WORDS:

. Chinese herb . insulin potentiating activity (IPA)
. glucose . insulin . cholesterol . G/I ratio

Insulin potentiating activity (IPA) was checked in twenty-four Chinese herbs (Cheng et al. 1988) which have been used as hypoglycemic and hypocholesterolemic agents for centuries in China. CH had the highest IPA among these Chinese herbs. However, there have been only a few reports of the effects of CH on lipid metabolism.

An effect of CH on cholesterol in eighty pigeons fed a hypercholesterolemic diet was reported by Guo and Song in 1986 (Guo and Song 1986). After 2 months of supplementation, the results showed that mean serum cholesterol of the CH or the nicotinic acid supplemented groups was significantly decreased compared with the control group.

The effects of CH on cholesterol, several enzymes, and pathological changes were investigated in thirty-nine rats fed either normal or hypercholesterolemic diets (Niou et al. 1988). After 17 days, serum cholesterol concentrations in both groups supplemented with CH extract or CH powder were decreased significantly compared to the group fed the hypercholesterolemic diet alone. The hepatic ATPase and SDH (succinate dehydrogenase) in CH supplemented groups were

lower than in the control group. The hepatic G-6-P (glucose-6-phosphate) in the CH powder supplemented group was lower than the other groups. There were many neutral fat drops in the lobules of the liver in the mice fed a hypercholesterolemic diet without CH supplementation. Both CH supplemented groups had very few fat drops and were almost like the normal diet group.

Effects of various doses of CH on lipidemic and atherosclerotic changes were tested in forty-six Japanese quail fed a hypercholesterolemic diet for 6 weeks (Wang and Jin 1984). The results showed that plasma HDL-cholesterol and HDL-cholesterol/total cholesterol ratios in groups supplemented with three levels of CH were increased compared to the control group. Plasma cholesterol, triglyceride, and cholesterol esters in the three CH-supplemented groups were decreased compared to the control group. Atherosclerotic changes in the aorta with the three levels of CH supplementation were less than in the control group, especially with the large dose of CH.

Two studies of mice fed either normal or hypercholesterolemic diets both showed that serum cholesterol was significantly decreased by CH extract (Cheng et al. 1988, Cheng and Stoecker 1990).

CH was further separated through G-25 Sephadex and three fractions were collected. High IPA was found in fraction 1 (Cheng and Stoecker 1992). Each fraction and the

combinations of fractions were investigated in mice fed hypercholesterolemic diets. The results showed that fraction 1 or the combinations containing fraction 1 caused a hypoglycemic effect instead of the hypocholesterolemic effect previously obtained from the crude CH extract (Cheng et al. 1988, Cheng and Stoecker 1990). The purpose of this study was to investigate a possible hypoglycemic effect with fraction 1 of CH in older obese mice.

Materials and Methods

Animals and diets. Nineteen obese male mice (51 days old) were randomly assigned to two groups: fraction 1 or control. The diet contained 20% casein, 0.3% DL-methionine, 48.5% sucrose, 5% cellulose (Celufil), 5% corn oil (Mazola), 3.5% AIN mineral mix or 3.5% modified AIN mineral mix, 1.0% AIN vitamin mix, 0.2% choline bitartrate, 1.0% cholesterol, and 0.5% cholic acid. Chinese herb (CH) was extracted with 0.1 N NH_4OH (1:20) and centrifuged (1,000 x g). The supernatant was applied to a Sephadex G-25 column (50-150 u of particle size, 60 x 2.6 cm). Three fractions were separated. The first fraction was collected and evaporated to one-third of its original volume at room temperature. Each mouse was given 100 ul of fraction 1 of CH or water orally by micropipette daily. The mice were maintained on a 12-h light-dark cycle and had access to diet and deionized water ad libitum. Trace metal contamination was reduced by use of plastic housing and feeding equipment. After 7 weeks,

mice were fasted 14 hr and then were given an oral glucose load (1 mg glucose/g body weight as a 50% solution) 60 minutes before sacrifice. Mice were anesthetized and exsanguinated by heart puncture. Tissues were weighed and plasma was prepared and frozen.

Analysis. Plasma glucose (Kadish et al. 1968) and cholesterol (Allain et al. 1974) were analyzed using enzymatic methods. Insulin was analyzed by radioimmunoassay (Crowley and Garbien 1974).

Results and Discussion

After 7 weeks, body weights and liver weight were not affected by feeding fraction 1 (Table 1). Both kidney and spleen weights were increased significantly in the supplemented group (Table 1). The group supplemented with fraction 1 of CH had higher spleen weight than the control group. A study reported that the supplementation of CH in mice caused proliferation in the T lymphatic cell-dependent area (including thymus and lymphatic cells around the central artery in the spleen) and in the B lymphatic cell-dependent area (including the white pulp and germinal center in the spleen) (Guo and Song 1986). These results suggested a positive effect of CH in the immune system. The spleen weight increase in the fraction 1 supplemented group might be caused by the proliferation of lymphatic cells. The basis for an effect on kidney weight by fraction 1 of CH is

unknown. No mention of such an effect was found in the literature, indicating a need for further study.

Mean plasma glucose, insulin and glucose/insulin (G/I) ratio were not affected by fraction 1 (Table 2). However, the mean plasma cholesterol in the group supplemented with fraction 1 (169 $\pm$ 39 mg/dl) was significantly lower ($P < 0.003$) than the control group (237 $\pm$ 47 mg/dl) (Table 2). In this study, fraction 1 played the same role as the crude extract of CH (Cheng et al. 1988, Cheng and Stoecker 1990) which was to produce a hypocholesterolemic effect. The same hypocholesterolemic effect by CH was reported in pigeons, rats and Japanese quail (Guo and Song 1986, Niou et al. 1988, Wang and Jin 1984). A study in rats showed that SDH decreased as serum cholesterol decreased with CH supplementation (Niou et al. 1988). The lower plasma cholesterol with the supplementation of CH also can be explained by the conversion of cholesterol to cholic acid (Xu and Li 1987). (7-^{3}H)-cholesterol was added to hepatic cells with 3 levels of CH in vitro. The results showed that the lowest concentration had the most effect on increasing conversion of cholesterol to cholic acid.

Fraction 1 did not have a hypoglycemic effect in the older obese mice as it did in the weanling lean mice. There were many differences, such as weight, age, diets, length of the experiment, and blood sampling times. More study needs to be done to understand the mechanism of hypocholesterolemic effect by CH in obese mice.

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TABLE 1

BODY, LIVER, KIDNEY, AND SPLEEN WEIGHT
IN OBESE MICE SUPPLEMENTED
WITH FRACTION 1 OF CH[1]

GROUP	WEIGHT GAIN (g)	LIVER (g)	KIDNEY (g)	SPLEEN (g)
FRACTION 1	6.2±1.6	3.95±.88	0.39±0.05[2]	0.08±0.01[2]
CONTROL	6.8±2.7	4.28±.95	0.35±0.04	0.07±0.01
P VALUE	0.6	0.4	0.04[2]	0.0006[2]

1. Values are means ± SEM; n = 10.

2. Significant difference from control group.

TABLE 2

MEAN VALUE OF GLUCOSE, INSULIN, G/I RATIO, AND CHOLESTEROL IN OBESE MICE SUPPLEMENTED WITH FRACTION 1 OF CH[1]

GROUP	GLUCOSE (mg/dl)	INSULIN (uU/ml)	I/G (ratio)	CHOLESTEROL (mg/dl)
FRACTION	393.6±72.3	63.7±22.3	7.3±4.0	168.7±39.0
CONTROL	417.3±36.1	79.3±25.6	5.7±0.1	237.1±46.9
P Value	0.4	0.2	0.6	0.003[2]

1. Values are means ± SEM; n = 9 -10.

2. Significant difference from control group.

CHAPTER VIII

CHROMIUM INTERACTION WITH NUCLEIC ACIDS IN RAT LIVER

Abstract

Effects of chromium on nucleic acid metabolism both at nutritional and toxicological concentrations have been observed. The chromium and nucleic acid concentration of rat liver extracts from rats fed 3 levels of dietary chromium were monitored to determine the effects of dietary chromium on nucleic acids in rats. Male Sprague Dawley rats were fed a low chromium diet (30 ug Cr/kg) or the same diet supplemented with 1 or 100 mg chromium as CrCl3 per kg of diet. Liver extracts from 8 week old rats were chromatographed on Sephadex G-15. Elution profiles of RNA, DNA and protein from rats consuming either the low chromium diet or the diet supplemented with 1 mg chromium per kg of diet were similar. Chromium was associated with both the RNA and DNA fractions as well as the high molecular weight (> 1500 daltons) material. Liver extracts of animals supplemented with 100 mg/Cr per kg diet displayed increased chromium associated with fractions of molecular weight greater than 1500 but low molecular weight fractions were

similar to those from animals fed the lower chromium diets. Liver chromium concentrations of animals fed the low chromium diet and of those supplemented with 1 mg chromium per kg of diet were similar. The mean DNA concentration of livers from animals fed the low chromium diet supplemented with 100 ug chromium was greater than DNA concentrations of livers from animals fed the low chromium diet or the low chromium diet plus 1 mg chromium/kg diet. The binding of chromium to DNA may have an effect on DNA stability leading to decreased degradation and therefore elevated concentrations. However, a direct effect of chromium on DNA synthesizing or degradative enzymes cannot be excluded. These results confirm that chromium is associated with nucleic acids. Dietary chromium alters the chromium in fractions with a molecular weight greater than 1500 daltons and dietary chromium also appears to have an effect on DNA concentration.

INDEXING KEY WORDS:

. Chromium . nucleic acids . RNA . DNA

Introduction

An interaction of chromium with nucleic acids was first proposed by Herrman and Speck in 1954. They suggested that the treatment of tissues with chromium or chromate reduced the amount of nucleic acids (Herrmann and Speck 1954). Later Wacker and Vallee found that chromium bound to nucleic acids

so tightly that even ethylenediamine tetraacetic acid (EDTA) (10-4 M) couldn't remove it. They also detected chromium concentrations in tissue (Wacker and Vallee 1959). Recently the interaction between chromium and nucleic acids has been investigated in terms of toxicology and physiology. Tamino and co-workers showed that Cr (III) in vitro bound to mammalian DNA and RNA (Tamino et al. 1981). Okada and collaborators have shown that trivalent chromium added to DNA and RNA synthesizing systems in vitro significantly stimulated RNA synthesis (Okada et al. 1982, 1983, 1984). They also postulated that chromium is present in regenerating rat liver in vivo in the form of a chromium-protein of approximately 70,000 daltons containing between 4 and 5 atoms of chromium, corresponding to approximately 3,600 ug chromium/g protein; the increase in RNA synthesis with Cr(III)-bound DNA is proportional to the molar binding ratio of Cr(III) to DNA (Okada et al. 1982, 1983, 1984). This enhancement is possibly due to an increase in the chromatin and causes an increase in the number of initiation sites on DNA (Okada et al. 1982, 1983, 1984). The present study was designed to reexamine the validity of reported chromium concentrations in tissue and to investigate possible associations between RNA, DNA and dietary chromium under carefully controlled conditions.

Materials and Methods

Animals and diets. Male Sprague Dawley rats, 45-50g, were divided into three groups and fed either a low Cr diet or a low Cr diet plus either 1 mg Cr or 100 mg Cr as chromium chloride per kg of diet. Chromium concentration of the low Cr diet was approximately 30 ng/g. After eight to ten weeks, rats were sacrificed and livers were removed, rinsed, and stored on ice or frozen at -20° C. Cold liver samples were homogenized with an equal weight of water and centrifuged at 10,000 rpm for 15 minutes. The supernatant was applied to a Sephadex G-15 column and eluted with water. RNA was determined by an orcinal methhod (Keleti and Lederer 1974), DNA by a diphenylamine procedure (Keleti and Lederer 1974) and protein by the Biorad method (Bio-Rad Protein Assay Kit). Chromium was determined by atomic absorption spectroscopy as previously described (Anderson and Kozlovsky 1985).

Results

The elution profiles of RNA, DNA, protein and chromium were all similar in liver extracts from animals consuming either the low Cr diet or the low Cr diet plus 1 mg Cr per kg of diet (Figure 1 & 2).

Chromium concentration of the hepatic nucleic acids was 5 $\pm$ 1 ug/g dry weight of liver in the animals fed the low Cr diet and the low diet supplemented with 1 ug Cr/g diet.

The increased amount of Cr was primarily associated with the high molecular weight material. The elution pattern and amount of the low molecular weight chromium were similar in the liver extracts from animals raised on a low Cr diet and also the low Cr diet supplemented with either 1 or 100 mg Cr per kg of diet (Figure 1 & 2).

The amount of DNA in the livers from animals fed the low chromium diet supplemented with 100 mg Cr/kg diet appeared to be greater than that of livers from animals fed the low Cr diet or the low Cr diet plus 1 mg Cr/kg diet (Figure 1 & 2).

Discussion

The results presented here support previous observations that chromium is associated with nucleic acids. The chromium concentrations obtained in the present study, under careful control of contamination, are lower than those reported earlier but are still much in excess of chromium concentrations in other materials. The chromium concentration in blood, for example, is close to 1 ng/g dry weight, approximately three orders of magnitude lower than the concentrations in nucleic acids reported here.

As shown in Figure 1, our procedure did not separate the large size nucleic acids from accompanying protein. A nucleoprotein from horse liver has been reported previously to contain very high chromium concentrations. Alternately, much of the chromium could be part of a specific, chromium-

binding protein, such as demonstrated by Okada and his co-workers (Ohba et al 1986).

The elution profile of RNA, DNA, protein and chromium were all similar in liver extracts from animals consuming either the low Cr diet or the low Cr diet plus 1 mg Cr per kg of diet. The elution of Cr was greater in the first peak of liver extracts from the animals supplemented with 100 mg Cr/kg diet compared to the low Cr and diet supplemented with 1 mg Cr/kg diet. Apparently the low molecular weight Cr compounds are regulated at relatively constant levels while the excess Cr is associated with complexes with a molecular weight of 1500 daltons or greater.

The elevated DNA concentration in the animals supplemented with the highest levels of chromium, although reproducible, needs to be studied further. The binding of chromium to DNA may have an effect on DNA stability leading to decreased degradation and therefore elevated concentrations. However, a direct effect of DNA on synthetic or degradative enzymes cannot be excluded.

These results confirmed that chromium is associated with nucleic acids amd that DNA and RNA fractions do contain substantial amounts of chromium. Dietary Cr alters the Cr in fractions with a molecular weight greater than 1500 daltons. Dietary Cr appears to affect DNA concentration.

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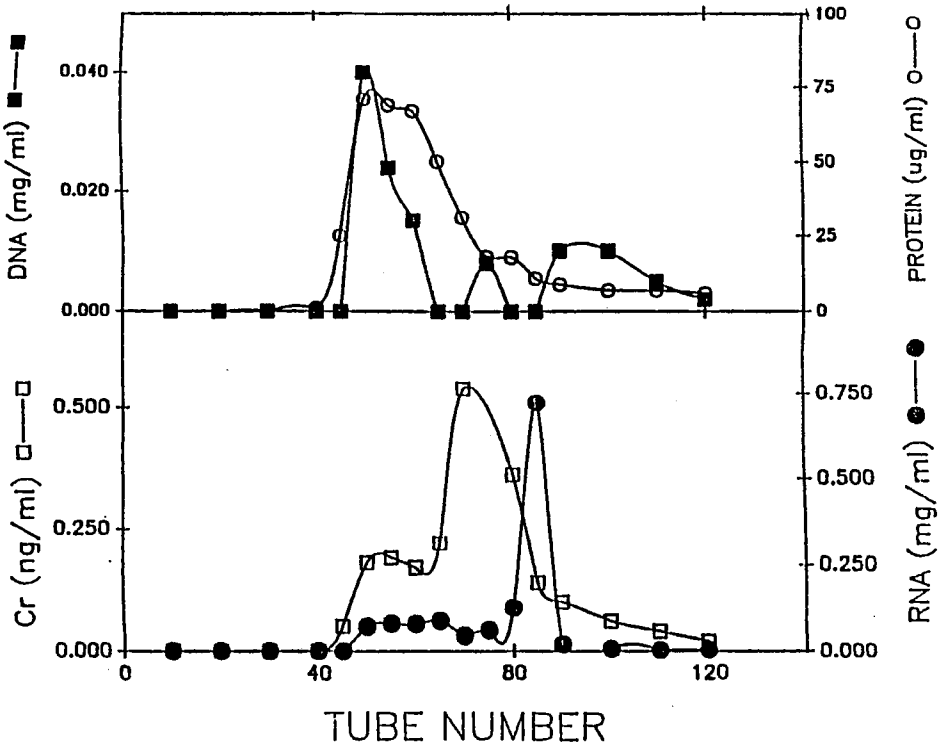

Figure 1. The Elution Profile of Liver Extracts From Rats Fed the Low Cr Diet

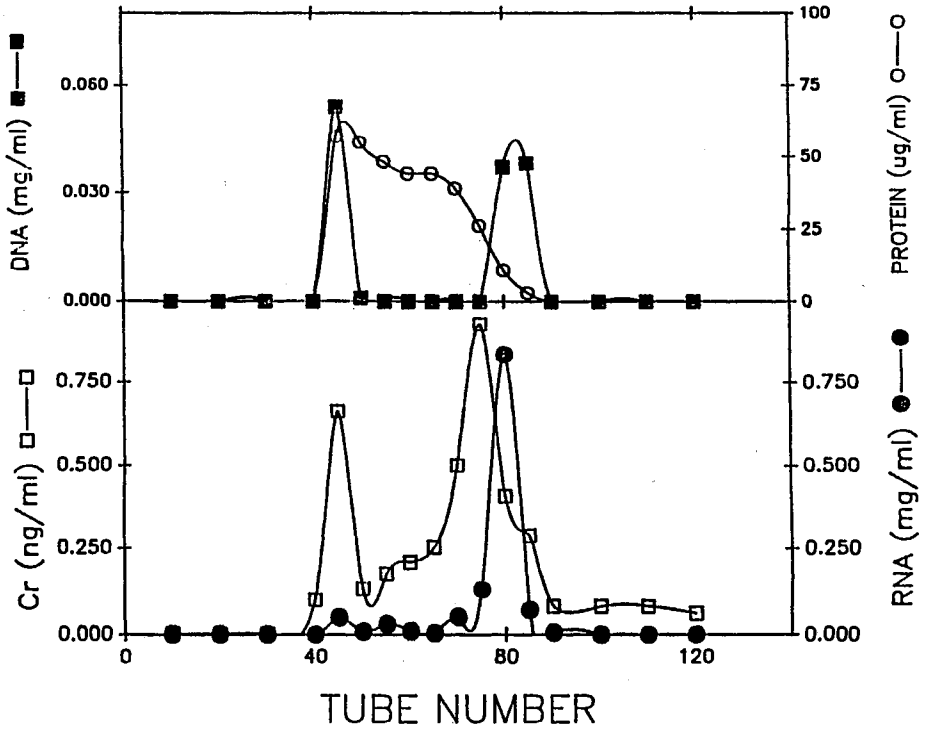

Figure 2. The Elution Profile of Liver Extracts From Rats Fed the Low Cr Diet Plus 100 ppm Cr

CHAPTER IX

EFFECTS OF CHROMIUM DEPLETION ON HEPATIC NUCLEIC ACIDS IN GUINEA PIGS

Abstract

Effects of dietary Cr and ascorbate on several parameters including growth and nucleic acids were investigated in guinea pigs. Thirty-six male weanling (181 g) Hartley animals were grouped in a 2 x 2 factorial design. Diets contained <60 ppb Cr (-Cr) or were supplemented with 2 ppm Cr as CrCl3 (+Cr). Animals were given an oral supplement of 1 mg (-C) or 10 mg (+C) ascorbate daily. Groups were -Cr-C, -Cr+C, +Cr-C, and +Cr+C. At 21 weeks the mean body weight of the Cr-depleted animals was significantly reduced ($P < 0.0003$). Mean weight of pancreas, liver, testes and epididymal fat were also significantly lower with Cr depletion. Ascorbate depletion, however, increased liver weight. Hepatic DNA of the Cr-depleted groups was lower (1.7 mg DNA/g liver) than the Cr-supplemented animals (2.58 mg/g) ($p < 0.005$). Hepatic RNA was similarly reduced by Cr depletion (8.89 mg/g vs. 10.57 mg/g) ($P < 0.03$). Chromium depletion decreased growth apparently through an effect on nucleic acids.

INDEXING KEY WORDS:

. chromium . nucleic acids . RNA . DNA . growth

Introduction

In 1954 Herrmann and Speck reported that the treatment of tissues with chromiun or chromate reduced the amount of nucleic acids (Herrmann and Speck 1954). Wacker and Vallee (1959) detected chromium in tissue and found that the concentration was very high. They also found that chromium tightly bound to nucleic acids and was not able to be removed by repeated treatment with ethylenediaminetetraacetic acid (EDTA). Edwards et al. reported that chromium accumulated in cell nucleic in rat liver (Edwards et al 1961). Andronikashivili and co-workers found that DNA isolated from rat sarcoma and human leukemic cells showed a high concentration of chromium (Andronikashivili et al. 1974, 1976). Tamino et al. indicated that chromium (III) in vitro binds to mammalian DNA and RNA (Tamino et al. 1981). Ohba and co-workers (1986) have reported that in vitro RNA synthesis was stimulated by the complex of Cr(III)-bound DNA. All these studies suggested an effect of Cr (III) on nucleic acid metabolism.

This study was designed to investigate the effects of dietary chromium and ascorbic acid depletion on several parameters including growth and nucleic acids. Guinea pigs

were used because they have a requirement for vitamin C in the diet.

Materials and Methods

Animals and diets. Thirty-six Hartley male guinea pigs (Sasco, Inc., Omaha, NE) with a mean body weight of 181 g were randomly assigned to chromium and ascorbate treatment groups in a 2 x 2 factorial design. The four groups were: ascorbate depletion and chromium supplementation; ascorbate supplementation and chromium supplementation; ascorbate depletion and chromium depletion; ascorbate supplementation and chromium depletion.

The diet contained (g/kg): 370 dextrose, 300 casein, 30 L-arginine HCl, 150 cellulose (Celufil), 70 corn oil, 50 mineral mix, 35 potassium acetate and 22 vitamin mix without ascorbate. The mineral mix contained (g/kg): 600 $CaHPO_4$, 80 NaCl, 100 MgO, 0.90 $ZnCO_3$, 1.8 $MnCO_3$, 0.25 $CuCO_3$, 0.035 KIO_3, 0.0044 $NaSeO_3.5H_2O$, 1.42 $FeSO_4$ and a quantity of sucrose sufficient to make 1 kg. Diets contained < 60 ug Cr/kg diet (-Cr) or were supplemented with 2 mg Cr as $CrCl_3$/kg diet (+Cr). The diet was mixed with deionized water to form balls approximately 35 mm in diameter. Animals were dosed orally with 1 mg ascorbate/day (-Cr) or with 10 mg/day (+Cr).

Animals had ad libitum access to food and deionized water from ceramic cups. They were housed in plastic cages to reduce trace mineral contamination.

Guinea pigs were weighed weekly. The study was terminated after 21 weeks. Prior to necropsy, animals were weighed and fasted overnight. Six hours before sacrifice, each guinea pig was dosed by micropipette with 90 ul (1.8 uCi of L-(carboxyl-^{14}C) ascorbate (for the ascorbate study) and placed in a sealed metabolic cage with a pumped air and exhaust. Animals were anesthetized in the metabolism cages with nitrous oxide gas and then exsanguinated by heart puncture. Liver samples were frozen immediately in liquid nitrogen. Hepatic RNA, DNA and protein were separated based on the preferential solubility of nucleic acids in hot trichloroacetic acid (Keleti and Lederer 1974). Hydrolyzed RNA was estimated by addition of orcinal reagent (Keleti and Lederer 1974). DNA was estimated using the diphenylamine reaction (Keleti and Lederer 1974).

Analysis. The Statistical Analysis System (SAS) was used to evaluate treatment effects. Data were analyzed as a 2 x 2 factorial design. The generalized linear model (GLM) was used for analysis of variance and least squares means determination.

Results and Discussion

The mean body weight of the chromium supplemented group was higher ($P < 0.0003$) than the Cr-depleted group. Roginski and Mertz suggested several years ago that chromium affected growth through protein synthesis (Roginski and Mertz 1967).

Mean hepatic RNA and DNA of the Cr-supplemented group

were higher than the Cr-depleted group ($P < 0.05$). The mean hepatic DNA of the ascorbate supplemented group tended to be higher than the ascorbate-deprived group ($P < 0.07$) (Table 1). The concentration of hepatic RNA (8-12 mg/g) in our study was higher than the hepatic RNA value (5-8 mg/g) reported by Vary and Kimball (Vary and Kimball 1992). Okada and co-workers reported that RNA synthesis was stimulated by the Cr(III)-complexes of DNA (Okada et al 1981). The effect of Cr(III) on RNA synthesis by DNA and chromatin isolated from mouse liver was investigated in comparison with seven other inorganic metals. Cr(III) showed significant stimulation on RNA synthesis when incubated with DNA or chromatin prior to the addition of RNA polymerase. The Cr(III)-complexes of DNA and chromatin also showed significantly enhanced template activities (Okada et al 1981) and caused an increase in the number of initiation sites (Ohba et al. 1986). The enhancement of RNA synthesis with Cr(III)-bound DNA is proportional to the molar binding ratio of Cr(III) to DNA, and is possibly due to an increase in the number of initiation sites on DNA (Okada et al, 1982). The enhancement of RNA synthesis in mouse liver was without altering the pool size of nucleotides (Okada et al. 1983). These results of previous studies might explain the increasing RNA content in our study.

In contrast with our result of DNA content, the previous study showed that the synthesis of DNA in mouse liver were not significantly changed by $CrCl_3$ administration

(Okada et al. 1983). But our guinea pig study lasted for 20 weeks instead of 48 hr as in Okada's study. Our previous study in rats for 8 weeks also showed DNA enhancement with Cr supplementation (Cheng et al. 1990). These results suggested that the Cr-bound DNA might play an important role in DNA stability causing the elevated concentrations. Our results suggested that long-term chromium supplementation in animals resulted in enhancement of DNA content.

In our study, body weights and RNA and DNA concentrations were all increased significantly in chromium supplemented groups. Combining these two results, one conclusion may be drawn: chromium may affect protein synthesis through an effect on nucleic acids.

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TABLE 1

WEIGHT GAIN AND HEPATIC RNA AND DNA OF GUINEA PIGS[1]

Group	Body Weight (g)	RNA (mg/g liver)	DNA (mg/g liver)
-Cr-C	653 ± 26	9.11 ± 0.72	1.68 ± 0.31
-Cr+C	618 ± 23	8.67 ± 0.67	2.07 ± 0.28
+Cr-C	752 ± 25	9.74 ± 0.67	2.15 ± 0.30
+Cr+C	715 ± 25	11.41 ± 0.67	2.88 ± 0.30
Source of Variation	Analysis of Variance P Value		
Cr	0.0003[2]	0.02[2]	0.05[2]
C	0.16	0.35	0.07
Cr*C	0.98	0.13	0.57

1. Mean ± SEM, n=8-10.

2. Significant difference.

CHAPTER X

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

This research evaluated the effects of chromium on the metabolism of lipids, glucose, and nucleic acids. CH, a Chinese herb which has high insulin potentiating activity, investigated for its effects on the metabolism of glucose, lipids, and insulin.

Eight objectives and hypotheses are listed in the introduction of this dissertation. The significance level is set at $P < 0.05$. Each hypothesis will be addressed individually; then other general conclusions and recommendations will be discussed.

Hypothesis one stated that there will be no significant differences in blood glucose or cholesterol between Cr-supplemented and Cr-depletion groups in mice. Hypothesis one is accepted because blood glucose and cholesterol were not changed by Cr-supplementation in mice.

Hypothesis two stated that there will be no significant differences in blood glucose or cholesterol due to CH-supplementation in mice. Hypothesis two is rejected because

blood cholesterol was significantly decreased by CH supplementation in mice.

Hypothesis three stated that there will be no significant differences in blood glucose or cholesterol due to CH- or Cu- supplementation in mice fed a hypercholesterolemic diet. Hypothesis three is rejected because blood cholesterol was decreased significantly by CH supplementation.

Hypothesis four stated that there will be no significant differences in the blood glucose or cholesterol of mice supplemented with individual fractions of CH and fed a hypercholesterolemic diet. Hypothesis four is rejected because plasma glucose of the fraction 1 supplemented group was significantly decreased compared with the control group.

Hypothesis five stated that there will be no significant differences in the blood glucose or cholesterol of mice supplemented with combinations of CH fractions and fed a hypercholesterolemic diet. Hypothesis five is rejected because plasma glucose of mice supplemented with the combination of fractions 1 & 2 or fractions 1 & 3 was decreased significantly compared with the control group.

Hypothesis six stated that there will be no significant differences in the blood glucose or cholesterol due to supplementation of fraction 1 of CH in the obese mice. Hypothesis six is rejected because plasma cholesterol was decreased significantly in the fraction 1 supplemented group.

Hypothesis seven stated that there will be no significant differences in hepatic RNA and DNA between Cr-supplementation and Cr-depletion rats. Hypothesis seven is rejected because the mean DNA concentration of livers from animals supplemented with 100 ug chromium/kg diet was greater than DNA concentrations of livers from animals fed the low chromium diet.

Hypothesis eight stated that there will be no significant differences in hepatic RNA and DNA between Cr- supplementation and Cr- depletion guinea pigs. Hypothesis eight is rejected because both hepatic RNA and DNA were increased significantly in Cr- supplementation group compared with Cr-depletion group.

Conclusions

This research investigated the effects of chromium on the blood concentrations of glucose and lipids and on hepatic nucleic acids. Based upon the hypothesis that GTF (glucose tolerance factor) affects glucose utilization, we did several studies on IPA (insulin potentiating activity) of Chinese herb to find one with high IPA (CH). Studies included IPA assays in vitro and animal studies with measurement of glucose, lipids, and nucleic acids.

The chromium studies showed no effects on the blood glucose and cholesterol, but the concentrations of hepatic nucleic acids did change with chromium supplementation. In these studies of chromium supplementation in mice and guinea

pigs, chromium had no effect on the metabolism of glucose and lipids, but chromium did affect nucleic acids.

The studies of Chinese herb (CH) showed different results between the crude extract from CH and the fractions from CH. The conclusions are that the crude extract from CH had hypocholesterolemic effect in mice and the fractions from CH had hypoglycemic effects in mice. Only older obese mice presented a hypocholesterolemic effect with fraction 1 supplementation.

Recommendations

Mice are so small that it is difficult to get enough blood and to do repeated measurements. It is important to check blood glucose and cholesterol initially during treatment. If larger animals are available, a complete glucose tolerance test can be done which is more accurate than fasting blood glucose or blood glucose only at 60 or 90 minutes after an oral glucose load. More blood can be drawn from the larger animal, so more indices such as triglycerides, HDL-cholesterol, and LDL-cholesterol can be further determined.

The finding that dietary chromium influences the chromium concentrations in a high molecular weight fraction of rat liver extracts from rats fed 3 levels of dietary chromium needs further study. Further purification needs to be done to identify the complex of chromium and nucleic

acids which might play an important role in the metabolism of nucleic acids.

Our studies showed that the crude extract from CH had a hypocholesterolemic effect in mice. Why the fraction(s) after purification didn't have the same effect as the crude extract did is a major question. Further study is needed to find the proper methods for better purification for CH.

Since fraction 1 had a hypoglycemic effect in animal studies, further study of its chemical structure is needed. More animal studies, especially with larger animal models are needed to test more biochemical indices in the blood. The metabolism of glucose needs to be further investigated in non-insulin dependent (type II) diabetic animal models. In addition to determinations of blood glucose and insulin, insulin receptors also need to be assayed.

In our study, fraction 1 showed a hypocholesterolemic effect in adult obese mice (models for NIDDM) instead of hypoglycemic effect. Further studies are needed in this older obese animal model.

Fraction 1 had the highest IPA among three fractions from CH. It also had a hypoglycemic effect on weanling lean mice and a hypocholesterolemic effect on older obese mice. Therefore studies of chemical structure and biological function of fraction 1 should be continued. After the animal studies have been done, finally more human studies are needed.

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APPENDIXES

APPENDIX A

ASSAYING OF INSULIN POTENTIATING ACTIVITY

Plastic containers were used exclusively for fat cell isolation and assay. Two rats were sacrificed by decapitation and their epididymal fat pads removed. The distal portion of the fat pads was rinsed with 0.9% NaCl, minced with scissors, and incubated at 37°C for 40 min in 6 mL of Krebs Ringer Phosphate (KRP) buffer containing 12 mg of collagenase in a water bath shaker at 200 rpm. The KRP contained 118 mM NaCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM KH_2PO_4, and 16.2 mM Na_2HPO_4-HCl (pH 7.4). The digested tissue was passed through a silk screen using a 10-mL syringe. The preparation was then washed three times by centrifugation with KRP containing 2% albumin. Both KRP and KRP-albumin were millipore filtered and gassed with O_2. The material below the floating fat cells and any fat above the adipocytes were removed by aspiration. KRP-albumin (10 mL) was added to disperse the washed fat cells. Fats cells remained viable for at least 4 h.

Insulin and indicated amount of sample to be tested were added to tubes that contained 1.9 mL of KRP-albumin, 0.4 uCi(U-^{14}C) glucose (313 Ci/mol), and 67.5 ug of dextrose. Adipocytes (0.06 mL) were added, and caps

containing center wells were used to seal the tubes. After incubation for 2 h at 37°C and shaking at 150 rpm, 0.2 mL of hyamine hydroxide (10X) was added to the center well, and 0.3 mL of 2 N H_2SO_4 was injected into the incubation mixture to stop the reaction. The tubes were incubated for 30 min to allow the hyamine to trap $^{14}CO_2$. The center wells were removed, carefully wiped, and added to 10 mL of Aquasol II and counted in a beta counter.

APPENDIX B

SEPARATION OF RNA AND DNA IN LIVER

1. 0.5 g of liver is diluted up to 5 ml with ice-cold sucrose reagent A.
2. Homogenize tissue at 4°C.
3. Add 10 ml of ice-cold TCA reagent B.
4. Let stand for 30 minutes or longer at 4°C.
5. Wash with 5 ml ice-cold reagent B. Centrifuge at 10,000 rpm for 20 minutes at 4°C. Discard the supernatant.
6. Suspend the precipitate in 5 ml of ethanol at room temperature.
7. Let stand for 30 minutes at room temperature.
8. Centrifuge at 10,000 rpm for 15 minutes at 4°C and discard the supernatant.
9. Repeat steps 6, 7 and 8.
10. Add 5 ml of ethanol:ether reagent C and heat in a 60°C water bath for 30 minutes.
11. Centrifuge at 10,000 rpm for 15 minutes at 4°C and discard the supernatant (save for the lipids).
12. Suspend the precipitate in 2 ml of 1 N KOH.
13. Incubaate in a 37°C water bath for 20 hours.
14. Add 0.2 ml of HCl reaagent D and 1 ml TCA reagent E.
15. Let stand for 30 minutes at 4°C.

16. Centrifuge at 10,000 rpm for 20 minutes at 4°C.
17. The supernatant contains the hydrolyzed RNA and can be estimated by the orcinol procedure.
18. Suspend the precipitate in 5 ml of TCA reagent E.
19. Heat in a 90°C water bath with occasional stirring for minutes.
20. Cool to 4°C and let stand for 30 minutes at 4°C.
21. Centrifuge at 10,000 rpm for 20 minutes at 4°C.
22. The supernatant contains the DNA and it can be estimated by the diphenylamine reaction.

Reagents

A. 8.55 g of sucrose is diluted up to 100 ml with water (0.25 M).
B. 10 g tricholroacetic acid is diluted up to 100 ml with water (10%).
C. 90 ml ethanol + 30 ml ethylether.
D. 25 ml concentrated HCL added to 25 ml water (6 N).
E. 5 g trichloroacetic acid diluted up to 100 ml with water.

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