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MECHANISM AND EFFECT OF EXCESS COPPER SUPPLEMENTATION ON BODY LIPIDS

Executive Summary

Rats were used in long term feeding experiments to determine the most bioavailable form of copper. Inorganic copper in the form of copper sulphate, copper gluconate and copper yeast were tested. Copper yeast was found to be the most bioavailable form.

Four rat experiments were done to determine the effect of copper supplementation, in excess of the rat's nutritional needs, on body weight and blood and liver lipids. Excess copper in the form of gluconate or yeast had no effect on weight gain. It significantly diminished the concentration of serum cholesterol and serum triglycerides. Excess copper raised the concentration of serum high density lipoprotein cholesterol. This form of cholesterol is hypothesised as a carrier of cholesterol from the plasma to the liver for ultimate excretion and is thus a beneficial form of cholesterol. Excess copper was also found to increase the level of liver cholesterol. Mechanistic studies with a radioactive cholesterol precursor also indicate that excess copper supplementation moves cholesterol from the plasma to the liver for ultimate excretion.

Thus, excess copper supplementation has a beneficial effect on the rat with respect to serum lipids. Extrapolation of these results to the human experience would indicate that excess copper supplementation is beneficial with respect to heart disease risk.

Technical Summary

The bioavailability of different forms of copper was determined by feeding weanling male rats a copper-deficient diet for one month and then supplementing with 4, 8 and 20 ppm of copper in the form of inorganic copper, copper gluconate and copper yeast. After one month of supplementation the rats were sacrificed and the blood and liver collected for analysis of copper by atomic absorption spectroscopy. From the slopes of the dose response curves, the order of bioavailability in blood is yeast > inorganic > gluconate and in the liver is yeast > gluconate > inorganic.

The yeast and gluconate were used in four different supplementation studies to determine the effect of excess copper supplementation. Weanling and mature male rats were supplemented for periods of time varying from one to four months at concentrations of copper of 5 ppm for the control group (copper sufficient) and 30 ppm for the experimental group (copper excess). In all but one experiment the control group was fed commercial rat chow which contained 5 ppm copper. Excess copper supplementation had no effect on weight or weight gain. In general, excess copper decreased serum cholesterol and triglycerides and elevated serum high density lipoprotein cholesterol and liver cholesterol. The statistical significance of the changes depended on the form of copper supplemented and on the duration of the supplementation. However, all changes in lipids as a result of copper supplementation were beneficial.

A rat study using C¹⁴ mevalonate, a cholesterol precursor, was done to determine how

copper affected lipid metabolism *in vivo*. The experimental group (copper excess) incorporated significantly less radioactivity in the plasma free cholesterol and cholesterol esters as compared to the control group (copper sufficient). These results are in the same direction as the copper sufficient vs. copper deficient groups studied by Klevay, who also used mevalonate. The liver results were also similar to Klevay's results. The mechanism study indicates that excess copper clears cholesterol from the plasma to the liver with the assistance of the high density lipoprotein cholesterol which is elevated due to excess copper.

Previous studies have compared copper deficient rats (fed 0 to 2 ppm copper) to copper sufficient rats (fed 5 to 18 ppm copper). These studies have shown that copper deficient rats were hypercholesteraemic. Mechanism studies have proven that copper deficiency has no effect on liver cholesterogenesis nor does it decrease the oxidation or excretion of cholesterol. The best rationale is that copper deficiency causes a shift of copper from the liver to the serum pool resulting in an increase in serum cholesterol. Our results confirm this hypothesis for groups of copper sufficient rats as compared to copper excess rats. Thus, our sufficient group is behaving in the same way relative to the excess group, as Klevay's deficient group behaves relative to his sufficient group.

Introduction

Ischaemic heart disease, formerly known as coronary heart disease, is the leading cause of death in the United States. It produces 35% of deaths, twice as many as are caused by cancer. The risk of heart disease increases with age, blood pressure and with the concentration of cholesterol in the serum. There have been many attempts, by means of diet and drugs, to lower the level of cholesterol in the blood. Within the last ten years literature reports have shown that a dietary deficiency of copper produced hypercholesteraemia in animals. Also it has been found that the human requirement for copper, 2 mg per day, is not met by conventional diets. Although ischaemic heart disease has many causes, it is hypothesised that copper deficiency contributes to the risk of human heart disease.

In our laboratory we have completed a small scale human study on the effect of 2 mg of copper, in the form of copper gluconate, taken daily. After 3 months of supplementation the five subjects had lower serum cholesterol and triglycerides. It is our hypothesis that excess copper supplementation is beneficial with respect to the risk of human heart disease.

Technical Report

Bioavailability study

In order to determine what form of copper should be used in supplementation studies, it is necessary to know what form of copper is most bioavailable. Bioavailability is best determined by a dose response curve in which different forms of the mineral are fed at different doses and the element determined in the blood and liver. Laboratory rats are good animal models for bioavailability studies because their diet can be easily controlled, they can be bred to be genetically similar, and large numbers can be easily studied for long periods of time.

Male weanling Sprague-Dawley rats were divided into ten groups of five rats. the average weight of each group was the same. All groups were fed a copper deficient

diet containing less than 0.05 mg of copper/kg of diet for a period of one month. One on the groups was continued on the copper deficient diet for the remainder of the study. Each of the other nine groups was fed one of three different forms of copper at one of the three different concentrations. The forms of copper used were copper sulphate (inorganic), copper gluconate (gluconate) in tablet form containing 0.5 mg of copper, and high copper yeast (yeast) in the form of powder containing 1% copper. The concentrations of copper added to the copper deficient diet were 4, 8 and 20 ppm. The supplementation period was one month. At the end of this period all the rats were sacrificed and the blood and liver collected for analysis. The blood and liver were ashed, reconstituted in 2N hydrochloric acid, and the copper was determined by atomic absorption spectroscopy. The results are shown in Tables 1 and 2.

Table 1: Copper concentrations in the blood of rats fed different forms of copper.

Form of Copper	Copper in Food (ppm)	Average Blood Copper (ppm)
Deficient food	Less than 0.05	1.72 ± 0.11
Inorganic	4	2.20 ± 0.30
Inorganic	8	2.47 ± 0.23
Inorganic	20	2.63 ± 0.23
Gluconate	4	2.19 ± 0.54
Gluconate	8	2.31 ± 0.18
Gluconate	20	2.42 ± 0.22
Yeast	4	2.37 ± 0.43
Yeast	8	2.69 ± 0.39
Yeast	20	2.88 ± 0.30

Table 2: Copper concentrations in the liver of rats fed different forms of copper.

Form of Copper	Copper in Food (ppm)	Average Liver Copper (ppm)
Deficient food	Less than 0.05	2.87 ± 0.53
Inorganic	4	2.96 ± 0.66
Inorganic	8	3.17 ± 0.38
Inorganic	20	3.60 ± 0.36
Gluconate	4	3.04 ± 0.53
Gluconate	8	3.37 ± 0.43
Gluconate	20	3.84 ± 0.32
Yeast	4	3.32 ± 0.25
Yeast	8	3.56 ± 0.38
Yeast	20	4.32 ± 0.34

It is evident from the data presented in Tables 1 and 2 that the yeast gives the highest concentration of copper in both the blood and liver at all doses. From this data the relative bioavailabilities of the different forms can be calculated. The value of the

slope of the plot of copper concentration in food (x-axis) vs. copper concentration in the blood or liver represents the bioavailability. The inorganic copper for comparison purposes is said to be 100% bioavailable. The relative bioavailabilities of the other forms can then be determined by comparing the slope to the slope of the inorganic form. The results are shown in Table 3.

Table 3: Comparison of the bioavailabilities of different forms of copper

Form of Copper	Slope of Plot	Relative Bioavailabilities
Blood		
Inorganic	0.0409	100%
Gluconate	0.0382	93%
Yeast	0.0505	124%
Liver		
Inorganic	0.0376	100%
Gluconate	0.0488	130%
Yeast	0.0697	195%

As can be seen, the yeast is the most bioavailable of the three forms in both the blood and the liver. The copper concentration in the blood represents the present copper status of an individual and the copper concentration in the liver represents the pool of available copper.

Copper Supplementation and Body Lipids in the Rat

Male Sprague-Dawley rats were used as the experimental animal to determine the effects of copper supplementation on weight and body lipids. The copper was supplemented to a diet already sufficient in copper. In other words, copper was given in excess of the amount minimally required for growth, gestation and lactation. The National Academy of Sciences in "Nutrient Requirements for Laboratory Animals" states in 1978 that the minimal concentration of copper in the diet of rats should be 5 ppm. In the following rat studies there were two groups of rats -experimental and control. The control group were fed normal laboratory rat chow from Ralston-Purina company which was sufficient in copper had been added. Both groups received the diet in a powder form. After supplementation body lipids were measured by an outside clinical laboratory using an enzyme technique.

Experiment A: Early in the project supplementation with copper gluconate was utilised for the experimental group at a level of 20 ppm. In this experiment mature male rats, two months of age, were used with a supplementation period of four months. The results are shown in Table 4.

Table 4: The effect of Copper gluconate supplementation for four months on weight and body lipids of mature rats.

Measurement	Control Group	Experimental Group	
Weight before supplementation	460 ± 75 g	435 ± 63 g	N.S.
Weight after supplementation	582 ± 69 g	550 ± 84 g	N.S.
Serum Cholesterol after supplementation	277 ± 276 mg/dl 7.15 ± 7.12 mm/l	144 ± 131 mg/dl 3.71 ± 3.38 mm/l	p<0.05
Serum High Density Cholesterol after supplementation	13 ± 10 mg/dl 0.34 ± 0.25 mm/l	18 ± 21 mg/dl 0.46 ± 0.54 mm/l	N.S.
Serum Triglycerides after supplementation	176 ± 143 mg/dl 2.46 ± 2.00 mm/l	135 ± 12 mg/dl 1.89 ± 0.17 mm/l	N.S.
Liver Triglycerides after supplementation	1045 ± 185 mg/dl 1.46 ± 0.26 mm/l	896 ± 370 mg/dl 1.25 ± 0.52 mm/l	N.S.
Liver Cholesterol after supplementation	2.8 ± 1.1 mg/g 7.22 ± 2.80 mm/g	6.8 ± 4.1 mg/g 17.5 ± 10.5 mm/g	p,0.05

N.S. = Not significant as determined by the student's t-test.

The average weight gain for the control group was 122 ± 64 g and for the experimental group 115 ± 65 g. It can be concluded that excess copper in the form of copper gluconate has no effect on weight gain at a level of supplementation of 20 ppm. Copper gluconate appears to significantly lower serum cholesterol and to significantly elevate liver cholesterol. Copper gluconate raises the level of beneficial serum cholesterol, that is, the high density lipoprotein. Copper gluconate also lowers serum triglycerides but not significantly.

Experiment B: A second experiment with mature male rats was similar to Experiment A except that only a one month supplementation period was used with 20 ppm copper as copper gluconate. The results are shown in Table 5.

Table 5: The effect of Copper gluconate supplementation for one month on weight and serum cholesterol of mature rats.

Measurement	Control Group	Experimental Group	
Weight before supplementation	469 ± 62 g	488 ± 54 g	N.S.
Weight after supplementation	528 ± 53 g	520 ± 53 g	N.S.
Serum Cholesterol after supplementation	52 ± 7 mg/dl 1.34 ± 0.18 mm/l	33 ± 9 mg/dl 0.85 ± 0.23 mm/l	p<0.005

Copper Gluconate again didn't have any significant effect on weight gain. As in experiment A, copper gluconate had a significant beneficial effect on serum cholesterol. Unfortunately the samples were lost and the other lipids could not be analysed.

Experiment C: This third experiment was done with a new commercial yeast. This material was found to be the most bioavailable form of copper and was used in all subsequent experiments. The yeast was found to have 85% of its copper in an organic form by solubilising the organic copper in a solution of 50% ethanol/50% water and determination of the soluble copper by atomic absorption spectroscopy. This product is manufactured by growing brewer's yeast in a nutrient medium containing copper. The yeast takes up the copper and converts it into an organic form which is probably a complex of copper with the amino acids of the yeast protein. The yeast is a tan powder, highly water soluble which contains 1% copper by weight. The yeast is suitable for human consumption.

Male weanling rats were fed a rat chow diet for two months. The control group was fed the rat chow alone. The experimental group received the rat chow supplemented with 20 ppm copper in the form of yeast. The results are shown in Table 6.

Table 6: The effect of Copper yeast supplementation for two months on weight and serum lipids of weanling rats.

Measurement	Control Group	Experimental Group	
Weight before supplementation	49 ± 5 g	50 ± 8 g	N.S.
Weight after supplementation	254 ± 3 g	255 ± 3 g	N.S.
Serum Cholesterol after supplementation	42 ± 6 mg/dl 1.10 ± 0.15 mm/l	34 ± 9 mg/dl 0.88 ± 0.23 mm/l	p<0.05
Serum High Density Cholesterol after supplementation	22 ± 4 mg/dl 0.57 ± 0.10 mm/l	33 ± 3 mg/dl 0.85 ± 0.08 mm/l	p<0.001
Serum Triglycerides after supplementation	116 ± 6 mg/dl 1.62 ± 0.08 mm/l	89 ± 15 mg/dl 1.25 ± 0.21 mm/l	p<0.01

The data shows less scatter and are thus more conclusive than in previous experiments. Yeast copper had no effect on weight gain but had a significant benefit with respect to body lipids. The yeast lowered the serum cholesterol and triglycerides significantly. It also significantly raised the beneficial high density lipoprotein cholesterol. The ratio of cholesterol/high density lipoprotein cholesterol is an excellent indicator for the risk of heart disease in humans. The lower the value of this ratio, the lower the risk of heart disease. In the control group, the ratio was 1.9 and in the experimental group, it was only 1.0. Thus the copper yeast had a profoundly beneficial effect on the risk of heart disease in this animal study.

Experiment D: The last rat supplementation experiment used weanling rats as in Experiment C with a difference. The control group was fed a copper deficient diet to which had been added 5 ppm copper in the form of yeast. Thus, these rats had a diet sufficient in copper. The experimental group were fed the copper deficient diet to which had been added 30 ppm copper in the form of yeast. In this experiment both groups received copper in the same form, yeast. In previous experiments the control group received copper from the rat chow which was in the inorganic form. The results of this experiment are shown in Table 7.

Table 7: The effect of Copper yeast supplementation for two months on weight and serum lipids of weanling rats.

Measurement	Control Group	Experimental Group	
Weight before supplementation	69 ± 2 g	68 ± 2 g	N.S.
Weight after supplementation	233 ± 16 g	238 ± 25 g	N.S.
Serum Cholesterol after supplementation	64 ± 9 mg/dl 1.65 ± 0.23 mm/l	70 ± 16 mg/dl 1.81 ± 0.41 mm/l	N.S.
Serum High Density Cholesterol after supplementation	41 ± 8 mg/dl 1.06 ± 0.21 mm/l	53 ± 12 mg/dl 1.37 ± 0.31 mm/l	p<0.1
Serum Triglycerides after supplementation	49 ± 20 mg/dl 0.69 ± 0.28 mm/l	58 ± 16 mg/dl 0.81 ± 0.22 mm/l	N.S.

The yeast had no significant effect on weight, serum cholesterol or serum triglycerides. It had a slightly beneficial effect on serum high density lipoprotein cholesterol. It appears that the high bioavailability of the yeast copper affected the results of this last experiment. The recommended concentration in the diet of rats is 5 ppm and this value was determined using inorganic copper. Thus, the recommended amount of yeast copper, which is better absorbed than inorganic copper, would be less than 5 ppm. Therefore the control group was receiving excess copper and was not a genuine control group.

Mechanism Study

This study was undertaken to determine the mechanism of copper's effect on body lipids. Previous mechanism studies have focused on the effect of copper deficiency. They have shown by radioactivity measurements that copper deficiency produces hypercholesterolaemia as a result of a shift of cholesterol from the liver to the blood.

The following mechanism study was designed to determine the effect of excess copper supplementation on cholesterol synthesis. This study used the same rats as described in Experiment D in which copper yeast was added to the control diet at 5 ppm, and the experimental diet at 30 ppm. The duration of feeding was two months. At the end of the supplementation the rats were fasted for 24 hours and injected subcutaneously with four microcuries of C¹⁴ mevalonate, a cholesterol precursor, four hours before sacrifice. The procedure for analysis of the various fractions was that of Allen and Klevay in: *Life Science*, **22**, 1691, 1978. The blood and liver were removed

after sacrifice and the blood converted to plasma. The total radioactivity in the organic solvent extracts of liver and plasma was measured. The neutral lipids in the extracts were fractionated by thin layer chromatography. The appropriate zones were eluted and the radioactivity corresponding to each class of neutral lipid was measured. There was no appreciable radioactivity in the triglyceride fraction. Total triglycerides and cholesterol in the liver were determined by fluorometry after extraction. The results are shown in Tables 8 and 9.

Table 8: Plasma cholesterol and specific activities of lipids in rats fed copper yeast for two months followed by injection of C₁₄ mevalonate.

Measurement	Control Group	Experimental Group	
Plasma Total Cholesterol	64 ± 9 mg/dl 1.65 ± 0.23 mm/l	70 ± 16 mg/dl 1.81 ± 0.41 mm/l	N.S.
Plasma High Density Cholesterol	41 ± 8 mg/dl 1.06 ± 0.21 g	53 ± 12 mg/dl 1.37 ± 0.31 mm/l	p<0.1
Specific Activity of Plasma Total lipids	984 ± 211 mg/dl	650 ± 181 mg/dl	p<0.05
Serum Activity of Plasma Free Cholesterol	439 ± 123 cpm/ml	243 ± 58 cpm/ml	p<0.05
Specific Activity of Plasma Cholesterol Esters	504 ± 143 cpm/ml	349 ± 128 cpm/ml	p<0.1

Table 9: Liver cholesterol and specific activities of lipids in rats fed copper yeast for two months followed by injection of C₁₄ mevalonate.

Measurement	Control Group	Experimental Group	
Liver Total Cholesterol	97 ± 54 mg/100g 250 ± 1.39 mm/l	148 ± 15 mg/100 g 3.81 ± 0.39 mm/l	p<0.1
Specific Activity of liver total lipids	3797 ± 999 cpm/g	3499 ± 481 cpm/g	N.S.
Specific Activity liver Free Cholesterol	1579 ± 163 cpm/g	1029 ± 196 cpm/g	p<0.00
Specific Activity of liver Cholesterol Esters	219 ± 65 cpm/g	280 ± 53 cpm/g	N.S.

Mevalonate is an obligatory intermediate in cholesterol synthesis and the formation of mevalonate from 3-hydroxy-3-methylglutarate is the principal control point in cholesterol biosynthesis. Thus, C₁₄ incorporation provides a measure of the net influx to and efflux from the plasma and liver cholesterol pools. Changes in C₁₄ incorporation in liver and plasma pools are dependent upon cholesterol synthesis, clearance to the plasma, cholesterol degradation into bile acids, biliary excretion and on the uptake of cholesterol by extrahepatic tissue.

The results in plasma represent an interesting parallel with previous copper deficiency rat studies. Our results indicate that copper sufficient rats, the control group, had a marked increase in C₁₄ incorporation in plasma total lipids (150% p<0.05), free

cholesterol (181% $p < 0.05$) and cholesterol esters (144% $p < 0.1$) as compared with the sufficient group which received excess copper, the experimental group.

The liver results are shown in Table 9. From this yeast study and our copper gluconate study in Table 4 it appears that excess copper supplementation significantly raises liver total cholesterol. This appears to be a result of the greater concentration of high density lipoprotein cholesterol in the blood of excess copper rats which transports cholesterol from the plasma to the liver for storage and ultimate excretion as bile acids. The radioactivity results are more difficult to explain, but it appears that there is a greater incorporation of radioactivity in liver free cholesterol (153% $p < 0.005$) but less incorporation in liver cholesterol ester (78% N.S.) for the copper sufficient, control group, as compared to the copper excess, experimental group. The seemingly contradictory results for free vs. esterified cholesterol may be explained on the basis of the time period chosen for analysis after injection of the radioactive malonate because Klevay found the same contradiction. Again the liver results gave the same trends for our sufficient group relative to our excess group, as Klevay's deficient group relative to his sufficient group.

Conclusions and Recommendations

Our results indicate that excess copper supplementation to rats significantly lowers blood cholesterol and triglycerides. Excess copper also raises the concentration of high density lipoprotein cholesterol. The decrease in cholesterol has been shown by a mechanism study to be due to a shift of cholesterol from the serum to the liver.

High density lipoprotein cholesterol is hypothesized to carry cholesterol from the blood to the liver for ultimate excretion. Thus, a decrease in total blood cholesterol and an increase in high density lipoprotein cholesterol should result in a decrease in the accumulation of cholesterol on the arterial walls and thus a decrease in the rate of atherosclerosis.

The next research should focus on the effect of excess copper on atherosclerosis using animal models. Also human studies with excess copper supplementation should be pursued.