Dietary copper, simple sugars, and metabolic changes in pigs

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Inadequate dietary copper is known to result in undesirable metabolic changes in rats and humans. Abnormal cardiac function, leading to sudden death, is a common finding when copper deficient rats are fed a 62% fructose diet. To further study the apparent mineral-carbohydrate relationship to cardiac physiology, 3 male and 3 female swine were randomly assigned to four groups (6 pigs per group) which were fed low copper (1.5 ppm) or copper supplemented (40 ppm) diets with 20% of calories from either fructose or glucose for 10 weeks. In agreement with results from other animal studies, copper deficient swine exhibited decreased plasma ceruloplasmin, erythrocyte superoxide dismutase and plasma lysyl oxidase activities and lowered serum copper. The copper deficient fructose group had the lowest aortic lysyl oxidase activity and hematocrit when compared to the other groups. The relative heart weight in the copper deficient fructose group was 93% greater than the other three dietary groups. The livers of copper deficient fructose fed pigs were also significantly larger. Two enzymes related to cardiac and hepatic function, aspartate and alanine aminotransferase were also measured. Copper deficiency significantly lowered alanine aminotransferase but there was no dietary effect on aspartate aminotransferase. The results of this project indicate that the pig is a sensitive model for the study of cardiovascular abnormalities which occur when fructose is consumed with a low copper diet.

Keywords: cardiomyopathy; fructose; copper; anemia; lysyl oxidase

Introduction

Human and animal experiments indicate that the type of dietary carbohydrate consumed can change the amount of dietary copper required to sustain optimal health.[1-3] An increased mortality due to severe cardiovascular abnormalities routinely occurs in rats after consuming a copper deficient 62% fructose diet.[4-7] Several studies have shown that an inadequate copper intake or availability in humans and animals can adversely affect a host of physiological and clinical indices associated with heart disease.[1,8-10] In animal and human studies, dietary fructose but not cornstarch, intensifies undesirable metabolic changes when copper intake is low or deficient.[4-7,10] In experiments using male rats, virtually every member of the copper deficient fructose fed group dies after about 7 or 8 weeks, while animals fed copper adequate or deficient diets with cornstarch survive.[4,5,7] In initial studies where this carbohydrate-mineral relationship was first identified, sucrose had the same terminal effect as fructose.[11] Therefore, in a 62% sucrose diet the amount of fructose available from that disaccharide would be a 31%, indicating that mortality in copper deficient rats can occur with a much lower intake of fructose. Based on the findings from the animal studies, it was initially concluded that dietary starch was protecting the animals. In a recent report, Lewis et al.[12] questioned this assumption. Their data indicate that when copper deficient diets are blended in proportions such that starch accounts for 100, 75, 50, 25, and 0% of carbohydrate with fructose added for an overall

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total of 62.7% carbohydrate, the harmful effects of fructose were proportional to its concentration.[12]

To more closely study the carbohydrate-copper relationship to vascular pathology, the pig, which has a circulatory system similar in size and morphology to humans, was the experimental animal chosen. A study in which 12 male and 12 female swine were fed a low copper (CuD) or copper supplemented (CuS) diet with 20% of calories as fructose or glucose was conducted for a period of 10 weeks. It was apparent that the measurement of blood lipids commonly associated with coronary heart disease in humans, would have little relevance since swine have an entirely different lipid metabolism than humans.[13,14] The factors which will be the major focus of this report are: cardiac and hepatic changes, severity of copper deficiency symptoms, and determination of the activity of copper dependent enzymes in blood and tissues.

Materials and methods

Twelve male and 12 female, 21-day-old pigs were fed a dried skim milk, 20% glucose or fructose, low copper (1.5 ppm), or copper supplemented (40 ppm) diet for a period of ten weeks. In all four treatment groups, dried skim milk provided about 65.4% of the diet composition (*Table 1*). The following sources were used for dietary ingredients: Holly Milk, Carlisle, PA, USA, dried skim milk; Hoffman-La Roche, Nutley, N.J., USA, fructose; Clinton Corn Processing Company, Clinton, IA, USA, glucose; CPC International, Inc., corn oil; International Filler Corp., North Tonawanda, NY, USA, Solka Floc (cellulose). Deionized water was provided throughout the study. Blood samples were taken before and at the 5th and 10th weeks of the study.

Table 1 Nutrient composition of experimental diets

Component	Percentage by Weight
Dried skim milk	65.4
Fructose or Glucose	20
Corn oil	5
Solka floc	4
Vitamin mix[a]	0.5
Trace mineral mix without Cu[b]	3
Dicalcium phosphate	1.8
Choline chloride	0.3
Copper[c]	1.5 or 40 ppm

The special swine vitamin[a] and trace mineral mixes[b] were purchased from United States Biochemicals Corporation, Cleveland, Ohio 44122 (catalog numbers: 850128DA3, 850128DA1).
[c]Copper was provided as pulverized copper sulfate. Dietary concentration was verified by atomic absorption spectroscopy and the recovery of Cu from National Bureau of Standard's SRM 1577a bovine liver was within 5% of the expected value.
The commercial sources of dietary components are included in the text.
Mineral mix ingredients are listed as percent of mix. NaCl, 16.7; KCl 16.7; Mg Sulfate, 34; Ca phosphate (dibasic) 24.2; Iron, 4.0; Zinc 2.2; Manganese, 1.5; Chromium, 0.65; Molybdenum, 0.015; Cobalt, 0.02; KI, 0.0044; Selenium, 0.002.
The following vitamins (amount/kg) were in the diet. A, 4400IU; D_3,880IU; K_3,2mg; B_1,11mg; B_2,8.8mg; B_6,15mg; B_{12},44ug; E,100IU; Niacin, 30.8mg; Calcium Pantothenate, 25mg; C,100mg; Folate, 6mg; Biotin, 5mg.

Some analyses were only conducted on the blood collected when the animals were sacrificed at week ten. All necessary precautions were taken to prevent mineral contamination of biological samples. Diet preparation and animal care were closely monitored by USDA scientists and technical staff. When the study was terminated at week 10, the animals were autopsied and heart and liver weights were determined. Blood and aortic tissue samples were also taken at this time for the analysis of lysyl oxidase (LOA) (EC 1.4.3.13) activity.[15] Growth rates ranged between 228 and 298 g/d. Hemoglobin and hematocrit were measured on the whole blood samples.[16,17] After 5 weeks, erythrocyte copper-zinc superoxide dismutase (SOD)(EC 1.15.1.1) activity was measured in saline washed, packed, erythrocytes by the method of Misra and Fridovich.[18] Since the SOD levels were below the assay detection limits for several CuD animals at week five, it was not measured at week 10. Plasma ceruloplasmin (CP) (EC 1.16.3.1) was analyzed based on its oxidase activity using o-dianisidine dihydrochloride as the substrate.[19] Alanine and aspartate aminotransferase (EC 2.6.1.2, EC 2.6.1.1) activities (ALT, AST) were measured in plama using enzymatic methods.[20] Normal and abnormal human control serum were used to verify precision and accuracy in the ALT and AST analyses. Copper content of serum and diets was determined by atomic absorption spectroscopy at week 10, using a combination of wet and dry ashing for sample preparation.[21] An appropriate standard reference material from the National Bureau of Standards was processed with the sample.[22]

Statistical methods included analysis of variance (ANOVA) using the SAS general linear models procedure.[23] Some data from analyses conducted at weeks 5 and 10 did not meet the criteria for homogeneity of variance.[24] In these instances, the data were log-transformed prior to the ANOVA. Duncan's multiple range test was used to determine significant differences between group means. Differences between values with $P < 0.05$ were considered statistically significant. The individual effects of sex, time, copper and carbohydrate and their multiple interactions were assessed statistically prior to choosing copper, carbohydrate and their interaction for the ANOVA at the three time points. The sex of the pigs was not a statistically significant factor with regard to the parameters measured. As expected time was statistically significant for many of the variables but the significance will be discussed rather than including it in the tables.

Results

Table 2 contains data on the body weight and relative heart and liver weights of the dietary treatment groups. The piglets weighed approximately 5.0 kg when the experiment began. Weight gain proceeded at

Table 2 Body, heart, and liver weights of experimental animals

	Body Weights (Kg)			
	Fructose +Cu	Fructose −Cu	Glucose +Cu	Glucose −Cu
Pretest	5.25	5.00	5.35	5.24
				RMSE = 1.27
Week 10	25.9	20.6	24.5	24.6
				RMSE = 4.93
	ANOVA: NS for both times.			
	Relative Heart Weights (g/Kg BW)			
	Fructose +Cu	Fructose −Cu	Glucose +Cu	Glucose −Cu
	5.58^{b}	9.56^{a}	$5.15^{b,c}$	4.12^{c}
	ANOVA*:			
	Cu	NS		
	CHO	$P < 0.0004$		
	Cu X CHO	$P < 0.0013$		RMSE = 1.41
	Relative Liver Weights (g/Kg BW)			
	Fructose +Cu	Fructose −Cu	Glucose +Cu	Glucose −Cu
	22.3	26.7	20.7	23.0
	ANOVA:			
	Cu	$P < 0.0017$		
	CHO	$P < 0.0083$		
	Cu X CHO	NS		RMSE = 2.17

ANOVA = Analysis of variance using the SAS general linear models procedure. RMSE = The square root of the mean square error term. NS = No statistically significant difference at $P < 0.05$. Means with different superscript letters are significantly different at $P < 0.05$ using the Duncan's Multiple Range Test.
ANOVA* = Conducted on log transformed values after heterogeneity of variance was established.

a similar rate for the four treatment groups until the seventh week. Weight increases for pigs in the 20% fructose low copper group (FrCuD) tended to be lower than the other groups from week seven to week ten. When the study was terminated at week 10, pigs in the FrCuD group were 4.6 to 5.9 kg lighter than the other groups, but this was not statistically significant. The relative heart weight was 93% greater in the FrCuD group than the average weight for the other diet groups. The heart weight data was statistically significant for the main effects and their interaction. Qualitative examination of the heart tissue in the FrCuD fed animals revealed hypertrophy unique to that group. The ANOVA revealed a significant copper and carbohydrate effect on relative liver weight. The FrCuD diet resulted in hepatic hypertrophy which was not observed with the other diets.

The effect on the direct indicators of copper status are presented in *Table 3*. Serum copper was significantly lower at weeks 5 and 10 in the copper deficient groups, regardless of dietary carbohydrate. Both CP and SOD were significantly lower in the CuD groups but there was no carbohydrate effect. Ceruloplasmin was also measured at week 10 and was extremely low in the CuD groups.

At the fifth week, there were clinical signs of anemia as measured by hematocrit and hemoglobulin (*Table 4*). Hematocrit was significantly lower at the tenth week in the FrCuD as compared to the other dietary groups.

Lysyl oxidase activity was determined in both

Table 3 Indices of copper status

	Serum Copper μg/dl			Red Cell SOD U/ml
	Pretest	Week 5*	Week 10*	Week 5*
FrCuS	171	176	171	819
FrCuD	157	22	4.0	37
GlcCuS	162	153	170	702
GlcCuD	174	28	4.7	71
ANOVA:				
Cu	NS	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
CHO	NS	NS	NS	NS
CHO X Cu	NS	NS	NS	NS
RMSE =	24.4	20.6	11.3	114
	Serum Ceruloplasmin ug/ml			
	Pretest	Week 5*	Week 10*	
FrCuS	198	255^{a}	264	
FrCuD	199	13.6^{b}	6.78	
GlcCuS	190	154^{a}	207	
GlcCuD	244	17.2^{b}	6.35	
ANOVA:				
Cu	NS	$P < 0.0001$	$P < 0.0001$	
CHO	NS	NS	$P < 0.008$	
Cu X CHO	NS	$P < 0.0404$	NS	
RMSE =	62.2	57.5	22.9	

*Indicates ANOVA was conducted on log transformed values. FrCuS = 20% fructose + 40ppm Cu, FrCuD = 20% fructose + 1.5ppm Cu, GlcCuS = 20% glucose + 40ppm Cu, GlcCuD = 20% glucose + 1.5ppm Cu. Red Cell SOD is the erythrocyte superoxide dismutase enzyme activity. The other terms are described in *Table 2*.

Table 4 Indices of anemia

	Hemoglobin g/dl			Hematocrit %		
	Pretest	Week 5	Week 10	Pretest	Week 5	Week 10
FrCuS	12.3	8.24	8.82[a]	43	29	32[a]
FrCuD	12.1	8.28	5.21[c]	42	29	19[b]
GlcCuS	12.0	7.26	7.51[a,b]	42	32	28[a]
GlcCuD	13.3	8.54	6.68[b,c]	46	32	26[a]
ANOVA:						
Cu	NS	NS	$P < 0.0041$	NS	NS	$P < 0.004$
CHO	NS	NS	NS	NS	NS	NS
CHO X Cu	NS	NS	$P < 0.0532$	NS	NS	$P < 0.025$
RMSE =	2.23	1.05	1.63	7.44	3.18	5.40

Abbreviations are described in *Tables 2* and *3*.

Table 5 Lysyl oxidase activity in plasma and aortic tissue at week 10

	Plasma	Aorta
FrCuS	11,304[a]	51,974[a]
FrCuD	6,176[b]	35,890[b]
GlcCuS	10,291[a]	51,610[a]
GlcCuD	7,109[b]	42,887[a]
ANOVA:		
Cu	$P < 0.05$	NS
CHO	NS	NS
Cu X CHO	NS	$P < 0.05$

Enzyme activity is expressed in plasma as disintegrations per minute from tritiated water per milliliter per hour. Enzyme activity in aortic tissue is expressed as disintegrations per minute from tritiated water per gram of dry tissue per hour.
The lysyl oxidase data has been published with permission of the FASEB Journal Editorial Office, Bethesda, MD 20814.

plasma and aortic tissue (*Table 5*). Plasma lysyl oxidase activity was significantly lower in the copper deficient pigs. Analysis of variance indicated a significant copper-carbohydrate interaction on aortic LOA. The FrCuD pigs had significantly less aortic LOA than the other dietary groups. There was a tendency for the fructose group to have lower plasma LOA than the other three groups.

Table 6 contains plasma ALT and AST activity data before and at weeks 5 and 10 of the study. The pretest enzyme activities did not differ for either ALT or AST in the dietary groups. At weeks 5 and 10, the ANOVA indicated a significant copper effect for ALT. AST and ALT activities significantly increased above pretest levels with time.

Discussion

A number of animal and human nutrition experiments indicate that the requirements for dietary copper may be higher if sucrose or fructose provide a significant percentage of calories.[1-3] In rodent experiments, severe cardiomyopathy resulting in sudden death from myocardial rupture frequently occurs after a seven week period of copper deficiency combined with a 62% sucrose or fructose diet.[4,7] Autopsy of the animals revealed necrosis and rupture at the apex of the heart, enlargement of the heart and what appears to be a viscous, soluble, collagen-like material coating the major blood vessels and cardiac tissues.[3-5,11] Since the dietary intake of fructose in the United States has increased eight fold since 1975 to approximately 10% of calories[25] and the intake of copper may be far below the estimated safe and adequate daily intake of 2 to 3 mg,[26,27] this project was conducted with an animal model which is similar in vascular architecture to humans. An absolute or relative copper deficiency has been proposed as a major factor in diseases of the heart, the primary cause of death in the United States.[28] In order to simulate a fructose-low copper effect in a relatively short period of time, as compared to the life span of a human, a 20% fructose level seemed appropriate.

Most metabolic and morphological changes which occurred in this experiment have been previously demonstrated with rodent studies where 0.6 to 1.0 ppm copper and 62% fructose were fed. The magnitude of increase in heart weight in the FrCuD group is consis-

Table 6 Alanine aminotransferase (ALT) U/l

	Pretest	Week 5	Week 10
FrCuS	34	72	63
FrCuD	36	48	47
GlcCuS	31	74	68
GlcCuD	36	62	57
ANOVA:			
Cu	NS	$P < 0.015$	$P < 0.022$
CHO	NS	NS	NS
Cu X CHO	NS	NS	NS
RMSE =	5.16	15.9	13.1
	Aspartate aminotransferase (AST) U/l		
	Pretest	Week 5	Week 10
FrCuS	42	51	51
FrCuD	37	47	41
GlcCuS	36	51	46
GlcCuD	38	56	46
ANOVA: NS at all times and diet treatments.			
RMSE =	6.42	11.7	9.81

Abbreviations used are described in *Table 2*.

tent with data from rat studies and a subsequent swine project with a similar diet.[29]

In that swine study,[29] cardiac tissue was dissected from the left ventricular wall, stained, and microscopically evaluated. Tissue from the FrCuD pigs had morphological abnormalities unique to that group including interstitial edema, the presence of vacuolated myocytes and perinuclear clearing. Edema was also noted in the initial study which is reported here; however, microscopic examination was not conducted.

Two recent pig experiments with similar but not identical dietary conditions demonstrated significant cardiac enlargement in the copper deficient groups regardless of the type of carbohydrate consumed.[30] The commercial source, proportions, and quantities of minerals provided were different when comparing those studies to the present study. A modification of the American Institute of Nutrition (AIN-76) mineral mixture[31] was used in the recent pig studies[30], while the U.S. Biochemical Corporation copper-free, special swine trace mineral mix was used in the study reported here. The amount of dietary zinc and iron measured in the diet of the earlier study was 260 ppm and 170 ppm, respectively, as compared to 90 ppm and 55 ppm on the recent studies.[30] These higher levels of zinc and iron may have magnified changes in the FrCuD group in the present study and the one reported by Steele et al.[29] by interfering with copper utilization.

Although the iron provided in the diets of the present study was considered adequate, hematocrit and hemoglobin levels were significantly reduced in all treatment groups by the 5th and 10th weeks as compared to pretest ($P < 0.0001$). This finding is consistent with the two recent swine studies in which dried skim milk was used as the protein source.[30] In one of those studies, three pigs were fed a "conventional," corn-soy diet[32] with 280 ppm iron and a higher overall mineral content than the refined diets. Pigs in that group had significantly higher hematocrit levels than the other ten groups listed. A higher than normal dietary calcium intake which may interfere with iron metabolism has been suggested as a possible cause of anemia when dried skim milk diets are fed.[30] If this theory is correct, then the AIN-76 mineral mixture,[31] which has twice the calcium content as the U.S. Biochemical Corp. swine mix (*Table 1*), would probably intensify the anemia. The more pronounced anemia seen in the CuD groups would presumably reduce the ability of erythrocytes to deliver and provide the same amount of oxygen to the tissues as the CuS animals. The additional metabolic stress resulting from fructose may have caused a continuous, mild tachycardia increasing the size of the heart muscle. Indeed, tachycardia did occur in two men who consumed a low copper (1.0 mg/day) diet with 20% of calories as fructose or cornstarch during an eleven week nutrition study.[1] Electrocardiogram abnormalities have also been seen in copper deficient rats.[33,34] Increased relative liver size may have resulted from edema. However, this was not verified.

Growth rates ranged from 228 to 298 g/d, with the FrCuD group tending to show a smaller weight gain. These weight increases are similar to those reported by Okonkwo et al. (275 g/d.) after a refined 50% glucose diet was fed with various levels of dietary coper.[35] Decreased growth rates in the FrCuD animals has also been reported in rat experiments.[4,5] This effect did not appear to be related to decreased caloric intake. In swine, copper supplementation has been shown to significantly improve weight gain, food efficiency and reduce hepatic oxidation as measured by lowered malondialdehyde reactivity.[36-38] However, if copper levels are increased, additional zinc and iron should also be provided.[35]

In this study, the dietary zinc and iron levels were somewhat higher than the amount which is usually fed,[35,39] but in the CuS groups the copper added was much more than that which is considered adequate for swine.[35]

The relatively high zinc levels fed may have had an impact on the degree of CuD seen in the low copper groups, since zinc is known to compete with copper for absorption. Divalent copper ions are known to effect specific hormones secreted by the anterior pituitary gland.[40,41] Copper deficiency aggravated by a 20% fructose diet may have also affected the release of growth hormone from the pituitary gland. In future animal studies with similar diets, growth hormone could be measured to test this hypothesis.

The measurement of erythrocyte superoxide dismutase activity has been suggested as one of the more sensitive indicators of copper status.[1] Reduced SOD activity after a low copper diet, observed in this study, is consistent with results of human and animal studies,[1,3] however, the activity is usually significantly lower in the FrCuD groups.

Serum CP was measured before and at the fifth and tenth weeks of the study. Although CP levels were very low in both CuD groups, only the FrCuD group had significantly reduced hematocrit levels by week 10. This finding would indicate that a mechanism other than a reduction in ferroxidase activity of CP is increasing the degree of anemia in the FrCuD group. The multiple range test indicated that the GlcCuS group had significantly less CP than the FrCuS group. Pigs in the GlcCuS began the study with 4.2% less CP than FrCuS and the difference increased to 66% by week 5. This trend was also apparent with serum copper and SOD at week 5. Although statistically significant, the difference between CP levels for the supplemented groups was much lower by the tenth week (28% vs. 66%). Apparently, the refined diet strongly affected the CP levels of this group initially but the pigs began to adapt by week 10.

Two transaminase enzymes that are clinically important in diagnosing chronic or acute liver disease and heart tissue damage were measured to assess the possible dietary effects on these organs. Significant increases in ALT have been reported when humans consume low copper diets with 20% of calories as fructose when compared to starch (42). The ALT enzyme activity was measured at pretest, week 5, and week 10.

The fact that ALT activity for the FrCuD group was the lowest of the four diet groups conflicted with the results of the previously mentioned human nutrition study.

As with lipid metabolism, the mechanisms responsible for these results may be very dissimilar to human hepatic function. The aspartate aminotransferase activity was measured at the same time points as the ALT, since the previous work with rats indicated heart tissue could be severely affected over time. The AST enzyme activities did increase with time and with exposure to the refined diet from pretest to week 5, and then leveled off by week 10. No significant differences were found.

A loss of lysyl oxidase activity due to copper deficiency has resulted in vascular abnormalities and increased mortality in several animal models.[43-48] Death has been attributed to a loss of strength or integrity of vascular tissue producing fatal aneurysms. These effects were clearly demonstrated by Hill and Davidson after young pigs were raised on a copper deficient diet.[49] It has also been suggested that human abdominal aortic aneurysms may be prevented by dietary copper supplementation in genetically susceptible individuals.[50] At the termination of the study, plasma and aortic tissue were processed for the analysis of LOA. The LOA data suggest that with continued exposure to the FrCuD diet the swine were at an increased risk for vascular aneurysms.

In conclusion, it appears that the pig is a sensitive model for the study of cardiovascular abnormalities associated with a low-copper, moderately high fructose diet. The results also indicate that the U.S. population consuming approximately 0.8 to 1.0 mg of copper daily may be advised to consume the higher estimated safe and adequate intake suggested for copper (2 to 3 mg/day), if fructose consumption continues to rise.

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