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Effects of changes in dietary energy density and the amount of fructose on indices of copper status and metabolic parameters in male rats

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This study was conducted to test the effects of changes in dietary energy density and fructose content on the signs of copper deficiency in rats. For 7 weeks male weanling rats were fed ad libitum diets prepared with adequate (95 μmol Cu/kg of diet) or low (9.5 μmol Cu/kg of diet) copper levels that were of different energy density and fructose levels. Rats fed the copper-deficient diet with a higher energy density and a high fructose level showed the characteristic abnormalities of copper deficiency. These include reduced body weight; enlarged heart, liver, and testes; atrophy of the pancreas; a low hepatic copper level; a low hemoglobin level; anemia; reduced red blood cell and hepatic superoxide dismutase activity; elevated serum cholesterol, triacylglycerol, glucose, and blood urea nitrogen levels; elevated hepatic iron; and mortality. Lowering the energy density and/or reducing the fructose level improved many of the signs associated with copper deficiency and prevented mortality. Reducing the energy density of the diet and the fructose level produced a marked improvement in the activity of the copper-dependent antioxidative enzyme, superoxide dismutase, in liver and red blood cells. It is possible that this improvement in copper-dependent antioxidative enzyme activity is partly responsible for the improvement in the signs of copper deficiency. (J. Nutr. Biochem. 7:118–124, 1996.)

Keywords: energy; fructose; copper deficiency; male rat; superoxide dismutase

Introduction

Copper is an essential trace metal that serves as a cofactor for several enzymes and proteins crucial for various biological and physiological functions in animals and humans.[1] Dietary copper deprivation in experimental animals leads to several abnormalities involving multiple organ systems.[2] In the rat the metabolic responses to the deficiency are dependent on the age,[3] gender,[4] and type of dietary carbohydrate.[5] Only weaned male, but not female, rats consuming a low-copper diet containing fructose, as compared with starch, exhibit severe copper-deficiency syndrome. This includes reduced body weight, anemia, hepatic and cardiac enlargement, atrophy of the pancreas, hypercholesterolemia, hypertriglyceridemia, elevated blood urea nitrogen (BUN) levels, and cardiac abnormalities that eventually lead to premature death due to heart rupture.[6–10]

Recent studies from our laboratory[11] and others[12] revealed that lowering the food intake ameliorates the severity of the copper deficiency and prevents mortality. These studies demonstrated that a 20 to 30% decrease in food intake was sufficient to improve the indices of copper and antioxidant status. These studies[11,12] also indicated that energy intake and fructose (sucrose) intake are two independent factors that may affect some of the known copper deficiency abnormalities. Energy restriction without malnutrition has been reported to prolong the maximum life span of laboratory rodents by mechanisms that retard age-related changes hypothesized to be associated with the activity of antioxidant enzymes, both copper-dependent and copper-

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independent, and free radical damage.[13,14] In contrast, copper deficiency impairs antioxidant status by decreasing the activity of copper-dependent and copper-independent antioxidant enzymes and enhancing free radical damage.[15,16] Thus, energy restriction may improve the deleterious symptoms of copper deficiency. Since fructose worsens copper deficiency in a dose-dependent manner,[17] it is possible that the reduction in fructose intake without reducing the energy intake may also improve the signs associated with the deficiency. The present study explores the hypothesis that in addition to decreased food intake,[11,12] lowering the energy density without restricting food intake, and reducing the content of fructose also reduce the syndrome of copper deficiency.

Methods and materials

Fifty weanling male Sprague–Dawley rats (Hilltop Lab Animals, Scottdale, PA USA) weighing 40 to 45 g were randomly divided into five groups of 10 rats each. The rats were housed individually in stainless-steel cages with wire-mesh bottoms in a room maintained at 22 to 24°C and 50 to 60% humidity with 12 hr periods of light and dark. The experimental protocol was approved by the Beltsville Area Institutional Animal Care and Use Committee of the Agricultural Research Service. The rats consumed diets ad libitum for 7 weeks that varied in copper, fructose, and energy (fat, protein, and fiber content) as indicated in *Table 1*. By analysis, the purified diets contained 96.7 and 9.7 to 10.8 μmol Cu/kg of diet and were considered copper-adequate and copper-deficient, respectively. All diets were adequate in all macro- and micronutrients except copper. Fructose was the sole source of carbohydrate. The energy density of the diets was altered by changing the amounts of egg white, corn oil, fructose, and fiber (cellulose). The diets were formulated to supply energy at two different levels: 17.35 mJ/kg (groups 1 to 3) and 13.61 mJ/kg (groups 4 and 5). The energy from fructose ranged from 45.2% (low) to 76.7% (high). The source of the diet ingredients were as follows: fructose (A.E. Staley Corp., Decatur, IL USA), egg white (ICN Biochemicals, Cleveland, OH USA), corn oil (CPC International, Inc., Englewood Cliffs, NJ USA), cellulose (ICN Biochemicals), AIN-76 mineral mixture prepared without copper (Teklad, Madison, WI USA), AIN-76A[18] vitamin mixture (Teklad), choline bitartrate (Teklad), and biotin (Aldrich Chemicals, Milwaukee, WI USA). Copper was added to the group 1 diet (copper-adequate) as copper carbonate (Fisher Scientific, Pittsburgh, PA USA). All animals were allowed free access to the diet and to distilled deionized drinking water. The level of copper in the drinking water was below the limit of detection (0.015 μmol/L).

At the end of the feeding period, the rats were fasted for 16 hr and decapitated. Trunk blood was collected in capillary tubes for packed cell volume (hematocrit) and in tubes for blood analysis. Whole blood was kept on ice before centrifugation at 1,500 *g* for 25 min at 4°C. Serum was separated and stored at –70°C until use. Erythrocytes were washed once in 5 vol of cold saline (9 g of sodium chloride/L), centrifuged, and used for the determination of superoxide dismutase (SOD) (EC 1.15.1.1) activity by the photochemical o-dianisidine riboflavin assay.[19] Heart, liver, pancreas, and both testes were quickly removed, blotted, and weighed. Liver samples were stored at –70°C for the measurement of SOD activity and for microelement analysis. To measure SOD activity, liver homogenates (10% wt/vol) were prepared in 0.2% (vol/vol) Triton X-100. The crude homogenates were treated with 0.25 vol of ethanol and 0.15 vol of chloroform. Following centrifugation at 6,000 *g* for 20 min, the supernatant obtained was used for the assay of SOD activity.[19] Diet and liver samples, 1 g each, were digested by dry heat and acid,[20] and copper and iron were measured by using flame atomic absorption spectrophotometry (Perkin Elmer, Norwalk, CT USA, model 5000). Bovine liver 1577a, obtained from the National Institute of Standards and Technology (Gaithersburg, MD USA), was digested and analyzed along with samples to verify accuracy.

Serum ceruloplasmin (EC 1.16.3.1) activity was determined with o-dianisidine dihydrochloride as substrate.[21] Serum cholesterol, triacylglycerol, glucose, and BUN were measured by the Centrifichem automated procedure (Trace-America, Miami, FL USA). Hemoglobin was determined spectrophotometrically as cyanmethemoglobin using kit 525 (Sigma Chemical Co., St. Louis, MO USA). Serum immunoreactive insulin was determined by radioimmunoassay[22] using rat insulin (gift from Eli Lilly Co., Indianapolis, IN USA) as the standard.

The data pertaining to the effects of dietary fructose level and

Table 1 Composition of experimental diets

Ingredients (g/kg)	Group 1	2	3	4	5
Fructose	625	625	470	625	470
Egg white	200	200	355	100	176
Corn oil	95	95	95	40	75
Cellulose	32	32	32	187	231
Mineral mixture*	35	35	35	35	35
Vitamin mixture†	10	10	10	10	10
Choline bitartrate	3	3	3	3	3
Biotin	0.002	0.002	0.002	0.002	0.002
Copper (μmol/kg)‡	96.7	10.4	10.8	9.76	10.1
Energy (mJ/kg)	17.35	17.35	17.35	13.61	13.61
Energy (%)					
Fructose (carbohydrate)	60.2	60.2	45.2	76.7	57.7
Protein	19.2	19.2	34.2	12.3	21.6
Lipids	20.6	20.6	20.6	11.0	20.7

*AIN 76 but prepared without copper.
†AIN 76A.
‡By analysis.

energy density were analyzed as a completely randomized design using the SAS GLM procedure.[23] The data from five groups were analyzed as a 2 × 2 factorial plus a control, and the main effects and the interactions were tested as linear contrasts. Duncan's Multiple Range Test was used to test the differences among the five groups. Groups 2 to 5 were analyzed by a 2 × 2 analysis of variance (ANOVA).

Results

During the seventh week of feeding, three rats from group 2 died. Necropsy showed clotted blood in the chest cavity and the rupture of the heart at the apex. Rats from all other groups survived until sacrifice.

Feeding copper-deficient diet with high fructose and higher energy density (group 2) significantly altered body weight and relative organ sizes (*Table 2*), the indices of copper status (*Table 3*), and the metabolic parameters (*Table 4*) compared with rats fed an adequate copper diet with high fructose and adequate energy (group 1). The results confirm our previous findings.

Table 2 summarizes the data on body weight and relative organ sizes in rats fed different diets with varying energy density and fructose content. Comparing only the rats fed copper-deficient diets (groups 2 to 5) by two-way ANOVA, there was a significant effect of the dietary fructose level on body weight and the relative sizes of the liver, heart, pancreas, and testes. Diets with a higher fructose content (groups 2 and 4) decreased body weight and pancreas size and increased relative liver size compared with diets with a lower fructose content (groups 3 and 5) at the same energy density. Changes in energy density affected the relative sizes of liver, heart, and testes. Thus rats fed diets with a lower energy density had lower relative sizes of liver, heart, and testes compared with those fed diets with a higher energy density. Significant interactions between the dietary fructose level and the energy level were observed on the sizes of the liver, heart, and pancreas. It is important to note that lowering the energy density and the amount of fructose (group 5) significantly improved the changes in body weight and organ sizes caused by the copper-deficient diet with high fructose and higher energy density (group 2). The values were not significantly different from those fed an adequate copper diet (group 1) except for testes size. Similar improvements were observed for liver, heart, and pancreas relative sizes when the level of dietary fructose was reduced (group 3).

Table 3 summarizes the data on the indices of copper status, hepatic iron, and copper-dependent enzymes with antioxidant properties. ANOVA of the data from rats fed copper-deficient diets (groups 2 to 5) showed a significant effect of energy level on all parameters reported, while the dietary fructose level significantly affected all parameters except the hepatic copper and iron levels. Similarly, significant interactions between the levels of energy density and fructose were observed for all parameters except hepatic copper and iron. It is important to note that the hematocrit, hemoglobin level, and SOD activities in red blood cells (RBCs) and liver were significantly higher in copper-deficient rats fed diets low in fructose and/or energy (groups 3 to 5) compared with those fed high fructose and adequate energy (group 2). The hematocrit levels were similar to those observed in rats fed the copper-adequate diet. There was a significant increase in the hemoglobin level and SOD activities in copper-deficient rats fed the diet with reduced energy density and/or fructose levels. However, they were still significantly lower than values observed for rats fed a copper-adequate diet. Among the rats fed low copper diets (groups 2 to 5), the hepatic iron concentration was significantly lower in those fed diets with a lower energy density (group 4). Their diet also had the lowest content of protein and fat but the high fiber content.

The changes in metabolic parameters as affected by different diets are summarized in *Table 4*. ANOVA showed that among the rats fed copper-deficient diets, the fructose level significantly affected the plasma cholesterol and triacyglycerol levels while the cholesterol, BUN, and glucose levels were significantly altered by the energy level. The highest levels of cholesterol, BUN, and glucose were observed in rats fed diets with a higher energy density and higher fructose content (group 2). Among the rats fed copper-deficient diets, the plasma triacylglycerol levels were higher in rats fed diets higher in fructose content regardless of the energy density. Significant interactions between levels of energy and fructose were observed for serum cholesterol and BUN levels. Compared with rats fed a copper-

Table 2 Body weight and relative organ sizes in male rats consuming diets with various energy and fructose levels*

	Group					ANOVA†		
	1	2	3	4	5	F	En	F × En
No. of rats	10	7	10	10	10			
Body weight (g)	280 ± 14^{a}	218 ± 26^{d}	$244 \pm 22^{b,c}$	$234 \pm 27^{c,d}$	$260 \pm 32^{a,b}$	S	NS	NS
Liver‡	3.50 ± 0.18^{c}	4.50 ± 0.19^{a}	3.65 ± 0.25^{c}	3.94 ± 0.23^{b}	3.65 ± 0.31^{c}	S	S	S
Heart	0.38 ± 0.04^{b}	0.58 ± 0.15^{a}	0.41 ± 0.06^{b}	0.40 ± 0.03^{b}	0.41 ± 0.03^{b}	S	S	S
Pancreas	0.65 ± 0.11^{a}	0.25 ± 0.06^{b}	0.64 ± 0.09^{a}	0.33 ± 0.11^{b}	0.58 ± 0.10^{a}	S	NS	S
Testes	1.13 ± 0.08^{c}	1.59 ± 0.16^{a}	1.47 ± 0.13^{b}	1.41 ± 0.14^{b}	1.34 ± 0.13^{b}	S	S	NS

F, fructose; En, energy.
*Values are means ± SD. Values not sharing the same superscript within a row are significantly different from each other at $P < 0.05$ according to Duncan's Multiple Range Test.
†2 × 2 ANOVA only for groups 2 to 5. S, significant; $P < 0.05$; NS, nonsignificant.
‡Relative organ size = (organ wt × 100)/body wt.

Table 3 Hematocrit, hemoglobin, hepatic copper, and iron concentrations and RBCs and hepatic SOD activity in male rats consuming diets with various energy and fructose levels*

	Group					ANOVA†		
	1	2	3	4	5	F	En	F × En
No. of rats	10	7	10	10	10			
Hematocrit (L)	0.46 ± 0.03^a	0.25 ± 0.04^b	0.41 ± 0.06^a	0.43 ± 0.05^a	0.41 ± 0.05^a	S	S	S
Hemoglobin (g/L)	140 ± 5^a	65 ± 11^c	109 ± 6^b	106 ± 4^b	112 ± 8^b	S	S	S
Hepatic Cu (nmol/g of WT)	85.6 ± 11.1^a	20.5 ± 2.5^c	27.8 ± 5.6^b	31.5 ± 4.6^b	30.8 ± 6.8^b	NS	S	NS
Hepatic Fe (μmol/g of WT)	1.79 ± 0.41^c	2.64 ± 0.24^a	2.68 ± 0.35^a	2.18 ± 0.32^b	2.51 ± 0.37^a	NS	S	NS
Ceruloplasmin (U/L)	128 ± 13	ND	ND	ND	ND			
RBC SOD (U/ml of PC)	685 ± 34^a	135 ± 26^c	255 ± 29^b	257 ± 28^b	260 ± 28^b	S	S	S
Hepatic SOD (U/g)	1875 ± 65^a	565 ± 41^c	978 ± 52^b	982 ± 48^b	985 ± 45^b	S	S	S

Cu, copper; Fe, iron; ND, nondetectable; WT, wet tissue; RBC, red blood cells; SOD, superoxide dismutase; F, fructose; En, energy; PC, packed cells.
*Values are means ± SD. Values not sharing the same superscript within a row are significantly different from each other at $P < 0.05$ according to Duncan's Multiple Range Test.
†2 × 2 ANOVA only for groups 2 to 5. S, significant; $P < 0.05$; NS, nonsignificant.

deficient diet with high fructose and adequate energy (group 2), rats fed either low fructose or reduced energy diets (groups 3 to 5) showed significant amelioration in levels of serum cholesterol, triacylglycerol, and BUN. There was no significant improvement in the plasma insulin levels by reducing either the fructose or energy levels.

Discussion

Previous studies by Saari et al.[12] and from our laboratory[11] have shown that food restriction partially ameliorates the severity of copper deficiency in male rats fed a copper-deficient diet with high fructose. The present study shows that lowering the energy density without restricting the food intake also partially ameliorates the severity of copper deficiency. A similar beneficial effect is also observed by decreasing the levels of fructose in the diet with or without lowering the energy density.

The fructose intake can be lowered without changing the source of carbohydrate by (1) isocaloric replacement of fructose with either protein or fat, (2) lowering the total energy intake by reducing the energy density of the diet without changing the percent energy contribution from fructose (carbohydrate), protein, and fat, or (3) total food restriction. The latter approach used in previous studies[11,12] involves meal feeding. It is not clear from these studies whether a lower fructose intake or a lower total energy intake produced the amelioration of copper deficiency syndrome. Furthermore, meal feeding per se affects several metabolic parameters compared with ad libitum feeding. Therefore, in the present study we used the first two approaches. Both these regimens produced varying degrees of amelioration of the effects on body weight, organ sizes, indices of copper status, antioxidant enzymes in the liver and RBCs, and various metabolic parameters. There was no significant additive or synergistic effect of lowering the fructose intake and energy density on any parameters studied. Most importantly, all three regimens (groups 3 to 5) prevented early mortality, the most detrimental effect of copper deficiency in male rats fed high fructose.[24]

Experimental copper-deficient animals show characteristic signs that have many similarities to those observed in age-related processes such as the abnormalities associated with free radical damage, oxidative stress, and altered physiological functions of various organs.[13–16] Toxic free radical reactions have long been thought to contribute to biological aging[25,26] by causing lipid peroxidation and free radical–mediated glycation which are known to play a major role in

Table 4 Serum parameters in male rats consuming diets with various energy and fructose levels*

	Group					ANOVA†		
	1	2	3	4	5	F	En	F × En
No. of rats	10	7	10	10	10			
Cholesterol (mmol/L)	2.08 ± 0.29^c	3.72 ± 0.39^a	2.88 ± 0.45^b	2.83 ± 0.39^b	2.82 ± 0.50^b	S	S	S
Triacylglycerol (mmol/L)	0.38 ± 0.11^b	0.68 ± 0.19^a	0.48 ± 0.12^b	0.75 ± 0.16^a	0.50 ± 0.12^b	S	NS	NS
BUN (mmol/L)	3.85 ± 0.25^c	9.01 ± 2.41^a	6.97 ± 1.23^b	4.69 ± 0.95^c	5.06 ± 0.79^c	NS	S	S
Glucose (mmol/L)	6.35 ± 0.34^c	8.03 ± 0.39^a	7.93 ± 0.32^a	6.59 ± 0.37^c	7.13 ± 0.45^b	NS	S	NS
Insulin (pmol/L)	25.6 ± 4.5^a	17.6 ± 3.8^b	19.8 ± 3.4^b	18.1 ± 3.6^b	16.5 ± 4.1^b	NS	NS	NS

BUN, blood urea nitrogen; F, fructose; En, energy.
*Values are means ± SD. Values not sharing the same superscript within a row are significantly different from each other at $P < 0.05$ according to Duncan's Multiple Range Test.
†2 × 2 ANOVA only for groups 2 to 5. S, significant; $P < 0.05$; NS, nonsignificant.

increasing the incidence of life-shortening diseases.[14] Evidence to support the theory that oxidative damage is related to signs of copper deficiency is the finding that the administration of exogenous antioxidants to copper-deficient rats inhibits the development of anemia and cardiac enlargement.[27] Further support for a possible role of hydroxyl radicals is the evidence that dietary chelation of iron and the promotion of hydroxyl radical formation by deferoxamine can also inhibit anemia and heart pathology.[28]

Oxygen-utilizing organisms have antioxidative defense mechanisms that involve many enzymes capable of destroying free radicals. One of the primary enzymes in the liver and RBCs is copper-containing superoxide dismutase. It catalyzes the dismutation of the superoxide anion into hydrogen peroxide, which is eventually converted to water. The sensitivity of copper-deficient rats to oxidant stress as expressed by increased lipid peroxidation, expiration of pentane and ethane, decreased body weight, and increased mortality[15] can be explained by the decreased activity of copper-containing antioxidative enzymes. Both SOD, by its dismutation of superoxide, and ceruloplasmin, by inhibiting iron-mediated catalysis of hydroxyl radical formation, may be regarded as antioxidant enzymes. The low levels of SOD in the liver and RBCs in the rats fed the high-fructose higher energy density copper-deficient diet suggest that oxidative damage could be the cause of the severe signs and morality observed in these rats.

In the present study, we demonstrated that feeding diets with a lower energy density and lower fructose level improved SOD activity in the liver and RBC but had no beneficial effect on the serum ceruloplasmin activity which remained below the detectable limit (*Table 3*). The improvement in the SOD activity may be related to an increase in the hepatic copper concentration. Though other antioxidative enzymes were not measured in the present study, Saari et al.[12] have shown that food restriction enhanced the activity of other hepatic antioxidative enzymes, cytochrome c oxidase, and glutathione peroxidase in addition to SOD. Thus, they suggested that the combined beneficial effects of food restriction on these enzymes could protect the rats from oxidative damage caused by copper deficiency. Energy restriction has also been found to enhance protection against reactive oxygen molecule-damaging action[29] by increasing the activity and mRNA levels of SOD, catalase, and glutathione peroxidase in the rat liver.[30]

Though the present and previous studies[11,12] provide support for the improvement of the copper status and the antioxidative defense by food restriction or lowering energy density and by reduced dietary fructose level, the exact mechanism responsible for modulation of the signs of copper deficiency in male rats is still uncertain. The absence of additive or synergistic beneficial effects in the current study suggest that the effects of diets with lower energy density and fructose concentration are probably unrelated in modulating the signs of copper deficiency.

In the present study we did not measure the food intake and hence it cannot be assumed that lowering the energy density of diet caused reduced energy intake. However, a recent study by Stubbs et al.[31] did demonstrate that lowering the energy density of the diet by substituting carbohydrate for fat content caused a reduction in the total energy intake. They further concluded that lower energy diets are more satiating than higher energy diets.[31]

Fructose is metabolized mainly in the liver and is known to increase the activity of enzymes of the pentose shunt[32] that generate reduced environment by forming reduced nucleotides. When fructose is the only dietary carbohydrate source, under reduced environment conditions, it is favorably converted to sorbitol by sorbitol dehydrogenase.[33] It is important to mention that Fields et al.[34] observed that sorbitol accumulates in the liver and kidneys of copper-deficient rats fed a high fructose diet. Sorbitol is a strong chelator of copper, and the complex is very stable.[35] The increased levels of sorbitol may chelate the limited amounts of copper in the copper-deprived rats making it even more nutritionally unavailable. Though the hepatic sorbitol concentration was not measured in the present study, it is possible that a 20% reduction in fructose intake could lower the hepatic sorbitol levels. Since the liver plays a major role in copper homeostasis, any decrease in the sorbitol levels will result in the increase of the hepatic copper level and should be beneficial to the animal.

It is important to note that diets with reduced energy density (groups 4 and 5) are low in total fat content and high in fiber content. In addition, group 4 has a very low protein concentration. It is possible that some of the effects observed in rats fed low copper diets with reduced energy density may be due to low protein and fat content and higher fiber content. The beneficial effects of a high fiber diet on lipid and carbohydrate metabolism, namely hypoglycemic and hypolipidemic effects, are well documented.[36–38] Similarly, the type and amount of dietary fat and carbohydrate also affects lipid metabolism and hormones involved in carbohydrate and lipid metabolism.[39–42]

As for food restriction,[11,12] we are uncertain whether modulations to life span and improvement in the signs of copper deficiency are due to the decrease in the intake or a particular dietary component or energy per se. In the present study, minerals and vitamins were not involved in the effects of copper deficiency since all diets contained the same amount. Regarding the manipulation of dietary protein and fat content, Yu[14] concluded that energy restriction rather than reduced protein or fat intake is the over-riding factor in modulating aging, age-related disease processes, and life span extension. Since copper deficiency and aging seem to share some common characteristics associated with decreased antioxidant enzyme activity and free radical damage, one may postulate that the mechanisms responsible for the beneficial effects of energy restriction on aging are similar to those on copper deficiency, and therefore decreased intake of protein and fat (group 4) may not be responsible factors in the observed ameliorating effect on the signs of copper deficiency.

Energy restriction, without essential nutrient intake deficiency, reduces the metabolic rate[43] that results in lowering the rate of electron transport in the respiratory chain, leading to decreased production of free radicals and cell damage.[44] Furthermore, energy restriction increases the ability to remove potentially toxic products of lipid peroxidation.[45] Masoro[43] also suggested that reduced energy in-

take involves altered characteristics of fuel utilization due to neural and/or endocrine responses. In the present study, rats fed low energy density copper-deficient diets exhibited a lower serum glucose level while insulin levels remained unaffected, suggesting that the characteristics of the use of fuel have been altered.

In conclusion, our previous data on reduced food intake and the source of carbohydrate (starch versus fructose) and the data in the present study provide support for the individual beneficial effect of either lowering the dietary energy density or fructose content on copper status and the status of copper-dependent antioxidant enzymes and more importantly in reducing early mortality in male rats fed a copper-deficient diet.

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