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Original Research

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Alterations in plasma lipids, glutathione and homocysteine in relation to dietary copper in rats

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ABSTRACT

Objective: This study aimed to investigate the effect of dietary copper either deficiency or excess on the plasma lipid profile, glutathione (GSH), and homocysteine levels. **Design:** Three groups of male albino rats were served as control (fed diet containing the required level of Cu; 5.40 mg/kg), copper-deficient (fed diet containing Cu; 0.60 mg/kg) and copper-excess (fed diet containing high level of Cu; 15.0 mg/kg). **Materials and Methods:** At the end of the experimental period (6 weeks), plasma levels of copper, lipid profile, GSH and homocysteine were estimated in all tested rats groups. **Results:** There was a significant increase in triacylgycerols, total cholesterol (T.Ch.), high density lipoprotein-cholesterol (HDL-C), very low density lipoprotein-cholesterol, and low density lipoprotein-cholesterol in rats fed a copperdeficient diet compared to control and those fed copper-excess one. In rats fed copper-deficient, HDL-C/T. Ch. ratio was significantly lower than that of the other tested animals. In addition, plasma GSH increased significantly, while plasma homocysteine decreased significantly in copper-deficient group compared to control and copper-excess groups. A significant negative correlation between plasma copper and each of T.Ch. and plasma GSH was observed in control, copper-deficient and copper-excess groups. On the contrary, plasma copper has shown a significant positive correlation with plasma homocysteine in all groups. **Conclusion:** the present results indicate that feeding of diets rich in bread and powdered milk develop copper-deficiency that resulted in an increase in plasma T.Ch. and triacylglycerols, while decreased HDL-C/T.Ch. ratio. This is representing a risk factor for the development of cardiovascular disorders. However, the body is able to protect itself against these risky changes by increasing the production of antioxidant (GSH) on the expense of homocysteine. Furthermore, copper supplementation can alleviate these degenerative changes.

KEY WORDS: Copper deficiency, glutathione, homocysteine, plasma lipids

INTRODUCTION

Copper is present in all tissues and is one of the critical components of the antioxidant system in the body. In the blood, plasma copper is attached to plasma transport protein, ceruloplasmin. In the blood cells, it is associated with superoxide dismutase [1].

Copper is also an essential cofactor for many enzymes including cytochromes, but it is toxic in unbound form. The vast majority of serum copper is transported bound to albumin, transcuprein and copper-amino acid complexes [1].

A role of low dietary copper may contribute to the pathogenesis of cardiovascular disease. In humans, some of the symptoms of copper deficiency are hypercholesterolemia, leucopenia, and demineralization of bones, anemia and demyelination of neural tissue [2].

Glutathione (GSH) appears to have marked antioxidant activities and therefore may prevent cardiovascular disease. It was suggested that reduced plasma GSH level is a risk factor for development of cardiovascular and cerebrovascular diseases [3].

Homocysteine is a sulfur-containing amino acid that is a demethylated derivative of methionine. Methionine is a component of protein of animal origin. Homocysteine is produced by intracellular demethylation of methionine and then exported to the plasma, where it circulates, mainly in its

oxidized form. Homocysteine is metabolized through two main pathways: Remethylation to methionine or transsulfuration to cystathionine and then to cysteine. A decrease in the rate of remethylation of homocysteine into methionine produces hyperhomocysteinemia.

It is recognized that hyperhomocysteinemia produces thrombogenesis, vasodilation and endothelial damage and is associated with cardiovascular and cerebrovascular diseases [4,5]. In addition, many other studies link high plasma homocysteine levels to cardiovascular diseases [6,7].

The present study aimed to investigate the effect of dietary copper either its deficiency or excess on the levels of different lipid constituents, GSH and homocysteine in plasma of rats.

MATERIALS AND METHODS

Experimental Animals

Forty-five healthy male albino Wistar rats (weighing 35-40 g) aged 2 weeks was used in this study. The rats were obtained from the animal house of Medical Research Institute Alexandria University, Egypt. The animals were maintained in stainless steel cages in a well-ventilated animal house at a normal temperature (25° ± 5°C) under 12:12 h light dark cycle. The rats were maintained under standard conditions in an animal house according to the guidelines of Beni-Suef University Committee for the purpose of control and supervision on experiments on animals. The rats were randomly divided into three equal groups (15 rats each). Every group was fed ad libitum on a certain diet and given bidistilled water free access. The first group served as control and fed a diet containing the required level of copper for rats (5.40 mg/kg). The second group was fed a diet deficient in copper (0.60 mg/kg), whereas the third group was fed a diet supplemented with a high level of copper (about 3 times the Cu level in the control diet; 15.0 mg/kg). The rats were fed the experimental diets for 6 weeks. The experimental diets were composed exclusively from wheat bread and whole powdered milk as these ingredients were very low in Cu content. Copper sulfate pentahydrate (containing 254,500 mg Cu/kg; Sigma-Aldrich, UK) was added at the rate of 20 and 60 mg/kg for the control and Cu-excess diets, respectively, to supply these diets with the designed levels of copper. The diets were formulated according to National Research Council [8] to meet the nutrient requirements of rats. Moreover, the different diets were analyzed according to standardized laboratory methods of Association of Analytical Communities [9]. The physical and chemical composition of diets is shown in Table 1. The rats were weighed at the start of the experiment and at the end of each week of the experimental period.

The blood samples were taken from the rats (8 weeks old) at the end of the experiment by heart puncture while they were under anesthesia. The animals were anesthetized with a 1.37:1 mixture of ketamine:xylazine (1 mL/kg b.wt., i.p; Sigma-Aldrich, UK). Blood samples were poured into tubes containing ethylenediaminetetraacetic acid (Sigma-Aldrich, UK). The samples were centrifuged at 1000×g for 15 min to separate blood plasma. Then, the plasma was stored at −20°C till the time of analysis.

Table 1: Composition of diets fed to the rats during the experimental period (as fed)

	Cu-excess	Cu-deficient	Control
Ingredients			
Bread; %	50.0	50.0	50.0
Milk powder; %	50.0	50.0	50.0
$CuSO_4.5H_2O$; mg/kg*	20.0	-	60.0
Chemical analyses			
ME (kcal/kg; calculated)	3788	3865	3633
CP; %	15.7	16.0	15.0
EE; %	15.3	15.6	14.7
Ca; %	0.53	0.54	0.51
P; %	0.40	0.38	0.39
Cu; mg/kg	5.40	0.60	15.0

*Copper sulfate pentahydrate contains 254,500 mg Cu/kg.
ME: Metabolizable energy, CP: Crude protein, EE: Ether extract

The experimental protocol was approved by Institutional Animal Ethics Committee.

Biochemical Studies

The following parameters were determined in the blood plasma of the three tested rats groups by T80 ultraviolet-visible spectrophotometer (PG Instruments Ltd., England) and by using Biodiagnostic assay kits (Biodiagnostic, Giza, Egypt) according to the instructions of their referred methods total cholesterol (T.Ch.)[10], triacylglycerols [11], high density lipoprotein cholesterol (HDL-C) [12], low and very low density lipoprotein-cholesterol (LDL-C and VLDL-C) [13] and GSH [14]. Copper was measured in the blood plasma of the three tested rat groups by atomic absorption spectrophotometer (Model 2300, Perkin Elmer, USA) [15]. Homocysteine was also measured in the blood plasma of the three tested rat groups by using Diazyme homocysteine enzymatic kit (Diazyme Laboratories, California, USA, cat. no. DZ568A-KH2) [16].

Statistical Analysis

All numerical data in the text and tables were expressed as the mean ± standard error of the mean. Statistical analysis was performed by using the Statistical Package for Social Sciences program, version 16 (Chigao, USA). Student's *t*-test and correlation coefficient were performed whenever needed. Differences were considered statistically significant at $P < 0.05$.

RESULTS

The body weight of rats was affected by the level of copper in the diet as indicated by a significant lower body weight of rats that were fed a copper-deficient diet throughout the experiment compared to the rats that were fed the control diet or copper-excess diet [$P < 0.05$; Table 2].

Measurement of lipid profile revealed a significant increase in the plasma levels of T.Ch., triacylglycerol, HDL-C, VLDL-C and LDL-C ($P < 0.05$) in copper-deficient group compared to that of control and copper-excess ones. In copper-deficient group, the HDL-C/T.Ch. ratio was significantly lower ($P < 0.05$) than that of control and copper-excess groups. On the other hand, there was no significant variation in the LDL-C/T.Ch. ratio among the three tested groups [Table 3].

There was a significant decrease in the plasma copper level in the copper-deficient group compared to control and copper-excess groups [Table 4]. In addition, the study of the plasma GSH showed a significant increase in its level in copper-deficient group compared to that of control and copper-excess ones ($P < 0.05$). In contrast, the plasma homocysteine level showed a significant decrease in rats that were fed the copper-deficient diet compared to those fed the control diet or copper-excess diet.

Investigation of the correlation coefficient between plasma copper and plasma T.Ch. had revealed a significant negative correlation between the two variables in the three tested groups. There was also a significant negative correlation between plasma copper and plasma GSH in all different treatments. On the contrary, plasma copper has shown a significant positive correlation with plasma homocysteine in the control, copper-deficient and copper-excess groups [$P < 0.05$; Table 5].

At the end of the experiment, the rats suffering from copper deficiency appeared smaller in size, but no signs of morbidity were observed throughout the experiment. Furthermore, no mortality was recorded in all treatments during the experiment.

Table 2: Body weight (g) of rats fed different experimental diets ($n=15$)

Week	Control	Cu-deficient	Cu-excess
2nd	36.1±0.90[a]	37.2±1.00[a]	38.4±0.80[a]
3rd	52.0±1.31[a]	47.3±1.05[b]	51.7±1.49[a]
4th	80.2±1.54[a]	69.1±1.36[b]	83.1±1.78[a]
5th	111.7±2.24[a]	87.5±1.85[b]	109.5±2.34[a]
6th	140.5±2.71[a]	104.1±2.50[b]	142.3±2.78[a]
7th	170.2±3.17[a]	123.5±2.86[b]	168.2±3.15[a]
8th	201.5±3.79[a]	138.2±3.40[b]	195.1±3.95[a]

[a,b]Means in the same row with different superscripts are significantly different ($P<0.05$)

Table 3: Lipids profile in plasma of rats fed the control, copper-deficient and copper excess diets ($n=15$)

	Control	Cu-deficient	Cu-excess
T.Ch. (mg/dL)	77.2±1.8[a]	110.1±2.5[b]	78.2±1.9[a]
TAG. (mg/dL)	38.2±1.1[a]	88.5±2.9[b]	41.9±1.8[a]
HDL-C (mg/dL)	21.1±0.5[a]	26.8±0.9[b]	22.9±1.6[a]
VLDL-C (mg/dL)	7.5±0.3[a]	17.3±0.4[b]	8.25±1.2[a]
LDL-C (mg/dL)	51.1±1.8[a]	65.5±3.1[b]	48.1±1.5[a]
LDL-C/T.Ch. ratio	0.62±0.02[a]	0.59±0.01[a]	0.63±0.03[a]
HDL-C/T.Ch. ratio	0.27±0.01[a]	0.24±0.01[b]	0.29±0.01[a]

[a,b]Means in the same row with different superscripts are significantly different ($P<0.05$). T.Ch: Total cholesterol, TAG: Triacylglycerol, HDL-C: High density lipoprotein-cholesterol, VLDL-C: Very low density lipoprotein-cholesterol, LDL-C: Low density lipoprotein-cholesterol

DISCUSSION

The diets were composed from ingredients of a low copper content. Thus, the diets were formulated exclusively from wheat bread and whole powdered milk. The copper sulfate was added to control and Cu-excess diets at 0.002% and 0.006%, respectively, to provide these diets with the needed amounts of copper. The copper levels were determined in the diets and were found, as indicated, to be 5.40, 0.60 and 15.0 mg/kg for the control, Cu-deficient and Cu-excess diets, respectively. The level of Cu used in the Cu-excess group was chosen to be not toxic for the rats as the toxic level of Cu was reported by Boyden *et al.* to be more than Cu 500 mg/kg [17]. In addition, the body weight of the rats fed excess-copper diet was similar to that of the control along the experiment [Table 2] and no symptoms of copper toxicity were observed on these animals.

Based on the obtained results, there was a direct effect of dietary Cu content on both body weight and plasma Cu level as indicated by lower body weight (138 g vs. 201 g) and plasma level (50 μg/dL vs. 129 μg/dL) in rats that were fed Cu-deficient diet compared to the control. However, excess of copper in the diet did not affect the body weight (195 g vs. 201 g) of rats, but increased the plasma level of copper (142 μg/dL vs.129 μg/dL).

In the present study, dietary copper deprivation reduced the growth of rats after about 1-2 weeks from the onset of the treatments ($P < 0.05$) and these animals showed also a highly significant lower body weight during the following weeks (3-6 weeks of the treatments; $P < 0.001$). The decrease in body weight of rats that were fed a copper-deficient diet indicates that copper is essential for growth as it is an integral part of many enzymes in the body and is also required for normal development [1].

The significant increase in plasma triacylglycerols of rats that were fed a copper-deficient diet compared with rats that were fed

Table 4: Concentrations of copper, GSH and homocysteine in plasma of rats fed the control, copper-deficient and copper excess diets ($n=15$)

	Control	Cu-deficient	Cu-excess
Copper (μg/dL)	128.6±5.8[a]	50.1±2.1[b]	141.6±6.53[c]
GSH (μmol/L)	3.4±0.4[a]	9.5±0.7[b]	3.7±0.5[a]
Homocysteine (μmol/L)	4.2±0.5[a]	1.9±0.3[b]	3.9±0.4[a]

[a,b,c]Means in the same row with different superscripts are significantly different ($P<0.05$). GSH: Glutathione

Table 5: Correlation coefficient between plasma copper and each of cholesterol, GSH, and homocysteine levels in the control, copper-deficient and copper excess groups of rats ($n=15$)

Correlation coefficient between	Control	Cu-deficient	Cu-excess
Plasma copper and cholesterol levels	$P<0.05$ $r=-0.57$	$P<0.05$ $r=-0.66$	$P<0.05$ $r=-0.68$
Plasma copper and GSH	$P<0.05$ $r=-0.4$	$P<0.05$ $r=-0.57$	$P<0.05$ $r=-0.4$
Plasma copper and homocysteine	$P<0.05$ $r=0.6$	$P<0.05$ $r=0.5$	$P<0.05$ $r=0.4$

GSH: Glutathione

control or copper-excess diet [Table 3] is supported by the results obtained by the previous studies on induced copper deficiency in rats [18-20]. This increase may result from enhanced hepatic lipogenesis, a reduction in lipoprotein lipase activity or both.

Plasma T.Ch. in rats which were fed a copper-deficient diet was increased significantly as compared with those which were fed a copper-excess diet ($P < 0.001$). This result comes in agreement with the previous finding of hypercholesterolemia in diet induced copper deficiency in rats [18-20]. The hypercholesterolemia obtained in the present result can be explained by the previous findings which suggested a shift of cholesterol from the liver to the plasma pool of copper-deficient rats [21]. The observed significant negative correlation between plasma copper and plasma T.Ch. in the present study [Table 5] confirmed that hepatic cholesterogenesis and lipogenesis were increased to sustain the hyperlipidemia associated with copper deficiency [22,23].

At the same time, plasma HDL-C, LDL-C and VLDL-C were increased significantly in rats that were fed a copper-deficient diet when compared with those which were fed the control diet or copper-excess diet ($P < 0.05$).

It was noted that T.Ch. and HDL-C were increased significantly in rats, which were fed a copper-deficient diet as compared with those which were fed the control diet ($P < 0.05$) or copper-excess diet [$P < 0.05$; Table 3]. This result coincided with the result obtained by Lefevre *et al*. [24]. Although the increase in HDL-C in copper-deficient rats is an advantage, the HDL-C/T.Ch. ratio showed a significant decrease as compared with the control and copper-excess groups ($P < 0.05$). Thus, the increase in HDL-C was not proportionate to the increase in T.Ch.

At the same time, LDL-C/T.Ch. ratio did not significantly differ among copper-deficient, copper-excess and control groups. Thus, redistribution of cholesterol occurred with a shift of cholesterol from HDL fraction to LDL fraction. This is hazardous as it will encourage accumulation of cholesterol in the tissues accelerating atherogenesis [25].

The results of the present study also revealed a significant increase in plasma GSH level and a significant decrease in plasma homocysteine level in copper-deficient group as compared with those of control and copper-excess groups ($P < 0.05$). This result agreed with the result obtained by Uthus *et al*. [26] who reported increased plasma GSH and a decreased plasma homocysteine in rats fed a low-copper diet. Our finding is also supported by the results of the previous studies, which reported that an increase in hepatic GSH and 3-hydroxy-3-methylglutaryl-coenzyme A "(HMG-CoA)" reductase activity can explain the hypercholesterolemia associated with copper deficiency in rats [27]. Increased GSH was supposed to enhance the activity of the rate-limiting enzyme of cholesterol biosynthesis, HMG-CoA reductase [27]. The enzyme possesses cysteine residue that, under oxidizing conditions, combine to form a disulfide-linked dimer. The fully reduced monomer showed positive cooperative in nicotinamide adenine dinucleotide phosphate binding, whereas the dimmer exhibits hyperbolic kinetics to this substrate and consequently is less active [28,29].

Significant copper deficiency in rats is associated with an unfavorable metabolic pattern of lipid profile changes, and copper supplementation might be recommended in view of its association with hypercholesterolemia and hypertriglyceridemia and markers of oxidative stress as GSH and homocysteine. These data indicate that copper deficiency can alter biochemical parameters relevant to copper function.

Although feeding on bread with powdered milk decreased homocysteine, it increased the lipid profile that may be attributed to the milk content, which is deficient in copper and high in lipids.

The observed a significant decrease in plasma homocysteine in copper-deficient group compared to that of control and copper-excess groups ($P < 0.05$), in addition to the observed significant negative correlation between plasma copper and plasma GSH level, and the significant positive correlation between plasma copper and plasma homocysteine could be explained on a metabolic basis.

In mammalian liver, two interesting pathways, remethylation and transsulfuration, compete for homocysteine that has been formed from methionine. Remethylation of homocysteine, employing either methyltetrahydrofolate or betaine as the methyl donor, forms a methionine cycle that functions to conserve methionine. In contrast, the transsulfuration sequence – cystathionine synthase and cystathionase – serves to irreversibly catabolize the homocysteine while synthesizing cysteine. The rate of homocysteine formation and its distribution between these two pathways are the sites for metabolic regulation and coordination. The mechanisms for regulation include both the tissue content and kinetic properties of the component enzymes as well as the concentrations of their substrates and other metabolic effectors [26].

All tissues possess the methionine cycle with methyltetrahydrofolate as the methyl donor, but only liver, kidney, pancreas, intestine, and brain also contain the transsulfuration pathway. The copper deficiency might have shifted homocysteine metabolism into the transsulfuration pathway as a result of up-regulation of the catalytic subunit of glutamate-cysteine-ligase, which catalyzes the rate limiting step in GSH biosynthesis producing cysteine, which was utilized for the synthesis of GSH [26,30].

High blood copper and homocysteine concentrations have been independently reported as risk factors for cardiovascular diseases. When they are simultaneously measured, a concomitant increase in both parameters in association with vascular dysfunction has been observed [31]. Copper itself has been shown to be beneficial to the cardiovascular system.

In addition, our results provide evidence that copper deficiency induced a decrease in homocysteine level, and this

is contradictory to high copper level that was associated with an increase in homocysteine level [31].

The current study provides novel data concerning the association among feeding of bread with powdered milk, copper deficiency, lipid profile, oxidative stress marker, and homocysteine.

CONCLUSIONS

Dietary copper deficiency has associated with an increase in plasma T.Ch. and triacylglycerols and a decreased HDL-C/T.Ch. ratio. The increase in plasma GSH and the decrease in plasma homocysteine may be due to a shift of homocysteine into the transsulfuration pathway producing cysteine, which was utilized for the synthesis of GSH.

These data may be important to those individuals who consume large quantities of bread with powdered milk in their foods where the supplement of copper is suboptimal. Copper deficiency was also associated with elevated lipids profile that is risk factor for the development of cardiovascular disorders. One of the novel mechanisms that is used by the body for protecting itself against these risky changes is the increase in the production of the antioxidant; GSH on the expense of homocysteine. However, copper supplementation can alleviate these changes.

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