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Serum lipoprotein profile and oxidative stress biomarkers in Wistar rats fed drinking water containing iron and copper

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Abstract: The aim of the research was to estimate the effect of different doses and combinations of iron and copper consumption with drinking water on lipid profile and oxidative stress biomarkers in albino Wistar rats serum. Rats were given drinking water containing 3 mg L^{-1} and 6 mg L^{-1} iron; copper 4.88 and 9.76 mg L^{-1}; a mixture of 3 mg L^{-1} iron and 4.88 mg L^{-1} copper. Control group obtained pure drinking water. Total cholesterol, lipoprotein spectrum and markers of lipid and protein oxidation were analyzed. It has been seen that administration of iron in concentration of 6 mg L^{-1} induces lipid peroxidation and protein oxidation, while copper given in the maximal doses leads only to protein oxidation. Free radical oxidation in rats obtaining combination of iron and copper with drinking water was more expressed than in case of administration of single metals in the same doses. Consumption of maximal doses of isolated metals leads to more expressed atherogenic changes, while combination of both metals in lower doses did not affect serum lipoprotein significantly. The data obtained show that chemical interaction of iron and copper in the organism has an additive effect on some vital parameters in comparison to isolated metal administration.

Key words: iron; copper; oxidative stress; dyslipoproteinemia

Introduction

It is well known than environmental pollution highly contributes to the development of a number of pathologies. In a large group of inorganic pollutants d-metals, also known as red/ox-metals, are of particular importance being widely spread in the environment. Iron and copper are essential d-metals and their maintenance in drinking water, foods and environments is controlled by hygienic monitoring. At the same time there is a possibility of excessive income of iron and copper into the organism due to their wide industrial use. Being able to act in red/ox reactions, including their own red/ox interaction, iron and copper induce free radical oxidation that leads to oxidative damage of macromolecules (proteins, lipids, nucleic acids) and cellular compounds (especially membranes) (Valko et al. 2005). It is known that oxidative modification of lipoprotein apoproteins causes impairment of lipid transporting system, resulting in forming of *circulus vitiosus* of atherogenic dyslipoproteinemia development (Witztum & Steinberg 1991). Despite of large number of works devoted to the studies of metal prooxidant action (Aust et al. 1985; Chevion 1988; Stohs & Bagchi 1995; Durackova 2010) and its role in atherogenesis (Lynch & Frei 1993; Stadler et al. 2004), the dependence of these effects on metal doses and combinations remains undefined.

The aim of the research was to estimate the effect of different doses and combinations of iron and copper consumption with drinking water on lipid profile and oxidative stress biomarkers in albino Wistar rats serum.

Material and methods

Animals and treatment

The current research was approved by the Local Ethics Committee. 36 female Wistar rats were divided into 6 groups with 6 animals per group. All groups of animals except the control one received $FeSO_4 \cdot 12H_2O$ and $CuSO_4$ with drinking water. All rats were given an unlimited access to the drinking water. The used concentrations of iron and copper were calculated on the basis of maximum permissible concentrations (MPC) for these chemicals in Russian Federation and consequently were ecologically relevant. MPCs for single chemicals Fe^{2+} and Cu^{2+} are 0.3 and 1.0 mg per liter of drinking water, respectively.

Rats of the first (control) group received high-quality drinking water with general mineralization less than 250 mg L^{-1} ("Aqua Vita", Orenburg, Russia; certified by the A. N. Sysin Research Institute of Human Ecology and Environmental Health, Moscow, Russia). Animals from the 2nd and the 3^{d} groups received drinking water containing 3 and 6 mg L^{-1} $FeSO_4 \cdot 12H_2O$, respectively. Rats from the 4th and the 5th groups consumed $CuSO_4$ with water in concentrations of 4.88 and 9.76 mg L^{-1}, respectively. Animals from the 6th group received the mixture of $FeSO_4 \cdot 12H_2O$ and $CuSO_4$ in concentrations of 3 and 4.88 mg L^{-1} in drinking water, respectively.

The light and the dark cycles in the animal room were 12 hours each. The rats had been acclimatized to the laboratory conditions for one week before the study. The animals from all groups were fed *ad libitum*. A granulated chow

Table 1. Serum concentration of oxidative stress markers in rats obtaining iron and copper with drinking water.

No.	Metal (salt dose, mg L^{-1})	TBARS μmol mg^{-1} protein	PC μmol mg^{-1} protein	Tryptophane RFU	Dityrosine RFU	Lys-LPO RFU
I	–	0.18 ± 0.01	11.96 ± 1.81	196.90 ± 10.29	1.46 ± 0.11	0.67 ± 0.04
II	Fe^{2+}/3	0.24 ± 0.03	12.62 ± 1.33	195.14 ± 12.23	1.61 ± 0.20	0.70 ± 0.14
III	Fe^{2+}/6	0.34 ± 0.05^{a}	17.49 ± 2.05^{ab}	152.21 ± 12.75^{ab}	2.51 ± 0.37^{ab}	0.83 ± 0.02^{a}
IV	Cu^{2+}/4.88	0.18 ± 0.01	12.25 ± 2.16	173.93 ± 10.32	2.16 ± 0.07^{a}	0.75 ± 0.13
V	Cu^{2+}/9.76	0.20 ± 0.02^{c}	12.02 ± 2.76	133.25 ± 8.64^{ad}	1.94 ± 0.22^{a}	0.70 ± 0.12
VI	Fe^{2+}+Cu^{2+}/3+4.88	0.24 ± 0.02^{ad}	20.17 ± 3.18^{abde}	161.86 ± 24.55^{a}	2.48 ± 0.36^{ab}	0.72 ± 0.02

Explanations: Data represent Means ± SEM; different letters indicate significant differences ($P < 0.05$): ^{a} statistically significant versus group I; ^{b} statistically significant versus group II; ^{c} statistically significant versus group III; ^{d} statistically significant versus group IV; ^{e} statistically significant versus group V; ^{f} statistically significant versus group VI.

("Orenburg food mixture factory", Orenburg, Russia) containing 270 kcal/100g (20% protein, 70% carbohydrate, 10% fat) was used as a diet in all groups of animals. After 90 days of experiment rats were sacrificed by decapitation.

Measurements

Serum total cholesterol (TC), triacylglycerides (TAG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) were determined with "Roche" commercial kits using Cobas Integra biochemical analyzer. Concentration of very low density lipoprotein cholesterol (VLDL-C) was calculated with the Friedewald equation: VLDL-C = TAG/5 (Friedewald et al. 1972).

Serum thiobarbituric acid reactive species (TBARS) content as a marker of lipid peroxidation was determined spectrophotometrically at 532 nm with Genesys 5 spectrophotometer (Ohkawa et al. 1979). Protein carbonyls (PC) were determined spectrophotometrically by the Levine's method at 363 nm with Genesys 5 spectrophotometer (Levine et al. 1990). Oxidative modification of amino acid residues was estimated fluorimetrically on Varian Cary Eclipse spectrofluorimeter. Concentration of oxidatively modified amino acids was expressed in relative fluorescent units (RFU) in 50 μl of serum. The formation of dityrosines was evaluated by measuring the emission spectra (380–440 nm) at excitation wavelength (λ_{ex}) 325 nm (slit width 5 nm) (Giulivi & Davies 1994). Fluorescence emission spectra in range 420–480 nm were measured in order to estimate the concentration of conjugates of lipid peroxidation products with free amino groups of proteins, primarily lysine (Lys-LPO) (Dousset et al. 1994) at $\lambda_{ex} = 365$ nm (slit width 5 nm). Decrease in tryptophan flurescence was determined at $\lambda_{ex} = 295$ nm and emission at 340 nm.

Serum protein concentration was determined spectrophotometrically by the method of Lowry (Lowry et al. 1951) using bovine serum albumin as a standard.

Statistical analysis

Data were expressed as mean values ± SEM (presented in the tables) and evaluated using Mann-Whitney *U*-test at the significance level 2 alpha = 0.05 (*).

Results

Effect of iron and copper on serum free-radical oxidation biomarkers

As it is seen from the data presented in Table 1 chronic intake of 6 mg L^{-1} $FeSO_4 \cdot 12H_2O$ and a combination of iron and copper salts results in significant increase in serum TBARS content by 89 and 33% in comparison to the control values, respectively. It is also seen that consumption of iron and copper with drinking water induces oxidative modification of serum proteins. Protein carbonyls content in serum was enhanced in rats obtaining 6 mg L^{-1} $FeSO_4 \cdot 12H_2O$ (III group) and a combination of 3 mg L^{-1} $FeSO_4 \cdot 12H_2O$ and 4.88 mg L^{-1} $CuSO_4$ (VI group) with drinking water exceeding control values by 46 and 69%, respectively. A 70% increase in dityrosines fluorescence was observed in animals treated with 6 mg L^{-1} $FeSO_4 \cdot 12H_2O$ (III group) and a combination of iron and copper (VI group) with drinking water. At the same time dityrosine fluorescence in serum of rats from IV and V groups exceeded a baseline by 48 and 33%, respectively. Fluorescence of tryptophan residues in serum proteins was decreased by 22, 32 and 17% in animals from III, V and VI groups, respectively. A significant increase in Lys-LPO conjugates fluorescence was observed only in rats obtaining maximal dose of iron with drinking water and enhanced control values by 24%.

Effect of iron and copper on serum lipoprotein profile

The obtained data in the Table 2 show that significant increase in TC was noted in animals obtaining maximal doses of iron and copper salts with drinking water exceeding controls by 23 and 17%, respectively. Hypertriacylglyceridemia was observed in serum of rats consumpting both concentrations of iron (II and III groups) and was more expressed than in control animals by 31 and 83%. At the same time copper-treated rats had a slightly elevated serum TAG level by 12% in both groups. Significant increase in VLDL-C was observed in rats obtaining 6 mg L^{-1} $FeSO_4 \cdot 12H_2O$ with drinking water and enhanced control values by 64%. Consumption of iron in concentration of 3 mg L^{-1} leads to non-significant increase in VLDL concentration by 18%. High copper intake primarily affected the β-lipoprotein levels. So LDL-C of IV, V and VI groups of animals exceeded control group values by 50, 130 and 65%, respectively. These data suggest that high copper consumption creates atherogenic situation. Chronic consumption of metals with drinking water leads to various changes in HDL-C. Significant decrease in serum HDL-C was observed in rats obtaining 6 mg L^{-1} $FeSO_4 \cdot 12H_2O$ with drinking water with values being lower than in control animals by 16%. At

Table 2. Serum lipoprotein spectrum in rats obtaining iron and copper with drinking water.

No.	Metal (salt dose, mg L^{-1})	TC (mmol L^{-1})	TAG (mmol L^{-1})	LDL-C (mmol L^{-1})	VLDL-C (mmol L^{-1})	HDL-C (mmol L^{-1})
I	–	1.63 ± 0.09	1.27 ± 0.06	0.1 ± 0.01^{a}	0.28 ± 0.01^{a}	1.25 ± 0.03^{a}
II	Fe^{2+}/3	1.55 ± 0.08	1.66 ± 0.14^{a}	0.15 ± 0.03	0.33 ± 0.03	1.23 ± 0.04
III	Fe^{2+}/6	2.00 ± 0.16^{abf}	2.32 ± 0.06^{abef}	0.09 ± 0.005	0.46 ± 0.01^{abef}	1.05 ± 0.05^{abf}
IV	Cu^{2+}/4.88	1.68 ± 0.04	1.40 ± 0.03	0.15 ± 0.01^{a}	0.28 ± 0.01	1.27 ± 0.04
V	Cu^{2+}/9.76	1.90 ± 0.05^{d}	1.43 ± 0.1	0.23 ± 0.02^{abcd}	0.29 ± 0.02^{c}	1.42 ± 0.01^{ade}
VI	Fe^{2+}+Cu^{2+}/3+4.88	1.63 ± 0.04	1.24 ± 0.11	0.165 ± 0.03^{a}	0.25 ± 0.02	1.25 ± 0.04

Explanations: Data represent Means ± SEM; different letters indicate significant differences ($P < 0.05$): ^{a} statistically significant versus group I; ^{b} statistically significant versus group II; ^{c} statistically significant versus group III; ^{d} statistically significant versus group IV; ^{e} statistically significant versus group V; ^{f} statistically significant versus group VI.
Abbreviations: MPC, maximum permissible concentration; TC, total cholesterol; TAG, triacylglycerides; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; VLDL-C, very low density lipoprotein cholesterol; TBARS, thiobarbituric acid reactive species; PC, protein carbonyls; Lys-LPO, conjugates of lipid peroxidation products with lysine; SEM, standard error of mean; RFU, relative fluorescent units

the same time a 14%-increase in HDL-C was noted in serum of rats receiving drinking water with 9.76 mg L^{-1} $CuSO_4$.

Discussion

The data obtained show activation of free radical processes in rats obtaining iron and copper in different doses with drinking water. It should be noted that administration of iron in concentration of 6 mg L^{-1} induces lipid peroxidation and protein oxidation, while copper given in the maximal doses leads to protein oxidation. Taking into account the chemistry of d-metals the basis of their prooxidant action is the ability to catalytically decompose in Fenton reaction (Fenton 1894):

$$Me^{n+} + H_2O_2 \rightarrow Me^{(n+1)+} + HO^- + HO^{\bullet},$$

leading to generation of a deleterious hydroxyl radical and subsequent induction of lipid and protein oxidation (Chaitanya et al. 2010).

It is seen that iron is a more potent prooxidant which induces both lipid and protein oxidation. Primary protein-oxidative effect of copper can be a consequence of protein-binding properties (Kato et al. 2001). It has been shown that copper ions localize on the protein surface, primarily on cysteine residues, where prooxidant action of copper leads to oxidation of amino acid residues in protein molecule (Letelier et al. 2010).

Observed free radical oxidation in rats obtaining combination of 3 mg L^{-1} $FeSO_4 \cdot 12H_2O$ and 4.88 mg L^{-1} $CuSO_4$ with drinking water was more expressed than in case of administration of single metals in the same doses. Potentiation of prooxidant action can be explained by redox interaction of iron and copper (Jomova & Valko 2011):

$$Cu^{+} + Fe^{+++} \rightarrow Cu^{++} + Fe^{++},$$

leading to generation of catalytic-active iron that is more active prooxidant as it has been showed in the recent research.

The obtained data also show that metal consumption affects serum lipoprotein spectrum. The most expressed influence is observed in case of isolated metal administration.

Atherogenic changes in lipoprotein profile can be a consequence of iron- and copper-mediated induction of hydroxymethylglutaryl-CoA reductase (EC 1.1.1.88) (Graham et al. 2010; Ohguchi et al. 1988). Subsequent elevation of intracellular cholesterol pool leads to enhanced synthesis of cholesterol transporting particles. Observed induction of free-radical reactions causes oxidative modification of LDL (Witztum & Steinberg 1991; Vogiatzi et al. 2009). Such modification from one hand elevates serum LDL due to impaired LDL-reception process and from the other hand it stimulates oxidized LDL phagocytosis by intimal macrophages via "scavenger-receptors" (Steinberg 1997). Systemic oxidative stress and oxidative liver damage leads to inhibition of cholesterol oxidation by 7α-cholesterol hydroxylase (EC 1.14.13.17) that also enhances serum total cholesterol forming "*circulus vitiosus*" (Meerson et al. 1988).

The observed increase in HDL-C concentration in copper-treated rats is of particular interest. The obtained data is consistent with the findings of other authors indicating copper-induced elevation in HDL concentration. It is supposed that metabolism of lipid hydroperoxides in presence of redox-metals can lead to increased generation of various products like short and long chain cholesterol esters and prospholipids (Abuja & Albertini 2001). A large proportion of these products can be transported in serum by HDL that can lead to increase in HDL concentration (Galhardi et al. 2004). At the same time, elevation in HDL concentration can be a consequence of beneficial effect of copper. However, the pathways leading to copper-induced increase in high density lipoprotein concentration should be investigated in details.

The above mentioned effects of red/ox metals administration are undoubtedly dependent on their cumulation in the organism. We have reported earlier that maintenance of iron and copper in the organism is dose-dependent in rats obtaining salts of metals with drinking water (Tinkov et al. 2012).

To conclude, chronic administration of iron and copper with drinking water induces atherogenic changes in serum lipoprotein spectrum and oxidative modification of serum macromolecules. Consumption of maximal doses of isolated metals leads to more expressed atherogenic changes, while combination of both metals in lower doses did not affect serum lipoprotein significantly. At the same time a potentiation of free-radical processes was observed in rats obtaining combination of iron and copper. The data obtained show that interaction of iron and copper in the organism has an additive effect on some vital parameters in comparison to isolated metal administration. At the same time the possibility of indirect interaction between iron and copper during absorption should also be taken into consideration (Sharp 2004). For example, it has been shown in a number of works that combined administration of iron and copper can lead to decrease in absorption (Arredondo et al. 2003; Arredondo et al. 2006) while we have not observed the mutual inhibition of iron and copper effects.

Taking into account the above mentioned effects of combined metal administration the mechanism of iron and copper interaction and effect *in vivo* should be investigated further.

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