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SERUM COPPER AND ZINC IN A REPRESENTATIVE SAMPLE OF BULGARIAN POPULATION

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Summary. Copper (Cu) and zinc (Zn) are essential for life. Body Cu and Zn content depends on variety of factors – age, gender, and diet, type of drinking water, geographical location and genetic predisposition. Copper status becomes even more relevant not only in rare genetic disorders such as Wilson disease but in diseases such as cardiovascular ones, impaired glucose tolerance and neuro-degenerative and tumor diseases. The study aimed to examine the distribution of serum Cu and Zn in a representative group of the Bulgarian population and to describe factors which influence metal content. It also aimed to describe the link between serum Cu levels and the frequency of Alzheimer's disease (AD) in Bulgarians. Cu and Zn in serum were measured in 379 individuals (172 males and 207 females) from 5 different regions in Bulgaria by flame atomic absorption using AAnalyst 400, Perkin Elmer. Statistical analyses were performed by SPSS, 19. Median and inert-quartile range (IQR) for blood Cu were 15.89 (13.87-7.89) μmol/L and for Zn – 13.00 (11.7-14.68) μmol/L in the examined group. Higher Cu levels in females than in males were found ($p < 0.001$). Decrease of Zn with aging was established ($p > 0.05$). Significant difference ($p < 0.05$) was found in serum Cu between young people (< 30 year old) and adults over 61 year old. Statistically significant difference in Cu and Zn was observed ($p < 0.05$) in respect of residences. Difference without significance was measured between serum lipids and serum Cu ($p = 0.541$) and Zn ($p = 0.741$).

Key words: *copper, zinc, trace elements, Alzheimer's disease*

INTRODUCTION

Cu and Zn are essential for many enzymatic reactions which are of a great importance for the oxide-reductive balance in the body. Interactions of metals and many physiological and non-physiological processes are described. It is well known that the impaired trace elements homeostasis may cause neurodegeneration mainly via oxidative stress [8]. Trace elements are involved in normal physiology of the brain. In the metal hypothesis of neurodegeneration, the trace elements dysregulation in the neuronal system is more important than the metals accumulation [13].

Body content of Cu and Zn depends on a variety of factors: age, gender, medicine intake, geographical location, diet and some special physiology conditions (pregnancy, newborn). Genetic factors are also involved [9]. Evaluation of Cu and Zn status in population may be a base for further studies of interactions between trace elements in health and disease.

We aimed to examine the distribution of serum Cu and Zn in a representative group of the Bulgarian population and to describe some factors which influence metal content. We also aimed to describe the link between serum Cu levels and the frequency of Alzheimer's disease (AD) in Bulgarians.

MATERIAL AND METHODS

Study design

The study comprised 379 individuals in apparently good health (172 males and 207 females), (47 ± 14 years) – medical employees, students, people in active age and retired individuals who went through prophylactic examination. Criteria for exclusion were: anemia, active inflammation, impaired glucose metabolism, renal and liver disorders, cardiovascular diseases, neurological and psychiatric disorders. Body Mass Index (BMI) was calculated for 57 individuals and questionnaires for diet, drinking and smoking habits, sleep duration and physical activity were done for 31 participants. Individuals from 5 different regions of Bulgaria were included: the city of Sofia (n = 204), Varna (n = 42), Rousse (n = 31), Pazardzhik (n = 50) and Radnevo (n = 52). All participants signed informed consent form in accordance with Helsinki declaration guidelines for experimentation with humans. Approval of Research Ethical Committee of Medical University – Sofia, Bulgaria, was obtained. The study was conducted in 2013-2015, in Clinical laboratory, "Sv. Ivan Rilski" University Hospital, Medical University – Sofia, Bulgaria.

Sample collection

Blood was drawn into VACUETTE®Serum Collection Tubes with Clot Activator, Gel Separator (Greiner Bio-One), 5 ml, Ref. № 456073 between 7:30-9:30 am by standard venipuncture after 12 h fasting pause overnight. Separated by centrifugation (≤ 1300 g for 10 min at 25°C) sera were transported to the Clinical laboratory of "St. Ivan Rilski" University Hospital, Sofia, in Eppendorf tubes and stored at 2-8 °C until laboratory examinations. All analyses were performed up to 24 h after blood collection.

Laboratory analyses

The following laboratory parameters were tested for each participant: serum lipids: total cholesterol (TChol), triglycerides (TG), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), serum Cu and Zn.

TChol, TG and HDL were determined by enzymatyc methods using Cobas Integra 400 Plus (Roche, Germany) following the recommended by the manufacturer procedures. Serum concentration of LDL was indirectly determined by calculation using Friedewald formula.

Serum Cu and Zn were measured by flame (air-acetylene) atomic absorption spectrophotometry (AAnalyst 400, Perkin Elmer). Three replicate determinations were made for each sample and mean value was calculated. Determination of metal concentration was done after 3 point calibration by standard calibration solutions for Cu (1000 mg/L, in 2% HNO_3, Lot No 19-09CUX1, Perkin Elmer Pure, USA) and for Zn (1000 mg/L, in 2% HNO_3, Lot No 19-08ZNX1, Perkin Elmer Pure, USA). Internal quality control scheme was applied using certificated reference materials: SeronormTM Trace Elements Serum, Sero, Norway; Level 1 Lot № 0903106. Intra-assay analytical imprecision (within a run) for Cu was 2.9 CV% and for Zn – 4.3 CV% (CV% – coefficient of variation in percent). Inter-assay analytical imprecision (between different runs) for serum Cu and Zn was 1.1 CV% and 2.7 CV%, respectively. Trueness in bias was 2.93% for serum Cu and 11.5 % for serum Zn.

Statistical analysis

The SPSS (Version 19.0) program for Windows was applied for statistical analysis. The Kolmogorov-Smirnov and Shapiro-Wilks tests were used to verify the normality of distribution. Mann-Whitney and Kruskal-Wallis tests were performed to compare serum Cu and Zn between groups (by gender, age and residence). The values $p < 0.05$ were considered statistically significant. To investigate correlation between two variables Pearson and Spearman correlation coefficients were used. Chi-squire test was used to determine statistical significance between type of diet, sleep duration, alcohol consumption, and physical activity and serum Cu and Zn levels.

RESULTS AND DISCUSSION

Serum Cu and Zn concentrations in examined individuals are presented in Table 1. This is the first research in Bulgaria with data about the distribution of serum Cu and Zn by regions. Similarly to Romero CD at al. [18], who reported significant difference in Cu and Zn contents among regions in Spain, we also found a significant difference in Cu and Zn contents in respect to the areas in Bulgaria ($p < 0.05$). Our data were contradictory to those of Bocca B et al. who found that residence had no effects on blood Cu level [6]. Individuals from Rousse and Pazardzhik showed the highest serum Cu levels with statistical significance ($p < 0.001$) in respect to all the examined individuals. In contrast, serum Cu levels in Sofia were statistically lower ($p = 0.002$) than that in the total group. Serum Zn in Radnevo ($p = 0.002$) and Sofia ($p < 0.001$) were statistically lower in comparison with the whole group. The differences between the examined areas could be due to their different geographical locations: Rousse and Pazardzhik are located on rivers, Varna is on the Black Sea, Sofia is nearby the Vitosha Mountain and Radnevo is in the valley. In general, people who live nearby mountain (Sofia) presented the lowest ranks of serum Cu and ones who live on the river side (Rousse and Pazardzhik) were with the highest Cu concentrations and in both cases with statistical significance in respect to the total group. The variances could be explained with geographical location, nutritional habits, and content of drinking water, composition of soils, traditional manner of cooking and the local industry. All these, together with factors of biological variation (age and gender), influence the concentrations of trace elements in the body [8] and must be taken into consideration when results are interpreted.

Table 1. Demographic data and distribution of serum Cu and Zn in individuals from Bulgarian population

	n	age	Cu (µmol/L)	Zn (µmol/L)
Overall	379	46 ± 14	15.89 (13.87-7.89)	13.00 (11.7-14.68)
Males	172	45 ± 15	14.56 (13.06-15.65)	13.08 (10.58-15.6)
Females	207	48 ± 14	16.64 (14.76-18.65)	12.85 (9.51-16.19)
Residences				
Pazardzhik	50	45 ± 16	17.27 (15.98-19.53)	19.85 (17.55-22.21)
Rousse	31	47 ± 16	18.09 (14.95-20.10)	12.47 (11.55-12.9)
Radnevo	52	48 ± 10	16.23 (14.67-17.72)	12.61 (11.18-13.27)
Varna	42	41 ± 11	15.10 (14.17-17.39)	13.46 (11.45-16.27)
Sofia	204	46 ± 14	14.94 (12.93-16.98)	12.77 (11.47-14.07)

Results are expressed as median and inter-quartile range (IQR)

In assessment with previous data for trace elements content in healthy Bulgarians (n = 345) [1], higher overall serum Cu and Zn levels were evaluated than in the recent study (Table 2). In other European countries, such as Spain [18], Italy [6] and Germany [14] serum Cu level was higher than that in Bulgaria. The same difference was observed in comparison to China [6, 30], Turkey [28] and Brazil [5]. For serum Zn we observed lower concentrations than that in Spain (17.74 µmol/L) [18] and slightly higher than that in Turkey (12.42 µmol/L) [28]. Obviously, serum metal levels differ among populations from different countries and possibly this might be due not only to geographical localization and diet, but also to certain genetics features. Genetic polymorphism is also important for the metabolism of trace elements [9].

Table 2. Comparison of serum Cu and Zn concentrations in Bulgarians

	Ivanova I. (2013-2015)			Tzatchev K. (1987)		
µmol/L	Males	Females	Overall	Males	Females	Overall
Cu	14.56	16.64	15.89	17.3	16.8	17.5
Zn	12.93	13.08	13.00	14.9	13.5	14.8

Results are expressed as medians

Most of the samples examined, 363 (95.8% of the total) for Cu and 362 (95.5% of the total) for Zn fell into the reference intervals for Cu (men 9.89-19.95 µmol/L; women 11.06–24.87µmol/L) and for Zn (men and women 8.87-23.48 µmol/L). Small number of all the samples exceeded the upper reference limits: for Cu 9 (2.4%) and for Zn 9 (2.4%) and respectively – below the lower reference limits: for Cu – 7 (1.8%) and for Zn – 8 (2.1%). The deficiency prevalence for Cu and Zn in Bulgarians was low and a bit higher for Zn than for Cu. These observations were similar to data of other authors: in Kuwait (560 Kuwaitis, aged 15-80 year old) – Cu deficiency – 0.36% and Zn deficiency – 0.53% [2]; in Canaraian population Cu and Zn deficiency were below 5% of the total population [18]. Clearly, the populations in those regions showed adequate dietary intake of Cu and Zn. In contrast, individuals from Santiago, Chili, pointed a high prevalence of Cu and Zn deficiencies in elder population over 60 years of age (for Cu 32.9% in women and 23.7% in men; for Zn 66.9% in women and 66.7% in men) [16].

Higher Cu concentration in females than in males was found ($p < 0.001$) by us. Serum Zn levels did not differ statistically between genders ($p = 0.67$). The same statistical relations between genders for Cu and Zn were reported in the literature [6, 11, 15, 16]. In comparison to previous investigation in Bulgarians [1] we observed higher serum levels for both Cu and Zn in women than in men (Table 2). Higher serum Cu in women was probably related to female sex hormones, estrogens particularly. During the time of menstrual bleeding, higher values were measured than during ovulation. The effect could be due to stimulation of estrogens on ceruloplasmin synthesis [5].

Contradictory data for age-related changes in serum Cu and Zn are available with [4, 11, 18] or without statistical significance [6, 30]. We found decrease of serum Zn with aging without statistical importance ($p > 0.05$) (Fig. 1). With aging serum Cu increases with statistical significance ($p = 0.003$) between young people (< 30 year old) and adults over 61 years old. The highest Cu levels were measured in the group over 65 years (median 16.57 µmol/L), as the highest levels were found in women (median 19.92 µmol/L). Serum Zn was higher in individuals up to 19 years (16.89

µmol/L). Similarly to other studies, we observed the highest levels of Cu [18] and Zn [5] in women up to 65 years with no difference for the later age. These data were consistent to those for other populations – Canarian population [13, 18].

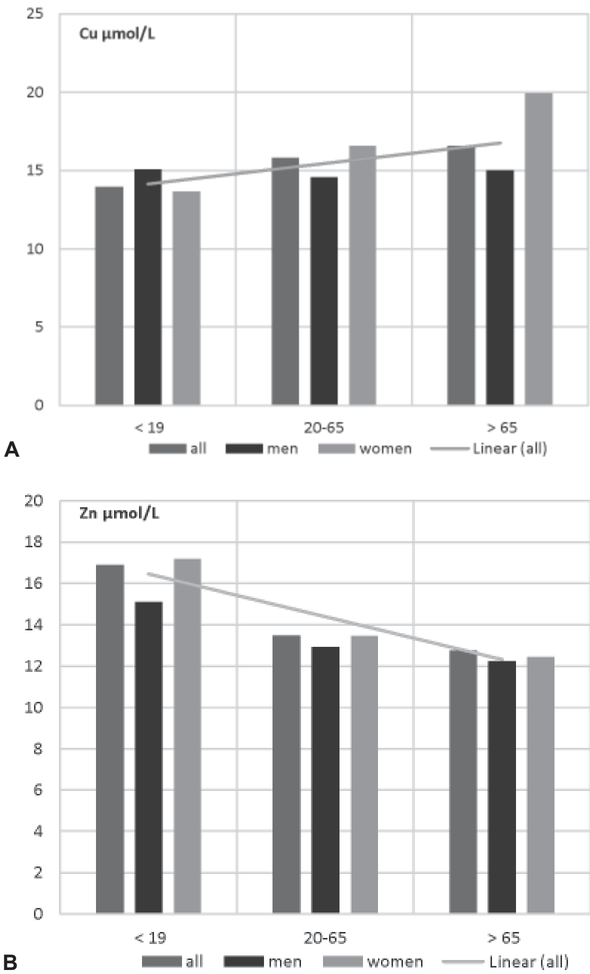

Fig. 1. Distribution of serum Cu (**A**) and Zn (**B**) concentrations for all examined individuals based on gender and age

Data for BMI were collected form 57 individuals of the whole studied group (men 26 and women 31) (43 ± 15 years). The results for BMI in the total group, males and females were: 23.44 ± 3.85; 24.75 ± 3.62 and 22.38 ± 3.75 respectively. In contrast to other authors [2, 4, 11] who established significant correlation ($r = 0.30$-0.85, $p < 0 = 0.001$) between serum Cu and BMI, our study did not find such relation for serum Cu (Spearman's correlation coefficient = 0.21) and Zn (Spearman's correlation coefficient = 0.10) concentrations.

Cu and Zn have an important role in modulating the serum lipid profile [17, 29]. A relation between serum lipids and serum Cu and Zn levels were reported [3]. Results for serum lipids in our research are presented in Table 3. Statistically significant difference in serum copper concentrations (p = 0.15) and zinc (p = 0.49) between control subjects and subjects with hyperlipidemia has not been established. No significant correlation between serum levels of lipids, and trace elements copper and zinc, was found in the control group patients and in those with hyperlipemia (Table 4). Studies for the link between serum lipids and serum Cu and Zn are contradictory (141). For a more detailed clarification of the correlation between lipid profile and status of trace elements it is necessary to conduct a study with a larger number of units involved.

Table 3. Serum lipids in the studied individuals

	Controls, (n = 31)	**Hyperlipemia, (n = 25)**	**p-value**
Parameter	4.6 ± 0.8	6.2 ± 1.02	< 0.001
TChol	1.5 ± 0.4	1.5 ± 0.3	0.84
HDL	2.6 ± 1.03	4.1 ± 0.6	< 0.001
LDL	0.91 ± 0.4	1.09 ± 0.4	0.098
TG	1.83 ± 0.9	2.8 ± 0.8	< 0.001
LDL/HDL	15.06 ± 1.8	15.9 ± 3.2	0.15
Zn µmol/L	13.27 ± 2.02	13.43 ± 2.09	0.49

TChol – total cholesterol; TG – triglycerides; HDL – high density lipoprotein cholesterol; LDL – low density lipoprotein cholesterol

Table 4. Statistical correlations between serum lipids in examined individuals – controls (normal lipid status) and individuals with hyperlipidemia

	Controls, n = 31		**Hyperlipidemia, n = 25**	
Parameter	**Cu µmol/L**	**Zn µmol/L**	**Cu µmol/L**	**Zn µmol/L**
TChol	r = –0.009 p = 0.963	r = 0.0.13 p = 0.945	r = 0.079 p = 0.712	r = –0.032 p = 0.88
HDL	r = –0.088 p = 0.663	r = 0.075 p = 0.710	r = 0.237 p = 0.301	r = 0.116 p = 0.615
LDL	r = –0.076 p = 0.712	r = 0.087 p = 0.671	r = 0.290 p = 0.202	r = 0.027 p = 0.907
TG	r = 0.277 p = 0.138	r = 0.107 p = .573	r = –0.099 p = 0.661	r = 0.071 p = 0.754
LDL/HDL	r = 0.027 p = 0.897	r = 0.038 p = 0.855	r = 0.053 p = 0.819	r = –0.049 p = 0.833

Totally 31 individuals form one and the same region (the city of Sofia) participated in questionnaire about the type of diet, sleep duration, alcohol consumption, smoking habits and physical activity.

Food and drinking water, predominantly the food [24, 26], are the main sources of trace elements for human body [10]. Foods, rich of copper, are liver, sea foods, oysters, legumes, nuts, pumpkin and cacao [26]. Contrary, milk and milk products, especially cow's milk, are poor of Cu [7]. Slightly acid water contents more Cu [7].

Consumption of food rich of Cu and reduced cow's milk products was related to significantly higher Cu (17.17 ± 1.9 μmol/L) ($p = 0.04$). Serum Cu concentration of 14.4 ± 1.6 μmol/L was measured in the group with predominant cow's milk diet.

Cu and Zn are antagonists of the N-methyl-D-aspartate glutamate receptor which mediates sleep [21]. Song CH et al. [21] suggests that serum Cu and Zn may be involved in sleep duration. We observed a tendency of higher serum Cu without significant difference ($p = 0.09$) for individuals with sleep duration less than 7 h a day and difficulty in falling asleep and waking up easily. Other authors observed normal serum Zn and Zn/Cu ration only in individuals with sleep duration over 7-9 hours a night [30].

Serum Cu and Zn concentrations that depend on drinking and smoking habits, and physical activity, are expressed on Table 5. Almost equal serum Cu concentrations in smokers and nonsmokers ($p = 0.51$) were observed in agreement with other authors [4, 18]. Zhang HQ and colleagues [30] have provided data for significant lowering of serum Cu and no significant lowering for serum Zn in smokers.

Table 5. Factors that influence serum Cu and Zn concentrations

	Cu μmol/L			Zn μmol/L		
	mean ± SD	**Min**	**Max**	**mean ± SD**	**Min**	**Max**
Smoking habits						
Smokers	14.94 ± 2.1	11.87	19.09	13.09 ± 1.81	9.56	15.91
Non smokers	14.44 ± 1.2	11.3	15.83	12.47 ± 1.26	10.56	15.38
Alcohol consumptions						
Never or 1-2 glass per week	14.72 ± 1.72	11.3	19.09	12.87 ± 1.47	10.56	15.91
Every day	14.09 ± 1.24	12.43	15.76	11.13 ± 1.57	9.56	12.7
Physical activity						
Daily activities	15.0 ± 1.8	11.87	19.09	12.87 ± 1.58	9.56	15.91
Active sport	13.7 ± 0.3	11.3	15.76	12.12 ± 1.27	10.56	14.22

min – minimal measured levels; max – maximal measured levels

Data are contradictory about the effect of alcohol on the serum levels of Cu. Some authors [4] assessed that alcohol influence on serum Cu levels as increased alcohol consumption was related to increase in serum Cu [4, 18]. According to our data, higher serum Cu was observed in people who rarely consume alcohol (a maximum 1-2 times a week for a cup of wine/beer) with no statistical significance (p = 0.27; p = 0.07) in respect to those who do not drink any alcohol. Serum levels of Zn did not differ significantly (p = 0.9) in respect of alcohol consumption. In the literature, some data about increasing of Zn and decreasing of Cu in relation with alcohol consumption were reported [30].

Some authors linked increased physical activity to decreased serum Cu concentration [18]. Contrary, Zhang HQ et al. [30] observed higher serum Cu and Zn in individuals with regular physical exercise but with no statistical significance. Our results pointed lowering of serum Cu and Zn levels in active sporting people without statistical significance (p = 0.3; p = 0.4, respectively).

Trace elements are of a great importance for normal functioning of brain [19, 24]. Recently, the interest about the role of trace elements in neurodegenerative disorders rises [6, 12, 24, 26, 27, 28]. Higher mortality because of AD in regions with soils rich of Cu and Zn [20] was reported in 2014 for China. AD is the most frequent cause of dementia [24, 25]. Pathogenesis of AD is multifactorial and many genetic and non-genetic factors are involved [23, 24]. Well known risk factors are age, gender, family history, carriers of ApoEε4 allele, blood pressure, BMI, serum TChol, physical activity and etc. [25, 26]. Nowadays, the importance of Cu dyshomeostasis becomes more relevant [24], even as a causative factor of AD [23]. Even small genetic defects in incorporation of Cu into ceruloplasmin structure are associated with slight but significant elevation of non-ceruloplasmin copper (“free” copper) in the serum of patients with AD [22]. Increased serum Cu levels lead to cognitive impairments and development of AD [24]. Squitti R and Polimanti R [24] reported significant increase in serum Cu concentrations, mainly of non-ceruloplasmin Cu, in AD.

National Center of Public Health and Analyses, Sofia, Bulgaria, reported 34.4 per 100 000 population frequency of AD for 2013 in Bulgaria. According to the data provided, the highest frequency of AD appeared in residences with the highest measured serum Cu levels (Rousse) and on the other hand – in Sofia, where the lowest serum Cu concentration was found, the lowest AD frequency appeared.

CONCLUSION

In this study we presented recent data for serum Cu and Zn concentrations in a representative number of healthy individuals from Bulgaria. The mean values and interrelation of serum Cu and Zn levels in dependence of age and gender, were similar to other researchers. Significant link between serum trace elements levels and LDL and a positive link between serum Cu levels and frequency of AD are a good reason for further studies on trace elements in health and disease.

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