Flame atomic absorption determination of serum copper and zinc in disordered bone metabolism

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This study presents the results from the atomic absorption analysis of the concentration of certain bone density-influencing microelements Zn and Cu in menopausal women with proven osteopenia or osteoporosis, and in a control group of menopausal women with normal bone density. In our preliminary measurements of copper and zinc levels we found no significant differences between patients with osteopenia and osteoporosis. That is why, we formed a new group of all patients with disturbances of the bone metabolism. We included the ratio copper/zinc as a new parameter. Serum samples were provided according to the requirements for quality assurance in pre-analytical phase of clinical laboratory testing. Serum Cu and Zn concentrations were measured by flame atomic absorption spectrometry (FAAS). Data about serum Cu and Zn and the ratio Cu/Zn in disordered bone metabolism were expressed as mean values ±SD. Evaluation was done by analysis of variance with non-paired t-test between different groups with $P < 0.05$ for statistical significance. These preliminary observations only imply for slight tendency of increase in serum Cu in disordered bone metabolism. The tendency seems like more pronounced for osteoporosis than osteopenia. Serum Zn does not differ significantly. Intriguingly, the Cu/Zn ratio follows the same statistical model as serum Cu. This study is encouraging with a potential for serum Cu and Zn and the ratio Cu/Zn to serve as useful biomarkers in laboratory diagnosis and treatment monitoring of disordered bone metabolism, especially in more severe forms of osteoporosis. In any case, more detailed research with bigger numbers of individuals in the tested groups is necessary to provide convincing evidence in this direction.

Keywords: Atomic Absorption Analysis, Cu Serum Levels, Zn Serum Levels, Bone Density

INTRODUCTION

Osteoporosis is a topmost socio-economic disease with severe psychological and economic consequences not only for the affected individuals, but also for their families and society as a whole. In the European Union, annual expenditures for osteoporotic fractures aggregate 37 billion euro [1]. The loss of bone mass is more commonly observed in women. Typically, the onset is at the age of 40, with the beginning of menopause. To date, there are a number of known risk factors for the development of osteoporosis. Its pathogenesis is well-studied, and there are a number of medications available for its pathogenetic treatment [2].

This is not the case with the serum levels of certain microelements. In recent years, a number of researcher teams have directed their research towards studying these links.

The mechanism for achieving a better bone metabolism and for improving physical impairments is yet unclear. Trace minerals such as Cu and Zn are important precursors for the biological process of bone health [3].

The serum levels of Cu, Zn and Ca were studied in postmenopausal women with osteoporosis (n = 23) and with osteopenia (n = 28). Considerably lower levels of Zn ($P = 0.001$) and Cu ($P = 0.05$) were detected. No statistically significant differences in serum levels were detected among osteoporotic patients [4].

Another trial included 50 participants (men and women with mean age 47.5 ± 5 years) with severe dental wear. Enamel biopsies showed reduced copper contents, which the authors associated with reduced mineral density of the spine. Copper deficit was seen as a potential factor for bone demineralization and dental wear [5].

Conflicting results were obtained from the measuring of serum levels of zinc and copper in 135 Iranian women. The mean levels of copper and zinc were 1.168 ± 0.115 and 1.097 ± 0.091 μg/mL in the control group (n = 51, T-score ≥ -1); 1.237 ±

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0.182 and 1.127 ± 0,176 μg/mL in patients with 1 > T-score > -1.7; 1.463 ± 0.174 and 1.327 ± 0.147 μg/mL in patients with T-score < –1.7. In this trial, higher serum levels of zinc and copper were measured in patients with reduced bone density than in the control group participants, even though the differences were insignificant [6].

The ratios between the levels of biogenic elements which are of metabolic importance are not yet fully understood. There are trials indicating a negative effect of high intakes of calcium and phosphorus (even from milk) on zinc absorption which has been demonstrated in adults and premature infants, as well as in animal models of human nutrition. The potential mechanisms by which calcium interferes with zinc absorption include competition for a divalent cation channel across the brush border membrane or calcium-stimulated excess loss of endogenous zinc. This type of interaction can be achieved in calcium to zinc ratio of 20:1 (in weight) to ≥ 50:1. The serum level of zinc is an important factor for bone health, as 29% of the zinc in the body is part of the bone mineral and is a cofactor for a number of enzymes that participate in bone metabolism and collagen degradation [7].

In a one-year continuous measuring of serum zinc, magnesium, iron, and copper in 21 patients with primary osteoporosis treated with the anti-resorption drug denosumab it was found that denosumab can improve the metabolism of Zn. The serum level of Cu did not change until the 8th month of the trial, but it decreased in the 10th and 12th months [8]. Hence, during continuous treatment with medications affecting bone resorption, it is not enough to do a single measurement of the serum microelements, but it is necessary to follow the inter-mineral interactions and to establish the long-term effects of the medication.

The optimal serum level of Zn is 12÷2.4 μmol/L and the optimal serum level of Cu in women is 13.2÷24.3 μmol/L [9]. In order to prevent impairments in bone metabolism, the provision of the necessary Cu and Zn *via* supplements or *via* a nutritionist-prepared diet should be considered. The recommended daily intake for women is 0.90 mg Cu and 8 mg Zn. [10].

A number of trials are directed towards researching correlational micronutrient dependences. According to some authors, after continuous intake of zinc (50 mg/day or more), a copper deficit was found. The higher intake of zinc increases the synthesis of intestinal cell proteins known as metallothioneins. They bind the metals and disallow their absorption by the intestinal cells. Metallothioneins have a higher affinity to copper than for zinc, hence higher levels of metallothioneins obtained due to an increase in zinc levels cause a decrease in copper absorption. On the other hand, higher doses of copper have not been found to affect the food status of zinc. No effect of copper supplements in humans has been established. The link between the serum levels of copper, zinc, iron, and oil-soluble vitamins A and E has also been studied. The increased intake of vitamin C also lowers the level of copper in the body. It has been found that the serum levels of zinc are strongly dependent on serum copper and iron [11].

Copper and zinc are essential microelements that play an important role in the human body as cofactors in a number of enzyme systems in the brain, muscles, bones, kidneys and liver. Copper participates in the synthesis of collagen and elastin. Cuproenzymes in the body act as antioxidants and prevent damage to the cells from free radicals. The daily intake of zinc is essential, as the body does not possess a system for its storage. There are data for low serum level of copper due to a high fructose diet [12]. Zinc is necessary for the catalyst activity of nearly 100 enzymes and for maintaining the immune system, protein syntheses, DNA-syntheses, and cell division. Zinc is responsible for the maintenance of taste and smell. Vegetarians typically have significantly lower doses of zinc intake. The zinc found in plant foods is in lower concentrations and is harder to assimilate. Zinc insufficiency presents itself with weakened immunity [10]. In some studies is reported that a lower zinc intake and lower serum concentrations of zinc are associated with disturbances of the metabolism [13]. There are publications in which a relation between serum levels of copper and zinc and the age at adults is reported [14, 15].

The important role of the microelements Cu and Zn and the non-equivocal trial results motivated us to conduct our own research, with the participation of patients from the region of Pleven.

MATERIALS AND METHODS

Study design

Included in the trial were 50 female patients settled into menopause (confirmed < 5 years ago), aged between 52 and 77 years (mean 59 years). In order to exclude the effect from any correlating risk factors such as tobacco smoking, coffee intake, motor activity, dieting, decreased levels of serum calcium and vitamin D, 24 out of these patients were specifically selected due to having this specific risk profile. Based on the T-score of measuring their bone density *via* the method of Dual X-ray Absorption (DEXA), the patients were separated into three groups: osteoporotic (T-score < -2.5) – 12 patients; osteopenia (T-score between -1 and -2.5) – 6 patients and a control group with patients of normal bone density (T-score > -1/-6).

Biological specimen

Serum samples were provided according to the requirements for quality assurance in the pre-analytical phase of clinical laboratory testing. Venous blood was drawn by a standard collection procedure. Collection tubes with clot activator and gel separator were used. Serum was separated by centrifugation <1200 *g* for 10 min at room temperature and kept frozen in small Eppendorf tubes of 1.5 mL before the measurement. To avoid possible evaporation, the tubes were kept closed during the storage.

Quantitative determination

Serum Cu and Zn concentrations were measured by FAAS (Perkin-Elmer AAnalyst 300). The instrumental parameters are presented in Table 1.

Table 1. Instrumental parameters for Cu and Zn measurements in serum samples

Instrumental parameters	Analysis of serum Cu	Analysis of serum Zn
Wavelength, nm	324.8	213.9
Slit, nm	0.7	0.7
Light Source	Hollow Cathode Lamp HCL-Lumina	Electrode Discharge Lamp EDL
Current, mA	15	250/modulated on
Atomizer	Flame air-acetylene	Flame air-acetylene

Before the quantitative analysis, serum samples were diluted by distilled water with proven acceptable purity (1:3 for copper with total volume of 1.5 mL and 1:5 for zinc with total volume of 2.5 mL).

The measurement of both elements was based on routine 1-point calibration using an aqueous standard solution with known concentration (Cu 7.87 μmol/L and Zn 6.12 μmol/L), prepared by proper dilution with distilled water of the stock standard: Titrisol Copper Standard 1000 mg Cu as $CuCl_2$ in H_2O and Titrisol Zinc Standard 1000 mg Zn as $ZnCl_2$ in 0.06% HCL).

Quality assurance

The quality of the patient results was guaranteed by the following: Internal Quality Control (IQC) and participation in External Quality Assessment (EQAS) programs. ICQ for serum Cu was applied using clinical assayed chemistry control material with declared value interval for atomic absorption and for serum Zn – using trace elements-certified reference material.

Statistical analysis

Data about serum Cu and Zn and the ratio Cu/Zn in disordered bone metabolism were expressed as mean values ± SD. Evaluation was done by analysis of variance with non-paired t-test between different groups with $P < 0.05$ for statistical significance.

RESULTS AND DISCUSSION

In our preliminary measurements of copper and zinc levels we found that there is not a significant difference between patients with osteopenia and osteoporosis. That is why we formed a new group of all patients with disturbances of the bone metabolism. We included the ratio copper/zinc as a new parameter.

Results

Serum zinc levels: The lowest mean level was found in the control group: 12.6 ± 2.7 μmol/L *vs* 13.6 ± 3.3 μmol/L in the osteopenia group and 13 ± 1.1 μmol/L in the osteoporotic group. However, there were no statistically significant differences between the measurements in the different groups ($p = 0.73$).

Serum copper levels: Again, the lowest mean level was found in the control group: 17.6 ± 2.6 μmol/L *vs* 19.5 ± 3.4 μmol/L in the osteopenia group and 20.2 ± 4.7 μmol/L in the osteoporotic group. However, once again there were no statistically significant differences between the measurements in the different groups ($p = 0.25$).

Serum copper/zinc levels: Again, the lowest mean level was found in the control group 1.4 ± 0.1 *vs* 1.6 ± 0.6 in the osteopenia group and 1.6 ± 0.3 in the osteoporotic group. The characteristics of the analytical performance are presented in Table 3.

Table 2. Grand Cu, Zn and Cu/Zn ± SD

Parameter, µmol/L	Control group	Osteopenia group	Osteoporotic group	Mixed Group
Cu	17.6 ± 2.6	19.5 ± 3.4	20.2 ± 4.7	19,9 ± 4.2
Zn	12.6 ± 2.7	13.6 ± 3.3	13 ± 1.1	13.2 ± 2.3
Cu/Zn	1.4 ± 0.1	1.6 ± 0.6	1.6 ± 0.3	1.6 ± 0.5

Table 3. Analytical performance of the determination of Cu and Zn in serum by FAAS

Analytical Performance	Serum Cu	Serum Zn
Laboratory imprecision within one run (CV%)	3.0	4.0
Laboratory imprecision day-to-day variation (CV%)	4.0	7.4
Trueness (Bias%)	2.8	4.0

Table 4. T-test, $P < 0.05$ statistical significance

Parameter, µmol/L	Osteopenia *vs* controls	Osteoporosis *vs* controls	Osteoporosis *vs* osteopenia	Combined disordered group *vs* controls
Cu	0.3	0.25	0.76	0.23
Zn	0.62	0.73	0.64	0.62
Cu/Zn	0.54	0.25	0.95	0.4

The applied systems for detection and elimination of contaminating factors and direct measurement of Cu and Zn in serum by FAAS provide effective control of contamination. Standardized sample preparation in the pre-analytical phase is an aspect of quality assurance [16, 17].

Atomic absorption analysis is a reference method for determination of copper and zinc in biological specimens. The methods in the present study were validated. The calibration pointed linear mode for Cu up to 157 µmol/L (r = 0.999) and up to 30 µmol/L (r = 0.999) for Zn. The calibration curves for both elements were based on aqueous calibration solutions with concentrations chosen to fall into 3 intervals with clinical significance: pathologically low, interval of reference values, pathologically high [16].

The analytical characteristics (calibration procedure, imprecision in one run, day-to-day variation, trueness) as part of the whole standardized operated procedure are related to preliminary sample preparation and to the same atomic absorption measurement.

Comments

1. Higher mean values for serum copper in patients with disordered bone metabolism are more pronounced in osteoporosis. T-test points a difference between controls, osteopenenia and osteoporosis: P = 0.30 osteopenia *vs* controls; P = 0.25 osteoporosis *vs* controls; P = 0.76 osteoporosis *vs* osteopenia. The difference between controls and combined disordered group (osteopenia + osteoporosis) is with P = 0.23. There is slight tendency for increasing serum copper in disordered bone metabolism, more pronounced in osteoporosis than in osteopenia.

2. The statistical difference in serum Zn between the three groups and between controls and the combined disordered group is: (osteopenia + osteoporosis) with P = 0.62 osteopenia *vs* controls; P = 0.73 osteoporosis *vs* controls, P = 0.64 osteoporosis *vs* osteopenia and P = 0.62.

3. The statistical difference for the ratio Cu/Zn between the three groups and between controls and the combined disordered group is: P = 0.54 osteopenia *vs* controls; P = 0.25 osteoporosis *vs* controls, P = 0.95 osteoporosis *vs* osteopenia and P = 0.4 combined disordered group *vs* controls. These preliminary observations only imply for slight tendency of increasing in serum Cu in disordered bone metabolism. The tendency seems like more pronounced for osteoporosis than for osteopenia. Serum Zn does not differ significantly. Intriguingly, the ratio Cu/Zn follows the same statistical model as serum Cu.

This study is encouraging with a potential for serum Cu and Zn and the ratio Cu/Zn to serve as useful biomarkers in laboratory diagnosis and treatment monitoring of disordered bone metabolism, especially in more severe form of osteoporosis. In any case, more detailed research with bigger numbers of individuals in the tested groups is necessary to provide convincing evidence in this direction.

Visible from the results is that in the control group the mean level of serum zinc and copper was significantly lower than that of the patients with osteoporosis and osteopenia, even if the difference was not statistically significant. This can be explained by the fact that copper and zinc participate as cofactors in a number of enzyme systems in bone metabolism. This might be the reason why the control group patients with normal bone metabolism did not possess these elements in high quantities in their serum, as they were engaged on an intracellular level. In order to find statistically significant results and to study this hypothesis, it will be necessary to increase the number of patients in all groups tested.

This is a preliminary investigation which will continue with extended numbers of patients. In case the found correlation is confirmed in future trials with a statistical significance of the results, the levels of copper and zinc will be able to be used as biomarkers for the efficiency of the bone metabolism.

High analytical reliability of the FAAS determination of Cu and Zn in serum, application of IQC schemes and successful participation in Trace Elements QAS programs provide evidence for guaranteed quality of the results. Such approach is a base for adequate clinical interpretations, also in the case of disordered bone metabolism.

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