Bulgarian Chemical Communications, Volume 55, Issue 4 (pp. 385-389) 2023 DOI: 10.34049/bcc.55.4.11NCC01

Analysis of serum antioxidant activity in women with impaired bone density and effect on it of serum concentrations of copper and zinc

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Received: November 15, 2022; Revised: November 21, 2023

This study aims to investigate the relationship between total antioxidant activity (AOA), serum copper and zinc levels, and their impact on bone mineral density (BMD) in menopausal and postmenopausal women. After measuring BMD through dual-energy X-ray absorptiometry (DEXA), participants were categorized into a control group, osteopenic patients, and osteoporotic patients. We determined the radical scavenging activity of serum samples (RSA%) and their trolox equivalent representing the total AOA, using a spectrophotometric ABTS-assay. The variance analysis of the results revealed a statistically significant difference among the groups based on the RSA% indicator, with mean values of 67.69±6.74 for osteopenia, 62.46±7.83 for osteoporosis, and 55.64±2.05 for the control group. Blood serum copper and zinc levels were measured *via* flame atomic absorption analysis. The results indicated a clear trend of increasing microelement concentrations as BMD decreased. The t-test demonstrated statistically significant results for copper concentration when comparing groups with reduced bone density and the control group. Additionally, a statistically significant difference was observed between the osteoporosis group and the control group concerning the Cu/Zn indicator, whose values consistently changed with the severity of the disease. The analysis of the results shows that patients with reduced bone density have higher RSA%, higher concentrations of copper and zinc, and higher Cu/Zn ratio values compared to the control group. The elevation of serum levels of Cu and Zn in patients with reduced bone density can be explained by the fact that copper and zinc are components of enzymes involved in bone metabolism, which degrade at increased levels of reactive oxygen species (ROS) in cells during osteoporosis. The increased concentration of copper ions in the serum initiates secondary radical processes and enhances its AOA. The results confirm the synergistic action of free radicals and redox-active metals such as copper on AOA and bone mineral density.

Keywords: serum antioxidant activity; copper; zinc; Cu/Zn; osteoporosis.

INTRODUCTION

Reactive oxygen species (ROS) are produced in the body as a result of cellular metabolism. Some of the ROS are free radicals that are generated primarily in the mitochondria. A main characteristic of ROS is their high reactivity, penetrating ability, and ability to participate in secondary chain-radical processes, leading to more aggressive radical and non-radical oxygen species. Small amounts of ROS are necessary for the human body as cell signaling substances. For example, they participate in the regulation of the processes of cell division and cell death, activate the expression of certain genes, initiate the renewal of tissues, the elimination of damaged cells as a protective mechanism of the body against DNA mutations, etc.

Endogenous ROS formation is a natural process. Concurrently, there are also biochemical mechanisms for scavenging free radicals through a combination of enzymatic and non-enzymatic antioxidants. When antioxidant defense mechanisms in cells are reduced and ROS production is increased, they react with lipids, proteins, DNA molecules and disrupt their structure. Mitochondrial DNA damage due to free radicals leads to the loss of organelle functions and, consequently, to a lack of cellular energy, which also leads to the loss of functions of the entire cell. As cells age, free radicals contribute significantly to both genome damage and mutations. The body can tolerate mild oxidative stress, but a greater one leads to numerous pathologies. The complex of occurring harmful and irreversible changes is observed at all levels of

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organization of the organism - molecules (DNA, proteins, lipids), cells and organs. These damages to biologically active molecules are the essence of oxidative stress. As the damage process progresses, the incidence of various diseases, such as osteoporosis, increases [1]. Oxidative stress affects a significant part of the population such as menopausal women, the elderly, obese people and those with long-term exposure to environmental pollutants.

Research has proven that uncontrolled production of ROS is associated with disruption of the structure of metalloprotein enzymes and disruption of homeostasis of redox-active metal ions. Upon incubation of Cu, Zn - superoxide dismutase with AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride), an azo compound that is a source of model peroxide radicals, the enzyme is oxidatively damaged by the radicals, inactivated, the protein is fragmented, and the copper ions are released [1, 2].

The increased concentration of redox-active metal ions, such as copper, accelerates the formation of free radicals, as well as disturbances of calcium and sulfhydryl homeostasis. Copper participates in free-radical processes with lipid peroxides formed by the attack of radicals on polyunsaturated fatty acid residues of phospholipids [1, 3].

The intake of low-molecular weight antioxidants such as vitamin C, vitamin E, carotenoids, flavonoids, and other antioxidants, which are capable of chelating metal ions, reduces free metal ions and the formation of free radicals. Oxidative stress is reduced [4, 5].

Zinc has no antioxidant effect, but as a component of some antioxidant enzymes, it supports their activity. Zinc has the following indirect roles in limiting oxidative damage to the body: protection against vitamin E depletion; stabilization of the membrane structure; contribution to extracellular antioxidant enzyme structure; maintenance of tissue concentrations of metallothionein; free radical scavenger. Depletion of zinc in cells increases DNA damage by disrupting DNA repair mechanisms [1, 4].

There is a limited number of studies in the literature on the role of oxidative stress in the pathogenesis of osteoporosis. Antioxidant activity of mesenchymal stem cells from people with osteoporosis was found to differ from that of healthy controls. Mesenchymal stem cells in women with osteoporosis adapt their functioning to a higher level of oxidation [1, 6].

Other research has found that the use of antioxidants in food suppresses the activity of osteoclasts and slows the development of osteoporosis. Evidence has been found for a positive relationship between the amount of ascorbic acid in the diet and bone mineral density [7].

More research is needed to establish the cellular and molecular mechanisms linking oxidative stress, antioxidants, and bone metabolism. The basis for the search for such a relationship is the significant decrease in plasma antioxidants with age. Oxidative stress alters the bone remodeling process, causing an imbalance between osteoclasts and osteoblasts and leading to the pathogenesis of the skeletal system characterized by low bone mass [8].

One study confirmed the antioxidant role of adding vitamins D3, K1 and B6 to the diet of postmenopausal women. After one year of supplementation, a reduction in oxidative stress and a significant increase in bone mineral density was reported [9].

Published studies on serum copper and zinc concentrations in women with reduced bone density deliver conflicting results. A potential relationship between the level of these trace elements, the level of oxidative stress and bone density has not been investigated. The aim of our study is to measure serum concentrations of copper and zinc and total antioxidant activity and to investigate their effect on bone density. Measurement and monitoring of these parameters may contribute to finding new aspects in the etiology, pathogenesis and treatment of osteoporosis.

MATERIALS AND METHODS

Data collection

The study included 66 menopausal and postmenopausal women. The included women were not receiving treatment for osteoporosis or osteopenia. Other exclusion criteria are concomitant endocrine disorders, intake of estrogens and biogenic elements with an impact on bone density. The study included participants who were close in age and BMI. This eliminates from the analysis the influence of these factors on BMD, as noted by many researchers. Bone density was measured using DEXA in all patients and controls. A T-Score was also determined from the BMD results. The T-score measurement is the ratio of the measured bone density compared to standard bone density, determined by measuring a large group of healthy 30-year-olds. The participants were divided into three groups, according to their T-scores: with osteoporosis (T-Score below -2.5 SD); osteopenia (T-Score between -1.0 and -2.5 SD); and control group with normal density (T-Score above -1.0 SD). The number of patients with a borderline T-Score value of -2.5 SD is small.

Venous blood was collected using a standard procedure in accordance with quality assurance requirements in the pre-analytical phase. RSA% and their Trolox equivalent, which reflects the total AOA, were determined in serum from the samples by spectrophotometric ABTS-test. Blood serum copper and zinc levels were measured by flame atomic absorption analysis.

Investigation of the antioxidant status of selected patients and controls

Experimentally, the antioxidant status of serum from patients was determined by ABTS-test [10]. The essence of the methodology is based on spectrophotometric recording of the change in absorption of the chromophore used in the system as a result of free-radical processes. Based on the changes in the measured indicator, after interaction with potential antioxidants from the blood serum, conclusions are made about the intensity of the ongoing processes and their influence on the patients' condition. In this model system, the stable radical cation of 2,2'-azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) is used.

The literature review demonstrated the applicability of the method to study the antioxidant properties of a wide variety of sample types, including multicomponent systems. This includes samples of animal origin. Therefore, it can be applied in the monitoring the antioxidant capacity of biological fluids taken from patients with various diseases [11]. The method is fast, simple, allows automated measurement of a large number of samples simultaneously. The radical cation has good solubility in both aqueous and organic solvents, indicating its applicability to both hydrophilic and hydrophobic antioxidants. The method exhibits no pH sensitivity over a wide range and little influence of ionic strength.

The experimental part of this study was divided into four main steps:

1) Preparation of radical stock solution. $ABTS^{\bullet+}$ is derived by the reaction:

$$2\ ABTS + K_2S_2O_8 \rightarrow 2\ ABTS^{\bullet+} + K_2SO_4 + H_2SO_4$$

Approximately 18 hours are required for the reaction to progress, as judged by the cessation of increase in absorbance of the solution – an indication that no more radical is generated.

The $ABTS^{\bullet+}$ solution is blue-green in color. It has a characteristic absorption spectrum with absorption peaks in the visible region at 420 nm, 734 nm and 829 nm.

The interaction of $ABTS^{\bullet+}$ with substances with radical scavenging activity in the samples leads to a decrease in its concentration, the color intensity of the solution and the measured absorbance. We performed the analysis of the serum samples at 734 nm to avoid the strong absorption at 420 nm of remaining traces of erythrocytes and haemoglobin in the serum. Extinction at this length allows testing and comparison of antioxidants.

2) Preparation of a working solution of the radical: The finished stock solution is diluted with water to obtain a working solution with an absorbance of 0.7 at 734 nm.

3) Evaluation of the interaction of the radical with potential antioxidants: The tested substance is added to 1 ml of working solution. The amount was determined so that no complete decolorization of the reaction mixture was observed after 1 hour of incubation. For the purposes of the study, the blood serum was diluted 20 times in PBS buffer and 50 microliters were added to the radical. 3 replicates were done for each sample. After one-hour incubation, the absorbance A(sample) and the absorbance A(control) of a sample containing only radical and 50 microliters of PBS buffer were measured. Mean value and SD were calculated for each sample. RSA% is calculated by the formula: RSA% = [1–A(sample)/A(control)].100 %. Complete decolorization corresponds to RSA% = 100 % and corresponds to maximum antioxidant capacity.

4) Construction of a calibration curve concentration of Trolox (the water-soluble analogue of vitamin E) – the radical scavenging effect, RSA%: The RSA% data of the Trolox referent standard solutions with different concentrations were used to construct a calibration curve with high correlation coefficient $R^2 = 0.9983$ and to estimate the total antioxidant capacity of the serum of the studied patients as μmol Trolox/μL serum - Trolox equivalent (TE). Based on the results obtained from the extinction of the samples, the effect of RSA% for the respective concentration was determined. Calculations of RSA% of the samples take into account that 1 mol Trolox captures 1.9 mol $ABTS^{\bullet+}$ [12].

Examination of serum concentrations of Cu and Zn

Serum concentrations of bioelements were measured using a Perkin-Elmer AAnalyst 300 flame atomic absorption spectrophotometer. Prior to analysis, serum samples were diluted with bidistilled water 1:3 for copper and 1:5 for zinc, respectively, to avoid transport interference. Measurement of both elements is based on routine calibration with aqueous standard solutions of known concentration prepared by suitable dilution with bidistilled water

of the stock standard [13]. The quality of the results was ensured through the implementation of internal quality control schemes, certified reference materials for the respective trace elements, and participation in external quality assessment programs.

Statistical analyses

Data obtained for each parameter are expressed as mean value ± SD. Assessment of statistical significance of differences in variables between groups was performed by analysis of variance with unpaired t-test. Significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

The first step of the experiments was to construct a calibration curve RSA% /Trolox, µmol/L to trace the radical-scavenging activity against $ABTS^{\bullet+}$ as a function of the concentration of the Trolox reference. This is an established practice for standardizing data obtained from studies in the ABTS system [14, 15]. Pooling the experimental results as Trolox equivalent concentration will allow them to be compared, if more data is obtained by other teams. We assessed the results using variance analysis and an unpaired t-test among the study groups, revealing statistical significance (Table 1).

Table 1. Average values of RSA %, $X_{average}$ ± SD

Parameter	Patients with osteopenia	Patients with osteoporosis	Control group
RSA %	67.69 ± 6.74 p* < 0.01	62.46 ± 7.83 p* = 0.01; p** < 0.05	55.64 ± 2.05

p* against controls; p** against osteopenia

Table 2. Mean serum levels of Cu, Zn, Cu/Zn, µmol Trolox/µL serum, X mean ± SD

Parameter	Patients with osteopenia	Patients with osteoporosis	Control group
Number of patients	20	36	10
Age	61.15 ± 9.22 p* > 0.05	63.67 ± 7.78 p* > 0.05, p** > 0.05	61.3 ± 9.96
BMI	24.42 ± 4.52 p* > 0.05	23.61 ± 3.49 p* > 0.05, p** > 0.05	25.57 ± 3.42
BMD (g/cm^2)	0.77 ± 0.11 p* < 0.01	0.70 ± 0.10 p* < 0.01 p** < 0.05	1.13 ± 0.12
Cu, µmol/L	23.13 ± 4.48 p* < 0.01	22.89 ± 4.20 p* < 0.01, p** < 0.05	18.22 ± 2.53
Zn, µmol/L	14.61 ± 2.92 p* > 0.05	13.14 ± 2.08 p* > 0.05, p** < 0.05	12.83 ± 2.24
Cu/Zn	1.63 ± 0.37 p* > 0.05	1.82 ± 0.51 p* < 0.05, p** > 0.05	1.45 ± 0.17
µmol Trolox/µL	6.81 ± 0.46	6.28 ± 0.49	5.60 ± 0.11

p* against controls; p** against osteopenia

The statistical significance of the mean values of all variables, assessed through an unpaired t-test between different groups with $p < 0.05$ and $p < 0.01$, is presented in Table 2. The results obtained by the age parameter confirm the accurate selection of the age group, and the BMD data validate the appropriate grouping of individuals. Regarding BMI, the groups do not have statistically significant differences, despite the clear trend of decreased BMI in patients with osteoporosis. Statistically significant results for Cu concentration were obtained when assessing the osteopenia and osteoporosis groups ($p^{**} < 0.05$), as well as when contrasting them with the control group ($p^{*} < 0.01$).

We observed elevated serum Zn levels in the groups with decreased bone density in comparison to the control group. However, a statistically significant difference in the Zn index was identified solely between the osteoporosis and osteopenia groups ($p^{**} < 0.05$). We obtained a statistically significant difference ($p^{*} < 0.05$) only between the groups with osteoporosis and the controls for the Cu/Zn indicator, the values of which naturally change with the degree of the disease.

CONCLUSIONS

Patients with reduced bone density exhibit higher RSA% values, elevated copper and zinc concentrations, and increased Cu/Zn ratio values compared to the control group. The rise in serum levels of copper and zinc among patients with decreased bone density can be attributed to the degradation of copper and zinc, which are components of enzymes involved in bone metabolism. This degradation is triggered by an elevated level of reactive oxygen species (ROS) within cells in the context of osteoporosis. The heightened concentration of copper ions in the serum initiates secondary radical processes, subsequently boosting its antioxidant activity (AOA). In contrast, patients with normal bone metabolism maintain lower serum concentrations of Cu and Zn because their intracellular function remains intact. The reduced generation of extra radicals accounts for the lower serum AOA observed in the control group. These findings substantiate the collaborative impact of free radicals and redox-active metals, like copper, on both AOA and bone mineral density.

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