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Original Article

Determınatıon of serum reference intervals for zinc and copper

Metin Demirel [1, 2] *, **Şahabettin Selek** [1], **Tuğçe Yildiz** [1], **Rana Sönmez** [3], **Ayşe Zehra Gül** [1]

[1] Department of Medical Biochemistry, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkey
[2] Health Sciences Institute, Bezmialem Vakif University, Istanbul, Turkey
[3] Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkey

* Correspondence to: Metin Demirel, Department of Medical Biochemistry, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkey. E-mail: medemirel@bezmialem.edu.tr

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Abstract

Zinc and copper are among the essential trace elements that have important roles in metabolism. These trace elements are also involved in developing and regulating female sex hormones. In this study, we aimed to evaluate the reference interval of copper and zinc elements, which have antagonistic relations with each other, in pregnant women, healthy women, and men. By direct sampling method, 120 women (60 pregnant and 60 non-pregnant) and 120 men were included in the study group. The reference interval was evaluated according to the parametric distribution, and the affair between zinc, copper, albumin, estradiol, and ceruloplasmin was evaluated by partial least squares analysis. Serum zinc and copper reference intervals in pregnant women were measured as 69.23–101.15 (μg/dL) and 79.01–193.34 (μg/dL), respectively. In non-pregnant women, serum zinc and copper reference intervals were measured as 66.40–104.67 (μg/dL) and 73.60–148.87 (μg/dL), respectively. According to the structural equation modeling, we determined that serum copper and zinc changed in the effect of estradiol and albumin. As a result, it was determined that it is important to consider albumin levels while investigating the reference interval of zinc and copper during pregnancy.

Keywords: zinc, copper, reference interval, pregnancy, trace elements.

Introduction

Because of their major roles in human metabolism, copper (Cu) and zinc (Zn) are considered essential trace elements. Some of the well-known functions of these elements involve maintaining normal growth, immunity response and providing antioxidant defense [1].

Cu is the component of certain metalloenzymes (superoxide dismutase, tyrosinase, cytochrome c oxidase, dopamine hydroxylase, lysyl oxidase, and ceruloplasmin) [2]. There are two inherited metabolic disorders related to Cu metabolism. One of these, Wilson's disease, develops due to increased free copper. Diversely, Menkes disease develops due to the lack of Cu transport to the tissues [3, 4].

Zn, the most abundant trace element after iron, is essential for growth, development, and immune function. It takes part as a cofactor for a wide variety of metabolic-functioning enzymes (alkaline phosphatase, carbonate dehydratase, thymidine kinase and carboxypeptidase). Its constitutional presence also regulates non-enzyme proteins, including the zinc finger proteins family. Zn loss is known to result in increased susceptibility to oxidative stress, improper preserving of the structure, and integrity of biomembranes [5, 6].

A proper reproductive system function also depends on these two trace elements. Cu has an important role in the regulation of female sex hormones. Zn is required, particularly in the female sexual development period, for oocyte maturation, ovulation, and luteolysis. Serum levels of these elements and reproductive functions are widely studied to elicit underlying associations. It has been reported that there is a correlation between plasma estradiol fluctuations and serum Zn-Cu levels [7]. Studies also showed that human plasma Cu and ceruloplasmin concentrations are higher in women using oral contraceptives [8].

Zn and Cu have been reported to have an antagonistic relationship [9]. Cu absorption occurs on the basolateral surface of enterocytes by the ATP7A protein. In addition, there are intracellular uptake mechanisms with metallothionein (MTO). MTO has a high affinity for transition metals forming mercaptide bonds with multiple cysteine residues. As a common process, absorption of Zn and Cu occurs through these metallothionein proteins. Excessive Zn levels induce intracellular ligand MTO synthesis in enterocytes, which then bind to Zn. This mechanism explains how Zn interferes with the absorption of Cu in the small intestine and affects Cu absorption [9].

Because of the antagonistic interaction between Cu and Zn, if estrogens affect Cu's metabolism, they might mediately influence the homeostasis of Zn withal.

Reference interval

Reporting the results together with reference intervals in clinical laboratories is mandatory. The reference interval can be affected by ethnic group, gender and age. The validity of the available reference intervals is controversial, as the ranges frequently cited in the literature are derived using older methodologies and tools. Besides, these intervals cover a limited age group or a relatively small sample size. For this reason, each laboratory must determine its own reference intervals according to the Clinical and Laboratory Standards Institute (CLSI) – EP28 guidelines to evaluate the results correctly [10].

In the process of determining reference intervals for a laboratory test, it is necessary to collect quantitative information about biological, preanalytical, and analytical sources of variation for the analyzed analyte. Determining the reference interval consists of sampling, analyzing and evaluating the results. The first stage, sampling, can be direct, indirect, priori, posteriori, random, and nonrandom. Sampling reference individuals apply the direct method. The indirect method is carried out by including reference individuals among the sampled individuals in the study. Although the direct method is recommended by The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), the indirect method is used more often because of the difficulty of its implementation [11]. Priori method requires screening before sampling in cases where a multi-factor specific reference sample must be selected and has a high-cost analysis. Posterior is used in cases with a large sample size and when the inclusion criteria can be applied after analysis. The random method is applied by creating a random subgroup from a large sampling group. It is usually carried out by national health centers. Since this method is only suitable for some laboratories, the nonrandom method is preferred.

Considering the preanalytical variations, reference individuals should be informed about factors such as fasting, exercise, drug use and sampling position. Reliability of the methods used in the analysis phase, calibrator, and control applications are also important in terms of analytical variations. A reference interval determination study should be performed, taking into account both preanalytical and analytical variations. Accordingly, the calculated measurement uncertainty can also be reported with the reference interval. By which statistical method (parametric or non-parametric) the data will be obtained as a result of the study should be analyzed. It is necessary to eliminate the outliers following the method decided. The suitability of the data to the gaussian distribution should be analyzed and checked with the parametric method. The data that do not conform to the Gaussian distribution are re-tested by transforming processes (log, square, square root).

It is important to determine the blood levels of trace elements because of their extensive use in various clinical conditions. The interpretation of laboratory results for those elements depends, to a large extent, on the use of reference intervals. Moreover, all the confounding factors must be scrutinized because reference interval values may vary due to sex and age. The reference interval for Zn and Cu elements should be determined by age, especially in pregnant women [8, 12].

This study aimed to establish the reference intervals of serum Cu and Zn in reference individuals selected by the direct sampling method from Turkish individuals (including pregnant) by determining the serum levels of these elements via spectrophotometric methods. Further, the related parameters to the trace element levels, such as ceruloplasmin, albumin, and estradiol, will be investigated to reveal any relationship.

Material and methods

Subjects

The reference population included 240 serum samples from healthy subjects (60 pregnant and 60 non-pregnant women and 120 men) ranging in age from 18 to 80 years attending Bezmialem Vakif University

Hospital from December 2020 to April 2021. This study was carried out by the declaration of Helsinki and the project was authorized by the Ethics Committee of Bezmialem Vakıf University (No: 18/351).

Collection of serum samples and analytical methods

Blood sampling was carried out after 12 hours of fasting. Samples were taken into biochemical tubes without separator gel and were centrifuged at 3000 rpm × 10 minutes. Serums were then aliquoted and stored at -86°C until further analysis.

Serum Zn concentrations were measured with a commercially available Rel Assay zinc measurement kit (Gaziantep, Turkey) using a fully automatic photometric method (Abbott ARCHITECT ci16200 clinical chemistry analyzer). The measurement of Zn levels is based on the principle that under alkaline conditions, the red-orange color of 5-Br-PAPS changes from the Zn contained in the sample to light pink. The change in absorbance at 548 nm is proportional to the total Zn level in the sample. The intra-assay coefficient of variation for Zn is 2.32%. The inter-assay coefficient of variation for Zn is 3.54%. The assay is calibrated with Zn sulfate dissolved in deionized water.

Serum Cu concentrations were measured with a commercially available Rel Assay copper measurement kit (Gaziantep, Turkey) using a fully automatic photometric method (Abbott ARCHITECT ci16200 clinical chemistry analyzer). The measurement of Cu levels is based on the principle that 3,5-DiBr-PAESA changes the red-orange color of the Cu in the sample to violet-blue under acidic conditions. The change in absorbance at 572 nm is proportional to the total Cu level in the sample. The intra-assay coefficient of variation for Cu is 1.85%. The inter-assay coefficient of variation for Cu is 2.74%. The assay is calibrated with copper sulfate dissolved in deionized water.

Statistical analysis

All statistical analysis was performed using R and Python programming languages. All data were initially assessed for normality by applying Shapiro–Wilk test. Grubb's test was conducted to identify and eliminate outliers in the data. Data were expressed as mean with ± standard deviation (SD), and a p-value less than 0.05 was considered statistically significant. The parametric method has been used to evaluate lower and upper reference limits as 2.5 and 97.5 percentiles of the reference interval distribution. Partial least squares (PLS) path modeling with the mediator and moderator analysis was performed using SmartPLS 3 software [13].

Results and discussion

All the data were checked for outliers using Grubb's test. No outliers were found in this study. In a study conducted on 240 (120 women and 120 men) subjects, similar results were obtained in studies conducted on other populations [14–18]. The mean results of age, BMI, zinc, and copper by gender of the subjects are presented in Table 1.

Serum Cu reference intervals were measured as 73.60–148.87 (µg/dL) and 56.91–143.76 (µg/dL) in women and men, respectively. Serum Zn reference intervals were found to be 66.40–104.67 (µg/dL) and 65.58–111.54 (µg/dL) in women and men, respectively. Serum Zn and Cu reference intervals in pregnant women were measured as 69.23–101.15 (µg/dL) and 79.01–193.34 (µg/dL), respectively (Table 2). Serum Zn levels were lower in pregnant women, while serum Cu levels were found to be higher, conversely.

Low blood levels of Zn have been linked to serious complications in pregnancy, such as prolonged labor, spontaneous abortion, hypertension, low birth weight,

Table 1: Age and BMI values of the study population. Results show the mean values obtained for serum copper and zinc concentrations (Data are means±SD).

	Women (n=120)		Men (n=120)
	Pregnant (n=60)	Non-pregnant (n=60)	
Age	34.0 ± 6.6	46.7 ± 16.1	49.6 ± 18.8
BMI (kg/m^2)	28.6 ± 3.1	26.4 ± 3.4	24.7 ± 3.9
Copper (µg/dL)	121.3 ± 38.0	120.08 ± 29.2	108.82 ± 37.5
Zinc (µg/dL)	82.6 ± 10.7	84.91 ± 13.5	81.66 ± 13.9
Cu/Zn	1.52 ± 0.6	1.47 ± 0.5	1.44 ± 0.8

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Table 2: Reference intervals for serum Cu and Zn concentrations.

	Women				Men	
	Pregnant		Non-pregnant			
	Lower	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
Copper (µg/dL)	79.01 (75.0–83.1)	193.34 (189.3–197.4)	73.60 (72.1–75.2)	148.87 (147.3–150.1)	56.91 (54.3–59.5)	143.76 (141.2–146.4)
Zinc (µg/dL)	69.23 (68.4–70.1)	101.15 (100.3–102.0)	66.40 (65.6–67.2)	104.67 (103.9–105.5)	65.58 (64.2–67.0)	111.54 (110.2–113.0)

postpartum hemorrhage and malformations [19–21]. When Zn and Cu metabolism is examined in pregnant women, the effect of estradiol comes to the fore. Estradiol causes an increase in ceruloplasmin serum levels [22]. In our study, a highly significant result was found with a critical correlation coefficient of 0.485 between estradiol and ceruloplasmin, supporting the former thesis. When we look at the correlation between ceruloplasmin and Cu, a strong relationship was found at the rate of 0.98 percent (Figure 1). The Cu level increase appears to be highly dependent on the ceruloplasmin induction. We consider this ceruloplasmin effect as the reason for increased Cu levels during pregnancy.

A number of 2380 samples were divided into five groups according to gestational weeks examined and serum Cu levels were found to increase with progressive weeks. In addition, it was determined that serum Zn levels between 13–27 weeks were significantly lower than those who were not pregnant [23]. In another study, 120 non-pregnant women and 128 pregnant women were grouped into 3 trimesters and as a result, blood Zn levels showed a decrease as the pregnancy progressed, although it was not statistically significant in the first trimester. The decrease was attributed to the disproportionate increase in plasma volume and maternal-fetal transfer [18].

According to structural modeling analysis, the decrease in Zn levels in pregnant women is related to the loss of albumin. First, a negative correlation was found between albumin and estradiol. The mean albumin level of 0.44 is explained by estradiol (Figure 1). The reduced albumin is compensated by ceruloplasmin, which then increases the Cu levels. At the same time, decreased albumin led to decreased Zn levels. In this regard, as one of the most important findings of this study, we suggest that albumin has a mediator effect on these correlations. In support of our results, several studies have observed an increase in the Cu/Zn ratio throughout pregnancy in women [14, 19, 24–27]. In addition, serum Cu levels increased with age and during pregnancy while serum Zn levels decreased with age and during oral contraceptive use [1, 12, 28]. Decreased serum Zn levels are explained by the need arising in fetal development and increased serum Cu levels were

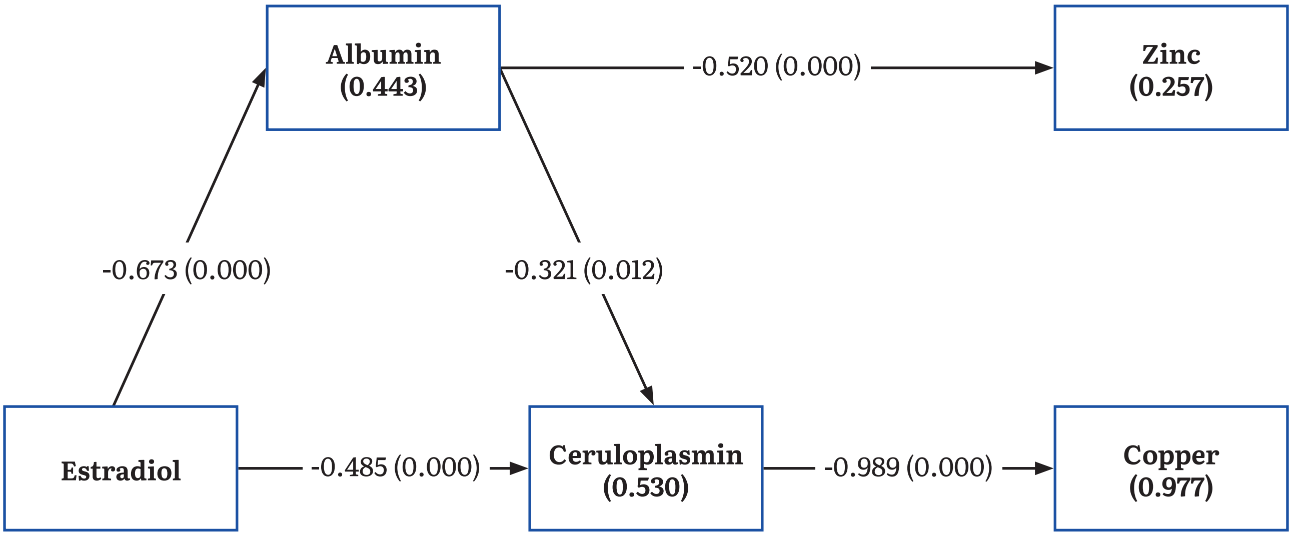

Figure 1: Structural equation modeling (SEM) of parameters (albumin, estradiol, and ceruloplasmin) that are likely to affect the reference interval (Zinc and Copper).

considered as the result of ceruloplasmin induction mediated by estradiol [27].

Researchers have noted that the differences in Cu metabolism between men and women may be due to the effect of female hormones, mainly estradiol [29]. Several studies have shown that plasma Cu and ceruloplasmin levels tend to increase during pregnancy and with the use of combined oral contraceptives [7, 29, 30]. Female steroid hormones also indirectly regulate Zn homeostasis through their influence on Cu homeostasis since Cu and Zn have an antagonistic effect on each other.

Further, we obtained similar results in serum Zn and Cu reference intervals with former studies in pregnant women. A study conducted on 31 healthy pregnant women and 51 non-pregnant women found that serum Zn levels were significantly low in pregnant women and serum Cu levels increased significantly in the 2nd and 3rd trimesters [27].

We obtained similar results with other populations in the study we conducted to determine the reference intervals of serum Zn and Cu levels in the adult Turkey population. When we examined the results for pregnant women, it was found that serum Zn levels were lower than in non-pregnant women. It was observed that serum Cu levels were in an antagonistic relationship with Zn and were at higher levels in this group. Therefore, it was concluded that the reference intervals of serum Zn and Cu in pregnant women should be evaluated separately.

According to the structural equation modeling results between estradiol, albumin, ceruloplasmin, Zn and Cu, it is understood that albumin has a mediator effect in the correlation observed between Cu, ceruloplasmin, and Zn. As a result, it was determined that it is important to consider albumin levels while investigating the reference interval of Zn and Cu during pregnancy.

This study provides an exquisite view of the mediator effect of albumin on the reference intervals of zinc and copper elements with structural equation modeling, which is a novel statistical method becoming frequently used in the field. Furthermore, it is the first study to determine reference intervals of trace elements zinc and copper in the population of Turkey, including pregnant individuals.

Conclusion

In summary, we demonstrated sex and gestational-specific reference intervals for blood Cu and Zn concentrations in the adult population of Turkey by obtaining the data from a large study sample, as recommended by the IFCC and CLSI guidelines. The obtained reference intervals for Cu and Zn represent considerable guidelines for screening trace elements in pregnant women.

Conflict of interest

The authors declare no conflict of interest.

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