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Original Article

Determination of plasma and erythrocyte levels of copper, magnesium and zinc by atomic absorption spectrometry in type-2 diabetes mellitus patients with metabolic syndrome

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Abstract

Background and purpose: Imbalance in blood levels of trace elements is independent risk factor for metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM), and its complications. This study investigated plasma and erythrocyte levels of copper, magnesium, zinc, and their correlations with biochemical components of the MetS in T2DM patients compared to the healthy controls.
Experimental approach: Forty men recently diagnosed T2DM with MetS without complications and thirty six age-matched healthy controls were enrolled in this cross-sectional study. Plasma and erythrocyte levels of selected elements were measured by graphite furnace atomic absorption spectroscopy.
Findings/Results: The results of the present study showed significantly lower plasma levels of copper, magnesium, and zinc and lower erythrocytes copper in the patients' group compared to the controls; while erythrocyte levels of magnesium and zinc were not significantly different between the two groups. Significant negative correlations were observed between plasma levels of copper with waist and hip circumferences, waist to hip ratio, systolic and diastolic blood pressures, fasting blood glucose, and glycated hemoglobin levels in all subjects; while erythrocyte copper levels showed significant negative correlation with triglyceride, and erythrocyte zinc was positively correlated with diastolic blood pressure and negatively with triglyceride.
Conclusion and implications: Alterations of trace elements may have a significant role in the pathogenesis of MetS and T2DM patients. It is suggested that the body status of copper, magnesium, and zinc might be significantly correlated with components of MetS in T2DM patients; and plasma copper levels may be correlated with complications of type 2 diabetes mellitus.

Keywords: Atomic absorption spectrometry; Copper; Diabetes mellitus; Magnesium; Metabolic syndrome; Zinc.

INTRODUCTION

Metabolic syndrome (MetS) is a disease developed from an accumulation of several risk factors of metabolic origin. These metabolic risk factors include abdominal obesity, dyslipidemia, increased blood pressure, and increased fasting serum glucose. MetS is characterized by an increased risk of several diseases such as type 2 diabetes mellitus (T2DM) and cardiovascular diseases. The prevalence of MetS in Iran is high, estimated to be 25 to 39% of the Iranian adult population depending on the criteria used (1). T2DM is a chronic heterogeneous disease characterized by hyperglycemia. International diabetes federation estimates the diabetic patient population to reach 592 million worldwide by 2035.

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Social industrialization, lack of physical activity, and age are some of the contributing factors to the increase in the rate of T2DM incidence. Diabetic patients in Iran are estimated to increase from 8.4% of the total national population in 2013 to 12.3% in 2035 (2,3). Hyperglycemia associated with T2DM evidently increases the production of reactive oxygen species, thus causing damage to a variety of tissues (4).

The disturbance in trace elements (TEs) status in T2DM could be instrumental in insulin resistance and the development of diabetic complications. On the other hand, the progression of T2DM may lead to the altered metabolic status of TEs in tissues (5). Trace elements may have a role in enhancing the action of insulin by serving as cofactors or components of enzyme systems of glucose metabolism. They may as well affect glucose metabolism through activation of insulin receptor sites, increasing insulin sensitivity, and acting as antioxidants that prevent tissue peroxidation (6).

Elements are paramount to human body functions. There is general accordance in the literature about the association of T2DM with alterations in trace elements status, although it is unclear whether any of the two factors is causal to the other (7-9).

Serum or plasma levels of the trace elements are normally regarded as eligible biomarkers for epidemiological studies and clinical investigations, even though the certainty of results obtained from these samples is controversial. Erythrocytes, on the other hand, may contain clinically valuable information, providing a better insight into trace elements' status and their correlations with the metabolic conditions (8).

Presence of the diabetic complications may be considerable in trace elements alterations. As demonstrated by Chen *et al*. diabetic patients with complications had higher plasma copper (Cu), and slightly lower plasma magnesium (Mg) and zinc (Zn) levels, when compared to diabetic patients without complications (10). Also, the MetS is known to be a disturbing factor on trace element status intrinsically (11). Thus, the presence of the above-mentioned factors should be noted when investigating the trace element levels in patients with T2DM.

Impaired metabolism of TEs like Cu has been reported in diabetes. Cu is an essential element in many enzymes that catalyze oxidation-reduction reactions, energy production, mitochondrial electron transport chain, detoxification of free radicals, and the formation of cross-links in connective tissue (12,13). The imbalanced status of Cu is also shown to have a pro-oxidant role and may contribute to the formation of free radicals (14).

Zn and Cu play an essential role in oxidant/antioxidant mechanisms, and to protect tissues from oxidative damages. An imbalance in these mechanisms may result in the further progress of T2DM or diabetic complications. Zn acts as an antioxidant by preserving the sulfhydryl groups of proteins and enzymes against free radical attacks in the body. Cu and Zn act as structural and catalytic constituents of some metalloenzymes such as Cu/Zn superoxide dismutase that protects cells from superoxide radicals (14). Alterations in the metabolism of Cu and Zn may be involved in several procedures which are related to oxidative stress. Several studies have reported either essentiality or toxicity of these elements in the pathogenesis of T2DM and diabetic complications (5).

Mg is also a cofactor in the glucose transporting mechanisms of the cell membrane, and various enzymes in carbohydrate oxidation. Also, at multiple levels, Mg is involved in insulin secretion and binding and enhancing the ability of insulin to activate tyrosine kinase. Mg deficiency has been implicated in insulin resistance, carbohydrate intolerance, dyslipidemia, and complications of diabetes (15).

Essential TEs such as Zn, Cu, chromium, and selenium take part in variety of enzymatic processes on molecular cellular level; this might be correlated to contrasts between TEs intracellular concentrations from their concentrations in plasma. TE measurements obtained from the plasma or whole blood samples analysis may fail to reflect the actual intracellular content of the elements. Investigation of TE concentrations in blood cells in addition to plasma may comprise significantly more information. Likewise in diabetes mellitus and other metabolic diseases,

TEs concentration in blood cells may contain more precise clinical information (8).

Although MetS is highly prevalent in T2DM patients (13), there are conflicting reports regarding trace elements status in T2DM patients with MetS.

In this cross-sectional study, the plasma and erythrocyte levels of Cu, Mg, and Zn in T2DM patients with MetS were investigated. This study aimed to determine the biochemical factors and levels of the selected elements in plasma and erythrocyte samples of the population of Iranian men with T2DM and MetS, and to further demonstrate the possible correlations among the variables, as well as contrasts between erythrocyte and plasma levels of the elements. Also, we aimed to discuss the possible disturbing factors and some of the controversies among other quantitative studies in this subject. As shown below, appropriate interpretation of the quantitative results obtained requires the identification and stratification of the different variables influencing these concentrations.

MATERIALS AND METHODS

Study subjects

Forty men were recently diagnosed with T2DM with MetS, and thirty normoglycemic apparently healthy men were age-matched and enrolled in this cross-sectional study. A thorough medical history was recorded through a private, face-to-face interview using a standard questionnaire. T2DM was established upon the American Diabetes Association criteria (fasting glucose ≥ 126 mg/dL or 2 h postprandial glucose ≥ 200 mg/dL, or if they were taking oral antidiabetic medication but not insulin (16). Oral antidiabetic medications for T2DM patients were metformin or combined formulations of metformin/glibenclamide. The subjects were excluded if they were cigarette smokers or used any other tobacco products. Taking lipid-lowering agents or insulin injection was also considered as exclusion criteria. However, the use of oral antidiabetes medications was assumed acceptable. Other conditions likely to alter trace elements status were added to the exclusion criteria for the present study, including acute diseases within a month prior to participation in the study, cardiovascular disease, stroke, neoplasia, chronic diarrhea, chronic inflammatory disorders, hepatic or renal diseases, defined coagulation deficiency, hypo- or hyperthyroidism, epilepsy, alcohol and substance abuse, vegetarianism, known occupational or environmental exposures to metals, metallic implants, as well as consumption of dietary supplements, anti-inflammatory and diuretic medications(11-13).

National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) criteria for MetS was utilized in the present study; according to which, MetS diagnosis is established when a patient has three of five of the following risk factors: hyperglycemia (having a fasting blood glucose (FBG) ≥ 100 mg/dL), hypertriglyceridemia (triglyceride (TG) ≥ 150 mg/dL), having low levels of high-density lipoprotein cholesterol (HDL-C < 40 mg/dL for men and < 50 mg/dL for women), increased blood pressure (≥ 130/85 mmHg), and increased waist circumference (WC ≥ 102 cm for men and ≥ 88 cm for women) (17).

This study was performed following approval by the Ethics Committee of Isfahan University of Medical Sciences in accordance with the principles of the Helsinki Declaration for medical research and its later amendments and informed written consent was obtained from all patients. The ethics approval code for this study was IR.MUI.RESEARCH.REC.1398.625.

Anthropometric measurements and blood pressure

Blood pressure (BP) was measured in a seated position after at least 15 min of rest, using a standard calibrated mercury sphygmomanometer according to a standard method. The mean of three measurements at 2-5 min intervals was recorded as the BP. Height and weight were scaled utilizing a portable calibrated electronic weighing scale and portable measuring inflexible bars, respectively; while participants were in the standing position with light clothes and without shoes. Subject' WC was determined at the umbilicus, and hip circumference (HC) at the

widest girth using a tape meter, and waist/hip ratio (WHR) was calculated accordingly. Body mass index (BMI) was computed as measured weight in kilograms divided by measured height in meters squared. All measurements mentioned above were carried out according to standard conditions, by one person to minimize the error.

Blood collection and processing

After at least 10 h of overnight fasting, blood samples were obtained *via* venipuncture and collected in ethylenediaminetetraacetic acid (EDTA) and plain test tubes. Samples were then centrifuged at 1500 *g* at 25 °C for 10 min and serum and plasma were separated into plain test tubes. FBG, TG, total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), HDL-C, fasting serum insulin (FINS), and high sensitivity C-reactive protein (hs-CRP) were measured in serum samples. EDTA blood samples were used for hemoglobin A1c (HbA1c) determination. Washed packed erythrocytes and plasma samples were then stored frozen at -20 °C and used for trace elements analysis after thawing.

Biochemical assays

Enzymatic colorimetric assay using the glucose oxidase method was adopted to measure FBG (Parsazmun, Iran). HbA1c and hs-CRP were measured using latex-enhanced immunoturbidimetric assays using an automated analyzer and commercial kits (Parsazmun, Iran). Serum TC, TG, and HDL-C were measured by enzymatic method kits (Parsazmun, Iran), utilizing a Hitachi 902 automatic analyzer (Hitachi, Japan). Serum LDL-C was calculated using Friedewald's formula. If serum TG concentration was > 400 mg/dL, LDL-C was determined directly by enzymatic method using the commercial kit. FINS was measured using an enzyme-linked immunosorbent assay kit (ELISA, Monobind, USA). Insulin resistance (IR) was estimated using the homeostatic model assessment for insulin resistance (HOMA-IR) index, according to the following equation (18):

HOMA-IR=fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5.

Analytical method

Apparatus

A Perkin-Elmer Zeeman-3030 (Perkin-Elmer & Co, GmbH, Germany) atomic absorption spectrometer with background correction and integrated screen, was equipped with an HGA-600 graphite furnace, auto-sampler AS-60, Perkin-Elmer recorder R-100, and used for all analyses. Cu, Mg, and Zn hollow-cathode lamps (Perkin-Elmer) were utilized as radiation sources.

Reagents and standard solutions

The solutions were prepared using ultrapure deionized water. Stock standard solutions (CertiPUR®, Merck) for Cu, Mg, and Zn containing 1000 mg/L, were used to provide working standards. Nitric acid (Merck Chemicals Co., Ltd. Germany) 65% (w/v) was used for the preparation of acid-washing solutions, automatic rinsing of auto-sampler bead, diluting the standards, and sample preparation, which will be further explained. Also, Triton® X-100 (Merck Chemicals Co., Ltd. Germany) was provided for Zn and Cu analyses sample preparation.

Trace elements analysis

A total of six series of analyses were carried out, which included estimation of Cu, Mg, and Zn in both the plasma and washed erythrocyte samples from each of the study subjects. Prior to the analysis, all laboratory ware were soaked in 15% (v/v) nitric acid for 24 h or more, then rinsed thoroughly 3 times with deionized water and dried at 80 °C for 2 h. . To enable a comparison of the results, an aliquot of washed erythrocyte was lysed with two volumes of deionized water and the erythrocyte hemoglobin concentrations were determined using cyanomethemoglobin method commercial kit (ZiestChem®, Tehran, Iran) (19). Trace elements in erythrocyte are reported as μg/gHb. For each analysis series, a blank sample and five working standards were prepared by stepwise dilution from stock standards solutions. Working standards solutions were constructed in order to cover at least a range from half the minimum to twice the maximum of expected concentrations in normal samples (20). Each standard solution

was carefully prepared and a calibration curve was considered acceptable only at $r^2 \geq 0.99$. After thawing and homogenizing by a vortex mixer and individual preparations, 20 µL of each sample was injected by the auto-sampler and the analysis was repeated three independent times, and the mean values were reported. Cu was analyzed by direct measurement against a calibration graph, *via* measuring the peak area. The blank solution, working standard preparation solution, and sample dilution solution were prepared by the same composition. Plasma Cu was analyzed after diluting two volumes of each of the samples with eight volumes of dilution solution including 1% nitric acid, 1% Triton® X-100, and deionized water (1:1:6). In order to prepare the erythrocyte samples, eight volumes of each sample were mixed with two volumes of the dilution solution of 1% nitric acid and 1% Triton® X-100 (1:1) (21). All temperature programs and general conditions are summarized in Table 1. Mg was also measured by peak height absorbance, using a calibration graph method, by diluting samples and standards with 0.05 mol/L nitric acid. Plasma and erythrocyte samples were diluted 600- and 1000-fold, respectively; and to resolve the probability of inhomogeneity errors, vortex mixing was applied for the preparation of washed packed erythrocyte (22). The temperature program used is shown in Table 1 (23). In order to minimize errors, dilution was arranged using a class 100 laminar flow hood, carefully calibrated micropipettes (Socorex®, Switzerland), and quality micropipette tips (Eppendorf®, Germany). Zn was analyzed in the peak height mode after diluting the samples *via* deionized water, by a factor of 100 and 500, for plasma and erythrocyte, respectively. The temperature program used is shown in Table 1 (19). In order to adjust for the matrix effects, the standard addition method was adopted to compose plasma Zn working standards (24), and for erythrocyte Zn measurements, major inorganic components of the erythrocytes were added to the standards as directed by Whitehouse *et al*. (19).

Accuracy and precision

To ensure the accuracy of each analysis, recovery tests at two concentrations of each element were carried out. This was accomplished by adding the two concentrations to an aliquot from each of the analysis series, followed by analyses of the concentration of the elements in replicates of three (19). Added concentrations for recovery tests were arranged to reach a final concentration within the range of working standards. Following the above-mentioned steps, post addition concentration values were obtained; and mean values, standard deviation, average recovery percentage, and coefficient of variation (CV) were calculated for each analysis. An analysis was presumed accurate only at CV rates below 5% and recovery percentage between 95 to 105%. To estimate the method precision, intra- and inter-day replicate analyses were performed, for which the same aliquots were analyzed 10 times in the same day and 5 times in 5 consecutive days, respectively (19). After which mean values, standard deviation, and CV were calculated, and the results were regarded as precise only at CV < 5%.

Statistical analysis

Statistical analysis was performed using the SPSS software (V.25, IBM Corporation). Data are expressed as means ± SD. Variables were checked for distribution normality by Kolmogorov-Smirnov test and equality of variances was computed *via* Levene's test. The student's t-test was utilized for the analysis of normally distributed independent data between groups and nonparametric variables were studied *via* the Mann-Whitney U test. Pearson's partial correlation and Spearman's rank-order correlation were used for quantitative and nonparametric data, respectively. All correlations were considered statistically significant at $P < 0.05$ (two-tailed).

Table 1. General conditions and the temperature program for the graphite furnace atomic absorption spectrometry analysis of elements.

Element	Lamp current (mA)	Gas	Steps	Wave-length (nm)	Slit width (nm)	Drying				Ashing				Pretreatment				Atomization				Flushing			
						Temperature (°C)	Ramp (s)	Hold (s)	Gas flow (mL/min)	Temperature (°C)	Ramp (s)	Hold (s)	Gas flow (mL/min)	Temperature (°C)	Ramp (s)	Hold (s)	Gas flow (mL/min)	Temperature (°C)	Ramp (s)	Hold (s)	Gas flow (mL/min)	Temperature (°C)	Ramp (s)	Hold (s)	Gas flow (mL/min)
Cu	15	Ar	6	324.8	0.7	1: 110 2: 130	1 5	25 25	250 250	600	10	20	250	1200	10	10	250	2100	0	5	0	2550	1	2	250
Mg	10	Ar	6	285.2	0.7	1: 110 2: 150	5 10	10 5	300 300	800	10	15	300	800	1	5	40	2500	0	3	40	2800	1	5	300
Zn	20	Ar	5	213.9	0.7	110	10	20	300	400	10	20	300		-			2100	0	4	Plasma:25 Erythrocyte: 100	2700	1	2	300

RESULTS

The characteristics of the study population are presented in Table 2. Significant differences in weight, WC, HC, WHR, BMI, SBP, FBS, HbA1C, HOMA-IR, TG, HDL-C, and FINS were observed between groups. No significant differences were observed between groups with respect to their DBP, TC, LDL-C, and hs-CRP.

The mean concentrations of the elements are presented in Table 3. Plasma Cu, Mg, and Zn levels were significantly decreased in patients compared with the controls. Accordingly, the Cu/Zn ratio showed a significant decrease in the patients compared with the controls. Mean erythrocyte Cu level was lower in patients compared to the controls, although for Mg and Zn differences were not statistically significant.

Accuracy and precision analyses were carried out *via* recovery test, and intra- and inter-day replicate analysis, respectively; the results of which are summarized in Table 4. All analysis series were accepted as accurate only at CV rates below 5% and recovery percentage between 95 to 105%. Regarding precision, a cut-off was considered at CV rates below 5%.

Table 2. Baseline characteristics and biochemical parameters of the study subjects. Data are expressed as means ± SD. Analysis was performed using independent samples t-test for normally distributed and Mann-Whitney U test for nonparametric values; Significant *P*-values are shown as bold font.

Parameters	Control (n = 30)	T2DM with MetS (n = 40)	*P*-values
Age (year)	54.12 ± 4.08	56.03 ± 6.9	0.174
Weight (kg)	75.69 ± 11.80	84.28 ± 13.67	**0.008**
Waist circumference (cm)	95.21 ± 8.45	105.71 ± 10.27	**0.000**
Hip circumference (cm)	99.00 ± 9.14	106.05 ± 7.21	**0.003**
Waist-hip ratio	0.97 ± 0.099	1.00 ± 0.053	**0.001**
Body mass index (kg/m^2)	22.67 ± 3.23	24.82 ± 3.75	**0.018**
Systolic blood pressure (mmHg)	123.28 ± 11.80	133.13 ± 13.55	**0.001**
Diastolic blood pressure (mmHg)	78.62 ± 10.26	79.33 ± 18.92	0.265
Fasting blood glucose (mg/dL)	95.93 ± 10.73	155.38 ± 44.56	**0.000**
Hemoglobin A1C (%)	5.45 ± 0.40	7.28 ± 1.46	**0.000**
Homeostasis model assessment of insulin resistance	2.69 ± 1.14	5.57 ± 3.98	**0.000**
Total cholesterol (mg/dL)	190.52 ± 22.41	190.97 ± 44.91	0.956
Triglyceride (mg/dL)	128.46 ± 32.50	198.41 ± 84.12	**0.000**
High density lipoprotein cholesterol (mg/dL)	46.14 ± 5.04	36.64 ± 5.69	**0.000**
Low density lipoprotein cholesterol (mg/dL)	116.03 ± 20.42	112.31 ± 31.79	0.559
Fasting serum insulin (mU/L)	11.11 ± 4.28	14.02±6.85	**0.041**
High sensitivity C-reactive protein (mg/L)	1.90 ± 2.07	2.22 ± 1.65	0.232

T2DM, Type 2 diabetes mellitus; MetS, metabolic syndrome.

Table 3. Comparison of the selected elements levels between groups. Data are expressed as means ± SD. Statistical analysis is done using independent samples t-test for parametric and Mann-Whitney U test for nonparametric values; Significant *P*-values are shown as bold font.

Parameters	Control (n = 30)	T2DM with MetS (n = 40)	P-values
Plasma Cu (mg/L)	0.87 ± 0.16	0.62 ± 0.14	**0.000**
Plasma Mg (mg/L)	21.61 ± 4.99	18.26 ± 6.30	**0.021**
Plasma Zn (mg/L)	1.16 ± 0.31	0.99 ± 0.28	**0.030**
Plasma Cu/Zn ratio	0.82 ± 0.27	0.68 ± 0.27	**0.020**
Erythrocyte Cu (µg/g Hb)	3.18 ± 1.83	2.08 ± 1.41	**0.007**
Erythrocyte Mg (µg/g Hb)	449.69 ± 193.38	435.41 ± 213.18	0.785
Erythrocyte Zn (µg/g Hb)	54.41 ± 14.14	51.41 ± 11.10	0.383

Cu, Copper; T2DM, type 2 diabetes mellitus; Hb, hemoglobin; MetS, metabolic syndrome; Mg, magnesium; Zn, zinc.

Table 5 shows the correlations between plasma and erythrocyte levels of selected elements with main characteristics among all study subjects. Disturbance in the selected trace elements may be interrelated within the patient groups; plasma Cu showed a significant positive correlation with erythrocyte Cu. Plasma Cu was also found to have a positive correlation with plasma Mg and plasma Zn, although not statistically significant. Erythrocyte Cu showed no correlation with plasma Zn, but had a significant correlation with plasma Mg.

There was a significant positive correlation between plasma Mg and erythrocyte Mg and Cu. Erythrocyte Mg showed a significant positive correlation with erythrocyte Zn.

It is worth to mention that plasma Zn showed no correlation with any other measured elements in this study, neither with the biochemical parameters measured in blood.

Plasma Mg and erythrocyte Mg showed significant positive correlations with HDL-C. The most pronounced trace element in correlation with biochemical factors of the study was plasma Cu. Plasma Cu showed significant negative correlations with WC, HC, WHR, SBP, DBP, FBS, HbA1C. Whereas erythrocyte Cu showed a significant negative correlation with TG, and erythrocyte Zn was positively correlated with DBP and negatively with TG. Among the measured factors, age, weight, TC, LDL-C, FINS, and hs-CRP revealed no significant correlation with any of the analyzed elements.

Table 4. Analysis of accuracy and precision.

Analyze	Accuracy study					Precision study					
						Intra-day			Inter-day		
	Before addition	Amount added (final concentration calculated)	Mean ± SD After addition	Average recovery (%)	CV %	n	Mean ± SD	CV %	n	Mean ± SD	CV %
Plasma Cu	0.68 mg/L	0.50 mg/L	1.18 ± 0.018	100.5	1.48	10	0.701 ± 0.027	3.9	5	0.702 ± 0.026	3.8
		1.00 mg/L	1.67 ± 0.014	99.02	1.38						
Plasma Mg	17.80 mg/L	15.0 mg/L	33.36 ± 0.574	103.8	1.72	10	17.802 ± 0.554	3.1	5	17.770 ± 0.825	4.7
		30.0 mg/L	47.83 ± 0.821	100.1	1.71						
Plasma Zn	0.85 mg/L	0.50 mg/L	1.37 ± 0.003	103.6	2.43	10	0.903 ± 0.035	3.8	5	0.931 ± 0.043	4.6
		1.00 mg/L	1.87 ± 0.007	101.5	3.85						
Erythrocyte Cu	3.105 µg/gHb	0.50 mg/L	6.322 ± 0.046	99.4	0.74	10	3.125 ± 0.076	2.4	5	3.148 ± 0.102	3.2
		1.00 mg/L	9.641± 0.350	101.0	3.62						
Erythrocyte Mg	431.91 µg/gHb	15.0 mg/L	529.41 ± 1.867	101.7	0.35	10	424.81 ± 11.674	2.7	5	440.59 ± 15.408	3.5
		30.0 mg/L	618.80 ± 3.686	97.5	0.60						
Erythrocyte Zn	44.27 µg/gHb	2.50 mg/L	59.13 ± 0.410	100.6	0.69	10	45.05 ± 1.105	2.5	5	45.77 ± 2.204	4.8
		5.00 mg/L	74.88 ± 2.518	103.7	3.36						

Cu, Copper; Hb, hemoglobin; Mg, magnesium; Zn, zinc.

Table 5. The correlation of blood levels of selected elements with main characteristics of all study subjects, *P*-values in parenthesis. Statistical analysis is done using Pearson's correlation for parametric and Spearman's rank-order correlation for nonparametric values.

Parameters	Plasma Cu (mg/L)	Erythrocyte Cu (µg/gHb)	Plasma Mg (mg/L)	Erythrocyte Mg (µg/gHb)	Plasma Zn (mg/L)	Erythrocyte Zn (µg/gHb)
Age (year)	-0.137 (0.289)	0.072 (0.600)	0.097 (0.449)	0.122 (0.363)	0.078 (0.547)	0.214 (0.131)
Weight (kg)	-0.231 (0.060)	-0.112 (0.392)	0.000 (0.999)	0.157 (0.219)	-0.147	-0.031 (0.824)
WC (cm)	-0.346 (0.004)*	-0.076 (0.569)	-0.075	0.188 (0.142)	-0.065	0.069 (0.622)
HC (cm)	-0.275 (0.026)*	-0.066 (0.617)	-0.116	0.178 (0.167)	-0.197	-0.033 (0.813)
WHR	-0.252 (0.041)*	-0.063 (0.638)	-0.070	0.113 (0.381)	0.111 (0.376)	0.120 (0.386)
BMI (kg/m^2)	-0.221 (0.077)	-0.095 (0.478)	0.034 (0.789)	0.190 (0.143)	-0.143	0.061 (0.664)
SBP (mmHg)	-0.363 (0.003)*	-0.195 (0.136)	-0.117	0.103 (0.421)	-0.221	0.048 (0.730)
DBP (mmHg)	-0.331 (0.006)*	-0.012 (0.925)	-0.208	0.180 (0.158)	-0.064	0.335 (0.012)*
FBG (mg/dL)	-0.442 (0.000)*	-0.113 (0.390)	-0.168	-0.013 (0.921)	-0.221	-0.235 (0.084)
HbA1C (%)	-0.496 (0.000)*	-0.253 (0.076)	-0.156	-0.120 (0.382)	-0.239	-0.110 (0.463)
HOMA-IR	-0.157 (0.221)	-0.053 (0.697)	-0.222	-0.132 (0.317)	-0.061	-0.245 (0.080)
TC (mg/dL)	0.114 (0.357)	-0.072 (0.584)	0.064 (0.604)	-0.065(0.612)	0.021 (0.863)	-0.183 (0.181)
TG (mg/dL)	-0.093 (0.460)	-0.253 (0.050) *	-0.136	-0.135 (0.294)	-0.089	-0.328 (0.016)*
HDL-C (mg/dL)	0.412 (0.001)*	0.195 (0.136)	0.311	0.280 (0.026)*	0.230 (0.061)	0.091 (0.507)
LDL-C (mg/dL)	0.031 (0.803)	0.074 (0.573)	0.084 (0.496)	0.088 (0.493)	0.075 (0.544)	-0.097 (0.482)
FINS (mU/L)	0.015 (0.908)	0.056 (0.684)	-0.169	-0.137 (0.300)	0.014 (0.916)	-0.090 (0.528)
hs-CRP (mg/L)	-0.026 (0.845)	0.081 (0.563)	-0.182	-0.180 (0.183)	-0.060	-0.021 (0.886)
Plasma Cu (mg/L)	1.000	0.289 (0.027)*	0.217 (0.077)	0.035 (0.787)	0.223 (0.072)	-0.068 (0.620)
Plasma Mg (mg/L)	0.217 (0.077)	0.315 (0.014)*	1.000	0.476 (0.000)*	-0.100	0.148 (0.280)
Plasma Zn (mg/L)	0.223 (0.072)	-0.002 (0.990)	-0.100	0.043 (0.740)	1.000	-0.107 (0.440)
Erythrocyte Cu (µg/g Hb)	0.289 (0.027)	1.000	0.315 (0.014)*	0.256 (0.055)	-0.002 (0.990)	0.220 (0.121)
Erythrocyte Mg (µg/g Hb)	0.035 (0.787)	0.256 (0.055)	0.476 (0.000)*	1.000	0.043 (0.740)	0.331 (.014)*
Erythrocyte Zn (µg/g Hb)	-0.068 (0.620)	0.220 (0.121)	0.148 (0.280)	0.331 (0.014)*	-0.107 (0.440)	1.000

*, Indicates significant differences between the groups of interest; BMI, body mass index; Cu, Copper; DBP, diastolic blood pressure; FBS, fasting blood glucose; FINS, fasting serum insulin; HbA1C, hemoglobin A1C; HC, hip circumference; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; Hb, hemoglobin; hs-CRP, high sensitivity C-reactive protein; LDL-C, low-density lipoprotein cholesterol; Mg, magnesium; MetS, metabolic syndrome; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglyceride; WC, waist circumference; WHR, waist-hip ratio; Zn, zinc.

DISCUSSION

In this study, we observed that plasma levels of Cu, Mg, and Zn were significantly lower in the T2DM group with MetS, while in erythrocytes, only Cu levels showed a significant decrease in these patients. This study was conducted to assess the relationship between selected elements in association with the biochemical components in subjects with T2DM and MetS, and also to evaluate further differences in levels of selected elements in blood compartments. It is worth to mention that the subjects who participated in the present study had no diabetic complications, which alongside the presence of the MetS, can be significant disturbing factors on trace elements levels in T2DM patients (10).

Elements are paramount to human body functions. There is general accordance in the literature about the association of T2DM with alterations in trace elements status, although it is unclear whether any of the two factors is causal to the other (7-9).

Serum or plasma levels of the trace elements are normally regarded as eligible biomarkers for epidemiological studies and clinical investigations, even though the certainty of results obtained from these samples is controversial. Erythrocytes, on the other hand, may contain clinically valuable information providing a better insight into trace elements' status and their correlations with the metabolic conditions (8).

The presence of the diabetic complications may be phenomenal in trace elements

alterations; as demonstrated by Chen *et al.* diabetic patients with complications had higher plasma Cu, and slightly lower plasma Mg and Zn levels, when compared to diabetic patients without complications (10). Also, the MetS is known to be a disturbing factor on trace element status intrinsically (11). Thus, the presence of the above-mentioned factors should be noted when investigating the trace element levels in patients with T2DM.

Regarding the role of Cu and Zn in oxidative stress, the antioxidant defense system might be affected by any change in the levels of Cu, Zn, and Cu/Zn ratio; which in turn would result in altered toxicity of metal-dependent free radicals, and ultimately to the progression of diabetic complications (5). On the other hand, superoxide dismutase enzyme functionality depends on the homeostasis of Cu and Zn, and alterations in the levels of these elements may further expose cells to superoxide radicals. Also, glycocholates may be produced from the interaction of Cu ions with glycated proteins under hyperglycemic conditions. Glycocholates will then be gathered in the vascular endothelium and participate in redox reactions, which may result in vascular complications of diabetes (25,26).

Mg deficiency may have a significant role in the progression of diabetes and metabolic syndrome through its association with a number of related risk factors such as insulin resistance, hypertension, obesity, dyslipidemia, and glucose intolerance. Impaired Mg levels may as well contribute to the progress of some diabetes-related shifts in the cellular level, including a decrease in tyrosine kinase activity, impaired post-receptor insulin signaling, and a decrease in cellular glucose utilization (27).

Cu plays a role in oxidative stress and many metabolic enzymes actions involved in the biochemistry of T2DM. Although controversial results have been reported regarding plasma Cu levels in diabetic patients compared to healthy controls (13). It was observed in our study that T2DM patients with MetS had significantly lower plasma and erythrocyte Cu levels. We identified 5 patients (12.8%) and 1 control (3.4%) to be Cu-deficient, with plasma Cu levels of 50 μg/dL or less. The mean plasma Cu concentration in control samples was found to be within the normal range for adult men (70-140 μg/dL); however, among T2DM group with MetS, it was measured slightly below the normal range (28). As mentioned above, Chen *et al.* reported a positive association between diabetic complications and plasma Cu levels (10). In contrast with our findings, Viktorínová *et al.* in plasma (5), Saha-Roy *et al.* in serum (9), Zhang *et al.* in serum (29), and Kazi *et al.* in whole blood (12) reported elevated levels of Cu in T2DM patients. The two latter studies were carried out regardless of the patients' diabetic complications status, and the other two excluded the patients with T2DM complications, without mentioning the MetS. The role of diabetic complications on trace element status could still be subject to further investigations. In line with our findings, Sobczak *et al.*(30), Basaki *et al.* (7), and Sohrevardi *et al.* (31) reported a decreased plasma Cu levels in T2DM patients without diabetic complications. Erythrocyte Cu levels were observed to be positively correlated with the plasma Cu among participants in the present study. Plasma Cu levels was found correlated with HbA1C, as well as many of the MetS components measured in the present study, including WC, HC, WHR, SBP, DBP, FBS, and HDL-C; most of which in turn, were found interrelated (Table 5).

Hypomagnesemia is assumed to be associated with MetS, hypertension, and T2DM. Mg is a crucial cofactor in carbohydrate metabolism and is involved in more than three hundred enzymes in the human body (11). There is general agreement in the literature regarding Mg levels in T2DM and MetS patients (5,9,11,29); although some investigations did not find significant differences in plasma Mg levels in T2DM patients compared to healthy controls (6,30). Consistent with other findings, we detected a significant decrease in plasma Mg in T2DM patients with MetS. Mean erythrocyte Mg levels were found to be slightly less in patients compared to the control group in the present study. Although, this difference was insignificant. We observed the mean plasma Mg levels of both groups to be within the normal range of 1.6-2.6 mg/dL (28). Another objective of the present study was to investigate

the correlations between erythrocyte Mg levels and biochemical components of MetS. Erythrocyte and plasma Mg levels were found to be correlated. However, we did not detect any significant correlations between Mg and other biochemical factors of MetS. HDL-C showed a significant positive correlation with Mg levels in both plasma and erythrocyte, while there was only a slight negative correlation between DBP and HOMA-IR and plasma Mg in all subjects (Table 5).

Zn is an essential trace element with antioxidant function and direct insulin-like effects. Zn is essential for the function of hundreds of enzymes in insulin metabolism and action (12). It has been found that serum and plasma Zn not to be related to the MetS (13,32,33). Regarding T2DM, there is accordance in the literature about decreased Zn levels in patients with T2DM (5,6,9,10,12). In line with these studies, we observed significantly lower plasma Zn levels in T2DM patients with MetS. These findings may be important to further clarify the contrasts of Zn-related metabolic pathways of the MetS and T2DM. Inconsistent with these findings, two studies by Zhang *et al*. in patients with T2DM and Yu *et al.* in patients with MetS, reported higher Zn levels compared to control groups (29,34). Since both the mentioned studies were carried out on the Chinese population, the ethnic association could be proposed for further investigations in the field. In the present study, the mean plasma Zn for controls and patients were within the normal range of 80-120 μg/dL and no individual showed Zn deficiency; while plasma Zn levels below the normal range were more common in the patients' group. The Cu/Zn ratio showed significantly lower levels in the patients' group compared to the controls in the present study. According to the results obtained from Pearson's correlation analysis on all participants of the present study, a slight negative association was observed between plasma Zn and SBP as a component of the MetS. DBP, another MetS component, was shown to have a significant positive correlation with erythrocyte Zn. Plasma Zn was also correlated with FBG and HbA1C, which may suggest an association between poor glycemic control and plasma Zn levels. In this study, TG was shown to have a significant inverse correlation with erythrocyte Zn levels, and HDL-C had a slight positive correlation with plasma Zn (Table 5), which are inconsistent with results from the study by Ahn *et al.* who found serum Zn positively correlated with TG and inversely associated with HDL-C in men with MetS (32). This contrast might be related to T2DM. TG is a MetS component that is in turn, associated with FBG, HOMA-IR, and HDL-C. Hypertriglyceridemia is proved to be further related to hyperinsulinemia, weight gain, and obesity (35). In contrast with our findings, Obeid *et al*. reported no correlation between Zn levels and components of the MetS in non-diabetic adults with MetS (13). Pizent *et al.* found a positive correlation between serum Zn and WC in non-diabetic women with MetS (33), which was not significant in our study. These findings may further mark the probable role of Zn in the progress of MetS and T2DM.

It is suggested by the authors of the present study for future studies in the field to regard the presence of T2DM complications, as well as ethnic and racial factors as grounds for contrasts in TE levels in different populations with T2DM or MetS. The weaknesses of this study were limited sample population and the absence of female subjects. It is also suggested for future studies to be carried out with samples of patients with T2DM but without MetS.

CONCLUSION

It seems that MetS may be an important factor determining the selected trace elements status in T2DM patients. The presence of the MetS should be noted in studies regarding the metabolic characteristics of diabetic patients. It may be suggested that the blood status of Cu, Mg, and Zn might be significantly correlated with components of MetS.

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Conflicts of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

F. Zadhoush participated in hypothesis generation, study design, data interpretation, and manuscript development (draft, revision, and final editing). A. Omidian conducted the experiments, participated in the statistical analysis, data interpretation, and manuscript development (draft and revision). M. Pourfarzam participated in the study design, data interpretation, and manuscript development (draft and revision). S.M. Ghanadian participated in the study design and technical support.

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