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Evaluation of Blood Concentrations of Copper and Molybdenum in Type 2 Diabetic Patients

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ABSTRACT

Diabetes mellitus is one of the most common metabolic diseases in humans. The blood concentrations of various trace elements, such as copper (Cu) and molybdenum (Mo), are altered in diabetes mellitus, which may have a significant role in the pathogenesis and progress of this disease. This study was aimed to evaluate the levels of copper and molybdenum concentrations in diabetic patients and healthy subjects. A total of 87 patients with type 2 diabetes and 45 participants with normal blood glucose were included in this study. The blood levels of Cu and Mo concentrations were measured by graphite furnace atomic absorption spectroscopy (GF-AAS). Statistical analyses were performed using SPSS 19.0 software. Biochemical factors were compared using student's t-test, Mann-Whitney test and Pearson correlation coefficient. The results showed a significant increase in mean Cu level in diabetic patients (394.11 ± 10.08) compared to healthy subjects (134.34 ± 3.66), however a significant decrease was found in mean Mo level in diabetic patients (8.41 ± 0.141) compared to healthy subjects (16.45 ± 0.72). Statistical analysis showed a significant positive correlation between the concentrations of each element with diabetes. The study indicates that type 2 diabetes can alter the blood levels of copper and molybdenum. The results of this study confirmed that the level of copper in diabetic patients is higher than healthy subjects, and that the level of molybdenum in diabetic patients is lower than that of healthy subjects. In addition, increasing or decreasing the copper concentration has no significant effect on the concentration of molybdenum in diabetic patients, meaning that there is no significant correlation between the two variables in diabetic patients.

Key words: Copper, Molybdenum, Type 2 Diabetes.

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1. INTRODUCTION

Type 2 diabetes is a metabolic disorder in the balance of energy that is characterized by hyperglycemia and altered lipid metabolism, which is due to the inability of the pancreatic islet beta cells to produce sufficient insulin in response to varying degrees of nutrition, inactivity, weight-gain or secondary obesity, and insulin resistance (1). The role of the trace elements in metabolisms and their importance in human health and diseases has attracted the attention of many researchers to study trace elements in normal and disease conditions. Impaired metabolism of trace elements, such as copper and iron, has been reported in diabetes (2). Cu is the third most abundant trace element in the human body; it is an essential element in the structure of many metalloenzymes that are involved in the oxidation and reduction processes, and provide a major portion of the energy required for metabolism (3). Cu is known as the cause of many diseases and plays an important role in the peroxidation mechanisms. Impaired balance in the process leads to an increased oxidative damage in tissue and ultimately to the progress of diabetes and its complications (4-9). Molybdenum participates in the structure of the three enzymes including xanthine dehydrogenase, aldehyde oxidase and sulfite oxidase (10). This element plays an important role in the health of living organisms as a constituent of the enzymes which are involved in the early stages of the metabolisms of compounds containing nitrogen, carbon and sulfur. The absorption of molybdenum in the human body is relatively high; the absorption rate of the element by the body is about 25-80%. It is absorbed mainly in the stomach and slightly in the

intestine. Copper and sulfates in the diet can decrease the molybdenum absorption. The highest amount of molybdenum has been found in the liver (0.57 µg g^{-1}) (11), while the lowest amount has been found in the human milk (5 - 25 ng g^{-1}) (12). Molybdenum has a number of metabolic roles in the body. Its antagonistic effect on copper metabolism is considerable (13). Although molybdenum is not directly associated with diabetes in human subjects, various thiomolybdate species have been used in the treatment of Wilson disease. Such compounds can eliminate excess of copper accumulated in liver and brain (14). The copper-molybdenum antagonism is very important in the progress of diabetes complications. However, it should be noted that, molybdenum needs to be administered in the form of thiomolybdate to chelate the protein-bound copper (15). Due to high costs required to manage and control the diabetes by health systems in Iran and around the world, it is very essential to identify and predict the factors to improve lifestyle b preventing the disease. The association and correlation of these elements with diabetes has not been studied so far. Therefore, the aim of this study was to prove the association and correlation of these trace elements with diabetes.

2. MATERIALS AND METHODS

2.1. Materials and reagents

The materials used in this study were Copper Nitrate (II) ($Cu(No_3)_2$), Sodium Molybdate (Na_2MoO_4), Nitric Acid (HNO_3), Hydrogen Peroxide (H_2O_2), Ethylenediaminetetraacetic acid (EDTA). All materials were obtained from Merck Company, Germany.

2.2. Apparatus

Trace elements were analyzed by an atomic absorption spectrometer, equipped with graphite furnace system (model GF3000, GBC, Australia).

2.3. Blood samples collection

Samples were collected from people who were referred to the laboratory of Shahid Fayaz-Bakhsh Hospital, Tehran, Iran. Based on the medical diagnosis, the referrals were comprised of the patients with type 2 diabetes and healthy individuals. Based on the sample size formula, to compare two ratios at 95% confidence interval, and a power of 80%, and desired outcome ratios in the case group (10%) and control group (30%), the sample size was calculated as 87 diabetic patients and 45 healthy subjects as the case and control groups, respectively. In case group, 87 samples were collected from diabetic patients (32 males and 55 females), who were referred to the laboratory based on diagnostic criteria and were under the control of a specialist physician. According to the diagnosis criteria of World Health Organization (WHO), diabetic patients are individuals who have been diagnosed with diabetes by a specialist physician (fasting plasma glucose greater than 126 mg/dl on two different occasions or 2-hour post-prandial (2-HPP) blood sugar greater than 200 mg/dl on two different occasions). The medical records of the patients were examined and their disease was confirmed by a physician based on the criteria provided by WHO. Most of the patients were treated with insulin or drugs to control blood glucose, except for those who control their blood glucose by diet. The inclusion criteria for the patients were fasting glucose greater than 126 mg/dl, and patients without other underlying diseases. In the other group, 45 healthy individuals (21 males and 24 females) were selected as controls that did not have any diabetic patients in their first-degree relatives. Two blood samples were obtained from each participant, including a fasting blood glucose sample and a 2-HPP blood sugar sample to ensure the absence of impaired fasting glucose (IFG) and or impaired glucose tolerance (IGT). In the other words, individuals were selected as control group that their fasting blood glucose and 2-HPP blood sugar were normal. As this research was a case-control study, the participants of control group were matched with the patient group in terms of all factors such as age, gender, race, etc. Blood samples were collected from all patients and control group with informed consent and the information documented on the questionnaire of the research. Clinical manifestations of the patients and demographic data including age, gender, onset of disease, and type and dosage of medications were determined based on the medical history of the patients and complete examination by a specialist physician and review of patients' test results and records. Four cc venous blood samples were taken from each individual in the case and control groups. Ethylenediaminetetraacetic acid (EDTA) was used as an anticoagulant for all blood samples. The solution was prepared as a 0.5 Molar solution of EDTA with pH = 8. An aliquot of the blood samples (2 cc) were poured in the tubes containing EDTA anticoagulant substance and other aliquot (2 cc) were poured in the tubes without EDTA. A volume of 100 µL of EDTA solution was used per 1 mL of blood sample. The blood samples without anticoagulant were centrifuged and used to measure the blood glucose. By analyzing the results of fasting blood glucose (FBS) test, it was established whether they were suffering from type 2 diabetes.

2.4. Samples preparation for atomic absorption spectrometry

Whole blood samples were used in this study. Although sample preparation by acid digestion is very time-consuming, this method was preferred because of the benefits of whole blood compared to the sera or plasma samples.

To prepare the samples a 300 µL venous blood from each individual in the patient and control groups was added to a 2 mL eppendorf tube, and 300 µL of nitric acid was added to it. The samples were then placed in a water bath (Memmert) at 60°C for one hour. Then the samples were removed from the water bath and their color was changed to pale yellow. Subsequently, 100 µL of H_2O_2 was added

to the samples and the color of the solutions was changed to transparent yellow. The samples were then shaken for 30 seconds and subsequently placed in a water bath at 37°C for 3 hours. Afterward, the samples were shaken for 30 seconds, and then the solutions were centrifuged rapidly at 12000 rpm for 3 minutes.

2.5. Making solutions

The stock solutions of copper and molybdenum were made using $Cu(No_3)_2$ and Na_2MoO_4, respectively. As the salts contain water, the molecular mass was calculated considering the number of water molecules. According to the stoichiometric equations, the quantity of copper and molybdenum were calculated from their salts. The stock solutions were prepared using copper salt (0.38 g) and molybdenum salt (0.215 g) at a concentration of 1000 mg L^{-1}. To plot the calibration curve and to determine its linear range, according to the stoichiometric equations, the stock solutions were diluted by serial dilution to yield a series of copper and molybdenum solutions with concentrations of 10 ppb, 20 ppb, 30 ppb, 40 ppb, 50 ppb.

2.6. Instructions to work with atomic absorption spectrometer, equipped with graphite furnace system

An atomic absorption spectrometer (model GBC SensAA) equipped with graphite furnace atomizer (model GF3000, GBC) was used in the present study. The instrument was equipped with a Hollow-cathode lamp (HCL) for copper and molybdenum (Model GBC) as radiation source, and a deuterium lamp for correction. In addition, a pyrolytically coated graphite furnace was used to measure the absorption of the prepared samples. The absorptions of copper and molybdenum were measured by a deuterium lamp at wavelengths of 193.73 and 313.3, respectively. Initially, the copper and molybdenum software were installed on a computer. The distilled water was used as blank sample and was first injected into the atomic absorption spectrometer each time it was run. After that, the standard solutions and then the supernatant of the prepared samples were injected into the atomic absorption spectrometer using a 10 µL sampler and their absorptions were read. Subsequently, the concentrations of Cu and Mo in the samples were calculated using the standard curve after measuring the absorption the samples.

2.7. Statistical analysis

Data entry and statistical analyses were performed using SPSS software (version 19.0). The data obtained in the present study were analyzed using student's t-test, Mann-Whitney test and Pearson correlation coefficient. The Kolmogorov-Smirnov test was used to verify that the data were normally distributed. The significant level was considered as $p \leq 0.05$.

3. RESULTS AND DISCUSSION

The results from determination and comparison of the mean values of biochemical factors between two studied groups of patients and controls are shown in Table 1. The results are presented in Table 1 at a significance level of 5%. All biochemical factors, other than low-density lipoprotein cholesterol (LDL-C) were significantly different between two groups (p-value < 0.05). Only the mean value of LDL-C was not significantly different between two groups (p-value = 0.503 > 0.05).

Table 1. Determination and comparison of the mean values of biochemical factors between two diabetic and healthy groups

Biochemical factors	Diabetic group	Control group (healthy)	p-value
FBS	183.84 ± 7.04	89.27 ± 2.09	0
Blood Sugar	275.33 ± 10.15	119.09 ± 1.95	0
Creatinine	0.971 ± 0.018	0.869 ± 0.017	0
Cholesterol	180.85 ± 4.92	161.29 ± 4.83	0.012
TG	170.52 ± 8.72	99.29 ± 9.15	0.001
LDL-C	97.88 ± 4.05	101.98 ± 3.18	0.503*
HDL-C	45.23 ± 1.23	56.78 ± 5.63	0.009
HA1C	7.68 ± 0.14	4.72 ± 0.05	0
Cu	394.11 ± 10.08	134.34 ± 3.66	0
Mo	8.41 ± 0.141	16.45 ± 0.72	0

The results from determination and comparison of the mean values of biochemical factors between males and females of two studied groups are presented in Table 2 at a significance level of 5%. There were no significant differences in the mean values of biochemical factors, except for creatinine between males and females in the control (healthy) group (p-value > 0.05). In the patient (diabetic) group, only the means of two variables including creatinine and triglyceride were significantly different between males and females (p-value < 0.05). The mean value of creatinine in diabetic females (0.916) was significantly lower than males with diabetes (1.06), and the mean value of triglyceride in diabetic females (189.2) was significantly higher than diabetic males (138.4). The mean values of the other biochemical factors were not significantly different between diabetic females and males (p-value > 0.05).

Table 2. Determination and comparison of the mean values of biochemical factors between males and females of two groups

	Diabetic group			Control group (healthy)		
	Men	Women	p-value	Men	Women	p-value
FBS	181.1 ± 10.77	185.4 ± 9.26	0.765	86.52 ± 4.03	91.67 ± 1.68	0.224
Blood Sugar	277.9 ± 17.43	273.8 ± 12.57	0.846	115.8 ± 2.87	121.9 ± 2.58	0.123
Creatinine	1.06 ± 0.029	0.916 ± 0.020	0	0.905 ± 0.212	0.837 ± 0.024	0.044
Cholesterol	170.6 ± 7.50	168.8 ± 6.36	0.111	151.7 ± 6.77	169.7 ± 6.52	0.063
TG	138.4 ± 13.99	189.2 ± 10.41	0.004	96.86 ± 11.25	101.4 ± 14.26	0.807
LDL-C	92.78 ± 6.63	100.8 ± 5.12	0.340	105.14 ± 4.42	99.21 ± 4.56	0.358
HDL-C	44.62 ± 2.29	45.58 ± 1.42	0.709	48.24 ± 2.66	64.25 ± 10.15	0.158
HA1C	7.49 ± 0.179	7.80 ± 0.204	0.307	4.64 ± 0.071	4.79 ± 0.074	0.157
Cu	403.51 ± 17.75	388.64 ± 12.21	0.480	133.54 ± 5.86	135.04 ± 4.67	0.840
Mo	9.12 ± 0.732	8.00 ± 0.477	0.187	17.02 ± 1.217	15.95 ± 0.851	0.467
Cu to Mo	57.75 ± 6.53	62.20 ± 5.13	0.596	8.66 ± 0.758	9.01 ± 0.585	0.715

The assumption was not confirmed that the data on Cu was normally distributed, therefore, the significant difference in Cu concentrations between healthy subjects and patients was tested using non-parametric Mann-Whitney test. Table 3 shows that the level of copper in healthy subjects was not the same as the diabetic patients. According to the mean ranks as presented in Table 3, the level of copper in diabetic patients was higher than the healthy subjects. The comparison of the mean copper levels between diabetic patients and healthy subjects is shown in Diagram 1.

Table 3. The results of Mann-Whitney test for differences of copper level between two diabetic and control groups

Cu Variable	N	Mean rank	Sum of ranks	Test Statistics	Significance (two-tailed)
Healthy subjects	45	23.11	104	5	0
Diabetic patients	87	88.94	7738		

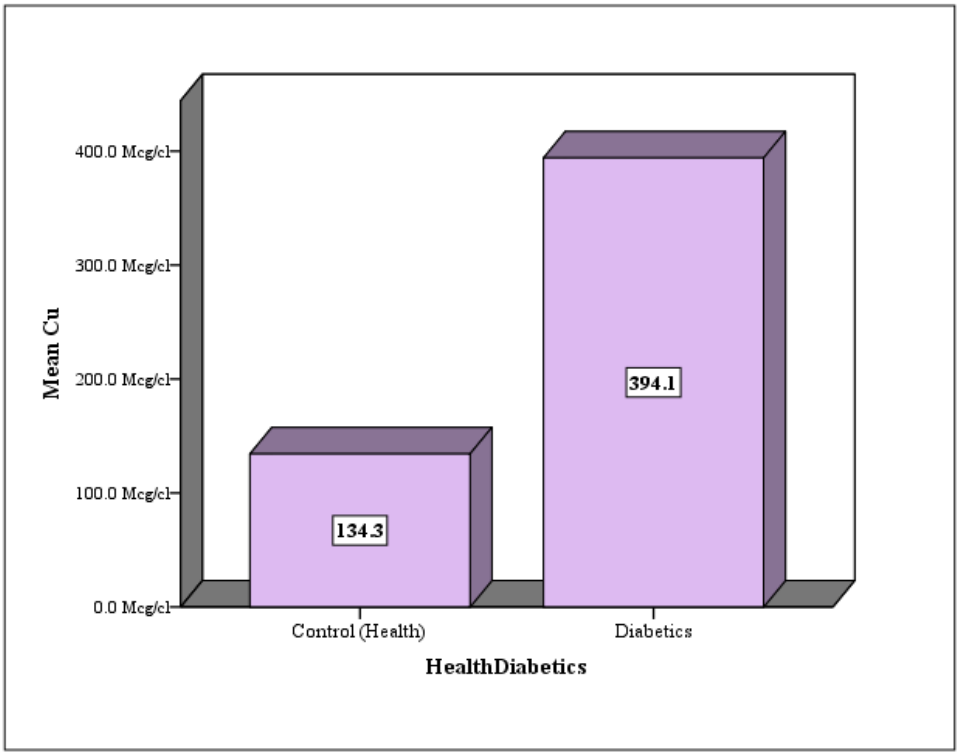

Diagram 1. Comparison of mean values of copper in diabetic and control groups

In the case of Mo, the assumption was confirmed that the variable was normally distributed. Therefore, an independent samples t-test (student's t-test) was used to test the second null hypothesis. Table 4 shows that the level of molybdenum in healthy subjects was not the same as the diabetic patients. According to the mean values of molybdenum for each group, the level of molybdenum in diabetic patients was lower than healthy subjects. The comparison of the mean molybdenum levels between diabetic and control groups is shown in Diagram 2.

Table 4. Independent samples t-test for molybdenum levels between two diabetic and control groups

Indices / Groups	Mean	The equality of variances assumption		t	Degrees of freedom	Significance (two-tailed)
		Statistics	Significance Level			
Healthy subjects	16.45	1.755	0.188	10.484	130	0
Diabetic patients	8.41					

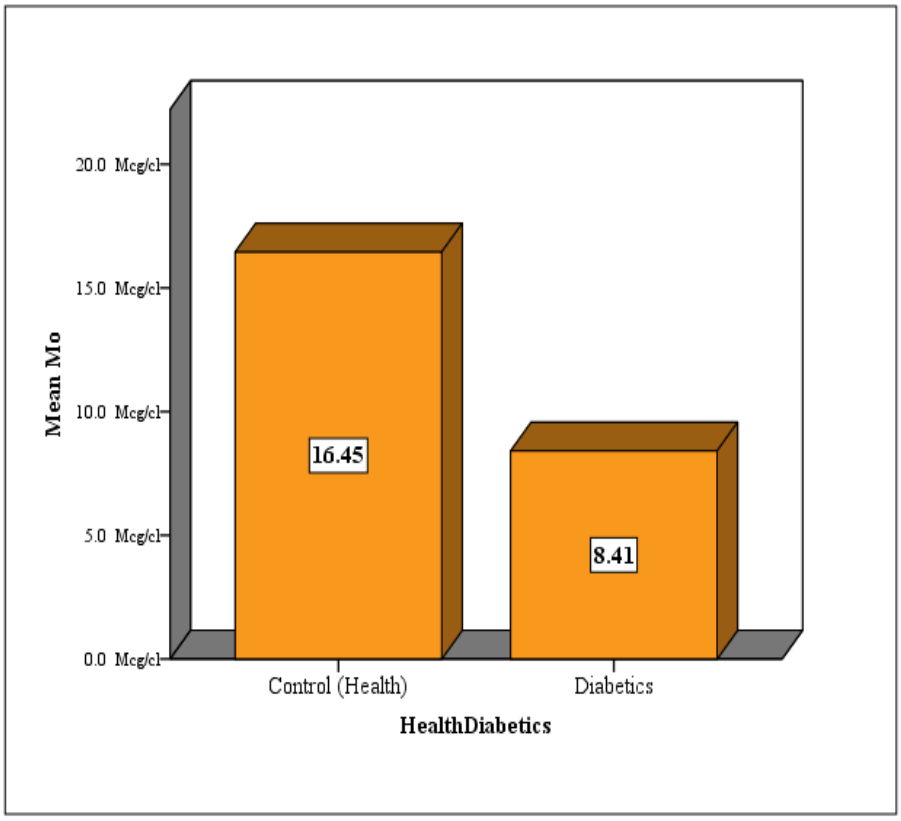

Diagram 2. Comparison of mean values of molybdenum in diabetic and control groups

The results of the Pearson correlation coefficient test (Table 5) showed that there was a correlation coefficient of -0.097 between the levels of copper and molybdenum in diabetic patients. As the significance level of this correlation coefficient (0.371) was higher than 0.05, it is not statistically significant. It means that increasing or decreasing of copper in diabetic patients does not have a significant effect on the increasing or decreasing of molybdenum in diabetic patients. It is also evident from the scatter-plot (Diagram 3) that there is no significant correlation between the two variables in diabetic patients.

Table 5. Correlation between copper and molybdenum in diabetic patients

index	Statistics	Level of copper in diabetic patients	Level of molybdenum in diabetic patients
Level of copper in diabetic patients	Pearson correlation coefficient	1	-0.097
	Significance (two-tailed)	*	0.371
	Covariance	8839.88	-34.564
Level of molybdenum in diabetic patients	Pearson correlation coefficient	-0.097	1
	Significance (two-tailed)	0.371	*
	Covariance	-34.564	14.342

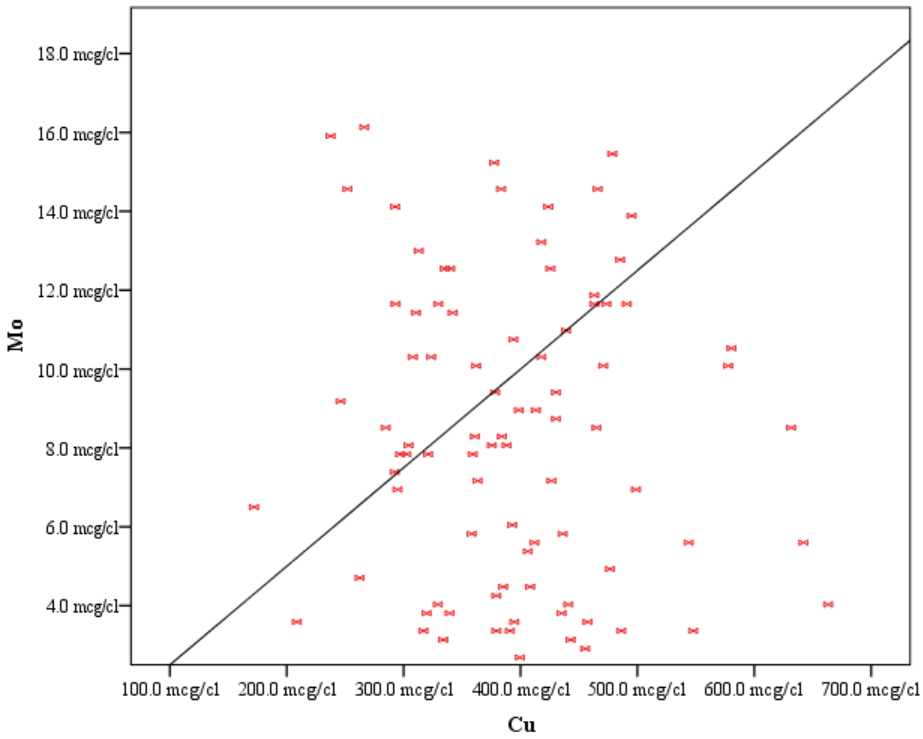

Diagram 3. Scatter-plot for copper versus molybdenum in diabetic patients

The results of this study showed that the level of copper in diabetic patients was higher than healthy subjects, and that the level of molybdenum in diabetic patients was lower than that of healthy subjects. Additionally, increasing or decreasing the copper concentration has no significant effect on the concentration of molybdenum in diabetic patients, meaning that there is no significant correlation between the two variables in diabetic patients. Excess amount of copper can disrupt the functioning of several enzymes and affects different body systems, including the digestive system, renal system, respiratory system, nervous system, skin and hair, etc. (16, 17). Many researchers believe that excess amount of copper induces apoptosis and cell death (18), causing lipid peroxidation, decreased expression of several genes and glutathione peroxidase (19, 20). The findings of the study by Wu et al. (1997) showed an increase in serum level of copper in diabetic mice which is consistent with the results of this study (21). Another study on mice with type 2 diabetes has also shown that a decrease in serum level of copper using copper chelating agent has led to improved blood glucose control (22). In another study, the level of copper and zinc were measured in the blood of diabetic patients at Zagreb University of Former Yugoslavia in 1992. The findings of the study showed that the serum levels of copper and zinc are decreased in type 1 and 2 diabetes which is not consistent with the results of present study (23). A research conducted by Kruse-jaress (2000) has found an increased levels of copper in red and white blood cells and plasma in diabetic patients. This increase was higher in the patients with poor diabetes control (HbA1c > 9%) (24). According to a study conducted by Galhardi et al. (2004) on diabetic mice, high level of copper can disrupt the functions of kidney (25). Flores et al. (2011) in their study on the possible roles of trace metals including copper and molybdenum in the progress of type 2 diabetes mellitus found that antagonistic interactions between molybdenum and copper might be involved in the progress of diabetes complications (26). The findings of this study and other studies indicate the role of trace elements such as copper and molybdenum in glucose homeostasis, and therefore the importance of their role in the incidence and progress of type 2 diabetes. Impaired balances of trace elements may be resulted from disorders such as diabetes. Therefore, copper and molybdenum are possibly useful to assess the risk of diabetes. Moreover, enhancing the level of these trace elements through medical interventions may help to reduce the risk of disease progression. However, these results require further investigation in the future on the correlation between trace elements and the progress of diabetes. In future studies, the correlation between other trace elements and type 2 diabetes should be considered.

4. CONCLUSION

In this study, the concentrations of Cu and Mo were investigated using a GF-AAS in the blood of 132 samples including 87 diabetic patients and 45 healthy subjects. The results showed an increasing in Cu level in diabetic patients compared to healthy subjects and decreasing in Mo in diabetic patients compared to healthy subjects. There was no significant relationship between Cu and Mo levels in the blood. It seems that Cu and Mo are two important factors in patients with type 2 diabetes. Therefore, according to the present study, the concentration of Mo and Cu affects the whole blood. The consumption of foods that are rich in Mo, or the

administration of supplements containing Mo in cases of its deficiency can be beneficial. In addition, Cu plays an important role in peroxidation mechanisms, and disruption of these mechanisms leads to increased oxidative damage to tissue and progression of diabetes. Therefore, prescribing medications that cause more Cu metabolism and avoid of eating foods with high Cu is recommended. Since, evaluation of these two elements have not been studied at the same time so far, wider studies and the using of more number of samples can further prove the synergistic effect or the lack of their synergistic effect on each other. Further studies are needed to demonstrate the precise role of these elements in the pathogenesis and involved mechanisms of type 2 diabetes. It seems that there is a need to pay more attention to the metabolism of these elements in patients with type 2.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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