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ORIGINAL PAPER

ASSESSMENT OF LEVELS OF SELECTED TRACE ELEMENTS AS PREDICTORS OF OXIDATIVE STRESS IN TYPE 2 DIABETIC PATIENTS USING MULTIVARIATE STATISTICAL ANALYSIS

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Abstract

Disturbances in levels of trace elements and oxidative stress are associated with the glycemic status, which is implicated in the development and progression of diabetes. The present study was conducted on 50 patients with type 2 diabetes, 40 years of age, gender and body mass index matched with healthy controls (Damanhur Educational Hospital, El Beheira, Egypt). Fasting blood glucose (FBG), serum catalase, paraoxonase activity, and malondialdehyde (MDA) levels were measured spectrophotometrically. The blood serum was digested and then used to determine the levels of seven trace elements, such as Cr, Fe, Cu, Zn, Cd, As and Se, using inductive coupled plasma mass spectroscopy (ICP-MS). The mean of serum catalase, paraoxonase activities and the concentration of Fe, Se, Zn, and Cd were significantly decreased, whereas the level of malondialdehyde (MDA) and the concentrations of Cu, Cd and As were significantly higher in type 2 diabetic group compared with the control group. The multivariate receiver operating characteristic (ROC) curve and variable importance in projection (VIP) scores were used to analysis the data. VIP score revealed that As, Fe and Se were strongly associated with the oxidative stress in type 2 diabetic patients. The best cut-off values for serum concentrations of Fe, Se and As were 46.78, 215 and 1.01 μg L^{-1}, respectively, which discriminated between diabetic patients with oxidative stress from the control group. The study showed that changes in trace element concentrations in diabetic patients may contribute to the lowering of antioxidant enzymes t, further leading to the progress of T2DM.

Keywords: type 2 diabetes, trace elements, oxidative stress, ROC curve, VIP score.

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INTRODUCTION

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Naqshbandi et al.2008). Oxidative stress plays an important role in the onset of diabetes (West et al. 2000). Reactive oxygen species (ROS), formed as a natural byproduct of oxidative stress, have been implicated in the pathophysiology of various diseases, including diabetes mellitus and long-term development of associated complications (Moussa et al. 2008). Diabetes increases production of free radicals and affects the capacity of antioxidant defenses, which in turn results in damage to cells in many ways. Damage to cells ultimately leads to secondary complications. Antioxidant enzymes, such as catalase, superoxide dismutase and paraoxonase, prevent the accumulation of oxidized lipids by converting reactive oxygen species before they can react or by removing the oxidized products from the endogenous proteins (Yildirim et al. 2004).

Trace elements are required in minute amounts for the health of our body; moreover, they have a significant influence on the activity of antioxidant enzymes, and thus on defense against oxidative stress (Pizent et al. 2010, Wołonciej et al. 2016). Trace elements act as cofactors for antioxidant enzymes, which protect body from damage due to oxidative stress (Koekkoek et al. 2016, Rehou et al. 2017). Any disturbance in the levels of trace elements may affect both antioxidant defense and glucose intolerance, thereby influencing the pathogenesis and progress of diabetes (Badran et al. 2016). However, there is little research on the influence of disturbances in levels of trace elements on the activity of antioxidant enzymes. Thus, the aim of this study has been to investigate relationships between Cr, Fe, Cu, Zn, Cd, As, Se, serum malondialdehyde (MDA) levels, catalase (CAT), paraoxonase (PON) activities in type 2 diabetic patients.

MATERIALS

Subjects

The present study was performed in Egypt, on 50 patients with T2DM and 40 healthy volunteers. Patients and controls were matched for age, sex and body mass index. Fasting blood glucose of the patients and the control group were 178.19±35.6 and 82.80±5.14 mg dl^{-1}, respectively. The mean age of the study and control group patients was 54.45±7.72 and 54.19±8.18 years, respectively, while the mean body mass index (BMI) of the study and control group was 23.5±1.95 kg m^{-2} and 20.24±0.86 kg m^{-2}, respectively. The patients were recruited from Damanhur Educational Hospital, El Beheira, Egypt.

METHODS

ICP-MS calibration

Determinations of the concentrations of seven selected trace elements: Cr, Fe, Cu, Zn, Cd, As and Se, were validated in terms of accuracy by using standard reference material (IAEA-A-13, freeze dried animal blood). Certified reference material was digested in triplicate and analyzed using (ICP-MS: Finnigan elements 2) to support quality assurance and control. Accurate results were obtained for the analyzed elements and were in satisfactory agreement with the certified value. Table 1 tabulates the recoveries of the analyzed elements together with the certified values.

Table 1

Quantitative analysis of determinations of trace elements in IAEA-A-13(μg g^{-1}) using ICP-MS

Element	IAEA-A-13(μg g^{-1})	
	specified	this work
Cd	0.07	0.07±0.003
Cr	0.26	0.25±0.007
Cu	4.30	4.39±0.12
Fe	2400	2352.78±56.45
As	6500	6292.02±163.59
Se	0.24	0.25±0.005
Zn	13.00	13.98±0.36

Samples digestion and treatment

Fast blood samples of 6-8 mL were withdrawn from a diabetic patient's vein; the samples were preserved in a clean tube with no anticoagulant agent. Blood samples were centrifuged at 3000 rpm for 10 min to separate serum. After centrifuging, 0.5 mL of serum was transferred to a 15 mL plastic centrifuge tube. To digest the sample, a mixture of 0.5 ml serum sample and 10 ml of nitric acid were placed in a beaker and heated at 120°C until complete wet acid digestion took place. After cooling to room temperature, the solution was diluted with double distilled water up to 10 mL. After complete wet acid digestion of the samples, concentrations of the selected trace elements were analyzed using inductively coupled plasma mass spectrometry (ICP-MS: Finnigan element 2).

Measurement of serum paraoxonase enzyme activity

Paraoxonase activity was measured by adding 15 μl serum to 285 μl Tris buffer (100 mmol L^{-1}, pH 8.0) containing 2 mmol L^{-1} $CaCl_2$ and 1 mmol L^{-1} paraoxon (O, O-diethyl-O-nitrophenylphosphate). The rate of the generation of P-nitrophenol was measured at 412 nm wavelength (Parsaeyan et al. 2012).

Measurement of serum malondialdehyde (MDA) level

The method was based on the binding of malondialdehyde (MDA) with thiobarbituric acid. A serum sample was diluted in phosphate buffer (20 mM, pH 7.4) and heated together with thiobarbituric acid TBA solution (375 mg ml^{-1}) in a boiling water bath for 15 min. The tubes were cooled and the absorbance was measured at 535 nm. 1,1,3,3-tetraethoxypropane was used as a standard (PARSAEYAN et al. 2012).

Measurement of catalase (CAT) activity

CAT was measured by measuring the constant rate of hydrogen peroxide decomposition in a reaction mixture prepared in an Eppendorf tube and composed of 500 µL 0.05 M phosphate buffer (pH 7.0), 300 µL distilled water, 50 µL 1.1 mM H_2O_2 in distilled water, and 50 µL serum samples. After 5 min of incubation at 25°C, 100 µL 50% trichloroacetic acid was added to each tube and the tubes were centrifuged (1,000 rpm). 10 µL titanium (IV) reagent was added to each tube and 200 µL supernatant were transferred into wells on a 96-well micro plate. The absorbance was read at 405 nm (SZUSTER et al. 2004).

Data analysis

Relationships between concentrations of the trace elements, catalase, paraoxonase activity and malondialdehyde (MDA) levels in serum samples from diabetic and control group were investigated using the Pearson's correlation. t-test for independent-samples was used to compare data between the two groups. The VIP score served to select relevant predictors according to their values. The ROC curve is frequently used for the selection of optimal cut-off values for trace elements as biomarkers of oxidative stress. A statistical package SPSS software, version 12, was used to calculate statistical parameters.

RESULTS

Serum catalase, paraoxonase activity malondialdehyde levels and trace element concentrations in healthy and diabetic patients

Trace element concentrations were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS: Finnigan element 2). The analysis of the samples revealed a significant difference (P>0.001) in the serum content of seven trace elements: Cr, Fe, Cu, Zn, Cd, As and Se, between the patient and control group. The mean concentrations of chromium (Cr), iron (Fe), zinc (Zn) and selenium (Se) were significantly lower than in the control group (p<0.001). In contrast, high mean values of Cu, Cd, and As were detected in the patient group. The serum catalase level was significantly decreased while the serum malondialdehyde (MDA) level was significantly higher in the

patient group than in the control group. No statistically significant differences were found in the paraoxonase level between the two groups. Descriptive statistics of the concentrations of the selected trace elements, the level of serum catalase, paraoxonase activity and malondialdehyde (MDA) levels in serum samples from diabetic and control group are tabulated in Table 2.

Table 2

Descriptive statistics of concentrations of the selected trace elements, serum catalase, paraoxonase activities, and malondialdehyde in patients with T2D and control

Parameters	Control group Mean±SD	Patients with T2D Mean±SD	*P*-value
Zn (μg L^{-1})	987.18±45.61	809.29±21.09	< 0.001
Cu (μg L^{-1})	129.35±7.43	180.00±2.36	< 0.001
Se (μg L^{-1})	242.03±17.48	203.30±6.16	< 0.001
Fe (μg L^{-1})	59.56±7.46	46.12±3.62	< 0.001
Cd (μgL^{-1})	0.43±0.19	1.35±0.37	< 0.001
Cr (μg L^{-1})	0.15±.010	0.11±0.01	< 0.001
As (μg L^{-1})	0.96±0.03	1.04±0.02	< 0.001
Catalase (U ml^{-1})	17.26±3.85	9.61±2.47	< 0.001
MDA(nmol mL^{-1})	2.28±.27	5.8414±.43	< 0.001
Paraoxonase (U ml^{-1})	43.25±10.22	37.89±10.06	0.08

The correlation analysis

Table 3 shows the correlation coefficients, calculated from the Pearson's correlation coefficient analysis. For the healthy group, a significant positive correlation was detected between paraoxonase with Fe and catalase (p<0.01, r=0.615, p<0.05, r=0.455, respectively), and Zn and As (p<0.05, r=0.498). In the type 2 diabetic patients group, a significant positive correlation in serum between catalase and paraoxonase (p<0.05, r=0.501), Fe with catalase and Paraoxonase (p<0.05, r=0.488, p<0.633, r=-0.01 respectively), FBG with Cu, As and MDA (p<0.05, r=0.507, p<0.05, r=0.496 and p<0.05, r=0.544 respectively). Meanwhile, significantly negative correlations were observed between Fe with FBG, As and MDA (p<0.01, r=-0.610, p<0.01, r=-0.695 and r=-0.584, p<0.01, respectively), As and paraoxonase (p<0.05, r=-0.523), Cu and Zn (p<0.001, r=-0.447), MDA with catalase and paraoxonase (p<0.01, r=-0.650, p<0.01, r=-0.682).

Partial least square regression VIP score

Partial least square regression (PLSR) is a statistical tool for modeling the predictor and the response datasets. It is used to select relevant predictors with respect to the response variable based on the partial least square regression. The score of variable important in projection (VIP) is an indicator

Table 3

Correlation coefficients for the selected trace elements, catalase, paraoxonase activities, and malondialdehyde in patients with T2D and control

Patients group										
	FBG	Cr	Fe	Cu	Zn	Se	As	Cd	catalase	MDA
FBG	1.000									
Cr	-0.110	1.000								
Fe	-0.610**	0.093	1.000							
Cu	0.507*	0.040	-0.031	1.000						
Zn	0.372	0.134	-0.020	-0.447*	1.000					
Se	-0.134	0.005	-0.052	-0.282	-0.303	1.000				
As	0.496*	-0.029	-0.695*	-0.161	-0.238	0.330	1.000			
Cd	-0.314	0.056	-0.171	0.145	-0.250	-0.247	0.204	1.000		
Catalse	-0.367	0.067	0.488*	0.223	-0.087	-0.131	0.428	0.175	1.000	
MDA	0.544*	-0.160	-0.584**	-0.372	0.104	-0.398	-0.380	-0.261	-0.650**	1.000
Paraoxonase	-0.424	-0.142	0.633**	-0.003	-0.128	-0.020	-0.523*	0.172	0.501*	-0.682**
Control group										
	FBG	Cr	Fe	Cu	Zn	Se	As	Cd	catalase	MDA
FBG	1.000									
Cr	-0.032	1.000								
Fe	-0.054	0.143	1.000							
Cu	0.122	-0.123	0.174	1.000						
Zn	-0.046	-0.101	0.294	-0.175	1.000					
Se	0.167	0.064	0.303	0.264	0.071	1.000				
As	0.306	0.003	-0.058	0.300	0.498*	0.084	1.000			
Cd	-0.126	-0.073	-0.309	0.199	-0.358	-0.179	-0.239	1.000		
Catalse	0.012	-0.063	0.234	0.314	-0.167	-0.049	-0.168	0.179	1.000	
MDA	0.243	0.206	-0.346	0.212	-0.124	-0.094	0.182	0.143	0.193	1.000
Paraoxonase	-0.049	0.012	0.615**	-0.050	0.188	0.001	0.041	-0.370	0.455*	-0.342

** – correlation is significant at the 0.01 level,

* – correlation is significant at the 0.05 level

that shows the effect of predictor variables change over response variables. It can be assumed that when VIP< 1, the influence of a predictor on response variable is very significant, when 1 <VIP <0.8, the influence is significant, and when VIP >0.8, the influence is not significant. The predictor with a higher value of VIP score shows that it is more relevant for prediction of the response variable. The selection of potentially important trace elements as biomarkers of oxidative stress in type 2 diabetes patients was done using variable importance on PLS projections (VIP). In the predictive model, the elements Cr, Fe, Cu, Zn, Cd, As and Se were used as explanatory factors where the response variables were the levels malondialdehyde (MDA), catalase and paraoxonase activity. Figure 1 shows the VIP values across the predictors with a dashed horizontal reference line at one.

Fe, As and Se were selected to be significant predictors which corresponded to higher VIP values (1.58, 1.48 and 1.06, respectively), while Cr, Cu,

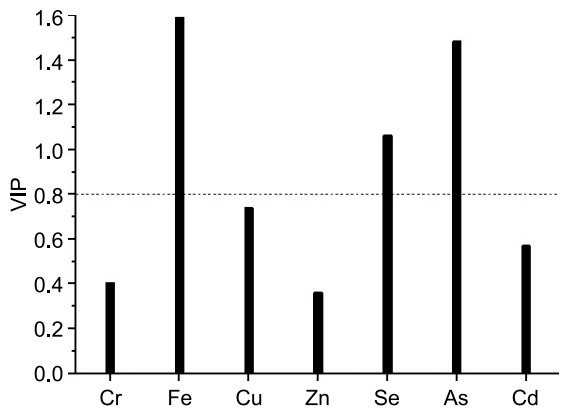

Fig. 1. Variable importance plot for PLS model: X – trace element concentration (Cr, Fe, Cu, Zn, Cd, As and Se), Y – paraoxonase activity (PON), catalase (CAT) activity and malondialdehyde (MDA) levels

Zn and Cd represented lower VIP values (0.40, 0.73, 0.56 and 0.56, respectively), and could be considered as unimportant for the general model.

Receiver operating characteristics (ROC) analysis

The ROC curve is used for the selection of an optimal threshold value for trace elements as a biomarker for the antioxidant enzymes in type 2 diabetic patients. Diagnostic cutoffs were calculated according to the Youden's index to investigate the potential diagnostic value for serum concentrations of Fe, Se and As, which may play a crucial role in counteracting oxidative stress in type 2 diabetic disease. Optimal cut-off points for these trace elements were determined based on the best balance of sensitivity and specificity which maximizes the Youden index. Figure 2 show the optimal cut off points for Fe, Se and As.

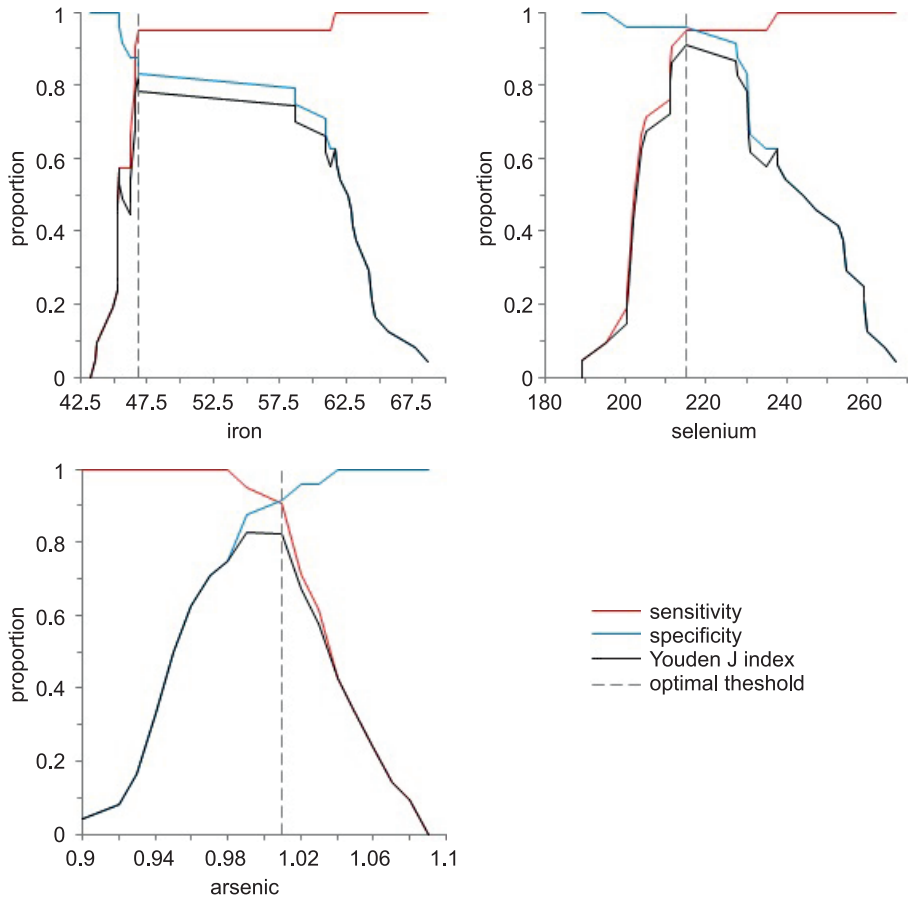

Fig. 2. The optimal cut-off points for iron, selenium and arsenic, based on the receiver operating characteristic curve

The mean concentrations of serum iron were significantly lower in diabetic patients (46.12±3.62 μg L^{-1}) than in the control group (59.56±7.46 μg L^{-1}). Iron cut-off point was 46.78 μg L^{-1} provided 95.2% sensitivity and 95.8% specificity with an AUC equal 0.935 (95% CI=0.865-1.004). The mean concentrations of serum selenium were significantly lower in diabetic patients (203.30±9.60 μg L^{-1}) than in the control group (242.03±17.48 μg L^{-1}). Selenium cut-off point was 215 μg L^{-1} provided 95.2% sensitivity and 87.5% specificity with an AUC equal 0.948 (95% CI=0.872-1.029 μg L^{-1}). The mean concentrations of serum arsenic were significantly higher in diabetic patients ((1.04±0.02 μg L^{-1}) than in the control group (0.96±0.03 μg L^{-1}). The arsenic cut-off point was 1.01 μg L^{-1} provided 90.5% sensitivity and 91.7% specificity with an AUC equal 0.966 (95% CI=0.921-1.012).

DISCUSSION

Oxidative stress is considered to be one of the major risk factors for the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance and type 2 diabetes mellitus – T2DM (Wright et al. 2006). In the present study Trace elements include Cr, Fe, Cu, Zn had a significant decrease, while Cd, As exhibit a highly significant increase in diabetic patient in comparison with the control group. Only Fe, Se and As had VIP score greater than one which is generally used as a criterion for predicting the antioxidant stress in type 2 diabetic patients. There was a significant decrease in the level of catalase, indicating a considerable reduction in the antioxidant status in the patient group (9.61±2.47 U ml^{-1}) compared with the control group (17.26±3.58 U ml^{-1}), which is in accordance with results of other studies on oxidative stress (Jandrić et al. 2004, Spanidis et al 2016). The deficient catalase activity can cause hemolytic anemia, which may be attributed to the deficiency of glucose-6-phosphate dehydrogenase; it may also damage heme proteins and produce highly toxic hydroxyl radicals (Tiwari et al. 2013). The paraoxonase levels were lower in the control group (37.89±10.06 U ml^{-1}) than the patient group (43.25±10.25 U ml^{-1}) but without statistically significant difference (P>0.05), which is consistent with a previous study (Suvarna et al. 2011).

The mean serum malondialdehyde (MDA) level was significantly higher in the patient group (5.48±0.48 nmol ml^{-1}) than in the control group (2.28±0.26 nmol ml^{-1}), which is in agreement with previous findings (Kaefer et al. 2012, Tangvarasittichai et al. 2017). The increase in the level of MDA may be attributed to hyperglycemia in these patients because of autoxidation of glucose, which results in the generation of free radicals (Kumawat et al. 2009). The VIP plot shows that iron, selenium and arsenic are the most important trace elements in the model. The area under the curve (AUC) for iron, selenium and arsenic was 0.935, 0.948 and 0.966, respectively, which reflects the high classification performance of these elements to distinguish between type 2 diabetic patients and control. The concentration of iron was significantly lower in the patient group (46.12±3.62 μg L^{-1}) than in the control group (59.56±7.46 μg L^{-1}). There was a significant negative correlation between malanodiadhyde (MDA) and iron, which is in accordance with earlier findings showing that the lipid peroxidation parameters of malondialdehyde were significantly increased in iron deficiency (Madhikarmi et al 2011).

Mitochondrial oxidative phosphorylation is affected by iron deficiency, leading to decreased ATP production, in addition to which it causes changes in the structural and functional integrity of the cell (Ganesh et al. 2012). Selenium is the main component of various selenoproteins, which have an important role in the protection against oxidative stress initiated by excess reactive oxygen species – ROS (Tinggi et al. 2008). In our study, type 2 diabetic patients showed significantly lower levels of selenium (203.30±9.60 μg L^{-1})

than the control group (242.03±17.48 μg L^{-1}). Selenium deficiency is an indicator of a metabolic response to oxidative stress in patients with type 2 diabetes (Karataş et al. 2006). The mean concentrations of arsenic in type 2 diabetic patients (1.04±0.02 μg L^{-1}) was significantly higher than in the control group (0.96±0.03 μg L^{-1}). Low or moderate arsenic exposure plays a positive role, while a high level of arsenic was associated with incidents of diabetes and abnormalities in glucose metabolism due to oxidative stress (Grau et al. 2017). Therefore, through a proper management strategy of trace elements and increasing antioxidant enzyme levels in type 2 diabetic patients, a therapeutic effect may be achieved.

CONCLUSION

The study underlines the role of trace elements as biomarkers for oxidative stress in type 2 diabetic patients. The results show that iron, selenium and arsenic are the most important predictive markers for oxidative stress in type 2 diabetic patients. The concentrations of iron and selenium were lower, but the concentration of arsenic was higher in patients with T2DM. The results also suggest that patients with iron and selenium concentrations less than 46.78 and 215 μg L^{-1}, respectively, and arsenic concentration above 1.01 μg L^{-1} can be suspected to be at high risk of oxidative stress. The cut-off values for Se, Fe and As are very useful biomarkers for the screening of oxidative stress in type 2 diabetic disease. The importance of using multivariate statistical techniques was obvious for the selection of the most important trace elements and for determination of the optimal cut-off values for these elements as biomarkers of oxidative stress in type 2 diabetic patients. In conclusion, our study confirmed that there is an association between the level of trace elements and oxidative stress in type 2 diabetic patients.

Conflict of Interest

The authors declare that there is no conflict of interest.

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