TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2017) 47: 1117-1123

doi:10.3906/sag-1601-95

Relationship between selected micronutrient deficiencies and oxidative stress biomarkers in diabetes mellitus patients with foot ulcers in Ibadan, Nigeria

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Received: 16.01.2016 • **Accepted/Published Online:** 17.03.2017 • **Final Version:** 23.08.2017

Background/aim: Micronutrients are indispensable in the prevention of diseases and maintenance of good health. Their deficiencies have been implicated in several diseases associated with oxidative stress (OS). This study aimed at investigating the levels of some vitamins and minerals in association with OS markers in diabetic foot ulcer (DFU) patients in Ibadan, Oyo State, Nigeria.

Materials and methods: Seventy DFU patients and 50 apparently healthy volunteers (controls) were recruited for the study. Blood samples of 10 mL were collected after a 10-h overnight fast from each participant after obtaining their consent. Levels of oxidative stress biomarkers such as lipid peroxide (LPO), 8-hydroxyl-2′-deoxyguanosine (8-OHdG), total antioxidant status (TAS), superoxide dismutase (SOD), and glutathione peroxidase (GPx) and micronutrients such as vitamin C, vitamin E, copper, selenium, and zinc were determined.

Results: Significant increases in the levels of LPO and 8-OHdG and GPx activity were found in DFU patients compared to controls ($P < 0.001$). Significant decreases in vitamin C ($P = 0.003$), selenium, vitamin E, and TAS concentrations were detected between DFU patients and controls ($P < 0.001$). However, nonsignificant decreases in SOD activity and copper and zinc levels were observed when DFU patients and controls were compared ($P > 0.05$). Vitamin C was significantly positively correlated with GPx and selenium was significantly negatively correlated with 8-OHdG in the DFU group. However, nonsignificant correlations were observed between other micronutrients and oxidative stress biomarkers of both the DFU and control groups.

Conclusion: Diabetes mellitus patients with foot ulcers may require micronutrient supplementation for proper control and maintenance of oxidant/antioxidant homeostasis.

Key words: Micronutrients, diabetic foot ulcer, oxidative stress

1. Introduction

Diabetic foot ulcer (DFU), a chronic complication of diabetes mellitus (DM), is a major cause of morbidity and mortality in patients with DM in Nigeria (1). It is the second leading cause of diabetes-related deaths, accounting for 24% of all diabetes mortalities in the country (2). DFU is associated with oxidative stress (OS), which arises in cells and tissues from excessive generation of free radicals in the presence of a decreased antioxidant defense system (3,4). The body's antioxidant defense is achieved through interaction between the nonenzymatic antioxidant micronutrients and the enzymatic antioxidants containing several metalloenzymes as components (5).

Micronutrients, including vitamins and minerals, are required by the body in small quantities for specific functions (6). They are essential in the prevention of deficiency diseases, regulation of metabolism and gene expression, deterrence of the development and progression of many chronic diseases, and the maintenance of good health (6,7). The vitamins, acting as direct antioxidants (7), neutralize and scavenge free radicals (3), while the minerals regulate enzyme activities (6) by serving as cofactors for antioxidant enzymes: selenium (Se) for glutathione peroxidase (GPx), iron for catalase, and copper (Cu), zinc (Zn), or manganese (Mn) for superoxide dismutase (5,7). Therefore, protective and scavenging effects are exerted on living systems by these micronutrients (8). Indeed, micronutrients have been considered as potential preventive and treatment agents for both type 1 and type 2 diabetes and for common complications of diabetes (9).

It has been demonstrated that persistently uncontrolled hyperglycemia can cause significant changes in the levels of micronutrients (9), and its deficiency state may lead to an increase in the production of free radicals (10).

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However, free radical measurement is difficult given their high reactivity, their very short biological half-lives, and their low concentrations. Therefore, indirect markers are commonly used to evaluate secondary products of lipid peroxidation. These markers include lipid peroxides (LPO) and 8-hydroxyl-2′-deoxyguanosine (8-OHdG), among others (11). Lipid peroxides result from excessive reactions of free radicals with polyunsaturated fatty acids in cell membranes (12), while 8-OHdG is a consequence of free radical damage of DNA (11).

Due to the various vital functions of micronutrients in living organisms, knowledge of their status and association with OS in people with DFUs is therefore necessary. There is a paucity of data as regards this in Ibadan, Southwest Nigeria. Therefore, this study investigated the relationship between selected micronutrient (vitamin C, vitamin E, Cu, Mn, and Zn) deficiencies and oxidative stress biomarkers (LPO, 8-OHdG, total antioxidant status (TAS), superoxide dismutase (SOD), and GPx) in DM patients with foot ulcers.

2. Materials and methods

2.1. Subjects

This case-control study included 70 type 2 DM patients with DFUs and 50 apparently healthy volunteers as controls. The DFU patients were on dietary restriction and oral hypoglycemic drug therapy. They were recruited from University College Hospital and Adeoyo Hospital, Ring Road, Ibadan. The controls were not on any medications and were selected from among the staff of the University of Ibadan. Males and nonpregnant and nonlactating females between the ages of 40 and 60 years were included in the study. Apparently healthy volunteers with fasting plasma glucose (FPG) levels of >5.6 mmol/L were excluded from the study.

Other recruitment criteria for DM patients with foot ulcers were as follows: participants with Wagner's Grade 2 ulcer classification (i.e. ulcer involving ligament, tendon, joint capsule, or fascia but no abscess or osteomyelitis) (13) were included in the study. Those with gangrene or severely impaired arterial supply in their feet, bone infection in the area of their ulcers, or immediate risk of major above-ankle/knee amputations were excluded from the study. Similarly excluded from the study were participants with renal, liver, and cardiac problems or impairment such as hypertension and other complications of DM or comorbid diseases. Informed consent was sought and obtained from all participants. Ethical approval was obtained from the joint University of Ibadan/University College Hospital Institutional Review Committee. Each participant's height in meters and weight to the nearest kilogram were measured. Body mass index (BMI) was calculated as weight (kg)/height^2 (m^2).

2.2. Collection of Blood

About 10 mL of venous blood was collected after a 10-h overnight fast into appropriate sample tubes and centrifuged at 3000 rpm for 10 min. Plasma, serum, and hemolysate were separated and stored in small aliquots at –80 °C until the day of analysis. Levels of FPG, glycated hemoglobin A1c (HbA1c), micronutrients (vitamin C, vitamin E, Cu, Se, and Zn), and oxidative stress biomarkers (LPO, 8-OHdG, and TAS) as well as erythrocyte SOD and GPx were determined.

2.3. Analytical methods

All tests were carried out using standard procedures with chemical reagents purchased from Sigma Aldrich (Germany). LPO concentrations were measured using the ferrous oxidation with xylenol orange (FOX VERSION II) assay according to the method of Nouroouz-Zadeh et al. (14). This method is based on the principle of rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions to form a Fe-xylenol orange complex that was measured using a spectrophotometer at wavelength of 560 nm.

The levels of 8-OHdG were measured at 450 nm on a microplate reader using a commercial kit obtained from the Japan Institute for the Control of Aging (Fukuroi, Japan). The technique, described by Patel et al. (15) and Shen et al. (16), is based on a competitive in vitro enzyme-linked immunosorbent assay (ELISA) for quantitative measurement of this DNA metabolite. The unknown samples or standards of 8-OHdG were first added to an 8-OHdG/bovine serum albumin conjugate preabsorbed ELISA immunoassay plate. After a brief incubation, an anti-8-OHdG monoclonal antibody was added, followed by horseradish peroxidase-conjugated secondary antibody. The 8-OHdG contents in unknown samples were determined by comparison with a predetermined 8-OHdG standard curve.

The determination of plasma TAS was based on the method of Miller et al. (17) using a commercial kit (Randox Laboratories, UK). In this method, 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) [ABTS$^{(R)}$] was incubated with metmyoglobin (a peroxidase) and H_2O_2 to produce the radical cation ABTS$^{(R)\bullet+}$. This reaction has a relatively stable blue-green color, which was measured at a wavelength of 600 nm. Antioxidants in the added sample cause the suppression of this color production to a degree proportional to their concentration.

Vitamin C determination was based on the method of Kyaw (18). In this method, plasma proteins were precipitated with phosphotungstic acid, producing a blue supernatant. The colors produced were measured using a spectrophotometer at a wavelength of 700 nm and are directly proportional to the level of ascorbic acid. Dehydroascorbic acid and diketogulonic acid do not

interfere with the phosphotungstic acid reagent. Thus, the reaction may be considered specific for ascorbic acid.

Vitamin E (alpha-tocopherol) levels in plasma were extracted using xylene. α,α-Dipyridyl was added to the xylene layer and allowed to react with the solution of ferric chloride for a specific time interval. The red colors produced were measured at 520 nm using a spectrophotometer and are directly proportional to the concentration of vitamin E. This method was developed by Quaife et al. (19) and modified by Baker and Frank (20).

Serum Zn and Cu were determined by the method of Kaneko (21) and plasma Se was determined by the method of Pleban et al. (22) using a 210/211 VGP atomic absorption spectrophotometer (Buck Scientific, USA).

Erythrocyte SOD activity was determined by the methods of Arthur and Boyne (23) as well as that of Suttle (24), using a commercial kit obtained from Randox Laboratories. This method uses xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The activities of SOD were then measured by the degree of inhibition of this reaction at a wavelength of 505 nm using a spectrophotometer.

The determination of erythrocyte GPx activity was based on the method of Paglia and Valentine (25) as modified by Kraus and Ganther (26) using a commercial kit (Randox Laboratories). This method involves the oxidation of glutathione (GSH) by cumene hydroperoxide catalyzed by GPx. In the presence of glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH), the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance is then measured at a wavelength of 340 nm using a spectrophotometer.

2.4. Statistical analysis

All values were expressed as mean ± standard error of mean (SEM). The data were subjected to statistical analysis using SPSS 20 (IBM Corp., Armonk, NY, USA). Statistical differences were determined using the independent Student t-test. Pearson correlation coefficients were used to determine the level of relationships between the variables. $P < 0.05$ was considered to be statistically significant.

3. Results

A total of 120 participants were recruited for the study. There was no significant difference in the mean age of DFU patients (51.63 ± 1.07 years) and the control subjects (51.64 ± 1.03 years) ($P = 0.994$). The mean values of BMI, FPG, and HbA1c respectively in the DFU group (26.08 ± 0.30 kg/m^2, 12.98 ± 0.43 mmol/L [233.93 ± 7.83 mg/dL], 8.63 ± 0.24%) and the control group (22.93 ± 0.24 kg/m^2, 5.09 ± 0.08 mmol/L [91.69 ± 1.35 mg/dL], 4.08 ± 0.11%) were significantly different at $P < 0.001$.

The oxidative stress biomarkers and micronutrients of DFU patients and control participants are given in Table 1. Significant differences in mean values of LPO, 8-OHdG, GPx, TAS, vitamin C ($P = 0.003$), vitamin E, and Se were found between the DFU and control groups ($P < 0.001$), while the mean values of SOD, Zn, and Cu in the DFU group were not significantly different from those of the control group ($P > 0.05$).

Correlation coefficients of micronutrients in relation to LPO, 8-OHdG, TAS, GPx, and SOD of DM patients with

Table 1. Oxidative stress biomarkers and micronutrients of diabetes mellitus patients with foot ulcers (DFU) and control participants.

Participants	DFU (n = 70)	Controls (n = 50)	P-value
FPG (mmol/L)	12.98 ± 0.43	5.09 ± 0.08	<0.001
HbA1c (%)	8.63 ± 0.24	4.08 ± 0.11	<0.001
LPO (μmol/L)	55.47 ± 1.55	31.41 ± 2.26	<0.001
8-OHdG (μmol/L)	49.96 ± 1.17	31.87 ± 1.64	<0.001
SOD (U/g Hb)	4264.26 ± 89.54	4408.61 ± 230.58	0.562
GPx (U/g Hb)	2747.18 ± 349.32	1055.49 ± 43.67	<0.001
TAS (mmol/L)	0.67 ± 0.01	1.42 ± 0.02	<0.001
Vit C (μmol/L)	3.76 ± 0.43	5.57 ± 0.43	0.003
Vit E (μmol/L)	19.57 ± 1.01	25.57 ± 0.27	<0.001
Se (μmol/L)	0.48 ± 0.01	0.81 ± 0.04	<0.001
Zn (μmol/L)	15.40 ± 0.24	15.97 ± 0.20	0.072
Cu (μmol/L)	14.59 ± 0.31	15.19 ± 0.35	0.203

foot ulcers and control participants are shown in Table 2. The data revealed a positive relationship between the micronutrients and TAS as well as an inverse association between LPO and 8-OHdG. Selenium was negatively and significantly associated with 8-OHdG (P = 0.029), while vitamin C was significantly and positively correlated with GPx (P = 0.001) in the DFU group. All other studied parameters did not show any significant association (P > 0.05) in either groups.

4. Discussion

Enhanced OS production is one of the leading factors in the pathogenesis of DM and its later complications including DFU, which remains the major cause of morbidity and mortality in people with DM (27). Production of OS is regulated and controlled by the body's antioxidant defense system orchestrated by micronutrients either as direct antioxidants (vitamins) or as components of antioxidant enzymes (minerals) (7). Indeed, micronutrient deficiencies have been implicated in diseases associated with OS such as DM and its later complications (28).

FPG and HbA1c were significantly higher in DFU participants. Similarly, the DFU participants were observed to be overweight. The increase in the glycemic parameters of DFU participants indicated poor glycemic control in these participants. Poor glycemic control has been hypothesized to stimulate the increased production of LPO, a marker of lipid peroxidation (12,29,30). This, in turn, leads to the excessive production of hydroxyl radical ($HO^{\cdot}$) through degradation of hydroperoxides (12,31,32). The $HO^{\cdot}$ produced then reacts with the C-8 position of DNA nucleosides to form 8-OHdG (33), which is a marker of DNA damage, thus causing further damage to cell and tissue membranes (34).

This hypothesis was confirmed by the significantly elevated values of LPO and 8-OHdG found in DFU patients compared to controls. These results are supported by Nouroouz-Zadeh et al. (14) and Martín-Gallán et al. (35) as well as Collins et al. (36) and Shin et al. (37). They respectively found significant increases in the concentrations of LPO and 8-OHdG of patients with DM compared to controls. The significant increases in LPO and 8-OHdG levels were also in support of our previous findings in DM patients with foot ulcers (38).

This increase in markers of OS subsequently led to a reduction in micronutrient concentrations, depletion of TAS and SOD enzyme activities, and activation of GPx enzyme activities in DFU patients. Furthermore, chronic hyperglycemia coupled with overweight and obesity has been reported to cause significant alterations in the status of micronutrients (8,9,39,40). Therefore, the lower micronutrient concentrations found in the DFU patients of this study were not surprising.

Vitamin C levels were significantly lower in DFU than control participants. This decrease may be due to insufficient intake of this vitamin ensuing from the restriction of diet practiced by our DFU patients. It may also be due to its involvement in warding off infection, maintaining structural position and skin integrity (41). Since vitamin C is known to be an essential cofactor in collagen synthesis (41), lower values of this vitamin, as found in the DFU patients of this study, may impair

Table 2. Correlation coefficient of micronutrients in relation to LPO, 8-OHdG, TAS, GPx, and SOD of diabetes mellitus patients with foot ulcers and control participants. *: Statistically significant.

	LPO	8-OHdG	TAS	GPx	SOD
	R (P-value)	R (P-value)	R (P-value)	R (P-value)	R (P-value)
		Diabetes mellitus patients with foot ulcers			
Se	–0.104 (0.393)	–0.261* (0.029)	0.231 (0.055)	0.242 (0.043)	–0.027 (0.827)
Cu	–0.210 (0.080)	–0.054 (0.655)	0.176 (0.145)	0.261 (0.029)	–0.003 (0.982)
Zn	–0.131 (0.279)	0.017 (0.888)	0.088 (0.470)	0.068 (0.576)	0.047 (0.697)
Vitamin C	0.107 (0.376)	0.144 (0.236)	0.011 (0.927)	0.399* (0.001)	0.109 (0.369)
Vitamin E	0.215 (0.074)	0.071 (0.557)	0.004 (0.974)	0.007 (0.952)	0.119 (0.327)
		Control participants			
Se	0.085 (0.559)	0.043 (0.769)	0.152 (0.290)	0.082 (0.571)	–0.185 (0.198)
Cu	–0.135 (0.350)	–0.018 (0.900)	–0.243 (0.089)	0.004 (0.977)	–0.059 (0.682)
Zn	0.014 (0.925)	0.027 (0.853)	–0.010 (0.944)	0.020 (0.891)	–0.097 (0.502)
Vitamin C	–0.013 (0.927)	–0.033 (0.820)	0.013 (0.928)	–0.051 (0.725)	–0.012 (0.935)
Vitamin E	–0.180 (0.210)	–0.206 (0.152)	0.171 (0.234)	–0.153 (0.290)	0.093 (0.520)

collagen synthesis and subsequently delay wound healing in this group of participants. In addition, as vitamin C is a water-soluble antioxidant (7), the decrease in levels could also be due to its involvement in scavenging free radicals generated by chronic hyperglycemia as well as recycling vitamin E from its radicals. The significant decrease in vitamin C is in agreement with other studies carried out on type 2 DM as compared to controls (42,43).

Similarly, vitamin E levels were found to be significantly lower in DFU patients when compared to control participants. This result corroborates the findings of Rema et al. (44) and Merzouk et al. (45). They reported lower plasma levels of vitamin E in patients with DM as compared to controls. The decrease in vitamin E may also be due to its inadequate intake emanating from dietary restrictions and its continuous scavenging role in neutralizing the free radicals produced.

Selenium is another micronutrient that was found to decrease significantly in DFU patients compared to the controls. This mineral is an important component of the antioxidant enzyme GPx (3,7). Its decrease may be due to lower dietary intake as a result of dietary restriction and its involvement in scavenging free radicals as well as regenerating vitamin E from its radical. Similarly, Se might have been extensively used up in the biosynthesis of GPx as depicted by the hyperactivity of this enzyme found in DM patients with foot ulcers. The lower Se levels observed here are supported by the studies of Kljai and Runje (46) and Kornhauser et al. (47), while the studies of Gupta and Chari (42) and Merzouk et al. (45) corroborate the increase in GPx enzyme activities.

Likewise, Cu and Zn are important components of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) (5,7). Their deficiencies may lead to reduced activities of this enzyme. The results obtained for Cu and Zn in DFU patients is comparable to those found in the control participants. This nonsignificant decrease of Cu and Zn is reflected in the activities of SOD, where a statistically nonsignificant depletion of SOD activity was observed in both groups. The decrease in SOD activities is supported by the study of Bhatia et al. (48), while the nonsignificant decreases in the levels of Cu and Zn respectively agree with the findings of Zargar et al. (49) and Babalola et al. (50) as well as Ugwuja et al. (51). Insufficient dietary intake coupled with involvement in the biosynthesis of SOD to mop up free radicals among other functions may be responsible for the marginal decrease obtained for these micronutrients in DFU patients.

TAS takes into consideration both known and unknown antioxidants present in the plasma as well as their mutual cooperation (52). However, in this study, a significant decrease in TAS was found in DFU patients when compared to the control participants, thus suggesting the existence of a low level of circulating antioxidants in these participants. This finding is supported by our previous study (53), where a significant reduction in TAS was found in type 2 DM patients. The decrease in TAS may be due to an increase in the utilization of antioxidants in removing excessive free radicals generated as a result of hyperglycemia (54,55). Similarly, it may have resulted from lower dietary intake of antioxidant micronutrients (56) due to dietary restrictions in this group of participants or the poor socioeconomic status of the participants (53).

The correlation results in the DFU group revealed negative associations whereby Se and Cu were correlated with LPO and 8-OHdG and Zn with LPO. Similarly, in the control group, Cu, vitamin C, and vitamin E shown negative associations with LPO and 8-OHdG. This implied that depletion in these micronutrient concentrations may subsequently lead to elevation of LPO and 8-OHdG levels. This further strengthened the results obtained from the mean comparisons, especially in the DFU group of this study. In addition, these micronutrients had a positive association with TAS, GPx, and SOD, with the exception of Cu and Se, where negative associations were found with SOD in the DFU group. However, in the control group, positive associations were observed of Cu, Se, and Zn with GPx; of vitamins C and E with TAS; and of vitamin E with SOD. This suggests that as the micronutrient decreases there is a corresponding decrease in TAS and the antioxidant enzymes (GPx and SOD). This supports the point that lower micronutrient concentrations may impair the scavenging ability of the body, therefore resulting in increased oxidative stress formation and weakening the antioxidant defense system. These correlation results were statistically not significant, except for Se and 8-OHdG, as well as vitamin C and GPx, and may not provide a precise conclusion.

In conclusion, this study revealed lower concentrations of micronutrients and a significant depletion of TAS in DM patients with foot ulcers when compared with apparently healthy volunteers. This suggests the existence of a low level of circulating antioxidants in DFU patients. Therefore, an increased intake of dietary antioxidants as found in leafy green vegetables and unsweetened fruits coupled with weight loss should be advised and encouraged in DM patients with foot ulcers.

Acknowledgments

We acknowledge the Organization for Women in Science for the Developing World (OWSD) for the award of Postgraduate Fellowship. We also extend our appreciation to the Chemical Pathology Department of the University of Limpopo (Medunsa Campus), Ga-Rankuwa, South Africa, and the technical assistance of the National Health Laboratory Services (NHLS) of the same university is appreciated.

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