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AMELIORATIVE ROLE OF ANTIOXIDANT MICRONUTRIENTS: SELENIUM, VITAMINS C AND E ON OXIDATIVE STRESS AND WOUND HEALING IN TYPE 2 DIABETIC PATIENTS WITH FOOT ULCER IN IBADAN

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ABSTRACT

Wound healing is a complicated process that requires several steps. The involvement of hyperglycaemia and oxidative stress (OS) in healing process has been reported. This study investigated the effects of selenium, vitamins C and E supplementation on OS indices such as lipid peroxides (LPO) and 8–hydroxy–2´–deoxyguanosine (8–OHdG) in wound healing in diabetic patients with foot ulcer. The study comprised fifty non-diabetics (Group A) and fifty diabetics (Group B) between 40 and 60 years. GroupB were divided into supplemented (1) and non-supplemented (2) subgroups. Patients in B1 were given 1000mg vitamin C + 400 mg vitamin E + 100 µg selenium for 16 weeks. Subgroup B2 received no supplementations. Wound healing process was assessed in Group B using 'ABDEFS´ tools of evaluating chronic ulcers. Blood samples of 10 ml were collected. 8-OHdG was determined by ELISA; LPO, TAS, SOD and GPx were measured spectrophotometrically. Data were analysed statistically using Wilcoxon tests at p≤0.05. Before supplementation, increases of 79.37 and 54.91% respectively in LPO and 8–OHdG levels with decrease of 53.52% in TAS were observed in Group B compared with Group A. After supplementation, reductions of 22.50 and 22.48% respectively in LPO and 8-OHdG with 10.77% increase in TAS were found in subgroup B 1 compared with subgroup B2. Decreased 'ABDEFS'-score of 22.91% was observed in subgroup B1 compared with subgroup B2 thus revealing better healing. Antioxidant micronutrient supplementation demonstrated ameliorative effect on oxidative stress and wound healing in Type 2 diabetes with foot ulcer.

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KEY WORDS

Diabetic foot ulcer; Oxidative stress; Antioxidant micronutrients; Type 2 diabetes mellitus

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[I] INTRODUCTION

Diabetes mellitus (DM) is a growing non-communicable disease worldwide. It is a metabolic disorder characterized by chronic hyperglycaemia with disturbances in carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both [1]. Among the associated morbidities of DM is impaired/delayed wound healing. Healing is a normal physiological process that proceeds through a series of co-ordinated cellular and cytokine-mediated events, culminating in the restoration of functional integrity of tissues [2]. Healing is delayed for several reasons under certain circumstances such as diabetes, starvation, ageing and in immunocompromised situations [2].The effects of diabetes on healing are divers, multifactorial, complex and inter-related. Diabetes mellitus, to some extent, affects almost all stages of wound healing [3]. Free radicals and their scavenging systems are known to have a very important role in healing of normal and/or delayed healing of wounds [4]. The magnitude of free radical generation and the antioxidant defence systems are also known to be altered in diabetic condition [2]. Hence, this present study aimed at investigating the possible ameliorative effect of antioxidant micronutrients on oxidative stress and wound healing in Type 2 diabetes mellitus with foot ulcer.

[II] MATERIALS AND METHODS

2.1. Patient Selection

Fifty Type 2 diabetic subjects with Wagner´s Grade 2 foot ulcer (i.e. ulcer without abscess or oesteomyelitis) attending the Medical Out–Patient (MOP) Unit and patients admitted in the Medical Wards (MW) of University College Hospital, Ibadan, and Adeoyo Hospital, Ring Road, Ibadan, Nigeria were recruited into the study as test group (Group B). This test group comprises male (54%) and non-pregnant/lactating female (46%) between the ages of 40 and 60 years (52.04±8.65). Fifty non-

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diabetic healthy subjects matched for age and sex were recruited as control group (Group A) after signing the informed consent form.

The diabetic subjects were on diet restriction and/or oral hypoglycaemic agents. They were divided into two groups of twenty-five each as follows: Supplemented Type 2 diabetes mellitus with diabetic foot ulcer (Group B1) and Non-supplemented Type 2 diabetes mellitus with diabetic foot ulcer (Group B2). Group B1 were given 1000 mg ascorbic acid (Ascomed, Kunimed Pharmchem Limited, Lagos), 400 mg α-tocopherol (α-tocopherol acetate, Korea United Pharm. Int'l Inc.) and 100 µg Se (Selenium ACE, Wassen International Limited, UK), dissolved in 100 ml of vanilla flavoured milky beverage while Group B2 received 100 ml of vanilla flavoured milky beverage only for 16 weeks (The amount of vitamins C and E in Selenium ACE has been incorporated in the daily dosages. Vanilla flavour was used to improve the taste of mixture). Patients were not given placebo because of their diabetic state. Both the supplemented and non-supplemented subjects were given the same medical management in terms of glucose control and wound dressing. In order to evaluate their compliance, they were asked to come back every fortnight to receive the next antioxidant supplements until the end of study.

This study was approved by the Joint Ethical Committee of the University of Ibadan and the University College Hospital Institutional Review Committee (UI/UCH IRC) (approval number UI/IRC/03/0096).

2.2. Blood Sample Collection

Ten millilitres aliquots of venous blood were drawn after a ten-hour overnight fast and collected into heparinised and EDTA sample tubes. The blood samples were collected at the beginning (0 week) of the study, 8 and 16 weeks after daily supplementation. All samples were centrifuged at 3000 rpm for 10 minutes. Plasma and haemolysate were stored at −80°C until the day of analysis and vitamin C samples were analysed within 3 hours of collection. Antioxidant enzymes (SOD and GPx) were measured in heparinised whole blood, total antioxidant status (TAS) was measured in heparinised plasma, while Se, vitamin C, Vitamin E and oxidative status parameters (LPO, 8-OHdG) were determined in EDTA plasma.

2.3. Biochemical Analyses

Lipid peroxide concentrations were measured spectrophotometrically at 560 nm using the ferrous oxidation with xylenol orange (FOX VERSION II) assay according to the method of Nourooz-Zadeh et al. [5]. This method is based on the principle of rapid peroxide-mediated oxidation of Fe2+ to Fe3+ under acidic conditions. Plasma levels of 8-OHdG were measured at 450nm on a microplate plate reader using a commercial kit from the Japan Institute for the Control of Aging (Fukuroi, Japan). The method is based on a competitive in-vitro enzyme-linked immunosorbent assay for quantitative measurement of this DNA metabolite in tissue, serum and plasma [6]. Erythrocyte SOD activity was determined by the method of Arthur and Boyne [7] using Randox kit (Randox Laboratories, UK). This method uses xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. Superoxide dismutase activity was then measured by the degree of inhibition of this reaction spectrophotometrically at 505 nm. The determination of erythrocyte GPx activity was based on modified method of Paglia and Valentine [8] using Randox kit (Randox Laboratories, UK). This method involves the oxidation of glutathione (GSH) by cumene hydroperoxide catalysed by GPx. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+ and the decrease in absorbance is then measured spectrophotometrically at 340nm. Total antioxidant status (TAS) in heparinised plasma was determined by the method of Miller et al. [9] using Randox kit (Randox Laboratories Limited, U.K). In this method, ABTS(R) (2,2´–Azino–di–[3–ethylbenzthiazoline sulphonate]) was incubated with metmyoglobin, a peroxidase, and H_2O_2 to produce the radical cation ABTS(R)˙+. This reaction has a relatively stable blue–green colour which was measured at wavelength of 600 nm. Antioxidant in the added sample caused suppression of this colour production to a degree which is proportional to their concentration. Vitamin C (ascorbic acid) was determined spectrophotometrically at wavelength of 700 nm in plasma by the acid phosphotungstate method of Aye Kyaw [10]. Vitamin E (α –tocopherol) in plasma was extracted using xylene, and its level was assayed spectrophotometrically at 520 nm according to the method of Baker and Frank [11]. Selenium in plasma was determined by the method of Pleban et al. [12].

2.4. Clinical assessment of ulcer

The 'ABDEFS' tool of evaluating chronic ulcers [13] was used in clinical assessment of wound healing progress. This system consists of scoring the ulcer based on certain features that begin with the first few English alphabets (excluding letter "c" but including letter "s") as follows:

A.	Aetiology:	1. Local, e.g. trauma, infection 2. Controlled systemic disease 3. Systemic disease, uncontrolled 4. Malignancy
B.	Base:	1. Soft, mobile 2. Hard fixed
D.	Discharge:	1. Slight to moderate 2. Copious, purulent, etc
E.	Edge:	1. Flat, shelving, punched out 2. Undermined, raised
F.	Floor:	1. Predominantly granulation 2. Predominantly sloughy
S.	Size:	1. Less than or equal to 2.5 cm in one dimension 2. Greater than 2.5 cm in one dimension.

Points corresponding to appropriate description were allocated to each feature on the ulcer. The minimum possible score of 6 denotes the best healing ulcers while the maximum score of 14 denotes the worst healing ulcers. The 'ABDEFS' assessing tool was selected because the six different aspects of 'ABDEFS' scoring system were various characteristics normally utilized in the description of an ulcer. This tool was easy to measure and no special training was required.

2.5. Statistical analysis

All data were presented as Mean ± Standard Deviation (SD) for 50 subjects in each group at baseline and 25 subjects in each subgroup after supplementation. The Statistical Package for Social Sciences (SPSS) (version 16) was used for statistical evaluation. Significance of differences was determined using Wilcoxon Test. Percentage (%)

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increase or decrease was used to interpret the differences between supplemented and non-supplemented subgroups. Statistical significance was set at $p \leq 0.05$.

[III] RESULTS

At the beginning of the study, significantly higher values were observed in fasting plasma glucose (13.01± 3.50 vs 5.09±0.53 mmol/L), glycated haemoglobin A1c (8.51±2.0 vs 4.08±0.75 %), body mass index (26.59±2.44 vs 22.93±1.69 kg/m2), lipid peroxides (56.34±10.04 vs 31.41±15.96 µmol/L) and 8–hydroxyl–2´–deoxyguanosine (49.37±7.78 vs 31.87±11.58 ng/ml ($p<0.001$) when patients with diabetic foot ulcer (Group B) were compared with non-diabetic subjects (Group A). The data also revealed lower activity of superoxide dismutase (4169.15±635.78 vs 4408.61±1630.42 U/g Hb; $p>0.05$) and higher activity of glutathione peroxidase (1281.64±240.36 vs 1055.49±308.79 U/g Hb; $p<0.001$) in Group B compared with Group A. In addition, decreases were observed in total antioxidant status (0.66±0.13 vs 1.42±0.17 mmol/L), vitamin C (0.003±0.002 vs 0.01±0.003 mmol/L) and vitamin E (0.05±0.02 vs 0.06±0.005 mmol/L) and selenium (0.46±0.12 vs 0.81±0.27 µmol/L) ($p<0.001$) when Group B patients were compared with Group A subjects.

However, supplementation with combined Se, vitamins C and E at 8 and 16 weeks resulted in decreases of 6.86, 7.33, and 6.05% at 8 weeks and of 9.07, 11.97 and 10.44% at 16 weeks respectively in BMI, FPG and HbA1c of the supplemented (B1) subgroup. Reductions of 4.57 and 6.95% in BMI, of 4.58 and 10.04% in FPG and 4.77 and 7.10% in HbA1c were observed at 8 and 16 weeks respectively in non-supplemented (B2) subgroup. Lipid peroxide and 8-OHdG levels were observed to decrease in subgroup B1 with percentage decreases of 12.51 and 10.43% ($p>0.05$) respectively at 8 weeks and of 22.50 and 22.48% ($p<0.01$) respectively at 16 weeks, but increased in the non-supplemented group (B2) by 8.45 and 18.73% in LPO and by 15.30 and 30.0% in 8–OHdG at 8 and 16 weeks respectively. Total antioxidant status increased by 6.15% ($p>0.05$) and 10.77% ($p<0.01$) respectively at 8 and 16 weeks of supplementation in the supplemented group, while decreases of 4.48 and 8.96% respectively were recorded at the same period of supplementation in the non-supplemented group. The ′ABDEFS′ tool was observed to decrease by 11.01 and 22.91% respectively at 8 and 16 weeks compared with baseline (8.08±1.38, 7.0±1.26 vs 9.08±1.61) in the supplemented subgroup while increase of 6.87 and 15.02% was found in the non-supplemented subgroup (9.96±1.27, 10.72±1.24 vs 9.32±1.44), at the same period of supplementation **[Table-1].**

Table: 1. Effect of 8 and 16 weeks of supplementation with Se, vitamins C and E on Oxidative stress indices in supplemented diabetic foot ulcer patients (Group B_1) and non-supplemented diabetic foot ulcer patients (Group B_2)

90Parameters (n=25)	Group B_1 (0 wk) (Mean±S.D)	Group B_1 (8 wk) (Mean±S.D)	Group B_1 (16 wk) (Mean±S.D)	‡Wilcoxon (p-value)	‡‡Wilcoxon (p-value)	Group B_2 (0 wk) (Mean±S.D)	Group B_2 (8 wk) (Mean±S.D)	Group B_2 (16 wk) (Mean±S.D)	#Wilcoxon (p-value)	#Wilcoxon (p-value)
BMI (kg/m^2)	26.68±2.72	24.85±2.90	24.26±2.84	2.839* (0.005)	3.377* (0.001)	26.49±2.17	25.28±1.76	24.65±1.77	1.722ns (0.085)	2.489† (0.013)
FPG (mmol/L)	12.92±3.57	11.98±3.36	11.38±3.40	2.059† (0.040)	2.570† (0.010)	13.10±3.50	12.50±3.52	11.78±3.63	0.605ns (0.545)	1.372ns (0.170)
HbA1c (%)	8.43±1.94	7.92±1.77	7.55±1.67	1.790ns (0.073)	2.287† (0.022)	8.59±2.10	8.18±1.90	7.98±1.86	0.600ns (0.549)	0.727ns (0.467)
LPO (µmol/L)	62.28±7.36	54.49±8.06	48.27±10.15	0.740ns (0.459)	2.600 (0.009)	50.41±8.85	54.67±13.24	59.85±13.45	0.283ns (0.778)	1.009ns (0.313)
8–OHdG (ng/ml)	53.51±5.23	47.93±8.38	41.48±9.82	0.969ns (0.333)	2.812 (0.005)	45.23±7.78	52.15±9.98	58.80±11.91	1.400ns (0.162)	2.812 (0.005)
SOD (U/g Hb)	4123.99±634.43	4128.62±632.42	4239.29±669.45	0.040ns (0.968)	0.578ns (0.563)	4214.33±644.93	4009.28±513.51	3789.33±469.20	1.413ns (0.158)	2.516† (0.012)
GPx (U/g Hb)	1325.28±282.90	2307.26±2559.90	2853.99±3434.48	1.413ns (0.158)	2.516† (0.012)	1237.99±184.26	3245.38±3349.87	3159.66±3329.29	2.193† (0.028)	2.408† (0.016)
TAS (mmol/L)	0.65±0.10	0.69±0.09	0.72±0.08	1.292ns (0.196)	2.705 (0.007)	0.67±0.15	0.64±0.13	0.61±0.14	0.772ns (0.440)	1.575ns (0.115)
Vitamin C (mmol/L)	0.003±0.002	0.01±0.005	0.01±0.005	3.296 (0.001)	3.956 (0.000)	0.003±0.002	0.003±0.002	0.003±0.003	0.229ns (0.819)	0.121ns (0.904)
Vitamin E (mmol/L)	0.05±0.02	0.06±0.02	0.07±0.02	2.759 (0.006)	3.297 (0.001)	0.05±0.02	0.05±0.02	0.04±0.02	0.071ns (0.943)	1.529ns (0.126)
Se (µmol/L)	0.47±0.12	0.53±0.12	0.58±0.12	1.884ns (0.060)	2.852 (0.004)	0.51±0.13	0.46±0.14	0.43±0.14	1.257ns (0.209)	1.924ns (0.054)

ns – Not significant ($p>0.05$) ; *Significant at $p<0.001$; †Significant at $p<0.05$; ‡8 weeks values were compared with baseline values (0 week); #16 weeks values were compared with baseline values (0 week)

[IV] DISCUSSION

Wound healing in diabetic patients is usually poor and slow. It is a complicated process that involves several steps and any alteration at any step may further slow down an already slow process. Factors affecting healing could either be 'general' resulting from lack of vitamin C, protein deficiency, ageing and other diseases such as diabetes, jaundice, uraemia, and Cushing´s disease; or it may be 'local' for example sepsis, impaired blood supply (ischaemia) and presence of dead or damaged tissues [2]. The possibility of altered free radical scavenging systems as a major cause for delayed healing cannot

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be ignored, since free radicals and antioxidant defence systems play an important role in many disorders including diabetes and it's complications [2]. Though poorly controlled metabolic disorders such as diabetes mellitus are associated with poor wound healing [14], the specific role of oxidative stress has received little attention. Our data indicate that optimum antioxidant status can significantly enhance healing of wounds.

At the beginning of this study, indices of diabetes were found to be higher in Type 2 diabetes mellitus with diabetic foot ulcer compared with non-diabetic control subjects. Elevated FPG has been shown to generate oxidative stress (OS) through several mechanisms [15]. An increase in FPG has been hypothesized to induce increase generation of lipid peroxide (LPO), a marker of lipid peroxidation, leading into excessive production of $HO^{\cdot}$ which then attack the C–8 position of deoxyguanosine and the subsequent loss of a hydrogen atom from the intermediate to form 8–hydroxyl–2´–deoxyguanosine (8–OHdG), a marker of DNA damage. Therefore, increase in FPG results in elevated levels of LPO and subsequently increase production of 8–OHdG. This hypothesis was indeed confirmed by the result obtained in this study as LPO and 8–OHdG levels were observed to increase in Group B compared with Group A. These increases in LPO and 8–OHdG levels in diabetic foot ulcer patients compared with non-diabetic control subjects resulted in lowering SOD activity and increasing GPx activity. These findings agree with the studies of Merzouk and Coll. [16] and Gupta and Chari [17]. The decrease in SOD activity may be due to the over production of peroxides which then resulted in increasing GPx activity as a compensatory mechanism to remove the excess peroxides generated from the dismutation process thus preventing further tissue damage in these patients.

Total antioxidant status (TAS), a useful indicator of risk from diseases associated with free radical activity, and which may indicate the need for antioxidant therapy was observed to be lower in diabetic foot ulcer patients compared with non-diabetic subjects. This finding in TAS of diabetic foot ulcer patients agree with the previous studies conducted in Type 1 and Type 2 diabetic patients [18] where significant decreases were reported. Significant decreases were similarly observed in non-enzymatic antioxidant micronutrients. These decreases in the antioxidant micronutrients are in accordance with the findings of Gupta & Chari [17] and Karatas et al. [19], they reported lower levels of vitamin C. Merzouk et al. [16] reported decreases in vitamin E levels while Kljai and Runje [20] and Kornhauser et al. [21], reported lower levels of selenium in diabetic subjects compared with non diabetic subjects. The low vitamin C level in diabetic foot ulcer patients may be due to its consumption in scavenging free radicals and/or its involvement in regenerating vitamin E from the generated α-tocopheroxyl radicals as well as its participation in collagen synthesis in these patients. The hyperactivity of GPx, a seleno–enzyme [22], indicated above in diabetic foot ulcer patients may justify the reduction of selenium. This micronutrient may be increasingly consumed in the biosynthesis of glutathione peroxidase enzyme in these patients.

Following 16 weeks of supplementation with combined Se, vitamins C and E coupled with diet restriction and oral hypoglycaemic agent administration, decreases in BMI, FPG and HbA1c were found in the supplemented subgroup. Similarly, in the non-supplemented subgroup, reductions in the diabetes indices were observed at the same period of supplementation. These reductions in diabetes indices in both subgroups B1 and B2 indicated good management of blood glucose in these patients. Although, good management of blood glucose was demonstrated in both subgroups as revealed by the reductions in diabetes indices, LPO and 8-OHdG levels were only observed to decrease in the supplemented group (B1) but increased in the non-supplemented group (B2). The TAS was observed to increase in the supplemented diabetic foot ulcer patients, as a result of the reduction in the oxidative stress indices while in the non-supplemented group a non-significant decrease was recorded. This findings suggested that supplementation with combined Se, vitamins C and E was able to reduce LPO and 8-OHdG levels thus improving the total antioxidant status while absence of supplementation exacerbate the antioxidant defence status.

Wound healing process was measured by the 'ABDEFS' assessing tool in diabetic foot ulcer group. This tool revealed improvement in wound healing in the supplemented group compared with the non-supplemented group. Vitamin C has been reported to be involved in two stages of wound healing. These are the inflammatory stage, in which vitamin C enhances neutrophil migration and lymphocyte transformation, and the proliferative stage where it is necessary for collagen synthesis [23]. Supplementation of diabetic ulcer with vitamin C therefore ensures the availability of this vitamin in these two phases thus speeding up the healing as depicted by the 'ABDEFS' result.

[V] CONCLUSION

Good glycaemic control, as indicated by reduction in levels of BMI, FPG and HbA1c in both supplemented and non-supplemented diabetic foot ulcer patients, could not improve oxidative stress and wound healing on its own. Reduction in oxidative stress indices coupled with better healing of wound was achieved by good glycaemic control and administration of antioxidant micronutrients. These findings signify the ameliorative role of the antioxidant micronutrients supplementation on oxidative stress and subsequently on wound healing in Type 2 diabetics with foot ulcer.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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