Plasma, Granulocyte and Mononuclear Cell Copper and Zinc in Patients With Diabetes Mellitus*

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The concentrations of Cu and Zn were determined in the plasma, granulocytes and mononuclear cells of 26 patients with diabetes mellitus and 26 age and sex-matched controls. In addition, Cu was measured in both washed and unwashed red blood cells, and Cu,Zn-superoxide dismutase (SOD) activity measured in washed red blood cells. Cu and Zn were determined by Zeeman-effect graphite furnace atomic absorption spectrometry following separation of plasma and red blood cells, and the white blood cell fractions (granulocytes and mononuclear cells) by density gradient centrifugation. There were no significant differences in any of the matching factors, or lipid profiles, between the groups. Plasma Zn was reduced by 17% in diabetics, compared with the controls ($P = 0.0001$). Neither the plasma nor the red blood cell Cu concentrations were significantly different. Of the white blood cell fractions, only mononuclear cell Cu was significantly different (30% lower in diabetics, $P = 0.0035$). The red blood SOD activity was reduced in diabetics by over 12%, but this difference was non-significant ($P = 0.0872$). There was a significant negative correlation between washed red blood cell Cu and the duration of diabetes ($r = -0.613$, $P = 0.0069$). In conclusion, the copper and zinc status of these diabetic patients was reduced, providing further evidence of a role for these antioxidant trace elements in this disease.

Keywords: *Copper; zinc; trace element; leucocytes; erythrocytes; diabetes*

Aim of Investigation

Diabetes mellitus is a complex disease involving either insulin deficiency or resistance to insulin action giving rise to abnormalities in glucose homeostasis and lipid metabolism. The long-term complications of diabetes (retinopathy, nephropathy, neuropathy and atherosclerosis) are insidious and devastating. A patient with diabetes has a 25-fold increase in the risk of blindness, a 20-fold increase in the risk of renal failure, a 20-fold increase in the risk of gangrene resulting in amputation and a 2 to 6-fold increased risk of cardiovascular disease.[1] Intensive treatment aimed at maintaining blood glucose concentrations close to the normal range delays the onset and slows the progression of these complications, but not completely.[2] Other factors may well play a role. There is considerable evidence of a disturbed antioxidant status in diabetes,[3] probably owing to the increased oxidative stress caused by the high circulating blood glucose concentrations.[4–6]

The essential trace elements copper and zinc function as antioxidants in biological systems, mainly through their associated enzymes (*e.g.*, Cu,Zn-superoxide dismutase or SOD, and caeruloplasmin). Nutritional copper and zinc deficiency may impair antioxidant status directly by decreasing the activity of these enzymes, and indirectly by altering other cell components such as iron, selenium and glutathione that influence antioxidant status.[7–10]

Significant changes in copper and zinc metabolism have been described in humans and animals with diabetes mellitus, and have been associated with certain complications of diabetes. Most of these investigators have focused on plasma and urine concentrations of copper and zinc to characterize these changes. However, plasma mineral concentrations are greatly influenced by stress, including inflammation, infection, and physical exertion. This may account for some of the contradictory results found in the literature.[11–18] The white blood cell content reflects whole body mineral status more effectively, and may be the best determinant of mineral deficiencies.[19]

The purpose of this study was to compare the copper and zinc status of patients with diabetes with healthy controls by determining granulocyte, mononuclear cell and plasma copper and zinc; and red blood cell copper and SOD activity.

Experimental

Results from 26 diabetic patients (age range, 17–83 years; duration of diabetes, 13.7 ± 12.7 years, mean ± standard deviation, *s*) and 26 controls (matched for age, range 19 to 78 years; sex, and smoking habit, see Table 1) are reported. This study was approved by the local medical ethics committee, and informed consent was obtained from all individuals.

A 30 ml volume of venous blood was collected directly into a plain plastic tube (10 ml) and tubes containing tripotassium ethylenediaminetetraacetic acid (K_3EDTA) as an anticoagulant (Monovette, Sarstedt, Leicester, UK). Data on smoking habit was asked of the patient; clinical data were obtained from hospital notes.

A 0.5 ml volume of whole blood was removed for analysis in an automatic cell counter (Minos STX, Roche Diagnostic Systems, Welwyn Garden City, Hertfordshire UK). The remaining blood was centrifuged at 1000*g* for 10 min. Plasma and serum were removed and either analysed immediately, or stored at −20 °C. A 0.5 ml aliquot of packed red blood cells (unwashed) was removed for copper determination. The white blood cell fractions were isolated from the remainder of the specimen using a density centrifugation technique within 2 h of collection as follows.

Cells remaining from 20 ml of EDTA whole blood were combined and mixed with a 6% (m/v) dextran 150 solution in saline. Following gentle inversion, the red blood cells were left to sediment at 1*g* for 45 min. The leucocyte-rich supernatant was washed once in phosphate-buffered saline (PBS, 120

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mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 10 mmol l^{-1} phosphate buffer, pH 7.4, Sigma, Poole, Dorset, UK), made up to 15 ml with PBS, then carefully layered over 10 ml of density gradient media, 1.077 g ml^{-1} (NycoPrep 1.077, Nycomed, Birmingham, UK) in an all-plastic Universal container (Life Technologies, Paisley, UK). The gradient media had residual trace element contamination removed by passing the solution several times through a Chelex resin-ion-exchange column (Biorad Laboratories, Hemel Hempstead, Hertfordshire, UK). The layered preparation was centrifuged at 800*g* for 20 min, and the mononuclear cell rich (upper) and granulocyte rich (lower) fractions collected using a plastic transfer pipette (Sarstedt). The following steps were carried out on both fractions. Platelet contamination was removed by a low-speed (200*g* for 10 min) wash with PBS. Residual red blood cells were removed by incubation of the washed cell pellets in 10 ml of freshly prepared TRIS-ammonium chloride solution (0.017 mol l^{-1} TRIS, 0.14 mol l^{-1} NH_4Cl, pH 7.2) for 10 min at 37 °C. This was followed by two PBS washes (450*g* for 6 min).

Each white blood cell fraction pellet was resuspended in 3 ml of PBS. Two 25 μl aliquots were taken for cell counting. The cells were then spun down, and the pellet stored at −20 °C. Red blood cells remaining from the dextran-sedimentation step were washed three times in PBS. Aliquots were taken for analysis of copper, SOD, and haemoglobin.

The mononuclear cell and granulocyte pellets were digested in 300 μl of 6 mol l^{-1} nitric acid in 7 ml Teflon vessels in a microwave oven (CEM, Buckingham, UK). The resulting digests were made up to 2 ml with de-ionized water (>18 MΩ) in calibrated flasks (final acid concentration of 0.9 mol l^{-1}). The acid digests, red blood cells, and plasma were assayed for copper and zinc by Zeeman-effect graphite furnace atomic absorption spectrometry (Zeeman 3030, Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) essentially by the method of Foote and Delves.[20] Details of the graphite furnace program are given in Table 2, instrument conditions and scheme for preparation of standards for the determination of mononuclear cell and granulocyte copper concentrations are given below. At all times throughout the whole procedure, close attention was paid to the avoidance of contamination. In addition, all solutions used were periodically assayed for copper and zinc to verify this.

Red blood cell SOD activity was determined by a commercially available enzyme assay kit (SOD-525, R&D Systems Europe, Abingdon, Oxon, UK). Mononuclear cell and granulocyte copper and zinc results were expressed per 10^9 cells. Red blood cell copper and SOD activity were expressed per gram of haemoglobin. Plasma lipids and glycosylated haemoglobin (HbA_{1c}) were determined by standard routine methods. For all methods, within- and between-run relative standard deviations (s_r) were less than 5 and 10%, respectively.

Comparisons were made by two-tailed unpaired *t*-tests and Pearson correlation analysis. Some data were positively skewed; this was corrected by log-transformation prior to analysis.

Procedure for Measurement of Mononuclear Cell and Granulocyte Copper

Instrument conditions

The instrumental conditions used were: argon gas (except for oxygen ash step); pyrolytically coated graphite tubes; wavelength, 324.8 nm; slit width, 0.7 nm; HDL lamp current, 15 mA; Zeeman background correction; peak-area measurements; injection volume, 25 μl; and number of replicates, 2.

Preparation of standards

Acid-washed grade A calibrated glassware was used throughout. A stock 20 μg ml^{-1} copper solution was prepared by diluting 1 ml of 1 mg ml^{-1} copper(II) nitrate standard solution (Merck, Lutterworth, Leicestershire, UK) and 0.5 ml of concentrated nitric acid (AristaR grade, Merck) to 50 ml with de-ionized water. This was further diluted to 400 ng ml^{-1} by diluting 1 ml of the stock solution and 0.5 ml nitric acid to 50 ml with de-ionized water. Working standards were prepared by adding 0, 250, 500, 750 and 1000 μl of the 400 ng ml^{-1} solution together with 1 ml of nitric acid to 20 ml calibrated flasks and making the volume up with de-ionized water. These aqueous standards contained copper at 0, 5, 10, 15 and 20 ng ml^{-1}, respectively.

Standards and samples were analysed neat. Two acid washed sample cups of each sample were analysed in duplicate, making a total of four replicate determinations.

Results and Discussion

There were no significant differences between the groups with respect to the matching characteristics (see Table 1), or lipid profiles (Table 3).

Table 1 Description of Subjects. All values are means ± *s*.

	Age/yrs	Sex, M : F	Height/m	Mass/kg	BMI*/kg m^{-2}	Current smokers	HbA_{1c} (%)
Controls (n = 26)	53.3 ± 14.3	10 : 16	1.69 ± 0.09	76.5 ± 14.3	26 ± 5	6	—
Diabetics (n = 26)	49.8 ± 17.6	14 : 12	1.70 ± 0.10	71.0 ± 12.8	24 ± 4	4	6.9 ± 1.9

* Body mass index (mass/height^2).

Table 2 Graphite furnace atomizer program

Step	Final Temperature/°C	Ramp Time/s	Hold Time/s	Gas flow/ ml min^{-1}	Notes
1	100	5	0	300	Dry I
2	120	60	0	300	Dry II
3	300	10	0	300	Oxygen Ash
4	900	10	30	300	Argon Ash I
5	900	1	2	0	Argon Ash II
6	2000	0	2	0	Read
7	2500	1	3	300	Clean
8	20	10	0	300	Cool

The concentration of copper in the mononuclear cells was 30% lower in diabetics, compared with that in the controls (Table 4). There were no significant differences in granulocyte copper, or zinc in the white blood cell fractions, although in all instances there was a tendency for concentrations in these fractions to be lower in diabetics. In the plasma of diabetics, however, zinc was significantly reduced (by 17% on average), compared with the controls. There was no significant difference in plasma copper between the groups. Similarly, the copper concentrations in washed or unwashed red blood cells was not different, nor was the red blood cell SOD activity (Table 4).

Correlation analysis showed a negative association between washed red blood cell copper and duration of diabetes (Fig. 1). The correlation between washed red blood cell copper and SOD activity did not reach statistical significance ($r = 0.3762$, $P = 0.1510$). The correlations between washed red blood cell copper and age of the subject were not significant for either the diabetic or control groups (data not shown).

The suggestion that diabetes is associated with a decreased status of copper and zinc has been investigated in several studies. For example, rats made diabetic by the injection of streptozotocin excrete more copper and zinc in their urine than untreated controls, and there are changes in tissue mineral concentrations. Furthermore, the administration of insulin normalizes these changes, although not completely.[21–23]

Several studies[13,15,16,24] have reported a decreased plasma zinc concentration in diabetes, which is in agreement with the results of this study. The situation for plasma copper is less clear: some authors report an increase,[11,24] some a decrease,[13] and others no change,[21] as reported here. Plasma copper is mainly bound to caeruloplasmin, an acute phase protein, which can change owing to a variety of conditions not directly related to copper metabolism. Zinc, however, is mainly bound to albumin, the concentration of which is much more stable.

Our results are consistent with those of other human studies where white blood cell copper and zinc were determined. Sjörgen *et al.*[16] studied a group of 18 adult diabetics, compared with 26 healthy controls. These authors reported an 11% reduction in plasma zinc, which compares well with the 17% reported here. The authors also measured skeletal muscle zinc, and found that this was reduced by 6% in diabetics. Again, this compares well with the zinc concentrations found in the present study: granulocyte (decreased by 13%, not significant) and mononuclear cell (decreased by 5%, not significant). A correlation between skeletal muscle and white blood cell (but not plasma) zinc has been reported.[19] Pai and Prasad[15] compared results from 16 diabetics with 19 controls. They reported decreased zinc concentrations in plasma (10%), granulocytes (21%), and lymphocytes (the main mononuclear cell, 23%) in diabetics, although this group reported white blood cell zinc concentrations which were lower than those reported here. They also reported a significantly decreased activity of lymphocyte nucleoside phosphorylase, a zinc-dependent enzyme. Nath *et al.*[14] studied 8 diabetic and 8 control subjects and found a 25% reduction in granulocyte zinc and 42% reduction in granulocyte copper (present data: 13 and 18% reduction, respectively; not significant). These differences are larger than those reported here, but Nath *et al.*[14] expressed the results per amount of protein in the cell, which might account for some of the difference. This group also measured the SOD activity in the granulocytes, and found it to be significantly reduced in the diabetics.

The mean SOD activity in the washed red blood cells was 13% lower in diabetics compared with the controls, although this failed to reach statistical significance (Table 4). The corresponding red blood cell copper concentration was only

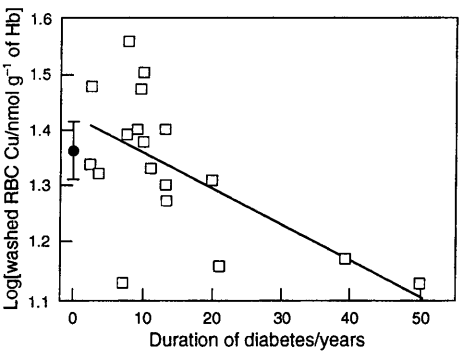

Fig. 1 Correlation between washed red blood cell (RBC) copper concentration and duration of diabetes. Filled circles, controls (mean ± 95% confidence interval); and open squares, diabetics ($r = -0.613$; $P = 0.0069^{**}$; and $n = 18$).

Table 3 Plasma lipids. Values are means (95% confidence interval)

	Total Cholesterol/ mmol l^{-1}	HDL Cholesterol/ mmol l^{-1}	LDL Cholesterol/ mmol l^{-1}	Triglycerides/ mmol l^{-1}
Controls ($n = 26$)	6.01 (5.61–6.44)	1.09 (0.98–1.21)	4.24 (3.89–4.63)	1.29 (1.09–1.52)
Diabetics ($n = 26$)	5.76 (5.18–6.41)	1.16 (1.03–1.30)	3.79 (3.37–4.27)	1.25 (0.91–1.71)

Table 4 Red cell, white cell, and plasma copper and zinc results. Values are means (95% confidence interval)

	Controls ($n = 26$)	Diabetics ($n = 26$)	P	
Plasma Cu/μmol l^{-1}	15.6 (14.3–17.1)	14.0 (12.3–15.8)	0.1472	NS*
Plasma Zn/μmol l^{-1}	12.5 (11.9–13.1)	10.4 (9.7–11.2)	0.0001	—
Granulocyte Cu/nmol per 10^9 cells	4.69 (3.60–6.12)	3.85 (2.74–5.41)	0.3470	NS
Granulocyte Zn/nmol per 10^9 cells	109.2 (93.5–127.7)	94.5 (78.4–113.9)	0.2239	NS
Mononuclear Cell Cu/nmol per 10^9 cells	11.34 (9.36–13.74)	7.95 (6.93–9.13)	0.0035	—
Mononuclear Cell Zn/nmol per 10^9 cells	158 (137–181)	150 (122–184)	0.6610	NS
Unwashed RBC Cu/nmol g^{-1} Hb	47.0 (42.4–52.1)	50.5 (45.8–55.6)	0.3034	NS
Washed RBC Cu/nmol g^{-1} Hb	23.0 (20.5–25.9)	21.7 (18.7–25.1)	0.4905	NS
RBC SOD/U g^{-1} Hb	1383 (1232–1535)	1209 (1070–1348)	0.0872	NS

* NS, Not significant.

reduced by 6%. Part of this difference, and indeed the lack of a correlation between these variables, might be explained by non-enzymic glycation of the SOD protein resulting in a reduced activity of this enzyme. However, we did not find a correlation between SOD activity and glycated haemoglobin (HbA_{1c}) concentration. Another possibility is that the SOD enzyme is vital for the red blood cell, as it is exposed to high oxygen tensions. Copper may be preferentially lost from other pools to spare the activity of this important antioxidant enzyme. SOD activity is not uniform for all tissues, for example, streptozotocin-diabetic rats show organ-specific changes in Cu,Zn-SOD activity: decreased in the liver and kidney, increased in the heart, and no change in the pancreas.[25] Therefore red blood cell SOD activity might not be ideal as a marker of copper status in diabetes. The SOD activities of the white blood cell fractions may provide better information.

The copper and zinc deficiencies reported here and elsewhere could be caused by the high urinary loss of these metals.[15–17,21] However, an impaired intestinal absorption of zinc has been reported in subjects with diabetes,[26] indicating that other mechanisms may contribute.

Decreased copper status may be extremely important for the long-term outcome of diabetes. For example, copper deficiency, as assessed by white blood cell copper concentrations, has been implicated as a risk factor in the development and progression of cardiovascular disease.[27]

If there is an increased oxidative stress in diabetes,[3] and a gradual but continual loss of the antioxidant trace elements copper and zinc in the urine as indicated above, then diabetics will be at increased risk of oxidative damage, this risk increasing with the duration of the disease. If the complications of diabetes are due at least in part to oxidative damage, then this sequence of events could explain the increased risk of complications that diabetic patients suffer. This hypothesis also opens the possibility of treating, or possibly even preventing, these complications by oral supplementation of the lost nutrients. Such studies would have to be long-term (lasting several years) and involve many patients (perhaps several hundred), but would be the only way to definitively answer these questions.

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