Response of putative indices of copper status to copper supplementation in human subjects

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No sensitive functional index is currently available to assess Cu status in healthy human populations. This study evaluated the effect of Cu supplementation on putative indices of Cu status in twelve women and twelve men, aged between 22 and 45 years, who participated in a double-blind placebo controlled crossover study. The study consisted of three 6-week supplementation regimens of 3 mg $CuSO_4$, 3 mg Cu-glycine chelate and 6 mg Cu-glycine chelate, each separated by placebo periods of equal length. Women had significantly higher caeruloplasmin oxidase activity ($P < 0{\cdot}001$), caeruloplasmin protein concentration ($P < 0{\cdot}05$), and serum diamine oxidase activity ($P < 0{\cdot}01$) at baseline than men. Erythrocyte and leucocyte superoxide dismutase activity, leucocyte cytochrome *c* oxidase activity, and erythrocyte glutathione peroxidase activity did not respond to Cu supplementation. Platelet cytochrome *c* oxidase activity was significantly higher ($P < 0{\cdot}01$), after supplementation with 6 mg Cu-glycine chelate in the total group and in women but did not change in men. Caeruloplasmin oxidase activity was significantly higher ($P < 0{\cdot}05$), in men after supplementation with 3 mg Cu-glycine chelate, while caeruloplasmin protein concentration was significantly lower in men after supplementation with 6 mg Cu-glycine chelate ($P < 0{\cdot}05$). Serum diamine oxidase activity was significantly higher after all supplementation regimens in the total group and in both men and women ($P < 0{\cdot}01$). These results indicate that serum diamine oxidase activity is sensitive to changes in dietary Cu intakes and may also have the potential to evaluate changes in Cu status in healthy adult human subjects.

Copper: Diamine oxidase

Cu has long been established as an essential micronutrient for health in both animals and humans. Clinically defined Cu deficiency in humans, however, remains rare and has only been described in a small number of patients (Danks, 1988). Severe Cu deficiency, as diagnosed by a marked depression in circulating Cu and caeruloplasmin concentrations, is accompanied by anaemia and neurological, arterial and myocardial problems (Solomons, 1979; Turnlund, 1994). Sub-optimal Cu status, however, may be present within populations and remain undetected as it is not associated with any overt clinical symptoms (Milne, 1994), and no suitable, sensitive biochemical index of Cu status is currently available (Olivares & Uauy, 1996).

The commonly used putative methods of Cu status assessment, serum and plasma Cu protein concentrations and caeruloplasmin oxidase activity, suffer from inherent problems in their interpretation. Serum or plasma Cu concentrations rise in conditions of stress. Caeruloplasmin is an acute-phase protein and concentrations are increased during inflammation, in smokers and in women using oral contraceptives and hormone replacement therapy (DiSilvestro, 1990; Fisher *et al*. 1990; Johnson *et al*. 1992).

Erythrocyte superoxide dismutase (SOD) activity is cited as an alternative, functional means of assessing Cu status, but may not be sensitive to supplementation and to small changes in Cu status (Turnlund *et al*. 1990). Moreover, erythrocyte SOD activity displays a high intra-individual variation (Gallagher *et al*. 1989), and may be affected by conditions other than Cu status (DiSilvestro, 1988; Nielsen *et al*. 1990). Other Cu-metalloenzymes, such as platelet cytochrome *c* oxidase (CCO) activity and serum peptidylglycine α-amidating monooxygenase activity, have also been proposed as alternative, sensitive indices of Cu status (Johnson *et al*. 1993; Milne & Forrest, 1996; Prohaska, 1997). Erythrocyte glutathione peroxidase (GPX), although not a Cu-dependent enzyme, appears to have lower activity

Abbreviations: CCO, cytochrome *c* oxidase; CuGC, copper-glycine chelate; DAO, diamine oxidase; GPX, glutathione peroxidase; Hb, haemoglobin; SOD, superoxide dismutase.

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during Cu depletion and has been suggested as an alternative to traditional indices of Cu status (Milne & Forrest, 1996).

Another Cu-metalloenzyme, which has shown promise as a functional index of Cu status in human subjects (Jones *et al*. 1997), is diamine oxidase (DAO). The latter enzyme is responsible for the oxidative deamination of diamines, such as putrescine and cadaverine, and histamine (Wolvekamp & de Bruin, 1994), and a sensitive colorimetric method for the assessment of serum DAO activity has been described (Takagi *et al*. 1994). Plasma DAO activity has been reported to be low in a human subject with Cu deficiency and to be able to differentiate between Cu-deficient and Cu-adequate rats (DiSilvestro *et al*. 1997; Kehoe *et al*. 2000). The current study was conducted to investigate the response of several putative indices of Cu status, including serum DAO activity, to Cu supplementation in young men and women. Data emanating from the study should indicate the potential of these putative indices as indicators of sub-optimal Cu status in healthy populations.

Subjects and methods

Subjects and supplementation regime

Twenty-four healthy free-living subjects (twelve female and twelve male), aged 22–45 years, were recruited from the staff and student population of the University of Ulster to participate in the double-blind, placebo controlled crossover study. Reasons for exclusion included smokers, those on medication, vitamin or mineral supplements, and the presence of disease such as diabetes or hypertension. The study protocol was reviewed and approved by the Ethical Committee of the University of Ulster. The study consisted of three separate 6-week supplementation regimens of 3 mg $CuSO_4$, 3 mg copper-glycine chelate (CuGC), and 6 mg CuGC/d, each separated by placebo periods of equal length. Habitual dietary information was collected from each subject by means of a diet history (Livingstone *et al*. 1992) by a trained investigator at the beginning and on completion of the study. The records were then analysed using food portion sizes (Crawley, 1992) and nutrient intake calculated using the computer package COMP-EAT (Nutrition Systems, London, UK). The subjects' profiles, including age, BMI, and average Cu and energy intake at baseline and Cu and energy intake at the end of the study are shown in Table 1.

Laboratory procedures

Blood samples were collected after an overnight fast by antecubital venepuncture into evacuated heparinized, citrated, and plain tubes from each subject at baseline (two samples, 2 weeks apart), and at the end of each separate supplementation or placebo period. Heparinized samples were centrifuged (1800 ***g***, 15 min at 4°C) and 200 μl plasma aliquots were stored at −80°C until analyses for caeruloplasmin oxidase activity and caeruloplasmin protein concentrations. The remaining blood sample was diluted to the original blood volume with 0·01 M-PBS pH 7·3 and centrifuged (2000 ***g***, 20 min at 25°C). The buffy layer was then aspirated from the sample, added to cold saline and centrifuged (2000 ***g***, 10 min at 25°C), the supernatant discarded and the leucocyte pellet resuspended in 6 ml distilled H_2O. A volume of 2 ml 3·4 % w/v NaCl (w/v) was added to the suspension, which was then centrifuged (2000 ***g***, 10 min at 25°C); this procedure was repeated three times. The resuspended pellet was divided into two, centrifuged (2000 ***g***, 10 min at 25°C), and then resuspended in 150 μl either 0·01 M-PBS buffer, pH 7·3 or 5 mM-PBS–EDTA buffer, pH 7·4, for leucocyte SOD and CCO determinations respectively. Leucocyte suspensions were stored at −80°C until analysis. Erythrocytes obtained after the aspiration of the buffy layer were resuspended in the original blood volume with 0·9 % NaCl solution and washed three times by centrifugation (2000 ***g***, 10 min at 25°C). A 500 μl aliquot of washed erythrocytes was stored at −80°C until analysis for haemoglobin (Hb), and for SOD and GPX activities. Citrated blood samples were centrifuged to remove the platelet-rich plasma (160 ***g***, 20 min at 25°C). This was further centrifuged to obtain the platelet pellet (730 ***g***, 10 min at 25°C). The pellet was resuspended to the original plasma volume with 5 mM-PBS–EDTA, pH 7·4, washed three times, and finally resuspended in 0·6 ml 5 mM-PBS–EDTA, pH 7·4. Analyses for CCO activity and protein concentrations were performed on the fresh platelet extractions. Serum samples were prepared by centrifuging clotted whole blood samples (2000 ***g***, 15 min at 4°C). These were stored at −80°C until analysis. All frozen samples were analysed in batch and in duplicate within 10 months of completion of the study.

Protein concentrations were determined using the method of Bradford (1976) (Bio-Rad Laboratories, Herts., UK), with bovine serum albumin as standard. The determination of Hb concentrations was performed using the cyanmethaemoglobin method. Erythrocytes (40 μl) were diluted

Table 1. Subject characteristics and dietary profiles
(Mean values and standard errors of the mean)

	Men (*n* 9)		Women (*n* 10)	
	Mean	SEM	Mean	SEM
Age (years)	31·3	8·23	33·1	8·23
BMI	23·2	3·47	25·0	1·88
Energy (kJ/d, week 0)	10280	640	8150	410
Energy (kJ/d, week 39)	11480	570	8810	530
Dietary copper (mg/d, week 0)	1·38	0·1	1·08	0·1
Dietary copper (mg/d, week 39)	1·59	0·1	0·98	0·1

with 20 ml ISOTON (Coulter Electronics Ltd, Luton, Beds., UK), in a plastic vial (Sterilin, BDH Ltd, Poole, Dorset, UK). The samples were mixed by inversion and six drops ZAPOGLOBIN (Coulter Electronics Ltd) added. The samples were left for 5 min at room temperature and the haemoglobin concentration was determined within 1 h, in g/dl, using a haemoglobinometer (Coulter Electronics Ltd).

Plasma caeruloplasmin oxidase activity was determined by a modification of the method by Henry *et al*. (1960), using *p*-phenylenediamine dihydrochloride (Sigma Aldrich Co Ltd, Poole, Dorset, UK) as substrate and measuring the rate of oxidation of *p*-phenylenediamine dihydrochloride at 37°C. Analysis was performed on the Cobas Fara automatic analyser (Roche, Basel, Switzerland). Caeruloplasmin protein concentrations were measured turbidimetrically using a modification of the method of Calvin & Price (1986). Using dilution buffer, pH 7·3, plasma was prediluted 1 : 41 (v/v) and mixed with excess rabbit anti-human caeruloplasmin (Dako, Glostrup, De nmark). The resulting antibody–antigen complex was stabilised with a phosphate buffer containing polyethylene glycol 6000 (BDH Ltd). The absorbance at 340 nm was measured on the Cobas Fara automatic analyser (Roche), and concentrations (U/l) were determined from a standard curve calculated using a human serum protein calibrator (Dako).

The activity of SOD was determined on the Cobas Fara automatic analyser (Roche) by a modification of the method of Jones & Suttle (1981), using a commercial kit, RANSOD. (Randox Laboratories, Co. Antrim, Northern Ireland). Activity of SOD was expressed per g Hb in erythrocytes, and per mg protein in leucocytes.

Erythrocyte GPX activity was assessed by a modification of the method of Paglia & Valentine (1967) using a commercially available kit, RANSEL (Randox Laboratories). The assay was performed on the Cobas Fara automatic analyser (Roche). GPX activity was expressed as U/g Hb.

The assay for CCO activity (U/g protein) was performed on fresh platelet suspensions and leucocytes using a modification of the method described by Smith (1955). Immediately prior to running the assay, 0·2 ml of the platelet suspension was sonicated at an amplitude of 10 μm/6 s. The substrate cytochrome *c* (Sigma) was reconstituted in 0·1 M-K_2PO_4 buffer, pH 7·0, and reduced with sodium dithionite (Sigma). The excess sodium dithionite was removed by separation using a G-25 Sephedex column (Pharmacia Biotech, Uppsala, Sweden), and the final concentration was standardised at 57·7 mM (±0·3 %) to ensure a concentration of 50 μM in the final reaction mix. The CCO activity was determined on the Cobas Fara automatic analyser (Roche) by measuring the loss of ferrocytochrome *c* at 550 nm at 37°C. A CCO (Sigma) buffered suspension, reconstituted in PBS buffer, was used as an in-house control for the CCO assay.

Serum DAO activity was determined on the Cobas Fara automatic analyser (Roche), using a modification of the method described by Tagaki *et al*. (1994). A substrate solution (36 mmol/l) was prepared by dissolving cadaverine dihydrochloride (Sigma) in a 25 mmol 1,4-piperazine-diethane sulphonic acid (PIPES) buffer/l (pH 7·2). The substrate solution, 130 μl, was incubated at 37°C for 5 min, after which 30 μl serum sample and 20 μl PIPES buffer were added, mixed and incubated for 30 min at 37°C. The colour solution was prepared by dissolving 10 μmol DA-67 (Wako Chemicals USA, Inc., Richmond, VA, USA) in 100 ml 25 mmol 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer/l (Sigma). Just before the addition of the colour solution to the assay, 6 U/ml peroxidase and 5 U/ml ascorbate oxidase (Sigma) were added to the colour solution. The development of methylene blue was initiated by the addition of 150 μl colour solution to the reaction mixture and the rate of colour development at 668 nm was measured over 12 min and quantified (U/l) by a standard curve prepared using DAO (Sigma) standards.

Data analysis

All data were analysed using the SPSS 6.1 for Windows statistical package (SPSS Inc., Chiago, IL, USA) and performed at each crossover only on subjects who completed both supplementation and placebo regimens. The presence of carryover was tested for at each crossover as outlined by Senn (1993). The effect of Cu treatment compared to placebo was analysed at each crossover by independent *t* tests of 'period differences' between supplementation sequences, a method that adjusts data for any possible period effects. One-way ANOVA was used to examine differences between the sexes at baseline. Unavoidable absences of subjects at particular blood sampling times account for differences in crossover numbers.

Results

Women had significantly higher plasma caeruloplasmin oxidase activity (725 (SE 59·1) U/l; $P < 0·001$), plasma caeruloplasmin protein concentration (0·24 (SE 0·2) g/l; $P < 0·05$), and serum DAO activity (0·24 (SE 0·29) U/l; $P < 0·01$) than men (552 (SE 44·4) U/l, 0·16 (SE 0·01) g/l and 0·10 (SE 0·13) U/l respectively) at baseline. Erythrocyte and leucocyte SOD, and erythrocyte GPX showed no changes in activity in the total group or in male and females separately after any Cu supplementation regimen (Table 2). Platelet CCO activity was significantly higher following supplementation with 6 mg CuGC than during the placebo regimen ($P < 0·01$) in the total group and in women but not in men (Table 2). Significant carryover effects were observed in platelet CCO activity in women during crossover with 3 mg CuGC ($P < 0·05$) and in leucocyte CCO activity in men during each crossover: 3 mg $CuSO_4$ ($P < 0·05$), 3 mg CuGC ($P < 0·01$), 6 mg CuGC ($P < 0·05$) (Table 2). No significant difference was observed in the response of plasma caeruloplasmin oxidase activity and caeruloplasmin protein concentration to any Cu supplementation regimen in the total group or in women. Men showed a significant increase in plasma caeruloplasmin oxidase activity after supplementation with 3 mg $CuSO_4$ ($P < 0·05$), and plasma caeruloplasmin concentration was significantly decreased after supplementation with 6 mg CuGC ($P < 0·05$). Serum DAO activity increased significantly during all three Cu-supplementation regimens. This increase was observed in the total group and in both men and women separately ($P < 0·01$) (Table 2).

Table 2. Response of putative indices of copper status to different levels of copper supplementation in the total group, and in men and women separately‡

(Mean values with standard errors)

	Crossover 1					Crossover 2					Crossover 3				
	3 mg $CuSO_4$/d		Placebo 1			3 mg CuGC/d		Placebo 2			6 mg CuGC/d		Placebo 3		
	Mean	SE	Mean	SE	*n*	Mean	SE	Mean	SE	*n*	Mean	SE	Mean	SE	*n*
Erythrocyte superoxide dismutase (U/g Hb)															
Total	1166	38	1077	33	23	1068	41	1032	29	21	1042	39	1049	48	19
Men	1107	50	1115	45	12	1123	77	1038	45	9	1096	53	1138	32	8
Women	1022	55	1035	48	11	1028	42	1040	38	12	1003	55	984	31	11
Leucocyte superoxide dismutase (U/g protein)															
Total	1·04	0·09	1·23	0·14	23	1·81	0·16	1·89	0·24	20	1·66	0·19	1·81	0·20	20
Men	0·98	0·13	1·15	0·14	12	1·98	0·27	2·39	0·49	8	2·09	0·25	1·76	0·27	9
Women	1·11	0·13	1·32	0·26	11	1·70	0·20	1·55	0·18	12	1·31	0·24	1·85	0·29	11
Platelet cytochrome *c* oxidase (U/g protein)															
Total	7·10	0·50	6·76	0·31	17	7·46	0·20	7·35	0·38	18	7·35**	0·25	6·33	0·15	14
Men	6·95	0·65	6·99	0·42	10	7·65	0·23	7·11	0·65	8	7·71	0·51	6·54	0·27	6
Women	7·32	0·81	6·67	0·46	7	7·31	0·30	7·55†	0·44	10	7·08**	0·19	6·17	0·17	8
Leucocyte cytochrome *c* oxidase (U/g protein)															
Total	1·96	0·29	2·31	0·37	21	4·37	1·26	4·26	1·44	17	3·97	0·60	4·56	0·77	20
Men	2·17	0·36	2·17†	0·64	10	2·84	0·85	3·36††	0·68	8	4·90	1·03	4·29†	1·07	9
Women	1·77	0·47	2·16	0·42	11	5·61	2·13	4·98	2·57	9	3·21	0·66	4·79	1·14	11
Erythrocyte glutathione peroxidase (U/g Hb)															
Total	45·6	2·0	46·2	1·9	23	47·5	2·4	47·4	2·1	21	48·2	2·2	48·4	2·4	20
Men	42·6	3·0	44·4	3·1	12	48·7	4·0	47·0	3·3	9	47·3	3·1	46·8	4·1	9
Women	48·9	2·4	48·6	2·1	11	46·6	3·2	47·6	2·8	12	49·0	3·2	46·8	3·0	11
Caeruloplasmin oxidase (U/l)															
Total	637	42·4	627	34·0	22	621	34·1	621	42·0	21	601	35·6	612	35·2	20
Men	545	36·9	566	25·2	11	537*	29·4	527	28·2	9	546	32·8	535	31·4	9
Women	729	67·0	687	59·1	11	639	48·9	691	64·1	12	647	56·7	676	52·3	11
Caeruloplasmin protein (g/l)															
Total	0·19	0·01	0·19	0·01	22	0·21	0·01	0·21	0·02	21	0·20	0·01	0·21	0·01	20
Men	0·17	0·01	0·18	0·01	11	0·18*	0·01	0·16	0·01	9	0·18*	0·01	0·19	0·01	9
Women	0·21	0·02	0·21	0·02	11	0·23	0·02	0·25	0·03	12	0·22	0·02	0·22	0·02	11
Serum diamine oxidase (U/l)															
Total	1·34**	0·31	0·30	0·29	16	1·63**	0·48	0·44	0·11	20	2·00**	0·11	0·89	0·22	17
Men	0·87**	0·27	0·27	0·17	7	1·21**	0·36	0·47	0·01	9	1·55**	0·49	0·44	0·28	7
Women	1·64**	0·47	0·33	0·17	9	1·92**	0·41	0·42	0·23	11	2·37**	0·79	1·16	0·32	10

CuGC, copper-glycine chelate; Hb, haemoglobin.
Mean value was significantly different from placebo group, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.
Significant carryover effect, $\dagger P < 0{\cdot}05$, $\dagger\dagger P < 0{\cdot}01$.
‡ For details of subjects and procedures see Table 1 and p. 152.

Discussion

The findings from the current study indicate that serum DAO activity shows a marked response to Cu supplementation in healthy subjects. This increase in DAO activity was evident in both men and women and after each Cu-supplementation period. These results suggest that serum DAO activity is responsive to Cu intake and has the potential to be a sensitive indicator of Cu status in human subjects.

Serum DAO activity, however, is raised in pregnancy (Kusche *et al*. 1974), uraemia (Tam *et al*. 1979), kidney-dialysis patients (DiSilvestro *et al*. 1997) and after gastrointestinal damage (Wolvekamp & deBruin, 1994), and is reportedly lowered in Crohn's disease and coeliac disease (Bayless *et al*. 1981). Tagaki *et al*. (1994) have questioned the significance of lowered serum DAO activity in these latter diseases as basal activity of serum DAO is very low in normal healthy individuals. We also found very low serum DAO activity in our subjects at baseline and at the end of the placebo periods. It is possible, therefore, that the rise in serum DAO activity after Cu supplementation is simply reflecting alterations in gut integrity caused by ingestion of the supplement. Our subjects, however, did not report any adverse side effects during the study. Moreover, DiSilvestro *et al*. (1997) have found significantly lower plasma DAO activity in Cu-deficient rats and low plasma DAO activity in a woman with spontaneous Cu deficiency who responded to parenteral Cu repletion by increases in plasma caeruloplasmin protein concentration and caeruloplasmin oxidase activity accompanied by an increase in plasma DAO activity. Data from our laboratory show that marginally Cu-deficient rats have significantly lower plasma DAO activity and liver Cu content but no differences in other indices of Cu status compared with Cu-adequate controls (Kehoe *et al*. 2000).

Taken together, these findings imply that serum or plasma DAO activity can also respond to changes in Cu status as well as to alterations in intestinal structural integrity.

Serum DAO activity was higher in women than men at baseline. This Cu-metalloenzyme showed similar sex differences to the well-documented higher caeruloplasmin oxidase activity and protein concentrations in women compared with men (Fisher *et al*. 1990; Johnson *et al*. 1992; Milne & Johnson, 1993). It is unlikely that the small increase observed in plasma caeruloplasmin activity after 3 mg CuGC or the small decrease in caeruloplasmin protein after 6 mg CuCG in our male subjects is of physiological significance. No changes were noted in plasma caeruloplasmin protein concentrations in sixteen healthy infants after supplementation with 9·1 μmol Cu/d for the first year of life (Salmenpera *et al*. 1989) and in seven adults receiving 10 mg Cu-gluconate for 12 weeks (Pratt *et al*. 1985). In contrast to the present study, in a similar placebo-controlled intervention, increases in serum caeruloplasmin protein concentration were observed in eight healthy male subjects after supplementation with Cu-gluconate (2 mg Cu/d) for 6 weeks (Medeiros *et al*. 1991).

The significant increase in platelet CCO activity (only after 6 mg/d CuGC supplementation) seen in our subjects, and which was largely driven by increased activity in women, is apparently at variance with the reported sensitivity of this enzyme as an indicator of Cu status based on depletion studies in women (Milne & Forrest, 1996), and men (Turnlund *et al*. 1997) where both mononucleated cell and platelet CCO activities were analysed. The significant carryover that was observed in several of the crossover regimens in the current study with this index, however, is a major confounding problem. In the absence of any effective method of adjusting data for carryover, this phenomenon can only be noted (Senn, 1993), and limits the interpretation of results of CCO activity, as the carryover effect may mask a possible treatment effect in the current study.

The responses of Cu-metalloenzymes, such as erythrocyte SOD activity, appear to be sensitive indicators of changes in Cu status in depletion studies (Milne & Forrest, 1996; Turnlund *et al*. 1997), but do not seem to respond to supplementation in healthy subjects. For example, Medeiros *et al*. (1991) found no significant difference in SOD activity of men supplemented with 3 mg Cu-gluconate/d for 6 weeks when compared to controls. Our observation that supplementation with different Cu compounds and at different Cu concentrations did not alter SOD activity is in agreement with these findings. It should be noted that mean dietary Cu intakes in our study were 1·38–1·59 mg/d for men and 1·08–0·98 mg/d for women and supplementation with 6 mg CuGC would increase intakes of Cu to greater amounts than those usually reported in western diets (Klevay *et al*. 1993).

In conclusion, the results of our study indicate that serum DAO activity increases after Cu supplementation in both healthy men and women and has the potential to be a sensitive functional indicator of Cu status in human subjects. As serum DAO activity has not been investigated extensively as an indicator of Cu status, future experimentation should further elucidate the specificity of the enzyme to changes in Cu status and the health consequences, if any, of low serum DAO activity.

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