

No effect of copper supplementation on biochemical markers of bone metabolism in healthy adults

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The influence of Cu supplementation of the usual diet for 6 weeks on biochemical markers of bone turnover and on putative indices of Cu status was investigated in healthy adults (twelve male and twelve female) aged 22–46 years, who participated in a double-blind placebo-controlled repeated crossover study. The study consisted of three 6-week supplementation regimens of 3 mg CuSO₄, 3 mg Cu–glycine chelate (CuGC), and 6 mg CuGC, each separated by placebo periods of equal length. During baseline and on the last day of each dietary period, fasting morning first-void urine and fasting blood serum, plasma and erythrocytes were collected. The habitual dietary Cu intakes in males and females were approximately 1.4 and 1.1 mg/d respectively. Females had significantly higher (50%) plasma caeruloplasmin (Cp) protein concentrations than males at baseline. Cu supplementation had no effect on erythrocyte superoxide dismutase (SOD, EC 1.15.1.1) activity or plasma Cp protein (putative indices of Cu status) in the total group. Similarly, serum osteocalcin (a marker of bone formation), urinary creatinine (Cr) concentration, urinary pyridinoline : Cr or deoxypyridinoline : Cr excretion (markers of bone resorption) were unaffected in either the total group or in males and females separately, by any Cu supplementation regimen. It is concluded that Cu supplementation of the usual diet in healthy adult males and females had no effect on biochemical markers of bone formation or bone resorption over 6-week periods.

Copper: Bone: Biochemical markers

Cu has an important role in the metabolism of the skeleton where it performs a key catalytic function as a cofactor for lysyl oxidase (EC 1.4.3.13), a Cu-dependent extracellular amine oxidase which is important in the first step of the maturation (crosslinking) of collagen to form stable fibrils (O'Dell, 1981; Tinker *et al.* 1988). Studies in experimental animals have shown that Cu deficiency can greatly reduce the activity of lysyl oxidase in bone (Siegel *et al.* 1970) and impair the crosslinking of collagen in the organic bone matrix, at least in some bones (Robins *et al.* 1985; Farquharson *et al.* 1989; Jonas *et al.* 1993a), leading to diminished tensile strength of bone (Jonas *et al.* 1993b). Cu deficiency during fetal and postnatal development has been shown to produce skeletal abnormalities and fragility in various species including the rat, chick, pig, horse and rabbit (Hurley, 1981; Fell, 1987; Dolwett & Sorenson, 1988). In human subjects, disorders of Cu deficiency with skeletal defects, such as osteopenia, cupping and flaring of metaphyses of long bones, submetaphyseal fracture, and spontaneous

fractures, most frequently in the ribs, have been noted in Menke's syndrome (Al-Rashid & Spangler, 1971; Seely *et al.* 1972; Ashkenazi *et al.* 1973), an inherited disease of Cu deficiency in young children where the gastrointestinal absorption of Cu is severely limited (Danks, 1987). Skeletal defects such as osteoporosis, multiple fractures and a variety of bone developmental defects observed in preterm infants, in whom Cu nutritional status can be impaired, have been reported to respond to oral supplementation with Cu which resulted in complete healing of fractures and improvement in the other bone defects (Allen *et al.* 1982; Patterson, 1990; Schmidt *et al.* 1991).

There is some evidence for a role for Cu deficiency in age-related osteoporosis. For example, serum Cu levels of forty-six elderly patients with fractures of the femoral neck were reported to be significantly lower than those of a group of controls matched for age and sex (Conlan *et al.* 1990).

Recently, we have shown that a low dietary Cu intake

Abbreviations: Cp, caeruloplasmin; Cr, creatinine; CuGC, copper–glycine chelate; Dpyr, deoxypyridinoline; Pyr, pyridinoline; SOD, superoxide dismutase.
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(0.7 mg/d) for 6 weeks significantly increased the rate of bone resorption, as assessed by the urinary excretion of pyridinium crosslinks, in healthy adult males aged between 20 and 59 years (Baker *et al.* 1999). An increased rate of bone turnover in adults may be a risk factor for fracture (Riggs *et al.* 1996), because it exacerbates bone loss (Hansen *et al.* 1991). This may have consequences for bone health of many adults because while mean daily Cu intakes of adults in Europe (1.0–2.26 mg for males, 0.9–1.79 mg for females; Van Dokkum, 1995) are greater than or close to the UK reference nutrient intake (1.2 mg/d for adults; Department of Health, 1991) and the EU population reference intake (1.1 mg/d for adults; Report of the Scientific Committee for Food, 1993), there may be significant numbers of individuals with marginal Cu intakes. For example, Klevay *et al.* (1993) suggest that greater than 30% of diets in North America and Europe provide less than 1.0 mg/d.

Despite this evidence of marginal Cu intake and its effects on bone health, the effect of increasing dietary Cu intake above usual levels on bone mass or bone metabolism has received little attention. Eaton-Evans *et al.* (1996) have shown that Cu supplementation with an additional 3 mg/d for 2 years in middle-aged women (45–56 years) with a usual dietary Cu intake of approximately 1 mg/d, led to a reduced rate of loss of bone-mineral density at the lumbar spine. We have shown that increasing dietary Cu intake in healthy adult males (aged 20–59 years) from a relatively low (0.7 mg/d) to a relatively high level (6.0 mg/d) for 6 weeks significantly decreased the rate of urinary pyridinium crosslink excretion (Baker *et al.* 1999). However, this was a depletion–repletion type study and therefore probably not representative of increasing dietary Cu intake above usual levels.

The objective of the present study, therefore, was to investigate the effects of increasing Cu intakes, above the usual dietary intake, on biomarkers of bone metabolism in healthy adults (aged 22–46 years) over a 6-week period.

Methods

Subjects

Twenty-four healthy adults (twelve females and twelve males, mean age 31.3 (range 22–46) years) were recruited from among staff and students at University of Ulster. Males were taller (1.80 (SD 0.01) m) and heavier (82.2 (SD 2.76) kg) than females (1.62 (SD 0.01) m and 61.3 (SD 2.77) kg respectively) and their mean BMI (25.4 (SD 0.7) kg/m²) was greater than that of females (23.2 (SD 0.8) kg/m²). The subjects were apparently healthy, without any history of bone or articular disease, and with no intake of medicine that could affect bone or cartilage metabolism. Additional exclusion criteria included smoking, chronic illness or taking nutritional supplements.

Ethical considerations

Before participation in this study, all subjects signed an informed consent document approved by the Ethical Committee of the University of Ulster.

Design

The study consisted of a double-blind, placebo-controlled repeat crossover trial of the effect of Cu supplementation of the usual dietary Cu intake, using various supplementation regimens, on biochemical markers of bone turnover in healthy adult subjects.

The Cu intervention trial was designed in six periods, i.e. three separate supplementation regimens of 3 mg elemental Cu as CuSO₄, 3 mg Cu as Cu–glycine chelate (CuGC), and 6 mg Cu as CuGC, each of 6 weeks duration and separated by placebo periods of equal length. Both organic (CuGC) and inorganic (CuSO₄) forms of Cu were used in the study to determine if there would be any alteration in the effect of Cu supplementation related to the form of Cu supplement used. Subjects were randomly assigned to the unsupplemented (placebo) or Cu-supplemented diet regimens for 6 weeks followed by crossover to the alternative dietary regimen for a further 6 weeks for each of the three separate Cu supplementation regimens. During the 6 week supplementation period each subject received, in addition to their usual diet (with self-selected Cu intake approximately 1 mg/d), either 3 mg elemental Cu/d, as CuSO₄, or 3 or 6 mg elemental Cu/d as CuGC (Thompson & Joseph Ltd, Norwich, Norfolk, UK). Subjects were requested to take the active capsule or matching placebo once daily with food. Subjects were questioned at each blood collection point to ascertain whether they had taken all supplements. Overall compliance of the subjects was excellent. Subjects were instructed to collect fasting first-void urine samples between 07.00 and 09.00 hours on the last day of each treatment period and during the week immediately preceding the trial (baseline). In addition, after an overnight fast, a blood sample (10 ml) was taken at 09.00 hours on the morning of the forty-second day of each treatment period and during the week immediately preceding the trial (baseline). Habitual dietary intake was assessed for each subject by means of a diet history at the beginning and on completion of the study.

Dietary analysis

Habitual dietary information (a typical month) was collected from each subject by means of a diet history (Livingstone *et al.* 1992) by a trained investigator. The records were then analysed using food portion sizes and a photographic album (Crawley, 1992) and the nutrient intake calculated using the computer package COMP-EAT (Nutrition Systems, London, UK).

Collection and preparation of samples

Portions of urine collected by each individual on each day of collection were stored at –20° from the morning of collection until required for analysis. Blood was collected by venepuncture into vacutainer tubes containing either no additive, citrate, or lithium heparin. Bloods collected in vacutainer tubes with no additive and with lithium heparin were processed to serum and plasma respectively, which were immediately stored at –80° until required. Whole bloods from vacutainer tubes with citrate were used on the

day of collection for isolation of erythrocytes. Erythrocytes were resuspended with 0.15 M-NaCl in the original blood volume and washed three times by centrifugation (2000 g for 10 min at 25°). A 500 µl portion of washed erythrocytes was stored at -80° until required for determination of haemoglobin concentration and superoxide dismutase (SOD; EC 1.15.1.1) activities.

Experimental techniques

Urinary pyridinoline and deoxypyridinoline. Samples were analysed in triplicate using a three-step procedure. Urine was first hydrolysed with an equal volume of 12 M-HCl at 110° for 18 h, the crosslinks were then extracted by CF1 cellulose chromatography with the use of an internal standard (acetylated pyridinoline; MetraBiosystems Ltd, Wheatley, Oxon., UK) and were measured using a reversed-phase HPLC method with fluorescence detection (Colwell *et al.* 1993). The acetylated pyridinoline (Pyr) was used in accordance with the method as described by Calabresi *et al.* (1994) and Robins *et al.* (1994). The crosslinks contents of urine samples were quantitated by external standardization using a commercially available Pyr/deoxypyridinoline (Dpyr) HPLC calibrator (MetraBiosystems Ltd). The intra-assay CV for Pyr and Dpyr measured as the variation between ten chromatograms obtained between column regenerations as described by Colwell *et al.* (1993) were 6% and 7% respectively. Inter-assay variation was avoided by analysing all samples from an individual in the same run.

Urinary creatinine. Fresh daily urine samples were analysed in duplicate by a colorimetric procedure using a diagnostic kit (catalogue no. 124, 192; Boehringer Mannheim GmbH, Mannheim, Germany). The intra- and inter-assay CV were 3.4% and 6.3% respectively.

Serum osteocalcin. Serum osteocalcin levels were measured using an ELISA (BRI-Diagnostics, Dublin 9, Republic of Ireland). The intra-assay CV was 10.2%. Inter-assay variation was avoided by analysing all samples from an individual in the same run.

Plasma caeruloplasmin content. Plasma caeruloplasmin (Cp) protein concentration was measured turbidimetrically using a modification of the method of Calvin & Price (1986). Plasma (pH 7.3) was diluted (1:41, v/v) with 0.01 M-PBS (pH 7.3) and mixed with excess rabbit anti-human Cp (Dako, Glostrup, Denmark). The resulting antibody-antigen complex was stabilized with a phosphate buffer containing PEG 6000 (BDH Ltd, Poole, Dorset, UK). The absorbance was measured on the Cobas Fara autoanalyser (Roche, Basel, Switzerland), and concentration was determined from a standard curve calculated using a human serum protein calibrator (Dako). The intra-assay CV was 1.7%. Inter-assay variation was avoided by analysing all samples from an individual in the same run.

Erythrocyte haemoglobin and superoxide dismutase. Determination of haemoglobin concentration was performed using the cyanomethaemoglobin method. Erythrocytes (40 µl) were diluted with 20 ml diluent (Iston; Coulter Electronics Ltd, Beds., UK) in a plastic vial (Sterilin, BDH Ltd). The samples were mixed by inversion and six drops of a lysing solution (Zapoglobin; Coulter Electronics Ltd) were added. The samples were left for 5 min at room

temperature, and the haemoglobin concentration was determined within 1 h, in g/l, using a haemoglobinometer (Coulter Electronics Ltd). Erythrocyte SOD activity 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, N. Ireland). Activity of SOD was expressed per g haemoglobin. The intra-assay CV was 2.7%. Inter-assay variation was avoided by analysing all samples from an individual in the same run.

Statistical analysis. Data are presented as means and standard deviations. Data for all variables were normally distributed and allowed for parametric tests of significance. Differences in age, height, weight and BMI between males and females were examined by unpaired Student's *t* tests. Differences in biochemical indices of Cu status and of bone turnover between males and females at baseline were examined by unpaired Student's *t* tests. Changes in the different biochemical indices (i.e. of Cu status and of bone turnover) during the placebo and Cu-supplemented periods were analysed at each crossover by the appropriate analysis for a crossover trial with continuous data as described by Jones & Kenward (1989) in which two-sample *t* tests are used to test hypotheses about direct treatments effects (i.e. Cu supplementation) and carry-over effects. Unavoidable absences of subjects at particular blood and urine sampling times account for differences in crossover numbers.

Results

Females (*n* 12) had significantly higher plasma Cp protein concentrations than males (*n* 12) at baseline (Table 1). There were no significant differences between males and females in baseline levels of urinary and serum biochemical indices of bone turnover or in erythrocyte SOD activity (a putative index of Cu status) (Table 1). The habitual dietary Cu intake in males was significantly greater than that in females, reflecting the higher energy intakes of the males (Table 1).

The effects of different Cu supplementation regimens on putative indices of Cu status and on serum and urinary biochemical indices of bone turnover are shown in Tables 2 and 3. Erythrocyte SOD, serum osteocalcin (a marker of bone formation), urinary creatinine (Cr) concentration, urinary Pyr:Cr or Dpyr:Cr excretion (markers of bone resorption) were unaffected in either the total group or in males and females separately, by any Cu supplementation regimen.

There was no significant difference in plasma Cp protein concentration between any of the unsupplemented (placebo) and Cu-supplemented dietary periods in the total group or in females. There was a significant decrease ($P < 0.05$) in plasma Cp protein concentration in males after supplementation with 6 mg Cu as the CuGC (Table 2).

Discussion

In the present study, the concentration of plasma Cp protein was higher in females than males at baseline whereas there was no difference in erythrocyte SOD activity, another putative index of Cu status, between males and females.

Table 1. Putative indices of copper status, biochemical markers of bone turnover and habitual dietary copper intakes in males and females at baseline*
(Mean values and standard deviations)

	Males (n 12)		Females (n 12)		Statistical significance of difference between means; P†
	Mean	SD	Mean	SD	
Blood:					
Plasma Cp (g/l)	0.16	0.03	0.24	0.07	< 0.001
Erythrocyte SOD (U/g Hb)	1133	111	1072	138	0.164
Serum osteocalcin (µg/l)	11.8	7.5	9.8	5.8	0.290
Urine:					
Pyr (nmol/mmol Cr)	34.4	16.1	35.3	13.0	0.867
Dpyr (nmol/mmol Cr)	11.7	6.2	11.3	4.8	0.856
Diet:					
Dietary Cu (mg/d)	1.38	0.30	1.08	0.32	0.042
Energy intake (MJ/d)	10.3	0.6	8.1	0.4	< 0.001

Cp, caeruloplasmin; SOD, superoxide dismutase (*EC* 1.15.1.1); Hb, haemoglobin; Pyr, pyridinoline; Dpyr, deoxypyridinoline; Cr, creatinine.

* For details of subjects and procedures, see pp. 284–285.

† Statistical analyses of biochemical indices of Cu status and of bone turnover between males and females using unpaired Student's *t* test.

This is in agreement with other studies which have shown a sex-related difference in Cp concentration (Fisher *et al.* 1990; Johnson *et al.* 1992; Milne & Johnson, 1993) but not in erythrocyte SOD activity (Johnson *et al.* 1992; Milne & Johnson, 1993). There was no effect of Cu supplementation with either 3 (as an organic or inorganic form) or 6 mg elemental Cu/d for 6 weeks on plasma Cp levels or on erythrocyte SOD activity in the total group or in the males or females separately, except for a small decrease in Cp protein after daily supplementation with 6 mg elemental Cu as CuGC in the males, which was probably not of any physiological significance. These findings are in general agreement with the findings of other Cu intervention trials in healthy adults which showed a lack of response of circulating Cp protein levels and erythrocyte SOD activity to daily supplementation with 2–3 mg Cu (Medeiros *et al.* 1991; Eaton-Evans *et al.* 1996).

Together, these data support the contention that circulating Cp concentration or erythrocyte SOD activity, which has been proposed as being more reflective of metabolically active Cu and Cu stores than is plasma Cp concentration (Milne, 1994, 1998), are not responsive to Cu supplementation of the usual diet in healthy subjects.

The findings of the present study showed that oral Cu supplementation of the usual diet (which contained approximately 1.4 and 1.1 mg Cu/d for males and females respectively) with either 3 or 6 mg elemental Cu/d for 6 weeks had no effect on urinary pyridinium crosslinks of collagen (Pyr and Dpyr, sensitive and specific biochemical markers of bone resorption (Eyre, 1992; Robins & New, 1997)) or serum osteocalcin (a sensitive biochemical marker of bone formation (Eyre, 1996)) in healthy adults. This is in contrast to the findings of our previous study which showed that increasing dietary Cu intake in healthy adult males from 0.7 to 6.0 mg/d over 6 weeks significantly decreased the rate of urinary excretion of pyridinium crosslinks (Baker *et al.* 1999). However, it should be noted that the later study was a depletion–repletion type study in which adult males were originally switched from a medium (1.6 mg/d) to low

(0.7 mg/d) intake of dietary Cu, and subsequently their Cu intakes were increased from the low to a high level (6.0 mg/d), each for 6 weeks. While decreasing the Cu intakes of the males significantly increased the urinary excretion of both Pyr (30%) and Dpyr (25%), this increased rate of urinary pyridinium crosslink excretion was reversed during the subsequent 6-week period of dietary Cu repletion (i.e. high-Cu diet) (Baker *et al.* 1999).

The findings of the present study agree with those of other Cu intervention trials which demonstrated that increasing dietary Cu intake above usual levels had no effect on the rate of bone formation, as assessed by biochemical markers (Pratt *et al.* 1985; Eaton-Evans *et al.* 1996). For example, Pratt *et al.* (1985) studied the effect of Cu supplementation of the usual diet in healthy adults on various biochemical variables, including serum alkaline phosphatase (*EC* 3.1.3.1), a biochemical marker of bone formation, and found that there was no significant difference in serum alkaline phosphatase activity between subjects who received 10 mg Cu/d (as copper gluconate) or placebo over a 12-week period. Similarly, Eaton-Evans *et al.* (1996) found that serum total alkaline phosphatase was unaffected by Cu supplementation (3 mg elemental Cu/d (as Cu–glycine chelate) for 2 years) in 45–56-year-old women. However, in the former study it is unclear whether the reported values refer to the activity of total alkaline phosphatase or the bone isoform of alkaline phosphatase which is a more sensitive and specific marker of bone formation (Delmas, 1992).

When taken together, therefore, the results of our present and previous studies suggest that while low Cu intakes (i.e. 0.7 mg/d) increase the rate of bone resorption, increasing dietary Cu intakes above approximately 1 mg/d (a level close to the recommended intake (Department of Health, 1991; Report of the Scientific Committee for Food, 1993)) had no effect on the rate of bone resorption or bone formation in healthy adults. While the underlying mechanisms for the osteoporotic-like lesions observed in bone in Cu deficiency disorders in animal species and human subjects are not

Table 2. Putative indices of copper status in the total group of healthy adults, and in males and females separately during unsupplemented (placebo) and copper-supplemented dietary periods*† (Mean values and standard deviations)

Dietary period ...	1st crossover			2nd crossover			3rd crossover					
	3 mg Cu (as CuSO ₄)			3 mg Cu (as CuGC)			6 mg Cu (as CuGC)			Placebo		
	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
Plasma Cp (g/l)												
Total	22	0.19	0.05	21	0.21	0.05	20	0.20	0.04	20	0.21	0.04
Males	11	0.17	0.03	9	0.18	0.03	9	0.18††	0.03	9	0.19	0.03
Females	11	0.21	0.07	12	0.23	0.07	11	0.22	0.07	11	0.22	0.07
Erythrocyte SOD (U/g Hb)												
Total	23	1166	182	21	1068	151	19	1042	170	19	1049	209
Males	12	1107	173	9	1123	206	8	1096	155	8	1138	90
Females	11	1022	182	12	1028	145	11	1003	182	11	984	103

CuGC, copper-glycine chelate; Cp, caeruloplasmin; Hb, haemoglobin.

* For details of subjects and procedures, see Table 1 and pp. 284–285.
 † Direct treatment effect (i.e. Cu supplementation) was analysed for each biochemical index at each crossover by two sample t tests of within group differences after testing for carry-over effects. No significant carry-over effects were found.
 †† Mean value was significantly different from that for the placebo period, †† P < 0.05.

understood, such abnormalities are generally considered to be owing to defective bone formation secondary to reduced lysyl oxidase activity (Danks, 1988). However, reduced lysyl oxidase activity with consequent reduction in collagen crosslinking does not readily explain the increased excretion of pyridinium crosslinks observed during the depletion period in our previous study (Baker *et al.* 1999). Similarly, an increased lysyl oxidase activity with consequent increase in collagen crosslinking does not readily explain the decreased excretion of pyridinium crosslinks during the repletion period in our previous study (Baker *et al.* 1999). It may be that changes in dietary Cu intake may have influenced osteoclastic bone resorption, as a consequence of faulty collagen crosslinking or by some other mechanism, e.g. direct effect on bone cell activity. An increased rate of bone resorption has been suggested as one explanation for the reduced bone mass observed in Cu-deficient rats (Strause *et al.* 1986). Furthermore, studies carried out *in vitro* have shown that Cu reduces active bone resorption, as measured by release of ⁴⁵Ca or hydroxyproline into the bathing medium from cultured explanted ⁴⁵Ca-labelled mouse calvaria bone grown in medium with or without the addition of Cu over a 48-h period (Wilson *et al.* 1981; Katz *et al.* 1984). The concentration of Cu used in media in these *in vitro* studies corresponded to physiological concentrations of Cu found in experimental mice. Wilson *et al.* (1981) suggested, based on their *in vitro* data, that a higher Cu concentration, but within the physiological range, reduces bone resorption. This may explain, at least in part, the findings of our previous study which suggest that the rate of bone resorption responded to changes in dietary Cu intake in the relatively short term (6 weeks) (Baker *et al.* 1999).

The findings from our present and previous studies (Baker *et al.* 1999), when taken together, suggest that there may be a threshold effect of dietary Cu on bone metabolism, whereby dietary Cu intakes above approximately 1 mg/d (a value similar to the recommended intake) are of no apparent benefit to bone metabolism in healthy adult males or premenopausal females. However, Eaton-Evans *et al.* (1996) reported that Cu supplementation of the usual diet with 3 mg/d for 2 years reduced the rate of loss of bone-mineral density at the lumbar spine in the 45–56 year-old-women whose usual dietary Cu intake was about 1 mg/d. There are several possible reasons why additional dietary Cu positively influenced bone mass in the study of Eaton-Evans *et al.* (1996). For example, as over half the females in the study were either perimenopausal or postmenopausal and therefore, likely to be of lower oestrogen status, this may have influenced the effect of Cu on bone. On the other hand, as the study was 2 years in duration it might be possible that bone collagen would be more crosslinked as a consequence of the higher Cu intake, and possibly more resistant to osteoclastic resorption. Alternatively, it may be that increasing dietary Cu intakes above the usual level does suppress the rate of bone resorption as a consequence of reduced osteoclastic activity, but that this may take up to 2 years before differences are apparent in bone-mineral density.

In conclusion, Cu supplementation of the usual diet in twelve healthy adult males (with a mean usual dietary Cu

Table 3. Urinary and circulating biochemical markers of bone turnover in the total group of healthy adults, and in males and females separately during unsupplemented (placebo) and copper-supplemented dietary periods*†
(Mean values and standard deviations)

Dietary period ...	1st crossover					2nd crossover					3rd crossover				
	3 mg Cu (as CuSO ₄)			Placebo		3 mg Cu (as CuGC)			Placebo		6 mg Cu (as CuGC)			Placebo	
	<i>n</i>	Mean	SD	Mean	SD	<i>n</i>	Mean	SD	Mean	SD	<i>n</i>	Mean	SD	Mean	SD
Blood:															
Osteocalcin (μg/l)															
Total	23	10.1	5.8	11.4	6.1	22	10.3	4.6	10.0	5.2	21	9.4	4.8	10.3	5.5
Males	12	12.2	6.3	13.1	5.0	9	12.3	3.7	13.1	5.2	10	10.9	4.9	10.5	5.8
Females	11	7.7	4.4	9.4	6.8	12	8.8	4.7	7.7	4.1	12	8.0	4.6	10.1	5.5
Urine															
Creatinine (mmol/l)															
Total	22	16.1	7.4	18.3	8.9	20	14.2	6.4	16.8	8.6	20	15.1	8.0	14.8	7.6
Males	11	15.7	7.3	18.3	9.1	9	13.8	5.1	17.7	9.0	10	15.7	6.9	14.8	6.5
Females	10	14.7	8.3	18.2	9.3	11	15.1	7.3	16.9	8.7	9	14.5	9.3	15.4	9.2
Pyr (nmol/mmol Cr)															
Total	22	32.4	15.6	39.9	16.1	21	42.1	15.2	34.5	13.1	20	34.1	14.4	36.2	16.4
Males	11	36.6	10.6	41.3	19.5	9	44.2	16.1	36.9	12.7	10	29.9	13.5	34.9	16.9
Females	10	40.5	18.0	39.1	12.4	12	40.6	12.7	36.6	13.6	10	37.8	18.2	37.5	15.1
Dpyr (nmol/mmol Cr)															
Total	22	10.2	6.2	13.0	6.8	21	12.7	7.7	10.9	4.9	20	9.8	4.4	11.1	6.8
Males	11	8.9	5.5	13.5	7.8	9	13.3	7.7	11.5	6.1	10	9.0	3.9	11.9	8.8
Females	10	11.4	6.8	12.4	5.8	12	12.3	7.9	10.4	3.8	10	10.6	5.0	10.2	4.3

CuGC, copper–glycine chelate; Pyr, pyridinoline; Cr, creatinine; Dpyr, deoxypyridinoline.

* For details of subjects and procedures, see Table 1 and pp. 284–285.

† Direct treatment effect (i.e. Cu supplementation) was analysed for each biochemical index at each crossover by two sample *t* tests of within group differences after testing for carry-over effects. No significant carry-over or direct treatment effects were found.

intake of 1.4 (SD 0.3) mg/d and twelve females (with a mean usual dietary Cu intake of 1.1 (SD 0.3) mg/d) had no effect on biochemical markers of bone formation or bone resorption over 6-week periods. However, more research is needed to examine the effects of increased Cu intake on bone metabolism and bone mass in older adults, particularly peri- and postmenopausal females, and especially in long-term studies.

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