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Vitamin C and bone markers: investigations in a Gambian population

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Vitamin C is an essential micronutrient. Absence from the diet will result in the deficiency disease scurvy, typically characterised by weakening of collagenous structures. High intakes of vitamin C have been associated with decreased incidence or severity of a number of diseases, including cancer and cardiovascular disease. These beneficial effects may be attributed to its antioxidant properties, although the exact mechanisms of action remain elusive. It is also unclear what intake levels are required for optimal health benefits. The task of defining optimal intakes is hindered by the lack of a reliable functional marker of tissue vitamin C status in man. Many different pathways have been investigated, but none of them have measurable outcome variables relating directly to scorbutic changes. The bone-collagen formation pathway has the potential to provide a functional index of tissue vitamin C adequacy. Vitamin C acts as a cofactor for the enzyme lysyl hydroxylase, which is required for the hydroxylation of lysine residues in procollagen chains. Pyridinoline is a mature collagen cross-link formed from three hydroxylysine residues, deoxypyridinoline is formed from two hydroxylysine and one lysine residue. Guinea-pig studies have shown an alteration in the pyridinium cross-link ratios in response to graded vitamin C intakes (Tsuchiya & Bates, 1998). In order to investigate whether these changes can be seen in a human population group, a study was carried out in rural Gambia, where there is a marked seasonal variation in dietary vitamin C. The present review discusses the rationale behind the study and presents some preliminary results.

Vitamin C: Pyridinium cross-links: Functional marker: Gambian population

Vitamin C

Most animal species are able to synthesise vitamin C from glucose, but man, along with other primates and guinea-pigs, lacks the final enzyme in the conversion pathway (Sato & Udenfriend, 1978).

An absence of vitamin C from the diet will result in scurvy; a disease that can be fatal if left untreated. Individuals with low vitamin C intakes may experience scorbutic changes, resulting in weakening of collagenous structures. The scorbutic changes manifest themselves as tooth loss, joint pains, poor wound healing and other bone and connective tissue disorders (Benzie, 1999).

The term vitamin C is often used synonymously with ascorbic acid. However, vitamin C is present in biological tissues in three forms: ascorbic acid; dehydroascorbic acid; and the intermediate free-radical form, semi- or mono- dehydroascorbate.

Vitamin C has been identified as acting as a cofactor for approximately eight enzymic reactions. These reactions are mainly involved in the biosynthesis of collagen, carnitine and neurotransmitters.

Dietary antioxidant

Potentially, the most important function of vitamin C is as a dietary antioxidant. There are many other dietary antioxidants, which work synergistically, but the potential importance of vitamin C is related to its ability to form a comparatively stable free-radical intermediate.

Despite the lack of direct evidence of a link between DNA oxidation and cancer, many epidemiological studies indicate that there is an association between increased intakes of dietary antioxidants and a decreased risk of certain cancers (Block, 1991; Poulsen *et al*. 1998). Vitamin C intake or, possibly more importantly, the intake of vitamin C-containing foods, is associated with protection against oxidative damage in some cases of cardiovascular disease (Frei, 1997) and in age-related cataract formation (Jacques & Chylack, 1991).

Abbreviations: dPyr, deoxypyridinoline; Pyr, pyridinoline.

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However, one of the major problems faced when interpreting results from epidemiological studies is that vitamin C does not act in isolation. Epidemiological studies are not designed to determine the exact role, or the mechanisms of action, of vitamin C *in vivo*.

Optimal intakes

As a result of the emerging multi-factorial role of vitamin C, focus has now changed from recommending intakes sufficient to prevent deficiencies to determining optimal intakes. Optimal intakes need to meet both the long-term and the short-term requirements. Short-term requirements need to be sufficient for all metabolic pathways that require vitamin C to function normally. The long-term requirements need to be high enough to prevent damage from oxidative stress.

High doses of vitamin C are thought to have few adverse side effects, which is possibly one of the reasons why it is the most commonly used vitamin supplement in the USA (Subar & Block, 1990). However, there is controversial evidence suggesting that intakes greatly exceeding the recommended daily amount (90 mg/d), which is set for male adults, can have detrimental health consequences, especially in vulnerable population groups (Herbert *et al*. 1996). Vitamin C can, in some situations, act as a pro-oxidant and it has been claimed that excessive intakes may stimulate free-radical reactions and increase mutagenesis (Podmore *et al*. 1998).

Work is ongoing to try to establish the levels of vitamin C required for maximum health benefits. More informed estimates of requirements could be made if a reliable functional marker of tissue vitamin C status were identified.

Molecular and biochemical markers

A number of potential molecular and biochemical markers have been investigated. Potential molecular markers may target DNA mutagenesis and cytokines, adhesion molecules and other gene products regulated by the redox state of the cell (Jackson *et al*. 1998). The use of molecular markers is restricted because of the difficulties involved in collecting suitable samples, the complicated analytical tools required to analyse them and the uncertainty about the specificity and interpretation of the results.

Biochemical markers may offer a more practical option. One of the pathways that have been extensively investigated is the carnitine biosynthesis pathway. Carnitine is required for the mitochondrial uptake of fatty acids. Vitamin C, along with Fe, acts as a cofactor for carnitine synthesis. Muscle carnitine is severely depleted in vitamin C-deficient guinea-pigs, which may account for the muscle weakness associated with scurvy (Hughes *et al*. 1980). However, neither guinea-pig nor human studies have been able to show a consistent relationship between vitamin C status and plasma carnitine levels (Johnston *et al*. 1996). Thus, plasma carnitine cannot be used as a marker for estimating vitamin C requirements.

The collagen formation pathway offers a well-defined biochemical pathway, with a measurable end point that can be related directly to the scorbutic changes seen as a result of severe vitamin C deficiency.

Collagen

Collagen is a very important protein, accounting for approximately one-third of the total body protein. It is found in many different connective tissues, including bone, skin, cartilage and in the internal organs. Its structural role makes it one of the few proteins that functions extracellularly.

Structure

Collagen, except in basement membranes, has its molecules built up into fibrils, grouped together into larger fibres. The collagen molecules have a triple helical formation, comprising α-1 and α-2 chains, with an N-terminal and C-terminal non-helical end. The helical structure is stabilised through a number of cross-links.

Currently, nineteen different forms of collagen have been identified, with different types being predominant in different tissues. Type I is the most abundant form overall. On the basis of their structure and function, the collagens are generally divided into two categories, fibrillar and non-fibrillar. However, all collagen types possess a common feature; they contain a repeat amino acid sequence of Gly-*x*-*y*, where *x* and *y* can signify most other amino acids. Proline residues make up approximately one-quarter of the remaining residues and lysine is another important constituent. The hydroxylated amino acids hydroxyproline and hydroxylysine are found exclusively in collagen and are formed from the parent amino acid by the action of the enzymes prolyl hydroxylase and lysyl hydroxylase respectively. The length of the chains that form the triple helix structure varies, but the α chain always follows the same Gly-*x*-*y* configuration. The location of the small glycine residue in every third position is necessary to facilitate the folding of the chains into the helical structure.

Formation

Collagen mRNA are transcribed in the nucleus where the intervening non-coding sequences are removed. The strands are then capped, polyadenylated and transported to the cytoplasm.

The organisation of the individual collagen chains into triple helical procollagen molecules requires post-translational hydroxylation and glycosylation steps (see Table 1). These processes are enzyme driven by lysyl and prolyl hydroxylases. Procollagen is synthesised initially with large C- and N-terminal peptide sequences. Disulphide bonds, which form at the C-terminal extensions, facilitate the assembly of the three α chains. Finally, the central segments self-assemble into a triple helical formation, and the resulting procollagen is transported through the Golgi pathway and secreted.

The conversion to the final collagen structure occurs following the cleavage and shortening of the N-terminal region and the removal of the C-terminal extension peptide. The final stage, resulting in the synthesis of mature collagen fibres, occurs in the extracellular matrix.

Following the removal of the extension peptides, the collagen monomers spontaneously aggregate into collagen

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Table 1. The collagen formation pathway

Site	Product	Process	Enzyme	Cofactor
Rough endoplasmic reticulum	Hydroxylysine and hydroxyproline	Hydroxylation and glycosylation	Lysyl and pyrolyl hydroxylases	Vitamin C, Fe^{2+}, O_2, α-Ketoglutarate
			Collagen galactosyl-transferase and glucosyltransferase	Mn
		Assembly of three α-chains		
	Procollagen	Triple-helix formation		
Extracellular space		Cleavage of C- and N-terminal telopeptides	Procollagen peptidases	Ca
Extracellular matrix	Collagen	Oxidative deamination of some lysines	Lysyl oxidase	Cu
		Fibril formation		
		Cross-linking in fibril		
	Mature fibre	Cross-links mature		
		Collagen degradation	Collagenases	

fibres, with the formation of covalent cross-links between the individual collagen chains.

Pyridinium cross-links

Although collagen cross-linking is an extracellular process, the type of mature cross-link that is formed depends on previous intracellular post-translational modifications, particularly the hydroxylation of the lysyl residue in the telopeptide region (Robins, 1988).

Pyridinoline (Pyr) and deoxypyridinoline (dPyr) are two naturally-fluorescent cross-links. Each Pyr molecule consists of three hydroxylysine residues and dPyr is composed of two hydroxylysine residues and one lysine residue. The main role of the pyridinium cross-links is to provide a stable matrix.

In healthy adult human bone collagen Pyr:dPyr is approximately 3:1. Any factor that causes a decrease in the extent of hydroxylation of the lysine residues will potentially alter this ratio. The enzyme lysyl hydroxylase catalyses the hydroxylation of lysine. This process requires ascorbic acid as a cofactor to ensure Fe^{2+}, another crucial cofactor, is maintained in its reduced form. A deficiency of vitamin C would affect the functioning of lysyl hydroxylase, resulting in a decrease in the number of hydroxylated lysine residues. This situation may in turn affect the formation of the pyridinium cross-links, particularly the hydroxylysine-rich Pyr cross-link.

Tsuchiya & Bates (1998) published a study in which weanling guinea-pigs were fed graded vitamin C diets for 46 d. At the end of the treatment period, tissue vitamin C concentrations, bone hydroxyproline, Pyr and dPyr concentrations, and urinary hydroxyproline, Pyr and dPyr concentrations were measured. There was a clear preferential formation of the dPyr cross-links in the four groups fed the lower amount of vitamin C, even up to intakes that were far in excess of their minimum daily requirements of approximately 1·5–3·0 mg. This finding suggests a response in the collagen cross-link ratio at physiological intakes, making it a strong candidate to facilitate determination of dietary vitamin C requirements.

Alteration of the normal cross-link ratios has also been seen in cases of the rare genetic connective tissue disorder Ehlers Danlos Syndrome type VI. This syndrome is characterised by muscle hypotonia, kyphoscoliosis, microcornea, joint laxity and osteoporosis without a tendency to fracture. The phenotypic traits result from reduced enzymic hydroxylation of lysine residues (Pinnell *et al*. 1972), resulting in a greatly increased dPyr:Pyr in urine samples of sufferers (Pasquali *et al*. 1994; Acil *et al*. 1995). Sufficient hydroxylation occurs to form dPyr, but Pyr formation is severely reduced. Supplementing these patients with vitamin C can increase the proportional formation of the Pyr cross-links and improve their clinical condition (Dembure *et al*. 1987).

Collagen turnover

Fibrillar collagens consist of very stable molecules. In healthy adult tissue they have a slow turnover rate and can exist for months, or even years. The rate of turnover in children is much higher, as the connective tissues are constantly being remodelled during times of growth. There is also a higher turnover rate following trauma or in response to mechanical stress. Collagen turnover is regulated in part by collagenases that recognise sequences on the triple helix that indicate cleavage sites. The degraded peptide fragments then either return to the cell for hydrolysis to amino acids or, in the case of the collagen cross-links, are excreted in the urine. The collagen cross-links are not metabolised before excretion, and concentrations can be quantified in urine or serum samples.

Cellular mechanisms

Vitamin C is known to be a necessary cofactor for collagen formation. *In vitro* studies have shown that ascorbic acid is required for type I matrix production, the expression of osteoblastic markers and bone mineralisation (Sugimoto *et al*. 1986; Franceschi & Iyer, 1992). Osteoblasts play a central role in bone formation by synthesising the bone matrix proteins and differentiating into bone cells, and there appears to be a reciprocal interaction between the osteoblast and the surrounding extracellular matrix (Masi *et al*. 1992). However, the mechanisms controlling osteoblast differentiation remain unclear. It is possible that osteoblast

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differentiation is adversely affected if there is a disruption to type I collagen matrix formation. Recent speculation has suggested that ascorbic acid affects the cell-signalling pathway, possibly through its role as an antioxidant and ion chelator.

Several epidemiological studies have attempted to determine whether there is a relationship between vitamin C, or other nutrients such as protein or Ca, and bone mineral density and bone turnover rates (New *et al*. 1997; Hall & Greendale, 1998). Although the main emphasis of these studies focused on women and osteoporosis risk, most of them showed a positive relationship between vitamin C intakes and bone mineral density.

Suboptimal vitamin C intakes may adversely affect the bone formation and degradation process, either by decreasing the rate of osteoblast differentiation or through the synthesis of abnormal collagen fibres that may be degraded before reaching maturity.

Bone formation and resorption markers

There are a number of biochemical markers that can be measured to determine the rate of bone formation and bone turnover.

Pyr and dPyr are present in nanomolar concentrations in both urine and serum. Pyr is released from numerous connective tissue sources, but dPyr comes almost exclusively from bone. The cross-links are not subjected to destruction following bone degradation, and measurements of dPyr are used to assess the rate of bone turnover. Measurements can be made of either free cross-links or total cross-links. Total cross-links are measured following acid hydrolysis, which hydrolyses peptide-bound fragments. Free cross-links account for approximately 40 % of the total cross-links, and there is a close correlation between the free and total levels (Robins *et al*. 1990).

During the course of bone resorption amino- and carboxyl-terminal fragments with attached cross-links are also released. These N-telopeptide and C-telopeptide fragments are excreted in the urine, and circulating levels can also be measured from serum and plasma samples.

Historically, hydroxyproline has been used as a measure of bone-turnover; however, it has been superseded by the other indices. The disadvantage of hydroxyproline is that it lacks specificity, as it is released from collagenous sources other than bone. It is also influenced by dietary intake and is partially and variably degraded before excretion.

Procollagen type I C-propeptides and N-propeptides are the end residues of the procollagen molecules which are enzymically cleaved before the maturation of the collagen molecules. Measurement of the release of C-propeptides and N-propeptides is used as a marker of bone formation. They are excreted intact and levels can be measured in serum, or plasma, and urine samples. Type I collagen is present in tissue other than bone, particularly skin, therefore the contribution from other tissues needs to be taken into consideration when using these markers.

Osteocalcin, a non-collagenous bone protein that is only produced in substantial amounts by osteoblasts, is another bone-formation marker. Osteocalcin is released into the circulation and a variable amount (60–90 %) is incorporated into the bone matrix during bone formation. Osteocalcin levels are affected by renal clearance (Blumsohn & Eastell, 1997) and intact osteocalcin is rapidly degraded in serum samples (Blumsohn *et al*. 1995). Careful sample collection and storage are necessary to ensure that the results are interpreted accurately.

Alkaline phosphatase has two predominant isoforms that are produced from bone and liver. Both forms are released into the circulation and can be measured in serum. Bone-specific alkaline phosphatase is produced by the osteoblast in extremely high amounts during the formation phase of the bone cycle. It is used as an indicator of bone formation activity.

Vitamin C intervention study

Seasonal variation in vitamin C

In many areas of Africa access to certain food types is limited by a seasonal variation in availability. This situation can result in severe deficiencies of key micronutrients, and total energy intakes, at certain times of the year. In the West Kiang region of The Gambia the main source of vitamin C is from mangoes (*Mangifera indica*), which are only available locally during the dry season months of March–June. Earlier in the dry season, about December, the citrus fruits ripen and these are eaten as snack foods. During the rainy season (July–November) the amount of vitamin C available from the diet is severely restricted. A study by Bates *et al*. (1994) showed a marked seasonal variation in vitamin C intakes, and plasma vitamin C levels, in a group of pregnant and lactating women in the West Kiang region. Intakes fell to approximately zero during the rainy season but peaked at levels of >100 mg/d during the dry season (see Fig. 1). Similar seasonal patterns have also been noted in other subgroups of the population, including children. The Bates *et al*. (1994) study is of particular interest because it spans an 18-month period, so the seasonal cycle between the dry and the wet seasons can clearly be seen.

As a consequence of a poor diet and high infection rates, especially during the latter part of the rainy season, the

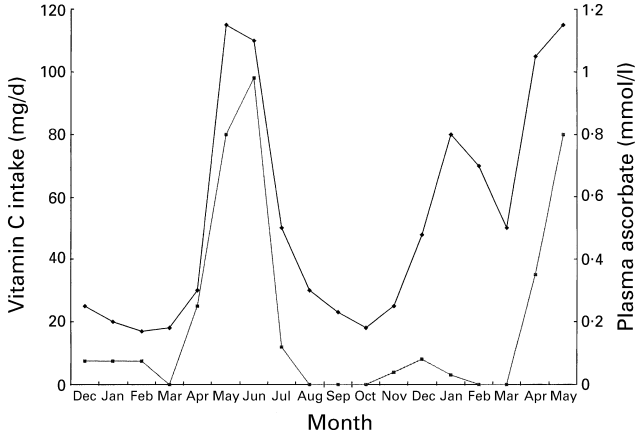

Fig. 1. Mean home-food vitamin C intake from fruit (■) and plasma vitamin C concentrations (◆) for lactating women in Keneba, The Gambia, during 1978–80. (From Bates *et al*. 1994; reproduced with permission from the *European Journal of Clinical Nutrition*.)

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children in the West Kiang region of The Gambia experience poor growth rates, delayed puberty and stunting. As vitamin C is required for normal collagen formation, a diet almost devoid of this vitamin for a number of months each year could have long-term detrimental consequences on their growth.

Ethical permission was obtained from the Cambridge Local Ethics Committee and the Medical Research Council Gambia Ethics Committee for a vitamin C intervention study. It was designed to run for 7 weeks during the rainy season, with repeat measurements 6 months later at the height of the mango (dry) season. The aim of the study was to address the question of whether or not the seasonal variation in vitamin C intake, resulting in severe biochemical deficiencies in a substantial proportion of school-age children, produces functional abnormalities in the biochemical reactions involved in bone formation *in vivo*. The primary focus was to attempt to detect differences in the pyridinium cross-link ratios, but it was also designed to investigate the relationship between vitamin C intakes and bone formation and resorption markers.

It was proposed that there might be a change in the cross-link ratios as a result of a controlled vitamin C intervention, or associated with the natural fluctuation of vitamin C intakes between the wet and the dry season.

Study design considerations

The recruitment of an initial study group of seventy-four individuals (thirty-seven subjects and thirty-seven controls) was calculated to be sufficient to detect a significant difference in the cross-links ratios (α 5 %, β 80 %). This calculation was based on values for within-subject variation of approximately 20 % in Pyr:creatinine in early-morning urine samples (Panteghini & Pagani, 1996). Three urine samples collected each week were pooled to decrease the influence of within-subject variation (Ginty *et al*. 1998).

A single gender group was used for the study. Prepubertal boys born between 1990 and 1994 were recruited (the final age-range was 5 years 8 months–10 years 5 months). During the recruitment process each boy's age was recorded. If the boy had no documentation to support his birth date, and the parents or guardian were not sure of the exact age, an estimate to the closest month was made with the aid of an events calendar, or by comparisons of their time of birth with that of boys with known birthdays.

The study involved the double-blind random vitamin C supplementation of the thirty-seven boys in the subject (intervention) group. These boys received 100 mg vitamin C/d in the form of a drink. The control (placebo) group received a drink containing 50 mg citric acid but no vitamin C. Urine samples were collected three times per week for 8 weeks, spanning the intervention period, and for a further 3 weeks during the follow-up period. The study started during the early rainy season in August 2000 and initial venous blood samples were taken at this period (baseline). Further samples were taken following the 7-week intervention, towards the end of the rainy season in October (end-intervention). Final blood samples were collected during the mango season in April 2001 (follow-up).

Interim results

In order to recruit sufficient subjects for the study, boys had to be recruited from two neighbouring villages, Kuli Kunda and Bajana. The lifestyle patterns in the two villages were similar, so no differences between the two village groups in any of the biochemical markers were expected. Individual subjects were matched, regardless of which village they came from, on an age, weight and height basis. The study involved double-blind random allocation to either the placebo or intervention group. Significance was defined as $P < 0{\cdot}05$.

Anthropometry

Weight, height and BMI were adjusted for age and gender by z-scores in order to draw comparisons with a reference population of British children (Cole, 1990).

The mean z-scores for the total group at each of the three time-points were negative for weight, height and BMI, indicating that the Gambian population group was both shorter and lighter than their British counterparts.

Differences between the placebo and intervention groups, and differences between the boys from the two villages, were also calculated for each of the time-points, but no significant differences were detected.

Vitamin C

Serum samples were collected and aliquots mixed 50:50 (v/v) with metaphosphoric acid (100 g/l) before being frozen at −80°. Vitamin C concentrations were determined using a Cobas Bio centrifugal analyser (Roche, Basle, Switzerland). The metaphosphoric acid deproteinises the serum and the ascorbic acid is oxidised to dehydroascorbic acid by ascorbate oxidase. The product undergoes a condensation reaction with *o*-phenylenediamine to form a fluorescent derivative (Vuilleumier & Keck, 1989).

Two-sample *t* tests were used to compare differences between the placebo and intervention groups at each of the three time points.

There were no significant differences detected between the placebo and intervention group at baseline (Table 2). However, as was expected, following the 7-week intervention the intervention group had significantly higher mean vitamin C levels than the placebo group ($P < 0{\cdot}0001$).

Measurements taken 6 months after the end of the intervention, during the follow-up study, again showed a significant difference ($P = 0{\cdot}036$) between the placebo and intervention groups. The differences remained significant ($P = 0{\cdot}04$) when adjustments were made for two confounding factors: dietary vitamin C intakes and inflammatory status. Vitamin C intakes were calculated from dietary recall questionnaires, which were specifically designed to assess the consumption of vitamin C-containing foods. Inflammatory status was determined through the measurement of α-1-antichymotripsin, an acute phase protein. At this time-point, the placebo group showed higher mean vitamin C levels than the intervention group. It was not clear why this difference occurred and further investigations are warranted to establish the reproducibility of this result.

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Table 2. Plasma vitamin C levels (μmol/l) for prepubertal boys born between 1990 and 1994 in two villages in the West Kiang region of The Gambia who received as a drink either 100 g vitamin C/d (intervention group) or 50 mg citric acid/d (placebo group)* (values are means and standard deviations for thirty-one subjects)

	Placebo		Intervention		Mean difference between placebo and intervention groups	Statistical significance of difference (*P*)
	Mean	SD	Mean	SD		
Baseline (August)	25·6	21·1	20·2	18·6	–5·4	0·534
End-intervention (October)	10·6	16·4	53·6	22·7	43·0	< 0·0001
Follow-up (April)	84·6	18·3	73·3	19·9	–11·3	0·036

*For details of procedures, see p. 433.

The pattern of vitamin C intakes, and serum concentrations, in this present study were was consistent with the result of the Bates *et al*. (1994) study on pregnant and lactating women (see Fig. 1). The lowest serum concentrations were recorded in the non-intervention group at the end of the rainy season in October, and the highest levels during the dry season.

However, the vitamin C intakes recorded during the rainy season were much higher in this present study than those reported by Bates *et al*. (1994). In the present study the mean daily intake recorded for the boys was approximately 30 mg, whereas the Bates *et al*. (1994) study estimated zero intakes during the months of August, September and October. It is possible that the pregnant and lactating women differed in their dietary intakes, but there are other factors that could account for these inter-study differences. The format of the questionnaires differed between the studies, with the Bates *et al*. (1994) study only recording intakes from foods believed to be the main contributors of vitamin C to the diet, such as mangoes, citrus fruit and leafy sauces. Also, since the publication of the earlier study, new information has become available about the vitamin C content of the leaves typically consumed by this population group. The present study found that both red chilli peppers (*Capsicum annum*) and leaf sauces were important sources of vitamin C in the boys' diets.

Bone markers

A number of assays are awaiting completion, including the HPLC pyridinium cross-link analysis. For this reason the bone marker data have not been reported in the present paper.

Discussion

The present study was designed to elucidate an effect on newly-synthesised collagen. Measurable changes in the cross-link ratios may not be detected if the children had adequate vitamin C intakes at the time of bone formation, regardless of their vitamin C states at the time of excretion of these bone degradation products.

Currently, there is no identifiable sub-fraction excreted in the urine that indicates levels of new collagen synthesis. It is not possible, therefore, to determine the proportion of the bone turnover products that was derived from new bone synthesis as opposed to old bone synthesis. Using a young population group, which has a higher rate of bone turnover than that of adults, increases the probability of detecting changes in newly-synthesised bone collagen in a shorter time.

Vitamin C is a water-soluble vitamin and, therefore, is poorly retained within the body. Plasma vitamin C levels of individuals with marginal status can fall below the deficiency cut-off point of 11 μmol/l within a very short period (< 2 weeks) if vitamin C is withheld from the diet (Johnston & Corte, 1999). Physiological changes can also be seen within a period of weeks after a change in vitamin C intake.

In the present study group the vitamin serum C levels were already low at the beginning of study (mean 27·8 μmol/l), with fourteen of the subjects falling below the deficiency cut-off point. A total of twelve subjects had marginal or deficiency vitamin C levels in both August and October. The results of the bone marker data from these subjects will be of particular interest.

Interactions with other micronutrients

For the interpretation of the results, the possible effect of overall nutritional status of the subjects should be considered. This rural Gambian population is known to have very low intakes of a number of micronutrients, either seasonally or throughout the year, including Ca, Fe, Zn, vitamin A and riboflavin (Bates *et al*. 1994; Dibba *et al*. 2000).

Fe^{2+} acts in conjunction with vitamin C as a cofactor for the enzymes lysyl and prolyl hydroxylase. A deficiency of Fe could therefore also affect the cross-link ratio.

Ca intakes of both adults and children are very low in this part of The Gambia (Prentice & Bates, 1993). Ca is required for normal growth and development of the skeletal system and calcium phosphate is a major component of mineralised tissue. Procollagen peptidases are Ca-dependent enzymes that cleave the terminal propeptides from type I procollagen. This step is followed by the oxidative deamination of some of the lysine residues, which requires the Cu-containing enzyme lysyl oxidase. This step precedes intermolecular cross-link formation, which results in the mature collagen molecule.

Although a guinea-pig study by Tsuchiya & Bates (1997) indicated that Cu intakes did not affect the cross-link ratio, deficiencies in either Ca or Cu could affect the formation of mature collagen molecules.

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Limitations of the study

The study was designed to look for changes in the cross-link ratios either as a result of vitamin C supplementation or through seasonal changes in vitamin C intakes. The collagen formation pathway is a complex biochemical pathway and there could be other factors not measured in the present study and therefore not adjusted for, such as intakes of other micronutrients that influence the cross-link ratio.

The dietary vitamin C intakes during the rainy season were much higher than expected, with the mean intake level corresponding to the recommended dietary allowance for this age-group. Whilst the subgroup of fourteen subjects with marginal or deficiency vitamin C concentrations at the two rainy season measurement points should prove the most interesting for the purpose of analysing the cross-link ratios, the small sample size would reduce the power to detect any significant differences.

The results of the present study will only allow conclusions to be drawn about the specific vitamin C-requiring bone collagen formation pathway. However, if it is possible to define the optimum intake of vitamin C necessary for maximum hydroxylysine-derived cross-link formation, then this value has the potential to be used as a surrogate measure for defining optimal intakes for other vitamin C-requiring functions.

Conclusion

Although the specific focus of the present study was to measure the pyridinium cross-links ratios, to test the hypothesis that low levels of vitamin C would result in the preferential formation of the dPyr cross-links, it has also provided a unique opportunity to investigate the effects of low vitamin C intakes on bone formation and degradation in a young Gambian population group.

There are still large gaps in our knowledge of the precise role of vitamin C *in vivo*, and in its exact role in bone synthesis. It is clear that ascorbic acid is required for osteoblast differentiation. This requirement is possibly related to the formation of a stable matrix within which the osteoblast cells can function optimally. Vitamin C may also have an effect on the cell-signalling pathway, either directly or through its role as an antioxidant and ion chelator. The mechanisms of action of vitamin C *in vivo* need to be investigated further.

Potentially, the most important function of vitamin C is as a dietary antioxidant. High intakes of vitamin C, and vitamin C-containing foods, have been associated with a decreased incidence or severity of numerous disease states. The extent to which the antioxidant properties of vitamin C are protective is unclear. Epidemiological-based evidence lacks the specificity to draw conclusions about the functioning of individual micronutrients in isolation. Thus, molecular or biochemical markers must be established in order to determine the intakes required for optimal short- and long-term health benefits for different population groups.

The establishment of a reliable functional index of vitamin C status in man would facilitate determination of the requirements, and possibly also the identification of individuals with increased disease risk because of suboptimal intakes.

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