Carboxylation of Osteocalcin in Post-menopausal Osteoporotic Women Following Vitamin K and D Supplementation

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The effect of vitamin supplements on bone metabolism indices in patients with osteoporosis has received scant attention in the literature. Over a 2-week period, vitamin supplements of K and K + D were given to 20 post-menopausal osteoporotic women with previous Colles fractures. Osteoporosis was confirmed by bone mass measurements that demonstrated that broadband ultrasound attenuation (os calcis) was almost as discriminatory as dual energy X-ray absorptiometry (spine and hip) in Colles fracture patients compared with matched controls. Vitamin K corrected the carboxylation defect in osteocalcin and while less marked 4 weeks later, the improvement was still detectable. The result after K + D was similar. The level of carboxylation became the same as in premenopausal women. Total osteocalcin level rose after vitamin K, this rise being due to carboxylated (bound) osteocalcin. While there was vitamin K correctable undercarboxylation of osteocalcin, simultaneously there was no evidence of undercarboxylation of prothrombin. (Bone 17:15–20; 1995)

Key Words: Bone mass; Carboxylation; Osteocalcin; Vitamin K; Vitamin D; Osteoporosis.

Introduction

After collagen, the most plentiful protein in bone is the vitamin K-dependent bone protein osteocalcin. Vitamin K mediates the carboxylation of glutamate and gamma-carboxyglutamic acid (Gla) confers calcium-binding properties to the vitamin K-dependent proteins whether in bone (osteocalcin) or blood (coagulation proteins). 28 The synthesis of osteocalcin in vitro is controlled by vitamin D 3 (calcitriol), 25 and vitamin K may have important implications in bone development. 18 There is evidence that the antagonism of vitamin K by long-term coumarin therapy causes reduction of bone mineral density. 5, 19, 27

The incidence of rickets peaks in the late winter and spring 1 and a similar seasonal pattern has been found for fractured neck of femur and vitamin K–dependent hemorrhagic disease of newborns. 3 These observations, together with laboratory evidence on vitamin D controlling osteocalcin synthesis, stimulated further work. 5, 6 In this study, supplements of vitamins K and D were given to osteoporotic women and the effect on biochemical bone markers measured.

Materials and Methods

Twenty women who had fractured their wrists between 1988 and 1990 were recruited. The following were exclusion criteria—cardiovascular disease, use of anticoagulant therapy, and use of other drugs known to interfere with bone metabolism. A further fracture since the initial event also excluded the patient. Twenty volunteers were matched into pairs by age and one of each pair chosen by random allocation. They were not matched in any other respect. There were 10 volunteers in group I and 10 in group II. The design of the study is shown in Figure 1; that is, a “crossover” pattern with a “washout” gap of approximately 3 months, allowing group I and group II to be used as “controls” at one time and “treated” at the other.

In the bone mass measurements, the comparison is with “controls” who have no specific role in the study being part of a community-based epidemiological study. These are referred to in the text as “community controls” with each of the 20 subjects age-matched with 2 controls. The study was approved by the local ethics committee and informed consent was obtained from the patient and their general practitioner.

For purposes of biochemical comparison of osteocalcin between the study’s post-menopausal women and younger premenopausal women, blood was collected from 10 premenopausal women on the staff. Their age range was 22–39 years (mean 29.6 years) and the collection was made at the time of D samples (see Figure 1) and at the same time of day as the blood from the patients.

Blinding

Patients and those carrying out the analysis were blinded in regard to medication, while the clinician seeing the patient was aware of the treatment group. Blinding of the patients was merited in the assessment of side effects. The outcome of the study was dependent on laboratory results, not clinical endpoints. Patients received matching placebo liquids for vitamin D and vitamin K.

Bone-Mass Measurement

Spine and hip bone mineral density was measured by dual energy X-ray densitometry (DXA) using a single Norland XR26 bone

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Blood and Urine Sampling

400 IU of vitamin D$_2$ as ergocalciferol was taken anytime in the morning or evening after lunch or after the evening meal. The placebo vitamin D was arachis oil.

Compliance

With the active and placebo preparations of vitamin K$_1$ and vitamin D$_2$, compliance was checked by the patient marking a chart for each daily dose taken. While open to abuse, inspection of the returned bottles showed that the remaining quantities matched the appropriate usage.

Coagulation Assays

The one-stage prothrombin time and the thrombotest technique are the standard laboratory methods that were used.

Measurement of PIVKA II

In the absence of vitamin K or during coumarin therapy, undercarboxylation of the prothrombin precursor results in the release of des-gamma-carboxyprothrombin (PIVKA II) into the circulation. This abnormal prothrombin is unable to form active thrombin in the normal coagulation mechanism. The test was used to establish evidence of lack of vitamin K in the coagulation system of the subjects. The levels were measured in the plasma of each patient using an enzyme immunoassay (EIA) test kit (EDO23) from Eisai Co. Ltd, Tokyo, and the procedure used followed the manufacturer's instructions. PIVKA II was expressed as arbitrary units (AU/mL). An AU is equivalent to 1 µg of prothrombin as measured by rocket electrophoresis.

Measurement of Serum Osteocalcin

Serum osteocalcin measurement consisted of a radioimmunoassay using the Incast RIA Kit (Incastor Corp., St. Paul, MN, USA). This was a one-site assay procedure and the manufacturer's instructions were followed. Radioactivity counts per tube were measured using the Cobra Auto-Gamma counting system (Packard Instruments Ltd.). The inter- and intraassay coefficients of variation were <10% and <6%, respectively.

Measurement of undercarboxylation of osteocalcin was based on the technique of Knapen et al. This technique estimates serum values for osteocalcin before and after adsorption on hydroxyapatite (hydroxyapatite “high resolution,” Fluka Chemika, Glossop, UK). Following centrifugation the supernatant and its supernatant contain undercarboxylated osteocalcin, which did not bind to hydroxyapatite in the test system. By subtraction of this value from the total the percentage of “carboxylated” osteocalcin was calculated. The serum was thawed at room temperature and a 500-µL aliquot transferred to an Eppendorf tube and 50 mg of hydroxyapatite was added. Following gentle mixing the tube was rotated end-to-end at 4°C for 30 min, and thereafter the hydroxyapatite was spun down at 3000 rpm for 5

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### Figure 1. Design of study.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>B</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
</tr>
<tr>
<td>3</td>
<td>D</td>
</tr>
<tr>
<td>4</td>
<td>E</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
</tr>
</tbody>
</table>

**Group I**

- (n=10)
- VT
- C

**Group II**

- (n=10)
- Placebo
- K+D

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**Blood and Urine Sampling**

Twenty milliliters of blood was collected without haemolysis. Five milliliters was citrated (3.8%) (nine parts blood, one part citrate) for coagulation studies and the remainder was collected as serum. The specimens were placed on ice and centrifuged within 90 min of collection at 1560g for 30 min. Plasma and serum were collected and stored in aliquots at -20°C until required.

For each parameter, all samples on the individual patient were measured at the same time following the end of the 6-week period.

**Vitamin K$_1$**

Each volunteer was instructed in the use of the small bottle with solubilized vitamin K$_1$ (Konakion) using an integrated dropper. One drop (approximately 1 mg) was taken in half a tumbler of water before breakfast. There was a detectable taste. The vitamin K is solubilized in distilled water with propyl-p-hydroxybenzoate, methyl-p-hydroxybenzoate, benzoic acid, and castor oil (polyethylene glycol ether) (Roche Nederland BV). The placebo vitamin K was 14031 quinolone yellow 0.0180% in de-ionized water before breakfast. There was a detectable taste.

**Vitamin D$_2$**

In the second phase of the study, 5 mL of arachis oil containing 400 IU of vitamin D$_2$ as ergocalciferol was taken anytime in the afternoon or evening after lunch or after the evening meal. The placebo vitamin D was arachis oil.

**Compliance**

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min using a bench centrifuge. The supernatant was decanted into a second Eppendorf tube and the osteocalcin measured, the hydroxyapatite pellet being discarded.

**Urinary Pyridinium Crosslinks, Calcium and Creatinine**

The pyridinium crosslinks were measured using the technique described by Pratt et al. Calcium and creatinine were analyzed by standard laboratory procedures.

**Bone Alkaline Phosphatase (ALP)**

The technique used was based on three publications, the Boehringer Mannheim kit (1087517) was used. Total ALP was measured and bone plus liver ALP were then assayed in the presence of phenylalanine which inhibits other ALP (e.g., intestinal or tumor derived) and only present in trace quantities. Bone ALP was inactivated by heat and remeasurement made for liver ALP.

**Statistical Analysis**

All sets of data were normally distributed and a paired t-test was used in comparison with subsequent values in the same individual, and an unpaired t-test used when comparison was made between different subjects. For key data, the Wilcoxon analysis was also applied (see Table 2).

For the bone mass measurements, a receiver operating characteristic (ROC) analysis was undertaken to investigate the most precise and specific method in discriminating Colles fractures and controls for each technique. The differences between techniques were assessed by calculation of the area under the curves (AUC) using Fig P software.

**Results**

**Subject Data**

The mean age of the patients was 61.7 years (range 52–73) and the mean time since the fracture was 31 months. There were no significant differences between two groups I and II when compared for age, time since fracture, weight, height, infertility, alcohol, cigarette smoking, exercise level, calcium intake (questionnaire measurement), and age of menopause. One placebo patient and one active patient had nausea at the start of therapy. Two patients had looseness of the stool settling within 2 days of stopping vitamin K1.

**Bone Mass Measurement**

Demographic details of patients and their controls are shown in Table 1. The community controls tended to be slightly heavier and taller than the patients, but the differences were not significant.

Highly significant differences between the fracture and control populations were found using a t-test for the BUA measurement, DXA of L2-L4, and DXA of Ward’s area of the hip (Table 1). The DXA of the neck of femur and the trochanteric region of the hip showed a significant difference, but to a lesser degree than the other areas measured. Significant differences between the z scores were found for all the bone measurements including BUA (Table 1).

The ROC (receiver operator curve) analysis (Figure 2) showed no clear difference in the discriminatory powers of any of the techniques. The AUCs were as follows: BUA = 0.67, DXA L2-L4 = 0.70, DXA of neck of femur = 0.66, DXA of trochanter = 0.62, and DXA of Ward’s area = 0.70.

**Coagulation Studies**

The mean clotting time of each group of 10 patients was established, before and after supplement and placebo, and compared with contemporaneous controls. All mean values (Thrombotest) for the patient groups were within 1 sec of the control. The less sensitive one-stage prothrombin time had a mean value within 1.5 sec of the control value. The one-stage test is insensitive close to the normal range, but the thrombotest less so. There was no shortening of clotting times as a consequence of giving vitamin K1.

There was no detectable PIVKA II in any of the 20 subjects. One coumarin plasma gave a figure of 6 AU/mL (AU = arbitrary).

![Figure 2. Receiver operator curve (ROC) analysis for Colles fracture (AUC in parentheses). Symbols are as follows: large square, BUA; triangle, DXA; inverted triangle, DXA of neck; small square, DXA of trochanter; and star, DXA of Ward’s area.]

**Table 1. Demographic and bone mass details of fracture patients and controls**

<table>
<thead>
<tr>
<th></th>
<th>Colles fractures (n = 20)</th>
<th>Controls (n = 40)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61.7 (52–73)</td>
<td>61.7 (52–73)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.59 (1.47–1.68)</td>
<td>1.60 (1.43–1.71)</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.8 (46–90)</td>
<td>67.6 (52–96)</td>
<td>n.s.</td>
</tr>
<tr>
<td>BUA (dB/MHz)</td>
<td>0.77 (±11.9)</td>
<td>0.90 (±21.7)</td>
<td>0.008</td>
</tr>
<tr>
<td>DXA L2-L4 (g/cm²)</td>
<td>0.68 (±0.16)</td>
<td>0.77 (±0.20)</td>
<td>0.031</td>
</tr>
<tr>
<td>DXA, neck of femur (g/cm²)</td>
<td>0.11 (±0.11)</td>
<td>0.11 (±0.19)</td>
<td>0.002</td>
</tr>
<tr>
<td>z score, BUA</td>
<td>-0.59 (±0.12)</td>
<td>0.0 (±0.18)</td>
<td>0.004</td>
</tr>
<tr>
<td>z score, L2-L4</td>
<td>-0.66 (±0.55)</td>
<td>0.0 (±1.00)</td>
<td>0.008</td>
</tr>
<tr>
<td>z score, neck of femur</td>
<td>-0.43 (±0.79)</td>
<td>-0.41 (±0.98)</td>
<td>0.031</td>
</tr>
<tr>
<td>z score, trochanter</td>
<td>-0.44 (±0.52)</td>
<td>-0.02 (±0.99)</td>
<td>0.028</td>
</tr>
<tr>
<td>z score, Ward’s area</td>
<td>-0.73 (±0.67)</td>
<td>0.00 (±1.00)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Mean values and range or 1 SD in parentheses.
Osteocalcin and Percentage Carboxylation

Osteocalcin and percentage carboxylation values are shown in Table 2. Total serum osteocalcin level was raised after vitamin K. This rise reached significance in the K + D phase and not in the K phase, but the magnitude of the changes was similar. The levels 4 weeks later were similar to the starting values. The degree of carboxylation was increased similarly in both phases and the rise after supplementation was still present at a lower level 4 weeks later. The mean carboxylation level after vitamin K was the same as that in the premenopausal women (72%).

Since the results showed no difference from adding vitamin D to vitamin K the 20 patients were examined together as a larger group before (10 group IA, 10 group IID) and after K supplementation (10 group IB, 10 group IIE). The mean value for osteocalcin after vitamin K (4.23 ng/mL) was significantly higher (p < 0.001) than 3.28 ng/mL before vitamin K. Four weeks later stopping vitamin K, the value was no longer greater at 3.45 ng/mL. The rise in percentage carboxylation was greater (p < 0.001) after vitamin K (73%) compared to before (57%). Four weeks later it was still significantly higher at 64% (p < 0.05). In group I, the improved carboxylation percentage had returned to baseline 10 weeks later, that is, at the beginning of the second phase of the study. There were no changes in the placebo samples.

Urinary Pyridinium Crosslinks, Alkaline Phosphatase, and Calcium Levels

These were not significantly different in the comparisons with placebo, or before and after vitamin K.

Discussion

Critical to the study was the choice of osteoporotic women. Bone mineral density was significantly reduced by approximately 15% in the group of 20 Colles fracture patients when compared with 40 controls. Women with these fractures have previously been shown to have a lower bone density than those without a fracture,4,8,10,17 and BUA has also been shown to be lower in those with Colles fractures.22

In the Colles fracture group there were significant differences in the fracture and control populations for all bone measurements undertaken, with DXA of Ward’s area showing only a slightly better discriminant value than BUA. DXA lumbar spine showed a more significant difference than the other areas of the hip, namely the neck of the femur and the trochanter. The ROC analysis shows that there were no real differences between DXA, at any site, and BUA in discriminating the fracture and control groups. Further studies are required to determine which technique is best at predicting future osteoporotic fractures.

Our finding of an undercarboxylation of osteocalcin in postmenopausal women has been reported previously.11,12 Others have reported a concomitant rise in total osteocalcin, especially in much older women, over 80 years of age.23

Vitamin K1 corrects the decreased level of carboxylated osteocalcin found in our postmenopausal women who had fractured their wrists and have diminished bone mineral density. The level of carboxylation after vitamin K became the same as that in premenopausal women. This confirms previous findings.11,12,20 When vitamin K1 was given for 3 months,11 the carboxylation improvement, after stopping, slowed at 1 month but was still detectable at 3 months after cessation. After 4 weeks, in our study (when vitamin K was only given for 2 weeks), the correction was markedly less and had disappeared at 14 weeks. This biochemical abnormality in osteoporosis can be corrected by ingesting vitamin K1, and might be expected to be of benefit by continued long-term therapy.

Because vitamin K brought the carboxylation level back to what found in premenopausal women, with the wisdom of hindsight, any greater change could not be expected as a consequence of adding vitamin D to vitamin K. After this work had been completed, Szulc et al.21 in 1993 reported on the effect of vitamin D and calcium on undercarboxylated osteocalcin in postmenopausal women with a mean age of over 80 years. Measurements of undercarboxylated osteocalcin made after 6 months and 1 year on vitamin D therapy showed a fall in the undercarboxylated osteocalcin percentage.
The plasma of our patients had no PIVKA II, the presence of which is a very sensitive indicator of vitamin K deficiency. There was no failure of carboxylation of prothrombin, yet there was failure of carboxylation of osteocalcin. All the clotting assays were within the normal range; there was no evidence of lengthening before vitamin K or shortening after vitamin K. It is not known why there is sufficient vitamin K to maintain normal vitamin K-dependent coagulation but an apparently insufficient quantity to maintain the carboxylation level of osteocalcin seen in younger women. There are already recognized differences between bone and liver with respect to vitamin K handling. In patients on coumarin therapy there is a reduction in total osteocalcin and there is a marked undercarboxylation similar to that in elderly patients over 85 years of age. In patients on long-term anticoagulant therapy there is a reduction in bone mineral density. Menaquinones (MK-7 and MK-8) are deficient in elderly patients over 85 years of age. In patients on coumarin therapy there is a reduction in total osteocalcin and there is a marked undercarboxylation similar to that in elderly patients over 85 years of age. In patients on long-term anticoagulant therapy there is a reduction in bone mineral density.

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There are technical problems in the measurement of osteocalcin and its carboxylation status. The literature on osteocalcin is full of apparent discrepancies between different authors, but these probably stem from the different measurement reagents and techniques. Osteocalcin binding varies with hydroxyapatite used, different amounts of hydroxyapatite, and different mixing times. The apparent degree of osteocalcin binding is dependent on the source of the hydroxyapatite used and, in common with the findings of Merle and Delmas, on the ratio of serum to hydroxyapatite used in the binding assay. Nevertheless, as all of the present studies were performed under standardized conditions, the results allow comparisons between groups, even though the absolute values may not be reliable. To compare with our results requires following the details of our study precisely. There were no important side-effects to the supplements of vitamins K and D. Minor looseness of the stool in two patients could have been due to castor oil in the excipient required in the vitamin K preparation. There is always the theoretical risk of "increased coagulability" following vitamin K, but no clinical or laboratory evidence is known to us in support of this. Patients with coronary heart disease on long-term anticoagulant therapy have an increased incidence of coronary events when this is stopped.

It is necessary to follow the lead by Szulc et al. If vitamin D on its own can return undercarboxylation of osteocalcin to the levels in premenopausal women then this could be the road forward.

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