Nutrient Requirements and Interactions

Vitamin C Supplementation Does Not Modify Bone Mineral Content or Mineral Absorption in Growing Pigs1,2

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ABSTRACT We have demonstrated that alkaline phosphatase activity and collagen synthesis are dose-dependently stimulated by ascorbic acid in differentiated pig osteoblasts. In this study we further examined the relationship between ascorbic acid and bone metabolism by feeding young pigs large amounts of ascorbic acid. Three groups of seven 47-d-old pigs were given no ascorbic acid supplement (control), 500 (500 AA) or 1000 (1000 AA) mg ascorbic acid/kg diet for 4 mo. Calcium and P absorption and retention were evaluated by a 14-d balance trial immediately before killing in control and 1000 AA groups only (n = 6). Bones were collected at death and the bone ash and bending moment (three-point bending test) determined. Various plasma and urine indices of bone metabolism, especially those reflecting collagen degradation (hydroxyproline, deoxypyridinoline) and synthesis (carboxyterminal propeptide of type I collagen) were monitored. The plasma ascorbic acid concentrations increased with time and paralleled the dietary concentrations (P < 0.01). The Ca and P balances and the bone ash and bending moments in the ascorbic acid–supplemented pigs did not differ from those of the controls. Plasma osteocalcin was elevated (P < 0.05), whereas the other bone formation markers, alkaline phosphatase and carboxyterminal propeptide of type I collagen, were not affected by ascorbic acid. The plasma concentrations of Ca, P and 1,25-dihydroxycholecalciferol did not differ among the three groups. The unaffected urinary excretion of deoxypyridinoline and hydroxyproline in the ascorbic acid–supplemented pigs indicates that ascorbic acid does not alter bone resorption. Thus, high intakes of ascorbic acid have no positive influence on bone metabolism and bone characteristics in pigs. The in vivo long-term effects do not correlate with the short-term in vitro effects previously reported. J. Nutr. 127: 1514–1518, 1997.

KEY WORDS: • ascorbic acid • pigs • bone • collagen markers • osteocalcin

There is considerable evidence (Franceschi 1992) that ascorbic acid is essential for type I collagen matrix synthesis, alkaline phosphatase activity, osteocalcin accumulation and matrix mineralization in osteblast cultures. Ascorbic acid also stimulates bone cell proliferation in culture (Franceschi 1992). We previously demonstrated that ascorbic acid alters the alkaline phosphatase activity and the collagen synthesis of bone cells isolated from pigs, which do not require vitamin C (Denis et al. 1994). Although it is known that bone disorders in vitamin C–deficient animals are cured by administration of vitamin C, the effects of ascorbic acid supplementation on bone metabolism in vivo are not known. Guinea pigs are sensitive to ascorbic acid deficiency and have reduced femur calcium and hydroxyproline contents when fed a low ascorbic acid diet (Tsuchiya and Bates 1994). Mineral density, assessed by densitometry, and bone volume, assessed by histomorphometry, of the femurs of scorbutic guinea pigs are also decreased (Kipp et al. 1996). Weiser et al. (1992) reported that chicks, another species not requiring vitamin C, had elevated plasma 1,25-dihydroxycholecalciferol and duodenal calbindin concentrations and increased bone strength when fed diets supplemented with ascorbic acid. The calcification of cultured fetal rat bones is not affected by ascorbic acid deficiency, but their dry weights and collagen contents are decreased (Chen and Raisz 1975). Osteogenic disorder rats (ODS rats) are genetically vitamin C deficient, and the bone formation variables estimated by histomorphometry are decreased, whereas bone resorption in femurs is reduced (Tsunenari et al. 1991). A recent clinical study showed that dietary ascorbic acid was positively associated with forearm bone mineral density in children and adolescents (Gunnes and Lehmann 1995). Dietary ascorbic acid supplements do not affect the growth rate of grower-finisher pigs (Cromwell et al. 1970, Mahan et al. 1994, Nakano et al. 1983) but may slightly improve the growth rate and/or feed efficiency in weanling pigs (Mahan and Saif 1983, Mahan et al. 1994, Yen and Pond 1981). Pigs can synthesize vitamin C from birth (Braude et al. 1950) and accumulate ascorbic acid when fed a high ascorbic acid diet (Mahan et al. 1994, Nakano et al. 1983, Yen and Pond 1981). There are presently no recommendations for vitamin C intakes for pigs (National Research Council 1988). We examined the relationship between ascorbic acid and bone metabolism by feeding young pigs large amounts of ascorbic acid. Because it has been suggested that vitamin C stimulates Ca absorption (Bourne 1972), we also measured mineral absorption in a balance study.

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1514
**TABLE 1**

<table>
<thead>
<tr>
<th>Diet ingredient</th>
<th>Amount (g/kg)</th>
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</thead>
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<tr>
<td>Wheat</td>
<td>360.0</td>
</tr>
<tr>
<td>Corn</td>
<td>370.4</td>
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<tr>
<td>Rice1</td>
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<tr>
<td>Soybean Meal</td>
<td>170.0</td>
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<tr>
<td>Corn oil</td>
<td>10.0</td>
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<tr>
<td>Hydrated dicalcium phosphate</td>
<td>20.0</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>13.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>3.0</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>2.0</td>
</tr>
<tr>
<td>Mineral-vitamin mix2</td>
<td>1.6</td>
</tr>
</tbody>
</table>

**Absorption and Retention**

Ascorbic acid and retentions were evaluated in a 14-d balance trial. The diet did not alter Ca and P absorption or retention (Pointillart et al. 1993). 

**Plasma Measurements**

Plasma Ca, inorganic P, alkaline phosphatase activity, 1,25-dihydroxycholecalciferol [1,25(OH)₂D₃], osteocalcin (BGP), and the carboxyterminal peptide of type I procollagen (PICP), a marker of collagen synthesis, were determined on venous blood samples collected at the time of death during exsanguination. The plasma concentration of hydroxyproline, a marker of collagen degradation, was also determined on blood samples obtained at death. The plasma concentration of ascorbic acid was monitored in blood samples from the anterior vena cava taken on d 8, 30, 60, 90, 100 and at killing (d 120). The Ca, inorganic P, alkaline phosphatase activity, 1,25(OH)₂D₃ and BGP were measured as described in Pointillart et al. (1995). Briefly, BGP was measured using a modified radioimmunoassay kit (Ostk-Fr, Oris, Gif sur Yvette, France) with purified bovine BGP as standard and tracer and rabbit antiserum to bovine BGP, adding an internal porcine standard (the bovine BGP standard inhibition curve and that of serial dilutions of the porcine plasma were parallel). The total alkaline phosphatase (EC 3.1.3.1) activity was measured (method from Bessey et al. 1946). We previously found that it was correlated with the plasma bone isoenzyme activity in pigs (r = 0.87, P < 0.001; Pointillart et al. 1995). Inorganic P was measured by colorimetry (Chen et al. 1956). 1,25-Dihydroxycholecalciferol was measured by radioimmunoassay using the kit from Nichols Institute Diagnostics (San Juan Capistrano, CA). Stock solution was made from and stored in 90% ethanol. After extraction on modified C₁₈OH columns, PICP was determined by radioimmunoassay (Orion Diagnostica, Espoo, Finland). The lowest concentration detected was 1.2 µg/L. The intra- and interassay coefficients of variation were 3% and 5%, respectively. Plasma hydroxyproline was determined by colorimetry (Jaeschke 1975). Plasma and dietary ascorbic acid were measured by colorimetry by the method of Omaye et al. (1979).

**Materials and Methods**

**Animals and diets.** Twenty-four 47-d-old male Large-White pigs (Cepra, Vermont, France), weighing 9.7 ± 0.1 kg, were randomly assigned to one of three groups: controls (no added ascorbic acid) 500 mg ascorbic acid/kg diet (500 AA) and 1000 mg ascorbic acid/kg diet (1000 AA). All pigs were fed similar amounts of basal diet, which consisted mainly of cereals and soybean meal formulated to meet the requirements for growing pigs (INRA 1989) (Table 1). These diets contained 17.8% protein (including 1.1% lysine and 13.8 M) digestible energy/kg, 1% Ca, 0.66% P and 25 µg cholecalciferol/kg. Some pigs, especially those receiving the 1000 AA diet, had diarrhea at the beginning of the experiment. Thus, we incorporated 2% rice in all diets. Because some cases of diarrhea persisted, we incorporated (at the expense of corn) 5% rice, and all diarrhea stopped. This diarrhea had no incidence on general health status as indicated by no differences among groups in mean body weights. The selected vitamin C supplementation levels were based on the findings of Yen and Pond (1981) and Mahan and Satl (1983), who found no adverse effects in pigs fed up to 990 mg ascorbic acid/kg diet. The ascorbic acid (L-ascorbic acid from Hoffmann-La Roche, Neuilly-sur-Seine, France) was added with the vitamin mixture which was incorporated with the other ingredients and pelleted. The ascorbic acid concentrations of the three diets were assessed on samples from six batches of each diet collected throughout the experiment. The pigs were kept in individual pens (1 × 2.5 m, concrete floor and walls) and received equal amounts of foods. The diets were fed for 4 mos. Pigs were stunned by electrocarnation and killed by exsanguination. The French instructions concerning rules in animal experimentation (no. 88-123 of December 13, 1988; no. 87-548 of October 19, 1987) were followed.

**Balance study and bone measurements.** The apparent Ca and P absorptions and retentions were evaluated in a 14-d balance trial immediately before killing. The balance was performed on six control pigs and six 1000AA pigs kept in individual cages as described (Pointillart et al. 1995). At time of death, the tibia and main metatarsals (fingers III and IV) were removed to determine the bending moment, ash content (metatarsals only) and apparent density (weight/volume, tibias only) (Pointillart et al. 1993). Hydroxyproline was used as a marker of collagen degradation and measured in urine samples collected during the balance trial (Pointillart et al. 1993). The concentration of deoxypyridinoline, mainly a marker of type I collagen degradation, in the urine samples was also measured (and in 48-h urine samples collected in the 500 AA pigs) using a kit (Pyrilinks-D, Metra Biosystems from Behring Diagnostic, Rueil-Malmaison, France).

**RESULTS**

Ascorbic acid supplementation had no effect on the general growth; the average daily gains were 0.59 ± 0.01 kg/d for controls; 0.58 ± 0.01 kg/d for the 500 AA pigs; 0.59 ± 0.02 kg/d for the 1000 AA pigs. The slaughter body weights did not differ (81.4 ± 1.5 kg, controls; 79.7 ± 1.7 kg, 500AA; 81.5 ± 2.2 kg, 1000 AA).

The plasma ascorbic acid concentrations increased (P < 0.001) with time in all three groups and were greater in pigs supplemented with vitamin C (P < 0.05). (Fig. 1) There was no difference in the plasma ascorbic acid concentrations of the two supplemented groups.

Supplementing the basal diet with 1000 mg ascorbic acid/kg diet did not alter Ca and P absorption or retention (Table 2). Urinary excretion of deoxypyridinoline did not differ among groups (19 ± 3, 22 ± 2 and 22 ± 2 nmol/mmol creatinine for controls 500 AA and 1000 AA pigs, respectively). Similarly, the urinary excretion of hydroxyproline was unaf-

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1 No rice was included in the first 50 d of the experiment, then 2% rice for 45 d, and 5% rice thereafter. This formula corresponds to the
2 Provided the following (mg/kg diet): Zn 105, Fe 100, Mn 42, Cu 10, I 0.6, Co 0.5, Se 0.1. Vitamins supplied (per kg diet); retinyl palmitate
3 of Yen and Pond (1981) and Mahan and Saif (1983), who found no
4 Abbreviations used: PICP, carboxyterminal propeptide of type I collagen; 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; BGP, osteocalcin; 500AA and
5 mg for control, 500AA and 1000AA diets, respectively (added at the expense of corn).
6 Abbreviations used: PICP, carboxyterminal propeptide of type I collagen; 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; BGP, osteocalcin; 500AA and
7 mg for control, 500AA and 1000AA diets, respectively (added at the expense of corn).
Ascorbic acid supplementation had no effect on plasma concentrations of calcium, inorganic P, 1,25(OH)2D3, PICP or hydroxyproline (Table 3). The plasma total alkaline phosphatase activity was unaffected by diet, but the BGP concentrations were elevated in both ascorbic acid–supplemented groups (P < 0.05). There was a significant correlation (r = 0.80, P < 0.01) between the plasma concentrations of ascorbic acid and those of BGP in the three groups of pigs throughout the experiment. The plasma (y: mmol/L) and urinary (x: µmol/mmole creatinin) concentrations of hydroxyproline were correlated (y = 11.44x + 29.6, r = 0.89, P < 0.01) in both the control and 1000 AA groups.

Ascorbic acid supplementation did not influence any of the bone measurements, including mineral contents, apparent bone density, or bending moments (Table 4).

**DISCUSSION**

The data demonstrate that dietary ascorbic acid supplementation of pigs, which do not require dietary vitamin C, does not affect the main variables of mineral and bone metabolism, including those reflecting collagen synthesis and degradation [hydroxyproline, deoxypyridinoline and carboxyterminal peptide of type I collagen (PICP)]. The ascorbic acid concentrations of the three diets were 116 mg ascorbic acid/kg for the control diet, 605 mg/kg for the 500 AA diet and 1128 mg/kg for the 1000 AA diet, indicating that the basal dietary ingredients provided approximately 100 mg ascorbic acid/kg diet. This amount may be sufficient to optimize bone function and may explain why additional dietary ascorbic acid had no effect.

Some of the dietary ascorbic acid supplement was absorbed by the pigs, as demonstrated by the significant differences in the plasma ascorbic acid concentrations. The 1000 AA pigs had the highest plasma concentrations of ascorbic acid, which were similar to those observed in previous ascorbic acid supplementation studies in pigs (up to 990 mg/kg diet) (Braude et al. 1950, Mahan et al. 1994, Nakano et al. 1983, Yen and Pond 1981), although others have found a nonsignificant increase in

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>500 AA</th>
<th>1000 AA</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca, mmol/L</td>
<td>2.25</td>
<td>2.30</td>
<td>2.28</td>
<td>0.02</td>
</tr>
<tr>
<td>Inorganic P, mmol/L</td>
<td>2.74</td>
<td>2.65</td>
<td>2.71</td>
<td>0.06</td>
</tr>
<tr>
<td>1,25(OH)2D3, pmol/L</td>
<td>84</td>
<td>91</td>
<td>84</td>
<td>2.4</td>
</tr>
<tr>
<td>Osteocalcin, µg/L</td>
<td>177</td>
<td>220*</td>
<td>213*</td>
<td>12</td>
</tr>
<tr>
<td>PICP, mg/L</td>
<td>2.8</td>
<td>2.4</td>
<td>2.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Alkaline phosphatase, µU/L</td>
<td>46</td>
<td>54</td>
<td>47</td>
<td>3</td>
</tr>
<tr>
<td>Hydroxyproline, µmol/L</td>
<td>59</td>
<td>63</td>
<td>57</td>
<td>2</td>
</tr>
</tbody>
</table>

1 Values are means, n = 7. *P < 0.05, significantly different from control value.
2 PICP, 1,25(OH)2D3 = 1,25-dihydroxycholecalciferol, PICP = carboxyterminal propeptide of type I collagen.

**TABLE 4**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>500 AA</th>
<th>1000 AA</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metatarsals2</td>
<td>Fresh weight, g</td>
<td>24.1</td>
<td>23.3</td>
<td>23.9</td>
</tr>
<tr>
<td>Ash weight, g</td>
<td>6.7</td>
<td>6.9</td>
<td>6.6</td>
<td>0.2</td>
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<tr>
<td>Ash, g/100 g dry matter</td>
<td>44.3</td>
<td>45.5</td>
<td>45.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Ash:bone volume, g/100 cm3</td>
<td>33.1</td>
<td>34.3</td>
<td>33.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Bending moment, N·m</td>
<td>10.2</td>
<td>11</td>
<td>10.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Tibias3</td>
<td>Fresh weight, g</td>
<td>149</td>
<td>145</td>
<td>156</td>
</tr>
<tr>
<td>Density, g/cm3</td>
<td>1.27</td>
<td>1.26</td>
<td>1.24</td>
<td>0.01</td>
</tr>
<tr>
<td>Bending moment, N·m</td>
<td>60</td>
<td>62</td>
<td>65</td>
<td>1</td>
</tr>
</tbody>
</table>

1 No significant differences in any variable among the three groups.
2 Means from four bones per pig (n = 7): two left, external (IV) and internal (III), metatarsals + two right, external (IV) and internal (III), metatarsals.
3 Left tibia, density: apparent density of the whole bone: weight/volume (n = 7).
4 Means from left and right tibias, n = 7.
plasma ascorbic acid (Cromwell et al. 1970, Mahan and Saif 1983). The correlation ($r = 0.83–0.92$) between the calculated ascorbic acid intakes and plasma ascorbic acid in each of the three groups of pigs in this experiment may explain why plasma ascorbic acid increased with time, as previously reported (Cromwell et al. 1970). Thus, the lack of change in the bone metabolism of ascorbic acid-supplemented pigs cannot be attributed to the lack of absorption of ascorbic acid.

Neither the direct measurements of bone health (ash contents and bending moments) nor the biochemical markers of bone remodeling (alkaline phosphatase and PICP) suggest that high dietary ascorbic acid has a positive effect on bone formation in pigs of this age group. Although there was a significant low correlation between plasma ascorbic acid and PICP ($r = 0.59$), there was no difference between treated and untreated groups for circulating levels of PICP, a marker of bone collagen synthesis.

Most of the effects of vitamin C on the mineral and hydroxyproline contents of bones have been described in animals that require vitamin C, such as guinea pigs (Bourne 1972, Kipp et al. 1996, Sergeev et al. 1990, Tsunenari et al. 1991). An in vivo study on broiler chicks (Weiser et al. 1992) showed that ascorbic acid supplementation (200 mg/kg diet) improved the bone weight, ash and hydroxyproline contents and the breaking strength of bones, but the protocols used suggest that there was a synergistic effect of dietary vitamin D and ascorbic acid supplements, rather than an effect of ascorbic acid alone. Another in vivo study on genetically altered rats requiring vitamin C showed that bone formation and resorption, evaluated by histomorphometry, were greatly decreased by vitamin C deficiency and restored by adding vitamin C (Tsunenari et al. 1991). Thus, there is direct evidence that a lack of vitamin C alters bone formation, but there are no data demonstrating that a dietary intake of ascorbic acid over the minimal requirements stimulates bone metabolism or mineral retention, as previously suggested (Bourne 1972).

Most of the published data suggesting that ascorbic acid stimulates bone formation are provided by in vitro studies, showing a dose-dependent stimulation of bone cell differentiation (Franceschi et al. 1994). However, we have found that ascorbic acid also blocks the differentiation of porcine osteoblastic cells in vitro, depending on the stage of differentiation of the cells (Denis et al. 1994). These opposing effects in vitro may balance each other in vivo or become negligible in a physiological situation.

The only bone-related variable that was increased in the ascorbic acid–supplemented pigs was the plasma concentration of BGP, a non-collagenous protein reflecting osteoblast activity. Increased circulating BGP has also been reported in a clinical study in women given 150 mg of ascorbic acid daily for 10 d (Cantatore and Carrozzo 1990) and in several in vitro studies with bone cells indicating that ascorbic acid influences the transcription of several bone proteins, including BGP (review Franceschi 1992). Our results confirm the link between ascorbic acid and BGP in vivo, with a good correlation between the plasma concentrations of ascorbic acid and osteocalcin observed throughout the experiment. However, the increased plasma BGP probably does not reflect enhanced bone matrix synthesis by osteoblasts, because the other markers of bone formation were unaffected. It may result from an isolated ascorbic acid effect on BGP synthesis or release by osteoblasts without any change in the size of their differentiated pool. Our protocol does not allow us to come to a firm conclusion; however, it emphasizes the fact that more than one marker is required to evaluate bone remodeling.

Mineral absorption and retention, assessed by the balance data, were not influenced by the ascorbic acid supplements. This contrasts with early studies (Bourne 1972), but it is consistent with the unchanged bone characteristics in the ascorbic acid–supplemented pigs.

None of the markers of collagen catabolism (urinary and plasma) hydroxyproline and deoxypyridinoline was modified by the dietary ascorbic acid supplementation, indicating that bone resorption was unaltered. This does not support a link between ascorbic acid status and bone resorption as previously suggested by studies in vitamin C–deficient animals (Kipp et al. 1996, Tsunenari et al. 1991).

Some studies suggest that vitamin C interacts with vitamin D metabolism by protecting or stimulating renal 1α-hydroxylase activity (Cantatore and Carrozzo 1990, Sergeev et al. 1990, Weiser et al. 1992), and the serum 25-hydroxycholecalciferol and 1,25(OH)2D3 in scorbutic guinea pigs are lower than those of pair-fed controls (Kipp et al. 1996). In contrast, Tsuchiya and Bates (1994) compared ascorbic acid–deficient and weight-matched ascorbic acid–replete guinea pigs and found that these changes in the vitamin D status (circulating vitamin D metabolites, kidney 1α-hydroxylase) did not result from the vitamin C deficiency per se. The present data also do not support a link between ascorbic acid and vitamin D, because the plasma 1,25(OH)2D3 concentrations in the ascorbic acid–treated and untreated pigs did not differ.

In conclusion, high intakes of vitamin C, which is not a required dietary vitamin for pigs, have no positive effects on bone or on Ca and P balance. They also indicate that extrapolation from in vitro results is questionable, because cultured pig osteoblasts respond to increasing ascorbic acid concentrations.

ACKNOWLEDGMENTS

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LITERATURE CITED


Cromwell, G. L., Hays, V. W. & Overfield, J. R. (1970) Effect of dietary ascorbic acid on bone or on Ca and P balance. They also indicate that extrapolation from in vitro results is questionable, because cultured pig osteoblasts respond to increasing ascorbic acid concentrations.


