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The effects of uremia, dietary phosphorus, the major vitamin D metabolites and aluminum on osteomalacia in rats

Lieuallen, Warren Grogger, Ph.D.

The Ohio State University, 1990
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THE EFFECTS OF UREMIA, DIETARY PHOSPHORUS,
THE MAJOR VITAMIN D METABOLITES AND ALUMINUM
ON OSTEOMALACIA IN RATS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Warren Grogger Lieuallen, B.S., D.V.M.

* * * * *

The Ohio State University
1990

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Approved by

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Adviser
Department of Veterinary Pathobiology
This Work is Dedicated

To My Family
ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my adviser, Dr. Steven E. Weisbrode, for his professional guidance throughout my research and residency training. I would also like to thank Drs. Charles Capen, Larry Nagode and Thomas Rosol for their valuable discussions and suggestions. The technical assistance of Owen Kindig, Evelyn Handley, Hendrik Colijn, and the staff of the Orthopedic Pathology Laboratory is also gratefully acknowledged. And finally, I would like to thank my wife Joanna, who made it all possible, and my children Tony and Laura, who made it all worthwhile.
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CHAPTER I
THE EFFECTS OF UREMIA AND DIETARY PHOSPHORUS
ON THE BONE OF RATS

Abstract:
In order to evaluate the effects of uremia and low levels of dietary phosphorus on bone, male Sprague-Dawley rats weighing 320±20 g (12 weeks old) were subjected to either a two-step, subtotal nephrectomy or sham-operation (SO), and a custom diet with either normal calcium (0.5%) and normal phosphorus (0.3%) (NP) or normal calcium and low phosphorus (0.03%). When compared to the NP SO group after seven days, only uremic rats fed the low phosphorus diet developed osteomalacia characterized by an increase in the osteoid thickness, surface and volume, a prolonged osteoid maturation time, and a decreased bone formation rate. No other groups developed these changes. This osteomalacia was also associated with hypophosphatemia, a reduced serum PTH and an elevation in the serum 1,25(OH)₂D₃. It was concluded that while neither this degree of uremia nor the low phosphorus diet alone had any significant effect, the combination of uremia and low dietary phosphorus resulted in the initiation of osteomalacia. This animal model should prove useful in investigations dealing with the influence of uremia on the mineralization process.
Introduction:

Among the many complications of chronic renal failure is a syndrome of skeletal abnormalities collectively referred to as renal osteodystrophy. Although usually manifested as fibrous osteodystrophy which results from renal secondary hyperparathyroidism, osteomalacia may be the exclusive component of renal osteodystrophy in 20-30% of these patients [1-3]. Recently, considerable effort has been focused on the causative role of aluminum in “dialysis osteomalacia” [4,5]. However, uremic osteomalacia independent of aluminum also exists [6-9].

The pathogenesis of uremic osteomalacia is uncertain, but focuses on the metabolic complications of uremia. Most important among these are: decreased levels of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) due to a loss of functional renal parenchyma and the mitochondrial 25-hydroxyvitamin D$_3$-1α-hydroxylase enzyme [10]; loss of 25-hydroxyvitamin D$_3$ (25(OH)D$_3$) and concurrent decreases in 1,25(OH)$_2$D$_3$ [11-13]; metabolic acidosis, which has been shown to potentiate the changes induced by uremia [14]; impaired mineralization due to altered local concentrations of calcium, pyrophosphate, magnesium, bicarbonate and other ions [15-18]; and increased bone resorption and calciuria [19-21], which accentuate abnormal calcium-phosphorus homeostasis.
Investigations into the role of uremia in the pathogenesis of osteomalacia have been hampered by the lack of a good in vivo or in vitro system. Although models of renal osteodystrophy do exist, they do not consistently reproduce the osteomalacic component, and are instead typically characterized by fibrous osteodystrophy [22-24].

We have shown previously that while rats made uremic by five-sixth nephrectomy develop a striking hyperostoidosis when fed a low calcium and low phosphorus diet, these lesions do not develop in non-uremic rats fed the same diet [25]. Although not directly analogous to the more common forms of spontaneous uremic osteomalacia in man or animals, this model could nonetheless be quite useful in investigations into the effects of uremia and vitamin D metabolites in the pathogenesis of osteomalacia.

In this experiment, our objectives were to evaluate the contributions of uremia and dietary phosphorus levels to the initiation of osteomalacia in the rat, and the influence of these factors on the plasma levels of the major vitamin D metabolites. In addition, we evaluated the correlations between these vitamin D metabolite levels and the light microscopic lesions of osteomalacia.

**Materials and Methods:**

Twenty-four (24) male adult Sprague-Dawley rats, weighing 320±20 g (twelve weeks old) were evaluated in this study. All rats were housed individually under
fluorescent lighting on a twelve-hour on, twelve-hour off lighting cycle throughout the entire study, and were initially fed a standard laboratory rodent chow (Agway Prolab Diet 3000) and water *ad libitum* for a seven day acclimation period. The rats were then randomly divided, half being placed into the 5/6 nephrectomized (Nx) group, and half into the sham operated (SO) control group. The Nx rats were anesthetized with intramuscular injections of 0.067 mg/kg fentanyl and 3.33 mg/kg droperidol (Innovar-Vet, Pittman Moore, Washington’s Crossing, NJ) and 1.2 mg/kg morphine (Eli Lilly & Co., Indianapolis, IN), and had both the cranial and caudal thirds of their left kidney removed via a left paracostal approach by using a silk ligature “cutting/crushing” technique. Seven days later, the rats were again anesthetized, and had their entire right kidney removed via a right paracostal approach. The SO rats were treated identically, but both of their kidneys were exteriorized only, and replaced intact after removal of the renal capsule.

Following surgery, both groups were maintained for a fourteen day recovery period on water *ad libitum*, and were pair-fed the standard laboratory rodent chow, such that all SO rats received an amount of food equal to the amount consumed the previous day by the Nx rats. Based on daily evaluations and subjective comparisons of food consumption, the surgical procedure was very well tolerated. The rats were then randomly divided into the following groups of six rats each:
NP Nx = 5/6 nephrectomized rats fed normal calcium (0.5%) and normal phosphorus (0.3%) diet (Teklad # TD 85343).

NP SO = sham-operated rats fed normal calcium (0.5%) and normal phosphorus (0.3%) diet.

LP Nx = 5/6 nephrectomized rats fed normal calcium (0.5%) and low phosphorus (0.03%) diet (Teklad # TD 85345).

LP SO = sham-operated rats fed normal calcium (0.5%) and low phosphorus (0.03%) diet.

All diets contained adequate levels of Vitamin D₃ (2200 I.U./kg diet). All rats were weighed at the onset of the custom diet feeding period.

The rats were maintained for seven days on these diets, with pair-feeding of the SO control rats based on consumption of the Nx rats, as before. Water continued to be offered ad libitum. During this time, all rats were treated on the first day (day 28), and again on the sixth day (day 34) with an intraperitoneal injection of 10 mg/kg calcein (Sigma Chemical Co., St. Louis, MO).

After this seven day period on the custom diets (day 35), all rats were weighed, and then killed via terminal exsanguination while under ether anesthesia. Two milliliters of blood was placed in a silicon-coated sterile glass tube, and a complete serum chemistry profile obtained using a Dacos automated serum analyzer (# 6701815). This
analysis included measurement of blood urea nitrogen (BUN), creatinine, calcium and phosphorus. All samples were refrigerated for no more than three hours before analysis. Measurement of the levels of several vitamin D metabolites \((1,25(OH)_2D_3, 24,25\text{-dihydroxyvitamin } D_3 (24,25(OH)_2D_3)\) and \(25(OH)D_3\)) on plasma samples from all rats were also obtained. These plasma samples were collected by adding 60 I.U. of heparin sodium to six milliliters of whole blood at the time of exsanguination. Following centrifugation at 1500 rpm for twenty minutes, the plasma was aspirated off, and frozen at -80 degrees centigrade until analysis. All metabolites were measured using previously published techniques [26]. Parathyroid hormone (PTH) levels were measured on selected groups using an N-terminal specific radioimmunoassay [27,28].

From the six rats within each group, the left tibia was removed, dissected free of soft tissue, and cut transversely two millimeters proximal to the distal end of the tibial crest. After shaving off the cranial and caudal edges of the tibial plateau, all specimens were fixed in cold (4 degrees centigrade), 40% ethanol, and subsequently dehydrated in increasing concentrations of ethanol and placed undecalcified in methyl methacrylate. These blocks were sectioned in a frontal plane at six microns on a Jung model K sledge microtome and stained with Von Kossa stain. Additional six micron thick sections were prepared unstained for evaluation of the calcein bone labels with a Zeiss epifluorescent microscope, and from selected groups for histochemical staining for aluminum [29].
On each of the stained undecalcified sections, the following parameters were evaluated within a 1 × 2 millimeter metaphyseal area, using the nomenclature proposed by the Report of the ASBMR Histomorphometry Committee [30]. The sampling area was located one-half millimeter below the last hypertrophied chondrocyte so as to avoid the primary and secondary trabeculae and centered within the metaphysis, based on the technique originally described by Baron [31]. This morphometric analysis was performed by using a semi-automated, microprocessor controlled Zeiss Interactive Digital Analysis System (ZIDAS) and a calibrated eyepiece:

Growth plate thickness (G.Pl.Th [μm]) - The average distance from the epiphyseal/physeal junction to the last hypertrophied chondrocyte, measured at four different locations.

Metaphyseal trabecular bone volume (BV/TV [%]) - The total volume of the sample occupied by osteoid and bone trabeculae, expressed as a percentage of the total tissue volume.

Osteoid volume (OV/BV [%]) - The total volume of osteoid within the sample area expressed per unit bone volume.

Metaphyseal trabecular surface (BS/TV [mm²/mm³]) - The total surface area of the bony trabeculae within the sample, expressed as a fraction of the tissue volume.
Osteoid surface (OS/BS [%]) - The fraction of the trabecular surface (BS) which is covered by osteoid.

Osteoid seam thickness (O.Th [μm]) - The average thickness of the osteoid seams, calculated as OV divided by OS.

Osteoblastic surface (Ob.S/BS [%]) - The area of the trabecular surface which is covered by cuboidal osteoblasts, expressed as a fraction of BS.

Osteoclastic surface (Oc.S/BS [%]) - The area of the trabecular surface which is covered by osteoclasts within Howship’s lacunae, expressed as a fraction of BS.

Mineralizing surface (MS/BS [%]) - We have calculated the mineralizing surface as the extent of double-labeled surfaces (dLS) only, as has been suggested for trabecular remodeling in the rat [31].

Mineral appositional rate (MAR [μm/d]) - The rate of mineralization of the osteoid matrix at sites of active mineralization, measured as the average distance between the calcein labels (corrected for obliquity by a factor of 0.79) in the double labeled areas, divided by the time in days between calcein administration.
Bone formation rate (BFR/BS [\( \mu m^3/\mu m^2/d \)]) - The amount of new mineralized bone formed per unit time, calculated as the product of MAR and MS/BS.

Osteoid maturation time (Omt [d]) - The average time between osteoid matrix formation and mineralization at sites of active mineralization, calculated as O.Th divided by MAR.

Unless otherwise indicated, all results are expressed as the mean of six values; the standard errors of the means are also indicated. Statistical analysis (performed with SAS on an IBM 3081-D mainframe) consisted of a Shapiro-Wilk normalcy test, followed by a non-parametric Wilcoxon Rank-Sum procedure and pairwise t-tests corrected for multiple comparisons, using a significance level of \( p < 0.05 \) [32]. Significant correlations between all variables (also at the \( p < 0.05 \) significance level) were determined with Spearman correlations coefficients.

For the diagnosis of osteomalacia, significant alterations in both the osteoid volume and the osteoid maturation time were required, to indicate an increase in unmineralized osteoid due to a delay in the mineralization process.
Results:

Body weight data are presented in Table 1. At both the beginning and end of the feeding of the custom diets, there were no significant differences in the weights between the different groups, regardless of renal function or dietary phosphorus level.

Static bone histomorphometry data are presented in Table 2. For purposes of comparison, all groups are compared to the NP SO control rats.

The width of the growth plate (G.Pl.Th) was significantly decreased in the LP SO group. The growth plate was not significantly thickened in any group.

The total bone volume (BV/TV) was not significantly different between the groups.

The osteoid volume (OV/BV) was dramatically increased in the LP Nx group, when compared to the baseline controls.

No significant differences were found in the total trabecular surfaces (BS/TV) in any group.
The fraction of the trabecular perimeter covered by osteoid (OS/BS) was significantly increased in the LP Nx group.

Osteoid thickness (O.Th) was markedly elevated in the LP Nx group.

Neither the osteoblastic surface (Ob.S/BS) nor the osteoclastic surface (Oc.S/BS) were significantly altered by uremia or the dietary manipulations in this study.

The aluminum-stained trabecular surfaces (Al.S/BS) were not detectable by the staining method used in both the NP SO controls and the osteomalacic LP Nx groups (the only two groups examined histochemically for aluminum).

The dynamic bone histomorphometry data are presented in Table 3. The absolute double labeled surface was significantly decreased only in the LP Nx rats.

The mineralizing surfaces (MS/BS) were decreased only in the LP Nx group.

The mineral appositional rate (MAR), which is the distance between double labels, was not significantly different between groups.

The bone formation rate (BFR/BS) was decreased only in the LP Nx group.
The osteoid maturation time (Omt) was increased in the LP Nx group.

Since increased osteoid may reflect not only a decrease in the mineralization rate, but also an increase in the rate of osteoid synthesis, we measured the rate of osteoid formation in selected groups. Measurements were made (in areas of double labeling) from the outermost (second) label to the osteoid-marrow interface, thereby indicating the thickness of osteoid formed in the day between the last label administration and euthanasia. LP Nx rats had a significantly elevated rate of osteoid apposition compared to the LP SO rats or the NP SO controls (LP Nx = 17.5 ± 3.61 μm/d, LP SO = 6.70 ± 2.77 μm/d and NP SO = 3.29 ± 2.05 μm/d; p<0.05).

Serum and plasma biochemical parameters are presented in Table 4. Serum calcium levels were elevated in the LP SO and LP Nx groups.

Serum phosphorus levels were decreased in all rats fed low phosphorus. It is interesting that the serum phosphorus is numerically lowest in the LP Nx group, which is the group with the highest elevation of serum calcium.

The BUN and serum creatinine values varied as expected; all nephrectomized rats had significant elevations of both BUN and creatinine, documenting this protocol’s ability to consistently produce a similar degree of uremia. In addition, the BUN was lower than
controls in the sham-operated groups fed low phosphorus; creatinine was mildly elevated in the LP SO rats.

The plasma levels of 1,25(OH)₂D₃ were found to be elevated in the LP SO and LP Nx groups. This directly parallels the groups which were found to have a lowered serum phosphorus.

24,25(OH)₂D₃ was decreased in all uremic rats tested, regardless of diet. It was also decreased in the LP SO rats. These changes are the inverse of those seen in 1,25(OH)₂D₃ levels, with the exception of the NP Nx group.

25(OH)D₃ was significantly elevated only in the LP Nx group.

PTH was decreased in both the LP Nx and LP SO groups.

Correlations between the vitamin D metabolite levels and the histomorphometric variables, which are presented in Table 5, revealed that 1,25(OH)₂D₃ correlated positively with OV/BV, O.Th and Omt, although none of the coefficients of correlation were greater than 0.64. 24,25(OH)₂D₃ correlated only with OV/BV, and 25(OH)D₃ had no significant correlations. PTH levels correlated positively with serum phosphorus and negatively with plasma 1,25(OH)₂D₃ quite well; PTH also correlated with MS/BS (0.64),
BFR/BV (0.63) and Oc.S/BS (-0.61). $1,25(OH)_2D_3$ correlated negatively (-0.69) with the serum phosphorus level.

**Discussion:**

Rats made uremic by five-sixth nephrectomy and subsequently fed low (0.03%) dietary phosphorus develop an osteomalacia characterized by an increased osteoid surface, volume and seam thickness, a prolonged osteoid maturation time, and a decreased bone formation rate. This osteomalacia is associated with hypophosphatemia, increased plasma $1,25(OH)_2D_3$ and decreased plasma $24,25(OH)_2D_3$ and PTH levels compared with sham-operated rats fed normal (0.3%) dietary phosphorus. The relative contributions of the low dietary phosphorus and the uremia to the osteomalacia in our model are unknown. Osteomalacia did not develop in LP SO or NP Nx rats. However, the data suggest that the low phosphorus diet may have been more critical. Values for MS/BS, MAR and BFR/BS in the LP SO rats were closer to those of the osteomalacic LP Nx rats than to those of NP SO rats. Groups of rats were also fed diets with low (0.05%) calcium, either alone or along with low phosphorus. Osteomalacia did not develop in the low calcium-fed rats; in addition, the rats fed both low calcium and low phosphorus were not significantly different from the rats fed low phosphorus alone (data not presented).
Dietary phosphorus deficiency has previously been implicated in the pathogenesis of osteomalacia [24,33-35]. Dietary phosphorus restriction is known to cause increased osteoid seam thickness and formation surfaces, and decreased mineral apposition [36,37]. The mechanisms whereby low dietary or serum phosphorus produce osteomalacia are not well characterized, but include an inhibition of cell-mediated actions necessary to initiate mineralization, and a demonstration that phosphorus incorporation is the rate-limiting step in the mineralization process [38].

A contributing factor to the low dietary phosphorus-induced osteomalacia in our model may be elevated plasma 1,25(OH)\(_2\)D\(_3\). It is known that dietary phosphorus, through serum phosphorus levels, can significantly affect 1,25(OH)\(_2\)D\(_3\) levels; as was found in this study, low phosphorus has been shown to elevate serum 1,25(OH)\(_2\)D\(_3\) [39]. Elevated levels of 1,25(OH)\(_2\)D\(_3\) have been associated with hyperosteooidosis and osteomalacia in rats [40-43]. The pathogenesis of this 1,25(OH)\(_2\)D\(_3\)-induced osteomalacia is unknown, but as was seen in this study, may involve both increased matrix production [44] as well as impaired matrix mineralization [45].

Surprisingly, in our study, plasma 1,25(OH)\(_2\)D\(_3\) levels were equally elevated in both uremic and sham-operated rats fed the low phosphorus diet. Although renal mass was reduced by 5/6 in the uremic rats, the remaining non-diseased remnant kidney was apparently able to respond to the low serum phosphorus stimulus to produce plasma 1,25(OH)\(_2\)D\(_3\) levels equivalent to those in rats with intact kidneys.
Since both the low phosphorus diet and uremia were required for the development of osteomalacia in our model, it is apparent that uremia also contributed to the osteomalacia. Uremia has long been implicated as a causative factor in the development of osteomalacia. In recent years, attention has been focused on the role of aluminum in the dialysis-related osteomalacia [46]. Uremic osteomalacia independent of elevated bone aluminum has also been described [47,48]. Factors commonly implicated in the pathogenesis of this form of osteomalacia include decreased $1,25(OH)_2D_3$ levels and the complications of chronic renal failure [49]. The degree of uremia produced by our nephrectomy technique is mild, with only moderate elevations of BUN and creatinine and no hyperphosphatemia or elevated PTH levels. The metabolic complications which attend advanced renal failure, usually thought to be responsible for uremic osteomalacia, may therefore not yet be involved in the genesis of the form of osteomalacia present in this study. The factors which may contribute to the osteomalacia seen in this study are: hypophosphatemia [49]; metabolic acidosis [50]; altered collagen formation and maturation [49]; and abnormal maturation of the amorphous calcium-phosphorus complexes into hydroxyapatite [49]. Although all of these factors were not specifically examined, it is likely that they play a role in the development of the osteomalacia produced by our protocol.

In summary, this study has established an easily manipulated model of the early pathogenesis of osteomalacia in uremic rats which should prove useful in investigating the influence of uremia on the mineralization process.
Table 1. Body Weights (mean ± S.E.)

<table>
<thead>
<tr>
<th></th>
<th>NP Nx</th>
<th>NP SO</th>
<th>LP Nx</th>
<th>LP SO</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 28</td>
<td>325.0±5.0</td>
<td>348.3±6.7</td>
<td>348.3±18.6</td>
<td>341.7±3.3</td>
</tr>
<tr>
<td>day 35</td>
<td>328.3±3.3</td>
<td>355.0±5.0</td>
<td>360.0±20.2</td>
<td>363.3±9.3</td>
</tr>
</tbody>
</table>

Note: No significant differences were noted at the p<0.05 significance level.

Table 2. Static Histomorphometry of Tibia (mean ± S.E.)

<table>
<thead>
<tr>
<th></th>
<th>NP Nx (μm)</th>
<th>NP SO (μm)</th>
<th>LP Nx (μm)</th>
<th>LP SO (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.Pl.Th (%)</td>
<td>192.3±9.0 AB</td>
<td>207.2±9.4 AB</td>
<td>237.1±30.4 A</td>
<td>166.5±13.0 CD</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>14.9±3.0 A</td>
<td>14.5±3.3 A</td>
<td>14.3±2.4 A</td>
<td>15.8±2.9 A</td>
</tr>
<tr>
<td>OV/BV (%)</td>
<td>2.0±0.3 B</td>
<td>1.2±0.4 B</td>
<td>67.2±18.0 A</td>
<td>1.4±0.7 B</td>
</tr>
<tr>
<td>BS/TV (mm²/mm³)</td>
<td>5.9±0.8 A</td>
<td>5.1±0.7 AB</td>
<td>5.4±0.5 A</td>
<td>5.6±0.9 A</td>
</tr>
<tr>
<td>OS/BS (%)</td>
<td>10.8±1.8 BC</td>
<td>8.9±2.6 BC</td>
<td>34.7±6.1 A</td>
<td>7.7±2.8 BC</td>
</tr>
<tr>
<td>O.Th (μm)</td>
<td>5.3±0.6 B</td>
<td>4.2±0.2 B</td>
<td>62±13 A</td>
<td>5.0±0.4 B</td>
</tr>
<tr>
<td>Ob.S/BS (%)</td>
<td>22.7±3.1 ABC</td>
<td>8.1±4.3 BC</td>
<td>24.8±3.6 ABC</td>
<td>16.1±4.0 C</td>
</tr>
<tr>
<td>Oc.S/BS (%)</td>
<td>0.4±0.2 AB</td>
<td>0.3±0.2 AB</td>
<td>0.7±0.2 A</td>
<td>1.4±0.6 A</td>
</tr>
</tbody>
</table>

Note: Unique letters horizontally denote significant differences between groups (viz. AB vs. CD). Any shared letters horizontally indicate no significant difference (viz. AB vs. BC).

Table 3. Dynamic Histomorphometry of Tibia (mean ± S.E.)

<table>
<thead>
<tr>
<th></th>
<th>NP Nx (6)</th>
<th>NP SO (5)</th>
<th>LP Nx (4)</th>
<th>LP SO (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dLS (μm)</td>
<td>1.1±0.2 A</td>
<td>0.7±0.2 AB</td>
<td>0.1±0.0 C</td>
<td>0.4±0.0 BC</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>11.1±2.3 A</td>
<td>9.2±2.5 A</td>
<td>1.5±0.5 B</td>
<td>4.2±1.7 AB</td>
</tr>
<tr>
<td>MAR (μm/d)</td>
<td>2.2±0.2 AB</td>
<td>2.4±0.2 A</td>
<td>2.8±1.1 AB</td>
<td>2.8±0.5 A</td>
</tr>
<tr>
<td>BFR/BS (μm²/μm²/d)</td>
<td>0.3±0.1 A</td>
<td>0.3±0.0 A</td>
<td>0.1±0.0 B</td>
<td>0.2±0.1 AB</td>
</tr>
<tr>
<td>Omt (d)</td>
<td>2.5±0.3 AB</td>
<td>1.6±0.3 B</td>
<td>21.1±11.3 A</td>
<td>1.7±0.3 AB</td>
</tr>
</tbody>
</table>

Note: Numbers in parentheses denote number of rats per group with double labels.

Unique letters horizontally denote significant differences between groups (viz. AB vs. CD). Any shared letters horizontally indicate no significant difference (viz. AB vs. BC).
Table 4. Serum and Plasma Biochemistry (mean ± S.E.)

<table>
<thead>
<tr>
<th></th>
<th>NP Nx</th>
<th>NP SO</th>
<th>LP Nx</th>
<th>LP SO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dl)</td>
<td>11.1±0.2</td>
<td>BC</td>
<td>10.7±0.1</td>
<td>CD</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>6.5±0.5</td>
<td>A</td>
<td>6.5±0.2</td>
<td>A</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>50±6</td>
<td>B</td>
<td>22±1</td>
<td>C</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.3±0.1</td>
<td>BC</td>
<td>0.7±0.0</td>
<td>EF</td>
</tr>
<tr>
<td>1,25D$_3$ (pg/ml)</td>
<td>62.8±6.4</td>
<td>D</td>
<td>78.5±9.1</td>
<td>CD</td>
</tr>
<tr>
<td>24,25D$_3$ (ng/ml)</td>
<td>8.73±1.75</td>
<td>BC</td>
<td>13.24±2.12</td>
<td>A</td>
</tr>
<tr>
<td>25D$_3$ (ng/ml)</td>
<td>25.2±4.7</td>
<td>AB</td>
<td>17.6±2.8</td>
<td>BC</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>34.2±4.5</td>
<td>A</td>
<td>29.0±6.5</td>
<td>A</td>
</tr>
</tbody>
</table>

Note: Unique letters horizontally denote significant differences between groups (viz. AB vs. CD). Any shared letters horizontally indicate no significant difference (viz. AB vs. BC).

Table 5. Histomorphometry and Biochemistry Parameter Correlation Coefficients

<table>
<thead>
<tr>
<th></th>
<th>OS/BS</th>
<th>OV/BV</th>
<th>O.Th</th>
<th>Omt</th>
<th>PTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)$_2$D$_3$</td>
<td>NS</td>
<td>0.44</td>
<td>0.42</td>
<td>0.49</td>
<td>-0.77</td>
</tr>
<tr>
<td>24,25(OH)$_2$D$_3$</td>
<td>NS</td>
<td>-0.46</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>25(OH)D$_3$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>-0.48</td>
<td>-0.35</td>
<td>NS</td>
<td>-0.42</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Note: NS = not significant at p < 0.05
References:


43. Boyce, R.W., Weisbrode, S.E. and Kindig, O.: Ultrastructural development of hyperosteoiodosis in 1,25(OH)$_2$D$_3$-treated rats fed high levels of dietary calcium. Bone 1985, 6:165-172


CHAPTER II

THE EFFECTS OF THE MAJOR VITAMIN D METABOLITES ON THE RESOLUTION OF OSTEOMALACIA IN UREMIC RATS

Abstract:

We have previously shown that an osteomalacia dependent upon both a low phosphorus diet and uremia (five-sixth nephrectomy) can be produced rapidly in rats. This is associated with hypophosphatemia and elevated 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). In order to investigate the role of exogenously administered vitamin D metabolites upon the resolution of this osteomalacia, seventy-two male Sprague Dawley rats weighing 320±20 g were subjected to a two-step, subtotal nephrectomy and subsequently fed a diet with low (0.03%) phosphorus for seven days. Groups of six rats each were then either continued on the low phosphorus diet, or switched to a nutrient-matched diet with normal (0.3%) phosphorus for an additional ten days. During this time, the rats were infused daily with either: 27 ng 1,25(OH)₂D₃; 81 ng 24,25 dihydroxyvitamin D₃ (24,25(OH)₂D₃); 135 ng 25 hydroxyvitamin D₃ (25(OH)D₃); both 27 ng 1,25(OH)₂D₃ and 81 ng 24,25(OH)₂D₃; or placebo. Dietary phosphorus repletion was found to reverse the osteomalacia by decreasing the growth plate thickness, the osteoid surface and volume, the osteoid maturation time, serum calcium and plasma
1,25(OH)₂D₃, and by increasing the mineralizing surface, bone formation rate and serum phosphorus. The osteomalacia was also reversed in phosphorus-repleted rats treated with 24,25(OH)₂D₃, with no additional effects attributable to the 24,25(OH)₂D₃ itself. Osteomalacia in phosphorus-repleted rats treated with 25(OH)D₃ or 1,25(OH)₂D₃ was only partially reversed; healing was interpreted to be impaired by the elevated plasma 1,25(OH)₂D₃ levels present in these rats. None of the rats maintained on the LP diet improved, despite various corrections in serum calcium and phosphorus and plasma vitamin D levels due to the administered vitamin D metabolites. It was concluded that in this model, no vitamin D metabolite was as effective as dietary phosphorus repletion in curing the osteomalacia. Exogenous 1,25(OH)₂D₃ and 25(OH)D₃ (via elevation of plasma 1,25(OH)₂D₃ levels) impaired mineralized bone formation, while 24,25(OH)₂D₃ was without a specific role in the bone mineralization process.

Introduction:

Since the elucidation of the metabolism of vitamin D nearly twenty years ago there has been debate over the physiologic and pharmacologic roles of each of the different forms of this hormone, particularly in the skeletal system. Considerable uncertainty remains concerning the action of these metabolites in the normal mineralization process. While many investigators have assigned specific roles to individual metabolites, no unifying mechanism has yet emerged. Studies with fluoridated vitamin D analogs (which prevent specific hydroxylation steps) suggest that 1,25-
dihydroxyvitamin D₃ (1,25(OH)₂D₃) is the only physiologically active metabolite [1,2]. Conversely, other studies have found that 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) is required for normal bone formation [3].

In attempting to treat the mineralization failure of osteomalacia, many researchers have found 1,25(OH)₂D₃ to have a beneficial role [4,5], although in other cases it was without an ameliorating effect [6,7]. 24,25(OH)₂D₃ has also been found to be able to cure some forms of osteomalacia [8], but is more often shown to be ineffective [5,9,10]. In addition, as the precursor to both metabolites, 25-hydroxyvitamin D₃ (25(OH)D₃), which is often decreased in patients with osteomalacia, has also yielded variable results [11,12]. Finally, some have suggested that both 1,25(OH)₂D₃ and 24,25(OH)₂D₃ play a role, and therefore act synergistically in the treatment of osteomalacia [13,14].

In previous work, we have shown that osteomalacia can quickly and consistently be produced by feeding a low (0.03%) phosphorus diet to rats made uremic by five-sixth nephrectomy [15]; the osteomalacia was dependent upon both the uremia and low phosphorus diet. The objectives of this study were to evaluate the effects of the major vitamin D metabolites upon the resolution of this model of osteomalacia, and to correlate alterations in plasma vitamin D metabolite concentrations with the light microscopic changes observed.
Materials and Methods:

Seventy-two (72) male adult Sprague-Dawley rats, weighing 320±20 g (twelve weeks old) were evaluated in this study. All rats were housed individually under fluorescent lighting on a twelve-hour on, twelve-hour off lighting cycle throughout the entire study, and were initially fed a normal laboratory rodent chow (Agway Prolab Diet 3000) and water ad libitum for a seven day acclimation period. The rats were then anesthetized with intramuscular injections of 0.067 mg/kg fentanyl and 3.33 mg/kg droperidol (Innovar-Vet, Pittman Moore, Washington’s Crossing, NJ) and 1.2 mg/kg morphine (Eli Lilly & Co., Indianapolis, IN), and had both the cranial and caudal thirds of their left kidney removed via a left paracostal approach by using a silk ligature “cutting/crushing” technique. Seven days later, the rats were anesthetized again, and had their entire right kidney removed via a right paracostal approach.

Following surgery, the rats were maintained for an additional fourteen days on the standard laboratory rodent chow and water ad libitum. Six rats were then fed a nutritionally complete, vitamin D replete diet (NP, # TD 85343, Teklad, Inc., Madison, WI) with normal calcium (0.5%) and normal phosphorus (0.3%), while the other rats were fed a nutrient-matched diet (LP, # TD 85345) with normal calcium (0.5%) and low phosphorus (0.03%) ad libitum for seven days.
Following this seven day period, the six NP-fed rats and six LP-fed rats were euthanatized prior to further treatment (these groups were therefore termed PT-NP and PT-LP respectively) to confirm the existence of osteomalacia, as previously described [15]. The remaining sixty LP-fed rats then were divided equally into these five treatment groups:

1,25-LP or NP — 27 ng 1,25(OH)₂D₃ per day.
24,25-LP or NP — 81 ng 24,25(OH)₂D₃ per day.
25-LP or NP — 135 ng 25(OH)D₃ per day.
1,25/24,25-LP or NP — 27 ng 1,25(OH)₂D₃ and 81 ng 24,25(OH)₂D₃ per day.
PL-LP or NP — Vehicle only (propylene glycol) placebo.

Vitamin D metabolites were kindly provided by Dr. M. Uskokovic (Hoffmann-La Roche, Inc., Nutley, NJ). As indicated, within each of these treatment groups of twelve rats, six were fed the LP diet and six were returned to the NP diet. This treatment regime lasted for ten days. Each of the above metabolites, as well as the placebo was administered as a constant infusion via a subcutaneous Alzet (model 2002, Alza Corp., Palo Alto, CA) osmotic pump, implanted on the first treatment day.

During this time, all rats in all groups were also treated on the third day and again on the ninth day with an intraperitoneal injection of calcein (10 mg/kg). All rats were then killed at the end of this ten day period via terminal exsanguination while under
ether anesthesia. A complete serum chemistry profile was obtained using a Dacos automated serum analyzer (# 6701815). This analysis included measurement of BUN, creatinine, calcium and phosphorus. All sera were refrigerated for no more than three hours before analysis. Measurement of the levels of three vitamin D metabolites (1,25(OH)₂D₃, 24,25(OH)₂D₃ and 25(OH)D₃) in plasma samples from all rats were also obtained. Plasma samples were collected by adding 60 I.U. of heparin to six milliliters of whole blood at the time of exsanguination. Following centrifugation at 1500 rpm for twenty minutes, the plasma was aspirated, and frozen at -80 degrees centigrade until analysis. All metabolites were measured using techniques previously described [16].

From the six rats within each group, the left tibia was removed, dissected free of soft tissue, and cut transversely two millimeters proximal to the distal end of the tibial crest. After shaving off the cranial and caudal edges of the tibial plateau, all specimens were fixed in cold (4 degrees centigrade) 40% ethanol, and subsequently dehydrated in increasing concentrations of ethanol and placed undecalcified in methyl methacrylate. These blocks were sectioned in a frontal plane at six microns on a Jung model K sledge microtome and stained with Von Kossa stain. Additional six micron thick sections were prepared unstained for evaluation of the calcein bone labels with a Zeiss (Carl Zeiss, Inc., Thornwood, NY) epifluorescent microscope.

On each of the stained undecalcified sections (one per rat), a 1 × 2 millimeter metaphyseal area was evaluated, using the nomenclature proposed by the Report of the
ASBMR Histomorphometry Committee [17]. The sampling area was located one-half millimeter below the last hypertrophied chondrocyte to avoid the primary and secondary trabeculae, and centered within the metaphysis, based on previously published techniques [18]. This morphometric analysis was performed by using a semi-automated, microprocessor controlled Zeiss Interactive Digital Analysis System (ZIDAS) and a calibrated eyepiece. The following parameters were evaluated:

Growth plate thickness (G.Pl.Th [μm]) - The average distance from the epiphyseal/physeal junction to the last hypertrophied chondrocyte, measured at four different locations.

Metaphyseal trabecular bone volume (BV/TV [%]) - the total volume of the sample occupied by osteoid and bone trabeculae, expressed as a percentage of the total tissue volume.

Osteoid volume (OV/BV [%]) - The total volume of osteoid within the sample area expressed per unit bone volume.

Metaphyseal trabecular surface (BS/TV [mm²/mm³]) - The total surface area of the bony trabeculae within the sample, expressed as a fraction of the tissue volume.
Osteoid surface (OS/BS [%]) - the fraction of the trabecular surface (BS) covered by osteoid.

Osteoid seam thickness (O.Th [μm]) - The average thickness of the osteoid seams, calculated as OV divided by OS.

Osteoblastic surface (Ob.S/BS [%]) - The area of the trabecular surface which is covered by cuboidal osteoblasts, expressed as a fraction of BS.

Osteoclastic surface (Oc.S/BS [%]) - The area of the trabecular surface which is covered by osteoclasts within Howship's lacunae, expressed as a fraction of BS.

Mineralizing surface (MS/BS [%]) - We have calculated the mineralizing surface as the extent of the double-labeled surfaces (dLS) only, as has been suggested for trabecular remodeling in the rat [18,19].

Mineral appositional rate (MAR [μm/d]) - The rate of mineralization of the osteoid matrix at sites of active mineralization, measured as the average distance between the calcein labels (corrected for obliquity by a factor of 0.79) in the double labeled areas, divided by the time in days between calcein administration.
Bone formation rate (BFR/BS [\(\mu m^3/\mu m^2/d\)]) - The amount of new mineralized bone formed per unit time, calculated as the product of MAR and MS/BS.

Osteoid maturation time (Omt [d]) - The average time between osteoid matrix formation and mineralization at sites of active mineralization, calculated as O.Th divided by MAR.

In addition, the osteoid appositional rate (OAR) was determined in selected groups by dividing the distance between the second label on doubled labeled surfaces and the osteoid-lining cell interface by the time between the last label administration and the animal's death (1 day).

Unless otherwise indicated, all results are expressed as the mean of six values; the standard errors of the means are also indicated. Statistical analysis (performed with SAS on an IBM 3081-D mainframe) consisted of a Shapiro-Wilk normalcy test, followed by a non-parametric Wilcoxon Rank-Sum procedure and pairwise t tests corrected for multiple comparisons, using a significance level of \(p < 0.05\) [20]. Significant correlations between all variables (also at the \(p < 0.05\) significance level) were determined with Spearman correlations coefficients.
For the diagnosis of osteomalacia, significant alterations in both the osteoid volume and the osteoid maturation time were required, to indicate an increase in unmineralized osteoid due to a delay in the mineralization process.

Results:

Bone histomorphometry data are presented in Tables 6 and 7. Serum and plasma biochemistry data are presented in Table 8.

As has been previously shown, compared to the PT-NP rats, the PT-LP rats had increased osteoid thickness, surface and volume, osteoid maturation time, serum calcium and plasma 1,25(OH)_{2}D_{3}, and decreased mineralizing surface, bone formation rate and serum phosphorus. These bone changes were interpreted as osteomalacia and were thought to be due to the combination of hypophosphatemia and uremia [15].

This osteomalacia persisted in the PL-LP rats. As in the PT-LP rats, PL-LP rats had increased osteoid thickness, surface and volume, osteoid maturation time and serum calcium, and decreased mineralizing surface, bone formation rate and serum phosphorus compared with PT-NP rats. Growth plate thickness, although not significantly different from PT-LP rats, was increased in PL-LP rats compared with PT-NP rats. The elevation in plasma 1,25(OH)_{2}D_{3} seen in PT-LP rats was not sustained in PL-LP rats.
By comparing the PL-LP rats to the PL-NP rats, it can be seen that the dietary phosphorus repletion reversed the osteomalacia by preventing the increase in growth plate thickness, causing a decrease in the osteoid surface and volume and osteoid maturation time, and an increase in the mineralizing surface and bone formation rate. These changes were associated with an increase in serum phosphorus and a decrease in serum calcium and plasma 1,25(OH)₂D₃. All these variables returned to their baseline (PT-NP) levels, with the exception of the plasma 1,25(OH)₂D₃ which fell to half the PT-NP level.

Exogenous 1,25(OH)₂D₃ treatment of LP-fed rats was only partially successful in reversing the osteomalacia, even though serum phosphorus was elevated to the same extent as in PL-NP rats. 1,25-LP rats had reduced osteoid surface, growth plate thickness and bone volume, and increased mineral appositional rate compared with PL-LP rats. However, although numerically lower, the osteoid mineralization time was not significantly different from that of the PL-LP rats. In addition, the OAR was significantly increased (14.5 ± 3.7 μm/d) when compared to PL-NP controls (2.8 ± 1.0 μm/d, p<0.05). These bone changes were associated with decreases in plasma 25(OH)D₃ and increases in serum calcium and phosphorus and plasma 1,25(OH)₂D₃.

The only bone parameter changed by the administration of 24,25(OH)₂D₃ to LP-fed uremic rats was the percent of osteoclastic bone surface, which was significantly elevated; this was not accompanied by changes in the total bone surface or volume compared to PL-LP rats. The osteomalacia persisted in 24,25-LP rats despite an
elevation in serum phosphorus to the same level as was present in the PL-NP rats. It is uncertain whether this elevation in serum phosphorus was due to 24,25(OH)_{2}D_{3} or an unexplained increase in uremia (increased serum creatinine) in this group. In addition, compared with PL-LP rats, 24,25-LP rats had increased plasma 24,25(OH)_{2}D_{3} and decreased serum calcium and plasma 1,25(OH)_{2}D_{3} and 25(OH)D_{3}.

25(OH)D_{3} was also unsuccessful in ameliorating the osteomalacia in LP-fed rats. Neither osteoid volume nor osteoid maturation time were reduced in 25-LP rats compared with PL-LP rats; osteoid seam thickness, mineral appositional rate and osteoclastic surface were increased and osteoblastic surface was decreased. An increase in serum phosphorus (which remained significantly lower than that of PL-NP rats) was the only difference in the serum and plasma parameters between 25-LP and PL-LP rats.

As with the 1,25-LP rats, the LP-fed rats treated with both 1,25(OH)_{2}D_{3} and 24,25(OH)_{2}D_{3} had only partial correction of the osteomalacia. Compared with PL-LP rats, 1,25/24,25-LP rats had decreased osteoid volume and surface and growth plate thickness, but no decrease in osteoid maturation time. Combined 1,25(OH)_{2}D_{3} and 24,25(OH)_{2}D_{3} treatment of LP-fed uremic rats elevated serum phosphorus compared to PL-LP rats, but not to the level of PL-NP rats. Plasma 1,25(OH)_{2}D_{3} and 24,25(OH)_{2}D_{3} were increased and plasma 25(OH)D_{3} was decreased in 1,25/24,25-LP rats compared with PL-LP rats.
Surprisingly, the osteomalacia was not completely reversed in 1,25-NP rats, even though serum phosphorus was elevated to the same level as that of PL-NP rats. Osteoid volume in 1,25-NP rats was between the values of PL-LP and PL-NP rats, and not significantly different from either one. Osteoid maturation time in 1,25-NP rats was significantly greater than in PL-NP rats and not significantly different from PL-LP rats. 1,25-NP rats also had increased mineral appositional rate and osteoblastic surface, and decreased osteoid surface, total bone surface and growth plate thickness compared with PL-LP rats. In addition, the OAR was significantly increased (15.2 ± 5.6 µm/d) when compared to the PL-NP controls (2.8 ± 1.0 µm/d, p<0.05). These changes were associated with increased plasma 1,25(OH)₂D₃ and decreased plasma 25(OH)D₃ in 1,25-NP rats compared with PL-LP rats.

As in the PL-NP rats, rats fed NP and treated with 24,25(OH)₂D₃ had total correction of the osteomalacia compared with PL-LP rats. In 24,25-NP rats, the decrease in osteoid volume, surface and maturation time and growth plate thickness, and increase in mineralizing surface and bone formation rate were like those in PL-NP rats compared with PL-LP rats. The bone volume and bone surface in 24,25-NP rats however were significantly less than either PL-LP or PL-NP rats; this was not associated with differences in osteoblastic or osteoclastic surfaces. Similar to PL-NP rats, 24,25-NP rats had decreased plasma 1,25(OH)₂D₃ and elevated serum phosphorus compared with PL-LP rats. The decrease in serum calcium and plasma 25(OH)D₃ in
24,25-NP rats compared with PL-LP rats was greater than that found in PL-NP rats. Plasma 24,25(OH)2D3 in 24,25-NP rats was greater than in either PL-LP or PL-NP rats.

The NP-fed rats treated with 25(OH)D3 had only a partial reversal of the osteomalacia, again despite elevated serum phosphorus. 25-NP rats had reduced osteoid volume and surface, and decreased growth plate thickness to the same extent as PL-NP rats compared with PL-LP rats. However, decreases in the osteoid maturation time and increases in mineralizing surface and bone formation rates were in between those of the PL-LP and PL-NP groups, and were not significantly different from either group. 25-NP rats had elevated serum phosphorus to a greater level than did the PL-NP rats. Likewise, plasma 24,25(OH)2D3 was greater in 25-NP rats than in either PL-NP or PL-LP rats. Plasma 1,25(OH)2D3 was the same in PL-LP and 25-NP rats, and greater in both groups than in PL-NP rats. Plasma 25(OH)D3 was lower in 25-NP rats than in either the PL-LP or PL-NP rats, which is unexplained and paradoxical.

The NP-fed rats given 1,25(OH)2D3 and 24,25(OH)2D3 also had only partial correction of the osteomalacia. Osteoid volume and surface and growth plate thickness were reduced, and bone formation rate was increased in 1,25/24,25-NP rats compared with PL-LP rats. These changes were of the same magnitude and direction as seen in PL-NP rats. However, osteoid maturation time, although reduced in 1,25/24,25-NP rats, was not significantly different from either PL-LP or PL-NP rats. The elevation in serum phosphorus and reduction in serum calcium compared to PL-LP rats were similar in
PL-NP and 1,25/24,25-NP rats. Plasma 1,25(OH)₂D₃ and 25(OH)D₃ were significantly lower in 1,25/24,25-NP rats than in either PL-LP or PL-NP rats. Plasma 24,25(OH)₂D₃ was higher than in PL-NP rats, but the same as in PL-LP rats.

Correlations between the vitamin D metabolite levels and the histomorphometric variables, which are presented in Table 9, revealed that plasma 1,25(OH)₂D₃ correlated positively with OS/BS, OV/BV, O.Th and Omt, and negatively with G.Pl.Th. Plasma 24,25(OH)₂D₃ correlated only negatively with OS/BS and OV/BV; plasma 25(OH)D₃ did not significantly correlate with any of the parameters used to detect osteomalacia, but did correlate positively with G.Pl.Th. Serum phosphorus correlated negatively with G.Pl.Th, OS/BS, OV/BV and O.Th; serum phosphorus also correlated negatively with plasma 1,25(OH)₂D₃ (-0.33). None of these individual correlations were a particularly good fit, however, the highest being plasma 1,25(OH)₂D₃ with OV/BV at 0.41.

**Discussion:**

In previous work, we have described an osteomalacia in uremic rats fed a diet low in phosphorus. This osteomalacia was associated with hypophosphatemia, elevated 1,25(OH)₂D₃ and decreased 24,25(OH)₂D₃ [15]. In this study we found that this osteomalacia persisted with an additional ten day feeding of low phosphorus, as demonstrated by maintenance of the elevated osteoid thickness, surface and volume, increased osteoid maturation time and lowered mineralizing surface and bone formation
rate in PL-LP rats. Furthermore, we have shown that this osteomalacia can be reversed by dietary phosphorus repletion for ten days. Exogenous 1,25(OH)_{2}D_{3} and 25(OH)D_{3} were shown to be only partially effective in reversing the osteomalacia in LP-fed rats, and actually deleterious in NP-fed rats at the dosages used. 24,25(OH)_{2}D_{3} was shown to have no additional effect on either impaired (24,25-LP) or improved (24,25-NP) bone mineralization.

Providing the LP-fed rats with a phosphorus-replete diet was the most effective means of reversing the osteomalacia. This agrees with previous results which demonstrated that this model of osteomalacia is dependent upon both uremia and dietary phosphorus restriction, developing only when both conditions are met [15,21,22]. Compared to the non-osteomalacic PT-NP rats, the PL-NP rats were found to be completely returned to baseline values with respect to bone histomorphometry. In addition, the PL-NP rats had neither the low serum phosphorus nor the elevated plasma 1,25(OH)_{2}D_{3} previously associated with this osteomalacia; it would seem that dietary phosphorus repletion allows for adequate serum phosphorus levels, thereby lowering plasma 1,25(OH)_{2}D_{3} and improving mineralization. Other studies have also confirmed the importance of serum calcium and phosphorus values in the genesis of osteomalacia [23], and have suggested a secondary role for the vitamin D metabolites in the face of adequate serum calcium and phosphorus levels [24,25]. Further, investigators have shown that in the rabbit, vitamin D deficiency leads to osteomalacia only in those animals
which develop hypophosphatemia; normophosphatemic, D-deficient rabbits remain normal with respect to bone histology [26].

The impaired bone mineralization in the 1,25-LP rats was improved, but not completely reversed. The improvement present is likely due to the normalization of serum phosphorus, under the influence of the high plasma 1,25(OH)_2D_3 level. In contrast, 1,25(OH)_2D_3 administration prevented a complete resolution of the osteomalacia in rats fed the NP diet, despite normalization of serum phosphorus. The mechanism of this dual "improved/impaired" mineralization is not known. Although some researchers have reported a histological improvement of osteomalacia following treatment with 1,25(OH)_2D_3 [10,27], other studies have noted that 1,25(OH)_2D_3 is primarily effective in reducing the thickened growth plates seen in osteomalacia [22,28], and have found that this metabolite may produce increases in metaphyseal osteoid tissue [28-30]. Previous studies have provided morphological evidence that this 1,25(OH)_2D_3-induced hyperosteoidosis results from both increased matrix production [31] as well as from decreased mineralization [7]; we have shown that the rate of osteoid production is increased in uremic rats fed the LP diet. There also seems to be agreement in both static and dynamic histomorphometric studies that despite this increase in bone matrix tissue formation, overall bone mineralization is depressed by elevated levels of 1,25(OH)_2D_3 [7,30]. This is demonstrated in our study by the impaired bone mineralization in all groups with elevated plasma 1,25(OH)_2D_3, and by the positive correlations of plasma 1,25(OH)_2D_3 with Omt and with OV/BV; no group with a plasma 1,25(OH)_2D_3 above
65 pg/ml had normal bone mineralization parameters. These findings emphasize the paradoxical nature of the action of 1,25(OH)\(_2\)D\(_3\) on bone mineralization; while 1,25(OH)\(_2\)D\(_3\) can improve the osteomalacia in LP-fed rats, it also precludes a complete response in NP-fed rats. Additionally, as has been seen under similar conditions, normal bone mineralization apparently cannot occur in the face of elevated plasma levels of 1,25(OH)\(_2\)D\(_3\) [32].

Administration of exogenous 25(OH)D\(_3\) produced results similar to those of 1,25(OH)\(_2\)D\(_3\) administration. In fact, the effects of 25(OH)D\(_3\) treatment in this study appear to be due to 1,25(OH)\(_2\)D\(_3\) itself, as both 25-LP and 25-NP rats had increased plasma 1,25(OH)\(_2\)D\(_3\) levels compared to PL-NP rats; previous studies have shown that 25(OH)D\(_3\) can “substitute” for 1,25(OH)\(_2\)D\(_3\), mimicking its actions [11].

24,25(OH)\(_2\)D\(_3\) was itself without beneficial effect on the osteomalacia; uremic rats fed the LP diet and treated with 24,25(OH)\(_2\)D\(_3\) continued to have osteomalacia, despite normalization of both serum phosphorus and plasma 1,25(OH)\(_2\)D\(_3\). As these biochemical changes were associated with cures in other groups, it is uncertain what prevented healing in these rats. The degree of renal failure (as demonstrated by BUN and serum creatinine levels) was significantly worse in this group; whether this is a coincidence of the surgical procedure or an effect of 24,25(OH)\(_2\)D\(_3\) administration to uremic, LP-fed rats is not known. The effects of exogenous 24,25(OH)\(_2\)D\(_3\) on impaired renal function have not been previously reported. Some researchers have found that 24,25(OH)\(_2\)D\(_3\) is
required for normal bone formation [3]. Others report “complete morphological and biochemical healing” with 24,25(OH)₂D₃ treatment of a vitamin D-deficient, low dietary phosphorus-induced model of osteomalacia [8], via a proposed mechanism of action involving enhanced differentiation and maturation of chondrocytes in the epiphyseal growth plate. However, most investigators seem to have concluded, as we did, that 24,25(OH)₂D₃ is without a significant curative effect on osteomalacia [1,2,9].

One potentially meaningful effect of 24,25(OH)₂D₃ treatment was that elevated 24,25(OH)₂D₃ seemed to blunt the rise in plasma 1,25(OH)₂D₃ seen in rats maintained on either diet. The mechanism of this interference may involve the induction of 1,25(OH)₂D₃-degradative enzymes [33] and an increased metabolic clearance rate of 1,25(OH)₂D₃ [34].

In summary, in our model of low dietary phosphorus and uremia dependent osteomalacia, repletion of dietary phosphorus reverses the osteomalacia by elevating serum phosphorus and lowering plasma 1,25(OH)₂D₃. In the dosages used, no vitamin D metabolite was as effective as dietary phosphorus repletion in correcting the osteomalacia. In addition, vitamin D metabolites sometimes exacerbated the osteomalacia by causing elevations in plasma 1,25(OH)₂D₃. The persistence of osteomalacia in vitamin D-treated rats in which serum phosphorus was elevated to control levels and plasma 1,25(OH)₂D₃ was reduced (the 24,25-LP and the 1,25/24,25-NP groups) remains unexplained.
## Table 6. Static Histomorphometry of Tibia (mean ± S.E.)

<table>
<thead>
<tr>
<th>Group</th>
<th>G.Pl.Th (µm)</th>
<th>BV/TV (%)</th>
<th>OV/BV (%)</th>
<th>BS/TV (mm²/mm³)</th>
<th>OS/BS (%)</th>
<th>O.Th (µm)</th>
<th>Ob.S/BS (%)</th>
<th>Oc.S/BS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT-LP</td>
<td>232±31.2 CD</td>
<td>14.3±2.4 ABCD</td>
<td>67.2±18 A</td>
<td>5.4±0.5 CDEF</td>
<td>34.7±6.1 A</td>
<td>61.7±13.3 A</td>
<td>24.8±3.6 ABC</td>
<td>0.7±0.2 A</td>
</tr>
<tr>
<td>PL-LP</td>
<td>319±22.4 A</td>
<td>22.1±3.0 A</td>
<td>10.5±1.9 AB</td>
<td>7.5±0.6 ABCDE</td>
<td>30.8±5.9 AB</td>
<td>11.0±0.8 CDE</td>
<td>21.1±3.0 BCD</td>
<td>0.3±0.2 BC</td>
</tr>
<tr>
<td>PT-NP</td>
<td>192±8.9 DEF</td>
<td>14.9±3.0 ABCD</td>
<td>2.0±0.3 FG</td>
<td>5.9±0.8 ABCDE</td>
<td>10.8±1.8 CD</td>
<td>5.3±0.6 F</td>
<td>22.7±3.1 ABC</td>
<td>0.2±0.1 BC</td>
</tr>
<tr>
<td>PL-NP</td>
<td>215±7.2 CD</td>
<td>17.8±1.3 ABC</td>
<td>4.0±1.7 EFG</td>
<td>6.2±0.3 ABCD</td>
<td>14.1±3.9 CD</td>
<td>7.6±1.2 EF</td>
<td>27.2±2.0 AB</td>
<td>0.2±0.1 BC</td>
</tr>
<tr>
<td>1,25-LP</td>
<td>165±8.6 F</td>
<td>12.8±1.9 CDE</td>
<td>7.1±2.7 BCDE</td>
<td>5.4±0.5 BCDE</td>
<td>12.7±3.1 CD</td>
<td>12.0±2.2 CD</td>
<td>17.1±2.8 CDE</td>
<td>1.4±1.3 BC</td>
</tr>
<tr>
<td>1,25/24,25-LP</td>
<td>183±9.1 EF</td>
<td>12.9±1.0 BCDE</td>
<td>3.6±1.3 DEFG</td>
<td>5.6±0.5 ABCDE</td>
<td>10.8±1.3 CD</td>
<td>9.3±2.6 DE</td>
<td>21.6±2.5 ABCD</td>
<td>1.5±0.8 AB</td>
</tr>
<tr>
<td>24,25-LP</td>
<td>346±66.1 AB</td>
<td>18.9±2.5 AB</td>
<td>12.3±4.4 BC</td>
<td>7.2±0.8 AB</td>
<td>24.8±7.4 ABC</td>
<td>15.3±1.9 ABC</td>
<td>12.0±3.7 DE</td>
<td>8.4±4.8 A</td>
</tr>
<tr>
<td>25-LP</td>
<td>325±58.1 AB</td>
<td>19.1±2.1 A</td>
<td>7.4±1.6 BCD</td>
<td>7.0±0.7 ABC</td>
<td>15.8±3.4 BC</td>
<td>15.8±0.6 AB</td>
<td>10.7±1.9 E</td>
<td>3.4±1.8 A</td>
</tr>
<tr>
<td>1,25-NP</td>
<td>183±9.7 EF</td>
<td>14.2±1.4 ABCD</td>
<td>5.1±0.7 BCDE</td>
<td>4.9±0.2 DEF</td>
<td>13.8±1.9 C</td>
<td>13.0±2.0 BCD</td>
<td>30.9±3.9 A</td>
<td>0.0±0.0 C</td>
</tr>
<tr>
<td>1,25/24,25-NP</td>
<td>224±6.5 BC</td>
<td>10.4±1.2 DE</td>
<td>4.4±1.3 CDEFG</td>
<td>4.4±0.5 EF</td>
<td>11.8±2.2 CD</td>
<td>9.7±1.6 DE</td>
<td>19.5±2.5 BCD</td>
<td>0.3±0.3 C</td>
</tr>
<tr>
<td>24,25-NP</td>
<td>204±11.2 CDE</td>
<td>7.5±1.5 E</td>
<td>1.7±0.8 G</td>
<td>3.3±0.6 F</td>
<td>6.5±2.3 D</td>
<td>5.1±1.1 F</td>
<td>25.7±4.5 ABC</td>
<td>0.0±0.0 C</td>
</tr>
<tr>
<td>25-NP</td>
<td>190±22.4 CDE</td>
<td>20.5±3.9 ABC</td>
<td>2.2±0.6 FG</td>
<td>5.5±0.6 BCDE</td>
<td>11.5±4.2 CD</td>
<td>9.0±1.0 DE</td>
<td>30.2±3.1 A</td>
<td>0.1±0.1 C</td>
</tr>
</tbody>
</table>

Note: Unique letters vertically denote significant differences between groups (viz. AB vs. CD). Any shared letters vertically denote no significant difference (viz. AB vs. BC).

Legend:
- PT-LP = pre-treatment low phosphorus diet
- PL-LP = placebo-treated, low phosphorus diet
- PT-NP = pre-treatment normal phosphorus diet
- PL-NP = placebo-treated, normal phosphorus diet
- 1,25-LP = 1,25(OH)2D3-treated, low phosphorus diet
- 1,25/24,25-LP = 1,25(OH)2D3 + 24,25(OH)2D3-treated, low phosphorus diet
- 24,25-LP = 24,25(OH)2D3-treated, low phosphorus diet
- 25-LP = 25(OH)2D3-treated, low phosphorus diet
- 1,25-NP = 1,25(OH)2D3-treated, normal phosphorus diet
- 1,25/24,25-NP = 1,25(OH)2D3 + 24,25(OH)2D3-treated, normal phosphorus diet
- 24,25-NP = 24,25(OH)2D3-treated, normal phosphorus diet
- 25-NP = 25(OH)2D3-treated, normal phosphorus diet
Table 7. Dynamic Histomorphometry of Tibia (mean ± S.E.)

<table>
<thead>
<tr>
<th>Group</th>
<th>MS/BS (%)</th>
<th>MAR (μm/d)</th>
<th>BFR/BS (μm²/μm²/d)</th>
<th>Omt (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT-LP (4)</td>
<td>1.5 ± 0.5</td>
<td>DEF</td>
<td>2.8 ± 1.1</td>
<td>A</td>
</tr>
<tr>
<td>PL-LP (6)</td>
<td>4.6 ± 1.7</td>
<td>CDEF</td>
<td>1.5 ± 0.2</td>
<td>CD</td>
</tr>
<tr>
<td>PT-NP (6)</td>
<td>11.1 ± 2.3</td>
<td>A</td>
<td>2.2 ± 0.2</td>
<td>D</td>
</tr>
<tr>
<td>PL-NP (6)</td>
<td>10.5 ± 2.2</td>
<td>AB</td>
<td>2.5 ± 0.2</td>
<td>AB</td>
</tr>
<tr>
<td>1,25-LP (5)</td>
<td>5.5 ± 2.2</td>
<td>ABCDE</td>
<td>2.3 ± 0.2</td>
<td>5.1 ± 1.4</td>
</tr>
<tr>
<td>1,25/24,25-LP (5)</td>
<td>2.1 ± 0.8</td>
<td>CDEF</td>
<td>1.9 ± 0.1</td>
<td>4.8 ± 1.6</td>
</tr>
<tr>
<td>24,25-LP (4)</td>
<td>0.9 ± 0.3</td>
<td>F</td>
<td>1.9 ± 0.4</td>
<td>7.8 ± 1.8</td>
</tr>
<tr>
<td>25-LP (5)</td>
<td>1.3 ± 0.3</td>
<td>EF</td>
<td>2.7 ± 0.5</td>
<td>6.9 ± 1.6</td>
</tr>
<tr>
<td>1,25-NP (6)</td>
<td>6.7 ± 3.0</td>
<td>ABCD</td>
<td>2.3 ± 0.2</td>
<td>5.8 ± 0.7</td>
</tr>
<tr>
<td>1,25/24,25-NP (4)</td>
<td>7.9 ± 3.8</td>
<td>BCDEF</td>
<td>1.8 ± 0.2</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>24,25-NP (6)</td>
<td>12.9 ± 3.4</td>
<td>A</td>
<td>2.4 ± 0.3</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>25-NP (6)</td>
<td>6.8 ± 1.3</td>
<td>ABC</td>
<td>2.1 ± 0.3</td>
<td>4.3 ± 0.3</td>
</tr>
</tbody>
</table>

Note: Unique letters vertically denote significant differences between groups (viz. AB vs. CD). Any shared letters vertically denote no significant difference (viz. AB vs. BC).

Numbers in parentheses indicate animals per group with detectable double labeling.

Legend: PT-LP = pre-treatment low phosphorus diet
PL-LP = placebo-treated, low phosphorus diet
PT-NP = pre-treatment normal phosphorus diet
PL-NP = placebo-treated, normal phosphorus diet
1,25-LP = 1,25(OH)₂D₃-treated, low phosphorus diet
1,25/24,25-LP = 1,25(OH)₂D₃ + 24,25(OH)₂D₃-treated, low phosphorus diet
24,25-LP = 24,25(OH)₂D₃-treated, low phosphorus diet
25-LP = 25(OH)D₃-treated, low phosphorus diet
1,25-NP = 1,25(OH)₂D₃-treated, normal phosphorus diet
1,25/24,25-NP = 1,25(OH)₂D₃ + 24,25(OH)₂D₃-treated, normal phosphorus diet
24,25-NP = 24,25(OH)₂D₃-treated, normal phosphorus diet
25-NP = 25(OH)D₃-treated, normal phosphorus diet
Table 8. Serum and Plasma Biochemistry (mean ± S.E.)

<table>
<thead>
<tr>
<th>Group</th>
<th>Calcium (mg/dl)</th>
<th>Phosphorus (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>1,25(OH)₂D₃ (pg/ml)</th>
<th>24,25(OH)₂D₃ (ng/ml)</th>
<th>25(OH)D₃ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT-LP</td>
<td>12.2±0.3</td>
<td>D</td>
<td>3.7±0.3</td>
<td>F</td>
<td>50±5</td>
<td>CD</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>PL-LP</td>
<td>13.0±0.1</td>
<td>BC</td>
<td>4.0±0.3</td>
<td>F</td>
<td>57±3</td>
<td>ABC</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>PT-NP</td>
<td>11.1±0.2</td>
<td>E</td>
<td>6.5±0.1</td>
<td>CDE</td>
<td>50±6</td>
<td>CD</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>PL-NP</td>
<td>11.3±0.1</td>
<td>E</td>
<td>7.6±0.4</td>
<td>BC</td>
<td>53±5</td>
<td>CD</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>1,25-LP</td>
<td>13.9±0.4</td>
<td>A</td>
<td>7.0±0.5</td>
<td>BCD</td>
<td>53±8</td>
<td>CD</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>1,25/24,25-LP</td>
<td>13.1±0.2</td>
<td>AB</td>
<td>5.7±0.3</td>
<td>E</td>
<td>51±4</td>
<td>CD</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>24,25-LP</td>
<td>12.1±0.4</td>
<td>D</td>
<td>7.6±1.1</td>
<td>BC</td>
<td>83±12</td>
<td>CD</td>
<td>2.7±0.6</td>
</tr>
<tr>
<td>25-LP</td>
<td>12.9±0.4</td>
<td>BC</td>
<td>5.8±0.8</td>
<td>DE</td>
<td>68±5</td>
<td>AB</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>1,25-NP</td>
<td>12.6±0.2</td>
<td>CD</td>
<td>8.0±0.3</td>
<td>AB</td>
<td>52±8</td>
<td>CD</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>1,25/24,25-NP</td>
<td>10.7±0.1</td>
<td>EF</td>
<td>6.9±0.1</td>
<td>BCD</td>
<td>53±4</td>
<td>BCD</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>24,25-NP</td>
<td>10.0±0.1</td>
<td>F</td>
<td>6.6±0.2</td>
<td>CDE</td>
<td>35±1</td>
<td>E</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>25-NP</td>
<td>12.6±0.2</td>
<td>BCD</td>
<td>9.1±0.1</td>
<td>A</td>
<td>49±11</td>
<td>DE</td>
<td>1.0±0.4</td>
</tr>
</tbody>
</table>

Note: Unique letters vertically denote significant differences between groups (viz. AB vs. CD). Any shared letters vertically denote no significant difference (viz. AB vs. BC).

Legend:
- PT-LP = pre-treatment low phosphorus diet
- PL-LP = placebo-treated, low phosphorus diet
- PT-NP = pre-treatment normal phosphorus diet
- PL-NP = placebo-treated, normal phosphorus diet
- 1,25-LP = 1,25(OH)₂D₃ -treated, low phosphorus diet
- 1,25/24,25-LP = 1,25(OH)₂D₃ + 24,25(OH)₂D₃ -treated, low phosphorus diet
- 24,25-LP = 24,25(OH)₂D₃ -treated, low phosphorus diet
- 25-LP = 25(OH)D₃ -treated, low phosphorus diet
- 1,25-NP = 1,25(OH)₂D₃ -treated, normal phosphorus diet
- 1,25/24,25-NP = 1,25(OH)₂D₃ + 24,25(OH)₂D₃ -treated, normal phosphorus diet
- 24,25-NP = 24,25(OH)₂D₃ -treated, normal phosphorus diet
- 25-NP = 25(OH)D₃ -treated, normal phosphorus diet
Table 9. Histomorphometry and Biochemistry Parameter Correlation Coefficients

<table>
<thead>
<tr>
<th></th>
<th>G.Pl.Th.</th>
<th>OS/BS</th>
<th>OV/BV</th>
<th>O.Th</th>
<th>Omt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)₂D₃</td>
<td>-0.26</td>
<td>0.32</td>
<td>0.41</td>
<td>0.41</td>
<td>0.38</td>
</tr>
<tr>
<td>24,25(OH)₂D₃</td>
<td>NS</td>
<td>-0.32</td>
<td>-0.30</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>25(OH)D₃</td>
<td>0.39</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>-0.24</td>
<td>-0.34</td>
<td>-0.38</td>
<td>-0.24</td>
<td>NS</td>
</tr>
</tbody>
</table>

Legend: NS = not significant at $p<0.05$
References:


CHAPTER III
ULTRASTRUCTURAL EFFECTS OF DIETARY PHOSPHORUS
AND THE MAJOR VITAMIN D METABOLITES ON MATRIX VESICLES
AND THE MINERALIZATION FRONT IN UREMIC RATS

Abstract:

We have previously described a uremia and low dietary phosphorus-dependent osteomalacia in rats that is characterized by elevated plasma 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and hypophosphatemia, and that can be reversed by dietary phosphorus repletion but not by treatment with vitamin D metabolites. In order to investigate the ultrastructural effects on mineralization induced by these treatments, twenty-seven male Sprague-Dawley rats (320±20 g) were subjected to either a two-step, subtotal nephrectomy or a sham-operation, and subsequently fed a diet with either low (0.03%) or normal (0.3%) phosphorus for 17 days. Fifteen of these rats were treated daily with 27 ng 1,25(OH)₂D₃, 81 ng 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) or 135 ng 25-hydroxyvitamin D₃ (25(OH)D₃) for the last 10 days. Transmission electron microscopy revealed that the osteomalacia in 5/6 nephrectomized rats fed low phosphorus was characterized by decreased numbers of young matrix vesicles present at an increased distance from the mineralization front, larger old matrix vesicles, a reversal of the ratio
of young to old matrix vesicles and an increased irregularity of the mineralization front when compared to normal phosphorus-fed, sham-operated controls. Dietary phosphorus repletion reversed these changes compared to rats continued on the low phosphorus diet for an additional 10 days, with the exception of old matrix vesicle size which continued to increase. Vitamin D metabolite administration did not produce significant alterations in the matrix vesicle size or distribution in low phosphorus-fed, nephrectomized rats, although the irregular appearance of the mineralization front did improve to various degrees. In addition, intra-matrix vesicle elemental Ca:P ratios as measured by energy dispersive analytical X-ray were similar in all groups, suggesting that the primary mineralization defect lies outside the matrix vesicles. It was concluded that this form of uremic osteomalacia has both cellular and matrical abnormalities, consisting of decreased matrix vesicle formation and decreased matrix vesicle incorporation into the mineralization front. It was also shown that while dietary phosphorus levels can modulate bone mineralization at the ultrastructural level, vitamin D metabolites appear to have no primary effect on the impaired matrix mineralization of uremic rats fed low dietary phosphorus.

Introduction:

Mineralization of bone is believed to occur initially in discrete membrane-bound structures called matrix vesicles [1,2]. These matrix vesicles are thought to arise from cytoplasmic processes of osteoblasts and chondrocytes [3,4], and have been shown to
selectively accumulate both calcium and phosphorus up to a 50-fold concentration over the surrounding matrix [5,6]. It is within the matrix vesicles that the amorphous calcium-phosphorus complex is crystallized into hydroxyapatite, beginning the mineralization process [7].

The role of vitamin D in this process is still not completely defined. Some studies suggest that 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) is required for normal bone mineralization, acting directly upon the bone matrix [8,9]. Others find that 1,25(OH)₂D₃ simply provides calcium and phosphorus in the necessary amounts and ratios [10,11]. Even less is known concerning other metabolites of vitamin D, such as 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃), 25-hydroxyvitamin D₃ (25(OH)D₃) or 1,24,25-trihydroxyvitamin D₃.

In previous work, we have established a uremic and dietary phosphorus-dependent model of osteomalacia [12], and have shown that this osteomalacia can be reversed by dietary phosphorus repletion [13]. The present study was designed to examine the changes in matrix vesicle structure and distribution in the development and resolution of this form of osteomalacia, and to evaluate any alterations caused by treatment with exogenous vitamin D metabolites.
Materials and Methods:

Twenty-seven (27) male adult Sprague-Dawley rats weighing $320\pm 20$ g were used in this study. Twenty-one of these rats were $5/6$ nephrectomized via a two-step, subtotal nephrectomy technique previously described [12]; the other six rats were sham-operated, being treated identically but having their kidneys exteriorized, stripped of the renal capsule and then replaced intact.

Following surgery, the rats were maintained for an additional fourteen days on the standard laboratory rodent chow and water ad libitum. Three nephrectomized (Nx) and three sham-operated (SO) rats were then fed a nutritionally complete, vitamin D replete diet (Teklad, Incorporated, Madison WI, # TD 85343) with normal calcium (0.5\%) and normal phosphorus (0.3\%; NP), while the other rats were fed a nutrient-matched diet (# TD 85345) with normal calcium (0.5\%) and low phosphorus (0.03\%; LP) ad libitum for seven days.

At the end of this seven day induction period, the six rats fed NP, the three SO rats fed LP and three Nx rats fed LP were killed humanely. These animals made up the following groups: NP$_7$SO; NP$_7$Nx; LP$_7$SO; and LP$_7$Nx. The remaining fifteen nephrectomized rats were equally and randomly divided into the following five treatment groups for an additional 10 day recovery period:
LP_{10}-Pl — switched to the NP diet, treated with placebo.

LP_{17}-Pl — continued on the LP diet, treated with placebo.

LP_{17-1,25} — continued on the LP diet, treated with 27 ng/d 1,25(OH)_{2}D_{3}.

LP_{17-24,25} — continued on the LP diet, treated with 81 ng/d 24,25(OH)_{2}D_{3}.

LP_{17-25} — continued on the LP diet, treated with 135 ng/d 25(OH)D_{3}.

Vitamin D metabolites were graciously provided by Dr. M. Uskokovic (Hoffmann-La Roche, Nutley, NJ). The vitamin D metabolites and vehicle placebo (propylene glycol) were administered via a subcutaneous osmotic minipump (Alzet model 2002, Alza Corporation, Palo Alto CA) implanted on the first treatment day. All treated rats were killed at the end of the 10 day period.

At both of the scheduled kill points, the right tibia was immediately removed and bisected. At least two 1 mm³ samples were removed from the center of the proximal metaphysis, approximately 0.5 mm below the growth plate to avoid the primary and secondary trabeculae [14].

For transmission electron microscopy, specimens were processed aqueously by being immersed in cold 3% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide with s-collidine buffer (pH 7.4). They were then dehydrated in increasing concentrations of ethanol and embedded in Med-Cast resin (Ted Pella Incorporated, Tustin CA). Thick (one micron) sections, stained with toluidine blue
were examined by light microscopy to evaluate and select appropriate areas from further processing. Within the smaller designated areas, thin (1,000 Å) sections were cut with a diamond knife on an LKB ultramicrotome and floated on a water bath at pH 7.4 as briefly as possible to prevent demineralization. The sections were stained with uranyl acetate and lead citrate and examined on a Philips 300 electron microscope to evaluate the relative number, structure and mineralization patterns of the matrix vesicles within the sampling area.

Within each group, photomicrographs were taken of 10 active osteoid seams (defined as an area of relatively electron-lucent, collagenous tissue bounded on one side by mineral and on the opposite side by an osteoblast with well-formed organelles; see Plate I) per rat, at a magnification of 23,000X. To insure that ten different osteoid seams were examined per rat, multiple specimens were photographed from each rat and the resulting photomicrographs were compared to eliminate any duplications. The remaining unique photomicrographs were then used to count the numbers of matrix vesicles within each of several defined morphologic categories, to assess the contour of the mineralization front, and to measure the area of the matrix vesicles present and their distance from the mineralization front via quantitative image analysis with a microprocessor-controlled digitizing tablet (BioQuant, Nashville, TN). The classification scheme devised by Sela [15,16] was used to group matrix vesicles, with the more electron-lucent empty and amorphous categories being combined ("young" vesicles) and
the more electron-dense crystal and rupture categories also being combined ("old" vesicles); see Plate II.

In addition, at least two other 1 mm³ samples from the same area of the tibial metaphysis were collected for electron dispersive analytical X-ray microprobe analysis and processed non-aqueously by using the following modification of previously published techniques [17,18]. The tissue samples were quickly quench-frozen in liquid nitrogen, then placed in anhydrous acetone at -72°C for 24 hours, and then at -40°C for another 48 hours. During this entire substitution process, the samples were in a desiccator along with an activated molecular sieve, in sealed vials. The samples were infiltrated at -40°C with a 50% acetone/50% ERL 4206 vinyl cyclohexane dioxide mixture for 4 hours, and then with 100% ERL 4206 for another 16 hours. Finally, the samples were infiltrated with 100% ERL 4206 for 3 hours while being gradually raised from -40°C to -15°C. Osmium post-fixation was specifically avoided to prevent any interference with the detection of calcium [19].

The samples were then infiltrated with 50% ERL 4206/50% Complete Resin Mix (10 g ERL 4206, 12 g dibutyl phthalate, 26 g nonenyl succinic anhydride and 1.6 g DMP-30) for 16 hours, while the temperature was slowly raised from -15°C to -4°C, and then with 100% Complete Resin Mix for 3 hours while the temperature was gently raised to 20°C. Finally, the samples were infiltrated with 100% Complete Resin Mix for 24 hours at 60°C, encapsulated and allowed to polymerize for another 24 hours at 60°C.
Thick sections were cut and evaluated and from the smaller, designated areas, thin sections cut as before. These thin sections were left unstained and examined on a JEOL 200 CX analytical electron microscope (Japanese Electron Optical Laboratory, Medford, MA) which was fitted with an energy dispersive X-ray microanalyzer. A Faraday cup assembly was utilized for monitoring the specimen beam currents, and a Tracor Northern fully quantitative analytical system was used to evaluate the X-ray data. Using the scanning/transmission (STEM) mode, a semi-quantitative, standardless analysis system was used to determine the concentrations of both calcium and phosphorus present within the matrix vesicles via weight percents and mass ratios. These analyses were performed using raster-scanning at 100 kV for 240 seconds, with a beam spot size sufficient to encompass the entire matrix vesicle. Matrix vesicles were again grouped into “young” and “old” categories, based primarily on their distance from the mineralization front (young matrix vesicles being further from the mineralization front), as the density differences in the non-aqueous specimens were difficult to appreciate. Equal numbers of young and old matrix vesicles (at least three of each) were examined in samples from two rats per group. The intrinsic error of this system has been estimated at 7%, whereas the error of collecting and processing biological specimens has been estimated at 10% [20,21].

The numerical data generated by this protocol were evaluated with a Shapiro-Wilk normalcy test, followed by a non-parametric Wilcoxon Rank-Sum procedure and pairwise t tests corrected for multiple comparisons, using a significance level of p < 0.05 [22].
Morphologic data for the matrix vesicles and mineralization front are presented in Table 10. Matrix vesicle concentrations and intra-matrix vesicle Ca:P ratios are presented in Table 11. Figure 1 illustrates the relative proportions of the different matrix vesicles’ morphologic categories between groups.

At the end of the seven day induction period, osteomalacia was present only in the LP₇Nx rats [12]. In these LP₇Nx rats young matrix vesicles were further from the mineralization front, reflecting the greater osteoid thickness in this group compared with the normal NP₇SO rats. The concentration (number per μm² of matrix) of young matrix vesicles was less and the ratio of young matrix vesicles without visible mineral (empty) to those with amorphous mineral (amorphous) was less in LP₇Nx rats than in NP₇SO rats. The size of young matrix vesicles was not significantly different between these two groups. The relative frequency of young to old matrix vesicles in LP₇Nx rats was less than 20% of the ratio in NP₇SO rats. Older matrix vesicles in the osteomalacic LP₇Nx rats were larger and had a greater ratio of ruptured matrix vesicles (rupture) to matrix vesicles with spicular mineral that had not yet ruptured (crystal) than in NP₇SO rats. Persistence of discrete matrix vesicles, and irregularity of the mineralization front was noted in the LP₇Nx rats, and it was interpreted that coalescence of old matrix vesicles into the mineralization front was much less than in the NP₇SO rats (see Figure 2). The
distance of the old matrix vesicles from the mineralization front and their concentration per unit area were similar in both groups, however.

Rats which were nephrectomized only (NP7-Nx) or fed the low phosphorus diet only (LP7-SO) did not have significant differences in any measured parameter compared to the NP7-SO rats. The ratios of young to old matrix vesicles in these two groups were intermediate between the two groups previously discussed.

Nephrectomized rats fed a phosphorus-replete diet for an additional 10 days (LP7-NP10-Pi) had reversed the osteomalacia compared with both LP7-Nx and nephrectomized rats continued on the low phosphorus diet (LP17-Pi) [12,13]. Young matrix vesicles in the LP7-NP10-Pi rats were closer to the mineralization front that in LP17-Pi rats, reflecting the reduced osteoid thickness in the phosphorus-repleted rats. Both the concentration of young matrix vesicles and the ratio of empty to amorphous young matrix vesicles were increased in LP7-NP10-Pi rats compared with LP17-Pi rats. The size of the young matrix vesicles was not significantly different between the groups however. The ratio of the relative frequency of young to old matrix vesicles was 6 times greater in the LP7-NP10-Pi rats than in the LP17-Pi rats. Older matrix vesicles in the LP7-NP10-Pi rats were larger, had a reduced ratio of ruptured to crystalline vesicles and were reduced in concentration compared to those in LP17-Pi rats. The distance of the older matrix vesicles from the mineralization front was similar in both groups. The mineralization front itself was much more regular in appearance in the LP7-NP10-Pi rats than in the LP17-Pi rats.
PI rats. The interpretation of these observations was that dietary phosphorus repletion improved the degree of matrix vesicle coalescence into the mineralization front.

Rats treated with 1,25(OH)₂D₃, 24,25(OH)₂D₃ or 25(OH)D₃ had few significant differences from the osteomalacic LP₁₇-PI rats. All of the vitamin D metabolite treated groups had a decrease in the irregularity of the mineralization front compared to that of the LP₁₇-PI rats; this change in old matrix vesicle incorporation was mild in LP₁₇-1,25 rats and moderate in LP₁₇-24,25 and LP₁₇-25 rats. In addition, LP₁₇-24,25 rats had young matrix vesicles closer to the mineralization front.

The intra-matrix vesicle calcium:phosphorus ratio was not different in any group, in either young or old matrix vesicles, regardless of treatment or dietary phosphorus level.

Discussion:

Rats subjected to 5/6 nephrectomy and fed a low (0.03%) phosphorus diet develop osteomalacia, as has been shown by both static and dynamic histomorphometry [12]; this osteomalacia is characterized by increased osteoid thickness, surface and volume, osteoid maturation time, and decreased bone formation rates, and is associated with hypophosphatemia and elevated plasma 1,25(OH)₂D₃. Ultrastructurally, this condition was represented by: young matrix vesicles being located farther from the mineralization
front; a paucity of young matrix vesicles; a reduced ratio of empty to amorphous vesicles; old matrix vesicles being larger in area; a reduced ratio of crystal to rupture vesicles; and a very irregular mineralization front. Despite these changes, the intra-matrix vesicle Ca:P ratio was not altered, and the individual matrix vesicles (both young and old) were normal in appearance.

The recovery from this osteomalacia caused by dietary phosphorus repletion [13] was associated with a reversal of all of the above changes, with the exception of the old matrix vesicles, which continued to increase in area but decrease in concentration. Treatments with 1,25(OH)₂D₃, 24,25(OH)₂D₃ and 25(OH)D₃ however were not successful in significantly altering these lesions.

The initial increase in the area of old matrix vesicles seen in the osteomalacic rats is thought to represent a decrease in matrix mineralization such that the individual mineralized matrix vesicles do not coalesce into the mineralization front. Instead, they continue to enlarge and remain as the discrete mineralized structures which were measured in this study as old matrix vesicles [23]. This increased persistence of old matrix vesicles resulted in the observed irregularity of the mineralization front (see Figure 2). It has been previously shown that matrix vesicle maturation involves a continuous increase in diameter [24]. Taken together, these changes support the concept of some defect within the osteoid matrix itself which delays extra-matrix vesicle mineralization. With a resolution of the osteomalacia due to dietary phosphorus repletion
however, the old matrix vesicles increased in area even further. We propose that this represents a "rebound" phenomenon of increased mineralization due to increased availability of calcium and phosphorus in the proper ratio. The reduced irregularity of the mineralization front and decreased concentration of old matrix vesicles in the phosphorus-repleted rats suggests increased incorporation of ruptured matrix vesicles into the ossified matrix. Since the size of the old matrix vesicles in the phosphorus-repleted rats was almost five times greater than baseline controls however, the matrix may not yet have fully supported mineralization and vesicle "fusion" into the mineralization front.

Another consistent change seen in the osteomalacic rats was a decrease in the ratio of young to old matrix vesicles and in the young vesicles' concentration within the matrix. While the baseline control rats had nearly twice as many young as old vesicles, the osteomalacic rats had less than half as many young as old vesicles. This may be in part the result of the mechanism described above in which the mineralization defect produced less "fusion" of the older matrix vesicles into each other, resulting in an increase in the number of old matrix vesicles due to continued vesicle maturation but less mineralized vesicle incorporation into the mineralization front. In addition, there appeared to be a decrease in new matrix vesicle synthesis, as there were fewer young matrix vesicles present in the osteomalacic rats. Even in osteomalacic rats however, the matrix vesicles that were formed appeared to be functioning normally. Neither the size of the young matrix vesicles nor their internal Ca:P ratio significantly changed over the course of this study, suggesting that once formed, the matrix vesicles functioned normally
in calcium and phosphorus accumulation and hydroxyapatite crystallization. Other studies have also demonstrated essentially normal matrix vesicle structure and function in cases of rickets [25]. The pathogenesis of this decrease in matrix vesicle formation is not known, and this finding has not been previously reported, but it suggests that there also exists in this form of low phosphorus dependent, uremic osteomalacia a defect within the osteoblasts, resulting in a decreased production of matrix vesicles.

The changes seen in the locations of the matrix vesicles from the mineralization front were not surprising. The young matrix vesicles were further from the mineralization front in osteomalacic rats, likely due to both the proposed decrease in matrix mineralization (thereby delaying the advancement of the front towards the new matrix vesicles), as well as to an increase in the rate of osteoid matrix formation in these rats [12,13]. On the other hand, the old matrix vesicles were always present at a similar distance from the mineralization front. This was probably due to the short time course of this study; the vesicles already embedded in the matrix at a fixed distance from the mineralization front were not affected by subsequent changes in the rate of matrix or vesicle formation.

As has been previously shown [12], uremia alone was not sufficient for lesion development; the 5/6 nephrectomized rats fed normal phosphorus were not significantly different from the sham-operated controls in any parameter other than the young:old matrix vesicle ratio, which was only partially affected. Although low phosphorus feeding
was also itself not sufficient for significant lesion development, it did affect the
appearance of the mineralization front as well as the matrix vesicle type distribution. In
addition, many of the numerical parameters, while not statistically different, were
intermediate between the baseline controls and the osteomalacic low phosphorus-fed,
nephrectomized rats. This emphasizes the importance of the serum (and presumably
osteoid matrix) phosphorus level, as both groups of rats fed low phosphorus developed
hypophosphatemia and an elevated serum Ca:P ratio during the induction period. During
the recovery period however, the rats treated with either 1,25(OH)₂D₃ or 24,25(OH)₂D₃
returned to normophosphatemia, as did the recovered normal phosphorus-fed rats. The
continued osteomalacia in the 1,25(OH)₂D₃-treated rats is felt to be due to the elevated
plasma 1,25(OH)₂D₃ level in these rats [13]; the lack of recovery in the 24,25(OH)₂D₃-
treated rats, in the face of a normalized serum phosphorus level and a lack of elevated
plasma 1,25(OH)₂D₃ remains enigmatic. Previous research has documented the lack of
beneficial effect of 24,25(OH)₂D₃ administration on osteomalacia, despite
normophosphatemia maintained via exogenous 1,25(OH)₂D₃ [26].

The exact nature of the mineralization defect seen in this model of uremic
osteomalacia is not known. The marked increases in osteoid tissue seen with light
microscopy are known to be due to both mineralization impairment and increased matrix
synthesis [12,13]. In this study, the role of the matrix vesicle in this mineralization
defect was examined, and it was concluded that the matrix vesicles per se were not
abnormal. The intra-matrix vesicle Ca:P ratios did not mirror the alterations in the
serum Ca:P ratios [12,13]; the matrix vesicles' calcium-phosphorus product was maintained despite the hypophosphatemia, and was similar to values previously published [6,27]. Mineralization, as represented by the presence of electron-dense crystals characteristic of hydroxyapatite [16], continued within the vesicles and in the immediately adjacent extracellular matrix. These findings, and the changes in old matrix vesicle size mentioned above all suggest a defect within the collagenous matrix itself, in addition to the observed decrease in new matrix vesicle formation in this model of uremic osteomalacia. While matrix vesicles are necessary for mineralization to occur, other factors are also required. The mineralization which begins within the matrix vesicle progresses and becomes self-sustaining within the extracellular matrix itself [28]. According to Anderson, “after providing for the site-specific deposition of a few seed crystals between collagen fibrils of the matrix, the work of the matrix vesicles is completed”. Among the many factors within the matrix known to contribute to the mineralization process are: calcium binding proteins, phosphoproteins and proteoglycans; phospholipids; pyrophosphates; and type X collagen [2,29-32]. Alteration of one or more of these factors may be the cause of the mineralization defect in this model. Other studies reporting similar findings have also documented abnormal osteoid matrix present in osteomalacic rats [33].

In summary, in this model of uremic osteomalacia, the mineralization defect appears to lie outside of the matrix vesicles, and is likely within the osteoid matrix itself. There also appears to be a decrease in the numbers of matrix vesicles formed during the
induction of osteomalacia. While dietary phosphorus repletion is able to ameliorate the condition, in the doses used, vitamin D metabolites are without beneficial effect.
Plate I: Active osteoid seam, illustrating relationship between osteoblast (Ob), osteoid matrix (Os) and mineralized osteoid (M). 23,000 X
Plate I: Active osteoid seam.
Plate II: Morphology of matrix vesicle types. Young matrix vesicles (arrow) were electron-lucent; old matrix vesicles (arrowhead) were electron-dense with spicular crystals. 23,000 X
Plate II: Morphology of matrix vesicle types.
Figure 1: Relative distribution of matrix vesicle types. The empty and amorphous categories are combined as “Young” matrix vesicles; the crystal and amorphous categories are combined as “Old” matrix vesicles. Numbers in parentheses indicate the total number of vesicles examined in each group.
Figure 1: Relative distribution of matrix vesicle types.

- Empty
- Amorphous
- Crystal
- Rupture
Figure 2: Morphology of the mineralization front. 23,000 X.

A) "Smooth" mineralization front of NP₂SO rat.

B) "Irregular" mineralization front of LP₂Nx rat, with foci of mineralization scattered throughout the osteoid.
Table 10: Ultrastructural Morphology of the Matrix Vesicles and Mineralization Front (mean ± S.E.)

<table>
<thead>
<tr>
<th>Group</th>
<th>Young Matrix Vesicles</th>
<th>Old Matrix Vesicles</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area (µm²)</td>
<td>Distance* (µm)</td>
<td>Area (µm²)</td>
</tr>
<tr>
<td>MF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP&lt;sub&gt;X&lt;/sub&gt; SO</td>
<td>0.3±0.1 A</td>
<td>3.6±0.3 B</td>
<td>0.4±0.1 B</td>
</tr>
<tr>
<td>NP&lt;sub&gt;X&lt;/sub&gt; Nx</td>
<td>0.4±0.1 A</td>
<td>4.2±0.6 B</td>
<td>0.4±0.1 B</td>
</tr>
<tr>
<td>LP&lt;sub&gt;x&lt;/sub&gt; SO</td>
<td>0.3±0.0 A</td>
<td>6.3±0.8 AB</td>
<td>0.6±0.1 AB</td>
</tr>
<tr>
<td>LP&lt;sub&gt;x&lt;/sub&gt; Nx</td>
<td>0.4±0.1 A</td>
<td>7.2±0.6 A</td>
<td>0.7±0.1 A</td>
</tr>
<tr>
<td>LP&lt;sub&gt;r&lt;/sub&gt;-Pl</td>
<td>0.5±0.1 AB</td>
<td>9.3±1.0 A</td>
<td>0.9±0.1 B</td>
</tr>
<tr>
<td>LP&lt;sub&gt;r&lt;/sub&gt;-NP&lt;sub&gt;se&lt;/sub&gt;-Pl</td>
<td>0.7±0.1 AB</td>
<td>4.7±0.6 B</td>
<td>1.9±0.7 A</td>
</tr>
<tr>
<td>LP&lt;sub&gt;r&lt;/sub&gt;-1,25</td>
<td>0.7±0.1 AB</td>
<td>9.5±1.0 A</td>
<td>0.7±0.1 B</td>
</tr>
<tr>
<td>LP&lt;sub&gt;r&lt;/sub&gt;-24,25</td>
<td>0.4±0.1 B</td>
<td>3.2±0.6 B</td>
<td>0.9±0.1 B</td>
</tr>
<tr>
<td>LP&lt;sub&gt;r&lt;/sub&gt;-25</td>
<td>1.5±0.5 A</td>
<td>5.8±0.8 AB</td>
<td>0.7±0.1 B</td>
</tr>
</tbody>
</table>

(a) Distance refers to the average distance of the specified matrix vesicle type to the mineralization front, and was measured for each matrix vesicle examined.

(b) MF Mineralization Front. Degree of irregularity: + mild; ++ moderate; +++ marked

Note: Unique letters vertically denote significant differences between groups at the same time period (viz. A vs. B).
Any shared letters vertically indicate no significant difference at the same time period (viz. A vs. AB).

Legend:
NP<sub>X</sub> SO = Sham-operated rats fed NCNP diet for seven days.
NP<sub>X</sub> Nx = Nephrectomized rats fed NCNP diet for seven days.
LP<sub>x</sub> SO = Sham-operated rats fed NCLP diet for seven days.
LP<sub>x</sub> Nx = Nephrectomized rats fed NCLP diet for seven days.
LP<sub>r</sub>-Pl = Nephrectomized rats fed NCLP diet for seventeen days, treated with placebo for the last ten days.
LP<sub>r</sub>-NP<sub>se</sub>-Pl = Nephrectomized rats fed NCLP diet for seven days, then NCNP diet for ten days, and treated with placebo for the last ten days.
LP<sub>r</sub>-1,25 = Nephrectomized rats fed NCLP diet for seventeen days, treated with 1,25(OH)₂D<sub>3</sub> for the last ten days.
LP<sub>r</sub>-24,25 = Nephrectomized rats fed NCLP diet for seventeen days, treated with 24,25(OH)₂D<sub>3</sub> for the last ten days.
LP<sub>r</sub>-25 = Nephrectomized rats fed NCLP diet for seventeen days, treated with 25(OH)D<sub>3</sub> for the last ten days.
Table 11: Matrix Vesicle Concentration and Ca:P Ratio (mean ± S.E.)

<table>
<thead>
<tr>
<th>Group</th>
<th>Matrix Vesicles' Concentration</th>
<th>Ca:P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young (#/mm²)</td>
<td>Old (#/mm²)</td>
</tr>
<tr>
<td>NP₅SO</td>
<td>0.36±0.17 A</td>
<td>0.20±0.12 A</td>
</tr>
<tr>
<td>NP₅Nx</td>
<td>0.23±0.11 AB</td>
<td>0.33±0.18 A</td>
</tr>
<tr>
<td>LP₅SO</td>
<td>0.20±0.14 AB</td>
<td>0.24±0.16 A</td>
</tr>
<tr>
<td>LP₅Nx</td>
<td>0.07±0.03 B</td>
<td>0.24±0.11 A</td>
</tr>
<tr>
<td>LP₁₇⁻Pl</td>
<td>0.12±0.08 B</td>
<td>0.30±0.11 A</td>
</tr>
<tr>
<td>LP₅NP₁₇⁻Pl</td>
<td>0.32±0.14 A</td>
<td>0.14±0.05 B</td>
</tr>
<tr>
<td>LP₁₇⁻1,25</td>
<td>0.08±0.03 B</td>
<td>0.37±0.19 A</td>
</tr>
<tr>
<td>LP₁₇⁻24,25</td>
<td>0.04±0.02 B</td>
<td>0.19±0.10 AB</td>
</tr>
<tr>
<td>LP₁₇⁻25</td>
<td>0.07±0.05 B</td>
<td>0.22±0.13 AB</td>
</tr>
</tbody>
</table>

Note: Unique letters vertically denote significant differences between groups at the same time period (viz. A vs. B). Any shared letters vertically indicate no significant difference at the same time period (viz. A vs. AB).

Legend:
- NP₅ SO = Sham-operated rats fed NCNP diet for seven days.
- NP₅ Nx = Nephrectomized rats fed NCNP diet for seven days.
- LP₅ SO = Sham-operated rats fed NCLP diet for seven days.
- LP₅ Nx = Nephrectomized rats fed NCLP diet for seven days.
- LP₁₇⁻Pl = Nephrectomized rats fed NCLP diet for seventeen days, treated with placebo for the last ten days.
- LP₅NP₁₇⁻Pl = Nephrectomized rats fed NCLP diet for seven days, then NCNP diet for ten days, and treated with placebo for the last ten days.
- LP₁₇⁻1,25 = Nephrectomized rats fed NCLP diet for seventeen days, treated with 1,25(OH)₂D₃ for the last ten days.
- LP₁₇⁻24,25 = Nephrectomized rats fed NCLP diet for seventeen days, treated with 24,25(OH)₂D₃ for the last ten days.
- LP₁₇⁻25 = Nephrectomized rats fed NCLP diet for seventeen days, treated with 25(OH)D₃ for the last ten days.
References:


CHAPTER IV
THE EFFECTS OF SYSTEMIC ALUMINUM ON THE RESOLUTION OF
A UREMIC AND DIETARY PHOSPHORUS DEPENDENT MODEL OF
UREMIC OSTEOMALACIA IN RATS

Abstract:

We have developed a model of osteomalacia which is dependent on both uremia and the feeding of a diet low in phosphorus, and which can be reversed by subsequent dietary phosphorus repletion. The objectives for this study were to use this model to investigate the role of aluminum (Al) in both the induction and resolution of osteomalacia. Adult male Sprague-Dawley rats were 5/6 nephrectomized and fed either low or normal dietary phosphorus, both with and without intraperitoneal Al injections. Uremic rats fed low phosphorus developed osteomalacia characterized by: increased osteoid surface, volume and thickness, and osteoid maturation time, and decreased mineralizing surface. Al-treated uremic rats fed low phosphorus were similarly affected, developing increased osteoid volume and thickness, and osteoid maturation time, and decreased osteoblastic surface, mineralizing surface and bone formation rate. In addition, they had a significantly increased Al-positive surface. Al-treated uremic rats fed normal phosphorus had only increased osteoid thickness and aluminum-positive
surface, and decreased osteoblastic surface. Osteomalacic rats continuously treated with Al during the induction and phosphorus repletion stages had increased growth plate thickness, osteoid volume and thickness and Al-positive surface, and decreased osteoblastic and mineralizing surface. Mineralization in these rats was impaired to such a degree that there were no detectable double labels present. Osteomalacic rats treated with Al during the induction phase but not during phosphorus repletion had increased osteoid surface and volume, and Al-positive surface, and decreased osteoblastic and mineralizing surface. Double labels were not detectable in these rats either. These results indicate that the osteomalacia induced in uremic rats fed low phosphorus was similar in rats with and without Al treatment. However, Al prevented the reversal of this osteomalacia by phosphorus repletion, even when Al administration was discontinued. The impairment of mineralization by Al in this model of osteomalacia suggests that Al positive surfaces are not an epiphenomenon in osteomalacia associated with uremia.

Introduction:

Elevated bone aluminum has been associated with cases of uremic osteomalacia [1,2]. In these patients, histochemical examination reveals linear aluminum deposits at the mineralization front [3,4], and the bone aluminum content is directly proportional to the degree of osteomalacia [5,6]. Some studies have therefore suggested that aluminum is the direct cause of the mineralization failure via: osteoblast toxicity [7-9]; matrix alterations [4,7,10]; decreased hydroxyapatite crystallization [11,12]; altered calcium and
phosphorus metabolism [13]; and decreased intestinal calcium absorption [14]. On the other hand, other studies have concluded that aluminum is deposited in bone already osteomalacic from other causes and represents an epiphenomenon [15-18].

We have developed a model of osteomalacia in rats which is dependent on both uremia and the feeding of a diet low in phosphorus [19]. This form of uremic osteomalacia can be reversed by dietary phosphorus repletion [20]. The objective of this study was to use this model to investigate the role of systemic aluminum in both the development and resolution of this uremic osteomalacia.

Materials and Methods:

Forty-two (42) male adult Sprague-Dawley rats, weighing 320±20 g (twelve weeks old) were evaluated. All rats were housed individually under fluorescent lighting on a twelve-hour on, twelve-hour off lighting cycle throughout the entire study, and were initially fed a standard laboratory rodent chow (Agway Prolab Diet 3000) and water ad libitum for a seven day acclimation period. All rats were then anesthetized with intramuscular injections of 0.067 mg/kg fentanyl and 3.33 mg/kg droperidol (Innovar-Vet, Pittman Moore, Washington’s Crossing, NJ) and 1.2 mg/kg morphine (Eli Lilly & Co., Indianapolis, IN), and had both the cranial and caudal thirds of their left kidney removed via a left paracostal approach by using a silk ligature “cutting/ crushing”
technique. Seven days later, the rats were again anesthetized, and had their entire right kidney removed via a right paracostal approach.

Following surgery, all rats were maintained for a fourteen day recovery period on the standard laboratory rodent chow and water *ad libitum*. The rats were then divided into the following seven groups of six rats each:

**Induction period:**

NP\(_7\) = normal phosphorus (0.3\%) diet (Teklad # TD 85343) for seven days, with placebo-treatment.

NP\(_7\)Al+ = normal phosphorus diet for seven days, with aluminum-treatment.

LP\(_7\) = low phosphorus (0.03\%) diet (Teklad # TD 85345) for seven days, with placebo-treatment.

LP\(_7\)Al+ = low phosphorus diet for seven days, with aluminum-treatment.

**Recovery period:**

NP\(_{10}\) = low phosphorus diet and placebo-treatment for seven days as above, and then normal phosphorus diet with placebo-treatment for ten additional days.

NP\(_{10}\)Al+ = low phosphorus diet and aluminum-treatment for seven days as above, and then normal phosphorus diet with aluminum-treatment for ten days.
NP_{10}Al- = low phosphorus diet and aluminum-treatment for seven days as above, and then normal phosphorus diet with placebo-treatment for ten days.

All diets contained adequate levels of Vitamin D₃ (2200 IU/kg diet).

Aluminum treatments consisted of intraperitoneal injections of 2.0 mg Al (as aluminum chloride) five days per week; placebo treatments consisted of intraperitoneal injections of an identical volume of saline vehicle alone at the same times. The cumulative dose of aluminum was 10 mg during the induction phase, and an additional 16 mg for the NP_{10}Al+ rats during the recovery phase. All rats were also treated twice with an intraperitoneal injection of 10 mg/kg calcein (Sigma Chemical Co., St. Louis, MO), six days apart, with the last label administration 24 hours before the rats were killed.

All rats were killed via terminal exsanguination while under ether anesthesia. Two milliliters of blood was placed in a silicon-coated sterile glass tube, and a complete serum chemistry profile obtained using a Dacos automated serum analyzer (# 6701815). This analysis included measurement of BUN, creatinine, calcium and phosphorus.

From the six rats within each group, the left tibia was removed, dissected free of soft tissue, and cut transversely two millimeters proximal to the distal end of the tibial
crest. After shaving off the cranial and caudal edges of the tibial plateau, all specimens were fixed in cold (4 degrees centigrade) 40% ethanol, and subsequently dehydrated in increasing concentrations of ethanol and placed undecalcified in methyl methacrylate. These blocks were sectioned in a frontal plane at six microns on a Jung model K sledge microtome and stained with a modified Von Kossa stain, using a MacNeil’s tetrachrome counterstain. Additional six micron thick sections were prepared unstained for evaluation of the calcein bone labels with a Zeiss epifluorescent microscope, and for histochemical staining for aluminum [21].

On each of the stained undecalcified sections (one per animal), the following parameters were evaluated within a centered 1 × 2 millimeter metaphyseal area, using the nomenclature proposed by the Report of the ASBMR Histomorphometry Committee [22]. The sampling area was located one-half millimeter below the last hypertrophied chondrocyte so as to avoid the primary and secondary trabeculae, and centered within the metaphysis based on the technique previously described [23]. This morphometric analysis was performed using a semi-automated, microprocessor controlled Zeiss Interactive Digital Analysis System (ZIDAS) and a calibrated eyepiece:

Growth plate thickness (G.Pl.Th [μm]) - The average distance from the epiphyseal/physeal junction to the last hypertrophied chondrocyte, measured at four different locations.
Metaphyseal trabecular bone volume (BV/TV [%]) - the total volume of the sample occupied by osteoid and bone trabeculae, expressed as a percentage of the total tissue volume.

Osteoid volume (OV/BV [%]) - The total volume of osteoid within the sample area expressed per unit bone volume.

Metaphyseal trabecular surface (BS/TV [mm²/mm³]) - The total surface perimeter of the bony trabeculae within the sample area, expressed as a fraction of the tissue volume.

Osteoid surface (OS/BS [%]) - the fraction of the trabecular surfaces (BS) which was covered by osteoid.

Osteoid seam thickness (O.Th [µm]) - The average thickness of the osteoid seams, calculated as OV divided by OS.

Osteoblastic surface (Ob.S/BS [%]) - The length of the trabecular surfaces which were covered by cuboidal osteoblasts, expressed as a fraction of BS.

Osteoclastic surface (Oc.S/BS [%]) - The length of the trabecular surfaces which were covered by osteoclasts within Howship's lacunae, expressed as a fraction of BS.
Mineralizing surface (MS/BS [%]) - We have defined the mineralizing surface as the extent of double-labeled surfaces (dLS) only, as has been suggested for trabecular remodeling in the rat [1,23].

Mineral appositional rate (MAR [μm/d]) - The rate of mineralization of the osteoid matrix at sites of active mineralization, measured as the average distance between the calcein labels at four equidistant sites (corrected for obliquity by a factor of 0.79) in the double labeled areas, divided by the time in days between calcein administration.

Bone formation rates (BFR/BS [μm³/μm²/d]) - The amount of new mineralized bone formed per unit time, calculated as the product of MAR and MS/BS.

Osteoid maturation time (Omt [d]) - The average time between osteoid matrix formation and mineralization at sites of active mineralization, calculated as O.Th divided by MAR.

Unless otherwise indicated, all results are expressed as the mean of six values; the standard errors of the means are also indicated. Statistical analysis (performed with SAS on an IBM 3081-D mainframe) consisted of a Shapiro-Wilk normalcy test, followed by a non-parametric Wilcoxon Rank-Sum procedure and pairwise t tests corrected for multiple comparisons, using a significance level of p<0.05.
For the diagnosis of osteomalacia, significant alterations in both osteoid volume and the osteoid maturation time were required, to indicate an increase in unmineralized osteoid due to a delay in the mineralization process.

Results:

Bone histomorphometry data are presented in Tables 12 and 13. Serum and plasma biochemistry data are presented in Table 14.

Compared to the baseline NP7 controls, rats fed low phosphorus had significant elevations of osteoid volume, surface and thickness. They also developed a decreased mineralizing surface, increased osteoid maturation time, and decreased serum phosphorus. These changes were interpreted as osteomalacia, which was associated with hypophosphatemia as has been previously described [19].

The rats fed low phosphorus and treated with aluminum were similarly affected, developing increased osteoid volume, thickness and maturation time, and decreased osteoblastic surface, mineralizing surface and bone formation rate. In addition, they had significantly increased aluminum-positive surfaces. They did not, however, develop a significant hypophosphatemia.
Rats fed normal dietary phosphorus and treated with aluminum had only increased osteoid thickness and aluminum-positive surfaces, and decreased osteoblastic surfaces when compared to the NP7 controls.

At the end of the 10 day recovery period (during which all rats were fed normal phosphorus), compared to the non-aluminum exposed, placebo-treated rats (NP10), the rats continuously treated with aluminum (NP10Al+) had increases in growth plate thickness, osteoid volume and thickness and aluminum-positive surfaces, and decreases in osteoblastic and mineralizing surfaces. Mineralization in these rats was impaired to such a degree that there were no detectable double labels present. The aluminum-positive surfaces were located at the osteoid-bone interfaces, rather than at cement lines. Although not significantly different, serum phosphorus was notably lower in the aluminum-treated rats.

The rats treated with aluminum during the 7 day induction period but then treated with placebo during the recovery period (NP10Al-) had increased osteoid volume and surface and aluminum-positive surfaces, and decreased osteoblastic and mineralizing surfaces. Double labels were not detectable in these rats either. As mentioned above, the aluminum-positive surfaces occurred at the osteoid-bone interface. As was true for rats continuously exposed to aluminum, these rats had numerically (but not significantly) lower serum phosphorus.
Discussion:

In this study of uremic rats, administration of 10 and 26 mg of aluminum over a seven or seventeen day period consistently produced aluminum deposition at the mineralization front, and a decrease in the relative extent of osteoblast-lined surfaces in the tibial metaphyses. While aluminum administration did not significantly affect the developmental phase of this uremic and dietary phosphorus dependent model of osteomalacia, it did prevent the reversal of the lesion brought about by dietary phosphorus repletion, even in the face of discontinuation of aluminum administration.

It is well established that aluminum is toxic to osteoblasts. Numerous studies have shown that elevated bone aluminum is associated with a decrease in the number of osteoblasts present [24-26], and can produce functional impairment of the mineralization process [4,10,27]. Many believe that these are the underlying mechanisms of aluminum-induced osteomalacia, as well as the aluminum-associated aplastic bone disease [7,10,28]. In this study, elevated bone aluminum was invariably associated with decreased osteoblastic surfaces, confirming the deleterious effect of aluminum on osteoblasts. However, administration of 10 mg of aluminum did not produce osteomalacia in uremic rats fed normal dietary phosphorus. Although the osteoid thickness was elevated in the NP7Al+ rats (perhaps indicating a partial degree of mineralization impairment), all other parameters of bone mineralization were similar to those of controls.
This aluminum dosage also did not produce a significant exacerbation of the osteomalacia induced in the uremic rats fed low phosphorus (perhaps because of the severity of the lesion produced independent of aluminum). However, elevated bone aluminum (due to administration of as little as 10 mg of aluminum) was able to prevent the resolution of the osteomalacia in uremic rats returned to normal dietary phosphorus. While some investigators have been able to produce significant improvements in bone mineralization with discontinuation of aluminum exposure for three weeks or longer [29,30], others have also found no improvement in the osteomalacia [27,31]. Furthermore, in a very comprehensive review of the pertinent literature, Vukicevic argues that studies reporting a “cure” of aluminum-induced osteomalacia in fact did not use aluminum dosages sufficient to produce toxicity in the first place [32].

The results of this study show that aluminum, once deposited in bone, is removed slowly; after 10 days of no aluminum exposure, NP10Al- rats had a numerically, but not significantly lower extent of aluminum-positive bone surfaces than NP10Al+ rats. Other studies investigating aluminum withdrawal also found a persistence of bone aluminum for three weeks or more [29,30].

Several related studies have suggested that aluminum accumulation in osteomalacic bone is an “epiphenomenon”, and that aluminum has no primary role in the development of osteomalacia [15,18]. They have shown that in non-uremic dogs and rats, vitamin D-deficiency osteomalacia is not worsened by aluminum administration, and
that vitamin D repletion can cure the osteomalacia despite continued aluminum treatment and elevated bone aluminum. Our data on the other hand, in agreement with that of many others \([3,24,30,31,33]\) clearly show that in uremic rats, aluminum does accumulate preferentially at the mineralization front in both normal (NP \(_7\) Al\(^+\)) and osteomalacic (LP \(_7\) Al\(^+\)) bone, and that the presence of this aluminum can have a profound effect on bone mineralization, completely inhibiting the mineralizing surface (NP \(_{10}\) Al\(^+\) and NP \(_{10}\) Al\(^-\)). This would suggest that the "epiphenomenon" conclusion is unique to the non-uremic, vitamin D deficiency model used, and that the situation is quite different in the uremic state.

The reasons for these differences are not known. It is known that uremia itself plays a role in the development of osteomalacia \([19]\). Although the exact mechanism is not known, some of the important factors are: altered local concentrations of pyrophosphate, magnesium, bicarbonate and other ions \([34-37]\); metabolic acidosis, which has been shown to potentiate the changes induced by uremia \([38]\); and increased bone resorption and calciuria \([39-41]\), which accentuate abnormal calcium-phosphorus homeostasis. Parathyroid hormone (PTH), thought to be elevated in the "epiphenomenon" studies \([15,18]\) is also known to play a role, although both elevated \([42]\) and decreased \([33]\) PTH have been shown to be protective against the development of osteomalacia. Although PTH was not measured in this study, a previous study has shown that this model of osteomalacia is associated with low PTH levels \([19]\). Finally, uremia also plays a role in influencing the development of aluminum toxicity. Renal
failure has been shown to increase the severity of aluminum-induced osteomalacia [1], and decrease the amount of aluminum necessary to produce toxicity (13.5 mg in uremic rats vs. 35 mg in intact rats) [32].

All of these factors emphasize that the dosage of aluminum, the duration and route of exposure, and the duration of aluminum withdrawal are all important factors in the development of aluminum-related bone disease, and greatly complicate inter-study comparisons. In addition, uremia itself plays an important role in the development of aluminum-related bone disease.
Table 12. Static Histomorphometry of Tibia (mean ± S.E.)

<table>
<thead>
<tr>
<th></th>
<th>NP7</th>
<th>LP7</th>
<th>LP7 Al+</th>
<th>NP7 Al+</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.Pl.Th (µm)</td>
<td>192±9.0</td>
<td>237±25.9</td>
<td>215±31.1</td>
<td>232±40.5</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>14.9±3.0</td>
<td>15.3±1.5</td>
<td>15.8±3.7</td>
<td>18.8±2.4</td>
</tr>
<tr>
<td>OV/BV (%)</td>
<td>2.0±0.3</td>
<td>37.2±1.9</td>
<td>10.0±2.7</td>
<td>3.4±1.1</td>
</tr>
<tr>
<td>BS/TV (mm²/mm³)</td>
<td>5.9±0.8</td>
<td>5.4±0.5</td>
<td>5.9±1.1</td>
<td>6.5±0.5</td>
</tr>
<tr>
<td>OV/BS (%)</td>
<td>10.8±1.8</td>
<td>34.7±6.1</td>
<td>16.0±4.2</td>
<td>6.0±1.9</td>
</tr>
<tr>
<td>O.Th (µm)</td>
<td>5.3±0.6</td>
<td>61.7±13.3</td>
<td>19.2±2.1</td>
<td>18.1±2.4</td>
</tr>
<tr>
<td>Ob.S/BS (%)</td>
<td>22.7±3.1</td>
<td>24.8±3.6</td>
<td>5.6±1.3</td>
<td>4.1±1.5</td>
</tr>
<tr>
<td>Oc.S/BS (%)</td>
<td>0.4±0.2</td>
<td>0.7±0.2</td>
<td>0.1±0.1</td>
<td>0.4±0.3</td>
</tr>
<tr>
<td>Al.S/BS (%)</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>35.0±8.2</td>
<td>28.2±0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>NP7</th>
<th>NP7 Al+</th>
<th>NP7 Al-</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.Pl.Th (µm)</td>
<td>215±7.2</td>
<td>430±56.7</td>
<td>310±36.1</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>17.8±1.3</td>
<td>10.6±2.0</td>
<td>12.2±2.1</td>
</tr>
<tr>
<td>OV/BV (%)</td>
<td>4.1±1.7</td>
<td>19.8±4.4</td>
<td>13.6±3.4</td>
</tr>
<tr>
<td>BS/TV (mm²/mm³)</td>
<td>6.2±0.3</td>
<td>5.3±0.6</td>
<td>4.7±0.6</td>
</tr>
<tr>
<td>OS/BS (%)</td>
<td>14.1±2.5</td>
<td>21.1±4.2</td>
<td>30.2±3.6</td>
</tr>
<tr>
<td>O.Th (µm)</td>
<td>7.6±1.2</td>
<td>16.7±2.3</td>
<td>14.7±1.9</td>
</tr>
<tr>
<td>Ob.S/BS (%)</td>
<td>27.2±2.0</td>
<td>6.2±1.6</td>
<td>7.5±0.9</td>
</tr>
<tr>
<td>Oc.S/BS (%)</td>
<td>0.2±0.1</td>
<td>1.2±0.7</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>Al.S/BS (%)</td>
<td>0.0±0.0</td>
<td>32.7±9.2</td>
<td>20.3±6.2</td>
</tr>
</tbody>
</table>

Legend:  
LP7  = low phosphorus diet for seven days  
NP7  = normal phosphorus diet for seven days  
LP7 Al+ = low phosphorus diet for seven days with daily aluminum treatments  
NP7 Al+ = normal phosphorus diet for seven days with daily aluminum treatments  
NP10  = low phosphorus diet for seven days, then normal phosphorus diet for ten days  
NP10 Al+ = low phosphorus diet for seven days, then normal phosphorus diet for ten days with daily aluminum treatments  
NP10 Al- = low phosphorus diet for seven days with daily aluminum treatments, then normal phosphorus diet for ten days with daily placebo treatments

Note: Unique letters horizontally denote significant differences between groups (viz. AB vs. CD). Any shared letters horizontally indicate no significant difference (viz. AB vs. BC).
Table 13. Dynamic Histomorphometry of Tibia (mean ± S.E.)

<table>
<thead>
<tr>
<th></th>
<th>NP,6</th>
<th>LP,6</th>
<th>LP,Al+3</th>
<th>NP,Al+4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS/BS (%)</td>
<td>11.1±2.3</td>
<td>1.5±0.5</td>
<td>0.7±0.4</td>
<td>10.5±2.2</td>
</tr>
<tr>
<td>MAR (μm/d)</td>
<td>2.2±0.2</td>
<td>2.8±1.1</td>
<td>0.8±0.3</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>BFR/BS (μm³/μm²/d)</td>
<td>0.3±0.1</td>
<td>0.1±0.0</td>
<td>0.0±0.0</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Omt (d)</td>
<td>2.5±0.3</td>
<td>21.1±11.3</td>
<td>18.2±3.9</td>
<td>3.0±0.3</td>
</tr>
</tbody>
</table>

Legend: LP7 = low phosphorus diet for seven days
NP7 = normal phosphorus diet for seven days
LP7 A1+ = low phosphorus diet for seven days with daily aluminum treatments
NP7 A1+ = normal phosphorus diet for seven days with daily aluminum treatments
NP,10 = low phosphorus diet for seven days, then normal phosphorus diet for ten days
NP,10 A1+ = low phosphorus diet for seven days, then normal phosphorus diet for ten days with daily aluminum treatments
NP,10 A1- = low phosphorus diet for seven days with daily aluminum treatments, then normal phosphorus diet for ten days with daily placebo treatments

Note: Numbers in parentheses denote number of rats per group with double labels.

Unique letters horizontally denote significant differences between groups (viz. AB vs. CD).
Any shared letters horizontally indicate no significant difference (viz. AB vs. BC).
Table 14. Serum Biochemistry (mean ± S.E.)

<table>
<thead>
<tr>
<th></th>
<th>NP7</th>
<th>LP7</th>
<th>LP7,A1+</th>
<th>NP7,A1+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dl)</td>
<td>11.1±0.2</td>
<td>12.2±0.3</td>
<td>11.5±0.6</td>
<td>10.6±0.6</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>6.5±0.5</td>
<td>3.7±0.3</td>
<td>5.3±0.8</td>
<td>7.4±1.1</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>50±6</td>
<td>50±5</td>
<td>55±8</td>
<td>54±9</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.3±0.1</td>
<td>1.8±0.1</td>
<td>1.2±0.1</td>
<td>1.3±0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>NP07</th>
<th>NP07,A1+</th>
<th>NP07,A1-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dl)</td>
<td>11.3±0.1</td>
<td>11.0±0.3</td>
<td>10.7±0.2</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>7.6±0.4</td>
<td>5.6±0.4</td>
<td>5.9±0.7</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>53±5</td>
<td>46±3</td>
<td>55±5</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.5±0.1</td>
<td>1.1±0.1</td>
<td>1.2±0.1</td>
</tr>
</tbody>
</table>

Legend:
- LP7 = low phosphorus diet for seven days
- NP7 = normal phosphorus diet for seven days
- LP7,A1+ = low phosphorus diet for seven days with daily aluminum treatments
- NP7,A1+ = normal phosphorus diet for seven days with daily aluminum treatments
- NP07 = low phosphorus diet for seven days, then normal phosphorus diet for ten days
- NP07,A1+ = low phosphorus diet for seven days, then normal phosphorus diet for ten days with daily aluminum treatments
- NP07,A1- = low phosphorus diet for seven days with daily aluminum treatments, then normal phosphorus diet for ten days with daily placebo treatments

Note: Unique letters horizontally denote significant differences between groups (viz. AB vs. CD). Any shared letters horizontally indicate no significant difference (viz. AB vs. BC).
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