Bone mineralization involves the deposition of apatite on the bone matrix which is responsible for the metabolic and biomechanic functions of bone. Mineralization is controlled by many types of cells, extracellular molecules and components of the organic matrix.

The mechanisms of mineralization are complex and many aspects are still unresolved. Calcification first occurs with the formation of a critical nucleus, the smallest combination of ions with a crystalline structure which is stable in solution. This nucleus is composed of calcium and phosphorus as apatite (1). Two mechanisms have been described for calcification, one predominant in cartilage and woven bone, the other in the lamellar bone (2).

Clinical data from patients with hypophosphataemic rickets have shown that during puberty the disease can go into remission. At this stage there is a cure for the rickets. With the disappearance of the X-ray lesions in the epiphyseal growth plate, suggesting an action of steroid hormones on the mineralization process. There is evidence that androgens play a role in the metabolism of bone cells, stimulating their formation (3). However, testosterone action on bone is not yet clearly defined, particularly in the process of bone mineralization (4).

The purpose of the present study was to analyse the action of testosterone on the bone mineralization process of hypophosphataemic rats through the histomorphometric study of the epiphyseal growth plate from the tibia and trabecular bone from the seventh caudal vertebra (5).

Material and methods

Forty-four male Sprague-Dawley rats, breast-fed for 21 days after birth were divided into six groups:

1) Control 15 d – Chow diet (7 rats) – For 15 days on a special diet for rats – Nuvilab CR1 – with free access to water and exposure to ambient light in isolated cages. On the 15th day the animals were sacrificed with removal of the right tibia and blood withdrawn from the abdominal aorta.

2) Low P + low vit D 15 d – Low phosphate and vitamin D diet (9 rats) – Rachitogenic diet – composed of 74% corn starch, 18% casein, 2% corn oil, 3% calcium carbonate, 2% of a mixture of mineral salts without calcium and phosphorus, 1% of a mixture of vitamins excluding vitamin D (6, 7). During the 15 days on this diet the animals had free access to water and were kept in complete darkness in isolated cages. On day 15 the rats were sacrificed, their blood collected and the right tibia removed.

3) Control 20 d – Chow diet (7 rats) – For 20 days, on the special diet for rats – Nuvilab CR1 – the rats had free access to water and exposure to ambient light in isolated cages. On days 15 and 18 every animal received 30 mg/kg body weight of tetracycline intraperitoneally. On the 20th day the animals were sacrificed, their blood collected and the right tibia and seventh caudal vertebra removed.

4) Low P + low vit D 20 d – Low phosphate and vitamin D diet (8 rats) – The animals in complete
darkness and with free access to water in isolated cages received the same diet as in the low P + low vit D 15 d group. From the 15th day onwards the rats were injected with soya bean oil subcutaneously. On days 15 and 18, 30 mg/kg of body weight of tetracycline was given intraperitoneally. On day 20, the animals were sacrificed, blood collected and the right tibia and seventh caudal vertebra removed.

(5) Low P + low vit D + Testosterone 20 d - Low phosphorus and vitamin D diet (6 rats) - For 20 days the animals, kept in darkness with free access to water and in isolated cages, received the rachitogenic diet as for the low P + low vit D 15 d group. From the 15th to the 20th day the rats were injected subcutaneously with testosterone propionate, in the dose of 0.5 mg/kg of body weight, diluted in soya bean oil. On the 20th day the animals were sacrificed, the blood collected and the right tibia removed.

(6) Low P + low vit D + Testosterone 25 d - Low phosphorus and vitamin D diet (7 rats) - For 25 days the rats received the same diet as the low P + low vit D 15 d group kept in isolated cages in complete darkness and with free access to water. From the 15th day onwards every animal received 0.5 mg/kg body weight of testosterone propionate diluted in soya bean oil given subcutaneously. On days 20 and 23 they were injected intraperitoneally with 30 mg/kg body weight of tetracycline. On the 25th day the rats were sacrificed, blood withdrawn and the seventh caudal vertebra removed.

At the end of every experiment the animals were submitted to ether anaesthesia and sacrificed by exsanguination from the abdominal aorta.

Histomorphometry

For the histomorphometric studies the bones were fixed in 70% ethanol and embedded in methyl methacrylate (8). Sections (8 µm thick) were stained with toluidine blue pH 6.4 (9). Fluorescent tetracycline labels were evaluated in undecalcified unstained 10 µm sections. The measurements were performed using a Photomicroscope Zeiss II with oculars I and II and Zeiss micrometer.

For analysis of the tibia growth plate the total thickness and hypertrophic zone thickness were determined with the micrometer. In each plate the mean of four equidistant measurements was taken. The total thickness measurement was taken from the superior limit of the resting to the provisional calcification zone and the hypertrophic zone from the upper limit of the hypertrophic chondrocytes up to the provisional calcification.

The following histomorphometric parameters were quantified in the trabecular vertebra diaphysis with Zeiss oculars I and II: bone volume (BV/TV, %) corresponding to the section fraction occupied by the trabecular bone expressed by the fraction of the volume occupied by the medulla plus the trabecular bone (magnification 126 ×); osteoid surface (OS/BS, %), which is the fraction of trabecular surface covered by the osteoid seam (magnification 126 ×); osteoid thickness (O.Th, µm) as the average of four separate and equidistant sites along the seam; and mineral apposition rate (MAR, µm/day), which estimates the rate of mineral deposition in the active surface of bone mineralization front and evaluated as the mean distance between the midpoints of the two fluorescent tetracycline labels divided by the interval of time between the two labellings (magnification 320 ×) (10).

Serum calcium was determined by atomic absorption spectrophotometry and inorganic phosphorus by the phosphomolibdate method (11).

Statistical analysis

The non-parametric tests of Mann–Whitney for independent variables expressed as percentages (12) and analysis of variance (13) to evaluate the differences among the groups were used. In all tests a level of 5% was established for rejection of the null hypothesis.

Results

After weaning, none of the 44 rats distributed in the six study groups showed any significant difference in weight by analysis of variance (Table 1). At the end of the experiment, the rats on a low phosphorus and vitamin D diet (the low P + low vit D 15 d and low P + low vit D 20 d groups) gained much less weight than animals on the chow diet (control 15 d and control 20 d, p < 0.05). The treatment of rats (low P + low vit D + testosterone 20 d and low P + low vit D + testosterone 25 d) with testosterone propionate did not induce significant weight gain.

<p>| Table 1. Weight of rats before and after the experimental period (mean ± sd). |
|--------------------------------------|--------------------------------------|--------------------------------------|
| Group | Control | Low P + low vit D | Low P + low vit D + testosterone |</p>
<table>
<thead>
<tr>
<th></th>
<th>15 d</th>
<th>20 d</th>
<th>15 d</th>
<th>20 d</th>
<th>20 d</th>
<th>25 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>49.4±6.7</td>
<td>54.5±8.6</td>
<td>47.6±8.9</td>
<td>53.2±11.5</td>
<td>48.1±4.7</td>
<td>53.0±6.5</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>125.1±19.6</td>
<td>148.2±14.4</td>
<td>72.3±12.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.7±12.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79.5±4.1</td>
<td>111.0±22.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>p<0.05 vs control 15 d; <sup>b</sup>p<0.05 vs control 20 d.
in comparison to those also kept on low phosphorus and vitamin D (low P + low vit D 20 d) (Table 1).

The low phosphorus and vitamin D diet, given for 15 and 20 days, induced severe hypophosphataemia. Testosterone propionate significantly increased the levels of serum phosphorus in low P + low vit D + testosterone 20 d compared to low P + low vit D 20 d rats (Table 2). However, in none of the low P + low vit D + testosterone 20 d animals did the serum phosphorus attain levels of 1.29 mmol/l. The values of serum calcium increased significantly in low P + low vit D 15 d compared to the control 15 d rats. However, in the low P + low vit D 20 d animals the levels of serum calcium despite being significantly higher than those in control 20 d were lower than in the low P + low vit D 15 d rats. Treatment of low P + low vit D + testosterone 20 d with testosterone significantly decreased serum calcium compared to the low P + low vit D 20 d rats (Table 2).

Table 3 indicates the histomorphometric measurements carried out in the growth plate of the tibias. As early as the 15th day of the low phosphorus and vitamin D diet the total thickness of the growth plate was significantly increased in relation to the control rats (control 15 d), this increment was predominantly in the hypertrophic zone. Besides these histological quantitative changes, a distortion of the columnar arrangement of the chondrocytes in the hypertrophic zone was noticed. The 20-day low phosphorus and vitamin D diet further increased the growth plate thickness and that of zona hypertrophica of low P + low vit D 20 d in comparison to the control 20 d rats, with accentuation of the columnar distortion of chondrocytes at that zone.

![Fig. 1. Photomicrographs of tibia growth plates of rats fed low phosphorus and vitamin D diet for 20 days (R20). A. Note the enlargement of the hypertrophic zone and distortion of columnar arrangement of the chondrocytes. B. Reduction of the thickness of the hypertrophic zone and columnar rearrangement of the correspondent chondrocytes after five days of testosterone propionate treatment (RT). Toluidine blue 100 x.](image-url)
Testosterone treatment for five days, while on the same rachitogenic diet, induced significant changes in the measurements performed in the tibia growth plate besides the rearrangement of the chondrocytes at the hypertrophic zone. In effect, the growth plate with the disappearance of the changes compatible with rickets had a normal aspect (Fig. 1B).

Osteomalacia features were evaluated in the trabecular bone of the seventh caudal vertebra of the rats on chow and/or low phosphorus and vitamin D diet for 20 days who had two tetracycline labels on the 15th and 18th days (control 20 d and low P + low vit D 20 d). Table 4 gives the measurements performed on the vertebra for evaluation of osteomalacia. After 15 days the rats on the low phosphorus and vitamin D diet developed intense osteomalacia, characterized by an increased osteoid seam area (OS/BS and O.Th) tetracycline labelling non-existent, indicating no mineral apposition. Testosterone propionate treatment (low P + low vit D + Testosterone 25 d) induced a significant reduction in the osteoid seam area relative to total bone area. However, tetracycline labelling was still non-existent, indicating no cure of osteomalacia. The rachitogenic diet reduced the trabecular volume which was not corrected by testosterone (Table 4).

Cortical bone was also examined but not quantified, an increase in the osteoid seam and no tetracycline labelling also being evident.

Discussion

Growing rats receiving a low phosphorus and vitamin D diet kept in complete darkness developed, after 15 days, hypophosphataemia and impaired bone mineralization. Because of increased bone remodelling during the growth phase in the presence of hypophosphataemia, there is an increase in bone reabsorption and hypercalcaemia develops, bone resorption being independent of PTH (14, 15).

After 15 days on the rachitogenic diet, tibia growth plates showed the characteristic changes of rickets, i.e. increased thickness of total as well as the hypertrophic zone with distortion of the columnar arrangement. Testosterone propionate treatment for five days on rats kept on the low phosphorus and vitamin D diet induced a significant reduction in the growth plate total thickness as well as in the hypertrophic zone. The cells in the hypertrophic zone returned to their columnar arrangement.

Dynamic histomorphometry performed in the trabecular bone of the seventh caudal vertebra allowed evaluation of the osteomalacia. After 20 days on the low phosphorus and vitamin D diet there was an enlargement of the osteoid seam area and an absence of tetracycline labelling, indicating a severe defect in bone mineralization. Cortical bone also presented an increase in osteoid seam width and no tetracycline labelling. The rats treated with testosterone and kept on the rachitogenic diet presented a reduction in the osteoid seam area, with deficient tetracycline labelling, indicating no cure of osteomalacia in the period of androgen treatment (10 days) in either the trabecular or the cortical bone.

Thus our data indicate that testosterone acts on the mineralization process in the hypophosphataenic rat, the action being different in the growth plate and trabecular bone during the period of our study. These findings are similar to those observed in hypophosphataenic human beings and rats receiving phosphate (16–18). These observations are in accordance with the tendency to restrict the term rickets to the mineralization defect at the growth plate, while defining osteomalacia as the changes which take place in the calcified bone-osteoid interface in the cortical and trabecular bone (17, 19).

The actions of testosterone on the mineralization process can be direct and/or indirect through changes in calcium and phosphorus or other hormones. In our study, the mineralization occurred independently of the levels of calcium and phosphorus. Serum calcium levels decreased with testosterone while phosphate values increased but not by more than 1.29 mmol/l. It is known that serum phosphate has an active role in the mineralization process when greater than 1.42 mmol/l (19). Thus, in our investigation, serum phosphate cannot be considered as responsible for curing rickets in rats.

On the other hand, physiological levels of testosterone increase the secretary rate of growth hormone in the rat and human being. Growth hormone acts directly on the growth plate of bone, stimulating the differentiation of the pre-chondrocytes that become responsive to IGF-1 produced locally or by the liver (20). However, the role of growth hormone and IGF-1 is still not well defined in the mineralization process. The role of testosterone on vitamin D metabolism is still undefined. However, Somjen et al. have suggested that the vitamin D level can modulate the bone tissue responsiveness to the steroid hormones, androgens included (21).

The direct action of hormones on bone mineralization is not clear. The mechanism of hormonal control of the epiphyseal cartilage is not well known particularly because of the difficulties in demonstrating their receptors in the cartilage cell (22). In relation to vitamin D the

### Table 4. Histomorphometric measurements in the diaphysis of the seventh caudal vertebra of the rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control 20 d</th>
<th>Low P + low vit D 20 d</th>
<th>Low P + low vit D + testos 25 d</th>
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<tbody>
<tr>
<td>BV/TV</td>
<td>16.1 ± 2.1</td>
<td>11.0 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.8 ± 1.7</td>
</tr>
<tr>
<td>OS/BS</td>
<td>15.5 ± 6.0</td>
<td>72.6 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.0 ± 6.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>O.Th</td>
<td>4.6 ± 1.6</td>
<td>21.6 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.1 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAR</td>
<td>2.3 ± 0.3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.05 vs control 20 d; <sup>b</sup> p < 0.05 vs low P + low vit D 20 d.
best studied hormone acting on bone mineralization there is no consensus whether its action is direct or indirect through the increase in serum calcium and phosphorus levels (23–27). In our study, testosterone acted on the growth plate of rachitic rats, independent of serum phosphorus levels. However, we were unable to show if the action of testosterone was a direct one or was through growth factor(s) induced by hormone treatment.

The period of development and growth in which the study was performed should be considered since there could be a variation in intensity of response of cartilagenous cells to the androgen (28, 29). Probably the difficulty in understanding the several effects of androgens in different stages of skeletal growth and maturation is due to the complexity of endochondral differentiation. The growth plate is composed of different types of cells in different stages of differentiation and difficult to separate for study.

Since osteoblasts have androgen receptors, Kasper et al. have shown that androgens have a direct effect on osteoblast line cells in vitro (30). The reduction of the osteoid seam area in the rachitic rats studied is an indication that testosterone acts in bone metabolism. However, the lack of change in tetracycline labelling suggests that there was no normalization of the calcification process in the lamellar bone.

In conclusion, testosterone action on bone, in our animal model, suggests that steroid hormones actively participate in the cure of rickets, as observed in human beings.

References

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