The effect of vitamin D and its metabolites on fracture repair in chicks

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(Received 10 January 1983; accepted 26 April 1983)

Summary

1. One-day-old chicks were depleted of vitamin D. At 3 weeks their right tibiae, and those of a control group given vitamin D₃, were fractured and pinned. After fracture the controls were kept on vitamin D₃. Another group was left vitamin D-deficient. The remaining depleted chicks, divided into four groups, were given vitamin D₃, 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃], 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] or a combination of 24,25(OH)₂D₃ and 1,25(OH)₂D₃.

2. The callus obtained after 9 and 14 days was subjected to torsional stress. The callus of chicks given vitamin D continuously showed the greatest resistance, whereas that of vitamin D-deficient chicks showed the smallest resistance. Repletion with either vitamin D₃ or its metabolites increased the strength of the callus. Repletion with the combination of 24,25(OH)₂D₃ and 1,25(OH)₂D₃ produced the most marked results, in that the callus was even stronger than that of chicks replete with vitamin D₃.

3. It is concluded that 24,25(OH)₂D₃ is essential for bone formation in addition to the known active vitamin D metabolite 1,25(OH)₂D₃, and the possible clinical implications of these findings are discussed.

Key words: callus, cholecalciferol, fractures.

Introduction

The important subject of fracture repair has been the focus of numerous investigations during the last few decades. Various hormones have been shown to influence the healing of fractures in experimental animals and in man [1]. The purpose of this study was to investigate the effect of various cholecalciferol (vitamin D₃) metabolites on fracture repair in chicks.

Cholecalciferol has long been known to be associated with bone metabolism and is used to cure rickets and osteomalacia [2]. In recent years, it has become clear that cholecalciferol is in fact a prohormone that is converted by the liver and kidney into 1α,25-dihydroxycholecalciferol [1,25-(OH)₂D₃], the active form of the vitamin. Views differ, however, as to whether 1,25(OH)₂D₃ is the only vitamin D metabolite needed for normal bone formation or whether other metabolites formed by the kidney, such as (24R)-24,25-dihydroxycholecalciferol [24,25(OH)₂D₃], are also essential [3]. Attempts to cure vitamin D-resistant rickets and renal osteodystrophy by administration of 1,25(OH)₂D₃ or its synthetic analogue, 1α-hydroxycholecalciferol [1α(OH)D₃], have been inconclusive and controversial [4]. Recent work has shown that high levels of 24,25-(OH)₂D₃ are found in the calluses of experimental fractures in chicks and that administration of 1α(OH)D₃ alone was unable to restore fracture repair to normal [5].

In the present study, the influence of various cholecalciferol metabolites, particularly on the mechanical properties of the callus during fracture repair, was examined.

Materials and methods

Seventy-four 1-day-old male chicks were used. Sixty-one were first depleted of vitamin D by feeding them on a vitamin D-deficient diet for
3 weeks [6]. An additional control group of 13 chicks were treated, from day 1, with daily injections of 0.5 μg of cholecalciferol for the entire experimental period. After 3 weeks, both the vitamin-depleted and the non-depleted chicks were subjected to operation and the right tibia of each chick was fractured by the following method. Under continuous halothane-inhalation anaesthesia, the right tibia was exposed by a longitudinal medial skin incision of 0.5 cm directly over the bone; the periosteum was also incised longitudinally and retracted. With a dental drill two holes were made at right angles in the mid-shaft of the right tibia. A Kirschner wire (1.0 mm diameter) was then threaded into the medullary cavity of the tibia through the skin and patellar ligament over the knee and was advanced down to the distal end of the tibia. The tibia was then gently broken by light manual bending while the Kirschner wire was in the medullary canal. The remainder of the Kirschner wire protruding from the knee was cut off and the incision closed with interrupted silk sutures. Within a few minutes after operation and recovery from anaesthesia the chicks were able to walk freely on both legs.

After surgery, the 13 non-depleted chicks (group 1) continued to be treated by daily injections of 0.5 μg of cholecalciferol. The 61 depleted chicks were divided into five more groups. Thirteen chicks were left on a vitamin D-deficient diet until the end of the experiment ('vitamin D-deficient chicks'; group 2); 12 chicks were given daily subcutaneous injections of 0.5 μg of cholecalciferol (vitamin D-replete chicks; group 3); 12 were given daily injections of 0.3 μg of 24,25(OH)2D3 (group 4); 12 were given daily injections of 0.1 μg of 1,25(OH)2D3 (group 5) and 12 were given a combination of 0.3 μg of 24,25(OH)2D3 and 0.1 μg of 1,25(OH)2D3 (group 6).

The chicks were killed at 9 or 14 days after operation. The plasma was prepared for estimation of calcium and inorganic phosphate [6]. Both tibiae were removed and cleaned. Care was taken not to damage the callus, by leaving a small layer of muscles around it. The bone length was measured by caliper. The non-fractured left tibia was used for analysis of bone ash content and the results were expressed as a percentage of the dry weight of each bone [6]. The ends of the fractured tibia were potted in a quick setting polyester resin and kept in NaCl solution (154 mmol/l) until subjected to torsion testing. Each bone specimen with its moulded ends was then subjected to torsional deformation with an Instron Universal testing machine (model TTK). The rate of deformation produced was 180°/min. The strength characteristics of each bone were analysed from the torque–angle graph obtained from each bone specimen. Four parameters are readily measured from each graph [7] (Fig. 1): 1, maximum torque; 2, angle; 3, initial stiffness; 4, stiffness. 'Maximum torque' is the torque at which the callus failed. 'Angle' is the degree of torsional deformation that caused failure of the callus. As the torque angle is non-linear, its slope varies and therefore the 'stiffness' also varies, and a single number cannot represent the true stiffness of the callus. Two parameters were therefore measured and termed 'initial stiffness', the torque/angle ratio at the elastic (linear) region, and 'stiffness', the torque/angle ratio at the point of maximum torque.

Results

Body weight

Continuous depletion of vitamin D resulted in retardation of growth and reduction in body weight (Table 1). Repletion with either vitamin D or its metabolites increased the body weight. But with the exception of the chicks given a combination of 24,25(OH)2D3 and 1,25(OH)2D3 for 14 days all the repleted chicks still had a significantly lower body weight compared with the chicks given vitamin D continuously.
TABLE 1. Body weight, plasma calcium and the inorganic phosphate and bone ash of the six groups of chicks

Bone ash is expressed as a percentage of the dry weight of the bones. Results are means ± SEM. Superscript symbols to the right of values (significances of results compared with those obtained for chicks given vitamin D continuously and for the same period of time): ***P<0.001, **P<0.01, *P<0.05, not significant. Superscript symbols to the left of values (significances of results compared with those obtained for vitamin D-replete chicks for the same period of time): †††P<0.001, ††P<0.01, †P<0.05, ‡ not significant.

<table>
<thead>
<tr>
<th>Vitamin D source</th>
<th>Period of treatment (days)</th>
<th>Time after fracture (days)</th>
<th>No. of chicks</th>
<th>Body wt. (g)</th>
<th>Plasma calcium (mg/100 ml)</th>
<th>Plasma inorganic phosphate (mg/100 ml)</th>
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<tbody>
<tr>
<td>Cholecalciferol (vitamin D continuously)</td>
<td>30</td>
<td>9</td>
<td>5</td>
<td>278 ± 20</td>
<td>12.07 ± 0.39</td>
<td>7.66 ± 0.28</td>
</tr>
<tr>
<td>None (vitamin D-deficient)</td>
<td>30</td>
<td>9</td>
<td>5</td>
<td>140 ± 12***</td>
<td>7.75 ± 0.30***</td>
<td>5.40 ± 0.66***</td>
</tr>
<tr>
<td>Cholecalciferol (repletion)</td>
<td>9</td>
<td>9</td>
<td>4</td>
<td>180 ± 10**</td>
<td>12.41 ± 0.66‡</td>
<td>7.09 ± 0.39‡</td>
</tr>
<tr>
<td>24,25(OH)_2D_3 (repletion)</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>183 ± 12**</td>
<td>10.58 ± 0.65‡</td>
<td>5.93 ± 0.35**</td>
</tr>
<tr>
<td>1,25(OH)_2D_3 (repletion)</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>183 ± 13**</td>
<td>11.79 ± 0.50‡</td>
<td>5.96 ± 0.10***</td>
</tr>
<tr>
<td>24,25(OH)_2D_3+1,25(OH)_2D_3 (repletion)</td>
<td>9</td>
<td>9</td>
<td>4</td>
<td>173 ± 8**</td>
<td>11.12 ± 0.38‡</td>
<td>6.69 ± 0.28*</td>
</tr>
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<td></td>
<td>14</td>
<td>14</td>
<td>8</td>
<td>~273 ± 3‡</td>
<td>11.11 ± 0.26‡</td>
<td>6.34 ± 0.30‡</td>
</tr>
</tbody>
</table>
Plasma calcium and inorganic phosphate

Depletion of vitamin D resulted in hypocalcaemia and hypophosphataemia (Table 1). Repletion restored the plasma calcium levels to normal except in chicks given 24,25(OH)2D3 alone for 14 days. Inorganic phosphate levels also returned to normal but only after 14 days, again apart from those chicks treated with 24,25(OH)2D3 alone.

Bone ash

Bones obtained from the vitamin D-deficient chicks had lower bone ash compared with the bones obtained from the chicks given vitamin D continuously (Table 1). Repletion for 9 days with either cholecalciferol or 1,25(OH)2D3 restored the bone ash to normal. With 24,25(OH)2D3, it was normal but significantly less than for cholecalciferol-replete chicks. A significant increase in bone ash was found in bones of the chicks treated for 14 days with a combination of 24,25(OH)2D3 and 1,25(OH)2D3 (Table 1).

The length ratio of tibiae

The difference between the length of the fractured and unfractured tibiae was expressed as a ratio for each individual chick. The fractured tibiae of the chicks given vitamin D continuously, the vitamin D-replete chicks and those given a combination of 24,25(OH)2D3 and 1,25(OH)2D3 were in general longer than the unfractured bones. Their index was greater than 1 (Fig. 2).

In contrast, the fractured tibiae of the vitamin-deficient chicks or chicks given either 24,25(OH)2D3 or 1,25(OH)2D3 were shorter (ratio <1) (Fig. 2).

Mechanical properties of the callus

Maximum torque. (a) The 9 days callus. The 9 days callus of vitamin D-deficient chicks failed at a low torque (Fig. 3). Repletion with either cholecalciferol or its metabolites caused a significant increase in the ‘strength’ of the callus, which failed at a higher torque than the vitamin D-deficient chicks. However, the 9 days callus of cholecalciferol-, 24,25(OH)2D3- and 1,25(OH)2D3-replete chicks failed at much lower torque than the callus of chicks given vitamin D continuously. Repletion by a combination of 24,25(OH)2D3 and 1,25(OH)2D3 gave highest maximum torque. The maximum torque of the callus of these chicks was similar to that of the callus of chicks given vitamin D continuously and significantly higher than that of vitamin D-replete chicks (Fig. 3).

(b) The 14 days callus. The 14 days callus of vitamin D-deficient chicks still failed at a very low torque (Fig. 4). Again repletion by cholecalciferol or its metabolites increased the maximum torque compared with vitamin D-deficient chicks. However, after repletion with either 24,25(OH)2D3 or 1,25(OH)2D3 alone, it was still significantly lower than for the chicks given vitamin D continuously and for the vitamin D-replete chicks, whereas now chicks given either cholecalciferol or a combination of 24,25(OH)2D3 and 1,25(OH)2D3 had a maximum torque similar to chicks given vitamin D continuously. The results with the combination were still the most marked, however.
The angle. The degree of rotational deformation (angle) needed to cause failure of the callus obtained from the vitamin D-depleted chicks was high (Table 2).

At 9 days after fracture, repletion by either cholecalciferol or its metabolites significantly decreased the degree of deformation needed to cause failure of the callus. But the callus of all the various replete chicks still failed at significantly higher angles than that of chicks given vitamin D continuously. There was, however, no difference between the cholecalciferol-replete chicks and other groups, except for the 24,25(OH)₂D₃-replete group, whose callus failed at a much higher angle (Table 2).

After 14 days, the angle had returned to near normal values. There was no significant difference between chicks given vitamin D continuously and

![FIG. 3. Maximum torque at which the 9 day calluses failed. Numbers within the columns are the numbers of calluses examined. Numbers beneath the columns are the six groups defined in the legend to Fig. 2. Mean results ± SEM are shown. For details of symbols etc. see the legend to Fig. 2.](image)

![FIG. 4. Maximum torque at which the 14 day calluses failed. For identity of symbols and all other details see the legends to Figs. 2 and 3.](image)

<table>
<thead>
<tr>
<th>Table 2. Degree of torsional deformation that caused failure of the callus (angle) and the torque/angle ratio at the point of maximum torque (stiffness) of the calluses obtained from the six groups of chicks</th>
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<tr>
<td>Results are means ± SEM. Superscript symbols to the right and left of the values are identified in Table 1.</td>
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<tr>
<td>Group no.</td>
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</tr>
<tr>
<td>1.</td>
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replete chicks, with the exception of 24,25-(OH)\(_2\)D\(_3\)-replete chicks. In the latter case the
callus still failed at a very much higher angle than for
calluses from chicks given vitamin D con-
tinuously and from cholecalciferol-replete chicks (Table 2).

**Stiffness.** At 9 days after fracture, the maximum
torque/angle ratio (stiffness) was highest for
the callus of chicks given vitamin D continuously
and lowest for the callus of the vitamin D-deficient
chicks (Table 2). Repletion for 9 days with
vitamin D or its metabolites resulted in increased
stiffness of the callus, but none of the repleted
groups had the same stiffness as the callus obtained
from the chicks given vitamin D continuously.
Repletion with a combination of 24,25(OH)\(_2\)D\(_3\)
and 1,25(OH)\(_2\)D\(_3\) gave the highest value and
repletion with 24,25(OH)\(_2\)D\(_3\) the lowest (Table 2).

At 14 days after fracture, further increase in
the stiffness of the callus was observed. There was
no significant difference between the callus of
chicks given vitamin D continuously and that of
those given cholecalciferol, 1,25(OH)\(_2\)D\(_3\) or a
combination of 1,25(OH)\(_2\)D\(_3\) and 24,25(OH)\(_2\)D\(_3\).
However, the callus of 24,25(OH)\(_2\)D\(_3\)-replete
chicks still exhibited very low stiffness (Table 2).

**Initial stiffness.** At 9 days after the fracture,
the callus of vitamin D-depleted and that of all
replete chicks had a significantly lower initial
stiffness than that of the chicks given vitamin D
continuously (Fig. 5). However, repletion with a
combination of 24,25(OH)\(_2\)D\(_3\) and 1,25(OH)\(_2\)D\(_3\)
gave relatively the highest initial stiffness
and 24,25(OH)\(_2\)D\(_3\) the lowest. The callus of chicks
treated with a combination of 24,25(OH)\(_2\)D\(_3\) and
1,25(OH)\(_2\)D\(_3\) had a significantly higher initial
stiffness than that of cholecalciferol-replete chicks.

At 14 days after fracture, the initial stiffness
of the callus of chicks given cholecalciferol or a
combination of 24,25(OH)\(_2\)D\(_3\) and 1,25(OH)\(_2\)D\(_3\)
had increased markedly. There was no significant
difference between their initial stiffness and that
of chicks given vitamin D continuously, whereas

that of the callus of chicks given 1,25(OH)\(_2\)D\(_3\)
alone, and particularly that of chicks given 24,25-
(OH)\(_2\)D\(_3\), was still significantly lower (Fig. 6).

**Discussion**

The experimental model for fracture used in this
study was found to be reproducible and easy to
perform. The internal fixation used secured the
two fractured bone ends and allowed the chicks
to use their legs immediately after surgery.
Controlling the extent of the initial trauma, the site
and the shape of the experimental fracture and
the mobility of the two fractured bones ends are
extremely important, as these factors were shown
to affect the process of fracture repair, the size of
the callus formed and its mechanical properties
[8-10].

Mechanical testing of the strength of a callus is
a well-accepted method for the estimation of the
extent of repair of fractures [7, 11]. The speed at
which the torsional deformation was applied in
this study was slower than that used by White and
his colleagues [7]. This would explain the higher
deformation needed to cause failure of the callus.
However, this does not influence the comparative
value of our results.

Previous experimental work gave evidence that
vitamin D and its metabolites are directly involved
in bone formation and fracture repair [3, 5].
Administration of 1α(OH)\(_2\)D\(_3\), the synthetic
analogue of 1,25(OH)2D3, to rachitic chicks restored the plasma calcium and inorganic phosphate to normal but the bones were found to be still rachitic and fracture repair was retarded [5]. Similar findings have been reported for man [4]. The finding that 24,25(OH)2D3, a metabolite of vitamin D that, like 1,25(OH)2D3, is formed in the kidney [12-13] and accumulates in foetal rat bones [14] and in chick calluses [5], suggests that this metabolite also is essential for normal bone formation [3]. The results of the present study tend to confirm these previous findings. Administration of 1,25(OH)2D3 to rachitic chicks restored the plasma calcium and inorganic phosphate to normal (Table 1), but the callus formed failed at lower torque and had lower initial stiffness than chicks given vitamin D continuously (Figs. 4 and 6). It also failed at lower torque than vitamin D-replete chicks but only at 14 days (Fig. 4). The initial stiffness was also lower but not significantly so (Figs. 5 and 6).

Repletion with 24,25(OH)2D3 was unable to restore the plasma calcium and inorganic phosphate to normal (Table 1) and the callus obtained had low resistance to torsional stresses (Figs. 3-6, Table 2). Repletion with a combination of 24,25(OH)2D3 and 1,25(OH)2D3 restored the plasma calcium and inorganic phosphate and increased the 'strength' of the callus even more rapidly than repletion with cholecalciferol alone (Figs. 3 and 5). Thus at 9 days after fracture the callus obtained from the chicks replete with 24,25(OH)2D3 plus 1,25(OH)2D3 failed at significantly higher torque and had a higher initial stiffness than the callus obtained from the vitamin D3-replete chicks (Figs. 3 and 5). For all the four strength factors tested, the callus of 24,25(OH)2D3 plus 1,25(OH)2D3 replete chicks was superior to that after giving 24,25(OH)2D3 or 1,25(OH)2D3 alone. It seems that 24,25(OH)2D3 and 1,25(OH)2D3 have a complementary, or even an additive, effect.

Since the strength of the callus increased in all the groups between days 9 and 14, it is of course possible that with prolonged administration of the various metabolites the callus obtained from the different groups would eventually achieve the same mechanical strength. However, the length of time needed to achieve this result is shorter when both 24,25(OH)2D3 and 1,25(OH)2D3 are given simultaneously.

The daily doses of vitamin D and its metabolites used in this study were based on previous experiments. When only 0.3 μg of 24,25(OH)2D3 was utilized, the chicks grew as well as those given 0.5 μg of vitamin D3 but were unable to maintain normal plasma calcium and inorganic phosphate levels, and the bones formed were abnormal [3, 15]. Administration of 0.15 μg of 1α(OH)D3 to rachitic chicks restored plasma calcium and inorganic phosphate levels to normal, but the bones formed were still abnormal [5]. The doses which restored the plasma calcium and inorganic levels to normal and resulted in normal bone formation were either (a) combination of 0.3 μg of 24,25-(OH)2D3 and 0.15 μg of 1α(OH)D3 or (b) 0.5 μg of cholecalciferol [3, 5].

Noff et al. [15] have shown that when 24,25-(OH)2D3 and 1α(OH)D3 are administered simultaneously to chicks there is no formation of 1,24,25-trihydroxycholecalciferol [1,24,25(OH)3D3]. Therefore the additive effect of 24,25(OH)2D3 is a direct one and not due to its conversion into 1,24,25(OH)3D3 in the body.

From the results obtained one can tentatively classify or arrange vitamin D and its metabolites according to their ability to increase the mechanical strength of the callus in vitamin D-depleted chicks in the following decreasing order: (1), 24,25-(OH)2D3 plus 1,25(OH)2D3; (2), cholecalciferol; (3), 1,25(OH)2D3; (4), 24,25(OH)2D3.

In addition to improvement of the mechanical strength, administration of the combination of 24,25(OH)2D3 and 1,25(OH)2D3 significantly increased the chicks' body weight and the bone ash content (Table 1). Therefore calcium retention in bone is increased. Other studies using administration of 1α(OH)D3 or 1,25(OH)2D3 to normal rats showed an increase in the rate of fracture repair [11, 16]. However, these studies were carried out on vitamin D-fed rats, in which administration of 1α(OH)D3 or of 1,25(OH)2D3 would induce endogenous formation of 24,25(OH)2D3. Thus a similar effect to that observed upon administration of the combination of 24,25(OH)2D3 and 1,25(OH)2D3 to vitamin D-deficient animals was obtained.

Fracture of a long bone in a growing animal and in man always results in acceleration of growth of the fractured bone [17, 18], owing to stimulation of the growth plates. The same was found in chicks given vitamin D continuously and in vitamin D- and 24,25(OH)2D3- plus 1,25(OH)2D3-replete chicks, and generally the fractured tibia was longer than the unfractured one. In contrast, the fractured tibiae of the vitamin D-deficient 24,25(OH)2D3-replete or 1,25(OH)2D3-replete chicks were shorter. Thus the growth plates of a fractured bone cannot react by increased proliferation of cells and new bone formation without the presence of both 24,25(OH)2D3 and 1,25(OH)2D3, and this is in agreement with a previously published study [3].

The results obtained in this study emphasize the importance of 24,25(OH)2D3 in addition to...
that of 1,25(OH)$_2$D$_3$ in the process of bone formation and provides further evidence that metabolites of cholecalciferol are directly involved in this process.

In a preliminary clinical investigation of the cause of non-union, the levels of vitamin D metabolites as well as that of other hormones known to affect bone formation (thyroid, parathyroid, adrenal and growth hormones at rest and under stress) were measured in eight patients. In three, 24,25(OH)$_2$D$_3$ was totally absent in spite of normal levels of all other hormones and vitamin D metabolites, as well as normal levels of calcium and inorganic phosphates. This finding is difficult to explain since in normal subjects the plasma levels of 24,25(OH)$_2$D$_3$ are 100-fold greater than those of 1,25(OH)$_2$D$_3$ and the clearance of 24,25(OH)$_2$D$_3$ in man is slower [19]. Administration of 4 μg of 24,25(OH)$_2$D$_3$/day for 6 weeks led to union of bone fractures. This, of course, cannot be considered as conclusive yet, since other therapeutic measures were also taken (plating or bone grafting). Therefore it may be that estimation of vitamin D metabolites in patients with non-union and adequate repletion, when necessary, would be worthwhile. Administration of 24,25(OH)$_2$D$_3$ to normal subjects increases calcium retention [20]. Its administration to patients with non-union and lacking 24,25(OH)$_2$D$_3$ may increase the retention of minerals in the callus and accelerate fracture repair.

References