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The action of various vitamin D_3 metabolites on calcium and phosphorus metabolism in chick embryo calvariae

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Summary. Chick embryos from vitamin D-deficient hens given physiological doses of 1,25-dihydroxyvitamin D_3 or 24,25-dihydroxyvitamin D_3 or both become severely hypocalcemic, hyperphosphatemic and fail to hatch as compared to those derived from hens given 25-hydroxyvitamin D_3 or 24,25-difluoro-25-hydroxyvitamin D_3. Calvariae from the former contain less mineral and on incubation in vitro produce significantly lower calcium and higher phosphate concentration in the medium than do the calvariae derived from the embryos of hens supported on 25-hydroxyvitamin D_3 or 24,24-difluoro-25-hydroxyvitamin D_3.
Key words. Embryonic development; bone; vitamin D.

Embryos from hens receiving physiological doses of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and/or 24,25-$(OH)_2D_3$ as their sole source of vitamin D do not mobilize shell calcium, consequently they do not form a fully mineralized skeleton and become severely hypocalcemic[1]. It is now quite clear that 1,25-$(OH)_2D_3$ normally induces the active transport of calcium through the chorioallantoic membrane and that this metabolite is not available to the embryos during the latter stages of embryonic life unless the hen has received one of the metabolic precursors of 1,25-$(OH)_2D_3$, namely vitamin D_3 or 25-OH-D_3[2,3]. Under normal circumstances, vitamin D_3 and 25-OH-D_3 are provided to the embryo from the yolk and the embryo has the enzymatic machinery necessary to produce 1,25-$(OH)_2D_3$[4].

Of considerable interest is the possible role of 1,25-$(OH)_2D_3$ in skeletal metabolism. Although one report has claimed that 1,25-$(OH)_2D_3$ directly stimulates bone formation in embryonic chick femurs in organ culture[5], studies in rats indicate that no vitamin D metabolite is directly required for normal skeletal growth and mineralization[6,7]. 1,25-$(OH)_2D_3$ injections into chick embryos produce hypercalcemia[8], even in the absence of the shell[9]. Moreover, 1,25-$(OH)_2D_3$ stimulates the release of ^{45}Ca from prelabeled embryonic chick long bones in culture[10]. These last two findings indicate that, as is the case in hatched chicks and other species, 1,25-$(OH)_2D_3$ is capable of stimulating bone mineral mobilization in embryonic chicks.

The experiment described in this report further examines the role of 1,25-$(OH)_2D_3$ in skeletal homeostasis in the chick embryo.

Materials and methods. Chick embryos were obtained from hens treated with either 25-OH-D_3, 24,24-F_2-25-OH-D_3, 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$ or a combination of both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ as their sole source of vitamin D. Extensive experimental details are provided in our two previous publications[3,4] and the doses of vitamin D_3 metabolites used are given in the table. Daily egg production was less than 5% in hens receiving no form of vitamin D and greater than 60% in hens receiving vitamin D_3 metabolites. Hatchability of fertile embryos from hens treated with 25-OH-D_3 or 24,24-F_2-25-OH-D_3 was greater than 90%, whereas fewer than 10% of the fertile embryos from hens treated with 1,25-$(OH)_2D_3$ and/or 24,25-$(OH)_2D_3$ hatched.

At 20 days of incubation, embryonic plasma was obtained and analyzed for calcium and inorganic phosphorus content[1]. Additional embryos were sacrificed at 20 days of incubation and their calvariae removed and incubated in 1.2 ml of medium in a shaker bath under an atmosphere of 95% O_2, 5% CO_2. The medium contained the following components: HEPES 30 mM, glucose 11.1 mM, NaCl 80 mM, KCl 5 mM, $MgSO_4$, 0.5 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 0.96 mM, Na_2HPO_4 2.24 mM and a mixture of the 20 major natural amino acids except asparagine at a total concentration of 29.4 mM; in addition, the medium contained 50 mg/l ascorbic acid, penicillin, streptomycin, the modified Eagles' Medium concentrations of vitamins, 5% horse serum and sufficient NaOH to adjust the pH to 7.4.

After 6 h of incubation the calvariae were removed, lightly blotted, dried overnight at 100 °C, weighed and then extracted with 2 ml of 2N nitric acid. This extract was diluted with water and analyzed for calcium content by atomic absorption spectroscopy as previously described[1]. The medium concentrations of calcium and inorganic phosphate were determined by atomic absorption spectroscopy and the colorimetric assay of LeBel et al.[11], respectively. Since the changes in medium calcium concentration during the course of the incubation resulted in a net uptake of calcium into the calvariae that was always less than 2% of the initial calvarial calcium content, this uptake of calcium was ignored in presenting the data on calvarial calcium content.

Results. The results are shown in the table. The plasma calcium and phosphorus data have been presented and discussed previously[1] and are included here for comparison to the medium calcium and phosphate data. The data for the five groups of

Chick embryo data

Vitamin D_3 compound administered	Dose (μg/day)	Embryos hatch	Embryo plasma calcium (mg/100 ml)	Embryo plasma phosphorus (mg/100 ml)	Calvarial dry weight (mg)	Calvarial calcium (% dry wt)	Medium calcium (mM)	Medium phosphate (mM)
25-OH-D_3	2.0	Yes	10.7 ± 0.1	4.8 ± 0.2	10.8 ± 0.9	12.7 ± 0.2	0.82 ± 0.02	1.88 ± 0.05
24,24-F_2-25-OH-D_3	2.0	Yes	10.6 ± 0.2	4.3 ± 0.3	11.1 ± 0.9	12.7 ± 0.2	0.83 ± 0.02	1.96 ± 0.07
1,25-$(OH)_2D_3$	0.4	No	4.2 ± 0.4	21.4 ± 2.9	6.8 ± 0.9	8.4 ± 0.6	0.68 ± 0.01	2.35 ± 0.10
24,25-$(OH)_2D_3$	2.0	No	4.7 ± 0.4	15.8 ± 1.5	8.9 ± 0.6	10.3 ± 0.4	0.68 ± 0.02	2.61 ± 0.06
1,25-$(OH)_2D_3$	0.4	No	6.5 ± 0.9	12.8 ± 3.0	6.7 ± 0.7	8.9 ± 0.4	0.66 ± 0.03	2.61 ± 0.10
plus 24,25-$(OH)_2D_3$	2.0							

All data are means ± SEM for 6–8 plasma determinations or six bone determinations. Vitamin D_3 metabolites were administered to the hens daily and the embryos were examined at 20 days of incubation.

embryos consistently fall into two distinct patterns. Treatment of hens with either 25-OH-D_3 or 24,24-F_2-25-OH-D_3 produced embryos that hatched and these embryos had normal levels of both plasma calcium and phosphorus and well mineralized calvariae. Incubation of calvariae from these embryos resulted in medium concentrations of calcium slightly greater than 0.8 mM and medium phosphate concentrations less than 2.0 mM. In contrast, embryos from hens treated with 1,25-$(OH)_2D_3$ and/or 24,25-$(OH)_2D_3$ did not hatch and these embryos were hypocalcemic, hyperphosphatemic and had poorly mineralized calvariae. Calvariae from these embryos supported medium calcium and phosphate concentrations below 0.7 mM and greater than 2.0 mM, respectively.

Discussion. The data on calvarial mineralization are in excellent agreement with the data previously reported[1] on embryo total body calcium content and mineralization of the tibia. We believe[1] that the reason for the poorly mineralized skeletons in the embryos from hens receiving only 1,25-$(OH)_2D_3$ and/or 24,25-$(OH)_2D_3$ as their sole source of vitamin D is that these vitamin D_3 metabolites are not available to the embryo during the latter stages of incubation and consequently these embryos are effectively vitamin D deficient. As a result of a lack of 1,25-$(OH)_2D_3$, these embryos cannot mobilize calcium from the shell and they become hypocalcemic and unable to form a fully mineralized skeleton. Under this hypothesis, embryos from hens receiving 24,24-F_2-25-OH-D_3 can form 24,24-F_2-1,25-$(OH)_2D_3$ and this last metabolite is an effective analogue of 1,25-$(OH)_2D_3$. 24,24-F_2-25-OH-D_3 cannot be hydroxylated to form 24,25-$(OH)_2D_3$[12]. Additional studies with the receptor for 1,25-$(OH)_2D_3$ have demonstrated that the fluoro groups do not act as a hydroxyl substitute and in fact is less like hydroxyl than hydrogen[13–15]. Further, injections of 1,25-$(OH)_2D_3$ into the eggs prior to incubation restores their ability to mobilize calcium from the shell[16]. Thus, it seems very clear that 24,25-$(OH)_2D_3$ is not required for embryonic development in the chick.

The major focus of this report is the effect of various vitamin D_3 metabolites on the ability of embryonic chick calvariae to support normal levels of calcium and phosphate in the medium during in vitro incubations. The calvariae obtained from embryos that do not hatch support lower levels of medium calcium but higher levels of medium phosphate than calvariae from embryos that hatch. This phenomenon occurs despite the large amount of mineral still present in the defective calvariae at the end of the in vitro incubation period, indicating a cellular failure caused by the vitamin D deficiency. These medium concentrations reflect the plasma levels of calcium and phosphorus in the embryos; that is, hypocalcemia is associated with low medium calcium concentrations whereas hyperphosphatemia is associated with high medium phosphate concentrations. Thus, the calvariae produce changes in medium calcium and phosphate levels that are in the same direction as those found in the abnormal embryonic plasma concentrations of these electrolytes.

These results indicate that in vitro incubations of chick embryo calvariae can be employed to examine skeletal changes produced by various vitamin D_3 compounds. Of particular interest is the possibility of using calvaria from embryos obtained from hens receiving 1,25-$(OH)_2D_3$ as their sole form of vitamin D. These embryos could be used effectively as a source of vitamin D-deficient bone for biochemical examination of the skeletal actions of 1,25-$(OH)_2D_3$ treatment in vitro.

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