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Vitamin D₃ and Its Metabolites Have No Role in Calcium and Phosphorus Metabolism in *Tilapia mossambica*

Devara SUNITA RAO and Namala RAGHURAMULU*

Department of Endocrinology and Metabolism,
National Institute of Nutrition, Jamai Osmania P.O.,
Hyderabad-500 007, A.P., India

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Summary The physiological function of vitamin D in fishes still remains uncertain. Earlier we observed no relationship between vitamin D₃ content of several freshwater fishes and their calcemic/phosphatemic status and bone mineral content. In the present study the effects of vitamin D₃ and its metabolites, 25-hydroxy vitamin D₃ (25-OH-D₃) and 1,25-dihydroxy vitamin D₃ [1,25-(OH)₂D₃], administration on serum calcium-phosphorus levels, intestinal calcium absorption, whole-body calcium-phosphorus uptake, and gill calcium binding protein (CaBP) activity in the freshwater fish, *Tilapia mossambica* (Tilapia) was examined. It was observed that vitamin D₃ and its metabolites could alter neither serum calcium-phosphorus levels nor intestinal calcium absorption and gill CaBP activity in fish at various doses. Further, the whole-body uptake of labelled calcium and phosphorus was also unaffected by vitamin D₃/1,25-(OH)₂D₃ at different levels and/or at various lengths of time. Thus these studies indicate that unlike in terrestrial vertebrates, vitamin D₃ or its metabolites are not needed for calcium-phosphorus homeostasis in fish.

Key Words fish, Tilapia, vitamin D₃, 1,25-(OH)₂D₃, 25-OH-D₃

In terrestrial animals, it is well established that vitamin D₃, which is produced by the photochemical process in skin (1-4) via its active hormonal form, 1,25 dihydroxy vitamin D₃ [1,25-(OH)₂D₃] (formed by two sequential hydroxylations of vitamin D₃, first in liver at C-25 position and second in kidney at C-1 position), along with parathyroid hormone (PTH), plays an important role in calcium-phosphorus homeostasis (5, 6), and vitamin D-dependent calcium-binding protein (CaBP) is important for mineral homeostasis. However, the role of vitamin D in fish is still confusing (7, 8). This may be because of their aquatic habitat, in which a constant supply of calcium and phosphorus is ensured by the environment.

Our earlier studies have shown that several freshwater fishes maintained vitamin

*To whom correspondence should be addressed. E-mail: icmrnin@ren.nic.in

D-dependent serum and bone parameters in the same range as in higher animals, in spite of the vast differences in their vitamin D contents (9). Also, vitamin D deficiency/hypervitaminosis D did not affect blood and bone mineral stores of fish (10, 11). All these findings indicate that the plasma calcium levels are closely regulated in fish. This now raises a question: Living in a calcium-phosphorus rich aquatic environment, do fish need vitamin D and its metabolites for mineral homeostasis?

We investigated the role of vitamin D and its metabolites on the levels of serum calcium-phosphorus, gill CaBP, and whole-body uptake of these minerals in the freshwater fish *Tilapia mossambica* (Tilapia) with a view to understanding the overall functions of vitamin D in calcium-phosphorus regulation in fish.

MATERIALS AND METHODS

Chemicals. ^{45}Ca (Spec. act. 105 mCi/g) and ^{32}P (carrier free) were purchased from Bhabha Atomic Research Centre (BARC), Bombay. They were supplied in solution form as calcium chloride and orthophosphoric acid, respectively. Dowex chelating resin (50–100 mesh) was obtained from Sigma Chemical Company, St. Louis, MO, USA. The details of all the other chemicals used was as described earlier (9).

Fish samples. Tilapia were purchased from the Department of Fisheries, Tank Bund, Hyderabad, Andhra Pradesh, India.

Effect of vitamin D and its metabolites on serum calcium-phosphorus levels and intestinal calcium absorption. Specimens of Tilapia (200 g) were maintained in tap water in plastic pools for a week and were fed vitamin D-deficient food containing all the nutrients except vitamin D_3 , which was prepared in the laboratory.

They were then divided into 9 groups (A–I) of 4 fish each and kept in identical glass aquariums. Air was slowly bubbled through the water to create turbulence. Fish of all the groups were given intraperitoneal injections of vitamin D_3 and its metabolites were dissolved in propylene glycol at the doses indicated below:

Groups A, B, C: 100 μL propylene glycol—(control); groups D and E: 240 ng and 2,400 ng vitamin D_3 /100 g body wt.; groups F and G: 100 ng and 1,000 ng 25-OH- D_3 /100 g body wt; and groups H and I: 130 ng and 1,300 ng of 1,25-(OH) $_2\text{D}_3$ /100 g body wt.

Fish that received vitamin D_3 were sacrificed 3 d after the administration. The fish injected with 25-OH- D_3 and 1,25-(OH) $_2\text{D}_3$ were sacrificed 2 d and 1 d after the treatments, respectively. The control fish were killed after 1 (group A), 2 (group B), and 3 (group C) d. Blood was drawn from the caudal veins of the fish and used to determine calcium and phosphorus, as described earlier (9). The in vitro active transport of calcium across the intestine was measured by the everted gut sac method (12), which is briefly described below.

Intestinal calcium absorption. The method used is essentially the one described by Larsson et al (32). The fish were sacrificed by decapitation, and the intestine distal to the pyloric valve was dissected out. It was immediately flushed with normal

saline, everted, and trimmed to a length of 5.5 cm. About 0.5 mL of the incubation buffer (pH 7.4) containing sodium chloride (125 mM), tris base (30 mM), calcium chloride (0.25 mM), fructose (10 mM), and ^{45}Ca (10,000 cpm/50 μL buffer) was injected into the segment and placed in 25 mL Erlenmeyer flasks containing 10 mL of the same buffer. Oxygen was continuously bubbled throughout the incubation period of 90 min at 37°C. After the incubation period, the sacs were taken out and the contents were drained into a clean test tube. An aliquot (50 μL) each, from the interior (serosal) and exterior (mucosal) of the sac was taken for radioactive counting in 10 mL of Bray's scintillation fluid [5 g of 2,5 diphenyloxazole (PPO), 0.5 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), 1.2 mg% EDTA and 120 g of naphthalene dissolved in 1 L of dioxane]. The results were expressed as the ratio of the counts in serosal medium "S" (inside the sac) to the counts of the tracer in the mucosal medium "M" (in the conical flask), i.e., S/M.

Whole-body uptake of calcium and phosphorus. This was studied by using ^{45}Ca and ^{32}P in water.

Dilution of the label. A sufficient quantity of either ^{45}Ca or ^{32}P was added to water to yield a count rate of 250,000 cpm/mL for ^{45}Ca and 20,000 cpm/mL for ^{32}P and allowed to equilibrate for another 2 h.

Acclimatized Tilapia (10–15 g) were distributed into several aquariums, each accommodating six fish, and given intraperitoneal injections of vitamin D_3 or $1,25\text{-(OH)}_2\text{D}_3$ dissolved in propylene glycol.

Vitamin D_3 was injected at various doses ranging from 6.25 pmol to 625.0 pmol (6.25, 31.25, 62.5, 125.0, 312.5, and 625.0 pmol), 2nd $1,25\text{-(OH)}_2\text{D}_3$ doses ranged from 3.15 pmol to 315.0 pmol/10 g body wt. of the fishes (3.15, 15.75, 31.5, 63.0, 157.5, and 315.0 pmol).

Fishes injected with vitamin D_3 were exposed to water containing ^{45}Ca or ^{32}P for 12 h (short term) or 4 d (long term). The $1,25\text{-(OH)}_2\text{D}_3$ -injected fishes were exposed to water containing $^{45}\text{Ca}/^{32}\text{P}$ for 1 d. The fish were then processed as follows.

Isotope uptake. After the experimental periods, the fish were removed from the labeled water, placed in a 1-L beaker, and allowed to swim for 5 min in running water to remove any adhering radioactivity. They were then killed and dried in an oven at 100°C for 72 h, ashed at 600°C, and the ash was dissolved in 6 N HCl and made up to 10 mL with water. Radioactivity was measured in a liquid scintillation counter by taking 100 μL aliquots in 10 mL of Bray's scintillation fluid. After appropriate correction for decay, the uptake was expressed as log mean dpm/g fish.

The whole-body calcium and phosphorus content was measured as described earlier by using appropriate dilutions.

Calcium binding protein (CaBP) in the fish tissues. The CaBP activity was quantitated by the method described by Wasserman and Taylor (13) with a few modifications and is briefly given below.

Preparation of CaBP rich fraction. Tilapia fasted overnight [approximately 200 g, $n=5$] were killed by a blow on the head, and the intestine (the first 10 cm), gills, liver, and kidney were dissected out immediately and processed as follows.

The intestine was flushed with cold saline. It was then cut open, the mucosa was scrapped, and a 20% homogenate was prepared in Tris-HCl buffer [containing Tris (13.7 mM), NaCl (120 mM), KCl (4.7 mM), and glucose (0.0985 mM); pH 7.4]. The entire gill arches were excised, and gill filaments were separated from the skeleton. The liver, kidney, and gill homogenates were prepared as described above.

The tissue homogenates were centrifuged at $38,000 \times g$ in a refrigerated centrifuge (Hitachi-Model No. 220S) for 30 min at 4°C . The supernatant was heated at 60°C for 10 min to denature the protein, which nonspecifically binds to calcium, and recentrifuged at $38,000 \times g$ for 20 min to yield a partially purified CaBP rich preparation. The protein content of this preparation was estimated by the Lowry method (14).

Measurement of CaBP activity. An appropriate aliquot of the homogenate containing 4 mg protein was taken, to which 0.1 mL of ^{45}Ca (50,000 cpm) was added, and final volume was adjusted to 1.2 mL with Tris-HCl buffer. After 20 min incubation at room temperature, 1 mL was added to tubes containing 50 mg chelex resin suspended in 0.2 mL buffer, and quickly centrifuged at 3,500 rpm. The radioactivity was monitored in 0.2 mL of the supernatant, and CaBP activity is expressed as nmol ^{45}Ca bound/mg protein.

Effect of $1,25-(\text{OH})_2\text{D}_3$ on CaBP activity in the gills of Tilapia. Tilapia (approximately 200 g) were divided into two groups of four each. One group was given a dose of $1,25-(\text{OH})_2\text{D}_3$ (1,300 ng/100 g fish) in 0.1 mL propylene glycol intraperitoneally, and the other group was given 0.1 mL of propylene glycol (controls). The fish were left in aerated tanks for 1 d. After the experimental period, they were killed and the gill arches were excised for the measurement of CaBP activity.

Statistical analysis. Data are expressed as the $M \pm \text{SE}$. The differences between experimental and control values were tested for statistical significance by using Student's *t*-test, one- and two-way analysis of variance (ANOVA) (15). One-way ANOVA-log transformations were used for the whole-body uptake studies (15).

The experiments were approved by the ethical committee on the use of animals for research.

RESULTS

Serum calcium and phosphorus

The effect of vitamin D_3 and its metabolites on the serum calcium-phosphorus levels and intestinal calcium transport are given in Table 1. It can be observed from the table that there were no significant differences in serum calcium and phosphorus levels at any dose of vitamin D_3 , when compared with the controls. Similarly, there was no difference in serum calcium and phosphorus levels of fish administered 25-OH-D_3 and $1,25-(\text{OH})_2\text{D}_3$. Moreover, no change was observed in serum calcium-phosphorus levels between the experimental groups (A-I) (two-way

Table 1. Effect of intraperitoneally administered vitamin D₃, 25-OH-D₃, and 1,25-(OH)₂D₃ on serum calcium-phosphorus levels and intestinal calcium absorption in Tilapia.

	ng/100 g body wt.	Serum		Intestinal calcium absorption (S/M)
		Calcium (mg/dL)	Phosphorus (mg/dL)	
Vitamin D ₃	Control	9.98 ± 0.2	19.0 ± 0.2	0.87 ± 0.01
	240	10.0 ± 0.2	19.3 ± 0.4	0.90 ± 0.01
	2,400	9.99 ± 0.2	19.0 ± 0.4	0.90 ± 0.02
25-OH-D ₃	Control	10.04 ± 0.2	19.3 ± 0.2	0.94 ± 0.02
	100	9.98 ± 0.2	19.1 ± 0.2	0.90 ± 0.01
	1,000	10.02 ± 0.3	19.3 ± 0.2	0.99 ± 0.03
1,25-(OH) ₂ D ₃	Control	9.99 ± 0.3	19.0 ± 0.4	0.92 ± 0.01
	130	9.98 ± 0.2	19.3 ± 0.2	0.94 ± 0.45
	1,300	10.00 ± 0.2	19.4 ± 0.3	0.95 ± 0.04

Results are M ± SE of four fishes. No significant differences were observed between the treatments (two-way ANOVA).

ANOVA).

Intestinal calcium absorption

As seen in Table 1, the intestinal calcium uptake remained unaffected in the fishes in response to any level of vitamin D₃, 25-OH-D₃, or 1,25-(OH)₂D₃ administration (two-way ANOVA).

Effect of vitamin D₃ and 1,25-(OH)₂D₃ on whole body ⁴⁵Ca and ³²P uptake

There was no significant difference in the whole-body ⁴⁵Ca/³²P uptake within a short period of 12 h (Fig. 1) or after 4 d (Fig. 2) at any level of vitamin D₃ administration, compared with the control fish. Moreover, the whole-body uptake of ⁴⁵Ca/³²P remained unchanged in response to doses of 1,25-(OH)₂D₃ (Fig. 3) (one-way ANOVA-log transformations).

Furthermore, the whole-body calcium/phosphorus concentrations of fishes were found not to vary significantly in different experimental groups given vitamin D₃ either for a short-term duration [(10.0 ± 0.4 to 10.8 ± 0.6 mg calcium/g fish) and (8.2 ± 0.2 to 8.9 ± 0.3 mg phosphorus/g fish)], or longer [(10.0 ± 0.2 to 10.5 ± 0.4 mg calcium/g fish) and (8.0 ± 0.8 to 8.7 ± 0.3 mg phosphorus/g fish)]. Similarly, the whole-body calcium/phosphorus levels of Tilapia injected with different doses of 1,25-(OH)₂D₃ remained unchanged in all groups [(8.3 ± 0.5 to 8.6 ± 0.7 mg calcium/g fish) and (8.0 ± 0.2 to 8.5 ± 0.6 mg phosphorus/g fish)] (one-way ANOVA).

Fig. 1. Whole-body uptake of ^{45}Ca (----) and ^{32}P (—) by Tilapia, 12 h after intraperitoneal administration of vitamin D_3 . Each value represents $M \pm \text{SE}$ of six fishes. The x axis is not to scale. No significant differences were observed between the various experimental groups when compared with the control value (one-way ANOVA log transformations).

Fig. 2. Whole-body uptake of ^{45}Ca (----) and ^{32}P (—) by Tilapia, 4 d after intraperitoneal administration of vitamin D_3 . Each value represents $M \pm \text{SE}$ of six fishes. The x axis is not to scale. No significant differences were observed between the various experimental groups when compared with the control value (one-way ANOVA log transformations).

Fig. 3. Whole-body uptake of ^{45}Ca (----) and ^{32}P (—) by Tilapia, 1 d after intraperitoneal administration of $1,25\text{-(OH)}_2\text{D}_3$. Each value represents $M \pm \text{SE}$ of six fishes. The x axis is not to scale. No significant differences were observed between the various experimental groups when compared the control value (one-way ANOVA log transformations).

Fig. 4. CaBP in the tissues of Tilapia. Values are $M \pm \text{SE}$ of five fishes. The variation in superscripts between mean values indicates the significance of difference ($p < 0.05$; one-way ANOVA).

Calcium binding protein in the tissues of Tilapia

Figure 4 shows the data for CaBP activity in the tissues of fish. As figure shows, CaBP was observed in all tissues studied, but the levels varied. Gills contained the highest activity, which was significantly different from the other tissues ($p < 0.05$).

Fig. 5. Effect of intraperitoneally administered 1,25-(OH)₂D₃ (13 µg/kg body wt. of fish) on gill CaBP activity in Tilapia. Each bar represents $M \pm SE$ of four fish. No significant differences were observed between the two groups (Student's *t*-test).

The CaBP activity was 0.45 nmol ⁴⁵Ca bound/mg protein in the intestine and did not significantly differ from that of kidney, but it was significantly different when compared with liver ($p < 0.05$), which contained the lowest CaBP activity among the tissues examined. The CaBP activity differed significantly between the kidney and liver ($p < 0.05$) (one-way ANOVA).

Effect of 1,25-(OH)₂D₃ on gill CaBP activity

It was observed that treatment with 1,25-(OH)₂D₃ (13 µg/kg fish) did not alter CaBP in the gills when compared with control fish that were injected with propylene glycol (Student's *t*-test). This data is shown in Fig. 5.

DISCUSSION

Vitamin D is known to occur abundantly in certain fishes (16–19), and this vitamin is mainly derived through dietary sources and not by photochemical synthesis (20–22). Several studies (23) showed the capability of fishes to convert this vitamin to the polar forms, 25-OH-D₃ and 1,25-(OH)₂D₃. But studies are limited and controversial with regard to the role of these vitamin D metabolites in calcium-phosphorus homeostasis in these aquatic vertebrates (7, 8, 24, 25).

We earlier observed no relationship between the calcemic/phosphatemic status and bone mineral content of several freshwater fishes and their vitamin D levels (9). This observation appears to dissociate vitamin D in fishes from its known functions in land animals, and it is further corroborated by the findings of the present study, which indicate that vitamin D₃ and its metabolites may not be needed for calcium-phosphorus homeostasis in fish. It is shown by the lack of any effect

of vitamin D₃/metabolites (given at various doses) on the various parameters studied: (i) maintenance of serum calcium-phosphorus levels; (ii) stimulating intestinal calcium absorption; (iii) inducing whole-body ⁴⁵Ca/³²P uptake and (iv) gill CaBP activity.

Recently, Sundell et al (8) have demonstrated that 1,25-(OH)₂D₃ (single injection of 10 µg/kg body weight) increased plasma concentrations of ionized calcium (Ca_i) in Atlantic cod, but the total plasma calcium and phosphorus concentrations were unaffected. Also, plasma calcium content was unaltered with other secosteroids [i.e., vitamin D₃, 25-OH-D₃, and 24,25-(OH)₂D₃]. But we observed that 1,25-(OH)₂D₃ given at a higher dose than that of Sundell et al (8) also had no effect on the total serum calcium-phosphorus levels in the freshwater teleost *Tilapia*. Though ionized calcium may be a good indicator of total calcium, we could not estimate ionized calcium in the present study to compare with the results of Sundell et al (8). Because gills are the organs in direct contact with the external environment and are known to be important in calcium absorption, it is understandable that vitamin D₃/metabolites may not affect the absorption of this mineral in the intestine. But it was surprising to find that even the gill CaBP activity appeared to be vitamin D-independent, in spite of CaBP being the highest in gills among the tissues studied. Taken together, these findings along with the observation of the whole-body uptake of calcium and phosphorus, not being influenced by vitamin D₃/1,25-(OH)₂D₃ in fish, are contrary to those of earlier studies with rat, in which intraperitoneal injection of 6.25 pmol of vitamin D₃ and 3.15 pmol 1,25-(OH)₂D₃ resulted in increased serum calcium and phosphorus levels (26). However, we found that even 100-fold higher doses of vitamin D and 1,25-(OH)₂D₃ did not induce calcium-phosphorus uptake in fish.

Our earlier studies in Rora (*Labeo rohita*) have shown that fish deficient in vitamin D (vitamin D-deficiency was judged by the absence of any detectable vitamin D in the liver of fish, grown in darkness and fed a diet devoid of vitamin D for six months) showed no significant differences in the vitamin D-related (bone and carcass mineral stores) and growth (carcass protein and lipid, hepatosomatic index, feed efficiency, and mortality rates) parameters when compared with the controls fed vitamin D₃, suggesting that this vitamin may not be an essential nutrient for fishes (27). This observation is contrary to similar studies in terrestrial vertebrates, where decreased calcification of bone and hypocalcemia is the classical manifestation of vitamin D deficiency (28–30).

Thus it is understandable that vitamin D₃ had no influence on any of the parameters examined in the present study, and this could not be because the fish already may have had adequate stores of the vitamin. This may not be the reason because the fish were fed a vitamin D-deficient diet for one week, which would have resulted in low vitamin D status, though it may not have resulted in total vitamin D deficiency. But surprisingly, even the active form of vitamin D₃, i.e., 1,25-(OH)₂D₃, could not alter any of the parameters studied, whereas in land vertebrates, even with adequate stores of vitamin D, 1,25-(OH)₂D₃ increases

serum calcium-phosphorus levels and intestinal absorption of these minerals (31). Therefore we suggest that contrary to the terrestrial vertebrates, the calcium-phosphorus homeostasis in fishes may not be vitamin D-dependent.

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