ZOOLOGICAL SCIENCE 14: 743–746 (1997)

Vitamin D Metabolites Affect Serum Calcium and Phosphate in Freshwater Catfish, *Heteropneustes fossilis*

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ABSTRACT—The effects of vitamin D_3, 24,25$(OH)_2$ vitamin D_3, 25(OH) vitamin D_3 and 1,25$(OH)_2$ vitamin D_3 were investigated on the serum calcium and phosphate levels of freshwater catfish, *Heteropneustes fossilis*. The fish were injected daily intraperitoneally with these secosteroids for 10 days. Blood samples were collected at day 1, 3, 5 and 10. Serum calcium and inorganic phosphate levels were elevated by all of the treatments except for 24,25$(OH)_2$ vitamin D_3.

INTRODUCTION

Bony fishes, particularly those inhabiting seawater, contain large hepatic stores of vitamin D (Copping, 1934; Urist, 1976; Takeuchi *et al.*, 1984). Vitamin D_3, which itself apparently lacks direct biological activity, produces a number of metabolites after sequential hydroxylations in liver and kidney (Norman *et al.*, 1982). Teleosts inhabiting freshwater and seawater are able to convert vitamin D_3 and 25(OH)D_3 to more polar metabolites (Hayes *et al.*, 1986; Takeuchi *et al.*, 1991). Moreover, fish contains circulating levels of vitamin D_3, 25(OH)D_3, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ (Hay and Watson, 1976; Nahm *et al.*, 1979; Avioli *et al.*, 1981; Takeuchi *et al.*, 1991; Sundell *et al.*, 1992; Rao and Raghuramulu, 1995). Furthermore, specific binding proteins for 1,25$(OH)_2D_3$ have been demonstrated in tissues from European eel and Atlantic cod (Marcocci *et al.*, 1982; Sundell *et al.*, 1992). These studies suggest a physiological role for vitamin D_3 system in fishes.

The effects of vitamin D_3 and its metabolites on calcium homeostasis have been studied in a few freshwater teleosts (*Amphipnous cuchia*; Srivastav, 1983: *Anguilla rostrata*; Fenwick *et al.*, 1984: *Clarias batrachus*; Swarup and Srivastav, 1982; Swarup *et al.*, 1984; Srivastav and Srivastav, 1988: *Cyprinus carpio*; Swarup *et al.*, 1991; Srivastav *et al.*, 1993: *Carrasius auratus*; Fenwick, 1984: *Heteropneustes fossilis*; Srivastav and Singh, 1992) and a few marine species (*Gadus morhua*; Sundell *et al.*, 1993: *Pagothenia bernachii*; Fenwick *et al.*, 1994). Nevertheless there is still considerable controversy regarding the physiological role of this vitamin and its metabolites in teleosts as many of the previous reports are contradictory. Administration of vitamin D or its metabolites has been reported to cause either (i) no significant change (Urist, 1962; MacIntyre *et al.*, 1976; Lopez *et al.*, 1977), (ii) increase (Srivastav, 1983; Fenwick, 1984; Swarup *et al.*, 1984, 1991; Fenwick *et al.*, 1984, 1994; Srivastav and Srivastav, 1988; Srivastav and Singh, 1992), or (iii) decrease (Sundell *et al.*, 1993) in the blood calcium content. Moreover, the effect of 24,25$(OH)_2D_3$ has been investigated only in *Sarotherodon mossambicus* (a freshwater species, Wendelaar Bonga *et al.*, 1983) and *Gadus morhua* (a marine species, Sundell *et al.*, 1993).

The present study was undertaken to investigate the effects of vitamin D and some of its major metabolites on serum calcium and phosphate of a freshwater catfish, *Heteropneustes fossilis*.

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MATERIALS AND METHODS

Freshwater catfish, *H. fossilis* of both sexes were procured and acclimated to laboratory conditions at 27 ± 2°C for one week prior to the experiment. The fish weighed between 45-64 g and were not fed following their capture. Blood samples from six fish was taken prior to the start of the experiment (zero hour). The remaining fish were randomly divided into five groups of 24 fish each. These groups received daily intraperitoneal injections of either vehicle (95% ethanol; group A), vitamin D_3 (5 μg; group B), 24,25$(OH)_2D_3$ (2 μg; group C), 25(OH)D_3 (1 μg; group D), or 1,25$(OH)_2D_3$ (0.5 μg; group E). The doses indicated are per 100 g body wt of fish/0.5 ml. The doses of various vitamin D metabolites used in the present study correspond more or less to the doses used in other teleosts by previous investigators (Wendelaar Bonga *et al.*, 1983; Sundell *et al.*, 1993).

Six fish from each group were anesthetized with MS222 and blood samples were collected 4 hr after the last injection (by a syringe from the caudal vessels) after 1, 3, 5 and 10 days of treatment. Sera were separated by centrifugation and total calcium and phosphate were measured according to Trinder (1960) and Fiske and Subbarow (1925) methods, respectively. Calcium from serum was precipitated as an insoluble orange red complex by an alkaline solution of naphtholhydroxamic acid. After centrifugation the precipitate was dissolved in alkaline disodium ethylenediamine tetraacetate, then treated with ferric nitrate and the resultant amber colour was measured colorimetrically. For phosphate, the serum was deproteinized by adding trichloroacetic acid. To the filtrate, ammonium molybdate was added followed by 1,2,4-aminonaphtholsulfonic acid. The resultant blue colour was measured colorimetrically.

Student's t test was used to determine statistical significance. In all cases, the experimental group was compared with the vehicle-injected group sampled at the same time. The data were also subjected to two-way ANOVA.

RESULTS

Serum calcium levels of fish treated with various vitamin D analogs are shown in Fig. 1. Both vitamin D_3 and $25(OH)D_3$ increased the serum calcium levels at day 3 and day 5. No changes were observed in calcium concentrations following $24,25(OH)_2D_3$ treatment. The serum calcium level of $1,25\text{-}(OH)_2D_3$ treated fish increased more rapidly and showed a significant increase on day 1 which progressively increased till day 5. All groups were normocalcemic by day 10.

Serum phosphate levels were unaffected through day 3 except for the $1,25(OH)_2D_3$ treated fish which were hyperphosphatemic. By day 5, all the treated groups were hyperphosphatemic with the exception of the $24,25(OH)_2D_3$ treated group (Fig. 2). Unlike the situation with calcium which return to normal values by day 10, the hyperphosphatemic effect, when stimulated, remained so up to day 10.

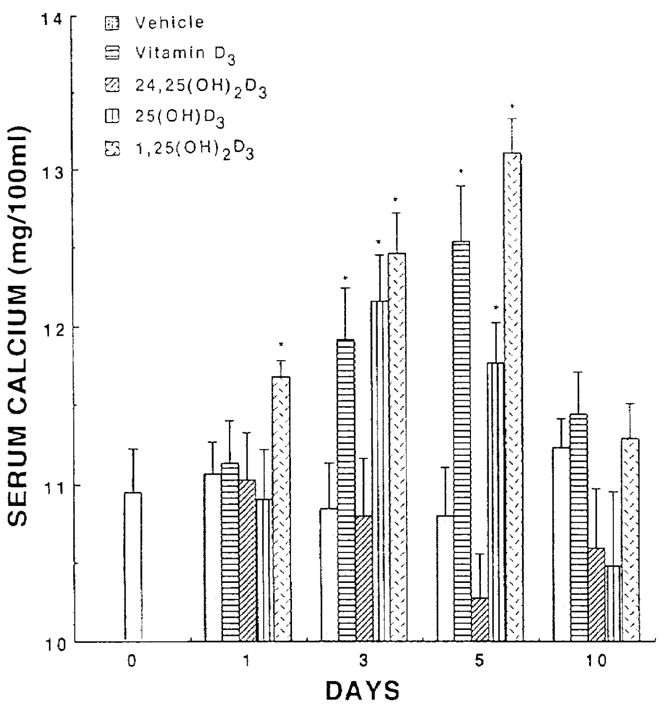

Fig. 1. Serum calcium levels of *H. fossilis* treated either with vehicle, vitamin D_3, $24,25(OH)_2D_3$, $25(OH)D_3$ or $1,25(OH)_2D_3$. Values are mean ± SE of six specimens. Asterisked values are significantly different ($P<0.05$) as compared to the vehicle-injected group.

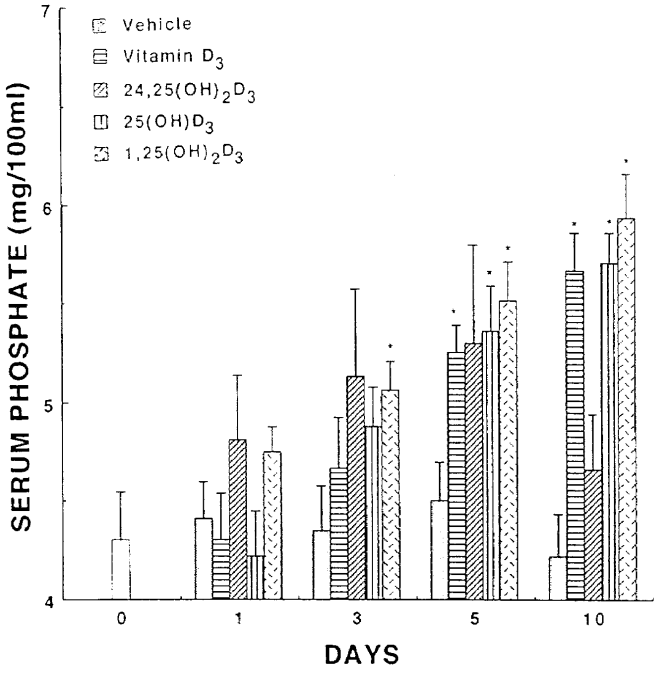

Fig. 2. Serum inorganic phosphate levels of *H. fossilis* treated either with vehicle, vitamin D_3, $24,25(OH)_2D_3$, $25(OH)D_3$ or $1,25(OH)_2D_3$. Values are mean ± SE of six specimens. Asterisked values are significantly different ($P<0.05$) as compared to the vehicle-injected group.

Comparing (two-way ANOVA) serum calcium and phosphate levels of *H. fossilis* treated with various vitamin D metabolites, it has been observed that these electrolytes differed significantly between the exposure period (for calcium $F=4.581$ and $P<0.01$; for phosphate $F=3.465$ and $P<0.04$), whereas between the various treatments used in this study, only phosphate levels differed significantly (for calcium $F=2.002$ and $P<0.16$, not significant; for phosphate $F=4.323$ and $P<0.02$).

DISCUSSION

The data shows that vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ affect calcium homeostasis in *H. fossilis*. These observations are in accord with the results of other investigations in which administration of vitamin D_3 and these metabolites elevated the serum/plasma calcium (total) concentrations in other fishes (Swarup and Srivastav, 1982; Srivastav, 1983; Fenwick, 1984; Swarup *et al.*, 1984, 1991; Srivastav and Srivastav, 1988; Srivastav and Singh, 1992; Fenwick *et al.*, 1984, 1994). Administration of $1,25(OH)_2D_3$ to marine fishes has been reported either to increase (*Gadus morhua*; Sundell *et al.*, 1993) or decrease (*Pagothenia bernachii*; Fenwick *et al.*, 1994) the ionized calcium concentration without altering the total plasma calcium levels. In contrast to the present study, daily injections (for seven days) of $25(OH)D_3$ to Atlantic cod lowered the total calcium levels (Sundell *et al.*, 1993). $25(OH)D_3$ treatment produced no significant effect on either ionized or total calcium concentration of *Pagothenia bernachii* (Fenwick *et al.*, 1994).

$24,25(OH)_2D_3$ injections to *H. fossilis* did not affect serum calcium levels and this agrees with the studies of Sundell *et* *al.* (1993) who have also noticed no change in calcium contents of 24,25$(OH)_2D_3$ treated Atlantic cod.

In fishes vitamin D_3 and 1,25$(OH)_2D_3$ increased calcium uptake (Chartier *et al.*, 1979; Flik *et al.*, 1982; Fenwick, 1984; Fenwick *et al.*, 1984). In Atlantic cod 25$(OH)D_3$ stimulated intestinal calcium absorption whereas vitamin D_3 and 1, 25$(OH)_2D_3$ did not affect the calcium influx across the intestinal mucosa (Sundell and Bjornsson, 1990). The observed hypercalcemia in *H. fossilis* may be explained by mobilization of calcium from internal stores and/or increased renal retention of calcium. Indeed, 1,25$(OH)_2D_3$ was shown to increase bone demineralization in teleosts (Lopez *et al.*, 1977; Wendelaar Bonga *et al.*, 1983). Moreover, an increased calcium uptake by the gills from the environment after treatment with these metabolites can not be ruled out.

The hyperphosphatemia evoked by the administration of vitamin D_3, 25$(OH)D_3$ and 1,25$(OH)_2D_3$ to *H. fossilis* is similar to that reported previously (MacIntyre *et al.*, 1976; Fenwick *et al.*, 1984; Swarup *et al.*, 1984, 1991; Srivastav and Singh, 1992). In contrast, Sundell *et al.* (1993) and Fenwick *et al.* (1994) have found no effect of these secosteroids on plasma phosphate content. It is of interest to note that in *H. fossilis* 24,25$(OH)_2D_3$ produced elevated phosphate levels although this increase was not statistically significant. The hyperphosphatemic response of vitamin D_3 and its metabolites in *H. fossilis* suggests that the nondietary phosphorus, possibly from the bone and/or from the soft tissues, can be mobilized. The increased renal retention of phosphate also can not be ruled out.

The different outcomes in the calcium and phosphate levels of *H. fossilis* at some time intervals in response to vitamin D_3 and its metabolites administration may be due to reported differences in the mechanism of actions of these metabolites—a slow genome-mediated and a rapid nongenome-mediated transcaltachic response (Larsson *et al.*, 1995).

In the present study serum calcium levels returned to normal at day 10 whereas phosphate levels were still increased. The recovery of serum calcium may be attributed to increased release of the hypocalcemic factor stanniocalcin from the corpuscles of Stannius after continuous hypercalcemic challenge induced by vitamin D_3 and its metabolites. Stanniocalcin has been reported to inhibit branchial Ca^{2+} influx (Lafeber *et al.*, 1988; Verbost and Fenwick, 1995). An increased activity of corpuscles of Stannius after vitamin D_3/ 1,25$(OH)_2D_3$ has been reported in a freshwater catfish (*Clarias batrachus*) (Srivastav *et al.*, 1985; Srivastav and Srivastav, 1988). The persisting increased serum phosphate levels at day 10 could be ascribed to the possible renal retention of phosphate by enhanced secretion of stanniocalcin which has been shown to stimulate the net renal phosphate reabsorption (Renfro *et al.*, 1996).

The present study concludes that vitamin D_3 and two of its prime metabolites, 25$(OH)D_3$ and 1,25$(OH)_2D_3$ can affect both calcium and phosphate metabolism in a freshwater teleost, *H. fossilis*. We also do not feel it unreasonable to speculate that vitamin D_3 and 25$(OH)D_3$ have to be converted to a more active form, probably 1,25$(OH)_2D_3$ as these secosteroids produced an effect only on day 3 whereas 1,25$(OH)_2D_3$ produced an effect in one day. The present results together with those of previous report (Sundell and Bjornsson, 1990) suggest that there exists different functional aspects in the actions of vitamin D_3 and its metabolites in freshwater teleosts (freshwater environment is hypotonic in relation to the blood—where vitamin D_3 analogs affect calcium homeostasis) and marine teleosts (sea water is hypertonic in relation to the blood — where vitamin D_3 and 1,25$(OH)_2D_3$ produced contradictory effects; Sundell *et al.*, 1993; Fenwick *et al.*, 1994).

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(Received April 23, 1997 / Accepted July 1, 1997)