1,25(OH)₂D₃ INDUCED ALTERATIONS IN PLASMA CALCIUM, INORGANIC PHOSPHATE, ULTIMOBRANCHIAL GLAND AND PARATHYROID GLAND OF THE GARDEN LIZARD, *CALOTES VERSICOLOR*

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Abstract. Garden lizards *Calotes versicolor* were procured and given daily intraperitoneal injections of 30 pmol of 1,25(OH)₂D₃/50 g body wt for 30 days. Lizards were sacrificed on the 1st, 3rd, 5th, 10th, 15th and 30th day of the experiment. The plasma calcium levels of *C. versicolor* remain unaffected after day 1 following 1,25(OH)₂D₃ treatment. After day 3 the levels increase significantly which progresses up to day 10. Thereafter, the plasma calcium level tends to decrease on day 15 and the levels become normal at day 30. In 1,25(OH)₂D₃ injected lizards the plasma inorganic phosphate levels remain unaltered up to day 3. After day 5, the value increases significantly. This increase progresses up to day 15. On day 30, the levels become almost normal. The ultimobranchial gland exhibits hyperactivity following 5 day 1,25(OH)₂D₃ treatment which is expressed by an increase in the nuclear volume and weak staining response of the cytoplasm of ultimobranchial cells. After day 10, the nuclear volume is further increased and some of the cells are exhausted. Following 15 days 1,25(OH)₂D₃ treatment the nuclear volume records an increase and many degenerating cells are discerned. After day 30, the nuclear volume is almost normal, most cells seem to be recovered and only a few degenerating cells are noticed. After day 10 and day 15 following 1,25(OH)₂D₃ treatment, the parathyroid glands of *C. versicolor* show reduced chromaticity of nuclei and a progressive decrease in the nuclear volume of parathyroidal cells. On day 30, the nuclear volume tends to become normal and a few degenerating cells are observed.

Key words: lizard, 1,25(OH)₂D₃, ultimobranchial gland, parathyroid gland

INTRODUCTION

Precise regulation of physiological levels of extracellular and intracellular ionized calcium is essential for vertebrate life. For this control several hormones have been implicated which are mainly parathyroid hormone (PTH), calcitonin (CT), stanniocalcin (STC), prolactin (PRL) and vitamin D-related steroid (GUERREIRO *et al.*, 2007; RIVERA and LOCK, 2008; TALMAGE and MOBLEY, 2008; SRIVASTAV *et al.*, 2008). In land vertebrates, three principal endocrine factors (PTH, CT and vitamin D-related steroid) act upon three major sites – the intestine, by the control of absorption; the kidney, by regulation of reabsorption from glomerular filtrate; and the bone, which serves as a storage site for calcium phosphate.

Reptiles are in many respects an interesting class of vertebrates as they represent the mainstream of tetrapod evolution (DACKE, 1979; SRIVASTAV *et al.*, 1995, 1998a). During water to land transition, probably the most important adaptation was the shelled amniotic egg, which eliminated the need for an aquatic habitat for egg laying, and the hazardous free living larval stage present in many amphibians. Moreover, double aortic heart of primitive amphibians was modified for life on land and two major groups evolved. One group developed an aorta on the right side of body and gave rise to modern reptiles, dinosaurs and birds whereas the other group developed aorta on the left side of the body and gave rise to synapsid reptiles which eventually produced mammals.

In reptiles, the parathyroid and ultimobranchial glands have been the subject of extensive research (CLARK 1965, 1971a, b, 1983, SEHE 1965, OGURO 1969, 1970, 1972, 1975, SENIOR 1974, CLARK and LAVERTY 1985, CLARK *et al.* 1986, KAGWADE and PADGAONKAR 1992, PADGAONKAR *et al.* 1992, YAMADA *et al.* 2001, SRIVASTAV *et al.* 1995, 1998 a, 2008), however, the effects of $1,25(\text{OH})_2\text{D}_3$ (biologically active metabolite of vitamin D) on these glands in reptiles have not been investigated. Hence, in this study we have made an attempt to investigate the effects of $1,25(\text{OH})_2\text{D}_3$ on the plasma calcium and inorganic phosphate levels as well as on the histological changes in the ultimobranchial and parathyroid glands of the Indian garden lizard, *Calotes versicolor*.

MATERIALS AND METHODS

Garden lizards *C. versicolor* (both sexes; body wt 28-35 g) were procured from commercial sources during April and acclimatized to the laboratory conditions for a week prior to use. Initial blood samples (from 10 specimens) were taken before the start of the experiment (zero hour). Then remaining animals were divided into two groups — A and B.

Lizards from both groups were given daily intraperitoneal injections of the following treatment for 30 days:

Group A: 0.1 ml/50 g body wt of vehicle (0.8% NaCl solution).

Group B: 30 pmol of $1,25(\text{OH})_2\text{D}_3$ /50 g body wt.

In all cases the injection volume was 0.1 ml/50 g body wt. $1,25(\text{OH})_2\text{D}_3$ was dissolved in 95% ethanol and diluted with 0.8% NaCl. The animals were not fed during the experiment.

Ten specimens from each group were anaesthetized with chloroform 4 h after the last injection on the 1st, 3rd, 5th, 10th, 15th and 30th day of the experiment and blood samples were collected. After collection of blood ultimobranchial and parathyroid glands were fixed.

Blood samples were taken by cardiac puncture at each experimental interval in heparinized tubes. Plasma was separated by centrifugation and analysed for calcium (Sigma kit) and inorganic phosphate (Sigma kit) levels.

After collection of blood, the ultimobranchial gland (the region anterior to the heart) and parathyroid gland (near the carotid bifurcation) from the lizards were removed. All these tissues were fixed in aqueous Bouin's solution. The material thus fixed was routinely dehydrated in graded series of alcohols, cleared in xylene and embedded in paraffin. Serial sections were cut at 6 µm and stained with hematoxylin/eosin (HE).

Nuclear (ultimobranchial and parathyroidal cells) indices (maximal length and maximal width) were determined with an ocular micrometer. Fifty nuclei were measured per animal. The nuclear volume was calculated as –

$$\text{Volume} = 4/3 \pi ab^2$$

where 'a' is the major semiaxis and 'b' is the minor semiaxis.

All data were presented as the mean ± S. E. of ten specimens and Student's t test was used to determine statistical significance. In all studies the experimental group was compared to its specific time control group.

RESULTS

The plasma calcium levels of *C. versicolor* remain unaffected after day 1 following 1,25(OH)₂D₃ treatment (Fig. 1). After day 3 the levels increase significantly which progresses up to day 10 (Fig. 1). Thereafter, the plasma calcium level tends to decrease on day 15 and the levels become normal at day 30 (Fig. 1).

In 1,25(OH)₂D₃ injected lizards the plasma inorganic phosphate levels remain unaltered up to day 3 (Fig. 2). After day 5, the value increases significantly. This increase progresses up to day 15. On day 30, the levels become almost normal (Fig. 2).

The ultimobranchial gland in control *C. versicolor* is present on the left side of the neck just anterior to the heart. Histologically the ultimobranchial gland contains follicles (one large follicle or few small follicles) and cell cords or cell clumps (Fig. 3). The follicles are lined by epithelium possessing simple cuboidal cells or pseudostratified columnar cells. The epithelium may also contain ciliated and goblet cells (Fig. 4). The lumen of the follicle may contain a variable amount of colloid-like material with desquamated cells and cell debris (Fig. 3). The cells, which form compact cell clumps are all alike. Their nuclei are ovoid having dense chromatin material (Fig. 5).

The ultimobranchial gland of 1,25(OH)₂D₃ injected lizards exhibits no change up to day 3. The gland depicts hyperactivity following 5 day 1,25(OH)₂D₃ treat-

Fig. 1. Plasma calcium levels of vehicle or 1,25(OH)₂D₃ treated lizards. Values are mean $\pm$ S.E. of 10 specimens. Asterisk indicates significant differences ($P < 0.05$) as compared with vehicle-injected specimens.

Fig. 2. Plasma phosphate levels of vehicle or 1,25(OH)₂D₃ treated lizards. Values are mean $\pm$ S.E. of 10 specimens. Asterisk indicates significant differences ($P < 0.05$) as compared with vehicle-injected specimens.

Fig. 3. Ultimobranchial gland of control *Calotes versicolor* exhibiting follicle and cell clumps. HE x 50.

Fig. 4. Ultimobranchial gland of control *Calotes versicolor* showing goblet cells in the follicular epithelium. HE x 400.

Fig. 5. Ultimobranchial gland of control lizard. HE x 200.

Fig. 6. Nuclear volume of ultimobranchial cells of vehicle or 1,25(OH)₂D₃ treated lizards. Asterisk indicates significant differences ($P < 0.05$) as compared with vehicle-injected specimens.

ment which is expressed by an increase in the nuclear volume (Fig. 6) and weak staining response of the cytoplasm of ultimobranchial cells (Fig. 7). After day 10, the nuclear volume is further increased (Fig. 6) and some of the cells are exhausted (Fig. 8). Following 15 days 1,25(OH)₂D₃ treatment the nuclear volume increases (Fig. 6) and many degenerating cells are discerned (Fig. 9). After day 30, the nuclear volume is almost normal, most cells seem to be recovered and only a few degenerating cells are noticed.

In control *C. versicolor* one pair of parathyroid glands occurs which is located near the bifurcation of the carotid arch in the cervical region. These glands are enclosed by connective tissue, spheroidal in shape and translucent yellow or pinkish in colour. In *C. versicolor* the cells of the parathyroid glands are arranged in compact cords separated by connective tissue strands containing blood vessels. The gland contains a single cell type which is oval, rounded or irregular in shape. These cells possess indistinct cell boundary (Fig. 10). The parenchymal cells contain scanty cytoplasm and a large centrally located ovoid nucleus with one or more nucleoli (Fig. 10). Occasionally, the occurrence of follicles has also been noticed in the parathyroid gland of *C. versicolor*.

Up to day 5 following 1,25(OH)₂D₃ treatment, the parathyroid glands of *C. versicolor* remain unaffected. After day 10 and day 15, reduced chromaticity of nuclei (Fig. 11) and a progressive decrease in the nuclear volume (Fig. 13)

Fig. 7. Ultimobranchial gland of five days 1,25(OH)₂D₃ treated *Calotes versicolor* showing weak staining response of cytoplasm. HE x 200.

Fig. 8. Ultimobranchial gland of 10 days 1,25(OH)₂D₃ treated *Calotes versicolor* showing exhausted cells. HE x 200.

Fig. 9. Ultimobranchial gland of 15 days 1,25(OH)₂D₃ treated *Calotes versicolor* showing degenerating cells. HE x 200.

Fig. 10. Parathyroid gland of control *Calotes versicolor* showing single cell type. Note indistinct cell boundaries and scanty cytoplasm. HE x 200.

Fig. 11. Parathyroid gland of 10 days $1,25(\text{OH})_2\text{D}_3$ treated *Calotes versicolor*. Note reduced chromaticity of nuclei. HE x 200.

Fig. 12. Degenerating cells in the parathyroid gland of 30 days $1,25(\text{OH})_2\text{D}_3$ treated *Calotes versicolor*. HE x 200.

Fig. 13. Nuclear volume of parathyroidal cells of vehicle or 1,25(OH)₂D₃ treated lizards. Asterisk indicates significant differences ($P < 0.05$) as compared with vehicle-injected specimens.

of parathyroidal cells have been noticed. On day 30, the nuclear volume tends to become normal (Fig. 13) and a few degenerating cells are observed (Fig. 12).

DISCUSSION:

Administration of 1,25(OH)₂D₃ to *C. versicolor* provoked hypercalcemia and hyperphosphatemia. This is in conformity with the earlier reports which describe hypercalcemia after administration of either calcium chloride, vitamin D or its metabolites in *Varanus flavescens* (SWARUP *et al.*, 1987) and *Natrix piscator* (SRIVASTAV and RANI 1992a, SRIVASTAV *et al.*, 1995). The present study also derives support from the reports of various investigators who have noticed similar effect of vitamin D/its metabolites in other vertebrates – fish (SRIVASTAV, 1983, 1989; FENWICK *et al.*, 1984; SWARUP *et al.*, 1984; SRIVASTAV and SINGH, 1989, 1992, SRIVASTAV *et al.*, 1993, 1997a,b, 1998a), amphibia (SRIVASTAV *et al.*, 1987, 1999), bird (SWARUP *et al.*, 1986-87) and mammals (SWARUP and SRIVASTAV, 1979).

Lizards in the present study were not fed during the experimental period, hence the possibility of enhanced intestinal calcium uptake can be ruled out. Thus, the calcium needed for the hypercalcemic effect after 1,25(OH)₂D₃ treatment might have derived from bone demineralization and/or enhanced tubular reabsorption of calcium by kidney.

In *C. versicolor* the hyperphosphatemic effect of $1,25(\text{OH})_2\text{D}_3$ suggests that non-dietary phosphorus, possibly from the bone or the soft tissues can be mobilized. There exists a single report regarding the effect of $1,25(\text{OH})_2\text{D}_3$ on blood phosphate level of a reptile – snake (SRIVASTAV *et al.*, 1995).

In *C. versicolor* the ultimobranchial gland became hyperactive after treatment with $1,25(\text{OH})_2\text{D}_3$ which is evident by the increased nuclear volume and loss of staining response of the ultimobranchial cells. This is in conformity with the reports of other investigators who have also noticed cellular hypertrophy and hyperplasia of ultimobranchial gland after experimentally induced hypercalcemia in lizards (DUBEWAR and SURYAWANSHI, 1977; DUBEWAR *et al.*, 1978) and snakes (SRIVASTAV and RANI, 1992a, SRIVASTAV *et al.*, 1994). The present study also derives support from the studies of Suzuki *et al.* (1982) who have noticed that in *Elaphe quadrivirgata* the ultimobranchial gland releases calcitonin granules in response to the high calcium treatment (as drinking water containing 100 mM calcium). In contrast, DAS and SWARUP (1975) have not observed any histological change in the ultimobranchial gland of *Varanus monitor* subjected to experimental hypercalcemia by administering daily injections of calcium gluconate.

SUZUKI *et al.* (1984) have reported that the small granular cells of ultimobranchial gland in the sea snake *Laticauda semifasciata* contain highly developed Golgi complexes which are surrounded by immature granules in the process of formation. They have noticed hyperactivity in these cells and related this with the high calcium content of the sea water where this snake species lives. The present results perhaps provide the first direct evidence of the role of $1,25(\text{OH})_2\text{D}_3$ in inducing the activity of ultimobranchial gland in lizards since DUBEWAR and SURYAWANSHI (1977) have treated their experimental specimens with calcium chloride and vitamin D_2 .

The transformation of 25-hydroxycholecalciferol to the active form $1,25(\text{OH})_2\text{D}_3$ has been reported to be facilitated by the parathyroid hormone (RASMUSSEN *et al.*, 1972, FRASER and KODICEK, 1973). Also, increased calcium ions can inhibit this transformation by the direct action on the parathyroid and calcitonin secretion rates (BOYLE *et al.*, 1971). In the present study the observed decrease in the plasma calcium levels on day 15 and day 30 may be explained on the basis that the elevated blood calcium has suppressed the secretion of parathyroid hormone, thus affecting the formation of $1,25(\text{OH})_2\text{D}_3$. The reduction in the parathyroid hormone release by enhanced blood calcium contents induced inactivity of the parathyroid gland leading to degeneration of few cells. Degeneration of parathyroid gland has also been noticed after experimental hypercalcemia in snakes (SRIVASTAV and RANI, 1992a, SRIVASTAV *et al.*, 1994) and other vertebrates (KAMEDA, 1970; NUNEZ *et al.*, 1974; SWARUP and SRIVASTAV, 1979; KOYAMA *et al.*, 1984).

The observed decrease in the plasma calcium levels on day 15 and day 30 in $1,25(\text{OH})_2\text{D}_3$ treated *C. versicolor* can also be attributed to the increased secretion of ultimobranchial gland which contains a hypocalcemic factor – calcitonin (Uchiyama *et al.* 1978, 1981, SUZUKI *et al.* 1982, 1984; SRIVASTAV *et al.*, 1998b).

WPLYW 1,25(OH)₂D₃ NA POZIOM WAPNIA I NIEORGANICZNEGO
FOSFORANU W SUROWICY, GRUCZOLE (CIAŁKU) POZASKRZELOWYM
(ULTIMOBRANCHIAL GLAND) I PRZYTARCZYCY U JASZCZURKI
OGRODOWEJ *CALOTES VERSICOLOR*

Jaszczurkom ogrodowym *Calotes versicolor* podawano dootrzewnowo dzienną dawkę 30 pmoli 1,25(OH)₂D₃ na 50 g wagi ciała przez 30 dni. Jaszczurki były preparowane po upływie 1, 3, 5, 10, 15 i 30 dnia eksperymentu. W 1 dniu po iniekcji stężenie wapnia w surowicy *C. versicolor* nie uległo zmianie. W 3 dniu nastąpił jego istotny wzrost utrzymujący się do 10 dnia. Od 15 dnia stężenie wapnia obniża się a około 30 dnia wraca do normy. Stężenie fosforanu nieorganicznego do 3 dnia nie uległo istotnym zmianom. Po 5 dniu następuje jego istotny wzrost który utrzymuje się do 15 dnia a następnie obniża się i w 30 dniu wraca do normy. Na preparatach histologicznych nadczynność gruczołu (ciałka) pozaskrzelowego (ultimobranchial gland), wyrażającą się zwiększeniem objętości jąder komórkowych i słabszym zabarwieniem cytoplazmy obserwuje się w 5 dniu eksperymentu. Po 10 dniach objętość jąder wzrasta w dalszym ciągu a w niektórych komórkach występują objawy degeneracji. Po 15 dniach obserwuje się dalszy wzrost wielkości jąder a liczba degenerujących komórek maleje. Po 30 dniach objętość jąder jest w granicach normy i tylko nieliczne komórki degenerują. Po 10 dniach eksperymentu komórki przytarczyc wykazują obniżone zabarwienie jąder i stopniowe zmniejszanie ich objętości. W 30 dniu objętość jąder wraca do normy a degenerujące komórki są obserwowane sporadycznie.

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