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Original Article

DOSE AND STAGE SPECIFIC EFFECTS OF THYROXINE ON THE TADPOLES OF DUTTAPHRYNUS MELANOSTICTUS (ANURA: BUFONIDAE)

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ABSTRACT

Objective: Aim of the study was to investigate the effects of thyroxine on the pre metamorphic tadpoles of the common Asian toad, *Duttaphrynus melanostictus* (Anura: Bufonidae), an ideal model for understanding the role of thyroxine during vertebrate development.

Methods: Tadpoles of two developmental stages, i.e., Gosner stege 30 and 34 was exposed to 50, 100 and 200 ng/ml of thyroxine by adding the hormone to the tadpole rearing water. Toxic and teratogenic effects of thyroxine on the tadpoles were recorded. Besides, effects of thyroxine on specific activity of acid phosphatase, a lysosomal marker enzyme in the tails of the tadpoles were investigated.

Results: A Dose and stage specific toxic effect of the hormone was observed in the treated tadpoles. There was precocious metamorphosis with incomplete differentiation of limbs, deformities of body and tail in the treated tadpoles. Biochemical investigation of tails showed a dose specific elevation in the specific activity of acid phosphatase up to 2.5 fold in 50 ng/ml and 3.15 fold in 100 ng/ml treated tadpoles as compared to corresponding control tadpoles.

Conclusion: Thus, the hormone thyroxine exhibited toxic as well as teratogenic effects on the developing tadpoles and also led to dose dependent elevation in specific activity of acid phosphatase *in vivo* in their tails. Keywords: Acid phosphatase, *Duttaphrynus melanostictus*, Tail regression, Thyroxine

INTRODUCTION

The thyroid hormones are known to regulate metamorphosis in anuran tadpoles where the level of the hormones are low during early development and peak at metamorphic climax [1, 2]. Amphibian metamorphosis is composed of three developmental periods i.e., premetamorphosis, prometamorphosis and climax [3]. Premetamorphosis extends from hatchling up to Nieuwkoop and Faber (NF) stage 54 [4] or Gosner stage 34 [5] when the developing thyroid gland begins to secrete thyroid hormones [6]. It is reported that during prometamorphosis (NF stage 55-57 or Gosner stage 37-40), endogenous thyroid hormone levels rise in the blood plasma, and an orderly succession of morphological changes occur, the most obvious of which, is the development of hind limbs. At the climax (NF stage 58-66 or Gosner stage 41-46), when the concentration of thyroid hormones reach peak levels, tadpoles undergo a rapid metamorphic transition, including the eruption of forelimbs from the opercular fold, the shrinkage of head, the resorption of gills, the remodeling of intestine and skin, and absorption of tail in a temporally predetermined order [7].

Role of exogenous thyroid hormone, thyroxine accelerating metamorphosis of anuran tadpoles has been a subject of interest since the early part of last century following the remarkable discovery of Gudernatsh [8] that equine thyroid extracts could lead to precocious metamorphosis of tadpoles into juveniles. Thyroxine induced metamorphosis of various organs such as tail [2, 9-11], gills [12] and intestine [13-15] have been studied *in vitro* in various anuran species. Out of the different organs investigated, anuran tail is an excellent index of metamorphic events as it disappears with the onset of metamorphosis. Moreover, resorption of tail is one of the finest examples of programmed cell death in higher vertebrates [16].

Various lysosomal enzymes like the cathepsins, phosphatases, ribonucleases, deoxyribonuclease and β-glucuronidase have been shown to exhibit higher levels of activity in the resorbing tail tissues of anurans during spontaneous metamorphosis [11, 17-22]. Out of the different enzymes, acid phosphatase has been described earlier to be an index of lysosomal activity [23] Even though thyroxine induced activities have been reported in several anuran species [1, 11, 24-30], studies on enzyme activity during tail regression of Indian anurans is limited to spontaneous metamorphosis [18-22, 31, 32]. Since regressing tail is an excellent model to study thyroid hormone induced cell death and lysosonal enzymes are known to be associated with tissue degradation, aim of the present study was to study the effects of thyroxine on the tadpoles of an Indian anura. This paper not only provides further evidence on the effects of thyroxine on anuran tadpoles, but also presents the common Asian toad, *Duttaphrynus melanostictus* (Anura: Bufonidae) as an ideal model to study the effects of thyroid hormones on vertebrate development. Besides describing the dose dependent elevation in specific activity of acid phosphatase in the tails in response to exogenous thyroxine treatment *in vivo*, the toxic as well as teratogenic effects of thyroxine on anuran tadpoles has been reported in this paper.

MATERIALS AND METHODS

Collection of egg mass and rearing of tadpoles: Egg nests in the form of strings of the common Asian toad, *Duttaphrynus melanostictus* were collected during the months of May within Utkal University campus, Bhubaneswar, India. Tadpoles were reared till an emergence of forelimbs in the laboratory following standardized procedure [33]) in plastic tubs (80 cm diameter and 10 cm height). Tap water aerated and stored for 72 h was used for rearing. Water of the rearing tub was changed on alternate days. Tadpoles were fed with boiled *Amaranthus* leaves and boiled eggs *ad libitum*.

Chemicals used: Chemicals used in the study were of the analytical grade. Para-nitrophenyl phosphate (pNPP), para-nitrophenol (pNP) and folin-phenol reagent were obtained from Sisco Research Laboratory, Mumbai, India. Thyroxine and bovine serum albumin (BSA) was obtained from Sigma Chemicals Co., USA. Other chemicals used in the investigation were of analytical grade.

Treatment with Thyroxine: The tadpoles of Gosner stage 30 and 34 [5] were selected for the investigation, because both the stages belong to premetamorphic period where the level of endogenous thyroid hormone in the plasma remains low. At stage 30, the hind limbs are in the form of elongated buds while the hind limbs are in the paddle form at stage 34. The experimental tadpoles were reared in glass pneumatic troughs (6 cm diameter and 6 cm height). Five tadpoles were kept in

each trough containing 500 ml of either conditioned tap water (control group) or thyroxine added conditioned tap water (experimental group). For treatment, 50 ng/ml, 100 ng/ml and 200 ng/ml thyroxine containing conditioned tap water was considered. The treated tadpoles were exposed to thyroxine for 24 h and following treatment they were reared in conditioned tap water till an emergence of forelimbs.

The forelimb emerged tadpoles were transferred to amphibious condition in terrarium till completion of metamorphosis. During experiment, the rearing medium was replaced with fresh conditioned tap water on alternate days. Each experimental group had two replicates and each experiment was repeated five times at room temperature keeping 12 h light and dark regime. The tadpoles, which died before the emergence of forelimbs, were fixed in 10% formalin. Photographs of tadpoles were taken using a Leica MZ6 stereomicroscope and Nikon FM10 camera.

For biochemical investigation of tails, similar experimental setup was followed. However, only stage 34 tadpoles treated with 50ng/ml and 100ng/ml of thyroxine were considered as, 80% of tadpoles from 200 ng/ml thyroxine treated group died within 72 h of treatment while 70 to 90% of tadpoles of stage 30 died following thyroxine treatment from all treated groups (table 1).

Biochemical analyses: For each assay a pool of amputated tails from ten tadpoles were taken. Tadpoles were anaesthetized with 0.0003 g/l MS222 (Tricaine methane sulfonate) prior to tail amputation through the base with a sharp sterilized blade, while maintaining the tadpoles laterally on a pre-sterilized porcelain plate. The specific activity of acid phosphatase of the whole tails of the control (without treatment) as well as treated tadpoles (50 ng/ml and 100 ng/ml) following 48 and 72 h post treatment was investigated.

A 10% (w/v) homogenate of tail tissue was made with 0.25 M sucrose. The homogenate was kept in ice cold condition and used for the assays for protein and acid phosphatases. The protein estimation was done according to Lowry *et al.* [34]. Acid phosphatase activity was determined according to the method of Guha *et al.* [35] with para-nitrophenyl phosphate as substrate.

The assay mixture consisting of 50 µl of 10% homogenate and 550 µl of buffer (0.1 M acetate buffer pH 5.0) and 300 µl of substrate (1% para-nitrophenyl phosphate solution prepared in 0.1 M acetate buffer pH 5.0) was incubated at 37 °C for 30 min. The reaction was stopped with 2.1 ml of ice cold 0.5 M NaOH. The intensity of the color was read at 410 nm with a Systronix 119 UV-Visible spectrophotometer. The unit of enzyme activity equals µmoles para-nitrophenol (pNP) liberated/mg protein/min at 37 °C.

Statistical analyses: Duncan's multiple range tests using SPSS package were used to study the significant difference in specific activity of acid phosphatase in the tails of the control and thyroxine treated tadpoles. Data where $p<0.05$ were considered significant. Same superscripts against specific activity of acid phosphatase, in table 2 represent the data that are not significantly different.

RESULTS

Mortality: In the control groups of stages 30 and 34, 100% tadpoles survived till an emergence of forelimbs. However, amongst the treated groups, 70 to 90% tadpoles from stage 30 and 30 to 80% tadpoles from stage 34 died before the emergence of forelimbs (table 1).

Table 1: Mortality and emergence of forelimbs in the control and thyroxine treated tadpoles of stage 30 and 34 of *Duttaphrynus melanostictus*

Groups N=50	Stages	Number (%) of tadpoles died before the emergence of forelimbs	Number of tadpoles (%) survived till emergence of forelimbs [Period of emergence of forelimbs in d]	Snout to tail tip length 48 h post experiment (mean±SD in mm)
Control	30	0	50 (100) [10-15]	11±0.01
	34	0	50 (100) [8-10]	16±0.01
50 ng/ml	30	35 (70)	15 (30) [4-6]	8.75±0.96
	34	15 (30)	35 (70) [2-5]	14±0.01
100 ng/ml	30	40 (80)	10 (20) [4-5]	9±1.41
	34	25 (50)	25 (50) [2-3]	13±1.41
200 ng/ml	30	45 (90)	5 (10) [4]	7.5±0.5
	34	40 (80)	10 (20) [2]	10±0.01

N= number of tadpoles experimented for morphological studied, SD=Standard deviation

Onset of metamorphosis: Onset of metamorphosis occurred with the emergence of forelimbs within 10 to 15days in the control group tadpoles of stage 30, and 8 to 10 d of stage 34 (table 1), respectively. Emergence of forelimbs occurred between 4 to 6 d after treatment in stage 30 tadpoles and 2 to 5d in stage 34 tadpoles from 50 n mg/ml treated group. With 100 ng/ml treatments, forelimbs emerged within 4 to 5 and 2 to 3 days in the tadpoles of stage 30 and 34, respectively. With 200 ng/ml treatment, forelimbs emerged within 4 d in the tadpoles of stage 30, and 2 d in the tadpoles of stage 34.

Morphological effects: The snout to tail tip length (STTL) of stage 30 tadpoles used in the experiment remained 10±0.1 mm. After 48 h, in the tadpoles of the control group, the mean STTL increased to 11±0.15 mm. In the treated tadpoles of stage 30, the STTL was reduced to 8.75±0.96, 9±1.41 and 7.5±0.5 mm following 50, 100 and 200 ng/ml thyroxine treatment, respectively after 48 h. The mean STTL of the stage 34 tadpoles remained 15±0.5 mm. In control tadpoles, the STTL increased to 16±0.01 mm 48 h post experiments.

But, in 50 ng/ml thyroxine treated tadpoles, the length decreased to 14±0.01 mm and in 100 ng/ml treated tadpoles the STTL decreased to 13±1.41 mm, respectively. In 200 ng/ml treated tadpoles, the STTL further decreased to 10±0.01 mm 48 h post treatment (table 1). Thyroxine treated tadpoles of both stage 30 and 34 were smaller in size at the emergence of forelimbs than the respective tadpoles of control groups (fig. 1-3).

Fig. 1: Control and 200 ng/ml thyroxine treated tadpoles (Stage 30) of *Duttaphrynus melanostictus* at the emergence of forelimbs A. Dorsal view of the control (left) and experimental (right) tadpoles four days post treatment. The control tadpoles are larger in size. The treated tadpoles show bent tail and emergence of partially differentiated forelimb (left side). B. Ventral view of both tadpoles. Scale bar = 2 mm

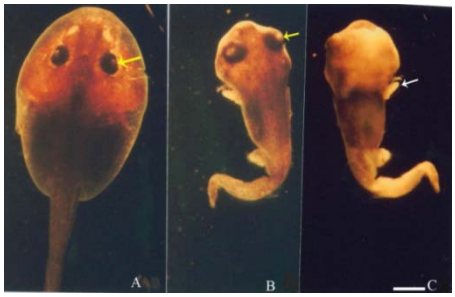

Fig. 2: Magnified view of the tadpoles of *Duttaphrynus melanostictus* shown in fig. 1, A. Dorsal view of the tadpole of control group, showing median position of eye (yellow arrow). B. Dorsal view of the 200ng/ml treated tadpole showing derso-lateral position of eye (yellow arrow). C. Ventral view of the 200ng/ml treated tadpole showing emergence of partially differentiated forelimb (left side, white arrow1), a bent and partially regressed tail. Scale bar = 1 mm

Fig. 3: Control and thyroxine treated tadpoles of stage 34 of *Duttaphrynus melanostictus* at the emergence of forelimb. A. Control tadpole of stage 34 B. Control tadpole after 10 d of experiment showing emergence of forelimb (right side) C. 50ng/ml thyroxine treated tadpole at the emergence of forelimb (right side), 2d post treatment D. 100ng/ml thyroxine treated tadpole at the emergence of forelimb (left side), 2d post treatment. E and F. 200 ng/ml treated tadpoles show emergence of forelimbs (left side) and partially regressed tail, 2 post treatment. Scale bar = 2 mm

Teratogenic activity was prominent in stage 30 tadpoles since the forelimbs were not fully differentiated at their emergence. All the tadpoles those survived till an emergence of fore limbs i.e. 30% tadpoles from 50 ng/ml treated group, 20% tadpoles from 100 ng/ml treated group and 10% tadpoles from 200 ng/ml treated groups, respectively showed undifferentiated fore and hind limbs (f2). However, the regressing tails were observed to be bent in all (100%) the tadpoles from the 200 ng/ml treated group. Similar bending of the tail was also observed in all (100%) the 200 ng/ml treated tadpoles of stage 34 (figs. 1 and 3E, F). Thus, abnormal bending of tail was observed with a higher concentration of thyroxine treatment.

Acid phosphatase (ACP) activity in the tails of tadpoles: The specific activity of acid phosphatase was estimated to be 3.048±0.26 and 3.054±0.13 µmol p-nitro phenol (pNP) formed/mg protein/min at 37 ^{o}C in the control tadpoles after 48 and 72 h of experiment, respectively (table 2). In the 50ng/ml treated group the level increased to 3.298±0.53 µmol p-nitro phenol (pNP) formed/mg protein/min 48 h post treatments. After 72 h, the level further increased to 7.75±0.26 µmol p-nitro phenol (pNP) formed/mg protein/min in this group. In the 100ng/ml treated group, the level of ACP in the tails was estimated to be 4.95±0.17 and 9.65±1.46 µmol p-nitro phenol (pNP) formed/mg protein/min after 48 and 72 h of treatment, respectively, which remained comparatively higher than the 50 ng/ml treated group.

Table 2: Specific activity of acid phosphatase (ACP) in µmoles para-nitrophenol (pNP) liberated/mg protein/minute at 37^{o}C in the tails of the control and thyroxine treated tadpoles of stage 34 of *Duttaphrynus melanostictus*

Group N=50	Specific activity of ACP 48 h post experiment mean±SD	Specific activity of ACP 72 h post experiment mean±SD
Control	3.048±0.26^{a}	3.054±0.13^{a}
50 ng/ml	3.298±0.53^{a}	7.75±0.26^{c}
100 ng/ml	4.95±0.17^{b}	9.65±1.46^{d}

N = Number of tadpoles experimented for biochemical investigation, SD = Standard deviation, Different superscripts against specific activities represent the data that are significantly different ($p<0.05$). Data are expressed as mean±SD of five observations.

DISCUSSION

Thyroxine treatment showed toxic effects on the tadpoles of *Duttaphrynus melanostictus.* Since stage 30 tadpoles were more sensitive than stage 34 tadpoles, the toxic effect of thyroxine was observed to be stage specific. The effect of thyroxine was also found to be dose specific as the tadpoles were more sensitive to 200 ng/ml treatment where 80 to 90% death occurred in the treated tadpoles before emergence of forelimbs. Prolong treatment of thyroxine (100 nmol/l) to prometamorphic tadpoles i.e., Nieuwkoop and Faber (NF) stage 55-56 (comparable to Gosner stage 37-39) of *Xenopus laevis* causing sickly appearance and leading to death of some tadpoles has been reported by Mukai *et al.* [36]

The control tadpoles showed an increase in the snout to tail tip length till an emergence of forelimbs. But, in the treated tadpoles there was reduction in snout to tail tip length, which was evident 48 h post treatment. Beachy [29] had reported the decrease in mass in the tadpoles of *Bufo americanus* following hormone treatment and correlated size reduction to the dehydrating effects of thyroxine [37, 38]. Emergence of forelimbs occurred within 10 to 15 (stage 30) and 8 to 10 days (stage 34) of the experiment in the control tadpoles. However, the emergence of forelimbs started from 2nd d onwards in all the treated tadpoles of stage 34 and 4th day onwards in the tadpoles of stage 30, respectively. Emergence of forelimbs was accompanied by tail regression in all the treated tadpoles. However, in the control tadpoles, tail regression was followed by an emergence of forelimbs (fig. 1 and 3).

The precocious shortening of tail leading to bent tails was observed in the 200 ng/ml treated tadpoles of stage 30 (f1) and 34 (f3). In the 50 and 100 ng/ml treated tadpoles, of both stages there was shortening of tail, but the tails were not bent (f3). Thyroid hormone induced tail regression has been observed in different anuran tadpoles namely *Rana catesbeiana* [25] *Rana japonica* [27] *Hyla versicolor* [28]; *Bufo americanus* [29] and *Xenopus laevis* [11, 39]). Another interesting finding of the present study was incomplete differentiation of limbs at the emergence of forelimbs in stage 30 tadpoles (f2) because of precocious onset of metamorphosis induced by exogenous thyroid hormone treatment. Similar thyroid hormone induced precocious metamorphic changes have been reported in the tadpoles of *Xenopus laevis* following addition of thyroid hormone to the tadpole rearing water [39].

Biochemical investigation of the tails showed the level of ACP to be low in the control group tadpoles than the respective treated ones. The level was found to be higher in the 100 ng/ml treated tadpoles than the 50 ng/ml treated tadpoles indicating dose specific effect of thyroxine on acid phosphatase activity. Up regulation of intracellular hydrolases have been reported in the tadpoles of *Xenopus laevis* following exogenous thyroid hormone treatment [40]. Expression of a lysosomal enzyme cathepsin D, in response to thyroid hormone treatment in the tail cells has also been observed in the tadpoles of *Xenopus laevis* [36]. In the present study, the level of ACP remained higher in the treated tadpoles than the controls 48 h post treatments. In comparison to the control, tails the increase was 0.75 fold in the 50 ng/ml treated group and 1.62 fold in the 100 ng/ml treated group. Following 72 h post treatments the level of ACP increased by 2.54 fold in 50 ng/ml treated group and by 3.15 fold in the 100 ng/ml treated tadpoles in comparison to the control. The specific activity of ACP, 72 h post treatment in 50 ng/ml and 100 ng/ml treated groups were comparable to the level of specific activity observed in the climax stage tadpoles of *Duttaphrynus melanostictus* [21] where at climax stage XXII (stage according to Taylor and Kollros [41], comparable to Gosner stage 43) the level of ACP remains 7.156±1.007 and at stage XXIV (stage according to Taylor and Kollros, comparable to Gosner stage 45) the level increases to 10.843±0.641. So, it is evident that thyroxine treatment leads to an elevation in the specific activity of acid phosphatase in a dose specific manner.

CONCLUSION

Thus, the present finding describes thyroxine as a toxic as well as teratogenic agent on the pre metamorphic tadpoles of *Duttaphrynus melanostictus*. It is also evident that the lysosomal enzyme acid phosphatase is involved during precocious tail regression and dose dependent elevation in specific activity of acid phosphatase *in vivo* occurs in the tails in response to exogenous thyroxine treatment.

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CONFLICT OF INTERESTS

Declared None

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