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Effect of Vitamin A on Development of Thyroid Gland in *Bufo melanostictus* Stage 25 & Stage 30

Dr. Kalpana Soni
Associate Professor (Zoology)
Raj Rishi Govt. (Autonomous) College, Alwar- 301001 (Rajasthan)
Email id: kalpanasoni2506@gmail.com

Abstract

The effect of vitamin A on development and differentiation of thyroid gland with particular reference of *Bufo melanostictus* (schneider) tadpoles of stage 25 and 30 of this toad species. Tadpoles of stage 25 does not give any obvious indication of presence of thyroid gland at the expected site but stage 30 show well developed thyroid gland. Vitamin A treatment to the tadpoles of stage 25 & 30 reduction in the size of thyroid gland as well as decrease in the size of colloid in such cases. Tadpoles of discontinuous treated group shows quite normal thyroid similar to the untreated group in stage 30 but tadpoles of stage 25 group C do not metamorphose after fifteen days.

Vitamin A has been found to affect differentiation of thyroid gland in the toad tadpoles and the effect is more severe on younger tadpoles as compared to the older ones.

Keywords: Colloid, follicles, vacuoles, thyroxine, vitamin A.

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Introduction

In the Vertebrates skin forms a safe protective layer around the body. As a result of remarkable adaptations of skin, vertebrates have managed to survive and to thrive in water, in land and in the air. Most information related to the skin of vertebrates have been obtained from land dwelling mammals. The skin of amphibians has a variety of different functions, as they inhabit a wide spectrum of different habitats. In amphibians, though the primary function of skin is protective, skin also serves for respiration, sensation and ion and water transport. Amphibians required a moist or aquatic environment for survival and reproduction.

The functional organization of amphibian skin is reflected in its complex cellular and extracellular structure. With the advancement of embryonic development the ectoderm and mesoderm differentiates into epidermis and dermis, respectively.

The structure of skin varies in different parts of the body. The distribution of skin glands also differs in different regions of the body. In young larvae of Anuran amphibians generally multicellular skin glands are scanty. Skin glands develop as a result of onset of metamorphosis. It appears that thyroxine plays an important role in differentiation of skin in amphibians. It has been observed by many investigators that exogenous administration of thyroxine induces differentiation of skin in the Anuran tadpoles. Contrary to the higher vertebrates, amphibian skin is generally naked i.e. skin derivatives are absent in amphibians. However, in the mouth region epidermal modifications causes formation of horny teeth and horny jaws, which represents skin derivatives in amphibians.

These epidermal derivatives are used by the Anuran larvae to chop food into sizes that can pass through the mouth. Keratinized oral armature also helps in scrapping the food from the surfaces of aquatic plants. Keratinized denticles make definite roles in the upper and lower lip regions. Thus, the distribution pattern of rows of horny teeth also helps in identification of Anurans in larval stages. Detailed structure and steps of differentiation of horny teeth and jaws are not yet known.

Vitamin A and its derivatives (Retinoids) are known to affect differentiation, morphogenesis and growth of vertebrates. Besides influencing morphogenesis in a wide variety of cells and tissues, retinoids have been observed to produce specific effect on cell differentiation particularly that of epithelial cells and appendages (Hardy, 1983).

A large number of studies have been done on the effects of vitamin A deficiency or excess on epithelial tissues on chick embryos. These studies have revealed that retinoids can modulate differentiation of epithelial tissues in vivo as well as in vitro. These studies have established the fact that retinoids play important role in proper differentiation and maintenance of epithelium. Vitamin A has been found to induce mucous metaplasia in the embryonic epidermis of treated chick embryos. Similar response has been found on mammalian epidermis in organ culture system. Vitamin A has been observed to cause complete suppression of epidermal keratinization and transformation of epidermal cell into ciliated and secretary type. The effect of vitamin A on skin differentiation has been found to be stage dependent. In a classical experiment Fell, (1957) found that 18 day old explants of chick embryos were showing less frequent, mucous metaplasia as compared to the 7 to 13 days old chick embryonic epidermis.

Vitamin A has been found to affect metamorphosis of Anuran tadpoles. Frog tadpoles when reared on vitamin A rich diet, their metamorphosis was either delayed or inhibited completely (Mc Carrison, 1923; Niazi and Saxena, 1972; Sharma and Niazi, 1983).It was found that thyroid glands of those tadpoles treated with vitamin A were adversely affected and this was suggested as one of the regions for delayed metamorphosis caused by vitamin A (Gupta, 1991).

Very little is known how vitamin A influences differentiation of skin and its glands in amphibians. Whether effect of vitamin A on differentiation of skin is a direct action or it is mediated by endocrine gland such as thyroid. In view of the above it is proposed to undertake studies on the effects of vitamin A on differentiation of skin of toad tadpoles with the following objectives.

To find the possible involvement of thyroid in regulating skin differentiation during ontogenesis and under the influence of vitamin A. On the stage 25 & stage 30.

General Material & Methods

The present studies were carried out on young and advance tadpoles of the common Indian toad, *Bufo melanostictus* Schneider (Bufonidae. Anura, Amphibia). This toad is found in abundance in and around Jaipur and Ajmer, It hibernates during winter and in other seasons it remains hidden during the day. From March onwards it comes out at dusk and can be collected easily during the nights. This toad species, like other many Anurans, breeds during monsoon. The spawning takes place shallow pool and ponds where the eggs are found in long double strings on the surface of water or entangled in between water plants. Generally these animals lay eggs in the early hours of the morning after a rain following a warm day. In laboratory conditions (29-32 °C) hatching takes place in less than 24 hours after spawning and the larval period lasts for about four weeks from hatching to the end of metamorphosis.

The spawn collected from the field hatched in the laboratory aquaria. The tadpoles were maximally fed with semi-boiled spinach every day. The young tadpoles were distributed in several tanks and plastic troughs to avoid overcrowding. The water of aquaria and troughs was also changed every day to avoid pollution. The tadpoles grew well in such conditions and there was negligible mortality.

All experiments were carried out on young tadpoles of stages 25 and 30(this toad species). The stagination was done according to the normal table of development of *Bufo melanostictus* (Khan, 1965).

Following is the brief description of the various developmental stages of tadpoles used in the present study (After Khan, 1965).

1. Stage 25 (Spirace stage or Hind limb bud stage).

At this stage of development the opercular folds are fused completely with the body wall on the right side, but on the left side the fusion is incomplete so that a spiracle is formed through which the gill filaments protrude out. The spiracle is situated on ventrolateral side of the body in the region where the first outer intestinal coil turns inwards to the mid ventral line. The preoral fleshy lip has an outer complete row of denticles and a second interrupted row. The post oral lip has got three complete rows of denticles. Mouth occupies subterminal position. The beak is well formed. Small hindlimb buds make their appearance at the junction of the belly and the tail on the dorsolateral side of the anal tube. The intestinal spiral is fully formed at this stage. Melanophores have developed in large numbers on the dorsal and lateral sides of the body.

2. Stage 30 (Length of Hindlimb 2½ times its breadth).

The general morphology of the body is well established at this stage. The hindlimb bud is 2½ times longer than it is broad. It is round near its base (stump) but its greater part is flattened from side to side (paddle). The free end is no longer round but somewhat narrow and conical and foreshadows the 4th toe. The constriction between paddle and stump marks the position of the ankle.

The studies consisted of two main lines of research:

1. Studies on differentiation of skin and keratinized oral armature of developing tadpoles under normal conditions and after treatment of tadpoles with vitamin A.
2. To study the effect of vitamin A on thyroid development during attention of skin differentiation.

Experimental design

Tadpoles at each developmental stage were divided into three experimental groups:

Group A Tadpoles of group A were reared in ordinary water throughout the period of experiment (control group).

Group B Tadpoles of this experimental group were reared in vitamin A palmitate (1 1U/ml-sigma).

Group C Tadpoles of group C were treated with vitamin A palmitate 1 IU/ml (sigma) for three days and then transferred to tap water for the remaining twelve days.

Schedule of Fixation

Tadpoles of different experimental groups were fixed at 1 day. 2 day. 3 day, 4 day. 5 day, 6 day, and 15 day following treatment.

Parameters of study

1. Temporal and spatial pattern of differentiation of skin and glands. Tadpoles fixed at different close intervals were sectioned serially and stained for visualization of various components of skin particularly the basement membrane and skin glands. The serial sections stained with modified Azan (Domagk, 1948) were also used for histo-chemical localization of mucin, collagen fibers etc.
2. Studies on keratinized oral armature under the influence of vitamin A, to find if vitamin A causes any degeneration of these structure and any possible recovery, once the treatment is withdrawn. morphological and histological observations were made on development of keratinized oral armature at selected stages of development.
3. Studies on development of thyroid glands of untreated and vitamin A treated tadpoles through serial sections to find the possible involvement of thyroid gland in differentiation of skin. For morphological studies, the tadpoles were examined under stereoscopic binocular microscope. They were sketched with the help of

camera Lucida and representative cases were photographed.

For histological examination, the tadpoles were processed through the steps of dehydration and clearing and then embedded in paraffin wax.

The tadpole was sectioned transversely and serially at 6µ thickness and then stained with aniline blue and orange G according to the modified Azanstaining technique (Domagk, 1948). The steps in sequence for this technique are given below.

1. Xylene	15 minutes
2. Xylene	15 minutes
3. Absolute Alcohol	10 minutes
4. 90% Alcohol	10 minutes
S. 70% Alcohol	10 minutes
6. 50% Alcohol	10 minutes
7. Distilled water	10 minutes
8. Nuclear fast red	30 minutes
9. Distilled water	Wash for 3-4 minutes
10. Phosphomolybdic Acid	1 minute
11. Distilled water	Wash for 2 minutes
12. Azan	5 minutes
13. Distilled water	Wash for ½ minutes
14. Differentiate in 90% alcohol	Few dips
15 Absolne alcohol	15 minutes
16. Xylene	15 minutes
17, Xylene	15 minutes

18. Mount in D.P.X using No.0 or No.1 cover glass

Working solution of Nuclear fast red, Azan (Aniline blue. Orange G and Oxalic acid) and Phosphomolybdic acid are prepared as follows:

Observation and Results

Development and differentiation of thyroid gland under the influence of vitamin A

Vitamin A is known to affect thyroid function and ultimately metamorphosis in Anuran tadpoles. In the present study development of thyroid gland was observed under the influence of vitamin A to understand, if there exists any correlation between thyroid functions and differentiation of skin and epidermal derivatives.

In the tadpoles of *Bufo melanostictus* thyroid glands are situated at the cartilage which is present below hyoid apparatus and above the carotid of heart. The developmental and functional status of thyroid reflects its requirement particularly during the metamorphosis.

Stage 25

Untreated (Control)

Group A

Internal, organization of thyroid as revealed by serial cross sections of the anterior region of tadpoles of stage 25 does not give any obvious indication of presence of thyroid glands at the expected site. Tadpoles of stage 25 reared in water for four days (equivalent to stage 29) show first indication of very small thyroid gland with few follicles.

Subsequent development for another three days, there is not much increased in the number of thyroid follicles as well as the quantity of colloid in these tadpoles

Vitamin A Treatment (Continuous) -

Group B

Vitamin A treatment for six days causes degeneration of thyroid gland. Very few follicles are present in the thyroid gland.

Vitamin A Treatment (Discountinous)

Group C

Tadpoles of stage 25 treated continuously do not metamorphose upto fifteen days. Similarly tadpoles of group C which receive vitamin A treatment for three days only then live in ordinary water also do not metamorphose after fifteen days.

Stage 30
Untreated (Control)
Group A

Thyroid gland is well developed in the tadpoles of stage 30 (group A reared for one day show thyroid glands which are situated at the cartilage present below hyoid apparatus and above the common carotids of the heart . The various developmental stages studied in the present thesis the first clear picture of thyroid is demonstrated in the tadpoles of stage 30. The thyroid gland looks like bunch of follicles. Each, follicle is made up of follicular cells which are made up of columnar epithelial cells. Colloid is present in the centre of the follicle which contains thyroxine hormone required for the development and metamorphosis of the tadpole. The characteristics of colloid and status of follicle indicates about the functional activity of the thyroid gland. The follicles and colloid present in the tadpoles of stage 30 shows an active state of thyroid. Vacuoles are present at the peripheral margins which are 3 to 4 in number in each colloid.

Empty follicles are absent at this stage. Tadpoles of stage 30 reared for 5 to 15 days show advancement in metamorphosis. In these tadpoles the number of peripheral vacuoles are increased and colloid recedes progressively towards the centre of the follicles.

Vitamin A Treatment (Continuous)
Group B

Vitamin A treatment to the tadpoles of stage 30 for 2 to 5 days causes significant reduction of the thyroid gland. There was total disintegration of thyroid follicles in the tadpoles treated for subsequent periods.

Vitamin A Treatment (Discontinuous)
Group C

Those tadpoles which were transferred to water after initial three days of vitamin A treatment shows gradual recovery from the inhibitory effects of vitamin A as indicated by reappearance of healthy follicles.

Although normal status of active thyroid is not restored in the tadpoles even after rearing for twelve days in water after initial three days of vitamin. A treatment in these, at least one follicle shows colloid and vacuoles in each thyroid gland.

Discussion

Thyroid gland

Vitamin A has been found to delay metamorphosis of tadpoles treated continuously as well as discontinuously. The effect is more pronounced and treatment given continuously.

Those tadpoles which were transferred to water after initial three days of vitamin A treatment recovered from the inhibitory effect of vitamin A. In these cases also there was delay in metamorphosis. Vitamin A has been found to delay metamorphosis in other Anuran tadpoles also (Sharma, 1982; Sharma and Niazi, 1983; Alam, 1983). These investigators have found that three days treatment with 15 IU per ml vitamin A palmitate delayed metamorphosis even after transfer of tadpoles to water. They had also found that the percentage of tadpoles metamorphosing during fifteen days period progressively decreased as the duration of treatment and/or the concentration of vitamin A increased. In most such studies vitamin A preparation used was an oily solution of vitamin A palmitate with the trade name Arovit (Roche). This preparation is relatively less toxic than the water dispersible vitamin A palmitate (sigma) used in the present studies.

It is interesting to note that vitamin A effect in delaying metamorphosis. is related to the stage of development of the tadpoles. Tadpoles of stage 25 treated continuously do not metamorphose upto fifteen days. Similarly tadpoles of group C which receive vitamin A treatment for three days only than live in ordinary water also do not metamorphose after fifteen days. On the other hand, tadpoles of stage 36 treated with vitamin A for three days undergo metamorphosis within twelve days. Similar observations were also made in Rana breviceps tadpole by Sharma and Niazi, (1983). Metamorphosis in tadpoles has been found to be directly related to functional state of thyroid. Those substances which inhibits thyroxine production have been found to inhibit metamorphosis of tadpoles. Treatment of Bufo tadpoles with potassium per chlorate has been found to inhibit thyroid functions and ultimately delay metamorphosis (Shivpal, 1976). Cross sectional studies carried out on thyroid of untreated and vitamin A treated tadpoles of stages (25, 30), clearly shows that vitamin A treatment adversely affects the thyroid glands.

Accumulation of follicles in making thyroid gland near the Hyoid cartilage was not observed in the very young tadpoles of stage 25. First clear picture of beginning of accumulation of thyroid follicles was noticed in the tadpoles of stage 30. Prominent peripheral vacuoles are observed in many colloids indicating functional state of thyroid gland. At metamorphic climax the colloids are reduced and gets concentrated in the centre and many follicles were found empty. The successive development of thyroid observed during metamorphosis Frog, stage 25

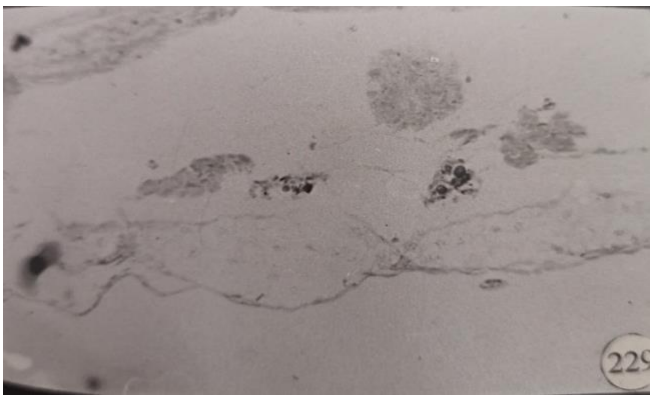
Continuous treatment of stage 25 and 30 tadpoles with IU/ml vitamin A for six days completely inhibited development of thyroid follicles. In these tadpoles either follicular development was completely inhibited or follicles were completely degenerated. This suggests that vitamin A retards growth of the thyroids primarily by inhibiting the formation of new follicles from the precursor cells. In the discontinuously treated group there was restoration of thyroid architecture on 15th day as indicated by beginning of formation of thyroid follicles.

Reversibility of vitamin A effect in the discontinuously treated group is a gradual process and the time needed for return to normal state depends upon the degree of severity of treatment and developmental stage of tadpoles. This explains why more time is needed to return to normal metamorphosis by the tadpoles of group C at stage 25 and 30 as compared to the stage 34 and 36. Prolonged exposure to vitamin A has been found to completely block growth and metamorphosis in other studies also (Sharma 1982; Alam1983; Gupta, 1991). The inhibitory effects of vitamin A on metamorphosis development tadpoles and growth of thyroid gland has been reported earlier in Frog tadpoles (Mc Carrison, 1923; Niazi and Saxena, 1972) and the results of present study on Bufo melanostictus tadpole further confirms this particular effect of this drug.

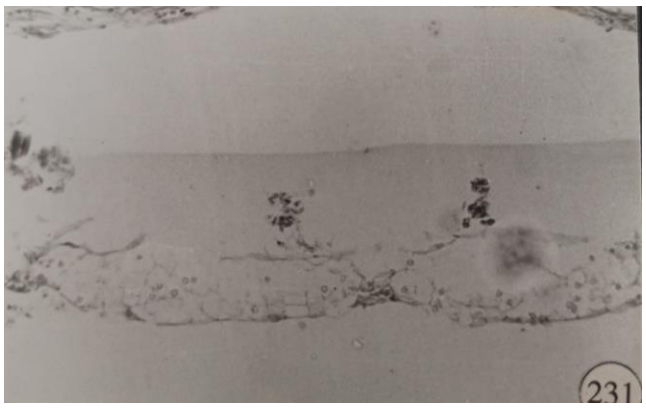
It is inappropriate to ignore the role of hypophysis in mediating the effect of vitamin A because TSH produce by hypophysis has been found to be responsible for normal development of thyroid. The complete inhibition of growth of young tadpoles in the present study could have been due to an adverse effect of this drug on pituitary particularly in releasing growth stimulating factors. Many years ago some workers found that injection of TSH to guinea pigs received excess of Vitamin A treatment partially reduced the severe affects of this drug on thyroid glands (Drill, 1943). Sadhu and Brody (1947) have suggested that vitamin A may be reducing thyroid size and its functions in rats by decreasing the level of TSH. This was later confirmed by Sadhu (1948) that TSH contents were reduced in the anterior pituitary of albino rats which were fed large quantity of vitamin A. Anurans can serve as best model to study the pituitary thyroid interaction related to the mode of action of vitamin A.

STAGE-25

1. Cross section of untreated (control) tadpoles of stage 25.



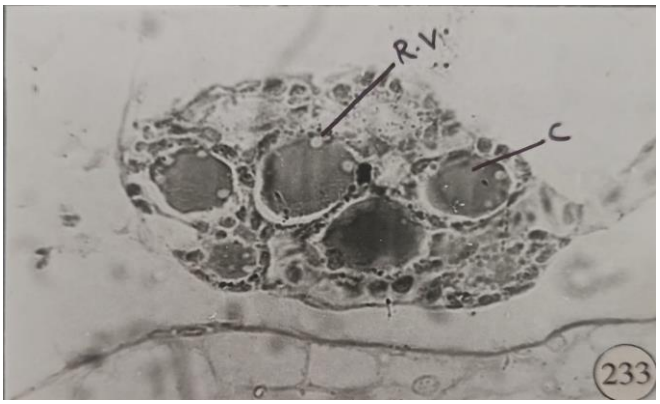
2. Cross section of Vitamin A treatment (continuous) tadpoles of stage 25.



3. Cross section of Vitamin A treatment (discontinuous) tadpoles of stage 25.

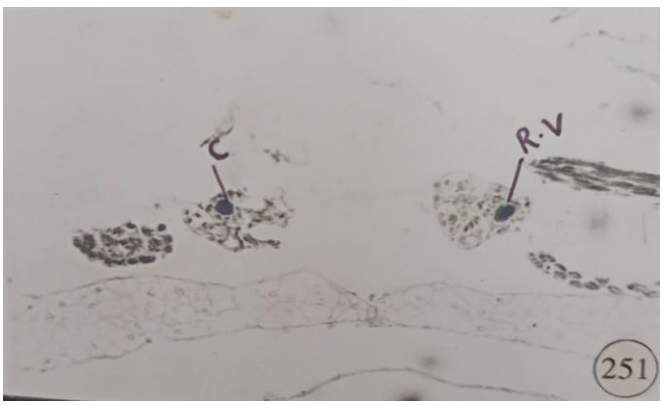
STAGE-30

1. Cross section of untreated (control) tadpoles of stage 30.



2. Cross section of Vitamin A treatment (continuous) tadpoles of stage 30.

3. Cross section of Vitamin A treatment (discontinuous) tadpoles of stage 30.



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