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EFFECT OF VITAMIN A ON THYROID GLAND DEVELOPMENT IN *Bufo melanostictus* STAGE 34 & 36

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Abstract

The effect of vitamin A on development and differentiation of thyroid gland with particular reference of *Bufo melanostictus* (schneider) tadpoles of stage 34 and 36 of this toad species. 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. In stage 34 and 36 untreated group shows well developed thyroid gland. Vitamin A treatment of stage 34 and 36 caused reduction in the size of thyroid gland as well as decrease in the size of colloid in such cases. These tadpoles show disorganization of epithelial cells of follicles. Tadpoles of discontinuous treated group shows quite normal thyroid gland similar to control. The colloid is thin, similar, and non-condensed

Keywords: - colloid, follicles, vacuoles, thyroxine, vitamin A

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Introduction

Adult amphibian skin like that of other vertebrates consists of two components, the epidermis and the dermis. Epidermis develops from the embryonic ectoderm whereas the dermal components are derived from the mesoderm. In the differentiation of skin these two components i.e., epidermis and dermis are mutually dependent on each other.

The dermis consists of stratum spongiosum which has a loose network of fibroblasts and pigment cells beneath the basement membrane. The further differentiation of skin involves differentiation of skin glands.

There are two types of skin glands commonly found in amphibians namely-

(1) Mucous gland and (2) granular glands.

These glands are invagination of the epidermis and their ducts open at the surface of epidermis. Below the stratum spongiosum, a layer of wavy sheet of collagen fibers is found which also possess fibroblasts cells; this layer is known as stratum compactum. Skin is not uniform in all the regions of the body. Its structural organization varies in different parts of the body. 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 secretory 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 34 & 36.

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 34 (1st Toe Indentation stage)

On the ventral side of the paddle like food appears another indentation marking the position of the first toe, below the indentation for the second toe. The various digits (2-5) are better demarcated and separated from each other. The three parts of the leg, thigh or stylopodium, shank or zeugopodium and the foot of the autopodium are clearly demarcated. The area between the second and third toes has also been invaded by melanophores.

2. Stage 36 (Full Anal Tube Stage).

The anal tube has extended up to the ankle. The tail piece is fully developed. The palmar region of the foot is closely applied to the ventral fin on lateral sides of it. Eyes are almost black. The forelimb rudiments can be seen through the transparent body wall on the ventral side. Melanophores can be seen on the dorsal side of all the five toes and the anterior side of the limb. Melanophores can be seen aggregated in a circle round the outer border of the intestinal loop. Small groups of melanophores are present on the dorsal fin also.

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
5. 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 skin in the tadpoles of stages (34 and 36 under the influence of vitamin A.

Normal development and differentiation of skin was observed in the tadpoles of Bufo melanostictus at stages 34, and 36 of development. Effect of vitamin A on skin gland differentiation was observed of these developmental stages. For vitamin A treatment tadpoles of different developmental stages were reared in 1 IU/ml solution of vitamin A palmitate (Sigma) for varying periods. Following experimental group were designed according to the mode of treatment.

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

Group B Tadpoles of this experimental group were reared in vitamin A palmitate (1 IU/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.

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 34

Untreated (Control)

Group A

The development of thyroid glands is progressive during metamorphosis. This feature is clearly indicated in the tadpoles of stage 34. Thyroid gland of those tadpoles of stage 34 which were reared in water for one day shows 6-7 follicles in each thyroid gland. Thyroid gland is functionally very active as indicated by large number of peripheral vacuoles and reduced size of colloid. The colloid has receded in the centre of the follicles. Tadpoles of stage 34 reared in water for two days show increase in the number of vacuoles in the colloid and there are open follicles without colloid.

During subsequent development of thyroid gland of those tadpoles of stage 34 reared in water from 3-15 days, there is significant increase in functional activity of thyroid as indicated by increase in number of follicles without colloid and presence of more vacuoles in the colloids.

Vitamin A Treatment (Continuous)

Group B

Vitamin A treatment to the tadpoles of stage 34 for two days causes reduction in the size of thyroid gland as well as decrease in the size of colloid in such cases. The colloid has become dark and opaque. Although it has receded in the centre but peripheral vacuoles are almost absent.

Subsequent treatment of vitamin A to the tadpoles causes disorganization of the epithelial cell of follicles. There are large intercellular spaces between the follicles. The follicles are either empty or a compact dark coloured colloid present in the centre.

Fifteen days treatment of vitamin A causes great deterioration of the thyroid glands. Follicles are either present remnants of thyroid or are badly disorganized in the form of clumped cell mass.

Vitamin A Treatment (Discontinuous)

Group C

Discontinuation of vitamin A treatment for two days i.e. rearing of tadpoles in water, after initial three days of vitamin A treatment shows further deterioration due to persistent effect of vitamin A.

Rearing of tadpoles for three days after initial three days of vitamin A treatment shows good recovery from the inhibitory effect of vitamin A on thyroid gland as indicated by follicles having colloid with peripheral vacuoles. Although colloids look like dense, dark droplets.

Tadpoles of discontinuously treated group reared in vitamin A for three days and transferred to water for twelve days show quite normal thyroid similar to the controls. The colloids are thin, non-condensed and having peripheral vacuoles.

Stage 36

Untreated (Control)

Group A

In the tadpoles of stage 36, there is progressive development of thyroid gland as indicated by empty follicles and colloid with vacuolization. There is significant increase in the size of follicles when compared with the tadpoles of stage 34.

Those tadpoles of stage 36 reared in water for six days metamorphosed and their thyroid gland was sectioned to study the changes in the internal organization. Most of the follicles are empty and whenever colloid is present it is dense.

Vitamin A Treatment (Continuous)

Group B

Vitamin A treatment does not show adverse effect on one day treated tadpoles. Vitamin A treatment for 2-6 days has adversely affected the thyroid gland of the tadpoles of stage 36. In these, thyroid follicles are greatly reduced. In some cases, follicles are empty and colloid is absent. Those tadpoles treated with vitamin A for nine days showed very reduced thyroid with disorganized follicles.

Vitamin A Treatment (Discontinuous)

Group C

Withdrawal of tadpoles from vitamin A solution causes gradual recovery in the thyroid gland from the inhibitory effect of vitamin A. In most such cases follicles are empty i.e. lack colloid. Whenever colloid is present it is highly vacuolated showing an active state of thyroid.

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 underga melamorphosis within twelve days. Similar observations were also made in Rama previceps ladpole 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 34 and 36) clearly shows that vitamin A treatment adversely affects the thyroid glands.

Accumulation of follicles in making thyroid gland near the Hyoid caftilage 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 (stage 36) 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 to 36 clearly indicates direct role of this endocrine gland on metamorphosis.

Continuous treatment of stage 34 and 36 tadpoles with 1 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.

In the advanced tadpoles vitamin A inhibits functions of thyroid gland as evidenced by the status of follicles, shape of cells, characteristic of colloid etc. In the well-developed thyroid gland of untreated tadpole follicular cells are generally cuboidal or columnar with vesicular nuclei and some cells even possess secretary granules. The contrary follicles of vitamin A treated thyroid shows flattened and squamous cells with dense nuclei with few secretion granule or without such granules. Quantity of resorption vacuoles and density of colloid also indicates about the functional status of thyroid. Untreated tadpoles of stage 34 and 36 show many resorption vacuoles in the colloid and many follicles of stage 36 tadpoles were found to be empty. However, in the vitamin A treated tadpoles of equal stage of development showed comparatively fewer resorption vacuoles in the colloid and in many cases colloids were dark and condensed. Completely empty follicles were not observed in any of the treated cases. Differential inhibitory effect of vitamin A on the development of thyroid glands of Anuran tadpoles has been observed, by other investigators also (Niazi and Saxena, 1972; Gupta, 1991). 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).

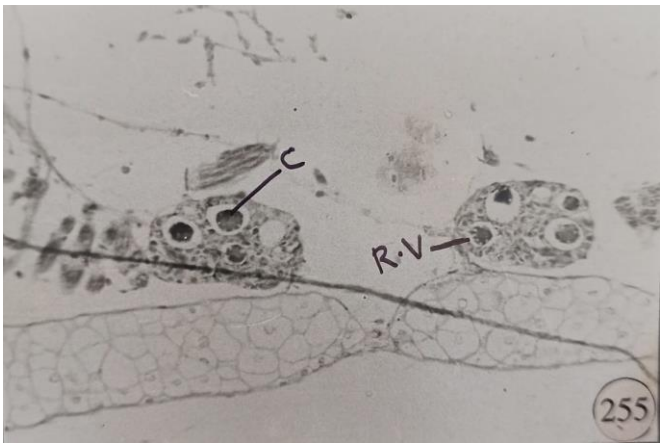
The inhibitory effects of vitamin A on metamorphosis of tadpoles and growth of thyroid gland has been reported earlier in Frog development tadpoles (Mc Carrison, 1923; Niazi and Saxena, 1972) and the results of present study on *Bufo melanostictus* tadpole further confirms this particular effect of this 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. This is also clear from the present study that vitamin A does not causes permanent effects on sensitivity of epidermis to undergo differentiation into skin glands as the process of skin gland differentiation is restituted once the treatment is withdrawn.

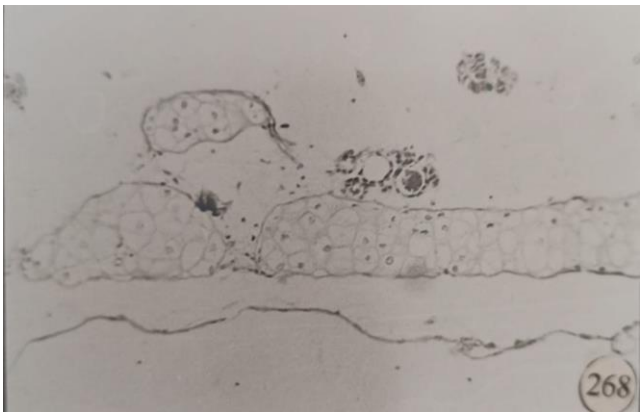
Is yet to be investigated whether vitamin A produces direct action on differentiating and developing skin and epidermal derivatives directly or the effects are mediated through systemic factors like hormones etc. Recently. molecular developmental biologists who are studying mode of action of vitamin A have suggested that vitamin A can activate or suppress homeobox genes which regulate pattern formation during development. Such alteration of Hox 4 genes is selective and specific in causing pattern alteration (Simeone et. al., 1990; Maden, 1996; Niazi, 1996; Okada, 1996). Involvement of homeobox genes in differentiation of skin is required to be investigated.

STAGE-34

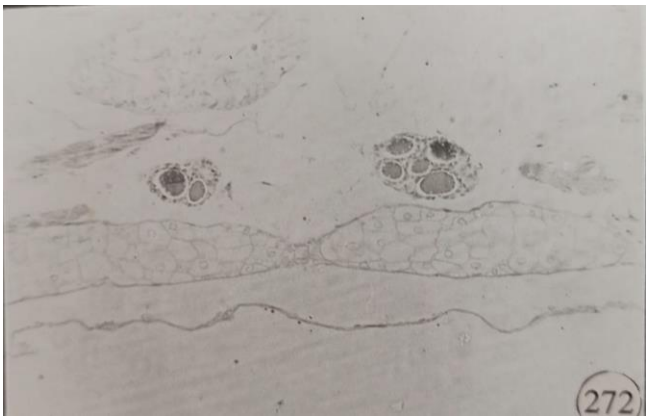
1. Cross section of untreated (control) tadpoles of stage 34.



2. Cross section of Vitamin A treatment (continuous) tadpoles of stage 34.

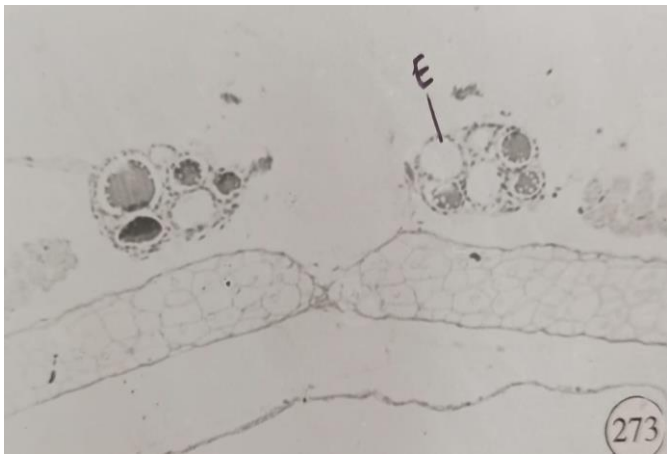


3. Cross section of Vitamin A treatment (discontinuous) tadpoles of stage 34.

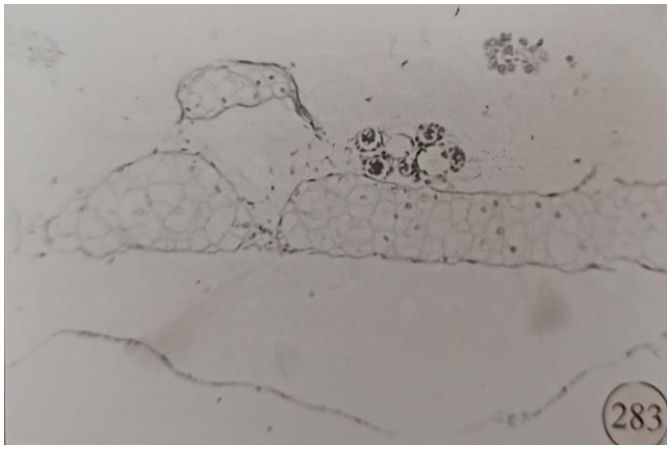


STAGE-36

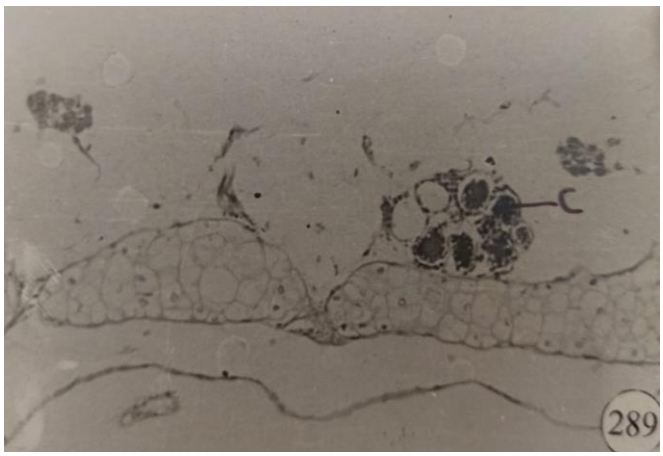
1. Cross section of untreated (control) tadpoles of stage 36.



2. Cross section of Vitamin A treatment (continuous) tadpoles of stage 36.



3. Cross section of Vitamin A treatment (discontinuous) tadpoles of stage 36.



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