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Influence of vitamin A on the development and growth of hind limbs in tadpoles of the frog, *Rana breviceps* (Schneider)

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Introduction

Vitamin A excess influences morphogenesis, differentiation and growth in vertebrates (FELL and RINALDINI, 1965). It is also a potent teratogen and if excess of this vitamin is given to expectant mothers early during pregnancy it produces a variety of malformations in the foetuses including those of limbs (TAKEKOSHI, 1964; KOCHHAR, 1973; KOCHHAR and AYDELOTTE, 1974). Several studies have been made on the effects of vitamin A excess on amphibian larvae. It is found to cause radical changes in the intestinal epithelium and resorption of the tail in *Xenopus laevis* tadpoles (WEISSMANN, 1961; WEISSMANN *et al.*, 1963). Rearing of *Bufo andersonii* tadpoles in vitamin A solution results in progressive emaciation and retarded body growth (SAXENA and NIAZI, 1977a). The same treatment given to the tadpoles of this species after tail and limb amputation adversely affects the regeneration of both these appendages (NIAZI and SAXENA, 1968, 1979; SAXENA and NIAZI, 1977b). The antagonism between vitamin A excess and thyroid hormone first indicated by experiments of McCARRISON (1923) on frog tadpoles has been confirmed by several authors in both anuran and urodele larvae (DRILL, 1943). NIAZI and SAXENA (1972) reported inhibition of the development of the thyroid glands by vitamin A excess in frog tadpoles, which were erroneously identified at that time to belong to *Rana*

cyanophlyctis, but were later found to be the tadpoles of *Rana breviceps*. The mistake occurred probably because of the usual difficulties in species identification of young tadpoles of frogs prior to metamorphosis.

The present paper reports the results of rearing of frog tadpoles in vitamin A solutions from an embryonic stage onwards on the morpohological and histological development of hind limbs.

Material and methods

A batch of eggs of the frog, *Rana breviceps* was collected from a local pool. At early gastrula stage they were divided into 7 groups of 25 each. Group 1 (controls) was reared in water and groups 2-7 were reared in media containing 1, 2.5, 5, 7.5, 10 and 15 I.U./ml concentrations of vitamin A palmitate (Arovit-Roche, India). The experiment was carried out at room temperature (30-32°C) and lasted 25 days. After hatching the larvae were fed boiled spinach and their medium was changed every two days.

At various intervals the larvae were anaesthetized in 1 : 4000 solution of MS 222 (Sandoz) and development of hind limbs recorded. On the 25th day post-hatching the surviving tadpoles of each group were fixed in Bouin's solution and representative cases were photographed. For histological examination the limbs were removed and processed for serial sectioning longitudinally at 7 microns thickness. The sections were stained with haematoxylin and eosin.

Observations and discussion

The vitamin treatment had no apparent effect on embryos of any group and all hatched normally at the same time as the controls. In the post-hatching period, however, growth of the treated tadpoles was retarded as compared to that of controls. The inhibiting effect was directly related to concentration of the drug in the rearing medium. The hind limb development was severely affected and this effect was also related to the vitamin A concentra-

tion. By the 5th day post-hatching the tad poles of groups 1 (control) and 2 (1 I.U./ml. vitamin. A treated) had attained the limb development equivalent to stages IV-VI of *Rana pipiens* according to TAYLOR and KOLLROS (1946). At this time the hind limbs were elongated buds or were at foot paddle stage. In groups-3-7 given 2.5, 15 I.U./ml vitamin A the hind limbs were still very small buds at stages I or II.

On the 24th day post-hatching the hind limbs of most control tadpoles (group 1) had reached developmental stages X-XII (fig.1); others were at stages VII-IX and one case was not developing properly (Table I). Histologically the limbs showed good differentiation of the skeleton and muscles in the thigh, shank and ankle; and even in the toes chondrogenesis was well indicated (fig. 2). In contrast, hind limb growth was retarded in all treated groups including those given the low amounts of 1 and 2.5 I.U./ml. vitamin A. As shown in the table, hind limbs in majority of tadpoles of groups 2 and 3 had reached only stages VII-IX (fig. 3) although a good number had attained as much development as the controls. In group 4, the hind limbs in majority were still at stages VI or lower and only in $\frac{1}{3}$ cases they had grown to stages VII-IX with most at stage VII (Table I; figs. 4, 6). Internally, the limbs showed only very early stage of beginning chondrogenesis in even the relatively better developed limbs (fig. 5). In groups 5-7 the limbs had not developed beyond stage VI in any case. In fact, in most of them they had remained small buds at stages I-II with no indication of histogenesis of internal tissues at all (Table 1; figs. 7,8). In tadpoles treated with 10 and 15 I.U./ml vitamin A, the minute buds contained only little mesenchyme with most cells appearing unhealthy (fig. 9).

The present results are in agreement with the findings of various authors on the inhibiting effects of vitamin A excess on the development of limbs in mammalian foetuses (TAKEKOSHI, 1964; KOCHHAR, 1973; MORRISS, 1972). JANGIR and NIAZI (1978) have also reported inhibition of morphogenesis of the limbs in tadpoles of *Bufo melano-*

stictus depending on the developmental stage of the limb at which the drug is administered. According to their finding the vitamin A has no effect if it is given after a certain degree of morphogenesis has been attained and histogenesis has begun. They had studied the effect of only 15 I.U./ml vitamin A concentration. In the present experiment the tadpoles were exposed to different concentrations of this agent from even before the appearance of limb buds. No amount of vitamin A prevented the appearance of initial limb buds in any case but their further development was affected in proportion to con centrations of the vitamin in the medium.

There was no appreciable difference between the epidermis of the controls and various treated groups but the mesoderm was profoundly affected. Mitosis occurred readily in the growing hind limbs of tadpoles given as much as 5 I.U./ml vitamin A, but its frequency was much reduced in groups treated with 7.5 I.U./ml vitamin A, and it was absent in groups given 10 and 15 I.U./ml vitamin A. High doses completely inhibited cell division and resulted in cell death and pycnosis in the limb bud area. This is also supported by the findings of MARIN-PADILLA (1966). She found complete inhibition of mitosis in the mesodermal cells in the embryos of hamster under excess of vitamin A. It has been found by several authors that embryonic mesoderm is the most vulnerable to vitamin A action (MARIN-PADILLA, 1966; JURAND, 1966 and MORRISS, 1973; KOCHHAR, 1973). Proper morphogenesis of limbs in vertebrates depends on mutual interaction between the ectoderm and mesoderm (FABER, 1971). Destruction of or an adverse eftect on the latter would naturally inhibit limb morphogenesis. Development of very small limb-buds in the larvae of groups 6-7, might be due to the primary damaging effects of the vitamin on mesoderm which ultimately caused insufficient accumulation and degeneration of mesenchyme in the future limb bud area. It is also known that vitamin A excess inhibits chondrogenesis (FELL and RINALDINI, 1965; LEWIS *et al.*, 1978).

Another factor responsible for retarded limb growth even with low concentrations of the vitamin must have been the antithyroid action of vitamin A (KOLLROS, 1961; NIAZI and SAXENA, 1972).

Summary

Tadpoles of *Rana breviceps* were reared in media containing 1, 2.5, 5, 7.5, 10 and 15 I.U/ml. vitamin A palmitate for 24 days. The treatment resulted in retarded body growth and inhibition of hind limb development both morphologically and histologically. Appearance of initial limb bud was not affected but its further growth, morphogenesis and histogenesis were severely affected in tadpoles given 5-15 I.U./ml. vitamin A and some retardation was observed with even lower amounts. High concentrations of the vitamin resulted in limb buds not growing at all and caused necrosis of the mesenchyme.

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Table 1. : Influence of vitamin A on hind limb development in the tadpoles of *Rana breviceps* (Schneider).

Group	Rearing medium	Total No. of larvae	Number showing various stages of limb development 24 days after hatching.		
			Stage VI or lower	Stages VII-IX	Stages X-XII
1. Water (Control)		21	1	6	14
2. 1 I.U./ml Vit. A		20	3	10	7
3. 2.5 I.U./ml. Vit. A		20	1	11	8
4. 5 I.U./ml. Vit. A		18	12	6	0
5. 7.5 I.U./ml. Vit. A		17	17	0	0
6. 10 I.U./ml. Vit. A		15	15	0	0
7. 15 I.U./ml. Vit. A		9	9	0	0

Note : Stages are equivalent to those of *Rana pipiens* according to TAYLOR and KOLLROS (1946).