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Influence of vitamin A on the regeneration of eye from damaged retinal pigmented epithelium cells in tadpoles of the toad, *Bufo melanostictus*

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In the present study complete eye was found to regenerate from a small part of retinal PECs in external gill stage tadpoles of *Bufo melanostictus*. More than half of the eye balls (including lens) were removed keeping a small part of neural retina intact in 40 tadpoles of external gill stage. Half (20) of the operated tadpoles were reared in tap water which served as control and remaining half (20) were reared in vitamin A (15 IU/ml) solution for five days and then transferred to water upto the day of termination of experiment (10th day after operation). Differences in retinal differentiation and the appearance of the new lens were noted between the two groups of tadpole. Although regeneration of eye was found in the both untreated as well as vitamin A treated tadpoles. However, vitamin A increased the percentage of eye regeneration. In the tadpoles of control group eye regeneration occurred only in 4 (20%) out of 20 whereas, it was in 16 (80%) out of 20 tadpoles of vitamin A treated group of the same age. Morphological and histological study revealed that newly regenerated complete eyes were similar to that of normal functional eyes.Similar experiment was performed on mature tadpoles of 5-toe stage. Vitamin A could induce eye regeneration in 12 (60%) out of 20 but regenerated eyes were found smaller in size. However, eye regeneration was not reported even in a single operated 5-toe stage tadpoles of untreated control group. This experimental model is the first to show that vitamin A can induce the developmental potency of neural retinal pigmented epithelial cells to regenerate not only the lost retinal cells but also the complete eye. Thus the results provide the basis for a new hypothesis concerning cytodifferentiation.

Key words: Complete eye regeneration, Vitamin A.

INTRODUCTION

REGENERATION is the ability of the fully developed organism to replace lost part/parts of the body by growth or remodeling of somatic tissues. It is a developmental phenomenon occurring during post embryonic period in an already formed and functional organism. One way or another, all animals possess the ability to regenerate damaged tissues. The degree of regeneration, however, varies considerably among tissues within a body and among species. Among vertebrates, amphibians have the ability to replace tails, limbs, a complete eye lens and a large portion of the retina from remaining pigmented epithelium.

(Niazi *et al* 1979, 1989; Niazi 1983; Jangir *et al* 1978,; Eguchi 1988, 1997; Reyer 1977, Okada 2000). However, examples of animals with the capacity for replacement of an entire eye are extremely limited (Rose 1964).

The analysis of eye tissue regeneration has been an important subject in developmental biology. Among the invertebrates, Eakin and Ferlatte (1973) found that Garden snail (*Helix aspersa*) had the ability to regenerate complete functional eye. The process of regeneration occurred through the mid-eye stalk and began by an invagination of integumentary epithelium at the apex of the stalk stump to produce a shallow cleft or "eye cup". Differentiation of all components of the eye occurred by transdifferentiation of these epithelial cells. Bever and Borgens (1988) also reported the ability to regenerate the eye completely after amputation through the mid eye stalk in the mystery snail. Eye regeneration in snail shares interesting similarities to the well studied regeneration of amphibian limbs, that is dependent on the intact nerve supply (Eakin and Ferlatte,1973;Bever and Borgens,1967,1988).

In the newt and some other limited animal species, the lens and neural retina can be regenerated completely through trans-differentiation of pigmented epithelial cells (PECs). Such a phenomenon, trans-differentiation, as observed in regeneration of ocular tissues seems to be highly powerful model for studying stability and instability in differentiation of tissue cells. The pigmented epithelium of vertebrate eyes is found to be most suitable tissue for such studies. Several workers reported retina regeneration from pigmented epithelium (Hasegawa 1958; Mitashov 1968, 69; Reyer 1971; Lopashov and Sologub 1972). It is reported that the removal of retina and iris in tadpoles of *Bufo viridis,* pigmented epithelium is not usually transformed into retina, but if it comes to lie between the margins of regenerating retina it is transformed into retina and lens as well (Lopashov 1949). Following the removal of retina in 4 day chick embryo it is not restored from the pigmented epithelium, but if a piece of retina from a chick embryo or a mouse embryo of the same age is transplanted into the cavity of such an eye, islets of retina arise in the pigmented epithelium (Coulumbre and Coulumbre, 1965, 1970). Lopashov and Sologub (1972) suggested that some retinal agent is essential for the trans-differentiation of pigmented epithelium cells into retina.

The capability of iris and retinal pigmented epithelial cells to transdifferentiate into lens is well documented in amphibians (Reyer, 1954, 1977; Eguchi & Itoh 1982; Eguchi 1988, 1997). However, this capability of iris and PECs is not restricted to amphibians only but widely conserved in almost all vertebrates (Eguchi 1997, Okada 2000). From this view point, trans-differentiation of PECs of vertebrates have revealed that dormant potential to transdifferetiate into retina and lens cells is widely conserved throughout vertebrate species and that cell type-specific genes are completely inactivated in the multipotent dedifferentiated cells originated from pigmented epithelial cells. Any factor that activates these specific genes may result the regeneration of complete eye.

Vitamin A is found to be an interesting model for its influence on such a transdifferentiation changes of one cell type to another. Excess of this vitamin A had been reported to destabilize cell membrane, stimulate synthesis and release of lysosomal

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enzymes into extra cellular environment and liberating cells capable of mitosis. Maden (1993) suggested that retinoic acid is a bioactive metabolite of vitamin A act on cells to establish or change the pattern of gene activity. Vitamin A is found to cause homoetic transformation of tail cells into limbs (Mohanty-Hejmadi *et al* 1992; Maden 1993). More recently Jangir *et al* (2001, 2005) also found that vitamin A induces and accelerate the transdifferentiation of pineal cells into median third eye in amphibian tadpoles.

These known properties of vitamin A motivated the present work to explore the influence of vitamin A on eye regeneration in toad tadpoles.

MATERIAL AND METHODS

For the present study, 80 tadpoles of the toad, *Bufo melanostictus* of two different stages *viz* 40 of external gill stage (Fig1) and 40 of five toe stage were employed. The operations were performed after anaesthetizing the tadpoles in 1:4000 solution of MS222(Ethyl-m-amino benzoate-methane sulphonate-sandoz) in tap water. This strength of MS222 solution narcotizes the toad tadpoles within a few minutes and the animals revived in 5-10 minutes on being transferred to water. The operations were performed under stereoscopic binocular microscopic. Under the anaesthetized condition a fine oblique cut was made through the eye ball and thus major part of eye ball including the lens was removed keeping the optic nerve intact (Fig.2).

Fig 1 : Photograph of the external gill stage tadpole of the toad, *Bufo melanostictus* employed for the experiment

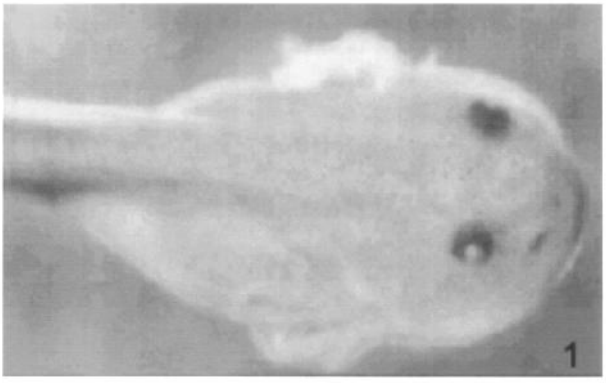
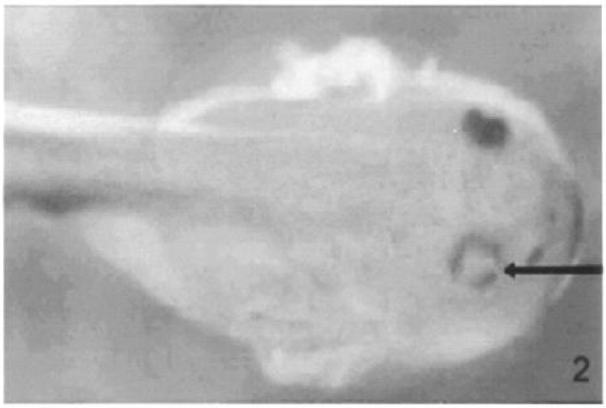


Fig 2 : Photograph of the operated tadpole from which major part of right eye ball was removed. Arrow ( ) indicates the operated right eye of which major part of retina/ retinal pigmented epithelium, iris and lens have been removed leaving behind a small part of retina/ retinal pigmented epithelium with optic nerve intact.



Operated animals of both age groups were further divided into two sub groups C1, C2 (controls) and V1, V2 (Vitamin A treated). Tadpoles of C1 and C2 were reared in tap water after their operation while those of V1 and V2 were reared in vitamin A solution for five days after operation and then transferred to tap water. The tadpoles were preserved in bouin's solution at different intervals of time (day 3,5,10) for histological study. The eyes were sectioned and stained with haemotoxylin and counter stained with eosin. The experiments were terminated on day 10 after operation.

RESULTS AND DISCUSSION

The results presented in the table 1 show that under experimental conditions complete eye can be regenerated from a small part of retinal PECs. The morphological features of regenerated eye are almost similar to that of intact eye. Their shape and size are identical to normal eyes. Anatomically, the regenerated eyes are consisting of all components like transparent cornea, lens and well-differentiated retina. The histological observations show that vitamin A can successfully induce eye regeneration from a small part of operated retinal PECs Vitamin A was found to enhance the percentage of eye regeneration in the tadpoles of the both age groups. But the percentage was found to decline with the age. In the external gill stage 80% eye regeneration was seen whereas it was 60% in the five toe tadpoles. In the

Table 1 : Complete eye regeneration in external gill stage tadpoles of Bufo melanostictus under the influence of vitamin A.

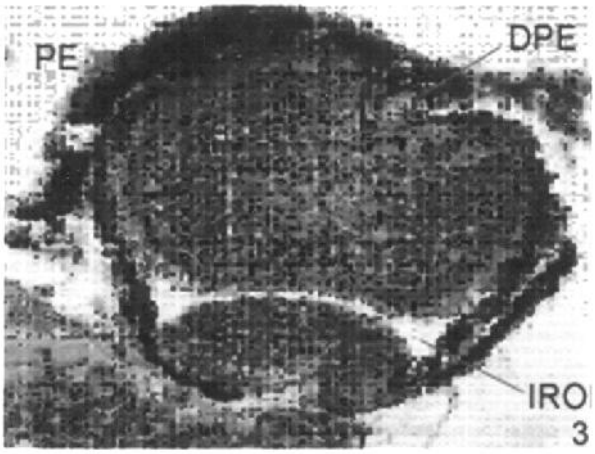
Age of animal on the day of operation	Group	No. of Animals employed	Day of preserva-tion	No. of preserved animals	Regenera-tion of Complete eye (no.)	% of eye regenera-tion
External Gill Stage	Control (C1)	20	3	3	-	
			5	3	1	
			10	14	4	20%
	Vit. A treated (V1)	20	3	3	-	
			5	3	2	
			10	14	1	80%
Mature Five toe stage	Control (C2)	20	3	3	-	
			5	3	-	
			10	14	-	00%
	Vit. A treated (V2)	20	3	3	-	
			5	3	2	
			10	14	10*	60%

*All regenerated eyes were of small size.

untreated tadpoles eye regeneration occurred only in 20% cases of external gill stage while it was not found even in a single case of five toe tadpoles.

Histological changes in regenerating eye of vitamin A treated and that of untreated tadpoles of external gill stage were found almost similar. During eye regeneration after removal of major part of eye ball including lens the transformation of the pigmented epithelium of operated eye balls into retina involves depigmentation, proliferation and then, formation of layers in newly formed retina. Beginning from the 3rd day after the operation, cells of pigmented epithelium start depigmented and flattened ellipsoid cell nuclei became rounded (Fig.3). On the 5th day after

Fig 3 : Microphotograph of a section (general view) through operated 3 days old regenerating eye of vitamin A treated external gill stage tadpole showing depigmentation of pigmented epithelium.(50X)
PE: pigmented epithelium, IROP: Intact retina left behind after operation, DPE: Depigmented cells of pigmented epithelium



the operation iris like regions arose and their intensive proliferation proceeded with the formation of retina (Fig. 4). By this time large masses of newly formed retina and iris appeared (Fig. 5 & 6). Later on the newly formed retina became subdivided into different

Fig 4 : Microphotograph of a section (general view) through operated 3 days old regenerating eye of vitamin A treated external gill stage tadpole showing transdifferentiation of pigmented epithelial cells into iris like structure. Arrow indicates the position of cells of pigmented epithelium are depigmented simultaneously in two region on both sides of the break in it. (50X)
PE: pigmented epithelium, RIS: Regenerating iris like structure

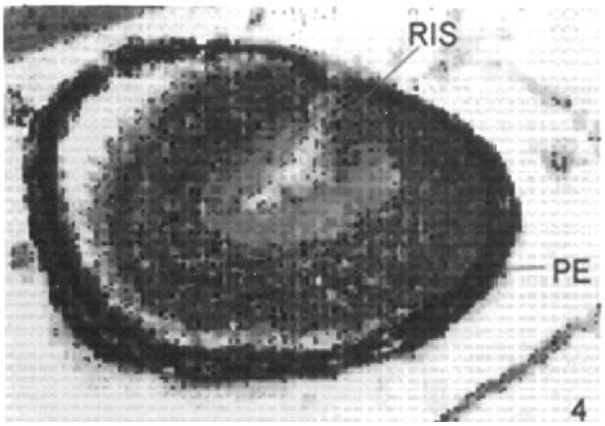


Fig 5 : Microphotograph of a section through operated 5 days old regenerating eye of vitamin A treated external gill stage tadpole showing transdifferentiation of pigmented epithelial cells into new retina (200X)
PE: pigmented epithelium, NR: New retina

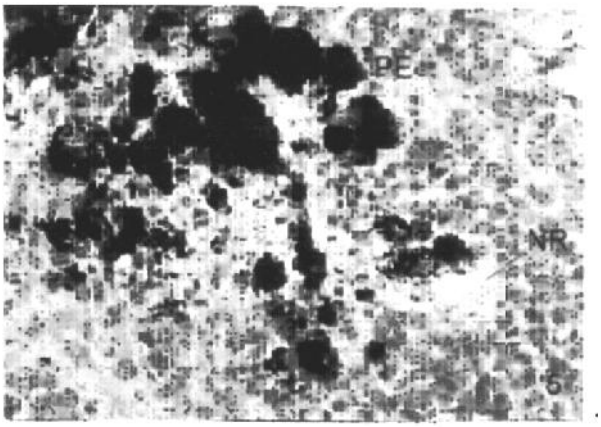
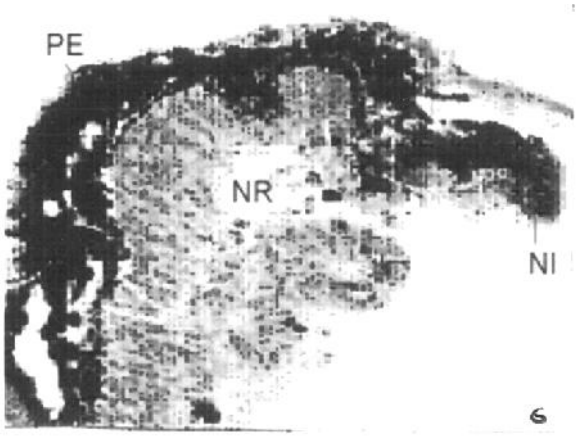


Fig 6 : Microphotograph of a section through operated 5 days old regenerating eye of vitamin A treated tadpole showing newly formed retina and iris. (50X)
PE: pigmented epithelium, NR: New retina NI: New Iris



layers. Simultaneously lens formation also began. The two layers of pigmented epithelium of dorsal iris begin to thicken and the nuclei of iris cells change their shape. The condensed chromatin of PECs nuclei become progressively disappeared. The nuclear volume increases. The PECs then discharge melanosomes, which appear around the dorsal margin (Fig.7). Mitosis is initiated in depigmented pigmented epithelial cells (Fig 8). The pupillary margin of the iris became knob like. The formation of this knob like structure continued until free margin became a swollen loop like structure. Dorsal iris cells continued to divide forming a vesicle like structure at the tip of dorsal iris. Now the vesicle differentiated into new lens (Fig 9). Once the new lens formed the cells of the dorsal iris ceased mitosis. By day 10 after operation the newly formed lens was surrounded by a lens epithelium. In addition, lens fiber formation was initiated in the inner surface of the vesicular lens. Cells began to elongate and entered the lumen of the vesicle. The lumen was filled by primary lens fiber nuclei before the secondary lens fibers begin to form. Later on the secondary lens fibers began to differentiate and grew around the central nucleus and thus newly formed lens became a better defined structure. (Fig.10) At last, the nuclei of the secondary lens fibers progressively disappeared. Simultaneously by 10 days after operation, newly formed retina became subdivided into main layers: ganglionar and inner nuclear ones, layer of

Fig 7 : Microphotograph of a section through regenerating eye of vitamin A treated tadpole (preserved on day 5 after operation) showing a swollen bulb like structure at the tip of dorsal iris. PECs discharging melanosomes (dedifferentiation) (100X)
PE: pigmented epithelium, DI: Dorsal Iris, ON: Optic Nerve, DM Discharged melanosomes, RR: Regenerating retina

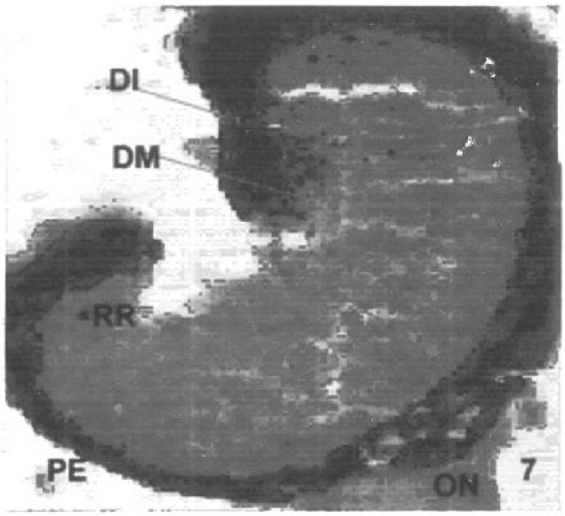


Fig 8 : Microphotograph of a section through regenerating eye of vitamin A treated tadpole (preserved on day 5 after operation) showing transdifferentiation of iris pigmented cells into lens. (100X)
PE: pigmented epithelium, M:Mitosis in developing lens, RR: Regenerating retina, LFC: Lens forming cells

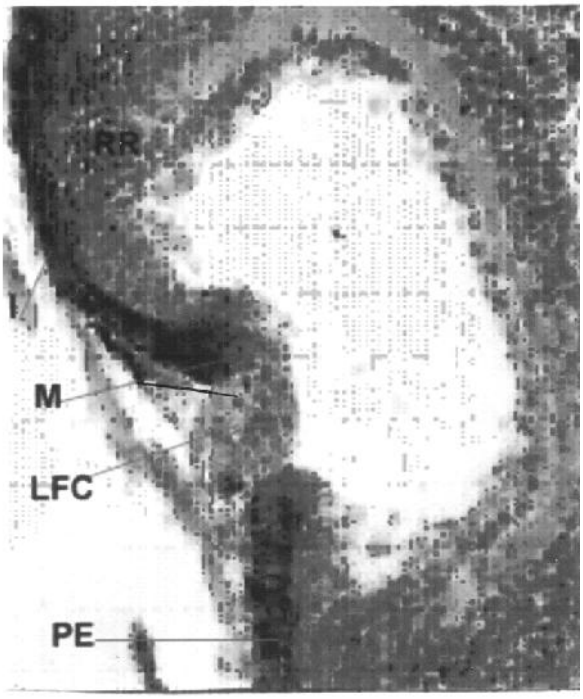


Fig 9: Microphotograph of a section through regenerating eye of vitamin A treated tadpole preserved on day 5 after operation showing developmental of lens vesicle from dorsal iris. (100X)
PE: pigmented epithelium, RR: Regenerating retina, LV: Lens vesicle, I:Iris

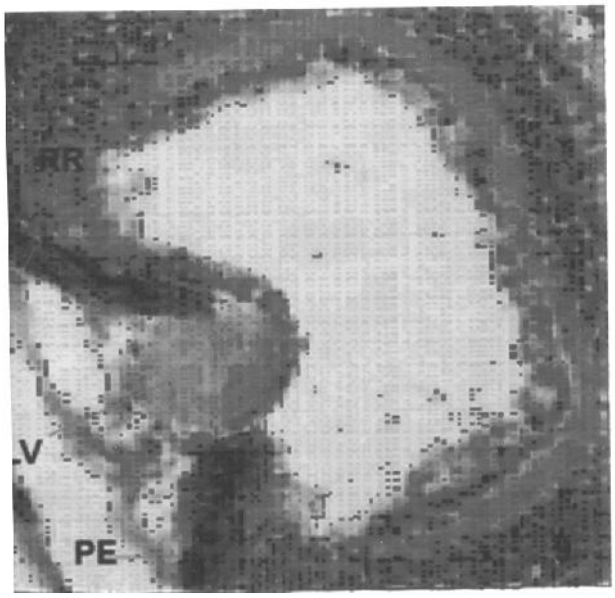
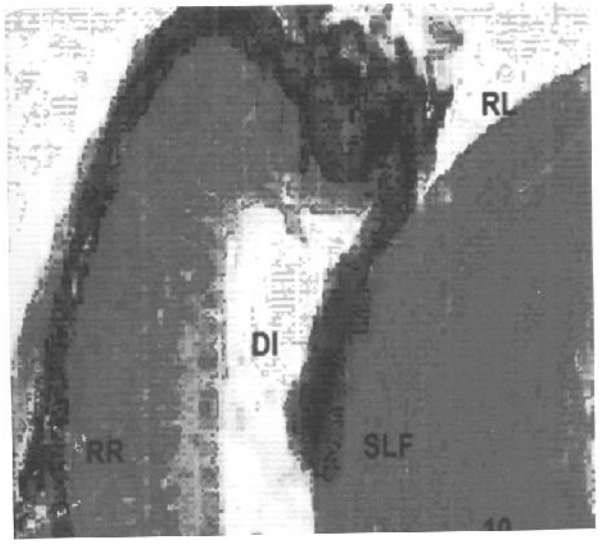


Fig 10 : Microphotograph of a section through regenerating eye of vitamin A treated tadpole preserved on day 10 after operation showing regenerated lens with well differentiated secondary lens fibers (200X)
RR: Regenerating retina, DI: Dorsal Iris, RL: regenerated lens, SLF: Secondary lens fibers



visual cells and reticular layers. Thus regenerated retinal layers become well differentiated similar to that of intact retina (Fig 10 &11b) the regenerated eye starts functioning as normal intact eye. Morphologically it gives normal look (Fig.11a)

In those cases where eye regeneration could not occur, cells of pigmented epithelium remained densely pigmented during the whole period of experiment (day 3,5,and 10). In most of the cases the layers of pigmented epithelium closed around the stump retina. No new retina and lens formed. (Fig 12b). Externally non-regenerated eye gives the impression of “black spot” in the eye orbit (Fig 12a)

Except a few where double retinas are reported otherwise

Fig 11a: Photograph showing normal looking regenerated eye of vitamin A treated tadpole preserved on day 10 after operation.
RE:Regenerated eye

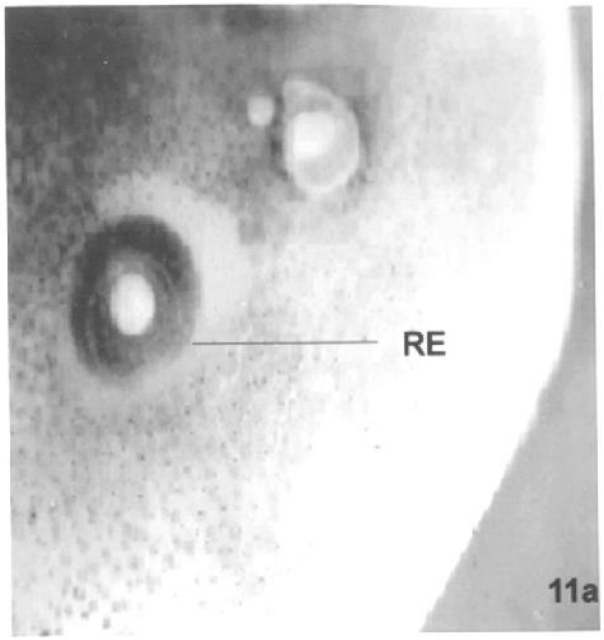


Fig 12 a: Photograph showing non:regenerating eye of untreated tadpole (control) preserved on day 10 after operation
NRE: Non:regenerating eye

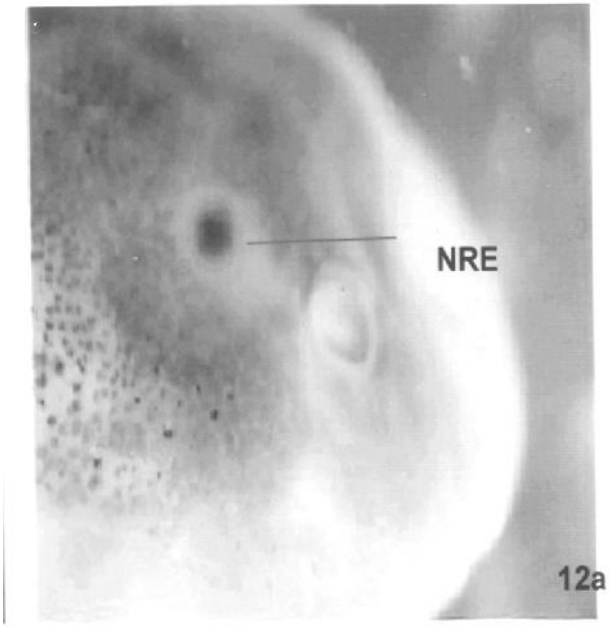


Fig 11 b: Microphotograph of a section through regenerating eye of vitamin A treated tadpole preserved on day 10 after operation showing well developed retina, iris and lens (general view)(50X)
RR: Regenerating retina, I: Iris, RL: regenerated lens

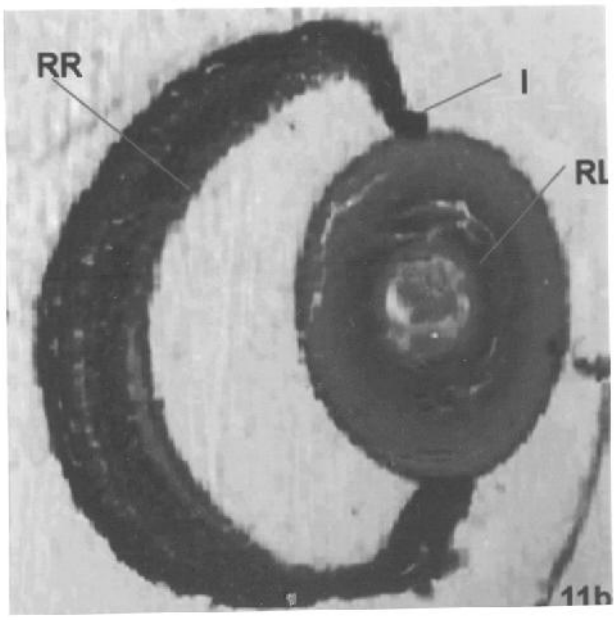
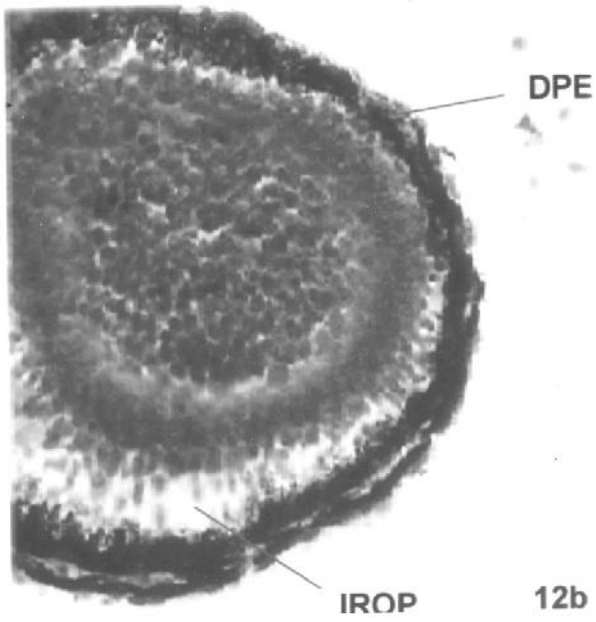


Fig 12 b: Microphotograph of a section through non:regenerating eye of untreated tadpole preserved on day 10 after operation showing pigmented epithelium completely closed around the remaining part of retina (200X)
DPE: Densely pigmented epithelium, IROP: Intact retina left behind after operation



the regenerated eyes of vitamin A treated tadpoles were found similar not only in shape and size but also in histological features to normal intact eyes.

The main finding of this study is that vitamin A can induce and accelerate the complete eye regeneration from remaining part of retina/retinal pigmented epithelium after removing major part of the eye ball including lens in external gill stage and five toe stage tadpoles of toad *Bufo melanostictus*. This is first report to show that even sensory organ like eye can regenerate in vertebrate animals. Several workers have studied the retina and lens regeneration in amphibians (Reyer 1954, 1956, 1971; Stone 1950 a,b, 1958; Yamada 1967; Itoh and Eguchi 1986, Okada 2000; Eguchi 1997) But the reports on regeneration of complete eye are extremely limited. Eakin and Ferlatte (1973) reported complete functional eye regeneration from the mid eye stalk in the Garden snail *Helix aspersa*. However, present study differs firstly the experimental animal employed was a vertebrate; secondly a small part of eye ball was left intact during eye

operation from which complete functional eye developed. Here retinal pigmented epithelial cells trans-differentiated into retina and iris. Later on iris pigmented epithelial cells become dedifferentiated and gave rise to a new lens. Thus a complete eye developed from the damaged small part of eye ball. Lopashov and Sologub (1972) discussed several factors regarding the regeneration of retina and iris from pigmented epithelium. During development the retina induces the formation of the lens from ectoderm, thus suggesting the presence of a lens forming agent, but it could be accepted that the development of the retina itself is directed by another agent as well (Lopashov, 1963). Indeed, at early stages of development the retinal anlage is able to induce both the formation of retina and lens in gastrula, ectoderm, depending on the type of contact (Lopashov & Hoperskaya, 1970). It is possible that a similar retinal agent is concentrated in the retinal anlage in the phase of its homotypic induction, its maximal concentration being attained by the beginning of its differentiation. It is possible that such an agent disappears from the pigmented epithelium during the development of animals incapable of regeneration. In this case the introduction of this agent from the retina would allow the metaplasia of pigmented epithelium into retina. The results of our experiment have shown that pigmented epithelium did not transform into retina in late five toe stage tadpoles whereas the PECs of external gill stage tadpoles could transdifferentiate in to retina, iris and lens.

It was also suggested that retinal agent is not the only agent formed by retina. It forms as 'retina factor' as well which is necessary for regeneration of lens from the newt iris (Reyer, 1962; Lopashov and Sologub 1972). Retinal agent induces the metaplasia of pigmented epithelium, exerting its effect both from the whole retina and its pieces, whereas lens regeneration can be stimulated by the whole closed retina (Eguchi, 1976). Thus the whole retina can stimulate both lens regeneration and metaplasia of pigmented epithelium into retina. However, it is known that the lens inducing ability of the retina decreases during the process of its differentiation and disappears at the time that the iris acquires its capacity for lens regeneration (Reyer 1950,1954)

In the present study lens was found to develop from iris pigmented epithelium (Fig 7,8,9 &10). Lens regeneration begins from the appearance of depigmented cells (de-differentiation) at the dorsal pupillary margin of the iris and these cells form lens vesicle. Cells of the inner wall of the vesicle elongate and protrude into the lumen forming primary lens fibers. The lens epithelium then proliferates into the secondary lens fibers from the equatorial zone. Thus by day 10 after operation complete functional eye developed from the damaged half part of eye ball left behind.

In external gill stage tadpoles, which have good ability to regenerate the lost parts of the body, an impression can be created that the damaged part/parts of the eye ball can also regenerate complete eye. But the experiment with non-regenerating stages- a stimulation is needed to start the definite set of events. To obtain metaplasia of the pigmented epithelium in five toe stage tadpoles who have lost this regenerative ability of eye, vitamin A was found beneficial. In any case, the loss of regenerative power in anurans is not totally irrevocable and by a variety of procedures several workers have induced or improved regeneration in the non-regenerating or poorly regenerating limbs of adults and advanced tadpoles of various species (Niazi, Jangir and Sharma 1979). It has been suggested that in order to induce regeneration in the normally non-regenerating organs it is necessary to increase destruction and dedifferentiation of stump tissues so that sufficient cells with reawakened morphogenetic potentialities are generated. Some recent studies have shown that treatment with vitamin A in lentectomized mice and pigs enhanced de-differentiation of PECs resulting lens regeneration in non regenerating cases. (Jangir *et al* 2004, 2005; Shekhawat *et al* 2001)

Excess of this vitamin had been reported to destabilize cell membrane, stimulate synthesis of lysosomal enzymes and thus liberating cells capable of mitosis (Fell and Rinaldini 1965). Increased mitosis in vitamin A treated explants *in vitro* was also observed by Fell and Rinaldini (1965). Tini *et al* (1993) also studied the effects of retinoids on lens development and reported that RA (retinoic acid) a natural endogenous morphogenetic agent which act as regulator of gene expression in the lens. Maden (2000) suggested that retinoic acid (RA)/ vitamin A act on cells to establish or change the pattern of gene activity. Recently Tsonis *et al* (2002) studied the effect of retinoids on urodelian lens regeneration. They found that lens regeneration was dramatically affected by inhibition of the synthesis of retinoic acid. Thus for vitamin A effect it can be suggested that in late five toe stage tadpoles, through mitogenic and dedifferentiative ability it could induce damaged retinal pigmented epithelium to regenerate complete functional eye.

McDevitt *et al* (1997) and Tsonis *et al* (1997) reported that mitogenic fibroblast growth factor (FGF) is useful in the initiation of lens regeneration from PECs. Any chemical that inhibits the activity of FGF retards the lens regeneration whereas the chemical that activates FGF accelerate lens regeneration. Vitamin A might have influenced FGF in some way so that eye regeneration could be induced. There is good evidence that vitamin A promotes and increases the proliferation through mitotic division and dedifferentiation in regenerating system (Niazi 1996, Jangir 2004, 2005).

A conclusive statement to be drawn from the present observation is that vitamin A can be very significant model for regeneration system. It enhances dedifferentiation which is prerequisite for regeneration to occur.

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