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Research Article

INJURY–INDUCED PLASTICITY OF CORNEA IN TADPOLES OF THE FROG, *RANA CYANOPHLYCTIS*

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ABSTRACT
This study demonstrates that besides *Xenopus laevis* some other anuran amphibian species like *Rana cyanophlyctis* also do have the ability to regenerate lens from cornea epithelium. This occurs during larval stages through transdifferentiation of the cornea epithelium. Vitamin A was found to accelerate the percentage of lens regeneration from cornea .Present findings provide valuable model of somatic cell plasticity influenced by injury and chemicals like vitamin A. The regeneration ability declined with the age of animal.

Key Words*: Plasticity, Cornea-lens, Vitamin A*

INTRODUCTION
The high regenerative ability of amphibians provides a valuable model system to gain basic information on plasticity and transdifferentiation. In homeostatic adult tissues, transdifferentiation is otherwise a rare event, but its frequency increases upon injury. One of the best examples of organ regeneration through transdifferentiation in vertebrates is the formation of lens from the pigmented epithelium of the newt iris (Eguchi and Kodama 1993, Grogg *et al* 2006, Galliot & Ghila 2010; Sharma *et al* 2010; Jangir *et al* 2012).However, in anuran amphibians regeneration of lens is reported from cornea (Reyer 1977, Bosco 1988a; Filoni 1980).Previously the only anuran amphibian known to regenerate the lens of the eye was *Xenopus laevis*. This occurs through transdifferentiation of the outer cornea epithelium under control of factors presumably secreted by the neural retina (Filoni *et al* 1982, 1983, 1997). Under normal circumstances the inner cornea endothelium and the lens serves as a key physical barrier that prevents retinal signals from reaching the outer corneal epithelium (Filoni *et al* 2006; Filoni 2008; Henry and Elkins 2001). Henry and Elkins (2001) reported that the process of lens regeneration triggered by factors allowed reaching the outer cornea epithelium following removal of barriers: the original lens and the inner cornea endothelium. This study demonstrates that these two structures do not prevent transdifferentaition via the production of inhibitors, but rather serve as physical barriers that block diffusion of the inducing substances thought to be made by the retina. It has also been shown that progressive loss of regenerative power with the age of animal, is not due to the diminished production of the inducing factors or the loss of competence of the outer cornea epithelium to respond to these factors, but rather to the more rapid rate at which the inner cornea heals back in older animals, which subsequently blocks the inducing factors from reaching the outer cornea epithelium. It is possible to reason that upon removal of the original lens, some anuran species do not exhibit the ability to regenerate new lenses via cornea-lens transdifferentiation due to precocious, rapid healing of the inner cornea endothelium (Henry 2003 and Filoni *et al* 2006; Filoni, 2008).

The present study attempts to explore the possibility that besides *Xenopus laevis* other anuran species like *Rana cyanophlyctis* shows lens regenerative ability from cornea in normal condition or it may be induced by some chemicals like vitamin A. Also larval *Rana cyanophlyctis* cornea is able to undergo lens formation when implanted into denucleated eye balls. Therefore, the present study has been under taken to report lens regeneration from injured cornea. An effect of vitamin A on transdifferentiation ability of cornea has also been studied.

MATERIALS AND METHODS

The experiments were carried out in young (3 toe stage) and mature (5 toe stage) tadpoles of the frog, *Rana cyanophlyctis* (Schneider). Tadpoles were raised from eggs collected from ponds of public parks of Bikaner, Rajasthan during monsoon season (July–September). Tadpoles were fed on half boiled spinach leaves. Experiments were conducted at room temperature (35^0C -37^0C.) .For control group operated tadpoles were reared in tap water while for treated group, tadpoles were exposed to 15 IU/ ml vitamin A solution for first 3 days after operation and then transferred to tap water. The experiments were terminated on day 20 after operation.

Plan of Experiment

Tadpoles used for experiment were anaesthetized with 1: 2000 MS 222 solution (Sandoz) for 3-5 minutes before operation and fixation. The experiments were designed into two series I and II. Series I was concerned with the study of transdifferentiation ability of injured cornea into lens under the influence of vitamin A *in situ* (*in vivo* study) .This series consisted of those tadpoles whose lenses were removed from right eyes. While series II was concerned with the study of the fate of meshed corneal tissue (as explant) into denucleated eye balls (eye balls from which lenses have been extracted) of host tadpoles.

Operation

For the series I operation (lentectomy) of anaesthetized tadpoles was done under stereoscopic binocular microscope. A longitudinal slit was made in the cornea of right eye extending across the middle of the pupillary space. The lens was extracted through incision. Half of the operated animals were treated with vitamin A solution (15 IU/ml) and remaining half were reared in tap water which served as control (For series II transplantation of meshed corneal extracted into denucleated eye balls of host animals) .

Preparation of explants

For this purpose eye balls were taken out from 10 donor young 3 toe stage tadpoles. Lenses were extracted from these eye balls. Anterior most part of these lensless eye balls (including cornea and limbal zone) was cut by a sharp sterilized blade under the stereoscopic binocular microscope. These operated corneas (including limbal zone) then pooled in 2 ml saline solution and meshed. This meshed tissue was used as explant.

Preparation of recipients

3 toe stage young and 5 toe stage mature tadpoles were prepared as recipient or host animals by extracting lenses from their right eyes. The animals were anaesthetized by MS 222 solution for 3-5 minutes before operation.

Implantation

About 0.01ml of meshed corneal tissue extract was implanted into the denucleated eye balls (eye ball from which lens has been removed).Operated animals with implants in their denucleated eye balls were kept immovable for 15-20 minutes by half submerging them in MS222 solution.

Half of the operated tadpoles were reared in vitamin A solution (15 IU /ml) and remaining half into tap water (control).

Operated tadpoles of both series I and II were fixed in Bouin's solution on day 3, 7 and 20 for histological evaluation. Experiment was terminated on day 20 after operation.

RESULTS

The results obtained from the series I and II are presented in the table 1.The results show that cornea –lens transdifferentiation ability is present in the employed anuran species. Vitamin A accelerated the percentage of lens regeneration from cornea. Results also revealed that lens regenerative ability declined with the age of animal in both treated as well as untreated control groups. In vitamin A treated cases it was 75% in young 3 toe stage tadpoles and 50% in mature 5 toe stage tadpoles. Similar declining trend was seen in untreated control group tadpoles. It was 45% in young and 25 % in mature tadpoles (Table 1).

Table 1: Plan of experiment and effect of Vitamin A on injury-induced plasticity of cornea tadpoles of the frog, *Rana cyanophlyctis*

Series	Developmental stage of tadpoles employed	Group	Day of prese-rvation	Number of operated animals preserved	Number of structure developed				
					Normal lens	Double lens	Lentoid	Unidentified structure	% of regeneration
SI		SI-A	3	5	-	-	-	5	
		Control	7	5	2	-	-	3	
	3 toe stage young tadpole		20	10	7	-	-	3	45%
		S I-B	3	5	-	-	-	5	
Lentectomized tadpole		Vit.-A treated	7	5	3	-	2	-	75%
			20	10	9	-	1	-	
		S I-C	3	5	-	-	-	5	25%
		Control	7	5	2	-	-	3	5%
	5 toe stage mature tadpoles		20	10	3	-	-	7	
		S I-D	3	5	-	-	-	5	
		Vit.-A treated	7	5	3	-	2	-	50%
			20	10	4	-	1	5	

SII				No. of implants employed	No. of successful implants						
			3	10	4	4	-	-	-	4	44.44%
		S II-E	7	10	6	6	2	-	-	4	
	Young	Control	20	10	8	8	4	-	2	2	
	3 toe stage host tadpoles	S II-F	3	10	4	4	-	-	-	4	77.77%
Tadpole with		Vit.-A treated	7	10	6	6	5	1	-	-	
meshed corneal tissue implants in lentectomized eye ball			20	10	8	8	7	1	-	-	
				10	4	4	-	-	-	4	33.33%
		S II-G	3	10	6	6	2	-	-	4	
	Mature	Control	7	10	8	8	2	-	2	4	
	5 toe stage host tadpoles		20								
		S II-H	3	10	4	4	-	-	-	4	66.66%
		Vit.-A treated	7	10	6	6	4	1	-	1	
			20	10	8	8	4	2	1	1	

Histological study clearly shows the transdifferentiation of injured cornea into lens forming cells (Figure 1) and then further differentiation into lens vesicle (Figure 2). Later on lens vesicle differentiates into lens with lens fibers. In both vitamin A treated and untreated control animals regenerates failed to develop beyond stage III in most of the cases (Figure 3).

Several of the regenerates were mis-shapen (Fig 3).However in some of the vitamin A treated animals, morphological features of regenerated lenses like shape size and transparency were found similar to that of normal intact lenses (Figure 4). As can be seen from Table 1, some implants had developed several foci of lens differentiation, giving rise to multiple lentoids (Figure 5) but in others, only a single lentoid had formed (Figure 6). In such transplantation experiments lens regeneration was also observed from the host cornea alone and in several cases, from the both host cornea and the corneal implant (Figure 7).

The majority of the regenerates formed from the host cornea had been arrested at stage III.In transplantation cases it is reported that lens initially occurs at multiple foci within this region, however, only one of these foci eventually forms a lens. The other foci appear to regress. The results of the series II as shown in the table also reveal that the percentage of lens regeneration declined with the age of animal, it was 77.8%, 66.7 % in young and mature tadpoles of Vitamin A treated groups and 44.4% and 33.3% in young and mature host tadpoles of control groups respectively.

DISCUSSION

The present findings give clear evidence of injury – induced plasticity of cornea to transdifferentiate into lens. Previous findings have shown that effective wounding of the inner cornea and presence of the eye cup are important factors in the expression of lens regeneration from the cornea (Reeve and Wild, 1978; Henry and Tsonis, 2010).

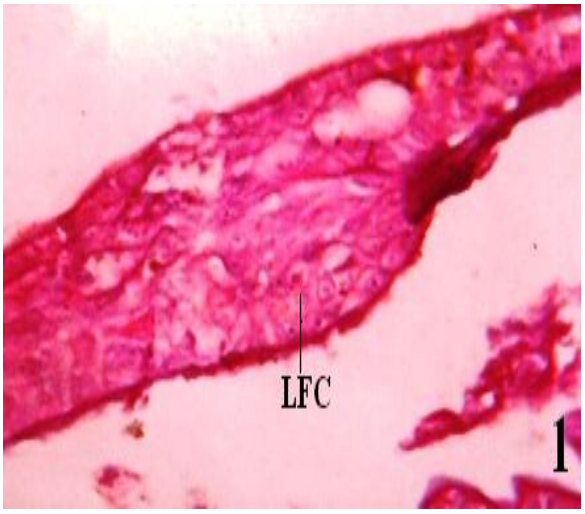

Figure 1: Microphotograph of a section passing through the lentectomized eye of vitamin A treated tadpole. Note lens forming cells originating from the inner layer of outer cornea (Dedifferentiation) (200X)During this stage cells of the inner corneal epithelium assume a cuboidal shape and then cells begin to assume thickened placoidal arrangement.

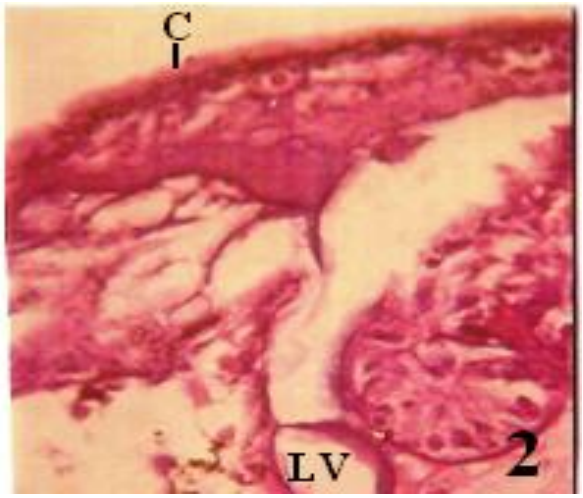

Figure 2: Microphotograph of a section passing through the lentectomized eye of 3 day vitamin A treated tadpole: Section showing formation of lens vesicle (200X). Vesicle is separated from the overlying corneal epithelium.

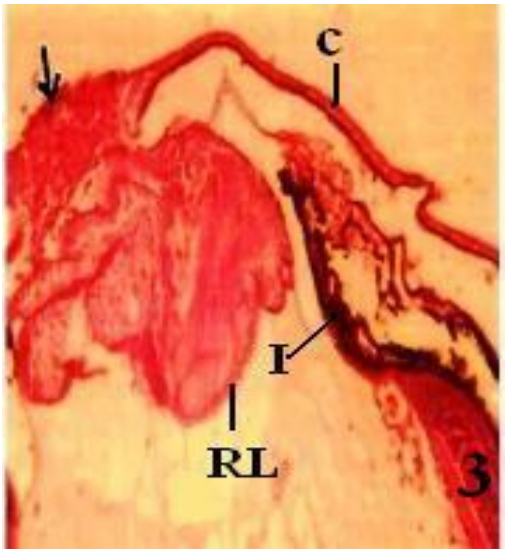

Figure 3: Microphotograph of a section passing through the lentectomized eye of 7 day vitamin A treated tadpole: photograph showing further differentiation of regenerated lens (100X). During this stage secondary lens fibers cells begin to disappear.

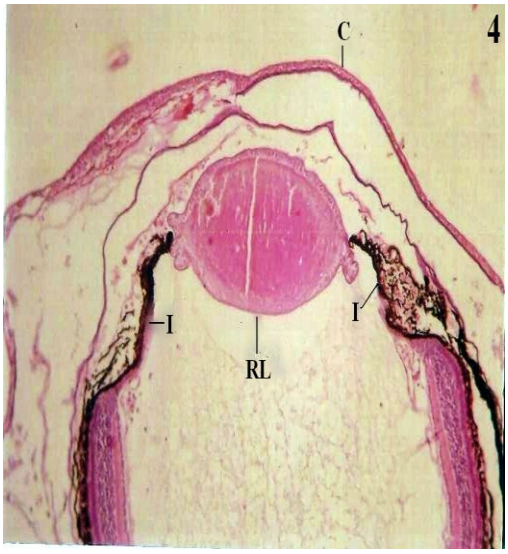

Figure 4: Microphotograph of a section passing through the lentectomized eye of 20 day vitamin A treated tadpole. Photograph showing well differentiated regenerated lens and lens positioned by the dorsal and ventral iris (100X).

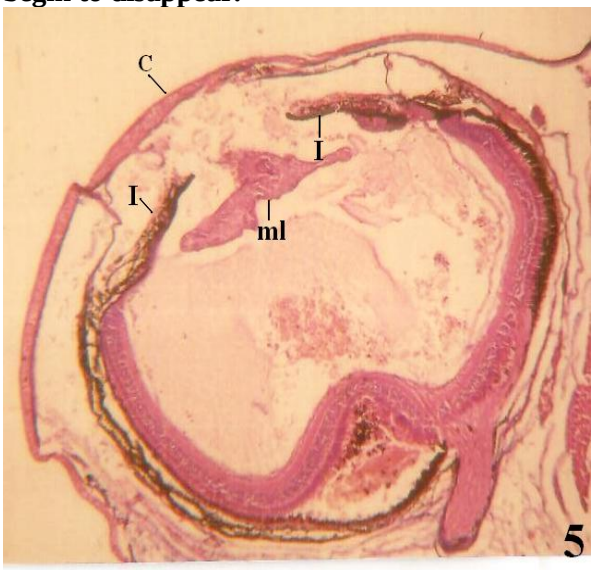

Figure 5: Microphotograph of a section passing through the lentectomized eye with corneal tissue implant of 3 day vitamin A treated host tadpole. Photograph showing transdifferentiation of corneal tissue into lentoid (40X).

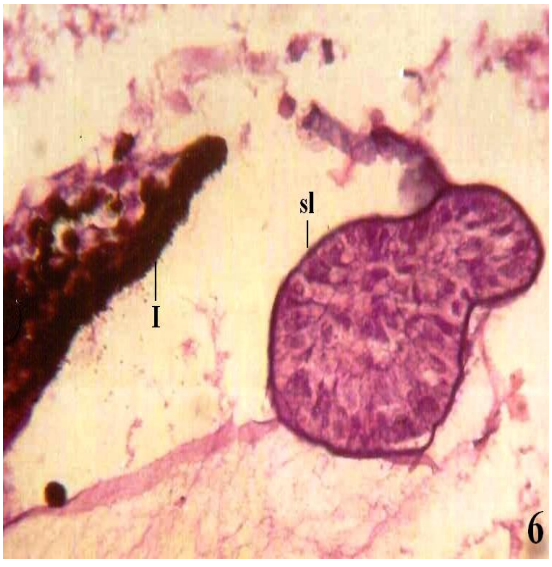

Figure 6: Microphotograph of a section passing through the lentectomized eye with corneal tissue implant of 7 day vitamin A treated host tadpole. Section showing formation of lens vesicle from implanted corneal tissue (200 X).

Abbreviations of Figures: LFC- Lens forming cells; C-Cornea; RL-Regenerated lens; LV-Lens vesicle; ml- multiple lentoid
sl- single lentoid

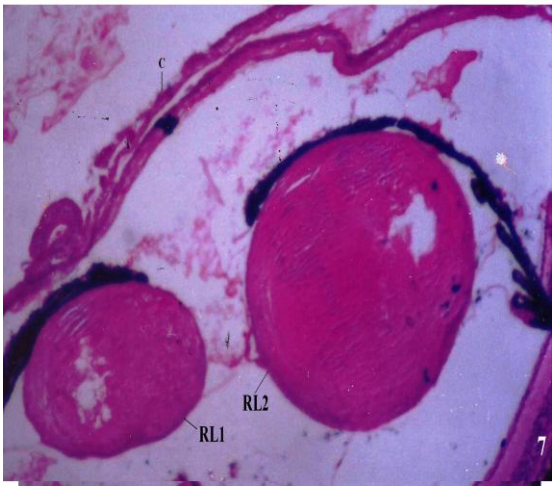

Figure 7: Microphotograph of a section passing through the lentectomized eye with corneal tissue implant of 20 day vitamin A treated host tadpole. Section showing formation of two lenses: One from coneal implant and one from cornea of host tadpole (100X).

Abbreviations of Figure: RL1 & RL2-Regenerated lens; I-Iris

Vitamin A was found to be a good model in signaling initiation of transdifferentiation of cornea into lens *in vivo* as well as in transplantation set up. However, the fact that many regenerates formed from *in situ* cornea had been arrested at stage III, suggests that with time the environment in the anterior eye region became less able to support regeneration. This is in marked contrast to those of transplantation cases where regenerates developing from implanted corneal tissue underwent lens fiber differentiation to give stage V (more advanced stage) regenerates. It can be suggested that a stimulatory factor is released by the eye cup into the posterior eye chamber which is able to reach the inner cell layer of the outer cornea via the wounded inner cornea and thus initiates lens regeneration. Reeve and Wild (1978) and Cannata *et al.,* (2003) also reported eye cup factor in *Xenopus laevis*. They reported successful differentiation of lens occurred in cornea transplanted to the posterior eye chamber, since the cornea in this position would not be exposed to the blocking action of the inner cornea. In present study tadpoles of the frog, *Rana cyanophlyctis* were also found capable of regenerating lenses .The success rate is much lower than that found in *Xenopus laevis*. For this rate it may be suggested that the inner corneal endothelium might healed more rapidly compared to that in *Xenopus laevis* and this tends to cut off the critical signaling factors required to support lens regeneration. Vitamin A was reported to accelerate dedifferentiation and mitotic division by several workers (Sharma *et al.,* 2010; Jangir *et al.,* 2012). In present study vitamin A might have induced injured cornea to transdifferentiate into lens. Therefore, the percentage of lens regeneration is high in both *in vivo* as well as in transplantation series.

Tsonis *et al.,* (2000) studied the role of retinoic acid on urodele lens regeneration. They reported that when synthesis of retinoic acid was inhibited by disulfiram or when the function of retinoid receptor was impaired by using RAR antagonist, the process of lens regeneration was dramatically affected. Thus results support the present accelerating effect of vitamin A on lens regeneration. Another pathway of RA action might be through the influence on fibroblast growth factor (FGF). FGF is supposed to initiate cell cycle events and cell division which are basis for lens regeneration (Mc Devitt *et al.,* 1997; Tsonis *et al.,* 2000). It is also observed that any chemical that inhibits the activity of FGF retards the lens regeneration. Whereas, the chemical that activates FGF accelerate lens regeneration. It is quite possible that vitamin A might have influenced FGF in some way so that lens regeneration occurred not only by transdifferentiation of injured cornea *in situ* but also by the meshed corneal implants is denucleated eye

balls. Thus it can be concluded that enhancing mitogenic and dedifferentiation activity of vitamin A might have accelerated the percentage of lens regeneration in the present study. Retinoids are thus a group of chemicals that can be employed for investigation of the molecular mechanisms responsible for homeotic transformation and /or transdifferentiation. Lens regeneration appears to be a suitable system for such investigation.

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