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RETINAL REGENERATION IN THE TADPOLES OF BUFO MELANOSTICTUS (SCHNEIDER) UNDER THE INFLUENCE OF VITAMIN A: AN OVERVIEW

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ABSTRACT
Retinal degenerative diseases have immense socio-economic impact. Millions of people worldwide are affected by retinal degenerative diseases, which lead to vision loss or blindness. Some non-mammalian species are known to have remarkable regenerative abilities and may provide the basis to develop strategies to stimulate self-repair/ or regeneration of damaged retina. In the present study, experiments were conducted on young (3 toe stage) and mature (5 toe stage) tadpoles of the toad *Bufo melanostictus*. The experiment was designed into three series I, II & III. Series I concerned with *in vivo* study, Series II concerned with retinal regeneration at ectopic site and Series III concerned with retinal regeneration *in vitro*. The one third part of the anterior retina (along with cornea and lens) was removed surgically *in situ* from the eye ball and operated animals were treated with Vitamin A (Series I). A similar surgical removal of the anterior portion of the retina and meshed it. The meshed retinal tissue treated as explant and implanted into a pit made on tail of host tadpole (Series II). It was used to assess the capacity of retinal regeneration at ectopic site (Outside of the eye ball). Operated tadpoles were treated with vitamin A. For *in vitro* study, retinal tissue was inoculated in culture medium and treated with vitamin A (Series III). The results obtained give clear evidence of the retinal regeneration ability in the toad tadpoles. In second and third part of experiment the results also showed that retinal regeneration could take place independently of the presence of eye ball. Vitamin A was found to accelerate retinal regeneration in situ as well as at ectopic site in transplantation set up and in culture medium.

Keywords: *Retinal Regeneration, Toad Tadpoles, Vitamin A*

INTRODUCTION
Retina regeneration is the process of restoring vision in vertebrates that have experienced retinal degeneration or lesions. A large number of people worldwide are affected by retinal degenerative diseases which lead to blindness. In this context, retinal regeneration research could lead to the development of new therapeutic strategies for the treatment of these pathologic disorders. For this type of study amphibian animals like toad tadpoles represent ideal animal models due to their outstanding regenerative capacity. A great deal of progress has been made in recent years towards the understanding of the molecular mechanisms that drive spontaneous retinal regeneration in these animals (Tsonis and Del Rio-Tsonis 2004; Jangir *et al.,* 2013; Tsonis PA 2000; Araki 2007; Vergara and Del Rio. Tsonis 2009; Chiba and Mitashov 2007; Middha *et al.,* 2017). In this system, the retinal pigmented epithelium (RPE) is able to regenerate an injured or lost neural retina through a process of transdifferentiation, which involves the dedifferentiation of these mature cells, their proliferation and subsequent differentiation into all the various cell types that constitute the normal tissue. The retinal pigmented-epithelium (RPE) is a partner of the neural retina and is indispensable for vision. In humans, proliferation and transformation of RPE cells after a transmatic injury of the neural retina causes a retinal disorder leading to loss of vision. In contrast, in certain adult amphibians such as *Xenopus laevis* a similar process in RPE cells leads to regeneration of the entire retina. Transdifferentiation of RPE into retina in this system requires the influence of certain

factors provided by the neural retina. If explants transplanted into the orbit of enucleated eye, as well as those transplanted into the anterior chamber of the host eyes, failed to transdifferentiate (Barbosa-Sebanero *et al.,* 2012; Chiba and Mitashov 2007; Lamba *et al.,* 2008; Tsonis and Del Rio-Tsonis 2004). The capacity of retinal pigmented epithelium to transdifferentiate into neural retina has also been demonstrated through the transplantation of RPE explants from the eyes of tadpoles into the eyes of tadpoles that had been lentectomized (Sakaguchi *et al.,* 1997; Vergara and Del Rio. Tsonis 2009) In general, embryonic or larval RPE cells in vertebrates have the capacity, to a greater degree to regenerate missing neural retinas through their proliferation and transformation, although such a capacity is lost during maturation. To find clues on how to treat RPE caused pathogenesis while promoting RPE-mediated retinal regeneration in a pathological condition, it would be a good strategy to uncover similarities and differences between regeneration competent and noncompetent systems. This review attempts to study retinal regeneration in situ as well as in transplantation set up at ectopic site. Integrated studies using present model system is certainly meaningful to understand the factors influencing the retinal regeneration. Vitamin A and its derivative retinoid have remarkable effects on different systems in developing embryos. Eyes are the organs whose development is largely dependent on retinoid and their receptors (Jangir *et al.,* 2012, Jangir 2013). The factors that regulate regeneration from the RPE are not well characterized in either amphibians or in birds and mammals. One of the first factors demonstrated to stimulate the process of regeneration from RPE was basic fibroblast growth factor (FGF) which has been shown to be effective in amphibian and chick embryos (Sakaguchi *et al.,* 1997). Tsonis PA (2000) presumed that possible pathway of vitamin A action might be through the influence of FGF. Reh & Nagy (1987) also reported inductive influence of retinoic acid on pigmented epithelial cells to proliferate and differentiate into neural retina. In the present review we will discuss our recent findings on retinal regeneration under the influence of vitamin A in young and mature toad tadpoles in situ as well as in transplantation set up at ectopic site and in culture medium.

MATERIALS AND METHODS

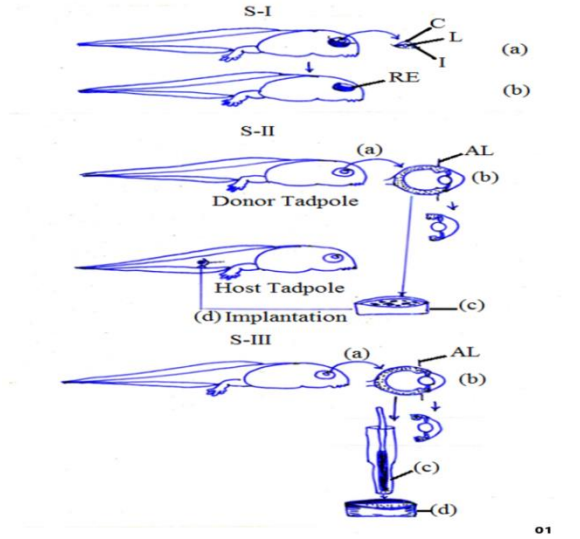
For the study of retinal regeneration, we employed two developmental stages: young (3 toe stage) and mature (5 toe stages) tadpoles of *Bufo melanostictus*. Tadpoles were fed on half boiled spinach leaves. They were then anaesthetized by immersion in 1:2000 MS222 solution before operation and fixation. Experiments were conducted at room temperature (35°C - 37°C) and designed into three series I, II and III. Series I was concerned with the study of retinal regeneration in retinectomized eyes in situ (*in vivo* study). Series I (Fig 1; S-I) consisted of those tadpoles whose one eye (right eye) was operated. The cornea was cut with a scalpel and sharp forceps. Lens was removed through the pupil with forceps. Then, anterior one third part of eye ball was removed by micro scissors. Animals were allowed to recover and then reared in respective solutions i.e. the tadpoles of control group were reared in water after surgery while treated group tadpoles were reared in 15 IU/ml vitamin A solution for first 3 days (Day 0 - Day 3) and then on day 4 animals were transferred into tap water upto the day of termination of experiment (Day 30). Series II (Fig 1; S-II) was concerned with the study of the fate of meshed retinal tissue explant at ectopic site (in a pit made in the tail of host tadpole). For this purpose twenty young tadpoles were anaesthetized in MS222 Solution (1:2000) before operation. One eye ball per animal was taken out. After removal of cornea and lens, the retinal tissue was taken out and pooled them in 2 ml saline solution and meshed. This meshed retinal tissue was used as explants. One hundred sixty tadpoles (80 young + 80 mature) were employed as recipients or host animals. For this purpose a pit of about 1mm deep and 1mm wide was made by a sharp sterile needle at mid lateral position of the tail (towards trunk) of anaesthetized tadpole and a pin head size meshed eye tissue was implanted into the pit. After insertion of explant (meshed retinal tissue) skin flap was covered over it (Fig 2). Half of the operated tadpoles were reared in 15 IU/ml Vitamin A solution for the first 3 days and then transferred into tap water and remaining half operated tadpoles were reared in tap water as control group.

Some of the operated tadpoles were fixed in Bouin's solution for 24 hours on day 5, 15 & 30 after operation for histological evaluation. Experiments were terminated on day 30 after operation.
While series III (Fig 1; S-III) was concerned with *in vitro* study of the fate of meshed ocular tissue explant inoculated in culture medium (Leibovitz L-15).

RESULTS AND DISCUSSION

Findings of present research provide substantial evidence for cell type conversion of pigmented epithelial cells into retinal structure in the anuran toad tadpoles. It is believed that complete retinal regeneration occurs only in urodele amphibians. However, contrary to this generally accepted notion, here we report that retinal regeneration also occurs in young as well as mature tadpoles of *Bufo melanostictus* from retinal pigmented epithelium (RPE) through its transdifferentiation. Present findings also revealed that the potency of regeneration declined with the age of animals.
Vitamin A was found to accelerate the percentage of retinal regeneration under all three modes of experiments employed. Findings based on results obtained from three modes of experiment: Series I: deals with the study of retina regeneration in situ (*in vivo* study). the percentage of retina regeneration in young (3 toe stage) tadpoles was 75% in vitamin A treated and 30% in untreated control group; whereas in matured tadpoles (5 toe stage) it was 65% into vitamin A treated and 25% in untreated group. Series II deals with the study of the fate of ocular tissue explant at ectopic site, Meshed ocular tissue explant was transplanted into a pit made on mid lateral position of tail of host tadpoles. This transplantation technique was found successful to study the retinal regeneration at ectopic site. The percentage of retinal regeneration was 45% in young and 40% in mature tadpoles of vitamin A treated group where as it was 15% in young and 10% in mature untreated group tadpoles. (Fig 3 S I; S II; S III)
Series III: deals with the *in vitro* study of the fate of meshed ocular tissue explant inoculated in culture medium. Results obtained revealed that the tissue explants inoculated in culture medium has transdifferentiation ability and vitamin A was found to accelerate the percentage of retinal regeneration; it was 27.77% in vitamin A treated explants and 15.55% in untreated control group explants. (Fig 3)
Histological evaluation revealed that the changes occurred during regeneration almost similar in treated and untreated cases except their percentage which was found much higher in vitamin A treated cases in all three modes of experiment.
During retinal regeneration, transdifferentiation of RPE cells begins on two days after operation. The retinal pigmented epithelial cells appear to be irregularly arranged on days 5 (Fig 4). Later on clusters of columnar neuroepithelial cells become dominant. Such cells give resemblance to the proliferative state of cells of the retinal margin. By this time RPE cells at the wounded site lose their cuboidal shape, discharge their pigment granules and detached from the underlying Basement membrane. By day 15, these cells proliferate to form a layer of pseudo stratified cells, typical of a germinative neuroepithelium. Later on some of the cells start migration which later on differentiates into the retina regenerates. By this time the retinal wound was closed by presumptive neural tissue. The inner boundary of the retina was re-established (Fig 5). By day 30, after operation particularly the regenerated retina of vitamin A treated animals become as good as intact retina. The regenerated retina differentiated into multilayer structure with typical ganglionar and inner nuclear ones, layer of visual cells and reticular layers (Fig 6). Thus retinal regenerates essentially become indistinguishable from an intact retina with respect to morphology and histological aspects.
In non-regenerating cases (in all three modes of experiment) there was dense accumulation of pigmented cells at the site of injury.
The pattern of histological changes occurred in explants of transplantation set up as well as *in vitro* models found to be almost similar. The sequential development of retina showed that meshed ocular tissue explants undergo depigmentation and proliferation and make a contact with the host's notochord. Notochord sheath become bulbous (thick) and might induce the implants to transdifferentiate into retina like structure. Histological examinations of inoculated meshed tissue in culture medium showed heavily

pigmented cells attached to the bottom of culture dish on 5th day of culture. Then RPE cells migrated out from the periphery of the explants and began to proliferate and become depigmented. Later on more apically located cells from a stratified epithelial structure. By day 30 newly formed epithelial structure differentiate into retinal regenerates



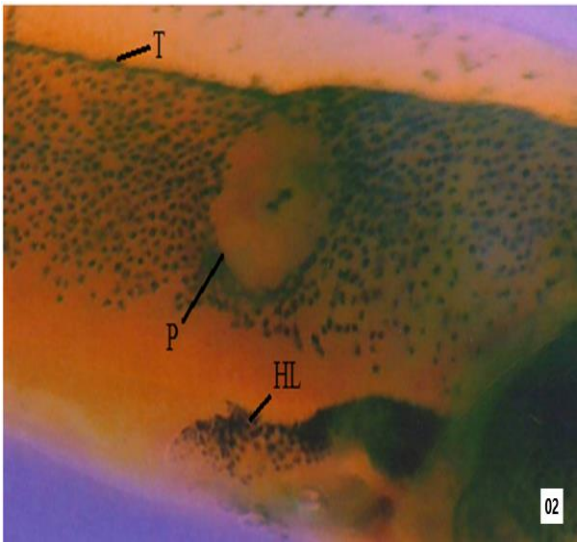
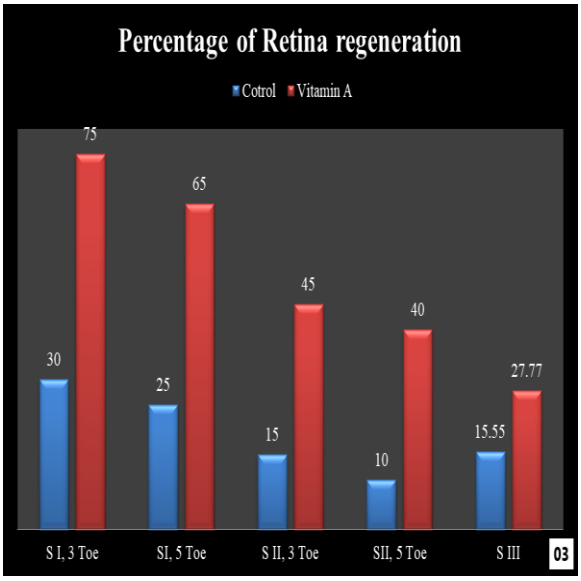


Figure 1: Line sketch diagram of the technique employed: SI, SII & SIII (No. of Series). AL- Amputation level; C- Cornea; I- Iris; L- Lens; RE- Removal of Eye ball

Figure 2: Microphotograph showing the position of a pit made on tail, where meshed ocular tissue explant was implanted. T – Tail; P- Pit; HL- Hind Limb



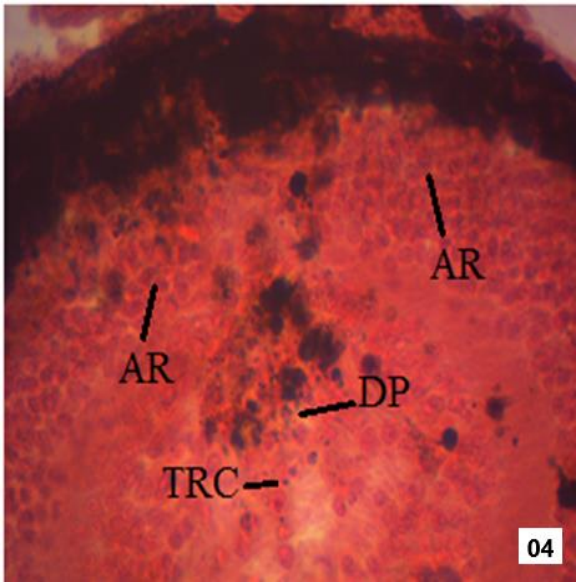
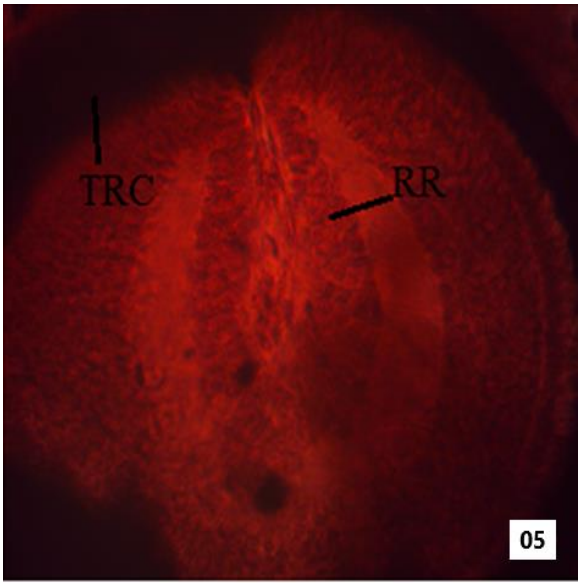


Figure 3: Showing percentage of Retinal regeneration;

Figure 4: Microphotographs of sections passing through retinectomized eye of treated group tadpoles. Figure showing occurring of retinal regeneration through transdifferentiation of RPE cells on day 5 after operation. Here, cells showing depigmentation and migration from RPE. RR- Regenerated retina; AR- Amputated retina; DP- Depigmentation of cells; TRC- Transdifferentiated retinal cells



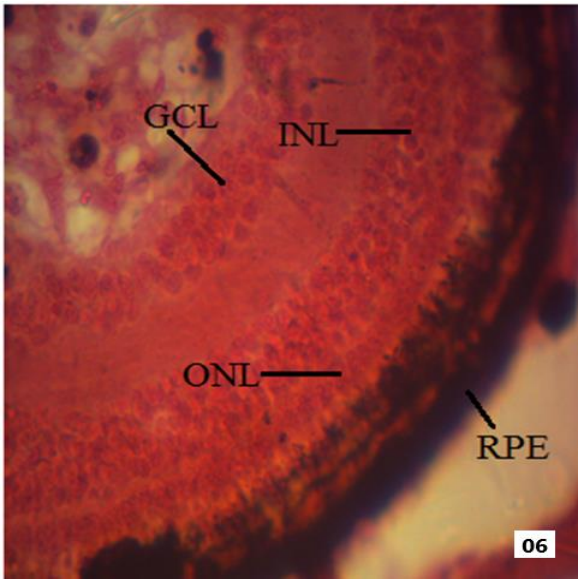


Figure 5: Microphotograph of a section passing through retinectomized eye of treated group tadpole. By day 15 post-operation, cells from RPE transdifferentiated into retinal cells and later on differentiated into multilayered, stratified retina regenerated (RR).

Figure 6: Microphotograph of a section passing through retinectomized eye of vitamin A treated tadpole. By day 30 post-operation, retinal regenerate become well differentiated with complete architecture of intact retina; the outer nuclear layer, the inner nuclear layer and the ganglion cell layer are clearly visible in the section. RPE- Retinal Pigmented Epithelium; ONL- Outer Nuclear Layer; INL- Inner Nuclear Layer; GCL- Ganglion Cell Layer

DISCUSSION

Research findings mentioned in the present review show that retinal regeneration in anuran toad tadpoles occurs through transdifferentiation of retinal pigmented epithelial cells. During this process RPE cells lose their cuboidal shape, death from the underlying basement membrane, disgorge their pigment granules and proliferate to from a layer of pseudo stratified cells; typical of a germinative neuroepithelium. Proliferation continues for several days and then differentiate into normal retina.

Tissue interaction plays an important role in RPE transdifferentiation as shown by the culture studies as well as in vivo observations (Araki 2007). The choroid makes a direct contact with RPE and appears to play a direct role in initial step of transdifferentiation. The choroid is supposed to be the source of FGF2, a trigger signal for transdifferentiation. FGF2 in the choroid is transmitted to RPE cells; this in turn up regulates several transcription factors including Pax 6 that are necessary for retinal regeneration (Azuma *et al.*, 2005; Spence *et al.*, 2007).

Araki (2007) reported that RPE when cultured on the Choroid only then RPE cells start to transdifferentiate to neuronal cells. This support the present results obtained in the second series experiments where meshed ocular tissue extract was implanted in to tail region along with choroid part of the eye resulting the retinal regeneration at ectopic site. Here also treatment with vitamin A was found beneficial for retinal regeneration.

In normal eyes, most RPE cells remain quiescent; probably in response to signals from the neural retina and small population located in the peripheral RPE undergo proliferation (Al-Hussaini *et al.,* 2008). Retinal detachment results in RPE cell proliferation (Anderson DH *et al.,* 1981). In addition, certain pathological conditions and chemical/physical stimulations can cause cells to proliferate. Several workers have reported multi sources for retinal regeneration in Xenopus sp. (Del Rio-Tsonis and Tsonis 2003; Araki 2014; Lenkowski and Remond 2014). Vitamin A and its active metabolites exert potent effects on

somatic cell plasticity particularly during regeneration (Jangir and Niazi 1978; Jangir *et al.*, 2012, 2013, 2014, 2015, Sharma *et al.*, 2010, Middha *et al.*, 2017).

The exact mechanism of vitamin A action is still not well known. Chytill and Ong (1984) and Maden *et al.*, (1988) suggested that retinoids enter the cells via some surface receptor and then binds to cellular retinoic acid binding protein (CRABP) and might accelerate dedifferentiation and hence retinal regeneration. Gudas and Wagner (2011) reported that retinoids regulate the cell differentiation. It is also reported that retinoids generated by one cell type can affect nearby cells and thus function in inter cellular communication. It was also reported that extra cellular matrix (ECM) play important role in the transdifferentiation of PECs into neural progenitors during retinal regeneration (Pittack *et al.*, 1991). Basic fibroblast growth factor (BFGF) has also shown to promote retinal regeneration in embryonic chick (Park and Hollenberg 1989). Vitamin A action might be through the influence on fibrobloast growth factor (FGF). FGF is found to control and induce the development and regeneration of lens and retina. It was reported that FGF2 can stimulate transdifferentiation of RPE explant cultures from embryonic rat (Zhao *et al.*, 1995) into neural retina. Sakami *et al.*, (2008) also reported that members of the FGF family can stimulate the RPE to generate neural retina.

In our previous findings we reported accelerating effect of vitamin A on lens, limbs, cornea and heart regeneration in different Species of vertebrates (Jangir & Niazi 1978; Shekhawat *et al.*, 2001; Sharma *et al.*, 2010; Jangir *et al.*, 2005, 2012, 2013, 2014). The exact mechanism, how vitamin A affects retinal regeneration is still not clear.

Several workers reported that retinoids, retinoic acid binding proteins and RA receptors are found throughout the retina, including the photoreceptor cell layer (Milam *et al.*, 1990; Stumpf *et al.*, 1991; Mc Caffery *et al.*, 1992). RA affects cell proliferation and differentiation is the retina of fishes, amphibians and Chick embryos (Reh *et al.*, 1993; Manns and Fritzseh 1991; Hyatt *et al.*, 1992). Exogenous treatment of retinoic acid to Zebra fish embryos induces a duplication of the retinas during development (Hyatt *et al.*, 1992). It has also been suggested that retinoids enter the cells via some surface receptors and then bind to cytoplasmic binding proteins the complex then transported to the nuclei where it ultimately alters the pattern of gene activity (Maden *et al.*, 1988; Nagal *et al.*, 2008, Middha *et al.*, 2017). Thus it can be speculated that vitamin A might affect cell surface properties on inter cellular space stabilizing factors of ocular tissue and induced the retinal regeneration. The findings of present study suggested that vitamin A plays important and evident role in retinal regeneration. Thus the study offers a new insight for further detail findings for therapies designated to repair the degenerated organs. Avasthi *et al.*, (2008) reviewed several research findings and mentioned that transplantation of retinal stem cells into damaged eye can restore normal vision. The present findings also offer new horizon of therapeutics in the form of organ development and replacement of lost tissue like retina and other damaged tissue.

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