International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

Effect of vitamin A on acid and alkaline phosphatase activities during tail regeneration in the tadpoles of the Asian toad

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Abstract

Anuran amphibians have the potential to restore their lost tail by regeneration, although, unlike urodeles their regeneration capacity is restricted to larval stages. Application of vitamin A or retinoids to tail amputated tadpoles causes abnormal tail regeneration and subsequently leads to development of ectopic organs (limbs and pelvic girdles) at the site of the tail, a phenomenon widely known as homeotic transformation. The present study focuses on the effect of different doses of vitamin A on acid and alkaline phosphatase activities during tail regeneration in the tadpoles of Asian toad *Duttaphrynus melanostictus*, a potential model for regeneration studies from Indian context. Application of vitamin A markedly increases the activity of both the phosphatases. Further, a comparatively higher dose of the vitamin leads to greater increase in activities of both phosphatases. A high level of both the enzymes in vitamin A treated regenerates suggests that the enzymes play an important role during abnormal tail regeneration, a prerequisite for ectopic organ formation.

Keywords: acid phosphatase, alkaline phosphatase, tail regeneration, vitamin A

Introduction

Among vertebrates, amphibians possess remarkable potential to regenerate lost body parts in a matter of days. The urodele amphibians can regenerate structures like limb and tail throughout their life cycle while in anurans this ability is restricted only to their larval period. In this context, vitamin A and its derivatives, also known as the retinoids bring about profound effects on amphibian (limb and tail) regeneration. In the regenerating amphibian limb, retinoids can proximalize, posteriorize and ventralize the axes of blastema (Niazi and Saxena, 1978; Maden, 1982, 1983a, b; Ludolph et al., 1990) whereas in regenerating tail their application causes development of abnormal, stunted or blunt structures (Niazi and Saxena, 1968), ectopic limbs and in some cases even pelvic girdles at the site of amputation (Mohanty-Hejmadi et al., 1992; Maden, 1993; Mahapatra et al., 2001; Mahapatra and Mohanty-Hejmadi, 1994; Muller et al., 1996; Pati et al., 2003; Mohanty-Hejmadi and Crawford, 2003). Biochemical investigation of vitamin A induced abnormally regenerated tails show elevated levels of oxidative stress (Mahapatra et al., 2002) and an elevation in specific activities of acid and alkaline phosphatase (Patnaik et al., 2012) in the Indian tree frog *Polypedates maculatus*. Molecular studies reveal up-regulation of certain retinoid receptors in the retinoid treated tail regenerates of the temperate frog *Rana temporaria* (Maden and Corcoran, 1996). However, the mechanism underlying the formation of such various forms of abnormalities is yet to be established.

Earlier studies suggest the involvement of two phosphatases – acid and alkaline in the process of regeneration (Ghiretti, 1950; Junqueira, 1950; Karczmar and Berg, 1951; Schmidt and Weary, 1962;

Schmidt and Weary, 1963) in different amphibians. Acid and alkaline phosphatases are basically phosphomonoesterases that cleave their substrates at an acidic and alkaline pH, respectively. While acid phosphatase is a prominent lysosomal marker enzyme (de Duve, 1983), alkaline phosphatase is mainly associated with plasma membrane and is considered as a marker for undifferentiated stem cells (O'Connor et al., 2008; Keeling et al., 2009). During limb (Miller and Wolfe, 1968; Inoue and Suzuki, 1969) and tail (Ghiretti, 1950) regeneration in urodele amphibians, there is elevation in the activities of both acid and alkaline phosphatase. Rise in the activity of alkaline phosphatase is reported during anuran tail regeneration (Junqueira, 1950). Further, treatment with retinoic acid leading to increased activity of acid phosphatase is evident during limb regeneration in urodeles (Ju and Kim, 1994) where it has been described to mediate dedifferentiation (Ju and Kim, 2010). Even though effect of vitamin A on activities of acid and alkaline phosphatase during tail regeneration has been studied in the tadpoles of Indian tree frog (Patnaik et al., 2012), such studies in other anuran species have not been investigated. This paper describes the effect of different doses of vitamin A on specific activities of acid and alkaline phosphatase during tail regeneration in another commonly available anura, the Asian toad *Duttaphrynus melanostictus*, a potential model for regeneration studies from Indian context.

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Materials and Methods

Chemicals

The chemicals used in this study were of analytical grade. MS222 (Tricaine methane sulphonate) was obtained from Himedia Laboratories, Mumbai, Maharashtra, India. Vitamin A tablets were procured from Piramal Healthcare Limited, Maharashtra, India. Sucrose, p-nitrophenyl phosphate, p-nitrophenol and folin-phenol reagent were obtained from Sisco Research Laboratory, Mumbai, Maharashtra, India. Bovine serum albumin was obtained from, Sigma, USA. Other chemicals used were of the highest purified grade available.

Collection of eggs and rearing of tadpoles

Egg strings of the Asian toad *Duttaphrynus melanostictus* were collected during the months of April to June in the years 2009-2013 from natural spawning grounds inside the city of Bhubaneswar, Odisha, India (20.2700° N, 85.8400° E). The hatchlings were reared in the laboratory following standardized procedure (Mohanty-Hejmadi, 1977) and were fed with boiled *Amaranthus* leaves *ad libitum*.

Tail amputation and vitamin A treatment

Hind limb bud stage (stages 27-29; Gosner, 1960) tadpoles were selected for tail amputation and grouped into three categories for the experiment. In the first group, designated as original group, non-amputated tails were taken for the enzyme assay. In the second group i.e. control group, regenerated tails of non-treated tadpoles were considered, while in the third group i.e. treated group, regenerated tails following vitamin A treatment were assayed. Based on different doses of vitamin A, the treated group was further subdivided into three categories i.e. T10 (10 IU/ml), T20 (20 IU/ml) and T30 (30 IU/ml). Prior to amputation, tadpoles were anaesthetized in 1:3000 solution of MS222 in conditioned tap water (tap water stored and aerated for 72 hours). Amputation was done with a sharp sterilized blade through the middle of the tail by keeping the tadpole laterally on a sterilized porcelain plate. After operation, the tadpoles were transferred to amphibian ringer solution for about 10 minutes to prevent further loss of blood. The tadpoles of treated group were treated with different doses of vitamin A palmitate for 72 hours (optimum treatment condition, present observation for this species). After treatment, tadpoles were reared in conditioned tap water whereas the control group tadpoles were directly reared in conditioned tap water following tail amputation.

Estimation of phosphatases

For estimation of specific activities of phosphatases, original and regenerated tails of control and treated groups after 5, 10 and 15 days post-amputation (dpa) were considered. A pool of 20 tadpoles was taken for each assay. Acid phosphatase (ACP) activity was determined according to Guha et al., (1979) and alkaline phosphatase (ALP) activity was determined according to Garen and Levinthal (1960) with p-nitrophenyl phosphate as substrate. The protein content of the sample was assayed as per Lowry et al., (1951). The specific activities of the enzymes were expressed as µmol p-nitrophenol (pNP) formed / mg protein / min at 37ºC.

Statistics

One way ANOVA test was used to assess the significant difference between pair of means. The level of significance was considered at 1% probability level ($p < 0.01$) and was calculated by Duncan's multiple range tests using SPSS package.

Photography

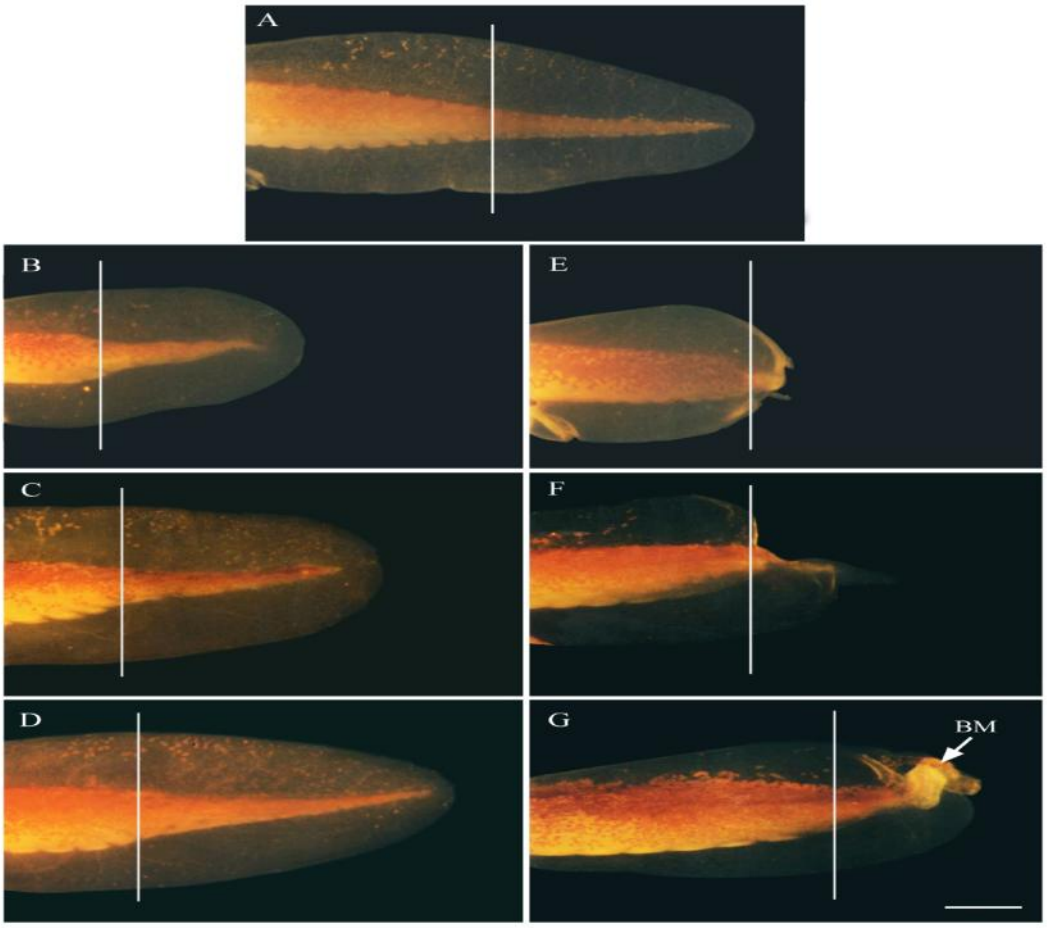
Photographs of tails were taken using a Leica MZ6 stereomicroscope and Pentax K1000 camera and further processed with Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, USA).

Results and Discussion

Morphology

The tadpoles of the control group regenerated normal looking tails whereas treatment with vitamin A led to abnormal tail regeneration with formation of bulbular masses at the cut end of the tail (Fig. 1). Formation of bulbular mass on application of vitamin A is reported earlier in the same species (Das and Mohanty-Hejmadi, 1998) and also other species of anurans (Maden, 1993; Mahapatra and Mohanty-Hejmadi, 1994).

Figure 1: A comparative account of original, control and vitamin A treated tails of tadpoles of *Duttaphrynus melanostictus*.



(A) Original tail. **(B)** Control tail 5 days post amputation (dpa). **(C)** Control tail 10 dpa. **(D)** Control tail 15 dpa. **(E)** Vitamin A treated abnormally regenerated tail 5 dpa. **(F)** Vitamin A treated abnormally regenerated tail 10 dpa. **(G)** Vitamin A treated abnormally regenerated tail 15 dpa. White line indicates plane of amputation. Scale bar (A-G) = 1 mm. Abbreviation: BM - bulbular mass.

Estimation of acid phosphatase (ACP)

ACP is a prominent lysosomal marker enzyme (de Duve, 1983) and is implicated in a variety of degradative processes, presumably autolytic in nature all of which results in conversion of endogenous or exogenous compounds into building materials for maintenance and repair (Osborne and Miller, 1963). In the present study, the specific activity of ACP was always higher in the regenerating tails (both control and treated groups) as compared to the non-amputated tails of the original group (Table 1). A comparative account of the specific activity of ACP in the original, control and the three treated groups has been presented in Fig. 2. High levels of ACP when compared to original tail is reported in the regenerating tail of the lizard *Podarcis muralis* (Alibardi, 1998). Similar elevation in the levels of ACP on regeneration is described in urodele limbs where they mediate cellular dedifferentiation and tissue remodelling processes (Miller and Wolfe, 1968; Ju and Kim, 1994). In the regenerated tails of the control group, the level of the enzyme increased significantly on 10 dpa followed by a decline on 15 dpa. High levels of ACP activity during first half of lens regeneration in the urodele *Triturus pyrrhogaster* suggests that ACP plays an important role in metabolism of nucleoprotein in the cells accompanying cellular multiplication, differentiation and growth (Setoguti, 1959). Likewise, in the present study, high ACP activity on 10 dpa suggests that ACP might help in differentiation and growth of the regenerate. In all the treated groups, specific activity of the enzyme remained higher than the corresponding control group. Here, the trend was similar to that of control with the activity peaking up at 10 dpa and then declining by 15 dpa and this increase in specific activity of ACP on 10 dpa was significantly different from 5 and 15 dpa tails. The T30 treated group showed maximum increase in enzyme activity which remained significantly higher than the corresponding tails of T10 treated group. Increase in activity of ACP on application of vitamin A is reported in the tail regenerates of *Polypedates maculatus* (Patnaik et al., 2012). Similar increase in ACP activity on treatment with retinoic acid (RA) is seen in regenerating limb of the urodele *Hynobius leechii* (Ju and Kim, 1994, 2010). Increase in ACP activity is thought to be due to increased synthesis of the enzyme or destabilization of lysosomal membrane or increased conversion of latent form of this enzyme into active form so as to maintain a prolonged and augmented state of dedifferentiation in the RA treated regenerates (Ju and Kim, 1994) for pattern duplication (Ju and Kim, 2010). Although there is no proof of dedifferentiation in the tails of *Duttaphrynus melanostictus*, up-regulation of ACP levels in the treated groups in the present study could be due to neosynthesis of the enzyme or release of the enzyme into extracellular matrix so that they can degrade tissue and liberate cells leading to formation of bulbular masses comprising of large number of undifferentiated cells (Das and Mohanty-Hejmadi,1998).

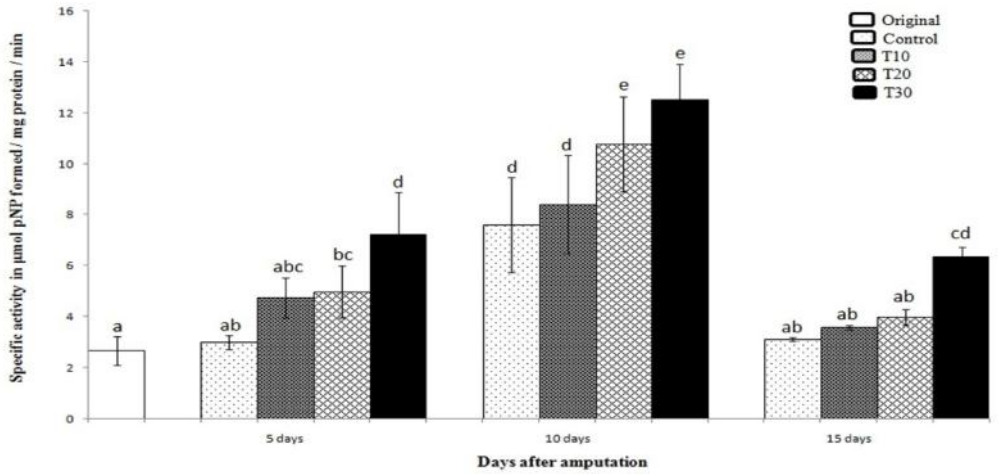
Table 1: Changes in specific activities of acid and alkaline phosphatase in regenerated tails of control and vitamin A treated tadpoles of *Duttaphrynus melanostictus*.

Phosphatases	Group	Days following tail amputation		
		5	**10**	**15**
ACP	C	1.129[¥]	2.861	1.171
	T10	1.784	3.160	1.349
	T20	1.871	4.051	1.498
	T30	2.715	4.696	2.380
ALP	C	1.769	3.047	1.432
	T10	4.488	7.484	3.994
	T20	5.224	9.093	4.858
	T30	6.080	10.337	5.780

[¥] Values in fold relative to original.

ACP - acid phosphatase; ALP - alkaline phosphatase; C – Control; T10 – 10 IU/ml treatment; T20 – 20 IU/ml treatment; T30 – 30 IU/ml treatment.

Figure 2: Specific activity of acid phosphatase in the original and regenerated tails of the control and vitamin A treated tadpoles of *Duttaphrynus melanostictus*.



Data are expressed as mean ± standard deviation of fine observations. Same superscripts over the bars represent data that are not significantly different.

Estimation of alkaline phosphatase (ALP)

ALP is considered as a marker for stem cells (O'Connor et al., 2008; Keeling et al., 2009). Though not much is known about its functional significance, it is suggested to be involved in cell division and differentiation associated with wound healing and initiation of regeneration process (Donachy et al., 1990). In the present study, specific activity of ALP showed a trend similar to ACP (Table 1). A comparative account of the specific activity of ALP in the original, control, T10, T20 and T30 treated groups has been shown in Fig. 3. In the control group, specific activity of the enzyme showed a significant increase by 10 dpa and then declined on 15 dpa. The findings of the present study support the view that ALP is intimately involved in the process of growth and regeneration (Junqueira, 1950; Osborne and Miller, 1963). In the urodele limb and tail regenerate, high level of ALP coincides with the blastema stage where dedifferentiation occurs (Ghiretti, 1950; Karczmar and Berg, 1951). The specific activity of the enzyme in the regenerated tails of the treated groups always remained significantly higher than the control group in the present study. In all treated groups, specific activity of the enzyme peaked on 10 dpa and then declined by the 15 dpa. Among the treated groups, the T10 and T30 groups were significantly different from each other. Increase in activity of ALP on application of vitamin A is reported in the anuran tadpoles of *Polypedates maculatus*, where it is correlated with the presence of undifferentiated cells in the tail regenerates (Patnaik et al., 2012). Involvement of undifferentiated cells during normal tail regeneration is known in anuran amphibians (Gurley and S´anchez Alvarado, 2008). Further, accumulation of loosely arranged undifferentiated cells in vitamin A induced abnormal tail regenerates is described earlier in the tadpoles of Asian toad, *Duttaphrynus melanostictus* (Patnaik, 2011). So, present observation of a higher level of ALP in vitamin A induced abnormal tails during mid of regeneration (10 dpa) is suggested to be related to presence of undifferentiated cells in the tail regenerates that later becomes a bulbular mass and subsequently differentiates into an ectopic organ.

Thus, it can be concluded that vitamin A elevates specific activities of both ACP and ALP during tail regeneration in the tadpoles of *Duttaphrynus melanostictus*. Considering the treated groups T10 and T30, it can be further concluded that a comparatively higher dose leads to a greater increase in activities of both the phosphatases.

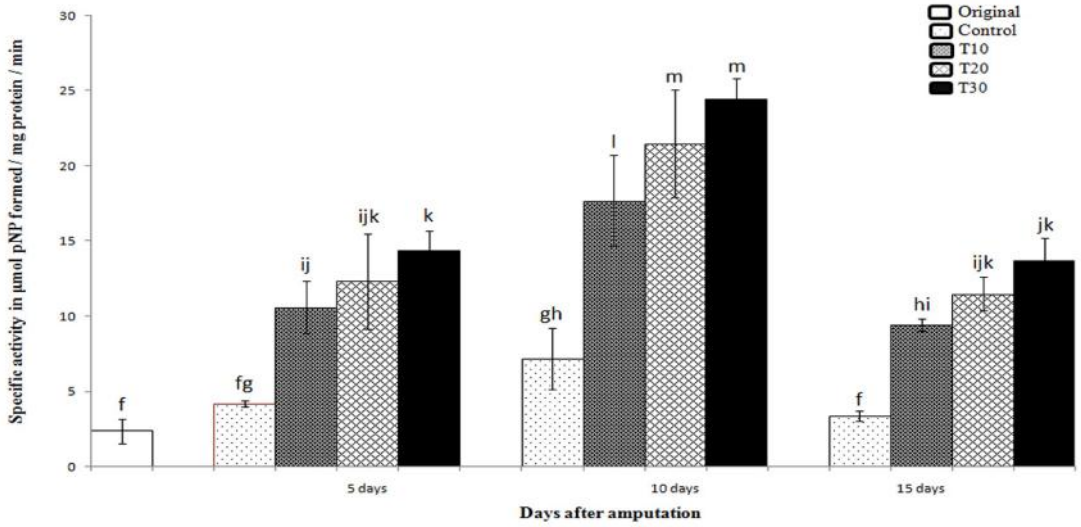


Figure 3: Specific activity of alkaline phosphatase in the original and regenerated tails of the control and vitamin A treated tadpoles of *Duttaphrynus melanostictus*. Data are expressed as mean ± standard deviation of fine observations. Same superscripts over the bar represent data that are not significantly different.

Acknowledgments

This work was supported by DST, Govt. of India (SR/SO/AS/41/2006) grant to Dr Mahapatra and DST PURSE grant to the Utkal University. NM would like to thank DST, Govt. of India for a Junior Research Fellowship and UGC for a research fellowship under UGC-BSR fellowship programme. The authors are indebted to the Head, P.G. Department of Zoology, Utkal University, India for providing necessary facilities.

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