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Amputation induced Reactive Oxygen Species regulate tissue regeneration in Clinotarsus curtipes tadpoles

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ABSTRACT

Regeneration is the ability of an organism to repair a wound which is partially destroyed tissue of animal's body or the damage involving the loss of an organ or the larger part of the body. Understanding the molecular mechanisms that promote tissue regeneration is necessary for advancements in regenerative medicine. Amphibian tadpoles' residents of aquatic systems, species Clinotarsus curtipes have the ability to regenerate their tails after amputation within 2 weeks effectively. Tail amputation induces a massive recruitment of inflammatory cells to the site of injury and produce high levels of ROS. Traditionally, ROS have been thought to have a negative impact on cells. But in this case they seemed to be having a positive impact on tail re-growth. Lowering levels of ROS impairs tail regeneration while sustained increased ROS are essential for downstream signaling pathway for proper tail regeneration. The findings demonstrate that injury-induced ROS production is an important regulator of tissue regeneration.

Keywords: Downstream signaling, Reactive Oxygen Species, Regeneration

I. INTRODUCTION

A large number of organisms are able to regenerate body parts. However tetrapods have the highest ability of regeneration. Amphibianshave long been used in the study of tail regeneration (Goss 1969). Urodeles regenerate their lost limbs and tails throughout their lives, but anurans do so only in a limited period in the tadpole stage.

Tadpoles have remarkable abilities to regenerate their tailsfollowing amputation via the coordinated activity of numerous growth factor signaling pathways, including the Wnt, Fgf, BMP, notch, and TGFβ pathways. Tail amputation induces a sustained production of reactive oxygen species (ROS) during tail regeneration. Sustained increased ROS levels are required for Wnt/β-catenin signaling and the activation of one of its major downstream targets, fgf20 7, which, in turn, is essential for proper tail regeneration (Love et al; 2013).

Oxygen derived free radicals are also very important mediators of cell injury and death (Joseph and Knight; 1995). Cells contain antioxidant defenses that respond to variations in cellular oxidant production. The imbalance between oxidants and antioxidants is termed oxidative stress (Sies; 1997). Evidence is present that implicates oxidants and antioxidants as a factor that can stimulate alteration in gene expression (Allen, 1991; Schulze-Osthoff and Baeurle, 1998) and the appearance of new tissue is preceded by the transcription of tissue specific genes and the concomitant suppression of transcription of genes that are specific to pluripotent stem cells(Allen, 1991). The aim of the present investigation was to study the

oxidative stress during amputation induced tail regeneration in C. curtipes.

C. curtipes, is endemic to the Western Ghats of peninsular India inhabiting the forest floors and sheltersunder the leaf litter (Dutta, 1997).An interesting feature of C. curtipes tadpoles is their size. Larval C. curtipes are the largest known anuran tadpoles inhabiting the Western Ghats. These tadpoles with long larval life are bulky, sluggish, andmove slowly in shoals making them vulnerable to diverse predators. Thus,evolution of large body size could be a morphological adaptation for survival especially since the tadpoleshave long larval period (Narahari P. et al, 2014). Oxidative stress was assessed by investigating lipid peroxidation (LPO). Furthermore, the antioxidant enzymes analyzed included superoxide dismutase (SOD) and catalase that are directly involved in processing of ROS. Reduced glutathione (GSH) a non-enzymatic antioxidant expressed during cell division and regeneration has been analyzed.

II. METHODS AND MATERIAL

A. Chemicals

Chemicals used in this study were of analytical grade. Thiobarbituric acid (TBA), bovine serum albumin (BSA), 5, 5'- dithiobis-2-nitrobenzois acid (DTNB), mercaptoethanol,nicotinamide adenine disodium salt (NADH), reduced glutathione (GSH), Folin-Ciocalteus reagent were obtained from Sigma Chemicals, India. All other chemicals were of the highest purified grade available.

B. Tadpoles collection and rearing

Tadpoles of *C. curtipes* were collected from a stream in the Western Ghats near Anamod, (15° 4′N and 74° 33′E) Karnataka, India during November–March, 2011 and transported quickly to the laboratory where they were maintained in plastic tanks with aged tapwater. The tadpoles were fed with boiled spinach leaves and the water was changed every alternate day.

C. Tail amputation

Limb bud stage tadpoles were taken for the experiment. Tadpoles were anaesthetized with MS222 prior to the tail amputation. Following amputation the experimental tadpoles were transferred to aged tap water. The control (original) tadpoles were transferred to aged water. 7 days, 15 days and 25 days post amputed tadpoles were re-amputed to study oxidative stress. Tail of non-amputated tadpoles were also taken after 7 days, 15 days and 25 days of the experiment. For each assay a pool comprising of 25 tadpoles was taken.

D. Estimation of oxidative stress

The original tail tips of limb – bud stage tadpoles were analyzed as the original group. Regenerated tail of 7 days, 15 days and 25 days post amputation were taken for analysis.

1) Lipid peroxidation: The lipid peroxidation was assayed as thiobarbituric acid reacting substance (TBARS) by the thiobarbituric acid (TBA) assay of Ohkawa et al., (1979). A 10% homogenate was prepared with 1.5% KCl. The homogenate was centrifuged at 4 ◦ C for 5 min at 1000 x g. The supernatant was used for the assay. The assay mixture contained 1 ml of homogenate mixed with 2ml of TBA-TCA-HCL complex. Solution was heated for 15 mins in boiling water bath. After cooling the absorbance was measured at 532nm. The protein was estimated by the Lowry et al (1951). The concentration of TBARS was calculated from the extinction co efficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Wills, 1969).

2) Superoxide Dismutase (SOD): SOD activity was measured using the method of Misra and Fridovich (1972). 10% homogenate was prepared in 0.25M sucrose. The homogenate was centrifuged at 12000

rpm for 20min. supernatant was used for the assay. The assay mixture contained 0.2 ml EDTA, 0.1ml NBT in 0.1ml of sample. Tubes were incubated for 5-8mins in light. Reaction was stopped by adding 0.05ml riboflavin after time interval of 1 min. Absorbance was measured at 560nm.

3) Catalase: Catalase activity was measured according to the method of Aebi (1974). A 10% homogenate was prepared in 0.25M sucrose and centrifuged at 12000 rpm for 20 min. Supernatant was used for the assay. The assay mixture contained 0.5ml of sample mixed with 2.5 ml of hydrogen peroxide. Tubes were incubated for 2mins. Reaction was stopped by adding 0.5ml of 1M HCl after time interval of 1min.Absorbance was measured at 240nm.

4) Reduced glutathione (GSH): The assay of GSH was done according to the method of Ellman et al (1959). A 10% homogenate was prepared in 5 % metaphosphoric acid. The homogenate was kept at room temperature for 15 min. Centrifuged for 30 min at 1000 x g at room temperature. 0.5ml supernatant was used. 2.5 ml of DTNB (5,5' dithiobis- 2 – nitrobenzoic acid) was added to the supernatant and centrifuged at 4500 x g for 5 min. Absorbance was measured at 412nm.

5) Statistical analysis: Statistical analysis was followed by the method of Gomez and Gomez (1984). The ANNOVA test to find out the significant difference between means was calculated by Duncan's multiple range test.

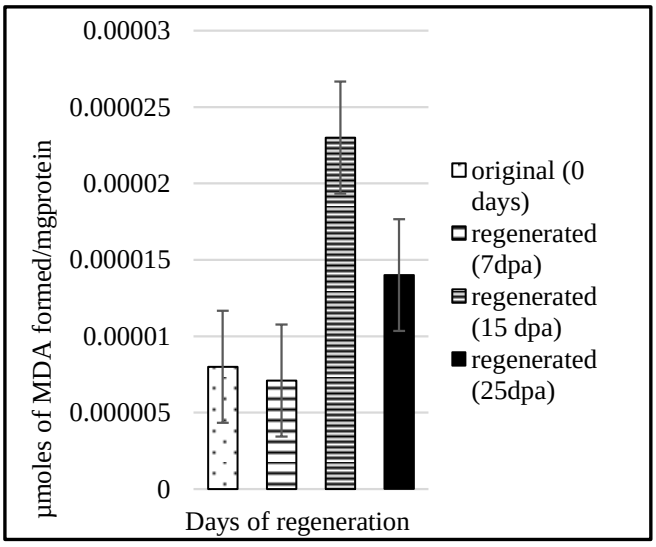
III. RESULTS AND DISCUSSION

The regenerated tail of 7 days post amputation were compared with the original tail. In the regenerated tail of 7 days amputated tadpoles LPO was lower than that of the original tadpoles (Fig. 1). The level of LPO had decreased to 0.7147 x 10^{-5}umoles MDA/mg protein in the regenerated tadpole tail from 0.8076 x 10^{-5}umoles MDA/mgprotein. An elevation was observed in the regenerated tail of 15 dpa and thereafter a reduction was observed in its level in the 25dpa tadpoles.

Table 1: Changes in oxidative stress parameters of the original and regenerated tails of tadpoles of *Clinotarsus curtipes*.

Oxidative parameter	Original (0 days)	Regenerated (7 days post amputation)	Regenerated (15 days post amputation)	Regenerated (25 days post amputation)
LPO[a]	0.8 x 10^{-5}	0.71 x 10^{-5}	2.3 x 10^{-5}	1.4 x 10^{-5}
SOD[b]	0.53	14	37.21	22.14
CAT[c]	1.89	7.80	21.3	13.3
GSH[d]	9.3 x 10^{-3}	10.71 x 10^{-3}	25.86 x 10^{-3}	16.6 x 10^{-3}

[a]level of lipid peroxidation (umoles MDA formed/mg protein).



[b] Activity of SOD (units of SOD/mgprotein)
[c]Activity of Catalase (umoles/ mg protein/ min)
[d] Level of Reduced glutathione (umoles/mgprotein)

Fig. 1. Lipid peroxidation (umoles of MDA formed/mgprotein)in the original and regenerated tailof tadpoles following 7, 15 and 25 days of post amputation in the tadpoles of *C. curtipes.*

In comparison to the original tail highest level in the activity of SOD was observed in the regenerated tails of 7 days post amputation. (Fig. 2). SOD activity showed significant increase in 7 and 15dpa tails of tadpoles, thereafter a decrease was seen in the 25dpa tails.

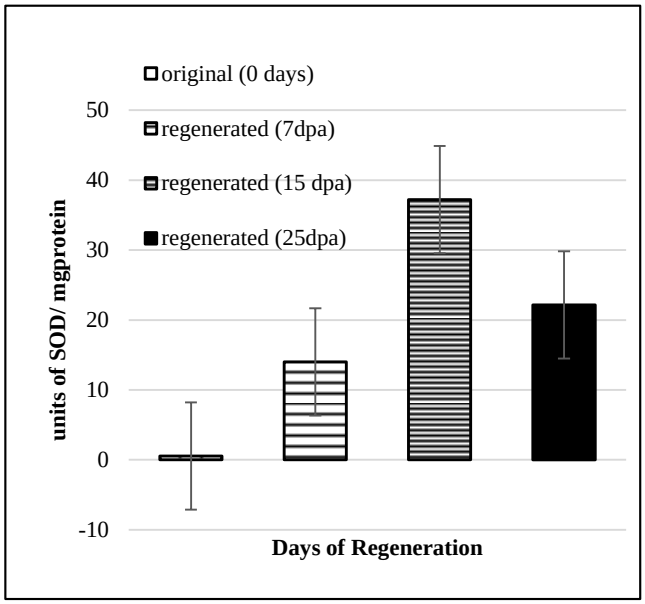


Fig. 2 Superoxide dismutase (units of SOD/mgprotein) in the original and regenerated tail oftadpoles following 7, 15 and 25 days of post amputation in the tadpoles of *C. curtipes.*

An elevation in the catalase activity was observed in the regenerated tail of 7 and 15 dpa tadpoles of *C. curtipes* in comparison to the original tail. (Fig. 3). The activity of catalase remained higher throughout and always higher than original tail. The Level of Catalase evaluated per milligram of protein was 1.89 umoles/mg protein in original and 7.8 umoles/mgprotein in the regenerated tadpoles of 7 dpa and 21.3 µmoles/mgprotein of 15dpa tadpoles of *Clinotarsuscurtipes* species. One time decrease was observed in the 25dpa tadpoles in comparison to 15dpa tadpoles.

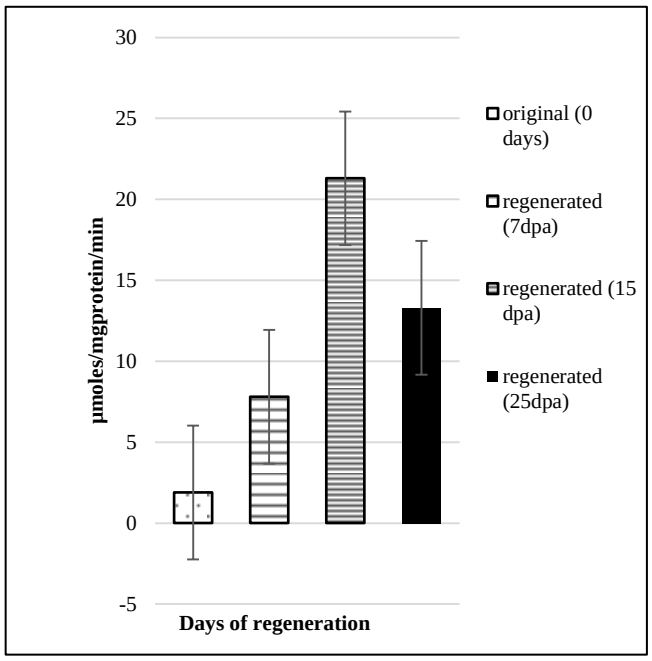
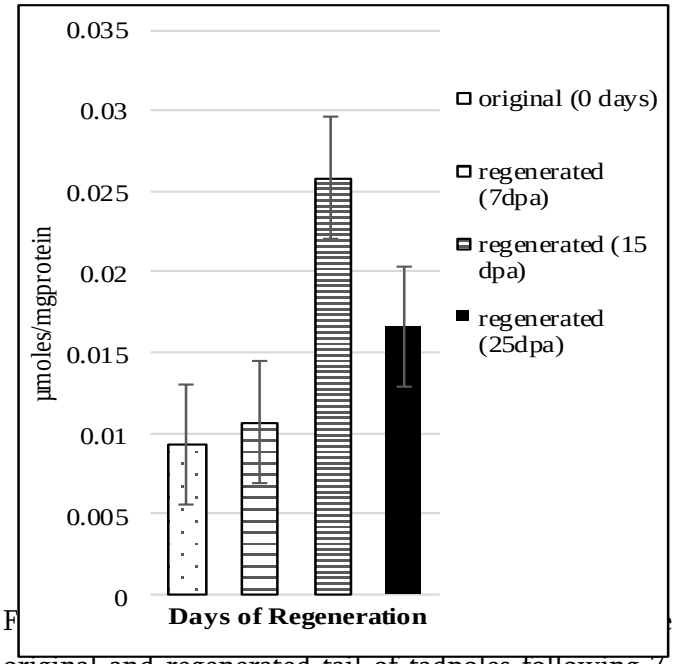


Fig. 3 Catalase activity (umoles/mgprotein/min) in the original and regenerated tail of tadpoles following 7, 15 and 25 days of post amputation in the tadpoles of *C. curtipes.*

The level of reduced glutathione (GSH) also showed elevation in the regenerated tail of 7 and 15 dpa in comparison to original tail of the *C. curtipes* tadpoles. While regenerated tail of 25dpa showed decrease in comparison to the 15 dpa tadpole tail.



F original and regenerated tail of tadpoles following 7,

15 and 25 days of post amputation in the tadpoles of *C. curtipes.*

A comparative account of the changes in oxidative stress parameters in the regenerated tail and original tail has been summarized in Table 1.

Different kinds of physiological factors can result in oxidative stress. For example, the antioxidant status of animals has been investigated in fish under anoxia and reoxygenation (Lushchak et al., 2001), in frogs under anoxia and recovery (Hermes-Lima and Storey, 1996,T.V. Bagnyukova, K.B. Storey, V.I. Lushchak, may 2002) and with respect to temperature changes (Perez-Campo et al., 1990; Joanisse and Storey, 1996).

We have observed a higher level of SOD and Catalase in the regenerated tails of 7 and 15 dpatadpoles (Fig. 2 and Fig. 3). Catalase enzyme is a scavenger of H_2O_2. H_2O_2acts as a secondary messenger molecule, affecting the proliferation and differentiation of cells. (Sehulze –Osthoff and Fiers, 1991).It has been observed in frog muscle that increased catalase activity minimizes cellular oxidative stress by lowering the concentration of H_2O_2. (Hermis-Lima and Story, 1998).In the present study the elevation in catalase activity is suggested to be due to increase in level of H_2O_2 in earlier stages i.e. 7 and 15dpa tadpoles.

A comparatively high activity of SOD (Fig. 2) was observed in the regenerated tails of 7 and 15 dpa tadpoles. SOD is widely distributed in aerobic organisms and plays an important role in the control of radical superoxide levels in the cellular compartments (Sasaki et al., 1997, Idowu Emmanuel Taiwo, AmaezeNnamdi Henry, Adie Peter Imbufe and OtubanjoOlubunmiAdetoro 2013.) An increase in the level of SOD has been observed during development in several amphibians (Bajra De Quiroga and Gutierrez, 1984). It has been explained that increase in SOD rather than the antioxidant properties of the enzyme seem to play a role in stimulating gene expression. (Allen and Balin, 1980). The level of LPO comparatively showed decrease at the same time in 7dpa whereas showed sudden increase in the 15dpa tadpoles. Our findings support the view of Allen and Balin. LPO disintegrates the biomembrane rich in polyunsaturated fatty acids (PUFA) which are susceptible to oxidation (Rady, 1993).

It is evident in amphibians that GSH concentration increases during mitotic phase of regeneration and decreases during redifferentiation of regenerating tissue. (Fig 4) This rise is due to the high level of cell division during this period. Decrease in GSH is seen in cells exhibiting a decline in mitotic acvity. In our observation there gradual increase in 7 dpa tadpole whereas a sharp increase in the 15dpa tadpole tail due to the high level of cell division during this period. The decline in the level in 25dpa tadpoles is due to the slowing of rate of cell division.

III. CONCLUSION

The remarkable observations of the present study includes higher level of SOD and Catalase and a lower level of LPO in the 7 dparegenerated tail of tadpoles. The above findings demonstrate that injury-induced ROS production is an important regulator of tissue regeneration and establishes a hyper oxidative stress condition in the regenerated tadpole tail in comparison to the original tail.

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