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# Studying the Effect of Amputation-Induced Oxidative Stress on Tadpole Tail Regeneration in *Clinotarsus curtipes*

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***Abstract***

***Background and Aim:*** *The role of oxidative stress, particularly reactive oxygen species (ROS), in tissue regeneration is increasingly recognized as crucial. This study aimed to investigate the role of ROS in the regeneration of tadpole tails in Clinotarsus curtipes following amputation, focusing on lipid oxidation, superoxide dismutase (SOD), catalase, and reduced glutathione (GSH) activity.*

***Material and Methods:*** *This prospective observational study was conducted in a controlled laboratory setting. The study population consisted of tadpoles subjected to tail amputation, and tissue samples were collected at regular intervals. Key biochemical markers, including lipid oxidation, SOD activity, catalase activity, and GSH levels, were measured to assess oxidative stress and antioxidant response.*

***Results:*** *Peak antioxidant activity was observed at 15 days post-amputation. Hydrogen peroxide was identified as a significant contributor to the regeneration process. ROS levels were elevated early in the regeneration process, and a decline in lipid oxidation and increase in antioxidant enzyme activity was noted as regeneration progressed.*

***Conclusion:*** *The findings suggest that ROS, particularly hydrogen peroxide, play a pivotal role in the regeneration of tadpole tails. Understanding the interplay between oxidative stress and tissue regeneration could have implications for developing therapeutic strategies for wound healing and regenerative medicine.*

***Keywords:*** *Tissue Regeneration, Oxidative Stress, Reactive Oxygen Species (ROS), Tadpole Tail Regeneration, Antioxidant Enzymes*

## INTRODUCTION

Regeneration is the ability of an organism to replace or restore damaged tissues. Tadpoles, especially species like *Clinotarsus curtipes*, exhibit remarkable regenerative capacity. Recent studies have shown that oxidative stress, induced by reactive oxygen species (ROS), plays a crucial role in tissue regeneration. ROS are traditionally considered harmful due to their ability to damage cells, but emerging evidence suggests they also serve as important signaling molecules in regeneration (He *et al.*, 2017).

Previous studies have shown that ROS are critical for regeneration in various animal models, including zebrafish (Yoo *et al.*, 2012), geckos (Zhang *et al.*, 2016), and axolotls (Carbonell *et al.*, 2021). However, the role of ROS in *Clinotarsus curtipes* tadpoles has not been well explored. This study aims to investigate how ROS affect tail regeneration in these tadpoles.

## METHODOLOGY

High-purity chemicals, including thiobarbituric acid (TBA), reduced glutathione (GSH), and other analytical grade reagents, were sourced from Sigma Chemicals and SRL, Chennai (Brehe & Burch, 1976). Tadpoles of *Clinotarsus curtipes* were collected from a stream in Papanasam, Tamil Nadu, India, during the months of November to March. Upon collection, they were transferred to the laboratory, where they were housed in plastic tanks under controlled conditions. The water in the tanks was changed every other day to ensure cleanliness, and the tadpoles were fed cooked spinach leaves as their primary food source. For the tail regeneration experiments, tadpoles were anaesthetized using MS222 (Tricaine Methanesulfonate) prior to amputation (Topic Popovic *et al.*, 2012). Tail amputations were conducted under sterile conditions, and the tadpoles were allowed to regenerate their tails. Samples of regenerating tails were collected at 0, 5, 15, and 30 days post-amputation for analysis. A control group of non-amputated tadpoles was also maintained throughout the study for comparative analysis.

### Assessment of Oxidative Stress

- **Lipid Oxidation Assay**: Tissue samples were processed to measure malondialdehyde (MDA), a biomarker of lipid peroxidation, using the TBARS method (Sørensen & Jørgensen, 1996).
- **Superoxide Dismutase (SOD) Activity**: Using the Misra and Fridovich method, SOD activity was measured to assess the enzyme's ability to reduce oxidative damage (Misra & Fridovich, 1977).
- **Catalase Activity**: Catalase activity was measured following Aebi's method by determining the breakdown of hydrogen peroxide into water and oxygen (Aebi, 1974).
- **GSH Assay**: Reduced glutathione (GSH) levels were measured using the Ellman *et al.*, method (Brehe & Burch, 1976).

## RESULTS

### Lipid Oxidation Assay

A lipid oxidation assay, also known as a TBARS assay, measures malondialdehyde (MDA) levels in a sample to detect oxidative stress. MDA is a biomarker for lipid peroxidation, which is the oxidative degradation of unsaturated fatty acids, cholesterol, and other components. MDA is a useful biomarker for lipid peroxidation that has been used extensively for a long time. The assay involves a reaction between MDA and thiobarbituric acid (TBA) to form MDA-TBA adducts, which are then measured spectroscopically (532 nm). Figure 1 displayed the standard curve for Lipid Oxidation Activity, measured in µmol/L of MDA. 15 days after amputation, the newly grown tails exhibited a higher level of MDA activity compared to the original tails (Figure 2). There was a significant rise in MDA activity in tadpoles' tails at 15 dpa, followed by a decline in the tails at 30 dpa.

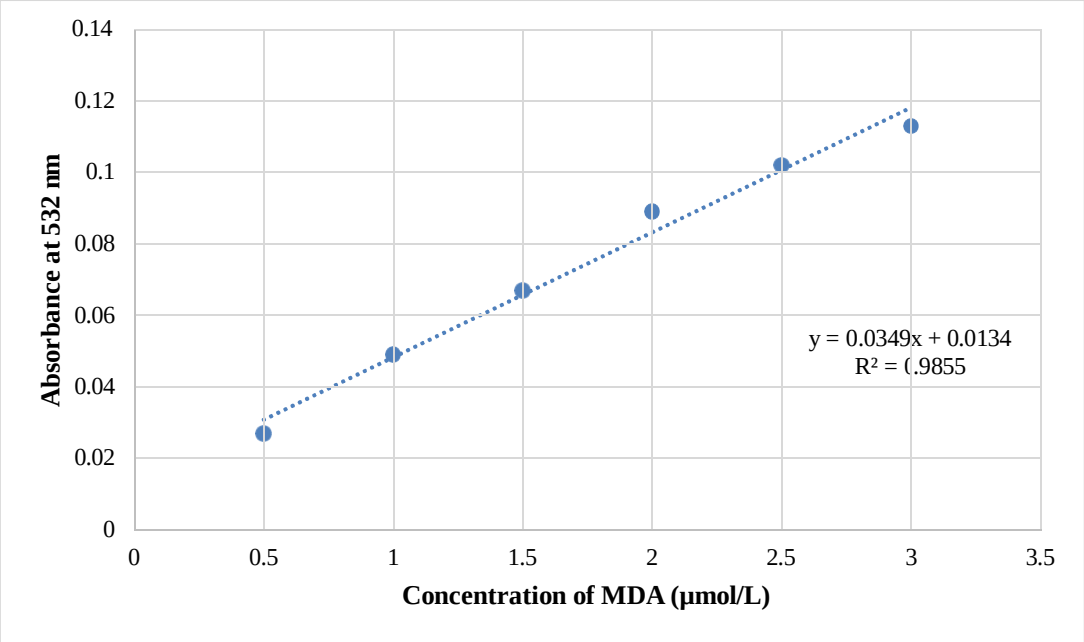


**Figure No. 1:** MDA Standard Curve for Lipid Oxidation Assay

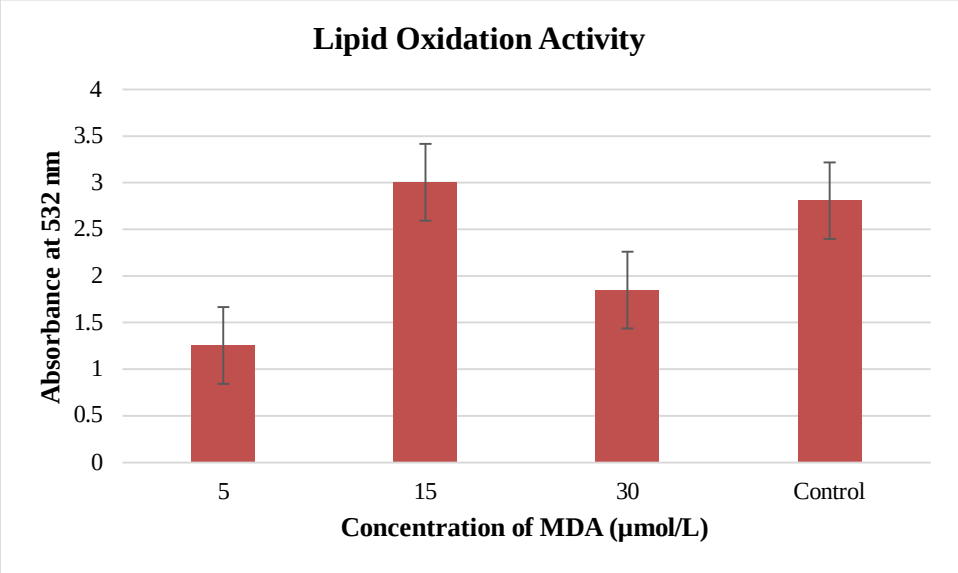


**Figure No. 2:** Lipid Oxidation Activity (MDA in µmol/L) in the control tadpoles (non-amputated) and regenerate tails at 0, 5, 15, and 30 days after amputation in *C. curtipes* tadpoles

**SOD Activity**

SOD enzymes function by transforming the superoxide (O2.-) radical into hydrogen peroxide and oxygen. This mechanism ensures a equilibrium between generating and scavenging biological oxidants in the body. Superoxide dismutase (SOD) enzymes facilitate the decomposition of the harmful superoxide (O2.-) free radical

into hydrogen peroxide and oxygen, contributing greatly to reducing oxidative stress in our body (Fridovich, 1975). SODs have been shown to provide protection against various skin conditions (Chen et al., 2012). In Figure 3, the standard curve for measuring Superoxide Dismutase (SOD) Activity in µM was shown. In Figure 4, the tails that were regrown after 5$^{th}$ day of amputation showed a higher SOD activity level than the original tail (Control). A notable increase in SOD activity was observed in tadpoles' tails at 15 dpa, which was then followed by a decrease in SOD activity in the tails at 30 dpa.

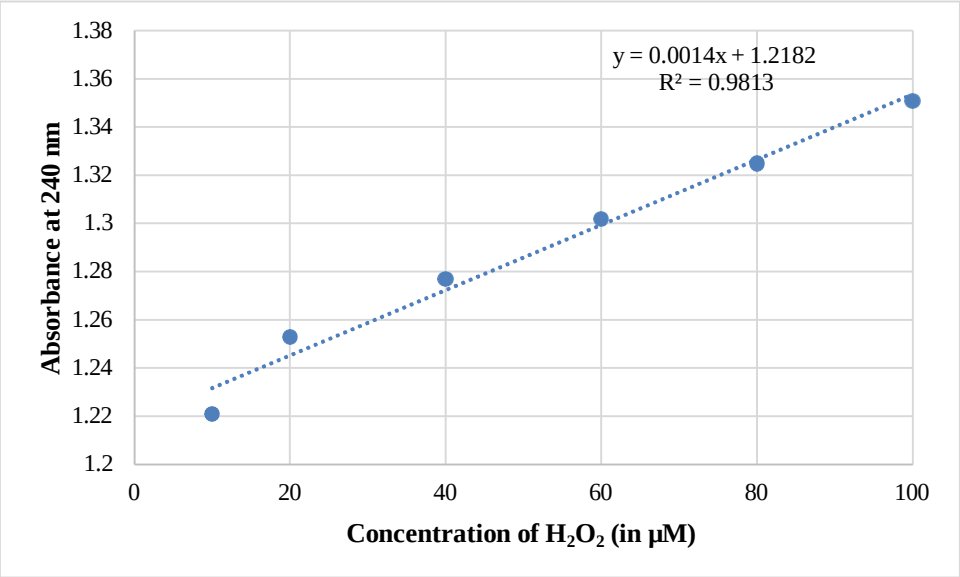


**Figure No. 3:** Standard Curve of $H_2O_2$ for the calculation of SOD and Catalase activity

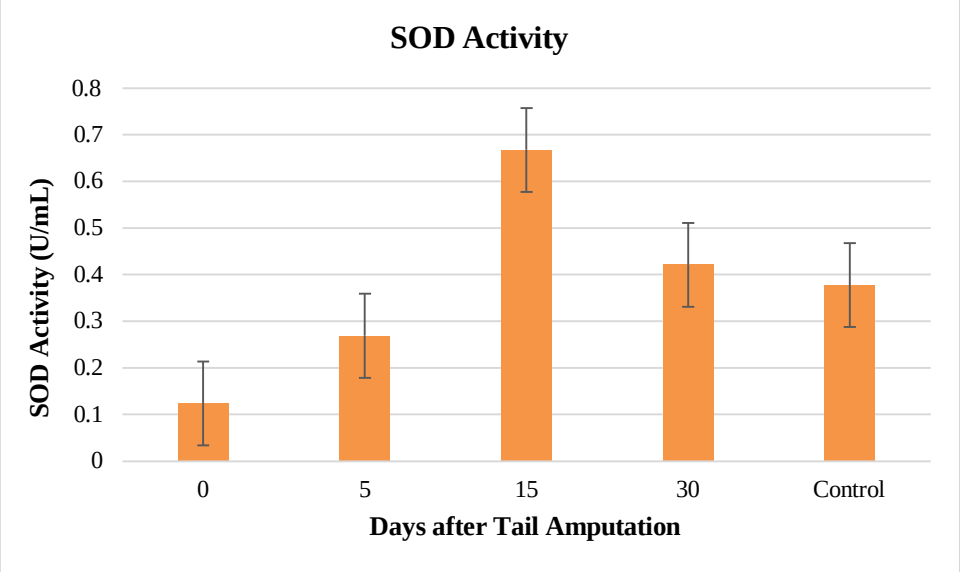


**Figure No. 4:** Superoxide Dismutase (SOD) Activity. Superoxide dismutase (U/mL) in the control tadpoles (non-amputated) and regenerate tails at 0, 5, 15, and 30 days after amputation in *C. curtipes* tadpoles.

### Catalase Activity

Catalase activity was found to be higher in the regenerated tails of *C. curtipes* tadpoles that were 5 and 15 dpa compared to the original tail (Figure 5). Throughout, the catalase activity was consistently higher than the control. Compared to the control group and 15dpa tadpoles, a decrease was seen in the 30 dpa tadpoles.

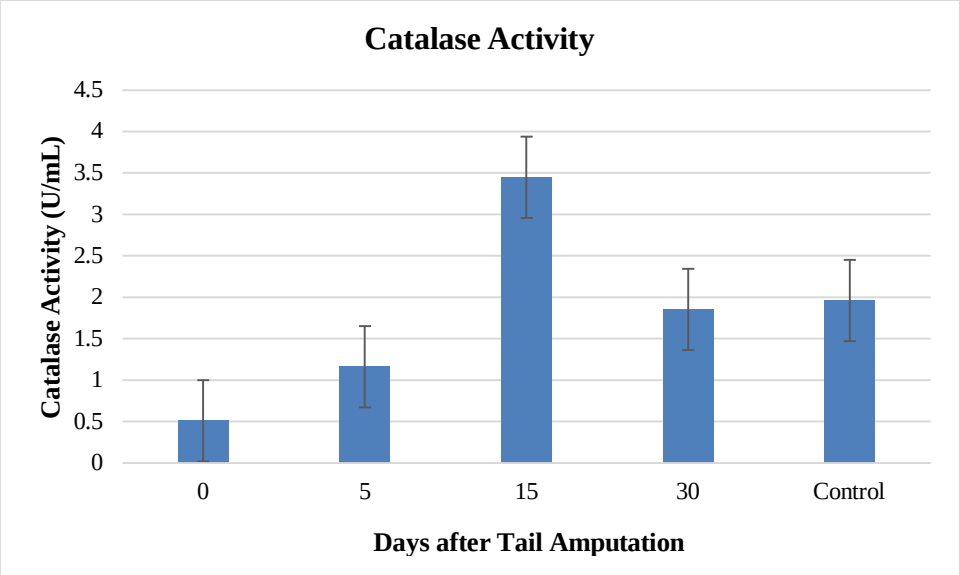


**Figure No. 5:** Catalase Activity (U/mL) in the control tadpoles (non-amputated) and regenerate tails at 0, 5, 15, and 30 days after amputation in *C. curtipes* tadpoles

### GSH Activity

During the course of its life, a tadpole can regenerate its amputated tail. According to some research, reduced glutathione (GSH), a non-enzymatic antioxidant, gradually increases in tadpoles with regenerating tails. In the original tails of the *C. curtipes* tadpoles, a proximo-distal gradient of reduced glutathione (GSH), a non-enzymatic antioxidant, was seen. The Standard Curve of GSH was shown in Figure 6. A progressive rise in GSH was observed in the regenerating tails at days 5 and 15 after amputation, as the tails grew longer. The GSH level in the proximal region of the freshly regenerated tails was likewise higher than in the control original tails (Figure 7).

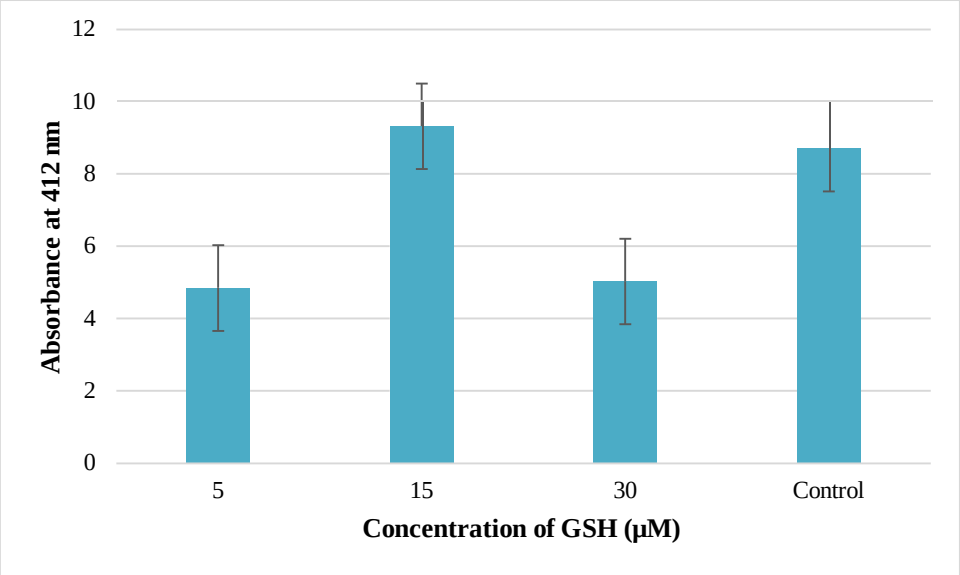


**Figure No. 6:** Standard Curve of GSH for the calculation of GSH activity

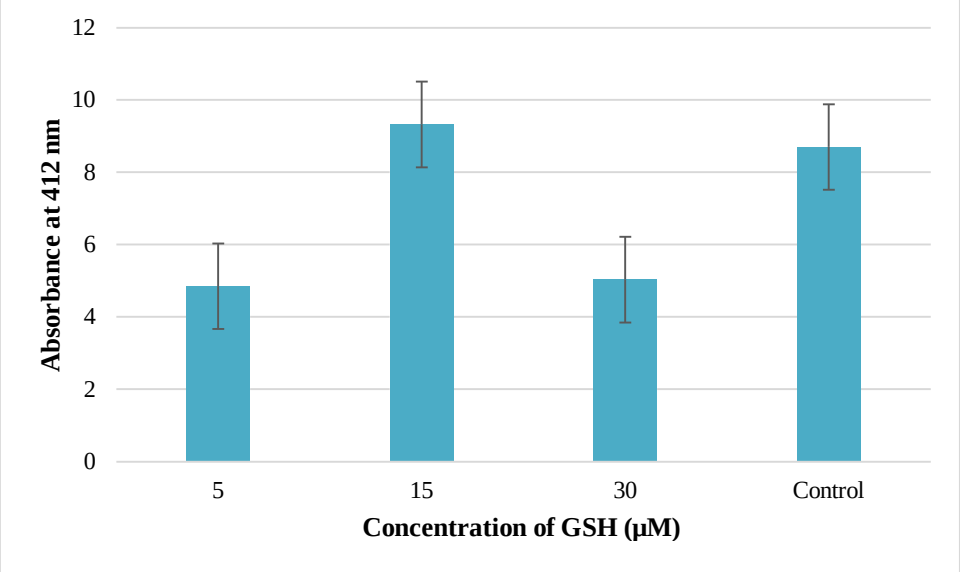


**Figure No. 7:** GSH activity (µM) in the control tadpoles (non-amputated) and regenerate tails at 0, 5, 15, and 30 days after amputation in *C. curtipes* tadpoles

## DISCUSSION

The process of replacing or returning a damaged cell, tissue, or organ to full function is known as regeneration, and it is limited by the degree of cell differentiation and the complexity of particular organ structures. This tissue replacement form is activated upon pathological stimuli such as injury and/or disease, which typically involves an inflammatory response, in compared to physiological cell turnover. The degree to which tissue heals itself is dependent on a variety of variables and processes (Iismaa *et al.*, 2018).

The anti-oxidant system's production and/or removal of ROS determines their intracellular concentration (He *et al.*, 2017). Numerous antioxidants are found in cells, where they function to both regulate redox-sensitive signalling pathways and prevent or repair damage caused by reactive oxygen species (ROS) (Genestra, 2007). Three of the main antioxidant enzymes found in mammalian cells—catalase, glutathione peroxidase (GPx), a substrate-specific peroxidase, and superoxide dismutase (SOD)—are considered to be essential for life in all oxygen-metabolizing cells (Weydert and Cullen, 2010). While catalase and peroxidases convert hydrogen peroxide into water and, in the case of catalase, oxygen and water, superoxide radical is converted by SODs into hydrogen peroxide and molecular oxygen (O2) (Augusto and Miyamoto, 2011). Hydrogen peroxide is converted to water and oxygen by catalase. The majority of catalase activity is found in peroxisomes, which are subcellular organelles. Superoxide and hydrogen peroxide are ultimately transformed into water (Sandalio *et al.*, 2013). While GPx requires multiple co-factors and proteins in addition to having five isoenzymes, SOD and catalase can function without co-factors (Ighodaro and Akinloye, 2018). The seleno-protein known as cytosolic glutathione peroxidase (GPx, GPx1) was initially identified as an enzyme that shields hemoglobin in red blood cells from oxidative degradation. GPx to operate as efficiently as possible, it needs a number of secondary enzymes, including reduced glutathione, NADPH, and glucose 6-phosphate dehydrogenase, as well as cofactors. As was previously mentioned, there are five GPx isoenzymes, with GPx1 being regarded as a key enzyme in the removal of $H_2O_2$. This enzyme's overexpression prevents oxidative damage to cells and inhibits $H_2O_2$-induced apoptosis (Ighodaro and Akinloye, 2018).

Tadpoles of *C. curtipes* have the remarkable capacity to regenerate a wide range of tissues, including the tail, among vertebrates. Given the complexity of this structure, tail regeneration is an intriguing process that serves as one of the primary and most widely used models for the study of morphogenesis control, regenerative response, and final patterning of the regenerated structure. In addition to the AEC's formation, the remnant tissue undergoes additional processes like histolysis and extracellular matrix remodeling that promote dedifferentiation and re-entry into the cell cycle. As a result, progenitor cells, including resident stem cells, accumulate between the remnant tissue and the wound epithelium to form blastemas (McCusker *et al.*, 2015). Ultimately, a phase of morphogenesis and growth of the regenerating structure occurs to form the lost or amputated tail after the blastema has grown and begun to pattern (McCusker *et al.*, 2015; Stocum, 2017). Many signals have been found to be involved in tail regeneration, ranging from the initial amputation to the blastema's formation, growth, and patterning, as well as the morphogenesis of structures derived from blastemas (Yokoyama, 2008; Stocum, 2017).

It's interesting to note that, despite being classified as harmful molecules for cellular and tissue homeostasis, reactive oxygen species (ROS) are now of great interest in the field of developmental biology and regeneration due to their ability to control a variety of cellular processes, including apoptosis, migration, cell proliferation, and differentiation (Covarrubias *et al.*, 2008; Hernández-García *et al.*, 2010; Meda *et al.*, 2018; Rampon *et al.*, 2018; Sies and Jones, 2020a). Interestingly, ROS can control the activity of many different molecules, such as kinases and transcription factors (Marinho *et al.*, 2014; Sies, 2017; Rhee *et al.*, 2018). Prior research has shown that the generation of ROS is a pro-regenerative signal that occurs in both vertebrates and invertebrates following the amputation of different structures (Pirotte *et al.*, 2015; Rampon *et al.*, 2018). ROS have been widely implicated in the regeneration of appendages in vertebrate models, including the tail fin in zebrafish (Yoo *et al.*, 2012; Gauron *et al.*, 2013; Romero *et al.*, 2018; Thauvin *et al.*, 2022), the tail of the gecko (Zhang *et al.*, 2016), and the tail regeneration in Xenopus (Love *et al.*, 2013; Ferreira *et al.*, 2016, 2018). Of particular note, it has been demonstrated in a number of these models that ROS control the activity of signals like kinases, Wnt, Fgf, and Shh, which are thought to be essential signals for limb regeneration (Yoo *et al.*, 2012; Love *et al.*, 2013; Romero *et al.*, 2018; Thauvin *et al.*, 2022). Furthermore, we have found that ROS, in particular $H_2O_2$, trigger tail regeneration in young axolotls by controlling blastema development and growth via activation of Yap1, a transcriptional co-activator of the Hippo signaling pathway, as well as by controlling Agr2 expression

and AKT kinase activation (Carbonell *et al.*, 2021). Zhang *et al.*, (2018) conducted a fascinating study in Xenopus that demonstrates the importance of reactive oxygen species (ROS) production triggered by melanocortin receptor 4 (also known as melanocortin four receptor, or Mc4r) in driving tissue regeneration.

## CONCLUSION

Since the production of reactive oxygen species (ROS) is necessary in several animal models to favour and promote tissue, organ, and appendage regeneration, ROS are molecules of great interest in the field of regenerative biology. It has recently been demonstrated that *C. curtipes* tail regeneration requires the generation of reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$). Nevertheless, whether ROS generation is required for tail regeneration in this animal model is still unknown. The bicolored frog *(Clinotarsus curtipes*) had its tails amputated proximally in order to study the dynamics of oxidative stress during regeneration. Enzymatic assays were employed to investigate the production of ROS. After that, exogenous hydrogen peroxide ($H_2O_2$) was used for all of the assays, including GSH, Catalase, SOD, and lipid oxidation. According to the findings of our investigation, *C. curtipes* produces reactive oxygen species (ROS) after amputation, which promotes the regeneration of a tiny tail. Furthermore, the tail amputation caused an increase in ROS production. Depending on the species, oxidative stress—a disrupted balance between free radicals and antioxidants in the body—may contribute to tail regeneration in various ways. Both detrimental and beneficial effects of oxidative stress on regeneration are possible. According to the current research, the tadpoles' regenerated tails exhibit a hyper-oxidative stress condition. We conclude that tissue growth or regeneration is most likely linked to an increase in oxidative stress and is achieved through increased cell division. According to our research, appropriate tissue regeneration and tail patterning require the early generation of ROS and $H_2O_2$.

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