UNDERSTANDING THE ROLE OF CYCLOOXYGENASE IN THE TEMPORAL REGULATION OF INFLAMMATORY MEDIATORS DURING WOUND HEALING IN LIZARD

[SYNOPSIS OF THE Ph.D. THESIS]

February-2021

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“There is something beautiful about scars, of whatever nature. A scar means the hurt is over, the wound is closed & healed, done with.”

-Harry Crews

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INTRODUCTION

Regeneration- The Reigning Resplendence

In the pursuit of successful existence on the planet, organisms endure a lot of physical trauma, through which life strives and arrives triumphant. Wounds turn to scars and the animal prepares itself for the next battles. Amongst these life forms, few are the chosen ones, which are bestowed with the exceptional capability to repair their wounds in the most efficient way. Some species give rise to new individuals from the severed parts, while others can regrow the entire lost organ, maintaining its functionality. Few others can simply cover the wounds but can't compensate for the lost organ in any way. Such observations across the animal kingdom prove that wound healing potential is scattered in the different species and the scale and dimensions of repair vary in all animal phyla (Bely & Nyberg, 2010; Tanaka, 2003). This subtle discrimination in the regenerative behaviour has been questioned and studied since generations yet, the mechanistic details and the molecular regulators of this mega developmental event are vaguely understood (Brockes & Kumar, 2008).

Regeneration realms in Animal Kingdom

Across the metazoan classes, regenerative potential is confined to few members while it is completely absent in others. Studying the mechanism underlying the classical losses of regeneration by certain species can highlight its ecological and evolutionary role (Bely & Nyberg, 2010). Scientists have tried severing the homologous organs of many model organisms simultaneously, to compare their restorative competence, which proved the distinct repair approaches followed by various species (Bely & Nyberg, 2010). Researchers witnessed challenges in comprehending the gain and loss of regeneration across animal phyla, as the homology problem persists amongst the different clades of metazoans (Brockes, Kumar, & Velloso, 2001). For an instance, comparing an arm of star-fish, with the limb of axolotls is illogical, although both elicit complete reformation of these organs, post amputation.

Such observations compelled the researchers to further explore the cellular and molecular details of regeneration, wherein both loss and gain of regeneration can be analysed spanning through the diverse classes of animals (Alvarado, 2000). Concentrating on the cellular participants of regeneration brought tissue localised stem cells under the lime light. Researchers speculated the unearthed calibre of the somatic stem cells in causing regeneration during post embryonic life stages in many species. Meanwhile, the possibility of differentiated cells regaining their stem cell like features, opened up new horizons of opportunities for scientists to address the ever-intriguing question- whether humans can use these cellular potentials to regenerate their lost body parts (Tanaka, 2003).

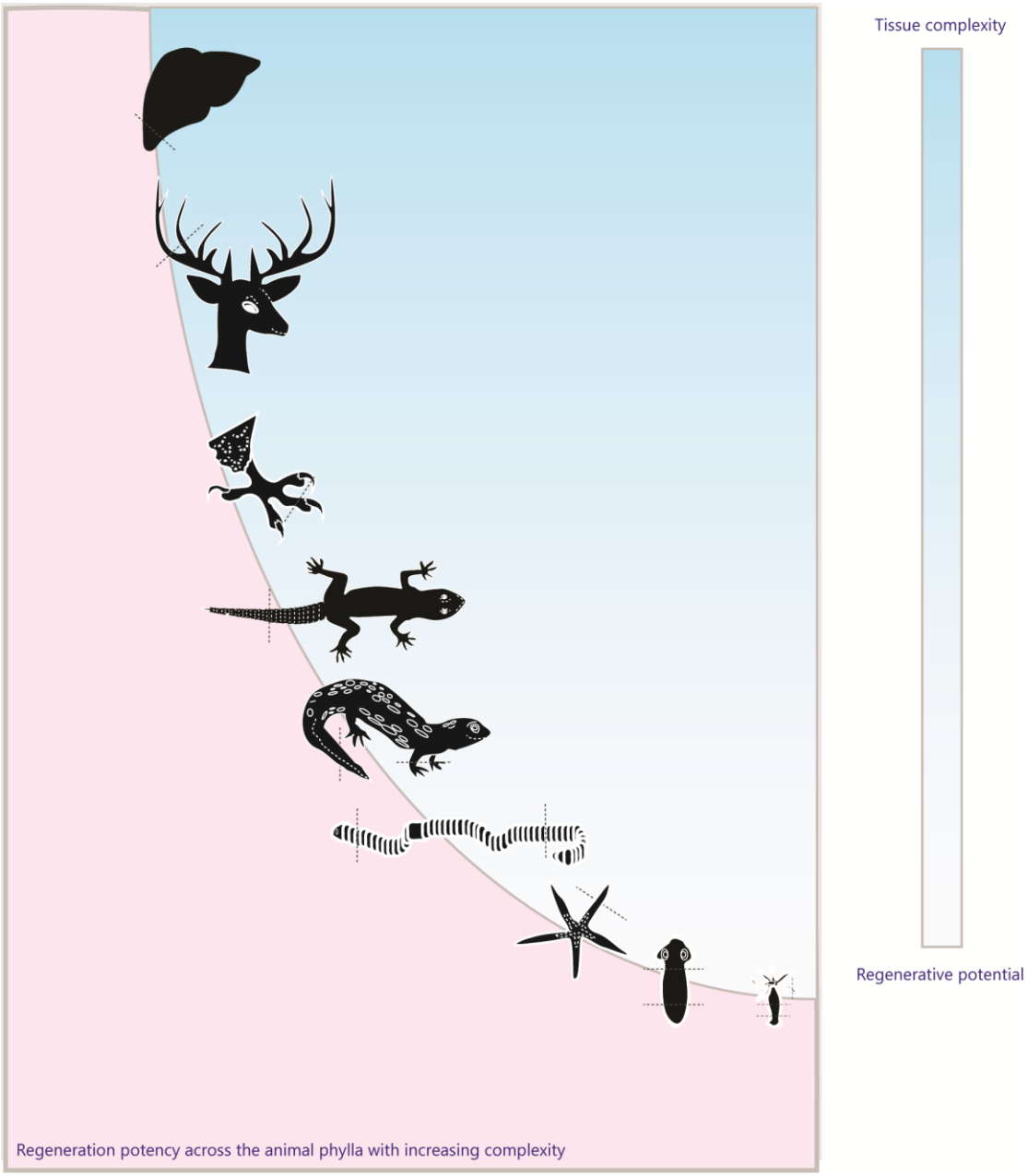


Figure 1. The progressively reducing regenerative potential across the animal phyla.

A huge array of animal models has been dissected to critically investigate and unearth the mechanisms of restoration and the aftermath of regeneration (Li, Yang, & Zhong, 2015). The take home lessons from all the detailed studies converges on a postulate, that with increasing complexity and developmental articulation, the organisms lose their knack on the regenerative front, or it is strictly confined to few body parts of these individuals. For an instance, planaria is capable of regenerating an entire organism from the broken parts, while physiologically most advanced humans can restore only few tissue types.

Humans have been curious to decipher the 'regenerative code', underlying the explicit healing and restorative potential of these apparently elementary organisms. Salamanders, Axolotls and many anuran species have been studied elaborately to unravel the cerebral components of regeneration at its molecular level (Wake and Dresner, 1967; Fior, 2014). Teleost fishes and mammals like mice provide a very elaborate account of all the organ systems which contribute to the platter of regenerating tissue systems. A large group of scientists have considered the *in vitro* study approach for deciphering the cellular and molecular intricacies of stem cell mediated regeneration as observed in the post-embryonic tissues of the adult mammalian model systems (Gawriluk et al., 2016).

Of the many classes studied under the label of regeneration, reptiles are the most fascinating and intriguing group due to their inexplicable regenerative bias, which leads to restoring behaviour being confined to only few tissues, in most of the members (Clause & Capaldi, 2006). Individuals, belonging to both Sauria and Squamata display varied levels of regenerative proficiencies; lizards can reform structural and functional replica of the tail, if broken, while members of crocodilian clade can replace the scutes, lost during any injury (Bellairs & Bryant, 1985).

Lizards- Boast a brilliant, yet biased regenerative mechanism

The regeneration of lost tail is accomplished by epimorphosis, a route of re-growth drafted via dedifferentiation of adult, well-differentiated cells into a proliferative mass of progenitor ones called as 'blastema', followed by redifferentiation into the structural components, once a part of the original organ (Cox, 1969; Brockes, Kumar, & Velloso, 2001; Delorme, Lungu, & Vickaryous, 2012). The wounded plane of the autotomised tail, is the site of action for all the molecular signalling, eventually leading to scar free healing, which culminates into a

reformed tail. It is interesting to observe that the capacity to reconstruct a functionally active organ is restricted only to tail in lizard; no other organ or appendage can be reformed in lizard. It is remarkable that the tissue architecture of tail and limbs are conspicuously similar, yet only tail elicits regenerative behaviour, while the limb shows no such response. This feature makes it a special case, where disparate wound healing mechanisms are operated in the tissue specific fashion to form regenerated tail while any other appendage, like a forelimb undergoes permanent scar formation (Alibardi, 2009; Ranadive et al., 2018).

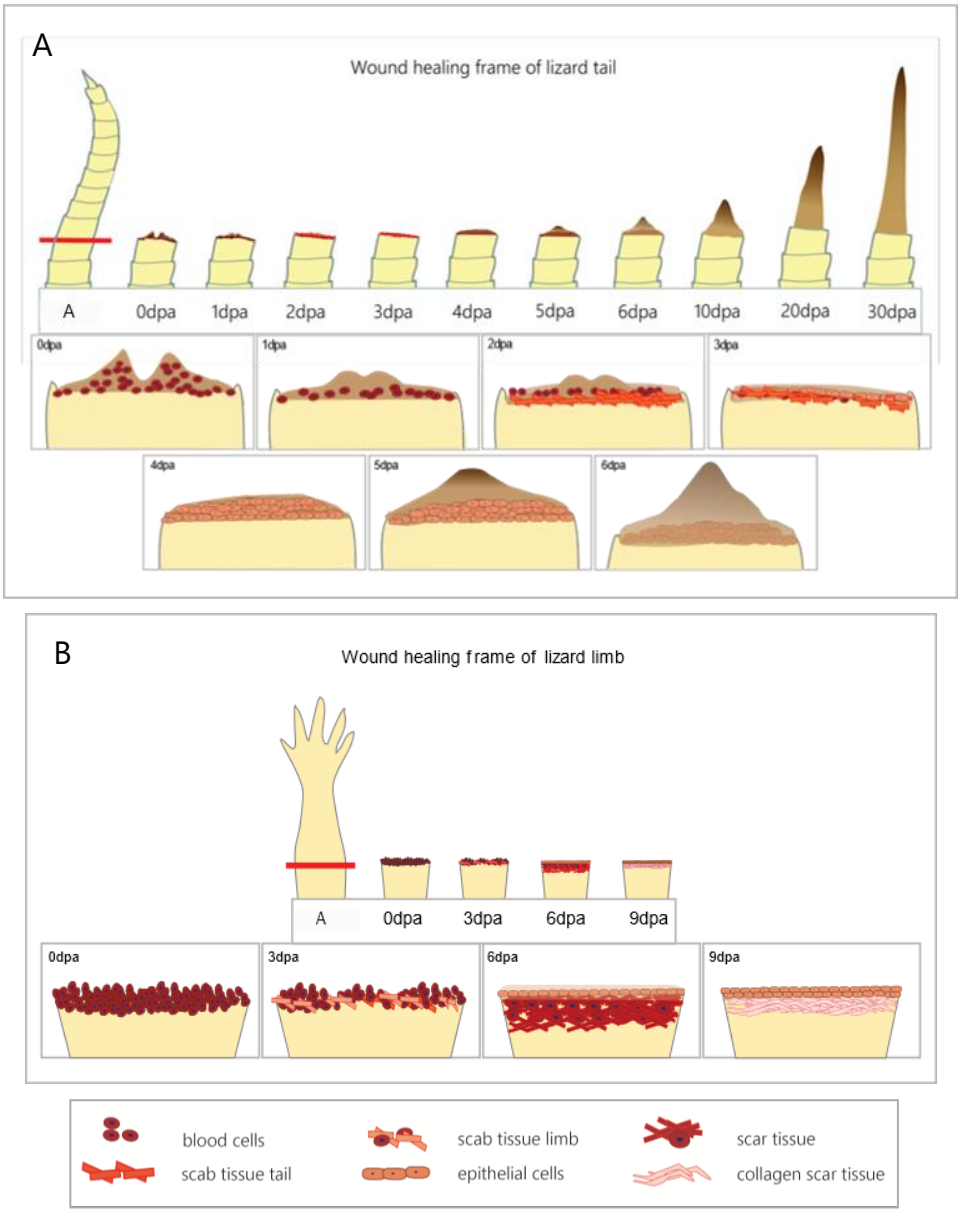


Figure 2. Disparate wound healing patterns in lizard: A-tail; B-limb

Of the many pivotal processes required for successful wound healing, inflammation plays a cardinal share, being the preliminary response to any injury. It is considered to be the

decisive factor in drafting the methods and outcome of the wound healing (Prisk & Huard, 2003; Eming, Krieg, & Davidson, 2007; Lawrence, 2009). It has been hypothesised that the tissues exposed to the external environment immediately induce inflammatory response to prevent any microbial invasion (Lawrence, 2009).

Unlike limb, autotomy of tail shows minimal blood loss and the tissue exposed, is immediately covered by the epithelial cells. Blastema formation follows, which further leads to replacement of the entire lost region of the tail (Moffat & Bellairs, 1964). Although the new tail formed is structurally different from the original organ, initially, it serves the same functions (Zani, 1996; Simou et al., 2008). An interesting finding about this regenerative phenomenon is that the inflammatory response in this organ is not as prolonged as found in limbs (Israeli et al., 2019). Various growth factors and inflammatory mediators are proposed to be having a crucial role in specific regeneration of tail, instead of limbs (Buch, Desai, & Balakrishnan, 2018; Vitulo et al., 2016). This differential regulation of wound healing is extensively studied in our lab and few other places around the world (Pilo & Suresh, 1994; Pilo & Anoop Kumar, 1995; Fitch, 2003; Sharma & Suresh, 2008; Alibardi, 2009).

The present project was envisaged to explore and understand the roles of various inflammatory mediators in formulating the tissue specific disparity of wound healing, if any, and to thereby decipher its contribution to the visible differential wound repair mechanism deployed in the tail and limb of lizard. Inflammation can be a double-edged sword as far as the wound healing is concerned. It is a physiological response, which can induce and boost wound repair, while its prolonged stay can also cause hindrances in achieving the same (Eming et al., 2007; Filbin, 2006; Mescher & Neff, 2006; Mescher, Neff, & King, 2017). Inflammatory response is annexed to many other signalling pathways and thus has a widespread effect on a plethora of physiological events like cell proliferation, migration, formation of extracellular matrix, apoptosis and even the clearance of debris from the site of action (Lu et al., 2012). Thus, the intensity and tenure of inflammation can determine the wound healing outcome and can pave the path for the discrete bias that is either causing restoration of the lost tail or permanent scar formation in limb.

COX-2- A puppeteer of inflammation

Across the globe, Cyclooxygenase regulates the status of inflammation in a wide spectrum of animals (Caughey et al., 2001). They are popularly known as COX, a family of enzymes

which regulates the levels of prostaglandins in the system (Caughey et al., 2001; Hill et al., 2012). Biologically active prostaglandins are derived from Arachidonic acid which is released from membrane phospholipids under the catalytic effect of Phospholipase A_2. The precursor of all the prostanoids - prostaglandin PGH_2 is formed from Arachidonic acid in the presence of COXs (Caughey et al., 2001; Simmons, Botting, & Hla, 2004; Korbecki et al., 2014). Two isoforms of COX namely - COX-1 and COX-2 are primarily known to mediate the synthesis of PGH_2 which is subsequently been converted into various prostanoids by the selective action of tissue specific enzymes on a need-based manner (Bos et al., 2004; Simmons et al., 2004). The general pathway of Cyclooxygenase is shown in Fig. 3.

COX-1 is constitutively expressed in many cells and is responsible for the maintenance of homeostasis by producing housekeeping prostanoids (Simmons et al., 2004; Rouzer & Marnett, 2009). COX- 2 is present at minimal levels but its production is induced and it increases manifold under inflammatory response (Williams, Mann & Dubois, 1999; Rajakariar, Yaqoob & Gilroy, 2006). Both isomers lead to formation of PGH_2, the common substrate for the tissue specific isomerases which form various Prostaglandins depending on the tissue forms present (Hata & Breyer, 2004).

Kuwano and colleagues (2004), have shown the positive correlation between the status of inflammation and level of COX-2. On the other hand, COX-2 plays pivotal role in causing angiogenesis (Kuwano et al., 2004), cell proliferation (Hashemi et al., 2019) and metastasis (Stolina et al., 2018, Hashemi et al., 2019).

An alteration in the inflammatory response has been studied previously by selectively inhibiting the production of COX-2, using specific NSAIDs (Non-Steroidal Anti-Inflammatory Drugs), which led to inhibition of various prostaglandins' production and hampered the normal immune response (Lee et al., 2009). The studies have underlined the retarding effect of these inhibitions on the regenerative capability of the lizard (Sharma et al., 2011; Buch et al., 2018).

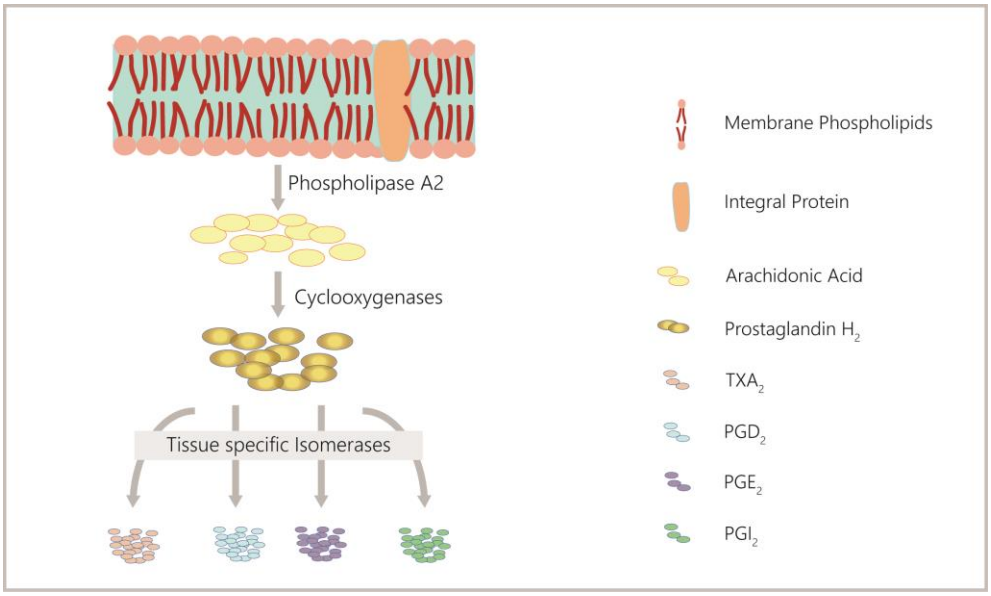


Figure 3. Schematic representation of prostanoids biosynthesis

Although, COX-2 has pivotal role in mediating immune response under the traumatic effects of injury, its elementary level in the resting tissue is unknown. As mentioned above inflammatory response differs in both the scar-free and scarred wound healing process and Cyclooxygenases (COX-1 and COX-2) are known to mediate these events, thus, fluctuations in their expressions (transcriptional and proteomic) are expected. Although previous studies have investigated only COX-2 as the pivotal regulator, role of COX-1 cannot be completely negated as opined by Crofford and colleagues (Crofford et al., 1994). Thus, knowledge of the tissue specific expression of both the Cyclooxygenases would provide a foundation to further investigate the varied inflammatory response in the two appendages (namely Tail and Limb) of lizard. For the present study, manifestation and activity of inflammation were observed through the compass of Cyclooxygenase expression and activity.

Hence, this study was envisaged to discover the primary status of COX-1 and COX-2 in resting tissues, followed by comparison of their expression in autotomised tail and amputated limb. Both the organs respond variably to an injury. Amputated tail shows clot formation followed by wound epithelium formation. Scar formation takes around 15-21 days post-amputation. Unlike limb, blastema is developed in case of autotomised tail, within 5-7 days (Ranadive et al., 2018). This physiological difference in the response is directed by the differing microenvironment; and various inflammatory mediators would contribute to it. Resultantly, examining the status of various inflammatory mediators would give an insight of the different prospective reasons for the varied physiological response.

The inflammatory response is initiated immediately after injury wherein, COX-2 induction leads to formation of various prostanoids. Prostaglandins is a family of lipid derived master regulators directing a wide range of cellular events such as function of Kidney, platelet aggregation, neurotransmitter regulation, inflammation etc (Goetzl et al., 1995). Of all the prostaglandins, E_2 plays a significant and definite role in regulation of inflammation (Funk, 2001; Rajakariar et al., 2006).

PGE_2 is synthesized under the catalytic effect of microsomal Prostaglandin E_2 synthase (mPGES). The two isomers found in the cells are mPGES1 and mPGES2. Another cytosolic isoform of this enzyme is cPGES (Murakami et al., 2000). The elementary levels of these enzymes and their activity would provide a clue about the status of PGE_2 expression in the two different organs i.e., tail and limb. The various responses elicited by this PGE_2 are a function of the particular receptors which mediate the tissue specific crosstalk. Their expression and abundance vary in different cells and tissues. EP2 and EP4 are known to be determining the inflammatory response (McCoy, Wicks, & Audoly, 2002). Thus, their expression profile also needs to be checked. This will further provide hints to understand the crosstalk of PGE_2 and different inflammatory mediators determining the fate of wound healing in tail and limb.

Various families of cells and their derivatives rush to the site of injury under the effect of cytokines. The defence mechanism thus prevents invasion of microorganisms through the tissues exposed by the injury (Kaiser et al., 2004). Immune cells like macrophages and neutrophils are known to be contributing to inflammation and thus fluctuation in their number and localisation at the site of injury is also prospected (Chazaud, 2014). As the response is induced, the early gene expression profile can provide information regarding the initial scenario in the microenvironment of the injured site. Thus, temporal change in the expression of various interleukins and cellular components is important to be checked. In order to highlight the disparity in wound healing routes taken by tail and limb, it was crucial to compare the inflammatory response immediately after injury in both appendages. Hence, the temporal status of the inflammatory mediators was checked in the healing tail and limb to address the major aim of this study, i.e., to understand the status and role of inflammation as a colossal systemic response, in devising the wound healing in both tail and limb.

The pivotal timepoints to observe and record the periodic changes in the status of COXs and other inflammatory mediators were determined based on the preliminary

knowledge of the healing stages of lizard tail and limb. From the previous studies in lab, the cerebral stages involved in the mega event of wound healing have been charted out in both tail and limb. These stages mark the accomplishment of the events namely Haemostasis, Inflammation, Granulation and Proliferation. In the tail, these milestones are achieved in the course of four to five days, in a chronological yet juxtaposed fashion, leading to the blastema formation that would culminate in the regenerating tail. In case of limb though, a prolonged inflammation masks the entire routine of wound healing, wherein the collagen deposition underneath the epithelium is conspicuously high and persistent. The microenvironment herein, leads to permanent scar formation instead of any regenerative developments. This basal difference in the healing pattern is a function of the molecular environment available at the site of amputation, which needs to be further explored for unravelling mystery of disparate wound healing in the different appendages of the same model organism.

The present study was envisaged as an effort to document the exact effects of inflammation on the wound repair by observing the expression status of major inflammatory modulators and their temporal alterations in the healing appendages. The prime focus, therefore is on inflammation not only as the preliminary response but also as the key director of the repair. To comment upon its role, the Cyclooxygenase mediated pathway has been selected for being the primordial contributor and controller of inflammation and many other developmental events.

OVERALL OBJECTIVE

Study the temporal expression pattern of Cyclooxygenase and to ascertain its role in the regulation of inflammatory response during wound healing in the appendages of lizard *Hemidactylus flaviviridis*.

This will be achieved by studying the following parameters that are treated herein as specific aims.

1. To examine the timed expression pattern of Cyclooxygenase as well as to study the turnover rate of Prostaglandin E_2 synthesis and hydrolysis in the appendages of lizard post-amputation.
2. To analyse the temporal expression profile of different PGE_2 receptors (EP1, EP2, EP3 and EP4) and the receptor associated intracellular mediators at the site of injury in both tail as well as limb.
3. To examine the temporal alteration in the expression pattern of various inflammatory mediators (cytokines and cellular components) during scarred (limb) and scar-free (tail) wound healing.

MATERIALS AND METHODS

The methodology deployed to address the objectives, was devised in accordance with the specific aim of the experiments.

Procurement of animals and maintenance

Hemidactylus flaviviridis (northern house gecko), both male and female, were caught from the nearby locality and caged in wooden chambers (15x15x10 in.). The animals selected for studies weighed around 10-12 g and were maintained at 36 ± 2 °C with light to dark cycle of 12:12. The experimental protocol (MSU-Z/IAEC/15-2017) was approved by the institutional animal ethics committee (IAEC) and all the experiments were performed as per the guidelines of CPCSEA, India.

Comparing the differential wound healing stages in the appendages of lizard

The scar-less, regenerative nature of the tail, can be dissected down in multiple sub stages, which are crucial to direct the path of tissue repair, that eventually reforms the lost one. These stages highlight the onset and stringent regulation of many physiological events, further causing the mega repair. Post injury, the immediate response is directed to prevent any form of pathogenic invasion, for which, the site of the amputation constricts and a single layer of epithelial cells, starts to cover the wound. Interestingly in tail, minimalistic or no blood loss is observed, which further boosts the epithelialisation process that is achieved by the end of the first day of amputation (1dpa). On the molecular level, the micro niche changes dynamically, with every passing day of healing, wherein inducing autotomy in the tail leads to start haemostasis at the site of injury and augments inflammation in the tissue (Delorme, 2011). Onset and immediate hike in inflammation ensures that the pathogens, which might have entered through the site of injury, will be dealt with to curb their further invasion. As per the reports, it is evidently proven that by the second day post amputation (2dpa), the levels of inflammation significantly rise and stay so till the third day following injury (3dpa). The changes occurring beneath the epithelial layer, lead to formation of a smooth, brown structure, which is visible as the tissue scab, is lost by the end of third day of healing. This stage is known as the wound epithelium stage and is marked by the presence of Apical epithelial cap or AEC. Hence the 4dpa is of cerebral importance for the further proceedings of epimorphosis. Further, this smooth surface bulges up and darkens in colour, also forming a dark dot like structure at the centre of this growing plateau like region. The structure formed here is called, ‘blastema’ and it is the characteristic feature of epimorphic type of regeneration. Underneath this dark external layer, is a pool of progenitor cells, which have the potential to reform the different tissue types, eventually remaking the entire tail. This stage of repair elicits elevated proliferation of cells, which is achieved by fifth day of amputation (5dpa). The darkened bulge then develops further to form a conical structure, which grows and reconstructs the tail (6dpa). It is interesting to observe the gradual morphological changes in this bulge which culminate to form the regenerated organ, that suffices for the function of the original one, despite being visibly distinct.

On the contrary, lizard limb, when amputated, undergoes a heavy blood loss, which forms a massive blood clot at the amputation region. The epithelialisation process is slow paced in this case as the cells have to form a monolayer, under the heavy mass of blood cells and fibrin filaments (Ranadive et al., 2018). Thus, a visible blood clot and the epithelial layer

are formed only by third day of amputation in limb (3dpa). Parallely, the limb elicits heightened inflammatory status for a longer duration of time and a thick scab of tissue is formed under the blood clot, which is shed only by the 6dpa. Meanwhile, the collagen deposition continues and the scar becomes firmer beneath the epithelial cells form primary layers, eventually leading to formation of a permanent, resilient scar by the end of 9dpa. This scar strengthens and stiffens further as the time progresses while no regenerative outcome is possible on the limb front.

In order to study this disparity in the types of wound healing, specific experiments were strategized wherein the animals were grouped in two main categories viz., Tail and Limb, to further design studies, revolving around the differential wound healing frames of these appendages.

For investigating the molecular events of inflammation in the scar-less tail and scar-forming limbs, the animals were randomly categorised into two main groups, namely tail and limb. These groups were further divided in various sub-groups based on the stages of the healing process to be targeted. For tail, 0, 1, 2, 3, and 4dpa and for limb, 0, 3, 6 and 9dpa were considered for the study, as these highlight the haemostasis, granulation along with the increased inflammation, culminating in scab formation, followed by appearance of wound epithelium. Ranadive and colleagues (2018), have reported the distinct time points for the attainment of the above-mentioned milestones that determine the path of repair in both limb and tail. The same timepoints are considered here for this investigation. For every experiment, six lizards were used in each sub-group, while three technical replicates were performed using pooled samples of six subjects. In the members of tail group, tail was forced to autotomise and the tissue was collected at the predetermined time points to further check the expression status of various inflammatory mediators. Autotomy causes the release of tail, from the fracture plane, where pressure is applied. This end was then observed daily for the specific changes and the segment was collected by applying pressure on the preceding fracture plane for further processing. Concurrently, for the subjects of limb group, their forelimbs were surgically amputated at the humerus as described previously and the tissue was then collected at particular time windows to proceed with the analysis. Around 3 mm of tissue chunk was harvested from the pre-amputated limb, to carry out various analyses as detailed in the following section.

Morphological Record

The periodic changes occurring during the diverse healing frames of tail and limb were recorded from both the groups. Pattern of repair is conspicuously different and the morphological panel created here just provides the supportive evidences for the same.

COX activity assay

Tissues were collected from both the groups at all the above-mentioned time points in 0.1 M Tris-EDTA buffer and 10% tissue homogenate was further used for a spectrophotometric kit-based COX assay (Cayman Chemical Co. USA; ID: 760151). The specific activity was calculated in nmol/min/100g tissue weight. The method deployed in this assay utilised the peroxidase activity of COX, wherein the appearance of the oxidised form of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) was measured at 590 nm. TMPD was provided as a ready to use substrate in the kit, while sample prepared from the isolated tissues acted as the enzyme cocktail.

PGE_2 estimation

Tissue samples were collected from all the subgroups and homogenised in double-distilled water (1 g/4 ml). 15% of v/v methanol was added to this homogenate, and the prostanoids present in it were allowed to be dissolved in the added alcohol for 1 hour. These homogenates were then centrifuged at 4 °C and 2000g for 5 min. The supernatant was collected for the kit-based estimation assay of PGE_2 (R&D Lab Systems, USA; ID: KGE004B). Herein, the PGE_2 present in the sample competes with the horseradish peroxide (HRP)-labelled PGE_2 in a sequential competitive binding immunoassay. The colour developed due to the competitive enzyme activity is measured at 450 nm. The estimated levels of PGE_2 were calculated in pg/ml.

Immunohistochemistry

Freshly collected tissue samples were processed in cryostat-microtome to obtain longitudinal cryo-sections of 8-10 μm. These were then fixed in chilled acetone for 15-20 min. Followed by which, the sections were subjected to rehydration with PBS-T (Phosphate buffered saline

+ 0.25% Tween-20) and blocked at room temperature for half an hour, with 0.5% bovine serum albumin in PBS (PBS-BSA) [GeNei, Merck, USA], followed by incubation with anti-COX-2 IgG rabbit (0.5 µg/ml, Sigma-Aldrich, USA). Further, the sections were washed thrice for 15 min. each, with the PBS-T. These sections were then subjected to FITC-conjugated secondary antibody (0.1 µg/ml, Sigma-Aldrich, USA). Three subsequent buffer washes of 15 min. each and then these sections were used to observe the distribution pattern of COX-2 in different compartments of the tissue. In order to observe the proper histology of the sections, DAPI was used to counterstain each section and the sections were then observed under a fluorescent microscope (Leica DM2500, Germany). The representative images were captured using the digital camera (Leica, EC3, Germany), mounted on the microscope.

Western blot

Samples from all sub-groups of tail and limb groups were collected in lysis buffer [50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM CaCl2 and 1% triton-X 100] and were processed at 10,000g for 10 min. Total protein was estimated by assaying 10% homogenate, using Bradford's method (Bradford, 1976). Further, equal amount of the protein extract was allowed to electrophorese on 12% gel of SDS-PAGE at 100 volts. The components of the gel were then allowed to be transferred on PVDF membrane via semi-dry western-blot transfer, at 100 mA for 25 min. This membrane was used to develop immunoblots using specific antibodies against the inflammatory mediators of interest. The blots were developed using Streptavidin conjugated ALP as an enzyme, and BCIP-NBT as substrate (Sigma-Aldrich, USA). The primary antibodies raised against the respective antigens in the rabbit and mouse, were used for IHC and Western blot, considering the stringently conserved genetic sequences of these molecules across all the classes of vertebrates (Kaiser et al., 2004; Murphy & Thompson, 2011). Also, the previous work from the lab establishes the cross reactivity of these antibodies amongst the species against which they are generated along with *Hemidactylus flaviviridis* (Buch et al., 2017, 2018; Ranadive et al., 2018).

Quantitative real-time PCR

Total RNA was isolated as per the manufacturer's guidelines, from all the tissue samples of both the groups, which were homogenised in TRIzol reagent (Applied Biosystems, USA). 1

µg of the total RNA was used then to synthesise the cDNA using one-step cDNA synthesis kit (Applied Biosystems, USA). Gene expression of COX-2 and other selected inflammatory mediators was checked using the PCR reaction performed in Light Cycler 96 (Roche Diagnostics, Switzerland), using specific primers designed through Primer-Blast tool of NCBI. For the analysis, 18srRNA was used as housekeeping control. Quantitative real-time PCR was performed with the following program: 100 s at 95 ºC followed by 40 cycles of 10 s at 95 ºC, 30 s at 60 ºC, and 30 s at 72 ºC. Fold change was calculated using method developed by Livak and Schmittgen using corresponding group's (limb's or tail's) 0dpa ΔCq values, for all the three technical replicates (Livak & Schmittgen, 2001).

RESULTS

Morphology

The panel presented in Fig. 3 underlines the crucial stages of wound repair in tail and limb. The panel provides visible proof of the differential repair, as the tail shows prompt covering of wound wherein the epidermal layer smoothens by the end of 4dpa. On the contrary in limb, the massive blood clot stays till 3dpa while the healing is visibly slow and takes up to 9dpa, ultimately forming a persistent scar (Fig. 4).

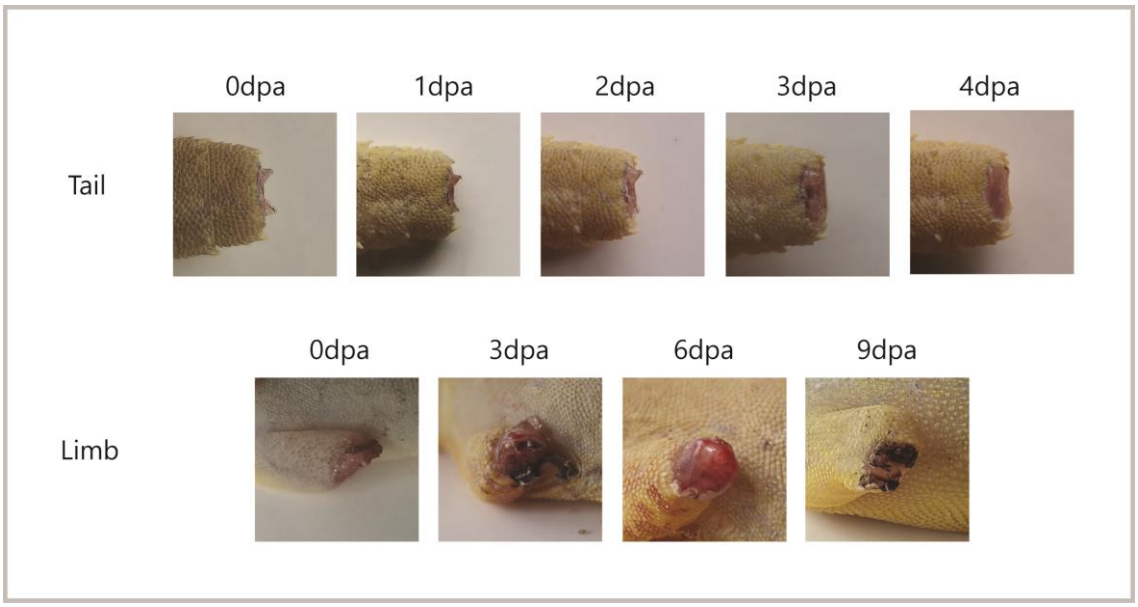


Figure 4. Diverse healing pattern in Tail and Limb observed morphologically

Temporal variation in the activity of COX-2 in the healing tail and limb

In this study, COX activity at various stages of scar-free and scarred wound healing in the tail (Fig. 5A) and the limb were compared (Fig. 5B). In the tail, the activity of COX-2 showed significant increase throughout the wound healing stages of tail from 1dpa to 4dpa, when compared to 0dpa (Fig. 5A). Similarly, in limb, COX-2 levels increased significantly at 3dpa, 6dpa and 9dpa when compared to 0dpa (Fig. 5B). Since the COX-2 activity levels were found to be high in both the appendages during their respective healing stages, pivotal mediator of inflammation downstream of COX-2, which actually controls the resultant modulations, namely PGE_2 (Prisk & Huard, 2003; Verma et al., 2021) was targeted.

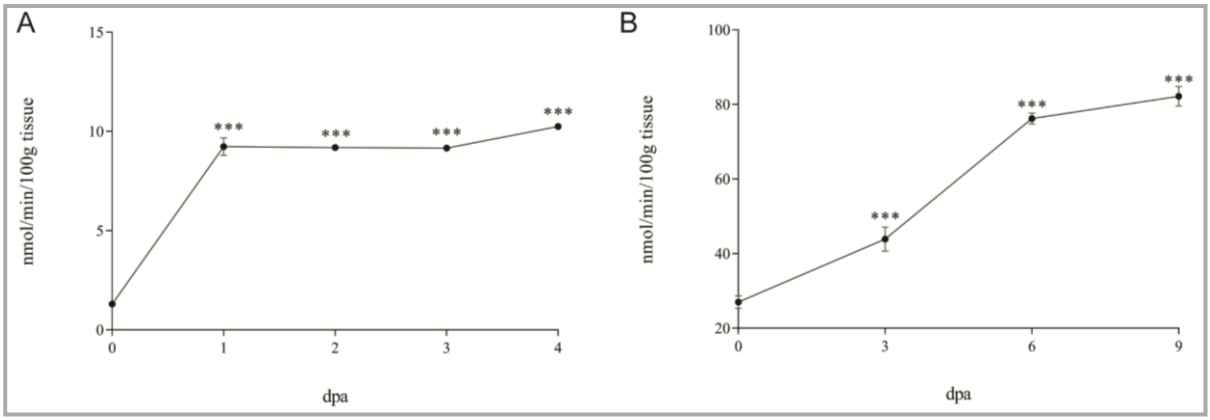


Figure 5. COX Activity assay- A. Tail and B. Limb.
*$p \leq 0.20$, **$p \leq 0.005$, ***$p \leq 0.001$; n=6.

PGE_2 level in the healing appendages

Prostaglandin E_2 is a pivotal contributor to inflammation, synthesis of which is initiated by the injury-induced activation of the enzyme COX-2 (Korbecki et al., 2014). Levels of PGE_2 were analysed at the selected time windows in both tail and limb. An increase in PGE_2 level was observed in tail tissue from 1dpa till 4dpa when compared to the resting stage (Fig. 6A). On the other hand, in limb, a reduction was observed in the level of PGE_2 starting from 3dpa till 9dpa as compared to 0dpa (Fig. 6B). The readable observation was the well-pronounced reduction observed in the PGE_2 level in limb as opposed to tail.

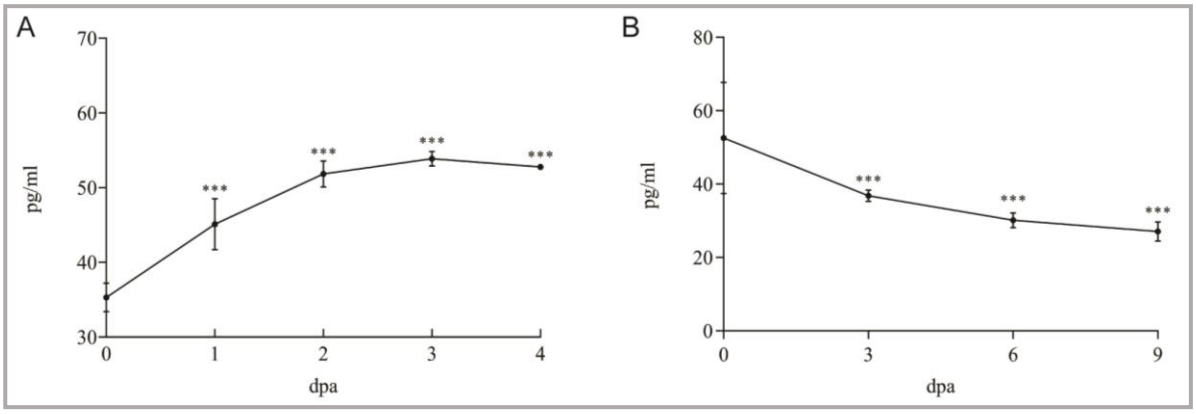


Figure 6. PGE_2 estimation- A. PGE_2 level in healing tail; B. PGE_2 levels in healing limb. *$p \leq 0.20$, **$p \leq 0.005$, ***$p \leq 0.001$; n = 6.

Immunohistochemistry

COX-2 was localised in the tissues collected at destined time points from both the appendages and the subsequent microscopic analysis vividly portrayed the temporal changes in its site of expression (Fig. 7). In tail tissue, initially at 0dpa, a faint expression of COX-2 at the site of autotomy was observed, although, at the following stages of 1dpa, 2dpa and 4dpa, its localisation significantly changed in the tail (Fig. 7A-D). It increased by the end of 1dpa, near the spinal cord region, while by 2dpa, it accumulated close to the epithelium covering the wound. The intensity of expression reduced evidently by the end of 4dpa, as the wound healing was achieved and the apical epithelial cover was well formed to initiate interactive signalling with the underlying mesenchymal tissues and initiate a regenerative response (Fig. 7A-D). Meanwhile, there was some visible expression of COX-2 in the tissue section, at the site of amputation at 0dpa stage in the limb. This area of expression is also dynamic, and the intensity of expression was found to be proficiently rising with every passing stage. At 3dpa stage, COX-2 was localised with high intensity over the injured surface of humerus immediately closer to the clot, which continued to rise further till 9dpa (Fig. 7E-H). At 6dpa, this expression increased as well as shifted distal to the chondrocytes, sandwiched between the former and the newly formed epithelial layer (Fig. 7G). A remarkable difference was observed in the site of COX-2 expression in both the appendages, wherein the area and intensity changed temporally. For instance, COX-2 localisation peculiarly shifted from the proximal region of progressive wound epithelium in dpa to closer to the dermal layer by 9dpa in case of limb, where the persistent scar is formed (Fig. 7G-H).

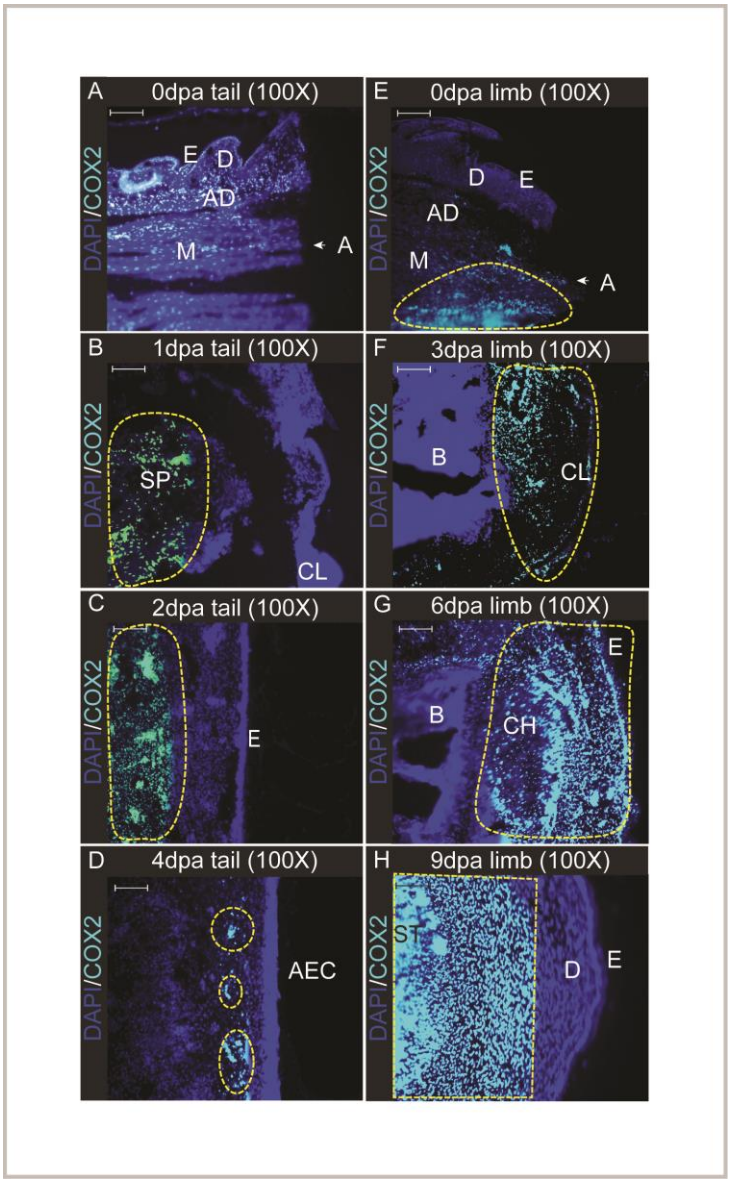


Figure 7. Immunohistochemical localisation of COX-2

Altering intensity and sites of COX-2 localisation in the healing Tail (**A-D**) and Limb (**E-H**). Yellow dotted line depicts the site of COX-2 expression in both the tissues. A-Amputation plane; AD-Adipocytes; AEC-Apical Epidermal Cells; B-Bone; CH-Chondrocytes; CL-Clot; E-Epidermis; D-Dermis, M-Muscles; SP-Spinal cord; ST-Scar tissue. Scale bar = 200 µm.

Protein expression pattern of the inflammatory mediators in healing appendages

Protein expression was checked for the major regulators of inflammation in both tail and limb. COX-2 protein levels in the tail went up for 1dpa, 2dpa, 3dpa and 4dpa in comparison to 0dpa (Fig. 8A). In limb tissue, the expression noticeably increased at 3dpa, maintained the same mark at 6dpa as compared to resting stage. At 9dpa, COX-2 protein levels were found to be highest in comparison to 0dpa (Fig. 8B). The protein levels of EP2 receptor were found to be constantly decreasing in tail whereas in limb its levels were significantly increased from 0dpa till 9dpa. Alongside, protein expression of EP4 receptor was found to be increased throughout the wound healing stages of tail in comparison to resting stage (Fig. 8A).

However, striking difference in the level of EP4 was observed in scarring limb wherein its level went down significantly from 0dpa and continued to do so till 9dpa (Fig. 8B).

Simultaneously, few pivotal proinflammatory mediators were checked for their expression, namely iNOS, TNF-α, IL-6, and IL-17. Expression levels of iNOS and TNF-α in tail were found to be reducing from 2dpa to 4dpa stage when compared to 0dpa (Fig. 8A). On the contrary, the protein levels of iNOS and TNF-α were found to be increased during scarring in limb (Fig. 8B). Expression level of one of the principal anti-inflammatory mediator IL-10, was also monitored and was found to be successively increasing from 0dpa to 4dpa in tail (Fig. 8A), while its levels stooped significantly in limb after 3dpa and remained low till 9dpa stage (Fig. 8B). Protein level of IL-6 in tail was decreased starting from 1dpa till 4dpa in tail whereas in limb it was found to be reduced at 3dpa however 6dpa onwards the level increased till 9dpa in limb. IL-17 protein levels were decreased noticeably from 1dpa to 4dpa in comparison to 0dpa in tail (Fig. 8A). In case of limb, IL-17 protein levels were found to be decreased significantly from 0dpa to 3dpa, followed by a sudden rise noted for 6dpa and 9dpa (Fig. 8B). IL-22 elicited a riveting result wherein, its protein expression increased from resting stage till 4dpa in tail. IL-22 showed marked decrease in the expression at 3dpa stage of healing limb but then gradually elevated at 6dpa followed by remarkable rise at 9dpa, when compared with 0dpa (Fig. 8B).

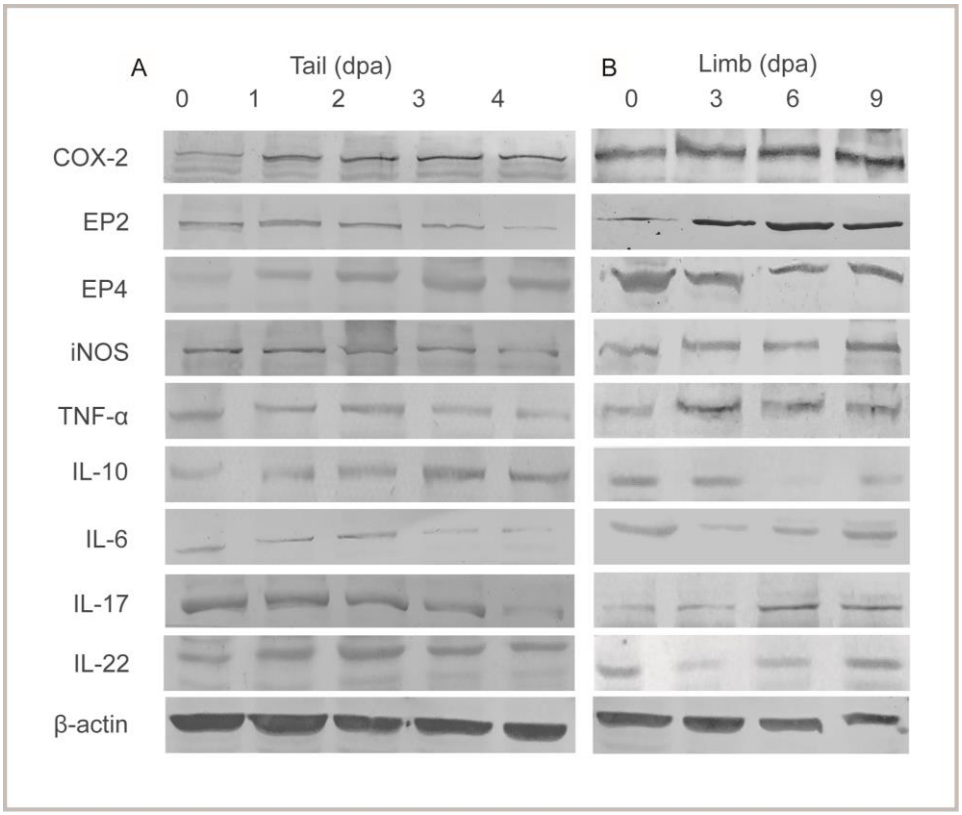


Figure 8. Western blot results

Temporal levels of COX-2, EP2, EP4, iNOS, TNF-α, IL-10, IL-6, IL-17 and IL-22 proteins in both, healing tail and limb. β-actin was used as loading control.

Gene expression pattern of inflammatory mediators in healing appendages

Quantitative real-time PCR was employed to further validate the expression status of various regulatory molecules, which organise the entire inflammatory response in these two varied appendages. These molecules were majorly considered for their distinct roles, either supporting or opposing inflammation. Both tail and limb groups showed major alterations in the expression of these molecules. The genes considered were, COX-2, EP2, EP4, iNOS, TNF-α, IL-10, IL-6, IL-17 and IL-22 (Fig. 9A and 9B). COX-2 is known to be upregulated under the effect of an injury, so was observed here, wherein significant elevation was observed in its transcript-level expression, under the impact of induced autotomy in the tail. The individuals showed a striking rise in COX-2 expression from 0 to 3dpa by almost 16-fold which then remained 8-fold at 4dpa in comparison to 0dpa for the tail (Fig. 3A). The subjects of the limb group also showed a progressive elevation in COX-2 mRNA from 0 to 9dpa (Fig. 9B).

Additionally, the level of EP2, a member of the PGE_2 receptor family, was checked, which showed noticeable variation in expression. The EP2 gene expression was in concurrence with its protein expression data wherein the levels were decreased throughout the course of healing in tail (Fig. 9A) and were upregulated in scarring limb (Fig. 9B). Another PGE_2 receptor, EP4 was checked and it elicited rise in gene expression at 1dpa and remained elevated till 4dpa, when compared to 0dpa (Fig. 9A). On the limb front though, it decreased significantly at 3dpa which remained persistent till 9dpa as well (Fig. 9B).

Various proinflammatory mediators, boosting the course of inflammation were checked, namely- iNOS, TNF-α and IL-6, for their temporal gene expression pattern. All these three genes showed prominent reduction from 1dpa till 4dpa as compared to 0dpa during lizard tail regeneration (Fig. 9A). However, during limb healing, iNOS, TNF-α and IL-6 transcript levels were upregulated remarkably in comparison to 0dpa (Fig. 9B). Thus, iNOS, TNF-α, and IL-6, all showed progressive reduction in expression during the course of wound healing in tail, while the anti-inflammatory mediators IL-10 showed increase of expression (Fig. 9A). Herein, conspicuous rise in its gene expression was recorded from 1dpa onwards, which progressed across 2, 3 and 4dpa stages to increase by 13-folds at the terminal time point, as compared to the resting one (Fig. 9A). On the contrary, a significant reduction in its expression was observed at the 6dpa of limb, after a marked elevation at 3dpa, followed by remarkable decrease at the final time point of 9dpa (Fig. 9B). Thus, subjects of the limb

group, showed exact contrast, to the tail group, with respect to the trend of gene expression, as the proinflammatory mediators displayed a prominent rise while the antiinflammatory molecule elicited evident reduction (Fig. 9A and 9B).

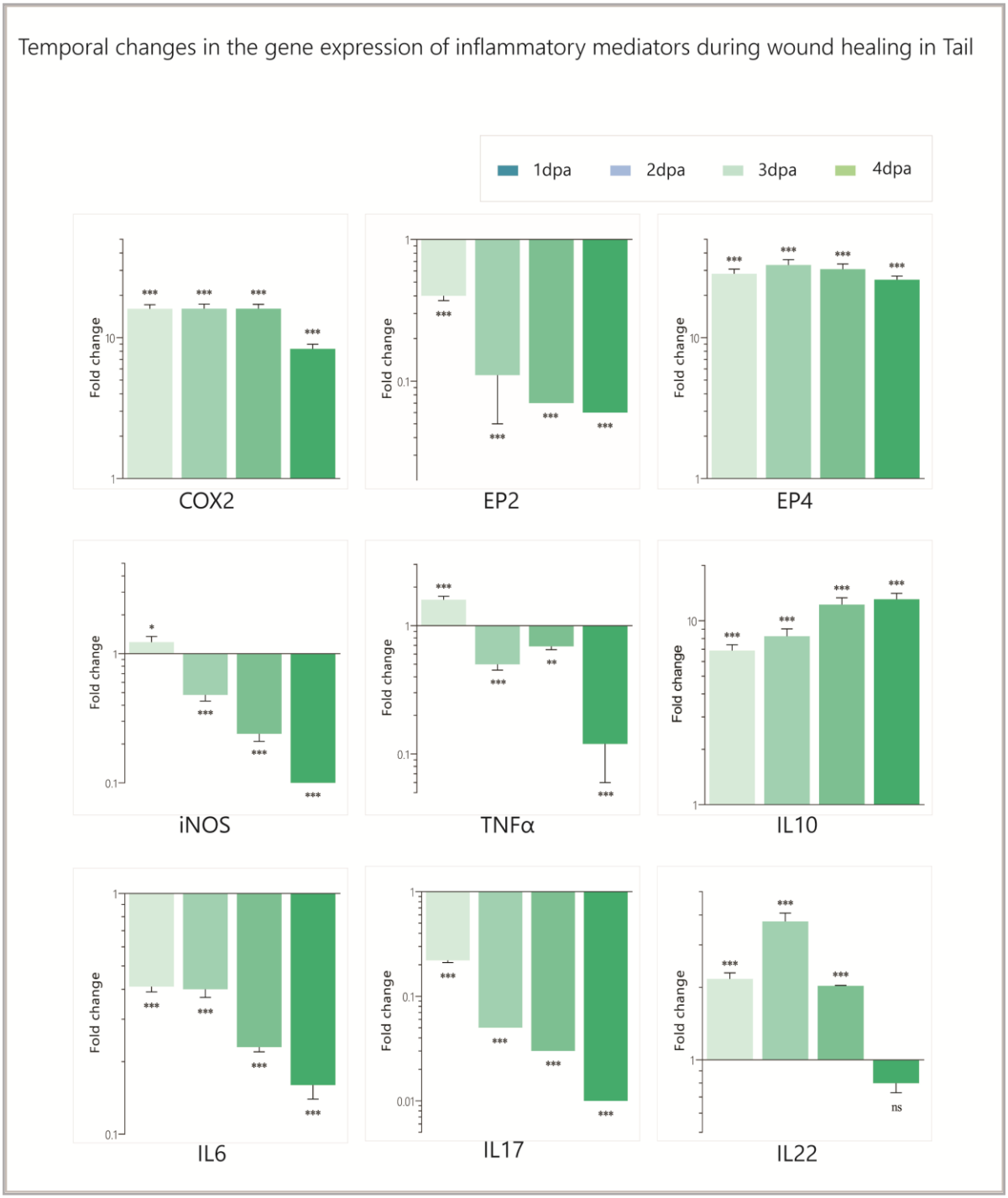


Figure 9A: qRT-PCR results (Tail)- Gene expression analysis of inflammatory mediators. *$p \leq 0.20$, **$p \leq 0.005$, ***$p \leq 0.001$; n = 6.

Apart from these genes, we found specific changes in the status of IL-17 and IL-22 gene expressions. During our study we found IL-17, to be behaving as a proinflammatory mediator, as it reduced in an ordered fashion, in the tail tissues (Fig. 9A). Initially, it promiscuously rose from 0 to 2dpa and suddenly stooped down by 3dpa, also staying down by the end of 4dpa. In such an environment, IL-22 portrayed a constructive character and supported the fast-healing process of the tail, possibly because its levels also increased till 2dpa as compared to the resting stage, followed by peculiar heightened numbers at 3dpa, which only reduced significantly at the last time point studied, i.e., 4dpa (Fig. 9A).

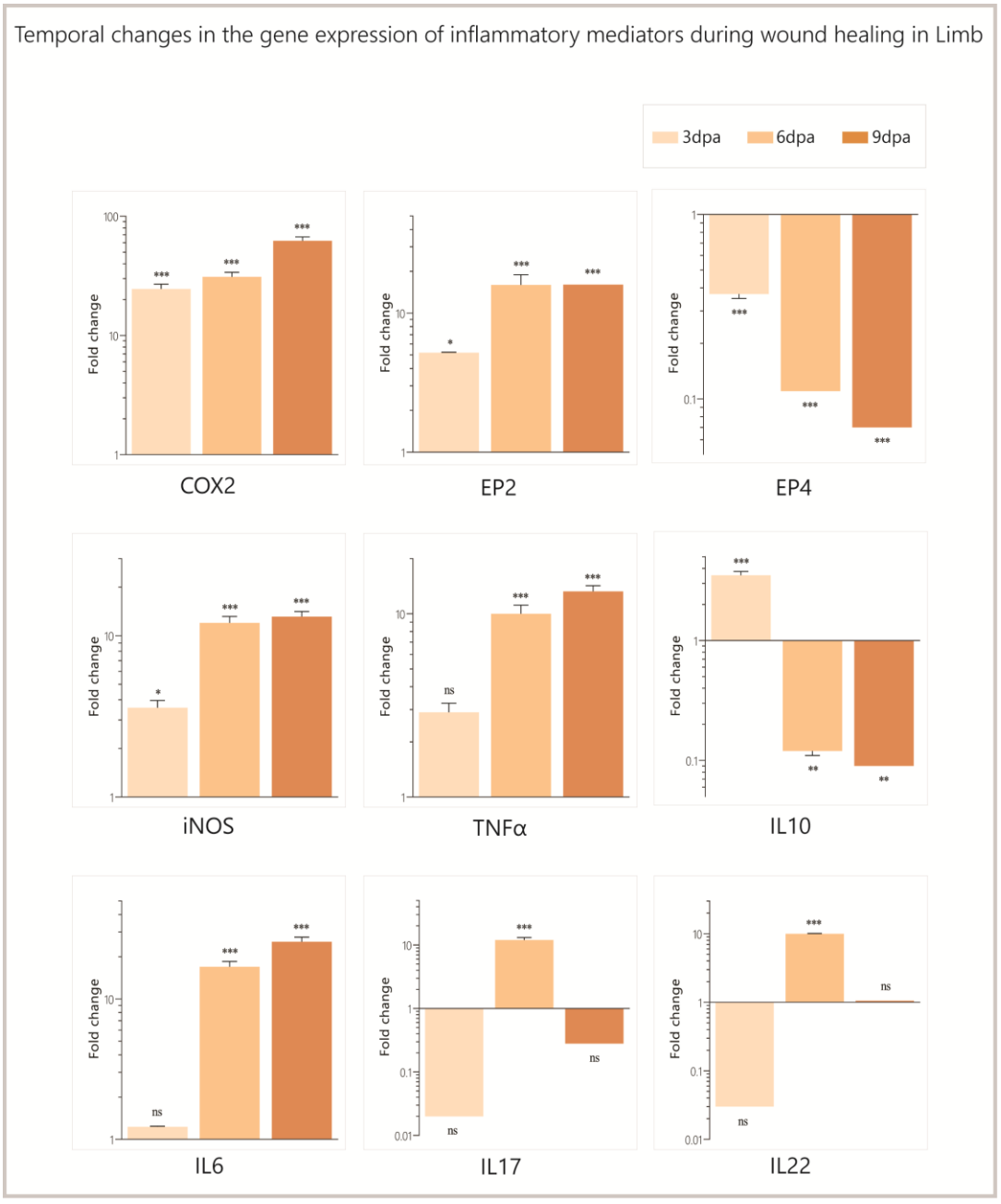


Figure 9B: qRT-PCR results (Limb)- Gene expression analysis of inflammatory mediators

DISCUSSION

This study is a diligent attempt to highlight the involvement and impact of inflammation, over the route of wound healing, in the two appendages, viz., tail and limb of the lizard. As per the prevalent knowledge of over many decades now, cyclooxygenase, is a family of enzymes, which regulates and fine-tunes multiple developmental programmes involving cell survival, proliferation and migration (Lu et al., 1995; Dubois et al., 1998; Simmons et al., 2004; Liou et al., 2007). The results obtained here suggest that COX-2, an inducible isoform of COX modulates inflammation through varied PGE_2-EP receptor signalling, wherein specific interleukins are recruited at the site of repair.

COX-2 activity elevated 2dpa onwards in lizard tail, while it was found to be high in the limb tissues, immediately after injury increasing progressively at the following time points. As COX-2 belongs to the family of early response genes and is strongly induced by mitogenic and proinflammatory stimuli (Lasa et al., 2000), in the next step, protein and transcript levels of COX-2 were checked. In resemblance to hiked activity, the protein and transcript levels of COX-2 were also found to be elevated till the 3dpa stage in tail, conforming its participation in modulating the early inflammation here, while they reduced at 4dpa, during proliferation and epithelialisation. On the contrary, in limbs COX-2 gene expression increased from the basal level till the terminal time point of 9dpa. This indicates mRNA stabilisation in limb tissue, under the effect of elevated proinflammatory interleukins as previously reported by Kang and colleagues (2007), in human bones, macrophages and granulosa cells.

Further COX-2 activity forms PGE_2 as an early response gene product, boosted by proinflammatory cytokines, which govern its transcriptional and post transcriptional levels (Kang et al., 2007). PGE_2 expression followed a trend of COX-2 activity in tail, while in limb, it showed significant decrease after 3dpa, until 9dpa. Although the basal level of PGE_2 in limb (0dpa) is higher than the terminal time point for tail (9dpa). This disparity in PGE_2 levels could be a function of COX-2 driving multiple signalling pathways in various tissues in a context specific manner (DuBois et al., 1998; Simmons et al., 2004; Tsatsanis et al., 2006). Also, other tissue specific inflammation curbing prostanoids might participate to cause early resolution and resultant super healing in tail, while the contrasting results are observed in limb (Bos et al., 2004; Korbecki et al., 2014). Meanwhile, a complete profiling of the prostanoids involved here, would indicate their roles in regulation of inflammation.

Aoki & Narumiya (2017), have established that PGE_2 and its interaction with the downstream receptor (EP1-4) (Minami et al., 2001), determines the course of inflammation in the healing tissue. Also, the intracellular messengers associated with these receptors are involved in the downstream signalling. For instance, cAMP and phosphor- CREB function under the EP2 and EP4 activation to cause the hike in the gene and protein levels of proinflammatory cytokines, which then support inflammation in the micro niche, regulating both, its manifestation and resolution (Smith, 1992; Bos et al., 2004).

Present results suggest a similar tissue specific PGE_2-EP receptor action, as in tail, along with EP2, IL-6, a major proinflammatory mediator got alleviated too. On the other hand, in limb, EP2 levels elevated for both protein and gene expression, along with steady rise in the levels of the major proinflammatory mediators such as iNOS, TNF-α and IL-6. The correlation of the EP2 receptors with the downstream proinflammatory mediators has been reported earlier too (Hinson et al., 1996; Aoki & Narumiya, 2017b). On the contrary, EP4 elicited clear temporal rise in the tail, whereas in limb, its level stooped significantly. The antiinflammatory action of EP4 has been reported in a wide array of systems (Heffron et al., 2020; Joshi et al., 2020; Yasui-Kato et al., 2020). In the present model too, this evident contrast in the EP2-EP4 expression pattern could be the primary reason for the exquisite dissimilarity in the status of inflammation, which is a direct function of interleukins present in the micro niche. This contrasting appearance and action of the two receptors, thus recruits a differential cluster of either anti or proinflammatory mediators at the site of wound healing in tail and limb, respectively.

PGE_2 along with its receptors, implements the systemic and tissue-specific inflammation, due to all the cytokines it deploys in these microenvironments (Portanova et al., 1996; Harris et al., 2002). Major proinflammatory mediators, iNOS, TNF-α, IL-6, etc work in congruence to promote the tissue-specific inflammation at the site of injury, while they function as per the COX-2 mediated PGE_2 expression and its binding with the downstream EP receptors (Hinson et al., 1996; Harris et al., 2002). It is the stark difference in the levels of these regulators (PGE_2 and EP receptors) and their periodic expression, which lays the foundation of biased wound healing in the two appendages.

TNF-α is a well-established proinflammatory mediator (Lawrence et al., 2009), that functions in coherence with PGE_2 and also recruits other inflammation boosting interleukins such as IL-6 (Hinson et al., 1996) at the site of action, further elevating the expression of this

prostaglandin as well (Harris et al., 2002). In the present study, TNF-α and IL-6 show distinct decline in the gene and protein expression in tail, overlapping haemostasis and epithelialisation stages of wound healing. These levels further stoop during the proliferative phase by the fourth day of amputation. On the contrary, in limbs, TNF-α and IL-6 showed continuous rise till 9dpa, confirming the prolonged high levels of inflammation in the microenvironment. These observations provide concrete support for the inflammation promoting feature of this interleukin.

Along with these proinflammatory cytokines, their antiinflammatory counterparts are also recruited, which form the necessary balance for the successful tissue repair (Renz et al., 1988; Hinson et al., 1996; Ricciotti and Fitzgerald, 2011). In the present results, gene and protein levels of a major antiinflammatory mediator IL-10 spiked up significantly in the tail but reduced during the healing course in limb. This substantial disparity in expression could be responsible for early and delayed epithelialisation found in tail (Fig. 2B-C) and limb (Fig. 2F-H), respectively. Peranteau and colleagues (2008) have reported the positive effect of IL-10 overexpression in an adult mice model of regeneration. The collaborative functions of the EP2 and EP4 receptors, which recruit and regulate these cytokines during wound healing (Hinson et al., 1996; Portanova et al., 1996; Harris et al., 2002; Harizi et al., 2003), could be responsible for the disparity in wound healing. Further experiments on the secondary messenger activation can reveal the mechanisms deployed by the receptor signalling.

However, with respect to the transcript and protein, the most striking observations were made for IL-17 and IL-22. IL-17 plays a major role in inducing inflammation that positively leads to tissue remodelling by studying IL-17 KO mice (Yang et al., 2008). Additionally, Yang and co-workers (2008) have proven its role in manifesting chronic inflammatory diseases but this is for the first time that its role is being revealed in a regenerating animal model. Its differential behaviour is studied here in two contrasting tissues, which have taken opposite paths of wound healing. IL-17 showed significant reduction in gene expression traversing all the time points, for tail group, after the early inflammation (2dpa). This supports the idea that reduction of chief proinflammatory mediators cause an overall decline of inflammation at tissue level in tail and promote the successful regeneration supportive type of wound healing (Fig. 2A-D). As opined by Veldhoen and group (2006), the reduction in IL-17 expression in tail is plausibly because of the coherent effect of another regulatory mediator like IL-6 which has shown a major hike here. It could even be due to the specific signalling dictated by the EP receptors (Hinson et

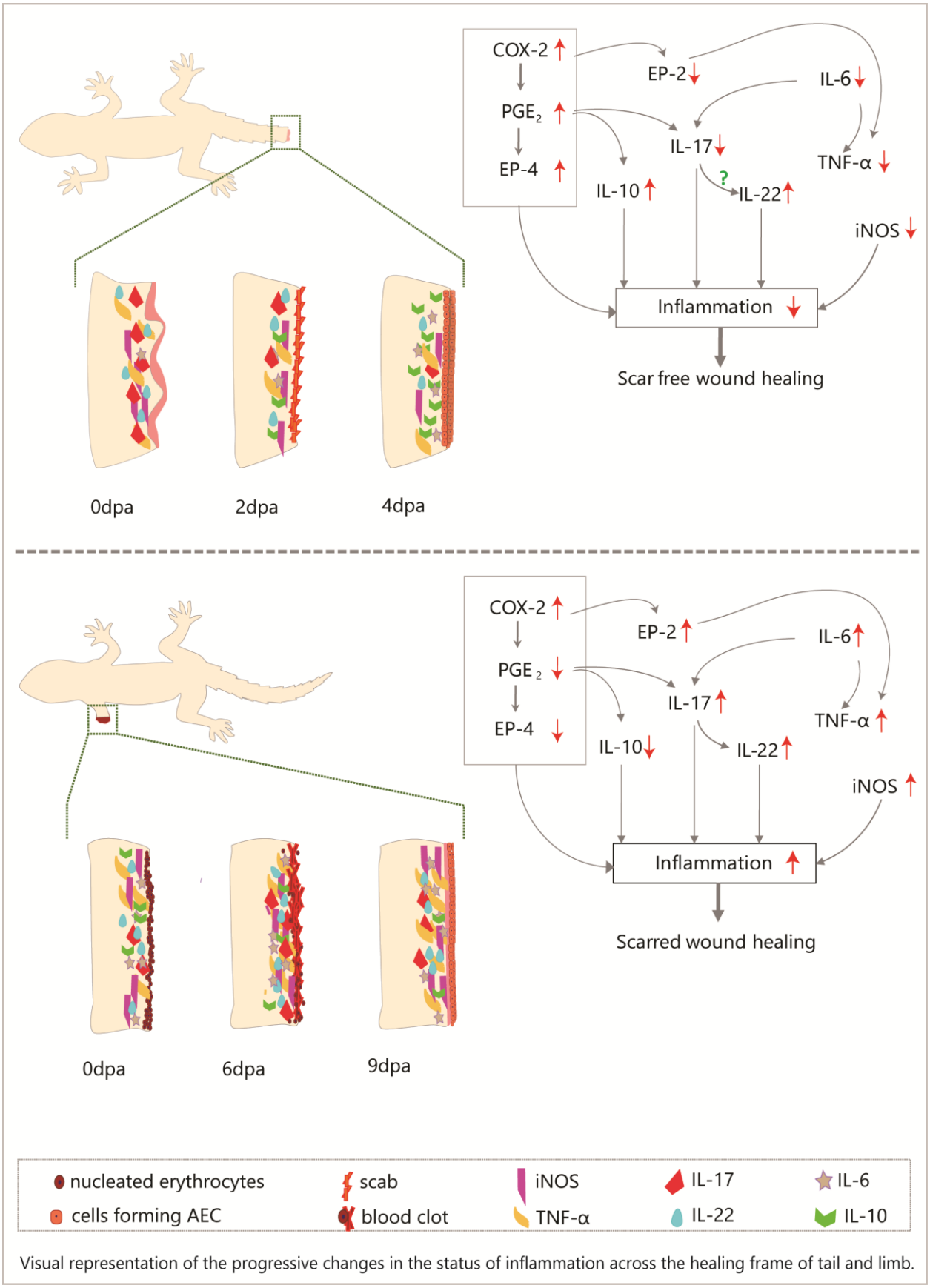
al., 1996). In limb tissue though, IL-17 was elevated, except at the time when scar formation and collagen deposition started at the site of healing (Fig. 2H). This disparity could be simply because by the end of scab formation, a permanent scar is constructed via collagen deposition and recruitment of fibroblast cells (Ranadive et al., 2018), while the tissue inflammation recedes to enhance the former process. Discovering this novel participation of IL-17 in the regeneration model recommends further investigation, where the performance of this cardinal inflammatory mediator can be explored.

IL-22, in the tail tissue showed well pronounced increase in its transcripts from 1dpa till 3dpa, after which its level reduced significantly till 4dpa. This ensures its participation in early epithelialisation, as achieved in tail. IL-22 elicits a protective role, when combined with IL-17, which specifically induces anti-microbial peptides in human keratinocytes (Sabat et al., 2013). Moreover, reduction of IL-17 could possibly influence the levels of IL-22 as observed in few other models like human T-cells (Veldhoen et al., 2006). This could be majorly because it is a cytokine of IL-10 superfamily, levels of which plunge under excessive inflammation (Zheng et al., 2007), as observed here in case of limb tissues. Herein, IL-22 followed the trend of IL-17, with noticeable rise in gene expression at the time of scab formation in limb tissue, in congruence with other proinflammatory mediators like IL-6, TNF-α, iNOS and IL-17. It is thus proved that IL-22 plays its part in repairing the wound in the two appendages, in synergy with IL-17 and reconstructs the framework for scar-free healing in tail (Fig. 2D), however, it supports scar formation (Fig 2H) under the prolonged inflammatory response in limb.

CONCLUSION

Overall, this is an inquisitive effort to deduce the crosstalk between inflammation and the colossal course of events leading to differential wound healing in lizard. These findings reinforce the concept that inflammation can hinder the restoration proceedings if its elevated levels remain persistent for a longer duration of time (Filbin, 2006; Mescher & Neff, 2006). The COX-2 mediated PGE_2 symphonises the entire event of the inflammation as it operates and directs the interleukin function at the site of tissue repair through a team of EP receptors, especially EP2 and EP4, and eventually, either reform the lost tissue via scar-free wound healing or form a permanent scar at the locale of injury. This study again establishes the dual role of inflammation in boosting and banishing the regenerative process by governing the fate of differential wound healing in the two appendages of the lizard.

Currently, under this ongoing project, the experiments are being conducted to identify the cellular components of inflammation coinciding with the humoral mediators. The effort is to localise macrophages and neutrophils at the site of wound healing, to explore their roles, if any, in contributing to the incongruent methods of repair as observed in the tail and limb of lizard. On the whole, the venture is designed to elaborate the participation of inflammation in modulating the course of disparate wound healing in the appendages of Northern House Gecko.



Visual representation of the progressive changes in the status of inflammation across the healing frame of tail and limb.

REFERENCES

Agata, K., Saito, Y., & Nakajima, E. (2007). Unifying principles of regeneration I: Epimorphosis versus morphallaxis. *Development, Growth & Differentiation*, *49*(2), 73-78.

Alibardi, L. (2009). Morphological and cellular aspects of tail and limb regeneration in lizards: a model system with implications for tissue regeneration in mammals.

Alibardi, L. (2010). Ultrastructural features of the process of wound healing after tail and limb amputation in lizard. *Acta Zoologica*, *91*(3), 306-318.

Alibardi, L. (2014). Histochemical, Biochemical and Cell Biological aspects of tail regeneration in lizard, an amniote model for studies on tissue regeneration. *Progress in Histochemistry and Cytochemistry*, *48*(4), 143-244.

Alvarado, A. S. (2000). Regeneration in the metazoans: why does it happen? *Bioessays*, *22*(6), 578-590.

Aoki, T., & Narumiya, S. (2017). Prostaglandin E 2-EP2 signaling as a node of chronic inflammation in the colon tumor microenvironment. *Inflammation and Regeneration*, *37*(1), 1-5.

Bellairs, A. D. A., & Bryant, S. V. (1985). Autotomy and regeneration in reptiles. *Biology of the Reptilia*, *15*(5), 301-410.

Bely, A. E. (2010). Evolutionary loss of animal regeneration: pattern and process. *Integrative and Comparative Biology*, *50*(4), 515-527.

Bely, A. E., & Nyberg, K. G. (2010). Evolution of animal regeneration: Re-emergence of a field. *Trends in Ecology & Evolution*, *25*(3), 161–170.

Bos, C. L., Richel, D. J., Ritsema, T., Peppelenbosch, M. P., & Versteeg, H. H. (2004). Prostanoids and prostanoid receptors in signal transduction. *The International Journal of Biochemistry & Cell biology*, *36*(7), 1187-1205.

Bradford, N. (1976). A rapid and sensitive method for the quantitation microgram quantities of a protein isolated from red cell membranes. *Analatycal Biochem*istry *72*(248), e254.

Brenneis, C., Coste, O., Altenrath, K., Angioni, C., Schmidt, H., Schuh, C. D., … Geisslinger, G. (2011). Anti-inflammatory role of microsomal prostaglandin e synthase-1 in a model of neuroinflammation. *Journal of Biological Chemistry*, *286*(3), 2331–2342.

Brockes, J. P., & Kumar, A. (2008). *Comparative Aspects of Animal Regeneration*.

Brockes, J. P., Kumar, A., & Velloso, C. P. (2001). Regeneration as an evolutionary variable.

Buch, P. R., Desai, I., & Balakrishnan, S. (2018). COX-2 activity and expression pattern during regenerative wound healing of tail in lizard *Hemidactylus flaviviridis*. *Prostaglandins & other Lipid Mediators*, *135*, 11-15.

Buch, P. R., Desai, I., & Balakrishnan, S. (2018). Prostaglandins and Other Lipid Mediators COX-2 activity and expression pattern during regenerative wound healing of tail in

lizard *Hemidactylus flaviviridis*. *Prostaglandins and Other Lipid Mediators*, *135*(January), 11–15.

Butin-Israeli, V., Bui, T. M., Wiesolek, H. L., Mascarenhas, L., Lee, J. J., Mehl, L. C., ... & Sumagin, R. (2019). Neutrophil-induced genomic instability impedes resolution of inflammation and wound healing. *The Journal of Clinical Investigation*, *129*(2), 712-726.

Caughey, G. E., Cleland, L. G., Gamble, J. R., & James, M. J. (2001). Up-regulation of endothelial cyclooxygenase-2 and prostanoid synthesis by platelets. Role of thromboxane A2. *Journal of Biological Chemistry*, *276*(41), 37839–37845.

Chamberlain, C. S., Leiferman, E. M., Frisch, K. E., Brickson, S. L., Murphy, W. L., Baer, G. S., & Vanderby, R. (2013). Interleukin expression after injury and the effects of interleukin-1 receptor antagonist. *PloS one*, *8*(8), e71631.

Chazaud, B. (2014). Immunobiology Macrophages: Supportive cells for tissue repair and regeneration. *Immunobiology*, *219*(3), 172–178.

Clause, A. R., & Capaldi, E. A. (2006). Caudal autotomy and regeneration in lizards. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, *305A*(12), 965–973.

Crofford, L. J., Wilder, R. L., Ristimäki, A. P., Sano, H., Remmers, E. F., Epps, H. R., & Hla, T. (1994). Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1 beta, phorbol ester, and corticosteroids. *Journal of Clinical Investigation*, *93*(3), 1095–1101.

Delorme, S. L., Lungu, I. M., & Vickaryous, M. K. (2012). Scar-Free Wound Healing and Regeneration Following Tail Loss in the Leopard Gecko, Eublepharis macularius. *Anatomical Record*, *295*(10), 1575–1595.

Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van De Putte, L. B., & Lipsky, P. E. (1998). Cyclooxygenase in biology and disease. *The FASEB journal*, *12*(12), 1063-1073.

Eming, S. A., Krieg, T., & Davidson, J. M. (2007). Inflammation in Wound Repair: Molecular and Cellular Mechanisms. *Journal of Investigative Dermatology*, *127*(3), 514–525.

Filbin, M. T. (2006). How inflammation promotes regeneration. *Nature Neuroscience*, *9*(6), 715–717.

Fior, J. (2014). Salamander regeneration as a model for developing novel regenerative and anticancer therapies. *Journal of Cancer*, *5*(8), 715–719.

Fitch, H. S. (2003). A Comparative Study of Loss and Regeneration of Lizard Tails. *Journal of Herpetology*, *37*(2), 395–399.

Font-Nieves, M., Sans-Fons, M. G., Gorina, R., Bonfill-Teixidor, E., Salas-Pérdomo, A., Márquez-Kisinousky, L., ... & Planas, A. M. (2012). Induction of COX-2 enzyme and down-regulation of COX-1 expression by lipopolysaccharide (LPS) control prostaglandin E2 production in astrocytes. *Journal of Biological Chemistry*, *287*(9), 6454-6468.

Funk, C. D. (2001). Prostaglandins and Leukotrienes: Advances in Eicosanoid Biology. *Science*, *294*(5548), 1871–1875.

Gilbert, E. A. B., Delorme, S. L., & Vickaryous, M. K. (2015). The regeneration blastema of lizards: an amniote model for the study of appendage replacement. *Regeneration*, *2*(2), 45-53.

Goetzl, E. J., An, S., & Smith, W. L. (1995). Specificity of expression and effects of eicosanoid mediators in normal physiology and human diseases 1. *The FASEB Journal*, *9*(11), 1051-1058.

Harizi, H., Grosset, C., & Gualde, N. (2003). Prostaglandin E2 modulates dendritic cell function via EP2 and EP4 receptor subtypes. *Journal of Leukocyte Biology*, *73*(6), 756–763.

Harris, S. G., Padilla, J., Koumas, L., Ray, D., & Phipps, R. P. (2002). Prostaglandins as modulators of immunity. *Trends in Immunology*, *23*(3), 144–150.

Hashemi Goradel, N., Najafi, M., Salehi, E., Farhood, B., & Mortezaee, K. (2019). Cyclooxygenase-2 in cancer: A review. *Journal of Cellular Physiology*, *234*(5), 5683-5699.

Hata, A. N., & Breyer, R. M. (2004). Pharmacology and signaling of prostaglandin receptors: Multiple roles in inflammation and immune modulation. *Pharmacology & Therapeutics*, *103*(2), 147–166.

Heffron, S. P., Weinstock, A., Scolaro, B., Chen, S., Sansbury, B. E., Marecki, G., ... & Fisher, E. A. (2021). Platelet-conditioned media induces an anti-inflammatory macrophage phenotype through EP4. *Journal of Thrombosis and Haemostasis*, *19*(2), 562-573.

Hinson, R. M., Williams, J. A., & Shacter, E. (1996). Elevated interleukin 6 is induced by prostaglandin E2 in a murine model of inflammation: Possible role of cyclooxygenase-2. *Proceedings of the National Academy of Sciences*, 93(10), 4885–4890.

Joshi, R., Hamed, O., Yan, D., Michi, A. N., Mostafa, M. M., Wiehler, S., ... & Giembycz, M. A. (2021). Prostanoid receptors of the EP4-subtype mediate gene expression changes in human airway epithelial cells with potential anti-inflammatory activity. *Journal of Pharmacology and Experimental Therapeutics*, *376*(2), 161-180.

Kaiser, P., Rothwell, L., Avery, S., & Balu, S. (2004). Evolution of the interleukins. *Developmental & Comparative Immunology*, *28*(5), 375–394.

Kang, Y. J., Mbonye, U. R., DeLong, C. J., Wada, M., & Smith, W. L. (2007). Regulation of intracellular cyclooxygenase levels by gene transcription and protein degradation. *Progress in Lipid Research*, *46*(2), 108–125.

Karin, M., & Clevers, H. (2016). Reparative inflammation takes charge of tissue regeneration. *Nature*, *529*(7586), 307-315.

Korbecki, J., Baranowska-Bosiacka, I., Gutowska, I., & Chlubek, D. (2014). Cyclooxygenase pathways. *Acta Biochimica Polonica*, *61*(4), 639–649.

Kuwano, T., Nakao, S., Yamamoto, H., Tsuneyoshi, M., Yamamoto, T., Kuwano, M., & Ono, M. (2004). Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. *The FASEB Journal*, *18*(2), 300-310.

Lasa, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J., & Clark, A. R. (2000). Regulation of cyclooxygenase 2 mRNA stability by the mitogen-activated protein kinase p38 signaling cascade. *Molecular and Cell Biology*, *20*(12), 4265–4274.

Lawrence, T. (2009). The nuclear factor NF-κB pathway in inflammation. *Cold Spring Harbor perspectives in biology*, *1*(6), a001651.

Lee, B.-S., Yuan, X., Xu, Q., McLafferty, F. S., Petersen, B. A., Collette, J. C., … Yu, J. S. (2009). Stimuli-responsive antioxidant nanoprodrugs of NSAIDs. *International Journal of Pharmaceutics*, *372*(1–2), 112–124.

Li, Q., Yang, H., & Zhong, T. P. (2015). Regeneration across Metazoan Phylogeny: Lessons from Model Organisms. *Journal of Genetics and Genomics*, *42*(2), 57–70.

Liou, J.-Y., Ellent, D. P., Lee, S., Goldsby, J., Ko, B.-S., Matijevic, N., … Wu, K. K. (2007). Cyclooxygenase-2-Derived Prostaglandin E 2 Protects Mouse Embryonic Stem Cells from Apoptosis. *Stem Cells*, *25*, 1096–1103.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, *25*(4), 402-408.

Lozito, T. P., Tuan, R. S., Alibardi, L., Bai, X., Wang, Y., Man, L., … Zhang, X. (2016). Lizard tail skeletal regeneration combines aspects of fracture healing and blastema-based regeneration. *Development (Cambridge, England)*, *143*(16), 2946–2957.

Lu, C., Xing, Z., Wang, X., Mao, J., Marcucio, R. S., & Miclau, T. (2012). Anti-inflammatory treatment increases angiogenesis during early fracture healing. *Archives of Orthopaedic and Trauma Surgery*, *132*(8), 1205-1213.

Lu, X., Xie, W., Reed, D., Bradshaw, W. S., & Simmons, D. L. (1995). Nonsteroidal antiinflammatory drugs cause apoptosis and induce cyclooxygenases in chicken embryo fibroblasts. *Proceedings of the National Academy of Sciences*, *92*(17), 7961-7965.

McCoy, J. M., Wicks, J. R., & Audoly, L. P. (2002). The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. *Journal of Clinical Investigation*, *110*(5), 651–658.

Mescher, A. L., & Neff, A. W. (2006). Limb regeneration in amphibians: immunological considerations. *The Scientific World Journal*, *6*, 1-11.

Mescher, A. L., Neff, A. W., & King, M. W. (2017). Inflammation and immunity in organ regeneration. *Developmental and Comparative Immunology*, *66*, 98–110.

Mescher, A., Neff, A., King, M. (2013). Changes in the inflammatory response to injury and its resolution during the loss of regenerative capacity in developing Xenopus limbs. PloS One. 8, e80477.

Minami, T., Nakano, H., Kobayashi, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., … Ito, S. (2001). Characterization of EP receptor subtypes responsible for prostaglandin E_2 -induced pain responses by use of EP_1 and EP_3 receptor knockout mice. *British Journal of Pharmacology*, *133*(3), 438–444.

Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., … Kudo, I. (2000). Regulation of Prostaglandin E 2 Biosynthesis by Inducible Membrane-associated Prostaglandin E 2 Synthase That Acts in Concert with Cyclooxygenase-2. *Journal of Biological Chemistry*, *275*(42), 32783–32792.

Murphy, B. F., & Thompson, M. B. (2011). A review of the evolution of viviparity in squamate reptiles: the past, present and future role of molecular biology and genomics. *Journal of Comparative Physiology B*, *181*(5), 575-594.

Nakanishi, M., & Rosenberg, D. W. (2013). Multifaceted roles of PGE2 in inflammation and cancer. In *Seminars in Immunopathology* (Vol. 35, No. 2, pp. 123-137). Springer-Verlag.

Pawel, K. (2012). Regulation of immune responses. Journal of Immunology, *188*, 21-8.

Peranteau, W. H., Zhang, L., Muvarak, N., Badillo, A. T., Radu, A., Zoltick, P. W., & Liechty, K. W. (2008). IL-10 overexpression decreases inflammatory mediators and promotes regenerative healing in an adult model of scar formation. *The Journal of Investigative Dermatology*, *128*(7), 1852–1860.

Pilo, B., & Anoop Kumar, B. (1995). Effect of chemical adrenalectomy and corticosterone administration on tail regeneration in the gekkonid lizard, Hemidactylus flaviviridis. *Indian Journal of Experimental Biology*, *33*, 917–920.

Pilo, B., & Suresh, B. (1994). Effect of EGF on progress of tail regeneration in the gekkonid lizard Hemidactylus flaviviridis. The *Journal of Animal Morphology and Physiology*, *41*, 61–66.

Portanova, J. P., Zhang, Y., Anderson, G. D., Hauser, S. D., Masferrer, J. L., Seibert, K., … Isakson, P. C. (1996). Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. *The Journal of Experimental Medicine*, *184*(3), 883–891.

Prisk, V., & Huard, J. (2003). Muscle injuries and repair: The role of prostaglandins and inflammation. *Histology and Histopathology*, *18*(4), 1243–1256.

Ranadive, I., Patel, S., Buch, P., Gowrikumari, U., Desai, I., & Balakrishnan, S. (2018). Inherent variations in the cellular events at the site of amputation orchestrate scar-free wound healing in the tail and scarred wound healing in the limb of lizard *Hemidactylus flaviviridis*. *Wound Repair and Regeneration*.

Renz, H., Gong, J. H., Schmidt, A., Nain, M., & Gemsa, D. (1988). Release of tumor necrosis factor-alpha from macrophages. Enhancement and suppression are dose-dependently regulated by prostaglandin E2 and cyclic nucleotides. *The Journal of Immunology*, *141*(7), 2388-2393.

Ricciotti, E., FitzGerald, G., (2011). Prostaglandins and inflammation. *Arteriosclerosis, Thrombosis and Vascular Biology.* 31, 986-1000.

Rouzer, C. A., & Marnett, L. J. (2009). Cyclooxygenases: structural and functional insights. *Journal of lipid research*, *50*, S29-S34. https://doi.org/10.1194/jlr.R800042-JLR200

Sabat, R., Witte, E., Witte, K., & Wolk, K. (2013). IL-22 and IL-17: an overview. *IL-17, IL-22 and their producing cells: Role in Inflammation and Autoimmunity*, 11-35.

Sharma, P., & Suresh, B. (2008). Influence of COX-2-Induced PGE2 on the Initiation and progression of tail regeneration in Northern House Gecko, Hemidactylus flaviviridis. *Folia Biologica*, *54*(6), 193–201.

Sharma, P., Anusree, P., Desai, I., & Suresh, B. (2011). Role of COX-2 induced PGE2 in epimorphosis: regulation of stage specific expression of proteins. *Journal of Cell and Tissue Research*, *11*(3), 2925.

Simmons, D., Botting, R., Hla, T., (2004). Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacological Reviews*. 56, 387-437.

Smith, R. S. (1992). A comprehensive macrophage-T-lymphocyte theory of schizophrenia. *Medical Hypotheses*, *39*(3), 248-257.

Steinfeld, B., Scott, J., Vilander, G., Marx, L., Quirk, M., Lindberg, J., & Koerner, K. (2015). The role of lean process improvement in implementation of evidence-based practices in behavioral health care. *The Journal of Behavioral Health Services & Research*, *42*(4), 504-518.

Stolina, M., Sharma, S., Lin, Y., Dohadwala, M., Gardner, B., Luo, J., ... & Dubinett, S. M. (2000). Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. *The Journal of Immunology*, 164(1), 361-370.

Sugimoto, Y., & Narumiya, S. (2007). Prostaglandin E receptors. *Journal of Biological Chemistry*, *282*(16), 11613–11617.

Suzuki, M., Yakushiji, N., Nakada, Y., Satoh, A., Ide, H., Tamura, K., 2006. Limb regeneration in Xenopus laevis froglet. Sci. *World Journal*. 6.

Tanaka, E. M. (2003). Regeneration: If they can do it, why can't we? *Cell*, *113*(5), 559–562.

Tsatsanis, C., Androulidaki, A., Venihaki, M., & Margioris, A. N. (2006). Signalling networks regulating cyclooxygenase-2. *The International Journal of Biochemistry & Cell Biology*, *38*(10), 1654-1661.

Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., & Stockinger, B. (2006). TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*, *24*(2), 179–189.

Verma, U., Gautam, M., Parmar, B., Khaire, K., Wishart, D. S., & Balakrishnan, S. (2021). New insights into the obligatory nature of cyclooxygenase-2 and PGE2 during early chick embryogenesis. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1866*(4), 158889.

Vervoort, M. (2011). Regeneration and development in animals. *Biological Theory*, *6*(1), 25-35.

Vitt, L. J. (1983). Tail loss in lizards: the significance of foraging and predator escape modes. *Herpetologica*, 151-162.

Vitulo, N., Dalla Valle, L., Skobo, T., Valle, G., & Alibardi, L. (2016). Transcriptome analysis of the regenerating tail versus the scarring limb in lizard reveals pathways leading to successful versus unsuccessful organ regeneration in amniotes. *Developmental Dynamics*, 116–134.

Yang, X. O., Chang, S. H., Park, H., Nurieva, R., Shah, B., Acero, L., … Dong, C. (2008). Regulation of inflammatory responses by IL-17F. *The Journal of Experimental Medicine*, *205*(5), 1063–1075.

Yasui-Kato, M., Patlada, S., Yokode, M., Kamei, K., & Minami, M. (2020). EP4 signalling is essential for controlling islet inflammation by causing a shift in macrophage polarization in obesity/type 2 diabetes. *Diabetes and Vascular Disease Research*, *17*(4), 1479164120945675.

Zheng, Y., Danilenko, D. M., Valdez, P., Kasman, I., Eastham-Anderson, J., Wu, J., & Ouyang, W. (2007). Interleukin-22, a TH17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature*, *445*(7128), 648–651.

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BBA - Molecular and Cell Biology of Lipids 1866 (2021) 158889

Contents lists available at ScienceDirect

BBA - Molecular and Cell Biology of Lipids

journal homepage: www.elsevier.com/locate/bbalip

New insights into the obligatory nature of cyclooxygenase-2 and PGE_2 during early chick embryogenesis

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ARTICLE INFO

Keywords:
Cyclooxygenase
Embryogenesis
Prostaglandins
Lipids
Development

ABSTRACT

Temporal expression patterns and activity of two cyclooxygenase (COX-1 and COX-2) isoforms were analysed during early chick embryogenesis to evaluate their roles in development. COX-2 inhibition with etoricoxib resulted in significant structural anomalies such as anophthalmia (born without one or both eyes), phocomelia (underdeveloped or truncated limbs), and gastroschisis (an opening in the abdominal wall), indicating its significance in embryogenesis. Furthermore, the levels of PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2 were assessed using quantitative LC-MS/MS to identify which effector prostanoid (s) had their synthesis initiated by COX-2. COX-2 inhibition was only shown to reduce the level of PGE_2 significantly, and hence it could be inferred that the later could be largely under the regulation of activated COX-2 in chick embryos. The compensatory increase in the activity of COX-1 observed in the etoricoxib-treated group helped to maintain the levels of PGD_2, $PGF_{2\alpha}$, and TXB_2. Though the roles of these three prostanoids in embryogenesis need to be further clarified, it appears that their contribution to the observed developmental anomalies is minimal. This study has shown that COX-2 is functionally active during chick embryogenesis, and it plays a central role in the structural configuration of several organs and tissues through its downstream effector molecule PGE_2.

1. Introduction

Cyclooxygenase enzymes, which exist in two isoforms (COX-1 and COX-2), convert arachidonic acid from the plasma membrane into various prostanoids such as prostaglandins (PGs) and thromboxanes (TXs) [1]. Typically, of these two isoforms, COX-2 is considered to be the inducible type, whereas COX-1 is considered as the constitutive one. Both these isoforms are stimulated during inflammation to initiate the production of prostanoids, whereupon specific prostanoids are produced with the help of tissue-specific prostaglandin synthases to perform particular functions regionally [1]. The roles of COX-2 and the derived prostanoids have been elucidated through the genetic modification and pharmacological inhibition of the enzyme [2,3]. The majority of investigations over the past decade have focused on the role of COX-2 in tumorigenesis, which has certainly helped in understanding the aetiology of several cancers [3,4]. On the contrary, only a handful of studies have explored the involvement of prostaglandins induced via COX-2 in embryogenesis and other developmental processes. Essentiality of COX-2 in facilitating appendage regeneration via modulation of the expression of WNT, FGF, and MMP has been recently identified in reptiles [5,6]. COX-2 induced PGs are also known to be actively functioning for ovulation, fertilisation, decidualisation, and implantation in mammals [7,8]. More recently, COX-2 was found to interfere with blastocyst hatching, a prelude to successful implantation, in hamsters [9]. Additionally, Loftin and colleagues reported COX-2 to be necessary for the closure of ductus arteriosus in mice [2]. Likewise, the ubiquitous expression of COX-2 in early stages of embryonic development has been shown to result in skeletal deformities in a novel COX-2 transgenic mouse model [10]. In an effort to understand what tissues might be affected, Stanfield and colleagues (2002) localised COX-2 in rat embryonic, and foetal tissues, wherein its presence was found in the heart, kidney, skin, and cartilage [11]. These studies indeed suggest that COX-2, either through its inhibition or activation, could have an effect on the development of a number of organs, organ systems or tissues in vertebrates.

In the present study, we ascertained the expression of COX-2 in the

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https://doi.org/10.1016/j.bbalip.2021.158889
Received 23 October 2020; Received in revised form 7 January 2021; Accepted 13 January 2021
Available online 15 January 2021

Received: 13 June 2020 | Accepted: 21 November 2020
DOI: 10.1002/tox.23074

RESEARCH ARTICLE

ENVIRONMENTAL TOXICOLOGY WILEY

Early embryonic exposure to chlorpyrifos-cypermethrin combination induces pattern deficits in the heart of domestic hen

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Funding information
Department of Biotechnology, Grant/Award Number: BT/PR11467/MED/31/270/2014

Abstract

Exposure to chlorpyrifos-cypermethrin combination during early development resulted in defective looping and ventricular noncompaction of heart in domestic chicken. The study was extended to elucidate the molecular basis of this novel observation. The primary culture of chicken embryonic heart cells showed a concentration-dependent loss of viability when challenged with this combination of technical-grade insecticides. Comet assay, DNA ladder assay, and analyses of appropriate markers at transcript and protein levels, revealed that chlorpyrifos-cypermethrin combination induced cell death by activating apoptosis. Parallelly, the tissues derived from control and experimental group hearts were checked for apoptotic markers, and the result was much similar to that of the in-vitro study. Further analysis showed that chlorpyrifos-cypermethrin combination deranged the expression pattern of the transcriptional regulators of cardiogenesis, namely TBX20, GATA5, HAND2, and MYOCD. This, together with heightened apoptosis, could well be the reason behind the observed structural anomalies in the heart of chlorpyrifos-cypermethrin poisoned embryos.

KEYWORDS
apoptosis, cardiogenesis, chlorpyrifos, cypermethrin, teratogenicity

1 | INTRODUCTION

Prenatal exposure to environmental chemicals is one of the leading causes of birth defects in humans. About four infants in one thousand live births globally possess malformed organs, resulting from chemical exposures during pregnancy.[1] Moreover, among the affected children, over a hundred thousand die every year from malformations and functional irregularities of heart worldwide.[2] It has been reported that newborns with congenital heart defects (CHD) represent a sizable portion of patients who are diagnosed with pediatric cardiovascular disease.[3] Interestingly, epidemiological evidence links maternal occupational exposure to agrochemicals as one of the leading reasons for CHD in infants.[4] In India, the preferred agrochemical to combat the insect pests of major crops is a combination of chlorpyrifos (CP) and cypermethrin (CM).[5] If used in combination, both the chemicals cause a synergistic toxic effect, eventually eliminating all the pest species, which otherwise are resilient to them, when used singularly.[6] Therefore, it was thought pertinent to evaluate the risk of chlorpyrifos-cypermethrin (CP-CM) combination for their potential to induce CHD.

It is well perceived that CP exerts its toxicity by phosphorylating Acetylcholinesterase, a key enzyme involved in the termination of neuronal stimulus, whereas CM acts by disrupting the neuronal membrane potential.[7] However, when used in combination, the organophosphate inhibits enzymes involved in the detoxification of pyrethroids, leading to a greater than additive toxicity.[8] A report from Taiwan suggests invasive toxic effects of CP and CM in young

