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Expression of inducible inflammatory mediators during early stages of regeneration

INTRODUCTION

Large scale regeneration follows an amputation injury in many organisms where a number of lost tissues are replaced with new ones. The greatest boost to our understanding of the regenerative process will come from the decipherment of the very initial events therein. It is rather interesting that the first responses to a wound in regeneration-competent and regeneration-incompetent systems are not entirely different (Bielefeld et al., 2013). The first course of action taken by a wounded organism is to protect the tissues exposed at the wound site from excessive blood-loss and pathogen invasion. Components of the immune system in almost all organisms of the animal kingdom achieve this through inflammation (Ohtola et al., 2008). The question which now remains is: If inflammation is the common early response to wounds in regenerating as well as non-regenerating systems, how then is the outcome not always the same? Two possible explanations may be given. First, the events following inflammation may be very different in the two types of wounds and that may divert one towards regeneration or otherwise. Second, the *nature* of inflammation itself may be different. Emerging research suggests that the nature of inflammatory response may indeed be different in the two cases and that the event following inflammation will depend on this nature.

Given that the nature of inflammatory responses in regenerating and non-regenerating systems is different, it is of great relevance to regeneration biologists to understand these differences. The greatest challenge in this respect, however, is the complexity of the process. Inflammation, although termed as a feature of the immune system, is really an outcome of a complex interaction between local tissue components and the immune cells and is mediated by a long list of secreted factors originating from both the sources. It has to be acknowledged that, over the decades, immunologists have managed to delineate the cellular and molecular mechanisms of cutaneous wound healing and inflammation in great detail. It is now well known that an injury initiates a cascade of signals locally, leading to recruitment of phagocytic immune cells in the area. These cells, in turn, activate local phagocytes to respond

to immune cytokines and join in the process of clearing damaged cells as well as fighting off microbes. After a dominating presence of the immune cells in the injured tissue, there follows a change in the phenotype of the response and anti-inflammatory signals from the immune cells lead the tissue to a state of homeostasis (reviewed by Bielefeld et al., 2013). The molecular signals involved in these cellular events are very well characterised. However, the said processes of healing are different from those in regenerative healing. The former results in production of fibrous tissue and an undefined extent of cell restoration, while the latter proceeds with negligible scar-formation and maximal structural and functional restoration of the original tissue. This scar-less wound healing, which leads to complete regeneration of tissue systems in many organisms, is now widely believed to be possible due to a restricted immune response to injury.

As such, the inverse relation between immunity and regenerative abilities is seen throughout the animal kingdom. As one moves up the ladder of evolution, the capacity to regenerate diminishes with a concomitant increase in the complexity of the immune system. The amniotes evolved very well developed immune systems owing to their true terrestrial habit. All amniotes indeed have almost no ability for large scale regeneration (barring a few exceptions). This relation is so strongly followed that among the amphibians themselves, there is a differential regenerative ability that relates to immune capacity. The salamanders, newt and *Xenopus* larvae have poorly developed adaptive immune features. When *Xenopus* (and other anuran members) metamorphose into adults, they develop an adaptive immune system comparable to that of mammals (Robert and Ohta, 2009) and anuran adults, as expected, are unable to regenerate appendages. Members of anura pose as handy organisms to study immune influence on regeneration, since they display the presence of two immune systems through their lifetime. As they undergo metamorphosis, the immune system undergoes a drastic change, more like a replacement, and they lose the capacity to regenerate (Flajnik et al., 2003).

It must be mentioned that even the regeneration-competent organisms are by no means spared from inflammatory action, but the action is a more controlled one. This makes timely resolution or removal of inflammation from the wound site extremely crucial. The hypothesis that timely resolution of immune response is inevitable for successful regeneration is validated by the extensive work on amphibians by the lab of Anthony Mescher (Harty et al., 2003; Mescher and Neff, 2006; King et al., 2009; Mescher et al., 2017). Moreover, regeneration experiments in Hydra and Planaria have revealed even in these lower

invertebrates, inflammation is the first response to amputation, before regeneration (Mastellos et al., 2013). Schmidt (1968) and Godwin et al. (2013) demonstrated that the infiltration of leukocytes at the wound site in newt limbs is comparable in magnitude to that in mammalian wounds (reviewed by Mescher et al., 2017).

We therefore understand that even as inflammation is an important event in both regenerating and non-regenerating wounds, the nature of this process is in some way different, and demands a closer look at. Some authors have observed very significant differences in the expression of immune-response genes between regenerating versus non-regenerating tissue (Harty et al., 2003; Mescher and Neff, 2005; 2006; Mescher et al., 2007; King et al., 2012). Mathew et al. (2007) showed that interference with glucocorticoid signalling in Zebrafish resulted in a failure of the expression of some regeneration-specific genes. The glucocorticoids are known to have an inhibitory effect on the acute inflammatory response. Further, some studies revealed that regeneration depends on components of both innate and adaptive immunity (Grow et al., 2006; King et al., 2012). Research on amphibians has established that precise regulation of innate and adaptive immunity helps create a favourable microenvironment in the tissue to lead it to epimorphic regeneration (Del Rio-Tsonis et al., 1998; Mescher and Neff, 2005; 2006; Grow et al., 2006; King et al., 2012).

What most complicates our understanding about the relation between immunity and regeneration is the appearance of reports showing a positive correlation therein. Eming et al. (2009) have reviewed the information available about the immune system promoting tissue repair and protection. The review cites examples of Salamanders, Zebrafish and even mammals where inflammation (or the presence of a well-developed immune system) not only allows tissue repair and regeneration but also promotes it (Breedis, 1954; Billingham and Russel, 1956; Godwin and Brockes, 2006; Cvejic et al., 2008).

A reasonable number of recent studies have helped identify the proteins promoting and also controlling inflammation during healing; however, as correctly pointed out by Mescher et al. (2017), a comparison of the expression profiles of the eicosanoids and other lipid mediators between regenerating wounds and mammalian wounds has still not been drawn. As part of a larger goal to decrypt the differences between regenerating and non-regenerating appendages in lizard, our lab had initiated work to check whether cyclooxygenase-2 mediated PGE_2 is required for regenerating the lizard tail. It was found that inhibiting COX-2 activity hampered the progression of various cellular processes essential for healing and regeneration (Sharma

and Suresh, 2008; Anusree et al., 2011). COX-2 derived PGE_2 is also reported to be a crucial mediator of cell-proliferation in various cancers. PGE_2 produced in tumour promotes the proliferation of head and neck squamous carcinoma (HNSC) cells by signalling through PGE_2 receptors expressed on most HNSC cells (Abrahao et al., 2010). Also, COX-2 expression is found upregulated in pancreatic cancer in humans (Tucker et al., 1999). Intestinal tumours in humans and experimental animals show an exaggerated presence of COX-2. Its induced expression also enables CaCo-2 cells to be more invasive than control cells lacking its expression (Tsuji et al., 1997). Inhibition studies in cancer systems further validate the importance of this enzyme in the process. A549 lung carcinoma cells with COX-2 knocked down demonstrated slow growth and reduced rate of colony formation (Li et al., 2011). Celecoxib administration delayed tumour growth in xenograft models (Leahy et al., 2002). In systems of regeneration, COX-2 and its major product PGE_2 have recently been implicated as essential promoters of healing, repair and proliferation of various tissue types. Stimulation of the PGE_2-EP2 pathway in rabbit enhanced the regeneration of cartilage tissues (Otsuka et al., 2009). The activity of this inducible enzyme was also discovered to be necessary for fracture healing. It was observed in histological sections that endochondral ossification in the healing of fractures required COX-2 expression (Simon et al., 2002). Indomethacin-mediated inhibition of COX-2 also caused delayed regeneration of the skeletal elements of tailfins in zebrafish (Bockelmann and Bechara, 2009). Further, almost all cancers and regenerating tissue are dependent on either resident or induced stem cells for repair and proliferation. Literature has evidence that PGE_2 induces proliferation of stem cells. PGE_2 treatment led to an expansion of the pool of hematopoietic stem and progenitor cells (HSPCs) in mice (Porter et al., 2013). With all these functions attributed to COX-2, our aim was to explore its specific role in the regenerating lizard tail.

COX-2, the inducible isoform of COX, shares many similarities with NOS-2 (or iNOS), the inducible isoform of Nitric oxide synthase (NOS). Both these enzymes have counterparts that are expressed constitutively and are required for the maintenance of homeostatic functions. Induction of the inducible isoforms of both enzymes results in a large amount of product, mounting strong immune reactions in response to the release of immune cytokines following pathogen attack or tissue injury. The nitric oxide synthases catalyse the oxidation of L-arginine to produce nitric oxide (NO) and citrulline. NOS exists in three distinct isoforms – constitutively expressed nNOS (neuronal NOS) and eNOS (endothelial NOS) and the inducible isoform iNOS (Moncada et al., 1991; Alderton et al., 2001). iNOS mediates

inflammatory reactions following infection and tissue damage. It can be expressed in almost every cell-type and its product may lead to either beneficial or harmful effects (Galea and Feinstein, 1999). As has been generally observed, cNOS (as the constitutive isoforms are collectively called) are associated with homeostasis, while iNOS mediates inflammation and tissue repair (Marletta, 1994; Nathan and Xie, 1994; Schmidt and Walter, 1994). iNOS is conceivably upregulated by inflammatory cytokines IL1, TNF-α and IFNγ, among others (Amin et al., 1995).

The enzymatic product of the NOS enzymes – nitric oxide – is a small and very simple, yet highly reactive molecule. It is intriguing that a simple reactive radical has the ability to regulate a whole range of physiological processes and that it has been conserved greatly through the course of evolution. It is found from arthropods right up to humans and is responsible for diverse functions like neurotransmission, blood-clotting, blood pressure regulation and immune fight-back (Feldman et al., 1993). NO can influence almost every cell type and has major implications on immune parameters. Its role is so diverse that it cannot be unambiguously classified as either pro- or anti-inflammatory (Cirino et al., 2006), which is also the case with COX-2.

Once secreted, NO freely diffuses through cell-membranes to activate guanylate cyclase by binding to its heme iron. The resulting increase in cGMP production is responsible for modulating the activities of various ion channels, kinases, etc. (Ignarro, 1992). By regulating these proteins, NO controls blood vessel behaviour, nerve signalling and defence responses in the immune system, as already mentioned (Moncada and Higgs, 1991; Alderton et al., 2001; Bogdan, 2001; Dawn and Bolli, 2002).

Much akin to COX-2/PGE_2 signalling, NOS/NO signals have been found to play a part in cancers. Reports show the involvement of NO in metastasis (Buttery et al., 1993; Dong et al., 1994). Gynecological tumours have been found with high local expression of NOS enzymes (Thomsen et al., 1994). Cancers induced by chemical carcinogens have shown iNOS expression (Chen and Lin, 2000). Interestingly, NO has also been found to bring about growth arrest and differentiation in cells of the CNS (Peunova and Enikolopov, 1995).

The process of wound healing also shows the involvement of NO. Human skin fibrobalsts are able to spontaneously produce NO through iNOS activation by cytokines (Wang et al., 1996). Skin keratinocytes can express all the NOS isoforms (Baudouin and Tachon, 1996; Bruch-Gerharz et al., 1996; Romero-Graillet et al., 1996; Sirsjo et al., 1996; Shimizu et al., 1997). In

mice, creation of an iNOS deficiency revealed an important role for iNOS-derived NO in wound healing. Animals in which iNOS was knocked out showed significantly delayed closure of excision wounds (Yamasaki et al., 1998).

Nitric oxide is thus found in processes where PGE_2 is implicated and the two pathways indeed show evidence of interaction at various levels. An important paper by Schoen-Smith and Lautt (2005) has proclaimed that both NO and PGE_2 are necessary triggers for the series of biochemical events that lead to liver regeneration. Studies on some systems make one believe that the NO and PGE_2 pathways must converge at the level of the secondary messenger cAMP. cAMP leads to an increase in iNOS mediated NO in macrophages, smooth muscle cells and mesangial cells, as observed by Hecker et al. (1999). Iimura et al. (1996) and Kunz et al. (1994) have shown that an increase in cAMP can stabilise iNOS mRNA, thus leading to increased NO production. Galea and Feinstein (1999) have however reviewed that influence of cAMP on iNOS expression may either be inhibitory or stimulatory and this depends on the cell types in question and also on the microenvironment in the tissue. For instance, PGE_2 inhibited the expression of mouse macrophages and, interestingly, this effect was mediated through cAMP (Hirokawa et al., 1994). LPS-induced production of NO was also found reduced in another piece of work on peritoneal macrophages and this was again relayed through cAMP (Raddassi et al., 1993). Tetsuka et al. (1994) have demonstrated inhibition of iNOS mRNA by COX-2/PGE_2 signalling in rat mesangial cells, which was not mediated through Adenylate cyclase activation. While all the above examples cite the regulation of NO by PGE_2, it is known in some instances that NO signalling also regulates the PGE_2 pathway and that this regulation can be either positive or negative in nature. NO production was found to increase the expression of COX-2 in pancreatic cells at the transcriptional level (Ling et al., 2005). In contrast, NO inhibited the expression of COX-2 in macrophages, as observed by Clancy et al. (2000).

The multifarious nature of the cross-talk between the two pathways is easy to appreciate but rather difficult to elucidate. Since the current work involves COX-2 as the central focus, we sought to first understand the normal expression of NO during early stages of regeneration, followed by the effect of COX-2 inhibition on iNOS expression.

With an aim to explicate the status of immune regulatory molecules during early regeneration, it was thought imperative to check the expression of key cytokines such as IL1β, IL6, IL10, NFκB, TNFα and IFNγ. While these regulators have been widely studied for

their immune effects, recent work even attributes functions of tissue regeneration to them. NFκB, for example, has a role in the regeneration of neurons (Haenold et al., 2014). It also plays an important part in liver regeneration along with TNFα (Iimuro and Fujimoto, 2010). TNFα activates IL6 signalling through its receptor TNFR1 to induce regeneration of the liver (Yamada et al., 1997). Muscle recovery following an injury may also depend on these immune molecules. TNFα expression was found high in injured myofibres (Collins and Grounds, 2001) and it promoted myogenesis through the p38 MAPK pathway (Chen et al., 2007).

Two interleukins of particular interest to our cause were the IL17 and IL22, on account of their significant contribution to organ regeneration in mammals. It was thought pertinent to study the presence of these lesser known interleukins in the lizard regeneration system, since there are no reports of their participation in epimorphic regeneration so far. You et al. (2005) for the first time reported the involvement of IL17 in organogenesis. IL17 promotes hepatic leukocytes to direct the regeneration of liver tissue (Rao et al., 2014). Many studies credit IL22 with a pro-regenerative role as well. It can induce proliferation in hepatocytes. Exogenously added IL22 could induce genes leading to regeneration in mouse liver (Rao et al., 2014). In a culture of intestinal organoids, proliferation and expansion of stem cells was significantly promoted by IL22 addition (Lindemans et al., 2015). Recombinant IL22 administered to mice gave a major impetus to regeneration of the damaged thymus after total body irradiation (Dudakov et al., 2012)

The current study therefore addresses the expression pattern of these inflammatory cytokines during the initiation of regeneration in lizard *Hemidactylus flaviviridis*. Moreover, since COX-2 was being tested for its position as a master regulator of epimorphosis, the influence of COX-2 inhibitor on the expression of these various signalling molecules was tested additionally. Blockage of COX-2 activity was brought about by means of a pharmacological inhibitor etoricoxib. Etoricoxib is specific for the inducible isoform of COX and has earlier shown potency in interfering with the cellular processes bringing about regeneration (Suresh et al., 2009).

MATERIAL AND METHODS

Lizards were divided into four groups for the study of early time points. Amputations were made at the junction of the second and third tail segment, following which the second (distal-most) segment was collected from the respective groups at 1, 2, 3 and 4 dpa. Tissue representing resting state (0 dpa) was collected from the above group animals during initial amputations. For inhibition studies, lizards were divided into two groups – control and etoricoxib. While control group animals received 50 µl of purified water each day, treatment group animals were given the same volume of etoricoxib suspension daily, as mentioned in Chpater 2: Material and methods.

For western blot experiments, 30 µg total protein was loaded. Anti-COX-2 IgG Mouse (Sigma-Aldrich, USA) or Anti-iNOS IgG Rabbit (Sigma-Aldrich, U.S.A.) were used for probing the proteins of interest at concentrations of 1 µg/ml and 2 µg/ml respectively. The same antibodies were used for immunohistochemical localisation experiments at 3 µg/ml each. Similarly, Anti-phospho-PI3K IgG Rabbit (SantaCruz Biotech, USA), Anti-phospho-CREB (Cell Signalling Technology, USA) IgG rabbit and Anti-phospho-p38 MAPK IgG Mouse (Thermo Fisher, USA) mouse were used at 0.1 µg/ml each for western blots of the respective proteins.

Quantitative real-time PCR was carried out to assess gene expression quantitatively. Data is represented as mean of normalised Cq values and also as fold change in expression (by the method of Livak and Schmittgen, 2001).

RESULTS

COX-2/PGE2 pathway

Cyclooxygenase-2 activity during early stages of regeneration

The activity of COX-2 was tested using a colorimetric kit-based method (Cayman Chemical Co.). The kit allowed differential assay of COX-2 activity by use of an inhibitor specific to COX-1. Since COX-2 is important in the orchestration of the inflammation, its activity was checked at the early stages of healing after tail amputation in lizard, the phase which corresponds to the inflammatory response. The time-period from amputation up to wound epithelium formation was divided into four equal intervals, and activity was measured at 0, 1, 2, 3 and 4 days post amputation (dpa). Wound epithelium formation was achieved at 4 dpa in

the ambient conditions of our set-up. COX-2 activity was seen to increase significantly ($p<0.001$) from the resting state at the first measured time-point 1 dpa (Figure 3.2). Activity remained high for the rest of the tested time-points. This reflects an early induction of the enzyme after amputation.

COX-2 protein expression

The change in activity of COX-2 after tail amputation in lizard was reflected in the previous experiment. However, it had to be determined whether this was due to post-translational activation of the protein or induction of the expression itself. For this, an immunoblot analysis was carried out at the said time-points. Total protein was transferred onto a PVDF membrane and COX-2 was probed as described in the material and methods section. Results showed COX-2 expression increasing early on in regeneration from the first measured time-point 1dpa (Figure 3.3). Protein expression remained high till it slightly tailed off after formation of the wound epithelium at 4 dpa.

COX-2 gene expression

Since western blots showed changes in the expression of COX-2 with the progress of healing, it had to be ascertained whether these changes were at the level of gene transcription, or they were post-transcriptionally brought about. For this, an RT-PCR analysis was employed on mRNA from regenerating tissue of lizard tail collected at the same time-points as mentioned above. Gene expression data (Figure 3.4) showed close relation to the pattern seen in western blot, with major elevation in COX-2 transcript apparent at 1 dpa and the expression remaining high (as compared to 0 dpa) till the attainment of wound epithelium.

Localisation of Cyclooxygenase-2

Previous work by Sharma and Suresh (2008) confirmed the presence and importance of COX-2 in the regenerating lizard tail and the above-mentioned experiment gave an understanding of its activity pattern as well. However, what remained to be seen is which tissues are expressing this enzyme. Localisation was carried out using immunohistochemistry on fresh frozen sections. Sections of 12 µm thickness were taken and probed as described in Chapter 2. Using the ALP substrate system for staining, the sites of enzyme expression were found (Figure 3.5, top). COX-2 was seen to be expressed mainly along the plane of amputation, i.e. at the interface of newly formed tissue and the stump. Faint staining was seen along the newly formed epithelium. At this stage the epithelium, although completely formed, further thickens into a multi-layered cap called the apical epithelial cap (AEC).

Localisation of this enzyme at the next milestone of epimorphosis – blastema – revealed its expression mainly at the original site of the cut nerve end (Figure 3.5, lower image).

Expression of PGE_2 receptors

COX-2 induced PGE_2 signals are relayed through its binding with the EP receptors. Four receptors in this group, *viz.*, EP1, EP2, EP3 and EP4 are found on the surface of cells responsive to PGE_2 and exhibit different functions, sometimes overlapping with each other. They participate in the autocrine and paracrine signalling of this pathway. If it is identified specifically which receptor/s is/are expressed in the system, a fair idea of the downstream signal and its effects can be obtained. Therefore, the expression of all four EP receptor subtypes was checked for and it was found that the EP2 receptor was expressed in the regenerating lizard tail. Next, the relative change, if any, in expression of this receptor was assessed at the early stages of regeneration. The time-points were as described above. Results show a reasonably high expression of EP2 receptor transcript in the resting state tail (Figure 3.4). This remained consistent before a slight decline was seen in transcript level at 2 dpa, which then remained constant. Apart from EP2, faint EP4 expression was also reflected in the PCR results. However, the results for EP4 were equivocal and it failed to consistently amplify in repetitions of the experiment.

iNOS/NO pathway

Nitric oxide levels during early stages of regeneration

Apart from the COX-2/PGE_2 pathway, the other well known inducible mediator of inflammation is the iNOS-induced nitric oxide (NO) signalling pathway. The levels of NO were estimated in regenerating tail tissue at 0, 1, 2, 3 and 4 dpa by a method of Greiss, as described by Moshage et al. (1995). The Greiss method is an appropriate tool used as a measure of iNOS expression since iNOS produces large amounts of NO continuously (Galea and Feinstein, 1999). Levels of NO obtained during the said time-points were rather interesting, with a sharp decline ($p<0.001$) following amputation at 2 dpa (Figure 3.6). The tissue concentration of NO remained low (as compared to resting tail) and was seen to be restored at the time of wound epithelium formation.

iNOS protein expression

It was found through western blots that iNOS protein expression went in agreement with the results of NO estimation. Protein levels decreased from the basal level and were restored only

at the time of complete formation of wound epithelium (Figure 3.7). It must be mentioned that the iNOS expression must be fairly low as bands were stained with very low intensity.

iNOS gene expression

Gene expression results for iNOS revealed that the level of transcripts did not change through the time-points significantly (Figure 3.8).

iNOS localisation

iNOS was localised in the regenerating tail at the wound healing and the blastema stages. The experiment was carried out in the same way as described for COX-2. The distribution of iNOS was similar to that seen for COX-2 in one of the previous experiments. iNOS expression at the wound healing stage was found in the cells which lined the original plane of amputation (Figure 3.9, top). Faint staining was also observed along the wound epithelium, which consists of cells actively proliferating. During the blastema stage however, iNOS was localised near the cut-end of the spinal cord (Figure 3.9, lower image). This is where the ependymal tube would originate from, a few hours subsequently.

Immune regulators in epimorphosis

The interleukins screened for gene expression in the current study were IL1β, IL6, IL10, IL17, IL22, NFκB, TNFα and IFNγ. Among these, gene expression of IL6, IL17 and IL22 was found in the regenerating tail of lizard at the early time-points. All the three interleukins followed a trend in expression similar to each other; their expression decreased from a basal level in the resting state to very low after 2 dpa (Figure 3.10).

Effect of COX-2 inhibition on immune mediators

Validation of COX-2 inhibition by Etoricoxib

COX-2 inhibition was achieved using pharmacological drug etoricoxib. Drug was administered to lizards at a dose of 25mg/kg body weight once daily. Dosing began a day prior to amputation and was continued till tissue collection at the required stage. To validate whether Etoricoxib was assimilated in the tail tissue, an LC-MS/MS analysis was employed. Tail tissue extracts were prepared in Acetonitrile and run on LC-MS/MS. The spectrum of Q1 MS-359 for etoricoxib is shown in Figure 3.11A. A calibration curve was prepared for concentrations of 1 ppb to 300 ppb etoricoxib for the MRM transition 359/279 (Figure 3.11B). Figure 3.11C and 3.11D respectively show the chromatograms of treated and control lizard samples run in MRM with positive-ion mode for m/z 359-280. The etoricoxib concentration was calculated at 32.8 ng/100mg tail tissue.

Next, a COX-2 activity assay was used to confirm its inhibition *in vivo* by etoricoxib. Etoricoxib was administered daily from a day prior to amputation (-1 dpa) and continued up to the day of tissue collection. Tissue at resting state, wound epithelium stage and blastema stage was collected from control and treatment groups and homogenised in Tris buffer. This was used for a microplate assay as described in Chapter 2: Material and Methods. Results obtained showed a very significant decrease in activity in samples from the groups treated with Etoricoxib as compared to the respective controls (Figure 3.12).

Effect of COX-2 inhibition on immune regulators

In an attempt to delineate the mechanisms of the initiation of regeneration, it was studied whether the inhibition of COX-2 had any effect on the functioning of other immune regulators. Control and etoricoxib treatment group animals were allowed to reach the wound epithelium stage (4 dpa) and the distal-most segment with the regenerating tissue was collected for gene expression studies. The expression of COX-2, EP2, EP4, iNOS, IL6, IL17, IL22 was analysed using a quantitative real-time PCR. Figure 3.13 depicts the mean Cq values of these genes for control and treatment groups. On a relative scale, higher Cq value reflects lower gene expression. Fold change in expression of treatment groups was calculated in relation to the respective controls. These values were calculated by the method of Livak and Schmittgen (2001) and are represented in Table 3.1. The expression of EP2, IL6, IL17 and IL22 increased significantly ($p<0.01$, $p<0.01$, $p<0.001$, $p<0.001$ respectively) in response to etoricoxib treatment.

Activation of secondary messengers

It was thus far not clear which was the intracellular pathway through which COX-2 signals were relayed. Western blot was performed in wound epithelium regenerates from control and etoricoxib group animals and probed for phospho-PI3K, phospho-CREB and phospho-p38 MAPK. Results revealed a visible decrease in the expression of phospho-CREB and no such change in case of the other secondary messengers (Figure 3.14).

DISCUSSION

That regeneration correlates negatively with the complexity of the immune function is evident from literature. However, the first response to tissue injury is always of an inflammatory type, regardless of whether healing is regenerative or otherwise. Immediate

protection against infection following injury is provided by cells of the innate immune system (Coussens et al., 2013). Infiltration of immune cells at the site of injury brings about major changes in the injured tissue. For example, activated leukocytes begin to secrete growth factors, which induce proliferation of local cells (Balkwill et al., 2005). Secretions from these leukocytes, particularly macrophages, may also stimulate re-organisation of the tissue matrix, thereby digesting components of the extra-cellular matrix (ECM) and subsequently liberating growth factors and other mitogens sequestered in the ECM (Lu et al., 2011). One of the major groups of immune cytokines linked with recruitment of leukocyte populations to tissue is the prostanoids. These lipid mediators may not always have received the attention deserved, but they are now being recognised for their far-reaching effects on immunity. Among the prostanoids, PGE_2 is produced in more abundance than any other and has been recognised for its modulation of immune responses (Balkwill and Mantovani, 2001). The expression of COX-2, which catalyses the production of PGE_2, also holds implications in processes such as cancer and regeneration – systems that involve extensive proliferative activity (Tsuji et al., 1997; Tucker et al., 1999; Leahy et al., 2002; Simon et al., 2002; Otsuka et al., 2009; Abrahao et al., 2010; Bockelmann and Bechara, 2009; Li et al., 2011).

Activity of COX-2 in regenerating lizard tail was assayed in the present study and there was an evident increase in the same after amputation. Activity remained high through the initial stages of healing, reflecting a role of COX-2-induced PGE_2 in the initiation of regeneration. To understand the regulation of COX-2 activity, the expression of this enzyme was studied at both protein and gene levels. It was found that there was induction of COX-2 gene at the level of gene expression itself. Even resting state did have expression of the gene, but this was increased manifold on the first day after amputation. The presence of fairly high amount of transcripts at the resting stage may be a characteristic feature of early response genes. This notion gains credence by the fact that the COX-2 gene is a compact one and genes for quick response proteins are generally small (Fletcher et al., 1992; Appleby et al., 1994). These results make one consider two documented outcomes of PGE_2 action – its ability to stimulate mitosis in cells and its possible role as an inhibitor of inflammatory reactions. Literature offers evidence for the anti-inflammatory properties of COX-2 induced PGE_2 and some authors categorically classify it, along with IL10, as an antagoniser of inflammation (Harizi and Gualde, 2006; Kalinski, 2012). It was found to inhibit NF-κB and its translocation into the nucleus (Poligone and Baldwin, 2001). It has to be acknowledged that the overall outcome of COX-2 is context dependent and the timing of its induction may be extremely

crucial. For instance, Gilroy et al. (1999) observed in a rat model of Carrageenan-induced pleurisy, that COX-2 expression peaked twice and exhibited a pro-inflammatory action during its first surge but helped decrease inflammation during the second. There are other studies that highlight the ability of PGE_2 to resolve inflammation. The PGE_2-EP4 pathway is suppressed in severe systemic inflammatory disease in humans (Duffin et al., 2016). This suppression has also been linked with an increase in neutrophil counts in inflammation. Also, pro-inflammatory cytokines like TNFα and IL6 were greatly upregulated in mice treated with Indomethacin (Duffin et al., 2016).

One important event in the early stages of healing and regeneration is the clearance of damaged cells by apoptosis. This process must be a finely regulated one and of the many factors affecting it, an influence of COX-2 has cannot be ruled out. PGE_2 produced by COX-2 has earlier been shown to protect mouse embryonic stem cells from apoptotic cell death through the EP2 receptor (Liou et al., 2007). Similar effect is seen in the case of prostate carcinoma cells wherein COX-2 specific inhibitor Celecoxib induces Caspase-2 activation and hence apoptosis (Hsu et al., 2013). The PGE_2 pathway may stimulate a range of downstream functions and the specific response elicited may depend largely on the receptor involved. With an aim to predict which signalling pathway is activated in epimorphosis, the four receptors of PGE_2, viz., EP1, EP2, EP3 and EP4 were tested for their expression. It was discovered that only EP2 is being expressed herein. There was some evidence of the presence of EP4 transcripts, however this was extremely low in abundance, (its amplification was not repeatable) while that of EP2 was present in high copy numbers. This is an interesting observation since EP2 and EP4 receptors are known to mediate the anti-inflammatory and neuroprotective effects of PGE_2 (Riciotti and FitzGerald, 2011). EP2 activation also helps control cytokine stimulation and synthesis of Prostanoids through a negative feedback system (Brenneis et al., 2011). Results from the present analysis help us narrow down to the role that may be played by COX-2 induced PGE_2 in early regeneration.

An immune regulator similar in many ways to COX-2 is the inducible Nitric oxide synthase (iNOS). Unlike the constitutive isoforms of NOS, iNOS once expressed, remains constitutively active (Ruan et al., 1996). NO production is therefore a direct reflection of its expression at the mRNA level, since post-translational regulation of activity is not well documented (Galea and Feinstein, 1999). Some evidence however does suggest modifications of iNOS protein post-translationally and these may present over-production of NO (Aktan, 2004). The enzymatic product NO is a small non-proteinaceous molecule and therefore not

open to regulation or modifications. NO diffuses in all directions from the cell that produces it and so its signalling can only be regulated at the level of its synthesis (Feldman et al., 1993).

Among the many functions of NO such as neurotransmission and blood vessel behaviour, the most notable one is that as an immune cytokine. In fact, macrophages, when activated, are a source of far more NO than that produced by neurons or vascular endothelial cells (Feldman et al., 1993). High expression of iNOS has been observed during the inflammatory phase of healing tissue in a mouse model of cutaneous injury (Frank et al., 1998; Reichner et al., 1999). Neutrophils and macrophages were found to be the major cells expressing iNOS in these studies. Among the much lesser known functions of iNOS-mediated NO is tissue repair and wound healing. Reports, however few, do point to a definitive role in this regard. NO protects liver tissue against injury and also from parasitic invasion (Suzuki et al., 1995; Ahn et al., 1999). Research on the molecular mechanisms of cutaneous wound healing has revealed that proliferating keratinocytes at wound edges express iNOS (Frank et al., 1998). Inhibition of iNOS activity affected wound healing in animal models through abnormal proliferation of the epithelial cells, which cover the wound (Stallmeyer et al., 1999). These were the major reasons for selecting the NO pathway for the current study, apart from its possible interactions with the COX-2 pathway. However, the effects of NO signalling are not very straight forward and it is suggested that concentration of this radical may be crucial in determining the outcome. The response of keratinocyte is two-pronged; with low NO concentration leading to their proliferation and high concentration pulling them out of mitotic phases of the cell-cycle (Krischel et al., 1998).

When tested in our model of scar-free wound healing, NO levels took a dive from the basal level found in a lizard tail shortly following amputation. Interestingly, a recovery of the tissue NO concentration was seen concomitant to the completion of wound epithelium formation. It seems from this result that the presence of NO above a certain concentration may be deleterious to scar-free healing and therefore its levels drop early on in the process. This may even be correlated with the requirement of immune suppression to allow regenerative healing. An early removal of pro-inflammatory molecules from the local environment is likely to result in reduced NO concentration, since the best known stimulators of iNOS mediated NO signalling are IL1, IL2, IFNγ and TNFα – all typically pro-inflammatory in nature (Feldman et al., 1993). One of the master regulators of immunity – NFκB – also features upstream of iNOS in immune responses (Xia et al., 2001). A relevant link between

immunity and wound healing comes to the fore at this point. Stimulation of iNOS expression by inflammatory cytokines occurs not only in immune cells, but also in skin-derived fibroblasts, as seen *in vitro* (Wang et al., 1996). NO may be responsible for the fibrotic nature of wound healing in mammals. Augmenting NO production increased collagen synthesis by cultured fibroblasts (Frank et al., 2000). This is mirrored in studies wherein NO synthesis inhibition resulted in decreased deposition of collagen in the healing wound (Schaffer et al., 1996; 1997; 1999). Another aspect of this versatile signalling molecule that can help support the current result is its effect on cell-cycle dynamics. It has been outlined by Chhatwal et al. (1996) that absence of NO in a cell could allow it to enter the cell-cycle and avoid terminal differentiation. This is important since in epimorphosis, a large pool of stem cells is maintained and it is this pool of cells that leads the process to completion.

Other interactions of NO that seem worthy of discussion here are those with reactive oxygen species (ROS). Although normal concentrations of NO show tissue protective effects, the presence of superoxides and peroxides bring about a dangerous turn in the behaviour of NO (Grisham et al., 1999). Deleterious effects are seen when NO binds to superoxide ion to form peroxynitrite. This can oxidise and nitrate biomolecules to disrupt their normal functioning (Beckman, 1996; Ahn et al, 1999). As stated earlier, concentration of NO must be crucial in determining the outcome of its signal. von Knethen at al. (1999) demonstrated that a high dose of NO resulted in the apoptosis of cultured macrophages. Interestingly, in the same study, COX-2 expression was found to protect the cells from such NO-induced apoptosis. It can be hypothesised that since the presence of ROS may be a requisite for proper wound healing (Love et al., 2013), NO levels must be subdued so as to prevent untoward effects on local cells, arising from the reactions of ROS and NO.

COX-2 and iNOS are known to be expressed at gene expression level in response to various stress parameters including pathogen invasion and tissue injury. They help mount strong inflammatory action in such cases. There are, however, other well characterised immune cytokines acting in all tissues to modulate immune function. Pro-inflammatory cytokines such as TNFα, NFκB, IFNγ, IL1, IL6, among others function to recruit and activate immune cells at a site of insult or injury. Anti-inflammatory cytokines such as IL10 act to resolve inflammation and are required to bring the tissue back to a state of homeostasis, lest prolonged inflammation causes damage even to local cells. Interleukins of particular interest were IL17 and IL22. IL22 has been implicated in the regeneration of the thymus (Dudakov et al., 2012) and also in tissue protection in inflammatory diseases such as colitis (Zheng et al.,

2008; Dudakov, et al., 2012; 2015; Sonnenberg and Artis, 2015). It is also able to induce cell proliferation as seen in small intestine organoid cultures (Lindemans et al., 2015) and hepatocytes (Ren et al., 2010). IL22 is known to affect cells of epithelial origin. Rutz et al. (2013) review that IL22 promotes wound healing driving proliferation of epithelial cells. It was also found to induce activators of cell-cycle c-Myc, Cyclin D1, Rb2 and CDK4 (Radaeva et al., 2004; Zenewicz et al., 2007; Sonnenberg et al., 2010). One of the functions attributed to IL22 is of great relevance to regeneration. Simonian et al. (2010) testified that IL22 prevents fibrosis in lung tissue. Similarly, IL17 also promotes regeneration in some tissues. As reported by Rao et al. (2014), γδT cells promote liver regeneration in mice through production of IL17. It also positively regulates wound healing through induction of keratinocyte proliferation (Lai et al., 2012). These interleukins are released from T helper cells and modulate a range of functions. While IL17 is produced by the T_H17 cells, discovered not long ago (Dong, 2008), IL22 is majorly produced by T_H1 cells in humans and T_H17 in mice (Rutz et al., 2013). The T_H17 cells have contrasting influence on cell proliferation and tumor progression, which depends on the local environment in the tissue (Wei et al., 2012).

Our analysis of the expression of the interleukins in regeneration revealed that IL6, IL17 and IL22 are produced in the regenerating tail of lizard. This is the first time that the expression of IL17 and IL22 has been shown in epimorphic regeneration. Although the selection of IL17 and IL22 for this study was made on the basis of their pro-regenerative effects reported in literature (Dudakov et al., 2012; Rao et al., 2014; Lindemans et al., 2015), their temporal expression pattern during the early stages of regeneration went well against our expectations. The gene expression of both these interleukins was appreciable in the resting state and remained so up to 1 dpa in lizard. However, from 2 dpa, their transcripts diminished and were not seen to be restored even when wound epithelium formation was complete at 4 dpa. It is plausible to think that the pro-inflammatory nature of IL17 and IL22 are more important to consider in this context than their regenerative effects; and literature offers support to this speculation. The major activity of IL17 assigned to it based on studies *in vitro* is the stimulation of the release of pro-inflammatory cytokines TNFα, IFNγ, IL1β, IL6 and granulocyte stimulating factor from monocytic cells (Li et al., 2000). Also, as stated by Rutz et al. (2013), IL17 is potent in eliciting a strong inflammatory reaction and it does so by inducing other pro-inflammatory cytokines and phagocytic immune cells. IL22, which has more regenerative and tissue protective effects attributed to it, also promotes the activation of

the innate immune system. While it does help maintain tissue homeostasis, the release of IL22 in excess can be harmful and can even have pathological implications (Rutz et al., 2013). This may help explain our gene expression results. Moreover, the presence of IL17 and IL22 together, results in a highly pro-inflammatory phenotype (Rutz et al., 2013). It aids the production of IL6 (Wolk et al., 2006). An interesting angle to our understanding of IL22 in epimorphosis is given by the observation that IL22 can induce the expression of anti-apoptotic factors of the Bcl-2 family (Radaeva et al., 2004; Zenewicz et al., 2007; Sonnenberg et al., 2010) and this is over and above the fact that it also induces cell-cycle promoting genes. It is therefore hypothesised that a consistently high level of IL22 may have the potential to push proliferating cells beyond controlled division and gain a tumorous phenotype. We are thus able to appreciate that while restoration of tissue through epimorphosis must involve reactivation of potency and induction of proliferation, it must be tightly controlled to prevent unwarranted growth and cancer.

The classical pro-inflammatory interleukin IL6 displayed an expression pattern that goes well with the hypothesis stated in the introduction of this chapter. A timely resolution of inflammation is key to the success of scar-free wound healing and IL6 expression drops from a basal level to almost untraceable levels at 2 dpa in the lizard tail. Apart from the three above-mentioned interleukins, other immune modulators were tested for their expression. These were IL1β, IL10, TNFα and IFNγ. Negative results were obtained in these cases. However, we exercise caution and do not assert the absence of these cytokines in our system of regeneration, since the respective positive controls for *H. flaviviridis* were unavailable and there could be a high possibility of primers not binding well. This uncertainty arises for interleukins, more than for any other regulatory factors, since the interleukins show very limited homology among the vertebrates, thereby making primer design a difficult process with low chances of success.

To gain clarity on our inference that COX-2 and its product are promoting regeneration by exerting anti-inflammatory effects, an inhibition study was taken up. Selective COX-2 inhibitor etoricoxib was used to inhibit the enzyme. The difference in a single amino acid between the active sites of COX-1 and COX-2 (Simmons et al., 2004) results in a hydrophobic pocket in the latter and this structural difference allows selectivity for this isoform (Kurumbail et al., 1996). Etoricoxib was administered to animals and the expression of the immune regulators mentioned above was tested at the wound epithelium stage in response to this. A very important observation was made in this experiment. It was seen that

the inhibition of COX-2 resulted in significantly high expression of IL6, IL17 and IL22 as compared to control (Figure X). This implies that the induction of COX-2 indeed plays a part in suppression of these interleukins early on in regeneration. This is therefore the strongest evidence in favour of COX-2 acting as a suppressor of inflammation to promote regeneration.

It is well-known that EP2 and EP4 receptors activate Gs and thereby stimulate cAMP production through Adenylate cyclase (Reimer et al., 1992; Ichikawa et al., 1996). EP1 mediated signals result in enhanced intracellular Calcium levels (Watabe et al., 1993; Funk et al., 1993) while signals passing through EP3 inhibit Adenylate cyclase via Gi protein (Nishigaki et al., 1993). In order to confirm the role of this pathway, immunoblots of phosphorylated PI3K, phosphorylated CREB and phosphorylated p38 MAPK were carried out in wound epithelium regenerates of control and etoricoxib-treated animals. COX-2 inhibition was found to have the most notable influence on the levels of phospho-CREB reflecting the involvement of the cAMP/PKA pathway. It is known from the work of Versteeg et al. (2000) that EP2 signalling leads to activation of CREB by its phosphorylation to induce CRE-dependent transcription of genes. This establishes that PGE_2 signals an increase in cAMP levels through the EP2 receptor to orchestrate the early events of regeneration.

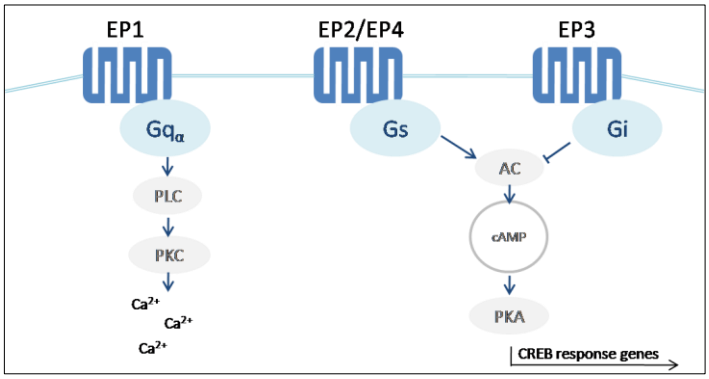

Figure 3.1: Schematic diagram showing signalling downstream of the EP receptor subtypes. AC: Adenylate cyclase, cAMP: Cyclic AMP, PLC: Phospholipase C, PKA: Protein kinase A, PKC: Protein kinase C. Adapted from Bos et al. (2004).

The present work establishes that COX-2 signalling helps in regenerative healing by imposing early resolution of inflammation in the amputated tail tissue and this is mediated through the EP2/cAMP pathway. Such a mechanistic insight into the early stages of epimorphosis will help us move towards the goal of replicating the process in mammals. Future considerations for work include identification of the sources of expression of the various immune molecules studied herein. Although COX-2 and iNOS are likely to be

majorly produced from resident and recruited macrophages, this must be confirmed since the induction of both these genes is possible in almost all cells, as mentioned earlier. Immune cytokines such as IL10, TNFα, IFNγ and NFκB also need to be studied in epimorphosis as they are the most influential regulators of inflammation.

Table 3.1: Relative gene expression data for COX-2, EP2 receptor and Interleukins in control and etoricoxib-treated animals at wound epithelium stage.

	Mean normalized Cq values		**Fold change in Etoricoxib group regenerates**
	Control	**Etoricoxib**	
COX-2	27.87 ± 0.19	27.22 ± 0.33	1.64 ± 0.32
EP2	28.87 ± 0.41	26.80 ± 0.26 **	4.35 ± 0.71 **
IL6	26.65 ± 0.46	25.08 ± 0.50 *	3.39 ± 1.06 *
IL17	28.40 ± 0.67	25.47 ± 0.11 ***	7.65 ± 0.59 ***
IL22	29.24 ± 0.35	26.29 ± 0.49 ***	8.71 ± 2.95 ***

Values are represented as mean±SEM. N=6. *p<0.05, **p<0.01, ***p<0.001 as compared to control. Fold change gene expression was calculated as relative to control.

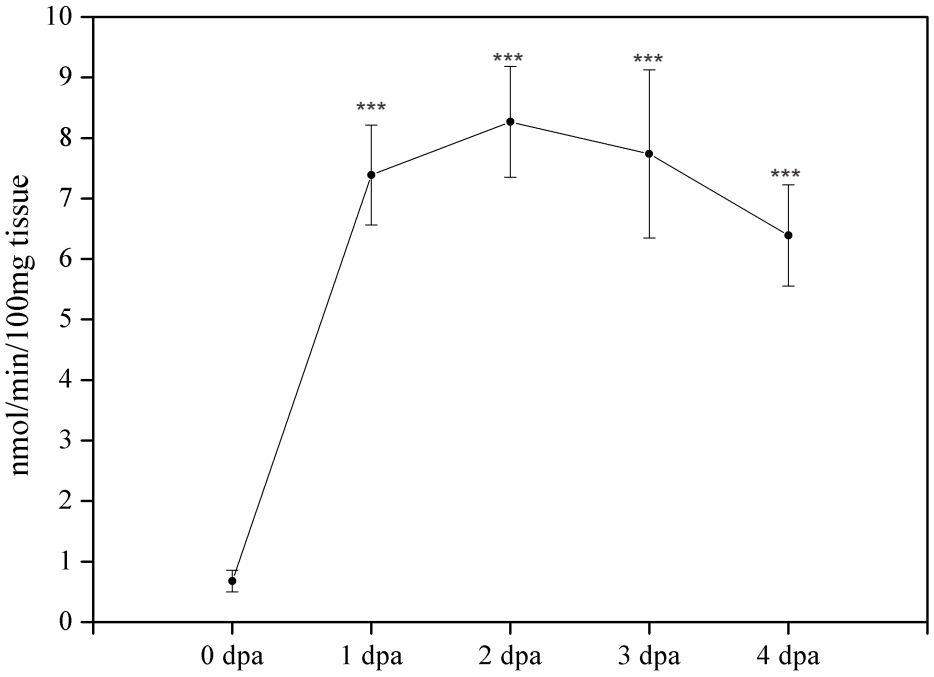

Figure 3.1: Activity of COX-2 in the regenerating tail at early time-points after amputation. ***p<0.001 as compared to 0 dpa.

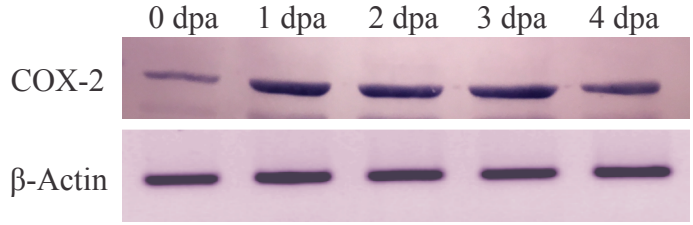

Figure 3.2: Western blot showing expression of COX-2 protein in the regenerating tail at early time-points after amputation. β-Actin was used as internal loading control

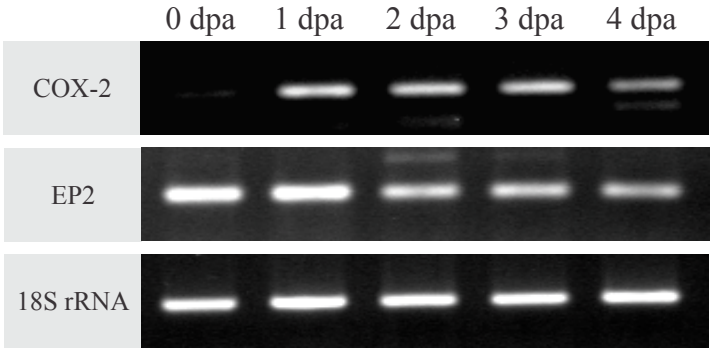

Figure 3.3: Trend in gene expression of COX-2 and EP2 receptor in regenerating tail at early time points after amputation obtained from RT-PCR. 18S rRNA was used as internal loading control.

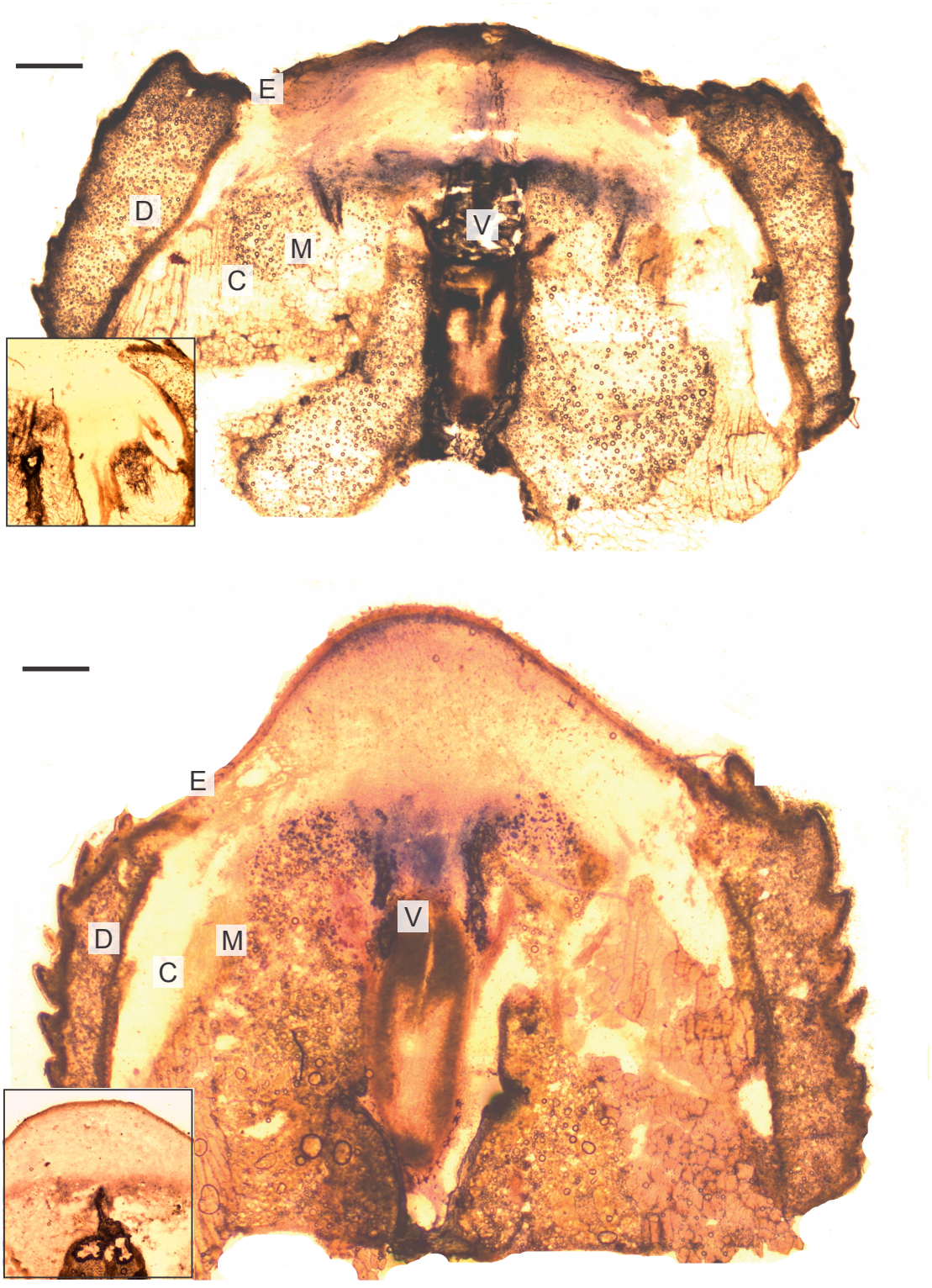

Figure 3.4: Immunohistochemical localisation of COX-2 in a longitudinal section of wound epithelium (upper) and blastema (lower) stage regenerates. Blue stained region indicates presence of COX-2 protein. Depicted here is a composite image of multiple micrographs. Negative controls for the primary antibody are shown inset. Scale bar represents 500 μm.

C: Connective tissue; D: Dermis; E: Wound epithelium; M: Muscle; V: Vertebra.

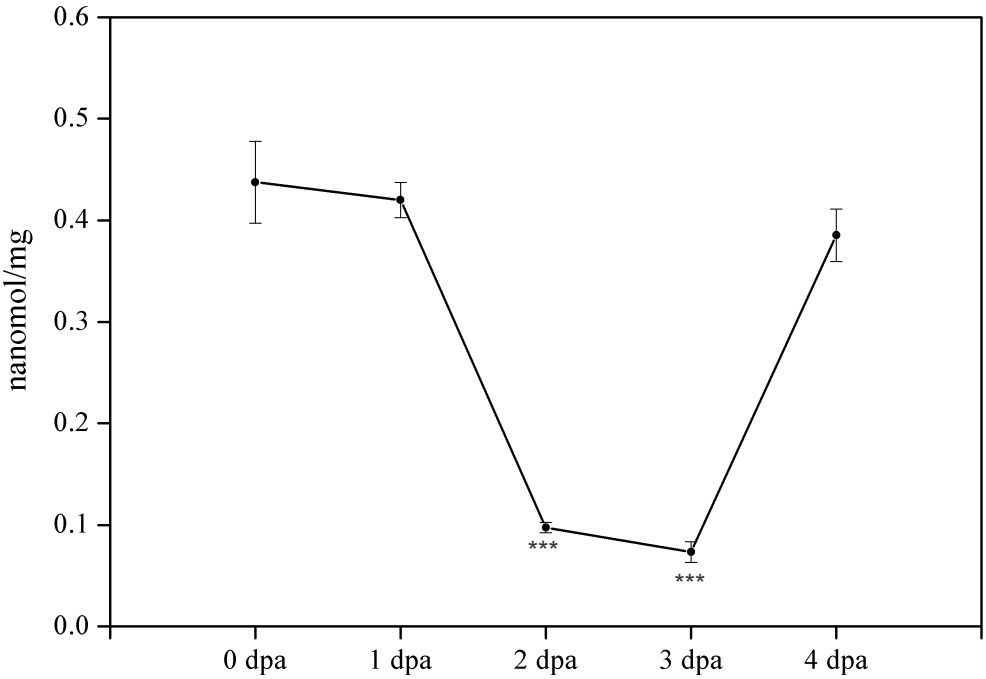

Figure 3.5: Levels of nitric oxide in the regenerating tail at early time-points after amputation. ***p<0.001 as compared to 0 dpa.

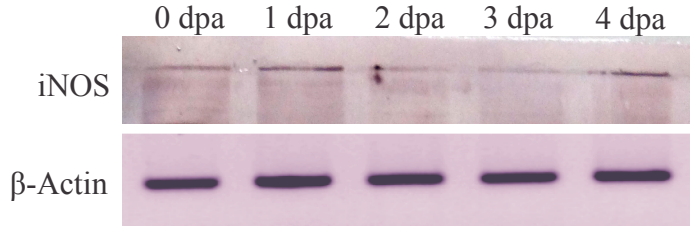

Figure 3.6: Western blot showing expression of iNOS protein in the regenerating tail at early time-points after amputation. β-Actin was used as internal loading control

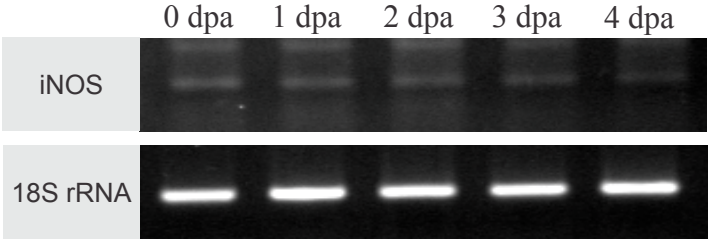

Figure 3.7: Trend in gene expression of iNOS in regenerating tail at early time points after amputation obtained from RT-PCR. 18S rRNA was used as internal loading control.

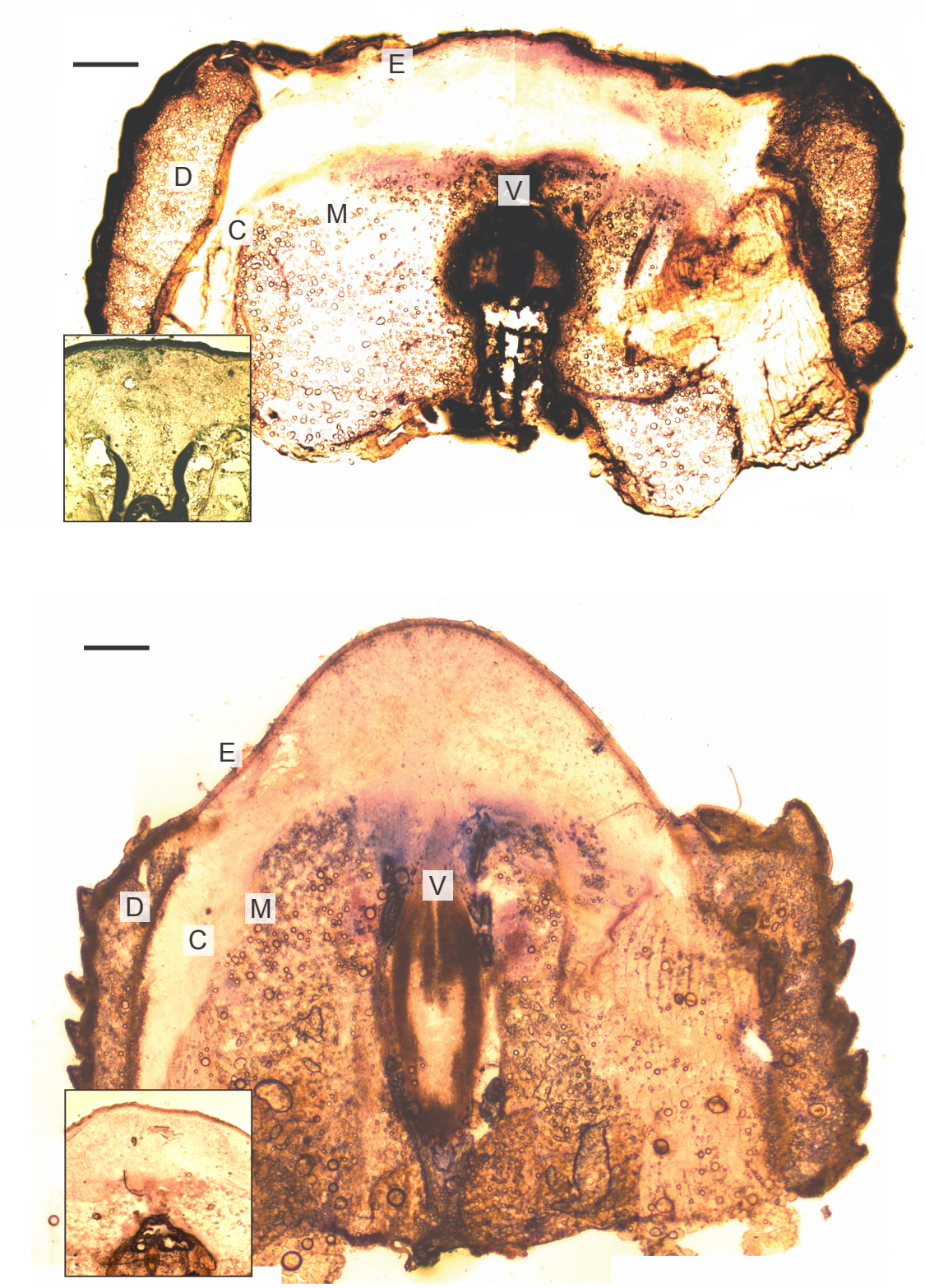

Figure 3.8: Immunohistochemical localisation of iNOS in a longitudinal section of wound epithelium (upper image) and blastema (lower image) stage regenerates. Blue stained region indicates presence of iNOS protein. Depicted here is a composite image of multiple micrographs. Negative controls for the primary antibody are shown inset. Scale bar represents 500 μm.

C: Connective tissue; D: Dermis; E: Wound epithelium; M: Muscle; V: Vertebra.

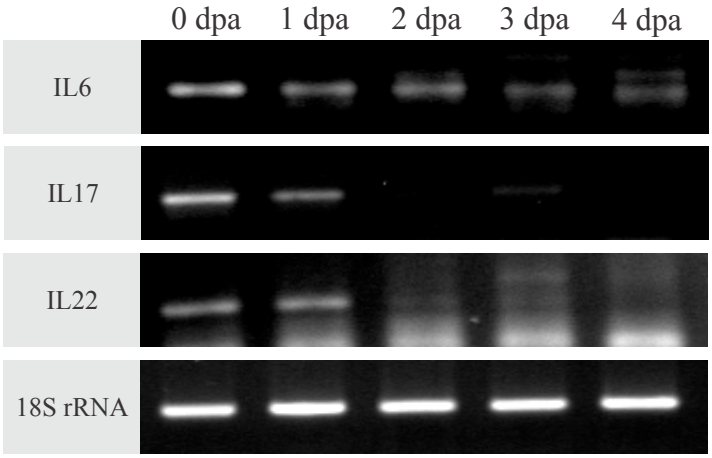

Figure 3.9: Trend in gene expression of interleukins in regenerating tail at early time points after amputation obtained from RT-PCR. 18S rRNA was used as internal loading control.

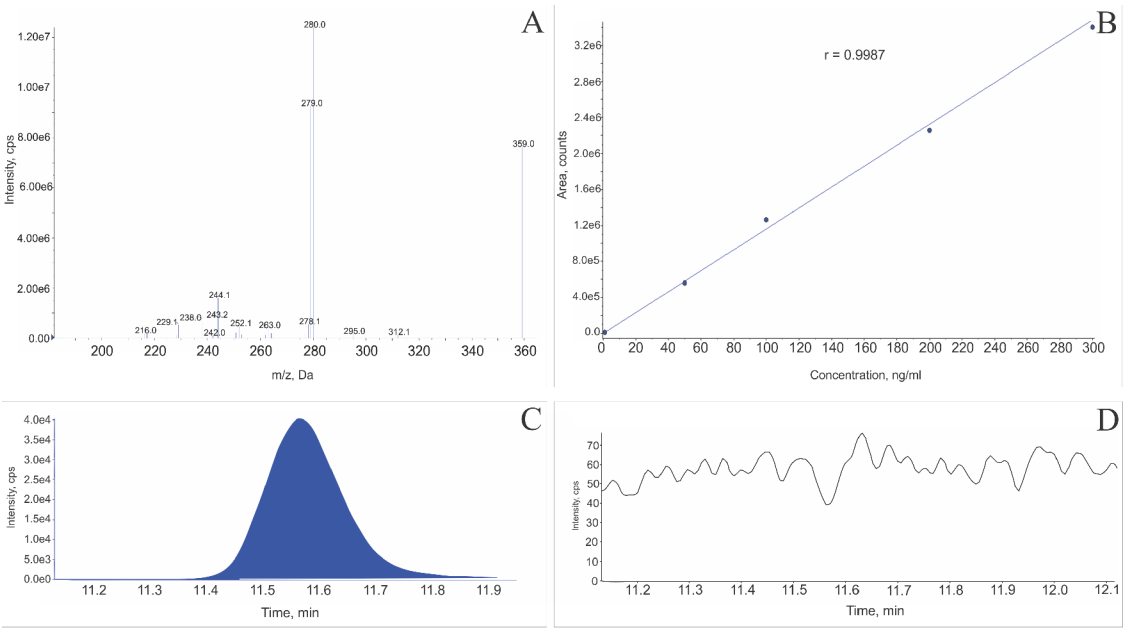

Figure 3.10: Representation of spectrum for product of Q1MS-359 (A) calibration curve of Etoricoxib from 1 ppb to 300 ppb with regression >0.998 for MRM transition 359/279 (B) representative chromatograms of treated (C) and control (D) samples obtained in MRM in positive ionisation mode at m/z 359 ➔280 for Etoricoxib

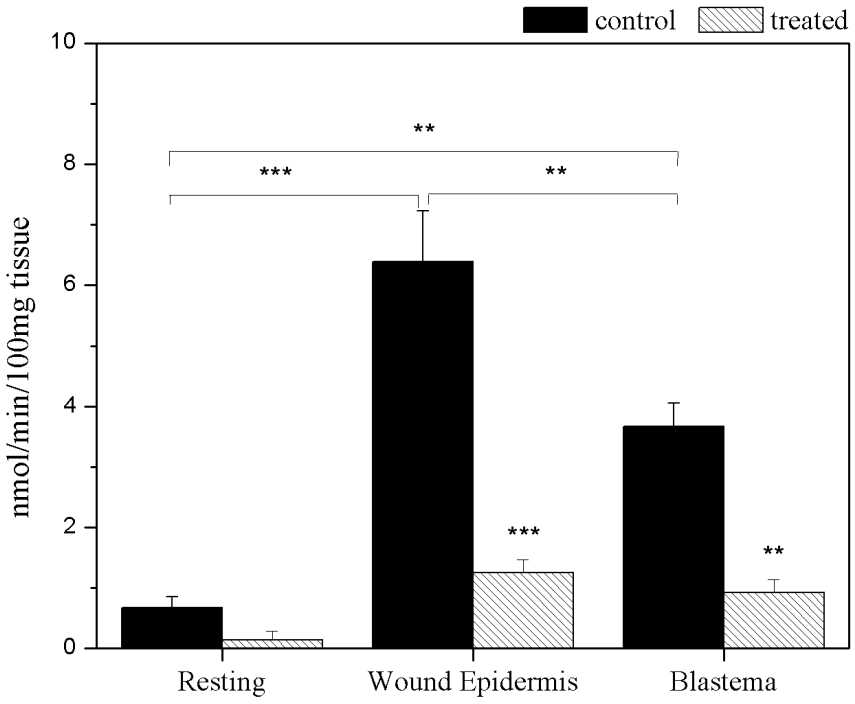

Figure 3.11: Activity of COX-2 at wound epithelium and blastema stages in control and etoricoxib treated animals.**p<0.01, ***p<0.001.

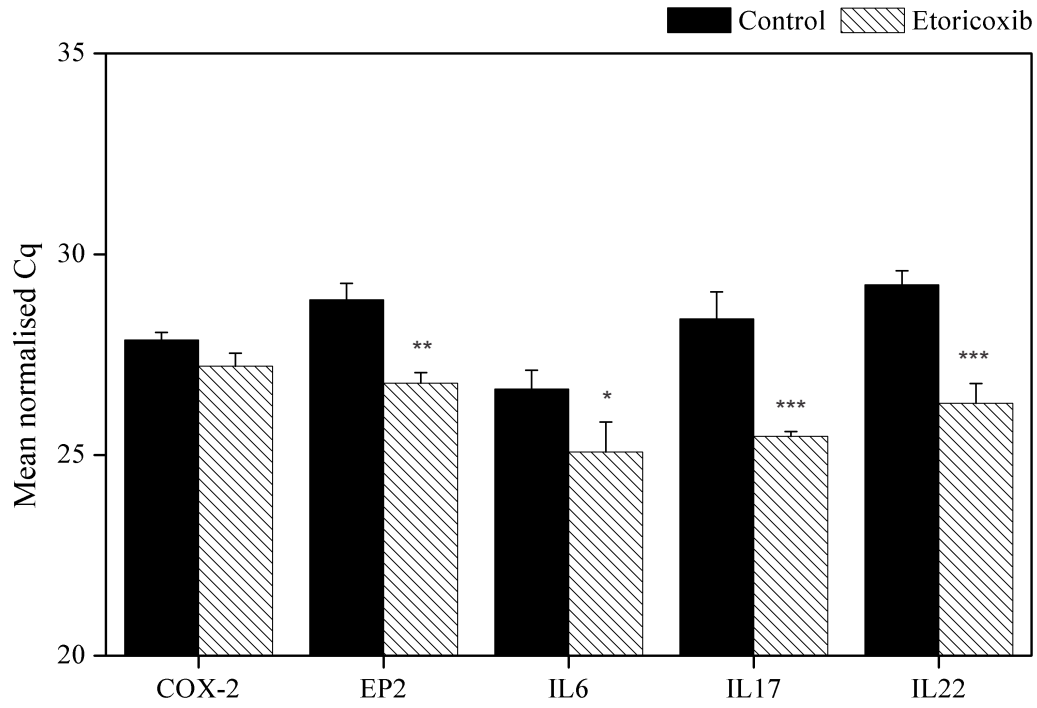

Figure 3.12A: Mean normalised Cq values for genes of immune regulators in wound epithelium stage regenerates. Cq value is inversely related to gene expression levels.

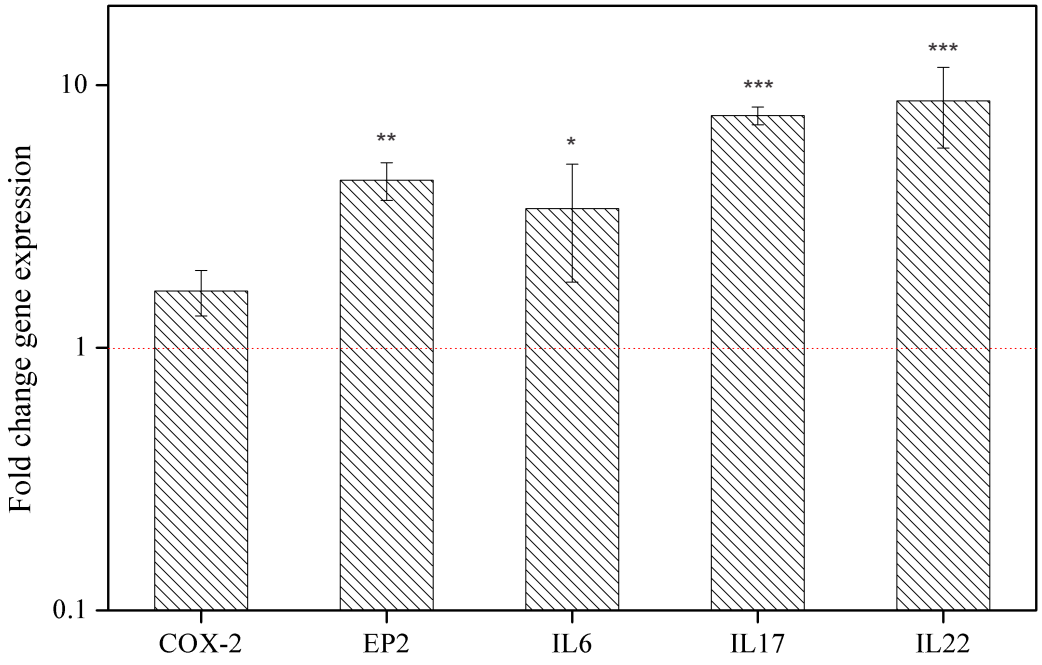

Figure 3.12B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of genes in wound epithelium stage regenerates with respect to intact tail tissue. Dashed red line indicates gene expression level of control group samples taken as 1.

Scale bars represent standard errors of means; *p<0.05, **p<0.01, ***p<0.001 as compared to controls

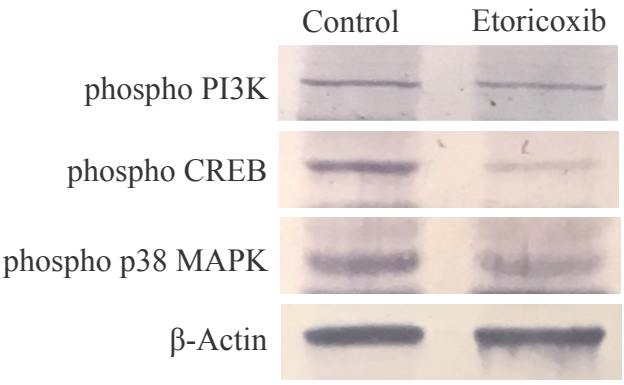

Figure 3.13: Western blot showing expression of intracellular signalling moelcules in control and etoricoxib group animals at wound epithelium stage (4 dpa). β-Actin was used as internal loading control