

Inhibition of Cyclooxygenase-2 Alters Wnt/ β -Catenin Signaling in the Regenerating Tail of Lizard *Hemidactylus flaviviridis*

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Abstract Epimorphic regeneration in vertebrates involves the restoration of lost tissue or organs through the formation of a regeneration blastema and occurs through a complex interaction of a number of molecular signaling pathways. Of the many effectors of successful tail regeneration in the lizard *Hemidactylus flaviviridis*, one crucial pathway is the cyclooxygenase-2 (COX-2) mediated PGE₂ signaling pathway. The current study was aimed at understanding whether COX-2 signaling plays any role in the expression of Wnt/ β -Catenin signaling components during regenerative outgrowth in *H. flaviviridis*. Etoricoxib—selective inhibitor of the inducible isoform of COX-2—was administered to lizards orally. We tested the expression of β -Catenin during wound epidermis and blastema stages in the regenerating tail and found a reduction in its expression in response to drug treatment. Further, it was observed that the expression of canonical Wnt ligands was greatly altered due to COX-2 inhibition. Our results provide evidence of a cross-talk between the COX-2 induced PGE₂ pathway and Wnt/ β -Catenin signaling in the regenerating lizard tail. An understanding of the interaction among various signaling pathways will help elucidate the mechanism underlying epimorphosis in lizards, the only amniotes capable of appendage regeneration.

Keywords Regeneration · Inflammation · Cyclooxygenase · Wnt · Beta-catenin

1 Introduction

Regeneration in vertebrates is a phenomenon that has been vastly studied since the mid 18th century and yet our understanding about the process in its entirety seems limited. Among the commonly studied vertebrate models, the Zebrafish and Salamanders are remarkable for their capacity to regenerate a wide variety of tissues. Beyond these, many lizards are also capable of appendage regeneration, albeit limited to the tail. Appendage regeneration

in all the above mentioned animals proceeds through the formation of a regeneration blastema, a population of progenitor cells responsible for regenerative outgrowth [1]. Blastema formation requires and follows the formation of a wound epidermis, which is a thick multilayer covering of the amputation wound by migrating epidermal cells [2, 3].

The current study makes use of northern house gecko *Hemidactylus flaviviridis*, which has been a subject of research for numerous parameters related to tail regeneration [4–8]. A complete wound epidermis in this lizard is formed at 4–5 days post amputation (dpa) and blastema at 7–8 dpa at 37 °C. Each of these stages results from drastic changes in the molecular environment that take effect immediately following amputation. Among the variety of regulatory factors induced during regeneration is cyclooxygenase-2 (COX-2), an inducible isoform of cyclooxygenase enzyme, which catalyses the formation of prostaglandin E₂ (PGE₂) from arachidonic acid [9]. COX-2 mediated PGE₂ is

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classically known to play crucial roles in pain, fever and inflammation [10]. More recently, its role has been acknowledged in the progression of cancers [11–13].

Reports of COX-2 having any role in initiation and progression of appendage regeneration are limited [7, 14–16]. Our lab has previously shown that inhibition of COX-2 activity, by the use of NSAIDs specific for the isoform, result in a retardation of wound epidermis formation and in its ability to give rise to a blastema [7]. We hypothesize that the PGE₂ pathway is functioning upstream of some developmentally important pathways, thereby ensuring at an early stage that an amputation wound takes its course towards regenerative repair. In the present study, we make use of Etoricoxib, a selective COX-2 inhibitor, to investigate whether COX-2 mediated PGE₂ pathway interacts with the Wnt/ β -Catenin pathway, which is attributed with numerous functions related to cell proliferation, specification and differentiation.

2 Material and methods

2.1 Animal housing and maintenance

Northern House Geckos *Hemidactylus flaviviridis* of both the sexes, weighing about 10–12 g were captured from the wild and maintained in wooden cages. All animals were screened for parasitic infections and the healthy ones were used for the experiment. Cockroach nymphs were fed once a day and purified water was provided *ad libitum*. A temperature range of 36 ± 2 °C was maintained with 12:12 hour light to dark cycles. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) constituted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (IACUC no. 984/07/2014-2).

2.2 Drug administration and experimental design

Animals were randomized and divided into two experimental groups, namely control and treatment, each group having 20 individuals. Treatment group animals were orally administered Etoricoxib (gift by Sun Pharma Advanced Research Centre, Vadodara, India) at 25mg/kg body weight once daily, based upon a dose-range study previously carried out in the lab. The drug was given as a suspension in purified water. Control animals were orally given the equivalent volume of purified water. Lizard tails were amputated a day after commencement of drug dosing by inducing autotomy after the third segment from the base of the tail. Regenerating tail tissue was collected for analysis at two major stages, *viz.*, Wound Epithelium and Blastema, from six animals per group at each stage.

2.3 Liquid chromatography–mass spectrometry

Tail tissue was collected after amputation and a 10% homogenate was prepared in Chloroform:Methanol (2:1 ratio), according to the method of Folch et al. [17]. After centrifugation at 10,000g for 10 min, the supernatant was collected and vaporized in a rotary vaporizer at 50 °C under high vacuum. The solid residues were washed twice with Acetonitrile, which was subsequently vaporized under the same conditions. Finally, the solid residue was dissolved in Acetonitrile, and filtered through a Nitrocellulose membrane filter of pore size 0.2 μ m. This solution was used further for LC/MS analysis. Separation was carried out on an Eksigent Ekspert UltraLC 100 machine coupled with an ABSciex 3200 Q Trap machine with Analyst software (version 1.6.2). The conditions for LC/MS were similar to those used by Bräutigam et al. [18]. A Reprosil 100 C18 RP column was used. Mobile phase was acetonitrile-water (90:10). Flow rate was 0.8 ml/min, with an injection volume of 20 μ l.

2.4 COX-2 activity assay

10% homogenates of tail tissue were prepared in cold Tris-EDTA from six samples pooled in each group. A microplate format COX activity assay kit (Cayman Chemical Co., USA) was used and results were reported in nmol/min/ml homogenate.

2.5 Western blot

Regenerating tail tissue at Wound Epithelium and Blastema stages was collected from six animals of each experimental group, pooled and homogenised in Tris-SDS lysis buffer with protease inhibitor (Sigma Aldrich, USA). 10% homogenates were assayed for total protein content by Bradford method [19]. Equal amount of total protein was loaded and separated by SDS-PAGE on 10% gels. Protein was transferred onto nitrocellulose membrane by semi-dry transfer at 100 mA for 30 min. The membrane was probed separately with Anti- β -Catenin and Anti- β -Actin (IgG antibodies raised in Mouse). The ALP system was used for staining the protein of interest.

2.6 Real time RT-PCR

Total RNA was isolated from regenerating tail tissue of six animals of each group using TRIzol reagent (Applied Biosystems, USA). 1 μ g total RNA was reverse transcribed to cDNA using a one-step cDNA synthesis kit (Applied Biosystems, USA). Primer sequences from 5' to 3' used for the subsequent real-time PCR were as follows: wnt1 fwd: AAGTCGGGAAGGAGAGGTGA; wnt1 rev: GAGCC

ATCTGAAACTGCCCT; wnt2b fwd: CCTGTTGGCTGGCTATGTCT; wnt2b rev: TGTCAGCCACCATAAAGCCA; wnt3a fwd: TCCTTTGTGCCAGCATACCA; wnt3a rev: TGGATGCCGATCTTTACCCC; wnt4 fwd: CGCAAGGTGGGATCTACCAA; wnt4 rev: TTCTGTTACTGGCGTCCC; wnt6 fwd: TTGGTCATGGACCACAACAG; wnt6 rev: CCTCGCTGACGATTTCTGGT; wnt7a fwd: CTGCGGAAGGGGATACAACA; wnt7a rev: TAACGTAGCAGCACCACAGG; wnt7b fwd: TAGCCAAGGCAACCTAAGCC; wnt7b rev: CCACGCCGTA CTGACATCT; wnt8a fwd: TAACAACGAGGCGGGAA-GAC; wnt8a rev: GCAACTTCCAGACACCCCAT; wnt10a fwd: CGAGGAGGCATTTCTGACTCA; wnt10a rev: ACCGTGAACCATCCCTTCC; wnt16 fwd: CTAAACAGTGACCAGTGCCG; wnt16 rev: GTCAGGGGTCAAGGACAGAAC; 18S rRNA fwd: GGCCGTTCTTAGTTGGTGGA; 18S rRNA rev: TCAATCTCGGGTGGCTGAAC. The suitability of 18S rRNA as an internal loading control was first checked. For this, the cDNA (which was prepared from equal amounts of total RNA) was itself quantified using the Qubit 3.0 fluorimeter assay (Life Technologies, USA) and was found to be not significantly different among samples. A qPCR of 18S rRNA was then performed and the Cq values were correlated with the total cDNA concentrations. It was found to correlate, with no variation among samples (data not shown). Quantitative real-time PCR was performed on a Lightcycler96 (Roche Diagnostics, Switzerland) with the following program: 3 min at 95 °C, 45 cycles (each cycle of 10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C). Melt curve analysis was used to confirm specific product formation. Data was represented as mean Cq values normalized with 18S rRNA levels and data was analysed by one-way ANOVA using Prism v5.03 (GraphPad Software Inc., USA). Fold change in expression was calculated using the $2^{-\Delta\Delta Cq}$ method of Livak and Schmittgen [20].

3 Results

To achieve inhibition of cyclooxygenase-2 activity *in vivo*, we orally administered Etoricoxib to *H. flaviviridis*. Before the effect of COX-2 inhibition on Wnt/ β -Catenin signaling could be tested, it was necessary to validate this inhibition. For this, we collected tail tissue after 24 hours of a single oral dose of Etoricoxib and prepared an extract in Acetonitrile (LC/MS grade). The sample was subjected to LC/MS and Etoricoxib was quantified in the tail tissue. A linearity graph was obtained using Etoricoxib standards of concentrations 1, 50, 100, 200 and 300 ng/ml (Fig. 1). Further, samples were run wherein control sample produced no peak while treated group sample produced a peak corresponding to 32.8 ng/ml. This corresponds to 32.8 ng

Etoricoxib in 100 mg of tail tissue. Next, to test the inhibition of COX-2 in tail tissue, an activity assay was performed. COX-2 activity in the wound epidermis and blastema stages was found to be significantly lower in the treated group animals ($p \leq 0.001$ and $p \leq 0.01$ respectively), as compared to controls (Fig. 2). Moreover, the activity assay revealed that COX-2 is most active in during the wound epidermis stage ($p \leq 0.001$ when compared to resting stage), followed by the blastema stage ($p \leq 0.01$ when compared to resting stage).

The present study was taken up to understand the effect of COX-2 inhibition on Wnt/ β -Catenin signaling. When Wnt signals are present, intracellularly β -Catenin is stabilized and allowed to translocate into the nucleus to induce the transcription of target genes. With this, it regulates various developmental processes including cell proliferation and differentiation. In the absence of the Wnt signal, however, β -Catenin is targeted for proteasomal degradation. We carried out a western blot to check the expression of β -Catenin in response to Etoricoxib treatment. It was found that drug treatment lead to a reduced level of β -Catenin in the tail tissue at both wound epidermis and blastema stages (Fig. 3).

Reduced β -Catenin signals prompted us to check the status of the Wnt ligands in wound epidermis and blastema tissue to understand whether COX-2 signaling is directly interacting with β -Catenin [21] or it is causing a change in the expression of Wnt ligands. Moreover, it was so far not known precisely which Wnt ligands are expressed during the specific stages of regeneration in lizards. Therefore, we screened for twelve Wnt ligands, classically known to induce the ‘canonical’ (β -Catenin dependent) pathway, for their expression in the two stages of tail regeneration. Of these, we found expression of transcripts of 10 ligands, *viz.*, wnt1, wnt2b, wnt3a, wnt4, wnt6, wnt7a, wnt7b, wnt8a, wnt10a and wnt16 in both wound epidermis and blastema stages. Further, real-time PCR results showed that wnt2b, wnt4 and wnt7a had heightened expression ($p \leq 0.001$, $p \leq 0.05$ and $p \leq 0.001$ respectively) in wound epidermis stage as compared to the resting tail and the blastema stages, whereas wnt1 and wnt6 showed significantly greater ($p \leq 0.001$) expression in the blastema stage (Table 1; Fig. 4).

For the current study, we analysed the expression of the abovementioned 10 Wnt ligands in response to COX-2 inhibition during the two stages, at transcript level using real time PCR. We found skewed expression of a number of these in both the stages. Among the two genes found majorly during wound epidermis stage, wnt4 showed an increase in Cq value pointing towards heavily decreased expression ($p \leq 0.001$) compared to control (Table 2; Fig. 5). wnt7a, on the other hand, was found to be upregulated ($p \leq 0.05$) as compared to control. During the

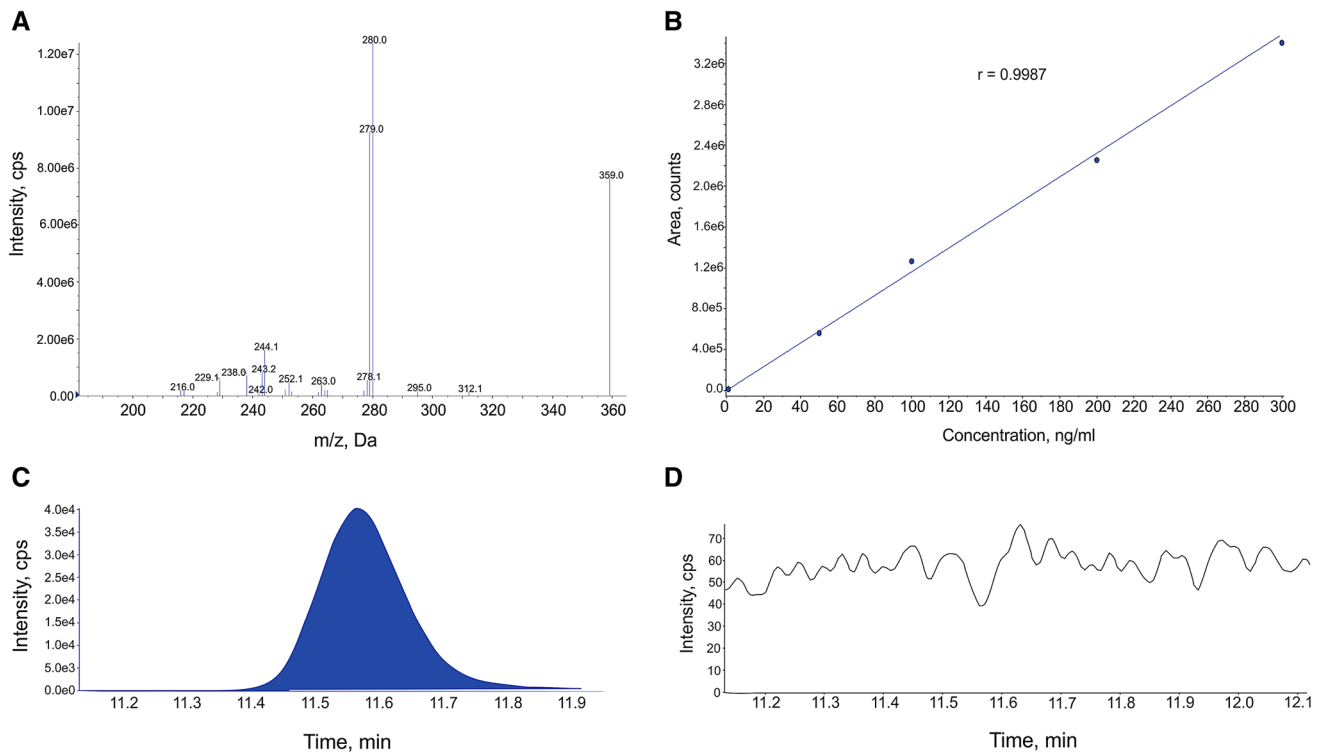


Fig. 1 Representation of spectrum for product of QIMS-359 **A** calibration curve of Etoricoxib from 1 ppb to 300 ppb with regression >0.998 for MRM transition 359/279 **B** representative chromatograms

of treated **C** and control **D** samples obtained in MRM in positive ionisation mode at m/z 359 \rightarrow 280 for Etoricoxib

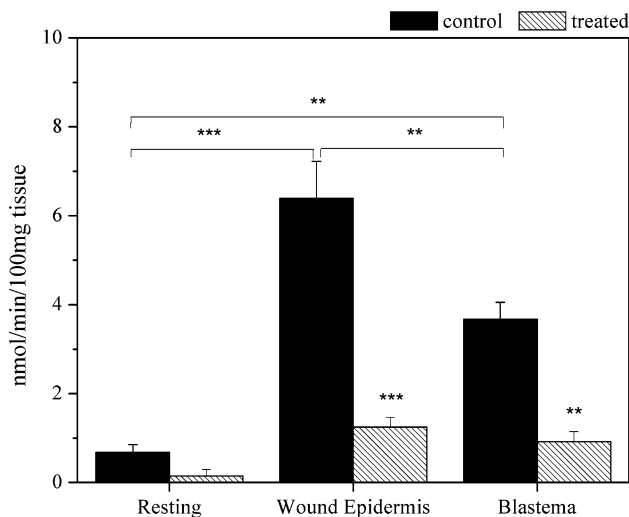


Fig. 2 Cyclooxygenase-2 activity in the tail tip during the resting (pre-amputation), wound epidermis and blastema stages for control versus Etoricoxib treated animals. Error bars represent standard errors of mean. ** $p \leq 0.01$; *** $p \leq 0.001$. $N = 3$

blastema stage, *wnt1* and *wnt16* showed sharp decrease ($p \leq 0.001$ as compared to controls) in expression in response to Etoricoxib treatment, as reflected by the high Cq values (Table 3; Fig. 6). Over and above these, a statistically significant increase in expression was found for

wnt2b, *wnt3a*, *wnt7a*, *wnt7b*, *wnt8a* and *wnt10a* in the blastema stage.

4 Discussion

Inflammation is an inevitable process immediately following an injury. In both regenerating and non-regenerating systems, an inflammatory response is essential to proper healing of the wound. It is well known that scar-free wound healing in regenerating vertebrate limbs involves immune molecules [22]. One such inducible mediator of inflammation is cyclooxygenase-2, the importance of which in regeneration has been highlighted in few earlier studies [7, 14–16]. Suresh et al. [23] demonstrated the impeding effects of COX-2 inhibition on angiogenesis, myogenesis and cell synthetic activities in the wound epidermal and blastemal cells of *H. flaviviridis*. However, the mechanism with which COX-2 mediated PGE₂ influences regenerative healing and outgrowth is still at large.

Of the molecular signaling pathways orchestrating the variety of processes which result in tail restoration, the Wnt/ β -Catenin pathway is vastly important for its role in cell migration, wound repair and proliferation in a wide variety of developing systems [21, 24, 25]. It has been identified as a regulator of growth and patterning during

Fig. 3 Immunoblot showing β -Catenin expression in the regenerating tail of control and Etoricoxib treated animals at wound epidermis and blastema stages

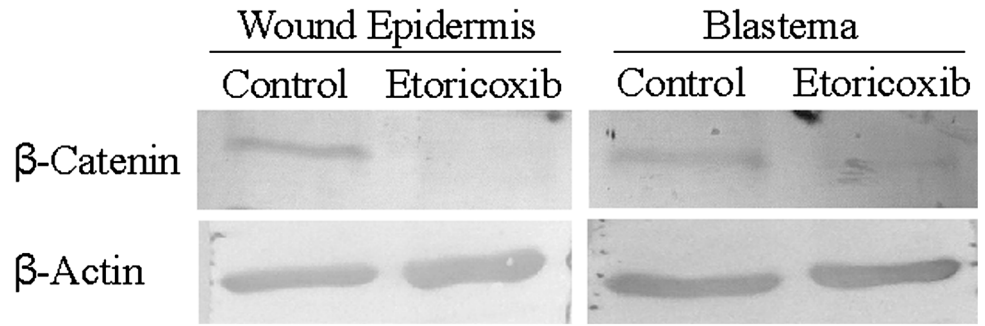


Table 1 Mean fold change in gene expression at wound epidermis and blastema stage as compared to resting tail

Gene	Fold change at wound epidermis stage	Fold change at blastema stage
wnt1	0.629961	725.7523
wnt2b	12.18189	2.434007
wnt3a	1.052145	1.132884
wnt4	4.756828	0.819794
wnt6	4.189176	10.70342
wnt7a	45.15039	2.901234
wnt7b	1.381913	2.694467
wnt8a	0.97041	3.863745
wnt10a	2.089754	6.916298
wnt16	2.037312	4.06522

Fig. 4 Normalised mean Cq values for wnt ligands expressed in a resting tail and during wound epidermis stage (WE) and blastema stage (BL). Error bars represent standard errors of mean. * $p \leq 0.05$; *** $p \leq 0.001$. N = 3

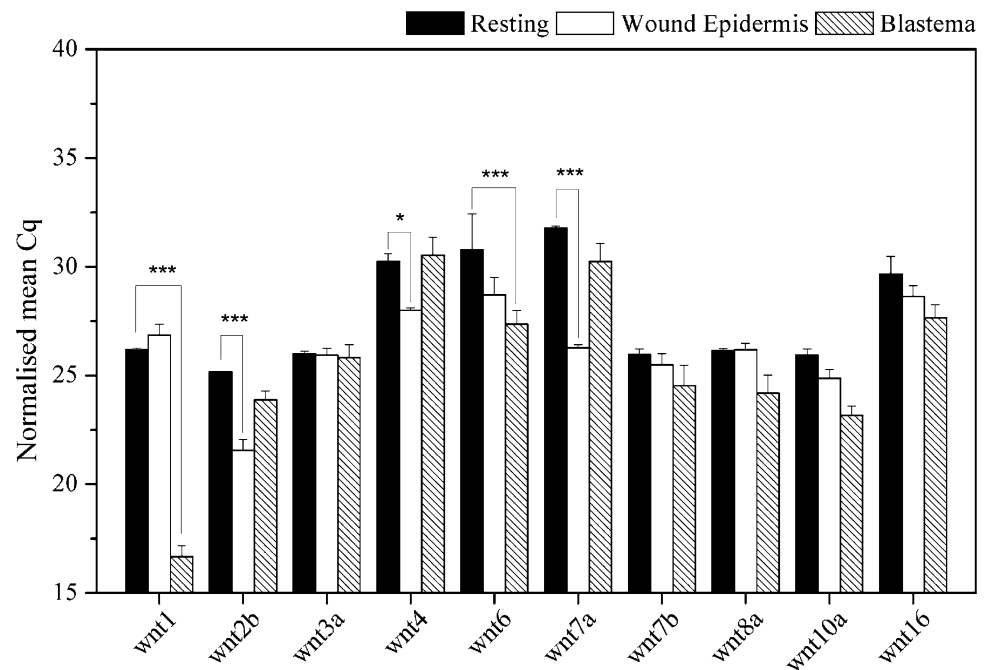


Table 2 Mean fold change in gene expression at wound epidermis stage in Etoricoxib treated samples as compared to control

Gene	Fold change
wnt4	0.015
wnt7a	6.19

appendage regeneration in amphibians [26–28]. Importantly, it has been demonstrated in a diverse set of biological systems that PGE_2 modulates Wnt signaling by altering the expression of various pathway components [21, 29–31]. Therefore, it was thought pertinent to test whether PGE_2 signals through Wnt/ β -Catenin to bring

Fig. 5 Normalised mean Cq values for wnt ligands expressed during wound epidermis stage in control and Etoricoxib treated animals. Error bars represent standard errors of mean. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N = 3

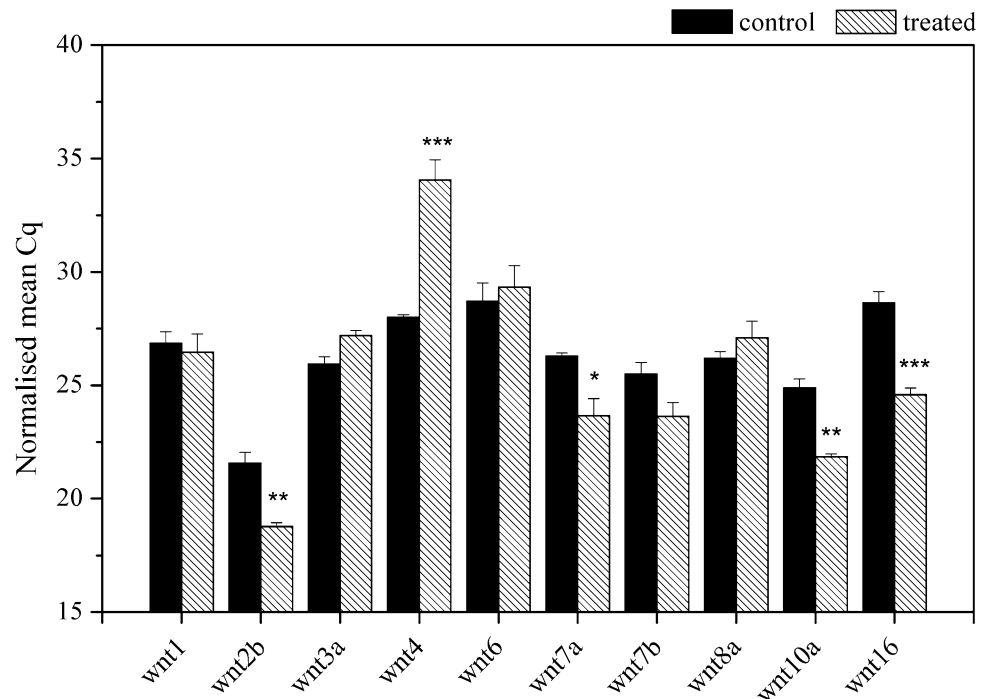


Table 3 Mean fold change in gene expression at blastema stage in Etoricoxib treated samples as compared to control

Gene	Fold change
wnt1	0.006
wnt2b	16.95
wnt3a	32.52
wnt7a	2043.27
wnt7b	12.12
wnt8a	8
wnt10a	6.11
wnt16	0.032

about successful regeneration of tail in *H. flaviviridis*. Our study involved the inhibition of COX-2 using its pharmacological inhibitor Etoricoxib and noting its influence on the expression of selected Wnt/ β -Catenin pathway genes.

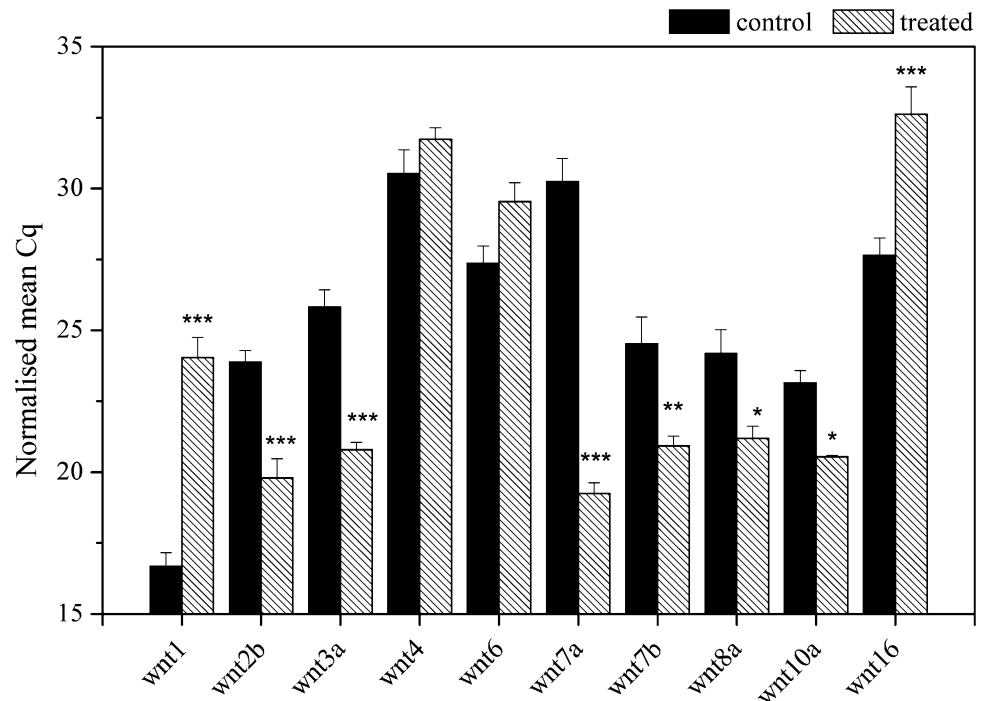
We orally administered Etoricoxib once daily to lizards up until the end of the experiment. Since there is no earlier record of oral treatment of animals with Etoricoxib, we first checked for its assimilation in the tail tissue by LC/MS and further assayed for COX-2 activity. We found significant inhibition of COX-2 activity in the tail after Etoricoxib treatment. Following commencement of drug treatment, the lizard tails were autotomised from a pre-determined point. Tissue sampling was done at two major stages of regeneration – wound epidermis and blastema, considering the importance of these stages and their implication in successful tail replacement.

Analysis of β -Catenin expression showed a definite reduction in both wound epidermis and blastema stages in

response to Etoricoxib. This points, at least partially, towards the lack of Wnt signal thereby allowing its proteasomal degradation. As a transcriptional coactivator, β -Catenin functions to regulate a battery of genes associated with cell proliferation, cell polarity and cell fate determination [32]. In order to ascertain whether altered expression of the Wnt ligands was responsible for the reduced β -Catenin expression, we performed a relative quantification of the transcripts of a set of Wnt ligands, classically attributed with the function of β -Catenin stabilization. A scan through literature, however, reveals that there has so far not been a screen identifying the Wnt ligands expressed during lizard tail regeneration and we are the first to list these. RT-PCR results show the presence of wnt1, wnt2b, wnt3a, wnt4, wnt6, wnt7a, wnt7b, wnt8a, wnt10a and wnt16 at both wound epidermis and blastema stages. At this stage, we may attribute the function of wound epidermis and blastema formation to the Wnt ligands showing heightened expression in these respective stages, although its ascertainment would need further detailed studies to be taken up.

Relative quantification of transcripts by real time PCR shows that among the Wnt ligand transcripts, wnt4 and wnt7a had significantly higher expression during the wound epidermis stage than the in the resting tail and blastemal stage. wnt4 gene expression in treated animals demonstrated a significant decrease, relative to control, during the wound epidermis stage. This may explain the incomplete healing which is seen in Etoricoxib treated animals since Wnt4 has been previously shown to be

Fig. 6 Normalised mean Cq values for wnt ligands expressed during blastema stage in control and Etoricoxib treated animals. Error bars represent standard errors of mean. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N = 3



important for timely wound healing and is upregulated in response to trauma [24]. During the blastema stage, wnt1 and wnt6 were expressed at a higher level than in the resting tail and the preceding wound epidermis stage. In samples treated with Etoricoxib, there was significantly reduced expression of wnt1 and wnt16 as compared to control. Wnt1 may be important in the late blastema owing to its property of inducing satellite cell proliferation [25]. It has to be acknowledged here that wnt2b, wnt3a, wnt7a, wnt7b, wnt 8a and wnt10a were all significantly higher in expression in treated samples versus control. While the work of Ramos-Solano et al. [33] supports the notion that Wnt7a may signal to decrease cell proliferation in some systems, one finds conflicting results from other studies [34, 35]. In order to draw a crisp conclusion about the role of individual Wnt ligands in tail regeneration, a lot more must be done in addition to the current work, since β -Catenin lies at the convergence point of multiple pathways and is not regulated by the Wnt ligands alone.

Our results here have nevertheless provided us with evidence that there is a cross-talk between the COX-2 induced PGE₂ signal and the Wnt/ β -Catenin pathway in the regeneration process and that together, they are essential for successful achievement of the wound epidermis and blastema stages. We hypothesize that this interaction could be through the Fibroblast Growth Factor (FGF) signaling pathway. This stems from a personal observation that the attenuation of COX-2-induced PGE₂ leads to reduced expression of FGF signaling components in both the mentioned stages of lizard tail regeneration (unpublished

data). Additionally, the Wnt/ β -Catenin pathway is known to be activated in the presence of the FGF signal [3, 36]. Thus deviant levels of Wnt ligands and β -Catenin in response to COX-2 inhibition by Etoricoxib could be a result of reduced FGF activation, leading to delayed and imperfect wound closure, reduced blastemal cell proliferation and hence retardation of the regenerative response. An understanding of the signaling pathways interacting with PGE₂ signaling will give us valuable information about many developmental processes, more so since COX-2 is also considered as a target for cancer therapeutics.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest whatsoever.

Ethical statement All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) constituted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (IACUC no. 984/07/2014-2).

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