Procurement of animals and maintenance

Hemidactylus flaviviridis (Northern House Gecko), both male and female, were caught from a nearby locality and caged in wooden chambers. Housing conditions were maintained as reported by Buch and colleagues (2017). Briefly, the animals selected for studies weighed around 10-12 g and were maintained at 34 ± 2 °C with light to dark cycle of 12:12 hours. 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 Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Animal grouping and experimental design

For investigating the molecular events of inflammation in the appendages, animals were randomly categorised into two main groups, namely tail and limb. These groups were further divided in various sub-groups based on the stage of the healing process to be targeted. For tail, 0, 1, 2, 3, and 4dpa (days post-autotomy) and for limb, 0, 3, 6 and 9dpa (days post-amputation) 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 these distinct time points for attaining the above-mentioned milestones that determine a path of repair in both limb and tail.

For every experiment, six lizards were used in each sub-group, viz., 0, 1, 2, 3, 4dpa for tail and 0, 3, 6 and 9dpa for limb 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. Induced autotomy caused the release of tail, from the fracture plane, where pressure was 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 right forelimbs were surgically amputated at the humerus under hypothermia as described previously by Ranadive and colleagues (2018). Ice pack was placed on the appendage and the animal was placed on a precooled tile. Tissue was then collected at particular time windows to proceed with the

analysis. Around 3 mm of tissue was harvested from the pre-amputated limb, to carry out various analyses as detailed in the following section.

Reagents and Buffers

Phosphate Buffered Saline (PBS)	
NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
NaH_2PO_4	1.8 mM
pH set to 7.4	

Tris Buffered Saline (TBS)	
Tris base	50 mM
NaCl	150 mM
pH set to 7.5	

Protein Isolation and Quantification

Tris-Triton Lysis Buffer	
Tris base	50 mM
NaCl	200 mM
$CaCl_2$	10 mM
Triton X-100	1%
Protease inhibitor (Sigma, USA)	1%
pH set to 7.5	

Bradford reagent

Bradford reagent (BioRad, USA) was diluted to 1X using double distilled water (Bradford, 1976). This reagent was filtered through Whattman filter paper (SRL Laboratories) and used for protein estimation. In order to obtain a standard curve, known concentration of Bovine Serum Albumin (BSA) (1 mg/ml) was used All experiments were performed in a 96 well microplate as the total system volume for this analysis is of 210 µl.

Protein Isolation Protocol

1. Homogenised the tissue chunk procured in lysis buffer, in a pre-chilled mortar pestle to till large parts of the tissue were broken in to small particles
2. Allowed this homogenised solution to stay at 4 °C for 45 minutes
3. The homogenate was spun at 8,000 rpm for 20 minutes at 4 °C.
4. Collected the supernatant and continue with quantification protocol.

Protein Quantification Protocol

	Sample (µl)	Bradford reagent (µl)	Water (µl)
BSA	1-10	200	9-0
Isolated Protein	0.5/1	200	9.5/9

1. Standard curve is plotted for the observed OD on adding known conc. of BSA protein to Bradford system
2. Use 0.5 or 1 µL of isolated protein and subject it to Bradford system. Observe OD at 595 nm wavelength using i-Mark Microplate reader (BioRad, USA).

SDS-PAGE

Sample Buffer/Loading Buffer (5X)	
Tris base	250 mM
SDS	10%
Glycerol	50%
Bromophenol blue	0.5%
β-Mercaptoethanol	Added freshly to make 100 mM final conc.
pH set to 6.5	

Tank Buffer	
Tris base	25 mM
Glycine	250 mM
SDS	0.2%

Gel Stock (30%)	
Acrylamide	29%

Bis-acrylamide	1%
Solution to be kept in dark overnight at room temperature and filtered before use	

Resolving Gel Buffer (12%; 5 ml)	
30% Gel stock	2 ml
Double distilled Water	1.6 ml
1.5 M Tris Cl (pH 8.8)	1.3 ml
10% SDS	0.05 ml
10% APS (freshly prepared)	0.05 ml
TEMED	0.003 ml
APS and TEMED were added just before pouring gel in PAGE assembly	

Stacking Gel Buffer (4% ; 3 ml)	
30% Gel stock	0.4 ml
Double distilled Water	1.8 ml
1 M Tris Cl (pH 6.8)	0.75 ml
10% SDS	0.03 ml
10% APS (freshly prepared)	0.02 ml
TEMED	0.002 ml
APS and TEMED were added just before pouring gel in PAGE assembly	

Coomassie Stain	
Coomassie Brilliant Blue-R250	0.5%
Methanol	40%
Acetic acid	10%

Destaining Solution	
Methanol	40%
Acetic acid	10%

SDS-PAGE Protocol

1. Post-quantification of protein, 2 µg/µl system was prepared using isolated protein, sample buffer, β-Mercaptoethanol. Sample was heated at 90 ℃ for 10 minutes and stored at -20 °C for further use.
2. PAGE assembly is set using glass plates (notched and normal) and spacers

3. The gels, both resolving and stacking, were prepared and poured in the PAGE assembly and allowed to polymerize for 30 minutes after placing comb for making uniform wells.
4. Tank buffer was introduced in the once the gel was polymerized, equal amount of protein system (2 µg/µl), was poured in wells along with dual stained marker in one well, and allowed to electrophorese at 100 V.

Western blot

Bjerrum-Schafer-Nielsen Buffer for Semi-dry transfer	
Tris base	48 mM
Glycine	39 mM
pH set to 9.2. Buffer was chilled before use	

Washing Buffer (TBS-T)	
Triton X-100 in TBS	0.1%

Blocking Buffer (TBS-MT)	
Skimmed milk powder in TBS-T.	5%

Antibody Dilution Buffer	
BSA in TBS-T.	2%
Sodium Azide in TBST-T	0.02%

ALP Substrate Buffer	
Tris base	0.1 M
NaCl	0.1 M
$MgCl_2$	0.05 M
pH set to 9.5	
BCIP-NBT Stock Solution (50X)	
BCIP	9.4 mg/ml
NBT	18.75 mg/ml
Prepared in 67% DMSO solution diluted in ALP substrate buffer fresh before use.	

Western blot Protocol

1. The components of the gel were transferred on PVDF membrane via semi-dry western blot transfer, at 100 mA for 25 minutes.
2. The membrane was used to develop immunoblots using specific primary antibodies against the protein of interest.
3. Host-specific biotinylated secondary antibodies were used for developing the blots. 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.

RNA Isolation and Quantification

DEPC Water	
DEPC in double distilled water	0.1%
Autoclaved DEPC water was used to make all reagents to prevent RNAse action on RNA	

TBE Buffer (10X ; 1 l)	
Tris base	108 g
Boric acid	55 g
0.5 M EDTA	40 ml
Volume made to 1 liter with DEPC water	

RNA Loading Dye (5X; 1 ml)	
500 mM EDTA	8 µl
40% Formaldehyde	72 µl
Glycerol	200 µl
Formamide	30 µl
2.5% BPB	30 µl
10X TBE	400 µl
Volume made to 1 ml with DEPC water	

RNA Isolation Protocol

1. TRIzol reagent (1 ml per 100 mg tissue), was used for tissue homogenisation. Homogenate was centrifuged at 8,000 g for 20 minutes at 4 °C.

2. 200 µl Chloroform was added to each vial and allowed to stand on ice for 20 minutes with intermittent shaking.
3. Mixture was then centrifuged at 12,000 g for 15 minutes at 4 °C, to receive aqueous and organic phases, separated with white interphase.
4. Upper layer was procured carefully and transferred in the vial having 500 µl isopropanol. This mixture was allowed to stand for 1 hour with intermittent shaking at every 15 minutes.
5. This mixture was then centrifuged at 12,000 g for 20 minutes at 4 °C.
6. Supernatant was discarded and the pellet was washed with 500 µl of 75% ethanol (made in DEPC), followed by centrifugation at 7,500 g for 5 minutes. Second wash of 200 µl ethanol (75%) follows with centrifugation at 7,500 g for 7 minutes.
7. Supernatant was discarded and pellet is allowed to dry, but over drying is avoided.
8. The RNA pellet obtained is dissolved in 30 µl DEPC water.

RNA Quantification Protocol

Dissolved RNA was diluted 1:10 times and quantified using Qubit Assay System as per the manufacturer's protocol. A quality check was performed on the RNA by running it through agarose gel.

cDNA Synthesis

1 µl of RNA from every sample was used to prepare cDNA using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The kit uses random primers and reverse transcriptase to form the cDNA

Semi-quant and q-RT-PCR

- TBE buffer prepared as mentioned earlier.

DNA Loading Dye (6X)	
Glycerol	30%
Bromophenol Blue	0.25%

Other Reagents Used

- 2X PCR master-mix (Sigma, USA)
- Primer pairs for the respective genes
- Nuclease-free water
- 2% Agarose in TBE buffer
- 25 mM Ethidium bromide

Primer Designing

Specific primers were designed through Basic local alignment search tool (BLAST) tool of NCBI using *Gallus gallus*, *Danio rerio*, *Poecilia spp* and *Anolis carolinensis* gene sequences. The regions with maximum homology were used to design these primers. While designing these primers, following criteria were considered:

- The amplicon size must be below 200 base pairs (bp)
- The melting temperature must be close to 60 °C
- Internal complementarity amongst the sequences must be minimum

Semi-quant PCR Protocol

The reaction mix comprised of following components:

Component	Volume
2X master-mix	5 µl
Forward primer (5 µM)	0.5 µl
Reverse primer (5 µM)	0.5 µl
cDNA template	1 µl
Nuclease free water	3 µl

The conditions for PCR were as follows:

3 minutes at 95 °C followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Semi-quant PCR was performed in thermal cycler T100 (BioRad).

Quantitative Real time PCR Protocol

The reaction mix comprised of following components:

Component	Volume
2X SYBR Green master-mix	5 µl
Forward primer (5 µM)	1 µl
Reverse primer (5 µM)	1 µl
cDNA template	1 µl
Nuclease free water	2 µl

1. Selected molecules were studied for their gene expression inflammatory mediators were checked using the PCR reaction performed in LightCycler 96 (Roche Diagnostics, Switzerland), using specific primers.
2. For the analysis, 18srRNA was used as housekeeping control. Quantitative real-time PCR was performed with the following program: 100 s at 95 °C followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C.
3. Quantification cycles (ΔCq) values calculated, for all the three technical replicates.
4. Fold change was calculated using method developed by Livak and Schmittgen (2001) using corresponding experimental 0dpa (resting stage).

Immunohistochemistry

Blocking Buffer	
BSA in PBS	0.5%

Immunohistochemistry Protocol

1. Freshly collected tissue samples were processed in cryostat-microtome to obtain longitudinal cryo-sections of 8-10 µm thickness.
2. Sections were fixed in chilled acetone for 15-20 minutes, followed by which, they 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).
3. Sections were incubated with anti-COX-2 IgG rabbit (0.5 µg/ml, Sigma-Aldrich, USA), followed by three buffer washes of 15 minutes each, using PBS-T.

4. Sections were then subjected to FITC-conjugated secondary antibody (0.1 µg/ml, Sigma-Aldrich, USA). Three subsequent buffer washes of 15 minutes. each and then these sections were used to observe the distribution pattern of COX-2 in different compartments of the tissue.
5. In order to observe the tissue architecture in these sections, DAPI was used to counterstain each one of these, which were then observed under a fluorescent microscope (Leica DM2500, Germany).
6. The representative images were captured using the digital camera (Leica, EC3, Germany), mounted on the microscope.

COX Activity Assay

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.

COX Activity Assay Protocol

1. 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 COX assay, following the manufacturer's protocol (Cayman Chemical Co, USA; ID: 760151).
2. The specific activity was calculated in nmol/min/100 g tissue weight.

PGE_2 Estimation

The PGE_2 present in the sample competes with the horseradish peroxidase (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.

PGE_2 Estimation Assay Protocol

1. Tissue samples were collected from all the subgroups and homogenised in double-distilled water (1 g/4 ml).
2. 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.
3. Homogenates were then centrifuged at 4 °C and 2000 g for 5 minutes. The supernatant was collected for the estimation assay of PGE_2 following the manufacturer's protocol (R&D Lab Systems, USA; ID: KGE004B).

Blood Slide Preparation

Blood was procured through two different methods based on the region of interest
Systemic Blood Smear: Animal was sacrificed through heart puncture method and immediately blood smear was prepared.
Tissue specific Blood Smear: Tissue specific injuries were infested and blood was collected from the organs (Tail and Limb).

Wright-Giemsa Stain

Wright Giemsa stain procured from HiMedia (Product No: S011) was diluted to 1:40 in distilled water and filtered before use.

Giemsa Staining Protocol

1. At all stages, blood smears were prepared to obtain a monolayer of cells.
2. The slides were allowed to air dry and fixed in methanol.
3. These fixed slides were allowed to air dry and subjected to Wright-Giemsa stain (1:40) for 24 hours.
4. Slides were observed under 10X and 40X objective lens of light microscope (Leica, Germany).
5. Images at 40X objective magnification were clicked using ISH300 inverted camera and ISCapture software.

For both systemic and tissue specific blood smears, same staining protocol was followed.

Fluorescent Activated Cell Sorting (FACS)

In order to analyse cells through FACS, single cell suspension is a preliminary requirement. Laser is beamed on the single cell suspension of tissues and scatter plot is developed based on specific gating method. BD Influx Cell Sorter System was employed with BD FACS Software. The gating method is based on regions identified on the scatter plot and stepwise gates are applied to sort cell population.

Reagents used

- PBS (1x)
- 4% Paraformaldehyde
- Trypsin-EDTA Collagenase
- PBS-T
- DMSO
- Blocking buffer (3-5% BSA)
- Primary antibody Human CD-14 raised in rabbit (Cloud Clone, USA) and Hoechst stain
- Secondary antibody – FITC conjugated anti-rabbit antibody (GeNei, Bangalore)

Single Cell Suspension Preparation

1. Tissue chunks were procured at 2dpa in tail and 3dpa limb in 4% PFA (in 100% methanol).
2. The homogenate was prepared and allowed to stay for 1 hour at 4 °C.
3. To this homogenate, Trypsin-EDTA (1x) and collagenase (1 mg/ml) were added, and the mixture was allowed to stand for 60 minutes.
4. The homogenate was then centrifugated at 3,000 rpm for 5 minutes and supernatant was removed.
5. The pellet is resuspended in fresh PBS and centrifugated at 3,000 rpm for 3 minutes in order to remove debris.
6. Formed pellet was subjected to PDT (2 ml PBST, 0.3% Triton-X , 1% DMSO) for 30 minutes.
7. To this mixture, 2 ml of blocking buffer was added and it was allowed to stay for 20 minutes at room temperature.

8. Primary antibody (CD-14) was introduced to this mixture and allowed to stay for 20 minutes at room temperature.
9. The mixture was spun at 3000 rpm for 5 minutes and remove the blocking buffers.
10. Secondary antibody FITC-conjugated anti rabbit antibody was introduced to the mixture and immediately images were captured for this group.

FACS Protocol

The single cell suspension from tail and limb tissues was allowed to enter the FACS setup. PBS was used as a sheathing fluid or medium for the standardisation process. Three main groups were considered for this analysis:

A. Tail (2dpa)
B. Limb (3dpa)
C. Systemic blood

Cell suspension formed for tail and limb samples were divided in four sub-groups

i. Unstained Cells
ii. Cells + Primary Antibody (CD-14)
iii. Cells + Primary Antibody (CD-14) + Secondary Antibody
iv. Cells + Primary Antibody (CD-14) + Secondary Antibody + Ho3342

Unstained blood cells were subjected to FACS analysis as no specific treatment was required here. Nucleus binding Hoechst stain was used for FACS analysis which stains the DNA specifically. It is excited by ultraviolet light and emits blue fluorescence at 460-490 nm. It effectively stains both, alive and fixed cells.

Blastema treatment

Blastema homogenate was applied to the healing limb, with a goal to check the effect of this extract on scar formation, if any. Blastema steers the process of pro-regenerative wound healing in tail, hence this part of the study tries to simulate ‘tail- like’ environment in limb. This is a pilot study which requires a series of follow-up experiments and analysis to reveal the modulators of repair process present in blastema.

Taking the first step in this direction, preliminary set of experiments were performed, wherein blastema was procured from the healing tails of six subjects. This pool of blastema was homogenised in PBS (1X) using a precooled mortar pestle and stored at 4 °C. Post-amputation, this blastema homogenate was applied on the healing limb for twelve days, at an interval of 12 hours, using a sterile round soft bristle paint brush (Faber-Castell, India) which was autoclaved prior to the experiment (Figure XI). At the predetermined time windows, healing limb of the test group subjects were observed morphologically. Hematoxylin and Eosin (H&E) staining were performed on limb tissue sections of both control and treated groups. Samples were out sourced to Unipath Laboratories, Baroda, for H&E staining. Sections were observed through 10X and 40X objective lens of light microscope (Leica, Germany) and images were captured using ISH300 inverted camera and ISCapture software.

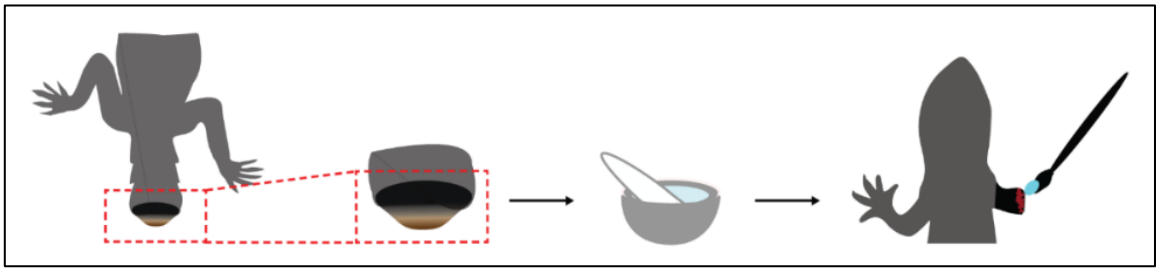

Figure XI: Blastema homogenate applied on healing limb.

List of Antibodies

Name	Clonality	Host	Molecular weight (kDa)	Catalogue number
COX-2	Polyclonal	Rabbit	70	SAB4200576
EP2	Polyclonal	Rabbit	35	PAA247Hu01
EP4	Polyclonal	Rabbit	53	PA5-111889
iNOS	Polyclonal	Rabbit	130	SAB4502012
TNF-α	Monoclonal	Mouse	50	WH0007124M2-100UG
IL-10	Polyclonal	Mouse	18	SAB1410712-100UG
IL-6	Polyclonal	Mouse	21.2	CPTC-IL6-1
IL-17	Polyclonal	Rabbit	16	PAA063Mu01
IL-22	Polyclonal	Rabbit	20	PAC032Mu01
β-actin	Polyclonal	Mouse	45	SC-69879

List of Primers

Gene	Forward (sequence 5'-3')	Reverse (sequence 5'-3')	Product length (bp)
COX-2	ACGTCTTACATCACGATCCC	GGAGAAGGCTTCCCAGCTTTT	86
EP2	AGTTCAGCCAGAGCGAGAAC	AAGACCCAGGGGTCGATGAT	85
EP4	CATTCCTCTGGTGGTCCGTG	GCTTGCAGGTCAGGGTTTTG	87
iNOS	AACATGCTCCTTGAGGTGGG	CAGCTCGGTCCTTCCACAAT	184
TNF-α	GGGTGTTCGCGTTGTGATTT	TCTCACTGCATCGGCTTTGT	171
IL-10	AAGGAGACGTTCGAGAAGATGG	TGATGAAGATGTCGAACTCCCC	70
IL-6	TATCTATGAAGGCCGCTCCG	CCATTCCACCAACATTCGCC	84
IL-17	ACAGGAGATCCTCGTCCTCC	CCTTTAAGCCTGGTGCTGGA	124
IL-22	AAGCGCTGAGTGCTGTAACT	CTTTTGGAGGTAGGGGGCTG	150
18s rRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGAAC	144