Title: Understanding the molecular mechanism of regeneration through Apoptosis Induced Compensatory Proliferation (AICP) studies

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Introduction

The lower animals (*Hydra vulgaris*, planarians, earthworms, etc.) have high wound healing and regeneration abilities than higher animals (mice, humans, etc.) in nature. The evolutionary progression of higher organisms can be traced back to their origins from lower organisms. However, it is noteworthy that over the course of evolution, the regenerative abilities that were once present in our ancestral forms have been progressively diminished. The loss observed can be attributed to a deficiency in signalling factors, a decrease in stem cell populations, and a reduction in the expression of crucial regenerative proteins [A. Zhao., 2016] In brief, higher animal cells are constructed as more specialized cells for sophisticated functions. An except embryogenesis process, they do not need that much demand from stem cells for all conditions but in lower animals have more number of unspecialized cells in their body and they have high demand on stem cells. Following the initiation of wound induction, it is observed that stem cells exhibit a tendency to amass in the vicinity of the wound site, originating from their respective stem cell niches [J.D.S. Christyraj., 2019]. These stem cells have acquired knowledge regarding the damage through communication with neighbouring cells in the vicinity of the wound. Suthira Owlarn et al., have elucidated the noteworthy roles played by the H signal and R signal in the intricate processes of wound healing and the remarkable regenerative capacities displayed by planarians. [S. Owlarn., 2017]. Adjacent cellular entities secrete signalling molecules to initiate a compensatory response, with the objective of counteracting tissue loss through the process of self-induced programmed cell death, known as apoptosis. Caspase-3, renowned for its crucial involvement in the intricate mechanism of apoptosis, additionally engages in the activation of Wnt3a, a fundamental modulator of stem cell dynamics. This context is called Apoptosis induced compensatory proliferation (AICP) [A. Bergmann, H.D. Ryoo, F.A. Martín, Li]. In order to acquire the regenerative ability and enhance wound healing potential in higher organisms, it is imperative to comprehend the molecular mechanisms underlying regeneration in lower organisms. The utilisation of the AICP context offers a comprehensive framework for understanding the intricate molecular processes that underlie the phenomena of wound healing and regeneration [Rajagopalan et al., 2022], because it covers apoptosis, stem cells and immune response, without any of these components, the attainment of successful regeneration would be hindered. AICP context have already reported in many animal models namely, *Caenorhabditis elegans*, *Hydra vulgaris*, newts, zebrafish, *Xenopus laevis,* and *Drosophila melanogaster* [David et al., 2000; Fujisawa, and David, 1984; Kobatake, and Sugiyama, 1986; Vlaskalin et al., 2004; Yamashita, 2003; Tseng, and Levin, 2008; Steller, 2008].

This thesis comprises of our findings includes the unravelling the regeneration capacity of earthworm blastema upon *in-vivo* and *in-vitro* condition, AICP importance and the hidden rulers (TCTP/p53) hypothesis for better way to understand regeneration, TCTP multifunctional role in earthworm regeneration, AICP in earthworm and ameliorating the regeneration and wound healing potential using clitellum factors and confirming that executed by AICP trigger.

Methodology

Culture and maintenance of earthworm

E. eugeniae worms were collected from a worm shop nearby Konga- nanchery, Chengalpattu, Tamil Nadu, 603,101, India. The collected worms were maintained on a worm bed which contains organic soil (70% humidity), leaf litter and cow- dung (60:30:10) in a plastic tub of size 60 × 40 × 15 cm2. The temperature was maintained at 24-26°C and water was sprinkled once every 4 d over the worm bed. This maintenance is common for all experiments.

Earthworm *E. eugeniae* amputation and maintenance for analyzing the regeneration capacity

Three groups of sexually matured *E. eugeniae* worms (age 4 months) were initially selected and their guts were cleaned. They were then taken for amputation studies with 20 worms in each group. Worms were amputated at the 10^{th}, 15^{th} and 30^{th} segments respectively to have 1–10, 1–15, 1–30, 11–anus, 16–anus and 31–anus segments. After amputation, the worms were washed with tap water to remove any blood from the amputation site and were maintained in the worm bed for 60 d. To analyse the unique ability of the clitellum in regeneration, another set of 20 worms were amputated at the 11th segment and 20^{th} segment to retain only the clitellum along with two body segments on both ends. For determining clitellum factor influence, another set of 20 worms were amputated at the 60th segment and allowed to form a blastema. After the 8th d of post amputation, the worm clitellum was removed by making an incision at the 30th segment and observed for the clitellum factor influence in worm survival. After amputation, all the amputated segments were observed for every 24 h and documented using a Canon digital camera (Tokyo, Japan. Model No: IXUS 285 HS).

In vitro maintenance of adult and juvenile *E. eugeniae* body segments and blastema

For *in vitro* maintenance, the 4th-d blastema (n = 20) was dissected out separately along with a small portion of the adjacent body segment and amputated further into two equal sections, namely anterior (ARB, 'Anterior part of Regenerative Blastema' (from the blastemal tip to the mid- portion of the blastema)) and posterior (PRB, 'Posterior part of Regenerative Blastema' (from the mid-portion of the blastema to the adjacent body segment)) portion. The anterior and posterior blastema portions (1st to 10th segments (n = 20) and 30th to 37th segments (n = 20)) of adult worms were individually maintained in the aseptic condition in Leibovitz's L15 medium (HiMedia; Thane, India. Catalogue number: AT011-10X1L) for 60 d. From juvenile worm (age 10 to 12 d), the segments from 30 to 37 (n = 20) were dissected using a sterile blade and maintained in the L15 medium. Before maintaining in the L15 medium, the dissected tissue portions were processed as fol- lows: after dissection, the tissue was immediately transferred to sterile ice-cold water and then, the tissues were washed 2 times in 1× PBS (phosphate buffer saline). For processing the tissues, 35-mm petri dishes and sterile blunt 1 mL tips were used. The sterile aseptic condition was maintained throughout the experiment by performing it in biosafety cabinet level II (Haier; Qingdao, China. Model No: HR40-IIA2). The tissues were

washed again with sterile-filtered ice-cold-filtered 1× PBS buffer and then, the amputated tissues were transferred into an antibiotic solution (Streptomycin (1µg/1ml) (IBI Scientific; Dubuque, Lowa. Catalogue Number: IB02180). Amphotericin B (1µg/1ml) (Thermo Fisher Scientific; Waltham, Massachusetts. Catalogue: 15290018) and Penicillin (1µg/1ml) (HiMedia; Thane, India. Catalogue: SD028), for 10 min. The tissues were washed thrice consecutively in the antibiotic solution for the successful elimination of bacterial and yeast contamination. Following that, tis- sues were transferred into filtered serum-free L15 medium for 5 min and then transferred into L-15 medium containing 10% FBS. Finally, tissues were incubated in an ambient air incubator and maintained at 28°C. The L15 10% serum medium was changed once every 3 d. The *in vitro* survival and regeneration ability were observed using an EVOS fluorescent microscope every 24 h until 1 month. All worms were subject to gut cleaning (maintained in wet tissue paper for 72 h) prior to the start of the experiments [Subramanian et al., 2017].

Histological analysis *in vitro* maintenance of *E. eugeniae*

Following 60 d of post-maintenance in *in vitro* conditions, the adult blastema tissue (n = 6) was care- fully taken out and subjected to histology. Initially, the tissue samples were fixed in 10% formaldehyde (formalin) (Cat. F0080; Rankem, Mumbai, India) for 24 h. Following that, the tissue samples were gently washed with distilled water and subjected to dehydration step for an hour using an increas- ing gradient in isopropanol (Cat.P0670; Rankem) from 60%, 70%, 80%, 90% and 100% each. After the clearing step using xylene (Cat. X0030; Rankem), the tissues were incubated overnight with paraffin wax (Cat. GRM1137; HiMedia) for impregnation and embedding. Using a microtome, the paraf- fin-embedded blocks were subjected to dissection (5 µm) and the collected sections were processed and stained by haema- toxylin (Cat. S059-Himedia) and eosin (Cat. S007-HiMedia). Following that, the slides were mounted using DPX solution (Cat.D0240; Rankem, Mumbai, India) and the tissue sections were visualised under an Epi-Fluorescence Microscope (Cat. FM-5000; Euromex, Chennai, India).

In-vivo* regeneration studies of *Perionyx excavatus

The process of *in-vivo* regeneration refers to the natural ability of an organism to restore and repair damaged or lost tissues and organs within its own. For the purpose of conducting amputation studies, a total of six worms were initially placed within a plastic container, where they were given a period of two days to acclimatise and adjust to their new environment. After a period of 48 hours, the annelids were subjected to a thorough rinsing using tap water, followed by submersion in cold water for a duration of 30 seconds, thereby inducing temporary immobilisation. Following that, the annelids were subjected to amputation at various locations utilising aseptic surgical instrumentation, specifically a sterile surgical blade manufactured by Surgeon, KEHR surgical private limited, located in Kanpur, Uttar Pradesh, India. The amputation procedure was conducted to obtain distinct fragments comprising segments ranging from the 1st to the 17th, the 18th to the 36th, the 37th to the 50th, the 51st to the 75th, and the 76th to the 98th segments. The amputated segments were subjected to observation in order to assess

the process of anterior and posterior regeneration on day 5, day 9, and day 12 following amputation. In the context of protein sample preparation, an additional six worms were subjected to amputation at the intersegmental regions spanning from the 23rd to the 37th segments. Subsequently, these worms were provided with suitable conditions to initiate the process of regeneration. The protein samples were obtained from the regenerating termini of the amputated worms at day 1, day 4, and day 6 post-amputation. The regenerative process of various worm species was meticulously documented using a Canon digital camera (model IXUS 285 HS) in Chennai, India. The amputation experiments were replicated in triplicate to obtain concurrent data.

BCIP/NBT Staining for stem cell tracking in *Perionyx excavatus*

The BCIP/NBT solution serves as a substrate for the enzyme alkaline phosphatase, which exhibits a higher concentration in stem cells. The BCIP/NBT stain solution, which was acquired from Sigma Aldrich (catalogue number B1911-100ml), India, was procured for use in the experiment. In the beginning, the worms underwent a thorough rinsing process with distilled water, followed by fixation using ice-cold methanol for a duration of 15 minutes. Following fixation, the nematodes underwent a thorough rinsing process using 1× TBST (1× Tris buffered saline with Tween-20) buffer. This buffer was applied three times, with each wash lasting for a duration of 5 minutes. The annelids were submerged in a solution of BCIP/NBT for a duration of 15 minutes, while being kept in a light-restricted environment at ambient temperature. Following this, the tissue samples underwent a subsequent washing step utilising 1× TBST buffer. Subsequently, the enzyme substrate reaction was brought to a halt by subjecting the samples to an incubation period in the stopping solution, consisting of a mixture of acetic acid (4), glycerol (1), and ethanol (2), for a duration of 15 minutes. The earthworm samples that had undergone processing were examined using a dissection light microscope (Optica Microscope Italy - Model No: SN621997), and meticulously recorded.

Cell Cycle Arresting Assay for unravelling the role of TCTP in regeneration principles

To block epimorphosis, 2mM thymidine was injected into the 24th segment of the worms (No: 10). Nuclease-free water injection was used for the control set of worms (No - 10). An injection was continued for up to 10 days. After the second day of injection, the worms were amputated on the 10th segment and observed for their regeneration kinetics with a 15cm scale and camera.

Buclizine injection and Pharmacological suppression of TCTP for unravelling the role of TCTP in regeneration principles

In this experiment, three dose levels of Buclizine (Mankind Pharma Ltd, Chennai, India), namely 140µg, 160µg, and 200µg, were injected into the post-clitellum regions (24th segment) of *P. excavatus* worms once per day throughout the experiments. The control worm was injected with 1X PBS. Following 2nd day of the initial injection, the worms were amputated at the 10th segment and observed for regeneration ability. The regeneration process was monitored and documented using a Canon digital camera (IXUS 285 HS).

Regeneration Studies for unravelling the role of TCTP in regeneration principles

We used the eight groups of mature *P. excavatus* worms in this study to understand the importance of TCTP in regeneration. Each group consisted of ten worms, and the treatment is as follows: 1. *in vivo* regeneration analysis, 2 & 3. Control and Buclizine treatment for TCTP silencing, 4 & 5. Control and Thymidine treatment for cell arrest analysis, and 6. Buclizine and thymidine treatment for combinatorial toxicology. 7 & 8. Control and Nutlin-3a treatment for TCTP silencing. For *in vivo* regeneration analysis, selected worms (group 1) were amputated in the 10^{th} segment (anterior region-head) using a cruzine carbon steel surgical scalpel blade (size 15) and maintained in the worm bed. Every 24 hours, the amputated worms were documented with the help of a canon digital camera (IXUS 285 HS).

Influence of Nutlin-3a in regeneration for unravelling the role of TCTP in regeneration principles

To study the importance of TCTP in regeneration, mature *P. excavatus* worms were selected. Two batches were selected, each containing 10 worms, 10 for control and another 10 for Nutlin-3a treatment. The first batch of ten worms acted as a control and was injected with DMSO. The second batch of ten worms was injected with Nutlin-3a in the 24^{th} segment at a dose of 5µg/g. The control and treated worms were amputated at the 10^{th} segment (anterior) and maintained in the soil. The worms were anesthetized by ice before amputation. The blastemal formation was carefully observed in both batches of worms with the help of a Canon digital camera (IXUS 285 HS).

Histology for unravelling the role of TCTP in regeneration principles

Thymidine-treated 7^{th} day regenerated worms, 5^{th} day Nutlin-3a treated samples and their respective control samples were subjected to histological sectioning. Both the regeneration blastema of the control and treated worms were cut using sterile scalpels along with the adjacent two segments and fixed in 10% formaldehyde (Cat.F0080; Rankem, Mumbai, India) for 24 hours. To remove formaldehyde, tissues were gently washed with distilled water and dehydrated with isopropanol and acetone after the tissue was cleared using xylene. Each step was performed for one hour at 50°C. Following the xylene removal step, tissue impregnation was incubated in paraffin wax, and the 5µm sections were made using a microtome [Subramanian et al., 2017]. Eosin and hematoxylin stains were used to distinguish the internal arrangements and were observed in Euromex bScope Epi-Fluorescence HBO Microscope (Catalogue Number: BS.3153-PLFi).

Western Blotting for unravelling the role of TCTP in regeneration principles

All experiment samples were quantified using Lowry's method, and an equal volume (80µg) of protein samples was loaded onto SDS page gel electrophoresis and applied to run at 60 volts for 2 hours 30 minutes using Bio-Rad gel systems. The resolved proteins in the SDS-PAGE gel were transferred to the PVDF membrane. The membrane was blocked with 5% BSA in TBST buffer and then incubated with either of the following primary antibodies of Anti-TCTP (Abcam, ab37506; dilution, 2.5:5000), Anti-p53 (Abcam, ab26; dilution, 2.5:5000) and Anti-

β-actin (Abcam, ab8226; dilution, 1:5000), Anti-Wnt3a (Abcam, ab19925; dilution, 0.5:5000), Anti-PCNA (Abcam, ab18197), Anti-H3 (Abcam, ab1791; dilution, 1:5000) and Anti-YAP1 (Abcam, ab62751; dilution, 0.5:5000) for overnight at 4ºC. After washing, the secondary antibody of Anti-rabbit IgG-HRP (1:10000) or Anti-mouse IgG-HRP (1:10000) was added to protein transferred membrane. The substrate ECL was used as a developer solution. ChemiDoc XRS documented the developed membrane, Bio-Rad, USA; the band intensity was analyzed using ImageJ analysis software (NIH, USA).

Homology Modelling of TCTP Protein

TCTP of *Lumbricus rubellus* (Humus earthworm) was selected as the target sequence for the study. The one-dimensional FASTA sequence of the target protein, TCTP, were retrieved from the UniProt protein sequence database with accession number 018477. The structural blast was performed with the TCTP sequence of *L. rubellus* against the PDB database to identify the template structure for homology modelling. The homology model of *L. rubellus* TCTP protein was generated by SWISS-MODEL homology modelling server with the crystal structure of human histamine releasing factor-translationally controlled tumor protein (HRF-TCTP) (PDB ID: 5O9m) as the template structure. The quality and reliability of the constructed model were evaluated by PROCHECK, ERRAT, and VERIFY3D servers.

Ligand Preparation for docking

The structure of the ligand, buclizine, was constructed using the 2D/3D ChemBio Draw Ultra software application, version 12 (Cambridge Soft), and then copied into the ChemBio 3D Ultra software application, version 12, to create the 3D structure. Then the structure was energy minimized by the MMFF94 method using the geometry optimization function in Chem Draw 3D software, and the lower energy conformations were selected for molecular docking studies.

Molecular Docking Studies

A molecular docking process was carried out using Autodock vina to reveal the binding analysis of *L. rubellus* (closely related species of *P. excavatus*) TCTP protein vs. Buclizine (PubChem ID: 6729). The protein's binding site was adjusted using a grid box and x, y, and z-axis values. The grid box (60ÅX 60Å X60Å) is centered at 4.066, 20.626, -11.479, and the spacing is 1. All other parameters were kept as default. An exhaustiveness of 10 was assigned for docking throughout the docking process, and 10 mode numbers were assigned to achieve reliable results. Post-docking analyses were carried out using Discovery Studio.

Amputation Studies for enhancing the wound healing and regeneration potential in mouse cell lines using Clitellum and regenerative clitellum factors

To prepare the Clitellum Factors (CF) and Regenerative Clitellum Factors (RCF), total 2 groups of worms were taken. Each contains 10 worms. In the first group, worms were amputated (n=10) at 10^{th} segment using sterile scalpel blade (Lister Surgical Blade; Size 15, Kanpur, India. Catalogue: IS:3319) for the preparation RCF. Following that, on the 4^{th} day of post-amputation, clitellum (n=10) was extracted from the regenerative worm. For the preparation of CF, non-amputated worm clitellum (n=10) was extracted.

Preparation of Clitellum factors (CF) and Regenerative Clitellum Factors (RCF) from earthworm *E. eugeniae*.

Both extracted whole clitellum parts were thoroughly washed with distilled water for thrice, then those were transferred into 1X Phosphate Buffer Saline (PBS) for surface sterilization. Following that, clitellum were kept in 10% DMEM (45ml of 0% DMEM media + 5ml Fetal Bovine Serum (FBS)) (DMEM - Sigma-Aldrich; United States. Catalogue: D1152-10L) (FBS - Gibco; United States. Catalogue: 10270106) and crushed with tissue homogenizer (Bel-Art Disposable Plastic Pestle; Spectra Services, Wayne County, Michigan. Catalogue: SKU: 19923-0000) at 4˚C for few minutes then the supernatant was stored in -20˚C freezer. The prepared mixture of CF and RCF used for cell culture studies and animal studies.

Cell Culture for enhancing the wound healing and regeneration potential in mouse cell lines using Clitellum and regenerative clitellum factors

Mouse myoblast C2C12 cells were purchased from National Center for Cell Science, Pune, India. The C2C12 cells were maintained in T-flasks containing DMEM supplement with 10% FBS and 1% antibiotics (such as penicillin [100µg/mL] (HiMedia; Thane, India. Catalogue: SD028), streptomycin [100µg/ml] (IBI Scientifc; Dubuque, Lowa. Catalogue: IB02180) and amphotericin B [100µg/mL] (Thermo Fisher Scientifc; Waltham, Massachusetts. Catalogue: 15290018). The cells were maintained in a CO2 incubator at 37˚C provided with 5% CO2. Cells were subcultured after the cells reach 90% confluence. The sterile aseptic condition was maintained throughout the culture maintenance by performing it in biosafety cabinet level II (Haier; Qingdao, China. Model No: HR40-IIA2).

MTT assay for enhancing the wound healing and regeneration potential in mouse cell lines using Clitellum and regenerative clitellum factors

To determine the cell viability, C2C12 cells (1×10^5 cells/ml) were seeded in the 96-well plate and incubated at 37˚C in CO2 incubator. After 24 hr of incubation, the plates were washed thrice with 0% media (no serum added) and CF and RCF treated with following percentage 5, 25, 50, 75, 100% with 10 DMEM medium. The plates were incubated for 48 hr at 37˚C in CO2 incubator and following that MTT (stock 5mg/10ml 1X PBS) (Sigma; Chennai, Tamil Nadu, India. Catalogue: 11465007001)) were added in 96 well plate and incubated for 2 hours in dark condition. After incubation, media was carefully removed and DMSO (Himedia; Mumbai, India. Catalogue: MB058-100ml) were added in plate for the purple color formation, which is directly proportional to the number of viable cells. The 96 plate was subjected to OD value at 570 nm using a microplate reader (BioTek Epoch Microplate Spectrophotometer; United States). The percentage of cell viability was calculated using the formulae namely (Treatment average OD/Control Average OD*100).

Wound healing assay for enhancing the wound healing and regeneration potential in mouse cell lines using Clitellum and regenerative clitellum factors

To determine the wound healing ability of CF and RCF, C2C12 cells were seeded in the 6 well plate and incubated at 37˚C in CO2 incubator. After 24 hr of incubation, the plates were washed thrice with 0% media (no serum added) and wound was created by manual method (scratch done by 200ul tips with help of 15cm scale). After that, media was removed which also contains dead cells from the scratch then CF and RCF were added in the following percentage 5, 25, 50, 75, 100% with 10% DMEM medium were added in 6 well plate and incubated for 24 hours at 37˚C in CO2 incubator. Wound healing activity were carefully observed for 0^{th}, 24^{th}, 30^{th} and 48^{th} hours by using EVOS microscope (EVOS-FL).

Immunofluorescence Assay for enhancing the wound healing and regeneration potential in mouse cell lines using Clitellum and regenerative clitellum factors

C2C12 cells were cultured in the six well plate with the supplement of CF (5%) and RCF (50%) along with control (10% FBS) and after attain the confluence cells were washed with ice-cold 1XPBS buffer for thrice. The cells were fixed with 4% paraformaldehyde (Himedia; Maharashtra, India. Catalogue: GRM3660-500mg) in 1% PBS for 10 min at room temperature, and then permeabilized with 0.5% Triton-X-100 (Triton™ X-100 Solution, 20% Sterile filtered; Himedia Labs, Maharashtra, India. Catalogue: TCL136) for 10 min on ice. The cells were blocked with 1% Bovine Serum Albumin (BSA) (SRL; Maharashtra, India. Catalogue: 9048-46-8) for 30 min and further incubation with primary antibody Anti-VEGF antibody (ab194806), Anti-P53 antibody (ab26), Anti-HoxD3 antibody (ab221101), Anti-Wnt3 antibody (ab19925), Anti-TCTP antibody (ab37506), Anti-H2AX antibody (ab20669), Anti-Caspase-3 (ab208161), Anti-H3 (ab1791) and Anti-β-actin (ab8227) for overnight at 4ºC. The cells were washed with 1XTBST and then treated with the appropriate secondary antibodies for 2 hours at room temperature under dark condition. After that, cells were washed with 1XTBST buffer for thrice and counterstained with DAPI (40,6-diamidino-2-phenylindole) (Sigma Aldrich, United States Catalogue: 10236276001), mounted by DPX mount (SRL; Maharashtra, India. Catalogue: 88147) and observed under EVOS fluorescence microscope.

Western Blotting for enhancing the wound healing and regeneration potential in mouse cell lines using Clitellum and regenerative clitellum factors

In order to understand the molecular mechanism behind the wound healing ability of CF and RCF, protein samples were prepared from the control, CF and treated C2C12 cells using RIPA buffer. Following that, samples were quantified by Lowry's method. Subsequently, 80µg of each protein samples were resolved in 12% SDS-PAGE. The resolved proteins in the SDS-PAGE gel are transferred to the PVDF membrane. Then protein membrane was treated with a 5% BSA in TBST buffer for blocking. Following that, membrane was treated with primary antibodies namely, Anti-VEGF antibody (ab194806), Anti-P53 antibody (ab26), Anti-HoxD3 antibody (ab221101), Anti-Wnt3 antibody (ab19925), Anti-TCTP antibody (ab37506), Anti-

H2AX antibody (ab20669), Anti-Caspase-3 (ab208161), Anti-H3 (ab1791) at the dilution of 1:5000 at 4°C for overnight. Following incubation, the membrane is washed with 1×PBST buffer thrice and incubated with secondary antibody, anti-rabbit IgG, HRP (produced in goat, Sigma-A0545), and Anti-Mouse IgG, HRP (produced in goat Abcam-ab6789) at a dilution of 1: 10,000 for 2 h at room temperature. The washing step was repeated for thrice then ECL substrate was added on the membrane and ChemDoc (Biorad ChemiDoc XRS+; United States. Catalogue: 170-8265) captured the luminescence signals from the membrane.

Enhancing the survivability in non-regenerative segments of *E. eugeniae* using CF and RCF

To ameliorate the survivability of non-regenerative segments of *E. eugeniae,* 1^{st} - 10^{th} segments were taken. Briefly, worms were categorized into three groups namely 1. Control, 2. CF treated, 3. RCF treated. Amputation were carried out on 1^{st} - 10^{th} segments for all three groups and amputated worms were treated with CF and RCF. Control and treated worms are carefully observed and documented using Canon Digital camera (Tokyo, Japan. Model No: IXUS 285 HS).

Statistical Analysis for enhancing the wound healing and regeneration potential in mouse cell lines using Clitellum and regenerative clitellum factors

Survival graph (Kaplan–Meier) for the CF, RCF treated worms were plotted using the statistical software- GraphPad Prism, Version 5.01. Briefy, CF and RCF applied of the earthworms, death, and live worms are counted and plotted in the (Kaplan–Meier) survival graph. All other experiments are repeated for at least three times to obtain the statistical signifcance. The data obtained were analyzed using the statistical software SPSS (version 22.0; IBM Corp., USA) and expressed as mean±SD. The results were considered as statistical significance when the p-value<0.05.

Results

Comparative analysis of the survival and regeneration potential of juvenile and matured earthworm, *Eudrilus eugeniae*, upon *in vivo* and *in vitro* maintenance

In this study elucidated that the survival and regeneration ability of *E. eugeniae* resided within the clitellum segments. In the absence of clitellum segments, the survival longevity and blastema formation were dependent on the presence of segment length and partial clitellum segments. But upon *in vitro* maintenance, the survivability was remarkably increased in both adult and juvenile worms. Notably, the blastemal forming ability was restored in adult ARB, in PRB and in juvenile posterior segments. The clitellum is important for the successful growth of blastema in *in vivo* conditions, but in *in vitro* conditions the FBS-containing L15 medium was responsible for blastemal growth. That data confirms that one of the functions of clitellum

might be the paracrine supply of growth factors. The manuscript was published in the journal of *In vitro* cellular and developmental biology Animal (Impact Factor: 2.72), Doi No of manuscript: **https://doi.org/10.1007/s11626-022-00706-6.**

Apoptosis Induced Compensatory Proliferation and hidden rulers behind AICP hypothesis

Caspase executes apoptosis and emits compensatory proliferative signals during regeneration. Both initiator and effector caspases were performed in Drosophila AICP, but other regenerative animal models predominantly followed effector caspase-dependent AICP. Unfortunately, in some animal models like planarians, earthworms, zebrafish, etc., AICP studies are not conducted directly. For instance, only the expressions of apoptosis during regeneration in adult earthworms have been reported; however, the stem cell expressions during regeneration are still unknown. Similarly, the movement of stem cells during regeneration in juvenile earthworms was observed, but the role of caspases during regeneration remains unknown. AICP studies will help us understand the molecular mechanism of regeneration and cancer. Collective reports indicate that caspase-3 is the backbone of AICP, but cell fate determinants like TCTP/p53 also play a vital role in AICP. TCTP and p53 can directly regulate the effector caspase and activate stem cells. In many animal models, TCTP/p53 reports have been revealed, but the aspect of AICP is not discussed. Meanwhile, TCTP/p53 are in a reciprocal repression relationship and, most importantly, TCTP can also regulate p53. Interestingly, TCTP can also be used as a regulator in the p35-undead cell model of AICP as it can regulate p35. So analysing the role of TCTP/p53 in AICP studies will help us to comprehend the AICP studies as well as in the development of therapeutic approaches in cancer and regenerative medicines. This article was communicated in the Journal of Differentiation; it is in under the review process (Manuscript No: DIFF-D-23-00104).

Understanding the molecular mechanism of regeneration using AICP in earthworms

The species *P. excavatus* exhibits a remarkable capacity for regeneration in their posterior segments, while this regenerative ability is absent in their anterior segments. During the process of regeneration, the worm exhibits a greater inclination towards anterior regeneration as opposed to posterior regeneration. This preference can be attributed to the fact that the anterior region encompasses a multitude of vital organs that are essential for the organism to carry out its normal physiological functions. The stem cell microenvironment of *P. excavatus* is primarily localised within the posterior segments, while being absent in the anterior segments, thereby accounting for the observed variations in regeneration capabilities (Figure 1). The process of normal regeneration adheres to the principles of AICP, which play a functional role in regulating the regenerative process and culminate in the restoration of specific structures. In the context of in-vitro maintenance of amputated small posterior segments, it is observed that the typical pattern of Apoptosis Induced Compensatory Proliferation (AICP) is not adhered to. Consequently, this deviation leads to the development of abnormal multiple buds, which originate at a distance from the site of amputation (Figure 2). In summary, our findings indicate

that the presence of stem cells alone is insufficient for their effective activation. Instead, it is the presence of specific physiological conditions that ultimately determine the stem cells' ability to regenerate tissue effectively. This article was communicated in the Journal of *In vitro* Cellular and Developmental Biology Animal, it is in under the review process (Manuscript No: IVAN-D-23-00169).

Understanding the multi-functional role of TCTP in the regeneration process of earthworm, *Perionyx excavatus*

Apoptosis, stem cell activation, cellular proliferation, and organ development are essential for regeneration. Our studies conclude that TCTP governs both epimorphosis and morphallaxis during regeneration. Inhibiting TCTP impairs the regeneration mechanism by inhibiting all keyframes of regenerative proteins, including PCNA (proliferation), Wnt3a (Stem cell activation), and YAP1 (Hippo signaling). The cellular stress following pharmacological suppression of TCTP also initiates the p53 expression in the context of anti-apoptotic responses (Figure 3). Collectively, the present studies reveal the regulatory role of TCTP in connection with all critical regenerative proteins. This article was communicated in the Journal of Tissue Engineering and Regenerative Medicine; it is in under the final revision process (Manuscript No: TERM-D-23-00063R5)

Enhancing the wound healing potential using earthworm clitellum factors and revealing its molecular level evidence on mouse myoblast cells C2C12.

The growing proof highlights the significance of the clitellum and its associated factors in the process of regeneration. In our study, extracts obtained from the clitellum of both non-regenerative and regenerative earthworm *E. eugeniae* have exhibited notable effectiveness in enhancing the process of wound healing in mouse myoblast cells C2C12 and enhancing the survivability in non-regenerative body segments of *E. eugeniae*. The results obtained from the MTT assay and wound healing assay indicate a significant enhancement in the viability and proliferation rate of cells treated with CF and RCF compared to the control group. The regenerative proteins, which play a vital role in the process of wound healing and regeneration, were found to have a favorable impact on cells that were subjected to CF and RCF. The veracity of this observation was supported by the outcomes acquired through immunofluorescence and western blotting methodologies (Figure 4 and 5). Significantly, the utilisation of CF and RCF demonstrated a noteworthy enhancement in the survival rate of non-regenerative anatomical segments in earthworms (Figure 6). This article was recently communicated in the Journal of Wound Repair and Regeneration (Manuscript No: WRR-23-08-0298)

Discussion

In the recent years, regeneration studies amongst the lower invertebrates like in annelids are more explored which adds more knowledge in the field of developmental and evolutionary

biology [Özpolat, and Bely, 2016]. The regeneration capacity is one of the main factors that determines survivability and it varies based on the depth of injury and the animal systems [Zhao et al., 2016]. In the comparative analysis study, all the amputated worm segments which possessed intact clitellum (11^{th} to anus and 1^{st} to 30^{th} segment) survived (100%) were able to heal, form blastema and differentiate segments. The results clearly indicate that, with intact clitellum, the worms were able to restore their lost structures which included the process of wound healing, blastema formation and blastemal differentiation ability. It was already reported that the expression of the pluripotent marker Oct-4 was observed on the 3rd d of blastemal cell of *E. eugeniae* [Kalidas et al., 2015] and that may be up-regulated only in the presence of intact clitellum, which in turn proceeds with successful regeneration. Besides the importance of intact clitellum in regeneration mechanism, the roles of clitellum factors that are released from clitellum and that act on the distant location of the amputation sites are also revealed through experiments. The results imply that even in the absence of clitellum, the already formed posterior blastema grew and differentiated into segments and new blastema are 100% formed at the anterior end. The *in vivo* and *in vitro* survival and regeneration potential of *E. eugeniae* are shown in Table 1. The data also clearly stated that other than intact clitellum segments, the clitellum factors that were released from clitellum are also very essential for regeneration at distant amputated sites. The movement of factors from the clitellum is one of the main reasons for successful regeneration and those factors are assumed to be stem cell factors (Sivasubramaniam 2021). Also, the stem cell niche might be present within the clitellum segments that may provide the microenvironment to support stem cell growth and maintenance. In planarians, after the wound induction, stem cells accumulate at the site of the wound and wait for the signals for healing and regenerating the lost tissue (Owlarn et al. 2017). A similar mechanism of regeneration was already reported in the juvenile worm of *E. eugeniae* in which a high number of alkaline phosphatase (ALP) positive cells in the clitellum migrated towards the amputation site and formed the regenerative blastema [Christyraj et al., 2019].

P. excavatus is a topsoil earthworm that is habitually subjected to injury by predators, and correlating it with its enormous posterior regeneration ability represents its evolution nature to survey following an injury [Banik, and Chaudhuri, 2017]. Regeneration studies show that the earthworm, *P. excavatus*, has an enormous regeneration ability. The worms amputated at the post-clitellum segment (30^{th} segment) can regrow as an individual worm. The data confirm that *P. excavatus* is a clitellum-independent worm that does not requires clitellum segments for their regeneration as it is necessary for clitellum-dependent worms [Christyraj et al., 2019]. The pre-blastema was observed within 48 hours, and within another 24 hours, the blastema developed into a well-developed structure and further developed rapidly in the following hours. The data represents that anterior head regeneration is more vigorous because it needs to restore all the vital organs like mouth, tubular heart, simple brain and other organ systems to survive and perform the normal functions. Following injection of 2mM Thymidine, the regeneration potential of the earthworm was suppressed to $1/3^{rd}$ level, representing the earthworm. *P. excavatus* can perform regeneration to a certain extent by utilizing morphallaxis when blocking epimorphosis. The epimorphosis mode of head regeneration was early reported in the earthworm *P. excavatus* [Bae et al., 2020], in which a high proliferative mass of cells forms the regenerative blastema. The blastema-like structure is observed in the arm

regeneration of starfish, which adopts the intermediate mechanism of both morphallaxis and epimorphosis for their regeneration [Yokoyama et al., 2007]. From histology, it confirms that the regenerative ability of the earthworm is suppressed in 2mM Thymidine injected worms with a lack of development in their internal structures like functional mouth, septum, and segment elongation, which indirectly implies the factors or signals necessary for regeneration are not regulated correctly [Michalopoulos, 2020].

Following the pharmacological suppression of TCTP using an antihistamine drug, buclizine, the worm shows reduced regeneration ability which implies the critical role of TCTP in determining the regeneration ability of the worm. TCTP determines the cell fate on both ends, on a positive side through the influence of DNA damage and on a negative side as regulated by p53 [Acunzo et al., 2014]. The positive side of TCTP in determining the cell fate is revealed upon regeneration in that TCTP is upregulated on succeeding days of regeneration, and their pharmacological suppression hinders regeneration. TCTP also plays a crucial role in promoting the pathways related to cancer progression [Bommer, and Kawakami, 2021]. Therefore more studies are needed to understand their regulatory mechanism in determining the cell fate with controlled (Regeneration) and uncontrolled (Cancer) regulation upon many extracellular stimuli [Seo et al., 2017]. The modelled TCTP protein using earthworm sequences and their close interaction with buclizine and *in-vivo* results conclude that antihistaminics are the potent lead compounds in inhibiting the TCTP, which have a broad medicinal scope in treating cancers [Seo et al., 2017]. TCTP is a multi-functional protein that plays a crucial role in cell proliferation, cell growth, and apoptosis by interacting with many regulatory proteins [Chen et al., 2020]. TCTP's part is also well documented in regenerative models involving epimorphosis or cell proliferation [Subramanian et al., 2017; Chen et al., 2020], but its role is not revealed in the aspect of morphallaxis. The combinatorial injection of 2mM Thymidine and Buclizine inhibit both epimorphosis and TCTP protein, respectively, resulting in complete regeneration loss, but the worms survived without any physiological stress. The data confirms that 2mM Thymidine injections block epimorphosis and conjoined inhibition of TCTP blocks morphallaxis, which can completely block the regeneration events in the earthworm, *P. excavatus.* The clitellum-independent worms have a vast regeneration ability because regeneration is not restricted or dependent only on the clitellum segments, and in such worms, TCTP governs both epimorphosis and morphallaxis. There are also high possibilities with more multi-functional ability of TCTP protein with animals with more regenerative ability and animals with less regenerative ability. In these aspects, research is needed to conclude it in the near future. Surprisingly in combinatorial injected amputated worms, the p53 level increased compared to the non-injected regenerating worms. p53 also reverses the cell cycle, allowing cells to repair their DNA and inducing apoptosis in severe DNA damage [Feroz, and Sheikh, 2020]. In the present study, the amputated worm is subjected to double stressful conditions, notably cell cycle arrest and TCTP suppression, and in that conditions, the worm expresses abundant p53, representing that p53 promotes cell survival to repair and rescue. Several *in-silico* and *in-vitro* research have examined buclizine as an inhibitor of TCTP, but neither study reported on *in-vivo* models [Seo, and Efferth, 2016; Kumar et al., 2017]. Here we reported the potential *in-vivo* interactions of Buclizine and TCTP with visible suppression of TCTP expression and regeneration in the earthworm model. TCTP is a multi-functional protein

inhibiting them with Buclizine targets TCTP and interplays with the TCTP interacting proteins [Bommer, and Telerman, 2020].

Unlike buclizine, the inhibitory effect of Nutlin-3a in targeting TCTP is well documented in many *in-vivo* models [Zuber et al., 2011; Subramanian et al., 2017; Kang et al., 2020]. The delay of posterior segment regeneration and wound closure following amputation was reported in Nutlin-3a injected clitellum dependent, *Eudrilus eugeniae* earthworm. Similarly, in these present studies, upon anterior regeneration, Nutlin-3a suppresses regeneration; histologically, it is evident with the improper cellular package. The data indicates that TCTP is linked with many regenerations-associated proteins, such as those involved in cell proliferation, cellular morphallaxis, cell differentiation, apoptosis, immune response, stem cell activation, and organ development. Inhibiting TCTP with Nutlin-3a suppresses the regeneration mechanism together with influences from other proteins like PCNA (cell proliferation), Wnt3a (stem cell marker), and YAP1 (organ formation ruler and Hippo signaling) (Figure 3). Following amputation, the microenvironment at the wound site provides signals for triggering regeneration and in which DNA damage-induced responses like apoptosis [Ryoo, and Bergmann, 2012], stem cell migration [Sahu et al., 2021] play a significant role in determining the regeneration potential. TCTP determines cell fate by regulating major cellular functions like apoptosis and proliferation [Hsu et al., 2007; Rho et al., 2011; Telerman, and Amson, 2017]. Notably, the TCTP protein is known for its anti-apoptotic role [Rho et al., 2011; Bommer, and Thiele, 2004; Lee et al., 2022] and also act as an apoptotic protein in some context of abrogate DNA repair [Omabe, 2022]. Like TCTP, Wnt3a is also known for its pleiotropic cellular functions regulating cell proliferation, cell renewal, cellular differentiation, apoptosis, and motility [He et al., 2015]. Compared to other WNTs, Wnt3a is remarkably important in determining regeneration potential in *in-vitro*, *ex-vivo*, and *in-vivo* conditions [Chang et al., 2020]. Following pharmacological suppression of TCTP, the Wnt3a expression decreases and directly indicates the tight regulation between TCTP and Wnt3a upon regeneration. The connective link between TCTP and β-catenin is reported in *in-vitro* and *in-vivo* cancer models [Gu et al., 2014], and in the present study the link between TCTP and Wnt3a upon regeneration is evident. The pharmacological inhibition of TCTP suppress the YAP1 signals upon regeneration and it represents the crosstalk between TCTP and YAP1. Regeneration occurs through a highly co-ordinated process and in that YAP/TAZ or Hippo pathway regulates the cell-cell interaction that determines the organ size and development [Halder, and Johnson, 2011]. YAP/TAZ complex also have a role in determining the cell fate by controlling the genes related with cell proliferation and apoptosis [Varelas, 2014].

The earthworm *E. eugeniae* is a highly esteemed animal model for studying regeneration. [Sivasubramaniam, 2021; Rajagopalan et al., 2022]. It's a clitellum dependent worm, without this specialized structure, earthworms would be unable to achieve successful outcomes in terms of their survival and ability to regenerate. [Rajagopalan et al., 2022; Paul et al., 2022]. In brief, amputated segments with intact of clitellum only survives and reach the successful regeneration under the *in-vivo* condition. The clitellum, a specialized region in certain organisms, serves as the origin of stem cells. Notably, during the process of juvenile

regeneration, a phenomenon known as clitellum factors have been observed, wherein stem cells migrate from the clitellum to the site of injury. [Christyraj et al., 2019].

Lower organisms such as *Hydra* and *Axolotl* exhibit a greater capacity for regeneration compared to higher organisms like mice and humans. Through the process of evolution, higher animals have experienced a loss of their regenerative capabilities [Zhao et al., 2016] because of the specialized cells (low stem cell numbers and less signalling factors) which are made for sophisticated functions [Bosch, 2008] but in lower animal has more unspecialized cells (high stem cell numbers and more signalling factors) which can create most of the mechanism based on the demand. Still higher animal has some exceptions like human liver [Michalopoulos, 2020] and african spiny mouse [Gaire et al., 2021]. It is intriguing to note that the expression of crucial regenerative proteins in lower organisms was significantly amplified during the process of regeneration. During the process of earthworm regeneration, it has been observed that over ten thousand genes exhibit differential expression. Notably, a total of 3986 genes were found to be significantly upregulated in the anterior regenerated blastema. [Paul et al., 2021]. The activation of crucial regenerative proteins holds the capacity to augment both the wound healing process and the intricate mechanisms implicated in regeneration. In this research article, the data is corroborating our hypothesis. The earthworm clitellum factors (CF) and regenerative clitellum factors (RCF) are prepared for ameliorating the wound healing and regeneration.

According to the results of wound healing, 25% and 5% RCF enormously provoked the wound healing activity. The 99.86% and 99.85% of wound was healed within 24 hours of post wound induction. In CF treated cells, 81.54% wound was healed in 5% CF and 78.81% wound was healed in 25% CF treated cells. Recently, Afshar A et al., have report that h 7d and 14d extracts of hydro-alcoholic extracts of Persian Gulf brittle star, *O. cynthiae*, revealed the significant wound healing potential [Afshar et al., 2023] *in vitro* on HFF cells (more than 99% wound healing observed in 30 hours of post wound induction) and *in vivo* on a rat wound healing model [Afshar et al., 2023]. Compare with hydro-alcoholic extracts of Persian Gulf brittle star, *O. cynthiae,* RCF and CF can heal the wound within 24 hours only. The supplementation of cell culture medium with fetal bovine serum is still usual practice in cell culture applications [van der Valk et al., 2018]. It's also one of the finest animal extract solution which have cell growth promoters, vitamins, minerals, amino acids, fatty acids, lipids, various small molecules, immune response activating factors (neutrophil activation factors, IL-8), metabolites, glucose etc., [van der Valk et al., 2018]. However, 10% FBS can heal the 100% wound on 48 hours only. The inquiry shall arise as to how the clitellum of the earthworm exhibits remarkable regenerative capabilities. The answer for the question we can reclaim it from the hydra [Bosch, 2008]. The interstitial stem cells of *Hydra* demonstrate a cell cycle duration that spans from 18 to 30 hours, while the proliferating stem cells of the epithelial lineages exhibit a doubling time of around 3 to 4 days. [Bosch, 2008]. The primary function of interstitial cells involves the intricate processes of steroidogenesis, spermatogenesis, and immune regulation. The clitellum plays a crucial role in various biological processes, including spermatogenesis, cocoon synthesis, and immune regulation. [Sivasubramaniam, 2021]. In our previous reports also we have observed the high intense stem cells in clitellum region [Christyraj et al., 2019]. However, there is no direct reports regarding the interstitial stem cells

in earthworms. Based on the collective reports, it has been suggested that the clitellum might possess regenerative capabilities, potentially inherited from *Hydra* interstitial stem cell lineages.

As previously elucidated, the initiation of pivotal regenerative proteins holds paramount significance in reinstating the aptitude for regeneration. In our western blot (quantitative analysis of protein expression) and immunofluorescence (protein expression in cellular level) results shows that the key regenerative proteins were successfully trigged by RCF and CF (Figure 4). The tumour suppressor protein p53 is regulated by the higher expression of TCTP [Rho et al., 2011]. In many cases reported that TCTP and p53 are in a reciprocal relationship [Amson et al., 2012; Rho et al., 2011; Choi et al., 2014; Acunzo et al., 2014]. Interestingly in our context, both TCTP and P53 protein expression was ameliorated in the CF and RCF-treated cells. Individually, TCTP and p53 are essential for wound healing and regeneration which has been confirmed in many studies but to reclaim the enormous wound healing and regeneration both protein has to work together. One of the most important key regenerative protein is Wnt3a. It is a stem cell activation marker and stem cell regulator [Shang et al., 2007; Lange et al., 2006; Scheller et al., 2008; Clevers et al., 2014; Chera et al., 2009]. Naturally, during the mouse myoblast cell (C2C12) proliferation, Wnt3a will be activated for proliferation [Zhang et al., 2012]. In our study, Wnt3a expression was moreover similar in CF and RCF treated cells. The known ruler of AICP is caspase-3. It can execute apoptosis as well as stem cell regulating signals like Wnt3a protein. For successful wound healing and regeneration, caspase-3 is mandatory. According to our results, the caspase-3 expression is notably increased in CF and RCF treated cells. Interestingly, in control samples there is no more caspase-3 activation is recorded. So it confirms that CF and RCF-treated cells provoke the AICP context ruler caspase-3 and other key regenerative proteins to attain successful growth in minimal time. In other context, all other key regenerative proteins of VEGF, HoxD-3, p53 notably increased in CF and RCF treated cells (Figure 5). It is shows that, activating the key regenerative proteins are the way to reclaim the wound healing capacity and may be regeneration capacity back.

As a step, we have applied the CF and RCF on non-regenerative earthworm segment 1^{st} to 10^{th}. Interestingly, the survivability of segments was notably increased in CF (upto 168 hours) and RCF treated segments (upto 216 hours) but in control within 60 hours all dead. While we applied the CF and RCF is may provoke them for regeneration because in the RCF treated segments on 120 hours, we have observed the bud kind of formation but unfortunately, it cannot successfully have regenerated but CF and RCF is increasing the survivability of 1^{st} to 10^{th} non regenerative segments (Figure 6).

Conclusion

Eudrilus eugeniae is a clitellum-dependent earthworm that requires intact clitellum segments for its survival and regeneration. The present study aims to interconnect the survival and regeneration ability that varies between *in vivo* and *in vitro* maintenance upon different sites of amputation. The amputated portion of the worm that possesses intact clitellum (13th–18th

segments) survived and had the potential to regenerate, whereas worms with partial or without clitellum segments only survived and were unable to regenerate. Besides segment length and clitellum segments, clitellum factors also determined the survival, blastemal initiation and differentiation potential. The survivability and regeneration potential of worms were augmented upon *in vitro* maintenance. Notably, the amputated segments (1st–10th segments) and posterior segments of similar length, which usually die within the 4th day *in vivo*, survived for more than 60 days *in vitro* but lacked the regeneration ability. On the other hand, the amputated posterior segments (30^{th} to 37^{th} segments) from juvenile worms, maintained in *in vitro* condition, survived and initiated blastema with multiple buds but lacked the ability to regenerate. Interestingly, the equal half of adult worm blastema that is maintained in *in vitro* conditions were able to form the blastema-like structure with the help of a unique stick. The anterior blastema failed to retain the regenerative structure but the posterior portion of the amputated blastema, which is also associated with a small portion of the body segment, showed the ability to retain the regenerative structure. Our results conclude that the survivability is enhanced upon *in vitro* maintenance and this condition favors the adult dedifferentiated blastemal and stem cell enriched juvenile posterior segments to form a regenerative blastema.

The earthworm, *P. excavatus* is used in these present studies to understand the AICP principles in different conditions like those in regeneration and in abnormal multiple bud formation. Initially, the worms are amputated into five equal portions and it is revealed that regeneration in *P. excavatus* is clitellum independent and it gives more preference for anterior regeneration than for posterior regeneration. The posterior segments of the worm possess enormous regeneration ability but it is lacking in anterior segments. Alkaline phosphate, a stem cell marker, shows strong signals throughout all the posterior segments but it is limited in the initial 1^{st} to 15^{th} anterior segments which lack the regeneration ability. While regenerating normally, the worm follows AICP principles that are evident with the increased expression of apoptosis signals throughout the regeneration process along with constant expression of stem cell proliferation response together with cellular proliferation. In *in-vitro* maintained amputated posterior segments, the apoptosis signals show extensive signals on the 1^{st} day but, upon 4^{th} and 6^{th} day H2AX expression is significantly suppressed which eventually alters the Wnt3a and histone H3 pattern that impairs AICP and resultant with multiple bud formation. Our results conclude that AICP pattern is crucial for initiating proper regeneration.

Apoptosis Induced Compensatory Proliferation is a process that maintain tissue homeostasis is followed by many regenerative animals such as Hydra vulgaris, Xenopus, Drosophila, and mice. In the past, cell death was viewed as a mechanism for discarding cells without functional consequences. However, current research has shed light on an additional layer of complexity in which dying cells provide physical or chemical signals with the aid of effector caspases to communicate with their neighboring cells. During regeneration, the effector caspase executes apoptosis and emits stem cell activation signals for compensation of tissue loss. But, some regenerative animal models, like planarians, earthworms, and zebrafish, have lacked in AICP studies. Many studies have recorded apoptosis in the early regeneration period in all three models, but the study of stem cell activation during the regeneration period is missing. AICP study helps to comprehend the molecular links of regeneration and cancer mechanisms in a better way. Here we report the current updates of AICP pathways in

regenerative animal models (lower to higher) and their research gaps. Substantially, effector caspase 3 is the centre of attention for AICP, but here we reveal the hidden rulers TCTP/p53 behind the complete process of AICP because both can regulate effector caspase 3 in many contexts. But, the study on TCTP/p53 on caspase 3 regulation during regeneration is a lack. Therefore, analyzing the role of TCTP/p53 will ameliorate the betterment of the AICP study.

To investigate the multi-functional role of TCTP in regeneration, the earthworm *Perionyx excavatus* was chosen. Through pharmacological suppression of TCTP, amputation, histology, molecular docking, and western blotting, the multi-function role of TCTP involved in regeneration is revealed. Amputation studies show that *P. excavatus* is a clitellum-independent regenerating earthworm resulting in two functional worms upon amputation. Arresting cell cycle at the G1/S boundary using 2mM Thymidine confirms that *P. excavatus* execute both epimorphosis and morphallaxis regeneration mode. The pharmacological suppression of TCTP using buclizine results in regeneration suppression. Following the combinatorial injection of 2mM Thymidine and buclizine, the earthworm regeneration is completely blocked, which suggests a critical functional role of TCTP in morphallaxis. The pharmacological inhibition of TCTP also suppresses the key proteins involved in regeneration: Wnt3a (stem cell marker), PCNA (cell proliferation) and YAP1 (Hippo signalling) but augments the expression of cellular stress protein p53. The collective results indicate that TCTP synchronously is involved in the process of stem cell activation, cell proliferation, morphallaxis, and organ development in the regeneration event.

This study is aim to evaluate the wound-healing potential of earthworm clitellum extracts in *in-vitro* and *in-vivo*. Earthworm *E. eugeniae* cannot survive and regenerate without clitellum segments. Here we have chosen the clitellum extracts from non-regenerative (Clitellum factors - CF) and regenerative worms (Regenerative Clitellum Factors - RCF) for analysing the wound healing potential and regeneration. The MTT, wound healing, immunofluorescence, western blotting assay were conducted to analyse the wound healing potential of CF and RCF and understanding their molecular mechanism behind the process. Based on the *in-vitro* findings, 5, 25 and 50% of CF was ameliorate the cell viability upto 20-28% than control and other side 5, 25, 50, 75% of RCF was ameliorate the cell viability upto 30-38% than control. The wound healing assay showed that the wound area within 24hours, 99.86% wound was healed in 25% RCF treated cells and 81.54% wound was healed in 5% CF but in control 40% wound only healed. The immunofluorescence result showed that the key regenerative protein expressions were notably expressed in p53, TCTP, Wnt3a, Caspase-3 and H2AX. The artichitechture of cellular internal arrangements were not affected by the treatment of CF and RCF. The western blotting results confirms that the key regenerative protein folds are notably increased in CF and RCF treated cells than control cells. Interstingly, the survivability of non-regenerative segments was ameliorated in CF and RCF treated segments than control. In conclusion, the CF and RCF factors are promoted the wound healing property in mouse myoblast C2C12 cells due to their wound healing and regeneration capacity.

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Figure and Figure legend

Figure 1

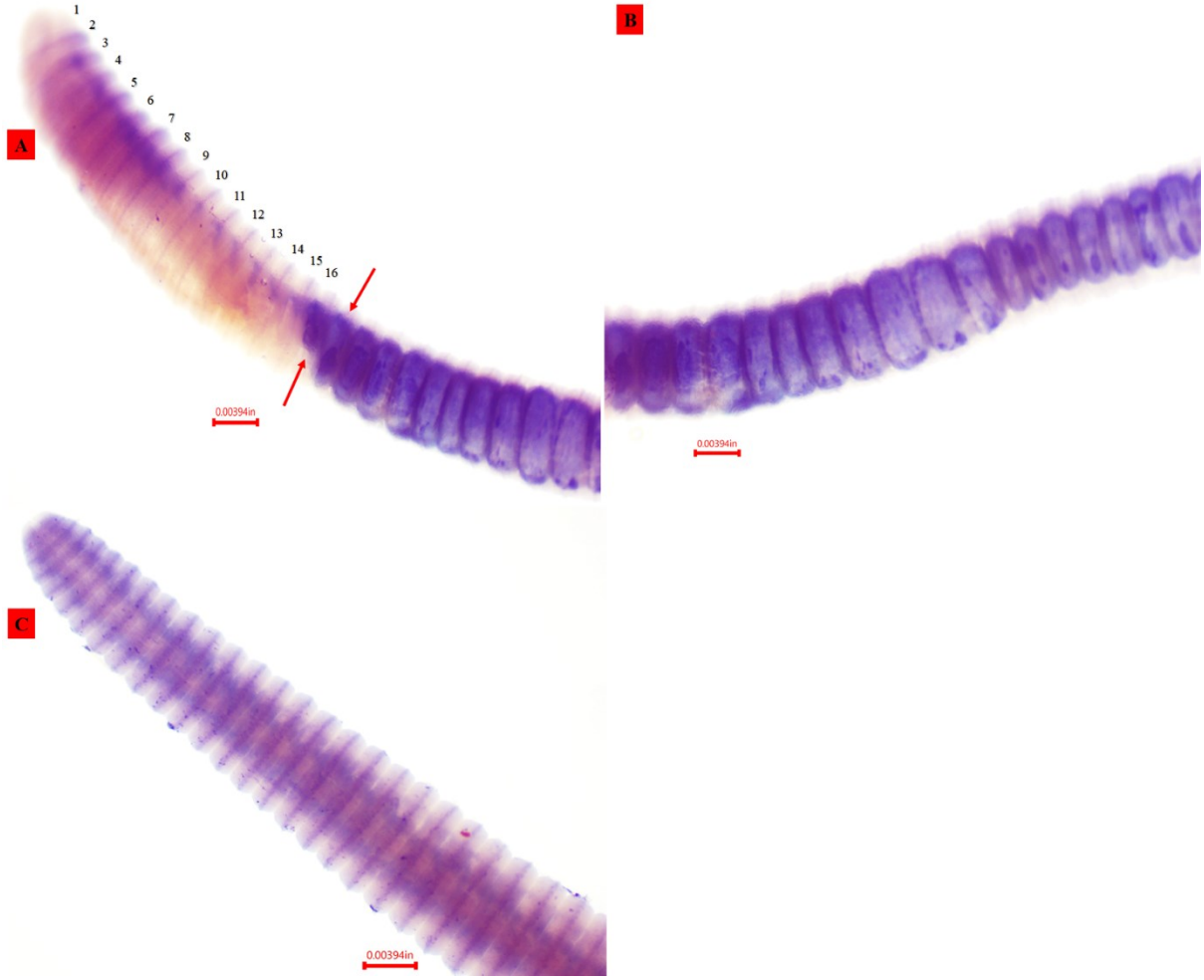

Fig. 1 Distribution of ALP signals throughout the body of *P. excavatus:* A. the first 15 segments lack the intense ALP signals but the presence of ALP signals was observed in ventral nerve cord of each individual segments. On the other hand, high intensity of ALP signals was observed in all posterior segments beyond 15th segments. B & C. the mid region of worm shows intense ALP signals throughout each segment. The ALP signal intensity is equally distributed in each individual segment's circumference i.e in dorsal, ventral and lateral sides of the worm. (Red arrow – high intense ALP signals observed on 16th segment), (Sky Blue arrow – prostomium), (Purple arrow – Mouth), (Yellow arrow – Ventral nerve cord).

Figure. 2

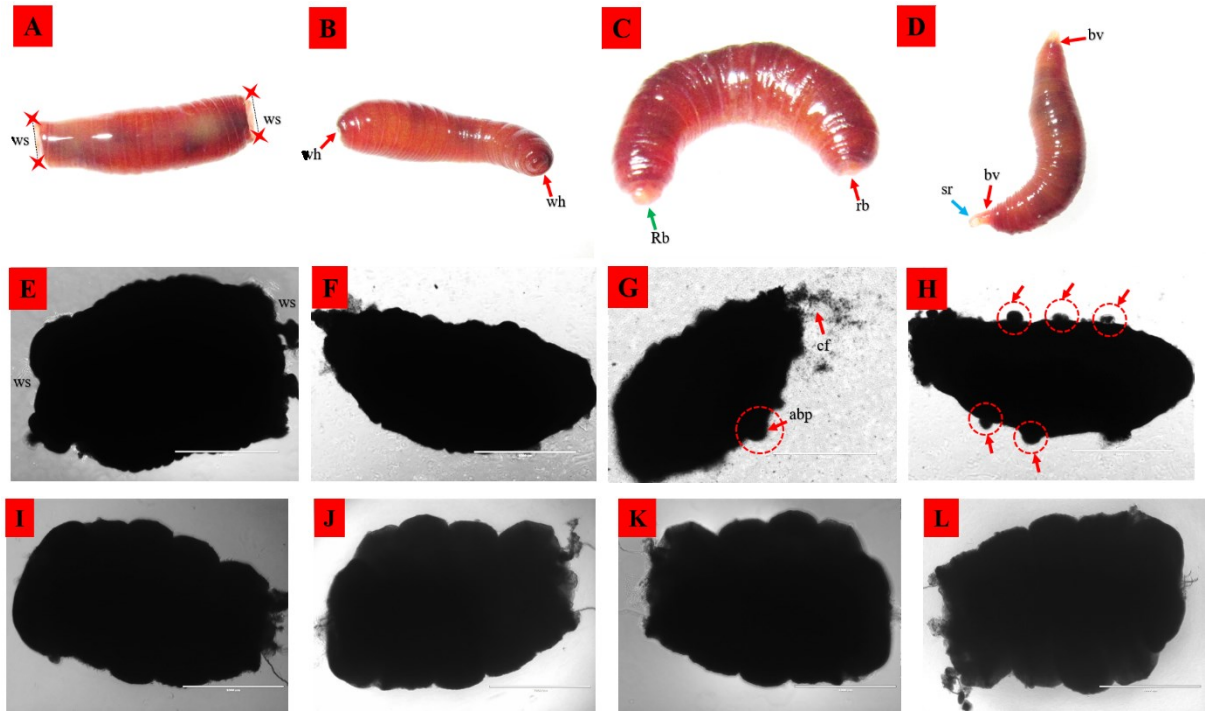

Fig. 2 Survival and regeneration potential of amputated segments that are maintained in In-*vivo* and *In-vitro* conditions. A. worms are amputated in-between 22nd-36th segment (15 segments) and observed on 0th hour with wound site. (B) Wound healing observed after 1st day of post amputation (24 hours). (C) on 4th day, blastema forms in both the amputated site (D) On 6th day of post amputation bud size elongation. (E) amputated 33rd to 37th body segments under *in-vitro* condition (10% L15 medium) at 0th hour observation. (F) Initiation of abnormal projections in the body segments was observed following 24 hours. The wound start to heal which is evident with the formation of curvature at the wound site. (G & H) Wound healing and multi-bud formation was observed after 36 and 48 hours respectively. I-L – amputated 33rd to 37th body segments that are maintained in in-vitro condition in L15 medium without 10% FBS and observed on 0th hour, 24 hrs, 36 hrs and 48 hrs respectively. Even though the worm survives, it is not able to heal the wound and unable to initiate abnormal bud formation. ws - wound site, wh - wound healing, rb - pre stage regeneration of blastema, Rb - regeneration of blastema, bv - blood vessel formation, sr - segment restoration, abp- abnormal patterning.

Figure 3

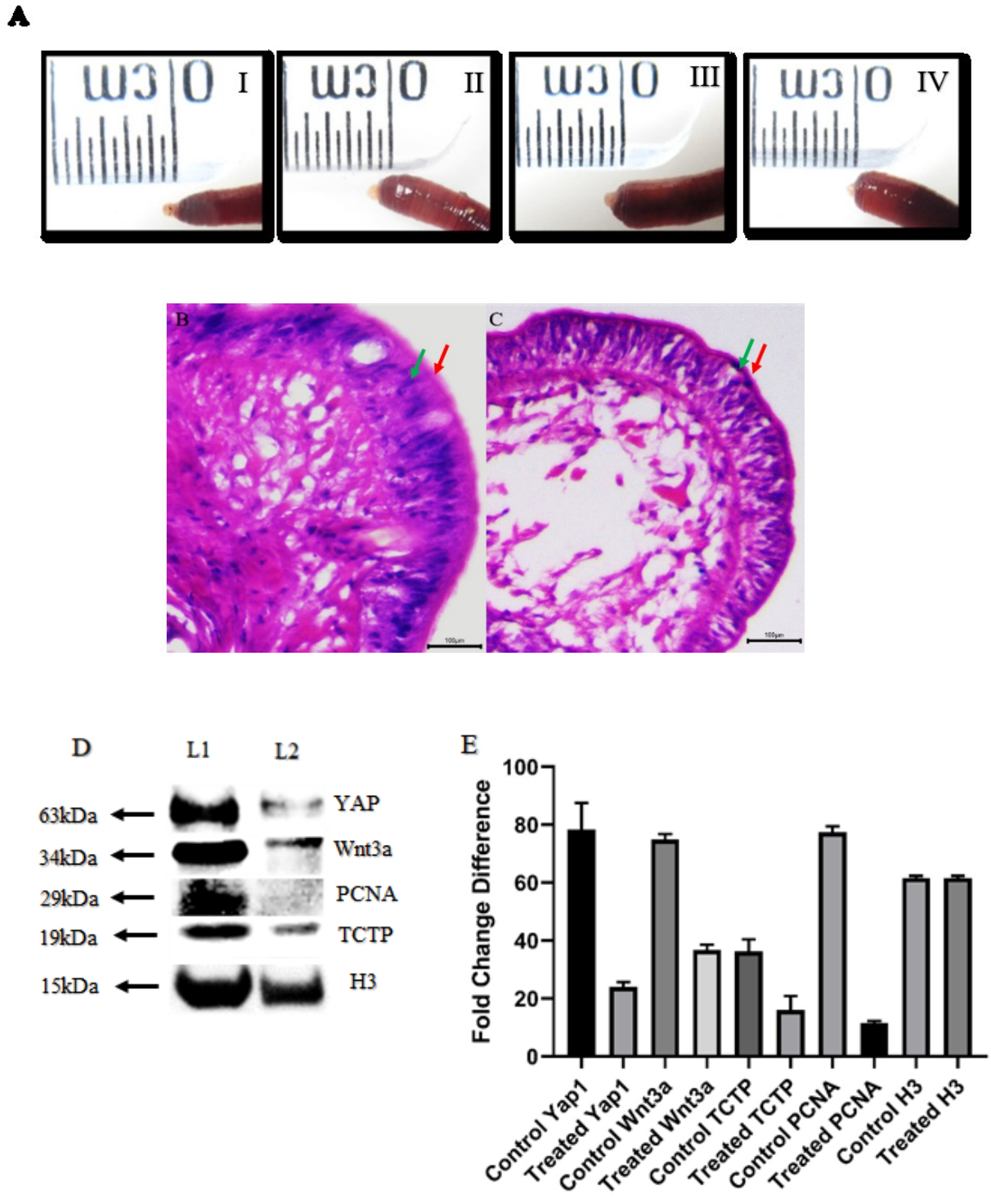

Fig. 3 TCTP and its connecting link with regenerative protein: A (I). Control amputated worm with 5th day bud. II, III and IV represent progressive regeneration suppression in Nutlin-3a injected worms with 5, 7, and 9µg, respectively. B. Histology of control 5th-day regenerative blastema with well-organized tissue structures has the most thickened outer and inner epithelial layer. C. In Nutlin-3a injected worms, the 5th day bud is not well organized, loosely packed

with internal bud tissues and thinner layers of the outer and inner epithelium. D. Western blotting image represents that when compared to the control samples (7^{th} day regeneration – Lane 1), all frame of regenerative key proteins was notably reduced in Nutlin-3a treated samples (7^{th} day regeneration – Lane 2). Nutlin-3a is known for TCTP silencing, and according to the result, TCTP silence influences organ formation (YAP1), stem cell activation (Wnt3a) and cell proliferation (PCNA). E. Quantification of YAP1, Wnt3a, TCTP, PCNA and H3 expression are done based on the band intensity and represented using bar diagram. The experiments were repeated in triplicate to analyze the statistical significance, representing their value as mean±SD. p-value<0.05 was considered statistically significant data. The red arrow represents the "Outermost epithelial layer"; the Green arrow represents the "Inner layer of epithelial tissue."

Fig. 4.

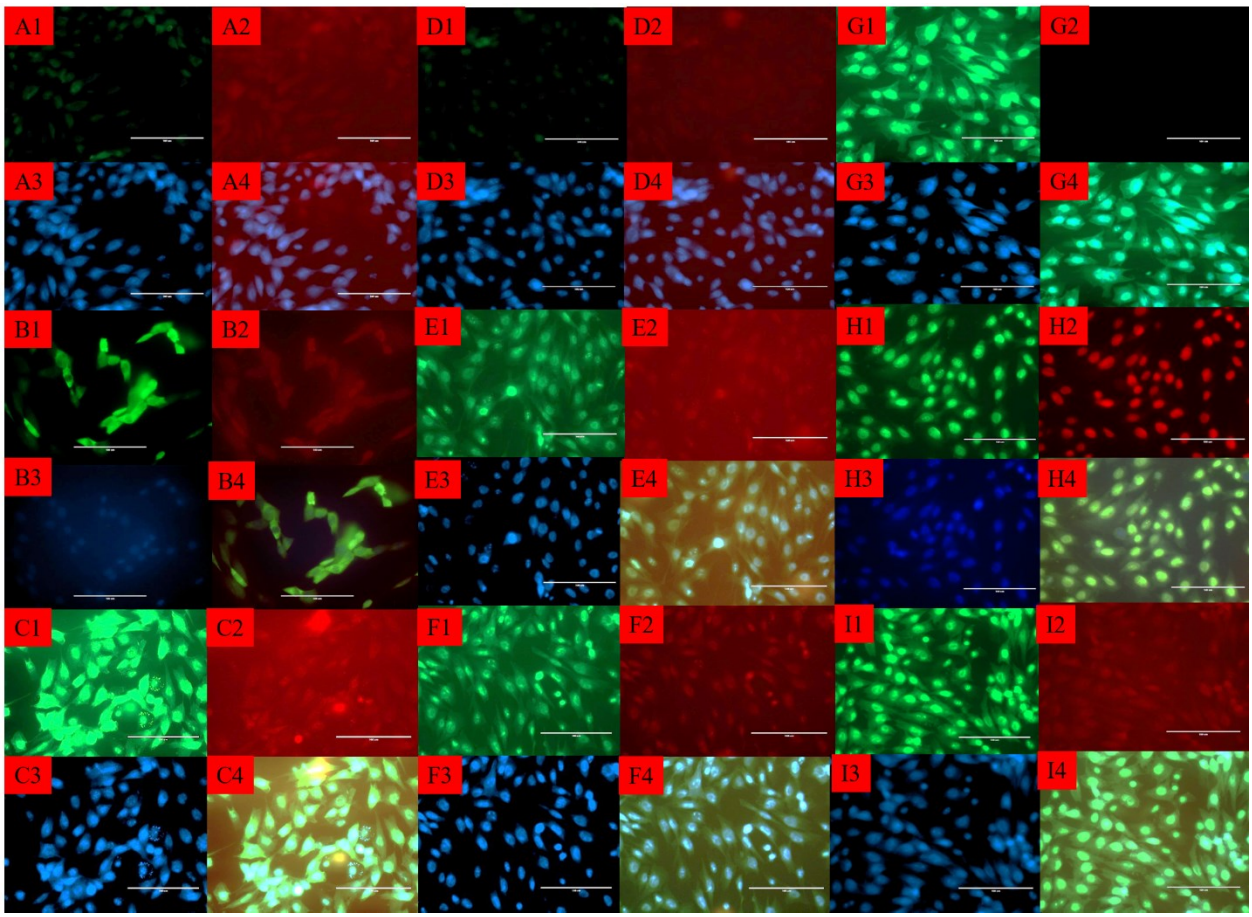

Fig. 4. Immunofluorescence with TCTP, p53, Wnt3a, Caspase 3a, H2AX and β-actin antibodies in mouse myoblast C2C12 cells. A1-A3. Control Cells (DMEM + 10% FBS) subjected to immunofluorescence. Green fluorescence indicates p53 while red indicates TCTP and blue indicates DAPI (counter stain for nucleus). A4. Merge image of control. B1-B3. Cells treated with CF subjected to immunofluorescence. Green fluorescence indicates p53 while red indicates TCTP and blue indicates DAPI. B4. Merge image of CF treated. C1-C3. Cells treated with RCF subjected to immunofluorescence. Green fluorescence indicates p53 while red indicates TCTP and blue indicates DAPI. C4. Merge image of RCF treated. D1-D3. Control Cells (DMEM + 10% FBS) subjected to immunofluorescence. Green fluorescence indicates Caspase-3 while red indicates Wnt3a and blue indicates DAPI. D4. Merge image of control. E1-E3. Cells treated with CF subjected to immunofluorescence. Green fluorescence indicates Caspase-3 while red indicates Wnt3a and blue indicates DAPI. E4. Merge image of CF treated. F1-F3. Cells treated with RCF subjected to immunofluorescence. Green fluorescence indicates Caspase-3 while red indicates Wnt3a and blue indicates DAPI. F4. Merge image of RCF treated. G1-G3. Control Cells (DMEM + 10% FBS) subjected to immunofluorescence. Green fluorescence indicates β-actin while red indicates H2AX and blue indicates DAPI. G4. Merge image of the control. H1-H3. Cells treated with CF subjected to immunofluorescence. Green fluorescence indicates β-actin while red indicates H2AX and blue indicates DAPI. X. H4. Merge image of CF treated. I1-I3. Cells treated with RCF subjected to immunofluorescence.

Green fluorescence indicates β-actin while red indicates H2AX and blue indicates DAPI. X. I4. Merge image of CF treated.

Fig. 5.

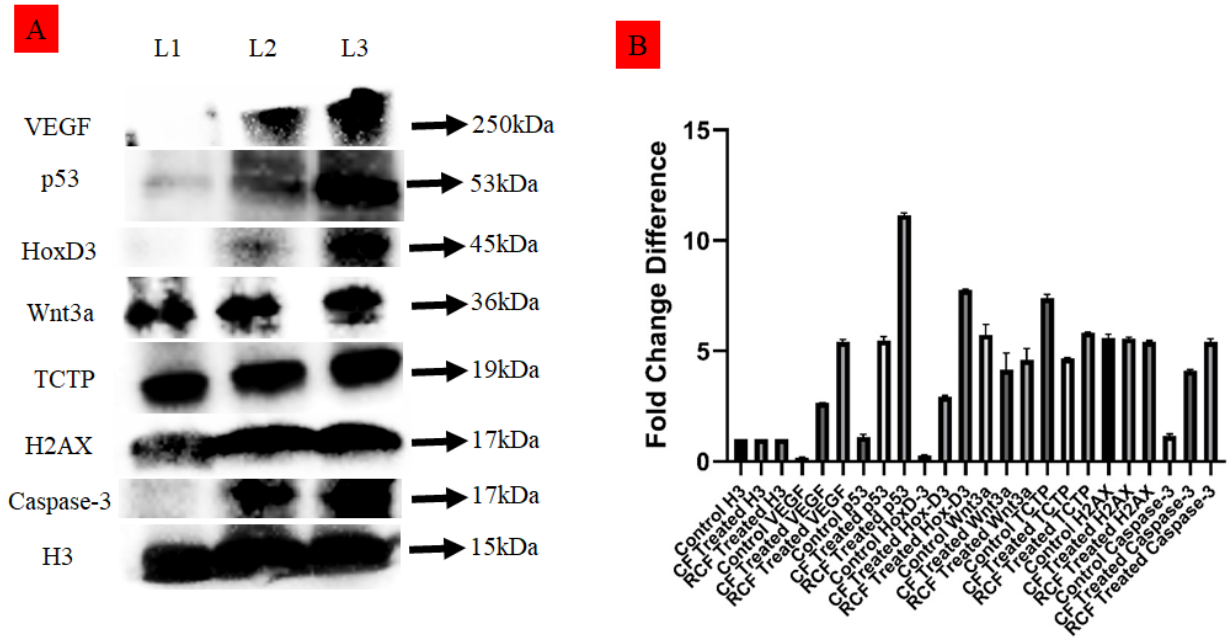

Fig. 5. Western blotting image represents VEGF (250kDa), P53 (53kDa), HoxD3 (45kDa), Wnt3a (36kDa), TCTP (19 kDa), H2AX (17 kDa), Caspase-3 (17kDa), H3 (15kDa) and β-tubulin (50 kDa) expression in Control, CF and RCF treated samples. L1 - Control C2C12 cells protein samples, L2 - CF treated, L3 - RCF treated. B. Graphical representation of Western blotting results shows the relative intensity of TCTP, H2AX, and β-tubulin in control, CF and RCF treated.

Fig 6.

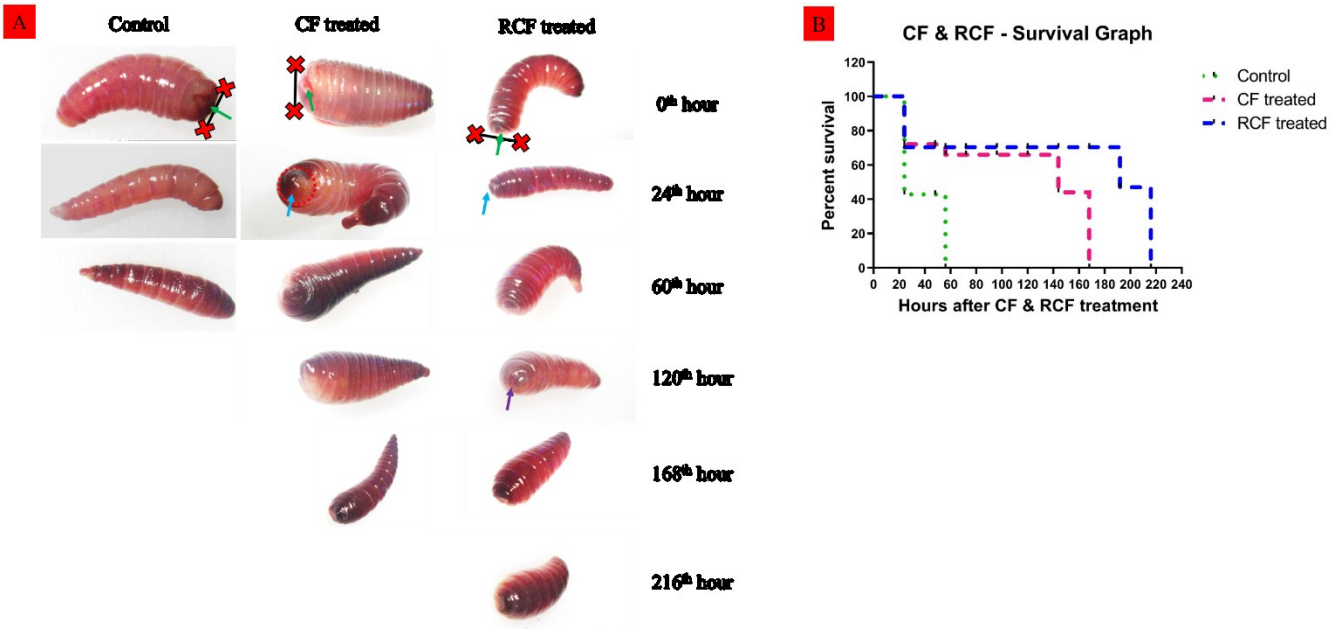

Fig. 6. Enhancing the survivability in 1st-10th segments of *E. eugeniae*. A. Control worms were survived until 60 hours of post-amputation and CF and RCF treated worms were survived until 168th hours and 216th hours of post amputation. The band intensity of the Western blotting image was quantified and depicted with a bar diagram. The p-value of 0.05 indicates statistical significance. Survival graph was plotted for the Control 1st-10th segments and CF and RCF treated 1st-10th segments. After treatment, the live and dead worms on different days are counted and plotted in the survival (Kaplan–Meier) curve. Statistical analysis was accomplished using the log-rank test (Mantel-Cox), and the obtained *p* values <0.05 were considered significant.

Table 1. In vivo and in vitro survival and regeneration ability of *E. eugeniae.*

E. eugeniae body segments ($n = 20$)	Medium of analysis	Clitellum	Survivability/mortality (median survival time)	Blastema formation		Segment restoration	Survival (days)
				Presence/absence	Percentage (%)		
1–10	In vivo	Non-clitellum	(-)#	(-)	0	(-)	3 ± 1
1–15	In vivo	Partial Clitellum	(-)#	✓	5	(-)	5 ± 1
1–30	In vivo	Intact of clitellum	(✓)#	✓	100	✓	90+
11–anus	In vivo	Intact of clitellum	(✓)#	✓	100	✓	90+
16–anus	In vivo	Partial clitellum	(-)#	✓	40	(-)	40
31–anus	In vivo	Non-clitellum	(-)#	(-)*	30	(-)	30
Only clitellum	In vivo	Intact of clitellum	(✓)#	✓	100	✓	90+
Clitellum influence	In vivo	Non-clitellum	(-)#	✓	100	✓*	50
1–10	In vitro	Non-clitellum	(✓)#	(-)	0	(-)	60+
Anterior Regeneration of Blastema	In vitro	Non-clitellum	(✓)#	✓	100	(-)	60+
Posterior Regeneration of Blastema	In vitro	Non-clitellum	(✓)#	✓	100	(-)	60+
Juvenile posterior body segments	In vitro	Non-clitellum	(✓)#	✓	100	✓	60+
Adult posterior body segments	In vitro	Non-clitellum	(✓)#	(-)	0	(-)	60+

✓, yes; (-), no; (-)*, tiny undifferentiated blastema formation; ✓*, only differentiated in prb; (-)#, high mortality rate with the median survival time (1–10 segment, 4; 1–15 segment, 4; 16–anus, 15; 31–anus, 30; clitellum influence, 50); (✓)#, no mortality rate with the median survival time (undefined)