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Heat-inactivated coelomic fluid of the earthworm *Perionyx excavatus* is a possible alternative source for Fetal Bovine Serum in animal cell culture

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Fetal bovine serum (FBS) is used as a major supplement in culturing animal cells under in-vitro conditions. Due to ethical concern, high cost, biosafety and geographical as well as batch wise result variations, it is important to reduce or replace the use of FBS in animal cell culture. The major objective of this work is to evaluate the feasibility of heat-inactivated coelomic fluid (HI-CF) of the earthworm, Perionyx excavatus as a possible alternative for FBS in animal cell culture experiments. The coelomic fluid (CF) was extruded from the earthworm using electric shock method and used for the experiments. Mouse primary fibroblast and HeLa cell lines were used in this study. The results show that by simple non-invasive techniques, electric shock it is possible to collect the CF without any harmful effect to the earthworm. Among HI-CF, autoclaved CF (A-CF) and crude CF (C-CF), the supplement of medium with HI-CF shows positive results. The processed HI-CF (90ºC for 5 min) at 10% supplement in cell culture medium promote maximum cell growth but cells need the initial support of FBS for the attachment to the culture flask. Microscopic observation and immunofluorescence assay with actin and lamin A confirm that the cellular and molecular morphology of the cells is maintained intact. The HI-CF of earthworm, P.excavatus has shown better cellular viability when compared with FBS and making it possible as an alternative supplement to minimize the use of FBS.

Keywords: *earthworm, Coelomic fluid, FBS, animal cell culture, MTT assay, immunofluorescence*

Conflict of interest

Authors declare no conflict of interest in any part of the manuscript.

Introduction

Culturing of animal cells is a prerequisite for various assays in the field of biomedical sciences. It is inevitable that the animal cell culture medium should be supplemented with Fetal Bovine Serum (FBS) for attaining optimum growth of animal cells in *in-vitro* conditions. Serum contains essential elements such as; macromolecules, enzymes, spreading factors, carbohydrates,

non-protein nitrogen's, fatty acids, lipids carrier proteins, low molecular weight nutrients, hormones, growth factors, micronutrients, trace elements and more importantly cell attachment promoting factors[1] which all supports cell viability. The increasing percentage of FBS in the cell culture medium usually helps to overcome stress in the *in-vitro* conditions.

In the current scenario, researchers are looking for alternative supplements for FBS to culture the animal cells, because of the following reasons; 1. Ethical and inhumane issue while harvests serum from bovine fetuses.[2,3] 2. Expensive by the rise in price up to 300% in the past few years due to high demand and lower supply.[4] 3. Scientific concern due to batch-wise result variation that occurs due to concentration variation in serum components.[5] The higher complex nature of serum-based media is difficult to replace with serum-free media, but it is scientifically validated and accepted.[6,7] Currently, different media supplements include human platelet lysate,[8,9] milk derived growth factors,[10] pooled human platelet lysate [11] have been used as an alternative supplement for the serum to obtain the better growth of cells.

Still the research community is in the need for better alternative substitute for FBS to culture variety of cell lines. Coelomic Fluid (CF) is the major circulatory system in earthworm and it occurs within the coelom. The major physiological function of CF is involved in respiration, body movement, innate immunity and circulating nutrient to different cells and organs.[12] In *P.excavatus,* the CF is greenish yellow in color with autofluorescence properties [13] and it is due to the presence of the high riboflavin content. The earthworms are able to eject the CF through their dorsal pores at the time of stressful condition such as mechanical, electrical, chemical, irradiation, heat or cool conditions.

The CF of earthworm has 70 to 80 % content of proteins and amino acids. Previous studies reported that CF proteins have notable antibacterial, anticancer, cytotoxic, proteolytic, hemolytic and hemagglutinating properties.[12] In the present experiment, an attempt was made to utilize the coelomic fluid of earthworm, *P.excavatus* as an alternative source of serum for culturing animal cells in *in-vitro* conditions.

Materials and methods

Culturing of earthworm, P.excavatus

The earthworm, *P.excavatus* was cultured and maintained in our laboratory at the Regeneration and Stem Cell Biology Unit, Centre for Nano-science and Nano-technology of Sathyabama Institute of Science and Technology, Chennai, Tamilnadu, India. For the experimental purpose, matured earthworm of average weight range from 0.8 to 1 gram was taken. The earthworms were maintained in a plastic tub containing the mixture of soil, cow dung and leaf litters at an ambient temperature.[14] Moisture and temperature of the medium were maintained at 60-70% and 18-25°C, respectively. The vermicompost was periodically removed from the plastic tub and compensated with the addition of soil mixture (soil, cow dung, and leaf litters).

Collection and processing of Coelomic fluid

The CF of the earthworm, *P.excavatus* was collected based on the following method as described earlier.[15] Briefly, Earthworms (20 Nos) were washed twice with distilled water, dried on a tissue paper, and then placed in a glass beaker. The earthworms are excited with 5V stimulation for 30 seconds, which induced them to extrude coelomic fluid through the epidermal

dorsal pores. The collected CF was mixed with equal volume of 1X Phosphate Buffered Saline (PBS) (pH 7.4), vortexed and filtered with 0.22 μm syringe filter (Millex-GP 33mm PES sterile filter). The aliquots of processed CF were subjected to heat inactivation (named as HI-CF) by incubating the tubes at different temperature viz. 70°C, 80°C, 90°C or 100°C for 5 min, respectively. In addition, another batch of CF was autoclaved (named as A-CF) and subjected to the experimental analysis. Aliquots of crude CF (named as C-CF) without any heat treatment was also used for the experiments. The processed and formulated CF was used as a supplement for the animal cell culture experiments.

Reagents used for the study

Dulbecco's Modified Eagle's Medium (DMEM), 3-(4, 5- Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) and antibiotics (Penicillin, Streptomycin and Amphotericin B) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) (South American Origin) was purchased from Cell Clone.

Cell Culture

HeLa and mouse primary fibroblast cells were purchased from National Center for Cell Science, Pune, India. The HeLa cell lines and mouse primary fibroblast were maintained in T-flasks containing Dulbecco`s Modified Eagle Media supplement with 10% FBS and 1% antibiotics (such as penicillin (100U/mL), streptomycin (100μg/mL) and amphotericin B (100μg/mL)). In the present study, for substitute of FBS, 10% HI-CF (90ºC for 5 min) was added to the DMEM medium. The cells were maintained in a CO_2 incubator at 37°C provided with 5% CO_2. Cells were sub cultured after the cells reach 90% confluence.

Microscopical Observation

HeLa and mouse primary fibroblast cells were cultured in four different media separately; M1 - DMEM with 10% FBS, M2 - DMEM with 10% HI-CF, M3 - DMEM with 20% HI-CF, and M4 - DMEM with 30% HI-CF. The cells were observed every day under the EVOS microscope (EVOS-FL). The morphological characteristics such as shape, size, the appearance of the cells in the medium and cells invading capability in the plate were analyzed from day 1 to 4.

Cell Viability Assay

To determine the cell viability, HeLa and mouse primary fibroblast cells (1×10^5 cells/mL) were seeded in the 96 well plates separately and incubated at 37 °C in CO_2 incubator. After 24 hrs of incubation, the plates were washed thrice with 1X PBS and supplemented with following percentage (viz., 10%, 20%, 30%, 40%, and 50%) of CF (C-CF, HI-CF or A-CF) in order to replace FBS. The plates were incubated for 48 hrs at 37°C in CO_2 incubator and further subjected to MTT assay. Briefly, after the incubation, the media was removed and DMSO was added to each well and subjected to OD value at 570 nm using a microplate reader. The percentage of cell viability was calculated using the formulae viz., (Treatment average OD / Control Average OD)*100). Similar experiments were performed with the following processed CF and FBS supplements.

Immunofluorescence assay

HeLa and mouse primary fibroblast cells were cultured in the 6 well plates with the supplement of 10% HI-CF along with control (supplemented with 10% FBS) and after attaining confluence the plates were washed twice with 1X PBS. The cells were fixed with 4%

Paraformaldehyde in 1X PBS for 10 min at room temperature, followed by permeabilization with 0.5% Triton-X-100 for 10 min on ice. The cells were blocked with 1% Bovine serum albumin (BSA) for 30 min and further incubation with primary antibody (anti-actin antibody and anti-lamin A antibody) for 2h at room temperature. After throughout washing with 1X TBST the cells were incubated with suitable secondary antibodies for 1h at room temperature. After washing with 1X TBST cells were counterstained with DAPI, mounted and observed under EVOS fluorescence microscope.

Statistical analysis

Statistical analysis, such as mean, standard deviation and standard error were performed using statistical software - GraphPad Prism, Version 5.01.

Results and Discussion

The earthworm, *P.excavatus* is reddish in color with distinguished body segments (80 to 100 segments) and it had a thick cylindrical collar like segments called the clitellum which is present in between 13^{th} to 17^{th} segments (figure 1). The major nutrients that are absorbed through the intestine of earthworm are transported to each cell through their coelomic fluid. Especially, all the internal organs like seminal vesicle, testis, ovary and prostate glands in earthworm are floated in the fluid system and obtained their nutrient for their optimal growth. Other than that the CF of earthworm is involved in respiratory activities, body movement, maintaining the moisture environment and play a key role in innate immunity.[16] CF has a different type of cell types like mucocytes, circular cells, chlorogogen cells, and coelomocytes. Similar to serum, CF contains watery matrix and plasma in the fluid. In this study, we show that the HI-CF of *P.excavatus*

supports the cellular growth and viability of animal cells under *in-vitro* conditions and thereby replacing or reduce the use of FBS. The major reason for using FBS is that it has lower levels of antibodies and hence utilized for the growth of cells cultured in *in-vitro* conditions. Since the earthworm is an invertebrate it mostly relies on the innate immune system and therefore the presence of antibodies is not a concern while using HI-CF as an alternative source for FBS in animal cell culture experiments.

The collection of CF from earthworms is very simple and it was achieved by either giving minimal electric shock and or by incubating the earthworms in an ice-cold buffer.[12] In the present study, we followed the electric shock method (5V for 30 seconds) which is enough to collect the CF from the earthworms. The extraction procedure is a non-invasive method and the earthworms are subjected to very minimal shock. Following the shock, the earthworms are returning to a normal state within 1 min without any harmful effect. Another advantage is that the nutrient content of CF is replenished within 10 days and it can be ready for next batch uses. The collected CF is greenish yellow in color, and the relevant reports suggest that it is due to the enriched level of vitamins specifically Vitamin B2 (Riboflavin). Reports clearly indicate that riboflavin is present in the earthworm, *Eisenia fetida* coelomocytes, which is the main source of autofluorescence.[17] Interestingly, the CF of the earthworm, *P.excavatus* has a broad spectrum anti-bacterial activity against both gram positive and gram negative organisms and this property helps to eliminate the use of antibiotics in the animal cell culture medium.[18] In addition, the earthworm CF has the following major macronutrients such as Na, K, Cl and Ca[19] and this ionic balance is important to inherit the property of FBS. Due to presence of micronutrients and various growth factors, CF was used as a supplement for plant tissue culture media.[20] Still, the

total chemical complexity of CF and its constituents is undefined so far. The pH of the CF was determined and found to be 7.2. The CF is more viscous in nature and hence to avoid the viscosity of CF, equal volume of 1X PBS was added, vortexed and stored at 4ºC. After adding 1X PBS, pH varies slightly and retains the neutral pH 7.4, which is very crucial for culturing the animal cells.

In order to use the CF as an alternative supplement for FBS, the CF must possess less or no cellular cytotoxicity. Hence, MTT assay was performed with all the three sets of CF (C-CF, H-CF, and A-CF) by supplementing the culture medium with increasing percentage of CF (10 to 50%). HeLa and mouse primary fibroblast cells were used to determine the cytotoxicity of the CF supplemented medium. Based on the MTT assay, we have identified that the HI-CF at 10% was ameliorating the percentage of cell viability when compared with A-CF and C-CF (figure 2). The supplement with C-CF has more cell cytotoxicity when compared with HI-CF and A-CF. The results of the MTT assay clearly show that the HI-CF of the earthworm, *P.excavatus* has less cell cytotoxicity and in addition, it supports maximum cell growth when compared to FBS. The result implies that the temperature of CF plays a major role in determining the cellular viability.

To understand the optimum temperature of CF, which supports maximum cell viability, the following experiments were performed. Briefly, CF aliquots were subjected to heating at 70°C, 80°C, 90°C or 100°C, separately for a time period of 5 min along with control (FBS). The individual samples are subjected to cell viability assay and the results are documented as shown in figure 3. The data clearly show that increasing the temperature reduces the cell cytotoxicity and vice versa. The data show that 90ºC is the optimum incubation temperature, for less cellular

cytotoxicity while comparing with 70°C, 80°C and 100°C. In addition, the result shows that the contents of HI-CF support more cellular viability when compared with FBS.

To understand the morphological characteristics of cells, culture medium supplement with different concentrations of HI-CF namely 10% (M2), 20% (M3) and 30% (M4) are subjected to microscopical observation. The result shows that the morphology of fibroblast cells supplemented with FBS (M1) and HI-CF (M2, M3, and M4), shows no morphological variations (figure 4). In addition, it was clearly observed that the HI-CF supplemented fibroblast cells shows a higher growth rate when compared with control (figure 4).

Upon clear observation under a microscope, the cell-cell interactions of HI-CF supplement cells were found to be intact when compared with the control. Also, the integrity between the cells was maintained as that of FBS supplemented cells. One drawback with HI-CF is that it lacks the ability to promote the attachment of cells in the cell culture flask. But FBS is having all essential nutrients including the fibronectin, which is a glycoprotein highly involving in the cell attachment process.[6] The comparative analysis clearly confirms that the CF of the earthworm *P.excavatus* does not have fibronectin rather reports clearly suggest that the earthworms have the ability to secrete fibrinolytic enzymes[21] which may be responsible for inhibiting the cell attachment. The fibrinolytic enzymes are inactivated in HI-CF and therefore it requires an initial supplement of FBS for cell attachment in cell culture flask. After attachment of cells, the old medium is replaced with fresh medium supplemented with HI-CF. The schematic representation of cell-cell interaction and cell-matrix interaction upon supplement of FBS and HI-CF on culturing adherent cells was shown in figure 5. We have also noted that for culturing of

suspension cells, initial supplement of FBS was not needed; rather an HI-CF supplement itself is enough for the growth of suspension cells in the cell culture flask.

Microscopic and molecular characterization of cells grown in HI-CF supplemented medium shows a uniform cellular morphological pattern with intact cell-cell and cell-matrix interaction. The experiments were repeated up to subsequent 10 passages and it shows no variation. The results confirm the HI-CF shows no harmful effect and it shows indistinguishable results when compared with FBS supplemented cells. Although HI-CF lacks the attachment factors the medium provide spreading factors that determine the cells to spread before they start to divide.

The basic cellular architecture and framework of cellular morphology were not disturbed in HI-CF supplemented cells (figure 4). Next, to that, we investigate the molecular morphology of cells in order to find out the intracellular integrity of cultured cells that are supplemented with HI-CF. Initially, the adherent cells were fixed and stained with anti-actin and anti-lamin A antibodies. The data show that the intact actin filaments along with nuclear lamin A were clearly visible as green and red fluorescence, respectively according to the secondary antibody conjugates under the EVOS fluorescence microscope in control as well as in HI-CF supplemented cells (figure 6). In addition, the DAPI was used as a nuclear stain and it is visualized as a blue fluorescence. The collective data clearly shows that there is no difference in the fluorescent intensity and in the appearance of actin filaments and nuclear lamin A in control and cells supplemented with HI-CF. The data confirm that the microenvironment inside the cells has not disturbed upon supplement with HI-CF.

In conclusion, the present research work reveals that the earthworm, *P.excavatus* coelomic fluid (heat inactivated) has been used as an alternative source for FBS and it cannot alter the

cellular growth and viability. In addition, further studies with different cell lines are essential to understanding the issues related to the attachment of cells while using DMEM/CF medium.

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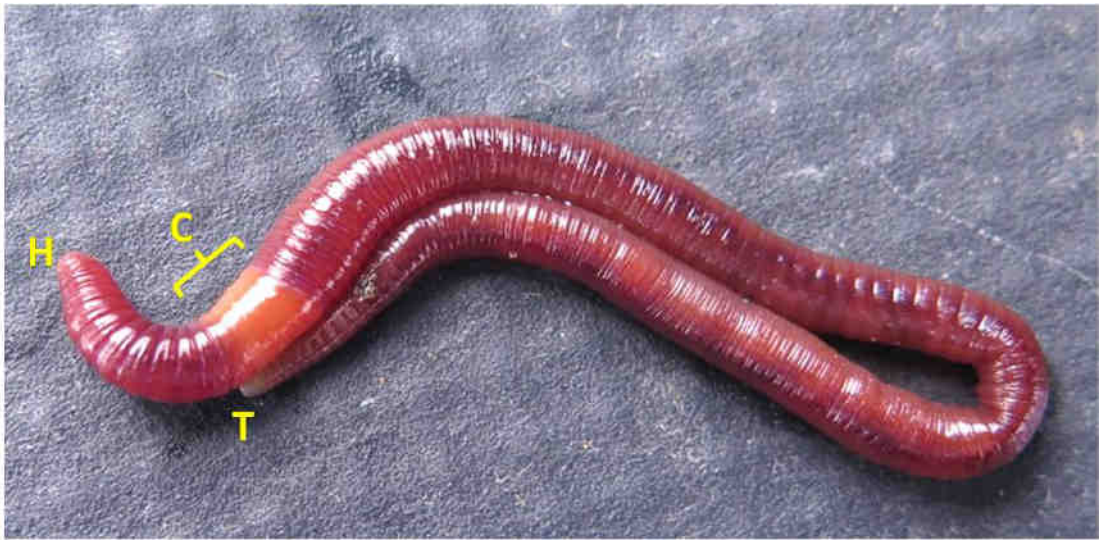

Figure 1. The earthworm, *P.excavatus* is a segmented worm with Head (H), Clitellum (C) and Tail (T) segments.

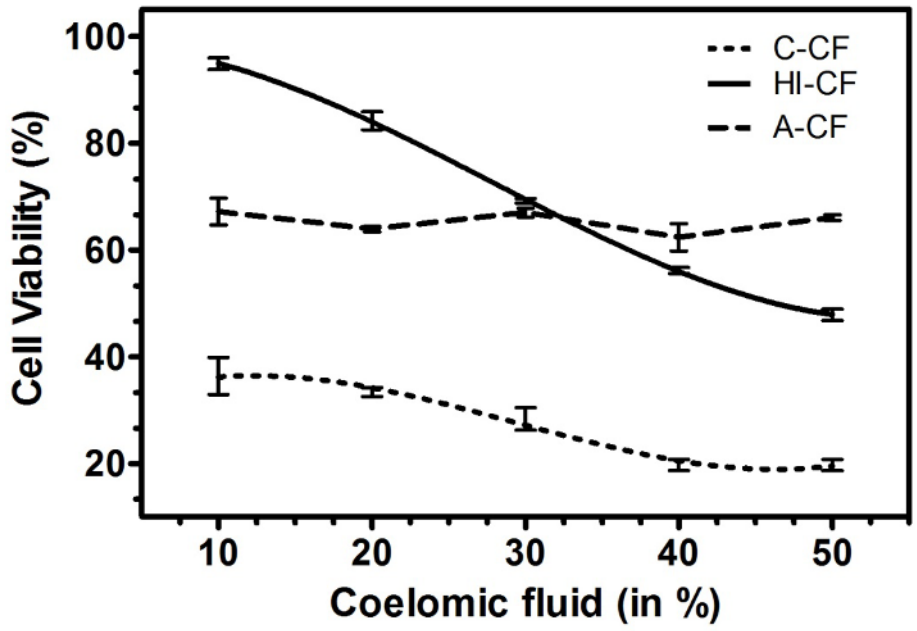

Figure 2. MTT assay. Cell viability assay was performed in HeLa cells upon a supplement of 10%, 20%, 30%, 40% and 50% of C-CF, HI-CF, and A- CF, respectively in DMEM medium.

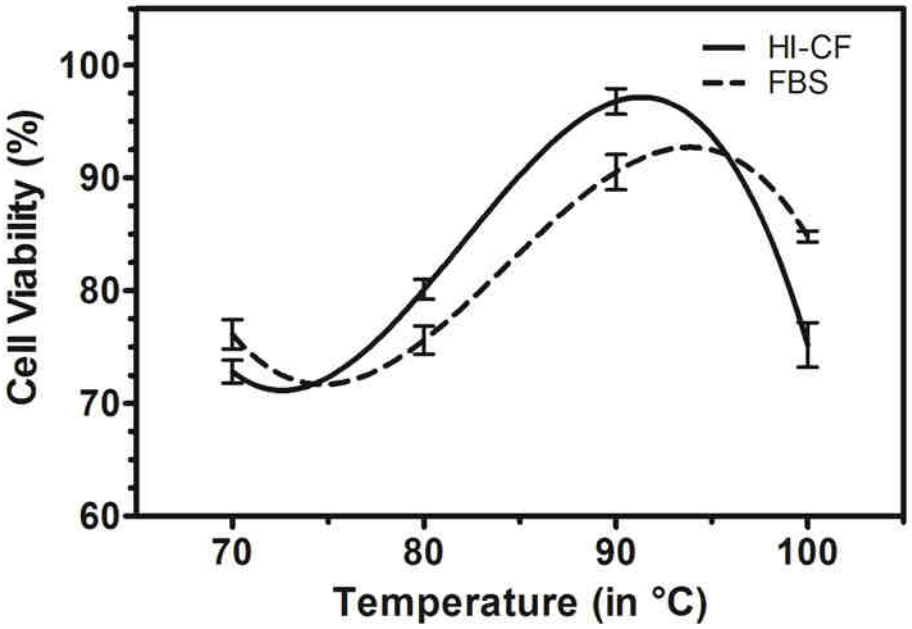

Figure 3. MTT assay. Cell viability assay was performed in HeLa cells upon supplement of HI-CF and FBS at the incubation temperature of 70°C, 80°C, 90°C and 100°C, respectively in DMEM medium.

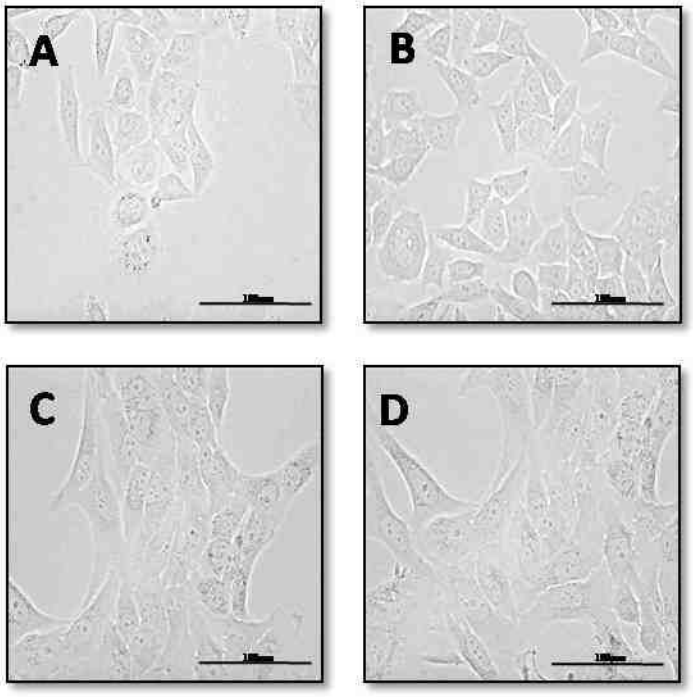

Figure 4. Microscopical observation of cells. Morphology of fibroblast cells supplemented with (A) M1 -

DMEM with 10% FBS, (B) M2 - DMEM with 10% HI-CF, (C) M3 - DMEM with 20% HI-CF, and (D) M4 – DMEM with 30% HI-CF with scale bars (100µm).

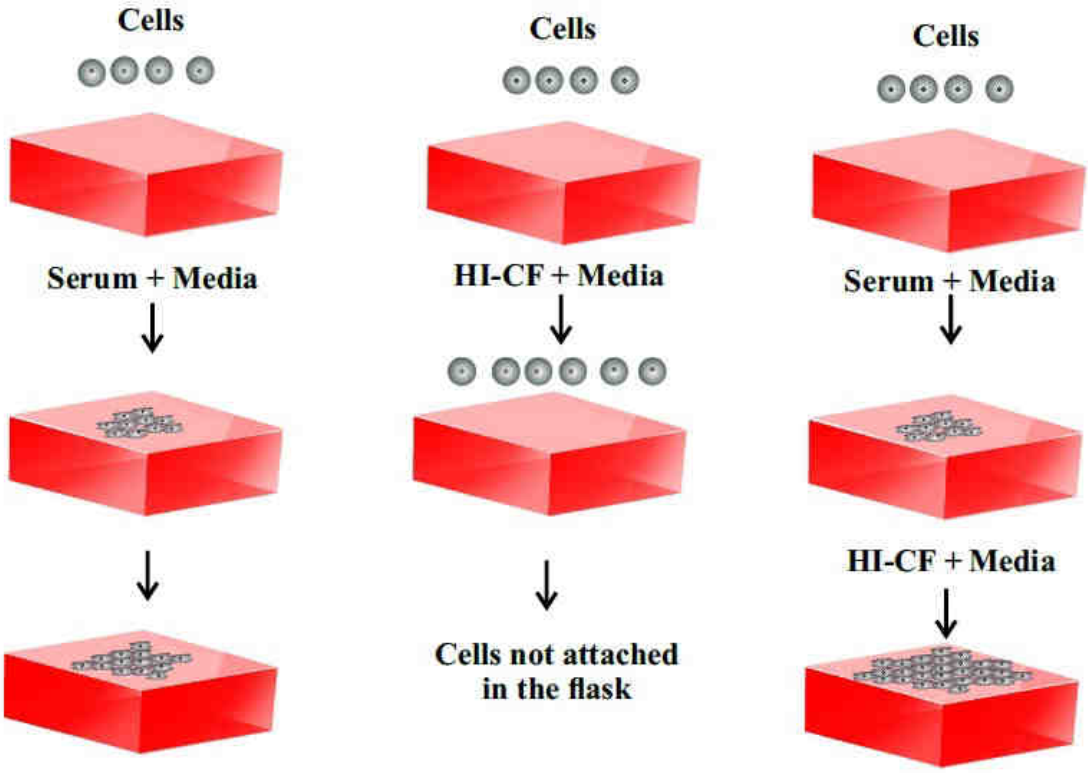

Figure 5. Schematic representation of cell-cell and cell-matrix interaction of FBS and HI-CF supplemented DMEM medium.

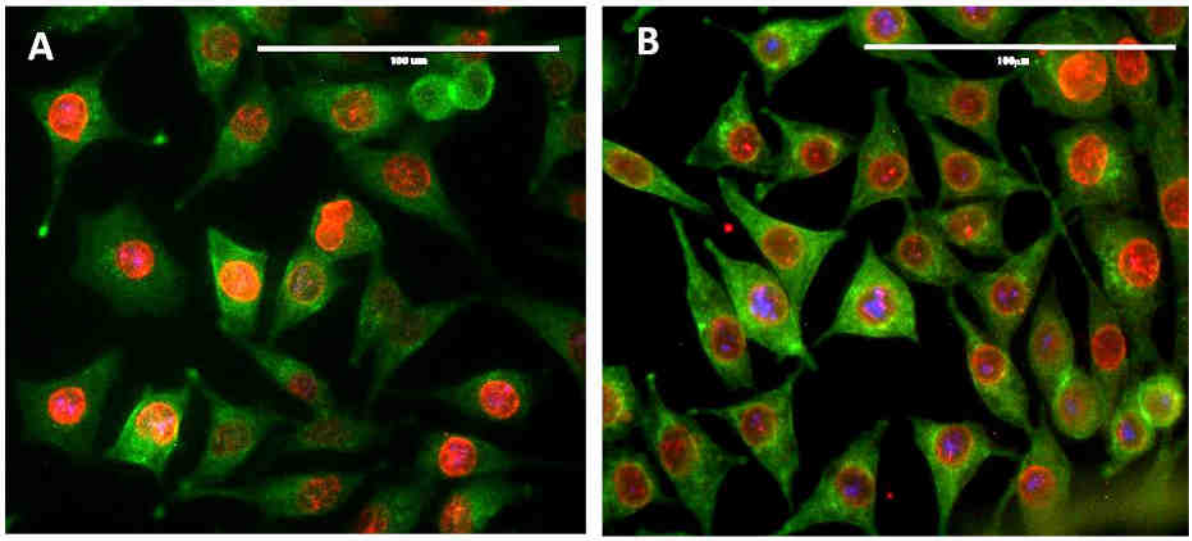

Figure 6. Immunofluorescence with actin and lamin A antibodies in mouse primary fibroblast cells. A. Cells supplemented with 10% FBS (control) were subjected to immunofluorescence B. Cells supplemented with HI-CF were subjected to immunofluorescence. Green fluorescence indicates actin while red indicates Lamin A and Blue indicates DAPI (counter stain for nucleus). Scale bar - 100µm.

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

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Abstract

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 (30th to 37th 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 favours the adult dedifferentiated blastemal and stem cell–enriched juvenile posterior segments to form a regenerative blastema.

Keywords Survivability · Regeneration ability · Clitellum · In vitro maintenance · *Eudrilus eugeniae*

Introduction

The matter of survival and death following tissue damage largely depends on the organism's individual ability to maintain homeostasis and regeneration ability (Reichman 1984; Goss 2013). In general, the survival and regeneration abilities are observed more in invertebrates than in vertebrate species (Luisetto *et al*. 2020). The regeneration and survivability depend on the presence of stem cells and an inflammatory microenvironment, the activation of regeneration-specific genes and proper epigenetic regulation (Zhao *et al*. 2016). Some of the invertebrates can regenerate the whole body like *Caenorhabditis elegans* (Srivastava *et al*. 2014), hydra (Vogg *et al*. 2021), jellyfish (Fujita *et al*. 2021) and corals (Luz *et al*. 2021). But axolotl and newt are the only known vertebrates that have the ability to regenerate the whole body parts (Farah *et al*. 2016).

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In earthworms, two phenomena of regeneration mechanisms were observed. The first is where the earthworms depend on the presence of intact clitellum for their successful regeneration as seen in *Eudrilus eugeniae* (Johnson Retnaraj Samuel *et al*. 2012). In the second case, the earthworms do not depend on clitellum for their regeneration like in *Eisenia fetida* (Gates 1949; Xiao *et al*. 2011). In adult *E. eugeniae*, amputation on any part of the clitellum will directly affect its survivability (Sivasubramaniam 2021). Studies conducted with juvenile earthworms of *E. eugeniae* showed that clitellum determines the fate of the regeneration mechanism and the survivability (Christyraj *et al*. 2019). However, all regeneration studies with *E. eugeniae* were carried out under in vivo conditions (Gopi Daisy *et al*. 2016; Subramanian *et al*. 2017; Paul *et al*. 2021) which represent different survival and regeneration abilities depending on the presence or absence of intact clitellum.

The cells, tissues or organs that are excised from the individual animals and maintained in in vitro conditions usually have high longevity but it also depends on the nature of tissue or organ dissected from the organism. For example, ant ovaries can be maintained in in vitro conditions for 8 months (Kral *et al*. 1986) whilst cockroach legs can only be maintained for 40 days (d) for in vitro regeneration studies (Marks and Reinecke 1964). This is similar to how the human colon can be maintained for 8 h (Mapes *et al*. 2014) whereas Guinea pig retina can be maintained for only 2.5 h in in vitro conditions (Wood 2011). In this context, it is important to know how invertebrates like earthworms which possess enormous regeneration ability respond in in vitro conditions. The stem cell niche of *E. eugeniae* resides in clitellum segments and the survival and regeneration ability of the organism in in vivo and in vitro conditions, in the absence of clitellum, needs to be investigated. To our knowledge, studies involving this aspect of regeneration have not been performed and would shed light on the functional properties of different tissues that are involved in cell fate determination. To explore this topic, the earthworm *E. eugeniae* was amputated at different sites to generate amputated segments with intact clitellum, partial clitellum, without clitellum and solitary clitellum. The amputated segments with varying lengths were observed for their survival and regeneration potential in in vivo and in in vitro conditions. A literature search in the PubMed database with the combined keyword of 'Earthworms + Survival' for the past three decades shows 846 articles, but when the terms 'Earthworms + Survival + Regeneration' were searched, only 13 articles were found, indicating that further studies are needed in this field.

Materials and methods

Culture and maintenance of earthworm *E. eugeniae* worms were collected from a worm shop nearby Konganchery, Chengalpattu, Tamil Nadu, 603,101, India. The collected worms were maintained on a worm bed which contains organic soil (70% humidity), leaf litter and cowdung (60:30:10) in a plastic tub of size $60 \times 40 \times 15$ cm^2. The temperature was maintained at 24–26°C and water was sprinkled once every 4 d over the worm bed.

Earthworm amputation and maintenance 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 10th, 15th and 30th 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 20th 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 follows: 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, tissues 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 Following 60 d of post-maintenance in in vitro conditions, the adult blastema tissue ($n=6$) was carefully 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 increasing 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 paraffin-embedded blocks were subjected to dissection (5 µm) and the collected sections were processed and stained by haematoxylin (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).

Statistical analysis The earthworms following amputation were carefully observed for survival conditions and correspondingly plotted in the survival graph (Kaplan–Meier) using GraphPad Prism software version 6 (GraphPad Software Inc., La Jolla, CA). Survival data were subjected to the Mantel-Cox test to determine the significant differences between the in vivo and in vitro survivability of earthworm segments in the Kaplan–Meier analysis (Christyraj *et al*. 2019). The results were considered statistically significant when the p value was < 0.05.

Results

In vivo survival depends on the presence of intact clitellum and segment length Following amputation at the 10th, 15th and 30th segments, the earthworms were observed for their survival and regeneration ability (Fig. 1*A–P*). The amputated anterior body segments that lacked intact clitellum (1st to 10th segments) (Fig. 1*A*) were able to heal their wounds by the 2nd d of post amputation (Fig. 1*B*). But these earthworms were unable to initiate the regenerative blastema and died within 4 d of interval. Similarly, the anterior portions of the amputated worm with partial clitellum (1st to 15th segments) (Fig. 1*C*) were able to heal their wounds by the 2nd d of post amputation. But only 1 out of 20 worms had the ability to form the regenerative blastema by the 8th d (Fig. 1*D*) and other amputated worms died within 5 ± 1 d. Importantly, 20% of the anterior portion of amputated worms with partial clitellum died within 3 d. The anterior portion of amputated worms with intact clitellum having 1st to 30th segments (Fig. 1*E*) was able to survive. They formed the regenerative bud on the 4th d and the formation of individual segments was noted by the 8th d of post amputation (Fig. 1*F*). Similarly, the amputated posterior portion of worms with intact clitellum was able to successfully regenerate from the differentiated segments by the 5th d post amputation (Fig. 1*H*). The worms having 16th to anus segments (Fig. 1*I*) with partial clitellum were able to survive for up to 30 to 40 d (Fig. 1*J*). About 3 ± 1 of 10 worms were able to form blastema in 6 ± 1 d but failed to differentiate and regain the lost parts (Fig. 1*J*). Interestingly, we observed that 20–30% of worms died within the 4th d post amputation. Similarly, 2 ± 1 out of 10 amputated worms without clitellum segments (31st to anus segments) (Fig. 1*K*) were able to form regenerative blastema (Fig. 1*L*) by the 7 ± 1 d. They were able to survive for up to 20 to 30 d. Notably, 10% of the worms died in 4 d post amputation. For in vitro maintenance of blastema, worms were initially amputated at the 10th segment (Fig. 1*M*). The worm formed the blastema on the 4th d (Fig. 1*N*). On the same day, the blastema with an adjacent segment was cut by a sterile surgical blade. The intact blastema was used for in vitro culturing.

Clitellum determines regeneration ability To further understand the influence of clitellum in survival and regeneration, the worms were amputated at the 11th and 20th segments (Fig. 1*O*). Following amputation, the intact clitellum containing a portion of the worm had two body segments in the anterior and posterior parts of the worm. The intact clitellum segment was able to survive and regenerate from both the ends (Fig. 1*P*) by the 4th d post amputation. The portions of the worm with intact clitellum were able to form regenerative blastema and differentiated into segments following the 6th d of post amputation (Fig. 2*A*, *B*), whereas the segments from 1 to 10th and from 21st to anus failed to regenerate successfully (Fig. 2*C*, *D*) and they died. This observation directly confirms the role of clitellum in animal survival and regeneration.

Figure 1. In vivo regeneration ability of different amputated segments of *E. eugeniae*. (*A*) Amputation at the anterior region of 1–10 segment. (*B*) Wound healing after 2nd d of post amputation of 1–10 segment. (*C*) Amputation site of anterior region of 1–15 (half clitellum) segment. (*D*) Regeneration of blastema (rb) of 1–15 (half clitellum) segment on 8th d of post amputation. (*E*) Amputation at 1–30 segment (whole clitellum). (*F*) Regeneration of blastema of 1–30 segment on 8th d of post amputation. (*G*) Amputation at 11–anus segment (whole clitellum). *H* Regeneration of blastema (rb) on 5th d of post amputation. (*I*) Amputation at 16th segment (half clitellum). (*J*) 16–anus segment forms regenerative blastema on 5th d of post amputation. (*K*) Amputation at 30th segment (clitellum free). (*L*) 31–anus segment forms small blastema following 8th d of post amputation. (*M*) Amputation at 10th segment. *N* 10–anus with defined blastema was observed in 4th d of post amputation and used for in vitro maintenance. (*O*) Amputation at 11 and 20th segment. (*P*) Regeneration of blastema was observed on both ends following 5th d of post amputation. ws, wound site.

Clitellum factor influences blastema formation and differentiation ability In order to determine the effects of clitellum factors that influence the survival and regeneration ability, the following experiments were carried out. The worms ($n = 20$) were initially amputated at the 60th segment (Fig. 3*A*) and allowed to form the regenerative posterior blastema on the 5th d (Fig. 3*B*). Following that, the worms were again amputated at the second position, i.e. at the 30th segment to detach the clitellum and anterior body segments (Fig. 3*C*), and observed for the new blastema formation at the amputated 30th segment. Surprisingly, even without the clitellum segments, blastema formation was initiated without any delay on the 4th d of post amputation at the 30th segment in 100% of worms (Fig. 3*D*). Following this, the already regenerated posterior blastema was elongated and differentiated into segments (Fig. 3*D*). Even though the regenerative anterior blastema cannot further develop into differentiated segments, the survivability of the amputated segments was enhanced to 40 to 50 d (Table 1).

In vitro maintenance enhances worm survivability and regeneration potential To understand the survivability in in vitro conditions, worms with the 1st to 10th segments following amputation were maintained in L15 medium and observed for their increase in survivability. As expected, a small portion of these segments died within 4 d in the in vivo condition (Table 1), but under the in vitro condition these segments survived for more than 60 d. As shown in Fig. 4*A*–*C*, 1st to 10th segments were maintained for 30 d in in vitro condition during which the worms were able to heal their wounds by the 2nd d post amputation but were unable to initiate the regeneration. The amputated tissue segments appeared normal without any morphological changes.

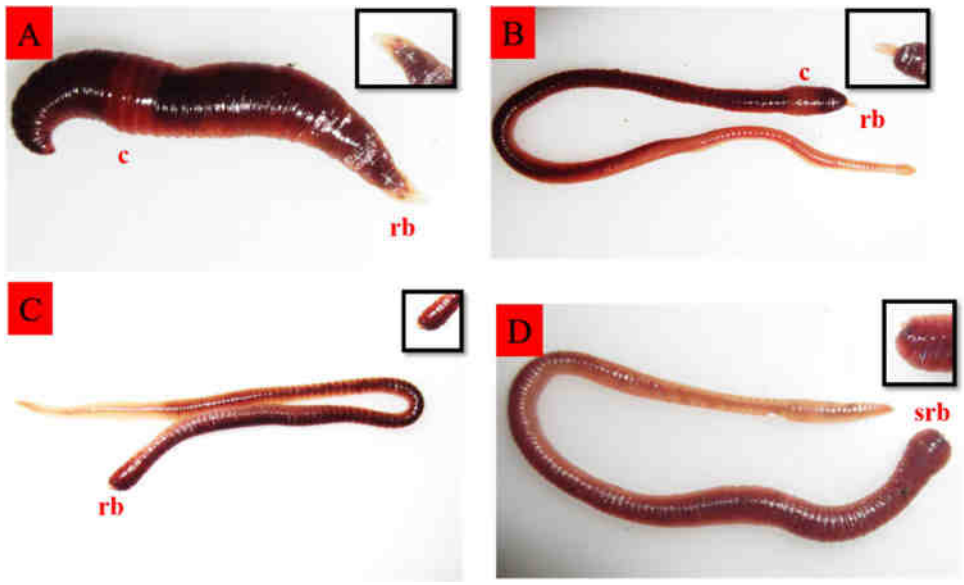


Figure 2. Comparative analysis of blastemal size that varies in between intact clitellum, partial clitellum and without clitellum. (*A*) Worm amputated at 30th segment forms posterior regenerative (rb) blastema with well-differentiated segments on 6th d. (*B*) Worm amputated at 10th segments forms anterior regenerative blastema (rb) with well-differentiated new segments on 6th d. (*C*) Partial clitellum segments form anterior blastema on 8th d but unable to grow and differentiate. (*D*) Worm amputated at 30th segment forms small anterior regenerative blastema (srb) on 8th d but unable to grow and differentiate.

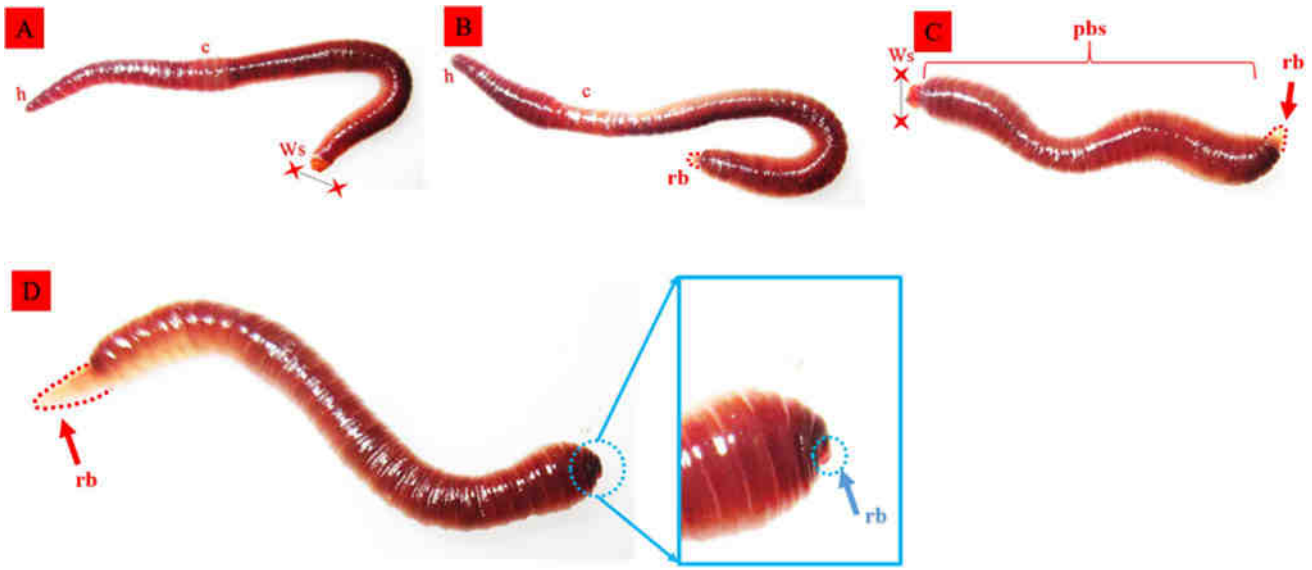


Figure 3. The importance of clitellum and clitellum factors in in vivo regeneration. (*A*) Worm amputated at 60th segment (with clitellum). (*B*) Regeneration of posterior blastema observed on 5th d of post amputation (with clitellum). (*C*) Incision of clitellum segments by amputation at 30th segment. (*D*) Observation of new blastema (*blue arrow*) after 5th d of post amputation; meanwhile, the regenerated rb grew and formed differentiated new segments (*red arrow*). Ws, wound site; pbs, posterior body segments; rb, regeneration of blastema.

To evaluate the increase in survivability, and to check their regeneration potential, the amputated 4th-d anterior blastema along with an adjacent body segment was dissected out from the in vivo worm (Fig. 5*A*–*H*) and the dissected blastema along with the adjacent half body segment was amputated into two equal halves and maintained in the L15 medium as mentioned in the "Materials and methods" section. The amputated anterior portion of blastema is called ARB (Fig. 5*A*–*D*) and similarly the amputated posterior blastema portion is called PRB (Fig. 5*E*–*H*). Both ARB and PRB survived in in vitro conditions for more than 60 d. In ARB, no changes were observed on the 2nd d (Fig. 5*A*) but the blastemal size increased gradually by the 10th d (Fig. 5*B*) and reached the final level of blastemal structure after 18th d (Fig. 5*C*). But on the 30th d, they lost their blastemal structure (Fig. 5*D*). In PRB (Fig. 5*E*), a novel pattern of changes was observed. They formed stick-like structures to which the cells were attached (Fig. 5*F*, *G*). The shape of

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 (ARB)	In vitro	Non-clitellum	(✓)#	✓	100	(-)	60 +
Posterior Regeneration of Blastema (PRB)	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)

the tissue elongated to form blastemal-like structure which looked similar to that of an in vivo regenerated blastema (Fig. 5*H*). The regenerated blastema could not further differentiate into segments but the blastemal structure was retained for up to 60 d.

Juvenile posterior body forms regenerative blastema uponin vitro maintenance The survivability of in vitro maintained adult posterior segments (30th to 37th segments) was 100% and is greater than the in vivo counterpart (Fig. 6*A–C*). The 30th to 37th segments exhibited 100% survivability in in vitro maintenance for up to 60 d of examination but in the in vivo condition, the same segments survived for only a maximum of 4 d. Even though the survivability was augmented upon in vitro maintenance, blastema formation was not initiated in any of the amputated adult segments. But remarkably, the amputated 30th to 37th segments in juvenile worms (age 10 d), upon in vitro maintenance (Fig. 6*D–F*), were able to form blastema on both the amputated ends by the 4th d of post amputation (Fig. 6*E*). These were able to grow their blastema size in the following 6 d but were unable to differentiate the blastema (Fig. 6*F*). The same segments in in vivo condition could not survive and died within 3 d. With the available data, a Kaplan–Meier survival curve was obtained for the survivability of earthworm *E. eugeniae* that are maintained in in vivo and in vitro conditions as

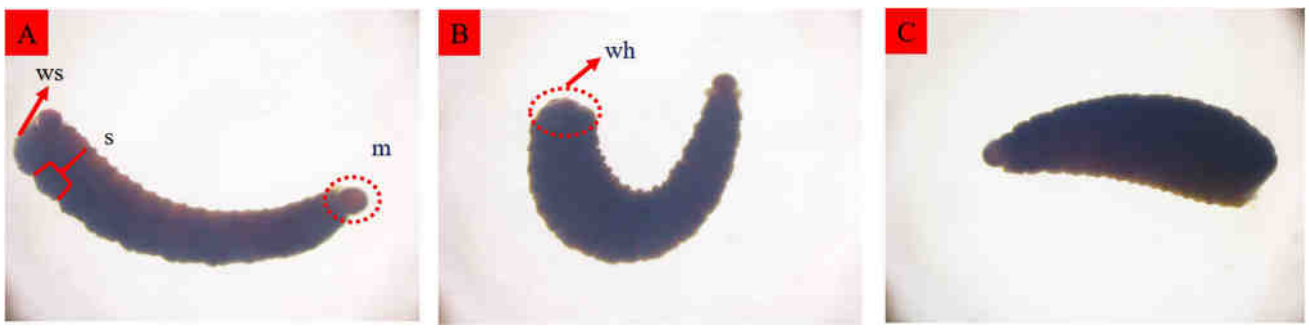


Figure 4. In vitro maintenance of head from 1 to 10th segments. (*A*) Anterior amputations at 1–10 segments (without clitellum) were maintained under the in vitro condition (ws, wound site; s, one segment; m, mouth). (*B*) Wound healing was observed in 2nd d of post amputation. (*C*) No morphological changes were observed after 30th d of post amputation (wh, wound healing).

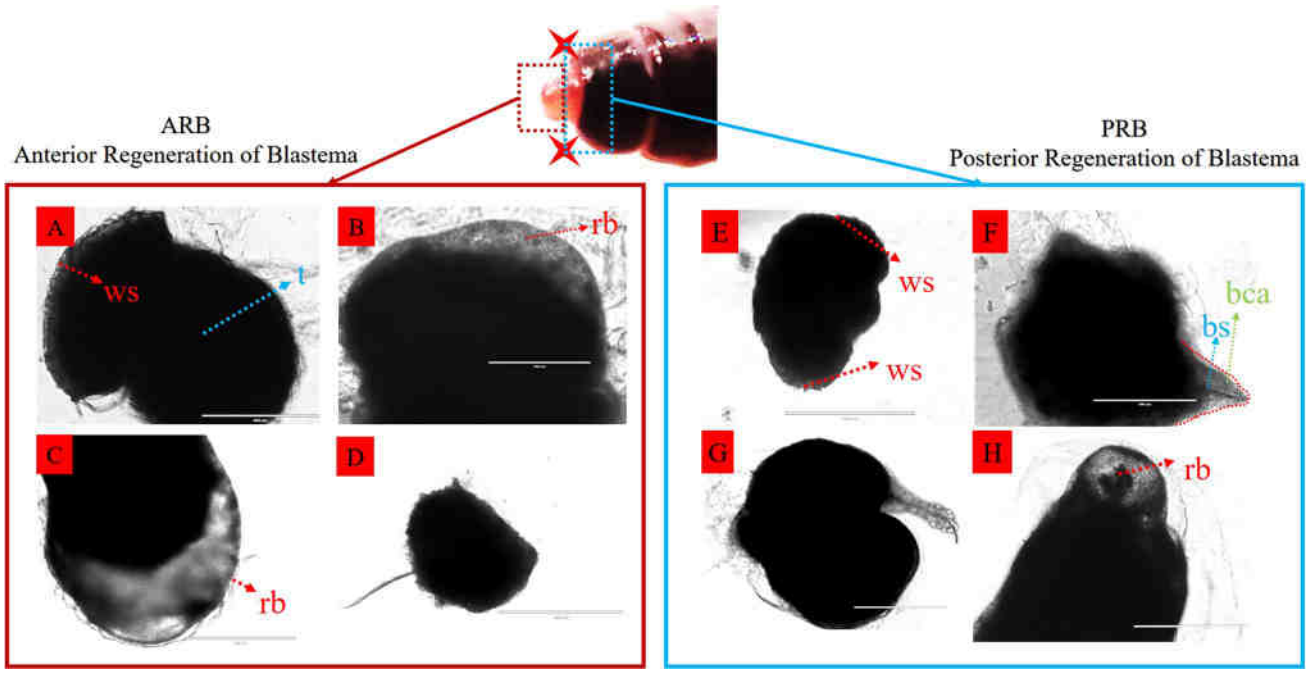


Figure 5. In vitro maintenance of adult 4th d blastema and their regeneration potential. The 4th d in vivo regenerated blastema was further amputated into two half and categorised into ARB (Anterior Regeneration of Blastema—only blastema region) and PRB (Posterior Regeneration of Blastema—blastema region with one body segment). In ARB: (*A*) 2nd d after amputation; (*B*) from the amputation site, the new blastema was formed on 10th d; (*C*) blastemal size was enormously increased on 18th d after amputation; (*D*) loss of regenerated portion of ARB on 30th d after amputation. In PRB: (*E*) 2nd d after amputation; (*F*) 10th d after amputation, the blastema regeneration was initiated with defined manner by the support of stick-like structure called blastema stick (bs) and cells are captured with their support; (*G*) elongation of blastema was observed on 18th d; (*H*) observation of reformatted structure of blastema (similar to in vivo regeneration) observed on 30th d. (ws, wound site; rb, regeneration of blastema; bs, blastema stick-like structure; bca, cells attached in blastema).

represented in Fig. 7*A* and *B*. In vivo amputated segments of 1–10 and 1–15 segments had a high mortality rate with a median survival time of 4 d. Following that, 16–anus, 31–anus and worms used in clitellum influence experiments of in vivo amputated segments have a median survival time of 15, 30 and 50 d respectively. On the other hand, all the in vitro maintained amputated segments had a median survival time of undefined period. The results shows the significant differences observed in between in vivo sets of clitellum influence with 1–10 and 1–15 segments. But in case of in vitro maintained amputated segments, no significant survival differences were observed in between the different amputated segments.

Blastema cell microscopic observation and histological analysis During in vitro maintenance of earthworm blastema (ARB and PRB), some of the cells from the blastema were detached from the tissue surface and attached to the bottom of a 35-mm petri dish (Fig. 8*A*–*C*). The attached cells showed different morphological structures which represented different cell types. Most of the cells looked like adipocytes morphologically. Some cells show pili-like structures as shown in Fig. 8*B* and *C*. The 60 d of in vitro maintained blastema was subjected to histology (Fig. 8*D*). The data shows the internal tissue layers are loosely packed, the cells are arranged in clusters and, in between the cellular clusters, lumen-like structures are observed (Fig. 8*D*. Live imaging of blastema tissue showed the active movement of cells and coelomocytes towards the newly forming blastemal sites as shown in the video file which represents the cellular movement involved in the formation of the blastemal structures.

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). Regeneration is a complex process and in annelids, following an injury, it starts with

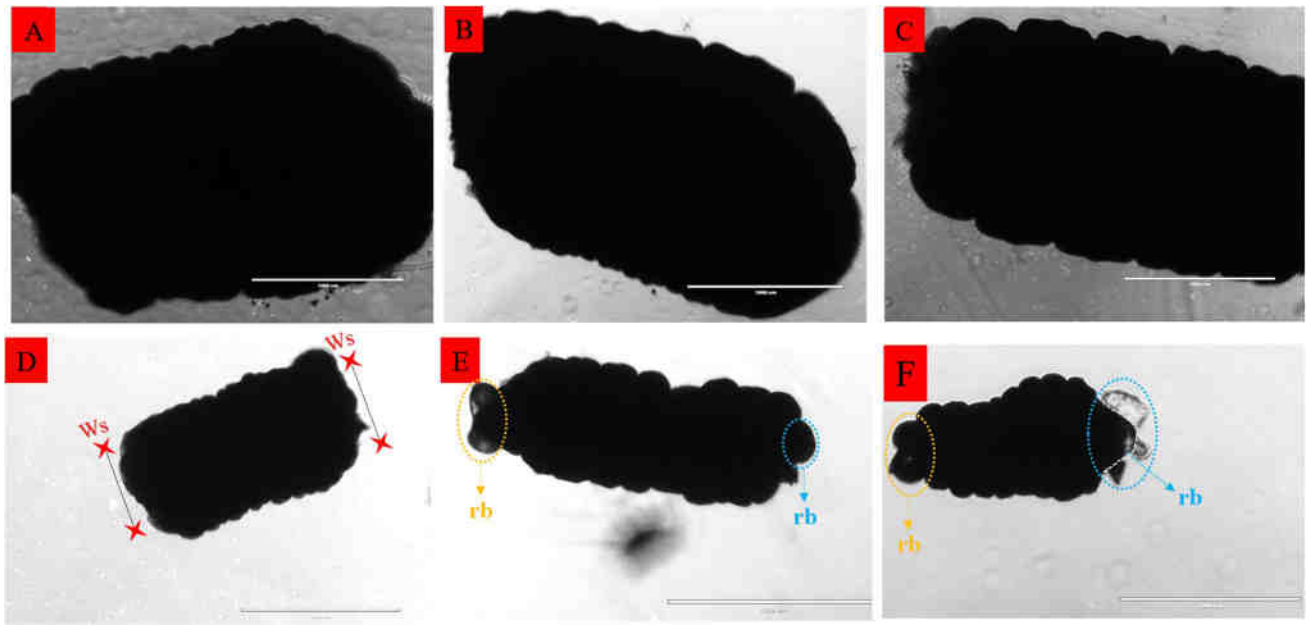
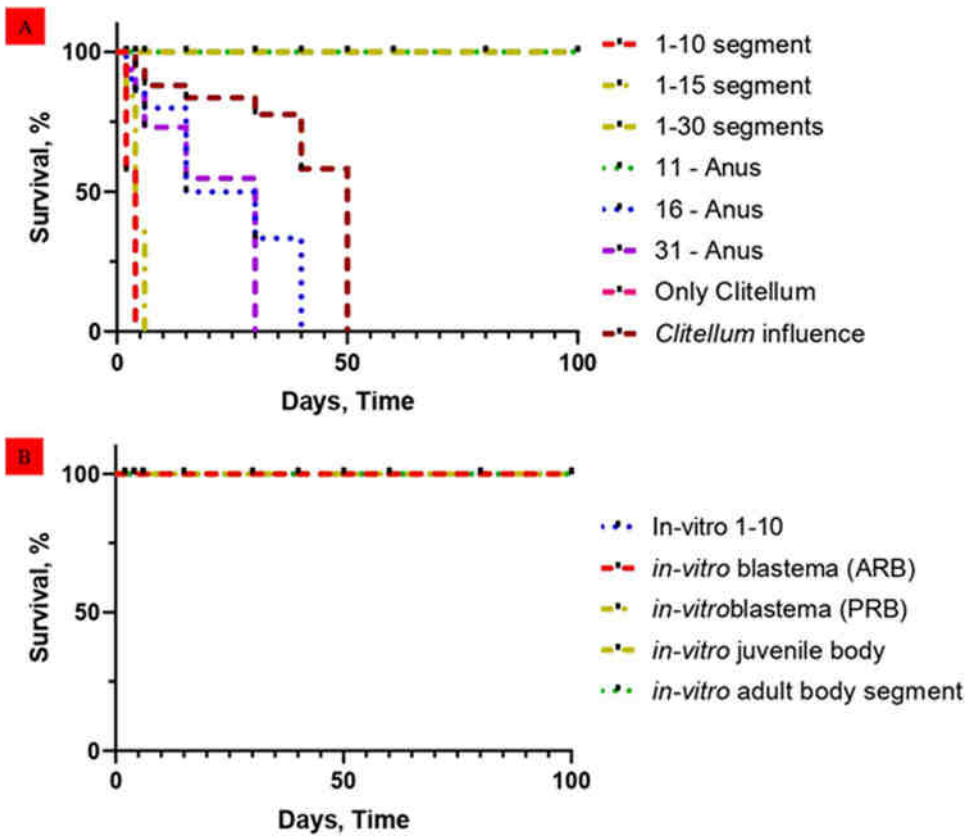


Figure 6. In vitro maintenance of adult and juvenile posterior segments (30 to 37th segments). *(A)* Adult *E. eugeniae* posterior segments on 0th d. (*B*), (*C*) No segment restorations were observed in day 15 and 30. (*D*) Juvenile *E. eugeniae* posterior segments on 0th d. (*E*) Regeneration of blastema (rb) was observed in both amputated ends after 4th d of post amputation in juvenile worm; yellow circle represents multiple blastema formations in one end which are transparent in nature and *sky blue circle* represents single blastema in another end. *F* Elongation of regenerative blastema in both wound sites; transparent tissue turns to dark (*yellow circle*) and the size of the blastema is elongated (*sky blue circle*).

Figure 7. Survival graph for the amputated worm with different segment lengths from the earthworm *E. eugeniae*. (*A*) In vivo segments survival ability. (*B*) In vitro segment survival ability. After amputation, the live and dead amputated segments in different days are counted and plotted in the survival (Kaplan–Meier) curve. Statistical analysis was performed using log-rank test (Mantel-Cox) and the obtained *p* values ˂0.05 were considered significant.



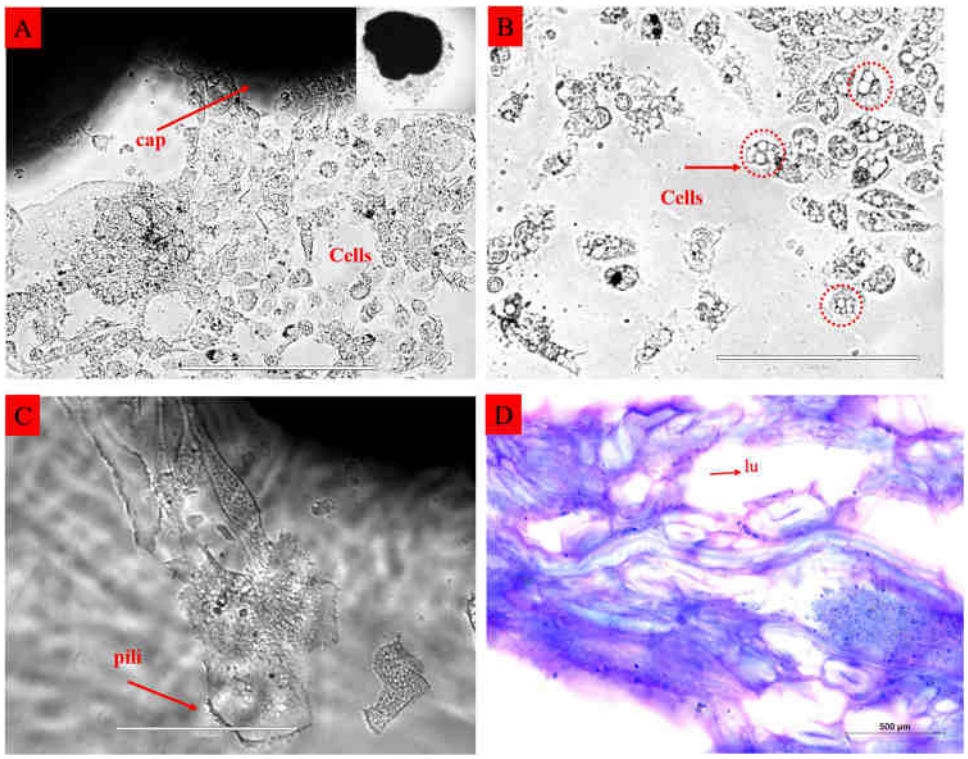


Figure 8. Nature of ARB and PRB in culture plate and their histology. (*A*) Different cell types are released from blastema and attached to the surface. (*B*) Immature adipocytes are the known predominant cells observed. (*C*) Cells with pili (marked in red *arrow*). (*D*) Histological sectioning of in vitro maintained blastema tissue showing tissue lumens (l) following staining with haematoxylin and eosin.

wound healing, cellular recruitment, cellular proliferation, cell differentiation and patterning (Kostyuchenko and Kozin 2020). In the present study, all the amputated worm segments which possessed intact clitellum (11th to anus and 1st to 30th 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.

The amputated worms with partial clitellum (1st to 15th segments and 16th to anus segments) survived for 5 ± 1 and 30 to 40 d respectively which directly implied that the segment length determined the survivability. However, in 20% of those worms, death occurred within 3 d, suggesting that partial clitellum may provide negative signals that discouraged cell proliferation. In worms with longer amputated segments with partial clitellum (16th to anus), 4 out of 10 worms initiated blastema formation by 6 ± 1 d but were unable to proceed further. The data clearly showed that in the absence of intact clitellum, both the survivability and regeneration potential were impaired but blastema initiation was observed in certain portion of worms (40%). Similarly, 3 out of 10 worms with 31st to anus segments were able to form regenerative blastema. In animals following any tissue damage, blastemal formation is initiated either by epimorphosis or by morphallaxis or by both (Reddy *et al*. 2019). The earthworm *E. eugeniae* follows epimorphosis in which cell proliferation is a key factor to form blastema (Johnson Retnaraj Samuel *et al*. 2012). Connecting with our results, it represents that worms with partial clitellum and without clitellum are able to initiate blastema but at the same time the worms fail to increase the blastemal size and lack differentiation ability without the presence of intact clitellum.

Another important point inferred from the experiment is that the longevity of survival increased in proportion to the amputated segment length. The amputated worms without clitellum segments (1st to 10th segments) and (31st to anus segments) survived for a maximum of 4 and 30 d respectively. The worms with longer segments, 31 to the anus, formed regenerative blastema though it occurred only in 2 ± 1 out of 10 worms. When compared with the partial clitellum residing worms, the survivability was greatly reduced in worms with no clitellum which directly implied that not only the segment length but also a portion of the clitellum determined the survivability and regenerative blastema forming capability. The amputated worms that possessed clitellum segments alone (13th to 18th segments) were able to survive, formed blastema and grew in size to form differentiated segments which confirmed that the regeneration ability of *E. eugeniae* that resided only within the clitellum segments. The clitellum differs from other body segments by showing the thickened structure, functionally producing cocoon and secreting albumin (Sivasubramaniam 2021).

Though its role in regeneration is known, the clitellum's exact molecular function in supporting regeneration has not been evaluated.

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 blastemas 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).

Even though segment length, presence of clitellum segment and clitellum factors influence the survivability in in vivo conditions, such a demand is not observed in the in vitro maintained earthworm tissues. All the in vitro maintained tissues survived irrespective of segment length, but variations were observed with regeneration potential. In adult worms, the in vitro maintained posterior segments (30th–37th segments) were able to survive for more than 60 d but lacked blastema forming ability. On the other hand, ARB and PRB, which were obtained from adult blastema, had the potential to form blastema-like structures due to the presence of undifferentiated cells and stem cells that resided within the blastema. Notably, PRB formed a stick-like structure and reinitiated the regeneration of the lost blastema structure. Despite ARB forming blastema-like structures, it was not able to retain it. The reason behind it may be that the undifferentiated blastema cells without the support of adjacent segments failed to keep the memory of the lost tissue pattern. Additionally, ARB lacked tissue-specific memory, shape memory and positional identity which are important to proceed with the complex process of regeneration.

Another interesting difference observed between adult and juvenile worms upon in vitro maintenance is that the posterior segments of juvenile worms (30th–37th segments) were able to form regenerative blastema on both ends in contrast to the adult worms. Even though the juvenile worms formed regenerative blastema, it was unable to develop into differentiated segments. But interestingly, multiple blastema formation was initiated at one end of amputated sites. The collective data demonstrated that the stem cells, usually enriched in juvenile worms, may be activated increasing their survival longevity upon in vitro maintenance. But such a regeneration initiation was not observed in in vivo conditions. Survival and regeneration potential are also determined by many factors; the worms maintained in in vivo conditions have some disadvantages because following amputation, the worms are maintained in an undefined worm bed composition without any aseptic conditions. On the other hand, the amputated worm segments that are maintained in in vitro conditions are held in defined medium in a sterile environment. Importantly, the FBS-supplemented L15 medium provided hormones, growth factors, vitamins, proteins, trace elements and embryonic growth-promoting factors that enhanced the growth and cellular reprogramming that led to enhanced survival and blastemal initiation (Kwon *et al*. 2016; van der Valk *et al*. 2018). Additionally, an artificially regulated environment was maintained (O_2 supply, pH 7.4, osmotic pressure 300 mOsm/L, temperature 30–34°C) in in vitro condition but non-regulated natural environment was maintained for in vivo conditions.

Earlier, we reported that in juvenile *E. eugeniae*, the ALP expression observed in the gut epithelium of each posterior segment (Christyraj *et al*. 2019) may support the phenomenon of regeneration upon in vitro maintenance. Though the juvenile worm retained the blastemal forming ability, the regeneration mechanism was not tightly regulated which resulted in the formation of multiple blastemas. The data concluded that without a proper signal from the clitellum, enhancement of blastemal formation will result in the impairment of cell cycle regulation that may lead to carcinogenesis. To understand the molecular mechanism of survival and regeneration potential of different amputation segments, the key proteins that determine the cell fate, like TCTP (Telerman and Amson 2017), Wnt (Chera *et al*. 2009) and caspases (Fogarty and Bergmann 2017), need to be investigated. These key proteins play an inevitable role in apoptosis, stem cell proliferation and differentiation (Fan and Bergmann 2008; Fogarty and Bergmann 2017; Telerman and Amson 2017). Generally, apoptosis-induced compensatory proliferation (AICP) is a process of compensation of tissue lost by cell proliferation which is induced by apoptotic cells (Bergmann and Steller 2010). AICP studies have been carried out in well-known regeneration models like Hydra (Chera *et al*. 2009), *Xenopus laevis* (Tseng and Levin 2008), *Drosophila melanogaster*

(Apidianakis and Rahme 2011) and mouse (Li *et al*. 2010). But in some animal models like zebrafish (Anand *et al*. 2021), earthworms (Bodó *et al*. 2021), axolotls, planarians (Almuedo-Castillo *et al*. 2014) and ascidians (Jeffery and Gorički 2021), such studies are lacking. AICP will aid to unravel the molecular mechanism of regeneration in which induction of apoptosis is the key step in activating stem cell proliferation and other events of regeneration. Notably, apoptotic cells have been observed in the first week of post amputation earthworm *Eisenia andrei* (Bodó *et al*. 2021). In the present study, different responses were observed in different amputated segments and correlating them with AICP will aid in unravelling the molecular mechanism of regeneration in *E. eugeniae*.

Conclusions

Overall, our 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 confirm that one of the functions of clitellum might be the paracrine supply of growth factors.

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Data availability All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval The experiments are carried out using lower invertebrate earthworm; therefore, ethical statement is not needed. Necessary care is taken in experimental procedure that is intended to avoid unnecessary pain and suffering to the experimental animals.

Conflict of interest The authors declare no competing interests.

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