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Blastema Tissue Regeneration in Immature Earthworms *Eisenia fetida*- a Prospective Wound Healing Agent

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Abstract. Research on immature nematode worm tissues and larval secretions have gained recent attraction of the scientific community due to their promising applications in regenerative medicine. Annelids, in general, and specifically earthworms, have remarkable capacity for regeneration. Extracts from earthworm tissues have demonstrated significant wound healing potential even centuries back in Chinese medicines. Clitellum is a specialized region of the body of earthworms which is primarily involved in mucus production, and reproduction, and in regeneration processes The present study aim to assess the amputation and regeneration potential of the blastema tissue in immature and mature earthworms before and after clitellar development. *Eisenia fetida* adults and immature worms were collected, acclimatized and made amputations on anterior and posterior segments of the young earthworms at the region pre and post clitellum and compared with adult worms amputed 8 segments from the posterior region. The regeneration capacity of both the adult and immature groups were observed and measured over a period of 12 days which led to the conclusion that, the young ones exhibited a relatable quantum of regeneration capacity compared with adult worms even before the formation of clitellum suggesting the regenerative tissue extract from immature earthworms can be a better choice as they contain both blastema specific regeneration factors as well as general growth factors of immature annelids giving a combinatorial effect on growth and healing.

Keywords: *Eisenia fetida*, Earthworm Regeneration, Wound healing, Segment Amputation

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1 Introduction

Immature nematode tissues and larval secretions has revealed promising wound-healing properties across several species. Excretory/secretory (ES) products from *Trichinella spiralis* newborn larvae were shown to stimulate collagen secretion and tissue repair by activating the TGF-β1/Smad3 pathway in host muscle cells, [1]. Similarly, a serine protease fraction (TS151c) from *T. spiralis* muscle larvae exhibited accelerating healing and collagen synthesis in fibroblast cultures, providing the best utility of nematode larval products in extracellular matrix formation. Together, the current research across the world highlights the utility of immature nematode-derived extracts and proteins in repair pathways, collagen deposition, and facilitating wound healing.

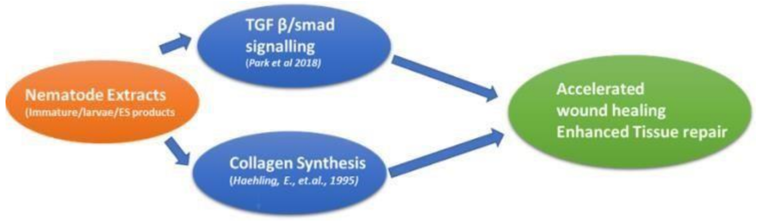

Fig. 1. Schematic diagram showing the reported molecular mechanisms by which immature nematode extracts promote accelerated wound healing by acting through TGF-β/Smad signalling (↑ collagen synthesis) and immune modulation (↓ inflammation) to

Among model organisms, earthworms represent a particularly valuable system for regeneration studies. *Eisenia fetida,* an earthworm species widely used in laboratory experiments, demonstrates remarkable regenerative potential. Experiments in *Eudrilus eugeniae* suggests that regeneration rates vary depending on the presence or absence of clitellum, thus clitellum has been shown to play a critical role in this process, as segments with an intact clitellum exhibit enhanced blastema formation and tissue differentiation, supported by histological and molecular analyses [2]. Furthermore, extracts derived from earthworm tissues have demonstrated significant wound-healing potential. For instance, there are reports stating earthworm metabolites extracts from anterior regenerated *Eudrilus eugeniae* accelerated ear lobe tissue regeneration in mice ,and identified a protein fraction termed G-90 from the regenerating tissue of *E. Fetida* that promoted proliferative responses in both *in vivo* and *in vitro* wound models [3]. These findings collectively underscore the importance of dissecting molecular and segment specific contributions to regeneration, which may serve as a foundation for novel biomaterial development in wound repair.

Regeneration is basically a survival mechanism which enables the animal to regenerate the lost or damaged body parts. Many of the organisms possess this ability to regenerate which includes simple invertebrates to higher vertebrates. It is essential for their survival as they are prone to predators or other accidents to loss their body parts. Research on regeneration in earthworms include experimental studies to ecological, molecular, and immunological investigations. It has also been demonstrated that it is the inherent ability of tissues differentiate and regenerate after an injury.[4] explained the positional control of regenerative capacity and explained the extrinsic and intrinsic factors regulating regeneration and suggested that the regeneration is not a uniform process by it is dynamic in nature.The regeneration also depends number of segments removed and the site of injury, with a high rate of regenerative potential in the anterior region than posterior region.

Such work established that regenerative ability is unevenly distributed within annelids. Another perspective is that regenerative potential differs across Annelida due to ecological pressures and phylogenetic history [5] and also explained regeneration as an ancient yet lineage-specific trait with a complex evolutionary trajectory. Regeneration of earthworm is widely studied on *Eisenia fetida* and found that anterior and posterior segments are easily regenerating. This regeneration power is quite interesting because it can pave ways to future research in different directions including regenerative medicine [6]. There are different mechanisms in which regeneration takes place in the animal kingdom, and the rate of regeneration varies from species to species. Some species have restricted capacity for regeneration which is restricted to some areas of the body, whereas others are able to regenerate entire limbs or organs. Investigations into the evolutionary background of regeneration have shown that this process stems from universally conserved cellular and molecular mechanisms. Regenerative blastema cells are activated after a wound epithelium forms, and these cells subsequently differentiate into new cells which further develops into lost body parts [7].

Eisenia fetida, the African species, is a more interesting earthworm because of its remarkable regenerative ability widely used in laboratory experiments. Amputation and survival capacity along with regenerative length was studied and noted that the greatest survival rates occurred for earthworms within most body segments remaining after amputation [8].

Regeneration studies at molecular genomic and immunological levels revealed that it is not only morphological but also a genetic and immunological process. There are other extrinsic factors which are having a direct regulatory role on regeneration efficiency of *Eisenia fetid*a, like, environmental factors, temperature and other abiotic components.

1.1 Life cycle of *Eisenia fetida*

Eisenia fetida passes through cocoon stage to egg lying stage and completes its Life span. Cocoons are much smaller and lemon-like shaped and it's yellow-coloured. Cocoon undergoes average 23 days of incubation at 25°C .Incubation periods ranges 14 to 44 days. Cocoons will gradually change its colour from golden yellow to deep red, much like maroon as 4 to 6 *Eisenia fetida* develop inside. The hatchlings come outside as earthworms, unpigmented and only a few millimeters in length , gain Their adult pigmentation within a few days shows its reproductive maturity. The clitellum may develop in 40 to 60 days to become a reproductive adult .

Mature individuals of different vermicomposting species can be differentiated by the presence of intact clitellum, the pale or dark coloured swollen belt located behind the genital pores. The clitellum secretes the fibrous cocoon, and the clitellar gland cells secrete a nutritive albuminous fluid that fills the cocoon. Earthworms display low degree of growth and can continue to grow after completing their sexual development although they do not add segments [9].Several experiments suggested the critical role of clitellum in the regeneration process of earthworms. The histological studies exhibited proliferation of layers of longitudinal cells at amputation sites is critical for successful regeneration, particularly in segments with intact clitellum. Transcriptomic studies identified key genes involved development of muscles in response to environmental cues, and patterning along the anterio-posterior axis during regeneration. By concluding cellular and molecular data, the study gives insights into how clitellar tissues contribute to the regeneration of complex body structures in earthworms, highlighting their aspects in evolution and ecology of tissue-specific regeneration mechanisms.

Comparative analysis of the survival and Regeneration potential of juvenile and mature *Eudrilus eugeniae* earthworms under both *in vivo* and *in vitro* conditions highlights the crucial role of the clitellum, in survival and regeneration of earthworms. Earthworms with intact clitellum segments are capable of surviving and regenerating, while those without it exhibit limited survival and regenerative ability. The study also notes that adult worms form a blastema-like structure under in vitro conditions, with the posterior portion showing more regenerative potential than the anterior [10].

A few research publications also discuss about the role of Apoptotic induced compensatory proliferation signals (AICP) is the basis of molecular mechanisms behind regeneration [11]. The study in *Perionyx excavatus* revealed the balanced act of AICPs and proliferation signals for its accurate regeneration.

Earthworms have been demonstrated as a source of wound regenerative bioactive molecules. One example is the glycoprotein extract from head tissue of earthworm *Eisenia fetida* have been demonstrated to exhibit mitogenicity, anticoagulation ,fibrinolysis , anti oxidation etc., making it a source of bioactive molecules with regenerative potential.[12]. There are also reports stating the wound healing effect of earthworm extract by analysing macroscopic, histopathologic, hematologic, and immunohistochemistry parameters . In a review the anti inflammatory ability has been illustrated and also the earthworm extract has been shown to reduce the blood glucose levels [13].Key genes and bio active molecules in earthworm extract have the ability to promote wound healing process are identified through LC -MS ,molecular docking and metabolism analysis.

The potentiality of AgNPs of earthworm extract in the effective treatment for preventing sepsis in mice has been experimented [14].AgNPs are effective against microbial infection due to the formation of free radicals, which destroy bacterial cell wall. At the same time, they exhibit anti-inflammatory ability by decreasing the release of pro-inflammatory cytokines in the body. It leads to the improved function of organs in the mice and it is confirmed by the histopathological studies.

There are also research which explored the potentiality of Rutin encapsulated earthworm granulation tissue (RdECM) from earthworm regenerated blastema against diabetic wound healing with special reference on angiogenesis [15].The study demonstrate the ability of earthworm regenerated tissue extract hydrogel (RdECM) can inhibit Nuclear Factor kappa B (NF-Kβ) pathway , reduction in free radicals induced DNA damage and down regulation of TNF receptor associated factor 1 (TRAF1)and lead to angiogenesis in diabetic rabbit.This study can perform a lead role in the diabetic wound healing instead of classical treatment like surgical debridement, hyperbaric oxygen therapy and use of antibiotics to control infection .

2 Materials and Methods

2.1 Materials Used

Eisenia fetida young and adult worms, Vermicompost, Cow dung, culture chambers lined with wet clothes, ice and dissection tools, Leica EZ 4 Stereozoom microscope.

2.2 Methodology

2.2.1. Collection and culturing of earthworms

Eisenia fetida, the African earthworm species was selected for the study because its robust regenerative capacity and easy culture conditions. The young immature animals of 3 weeks were collected in vermicompost from Kerala Agricultural University ,Mannuthy, Trissur, Kerala. Earthworms were well maintained and acclimatized in laboratory condition for one week prior to the experiments.

2.2.2. Amputation Procedure

The adult animals with intact clitellum and juvenile worms of 3 weeks age lack clitellum are taken for the experimentation and kept under optimal conditions at 25 degrees Celsius for one week for their acclimatization and wet cow dung was added intermittently to the vermicompost. To study the regenerative potential of earthworms, the animals were grouped in three, based on the amputation performed namely “immature anterior” (IA) and “immature posterior” (IP) and “adult posterior” (AP) respectively. The amputations were performed under sterile conditions. Five anterior segments were removed from the group of animals under the IA and eight segments removed from the posterior edge of the worms under the category IP and AP. Individual worms were kept in separate boxes containing wet vermicompost and kept at optimal conditions also ensures the temperature by periodic checking and maintained moisture by adding water .

2.2.3. Observation and quantification of regeneration in Amputed earthworms

After amputation, earthworms were cultured in optimal growth conditions for Twelve days for regeneration experiments and observed individually under Leica Stereo zoom EZ 4 microscope every day and length measurements of newly formed blastema were taken individually for 12 days intermittently (3rd , 6th ,9th and 12th day of experiments) for all animals under the groups IA,IP and AP using the software Leica Application Suite.

On Day 3: Initial signs of regeneration were observed at the site of amputation, characterized by the formation of a blastema (a mass of undifferentiated cells). The growth at this stage was limited to undifferentiated tissue formation.

On Day 6 : Visible regeneration with the newly formed segments beginning to differentiate into specialized tissues.

On Day 9 : Specialization of tissue structures were observed, such as nerve cells and skin like epidermal layers.

On Day 12: The regenerated anterior segments continued to grow in size significantly and differentiate, restoring most of the original tissue functions and structures .

2.2.4. Statistical Data Analysis

Statistical analysis has been conducted to validate the regeneration rates - mean length, its standard deviation and standard error of IA, IP and AP groups in both adult and immature earthworms. The difference in the growth rates were analysed from observations on 3rd ,6th ,9 h and 12th day of regeneration after amputation.
For each group (AP, IA, IP) and day (3, 6, 9, 12), descriptive statistics were computed as mean with standard error of the mean (SEM); figures display upper-only SEM error bars for clarity. Between-group differences within each day were tested using two-sided Welch's t-tests (unequal variances), comparing AP vs IA, AP vs IP, and IA vs IP. Exact p-values are shown above brackets in the plots, with significance codes: ns (≥0.05), * (<0.05), ** (<0.01), *** (<0.001), **** (<0.0001).

3 Results and Discussion

Regenerative potential of juvenile worms of *Eisenia fetida* was experimented by amputating anterior Five segments (IA) posterior eight segments (IP). The regeneration potential and blastema formation was monitored daily for a period of Twelve days measuring the length of newly regenerated blastema. By the end of experimentation, there observed remarkable difference in regeneration of both anterior and posteriorly amputated worms (IA &IP) as anterior region exhibited an increased potential to regenerate compared to posterior region.

Immature anterior (IA): On day 3, the average regeneration length was 0.30 Cm, which increased to 0.87 Cm by day 12 (Fig 2 A).
Immature posterior (IP): Immature posterior segments exhibited relatively slow regeneration rates compared among groups. The mean regeneration length on day 3 was 0.17 Cm, increasing to 0.50 Cm by day 12 (Fig 2 B).
Adult Posterior (AP): Adult posterior segments exhibited relatively higher-level regeneration rates compared among groups. The mean regeneration length on day 3 was 0.25 Cm, increasing to 0.650 cm on day 9 and 1.04 Cm by day 12 exhibiting maximum capacity of regeneration among the groups (Fig 2 C). Figure 3A-D express the actual difference in average regeneration in Immature Anterior and Immature Posterior groups of *Eisenia fetida* in day 3,6,9, and 12. The pairwise comparison using two-sided Welch's t-tests and statistical significance analysis revealed that, across time, IA exhibited the highest mean growth and IP the lowest (Fig 4); AP vs IA was not significant at Day 9 and Day 12, whereas AP vs IP and IA vs IP were significant at all days ($p < 0.05$) .

The lack of well-developed structures in immature worms may contribute to this reduced regenerative capacity, particularly in the posterior region, which is less complex than the anterior region. This indicated that, even though the overall regenerative capacity is not as robust or polarized as that of adults, the immature worms regenerated anterior segments relatively faster compared to posterior segments. This may possibly due to the incomplete development of the neural and organ systems, even then there is a polarization in regeneration in immature earthworms, even before the clitellar formation or reproductive maturity.

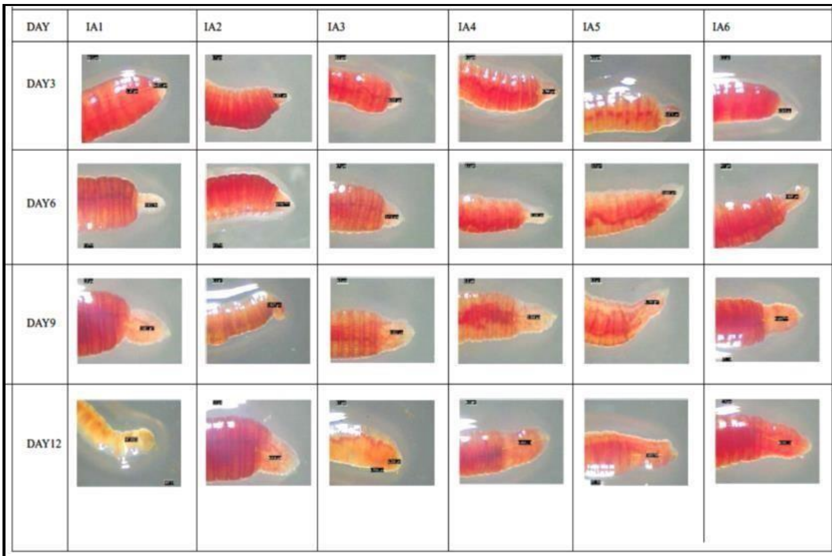

Fig. 2. A Image showing the regenerated anterior region of Immature *Eisenia fetida* on Day 3, 6, 9 and 12.

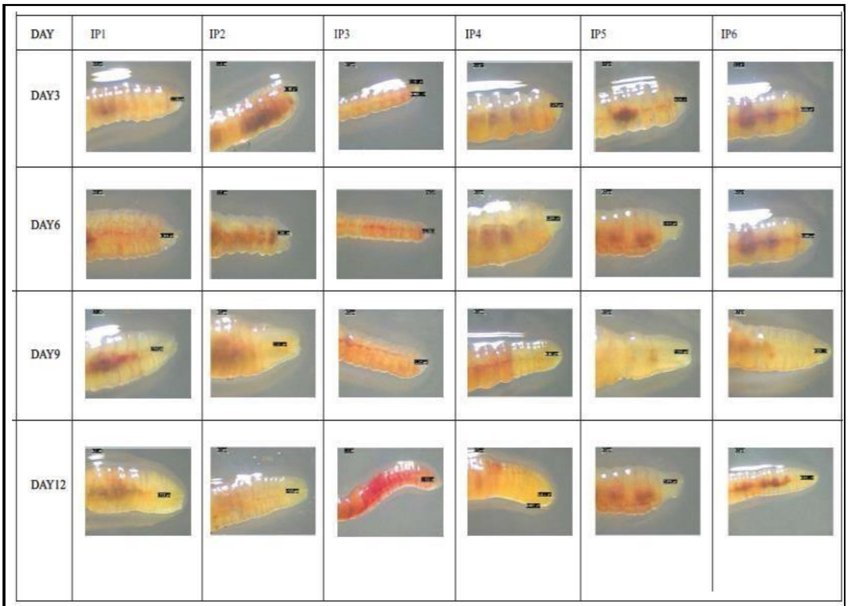

Fig. 2. B Image showing the regenerated posterior region of Immature *Eisenia fetida* on day3, 6, 9 and 12

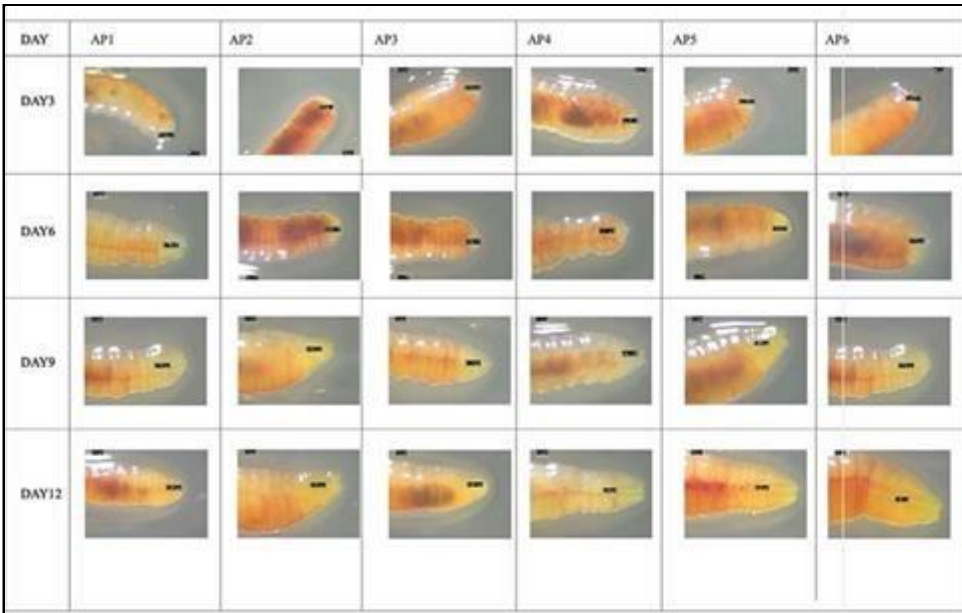

Fig. 2C. Image showing regenerated posterior region of adult *Eisenia fetida* on day 3, day 9 and day 12.

Fig. 2. (A - C) showing the regenerated amputed regions of Immature and mature *Eisenia fetida* on day 3, day 9 and day 12; Figure 2(A): Immature Anterior (IA) group ;Figure 2(B): Immature Posterior (IP) group; Figure 2(C) :Adult Posterior group in day 3,6,9 and 12 using the software Leica application suite and plotted graph using their average regeneration.

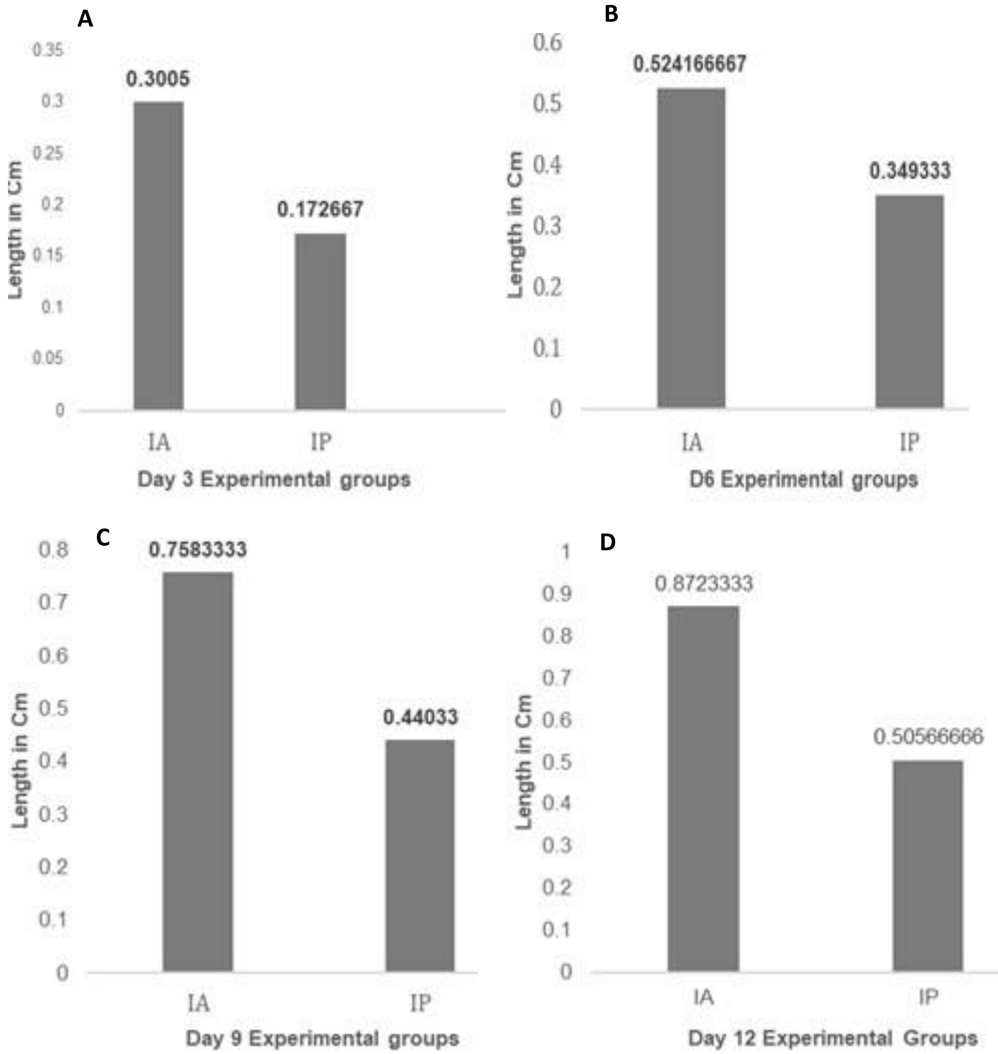

Fig. 3. (A-D) Graph showing difference in average regeneration in Immature Anterior and Immature Posterior groups of *Eisenia fetida* in day 3,6,9, and 12.

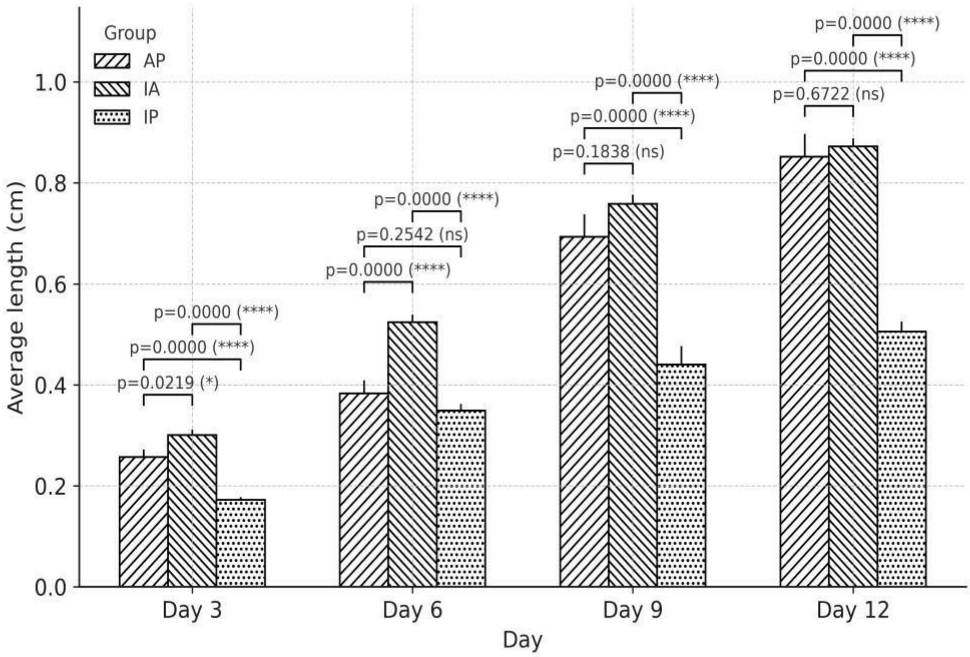

Fig. 4. Graph showing difference in average regeneration Bars show mean regeneration length (cm) ± SEM (upper-only) for AP, IA, and IP at Day 3, 6, 9, and 12. Pairwise comparisons were performed within each day using two-sided Welch's t-tests; p-values are shown above brackets (significance codes: ns ≥ 0.05; * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001). Across time, IA exhibited the highest mean growth and IP the lowest; AP vs IA was not significant at Day 9 and Day 12, whereas AP vs IP and IA vs IP were significant at all days ($p < 0.05$).

4 Conclusion

Immature nematode tissues and larval secretions has revealed promising wound-healing properties by enhance collagen deposition, activating growth promoting cytokines and tissue regeneration across several species. The study of regeneration in immature *Eisenia fetida* suggests that regeneration before attaining sexual maturity is comparable with the regeneration of its adult. At the same time the regeneration potential is polarized giving a better growth in the anterior blastema than posterior segments.

This indicated that, even though the overall regenerative capacity is not as robust or polarized as that of adults, the immature worms regenerated anterior segments relatively faster compared to posterior segments. These projects the better utility of blastema tissues formed from the anterior region of amputated immature earthworms over mature ones in wound healing applications or regenerative medicine. In conclusion, the study paves a promising foundation for further research into earthworm-derived bioactive compounds, Nano formulations and their clinical applications in regenerative medicine and wound care.

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