

**Augmentation of regeneration potential using Low energy electric shock in
earthworm, *Eudrilus eugeniae***

*Submitted in partial fulfilment of the requirements for the award of Master of
Science in Medical Biotechnology and Clinical Research*

By

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INTERNATIONAL RESEARCH CENTRE**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ms. NIVEDHA B (42101010)** who carried out Project entitled "**Augmentation of regeneration potential using Low energy electric shock in earthworm, *Eudrilus eugeniae***" under the supervision of **Dr. S. JACKSON DURAIRAJ**, Associate Professor (Research) from September 2023 to April 2024.

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DECLARATION

I, **Ms. NIVEDHA B (42101010)** hereby declare that the Project Report entitled "Augmentation of regeneration potential using Low energy electric shock in earthworm, *Eudrilus eugeniae*" done by me under the guidance of **Dr. S. JACKSON DURAIRAJ, Associate Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

DATE: 18/04/2024

PLACE: Chennai

A handwritten signature in blue ink, reading 'Nivedha B.', is written over a horizontal line.

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to **The Board of Management of Sathyabama Institute of Science and Technology**, Chennai, for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

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ABSTRACT

Biological electric field is basic for many body functions like conducting nerve impulses, heartbeat, metabolism, regulating tissue and organ functions. The present study aims to understand the effect of low energy electric shock on regeneration ability and their underlying regulatory proteins. Amputation study, blocking epimorphosis, histology, western blotting and in-vitro wound healing assay are used to assess the functional effect of low energy electric shock upon regeneration. Amputation studies following low energy electric shock confirms, the regeneration was accelerated to 1.5 to 2 folds on 3rd, 5th, 7th and 9th day of post amputation. The histological studies reveals that the internal structures and organogenesis are augmented and visibly observed on electric shock treated 8th day regenerating worms than the control worm. Following 2mM thymidine injection, the epimorphosis mechanism was blocked which is evident with the 1/3 level inhibition of regeneration kinetics. Combinatorial effect of 2mM thymidine injection and low electric shock represents augmented regeneration ability which directly represents, low energy electric shock accelerates the morphallaxis phenomenon of regeneration. Western blotting assay shows that the expression of VEGF, YAP, Wnt-3a are increased notably and c-Myc and COX-3 shows marginal overexpression upon treatment with low energy electric shock and following regeneration. In thymidine and electric shock treated worms, VEGF, YAP, Wnt-3a and c-Myc are overexpressed than only with electric shock treated worms. The in-vitro wound healing assay following electric shock represents augmented wound closure ability. In overall, our results concludes that low energy electric shock induces wound healing, regeneration, angiogenesis and associated key proteins which would be an ideal target for regenerative therapy in near future.

Keywords : Earthworm, Electric shock (1.5V), Thymidine, Wound Healing.

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LIST OF ABBREVIATIONS

APS	Ammonium Per Sulphate
BSA	Bovine Serum Albumin
Ca ²⁺	Calcium
Cl ⁻	Chloride
EA	Electro-Acupuncture
ECL	Enhanced Chemiluminescence
HVGC	High-Voltage Galvanic Current
K ⁺	Potassium
Na ⁺	Sodium
NMES	Neuromuscular Electrical Stimulation
PBS	Phosphate Buffered Solution
PVDF	Poly (vinylidene fluoride)
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TBST	Tris-buffered Saline with Tween 20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TENS	Transcutaneous Electrical Neuromuscular Stimulation

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AUGMENTATION OF REGENERATION POTENTIAL USING LOW ENERGY ELECTRIC SHOCK IN EARTHWORM, EUDRILUS EUGENIAE

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BSA	Bovine Serum Albumin
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EA	Electro-Acupuncture
ECL	Enhanced Chemiluminescence
HVGC	High-Voltage Galvanic Current
K ⁺	Potassium
Na ⁺	Sodium
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CHAPTER 1

INTRODUCTION

1.1 ELECTRIC SHOCK IN OUR HUMAN BODY SYSTEM

Electricity is required for the nervous system to send signals throughout the body and to the brain, making it possible to make us move, think and feel. Electrical currents are the specific function of our cells. The body of scientific knowledge regarding human interaction with electricity is constantly growing. Human skin has shown to be a naturally protective barrier against the flow of electricity, which is to our advantage. Humans have the potential to integrate with the electric circuits and processes in their surroundings given the correct circumstances. Electrical current passing through tissues is usually what causes electric shocks in humans. The conductive qualities of tissues and the presence of electrolytes in body fluids like blood allow them to transmit electricity quite effectively. People are, in the best sense of the word, energized. Our bodies generate energy with muscle contraction, and cell response. The average power produced by the human body at rest is 100 watts. It reaches 300–400 watts during physical activity. This equates to burning two thousand calories every day. Different tissues have varying levels of conductivity, therefore some regions of the body may experience preferential current flow. About 10 muscle cells contract and relax rhythmically to produce the heart's pumping motion. This mechanism is governed by a complicated pattern of electrical activation. Cardiac function depends on electrical activity, and disruptions in this activity are intimately associated with numerous cardiac problems. There is an abundance of information regarding the small-scale processes that take place in the cells as well as the organ-level patterns of electrical activation. The electrical activity of the heart is a thoroughly researched process. Many physiological processes, such as nerve signaling, muscle contraction, and cell communication, depend on this electricity. The action potential is one of the most basic types of electricity. Rapid variations in the electrical charge across the cell membrane of muscle and neuronal cells are known as action potentials. Movement of ions, such as sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and chloride (Cl^-), across cell membranes is the main cause of electric currents in the

body. Entered and exited cells by means of specialized channels, pumps, and exchangers, these ions can be positively or negatively charged. Electric currents in neurons are essential for signal transmission throughout the neurological system. An electrical gradient, or membrane potential, is maintained by the transport of ions across cell membranes and is critical to both cell function and general health. The skin contains more than 99 percent of the body's resistance to the flow of electric current. Electric currents are essential to the human body's operation because they enable many different physiological activities, including muscle contraction and nerve communication.

1.2 ELECTRIC IMPULSES THERAPY

The electrical impulse is what initiates the mechanical force, the electrical event occurs before the heart contracts. Neurons use the action potential to conduct electrical impulses. The passage of positively charged ions across the neural membrane causes this event. Electrical stimulation is an amazing technology that has the potential to revolutionize how we restore and enhance the human body. Electrical stimulation is the process of stimulating muscles by passing electrical currents through the skin. This could promote tissue healing, lessen discomfort, and build muscle.

An electrical current can be used to increase blood flow, inhibit pain signals, and strengthen muscles.

1.2.1 Types of electric stimulation technology

- Transcutaneous electrical nerve stimulation
- Iontophoresis
- Neuromuscular electrical stimulation
- Russian stimulation
- Interferential current
- High – Voltage Galvanic Current.

Transcutaneous electrical nerve stimulator

Transcutaneous electrical neuromuscular stimulation (TENS) is a pain management technique used to treat both acute and chronic pain. Your body will send pain signals to your brain, and the electricity's intensity will be changed to block those signals.

Iontophoresis

Iontophoresis most likely makes use of medication to reduce muscle spasms or inflammation. Different drugs are forced into your body through your skin by the electrical current. Iontophoresis is a technique that is used with various medications to achieve various objectives.

Neuromuscular electrical stimulation (NMES)

Neuromuscular electrical stimulation (NMES) to contract a single muscle or a group of muscles. A suitable muscle fiber can be activated by the physical therapist by applying electrodes to different parts of the skin. The contraction of the muscle not only improves muscle function but also increases local blood flow. This promotes healing of the wound.

Russian stimulation

It enhances the contraction of your muscles. Simply put, Russian stim makes use of a distinct waveform that might be a little easier for you to handle.

Interferential current

Usually, a crisscross pattern of four electrodes is used for interferential current. By using an alternating current, one can reduce pain, relax spasms in the muscles, or enhance blood flow to different parts of the body. It's frequently applied to lower back pain.

High-voltage galvanic current (HVGC)

High-voltage galvanic stimulation (HVGC) is a technique that targets deep tissue penetration by using low-frequency, high-voltage electricity. It helps with pain relief, blood flow enhancement, muscular spasm relief, and joint mobility improvement.

Electro-acupuncture:

Electro-acupuncture does not require the use of needles, it is remarkably comparable to traditional acupuncture in that the same locations are frequently used throughout treatment. A little current, either DC or AC, is given via point stimulation directly into acupuncture/trigger points during electro-acupuncture (EA), a type of acupuncture electro-therapy, without the need for skin puncture. The advantage of EA over traditional acupuncture is that a practitioner does not need to be as accurate as they would be with needles because practically all available devices contain a "point locator" that "detects" trigger points and acupuncture by measuring variations in skin resistance.

1.3 REGENERATION AND THEIR PHYSICAL FACTORS

Repairing or replacing damaged or absent cells, tissues, organs, and even whole body parts to their original state of function is the process of regeneration. Every living thing, including plants and animals, has the capacity to regenerate in some amounts as a part of their normal organ and tissue maintenance. When an organism is injured, it can regenerate by initially generating a mass of different types of proliferating cells, including adult stem cells. The structure is referred to as a blastema in certain animals, including salamanders. After an amputation, the skin, scales, muscle, bone, or cartilage required for the growth of a new leg, fin, or tail are formed by the fast division of blastema cells. Adult stem cells have limited regeneration capacities because they can differentiate into the cell types of the tissue or organ in which they are situated. This process makes it possible to generate the portions that age or are injured and require replacement. Cells were compelled by physical causes to reprogramme their functions in order to dynamically adapt to their

surroundings. Advances in tissue engineering through biotechnological understanding of the physiological principles of tissue regeneration and repair.

1.3.1 Types of regeneration:

- Epimorphosis
- Morphallaxis
- Compensatory regeneration

Epimorphosis: The growth of new cells from the surface of the wounded portion causes this kind of regeneration. At the lost or injured portion, the adult structure dedifferentiates, multiplies to produce more dedifferentiated cells, and eventually forms blastema.

Morphallaxis: The primary process of regeneration is the reorganization of the preexisting tissues. The morphallaxis is characterized by a low rate of new cell growth. Every part of the body remodels into a new organism during this kind of regeneration.

Compensatory regeneration: This kind of regeneration happens when an organ is damaged in part and is restored by the multiplication of the original tissue. The cells in this split, but they do not dedifferentiate. While similar cells are generated, they do not combine to form an undifferentiated tissue mass.

The different kinds of physical stimulation :

- Mechanical stimulation
- Electrical stimulation
- Thermal stimulation
- Ultrasound stimulation
- Light Therapy.

Mechanical stimulation : Cell behavior and tissue regeneration can be directly impacted by mechanical forces such stretching, compression, and shear stress.

Electrical stimulation : Electrical signals have the ability to control tissue regeneration and alter cellular activity.

Thermal stimulation : Temperature variations can impact tissue regeneration and cellular metabolism.

Ultrasound stimulation : Tissue-penetrating ultrasound waves can cause a variety of biological responses that encourage tissue regeneration.

Light therapy : Light-based treatments that affect cellular activity and tissue regeneration include photo biomodulation and laser therapy.

1.4 REGENERATION IN ELECTRIC SHOCK

The potential of electric shock to promote healing or regeneration in living things has been investigated in some settings, especially in relation to medical interventions. The recovery of muscle sensitivity and functionality following a nerve damage is accelerated by low-energy shock wave therapy. The impact of electric shock on epithelial regeneration, given that evidence suggests that shock circumstances have a significant impact on mitotic activity. Many studies have been conducted on low-frequency electrical stimulation of the proximal nerve stump following initial repair. The regenerative effects of acute, short, low-frequency electrical stimulation on axonal regeneration have a biological foundation. Recent clinical uses of the electrical stimulation procedure to show this modality's substantial translational potential for healing lesions to peripheral nerves. Low-frequency electrical stimulation has been shown to maximize functional recovery in a variety of peripheral nerve injuries, and it can effectively encourage axonal regeneration following surgical repair. Studies have looked into the possibility of electrically stimulating damaged peripheral axons, with promising results for improved nerve regeneration and functional recovery. Electrical stimulation of a peripheral nerve is used to encourage nerve regeneration.

1.5 REGENERATION IN EARTHWORM *EUDRILUS EUGENIAE*

Eudrilus eugeniae is a clitellum-dependent earthworm that requires intact clitellum segments for its survival and regeneration. The 13–17 segments of *E. eugeniae* contain the clitellum, a discolored, somewhat enlarged organ that creates the cocoon

that contains the earthworm's eggs. In addition to secreting albumin, the clitellum includes two copulation setae. According to our research, during regeneration, the cells in the clitellar segments migrate. The stem cells that are migrating clitellar cells should finally reach the site of amputation and enter the posterior regenerative blastema. The ability to regenerate varies among species of earthworms, so it's critical to investigate the mechanism underlying the process. It's interesting to note that clitellum segments may support regeneration in earthworms. Different species of earthworms have varying capacities for regeneration, thus it's critical to investigate the mechanisms underlying these processes. Stem cells are responsible for the development of regeneration and play a critical role in it. The Suthira Owlarn assumption describes how regeneration begins in amputated tissue conditions. According to their assumption 1, every time a wound heals, it is referred to as an H-wound, and every time a wound regenerates, it is referred to as an R-wound. The indications of H-wound and R-wound are predominantly used in the healing and regeneration mechanisms. It was looked at how *E. eugeniae* regenerates its mouth and anus. The gut microbiota of the earthworm undergoes riboflavin synthesis in response to hunger, and this activity was observed in the worm for five days. During the hungry phase of early regeneration, *Bacillus endophyticus*, one of the bacteria that produces green riboflavins, has been observed in the intestine by 16S rRNA sequencing to maintain homeostasis.

CHAPTER 2

LITERATURE SURVEY

Regenerating both anterior and posterior segments is a capability of *Eudrilus eugeniae* (Sayan Paul et al., 2020). Amputated earthworms were housed in habitat soils and decomposing cow dung in different experimental group (Banik,D et al., 2017). The posterior regeneration lengths were not connected with either the number of remaining segments or the site of the amputation; the anterior regeneration lengths were correlated with the amputation position but not with the number of remaining segments (Nengwen Xiao et al., 2011).The earthworm would effectively survive even without its brain if the anterior portion of the worm—which comprises the brain—were removed (Nino Gopi Daisy et al., 2016). According to recent research, free-floating coelomocytes move early to the site of wounds in earthworms and, through their phagocyte activity and distribution of nutrients and growth-stimulating chemicals, play a role in the creation of the wound plug and subsequent tissue rearrangement (D Banik et al., 2016). 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 (Kamarajan Rajagopalan et al., 2022).

Comparing soil aggregates and casts in terms of swelling, water-stable aggregation, and dry and moist porosity allowed researchers to assess the impact of earthworms (O.Larink et al.,2001). Earthworm abundance and ecological diversity interact to alter soil functioning; two distinct mesocosm studies were set up to investigate this relationship (N. L. Schon et al., 2021). It was discovered that the epithelial layer of the skin is the only place where the protein is expressed. The tip of the blastema was found to have the highest level of protein expression during regeneration (Elaiya Raja Subramanian et al., 2017).The process of cell division in eukaryotic organisms is intricate, and little is known about the part fatty acids play in stem cell-mediated mitosis (Chandran Rajesh et al., 2021). Stem cells have the ability to release strong combinations of trophic factors that alter the environment's molecular makeup and cause resident cells to react. In light of this new understanding, efforts are currently

being made to better understand, enhance, and utilize stem cell paracrine pathways for tissue regeneration (Priya R Baraniak et al., 2009). Tissues that continuously lose cells due to senescence and maturation are renewed by stem cells (Donald Orlic et al., 2002). Coelomocytes assume pivotal functions in wound healing and regeneration subsequent to physical trauma. The phylum of annelids has very different regenerating capacities (Gerhard Paul Aigner et al., 2023).

The impact of earthworm extract (EE) on the skin wound healing process in comparison to Jingwanhong (JWH), a reputable and extensively used ointment for external wounds, and secondly investigate EE's possible mechanism of action (Zhenhan Deng et al., 2018). Although they are mutually exclusive biological processes, cell division and proliferation are necessary. Interactions between these mechanisms limit the growth and survival of cells carrying cancerous mutations while fostering a balance between proliferation and death (Ming Guo et al., 1999). It is clear that more new cells and their differentiation into tissue cells are required for the production of a greater number of regenerated segments (Mahmoud H. Abufadda et al., 2021). Researchers have been studying the medical effects of earthworm extracts for the past few decades, and they have been found to have antibacterial, antipyretic, anti-inflammatory, fibrinolytic, and antioxidant characteristics. Because a variety of immune protective leukocytes are produced and released, earthworms have both innate and adaptive immunity (Dominic Augustine et al., 2019). The important genes that are differently expressed and the pathways and functions that are linked to them that regulate the clitellar tissue alterations in the earthworm *Eudrilus eugeniae* during both anterior and posterior regeneration (Sayan Paul et al., 2022). During the early stages of regeneration, there was a higher expression of TCTP, which eventually decreased. Regeneration was stopped by the selective and pharmacological inhibition of TCTP (Elaiya Raja Subramanian et al., 2017). The antiproliferative impact of ECF of several earthworm species found in this work is encouraging and calls for advanced anticancer research on oral cancer cell lines (Dominic Augustine et al., 2017). By applying different concentrations of chloroauric acid, gold nanoparticles were created by the citrate reduction technique. Using UV-Vis, SEM, and AFM investigation, the generated particles were described (Antony V. Samrot et al.,

2018). CF increased the resilience of rice seedlings against the fungal disease *R. solani* and stimulated rice seedling growth. Research should be done to determine the degree of protection against this fungal infection provided by combining transgenic rice plants expressing PR proteins with vermicompost and CF (Ganapathy Raja Vadivu Nadana et al., 2020). A defining feature of the tissue remodelling processes that underpin cancer invasion, wound healing, and embryonic morphogenesis is the collective movement of cells as a cohesive unit (Peter Friedl et al., 2009). Promote the migration of endogenous reparative cells to restrict inflammatory cell invasion and potentially improve the development and regeneration of joint tissue (Feini Qu et al., 2019). The impacts on Schwann cell migration, the different treatments effects on non-neuronal cells were assessed using immunohistochemistry staining with S-100 antibodies (S. B. Bailey et al., 1993). Improving directed cell migration and migration inhibitory treatments against inflammatory illnesses and cancer may be possible with a deeper understanding of this molecular route and other similar pathways in regenerative medicine (Beatriz de Lucas et al., 2017). Understanding the roles of stem cells in both normal development and various disease scenarios requires an understanding of CXCR4 signaling (Richard J. Miller et al., 2009). Efforts have been made to colonize pre-28-somite hepatic explants in vitro utilizing an external supply of yolk-sac-derived HSC, with the emergence of granulopoiesis serving as a sign of successful recombination (G. R. JOHNSON et al., 1975). The galvanotactic response of cells can be influenced by cell-specific parameters such as cell type and differentiation status, time in culture, cell-matrix, and cell-cell contact (Stephanie N. Iwasa et al., 2017). Hematopoietic cells proliferate when hematopoietic growth factors are mobilized; this might indirectly alter the local cytokine and chemokine milieu, the expression of adhesion molecules, and ultimately the mobilization of hematopoietic progenitor cells (Robert Möhle et al., 2009). The amount of knowledge on stem cell research that has been done on cellular migration and angiogenesis in order to examine the viability of various tactics and cell type variants (Aziz et al., 2020). Cytokines promote migration toward SDF-1, in vivo homing, and repopulation, as well as surface expression of CXCR4. We talk about the pleiotropic functions of SDF-1/CXCR4 interactions in transplanted immune-

deficient mice for human stem cell migration, development, and repopulation (Tsvee Lapidot et al., 2006). A stem cell population moves net away from its source due to dynamic interactions between directed migration, proliferation, and diffusion (Eric D. Miller et al., 2011). That polarization of cell membrane proteins and activation of intracellular signaling molecules are involved in the process of EF-directed axonal development and cell migration, albeit the exact mechanism is yet unknown (Li Yao et al., 2016). The basic characteristics of hematopoietic stem and progenitor cells has expanded the possible uses of these cells in medical interventions (Motonari Kondo et al., 2003). Based on the results of the toxicity experiment, we hypothesized that the concentration of malathion pesticide would cause a little increase in its toxic level (Archana Jeyaprakasam et al., 2021). Stem cells enable the worm to regenerate lost organs, the anterior-posterior axes, which guide the formation of fetal organs along this axis, have the potential to be renewed. The nervous, digestive, and respiratory structures, on the other hand, are absent from the worm's tail segments (Latifeh Karimzadeh et al., 2014). Antioxidative activity served as support for the anabolic response that maintained the mitogenic activity, so averting needless oxidative reactions during wound healing, regeneration, and cell proliferation following surgery or injury (Kasahun Gudeta et al., 2020). The antioxidant capacity of the earthworm's defensive mechanisms was negatively impacted by CPF (Arezoo Torabi Farsani et al., 2021). The electrical treatment decreased the biomass and number of earthworms, which goes against the notion of soil conservation techniques in agriculture (Th. Ruf et al., 2023). Earthworms undergo oxidative damage and apoptosis, which stresses them and interferes with their regular physiological processes (Jingwen Zhang et al., 2023). It is hypothesized that Ef and Ea have different mechanisms governing the expression of genes linked to stress and immunity; hence, additional research on these genes and how they interact in hybrids is required (Marta Jaskulak et al., 2021). The bioworld's electrical conduction mechanisms suggest that it is possible to incorporate the fundamental bioworld principles into created items (Allan P. Wandall et al., 2022).

In *E. eugeniae*, the evolutionarily conserved gene TCTP was found and sequenced. Its homology with vertebrates and invertebrates is considerable. Early regeneration

was associated with a greater expression of TCTP, which subsequently diminished. Regeneration was stopped by pharmacological and targeted inhibition of TCTP. We can infer from the experiment's findings that TCTP is essential for the cell differentiation process in the clitellum (Elaiya Raja Subramanian et al., 2017). Ethanol-extracted fractions improved the worms' ability to regenerate. Our findings show that *Acorus calamus* rhizomes contain proliferative and antimitotic chemicals. Therefore, we propose that earthworms may be employed as an *in vivo* screening system for proliferative and antimitotic substances originating from unidentified origins (Kamaraj Rajamanikkam et al., 2019). In order to optimize BMSC behaviour, our study identifies the type and working intensity of fESW-derived mechanical stimuli. The patient, the experimental setup, and—most importantly—the ESW device employed will all influence the ideal application time and intensity. This study describes the ways in which hBMSCs respond to mechanical stress by altering their migration, proliferation, and survival as well as, in conjunction with other research, their paracrine and proteolytic activities (Frank suhr et al., 2013). Stem cells are responsible for the development of regeneration and play a critical role in it (Thorgeirsson S.S., 1996).

The Suthira Owlarn assumption describes how regeneration begins in amputated tissue conditions. According to their assumption 1, every time a wound heals, it is referred to as an H- wound, and every time a wound regenerates, it is referred to as an R- wound. The indications of H- wound and R- wound are predominantly used in the healing and regeneration mechanisms. Assumption 2 states that only when the amputated tissue contexts have been repaired might help the regeneration process to begin; without the missing tissue contexts, just the wound-healing process will be performed (Owlarn s. et al., 2017). Animal adaptability and organogenesis are important events in regeneration, (Subramanian et al., 2016). The greater parts of the amputated segments (from segments 15 and 30 to the anus) that did not have an intact clitellum healed the incision, but did not regenerate. Alkaline phosphatase (ALP) signals were detected in the gut epithelium, clitellum, and anterior tip of control worms. In contrast, amputation reduced the enriched clitellum signals, but left the amputation site and regenerating blastema with strong signals. Remarkably, the

regeneration blastema's differentiation process started on days 3 and 4, which is why blastemal tips did not exhibit ALP signals during those days. In conclusion, the use of a foldscope microscope revealed that ALP activity showed the clitellum's function in the regeneration mechanism (Jackson Durairaj Selvan Christyraj et al., 2020). When the regenerated tissue was examined for total antioxidant capacity and free radical scavenging activity, it produced impressive results with high levels of both. Using the DPPH method, it was discovered that the free radical scavenging activity of *Eudrilus eugeniae* tissue increased as sample concentration increased. *Eudrilus eugeniae* is therefore a good source of antioxidant potential and free radical scavenging activity, according to the results of the current investigation (Puspa Reddy et al., 2019).

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To enhance the regeneration ability using Low energy electric shock in earthworm and understanding its mechanism.

3.2 OBJECTIVES

1. To Standardize the dosage of Low energy electric shock in earthworm, *Eudrilus eugeniae*.
2. To analyze the regeneration kinetics following Low energy electric shock.
3. To determine the expression pattern of key regenerative proteins following Low energy electric shock.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

- 1.5V Battery (Eveready)
- Red wire – Positive
- Black wire – Negative



Fig 1.1 Electric shock is given by the carbon zinc battery, Heavy duty, D size, 1.5V.

4.2 EARTHWORM MAINTENANCE

The earthworm, *Eudrilus eugeniae* are obtained from the stock culture that are maintained in the confined environment of Molecular Biology and Stem Cell Research Laboratory, International Research Centre, Sathyabama Institute of Science and Technology, Chennai as described earlier . Worms are cultures in the plastic tub that contain soil, leaf litters and cow dung. The moisture condition of the worm was maintained in between 22-25°C with good ventilation. Every week, jaggery filtrate was sprayed over the earthworm bed for effective culturing of the worm and once in a month, the vermicompost that formed in the culture bed is replaced with fresh feed. The day and dark rhythm were maintained 12 hrs throughout the experiments.

4.3 ELECTRIC SHOCK AND 2 mM THYMIDINE INJECTION

4.3.1 Worm selection

The mature worms which are evident with the buffy clitellum with the average weight of around 900-950 mg were chosen for the present experiments. Worms are divided into 4 groups namely as control, electric shock treated, 2 mM thymidine treated and 2 mM thymidine + electric shock treated.

4.3.2 Shock Treatment

For performing electric shock, initially the setup is made by connecting the cathode and anode of Eveready carbon zinc battery (Heavy duty, D size) of 1.5 V to a circuit using an electric wire. group of worms are picked separately from the culture bed and washed thoroughly in a distilled water, dried using filter paper and kept inside the glass beaker. The cathode and anode end of the electric wire that are connected to the 1.5 V battery is placed simultaneously over the worms for approximately 40 times in a minute to evenly distribute the electric shock to the individual worms in a group. Following electric shock, worms are carefully placed in the culture bed and monitored every 24 hrs.

4.3.3 2 mM Injection

To block cell proliferation, 2 mM thymidine was prepared in distilled water and injected into the earthworm, *E. eugeniae*. The worms are initially placed in ice cold water for few seconds to cease their active movement, then the worms are placed in dorsal orientation and 2 mM injection (10 μ l 2mM thymidine/gram of worm) were made in the juncture of segments (25-26th) of the worm. Following injection, the worms are returned to the culture bed, the 2 mM injection was continued for every 24 hrs to synchronize and arrest the cell cycle at G1/S boundary.

4.3.4 2 mM Thymidine injection + Shock treatment

To block the cell proliferation we injecting the 2 mM thymidine was injected. After 2 hrs of injecting the 2 mM thymidine, group of worms are picked separately from the culture bed and washed thoroughly in a distilled water, dried using filter paper and

kept inside the glass beaker. The cathode and anode end of the electric wire that are connected to the 1.5 V battery is placed simultaneously over the worms for approximately 40 times in a minute to evenly distribute the electric shock to the individual worms in a group. Following electric shock, worms are carefully placed in the culture bed and monitored every 24 hrs.

4.4 AMPUTATION STUDIES:

Following electric shock or 2 mM thymidine injection or 2 mM thymidine injection + electric shock for initial two days, the worms are amputated at the junction of 9-10th anterior segments on 3rd day. Before amputation, the worms are initially immobilized by dipping in the cold water for 30 seconds and aseptically amputated using the sterile surgical blade by placing it in the dissection board. Following amputation, the blood that oozes out from the wound are washed out using tap water and the worms are placed back in the culture bed. It is to note that following amputation likely the worm that are receiving injection or electric shock is continued for another nine days. The amputated worms are observed for the regeneration ability on 3rd, 5th, 7th and 9th day of post amputation and their respective images are captured under the measuring scale.

4.5 PROTEIN ISOLATION:

Control and regenerated portion of the treated worms following the 7th day of post amputation along with one adjacent segment are dissect from the worm. After dissect from the worm, the regenerate portion will be washed with distilled water and 2 mins in 1X PBS. Then the sample homogenized immediately with 2X sample buffer in an ice-cold condition. The processed samples are heated at 100°C for 5 mins and further estimated using Lowry method of protein estimation.

4.6 SDS-PAGE

SDS PAGE is a method of separating proteins by their molecular weight. Predominantly the study of the movement of charge molecules in an electric field is known as electrophoresis. The strength of the field, net charge, size and shape of the molecules, as well as the ionic strength, viscosity, and temperature of the medium in

which the molecules are traveling, all influence their rate of movement. Larger molecules, such as proteins, are commonly separated using polyacrylamide gels. Proteins are sorted based on their resistance to the gel matrix, with smaller proteins having less resistance and larger proteins having more. When proteins are electrophoresed in the presence of a detergent sodium dodecyl sulfate and a reducing agent -Mercaptoethanol, the detergent can bind to the protein backbone and the denaturing effect of SDS is very strong, disrupting the secondary, tertiary, and quaternary structure of the protein, resulting in a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol aids in the denaturation of proteins by decreasing all disulfide linkages (both inter-disulfide and intra-disulfide bonds). Polyacrylamide forms a mesh-like matrix that aids in protein separation. During PAGE electrophoresis, the proteins' linear or primary structure was stacked or placed in the appropriate order; as the proteins reached the separating gel, they began to separate based on their size.

4.6.1 Materials

Eppendorfs; Micro-pipettes; Micro-tips; Centrifuge; Water bath; Vortex and Distilled water.

4.6.2 Chemical preparation of SDS-PAGE

30% Acrylamide and 0.3% Bis acrylamide:

Acrylamide	- 30 g
N,N' Methylene Bisacrylamide	- 0.3 g
Make up into 100 ml with distilled water.	

4.6.2.1 4X Separating Buffer

Tris HCL	- 18.2 g
Distilled water	- 60 ml
pH was adjusted to 8.8 with 10N NaOH	
Sodium Dodecyl Sulphate (SDS)	- 0.4 g
Make up into 100 ml with distilled water.	

4.6.2.2 4X Stacking Buffer

Tris HCl	- 6.05 g
Distilled water	- 40 ml

pH was adjusted to 6.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4 g

Make up into 100 ml with distilled water.

4.6.2.3 2X Sample Buffer

Tris stacking buffer (pH 6.8) - 25 ml

Sodium Dodecyl Sulphate (SDS) - 4 g

20% Glycerol - 20 ml

Make up into 100 ml with distilled water. During protein sample preparation, 5% β -mercaptoethanol and during loading 0.001% (w/v) of Bromo-phenol blue was added.

4.6.2.4 10% Ammonium Per Sulphate (APS)

APS - 100 mg

Make up into 1 ml with distilled water.

4.6.2.5 1X Running Buffer

Tris HCl - 3.94 g

Glycine - 15.014 g

SDS - 1 g

Make up into 1L with distilled water.

4.6.3 Staining Solution

Coomassie Brilliant Blue R-250 - 200 mg

Methanol - 50 ml

Glacial Acetic acid - 7 ml

Make up into 100 ml with distilled water.

The above mixture was filtered using Whatman No 1 filter paper.

4.6.4 Destaining Solution

Methanol - 30 ml

Glacial Acetic acid - 7 ml

Make up into 100 ml with distilled water.

Table 4.1 Separating gel concentration.

Sl. No	Stock Solution	5%	6%	7%	7.5%	8%	9%	10 %	12 %	13 %	15 %
1.	30% Acrylamide + 0.3% Bis-acrylamide (ml)	5.0	6.0	7.0	7.5	8.0	9.0	10.0	12.0	13.0	15.0
2.	4X Tris Separating Buffer (ml) pH 8.8	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
3.	Distilled water (ml)	17.5	16.5	15.0		14.5	13.5	12.5	10.5	9.5	7.5
4.	10% APS (μl)	200	200	200	200	200	200	200	200	200	200
5.	TEMED (μl)	20	20	20	20	20	20	20	20	20	20

Table 4.2 Stacking gel concentration.

Sl.No	Stock Solution	5 ml	10 ml	15 ml	20 ml
1.	30% Acrylamide + 0.3% Bis-acrylamide (ml)	0.65	2.5	3.75	5.0
2.	4X Tris Stacking Buffer (ml) pH 6.8	1.25	2.5	3.75	5.0
3.	Distilled water (ml)	3.05	5.0	7.5	10.0
4.	10% APS (μl)	100	100	100	100
5.	TEMED (μl)	10	10	10	10

4.6.5 Preparation of gel

The glass plates were cleaned by using ethanol and manually assembled. Glass plates fixed in the proper equalize manner with each corners covered by using spacers so that used as a primary baseline on gel which helps in a process of agarose sealing or second level baseline system. 1% agarose was prepared and boiled until liquidy then corners sealed in both surface and bottom area of spacer. Separating gel was manually prepared and loaded on the gel with careful manner. The gel was overlaid with isopropyl alcohol so that it excludes the air. Then IPA was removed and washed with distilled water, after drain stacking gel was prepared and added, instantly comb was fixed on the stacking gel. The gel was allowed few minutes to solidifying then comb were carefully removed and wells manifestly washed with water to eliminate the bubbles and the bottom of the spacer were carefully removed and setup was fixed in the bottom chamber which carried 1X running buffer as well as upper chamber.

4.6.6 Sample loading and electrophoresis

Protein sample of 10 µg and the appropriate molecular markers were loaded in the wells. The apparatus was connected to the power pack with proper polarity and 5 to 6 v/cm was applied. After electrophoresis, the glass plate assembly was taken from the buffer chamber. The spacer was gently twisted so that the upper glass plate pulls away from the gel.

4.6.7 Staining and Destaining the gel

The gel was carefully taken out from the glass plate and the stacking gel was removed by using cleaver. Separating gel was removed by gently grasping the two corners of the gel and placed in the container containing the commassie brilliant blue R-250 (stainer solution). The gel should be fully submerged in the staining solution for 30 minutes by gentle agitation on a Rocker. Then it was washed with distilled water and transferred to destaining solution until protein bands are visualized clearly. Commonly first destainer wash is about 30 minutes then second wash is about overnight.

4.7. WESTERN BLOTTING

4.7.1 Running the gel

- Prepared 8% gel for high molecular weight and prepared 12% gel for low molecular weight, and then place it in SDS page unit.
- Add 1X Running buffer, and then add the protein ladder in the first lane of the gel for molecular weight determination.
- Then load the protein sample for 80µg concentration in each well. Set the gel to 65V until the protein run successfully.

4.7.2. Buffer preparation

10X Transfer Buffer (Stock)

Glycine - 72.1g

Tris-base - 15.2g

Distiller water- 500ml

The 10X transfer buffer was stored in the room temperature, and during blotting the 1X transfer buffer were prepared from the 10X in the ratio of preparation viz.,

For Total volume 1 liter

100ml - 10X Transfer Buffer

200ml - Methanol

700ml - Distilled water

4.7.3 Transfer into PVDF membrane

Gel were transfer in to the 1X transfer buffer for 15 minutes. Blotting apparatus was soaked in to the transfer buffer, from the basement sponges and front the bottom onwards tissue papers and then gel was placed on it. Activate the PVDF membrane with carbinol for 3 minutes and then the membrane was transfer into the transfer buffer. Smooth surface of the PVDF membrane was placed on the gel. Then double tissue papers and sponge were applied and closed by hands. Blotting apparatus was

transferred into the box contains 1X transfer buffer with ice packs maintained in the temperature 4- 8°C.

4.7.4. Blocking

Blocking buffer was used to block all proteins which would bind with targetable and non-targetable proteins. 5% of Bovine serum albumin (BSA) with 10ml of 1X TBST buffer was prepared for Blocking.

4.7.5. Primary Antibody

Primary antibody H2AX (produced from Rabbit polyclonal) was added in the 5% BSA solution (1:5000 dilutions). Only could be binds with H2AX. Incubate for 3 hours at room temperature, and then incubate overnight in refrigerator. Washed 3 times with 1X TBST buffer for 30 minutes.

4.7.6 Secondary Antibody

Secondary antibody H2AX was added in the 5% BSA solution. Anti Rabbit IgG (Produced in goat) (1:10000 dilutions). Only could be binds with primary antibody. Incubate for 35 minutes or 1 hour. Washed 3 times with 1X TBST buffer for 30 minutes.

4.7.7. Chemi-doc imaging of the membrane

Advanced Bio-Rad Chemi-Doc TM XRS+ imaging equipment was used to perform gel imaging for faster and more accurate western blot quantification. It is the only stain-free equipment capable of chemiluminescent, multiple fluorescence and routine gel imaging. After that, an ECL substrate solution was made by mixing equal amount of ECL solution A and ECL solution B in a (1:1) ratio. Then Activate the BioRad ChemiDoc TM and hook up with PC. The membrane is kept inside within the correct position after wiping the place with the tissue where they provided to putting the membrane. Add the prepared ECL substrate over the membrane in drops/spraying covering all the areas of membrane. Open Bio-Rad image Lab 6.1 software and choose new protocol a application, a get a blots a chemi, imaging area a get gel A Bio-Rad criterion gel. Within the image exposure area, manually set

exposure time to 150 seconds and choose run protocol. The results are going to be displayed within the monitor with clear bands supported the protein luminanace in several exposure times. The results were documented by File a export for publication a group the resolution and save. The whole procedure was administrated under the dark environment.

4.8 HISTOLOGICAL STUDIES OF REGENERATION BLASTEMA

The Regenerated blastema along with two adjacent segment are amputated and washed with distilled water, and the amputated section was kept in 10% formaldehyde for overnight. After the incubation of blastema was washed with water for removal of formaldehyde and then tissue transferred in to the 50% IPA for 1 hour at 65°C. Then tissue transferred in to xylene to remove the IPA. Finally, tissues immigrated in wax for overnight (Temperature supposed to be maintained in 50°-60°C). After incubation of tissues rottenly transferred in to the second wax and after 3 hours tissue placed on the final wax. Tissues transferred into the boxes for sectioning. Thin sections of 7µm size taken from the roto-microtome and placed on the albumin-coated clean glass slide, which carry warm water. After the formation of the liquid state of wax from the sections, slides are kept in xylene jar for 5 minutes for removal of wax. Then slide were transferred into the 100% IPA jar for 3 minutes. Hematoxylin and eosin stains were used for distinct visisble and slides were mounted with DPX mountant.

CHAPTER 5

RESULTS

5.1 EARTHWORM MORPHOLOGY

- Earthworm have cylindrical shaped and reddish brown segmented body.
- Earthworm are smooth skinned, with a body made of many small segments. Each segment has stiff little hairs that help the worm move.
- It has a central and pheripheral nervous system.
- A Glandular tissue called clitellum is present in segments 14-16 of a matured earthworm.
- They have no skeleton or other rigid structures to interfere with their movement.

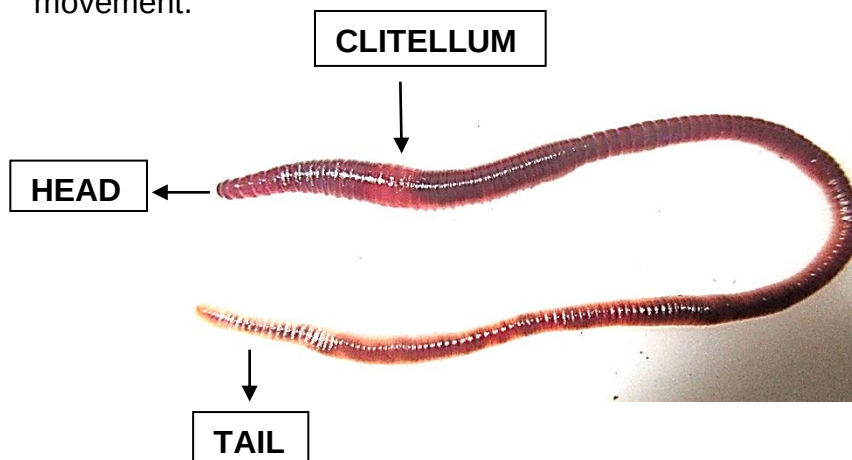


Fig 5.1 Morphology of earthworm (Eudrilus eugeniae).

5.2 REGENERATION IN EARTHWORM

Earthworm have remarkable power of regeneration. In earthworm, regeneration capability varies among species. Clitellum plays the vital role in regeneration. Earthworm regeneration starts with the production of blastemas at the site of amputation, then cells proliferate and differentiate into head or tail buds.

5.3 MORPHOLOGY OBSERVATION OF ELECTRIC SHOCK EFFECT ON REGENERATION

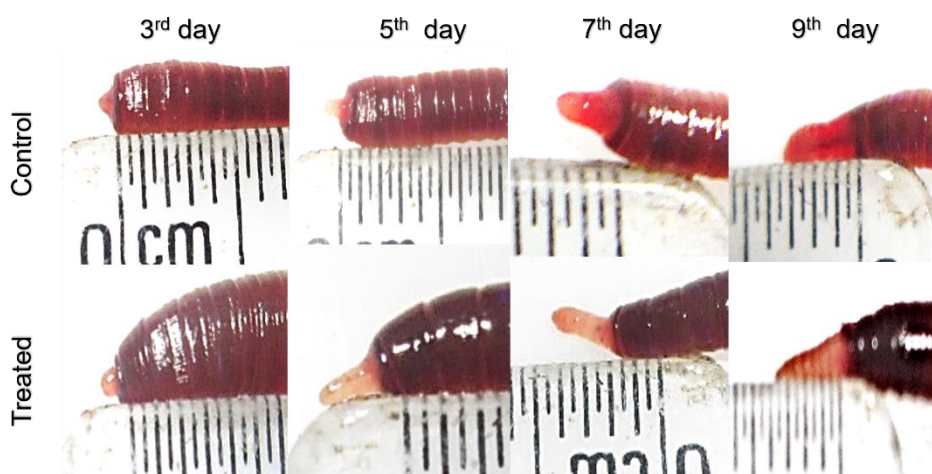


Fig 5.2 Morphological observation of control and electric shock.

In order to understand the exogenous application of electric shock (1.5 V) on earthworm regeneration ability, the worms are subjected to electric shock for 1 min for initial 2 days and amputated on the 3rd day as described in materials and methods. The electric shock was continued for every 24 hrs up to 9 days of post amputation and observed for the regeneration ability. The control worm that are not received any electric shock shows the formation of blastema on day 3 of post amputation. The blastema enlarges on 5th day and form visible differentiated segments on 7th day and almost restore the lost segments on 9th day of post amputation. In response to electric shock, the amputated worm forms blastema on 3rd day on the injury site but the proliferated cell mass is transiently higher which is evident with the enlarged blastemal size. when compared with the 3rd day control worm blastema. The regeneration kinetics is double the fold increased in electric shock given worm on 5th day of post amputation. when compared with its respective 5th day control. Subsequently, following 7th and 9th day of post amputation, the electric shock given shows 1.5 times more augmented growth when compared with their respective 7th and 9th day control worms.

5.4 MORPHOLOGY OBSERVATION OF ELECTRIC STIMULATION EFFECT ON EPIMORPHOSIS BLOCK

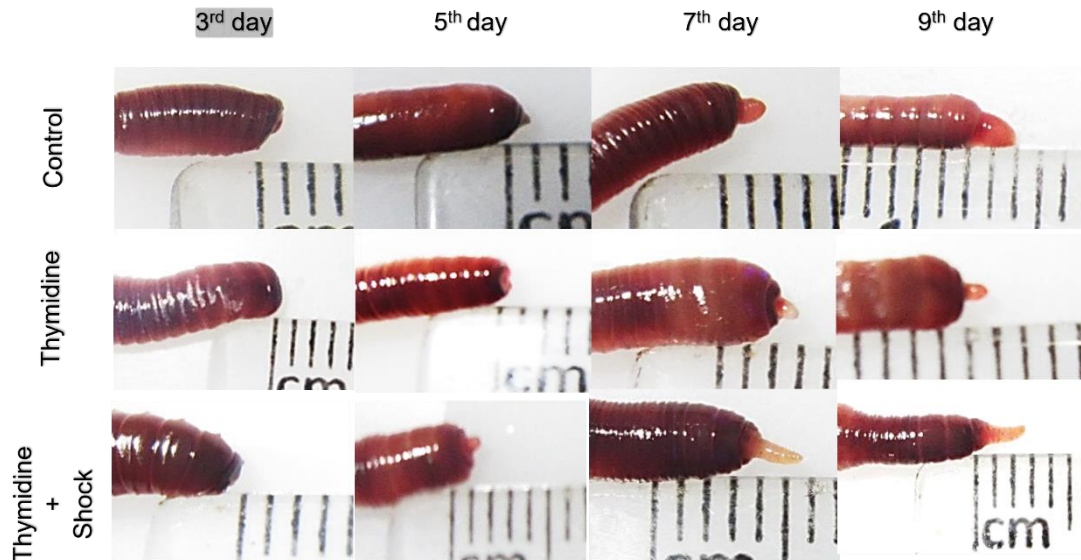


Fig 5.3 Morphological Observation of control, thymidine injection and Thymidine + electric shock.

To understand the regeneration mechanism of the worm, the worms were injected with 2 mM Thymidine and 2 mM Thymidine + shock will apply, then the control worm is injected with distilled water. Initially 2 days will injection and shock will given. Worms are amputated on 3rd day and observe the regeneration ability on the 3rd, 5th, 7th, and 9th day. The results shows that the thymidine block the epimorphosis, but the electric shock trigger the worm to regenerate. In this Thymidine + shock morphallaxis occur for their regeneration. Comparatively the bud formation for control and Thymidine + shock moreover similar. When compared to respective 7th and 9th day of Thymidine and Thymidine+shock. The Thymidine + shock treated only increased because fo cell movement.

5.5 HISTOLOGICAL ANALYSIS FOR ELECTIC SHOCK

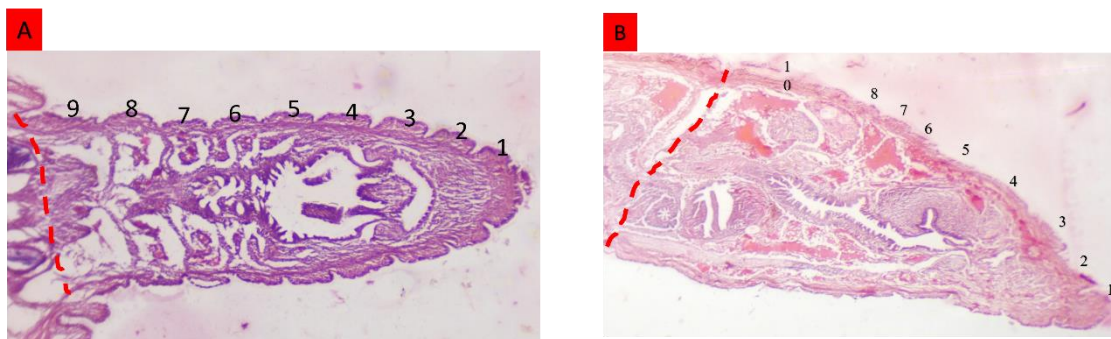


Fig 5.4 Histological analysis of control sample (A) and electric sample (B).

The control and electric shock stimulated 8th day regenerating worms are subjected to histological observations. The histological results strongly correlate with the morphological observation in which ingrowth regenerative tissue development is extensively increased upon electric shock stimulation as shown in the Fig. 2A-B. In control regenerative tissue out of ten amputated segments, the worms are able to regenerate only nine segments. The development of larger organs like seminal vesicles, testis is not distinguishably visible but the development of intestinal track and formation of septum are distinctly visible in control worms. On the other hand, following electric shock stimulation, the worm regenerated all the ten amputated segments and in perhaps the development of internal structures like seminal vesicles and testis are clearly visible.

5.6 KEY REGENERATIVE PROTEINS AND ITS INFLUENCE FOLLOWING ELECTRIC SHOCK

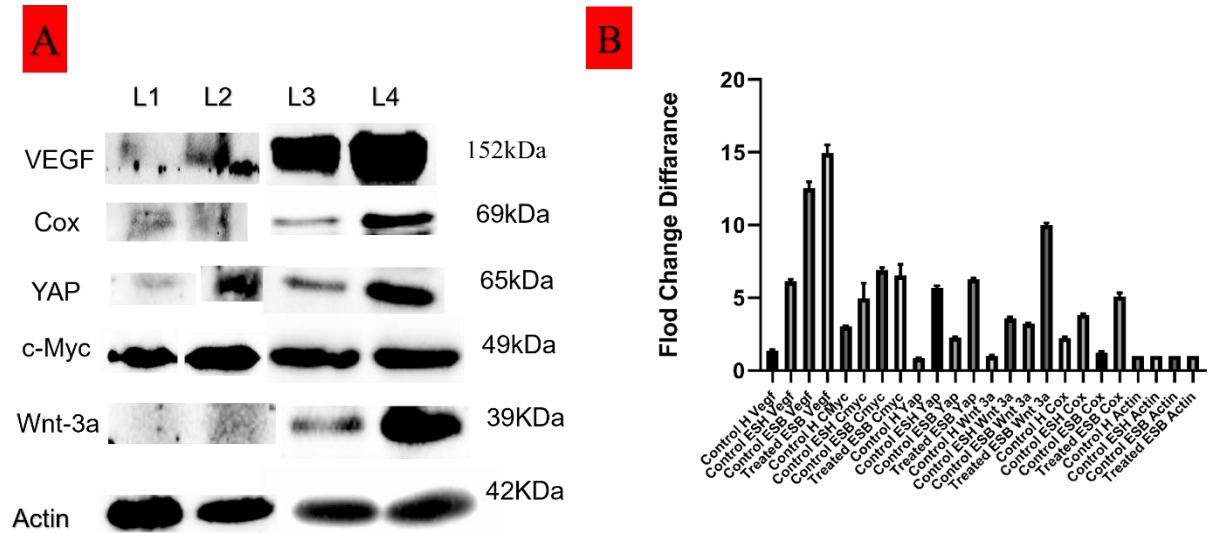


Fig 5.5 (A) Protein Expression Studies, the western blotting images showcasing the expression of VEGF (142kDa), cox (69kDa), YAP (65kDa) and wnt3a (39kDa). (B) Band intensity of the western blotting Image is quantified and represented as graph.

To understand the expression of VEGF, Cox, YAP, c-Myc, and wnt-3a. Compare to the control head(L1), control ES head(L2), Control Amp (L3) and treated (L4) in the treated has high expression i.e., L4. All the regenerative proteins are expressed. VEGF and Wnt-3a acts as a stem cell activator, this shows that the regeneration is increased in electric shock treatment compare to the control Amputation. The shown that the electric shock give stress to the earthworm to proliferate the cells through blastema.

5.7 ELECTRIC STIMULATION ON EPIMORPHOSIS BLOCK

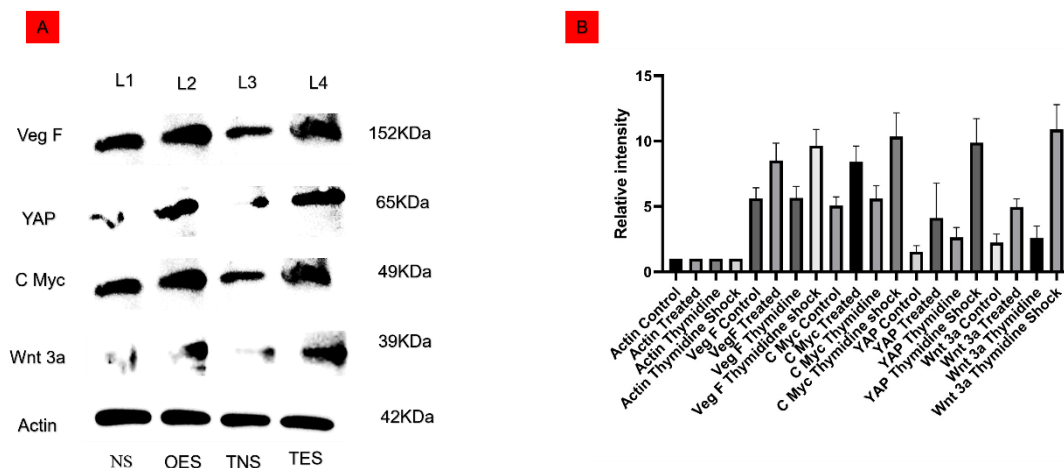


Fig 5.6 (A) Protein Expression Studies, the western blotting images showcasing the expression of VEGF (142kDa), c myc (49kDa), YAP (65kDa) and wnt3a (39kDa). (B) Band intensity of the western blotting Image is quantified and represented as graph.

Electric stimulation effect on epimorphosis block – key regenerative protein expression pattern. In Epimorphosis blocked also morphallaxis takes place and activate the cell proliferation. In this protein expression, VEGF, Wnt3a and cMyc are expressed in high intensity.

5.8 WOUND HEALING IN 3T3 CELLS

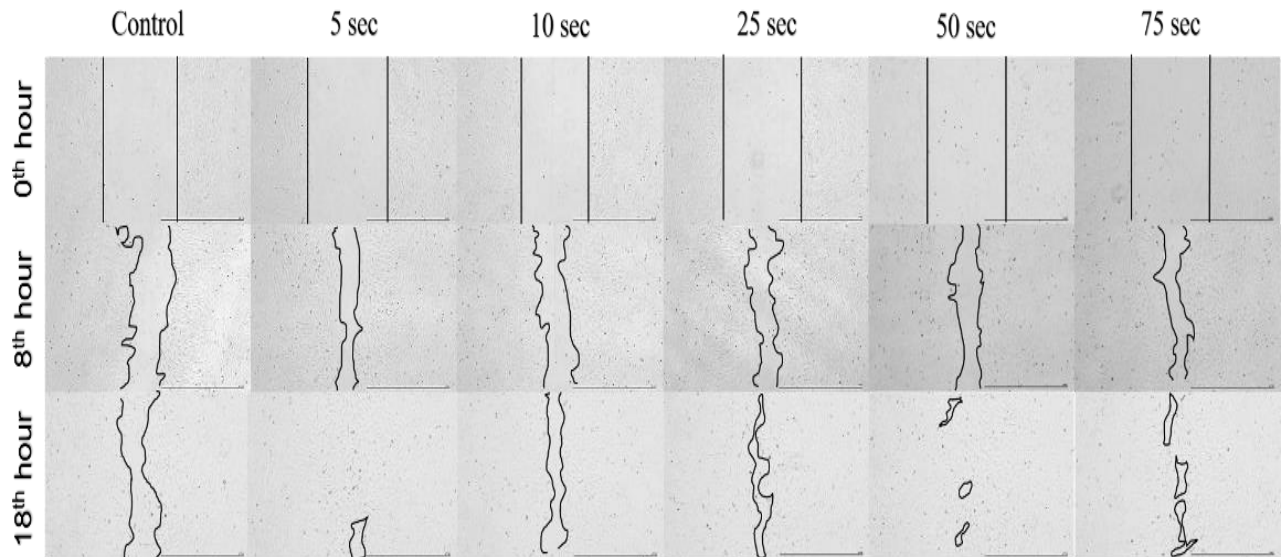


Fig 5.7 Potential of electric impulsion in wound healing.

In order to understand the wound healing potential of electric impulsion effect on cells, wound healing experiment was conducted. According to the results, compare to the control wound, the healing process were ameliorated in electric impulsion treatment, Notably 90% of wound were healed in 5 and 25 second electric impulsion on 18th hours.

DISCUSSION

In recent years, regeneration studies amongst the lower invertebrates like in annelids are more explored with add more knowledge in the field of developmental and evolutionary biology (Ozpolat and bely 2016). Regeneration studies show that earthworm *Eudriius eugeniae*, has an enormous regeneration ability. *Eudrilus eugeniae* is a clitellum dependent. The worms are amputated at the pre-clitellum part (10-15 th segments). The Pre-blastema was observed within 48 hours.

The rate of regeneration was studied in control and VPA treated earthworms. Subsequently, the healing of severed nerve cord was also studied by Rapid Escape Response (RER) Assay. That VPA significantly reduced the rate of segmental regeneration but promoted ventral nerve cord healing as exhibited by RER assay (Tanvi singh et al., 2023). In this study the rate of regeneration and wound will be increased and healed respectively with the treatment of low energy electric shock. The morphology data represents that compare to the control, the treated is well developed.

We utilized boric acid treatment, which blocks regeneration of segments in amputated fragments and block architomic fission during axesual reproduction, to investigate mechanistic relationships and differences between these two modes of development(Veronica G Martinez et al., 2006). Injection of 2mM Thymidine injected , the regeneration potential of the earthworm was suppressed. *Eudrilus eugeniae* can perform regeneration to a certain extent by utilizing morphallaxis. Epimorphosis mode of regeneration was early reported in the earthworm *Eudrilus eugeniae*. After blocking the epimorphosis, will give some stress by applying shock in the thymidine injected worm it will enormously increasing the regeneration. It shows the epimorphosis blocked and the morphallaxis is takes place in the regeneration.

The earthworm's capacity to regenerate itself is inhibited at 2 mM, according to histology. Intraintestinal features such as the septum, mouth, and segment elongation of thymidine-injected worms are not developed, which suggests that the signals or components required for regeneration are not properly regulated (Kamarajan Rajagopaln et al., 2023). In histology studies, the regeneration of the control and

electric shock stimulated worm. In electric stimulated sample, the internal organs are well developed but in the control sample not that much organs are developed.

During muscle repair, several growth factors are produced, but only insulin-like growth factors (IGFs) support muscle cell proliferation and differentiation, which is crucial for muscular hypertrophy and regeneration (Nadege zanou et al., 2013).

While giving electric stimulation the growth factors are activated. Wnt-3a (Stem cell marker), YAP (Organ formation ruler and Hippo signalling) ,and VEGF

(Vascular Endothelial Growth Factor) are the proteins to proliferate the stem cells.

In thymidine injection and thymidine and electric shock is regulating cell proliferation and cell differentiation, the expressed growth factors are Wnt3a, VEGF and YAP. All these growth factors are proliferate the stem cells for regeneration.

In this work, we report for the first time on the highly effective killing capacity of ZnO nanocrystals (ZnO NCs) functionalized with aminopropyl in combination with high-energy ultrasound shock waves (SW) for the treatment of cancer cells (luisa racca et al., 2020). Electric shock is given to cells with different seconds. Wound was healed at few seconds of electric impulsion in the cells. Electric current or any electric impulsion will heal the wound in less time.

CHAPTER 6

SUMMARY & CONCLUSION

Our results conclude, low energy electric shock augments regenerate and organogenesis ability in earthworm, *Eudrilus eugeniae*. Blocking epimorphosis through 2mM thymidine inhibit regeneration but combinatorial application of low energy electric shock and application of 2mM thymidine provoke the regeneration ability. The results conclude, low energy electric shock is able to accelerate both epimorphosis and morphallaxis. Molecular studies confirm, applying low energy electric shock induces angiogenesis (VEGF), YAP (organogenesis), Wnt-3a (Stem cell activation) and which are also highly triggered while blocking with epimorphosis. The obtained results pay a way for the application of low energy electric impulses in regenerative therapy.

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