

# **MOLECULAR EFFECTS OF SODIUM SELENITE DURING REGENERATION IN EARTHWORMS**

## **PROJECT WORK**

Submitted in partial fulfilment of the requirements for the award of Bachelor of  
Technology degree in Biotechnology

By

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**BONAFIDE CERTIFICATE**

This is to certify that this Project Report is the bonafide work of **Mirdushi Vasu. M (40230027)** and **Priyadarshini Senthilkumar (40230036)** who carried out a Project entitled “**Molecular effects of sodium selenite during regeneration in earthworms**” under my supervision from September 2023 to March 2024.

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## DECLARATION

We, **Mirdushi Vasu. M (40230027)** and **Priyadarshini Senthilkumar (40230036)** hereby declare that the Project Report entitled “**Molecular effects of sodium selenite during regeneration in earthworms**” done by us under the guidance of **Dr. Prakash Pandurangan** (Internal) and **Dr. Jayshree Nellore** (Internal) at the Department of Biotechnology and **Dr. S. Johnson Retnaraj Samuel** (External) at the International Research Centre, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfillment of the requirements for the award of **Bachelor of Technology degree in Biotechnology**.

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## ABSTRACT

The process of regeneration, which is fundamental to biology, remains inadequately understood across various organisms. This study aimed to elucidate the regenerative capabilities of annelids, particularly focusing on the earthworm species *Perionyx excavatus*. Additionally, we investigated the effects of sodium selenite injection on the regeneration process in earthworms. In our research, we conducted a thorough examination of the effects of sodium selenite injection into the head region of earthworms, a site known for its heightened regenerative activity. Our experimental protocol involved a five-day observation period after amputation of earthworm's head following the administration of sodium selenite, to assess bud growth. This experimental design allowed us to analyze the impact of sodium selenite on the regenerative process over a defined period. Motivating our choice of sodium selenite was its relatively unexplored potential in the context of regeneration, particularly its observed positive effects on regeneration patterns in miRNA. Despite its promising attributes, sodium selenite had not been thoroughly studied in the context of earthworm regeneration prior to our investigation. Therefore, we aimed to fill this gap in knowledge by systematically examining its influence on regenerative processes in earthworms. Our experimental workflow encompassed several essential techniques and methodologies, including RNA isolation and quantification, cDNA synthesis, primer designing, primer dilution, miRNA and mRNA target prediction, qPCR studies, and histology. Upon analyzing the data obtained from our experiments, we made a significant observation regarding the correlation between miRNA and its target genes in response to sodium selenite exposure. Specifically, we found a positive correlation between the expression levels of miRNA and its target genes, indicating a potential regulatory role of sodium selenite in modulating the expression of genes involved in the regenerative pathway.

**Keywords :** Regeneration, *Perionyx excavatus*, Sodium Selenite, Gene expression studies, Earthworms.

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## LIST OF ABBREVIATIONS

| ACRONYM | EXPANSION                                                 |
|---------|-----------------------------------------------------------|
| mRNA    | Messenger ribonucleic acid                                |
| miRNA   | Micro ribonucleic acid                                    |
| RT PCR  | Real time reverse transcriptase polymerase chain reaction |
| qPCR    | Quantitative polymerase chain reaction                    |
| Pex     | <i>Perionyx excavatus</i>                                 |
| DNTP    | Deoxynucleoside triphosphate                              |
| Ds      | Dachsous                                                  |
| USP     | Universal stress proteins                                 |
| Celsr   | Cadherin EGF LAG Seven-Pass G-Type Receptor               |
| Yurt    | Erythrocyte membrane protein band                         |
| RT      | 4.1 like 5 Reverse Transcription                          |
| H&E     | Hematoxylin and Eosin                                     |
| PBS     | Phosphate-buffered saline                                 |
| DMEM    | Dulbecco's modified eagle medium                          |
| FBS     | Fetal bovine serum                                        |
| EDTA    | Ethylenediamine tetra acetic acid                         |
| DNA     | Deoxyribonucleic acid                                     |
| dsDNA   | Double stranded DNA                                       |
| ssDNA   | Single stranded DNA                                       |
| Tm      | Melting temperature                                       |
| NFW     | Nuclease-free water                                       |
| BLAST   | Basic local alignment search tool                         |

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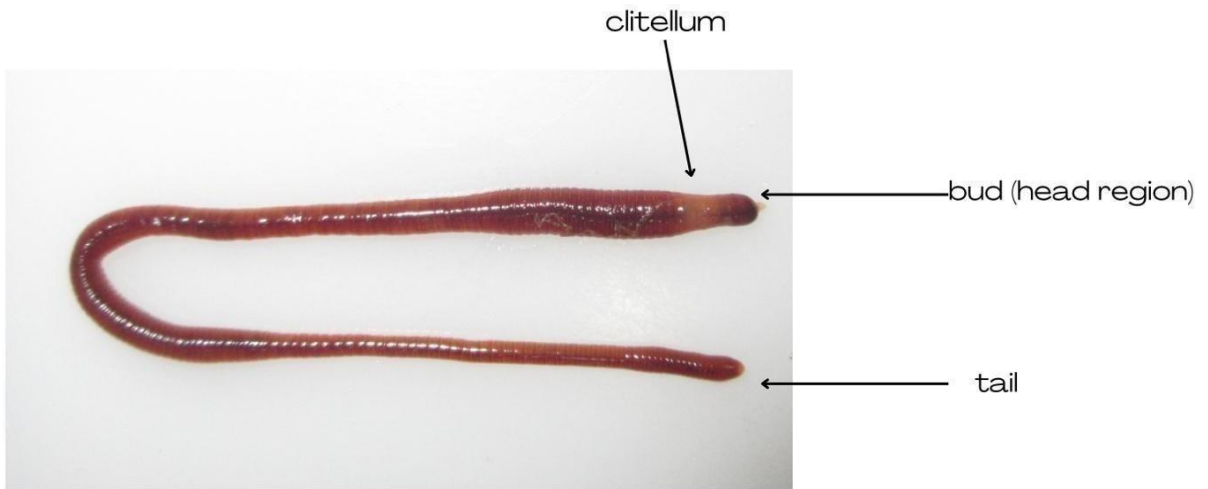
# 1. INTRODUCTION

Annelids, encompassing diverse species like earthworms and sea polychaetes, are renowned for their segmented bodies and varied habitats, exhibiting remarkable adaptability and ecological significance (*Rouse & Pleijel, 2001*). Their distinguishing feature lies in their segmented bodies, often equipped with lateral appendages called parapodia in marine species, aiding in movement and other functions. Annelids also possess a hydrostatic skeleton, providing support and mobility through fluid-filled chambers (*Brusca & Brusca, 2003*). Evolving over millions of years, annelids diversified into classes like Polychaeta, Oligochaeta, and Hirudinea, as evidenced by fossil records and molecular phylogenetic studies (*Martin.P, 2003*). They play vital roles in ecosystems, regulating nutrient cycling, soil health, and sediment dynamics. Earthworms enhance soil fertility, while polychaetes serve as keystone species in marine environments, influencing benthic community structure (*Londoño-Mesa & Carranza, 2017*). Some annelids, such as leeches, offer medical benefits and contribute to scientific research. Annelids' unique traits, including diverse reproductive systems and feeding habits, underscore their adaptability and resilience (*Ziegler & Faber, 2020*).

Regeneration, observed across the animal kingdom, is particularly remarkable in annelids, a diverse group of segmented worms encompassing species like earthworms, leeches, and marine polychaetes (*Bely AE., 2006*). Their exceptional regenerative ability, capable of restoring lost segments or even entire organisms from body parts, has fascinated scientists for centuries and remains a subject of intensive study. Annelids exhibit a wide spectrum of regenerative capacities, varying between species and even within the same organism (*Galliot B, Chera S., 2010*). Earthworms can regenerate segments posterior to the clitellum, while some polychaetes can regenerate complete body segments, including complex systems like the nervous and digestive systems. These regenerative processes involve intricate biological events, including wound healing, cell dedifferentiation, proliferation, and differentiation, orchestrated by complex molecular signaling

networks (*Rouse GW, Pleijel F., 2001*). Understanding these processes not only elucidates fundamental principles of regeneration but also holds promise for regenerative medicine applications in human health. Despite significant advancements, many questions remain unanswered, including the molecular signaling mechanisms governing regeneration, the role of stem cells in tissue regeneration, and factors influencing regenerative capacity among different annelid species. Further research in these areas will continue to deepen our understanding of regenerative biology and its potential applications (*Stocum DL., 2017*).

Earthworms, often overlooked yet vital to soil ecosystems, play a crucial role in soil health and fertility worldwide. As members of the Annelida phylum and Clitellata class, they quietly burrow beneath the soil's surface, contributing significantly to ecosystem engineering humans (*Kumar D, Kumar V., 2012*) (*Bely AE.,2010*). Their aerating activities improve soil structure, facilitating oxygen and water penetration, while bioturbation processes enrich soil fertility by converting organic matter into nutrient-rich castings (*Karthik, R.,et al., 2018*). Earthworms also enhance soil microbial activity, fostering beneficial relationships that aid in nutrient breakdown and mineralization. However, these essential ecological contributors face threats from human activities such as deforestation, agriculture, and urbanization, leading to habitat loss and pollution. Conservation efforts, guided by an understanding of earthworm ecological roles, are crucial for safeguarding soil ecosystem health and resilience against these challenges (*Gajalakshmi, S., Ramasamy, E.V., 2008*) (*Dash, M.C., et al., 2013*).



**Fig 1.1: *Perionyx excavatus*,**

*Perionyx excavatus*, also known as the Indian blue worm, showcases remarkable regenerative abilities within the Annelida phylum, offering valuable insights into fundamental biological processes and potential applications in regenerative medicine (Salama & Saber., 2018). Thriving in diverse environments like soil and compost heaps, *P. excavatus* plays a crucial role in nutrient cycling and soil health, relying on its ability to swiftly replace damaged tissues to survive in dynamic conditions (Reyes & Munir., 2020). While the specific molecular mechanisms driving its regeneration remain largely unknown, comparative studies with other regenerative organisms offer promising avenues for understanding potential regulatory pathways (Giani *et al.*, 2011). This species serves as an exceptional model for research across developmental and evolutionary biology, as well as biomedical fields, aiming to enhance tissue repair and regeneration in various organisms (Antwis *et al.*, 2017). Exploring regeneration in *P. excavatus* not only sheds light on its evolutionary significance but also underscores the ecological importance of regeneration in natural ecosystems.

Regeneration, a fascinating phenomenon across the biological spectrum, relies on intricate genetic circuits orchestrating cellular responses to injury or stress. Critical to this process are regeneration-specific genes, which initiate and

coordinate tissue repair and renewal (Seifert & Muneoka,., 2017); (Tanaka & Reddien, 2011). Deciphering the molecular mechanisms governing these genes holds immense potential for unlocking the full regenerative capacity of diverse organisms, including humans. By unraveling these genetic blueprints, researchers aim to revolutionize tissue repair and regeneration, paving the way for innovative therapies to address injuries, diseases, and age-related degeneration (Knapp & Tanaka, 2010); (Gawriluk et al., 2016).

Regeneration is a multifaceted biological process where organisms restore damaged tissues, driven by the intricate orchestration of various genes like Yurt, Dashous, Actin, SOX C, CELSR, and Notch. Yurt and Dashous, identified in *Drosophila melanogaster*, regulate cell polarity and tissue development, crucial for coordinating cytoskeletal dynamics during regeneration (Pilling et al., 2009). Actin, a pivotal cytoskeletal protein, contributes to cellular architecture, mobility, and wound healing across diverse animal species (Ma et al., 2009). SOX C, a member of the SOX transcription factor family, maintains progenitor cell populations and modulates cellular responses to damage stimuli, essential for tissue regeneration (Kim et al., 2009). CELSR genes, encoding cell surface receptors, facilitate tissue patterning and remodeling by regulating cell-cell contacts and signaling processes (Dohn & Waxman, 2005). Notch receptors and ligands govern cell fate decisions and tissue homeostasis, crucial for stem cell maintenance, lineage commitment, and the creation of regenerative niches during tissue regeneration (Bray, 2006). These genes collectively orchestrate the complex cellular processes underlying regeneration, offering insights into potential therapeutic strategies for enhancing tissue repair and regeneration.

Sodium selenite, containing selenium, demonstrates potential in enhancing regeneration by modulating cellular signaling, antioxidant mechanisms, and inflammation. It regulates enzyme activity crucial for tissue repair, stimulates growth factors and cytokines promoting cell growth and differentiation, and exhibits anti-inflammatory properties that mitigate tissue damage (Rahimi et al., 2023); (Yan et al., 2019). Our current research delves into the effects of sodium

selenite on *Perionyx excavatus* earthworms, focusing on understanding its influence on cellular signaling pathways, antioxidant defenses, and regenerative capacities specific to this species. Through this study, we aim to uncover valuable insights into the regenerative potential of sodium selenite and its applications in regenerative medicine.

## 2. LITERATURE REVIEW

### 2.1 ANNELIDS

Annelids, commonly known as segmented worms, constitute a diverse phylum with over 22,000 species, including earthworms, ragworms, and leeches, inhabiting freshwater, terrestrial, and marine environments. Bilaterally symmetrical and coelomate, they rank among the most diverse phyla within Metazoa (*Bandyopadhyay & Munshi, 2023*). Early annelids, dating back to the Early Cambrian period, displayed primitive characteristics like head appendages and biramous parapodia. Crucial to marine ecosystems, they inhabit various habitats and play roles as deposit feeders, filter feeders, carnivores, herbivores, and parasites, influencing the food chain. Certain species, notably earthworms, contribute to sediment mixing and organic matter breakdown, although they may accumulate pollutants. Annelids exhibit diverse reproductive strategies and lifespans, serving as bait, food, or aquaculture feed. Their regenerative capacity, a captivating biological aspect, is studied for its potential applications. Annelids also yield bioactive compounds useful in various fields such as anesthetics, antibiotics, and insecticides (*Purschke et al., 2019*);(*Capa & Hutchings, 2021*).

### 2.2 REGENERATION IN EARTHWORMS

Earthworms play a crucial role in soil ecology, contributing to soil formation, maintenance, and organic material turnover. Annelids, including earthworms, are studied for their regenerative capabilities, with some species capable of rapid restoration post-amputation. Earthworms' regenerative ability varies based on species, location of incision, and cut cleanliness (*Xiao et al., 2011*). Typically, they can regenerate tails and even segments from the head end, although regenerative success may diminish with increased segment loss. Certain species employ tail autotomy as a defense mechanism against predators, detaching tails to escape. Additionally, some earthworms discard waste by amputating their tails when full. Comparative studies of regeneration patterns in earthworms can provide valuable insights into the evolutionary conservation of regeneration mechanisms.

### **2.3 PERIONYX EXCAVATUS**

*Perionyx excavatus*, a commercially utilized earthworm species, falls under the Eukaryota domain and Annelida phylum, specifically Clitellata, distinguished by its clitellum. Further taxonomic classification places it in the Opisthopora order and Lumbricina suborder, within the Megascolecidae family. This genus of earthworms is extensively employed in vermicomposting, organic farming, and as feed for livestock and fish due to its high nutritional content. Rich in protein, fats, carbohydrates, and essential nutrients, earthworms are valued as a dietary supplement. However, natural habitat populations are insufficient to meet demand. *P. excavatus* possesses the capacity to accumulate hazardous substances like pesticides and heavy metals. Its growth and reproductive rates are influenced by factors such as population density and temperature, with optimal conditions enhancing growth and reproduction, as observed in studies on various organic substrates and temperature ranges (Edwards, C.A et al., 1998).

### **2.4 REGENERATION IN PERIONYX EXCAVATUS**

*Perionyx excavatus*, a tropical earthworm species, exhibits remarkable anterior regeneration capabilities, with over 90% success even after removal of the central nervous system-containing head, making it an ideal model for studying regeneration mechanisms (Bae, Y.S., et al., 2020). This species can regenerate reproductive organs within two weeks post-amputation, providing valuable insights into the molecular mechanisms underlying cell identity changes during anterior regeneration (Cho SJ., et al., 2009). While anterior regeneration is prevalent in *P. excavatus*, it is not universally observed across all earthworm species.

### **2.5 REGENERATION SPECIFIC GENES**

Research on earthworm regeneration has primarily focused on determining the extent of regenerative capacity, with limited investigation into gene expression during the process. Unlike other regenerative model organisms like zebrafish, planaria, and hydra, comprehensive transcriptomic analyses of

earthworm regeneration are lacking. Studies on these model organisms have highlighted the importance of growth factor signaling and reprogramming factors in regeneration (*Bhambri A, et al., 2018*). Comparative genomic and transcriptomic analyses among closely related species with varying regenerative potentials may uncover novel genes, regulatory components, or genomic rearrangements underlying regenerative ability evolutionarily. Recent findings suggest that earthworm regeneration induces genes associated with cognitive regeneration, indicating the involvement of conserved molecular processes in the gene expression program.

### **2.5.1 Actin**

Actin, a fundamental component of the cytoskeleton, plays diverse roles in cellular functions such as cell division, shape maintenance, chromatin remodeling, transcription and motility (*Rzadzinska et al., 2004*). Despite the stable architecture of filament structures, actin undergoes regular turnover. Moreover, actin provides binding sites for numerous proteins essential for various biological processes. Recent studies have highlighted the critical role of actin in guiding axon development in neurons through the cytoskeleton. Although *Lumbricus terrestris* has several actin genes, little is known about their expression patterns.

### **2.5.2. Yurt - Erythrocyte membrane protein band 4.1 like 5**

The Erythrocyte membrane protein band 4.1 like 5 (EPB41L5) gene is implicated in earthworm regeneration, potentially regulating cellular dynamics crucial for tissue repair. Its role in cellular structure and organization suggests involvement in migration (*Hoover KB, et al., 2006*), proliferation, and differentiation during regeneration. EPB41L5 likely influences adhesion and signaling pathways, vital for coordinated cellular activities in tissue regeneration. Insights into its function may offer novel therapeutic avenues in regenerative medicine.

### **2.5.3. Celsr - Cadherin EGF LAG Seven-Pass G-Type Receptor 1**

The Celsr gene, known for its role in cell polarity and tissue organization, has been identified as a key player in earthworm regeneration.

Earthworm's remarkable ability to regenerate damaged segments has made them valuable for studying regeneration (*Joshi B, et al., 2022*). Recent research has shown that the *Celsr* gene is involved in this process, regulating cell polarity and tissue patterning essential for effective regeneration. Manipulating *Celsr* gene expression influences the rate and extent of regeneration, highlighting its importance in this biological phenomenon.

#### **2.5.4. Notch**

Utilizing the notch gene in earthworm regeneration studies provides valuable insights into the molecular mechanisms of tissue regeneration. The notch gene's role in cell fate determination and tissue patterning offers a promising avenue for understanding regeneration processes and advancing regenerative *medicine* (*Hrouda, M., & Janssen, R., 2018*).

#### **2.5.5 Hedgehog**

The hedgehog gene is crucial in embryonic development across species, including humans, initially discovered in the fruit fly *Drosophila melanogaster*. Its mutations led to the appearance of spiky projections resembling hedgehog spines, hence the name (*Sasai N, et al., 2019*). This gene family is highly conserved, encoding signaling proteins essential for tissue and organ formation, from insects to mammals. In earthworms, the hedgehog gene regulates cell proliferation, differentiation, and patterning, crucial for development and regeneration.

#### **2.5.6 Universal Stress Protein**

Universal stress proteins (USPs) are a widely distributed family of proteins found in organisms ranging from bacteria to humans. These proteins aid cells in responding to diverse stress conditions like heat shock, nutrient deprivation, and oxidative stress. In earthworm regeneration, USPs serve to protect cells from stress, regulate cellular processes, and maintain cellular homeostasis. During regeneration, earthworms encounter physiological stress from tissue damage and the need for rapid rebuilding (*Boes N, et al., 2011*).

### **2.5.7 SoxC**

As a transcription factor, SoxC regulates cellular differentiation and proliferation during regeneration, making it an ideal option for studying the molecular mechanisms underlying earthworm regeneration. Researchers can obtain insights into the genetic basis of earthworm regeneration by analysing the SoxC gene's expression patterns and regulatory networks, perhaps revealing new pathways and targets for therapeutic applications in regenerative medicine. Understanding the role of SoxC in earthworm regeneration could also provide insights into evolutionary conserved mechanisms of tissue repair and regeneration, with implications for broader biological study and biotechnological breakthroughs (*C Voskamp et al., 2020*).

### **2.5.8 Dachsous**

Dachsous (Ds), a conserved transmembrane protein, is a key regulator of tissue growth and patterning across various species. Dachsous, which was first discovered in *Drosophila*, is thought to regulate key signalling pathways involved in tissue repair and regeneration. Given the conservation of genetic pathways across species, including earthworms, studying Dachsous' role in these animals could shed light on fundamental principles of regeneration. Understanding how Dachsous regulates cellular processes such as proliferation, differentiation, and wound healing in *Drosophila* and earthworms allows researchers to identify conserved genetic pathways and mechanisms that promote regeneration across species. Thus, applying the knowledge learned from *Drosophila* investigations to Dachsous should provide vital insights into improving earthworm regeneration ability, with potential applications in regenerative medicine and tissue engineering (*Lefebvre V, Bhattaram P, 2016*).

### **2.5.9 Synaptotagmin - 7b**

The interaction between synaptotagmin-7b and calmodulin results in the formation of a complex, indicating their role in regulating the calcium-dependent replenishment pathway. This binding suggests a crucial mechanism by which calcium signaling influences synaptic vesicle replenishment.

Synaptotagmin 7, a calcium-binding protein primarily located in synaptic vesicles, is known to play a pivotal role in synaptic vesicle exocytosis ( *Saghian R, Wang LY; 2022*). Calmodulin, on the other hand, is a calcium sensor protein involved in numerous cellular processes, including neurotransmitter release. The formation of the synaptotagmin 7-calmodulin complex likely modulates the calcium sensitivity of synaptic vesicle replenishment, highlighting its significance in synaptic function and neurotransmission regulation. This interaction underscores the intricate molecular mechanisms governing synaptic vesicle dynamics and neurotransmitter release at synapses. ( *Huson V, Regehr WG., 2020*), ( *Xue R, Meng H et al., 2021*)

#### **2.5.10 Transcription factor Su(H)**

The transcription factor Suppressor of Hairless (Su(H)) plays a pivotal role as a molecular switch within the Notch cell-cell signaling pathway. In the absence of signaling, Su(H) functions as a repressor, regulating gene expression accordingly. However, upon activation of the Notch receptor and subsequent signaling, Su(H) undergoes a transformation into an activator ( *Sprinzak D, Blacklow SC et al.,2021*). This switch in functionality enables Su(H) to modulate gene expression patterns in response to Notch signaling, thereby exerting precise control over cellular processes such as development, differentiation, and homeostasis. The dynamic interplay between Su(H) and the Notch receptor highlights the intricacies of the Notch signaling pathway and its profound impact on various biological phenomena.

#### **2.5.11 Transcription factor Sox F**

Sox proteins constitute a family of transcription factors renowned for their pivotal role in orchestrating gene expression within stem cells. These proteins are instrumental in governing critical cell fate determinations throughout development, exhibiting profound influence over the trajectory of cellular differentiation ( *Pouremamali F, et al., 2022*). Beyond developmental processes, Sox proteins are increasingly recognized for their potential involvement in adult tissue regeneration and maintenance of tissue homeostasis. Their multifaceted roles underscore their significance as key regulators in both embryonic and adult contexts, offering insights

into fundamental mechanisms underlying cellular plasticity and tissue dynamics. (Ye P, *et al.*, 2023)

## 2.6 SODIUM SELENITE IN REGENERATION

Sodium selenite is a chemical compound containing sodium and selenium, an essential trace element for humans. Sodium selenite has the chemical formula  $\text{Na}_2\text{SeO}_3$ . It consists of sodium ions ( $\text{Na}^+$ ) and selenite ions ( $\text{SeO}_3^{2-}$ ) (Acta Cryst. E58, i103-i104). Sodium Selenite, water-soluble inorganic form of selenium, exerts profound effects on human physiology at the molecular level owing to its interaction with various biological systems (Wickleder, M. S.; 2002). It is an essential micronutrient for humans, meaning it must be obtained from the diet. At the molecular biology level, the actions of sodium selenite primarily revolve around its role as a precursor for the synthesis of selenoproteins, which are pivotal in modulating cellular redox balance, DNA repair mechanisms, and immune responses. (Patterson BH, *et al.*, 1989)

Selenoproteins are a distinct class of proteins characterised by the incorporation of the amino acid selenocysteine, encoded by the UGA codon, which typically serves as a stop signal during translation. However, in the presence of specific RNA structures called selenocysteine insertion sequences (SECIS), translation termination is bypassed, facilitating the incorporation of selenocysteine into the nascent polypeptide chain. These selenoproteins encompass a wide array of enzymes and structural proteins crucial for cellular function.

Selenium compounds, including sodium selenite, have been shown to modulate various cell signalling pathways implicated in regeneration. For instance, sodium selenite can influence the activity of transcription factors such as nuclear factor-kappa B (NF- $\kappa$ B) and activator protein-1 (AP-1), which regulate the expression of genes involved in inflammation, cell proliferation, and tissue remodelling—all processes integral to regeneration. Selenium plays a crucial role in DNA repair processes, which are essential for maintaining genomic integrity and facilitating tissue regeneration (Lu J, Holmgren A., 2009).

Sodium selenite possesses potent antioxidant properties, primarily mediated through its incorporation into selenoproteins such as glutathione peroxidases (GPx) and thioredoxin reductases (TrxR). By scavenging reactive oxygen species (ROS) and mitigating oxidative stress, sodium selenite can protect cells and tissues from oxidative damage, creating a favourable microenvironment for regeneration to occur.

Sodium selenite has been implicated in epigenetic modifications, influencing gene expression patterns without altering the DNA sequence. Selenium compounds, including sodium selenite, can affect DNA methylation patterns and histone modifications, thereby modulating chromatin structure and gene accessibility. By exerting epigenetic regulatory effects, sodium selenite can influence cellular differentiation, development, and disease processes, contributing to its broader impact on human health and physiology (*El-Magd NF et al., 2022*)

Proper remodelling of the extracellular matrix (ECM) is essential for tissue regeneration and functional restoration. Selenium compounds like sodium selenite can modulate ECM remodelling by influencing the synthesis and degradation of ECM components, such as collagen and proteoglycans. This regulation of ECM dynamics contributes to tissue restructuring and regeneration (*Carlson BA, Yoo MH et al., 2009*) We aim to delve deeper into understanding the precise mechanisms through which sodium selenite influences regeneration and to investigate its potential therapeutic uses in the field of regenerative medicine. This is why we have chosen to focus our research on utilising earthworms as a model organism to study regeneration.

## **2.7 SODIUM SELENITE AND miRNA INTERPLAY**

miRNAs could serve as mediators for certain regenerative properties elicited by sodium selenite, potentially through the regulation

of pivotal genes and signalling cascades pertinent to the regenerative process. Such as (Lendvai G, et al., 2020), Selenoprotein Synthesis Pathway: Selenium is an essential component of selenoproteins, a group of proteins that contain the amino acid selenocysteine (Sec) in their active sites. The Synthesis of selenoproteins involves a unique mechanism that requires specific sequences in their mRNA called selenocysteine insertion sequence (SECIS) elements. Some miRNAs have been found to regulate the expression of selenoproteins by targeting components of this pathway. For example, miR-185 has been shown to downregulate selenoprotein expression by targeting the 3' untranslated region (UTR) of selenoprotein mRNAs.

Redox Signaling Pathway: Selenium is a component of selenoproteins with antioxidant functions, such as glutathione peroxidases (GPx) and thioredoxin reductases (TrxR). These enzymes play crucial roles in maintaining cellular redox balance and protecting cells from oxidative stress. Some miRNAs have been implicated in the regulation of antioxidant selenoproteins, affecting cellular responses to oxidative stress. For instance, miR-34a has been reported to target the mRNA of selenoprotein H, thereby reducing its expression and compromising cellular antioxidant defenses. Sodium selenite may exert an influence on the expression profiles of miRNAs, consequently altering the regenerative capacity of cells and tissues. For example, selenium supplementation has been shown to alter DNA methylation patterns and histone modifications, which can indirectly affect miRNA expression by modulating the accessibility of miRNA genes to transcription factors and RNA polymerase. All the miRNA's chosen for our studies exhibit strong regeneration properties.

### **2.7.1 miR – 1**

miR-1 plays a vital role in regulating myogenesis, the process of forming and differentiating muscle cells. It facilitates the transition of myoblasts, precursor cells of muscle cells, into fully mature muscle cells, essential for tissue regeneration following injury (Rao PK, et al., 2006). By targeting and inhibiting the expression of

specific genes involved in cell proliferation, miR-1 orchestrates a shift towards muscle cell differentiation, a pivotal aspect of the regeneration process. Notably, miR-1 is prominently expressed in both cardiac and skeletal muscle tissues (*Zhang D, 2012*)

### **2.7.2 miR – 219**

miR-219 has been implicated in the regulation of neural development. It may influence processes such as neuronal differentiation, maturation, and synaptic plasticity. miR-219 has been identified as a regulator of oligodendrocyte differentiation, which is crucial for the formation of myelin, a protective sheath around nerve fibres. Myelin plays a significant role in neural function, and the regulation of oligodendrocyte differentiation is important for neural repair and regeneration. Some studies have suggested that miR-219 may play a role in axonal regeneration. Axons are the long projections of nerve cells responsible for transmitting signals, and the ability to regenerate axons is vital for neural repair after injury. (*Rao PK, et al., 2006*).

### **2.7.3 miR – 29 b**

MiR-29b has garnered significant attention for its multifaceted roles in various physiological and pathological processes. In fibrosis regulation, it is recognized for targeting genes responsible for the excessive production of extracellular matrix components, thereby offering potential therapeutic avenues for managing fibrotic diseases (*Cushing L, et al., 2015*). Additionally, dysregulation of miR-29b has been implicated in cancer progression, where it acts as a tumour suppressor by inhibiting genes involved in cell proliferation and metastasis. (*Yin H, et al., 2020*) Its involvement extends to cardiovascular diseases, where it can influence genes associated with cardiac fibrosis, hypertrophy, and apoptosis, potentially impacting heart-related conditions. Moreover, studies have explored its relevance in neurological disorders, suggesting a role in modulating neuroinflammation and neuronal apoptosis, thus contributing to the pathophysiology of conditions like Alzheimer's disease (*Shen Y, et al., 2022*).

#### **2.7.4 miR – 34**

MicroRNA-34 (miR-34) is a family of microRNAs that includes three members: miR-34a, miR-34b, and miR-34c. (Jauhari A, et al., 2018) MiR-34, recognized as a tumour suppressor microRNA, is regulated by the tumour suppressor protein p53, typically activated following DNA damage. It functions by inhibiting the expression of genes that facilitate cell cycle progression, cell survival, and avoidance of apoptosis. Through its actions in inducing cell cycle arrest and apoptosis, miR-34 serves as a deterrent against the uncontrolled proliferation of cancer cells. Moreover, miR-34 plays a role in regulating apoptosis and cellular senescence by targeting anti-apoptotic genes and promoting senescence—a condition of irreversible cell cycle arrest triggered by various stressors (Hermeking HJ., 2010).

### **3 AIM AND SCOPE:**

#### **3.1 AIM**

The aim of our project is to explore alterations in miRNA expression in *Perionyx excavatus* upon sodium selenite injection and predict mRNA targets to elucidate the associated molecular mechanisms.

#### **3.2 SCOPE**

- Evaluate miRNA expression changes in *Perionyx excavatus* post-sodium selenite injection, identifying and quantifying specific miRNAs with significant alterations.
- Employ RNAhybrid tool to predict mRNA targets for sodium selenite-influenced miRNAs in *Perionyx excavatus*.
- Perform qPCR experiments to quantify selected miRNA expression in response to sodium selenite, concurrently evaluating the expression of predicted mRNA targets for correlation with miRNA modulation.
- The project's novelty lies in the investigation of molecular regeneration patterns in *Perionyx excavatus*, coupled with an examination of regeneration-specific microRNAs following exposure to sodium selenite.

## 4 MATERIALS AND METHODS

### 4.1 MATERIALS

- Earthworms
- Distilled water
- Tissue paper
- Forceps
- Scalpel blade
- Eppendorfs
- Plastic box
- 1 x PBS
- Trimpure
- Isopropanol
- Chloroform
- Liquid nitrogen
- Cutting board
- Mortar and pestle
- 75% ethanol

## 4.2 METHODOLOGY

### 4.2.1. MAINTENANCE OF PERIONYX EXCAVATUS - VERMICULTURE

Adult *P. excavatus* specimens taken in Chennai were bred in International Research Centre Laboratory at Sathyabama Institute of Science and Technology. Adults were reared in 60 × 40 × 15 cm<sup>2</sup> plastic containers filled with organic soil with at least 70% humidity provided by The University. Organic soil was changed every two weeks. The incubation temperature was regulated between 22 and 26 degrees Celsius. Incubation is done in dark conditions for vermiculture because earthworms are light-sensitive organisms (Abesekara CP et al., 2007).

#### 4.2.1.1 Gut cleaning and amputation

The adult earthworms are carefully selected and subjected to gut cleaning to obtain a clean sample for analysis or testing. After removing the contents of the digestive tract, the worms are washed in distilled water and placed on a water-soaked paper towel to minimize stress. They are then individually housed in plastic boxes filled with paper bits, with regular sprinkling of water for moisture. Over the next 24 hours, the boxes are monitored for any signs of worm mortality or contamination. Amputation is performed on the earthworm's posterior end, typically after the ninth segment, ensuring preservation of vital sensory organs and reproductive structures. The amputated segments are placed in plastic boxes with moist soil for bud formation over five days, while the removed segments are stored in centrifuge tubes containing cold 1X PBS for further analysis (Rajagopalan K et al., 2022).

### 4.2.2 RNA ISOLATION

#### 4.2.2.1 TriMPure protocol :

The TriMPure RNA extraction reagent was utilized for the rapid extraction and isolation of total RNA from various cell types, including animal and plant tissues, cultured cells, and microbial cell masses. This specially formulated solution, Trimpure Clear, was a ready-to-use, red solution designed to facilitate single-step RNA

isolation alongside the recovery of whole DNA and proteins from cell lysate. Moreover, the reagent's procedures incorporated provisions for miRNA purification, enhancing its versatility. In the extraction process, frozen tissue was transferred into a mortar and pestle and homogenized until powdery to ensure optimal RNA quality and yield. Trimpure was added in an amount corresponding to the homogenized tissue. For fresh samples, Trimpure was added immediately after tissue collection and homogenization. The homogenized sample was then transferred into a centrifuge tube and kept at room temperature (15-30°C) for 5 minutes. Subsequently, the tube was centrifuged at 12,000g for 5 minutes at 4°C, and the supernatant was collected and transferred to a new centrifuge tube, while the pellet was not collected (*Hosking E et al., 2020*).

#### **4.2.2.2 Extraction of total RNA**

0.2ml of Chloroform was added per 1ml of Trimpure used for homogenization. The centrifuge tube was capped and mixed until the solution became milky. The solution was kept at room temperature for 5 minutes. Subsequently, it was centrifuged at 12,000g for 15 minutes at 4°C. The solution separated into three layers: the top layer containing a semisolid layer, the middle layer mostly comprising RNA or DNA, and the bottom organic solvent layer containing proteins, cells, etc. The top liquid layer was carefully transferred to a new centrifuge tube without disturbing the middle layer. Then, 0.5-1 ml of Isopropanol was added per 1ml of Trimpure used for homogenization, and the mixture was thoroughly mixed. The mixture was kept at room temperature for 10 minutes. Finally, it was centrifuged at 12,000g for 10 minutes at 4°C to precipitate the RNA (*Hosking E et al., 2020*).

#### **4.2.2.3 Cleaning RNA precipitate**

The supernatant was carefully removed without disturbing the pellet, ensuring that any remaining isopropanol posed no issue. An amount of 75% ethanol equivalent to the volume of the supernatant was added to the pellet. The precipitate was then cleaned by vortexing. Subsequently, the solution was centrifuged at 7500g for 5 minutes at 4°C, and the supernatant was discarded while being cautious not to disturb the precipitate.

#### **4.2.2.4 Dissolving RNA**

The precipitate was dried by leaving the tube open for several minutes. It's important to note that centrifuging to dry the precipitate or heating it for drying should be avoided as it may cause difficulties in dissolving the RNA. Once the precipitate was dry, an appropriate amount of RNase-free water was added to dissolve it, ensuring optimal conditions for RNA dissolution (*Hosking E et al.*, 2020).

#### **4.2.2.5 RNA quantification**

The required number of Qubit™ tubes for standards and samples were set up, ensuring the use of thin-wall, clear, 0.5-mL PCR tubes or 8 × 200-µL tube strips for the Qubit™ 4 Fluorometer or Qubit™ Flex Fluorometer, respectively. Care was taken to label the tube lids, avoiding labeling the sides to prevent interference with sample reading. Next, the Qubit™ working solution was prepared by diluting the Qubit™ RNA HS reagent 1:200 in Qubit™ RNA HS buffer in a clean plastic tube, with 200 µL of the working solution added to each tube. Subsequently, 10 µL of each Qubit™ standard was added to the appropriate tube, followed by 1-20 µL of each user sample. The tubes were vigorously vortexed for 3-5 seconds and then incubated at room temperature for 2 minutes. Following the incubation, the standards and samples were read. For the Qubit™ 4 Fluorometer, the RNA High Sensitivity assay type was selected, and the standards were read, followed by running the samples according to the provided instructions. For the Qubit™ Flex Fluorometer, the same procedure was followed, ensuring to load the tube strips with samples as indicated. The interpretation of results could be found in the respective user guides available for download. These steps were repeated as necessary to read all samples.

The Qubit® RNA BR Assay Kit protocol involves several steps for setting up assay tubes, preparing Qubit® Working Solution, and performing the assay. Firstly, assay tubes are set up for standards and user samples, with specific tubes designated accordingly. Qubit® Working Solution is then prepared by diluting the Qubit® RNA BR Reagent in Qubit® RNA BR Buffer at a ratio of 1:200, resulting in 200 µL prepared. Assay tubes are prepared with the appropriate volumes of BR

solution and BR dye, along with the user's RNA sample. After vortexing and incubation, the RNA Broad Range Assay is selected on the Qubit® 2.0 Fluorometer for calibration with standards and reading the samples. This standardized procedure ensures accurate quantification of RNA samples using the Qubit® system (Wang J et al., 2023).

### 4.3 cDNA SYNTHESIS

The iScript reverse transcriptase is RNase H<sup>+</sup>, offering more sensitivity than RNase H<sup>-</sup> enzymes in quantitative PCR. iScript is a modified MMLV-derived reverse transcriptase that can reliably synthesize cDNA from a wide variety of input RNA. The enzyme is supplied pre-blended with RNase inhibitor. The iScript reaction mix, which combines oligo(dT) and random hexamer primers, is effective for a wide range of targets. This mix is optimized for producing targets under 1 kb in length. The iScript cDNA synthesis kit yields good results in both real-time and traditional RT-PCR.

**Table 4.1 : Components present in iScript cDNA synthesis kit**

|                                             |       |
|---------------------------------------------|-------|
| 5x iScript reaction mix                     | 4 µl  |
| iScript reverse transcriptase               | 1 µl  |
| Nuclease-free water                         | x µl  |
| RNA template<br>(100 fg to 1 µg total RNA)* | x µl  |
| Total volume                                | 20 µl |

The quantification of cDNA levels across different tissue types reveals varying concentrations, indicative of differential gene expression profiles. The head and head blastema samples exhibit relatively higher cDNA quantities, with 3.88 ng/l and 4.14 ng/l, respectively. Contrastingly, the clitellum and clitellum blastema samples demonstrate lower levels, at 0.74 ng/l and 0.37 ng/l, respectively. Similarly, the tail and tail blastema samples also exhibit lower cDNA concentrations, measured at 0.44 ng/l and 0.37 ng/l, respectively. These findings suggest tissue-specific differences in gene expression dynamics during regeneration processes, highlighting the importance of further investigation into the molecular mechanisms underlying tissue regeneration in earthworms.

**Table 4.2: Reaction protocol**

|                       |                 |
|-----------------------|-----------------|
| Priming               | 5mins at 25 °C  |
| Reverse transcription | 20mins at 46 °C |
| RT inactivation       | 1min at 95 °C   |
| Optional step         | Hold at 4 °C    |

#### **4.3.1 cDNA quantification**

To prepare the Qubit™ working solution for the required number of samples and standards, follow a systematic procedure. Start by ensuring the correct number of tubes or wells are designated for each sample and standard. Then, dilute the Qubit™ assay reagent according to the manufacturer's instructions, typically by mixing with an appropriate buffer solution to form the working solution. This working solution is specifically designed to interact with the target molecule, such as dsDNA, ssDNA, RNA, miRNA, or protein, causing fluorescence only when bound. The Qubit™ assays cover a range of nucleic acid and protein types, offering flexibility in quantification. For example, the Qubit dsDNA HS Assay Kit is optimized for sensitive DNA samples and

provides accurate quantitation within a specified concentration range. Once the working solution is prepared, it can be used with the Qubit™ fluorometer, a highly sensitive instrument that measures fluorescence intensity emitted by the fluorescent dye bound to the target molecule. This method ensures accurate and selective quantitation of nucleic acids or proteins, making it particularly useful for assessing DNA quality before downstream applications such as next-generation sequencing. Comparatively, other methods like NanoDrop, based on UV absorbance, and quantitative PCR (qPCR), which measures target gene abundance, offer alternative approaches for DNA quantification, each with its advantages and limitations. Therefore, the choice of method depends on the specific requirements of the experiment and the type of nucleic acid or protein being quantified.

To prepare the Qubit™ working solution, label 0.5mL PCR tubes for each sample and standard. Dispense 190 µL of Qubit™ working solution into standard tubes and 180-199 µL into sample tubes to reach a final volume of 200 µL. Add 10 µL of standard and 1-20 µL of each user sample to their respective tubes. Vortex vigorously for 3-5 seconds and incubate at room temperature for 2 minutes. Utilize the Qubit™ 3 Fluorometer to read standards and samples, selecting the appropriate assay type and volume. Record concentration results for each sample. Save the run file for future reference. Check negative controls, ensuring concentrations are ideally <1 ng/µL; if not, contamination may have occurred, requiring repetition of the steps (*Won J, et al., 2020*).

#### **4.4 PRIMER DESIGNING**

We accessed the IDT PrimerQuest Tool by visiting the Integrated DNA Technologies (IDT) website and navigating to the PrimerQuest Tool section. We designed forward primers for the following miRNA's : miR- 1, miR - 29b, miR- 219, miR- 34 and the reverse primer was commercially bought, then we designed primers for the following genes : Dachous, TF Su(H), Synaptotagmin - 7b, TF Sox F (*Klymus KE et al., 2020*).

**Table 4.3: miRNA primers for qPCR analysis**

| Serial no | miRNA     | Forward primer sequence   |
|-----------|-----------|---------------------------|
| 1.        | miR - 1   | AAGCGACCTGGAAGACTTGTGATT  |
| 2.        | miR - 29b | CGCGGCCTAGCACCATTTAAAATCA |
| 3.        | miR - 219 | AACACGCTGATTGTCCAAACGCAGT |
| 4.        | miR - 34  | AACAAGTGGCAGTGTGGAAAGCTG  |

Reverse primer is commercially bought

**Table 4.4: List of primers used for qPCR analysis**

| Serial no. | Genes              | Forward primer sequence    | Reverse primer sequence    |
|------------|--------------------|----------------------------|----------------------------|
| 1.         | Dachsous           | TCCCTGAGTCTGACTTCCA<br>A   | GAGATGAGAACGGAAGG<br>GAAAG |
| 2.         | TF Su(H)           | GGACGATCATTAGCACCG<br>ATAG | AACATCTCCGCCTCCATT<br>TAG  |
| 3.         | Synaptotagmin - 7b | CTCAAGGCCAAAGACCTC<br>AA   | GAACCACCGTCTTCTTCT<br>TCTC |
| 4.         | TF Sox F           | GAACGCCTTCATGGTTTG<br>G    | TCACTCACCCAGCATCTT<br>G    |

We entered the sequence information by inputting the target DNA sequence for which we wanted to design the primer. We typed the sequence directly into the designated box. Then we define the primer parameters, We specified the desired length for our primers, typically ranging around 18-30 nucleotides. Additionally, we also set the desired GC content percentage, typically between 40% and 60%, and enter the desired Tm range, commonly set around 50-65°C. We also specified

additional parameters such as maximum self-complementarity, maximum 3' self-complementarity, maximum hairpin stability, and maximum 3' stability. Once the primer design phase concluded, we received a compilation of primer pairs that met our predetermined criteria. It was crucial to meticulously scrutinise the outcomes, focusing on factors such as melting temperature ( $T_m$ ), GC content, and the possibility of secondary structures. We assessed the specificity of the primers by examining any potential off-target binding sites. Moreover, we took into account primer interactions and the likelihood of primer-dimer formation. This comprehensive evaluation ensured the efficacy and accuracy of the chosen primer pairs for our intended application.

We accessed the NCBI BLAST website (<https://blast.ncbi.nlm.nih.gov/>) to utilize the BLAST search tool for checking the specificity of our primers. We selected the "Nucleotide BLAST (blastn)" program, suitable for PCR primer sequences. Entering the sequences of our forward and reverse primers in the "Enter Query Sequence" box, ensuring each primer sequence was in FASTA format or pasted in separate lines.

Adjusting the search parameters as needed, such as the expect (E) value threshold and word size, we initiated the search by clicking the "BLAST" button. Upon completion, we reviewed the list of hits, focusing on the "Descriptions" column for information about sequences matching our primers. Evaluating the specificity by examining alignment results, we looked for matches with our target organism's genome or other relevant sequences. Considering alignment length, percent identity, and E-value, we gauged the level of similarity.

To analyse alignment in detail, we clicked on the alignment link to inspect alignment visually and identify potential mismatches or non-specific binding sites. We interpreted the results, considering the significance of any matches found. A perfect match to our target sequence indicated potential specificity, while matches to unintended sequences suggested non-specific binding or off-target effects. Finally, we selected the primer pairs that best met our requirements and objectives, proceeding to order them directly through the IDT website.

#### 4.5 PRIMER DILUTION

The protocol for diluting primers involves utilizing ultra-pure water that is devoid of DNA, RNA, enzymes, salts, and any other forms of contamination. Upon receiving, the primers are typically contained within an envelope. To prepare the primer solution, centrifuge the primer tubes at 8000 rpm for 30 seconds to ensure proper settling. Then, dissolve the primer in nuclease-free water (NFW) following the specifications outlined in the primer datasheet. Vortex the tubes thoroughly to ensure homogeneity and incubate them for 2 minutes at room temperature to allow for proper reconstitution. Next, incubate the mixture at 60°C for 10 minutes in a water bath to facilitate any necessary annealing processes. After this step, centrifuge the tubes again at 8000 rpm for 30 seconds to pellet any residual debris. Finally, prepare aliquots by combining 10 µL of the primer solution with 90 µL of NFW in labeled tubes. Store the aliquots at -20°C for long-term preservation, ensuring the integrity of the primer solution for future use in molecular biology applications (*Nalla AK et al., 2020*).

#### 4.6 qPCR STUDIES

To commence the procedure, the necessary number of Qubit™ tubes for standards and samples were arranged, ensuring the utilization of thin-wall, clear, 0.5-mL PCR tubes or 8 × 200-µL tube strips for the Qubit™ 4 Fluorometer or Qubit™ Flex Fluorometer, respectively. Tube lids were labeled meticulously, avoiding placement of labels on the sides to prevent any interference with sample reading. Subsequently, the Qubit™ working solution was prepared by diluting the Qubit™ RNA HS reagent 1:200 in Qubit™ RNA HS buffer in a clean plastic tube, with 200 µL of the working solution added to each tube. Following this, 10 µL of each Qubit™ standard was introduced into the appropriate tube, succeeded by the addition of 1-20 µL of each user sample.

The tubes underwent vigorous vortexing for 3-5 seconds and were then left to incubate at room temperature for 2 minutes. After the incubation period, both the standards and samples were read. For the Qubit™ 4 Fluorometer, the RNA High Sensitivity assay type was chosen, and the standards were read first, followed by

running the samples according to the provided instructions. Similarly, for the Qubit™ Flex Fluorometer, the same protocol was followed, ensuring that the tube strips were loaded with samples as instructed. Interpretation of the results are found in the respective user guides available for download. These steps were repeated as necessary to read all samples

**Fig 4.5: Reaction setup**

| COMPONENT                     | VOLUME FOR 20 $\mu$ L REACTION | VOLUME FOR 10 $\mu$ L REACTION |
|-------------------------------|--------------------------------|--------------------------------|
| iTAQ Universal SYBR Green Mix | 10                             | 5                              |
| Forward primer                | Variable                       | Variable                       |
| Reverse primer                | Variable                       | Variable                       |
| DNA template                  | Variable                       | Variable                       |
| Nucleus free water ( NFW )    | To 20 $\mu$ L                  | To 10 $\mu$ L                  |
| Total reaction mix volume     | 20 $\mu$ L                     | 10 $\mu$ L                     |

To calculate the expression fold change, begin by determining the average Ct values for both the gene being tested and the housekeeping gene in both experimental (E) and control (C) conditions: TE, TC, HE, and HC. Then, compute the differences between experimental and control values for both the gene being tested ( $\Delta CTE = TE - HE$ ) and the housekeeping gene ( $\Delta CTC = TC - HC$ ). Next, calculate the difference between the  $\Delta\Delta CT$  values for the experimental and control conditions ( $\Delta\Delta CTE - \Delta\Delta CTC$ ) to determine the double delta Ct value ( $\Delta\Delta\Delta CT$ ). Since all calculations are performed using a logarithm base 2, remember that every time there's twice as much DNA, the Ct values decrease by 1, rather than halving. Hence, to obtain the expression fold change, compute the value of 2 raised to the power of negative  $\Delta\Delta\Delta Ct$  ( $2^{-\Delta\Delta\Delta Ct}$ ). This value represents the fold change in gene expression between the experimental and control conditions.

To calculate expression fold change, first, find the average Ct values for the gene being tested and the housekeeping gene in both experimental and control conditions. Then, compute the differences between experimental and control values for both genes. Next, find the difference between the  $\Delta\Delta CT$  values of experimental and control conditions to get the double delta Ct value. Finally, raise 2 to the power of negative double delta Ct to obtain the fold change, as Ct values decrease by 1 for every doubling of DNA. Optimizing the annealing temperature in qPCR assays is crucial for ensuring specificity and yield of desired PCR products. Setting it too low can lead to nonspecific amplification, while setting it too high may reduce product yield. Even after calculating the primer's melting temperature ( $T_m$ ), empirical testing across a range of temperatures may be necessary. Bio-Rad real-time PCR detection systems offer a thermal gradient feature, facilitating annealing temperature optimization in a single experiment. By testing a temperature range above and below the calculated  $T_m$ , the optimal annealing temperature can be determined. This optimal temperature is identified by the lowest Cq value without nonspecific amplification. Using this approach, researchers can efficiently determine the most suitable annealing temperature for their qPCR assay. To ensure the specificity of your qPCR assay, it's essential to analyze the reaction products using both the melt curve function on your real-time instrument and agarose gel electrophoresis. An optimized SYBR® Green qPCR assay should exhibit a single peak in the melt curve analysis, indicating the presence of a specific PCR product. This peak should correspond to a single band observed on the agarose gel. The absence of additional peaks in the melt curve and bands on the gel suggests that the assay is specific, with minimal nonspecific amplification. By comparing the melt curve and agarose gel analyses, researchers can confirm the specificity of their qPCR assay and ensure reliable detection of the target DNA sequence (Barra GB, et al., 2020).

#### **4.7 IN VITRO STUDIES**

For this experiment, we utilised the C2C12 mouse muscle cell line cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS). We treated the cells with sodium selenite ( $Na_2SeO_3$ ) for the experimental conditions. To observe cellular responses, we employed the Celloger cell imaging

system or an equivalent imaging system. Cell culturing was conducted on appropriate cell culture plates using pipettes and pipette tips for handling. The cells were maintained in a controlled environment within a cell culture incubator. For cell passaging and detachment, we utilised Trypsin-EDTA solution. Additionally, phosphate-buffered saline (PBS) was used for washing and resuspending cells during various experimental procedures.

In the initial step of the procedure, C2C12 cells were thawed and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) within cell culture plates. The cells were maintained under optimal conditions in a humidified cell culture incubator set at 37°C with 5% CO<sub>2</sub>. Subsequently, when the cells reached 70-80% confluence, they were passaged using trypsin-EDTA solution in accordance with standard cell culture protocols.

Following the cell culture stage, the treatment setup phase commenced. Initially, sodium selenite stock solutions were prepared at varying concentrations including 500nM, 1µM, 100µM, 200µM, and 300µM through appropriate dilutions. Subsequently, the growth medium within the cell culture plates was replaced with fresh DMEM supplemented with 10% FBS. To initiate the treatment, sodium selenite stock solutions were added to designated wells, ensuring the attainment of desired final concentrations. Control wells, left untreated, were included for comparative analysis.

Following the treatment phase, the cells were subjected to incubation within the cell culture incubator for a duration of 24 hours, as dictated by the experimental parameters. During this incubation period, the treated cells were allowed to interact with the sodium selenite compounds, facilitating the observation of any resultant cellular responses or changes.

Upon completion of the designated incubation period, the observation and imaging step ensued. Initially, the culture medium from the wells was carefully removed to prepare the cells for subsequent analysis. Subsequently, the cells were

gently rinsed with phosphate-buffered saline (PBS) to effectively eliminate any residual culture medium, ensuring a clean substrate for observation. This step aimed to minimise any potential interference or artefacts during the subsequent imaging process.

After the fixation of the cells, we proceeded to observe the results using the Celloger imaging system. This system allowed for high-resolution imaging of the fixed cells, enabling us to visualise any morphological or functional changes induced by the treatment with sodium selenite. The Celloger system provided detailed insights into cellular responses, facilitating the analysis and interpretation of experimental outcomes (Zhou D, et al., 2021)..

#### **4.8 TARGET PREDICTION**

Our target prediction protocol involves employing three distinct tools: RNAhybrid, RNA22, and miRNanda. Firstly, we input our miRNA sequence along with the target mRNA sequence into each tool separately. RNAhybrid utilizes thermodynamic calculations to predict miRNA-mRNA interactions, considering factors such as hybridization energy and seed region complementarity. RNA22 employs a pattern-based approach to identify potential miRNA binding sites within the mRNA sequence, utilizing statistical models to predict target sites. Lastly, miRNanda employs dynamic programming algorithms to identify potential miRNA targets based on sequence complementarity and secondary structure accessibility. After running our sequences through each tool, we carefully analyze the results, considering factors such as binding site accessibility, sequence complementarity, and predicted binding energy. By employing multiple target prediction tools, we aim to enhance the reliability and comprehensiveness of our miRNA target predictions, facilitating a more thorough understanding of miRNA-mRNA interactions and their biological implications (Rawoof A et al., 2021).

#### **4.9 PREPARATION OF MODEL EARTHWORMS FOR INJECTION WITH SODIUM SELENITE**

Adult *Perionyx Excavatus* earthworms, totaling approximately 76 individuals, were collected for the experiment, with each concentration group comprising 12 to 14 worms. Before commencing the experiment, the earthworms underwent gut cleaning procedures over the course of one day, followed by the amputation of their heads. The experiment involved testing six different sodium selenite dosage concentrations, including a control group, ranging from 500 nm to 300  $\mu$ m.

The average weight of the earthworms fell within the range of 70-80 mg, which necessitated a sodium selenite injection dosage of 6  $\mu$ m. Throughout the experiment, daily photographic documentation was conducted over a five-day period to monitor and record the growth progression of the head region in the earthworm specimens. This meticulous observation allowed for the assessment of any changes or responses induced by the sodium selenite treatments across the different dosage concentrations (Xu Q, *et al.*, 2022).

#### **4.10 HISTOLOGY**

In fixation process, we submerged the earthworm heads, which had undergone sodium selenite treatment following amputation, in a fixative solution immediately upon collection. We opted for a commonly used fixative, 10% neutral buffered formalin, to preserve the soft tissues. The specimens were then left to fixate for 24 to 48 hours at room temperature, with longer fixation times possibly necessary for larger specimens.

After fixation, we transferred the specimens to isopropyl alcohol (IPA) with increasing concentrations, ranging from 60% to 100%, for dehydration. Each ethanol solution was carefully prepared, and the specimens were left in each solution for approximately 1 to 2 hours to ensure thorough dehydration. It was crucial to fully submerge the specimens in each ethanol solution to facilitate proper dehydration.

Following dehydration, we moved the specimens to a clearing agent, such as xylene, to remove any remaining ethanol and make the specimens transparent. The

specimens were left in the clearing agent for approximately 30 minutes to ensure effective clearing.

For infiltration, we prepared a paraffin wax embedding medium by melting paraffin wax in a suitable container. Once the specimens were adequately cleared, we transferred them to a container filled with molten paraffin wax. The specimens were allowed to infiltrate with the paraffin wax for several hours to ensure proper embedding. This process was crucial to prepare the specimens for subsequent sectioning and histological analysis.

In the embedding stage, we carefully oriented the specimens within embedding molds filled with molten paraffin wax to ensure proper sectioning orientation. This step was crucial to obtain accurate views during subsequent analysis. Subsequently, we allowed the paraffin wax to solidify, forming blocks containing the embedded specimens.

Next, we used a microtome to cut thin sections, approximately 5  $\mu\text{m}$  thick, of the embedded specimens. It was essential to ensure that the sections were cut perpendicular to the long axis of the earthworm to obtain cross-sectional views, facilitating detailed examination. To ensure proper adherence of the tissue sections to the slides, we applied an adhesive solution comprising egg albumin, glycerol, and thymol.

Following sectioning, we floated the sections on a warm water bath, maintained at approximately 40-45°C. This process helped to flatten and expand the sections before transferring them to glass slides for mounting. Each step was carefully executed to prepare the tissue sections for subsequent staining and microscopic analysis, ensuring accurate and reliable results in our histological investigation.

In our staining procedure, we utilised Hematoxylin and Eosin (H&E) staining, commonly employed for general tissue morphology and cellular details. Initially, we placed the slide in a slide warmer set at 70 - 80 degrees to remove the wax.

Subsequently, the slide was immersed in xylene for 5 minutes to further eliminate wax residues. Following this, we immersed the slide in 100% IPA for 3 minutes to remove xylene residues, followed by a water wash.

Next, we applied the Hematoxylin stain to the slide and allowed it to incubate for 10 minutes to ensure proper nuclear staining. Subsequently, we dipped the slide once in a 1% acid-alcohol solution (HCl : IPA) for brief immersion. Afterward, the slide underwent blooming for 13 minutes to enhance staining intensity. Following this, Eosin stain was applied to the slide for 55 seconds, followed by an immediate water wash.

Upon completion of staining, we proceeded to mount the sections onto glass slides. Firstly, the sections were dehydrated by passing them through 100% IPA, followed by clearing in xylene or a xylene substitute. Subsequently, we mounted the dehydrated and cleared sections using a mounting medium, specifically DPX, onto glass slides. Finally, we covered the mounted sections with coverslips and sealed the edges to preserve the specimens.

In our visualisation process, we ensured that the slides were allowed to dry completely before viewing them under a light microscope. We captured images of the histological sections using an inverted fluorescence microscope equipped with a digital camera. This equipment allowed us to visualise the tissue sections at a magnification of 10x, providing detailed and high-resolution images for analysis (*Li M et al.*, 2020)..

## 5 RESULTS

### 5.1 PRIMER DILUTION

The functional roles of various genes implicated in earthworm regeneration and development. Actin, a cytoskeleton protein, is crucial for maintaining cell structure and enabling cellular motility. Its highly conserved nature underscores its fundamental importance across different organisms. Dachshaus, associated with polarity complex proteins, contributes to establishing and maintaining cellular organization, crucial for coordinated development and regeneration. Hedgehog's involvement in cell pattern and fate highlights its pivotal role in directing developmental processes, ensuring proper tissue formation. Sox-C's role in cell fate determination underscores its significance in regulating gene expression patterns that drive cell differentiation, critical for tissue regeneration. Tenurin, a cell adhesion molecule, is highlighted as a housekeeping gene due to its stable expression across earthworm species, ensuring reliable normalization in qPCR experiments. The universal stress gene provides tolerance to various stresses, reflecting its role in the earthworm's adaptive response to environmental challenges. Wnt-11's function in cell growth, function, and differentiation underscores its involvement in signaling pathways crucial for regulating these vital processes during regeneration. Yurt, similar to Dachshaus, plays a role in cell polarity, contributing to the spatial organization of cells, which is essential for coordinated tissue regeneration. The notable high expression of Dachshaus in the head region suggests its specific involvement in directing regeneration processes in this anatomical region, possibly indicating its importance in initiating or orchestrating regeneration events. Overall, these genes collectively contribute to the complex molecular machinery governing earthworm regeneration and developmental processes.

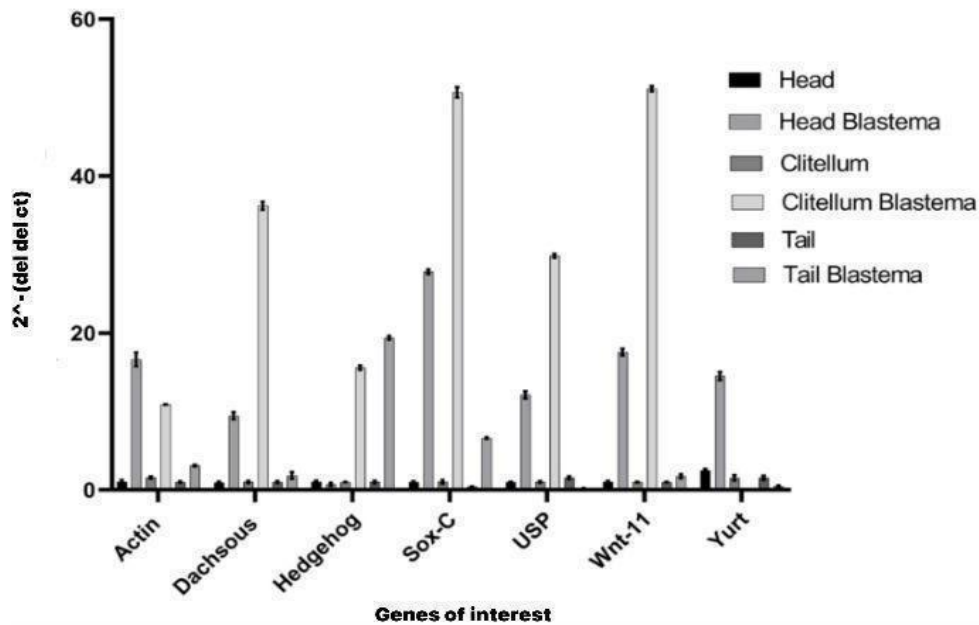
**Table 5.1: Details of primers that has been used for q-PCR amplification.**

| Sl.No | Gene                       | Forward Primer         | Reverse Primer          | Function of the gene                    |
|-------|----------------------------|------------------------|-------------------------|-----------------------------------------|
| 1     | Actin                      | GTAAGGATCTCTACGCCAACAC | CTGGTGATCTCCTTCTGCATAC  | Cytoskeleton protein                    |
| 2     | Dachsous                   | ACGACCATTGGGTGTCTTTAC  | GAGCATTCCAGTTCCCAGTTAT  | Polarity complex proteins               |
| 3     | Hedgehog                   | TTCGTTCTCCTGGTACTCCT   | CGTTCGGAATGTGCTGTTTG    | Cell pattern and fate                   |
| 4     | Sox-C transcription factor | GATTCGTTCAAGGACCGATGTA | GGAAGAGGATGATGAGGATGAAG | Cell fate determination                 |
| 5     | Teneurin                   | GGCGATTCAAGTCCAGATATT  | GACCCAGTTGCATGTCATAGA   | Cell adhesion molecules                 |
| 6     | Universal stress protein   | CATCACCATGACCACGAAGT   | CCCTTCGTCACAGCATCAA     | Tolerance to multiple stresses          |
| 7     | Wnt-11                     | GAGAACCGCAAAGTAGGAAAGA | CGGAAGACAATAATCGGGAGAC  | Cell growth, function & differentiation |
| 8     | Yurt                       | TCTCAGTGGTCGACTTGAATG  | AGGATTGTGGATGCCTTCTT    | Polarity complex proteins               |

## 5.2 qPCR analysis

qPCR analysis is used in regeneration studies to quantitatively measure changes in gene expression levels at various stages of regeneration, providing insights into the molecular mechanisms underpinning tissue repair and growth. It helps to understand the dynamics of important regulatory genes involved in regeneration pathways, which aids in the discovery of possible therapeutic targets for improving regenerative processes. This graph measures the relative expression levels of certain genes across samples. The x-axis shows the following genes of interest: Actin, Dachsous, Hedgehog, Sox-C, USP, Wnt-11, and Yurt. The y-axis shows the relative expression levels of these genes. The samples are divided into six categories, each indicated by a different shade of grey in the bars: Head, Clitellum, Clitellum Blastema, Tail, and Tail Blastema. Teneurin is used as control as it is expressed neutrally in all parts. Actin and Hedgehog are normally present in body and USP is a stress protein stimulator, these are generally used to build up tissues.

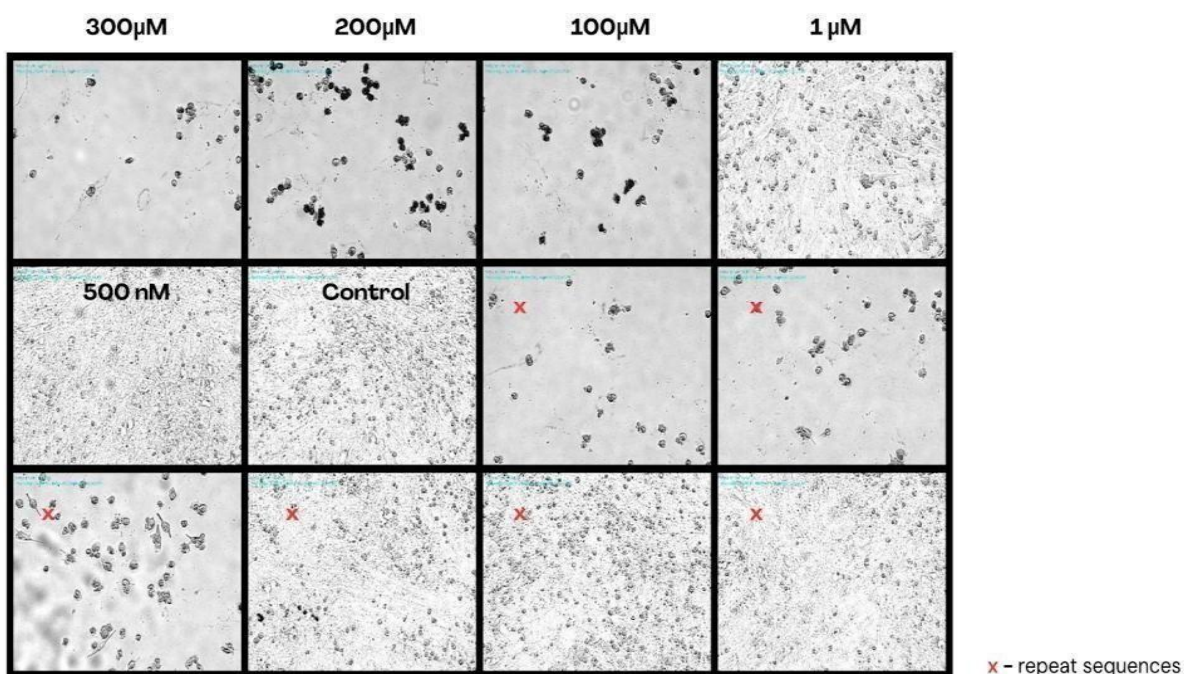
Actin is mostly used as housekeeping genes but here it is not used because it shows different expression levels in different samples during regeneration. Also Actin appears to be expressed at very similar amounts in all samples. Sox-C and Wnt-11 help in proliferation of stem cells and they are over expressed in most tissue types, with their expression levels notably higher than those of the other genes. This is evident from the tall bars associated with these genes across different tissue types, particularly in the blastema tissues, where their expression reaches the highest levels observed in the graph. Dachshaus and Yurt are usually observed in developmental state of drosophila and also in earthworms. It helps in organ formation. Dachshaus is the only gene that is expressed within normal or expected ranges. Dachshaus expression is higher in the Head Blastema and Clitellum Blastema compared to other samples. Hedgehog is highly expressed in the Clitellum Blastema. Sox-C is highly expressed in the Head Blastema, but also in the Clitellum Blastema. USP has the maximum expression in the Head Blastema, followed by the Clitellum Blastema. Wnt-11 is most strongly expressed in the Clitellum Blastema, but it is also present in the Head Blastema. Yurt expression is generally low in all samples, but slightly higher in the Head Blastema and Clitellum Blastema.



**Fig 5.1: Gene Expression Profile during Earthworm Regeneration**

### 5.3 IN VITRO STUDIES

In vitro experimentation was conducted using the C2C12 cell line, derived from mouse muscle cells, serving as a model system to assess the impacts of varying concentrations of sodium selenite. The experiment encompassed a control group devoid of sodium selenite treatment, alongside doses of 500nM, 1μM, 100μM, 200μM, and 300μM. Results were obtained through observation utilizing the celloger cell imaging system, revealing toxicity at concentrations of 100μM, 200μM, and 300μM. Despite these initial toxic outcomes, the experiment proceeded with the same concentrations applied to earthworms, where no adverse effects were observed.

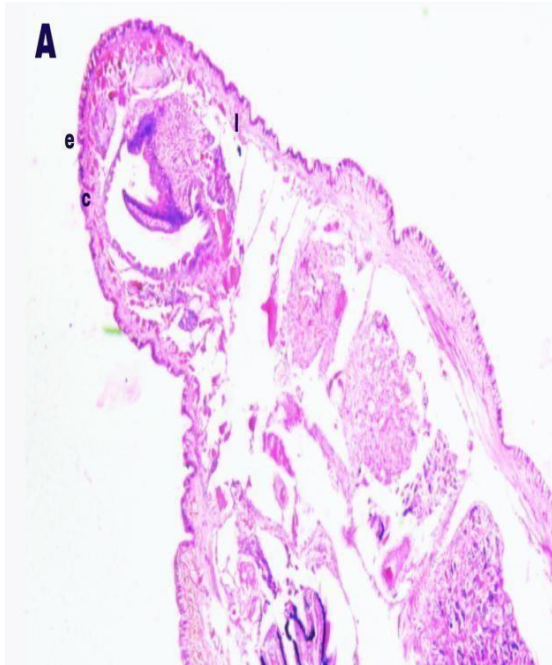


**Fig 5.2: C2C12 cell line with sodium selenite treatment with different concentrations (control, 500 nM, 1µM, 100µM, 200µM, and 300µM)**

In the figure above, the C2C12 cell line, derived from mouse muscle cells, was utilized as a model system to investigate the effects of varying concentrations of sodium selenite. The concentrations tested included a control group without sodium selenite treatment, as well as doses of 500nM, 1µM, 100µM, 200µM, and 300µM.

#### 5.4 CELLULAR MORPHOLOGY STUDY – HISTOLOGY

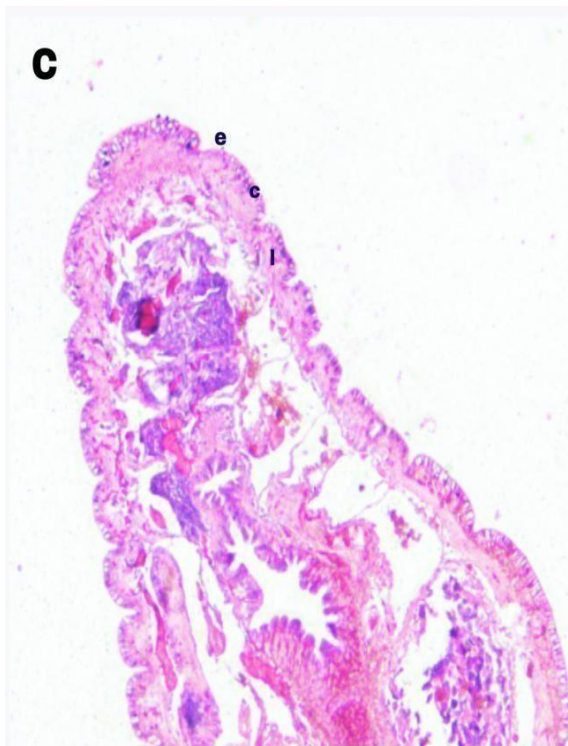
A longitudinal section of *Perionyx excavatus* subsequent to a five-day administration of sodium selenite, imaged at a magnification of 10X, revealed distinct outcomes across various concentrations of the administered compound. The treatments included a control group devoid of sodium selenite (A), along with concentrations of 500 nM (B), 1 µm (C), 100 µm (D), 200 µm (E), and 300 µm (F) of sodium selenite. Administration occurred at the head region of three segments before the clitellum. Histological examination indicated notable differences among the treatment groups. Particularly, the 300 µm concentration exhibited the most distinctively segmented head compared to the control, where only a bud was evident.



**A. Control**



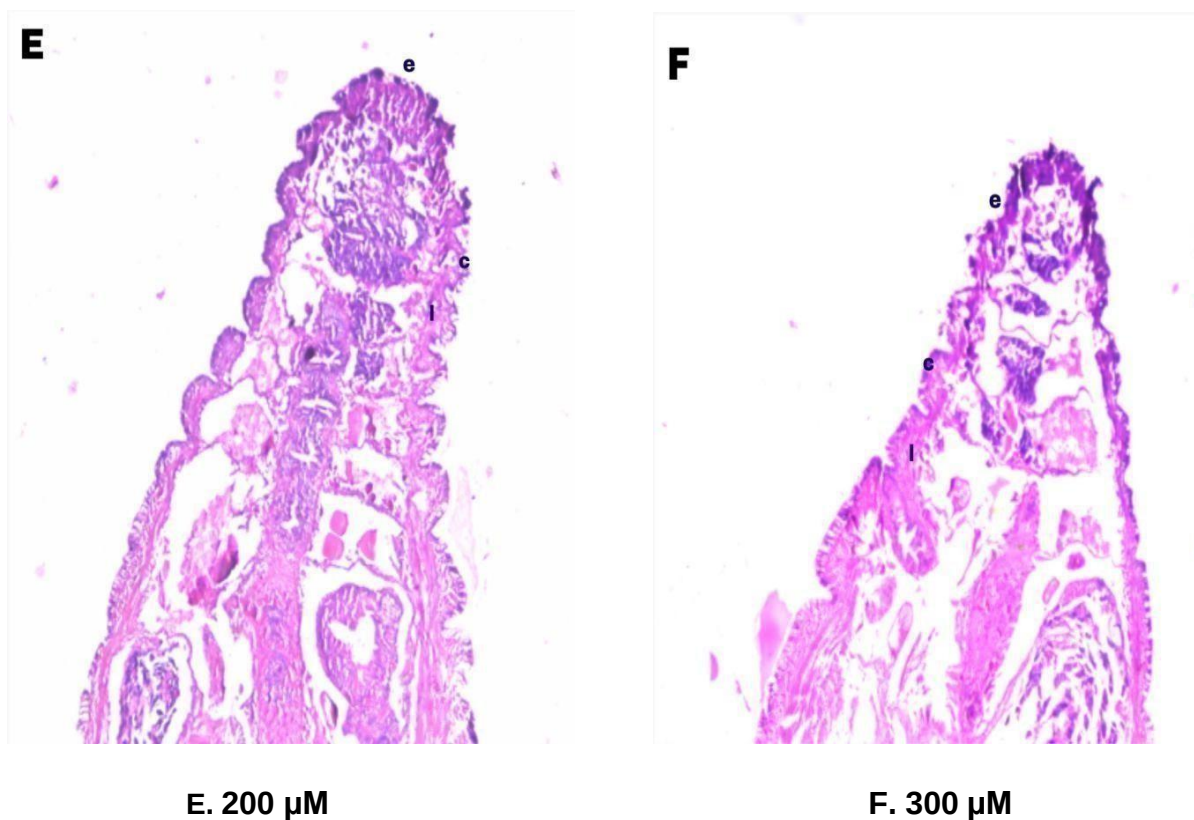
**B. 500 nM**



**C. 1 μM**



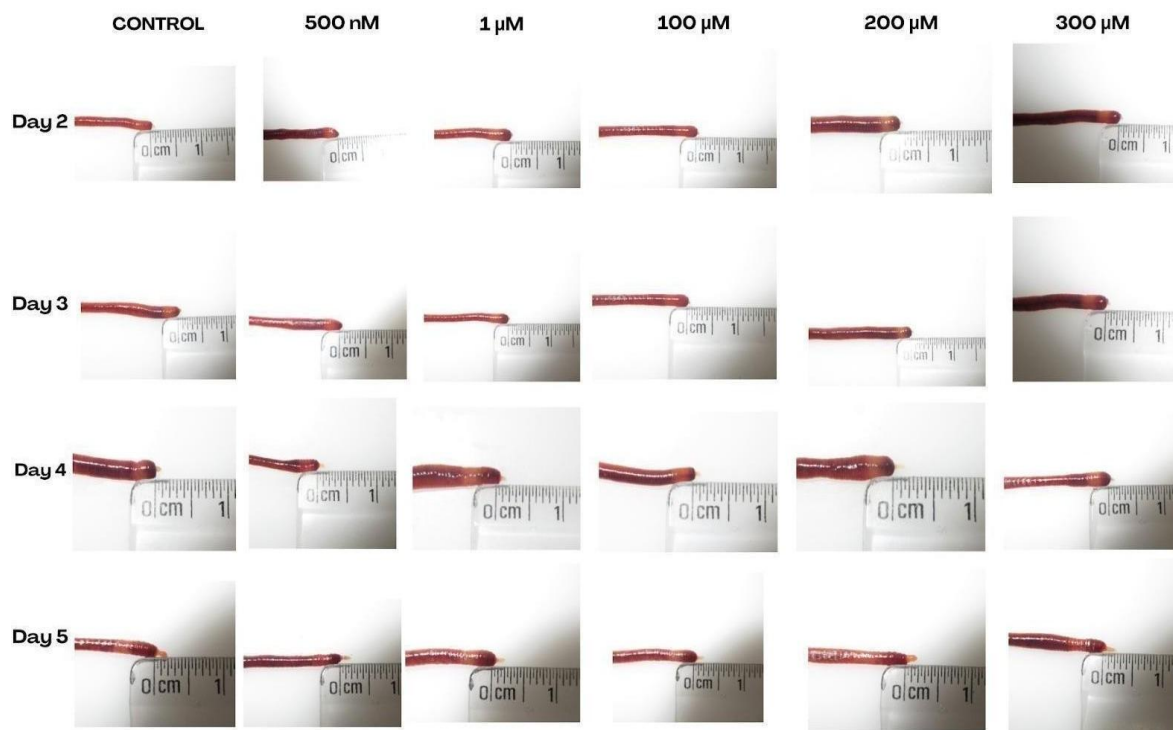
**D. 100 μM**



***Fig 5.3: Longitudinal section of Perionyx excavatus subsequent to a five-day administration of sodium selenite, captured at a magnification of 10X. Epidermis (e), Circular muscle layer (c) and Longitudinal muscle layer (l).***

## **5.5 BUD GROWTH OBSERVATION POST SODIUM SELENITE INJECTION**

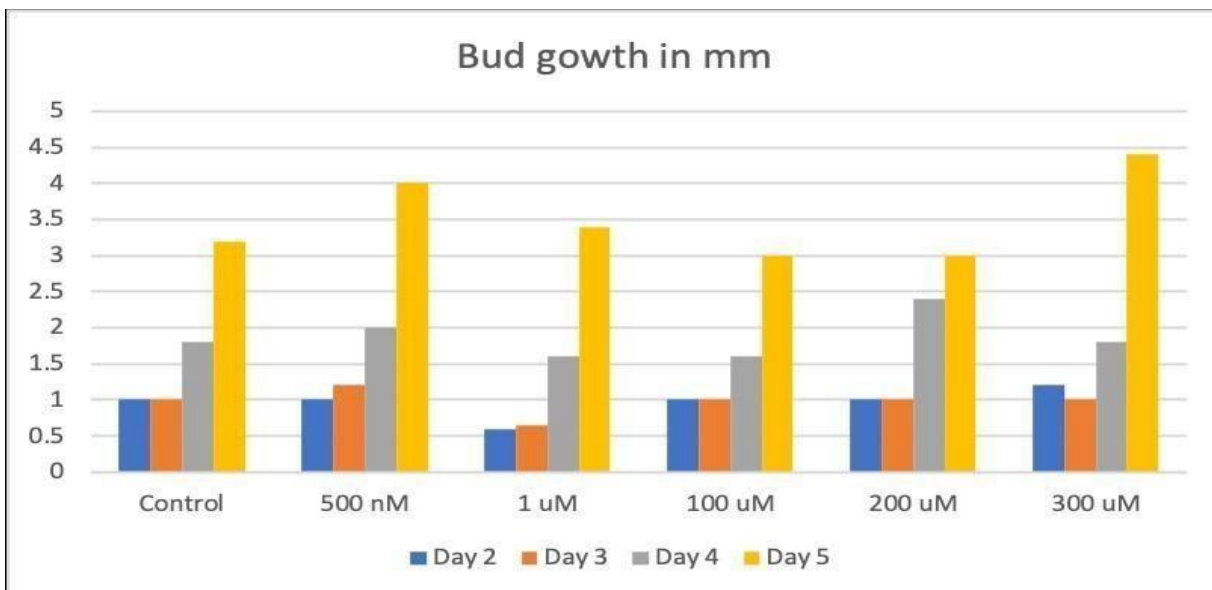
An apparent pattern is observed from the analysis, showing a much faster growth of the bud in response to the 300  $\mu\text{M}$  concentration of sodium selenite compared to the control group. This suggests that the 300  $\mu\text{M}$  concentration has a strong stimulating effect, speeding up the growth of the bud in our experiment. These findings highlight how sodium selenite at this concentration could influence cell growth and development, acting as a regulator of growth in our study.



***Fig 5.4: Detailed daily record of picture data starting from the second day following sodium selenite administration.***

**Table 5.2: Bud growth length was measured after five days of sodium selenite treatment across six concentrations.**

| CONC.   | Day 2  | Day 3   | Day 4  | Day 5  |
|---------|--------|---------|--------|--------|
| Control | 1 mm   | 1 mm    | 1.8 mm | 3.2 mm |
| 500 nM  | 1 mm   | 1.2 mm  | 2 mm   | 4 mm   |
| 1 uM    | 0.6 mm | 0.65 mm | 1.6 mm | 3.4 mm |
| 100 uM  | 1 mm   | 1 mm    | 1.6 mm | 3 mm   |
| 200 uM  | 1 mm   | 1 mm    | 2.4 mm | 3 mm   |
| 300 uM  | 1.2 mm | 1 mm    | 1.8 mm | 4.4 mm |



***Fig 5.5 : Bud growth observation after sodium selenite injection in 6 different concentrations graph in mm scale.***

Bud growth observation after sodium selenite injection in 6 different concentrations graph in mm scale concentrations being - control, 500 nM, 1 uM, 100 uM, 200 uM, 300 uM of sodium selenite on the amputated head region for five days, growth was measured in mm scale. At concentrations of 100 uM, 200 uM, and 300 uM, the growth was significantly higher compared to the control group.

## 5.6 GENE EXPRESSION ANALYSIS – TARGET PREDICTION

In this study, we used Rna22 ([https:// cm.jefferson.edu/rna22/Interactive/](https://cm.jefferson.edu/rna22/Interactive/)), RNAhybrid ([https://bibiserv2.cebitec.uni-bielefeld.de/ rnahybrid](https://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid)) and Miranda ([http://cbio.mskcc. org/](http://cbio.mskcc.org/)) software's for the target prediction of mir-219 because it shows the concentration depended expression. We selected the target genes with high prediction scores, which could play regeneration, which were Dachsous, Synaptotagmin 7b, TF Sox F, TF Su(H).

**Table 5.4: Results of prediction scores of miR-219 target genes**

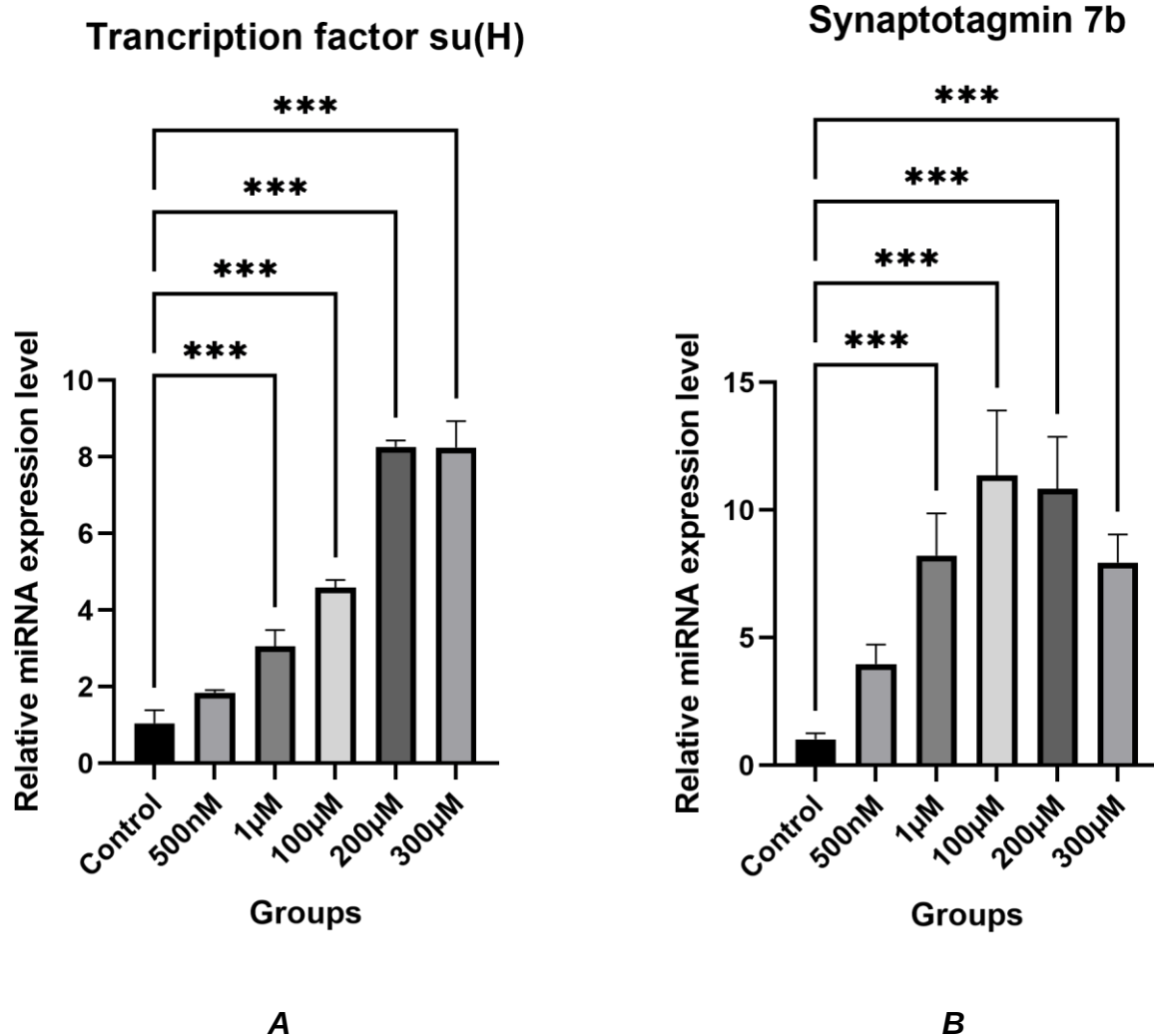
| miRNA       | Gene                                               | RNAhybrid | RNA22  | miRanda |
|-------------|----------------------------------------------------|-----------|--------|---------|
| Pex-miR-219 | Dachsous                                           | (-33)     | (-12)  | (-27)   |
| Pex-miR-219 | CCT-epsilon (T- complex protein 1 subunit epsilon) | (-31)     | -13    | -20.8   |
| Pex-miR-219 | Synaptotagmin 7b                                   | (-32)     | -10.40 | -31.30  |
| Pex-miR-219 | Uncx                                               | -28       | -7.80  | -18.34  |
| Pex-miR-219 | Protein NDNF                                       | -28       | -6     | -27.45  |
| Pex-miR-219 | Transcription factor SoxF                          | -31       | -24.98 | -27.87  |

|               |                             |     |        |                             |
|---------------|-----------------------------|-----|--------|-----------------------------|
| Pex-miR- 219  | Protein DPCD                | -27 | -9.40  | -18, -14, -16, -9.44, -9.72 |
| Pex-miR- 219  | Transcription factor Su(H)  | -27 | -7.60  | -22.79, -15.59              |
| Pex-miR- 219  | Optomotor blind (Fragment)  | -27 | -7.70  | -17.09                      |
| Pex- miR- 219 | Decapentaplegic             | -26 | -15.60 | -22.57                      |
| Pex- miR- 219 | Protein tweety homolog      | -26 | -13.40 | -16.03                      |
| Pex- miR- 219 | Nipped-B protein            | -26 | -10.26 | -18                         |
| Pex- miR- 219 | Cortactin-binding protein 2 | -26 | -10.80 | -24.50                      |
| Pex- miR- 219 | Gsx (Fragment)              | -26 | -13.70 | -21.96                      |

## 5.7. qPCR STUDIES - miRNA AND mRNA

### 5.7.1 mRNA

After conducting qPCR and obtaining Ct values, we utilized the formula  $2^{-\Delta\Delta Cq}$  to calculate the relative gene expression values. Analysis of the obtained data revealed that the concentrations of 100  $\mu\text{m}$ , 200  $\mu\text{m}$ , and 300  $\mu\text{m}$  exhibited the most pronounced effects, as depicted in the provided figure. This suggests a potentially significant impact of these concentrations on the gene expression levels under investigation. To further assess the statistical significance of these findings, a one-way ANOVA was performed. The results of the ANOVA analysis indicated a statistically significant difference among the various concentrations, with a p-value denoted by three stars (\*\*\*) suggesting a highly significant result. This implies that the observed variations in gene expression among the different concentrations are unlikely to have occurred by chance alone, further supporting the notion of a substantial effect exerted by the last three concentrations.

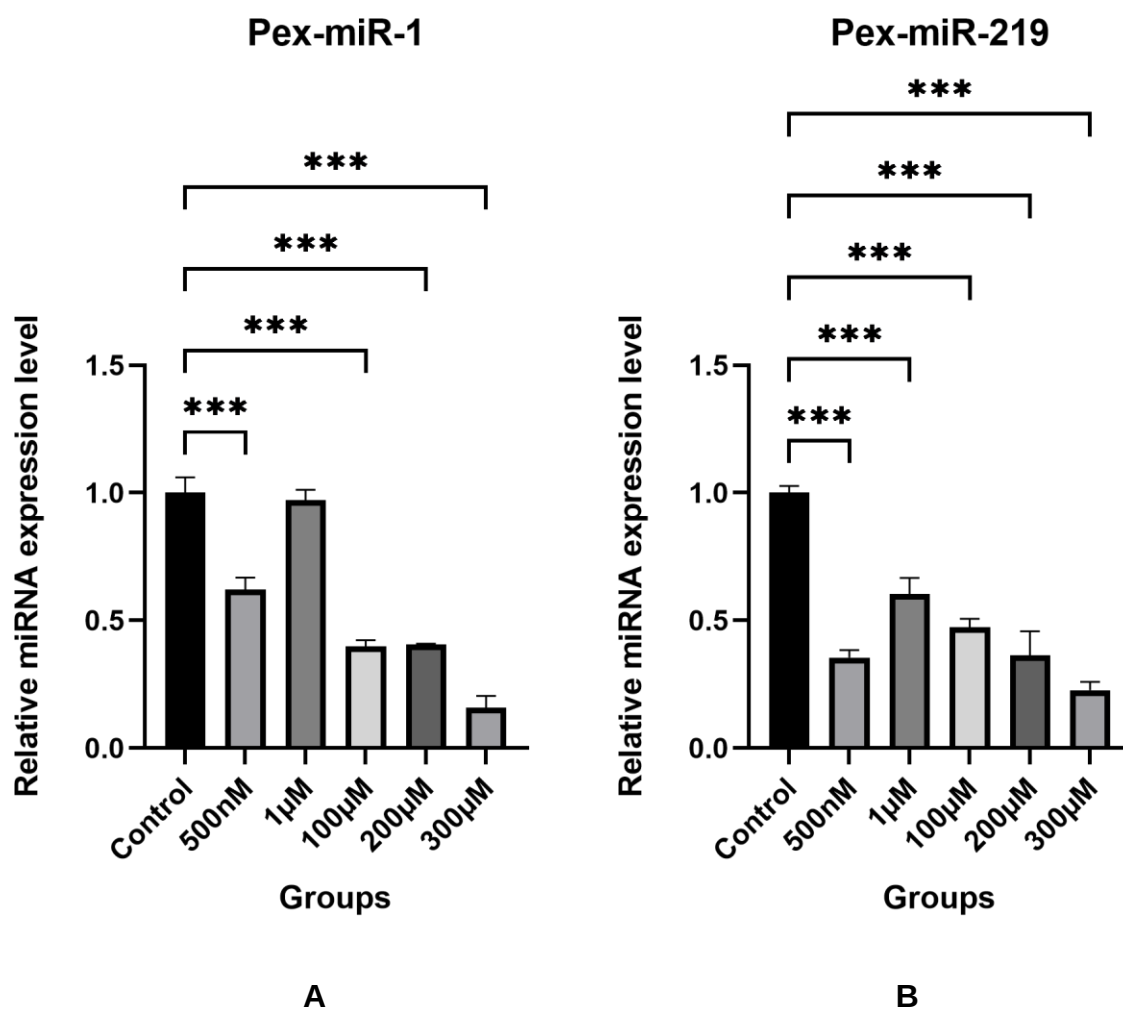


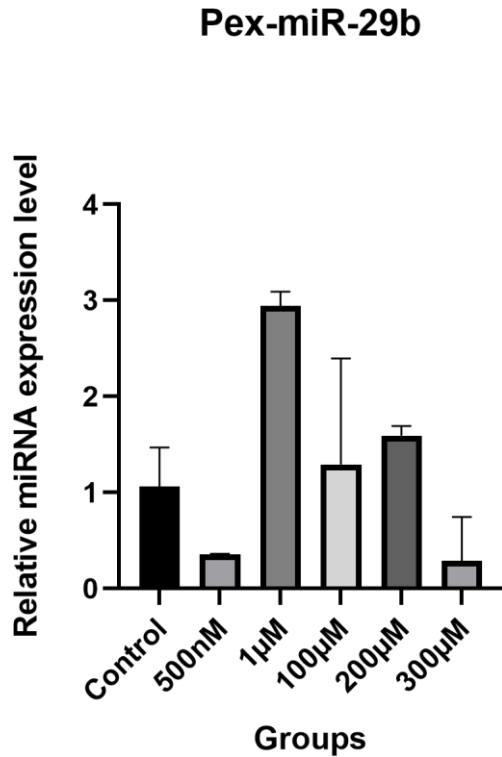
**Fig 5.6: The qPCR graphs depicting the  $2^{\Delta\Delta Cq}$  values of transcription factors Su(H) and Synaptotagmin-7b illustrate the effects of different concentrations, particularly emphasizing the significant impact of the last three concentrations (100  $\mu$ m, 200  $\mu$ m, 300  $\mu$ m).**

The graph displays data obtained from triplicate experiments, which were analysed using one way ANOVA test. \* Represents significant difference  $p < 0.05$ , \* represents significant difference  $p < 0.01$ , \* represents significant difference and  $p < 0.001$ , \*\* represents significant difference  $p < 0.0001$ .

### 5.7.2 miRNA

Following qPCR analysis, Ct values were obtained and utilized to calculate relative gene expression values using the formula  $2^{-\Delta\Delta C_q}$ . Observation of the figures revealed a notable downregulatory effect associated with the last three concentrations (100  $\mu\text{M}$ , 200  $\mu\text{M}$ , 300  $\mu\text{M}$ ). Moreover, a one-way ANOVA was conducted, indicating significance with three stars, indicating a notable difference between the groups. Conversely, miR29b qPCR values did not demonstrate significance, as reflected by the absence of stars in the ANOVA analysis. Furthermore, slight deviations were observed in triplicate values. Delta CT values have a reverse relationship with MiRNA's and target genes expression levels.





C

**Fig 5.7: Graphs revealed a downregulatory effect of the last three concentrations (100 µm, 200 µm, 300 µm).**

The q-PCR validation of the miRNA of *P. excavatus* were analysed in anterior head. The x-axis denotes the relative miRNAs expression and the y-axis denotes the concentration groups of sodium selenite. The graph displays data obtained from triplicate experiments, which were analysed using one way ANOVA test. \* Represents significant difference  $p < 0.05$ , \* represents significant difference  $p < 0.01$ , \* represents significant difference and  $p < 0.001$ , \*\* represents significant difference  $p < 0.0001$ . Graphs revealed a down regulatory effect of the last three concentrations (100 µm, 200 µm, 300 µm).

The differential expression of various earthworm tissues and their subsequent regeneration response following the injection of sodium selenite was investigated. The study employed quantitative polymerase chain reaction (qPCR) techniques to

analyze gene expression levels in different earthworm tissues and assess the impact of sodium selenite on regeneration processes. The research began by designing specific primers and optimizing their dilution for qPCR studies, crucial for amplifying target genes associated with regeneration pathways in earthworm tissues. The primer dilution process ensured the accuracy and efficiency of gene expression analysis during qPCR experiments. Subsequently, the study involved the collection and isolation of various earthworm tissues, including segments such as the anterior, posterior, and clitellum. Through qPCR studies, the expression levels of specific genes related to regeneration were quantified in each tissue type. Following the baseline gene expression analysis, earthworms were injected with sodium selenite, a compound known for its potential impact on regeneration processes. The injection allowed for the examination of how sodium selenite influenced the differential expression of genes associated with regeneration in earthworm tissues.

The results obtained from qPCR analyses, in conjunction with insights from referenced literature (*Paul, S., Balakrishnan, S., et al., 2022*). (*Zheng P et al., 2016*), provided valuable information regarding earthworm tissue regeneration in other earthworm species. Our study explored the correlation between gene expression levels and the earthworms' regeneration capabilities post-treatment. Notably, the exposure to sodium selenite exhibited a positive correlation with the miRNA, mir-219, and its target TK Su(H), indicating involvement in the Notch signaling pathway, which is significant for regenerative properties. Overall, this research contributes to our understanding of earthworm regeneration processes and sheds light on the potential effects of sodium selenite on tissue-specific gene expression and regeneration responses. These findings hold implications for both basic research in regenerative biology and applied studies aimed at understanding the environmental impacts of selenium compounds on earthworm populations.

## 6. SUMMARY AND CONCLUSION

In summary, we conclude that the head (anterior) part of *Perionyx excavatus* exhibits a notable capacity for regeneration. Our investigation revealed that this specific region of the earthworm possesses a heightened ability to undergo tissue regeneration processes. Through careful experimentation and analysis, we observed significant regeneration activity concentrated in the anterior segment, suggesting a focal point for further studies on regenerative mechanisms in *Perionyx excavatus*. We also delved into the potential effects of sodium selenite on tissue-specific gene expression and regeneration responses in *Perionyx excavatus*. By subjecting earthworms to sodium selenite exposure, we aimed to discern its impact on gene expression patterns and subsequent regeneration capabilities. Our findings underscore the importance of understanding how environmental factors, such as sodium selenite, can modulate gene expression profiles and influence regenerative processes in earthworms, particularly in species like *Perionyx excavatus*. Our study identified a significant correlation between the exposure to sodium selenite, the expression of miRNA mir-219, and its target gene TK Su(H), indicating a potential pathway underlying regenerative properties. Specifically, our results suggest an association with the Notch signaling pathway, a critical regulator of tissue regeneration. This correlation sheds light on the molecular mechanisms through which sodium selenite may exert its effects on regenerative processes in *Perionyx excavatus*, providing valuable insights into the interplay between environmental factors, gene expression, and regeneration pathways.

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# ANNEXURE

## JOURNAL SUBMISSION

### Mirdushi

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6:49 PM (0 minutes ago) ☆ ↶ ⋮

Re: Unlocking the potential of mRNA therapeutics  
by MIRDUSHI VASU MANOHARAN; Srinidhiy Chittibabu anitha  
Short Communication

Dear MS MIRDUSHI VASU MANOHARAN,

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### Priyadarshini

JBIOTEC-D-23-00726 - Confirming your submission to Journal of Biotechnology Inbox x



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Re: MicroRNA Biogenesis, Regulation, and Therapeutic Implications in Tissue Regeneration and Tissue Engineering  
by PRIYADHARSHINI KUMAR  
Review Article

Dear Priyadarshini Kumar PRIYADHARSHINI KUMAR,

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Centre Number **IN855** Date **09/MAR/2024** Candidate Number **572646**

### Candidate Details

Family Name **MANOHARAN**

First Name **MIRDUSHI VASU**

Candidate ID **B6480537**

UKVI Number **IEL/09032024/IN855/572646**



Date of Birth **30/04/2003** Sex (M/F) **F** Scheme Code **Private Candidate**

Country or Region of Origin

Country of Nationality **INDIA**

First Language **MALAYALAM**

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## HIGHER STUDIES ADMISSION LETTER



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Dear Mirdushi Vasu Manoharan

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| Student details                       |                         |
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| Full name (as shown on your passport) | Mirdushi Vasu Manoharan |
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Name of the student : MIRDUSHI VASU. M  
Register Number : 40230027  
Title of the Thesis : MOLECULAR EFFECTS OF SODIUM SELENITE DURING REGENERATION IN EARTHWORMS

Internal Guide Name : Dr. PRAKASH PANDURANGAN

External Guide Name : Dr. S. JOHNSON RETNARAJ SAMUEL

| PO / PSO / PEO / SDG | Justification                                                                                                                                                                                                                                     |
|----------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| PO 2                 | It demands a nuanced understanding of environmental science, as it assess the broader implications of introducing this substance into ecosystems, emphasizing a holistic approach to research and inquiry                                         |
| PO 4                 | Conducting complex investigations fits the study of "Molecular effects of sodium selenite during earthworm regeneration" by analyzing data to draw conclusions about its impact.                                                                  |
| PSO 2                | It involves validating techniques relevant to biological research, aligning with the investigation of cellular responses to sodium selenite in earthworms.                                                                                        |
| PSO 4                | Investigating the intricate molecular interactions between sodium selenite and earthworm regeneration, designing experiments, analyzing data.                                                                                                     |
| PSO 7                | The environmental impact of sodium selenite on earthworms, recognizing its broader implications for ecosystem health and sustainability, thereby demonstrating my understanding of the societal context and the need for sustainable development. |
| PEO 2                | Our objective incorporates into our research when we investigated the molecular effects of sodium selenite on earthworm regeneration with research skills and multidisciplinary thinking.                                                         |