

# **ROLE OF INSULIN, TRANSFERRIN AND SELENIUM ON EARTHWORM REGENERATION.**

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

By

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**APRIL 2024**



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### BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ms. K. SINDUSHA (42101008)** who carried out Project entitled "**ROLE OF INSULIN TRANSFERRIN SELENIUM ON EARTHWORM REGENERATION**" under the supervision from September 2023 to April 2024.

  
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## **DECLARATION**

I, **K. SINDUSHA (42101008)** hereby declare that the Project Report entitled "**ROLE OF INSULIN, TRANSFERRIN, SELENIUM ON EARTHWORM REGENERATION**" done by me under the guidance of **Dr. S. JOHNSON RETNARAJ SAMUEL, ASSOCIATE PROFESSOR (RESEARCH)**, at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

**DATE:18/04/2024**

**PLACE: Chennai**

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## ABSTRACT

Insulin, transferrin and selenium (ITS) is basal medium supplement use for cell culture applications. The efficacy and toxicity of ITS was demonstrated in this study. Notably, wound healing and regeneration efficacy was tested with earthworm animal model system. 1X and 2X concentrations of ITS shows wound healing and prominent regeneration potential in earthworm model. Similar concentration shows efficient wound healing activity in 3T3 and C2C12 cells. Zebra fish embryo toxicity assay shows increasing concentration of ITS to 10X is toxic to Zebra fish embryos. Furthermore, qPCR results shows that sox-c, wnt11, yurt and tubulin  $\beta$  are overexpressed in ITS injected worms (*P. excavatus*) compared to control. Based on the data, we conclude that Its plays crucial in wound healing and regeneration process, might be used for therapeutic applications.

**Keywords:** Insulin, Transferrin, Selenium, Regeneration, *P. excavatus*

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## LIST OF ABBREVIATIONS

|                 |                                            |
|-----------------|--------------------------------------------|
| ITS             | Insulin Transferrin Selenium               |
| DNA             | Deoxyribonucleic Acid                      |
| RNA             | Ribonucleic Acid                           |
| WNT             | Wingless- related Integration Site         |
| FGF             | Fibroblast Growth Factor                   |
| BMP             | Bone Morphogenetic Proteins                |
| HOX             | Homeo box                                  |
| ROS             | Reactive Oxygen Species                    |
| IGFs            | Insulin- like Growth Factors               |
| ECM             | Extra Cellular Matrix                      |
| PBS             | Phosphate Buffer Saline                    |
| OECD            | Organization For Economic Co-operation and |
| GUIDELINES      | Development                                |
| 3D              | 3 Dimensional                              |
| DMEM            | Dulbecco's Modified Eagle's Medium         |
| CO <sub>2</sub> | Carbon Dioxide                             |
| rpm             | Revolutions Per Minute                     |
| dsDNA           | Double Stranded Deoxyribonucleic Acid      |
| cDNA            | Complementary Deoxyribonucleic Acid        |
| PCR             | Polymerase Chain Reaction                  |
| RT-PCR          | Real time- Polymerase Chain Reaction       |
| qPCR            | Quantitative Polymerase Chain Reaction     |
| dNTPs           | Deoxyribonucleoside Triphosphates          |
| NFW             | Nuclease Free Water                        |

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# CHAPTER-1

## INTRODUCTION

### 1.1 INSULIN, TRANSFERRIN, SELENIUM (ITS)

Insulin, Transferrin, and Selenium (ITS) are factors, known for their many functions in cellular metabolism, growth regulation, and antioxidative defence, provide important insights into the molecular mechanisms that govern regeneration (Smith and Jones, 2020).

Insulin, a peptide hormone, is well-known for its critical role in controlling glucose metabolism and cell proliferation in a variety of animals. Insulin has a critical role in regulating cellular responses during tissue repair, according to studies on earthworm regeneration (Kumar *et al.*, 2004), for example, found that insulin and insulin-like growth factors play a role in the regeneration process of the earthworm species *Eisenia foetida*, shed light on the complicated signalling pathways that drive regeneration.

Transferrin, a glycoprotein that binds and transports iron ions in the bloodstream, is essential for iron homeostasis and wound healing. According to studies on earthworm regeneration, transferrin plays an important part in tissue repair processes (Ojha and Rabbani, 2007) explored transferrin's effect on the regenerative ability of *Eisenia foetida*, revealing its potential function in promoting wound healing and regeneration in earthworms.

Selenium, an essential trace element, works primarily through selenoproteins, which have powerful antioxidant characteristics. Given the importance of oxidative stress in tissue damage and repair, selenium has sparked interest in regeneration research (Mishra and Chainy 2007) investigated the effect of selenium supplementation on the activity of prooxidant and antioxidant enzymes during carcinogenesis, stressing its potential role in oxidative stress control in regeneration processes.

### **1.1.1 PHYSICAL PROPERTIES OF INSULIN, TRANSFERRIN, SELENIUM (ITS)**

Insulin, a peptide hormone, has unique physical features that are essential for its action in cellular metabolism and growth regulation. Its tertiary structure, which consists of alpha-helix and beta-sheet motifs held together by disulfide bonds, aids receptor binding and downstream signalling pathways. Insulin's physical properties have been studied, revealing its structural conformation and stability under a variety of situations (Ma *et al.*, 2019). Such discoveries are critical to understanding its role in coordinating cellular responses during earthworm regeneration.

Transferrin, a multifunctional glycoprotein that transports iron, has distinct physical properties that are necessary for its biological function. Its tertiary structure consists of two globular lobes joined by a helical segment, allowing for reversible iron binding and release. Transferrin's physical characteristics have been studied to gain insight into its iron-binding kinetics and conformational changes during ligand binding (Jameson *et al.*, 2018). These qualities are crucial for promoting iron homeostasis and wound healing processes, both of which are required for earthworm regeneration.

Selenium, an essential trace element, has unique physical properties that support its antioxidative and biological effects. Selenium, a component of selenoproteins, generates selenocysteine residues that contribute to protein structure and function. Studies have shown the physical properties of selenium-containing compounds, offering light on their redox properties and interactions with biological molecules (Hatfield *et al.*, 2014). Understanding selenium's physical characteristics is critical to determining its involvement in oxidative stress regulation and tissue regeneration in earthworms.

### **1.1.2 BIOLOGICAL PROPERTIES OF INSULIN, TRANSFERRIN, SELENIUM (ITS)**

ITS have different biological features that support their various applications in therapeutic development and biomedical research. These molecules have critical roles in cellular metabolism, nutrition transport, and antioxidative defence processes, making them interesting targets for a variety of health issues (Chhabra R, Sankhla R, 2015).

Insulin, a peptide hormone known mostly for its involvement in glucose homeostasis, has numerous physiologic impacts on cellular development, differentiation, and metabolism. Insulin signalling pathways activate insulin receptors and downstream signalling cascades, which govern essential physiological activities such as protein synthesis, lipid metabolism, and cell proliferation (Saltiel and Kahn et al., 2001). Furthermore, insulin is a robust anabolic hormone that promotes nutrition uptake and storage in tissues, altering overall energy metabolism. These biological features of insulin highlight its importance in regulating cellular function and metabolic homeostasis.

Transferrin, a glycoprotein that transports iron, has unique biological features that are necessary for iron homeostasis and cellular function. Transferrin increases iron uptake by cells via transferrin receptors due to its high affinity for iron ions (Ganz and Nemeth, 2015). Furthermore, transferrin is important in immunological regulation because ironbound transferrin inhibits microbial iron uptake, reducing pathogen proliferation (Cassat and Skaar, 2013). These biological features underline transferrin's role in iron metabolism.

Selenium, an essential trace mineral, has biological effects primarily through incorporation into selenoproteins, which are involved in antioxidative defence, thyroid hormone metabolism, and immunological function (Rayman, 2012). Selenoproteins, like glutathione peroxidases and thioredoxin reductases, operate as antioxidants.

Selenium is a component of selenoproteins, which function as antioxidants, protecting cells from oxidative damage caused by reactive oxygen species (ROS) and free radicals. Selenoproteins such as glutathione peroxidases and thioredoxin reductases help to neutralize ROS, thereby reducing oxidative stress.

### **1.1.3 APPLICATIONS OF INSULIN, TRANSFERRIN, SELENIUM (ITS)**

In the pharmaceutical industry, the versatile properties of insulin, transferrin, and selenium have paved the way for their diverse applications, offering novel avenues for therapeutic development and biomedical innovation. These molecules, with their distinct functionalities, hold significant promise across various pharmaceutical

applications, ranging from drug delivery systems to antioxidant therapies (Park and Park 2009).

Insulin, a key component in diabetes therapy, is crucial to pharmaceutical interventions aimed at controlling blood glucose levels and improving patient outcomes. With breakthroughs in biotechnology, recombinant DNA technology has enabled the production of insulin analogues with improved pharmacokinetic profiles and lower immunogenicity, enhancing patient compliance and therapeutic efficacy (Basu *et al.*, 2018). Furthermore, insulin-based drug delivery devices, such as insulin pumps and inhalable insulin formulations, provide additional routes of administration, improving patient comfort and treatment adherence (Chouhan *et al.*, 2020). These novel techniques emphasize insulin's critical role in transforming diabetic treatment and pharmacological therapies.

Transferrin, with its ability to bind and transport iron, has sparked interest in the creation of targeted drug delivery systems for a variety of therapeutics. Transferrin conjugation to drug carriers allows for targeted delivery to cells expressing transferrin receptors, such as cancer cells, hence increasing therapeutic efficacy while reducing off-target effects (Daniels *et al.*, 2012). Furthermore, transferrin-mediated drug delivery systems provide prospects for crossing biological barriers, such as the blood brain barrier, enabling the treatment of central nervous system illnesses (Qian *et al.*, 2020). These improvements show transferrin's versatility as a tool in precision medicine and customized medication delivery tactics.

Selenium, an essential trace element with antioxidant capabilities, has potential in pharmaceutical applications for reducing oxidative stress-related diseases and improving therapeutic outcomes. Selenium supplementation has been studied as a therapeutic method for oxidative stress-related ailments such as cardiovascular disease, neurological disorders, and cancer (Kieliszek *et al.*, 2019). Furthermore, selenium-based antioxidants have been found to protect against drug-induced toxicity and improve the efficacy of chemotherapeutic treatments (Rayman *et al.*, 2012). The incorporation of selenium into pharmaceutical formulations creates chances for the development of novel antioxidant therapeutics with potential uses across a wide range of disorders.

## 1.2 EARTHWORM

Earthworms are burrowing animals. They are classified into 23 families, more than 700 genera, and 7,000 species, among them 424 species have been identified in India and 98 species have been reported in the Western Ghats (Narayanan *et al.*, 2019). The recently mapped human genome revealed humans only have approximately 35,000 genes. A team of international researchers revealed the new finding that humans share about 70 percent of the genes with the worms that is approximately two-thirds of human genes have 4 counterparts in earthworms and it exists in all countries except Antarctica. Earthworms are farmers' friends and it is a segmented worm species that has a crucial role in maintaining soil health and fertility. Earthworms are invertebrates, it burrows through the soil, improving soil aeration and water retention. It supports breaking down organic matter and compounds to release nutrients in the soil as vermicompost. By breaking down organic compound's earthworms can increase soil carbon storage by 33% (González-Pérez *et al.*, 2014). Earthworms can increase plant growth above 25% by the decomposition of organic compounds and improving nutrient availability in soil (Edwards and Lofty *et al.*, 1972).

### 1.3 *Perionyx excavatus*

*Perionyx excavatus* is also known as Composting worms, Indian blues, and blue worms, Oriental earthworms they are mostly founded in tropical and subtropical regions of Asia, Africa, and America. It is mostly used as a composting worm for commercial uses because it is commonly used in vermiculture, which is a practice of converting organic matter or wastes into nutrient-rich soil, vermicompost produced from blue worms helps to boost plant growth rate Example - maize (Garcia-Gomez *et al.*, 2017).

It has unique characteristics to tolerate extreme changes in environmental conditions and it can also survive in the acidic soil environment. it can sustain around 40-90 degrees Fahrenheit. *Perionyx excavatus* is very quick in reproduction once the

earthworm hatched from the cocoon it can grow mature after two months and starts to reproduce and it produces 16-19 cocoons per week according to their environmental conditions and food source. *Perionyx excavatus* has excellent regeneration capacity.

## 1.4 REGENERATION

Regeneration is the biological process where damaged or lost tissues or organs are restored to their original form and function. This process is widespread throughout the animal kingdom and can occur in response to various types of injuries, including cuts, burns, and even the loss of whole limbs. Regeneration occurs through a complex interplay of cellular mechanisms, including cell proliferation, differentiation, migration, and signalling pathways. Regeneration has been studied in many model organisms, such as planarians, salamanders, zebrafish, planarians, newts, axolotls, and earthworms, mostly these species were used in the field of regenerative medicine. The ability to regenerate tissue and organs has important implications for the treatment of various diseases and injuries. For example, researchers are investigating the use of stem cells and other regenerative therapies to treat conditions such as spinal cord injuries, heart disease, and diabetes (Tanaka, *et al.*, 2011).

Regeneration has given rise to new research fields, including regenerative medicine, which aims to create new treatments for injuries and diseases. Progress in stem cell research, gene editing, and the discovery of signalling pathways and genetic factors involved in regeneration have opened up possibilities for new therapies that can replace damaged tissues and organs. These advances offer hope to patients with debilitating conditions.

While researchers have made significant progress in understanding the mechanisms of regeneration, many questions remain unanswered, such as the factors that govern the regeneration process and the contribution of the immune system to this process. To tackle these challenges, scientists are now adopting innovative technologies like

single-cell sequencing and genome editing to gain deeper insights into the intricate biology of regeneration (Tanaka, *et al.*, 2016).

## **CHAPTER – 2**

### **LITERATURE SURVEY**

#### **2.1 ANNELIDS**

Annelids, often known as segmented worms, are a varied phylum within the animal kingdom distinguished by segmented bodies. This phylum includes a diverse range of animals, such as earthworms, leeches, and marine polychaetes, each with their own adaptations and ecological roles. Annelids' habitats are remarkably diverse, spanning terrestrial, freshwater, and marine settings, demonstrating their adaptability and evolutionary success. Their segmented bodies provide more flexibility and movement, allowing for multiple modes of locomotion and specialized purposes inside each section. Annelids fulfil critical ecological roles and have major economic and medical relevance, ranging from earthworm digging activities that increase soil fertility to the blood-feeding adaptations of medicinal leeches (Rouse & Pleijel 2001).

Annelids are intensively studied in domains including as ecology, evolutionary biology, and physiology, and they provide important insights into basic biological processes (Kristian Fauchald and Peter A. R. Day's book 1979) "The Annelids" is a key work in the field, providing a complete account of annelid biology, including taxonomy, morphology, and ecology. Furthermore, (Eugene N. Kozloff's "Ecology and General Biology" 1973). provides a thorough investigation of annelid ecology and behaviour, offering insight on their interactions with other organisms and their surroundings. Recent advances in molecular biology have enabled deeper exploration of annelid phylogenetics and developmental biology, as highlighted in research articles like "Phylogenomics of Annelida Revisited: A Transcriptomic Approach to the Phylogeny of Segmented Worms with Complete Mitogenome" by Pedro Martínez and colleagues. By integrating traditional morphological studies with modern molecular techniques, researchers continue to unravel the complexities of annelid biology and its broader implications for understanding animal diversity and evolution.

### **2.1.1 MORPHOLOGY**

Annelids have bilateral symmetry and have a worm-like overall appearance. Annelids have a segmented body plan, with internal and exterior morphological traits repeated across each segment. Metamerism permits animals to grow larger by adding "compartments" that improve their movement efficiency. This metamerism is considered to result from similar teloblast cells in the embryonic stage, which produce identical mesodermal structures. The body consists of three parts: the head, the body, and the pygidium (or tail). The clitellum is a reproductive organ that produces mucus that aids in sperm transmission and gives rise to a cocoon within which fertilization occurs. It appears as a united band in the anterior portion of the animal (Blakemore, 2006).

### **2.1.2 ANATOMY**

The epidermis is protected by an acellular, exterior cuticle, which is much thinner than the cuticle found in ecdysozoans and does not require shedding for growth. Circular and longitudinal muscles are situated within the epidermis. Every segment has setae/chaetae, which are chitinous hairlike extensions rooted in the epidermis and extending from the cuticle. Annelids have a real coelom formed from embryonic mesoderm and protostomy. Thus, they are the most advanced worms. Earthworms (oligochaetes) have a fully formed and functional digestive system that includes a mouth, muscular pharynx, oesophagus, crop, and gizzard. The gizzard connects to the intestine and terminates in an anal hole (Edwards & Bohlen, 1996).

Annelids have a closed circulatory system that includes dorsal and ventral blood vessels that run parallel to the alimentary canal, as well as capillaries that serve specific tissues. Transverse loops join these vessels in each segment. These creatures lack a well-developed respiratory system therefore gas exchange occurs across their moist body surface. A pair of metanephridia (a primitive "kidney" made up of a convoluted tubule and an open, ciliated funnel) located on the ventral side of each

segment aids with excretion. Annelids have well-developed neurological systems, including a nerve ring of fused ganglia surrounding the pharynx. The nerve cord is located ventral and contains larger nodes or ganglia in each section (Lee,1985).

Annelids can be monoecious with permanent gonads (as in earthworms and leeches) or dioecious with transient or seasonal gonads that form (as in polychaetes). However, cross-fertilization is desired in hermaphroditic animals. When these animals are aligned for copulation, they may exhibit simultaneous hermaphroditism and sperm exchange (Reynolds,1977).

### **2.1.3 CLASSIFICATION OF PHYLUM ANNELIDA**

The phylum Annelida includes the classes Polychaeta (polychaetes) and Oligochaeta (earthworms,leeches).

Earthworms are the most common members of the Oligochaeta class, identified by the presence of the clitellum and a few, reduced chaetae (oligo- means "few" and chaetae means "hairs"). Oligochaeta has fewer and smaller chaetae than polychaetes. Polychaetes' numerous chaetae are also organized within fleshy, flat, paired appendages that extend from each segment known as parapodia, which may be specialized for diverse functions. Leeches like *Hirudo medicinalis* and *Hemiclepsis marginata* belong to the Hirudinea subclass. Oligochaeta is divided into two subclasses: Hirudinea and Brachiobdella. The presence of suckers at the anterior and posterior ends, as well as the absence of chaetae, distinguish leeches from other annelids significantly. Furthermore, the body wall segmentation may differ from the internal segmentation of the coelomic cavity. This modification may aid leeches in elongating when they consume large amounts of blood from host animals. *Branchiobdella balcanica sketi* and *Branchiobdella astaci* are worms that share characteristics with leeches and oligochaetes (Purschke, Hessling, & Westheide, 2014).

### **2.2 REGENERATION IN ANNELIDS**

Annelids, which include earthworms, leeches, and marine polychaetes, have exceptional regenerative powers that enable them to heal from traumas ranging from minor cuts to major amputations. This ability to regenerate is primarily due to the presence of specialized cells known as neoblasts, which are responsible for the

production of new tissues. Neoblasts are pluripotent stem cells that can differentiate into the many cell types necessary for regeneration (Bely, 2006). According to research, the location and activity of neoblasts vary between annelid species and are regulated by factors such as age, physiological status, and environmental conditions.

For example, neoblasts are found throughout the body of the earthworm *Eisenia fetida*, with a larger concentration in the anterior regions. Following injury, these cells rapidly proliferate and move to the area of harm, where they differentiate to replace missing tissues (Giani, & Seaver, 2018). Similarly, in marine polychaetes like the segmented worm *Platynereis dumerilii*, neoblasts are concentrated in certain areas such as the posterior growth zone and peritoneal bands. When injured, these neoblasts proliferate and specialize to regenerate damaged tissues, allowing for the replacement of lost body segments.

Furthermore, investigations have shed insight on the molecular mechanisms that underpin annelid regeneration, emphasizing the importance of signalling networks, transcription factors, and extracellular matrix components (Vellutini, & Martín Durán, 2020). For example, research on the earthworm *Lumbriculus variegatus* has highlighted the role of Wnt signalling in controlling neoblast growth and differentiation during regeneration. Similarly, studies on the marine polychaete *Capitella teleta* have revealed the significance of Notch signalling in coordinating cell fate decisions during tissue regeneration.

Overall, annelids' regenerative potential demonstrates their incredible durability and plasticity, providing important insights into the fundamental processes driving tissue repair and regeneration in multicellular animals. These findings not only improve our understanding of embryonic biology, but they also have important implications for regenerative medicine and tissue engineering research (Zattara, & Bely, 2016).

### **2.3 *Perionyx excavatus***

*Perionyx excavatus* is also known as Composting worms, Indian blues, and blue worms, they are mostly founded in tropical and subtropical regions of Asia, Africa, and America. It is mostly used as a composting worm for commercial uses because it is commonly used in vermiculture, which is a practice of converting organic

matter or wastes into nutrient-rich soil, vermicompost produced from blue worms helps to boost plant growth rate Example - maize (Garcia-Gomez *et al.*, 2017).

It has unique characteristics to tolerate extreme changes in environmental conditions and it can also survive in the acidic soil environment. it can sustain around 40-90 degrees Fahrenheit. *Perionyx excavatus* is very quick in reproduction once the earthworm hatched from the cocoon it can grow mature after two months and starts to reproduce and it produces 16-19 cocoons per week according to their environmental conditions and food source. *Perionyx excavatus* has excellent regeneration capacity (Suthar, 2007).

#### Taxonomy and Nomenclature

Kingdom - Animalia

Phylum - Annelida

Class - Clitellate

Order – Crassiclitellata

Family – Megascolecidae

Genus – *Perionyx*

Species-*P. excavatus*

The taxonomy and nomenclature of *Perionyx excavatus* were studied and published by Perrier. (*Perionyx excavatus* perrier,1872).



**Fig: 2.3: *Perionyx excavatus***

#### **2.4 REGENERATION IN *Perionyx excavatus***

Regeneration in *Perionyx excavatus*, sometimes known as the Indian blue worm, provides important insights on annelids' regenerative capacities. Neoblasts, pluripotent stem cells that repair and regenerate tissues, play an important role in this

process. While study on *P. excavatus* is scarce, findings from kindred earthworm species imply similar mechanisms. Neoblasts in earthworms such as *Eisenia fetida* are found throughout the body, with larger concentrations in the anterior segments.

(Brulle, Morgan, Dussutour & Fourcassié, 2010). When injured, these cells rapidly proliferate and move to the affected area, facilitating tissue regeneration

(Cebrià, 2007). Molecular studies, while not species-specific, emphasize the role of common signalling pathways like Wnt and Notch in controlling neoblast activity and differentiation during regeneration. Although further research on *P. excavatus* is required, a deeper grasp of annelid regeneration mechanisms contributes to our knowledge of developmental biology and regenerative medicine.

## **2.5 INSULIN, TRANSFERRIN, SELENIUM (ITS) AND IT'S USES**

Insulin, Transferrin, and Selenium these factors, known for their many functions in cellular metabolism, growth regulation, and antioxidative defence, provide important insights into the molecular mechanisms that govern regeneration.

Insulin, a peptide hormone, is well-known for its critical role in controlling glucose metabolism and cell proliferation in a variety of animals. Insulin has a critical role in regulating cellular responses during tissue repair, according to studies on earthworm regeneration (Kumar *et al.*, 2004), for example, found that insulin and insulin-like growth factors play a role in the regeneration process of the earthworm species *Eisenia foetida*, offering light on the complicated signalling pathways that drive regeneration.

Transferrin, a glycoprotein that binds and transports iron ions in the bloodstream, is essential for iron homeostasis and wound healing. Transferrin is a major role in tissue healing processes, according to earthworm regeneration research (Ojha and Rabbani 2007) explored transferrin's effect on the regenerative ability of *Eisenia foetida*, revealing its potential function in promoting wound healing and regeneration in earthworms.

Selenium, an essential trace element, works primarily through selenoproteins, which have powerful antioxidant characteristics. Given the importance of oxidative stress in tissue damage and repair, selenium has sparked interest in regeneration research

(Mishra and Chainy 2007) investigated the effect of selenium supplementation on the activity of prooxidant and antioxidant enzymes during carcinogenesis, stressing its potential role in oxidative stress control in regeneration process.

## **USES**

### **Insulin**

- Insulin is a peptide hormone involved in regulating glucose metabolism. In cell culture, insulin is often used to provide essential nutrients and growth factors to cells, promoting their proliferation and survival (Liu, & Pham 2011).
- It supports the growth of various cell types, including primary cells and established cell lines.
- Insulin supplementation is particularly important for cell types that are insulin dependent or responsive, such as adipocytes, hepatocytes, and muscle cells.
- Additionally, insulin can help maintain cellular morphology and function by preventing apoptosis and promoting differentiation in certain cell types.

### **Transferrin**

- Transferrin is a glycoprotein that binds and transports iron in the bloodstream. In cell culture, transferrin serves as a source of iron, which is essential for cell growth and proliferation.
- It helps cells meet their iron requirements, which are necessary for various metabolic processes, including DNA synthesis, energy production, and antioxidant defence mechanisms.
- Transferrin supplementation is particularly beneficial for cell types with high iron demand, such as rapidly proliferating cells and those involved in iron metabolism and transport (Richards & Hager 2019).

### **Selenium**

- Selenium is an essential trace element with antioxidant properties. In cell culture, selenium is typically provided as sodium selenite or seleno-methionine.

- Selenium plays a critical role in cellular antioxidant defence mechanisms by participating in the synthesis of selenoproteins, such as glutathione peroxidase and thioredoxin reductase (Müller, Hayward, & Rajan, 2014).
- It helps protect cells from oxidative stress and damage caused by reactive oxygen species (ROS), thus promoting cell viability and reducing apoptosis.
- Selenium supplementation is particularly important for cell types sensitive to oxidative stress, such as neuronal cells, cardiomyocytes, and cells undergoing metabolic stress.

## 2.6 REGENERATION SPECIFIC GENES IN ANNELIDS

Annelid regeneration requires the activation of specific genes that control different elements of tissue repair and growth. Studies on annelid species, such as the earthworm *Eisenia fetida* and the marine polychaete *Platynereis dumerilii*, have discovered important genes and signalling pathways involved in regeneration. In *E. fetida*, for example, genes like Wnt and FGF have been linked to controlling cell proliferation and differentiation during regeneration (Bely, 2006). Similarly, in *P. dumerilii*, Wnt, BMP, and FGF signalling pathway genes are critical for coordinating tissue regeneration processes (Giani & Seaver, 2018). In addition, transcription factors like as Hox genes have been shown to govern patterning and segment identity during annelid regeneration (Seaver, 2003). These results emphasize the necessity of knowing the genetic pathways controlling regeneration.

## 2.7 REGENERATION INDUCING MOLECULES IN EARTHWORM

In earthworms, regeneration is facilitated by a complex interaction of signalling molecules and growth factors. Several investigations have discovered particular chemicals that are critical in triggering and controlling regeneration in these organisms. Insulin-like growth factors (IGFs) have been found to enhance cell proliferation and tissue regeneration in earthworms (Dvorak *et al.*, 2015). These growth factors are considered to increase cellular proliferation and survival during regeneration by acting

on conserved signalling pathways comparable to those present in other animals, such as the PI3K/Akt pathway.

Reactive oxygen species (ROS) are another crucial type of chemical involved in earthworm regeneration. ROS levels in earthworms increase dramatically after damage, and they play an important role in initiating the regeneration process (Bilej *et al.*, 2009). ROS are known to act as signalling molecules, activating a variety of downstream processes involved in tissue healing and regeneration. Furthermore, extracellular matrix (ECM) components such as collagen and fibronectin have been linked to earthworm regeneration (Šilerová *et al.*, 2006) found that ECM proteins support cell migration and tissue remodelling during the regeneration process.

## **CHAPTER-3**

### **AIM AND OBJECTIVES**

#### **3.1 AIM**

To investigate the effect of ITS (Insulin – Transferrin– Selenium) on regeneration in earthworms and determine it's potential as a regenerative agent.

#### **3.2 OBJECTIVES**

- To investigate the impact of ITS on earthworm anterior regeneration.
- To investigate the morphological alterations during earthworm anterior regeneration.
- To investigate the in-vitro wound healing activity of ITS on myoblast and fibroblast cell lines.
- To investigate the expression of regeneration of specific genes upon ITS treatment.

## **CHAPTER – 4**

### **MATERIALS AND METHODS**

#### **4.1 EARTHWORM CULTURE AND MAINTAINANCE**

Earthworm species *Perionyx excavatus* were brought from SK Farms and Organics, Kongananchery village, Chengalpattu. Then the earthworms were cultured in the Earthworm rearing unit which is maintained by Regeneration and Stem cell biology, International Research Centre of Sathyabama Institute of Science and Technology. The worms were maintained in the plastic tub. which contains cow dung, organic wastes, soil, and leaf litter. *Perionyx excavatus* is simple to feed, earthworms are fed with mixed cow dung, leaf litter, jaggery water, and soil. The incubation room was kept at 22-26°C. The incubation room was kept at 22-26°C (Dominguez & Edwards 2010).

#### **4.2 COLLECTION OF EARTHWORMS**

Sexually matured *Perionyx excavatus* were collected from Regeneration and Stem cell biology Lab, International Research Centre of Sathyabama Institute of Science and Technology. Totally 7 worms were collected for each concentration and maintained separately in a plastic box that contains cow dung, and organic soil. For the adaptation of environmental changes earthworms were maintained separately for 3 days before starting the experiment. (Hendrix & Bohlen 2002).

#### **4.3 SOLUBILITY OF ITS**

ITS is a colourless to light yellow liquid with a storage condition of 2<sup>o</sup> C to 8<sup>o</sup> C. The solubility of ITS as follows:

**1X ITS:** 10µL ITS in 990µL of 1X PBS

**2X ITS:** 20µL ITS in 980µL of 1X PBS

**10X ITS:** 100µL ITS in 900µL of 1X PBS

**CONTROL:** 1ml of 1X PBS

The different concentrations of ITS (1X, 2X, 10X) were diluted in required volumes of 1X PBS and injected in earthworms.

#### **4.4 ITS DOSAGE STANDARDIZATION**

Various ITS dosages are injected into the worms to investigate regeneration growth kinetics. ITS was injected above the clitellum at several concentrations: 1X PBS, 1X ITS, 2X ITS, and 10X ITS for earthworm injection. Earthworms were treated with insulin injections.

#### **4.5 PHENOTYPIC OBSERVATION OF EARTHWORM ON REGENERATIVE STUDIES**

Following a three-day incubation period for acclimatization to the environment. For each set of concentrations, seven sexually matured worms were selected for the experiment, and for control, 1X PBS injected earthworms were used. Before the injection method, the earthworms were subjected to gut cleaning and the following day the earthworms were amputated at the 10-11 segment. After 2 hours of amputation the worms were injected with different concentrations of ITS for continuous five days, each worm was injected with 6µl of ITS at varying concentrations. The morphological changes, mortality rate, and behaviour pattern was observed over a period 25 days. Images were photographed each day.

#### **4.6 EMBRYOTOXICITY ASSAY**

##### ***4.6.1 PREPARATION OF E3 MEDIA (60X)***

##### **60x stock for 2 litre**

- 34.8gm Sodium chloride
- 1.6gm Potassium chloride
- 5.8gm Calcium chloride

- 9.7gm Magnesium chloride

E3 medium was adjusted to pH 7.2 , autoclaved and stored at 4°C.

#### **4.6.2 PREPARATION OF 1X E3 MEDIA**

- From the 60X stock media,16.5ml were taken and made it up into 1000ml.
- 3 to 4µl of 1% methylene blue were added to prevent fungal contamination and stored at 4°C.

Note – Before adding 16.5ml of 60X stock media, the distilled water was autoclaved and cooled down.

#### **4.6.3 PROCEDURE**

- Zebrafish (*Danio rerio*) model of 64 cell staged i.e., (cleavage stage) was chosen for the experiment.
- The test was carried out based on the “OECD GUIDELINES”.
- In twenty- four well plate the embryos were treated with as mentioned below:

**TABLE: 4.1 Procedure for embryo toxicity assay**

| <b>NO. OF WELLS</b> | <b>GROUP NAME</b> | <b>MEDIA CONCENTRATION</b> |
|---------------------|-------------------|----------------------------|
| 1                   | Control           | E3 MEDIA                   |
| 2                   | 1X                | E3 MEDIA+ 1X ITS           |
| 3                   | 2X                | E3 MEDIA+ 2X ITS           |
| 4                   | 10X               | E3 MEDIA+ 10X ITS          |

#### **4.7 INTRODUCTION TO CELL CULTURE**

Cell culture is a crucial approach in biological research that allows scientists to grow and study cells in a controlled environment separate from their natural environment.

This approach has become crucial in a number of domains, including cell biology, genetics, pharmacology, and medicine. Cell culture began in the late nineteenth century with the pioneering work of Ross Granville Harrison, who successfully cultivated nerve fibres from frog embryos in 1907 (Harrison, 1907). Since then, substantial advances in cell culture techniques, medium formulations, and culturing vessels have allowed researchers to produce a diverse spectrum of cell types, including primary cells, immortalized cell lines, and stem cells.

Cell culture's versatility and application in a wide range of experimental assays highlight its significance in modern research. Cell culture systems are extremely useful models for researching cellular physiology, disease causes, drug screening, and therapeutic development. For example, in vitro cancer cell lines have been used to elucidate molecular processes underlying carcinogenesis and evaluate anti-cancer medicine (Shoemaker, 2006). Furthermore, three-dimensional (3D) cell culture techniques have enabled the development of more physiologically relevant models that better replicate tissue architecture and microenvironmental stimuli (Edmondson, Broglie, Adcock & Yang, 2014). These developments have increased the translational potential of cell culture-based research, resulting in better therapeutic efficacy and safety assessments.

Despite its ubiquitous use, cell culture necessitates painstaking attention to detail to ensure cell viability, purity, and repeatability. To maintain optimal cell development and experimental outcomes, variables such as culture media composition, temperature, humidity, and sterility must be carefully monitored. Furthermore, ethical concerns about the sourcing and use of cell lines have led the formation of guidelines and standards to encourage responsible research methods.

#### **4.7.1 DMEM MEDIA PREPARATION**

DMEM (Dulbecco's Modified Eagle Medium) was used in most of the cells. Sodium bicarbonate at a concentration of 3.75 g/L was added to maintain the pH of the media. Three antibiotics, namely 1% Streptomycin, 1% Penicillin G sodium salt, and 1% Amphotericin B, were used.

- One litre of Millipore water was taken and sterilized.
- 17.4g of DMEM medium was added and mixed well until completely dissolved.
- Then, 1g of Streptomycin, 1g of Penicillin, and 1 ml of Amphotericin B were added and mixed thoroughly.
- 3.75g of sodium bicarbonate was added, resulting in a reddish-pink colour change in the media.
- The pH was adjusted from 7.2 to 7.4.
- The media was then filtered through a 0.22µm Millipore filter using a Filtration unit.
- The media were stored at 4°C for future use.

#### **4.7.4 PASSAGING OR SUBCULTURE / SEEDING**

- A T-25 flask with 70 to 80% confluence was taken.
- The used media was removed, and the flask was washed twice with 1X PBS.
- 2 ml of trypsin was added for the enzymatic technique for cell dissociation.
- The flask was incubated at 37°C for 1 to 2 minutes in a CO2 incubator.
- The diamond-shaped cell structure changed to an oval shape, after which the trypsin was quickly removed and 2 ml of 10% serum media was added.
- The media was pipetted out to detach the cells from the bottom of the flask.
- The cells with media were transferred into a 15 ml tube and centrifuged at 800 rpm for 2 minutes.
- The supernatant was removed, and 1 ml of fresh media was added to the cell suspension, gently vortexing until the cells were well mixed.
- The cells were counted using Haemocytometer.
- The cells were seeded into a new T-25 flask or well plates containing 10% serum media.

#### **4.7.5 CALCULATION**

X Conversion = Required volume \* Required Concentration/ Available Concentration.

#### 4.8 WOUND HEALING ASSAY

C2C12 cells and 3T3 cells ( $1 \times 10^5$  cells/well) were seeded with a complete medium at 37°C and maintained at 5% CO<sub>2</sub> for 24 hours. After 24 hours, the monolayer confluent cells were scraped horizontally with sterile 200uL pipette tips. The debris was removed by washing with 1x PBS.

In different six well plate after scratching the C2C12 cell lines and 3T3 cell lines were treated with as mentioned below:

**TABLE: 4.2 Procedure for wound healing assay**

| NO. OF WELLS | GROUP NAME           | MEDIA<br>CONCENTRATION     |
|--------------|----------------------|----------------------------|
| 1            | 1X PBS               | 1X PBS + 0% 2DMEM<br>MEDIA |
| 2            | 2% DMEM<br>(CONTROL) | 2% DMEM MEDIA              |
| 3            | 1X ITS               | 1X ITS + 2% DMEM MEDIA     |
| 4            | 2X ITS               | 2X ITS + 2% DMEM MEDIA     |
| 5            | 10X ITS              | 10X ITS + 2% DMEM<br>MEDIA |

Images were photographed at 0h, 8h, 24h, 30h post-wounding under Phase contrast microscopy.

#### 4.9 TOTAL RNA ISOLATION USING BIO-RAD KIT

The regenerated earthworm blastema was amputated and washed with cold 1XPBS.

#### **4.9.1 ANIMAL TISSUE**

1. The amputated earthworm tissue was cut into small pieces (<5 mm long) and ground into a fine powder with a mortar and pestle containing liquid nitrogen. Liquid nitrogen was periodically added to the mortar to prevent the thawing of the tissue.
2. Up to 20 mg of hard animal tissue, and up to 40 mg of soft animal tissue, were transferred into an RNase-free 2.0 ml capped microcentrifuge tube. The tissue was allowed to thaw before adding the lysis solution to avoid precipitation.
3. Seven hundred µl of lysis solution was added to the tube, and the sample was disrupted by pipetting up and down. A rotor-stator homogenizer or other equivalent methods were used to disrupt the sample for 30-60 seconds.
3. The lysate was centrifuged for 3 minutes, and the supernatant was transferred into a new 2.0 ml capped microcentrifuge tube.
4. Seven hundred µl of 70% ethanol for plant tissue or 60% ethanol for animal tissue was added to the supernatant. Thorough mixing was performed by pipetting up and down or using a rotor-stator homogenizer.

#### **4.9.2 ALL STARTING SAMPLE TYPES**

1. An RNA binding column was inserted into a 2 ml capless wash tube.
2. The homogenized lysate was decanted or pipetted into the RNA binding column. It was then centrifuged for 30 seconds (for cultured, bacterial, or yeast cells) or 60 seconds (for animal or plant tissue). The RNA binding column was removed from the wash tube, the filtrate from the wash tube was discarded, and the column was replaced with the same wash tube.
3. The low stringency wash solution, provided as a 5x concentrate, was prepared by adding 4 volumes (80 ml) of 95-100% ethanol to the concentrate.
4. Seven hundred µl of low stringency wash solution was added to the RNA binding column. It was centrifuged for 30 seconds. The low-stringency wash solution was discarded from the wash tube, and the column was replaced with the same wash tube.

5. The RNase-free DNase I, provided as a lyophilized powder, was reconstituted by adding 250µl of 10 mM Tris, pH 7.5 (not provided), to the vial and briefly pipetting up and to mix.
6. For each processed column, 5µl of reconstituted DNase I was mixed with 75µl of DNase dilution solution in a 1.5 ml microcentrifuge tube (not provided). If processing more than one column, the proportions were scaled up accordingly. 80µl of diluted DNase I was added to the membrane stack at the bottom of each column. The digest was allowed to incubate at room temperature for 15 minutes (25 minutes for animal tissue).
7. Seven hundred µl of high stringency wash solution was added to the RNA binding column. It was centrifuged for 30 seconds. The high-stringency wash solution was discarded from the wash tube, and the column was replaced in the same wash tube.
8. Seven hundred µl of low stringency wash solution was added to the RNA binding column. It was centrifuged for 1 minute (for cultured, bacterial, or yeast cells) or 30 seconds (for animal or plant tissue). The low-stringency wash solution was discarded from the wash tube, and the column was replaced in the same wash tube.
8. An additional centrifugation step was performed for 2 minutes to remove the residual wash solution.
10. The RNA binding column was transferred to a 1.5 ml capped microcentrifuge tube. 80µl (or 40µl) of the elution solution was pipetted onto the membrane stack at the bottom of the RNA binding column, and 1 minute was allowed for the solution to saturate the membranes. The column was centrifuged for 2 minutes to elute the total RNA.
11. The eluted total RNA samples could be used immediately in downstream applications. Alternatively, the total RNA could be stored at -20°C or -80°C for later to use.

## **4.10 RNA QUANTIFICATION USING QUBIT FLUOROMETER**

### **4.10.1 PREPARING SAMPLES AND STANDARDS**

This protocol assumed that you were preparing standards for calibrating the Qubit Fluorometer. If you planned to use the last calibration performed on the instrument, fewer tubes (step 1) and less working solutions (step 4) were needed.

1. The required number of Qubit tubes for standards and samples was set up.
2. Thin-wall, clear, 0.5-mL PCR tubes were used for the Qubit 4 Fluorometer. Note: Only thin-wall, clear, 0.5-mL PCR tubes should be used for the Qubit 4 Fluorometer.
2. The Qubit working solution was prepared by diluting the Qubit RNA BR Reagent 1:200 in Qubit RNA BR Buffer. A clean plastic tube was used each time the Qubit working solution was prepared.
3. Qubit working solution was added to individual assay tubes so that the final volume in each tube after adding the sample was 200µL.

**TABLE: 4.3 Preparing samples and standard**

|                                 | <b>Standard Assay Tubes</b> | <b>User Sample Assay Tubes</b> |
|---------------------------------|-----------------------------|--------------------------------|
| Volume of working solution      | 190µL                       | 180-199µL                      |
| Volume of standard              | 10µL                        | —                              |
| Volume of user sample           | —                           | 1-20µL                         |
| Total volume in each assay tube | 200µL                       | 200µL                          |

4. Ten µL of each Qubit™ standard was added to the appropriate tube.
5. One to twenty µL of each user sample was added to the appropriate tube. The tubes were vigorously vortexed for 3–5 seconds.
6. All tubes were allowed to incubate at room temperature for 2 minutes, and then the standards and samples were read to quantify the RNA using the Qubit Fluorometer.

## **4.11 cDNA SYNTHESIS USING BIO-RAD KIT COMPONENT**

|                                  |                |
|----------------------------------|----------------|
| 5x iScript Reaction Mix          | - 4µl iScript  |
| Reverse Transcriptase            | - 1µl          |
| Nuclease-free water              | - Variable RNA |
| template (100fg – 1µg total RNA) | - Variable     |
| <b>Total Volume</b>              | <b>- 20µl</b>  |

### **4.11.1 REACTION PROTOCOL**

Incubate the complete reaction mix in a thermal cycler using the following protocol:

1. Priming - 5 min at 25°C
2. Reverse transcription - 20 min at 46°C
3. RT inactivation - 1 min at 95°C
4. Optional step - Hold at 40°C

Note- When using larger amounts of input RNA (>1µg), the reaction should be scaled up (for example, 40µl reaction for 2µg, or 100µl reaction for 5µg) to ensure optimum synthesis efficiency.

## **4.12 QUANTIFICATION OF cDNA USING QUBIT FLUOROMETER**

### **4.12.1 PREPARING STANDARD AND SAMPLES**

1. The required number of assay tubes (or tube strips) for standards and samples was set up.
2. 10µL of each Qubit standard was added to the appropriate tube.
3. 1–20µL of each user sample was added to the appropriate tube.
4. The Qubit 1X dsDNA working solution was added to each tube, ensuring a final volume of 200µL.
5. Each sample was vigorously mixed by vortexing for 3–5 seconds.

6. All tubes were allowed to incubate at room temperature for 2 minutes. The cDNA was quantified using the Qubit Fluorometer.

#### **4.13 GENE EXPRESSION USING qPCR**

Conventional PCR involves running DNA on an agarose gel once the reaction is complete in order to detect the amplified product or amplicon. In contrast, real-time PCR (RT-PCR) allows the accumulation of the amplified product to be detected and measured as the reaction progresses that is, in real-time.

##### **4.13.1 PRINCIPLE**

Real-time detection of PCR products is made possible by incorporating a fluorescent molecule in the reaction that reflects an increase in DNA amount with a corresponding increase in fluorescent signal. The fluorescent chemistries used for this purpose include DNA-binding dyes and fluorescently labelled sequence-specific primers or probes. Fluorescence is monitored when amplicon occurs using specialized thermal cyclers that have fluorescence-detecting modules. The amount of amplified product in each cycle is represented by the measured fluorescence. Real-time PCR has several advantages over traditional PCR, but its ability to estimate the beginning template copy number with precision and excellent sensitivity over a broad dynamic range is its key benefit. Results from real-time PCR might be quantitative or qualitative. Quantitative RT-PCR is also referred to as qPCR.

Contrarily, traditional PCR is only semi-quantitative. Additionally, gel electrophoresis is not necessary for the evaluation of real-time PCR results, reducing experiment time and increasing throughput. Also, chances for contamination are very less because reactions are carried out and data are analysed in a closed tube system.

##### **4.13.2 SYBR GREEN DYE**

SYBR Green is the most commonly used double-stranded binding dye for RT-PCR. The dye exhibits minimal fluorescence when free in solution, but when bound to dsDNA, its fluorescence increases up to 1000-fold. In RT-PCR, its fluorescent signal increases in proportion to the amount of dsDNA generated during amplification. The excitation and emission maxima of SYBR Green dye are 494nm and 521 nm

respectively. This SYBR green Supermix is a ready-to-use 2x reaction cocktail that contains an optimized concentration of SYBR Green I dye, Taq DNA polymerase, dNTPs, and buffers for detecting a wide range of target DNA.

DNA-binding SYBR Green dye in RT-PCR. Fluorescence dramatically increases when SYBR Green binds to dsDNA.

#### **4.13.2 MATERIALS REQUIRED FOR SETTING qPCR**

- SYBR Green SuperMix
- Template DNA (cDNA)
- Forward and Reverse Primers
- Pipettes, Pipettes tips
- Sterile Microcentrifuge tubes
- Vortex, Microcentrifuge
- qPCR microplates or tubes
- Nuclease free water

#### **4.13.3 PROTOCOL FOR GENE EXPRESSION ANALYSIS USING qPCR**

The important application of RT-PCR was the study of gene expression. Studying gene expression was of fundamental importance in many areas of modern biomedical research, ranging from basic science to practical applications in industry and medicine. Most of the gene expression studies were determined by comparing the expression level of test samples vs control samples. For studying the gene expression studies, the first step was to isolate RNA from the particular samples, which was followed by the reverse transcription of isolated RNA to cDNA. The expression of the target genes and the reference genes (Actin) was determined in each cDNA sample using a qPCR assay. Further data was analyzed, and the relative expression of the target genes in the samples was calculated.

#### **4.13.4 IMPORTANT POINTS WHILE DESIGNING PRIMERS**

1. The primers were designed in such a way that they had a GC content of 50-60%.
2. The melting temperature ( $T_m$ ) was best kept between 50 and 65°C.
3. Repeats of G's or Cs longer than 3 bases were avoided, and G's and C's were placed on the ends of the primers.
4. The sequence of the forward and reverse primers was checked to ensure no 3' complementarity to avoid primer-dimer formation.

#### **4.13.5 PRIMER DILUTION**

1. The primer tubes were centrifuged at 8000 rpm for 30 sec.
2. The primers were dissolved with the appropriate volume of (NFW) 4 nuclease-free water as mentioned in the primer datasheet.
3. The tubes were vortexed and allowed to settle for 2 minutes at room temperature.
4. The tubes were kept in a boiling water bath for 10 minutes at 60°C.
5. Afterward, the tubes were centrifuged at 8000 rpm for 30 sec.
6. To aliquot the primers, 10 $\mu$ L of the primer was taken and mixed with 90 $\mu$ L of nuclease-free water in a sterile centrifuge tube. The tubes were properly labelled and stored at -20°C.

#### **4.13.6 PREPARATION OF REACTION MIXTURE**

1. The frozen 2X SYBR Green Supermix, template DNA, and primers were thawed on ice. The tubes were briefly vortexed and spun to collect the contents at the bottom.
2. The components were added to each reaction tube as given in Table 1. Each reaction mix was briefly vortexed and the tubes were spun at low speed in microcentrifuge (2000 rpm for 2 min).

**TABLE: 4.4 Reaction mixture – for 10µl**

| <b>STEP</b>                      | <b>VOLUME</b> | <b>FINAL<br/>CONCENTRATION</b>                  |
|----------------------------------|---------------|-------------------------------------------------|
| Nuclease Free Water              | 1µL           | -                                               |
| Forward Primer                   | 1µL           | 300nM (100-500nM)                               |
| Reverse Primer                   | 1µL           | 300nM (100-500nM)                               |
| 2X SYBR Green<br>Supermix        | 5µL           | 1X                                              |
| Template DNA                     | 2µL           | cDNA equivalent to 1pg to<br>100ng of total RNA |
| <b>Total Reaction<br/>Volume</b> | <b>10µL</b>   |                                                 |

**TABLE:4.5 RT-PCR protocol**

| <b>CYCLING STEP</b>                           | <b>TEMPERATURE</b>                         | <b>HOLD<br/>TIME<br/>(min:sec)</b> | <b>NO. OF<br/>CYCLES</b> |
|-----------------------------------------------|--------------------------------------------|------------------------------------|--------------------------|
| Initial denaturation and enzyme<br>activation | 95 <sup>0</sup> C                          | 02:00                              | 1                        |
| Denaturation                                  | 95 <sup>0</sup> C                          | 00:15                              | 40                       |
| Annealing / Extension                         | 55-60 <sup>0</sup> C                       | 00:20                              | 40                       |
| Melt Curve (Optional)                         | 65-95 <sup>0</sup> C (in<br>0.5increments) | 00:05                              | 1                        |

#### **4.13.7 qPCR ANALYSIS METHODS**

The choice of reaction conditions for qPCR was important to ensure reliable and effective amplification for both relative and absolute measurements. The CT or Cq values obtained during qPCR were used for absolute and relative quantification.

**In absolute quantification**, the CT or Cq values of test samples were compared to those of standards of known quantity using a standard curve. This analysis provided the copy number or concentration of the sample. This method was widely used for the detection of pathogens or copy number analysis.

**In relative quantification**, the CT or Cq values of the test samples were compared to those of the control samples. This analysis yielded the ratio of the relative amount, i.e., the fold difference of the target gene in the test vs control samples.

It was commonly used for gene expression studies. This analysis could be done using software, and manual calculations could also be performed in an Excel sheet using formulas.

## CHAPTER-5

### RESULTS AND DISCUSSION

#### 5.1 EARTHWORMS TREATED WITH DIFFERENT CONCENTRATIONS OF ITS

The matured worms were taken for the experiment (Fig:5.1). For each set of concentrations, seven sexually matured worms were selected for the experiment, and for control, 1X PBS injected earthworms were used. Before the injection method, the earthworms were subjected to gut cleaning and the following day the earthworms were amputated at the 10-11 segment (Fig:5.2). After 2 hours of amputation the worms were injected with different concentrations of ITS.

*Perionyx excavatus* at 1X, 2X, and 10X concentration of ITS, and 1X PBS as a control injected for continuously five days respectively. The experimental worms were maintained for up to 25 days for observation. The amputated worm started regenerating buds and it was measured with a scale every day and documented neatly.

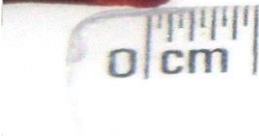
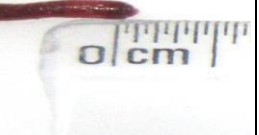


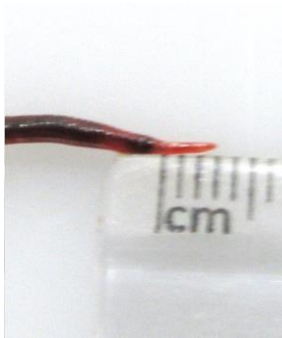
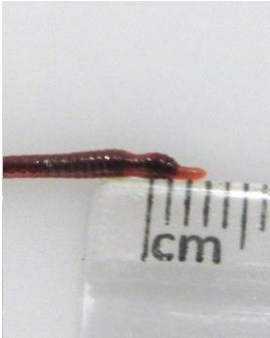
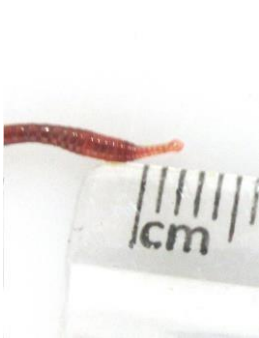
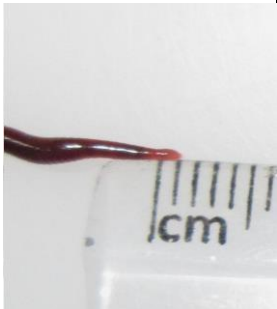
**Fig: 5.1: Sexually matured *Perionyx excavatus* (Day-1)**



**Fig: 5.2: Amputated worm (Day-2)**

**TABLE: 5.1 Phenotypical observations of the treated samples**

|           | CONTROL                                                                             | 1X ITS                                                                              | 2X ITS                                                                               | 10X ITS                                                                               |
|-----------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| DAY<br>0  |    |    |    |    |
| DAY<br>5  |    |    |   |   |
| DAY<br>10 |  |  |  |  |
| DAY<br>15 |  |  |  |  |

|           |                                                                                   |                                                                                    |                                                                                    |                                                                                     |
|-----------|-----------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| DAY<br>20 |  |   |  |  |
| DAY<br>25 |  |  |  |  |

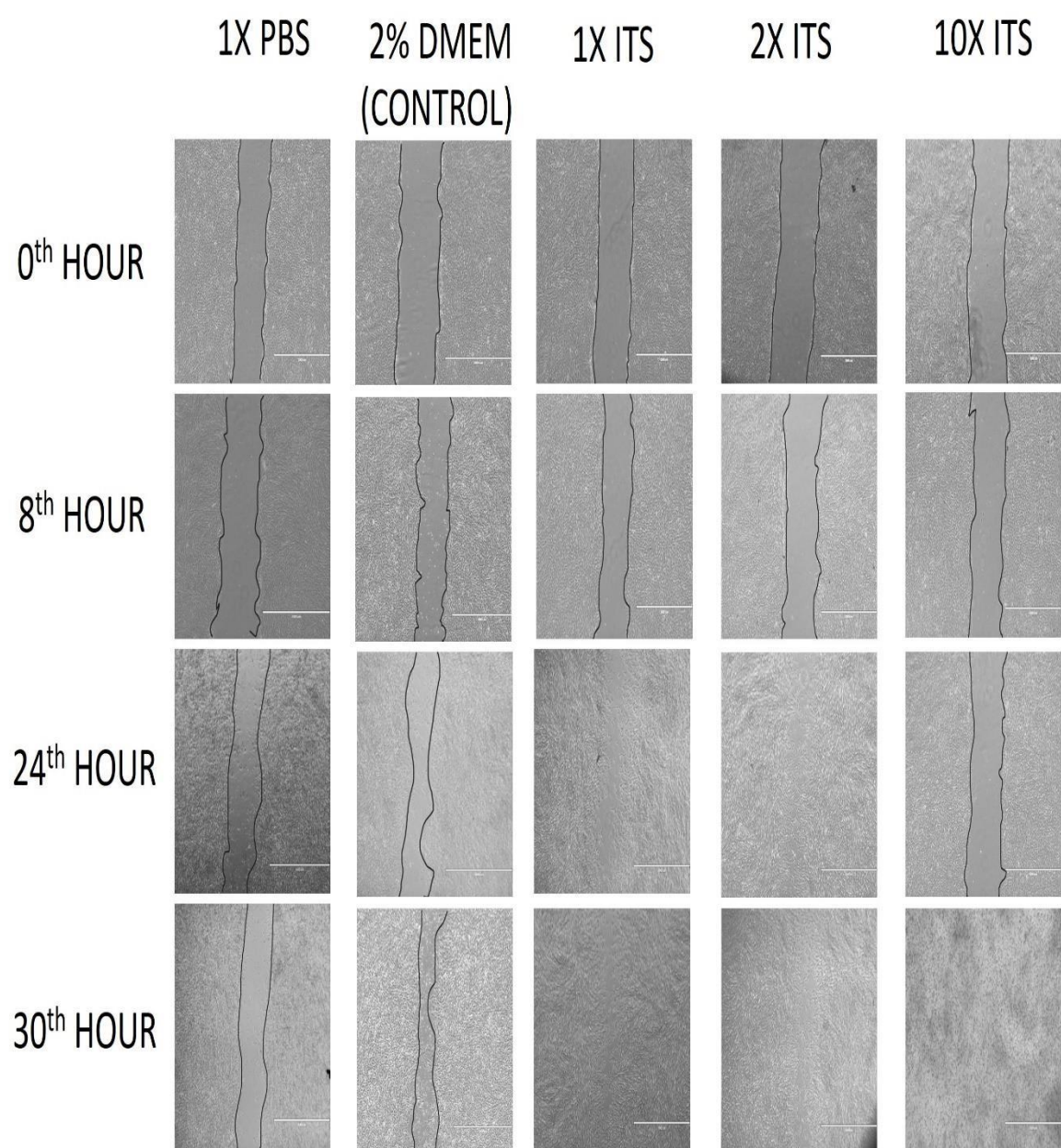
Control and ITS injected worms were measured and the data was shown in the (Table: 5.1). ITS injected worms were compared with control worms. On the 25<sup>th</sup> day, a significant difference was observed in the regenerated bud of the earthworm. When comparing to control 1X, 2X, 10X concentration, 1X, 2X concentration of ITS injected groups promotes the regeneration process based on the bud observation probable reason for increasing regeneration growth kinetics in earthworm is due to selenium. Low and optimum concentrations of selenium induce cell proliferation at the same time it is evident that increasing concentration of selenium leads to retard in the regeneration growth kinetics. For further molecular evidence, wound healing, qPCR and immunoblotting studies were carried out with the regenerated bud protein samples (Blakemore et al., 2010).

## 5.2 WOUND HEALING ASSAY

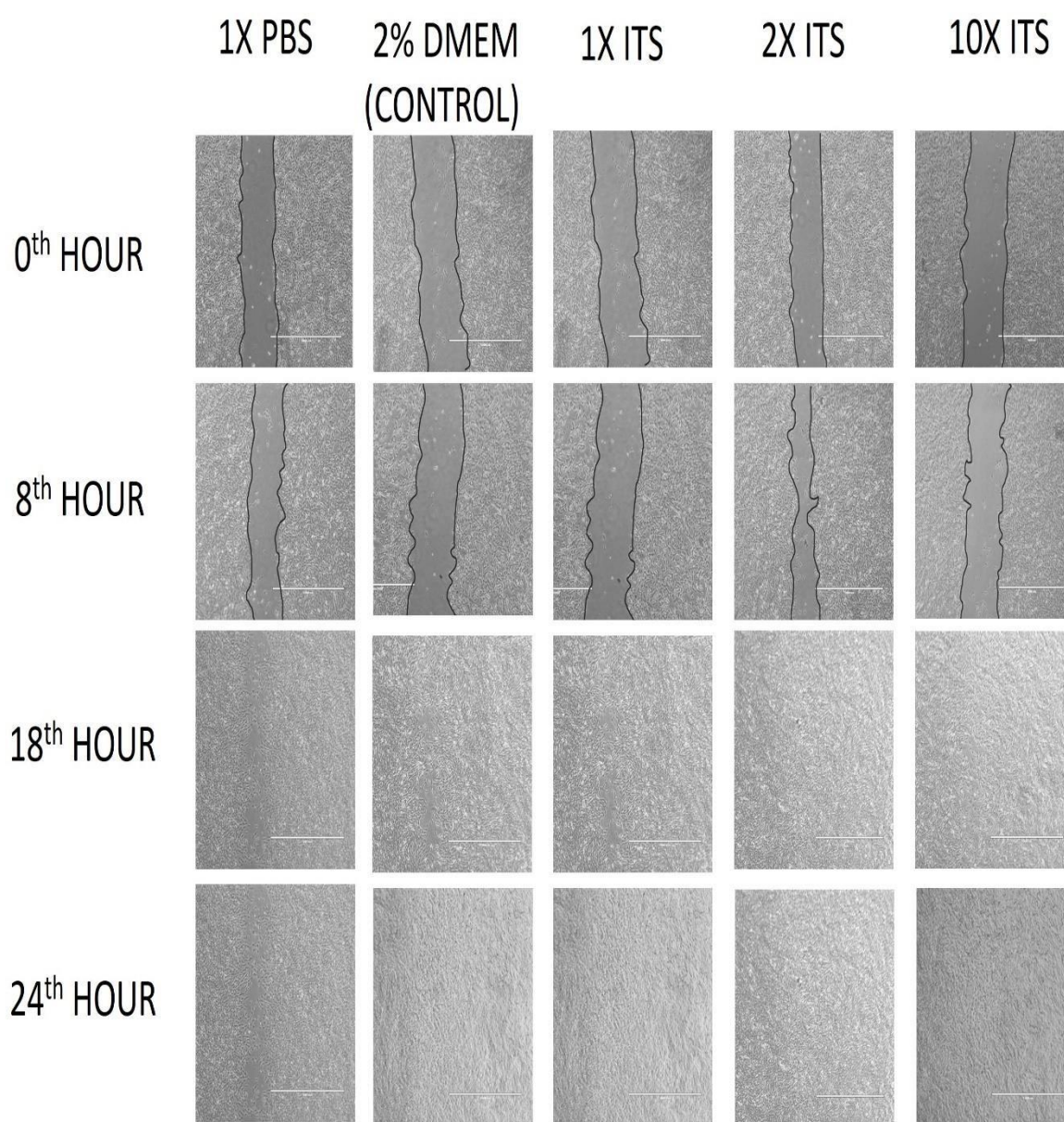
C2C12 cells ( $1 \times 10^5$  cells/well) were seeded with a complete medium at 37°C and maintained at 5% CO<sub>2</sub> for 24 hours. After 24 hours, the monolayer confluent cells were scraped horizontally with sterile 200uL pipette tips. The debris was removed by washing with 1x PBS.

The cells were treated with various concentrations of ITS (1X, 2X, 10X) by diluting with a complete medium. The cells along with 2% DMEM media were used as control for this experiment. Images were photographed at 0h, 8h, 24h, and 30h post-wounding under Phase contrast microscopy. Wound-healing migration assay was applied to assess the migration. C2C12 mouse myoblast cell line and 3T3 mouse embryonic fibroblast cell line was used in this study. ITS at the concentration of 1X,2X,10X were compared with the control group. The migration of C2C12 cells and 3T3 cells were observed at different times (Fig:5.3 and Fig:5.4). The results indicate that ITS enhances the migration of C2C12 cells and 3T3 cells at 30h, whereas the 10X ITS treated cells were not healed, which proves that it promotes wound healing in mouse myoblast cells and concentration which exceeds 10X are not fully healed at 30th hour (Räsänen and Vaheri et al., 2010).

Reason for the detached of cells at high concentrations (10X) of ITS might be due to the presence of selenium. Selenium at higher concentrations is toxic to the cells.



**Fig: 5.3: Wound healing assay (C2C12) of ITS treated**

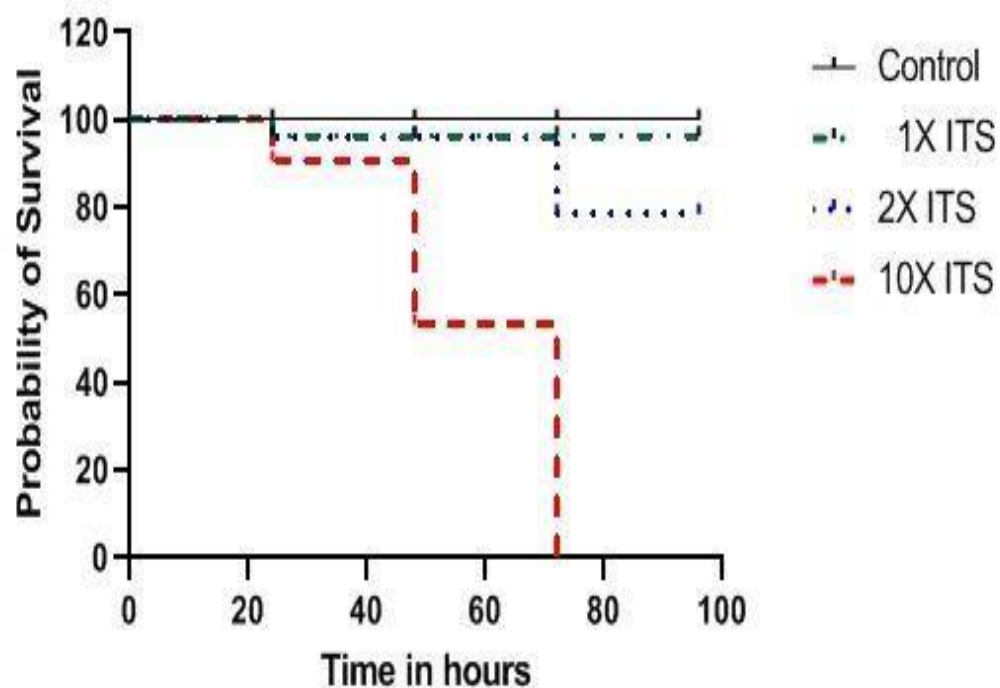


**Fig: 5.4: Wound healing assay (3T3) of ITS treated**

### 5.3 EMBRYO TOXICITY ASSAY

Embryotoxicity studies were carried out in zebrafish embryos. After the fertilization of embryos, for analysis of embryotoxicity, the embryos were treated with ITS of various concentrations (1X, 2X, 10X). Hence, it is observed that as the concentration of the ITS increases gradually the toxicity also increases gradually as mentioned below in

(Fig 5.5). After 72 hours it is observed that the embryos in 10X concentration were dead and the embryos in 1X ,2X concentration were alive after 72 hours while compared with the control. So, reason for the toxicity might be the concentration of selenium in the 10X ITS. Selenium at higher concentration is toxic to the embryos. However, insulin and transferrin are growth factors which promotes cell proliferation. So, it implies that the increased concentration is directly proportional to the increased toxicity (Foran *et al.*,2013).



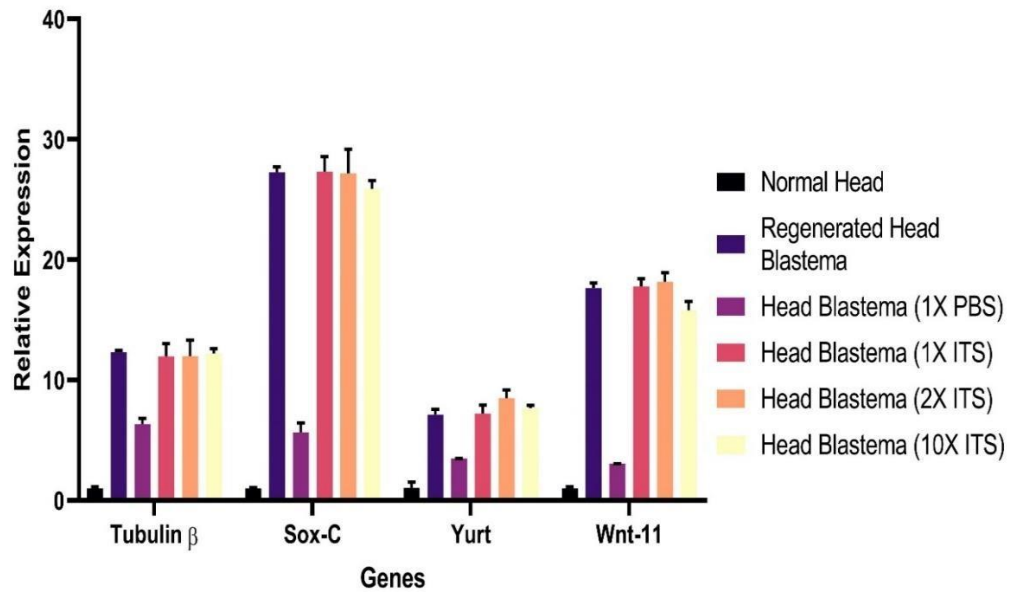
**Fig: 5.5: Embryotoxicity Assay treated with ITS**

## 5.4 GENE EXPRESSION STUDIES

Gene expression studies were performed from the following samples viz control, 5<sup>th</sup> day regenerated control, 1X, 2X, 10X ITS treated groups. The gene expression studies were resolved with the following gene, TUBULIN  $\beta$ , YURT, WNT-11, SOX-C. Results show that (Fig 5.6) compared to the control, TUBULIN  $\beta$ , YURT, WNT-11, SOX-C the gene expression in the 5th-day regenerated control were increased in TUBULIN  $\beta$ , YURT, WNT-11, SOX-C.

- TUBULIN  $\beta$  - functions for various cellular processes, including cell division, intracellular transport, cell shape maintenance, and cellular signalling. (Ludueña *et al.*, 1993).
- YURT - it plays a role in the head and tail polarity axis.
- WNT11 - important roles in embryonic development, tissue morphogenesis, organogenesis, and disease processes. wnt11 expression is highly increased at 5 $\mu$ g compared to 5th-day regenerated blastema control and ITS-treated other samples 25 and 50 $\mu$ g, by analysing the gene expression wnt11 plays an important role in an early stage of embryo development. (Mikels and Nusse 2006).
- SOX C - important roles in embryonic development, tissue homeostasis, and disease processes such as cancer.

Hence, in this study TUBULIN  $\beta$ , YURT, WNT-11, SOX-C were overexpressed.



**Fig: 5.6: Relative Gene Expression of ITS exposure in *Perionyx excavatus***

ITS treated sample has a high expression of 1X, 2X concentration compared to the Head Blastema (1X PBS) and Regenerated Head Control, interestingly similar to other genes SOX- C and Wnt 11 has a high expression on 1X, 2X concentration of ITS treated groups compared to a Head Blastema sample.

## CHAPTER-6

### SUMMARY AND CONCLUSION

Insulin, transferrin and selenium (ITS) is a growth supplement used for culturing various cell types. Here, in this study we have checked ITS for therapeutic applications, notably for wound healing and regeneration. Earthworms, *Perionyx excavatus* was used as an animal model system for evaluating the efficacy of ITS, Briefly, earthworms were injected with 1X, 2X and 10X concentrations of ITS and studied for its wound healing and regeneration potential.

Interestingly, 1X and 2X concentration of ITS shows prominent wound healing and promotes regeneration process efficiently compared to the control. To validate the results, we have performed the *in-vitro* wound healing assay with mouse primary cells (3T3 and C<sub>2</sub>C<sub>12</sub>) which shows 1X and 2X concentration of ITS shows prominent wound healing activity. In addition, embryo toxicity also performed with ITS using zebra fish embryos. Our results conclude that 1X and 2X concentrations of ITS is the optimum concentration shows no embryo toxicity, might be used for therapeutic applications. Furthermore, qPCR studies perform to find the expression of regeneration of specific genes in earthworm model shows that sox-c, wnt 11, yurt and tubulin  $\beta$  were over expressed in ITS treated worms compared to control. Our findings clearly conclude that ITS plays important role in wound healing and regeneration process which might used for therapeutic applications.

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