

ACCESSING THE KEY ROLE OF HOXD3, WNT3A, YAP IN *EX-VIVO* MAINTAINED REGENERATING PROSTATE GLANDS

(PROJECT –REPORT)

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

By

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DEPARTMENT OF MEDICAL BIOTECHNOLOGY & CLINICAL RESEARCH

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade “A” by NAAC | 12B | status by UCG | Approved by AICTE

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DEPARTMENT OF MEDICAL BIOTECHNOLOGY AND CLINICAL RESEARCH

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Puja Das (411010009)** who carried out the project entitled "**ACCESSING THE KEY ROLE OF HOXD3, WNT3A, YAP IN EX-VIVO MAINTAINED REGENERATING PROSTATE GLANDS**" under our supervision from December 2022 to May 2023

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DECLARATION

I Ms. Puja Das (Reg. No. 41101009) hereby declare that the Project Report entitled “Accessing the key role of HOXD3, WNT3A, YAP in *ex-vivo* maintained regenerating prostate glands” was successfully done by me under the guidance of Dr. S. Jackson Durairaj submitted in partial fulfilment of the requirements for the award of Master of Science Degree in Medical Biotechnology and Clinical Research.

Date: 05.05.2023

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ABSTRACT

Ex-vivo maintenance of any organ or tissue is challenging to achieve, and maintaining it for a longer length of time is even more difficult. Ex-vivo-maintained organs or organoids provide numerous advantages in modern science, including drug screening, disease modeling, drug discovery, organ regeneration, and basic molecular biology research. The earthworm *Eudrilus eugeniae* is a well-known model animal for regeneration studies as it can regenerate a variety of organs. The main goal is to look into the earthworm prostate gland's ability to regenerate in ex-vivo conditions. At first, the dissected intact prostate glands were effectively maintained in ex-vivo condition for two days in the L15 medium. After that, the prostate glands were amputated at the tip of the posterior region, and the regeneration potential was evaluated under the influence of valproic acid (10mg/ml). Following 15 days of post-amputation, the valproic acid-treated prostate gland which is maintained in ex-vivo condition shows augmented regeneration potential and it almost regains its lost structures. We also observed the rapid beating of the prostate gland in the valproic acid-treated samples. Western blotting results show that Wnt3a (Stem cell marker), HOXD3 (organ patterning), and YAP1 (Hippo signaling) expression are upregulated in the valproic acid-treated regenerating prostate glands. COX2, an inflammatory marker shows decreased expression in valproic acid-treated samples than the control samples. In overall, we successfully maintained the prostate gland in ex-vivo condition and augmented the prostate gland regeneration using valproic acid which is evident with the overexpression of Wnt3a, HOXD3, and YAP1 expression.

Keywords: Prostate gland, valproic acid, Wnt3a, HOXD3, YAP1

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ABBREVIATIONS

1. 1XPBS	1X Phosphate Buffer Saline
2. BCIP/NBT	Nitro-blue tetrazolium and 5-bromo-4-chloro-3'indolyphosphate
3. β -ME	Beta Mercapto Ethanol
4. SDS	Sodium Dodecyl Sulfide
5. 1X TBST	1X Tris Buffered Saline with Tween
6. ECL	Enhanced Chemiluminescence
7. IgG	Immunoglobulin G
8. IPA	Iso-Propyl Alcohol
9. RPM	Revolutions Per Minute
10. L-15. Medium	Leibovitz's Medium
11. DPX	Distyrene Plasticizer Xylene
12. SCN	Stem Cell Niche
13. NC	Nerve cord
14. ALP	Alkaline Phosphatase
15. RFP	Red Fluorescent Protein
16. DAPI	4',6-diamidino-2-phenylindole
17. PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
18. BSA	Bovine Serum Albumin
19. PSCA	Prostate Stem Cellular Antigen
20. Oct-4	Octamer-binding transcription factor 4
21. GFP	Green Fluorescent Protein

22. pH	Power of Hydrogen
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- 2.10
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UNITS

1. **mm** - milli meter
2. **μg** - micro gram
3. **μM** - micro molar
4. **g** – grams
5. **m²** - meter square
6. **ml** – milli litre
7. **l** – litre
8. **V** – volts
9. **kDa** - kilodalton

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CHAPTER – 1

INTRODUCTION

1.1 Overview of earthworm *Eudrilus eugeniae*

Eudrilus eugeniae is one of the most abundant earthworm species in West African soils. The "African nightcrawler" is the popular name for this worm. It is also found in other regions of the world (Blakemore, R.J., 2015). This species is also earning more interest as a potential waste decomposer and protein source. More fundamental research into its general biology is required to properly confirm this species' significance as a waste decomposer (Reinecke, A. J., & Viljoen, S. A. 1988). Earthworms are the most prevalent soil invertebrates and can be found in many parts of the world, including temperate and tropical zones (Byambas et.al, 2019). *Eudrilus eugeniae* is a clitellum-dependent earthworm that requires intact clitellum segments for its survival and regeneration. The worm is an excellent model for cell and molecular biology research. It also has an exceptional ability to regenerate, the worm can be used to study the complicated cell and molecular biological events of regeneration. To survive and regenerate, the clitellum-dependent earthworm requires undamaged clitellum segments. It is an excellent source of protein and has great pharmaceutical potential. This species of earthworm is endemic to tropical West Africa, but it is now common in warm places because of vermicomposting.

<i>Eudrilus eugeniae</i> scientific taxonomy	
Kingdom	Animalia - Animal
Subkingdom	Bilateria
Infrakingdom	Protostomia
Superphylum	Lophozoa
Phylum	Annelida - annelids,
Class	Clitellate Superorder
Superorder	Metagynophora

Order	Opisthopora
Suborder	Crassiclitellata
Family	Eudrilidae
Genus	Eudrilus
Species	<i>Eudrilus eugeniae</i> - African night crawler, African red
Geographic division	Africa, Australia, Caribbean, America and Southern Asia

Table 1.1: Scientific taxonomy of the earthworm *Eudrilus eugeniae*

1.1.1 Description of an earthworm *Eudrilus eugenia* (Blakemore, R.J., 2015)

a) *External morphological characteristics*

- ❖ Body length: Complete matures, 90–185 mm, or up to 250–400 mm under excellent cultivation conditions; posterior tapering, becoming thinly flattened in terminal 'zone of growth'.
- ❖ Width: Approximately 4–8 mm in width.
- ❖ Mass: Average adult mass is around 1.0 grams, with an ideal maximum of 5.0– 6.0 grams.
- ❖ Color: Red-brown dorsum fading posteriorly; anterior with dazzling blue/green iridescent sheen from cuticle diffraction, ventrum beige, clitellum darker (occasionally lighter) than surroundings;
- ❖ Prostomium: A small, open epilogues prostomium.
- ❖ Clitellum: present in the 13, 14, 15–18th segments, and ventrally interrupted.

b) *Internal morphological characteristics*

- ❖ **Septa:** thickened from 4th to 5th segments; and from 6,7,8,9 and 14,15th segments.
- ❖ **Dorsal blood vessel:** Single, truncated before anterior hearts in the 7th segment; connect to paired supra-oesophageal in 7–14th segment and paired extra-oesophageal medial to the hearts.
- ❖ **Hearts:** 7th segment is lateral, 8–11th segment is later-oesophageal, all distended with blood in certain specimens, the anterior hearts were undistended.
- ❖ **Gizzard:** Weakly muscular in 5th segment present just behind the pharyngeal bulk.
- ❖ **Intestine:** Origination in 14th or 15th or close to it. Caeca and typhlosome are not present. Small supra-intestinal glands seen in the 8th – 42nd segment of sections may aid digestion and/or play a role in worm immunity.
- ❖ **Nephridia:** Each segment from 4 has paired, big coiled holo nephridia that are not visibly vesiculate.
- ❖ **Prostates:** A big pair of digitiform prostates with a white muscular sheen

from 18 to 23; sharply muscular enlargements of loops of paired sperm ducts that join to the apex of the copulatory chamber mound in the center. Although 3 it consists of two such glands, a smaller blind duct - the Y-shaped gland - joins to the base of the mound mesially.

❖ **Reproductive System:** Earthworms have two sexes. As a result, each individual has both male and female reproductive systems.

❖ **The male reproductive system:** It is made up of two pairs of testes (10-11 segments), a vasa deferentia (until the 18th segment), and two pairs of accessory glands (17th and 19th segments). A pair of male genital holes allow access to the prostate and spermatic ducts (18th segment). The spermatozoa are held in four pairs of spermathecae (6-9 segments).

❖ **The female reproductive system:** It is made up of a single set of ovaries and an oviduct. The ovaries open into an ovarian funnel that runs beneath them, joins the oviduct, and opens at the female genital aperture (14th segment).

1.2 Regeneration

1.2.1 *Regeneration and stem cell activities*

Regeneration is the process of renewing or reviving damaged or amputated bodily parts of certain organisms (Shin SY et al., 2015). Regeneration can either return the entire organism or just the amputated body parts to their pre-amputation state (Santos Jr AR et al., 2014). The majority of this process will be carried out with the assistance of stem cells. It only happens in an organism with a simple structure and specialized cells. These specialized cells may drastically divide nature and produce a big number of cells in a short period of time. Every living body has the tendency to revive and nourish its tissues and organs as part of natural processes (Kolios G & Moodley Y., 2013). Earthworms have a strong capacity for regeneration. Unfortunately, mammals' regenerative abilities are limited, consisting of thick scar development, new skin and hair regeneration, bone fracture repair, and certain important organ regeneration. While stem cells (i.e pluripotent or tissue-specific stem cells) can help in regeneration, regeneration can also happen without them. Cell revival, restoration of actual differentiated cells, activation of native adult stem cells, and conversion of one cell type to another are the four components of 4 regeneration. Tissues or organs that are regenerating need one or more of the components listed above to restore their structure (Stocum DL., 2012). The regeneration mechanism includes a number of signaling components. Factors that alter cell cycle via various signaling transduction pathways, allow cell viability, apoptosis, or cellular destruction to be determined.

1.2.2 *Stem cell niche*

The in vivo or in vitro local microenvironment within the specific tissue is broached by the stem cell niche (Ferraro F., 2010). In which stem cells cohere with extracellular matrix and other cell types, prompting cell-to-cell adhesion as

a main function (Lodish H et al., 2000). This interaction aids the niche's cells in maintaining their population or stimulating differentiation (Morrison SJ & Spradling AC., 2008). The stem cell niche is a large space that comprises a complex arrangement of biochemical and physical signals that aid stem cells in their proliferation, migration, and differentiation processes. It leads cancerous cells to arise as a result of excessive niche proliferation (Li X., 2017). As a consequence, there should not be a big number or a small number of stem cells for proper functioning, as this leads the niche to become unbalanced (Clark DP & Pazdernik NJ., 2016).

1.2.3 Activation of stem cells

Stem cell activation is a useful distinction in a variety of stem cell biology-related perspectives (Biehl JK & Russell B., 2009). When a stem cell is activated, it navigates to the aberrant area and performs anti-inflammatory and other biological activities (Fontes PA & Thomson AW., 1999). Wound healing, organogenic, and tissue revitalization are all involved in this process. Three major steps are involved in stem cell activation: Cell migration, proliferation, and revival are all processes that cells go through. Migration refers to the movement of cells from one location to another, whereas Proliferation refers to the doubling of cell numbers and cell revival due to the DNA methylation process that affects gene expression (Zakrzewski W et al., 2019). Organs such as the skin, liver, and intestines regenerate frequently, but body components such as nails and hair follicles remain dormant until they receive signals to activate. As a result, stem cell activation aids in adult stem cell replenishment (Cheung TH & Rando TA., 2013)

1.2.4 Organ-specific stem cells

Stem cells are self-renewing, clonogenic progenitor cells that can differentiate into one or more specialized cell types. Stem cells in vertebrates have typically been split into two categories. Only embryonic stem (ES) cells form the first group. These pluripotent stem cells, which are produced from the blastocyst's inner cell mass, are capable of producing all differentiated cell types in the body. The second kind of stem cells is created by ES cells and is known as organ- or tissue-specific stem cells. In embryos and, in certain situations, adults, such multipotent stem cells generate the cell types that make up a specific tissue. The hematopoietic stem cell, which generates all of the blood and immune system's cell types, is the prototypic example of this second group. In addition to the cells that make up blood, stem cells can be found in a variety of other organs throughout a mammalian's life. The presence of stem cells in organs like the intestine and skin is maintained by continual cellular regeneration. The presence of stem cells in other organs, such as the brain and heart, is confirmed by their separation from these tissues and subsequent growth and differentiation in culture, either in vitro or after transplantation in vivo (Anderson David J et al., 2001). However, it is questionable if the body truly uses these cells to replace diseased or damaged cells.

1.2.5 Regeneration in Organs

When compared to other organisms around the world, mammalian' regenerative powers are notably superior. Assuming that if a bodily cell is injured, it will either heal itself or not respond at all. Humans, unlike other creatures, do not tend to regenerate the entire missing section of the body (Shieh S & Cheng T., 2015). Humans, on the other hand, have the ability to regenerate partial organs, repair wounds, and regenerate big organs such as the liver and kidney (Krafts KP., 2010). As previously indicated, there are several primary phases of regeneration: first, homeostasis, then inflammation, proliferation, and ultimately remodeling. Starting with the injury, homeostasis commences coagulation within a few minutes; following that, inflammation occurs between hours and days for immune infiltration. Proliferation also causes scar formation, collagen synthesis, and angiogenesis, which takes weeks to months; finally, tissue or organ remodeling takes months to years (Grayson WL et al., 2015). Organ regeneration, on the other hand, can occur naturally or be produced in 6 many wounded places by transplanting stem cells, lab-grown tissues, and organs (Mao AS & Mooney DJ., 2015).

1.2.6 Prostate gland regeneration

Androgen deprivation causes death in most luminal cells in the rat and human prostate, although the majority of basal cells survive (English et al., 1987). Apoptosis cells are preferentially distributed to the distal sections of prostatic ducts, while proximal ducts are less impacted, which is the inverse of the ductal expansion process. The prostate gland varies from other tissues (e.g., the epidermis, intestinal epithelium, and hematopoietic system) in which stem cell activity is required on a continuous basis to maintain tissue homeostasis. The prostate, on the other hand, looks to be more like growth-quiescent tissues like the lung, where stem cell activity is linked to wound repair and regeneration (Shen, M. M. et al., 2008)

1.3 Valproic acid

VPA was first produced in 1882 as an organic solvent [2]. The clear, colorless, eight-carbon branched-chain fatty acid derived the name 'valproic acid' from the chemical name of 2-propyl valeric acid. The therapeutic potential of VPA was fortuitously discovered in 1962 when Carraz and colleagues used VPA as a solvent for experimental anticonvulsants and recognized that VPA itself had anticonvulsant activity. The fatty acid structure of VPA explains a number of its characteristics, including its distribution, sites of action, and routes of elimination. VPA is a short-chain fatty acid that is used in the treatment of epilepsy, BPD, and migraine headaches, and is being investigated as a therapeutic agent in the treatment of certain forms of cancer. The mechanism by which VPA produces both its therapeutic and side effect profile is unknown, but likely involves multiple actions including alterations of neurotransmitters, inhibition of HDACs, and effects on intracellular signaling pathways.

1.4 Organoid structures

In the last decade, stem cell–derived organoids have emerged as a powerful new model for studying human disease. These small organs are made out of three-dimensional structures that mimic the architecture of the actual product. Organoids provide new possibilities for medical research, including individualized therapy, 7 toxicological testing, and drug development, because they can be created by any patient. With the advancement of gene editing technologies and the development of organoids that mimic an increasing number of tissues, including the brain, the female reproductive tract, and early embryos, the scientific community and the general public are beginning to consider the ethical implications of organoid technology (Bartfeld, S., 2021). Yoshiki Sasai established the first organoids with the iPS/ES system in 2008. Previously, ES and iPS cells only desired to produce a single cell type, such as additional stem cells, beta cells, liver cells, or neurons. Pluripotent stem cells aid in the formation of cell clusters known as embryoid bodies, which are nothing but organoid structures (Eiraku M, et al., 2021). Maintaining any animal organ in the ex-vivo condition is very difficult. The prostate is usually associated with other male reproductive organs like the testis and seminal vesicle, and maintaining them individually is tedious. Earthworm has a regeneration ability and it is important to investigate the regeneration potential of the prostate gland in ex-vivo condition. If the regeneration mechanism of the prostate gland is revealed it helps to sort out and treat injuries, inflammation, and any other disease condition of the prostate gland. There is no report on prostate gland regeneration and organoid formation using earthworms as a model. The importance of organoids is they may give better insight into the cell communication between cell types, and thereby, increase the efficiency of ex-vivo testing. Organoid models are used in stem cell developmental biology, toxicological studies, diseased models, infectious diseases, and regenerative medicine. This study could find a way for future regenerative studies and organoid models.

CHAPTER – 2

REVIEW OF LITERATURE

2.1 Case Histories on Earthworms

Perrier (1872), Horst (1886), Ude (1894), and Beddard (1886a, 1886b, 1887, and 1888) investigated the reproductive organs of *Eudrilus euginae*, and they correctly pointed out that they differ from those of the other Oligochaeta. In his last series of papers, Beddard (1888) talks about the male and female reproductive organ specifications and their morphology. Gates (1942) made a number of observations based on his dissections. Later on, Vidya Vati updated the above studies in 1947 and made some interesting observations about the morphology and arrangement of the earthworm's reproductive organs (Vidya vati., 1942). According to A.J. Reinecke, researchers focused more on earthworms for decomposition (Reinecke, A.J. et al., 1992). According to several experts, the earthworm species *Eudrilus eugeniae*, *Perionyx excavatus*, and *Eisenia fetida* are potentially suitable for decomposing organic waste (Hink, W.F., 1979, Hartenstein, R. et al., 1979, Haimi, j. and Huhta, V., 1986). Worms failed to survive at temperatures below 12 degrees Celsius, according to S.A Viljoen and A.J Reinecke. They further claim that high growth rates and biomass production were recorded at high temperatures up to 29°C, while high maturation rates and cocoon creation were observed at an ideal temperature of 22 to 25°C. As a result, they both agreed that earthworms are extremely sensitive to temperatures below 12°C and can survive up to 30°C (S.A Viljoen and A.J Reinecke., 1992).

2.2 Stem Cells and Regeneration in Earthworms

Stem cells are responsible for the development of regeneration and play a critical role in it (Thorgeirsson S.S., 1996). The Suthira Owlarn assumption describes how regeneration begins in amputated tissue conditions. According to their assumption 1, every time a wound heals, it is referred to as an H- wound, and every time a wound regenerates, it is referred to as an R- wound. The indications of H- wound and R- wound are predominantly used in the healing and regeneration mechanisms. Assumption 2 states that only when the amputated tissue contexts have been repaired might help 9 the regeneration process to begin; without the missing tissue contexts, just the wound-healing process will be performed (Owlarn s. et al., 2017). As per Kalidas R.M. and colleagues, Oct-4 is highly expressed within the regeneration blastema. Lamin A was no longer detectable in regeneration blastema, confirming the immunohistochemical findings. The prostate tissue pattern, on the other hand, revealed that lamin A had a highOp; loiku7jy6htgrfdsaxZeTFDX r content material. Finally, they discovered that differentiated cells express lamin A while undifferentiated cell does not. Lmn A1 and A2, which are unique antibodies to lamin A, are effective tools for observing regeneration and stem cells (Kalidas R.M et al., 2015). Animal adaptability and organogenesis are important events in regeneration, according to Subramanian et al., (2016). The regeneration of

the mouth and anus in *E. eugeniae* was investigated. For five days, the earthworm was detected in a specific starving behavior; hunger causes the gut microbial flora to synthesize riboflavin. *Bacillus endophyticus*, one of the green riboflavins-producing bacteria, has been seen in 16S rRNA sequencing to be in the intestine to maintain homeostasis throughout the starving phase of early regeneration. In humans and mice, Watabe, T and his colleagues examined if PSCA (prostate stem cell antigen) is a cell surface molecule associated with prostate cancer. They generated a transgenic mouse strain that uses the human PSCA promoter area to modify the expression of brighter green fluorescent protein to help them better understand how this molecule is regulated (GFP). GFP expression was discovered in the middle of the pregnancy. GFP expression was restricted to a subset of cells within the distal terminals of the glands in adult mice. At some point during puberty, GFP expression increased, and regeneration was pushed by androgen, which was linked to prostatic growth. In the human prostate, GFP-advantageous cells co-expressed markers associated with both basal and secretory cells. Prostate carcinogenesis induced by T antigen in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model led to a higher percentage of PSCA promoter-driven GFP-advantageous cells and a higher intensity level. This transgenic system allows researchers to examine a variety of cell modifications linked to altered PSCA expression, implying that transcriptional control is a key factor in regulating PSCA levels. It also provides a useful tool for examining subpopulations of prostate epithelial cells and factors that modify the PSCA promoter (Watabe, T et al., 2002)

2.3 Valproic acid and its importance in regeneration

Valproic acid (VPA) is an antiepileptic drug approved in 1978 by the United States Food and Drug Administration (FDA) for the treatment of absence seizures. VPA is a simple eight-carbon branched-chain fatty acid, which is structurally unrelated to any other marketed drug. Valproic acid (VPA) is an anticonvulsant and mood stabilizer medication. It is widely used in the adult population to treat convulsions, migraines, and bipolar disorders. Valproic Acid (VPA), a well-known HDAC (Histone deacetylase) inhibitor, was applied to the site of amputation to see how it affected the regeneration process. Valproic acid also influences the regeneration process. It has recently been demonstrated that valproic acid (VPA) robustly promotes neurite outgrowth, activates the extracellular signal-regulated kinase pathway, and increases growth cone-associated protein 43 and bcl-2 levels in cultured human neuroblastoma SH-SY5Y cells. We hypothesized that VPA could also enhance regeneration in prostate glands. To test this hypothesis, we examined the effects of VPA on prostate gland regeneration after being maintained in *ex-vivo* conditions. Valproic acid (VPA) possesses the natural characteristic of penetrating rapidly through the membrane because of its small molecular weight.

2.4 Organoid formation

Organoids can be formed from adult organs and pluripotent stem cells, according to T. Takebe and J.M Wells. The age of organoid formation can be substantiated by using a powerful architect of cells to create a fictional design

for growth, assembly, morphogenesis, and function utilizing tissue-specific stem cells (T. Takebe and J.M Wells et al., 2019). o and Knoblich defined organoids as structures that include a larger number of cells to produce an organ that has the same structural and functional characteristics as its natural equivalent. Organoid structures are formed as a result of an understanding of natural organ developmental processes. Organoids have a lot of potential for mimicking human development and disease, as well as biomedical research and regenerative medicine (Lancaster, M. A., & Knoblich, J. A. 2014)

CHAPTER – 3

AIM AND OBJECTIVE

3.1 Aim of the study

Accessing the key role of HOXD3, WNT3A, YAP in *ex-vivo* maintained regenerating prostate glands

3.2 Objective:

- To standardize Leibovitz's L-15 medium condition that influence functional maintenance of the dissected earthworm prostate gland.
- Establishment of successful whole organ culture (*ex-vivo*) and functional maintenance of earthworm prostate gland in *Ex-vivo* condition for 3 weeks.
- Performing prostate gland regeneration studies by amputating at the posterior ends and analyzing regeneration augmentation using valproic acid (increases stemness property).
- To assess the successful regeneration based on morphological (gland regeneration and elongation) and functional examination (beating behavior).
- Molecular level characterization of prostate gland regeneration using YAP (organ formation ruler and Hippo signaling), HOXD3 (organ patterning), Wnt3a (Stem cell marker), and COX2 (Inflammatory marker).

CHAPTER – 4

MATERIALS AND METHODS

4.1 Earthworm culture and maintenance

Earthworms were collected from the International Research Centre of Sathyabama Institute of Science and Technology's Regeneration and Stem Cell Biology Lab. The worms were kept in a plastic container with leaf litter, cow dung, and soil. A diet with an equal ratio of cow dung and jaggery is provided three times per week.

4.2 Dissection and organ separation

Worm selection:

- ❖ For dissection of the prostate gland, sexually matured earthworm species of *E. eugeniae* worms were chosen.
- ❖ In the 18th to 22nd segment of the earthworm, a pair of prostate glands will be located.

Dissection:

- ❖ A sterile scalpel blade was used to dissect the gland in the ventral side of a worm.
- ❖ The cut was made along the worm's axis on the ventral side, using the sterile scalpel.
- ❖ The pair of prostate glands were carefully dissected out from the 18th segment using a scalpel and finer forceps to avoid disturbing the glands.

Washing:

- ❖ The glands were properly cleaned with ice-cold distilled water after dissection to remove all dust and debris. (Note: From this point onwards, every step should be undertaken in an ice-cold environment.)
- ❖ The glands were then washed twice mostly for 4 to 5 minutes in ice-cold 1X PBS

4.3 Ex-vivo organ maintenance

Processing:

- ❖ *After dissection* and washing, the prostate glands were placed to a sterile Petri dish containing filtered ice-cold 1X PBS and rinsed for 5 to 7 minutes. (All subsequent processing stages should be carried out in sterile circumstances.)
- ❖ Followed by washing, 0.1% antibiotic solution Streptomycin, Amphotericin B and Penicillin were added and incubated for 7 to 10 minutes.
- ❖ The glands were then moved to a new plate containing serum-free L15 medium (0% media) and allowed to fix for 7 to 10 minutes after incubation.
- ❖ Finally, the prostate was transferred to a new plate containing filtered 10% L15 media (10% FBS + L15 medium) for ex-vivo maintenance and stored at 28°C in an incubator.
- ❖ To avoid contamination, the ex-vivo maintenance glands were examined and tested for contamination on a daily basis, and the media should be changed every 2 to 3 days.

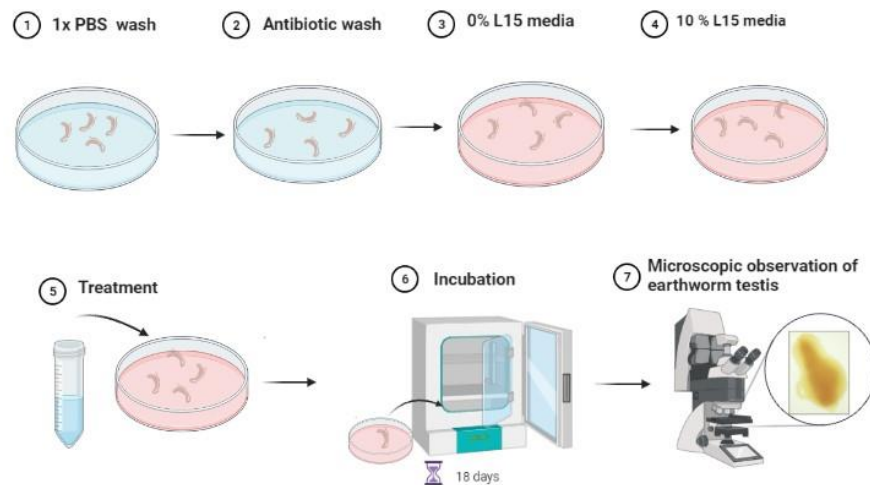


Figure: 4.3 Ex-vivo maintainance process

4.4 Prostate gland regeneration

- ❖ Followed by successful dissection and maintenance of prostate gland in ex-vivo condition for about 48 hours, the posterior end tip of the gland was amputated using a sterile scalpel and documented the 0th-day observation using a fluorescence microscope TM.
- ❖ The amputated glands were processed with 1X PBS, 0.1% antibiotic solution, and L15 media (10% and 0%) in a sterile condition for ex-vivo maintenance to observe the regeneration from the wound site.
- ❖ After 24 hours from amputation, the gland is observed under a microscope to analyze the morphology of the amputated site after amputation.
- ❖ Finally on the 3rd day the regeneration blastema was observed and carried out for histological studies, staining procedures, and Western blotting techniques.

4.5 Induction of caffeine for regeneration

- ❖ After successful dissection and maintenance of prostate gland in ex-vivo 14
- ❖ condition using L15 media for about 48 hours, the glands were set to adapt in different groups of caffeine concentrations 60 μ M, 80 μ M, and 100 μ M for 2 days in the incubator at 28°C.
- ❖ Then the amputated glands were treated with 4 experimental groups.

4.5 Protein isolation

For weakening protein di-sulfide bonds, β -Mercaptoethanol (β -ME-reducing agent) is utilized. 5% β -Mercaptoethanol (reducing agent) was added to 2X sample buffer (stock 1). At 4°C, the samples were transferred into a new 1.5ml vial containing an equal amount of sample buffer. Manual homogenization was performed using a homogenizer, following which the samples were transferred

to a dry bath at 100°C for 5 minutes, centrifuged at 10000 rpm for 5 minutes, and the supernatant was transferred to a fresh 1.5ml via and stored at -20°C.

4.6 Induction of valproic acid for regeneration

- ❖ After successful dissection and maintenance of prostate gland in ex-vivo condition for more than 2weeks.
- ❖ condition using L15 media for about 48 hours, the glands were set to adapt in valproic acid concentrations 10mg/ml for 2 days in the incubator at 28°C.
- ❖ Then the amputated glands were treated with valproic acid (10mg/ml) for continuous days.

4.7 SDS-PAGE

4.7.1 Principle

SDS PAGE is a method of separating proteins by their molecular weight. Predominantly The study of the movement of charge molecules in an electric field is known as electrophoresis. The strength of the field, net charge, size and shape of the molecules, as well as the ionic strength, viscosity, and temperature of the medium in which the molecules are travelling, all influence their rate of movement. Larger molecules, such as proteins, are commonly separated using polyacrylamide gels. Proteins are sorted based on their resistance to the gel matrix, with smaller proteins 15 having less resistance and larger proteins having more. When proteins are electrophoresed in the presence of a detergent sodium dodecyl sulphate and a reducing agent -Mercaptoethanol, the detergent can bind to the protein backbone and the denaturing effect of SDS is very strong, disrupting the secondary, tertiary, and quaternary structure of the protein, resulting in a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol aids in the denaturation of proteins by decreasing all disulfide linkages (both inter-disulfide and intra-disulfide bonds). Polyacrylamide forms a mesh-like matrix that aids in protein separation. During PAGE electrophoresis, the proteins' linear or primary structure was stacked or placed in the appropriate order; as the proteins reached the separating gel, they began to separate based on their size.

4.7.2 Materials

- ❖ Eppendorf;
- ❖ Micro-pipettes;
- ❖ surgical blade;
- ❖ syringe;
- ❖ Petri-dishes;
- ❖ Micro-tips;
- ❖ Centrifuge;
- ❖ Water bath;
- ❖ Vortex
- ❖ Distilled water.

❖ Valproic acid.

4.7.3 Chemical preparation of SDS-PAGE

30% Acrylamide and 0.3% Bis acrylamide:

Acrylamide - 30 g

N,N' Methylene Bis-acrylamide - 0.3 g

Make up into 100 ml with distilled water

4X Separating Buffer:

Tris HCL - 18.2 g

Distilled water - 60 ml

pH was adjusted to 8.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4 g

Make up into 100 ml with distilled water.

4X Stacking Buffer:

Tris HCl - 6.05 g

Distilled water - 40 ml

pH was adjusted to 6.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4 g

Make up into 100 ml with distilled water.

2X Sample Buffer:

Tris stacking buffer (pH 6.8) - 25 ml

Sodium Dodecyl Sulphate (SDS) - 4 g

20% Glycerol - 20 ml

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

10% Ammonium Per Sulphate (APS):

APS - 100 mg

Make up into 1 ml with distilled water.

1X Running Buffer:

Tris Hcl - 3.94 g

Glycine - 15.014 g

SDS - 1 g

Make up into 1L with distilled water.

Staining Solution:

Coomassie Brilliant Blue R-250 - 200 mg

Methanol - 50 ml

Glacial Acetic acid - 7 ml

Make up into 100 ml with distilled water.

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

De-staining Solution:

Methanol - 30 ml

Glacial Acetic acid - 7 ml

Makeup into 100 ml with distilled water.

Sl no	Stock Solution	5%	6%	7%	7.5%	8%	9%	10%	12%	13%	15%
1	30% Acrylamide +0.3% Bis-acrylamide (ml)	5.0	6.0	7.0	7.5	8.0	9.0	10.0	12.0	13.0	15.0
2	4X Tris Separating Buffer (ml) pH	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5

	8.8										
3	Distilled water (ml)	17.5	16.5	15.5	15.0	14.5	13.5	12.5	10.5	9.5	7.5
4	10% APS (μl)	200	200	200	200	200	200	200	200	200	200
5	TEMED (μl)	20	20	20	20	20	20	20	20	20	20

Table 4.8: Separating gel concentration

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

Table 4.9: Stacking gel concentration

4.7.4 Gel preparation

The glass plates were cleaned with ethanol and assembled by manually. Glass plates were attached in the appropriate uniform manner with each corner covered by spacers so that they could be utilized as a primary baseline on the gel to aid in the agarose sealing process or as a second level baseline system. 1% agarose was prepared and boiled until liquid then corners were sealed in both surface and bottom area of the spacer. Separating gel was hand produced and carefully put onto the gel. Isopropyl alcohol was used to cover the gel and keep the air out. After removing the IPA and washing it with distilled water, drain stacking gel was produced and poured, and the comb was immediately fastened to the stacking gel. The gel was allowed to solidify for a few minutes before the comb was carefully removed and the wells were clearly washed with water to remove any bubbles. Finally, the bottom of the spacer was

carefully removed and the setup was fixed in the bottom chamber, which contained 1X running buffer, as well as the upper chamber.

4.7.5 Sample loading and electrophoresis

The wells were loaded with a 60µg protein sample and the relevant molecular markers. The equipment was properly polarized and 5 to 6 v/cm was applied to the power pack. The glass plate assembly was removed from the buffer chamber after electrophoresis. The spacer was gently twisted away from the gel, causing the upper glass plate to pull apart.

4.7.6 Staining and De-staining Procedure

The stacking gel was carefully removed from the glass plate and the gel was carefully removed from the glass plate using a cleaver. By carefully gripping the two corners of the gel, the separating gel was extracted and placed in the container containing the Coomassie brilliant blue R-250 (Stainer solution). By gently agitating the gel on a Rocker for 30 minutes, it should be entirely submerged in the staining solution. It was then rinsed with distilled water and transferred to a de-staining solution until the protein bands were clearly visible. The first detainer wash usually takes approximately 30 minutes, and the second wash takes roughly overnight.

4.8 Western blotting

4.8.1 Running the gel

- ❖ Prepared 12% gel, and then placed it in the SDS page unit.
- ❖ Add 1X running buffer, and then add protein ladder at the first lane of the gel for molecular weight determination.
- ❖ Then add a protein sample for 60µg concentration in each well. Set the gel to 80V until the protein run successfully.

4.8.2 Buffer preparation

❖ 10X Transfer Buffer (Stock)

Glycine - 72.1g

Tris-base - 15.2g

Distiller water- 500ml

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

For Total volume 1 liter,

❖ **1X Transfer buffer:**

100ml - 10X Transfer Buffer

200ml - Methanol

700ml - Distilled water

4.8.3 Transfer to membrane

❖ Cut the PVDF membrane by the same as the gel size. Mark which side is smooth (smooth side only we should place the gel to transfer protein).

❖ Soaked the PVDF membrane in methanol for 1-2 minutes (for activation of membrane). Then placed the PVDF membrane into 1X transfer buffer until prepare the sandwich.

❖ Then prepared the blotting apparatus, sponge, and filter paper to arrange the sandwich setup. Before arranging soak the apparatus, sponge, and filter paper in the 1Xtransfer buffer.

❖ Placed the blotting setup in the blotting unit, and added transfer buffer and 2 gel packs in the unit to maintain it at 4°C until the full transfer.

❖ Transfer the gel at 85V for 65 minutes.

4.8.4 Blocking

A blocking buffer was used to block all proteins which would bind with targetable and non-targetable proteins. 5% of BSA with 20ml of 1X TBST buffer was prepared for Blocking.

4.8.5 Primary Antibody

❖ Primary antibody OCT4 (Abcam - ab181557, a monoclonal antibody produced from Rabbit) was added in the 5% BSA (1:3500 dilutions) and incubated for 2 hours. It binds only to the specific protein.

❖ Washed 5 times with PBST for 30 minutes.

4.8.6 Secondary Antibody

❖ Secondary antibody (Abcam - ab150077, Anti Rabbit IgG produced in goat) (1:1000 dilutions) was added in the 5% BSA and incubated it for overnight. It binds with the primary antibody.

❖ Washed 5 times with PBST for 30 minutes

4.8.7 Chemi-Doc imaging of the membrane:

The advanced BioRad ChemiDoc™ XRS+ imaging equipment was used to perform gel imaging for faster and more accurate western blot quantification. It is the only stain-free equipment capable of chemiluminescent, multiplex fluorescence, and routine gel imaging, and it is used to quantify total protein after transfer to the membrane for normalization purposes. avoids the mistakes that can occur when utilizing housekeeping proteins for normalization. After a 2-hour incubation with a secondary antibody, the PVDF membrane was washed 5 times with 1X PBST every 5 minutes. After that, an ECL substrate solution was made by mixing equal amounts of ECL solution A and ECL solution B in a (1:1) ratio, and the blot was washed more than 5 times in the same box before being transferred to a new one with only a few washes to avoid patchy areas on the blot. Then, connect the BioRad ChemiDoc™ to the PC by turning it on. After wiping the place specified for putting the membrane with tissue paper, the membrane is kept within the precise position. Apply the prepared ECL substrate to the membrane by drops or sprays, ensuring that all portions of the membrane are covered. Select New protocol → Application → select → blots → chemo, in imaging area → select gel type → Bio-Rad criterion gel in the BioRad image Lab 6.1 software. Set the exposure time to 150 seconds in the Image exposure area and choose Run protocol. The results will be shown in clear bands on the monitor based on the protein luminescence at various exposure durations. File → export → export for publication → set the resolution → save was used to document the results. The complete procedure was performed in complete darkness.

CHAPTER – 5

RESULT AND DISCUSSION

5.1 Ex-vivo maintenance of earthworm prostate gland

5.1.1 Dissection

The earthworm *Eudrilus euginae* has well-developed male and female reproductive organs and it is easy to study its morphology. An adult worm was picked, anesthetized, and dissected to find the organs present in it **Figure 5.1. A** ventral section of cut was made without disturbing the organs inside the worm. The prostate gland was found at the 18th to 24th segments. A big pair of digitiform, euprostates with a white muscular sheen and it is sausage in shaped morphological identification helps to find it out easily. Prostatectomy was made surgically and washed with the following procedures. The dissected-out prostate gland was washed in ice-cold condition to remove the debris and blood from the gland and after washing, the gland was further carried out for ex-vivo processing in a sterile condition.

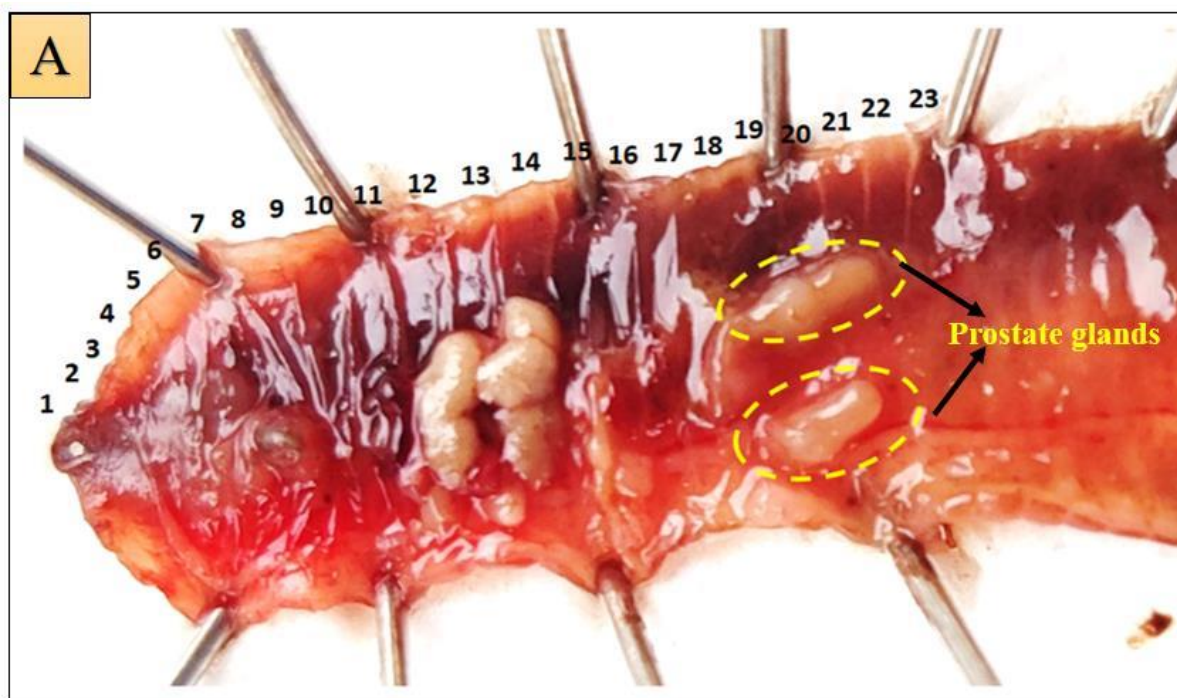
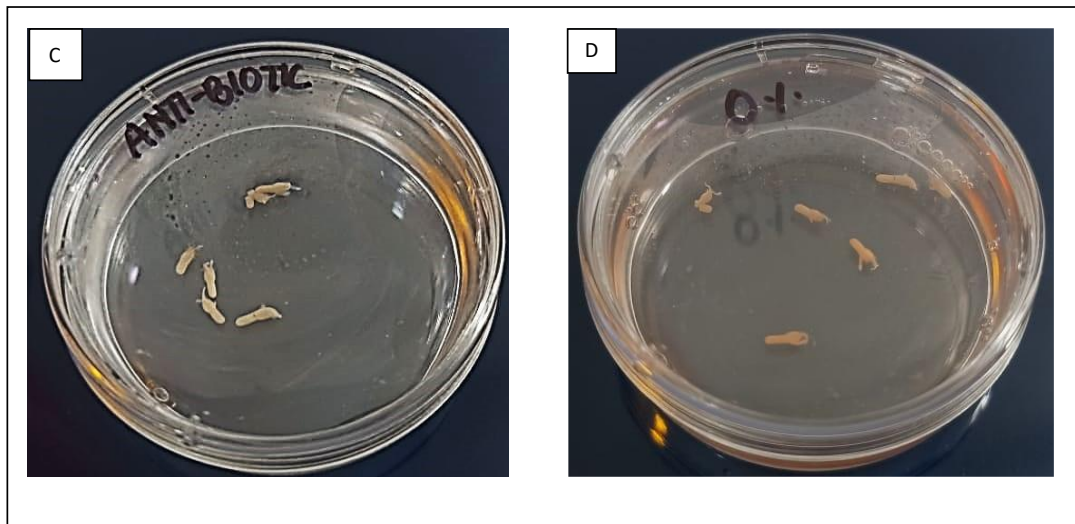
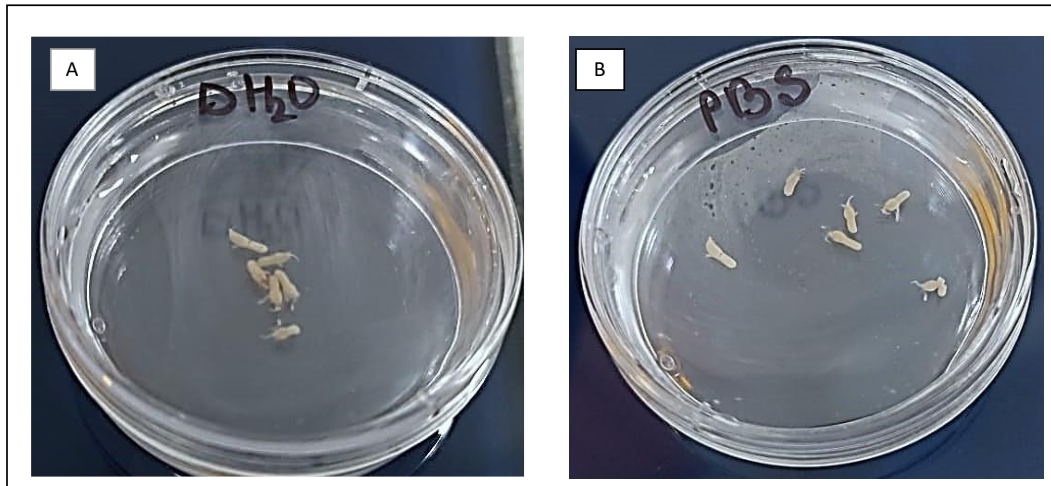


Figure: 5.1 Dissection of earthworm prostate gland

5.1.2 Processing of prostate gland and Amputation

After dissection and washing in ice-cold conditions the earthworm prostate gland was processed in ex-vivo condition. The processing was carried out in a sterile and ventilated workspace called a biosafety cabinet (BSC) for performing the process. This BSC is used to get rid of contamination from the ex-vivo-maintained prostate gland. The prostate gland was firstly washed with distilled water(A) and then ice-cold 1X PBS (B), followed by antibiotic wash (C) to control the growth of microorganisms, lately the

prostate gland is allowed to adapt in 0% L 15 media (D) to bare the pH changes and finally, the prostate gland is processed in 10% L 15 media (E) Figure 5.2 and stored at 28°C in the incubator though it is the ideal temperature to maintain the glands. After maintaining for 2 days, we amputated the posterior part of the glands very carefully in a sterile condition at the ideal temperature to maintain the gland's growth and observe the regeneration with valproic treatment and without treatment.



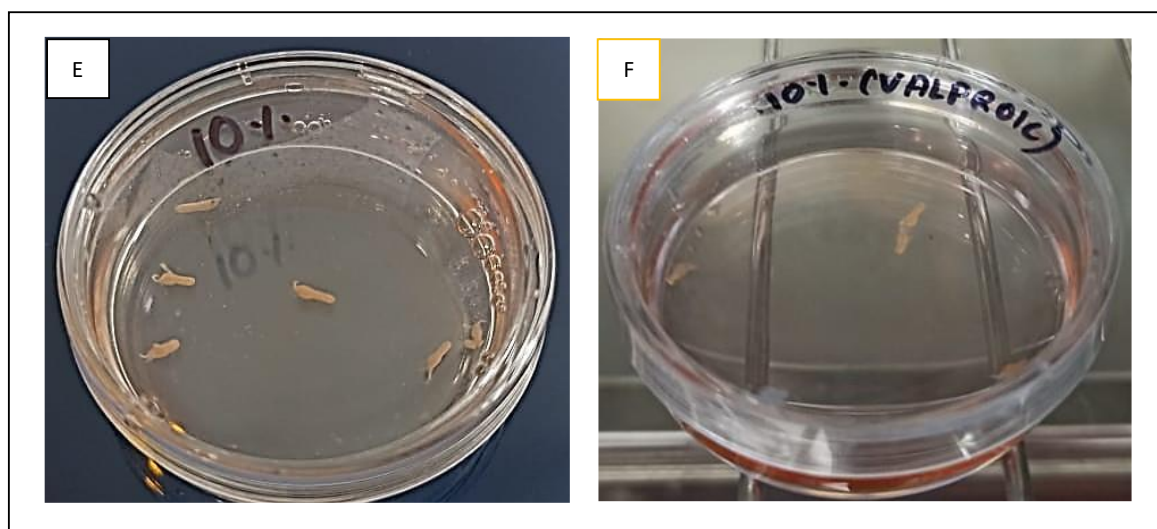


Figure 5.1.2: Ex-vivo processing of the earthworm prostate gland; A) Distilled Water wash B) 1X PBS wash C) Antibiotic wash D) 0% L15 Media E) 10% L15 Media F) Valproic acid treated

5.1.3 Maintenance

In this study, 28°C temperature is suitable for prostate gland maintenance in ex-vivo conditions in the incubator Figure 5.3. The incubator is filled with 1 liter of copper sulfate solution its fumes help to maintain the sterile environment. After two days of maintenance, the L15 media has a decline in pH so the media was changed every two days. The worms were usually maintained at ideal temperatures of up to 28° Celsius to attain high growth rates and biomass production (S.A Viljoen and A.J Reinecke., 1992).

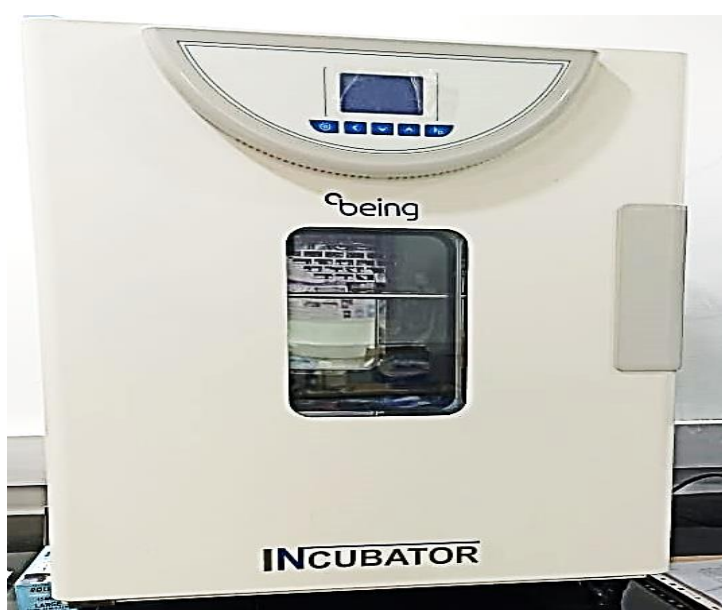




Figure 5.1.3: Ex-vivo maintenance of prostate gland in the incubator

5.2 Valproic treatment

The purpose of this study is to see whether valproic speeds up the regeneration of prostate-specific stem cells. As valproic acid triggers the stem cell development so it might help to regenerate. The assumption is that the valproic treatment will promote the regeneration of the prostate gland after amputation. Microscopic observations were made using Euromex fluorescence microscope TM to determine the regeneration rate from the number of days between amputation and complete regeneration. The goal of this study is to investigate how the valproic treatment concentrations influence the regeneration of the complete prostate gland Figure 5.2 when compared to the control group.

5.2.1 Prostate gland regeneration upon treated with Valproic acid

The experimental results treated with valproic acid show a speedy regenerative prostate gland which was observed clearly. After 2 days of maintenance in 10% L15 media the prostate gland was amputated at the posterior end and the treatment begins from that point every day. Surgical amputation was performed promptly at the posterior end of the prostate gland. The number of days between amputation and full regeneration was determined by visual inspection during a continuous period. This study claims that valproic acid is used as a therapy to enhance regeneration. When compared to a control treatment with 10% L15 medium, the regeneration Figure 5.6 accelerates the stages of regeneration beginning with blastema formation, growth, and differentiation but was comparatively slower than the treated. The findings suggest that the regeneration rate was higher in valproic acid-induced prostate glands when compared to the control.

5.3 Regeneration analysis

The amputated prostate glands were thoroughly washed and processed in *ex-vivo* conditions for continuous to analyze the regeneration rate. The regeneration process was carried out with two different processes; first is the control gland and which is processed normally only with the 10% L15 media and the next is the valproic acid-induced treatment(10mg/ml). The rate of regeneration was analyzed using Euromex fluorescence microscope TM Figure 5.5.

5.3.1 Regeneration Analysis from the experimental group

In this analysis, the regeneration on the amputated prostate gland with control was observed Figure 5.6 from day 0 to continuous days. On the 0th day, the prostate glands were amputated equally. On the next day, that is from day 1 the initial regeneration stages from each group were different from the control, valproic treated concentrations respectively. And finally, on day 15th the results based on the regeneration rate were confirmed based on the difference in morphological changes from each group. Their beating behaviors were noted and observed. After 15 days we can observe the fully regenerated prostate gland in the valproic-treated segment whereas in control the regeneration was not fully developed.

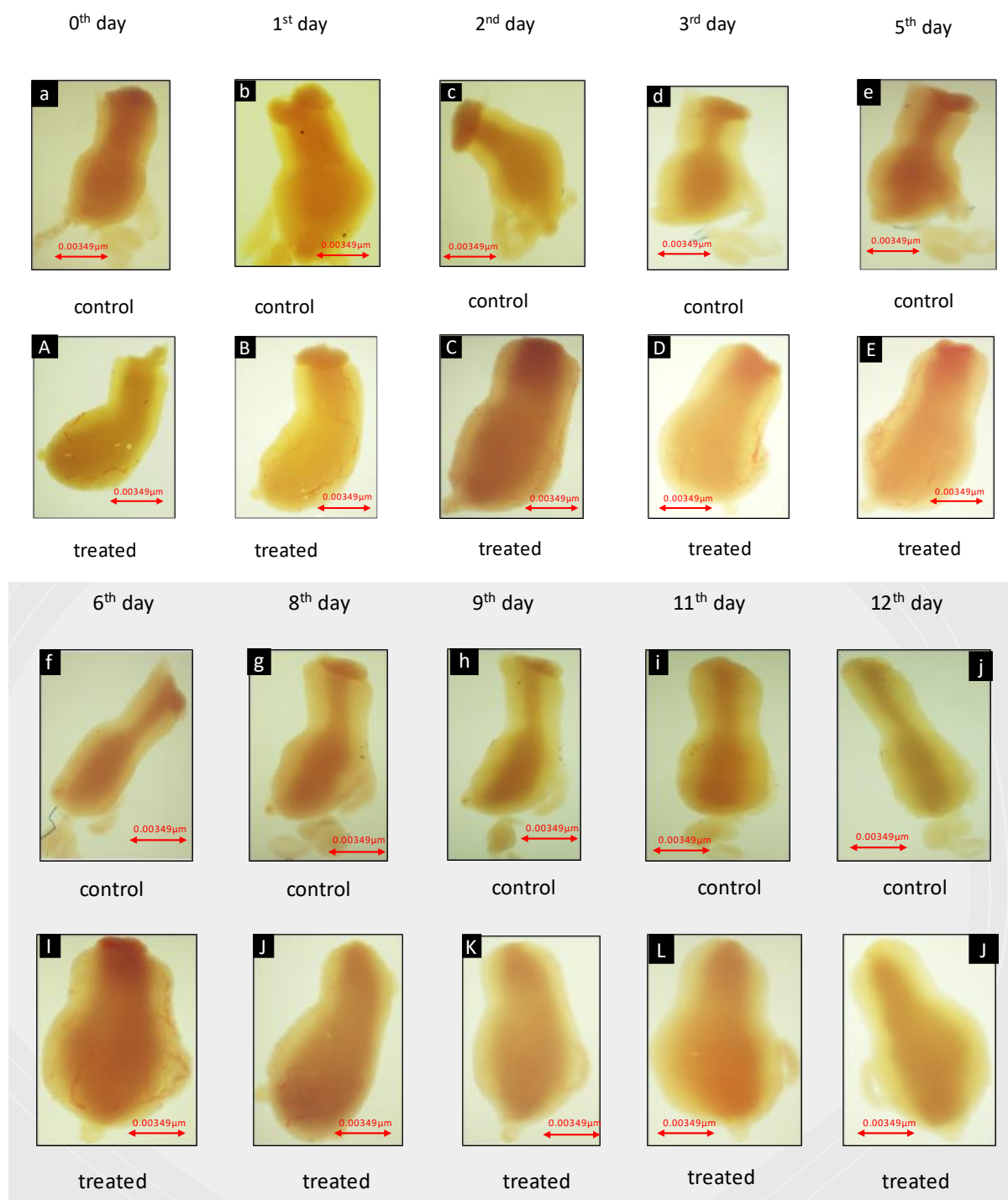


Figure: **5.3.1 Regeneration Analysis for continuous days with valproic treated**

5.3.2 Regeneration analysis on the 15th day

On the 15th day of regeneration, as shown in Figure 5.3, morphological alterations were found in the control prostate gland and treated prostate gland. The valproic acid-induced prostate gland began to regenerate with blastema formation, growth, and differentiation faster than the control sample. The prostate gland grew healthily in the

ex-vivo condition and the beating behavior was checked which was much more active than the control sample.

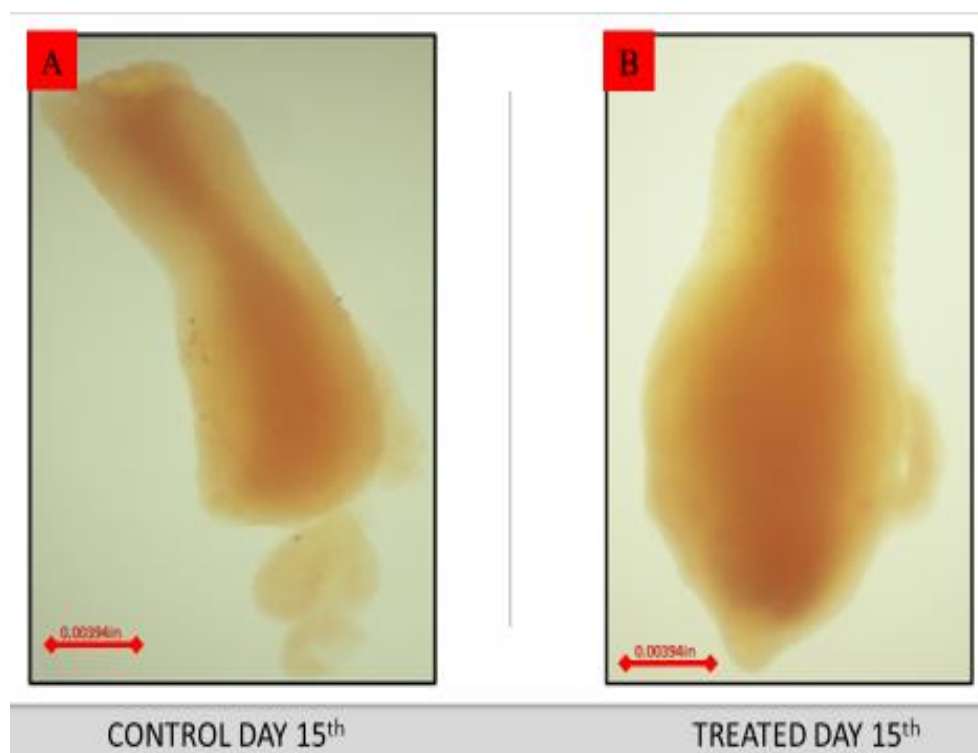


Figure: 5.3.2 Regeneration on the 15th day

5.5 Protein separation from control and valproic-treated prostate samples.

The protein sample was prepared from the regenerated prostate gland of the earthworm *E. eugeniae*. Using a pre-chilled mortar and pestle, the tissues were homogenized with 2X protein sample buffer. The tissue homogenate was heated for 5 minutes at 100°C. Following that, 60µg of each protein sample was separated in 12% SDS-PAGE. The protein content of the earthworm *E. eugeniae*'s regenerating prostate gland was analyzed quantitatively. To separate the proteins, we employed two lanes Figure 5.5 Proteins from each of the two lanes were segregated by their molecular weight. The separated proteins in the SDS PAGE gel were uniformly quantified before being transferred to the PVDF membrane for immunoblot.

5.6 SDS PAGE

The protein sample was prepared from the regenerated prostate gland of the earthworm *E. eugeniae*. Using a pre-chilled mortar and pestle, the tissues were homogenized with 2X protein sample buffer. The tissue homogenate was heated for 5 minutes at 100°C. Following that, 60µg of each protein sample was separated in 12% SDS-PAGE. The protein content of the earthworm *E. eugeniae*'s regenerating prostate gland was analyzed quantitatively. To separate the proteins, we employed five lanes Figure 5.8 Proteins from each of the five lanes were segregated by their molecular

weight. The separated proteins in the SDS PAGE gel were uniformly quantified before being transferred to the PVDF membrane for immunoblot.

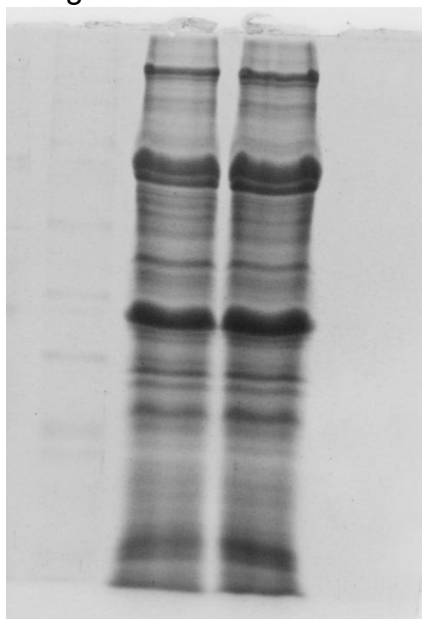


Figure: 5.6 Regeneration on the 15th day

5.7 Western blotting to analyse HOXD3, COX-3, YAP, and WNT3 expression

The earthworm prostate gland was amputated and the different stages of regeneration were examined to investigate HOXD3, COX-3, YAP, and WNT3 expression in *E. eugeniae* during regeneration. On the fifteenth day, the regeneration was complete so the sample was collected from there. The longitudinal cells functioned as progenitor cells, resulting in blastema regeneration. It eventually recovered the tissues from the prostate gland that had been amputated and glands were taken and protein samples were prepared and then blotting was carried. As we know wnt3a is responsible for organ patterning so we can observe its expression is higher in treated as compared to control, in yap1 which is responsible for hippo signaling is much higher in treated than control from this we can say valproic acid is triggering the regeneration process. The interesting factor we can notice is that cox expression has decreased in the valproic acid treated which indicated that the gland is growing healthily without stress condition

in the *ex-vivo* condition.

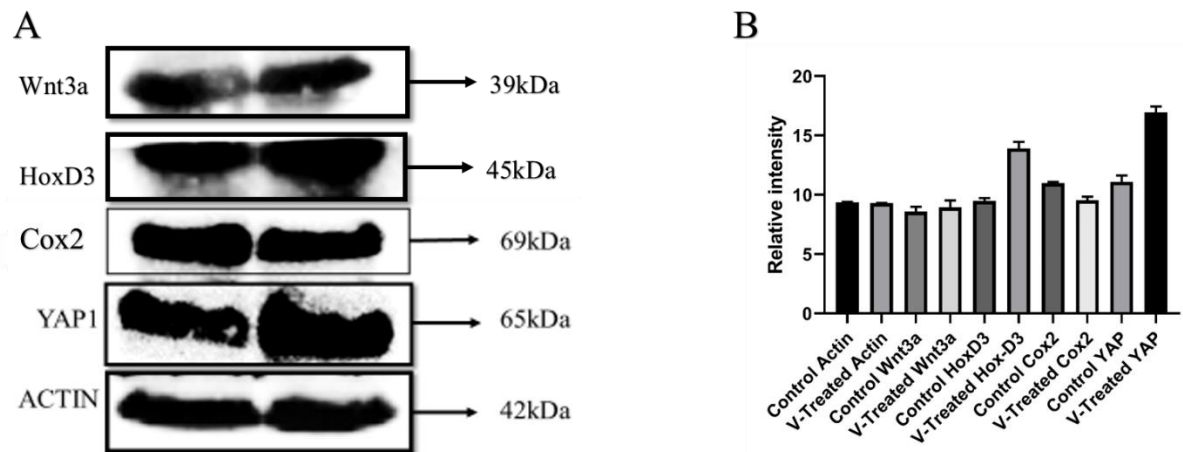


Figure 5.7: Protein expression studies on regeneration key proteins such as Wnt3a, HoxD3, Cox2, YAP1. A. L1: Control in-vitro regeneration of gland, L2: valproic acid treated in-vitro regeneration of gland.

CHAPTER – 6

SUMMARY AND CONCLUSION

- The earthworm *Eudrilus eugeniae* has a remarkable ability to regenerate, and it can be utilized to examine the complex cell and molecular biological processes that occur during regeneration. The current study examines the effects of valproic acid-induced treatment scenarios on the regeneration of the posterior prostate gland in this species. The prostate gland from earthworm was successfully maintained in ex-vivo condition and following amputation, its regeneration ability is augmented using valproic acid treatment. The treatment results in full regeneration, while the control results in only limited regeneration ability. Valproic acid not only augments the prostate gland regeneration but it also increases the expression of YAP (organ formation ruler and Hippo signaling), HOXD3 (organ patterning), and Wnt3a (Stem cell marker) together with the downregulation of COX2 (Inflammatory marker). As we know wnt3a is responsible for organ patterning so we can observe its expression is higher in treated as compared to control, in yap1 which is responsible for hippo signaling is much higher in treated than control from this we can say valproic acid is triggering the regeneration process. We can also notice that cox expression has decreased in the valproic acid treated which indicated that the gland is growing healthily without stress condition in the ex-vivo condition. From my study, we can get that organs can be maintained and regenerated healthily in ex-vivo conditions and we can go to higher levels of studies for the regeneration process.

CHAPTER – 7

REFERENCE

1. Anderson, David J.; Gage, Fred H.; Weissman, Irving L. (2001). Nature Medicine, 7(4), 393–395.
2. Bartfeld, S. (2021). Realizing the potential of organoids—an interview with Hans Clevers. Journal of Molecular Medicine, 99(4), 443-447.
3. Biehl JK, Russell B. Introduction to stem cell therapy. J Cardiovasc Nurs. 2009;24: 98.
4. Blakemore, R.J. (2015). "Eco-taxonomic profile of the iconic vermicomposter - the 'African Nightcrawler', *Eudrilus eugeniae* (Kinberg, 1867)". African Invertebrates 56: 527-548. Archived from the original on 2016-10-22.
5. Byambas, Patrick, Jean Luc Hornick, Didier Marlier, and Frederic Francis. "Vermiculture in animal farming: A review on the biological and nonbiological risks related to earthworms in animal feed." Cogent Environmental Science 5, no. 1 (2019): 1591328.
6. Cheung TH, Rando TA. Molecular regulation of stem cell quiescence. Nat Rev Mol cell Biol. 2013;14: 329–340.
7. Clark DP, Pazdernik NJ. Chapter 18 - Cloning and Stem Cells. In: Clark DP, Pazdernik NJBT-B (Second E, editors. Boston: Academic Cell; 2016. pp. 565–591.
8. Collins, E. L. (2007). The effect of caffeine and ethanol on flatworm regeneration (Doctoral dissertation, East Tennessee State University).
9. Daisy, Nino Gopi, Elaiya Raja Subramanian, Jackson Durairaj Selvan Christyraj, Dinesh Kumar Sudalai Mani, Johnson Retnaraj Samuel Selvan Christyraj, Kalidas Ramamoorthy, Vaithilingaraja Arumugaswami, and Sudhakar Sivasubramaniam. "Studies on regeneration of central nervous system and social ability of the earthworm *Eudrilus eugeniae*." Invertebrate Neuroscience 16, no. 3 (2016): 1-13.
10. Eiraku M, Watanabe K, Matsuo-Takasaki M, Kawada M, Yonemura S, Matsumura M, Wataya T, Nishiyama A, Muguruma K, Sasai Y (2008) Selforganized formation of 446 J Mol Med (2021) 99:443–447 polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. Cell Stem 42 Cell 3:519–532.
11. English, H.F., Santen, R.J., and Isaacs, J.T. 1987. Response of glandular versus basal rat ventral prostatic epithelial cells to androgen withdrawal and replacement. Prostate 11: 229–242
12. Ferraro F, Celso C Lo, Scadden D. Adult stem cels and their niches. Cell Biol Stem Cells. 2010; 155–168.
13. Fontes PA, Thomson AW. Stem cell technology. Bmj. 1999;319: 1308
14. Grayson WL, Bunnell BA, Martin E, Frazier T, Hung BP, Gimble JM. Stromal cells and stem cells in clinical bone regeneration. Nat Rev Endocrinol. 2015;11: 140–150.
15. Haimi, J. and Huhta, V., Capacity of various organic residues to support adequate earthworm biomass for vermicomposting. Biology and fertility of soils, 2 (1), 1986, pp. 23-27.
16. Hartenstein, R., Neuhauser, E.F. and Kaplan, D.L., Reproductive potential of the earthworm *Eisenia foetida*. Oecologia, 43 (3), 1979, pp. 329-340.
17. Hink, W.F, Cell lines from invertebrates. In Methods in enzymology, 58, 1979, pp. 450-466 Academic Press.
18. Hwang JS, Kobayashi C, Agata K, Ikeo K, Gojobori T. Detection of apoptosis

- during planarian regeneration by the expression of apoptosis-related genes and TUNEL assay. *Gene* 2004; 333: 15–25.
19. Kalidas, R. M., Raja, S. E., Mydeen, S. A. K. N. M., Samuel, S. C. J. R., Durairaj, S. C. J., Nino, G. D., ... & Sudhakar, S. (2015). Conserved lamin A protein expression in differentiated cells in the earthworm *Eudrilus eugeniae*. *Cell biology international*, 39(9), 1036-1043.
 20. Kim, J., Koo, BK. & Knoblich, J.A. Human organoids: model systems for human biology and medicine. *Nat Rev Mol Cell Biol* 21, 571–584 (2020).
 21. Kimble JE, White JG. On the control of germ cell development in *Caenorhabditis elegans*. *Dev Biol.* 1981;81(2):208–219.
 22. Kolios G, Moodley Y. Introduction to stem cells and regenerative medicine. *Respiration.* 2013;85: 3–10.
 23. Krafts KP. Tissue repair: The hidden drama. *Organogenesis.* 2010;6: 225–233
 24. Lancaster, M. A., & Knoblich, J. A. (2014). Organogenesis in a dish: modeling development and disease using organoid technologies. *Science*, 345(6194), 1247125.
 25. Lazorik, O. (2019). The effect of caffeine on the regeneration of Brown Planaria (*Dugesia tigrina*). *Journal of Emerging Investigators*• www. emerginginvestigators, 2, 1.
 26. Li X, Cheng Y-H, Roman J, Mao H-Q. Biomimetic Nanofibers as Artificial Stem Cell Niche. *Biology and Engineering of Stem Cell Niches*. Elsevier; 2017. pp. 411–427.
 27. Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. *Molecular cell biology* 4th edition. Natl Cent Biotechnol Information, Bookshelf. 2000;9.
 28. Mao AS, Mooney DJ. Regenerative medicine: current therapies and future directions. *Proc Natl Acad Sci.* 2015;112: 14452–14459.
 29. Millane, R. C., Kanska, J., Duffy, D. J., Seoighe, C., Cunningham, S., Plickert, G., & Frank, U. (2011). Induced stem cell neoplasia in a cnidarian by ectopic expression of a POU domain transcription factor. *Development (Cambridge, England)*, 138(12), 2429–2439.
 30. Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell.* 2008;132: 598–611.
 31. O'Neil MJ, Smith A, Heckelman PE, Budavari S, Merck. 2001. Merck Index: An encyclopedia of chemicals, drugs, and biologicals. John Wiley & Sons.
 32. Owlarn, S., Klenner, F., Schmidt, D., Rabert, F., Tomasso, A., Reuter, H., Mulaw, M.A., Moritz, S., Gentile, L., Weidinger, G. and Bartscherer, K, Generic wound signals initiate regeneration in missing-tissue contexts. *Nature communications*, 8 (1), 2017. 2282.
 33. Reinecke, A. J., & Viljoen, S. A. (1988). Reproduction of the African earthworm, *Eudrilus eugeniae* (Oligochaeta)—cocoons. *Biology and fertility of soils*, 7(1), 23–27.
 34. Reinecke, A.J., Viljoen, S.A. and Saayman, R.J, The suitability of *Eudrilus eugeniae*, *Perionyx excavatus* and *Eisenia fetida* (Oligochaeta) for vermicomposting in southern Africa in terms of their temperature requirements. *Soil Biology and Biochemistry*, 24 (12), 1992, pp. 1295-1307. 44
 35. Santos Jr AR, Nascimento VA, Genari SC, Lombello CB. Mechanisms of cell regeneration—From differentiation to maintenance of cell phenotype. *Cell Biomater Regen Med.* 2014; 37–69
 36. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells.* 1978;4(1–2):7–25.

37. Shen, M. M., Wang, X., Economides, K. D., Walker, D., & Abate-Shen, C. (2008, January). Progenitor cells for the prostate epithelium: roles in development, regeneration, and cancer. In Cold Spring Harbor Symposia on Quantitative Biology (Vol. 73, pp. 529-538). Cold Spring Harbor Laboratory Press.
38. Shieh S, Cheng T. Regeneration and repair of human digits and limbs: fact and fiction. *Regeneration*. 2015;2: 149–168.
39. Shin SY, Rios HF, Giannobile W V, Oh T-J. Periodontal regeneration: Current therapies. *Stem Cell Biology and Tissue Engineering in Dental Sciences*. Elsevier; 2015. pp. 459–469.
40. Stocum DL. An overview of regenerative biology. *Regen Biol Med*. 2012;2: 3– 20
41. Takebe, Takanori; Wells, James M. (2019). Organoids by design. *Science*, 364(6444), 956–959.
42. Thorgerirsson, S.S., Hepatic stem cells in liver regeneration, *The FASEB Journal*, 10 (11), 1996, pp. 1249-1256.
43. Vidya vati., The reproductive organs of the oligochaete *Eudrilus eugeniae* (KNIB),
44. Viljoen, S. A., & Reinecke, A. J. (1992). The temperature requirements of the epigeic earthworm species *Eudrilus eugeniae*
45. (Oligochaeta)—a laboratory study. *Soil Biology and Biochemistry*, 24(12), 1345-1350.
46. Vlaskalin T, Wong CJ, Tsilfidis C. Growth and apoptosis during larval forelimb development and adult forelimb regeneration in the newt (*Notophthalmus viridescens*). *Dev Genes Evol* 2004; 214: 423–431.
47. Watabe, T., Lin, M., Ide, H., Donjacour, A. A., Cunha, G. R., Witte, O. N., & Reiter, R. E. (2002). Growth, regeneration, and tumorigenesis of the prostate 45 activates the PSCA promoter. *Proceedings of the National Academy of Sciences*, 99(1), 401-406. (M.Sc., research fellow, Department of zoology, University of Lucknow, India.) 1945.
48. Xie T, Spradling AC. decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. *Cell*. 1998;94(2):251– 260.
49. Zakrzewski W, Dobrzyński M, Szymonowicz M, Rybak Z. Stem cells: past, present, and future. *Stem Cell Res Ther*. 2019;10: 1–22
50. Browne, T.R., 1980. Valproic acid. *New England Journal of Medicine*, 302(12), pp.661-666.