

DEVELOPING AN *EX-VIVO* TESTIS MODEL FOR ACCESSING MALE REPRODUCTIVE TOXICITY

(PROJECT –REPORT)

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

By

G. SARITHA (41101012)



**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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BONA FIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **G.Saritha (41101012)** who carried out the project entitled "Developing a *ex-vivo* testis model for accessing male reproductive toxicity" under our supervision from December 2022 to January 2023

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DECLARATION

I, **G. Saritha (41101012)** hereby declare that the Project Report entitled “**DEVELOPING A *EX-VIVO* TESTIS MODEL FOR ACCESSING MALE REPRODUCTIVE TOXICITY**” done by me under the guidance of Dr. S. JACKSON DURAIRAJ, Scientist - D, International Research Centre at Sathyabama Institute of Science and Technology (Deemed To Be University) Accredited With A Grade “A” By Naac, Jeppiaar Nagar, Rajiv Gandhi Salai Chennai- 600119 is submitted in partial fulfillment of the requirements for the award of Master of Medical Biotechnology and Clinical Research

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ABSTRACT

In recent years, the Food and Drug Administration (FDA) has withdrawn hundreds of drugs from the market due to male reproductive toxicity. For male reproductive toxicology studies, animal experiments are more often used which requires long-term research experiments and requires large quantities of compounds. Developing an *ex-vivo* testis model can aid to overcome this drawback. But developing an intact *ex-vivo* testis model and maintaining its viability for long duration is difficult to achieve. Some studies show that maintaining the intact testis from Zebra fish in *ex-vivo* condition for only 2 days. Earthworm, *Eudrilus eugeniae* has two pairs of testes in the pre-clitellum segment and which are exploited here for studying male reproductive toxicity studies by maintaining in *ex-vivo* conditions (Wang et al. 2012). Initially, we successfully maintained the dissected earthworm intact testis in L15 medium for 10 days. To further increase the viability in *ex-vivo* condition either Testosterone, Forskolin or valproic acid or their different combination were tested. Our studies show that *ex-vivo* testis supplemented with Testosterone alone are able to increase the viability and we successfully maintained it for 3 weeks. When treating the *ex-vivo* testis with suspected FDA withdrawal drug, pramipexole dihydrochloride a drug for Parkinson's disease shows aberration in their functions. Histopathology studies show that when compared with the control, Testosterone and Testosterone + pramipexole dihydrochloride treated *ex-vivo* testis shows tissue degeneration and stiffness. Sperm analysis (number, shape, motility) shows enhanced sperm viability observed in testosterone treated samples. H3 (proliferation marker) and OCT4 (pluripotency regulator) shows upregulated expression in testosterone treated samples whereas COX2 (marker for oxidative stress) and H2AX (DNA damage response) shows increased expression in control and pramipexole dihydrochloride treated samples. In overall, we successfully maintained the earthworm testis in *ex-vivo* condition for 3 weeks and which are used to assess the toxicity of drug which are suspected to withdraw from the market.

Keywords: *Ex-vivo* testis, testosterone, Sperm analysis, pramipexole dihydrochloride, OCT4

TABLE OF CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
	ABSTRACT	v
	List of Abbreviations	vii
	List of Figures	viii
1	INTRODUCTION	1
	1.1 Role of testis in reproductive toxicity	2
	1.2 Earthworm <i>Eudrilus eugeniae</i>	3
	1.3 Male reproductive system of earthworm (Testis model)	2
	1.4 Testicular toxicity studies	4
2	REVIEW OF LITERATURE	7
	2.1 Ex - vivo organ culture	7
	2.2 Testis	8
	2.3 Testosterone	8

TABLE OF CONTENTS

	2.4 Valproic acid	9
	2.4 Pramipexole dihydrochloride drug	9
3	AIM AND OBJECTIVE	10
4	MATERIALS AND METHODS	11
	4.1 Earthworm culture and maintenance	11
	4.2 Dissection of Earthworm testis	11
	4.3 L15 media preparation for <i>ex-vivo</i> maintenance:	12
	4.4 Processing of Earthworm testis model for <i>Ex-vivo</i> maintenance:	13
	4.5 Drug treatment – suspected to withdraw from the market	14

TABLE OF CONTENTS

	4.5.1 Determining the concentration of Sperm with a Hemocytometer	14
	4.6 Protein sample isolation:	14
	4.6.1 Lowery's Method	14
	4.7 SDS-PAGE:	15
	4.8 Immunoblotting	22
	4.9 Histopathology	23
5	RESULTS AND DISCUSSION	26
6	SUMMARY AND CONCLUSION	30
7	REFERENCES	31

Abbreviations

L15	Leibovitz medium
1X PBS	1X Phosphate Buffered Saline
APS	Ammonium per sulphate
TEMED	Tetramethylethylenediamine
NaOH	Sodium Hydroxide
SDS - PAGE	Sodium dodecyl sulfate and polyacrylamide gel electrophoresis
1X TBST	1X Tris Buffered saline with Tween 20
BSA	Bovine serum albumin
COX2	Cyclooxygenase 2
BCIP/NBT	Nitro-blue tetrazolium and 5-bromo-4-chloro- 3'indolyphosphate
β -ME	Beta Mercapto Ethanol
ECL	Enhanced Chemiluminescence
IgG	Immunoglobulin G

RPM	Revolutions Per Minute
ALP	Alkaline Phosphatase
IPA	Iso-Propyl Alcohol
DPX	Distyrene Plasticizer Xylene+
SCN	Stem Cell Niche
RFP	Red Fluorescent Protein
PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
Oct-4	Octamer-binding transcription factor 4
PSCA	Prostate Stem Cellular Antigen
pH	Power of Hydrogen
GFP	Green Fluorescent Protein

LIST OF FIGURES

FIGURE NO.	FIGURE TITLE	PAGE NO.
Figure 4.1	schematic illustration of <i>ex-vivo</i> maintenance of earthworm testis	11
Figure 5.1	Adult earthworm <i>Eudrilus eugeniae</i>	25
Figure 5.2	Morphology of earthworm <i>E. eugeniae</i>	26
Figure 5.3	Sperm viability calculation (% motility)	27
Figure 5.4	Histopathological analysis	28
Figure 5.5	Western blotting analysis	30

CHAPTER – 1

1. OVERVIEW

1.1 INTRODUCTION

Male infertility refers a male's inability to result in pregnancy in a fertile female. "Male factor" infertility is seen to as an alteration in sperm concentration, motility, or morphology in a minimum of one of two sperm analyses collected 1 and 4 weeks apart (Kumar N, Singh AK., 2015). The incidence of male infertility is increasing in recent decades due to increasing account of pesticides, pharmaceutical drugs, chemotherapy, radiotherapy, life style changes, obesity which are directly affecting human reproductive systems (Stukenborg JB, et al., 2021). For male reproductive toxicology studies, animal experiments are more often used, which necessitate long-term research experiments and large quantities of compounds. A clinical study is a scientific investigation in which human volunteers receive a medical intervention or are observed under controlled conditions, which is extremely expensive. Clinical studies may involve the testing of new drugs, vaccines, and medical devices that are undergoing FDA approval. Following FDA approval, it undergoes to post-marketing surveillance. After a few years, the FDA withdrawn drugs or medicines from commercial markets due to patient safety concerns, but also for commercial reasons (e.g., a lack of demand and relatively high production costs), resulting in a massive loss. As therefore, initial per-screening is improving to describe activities prior to obtaining informed consent (i.e., prior to enrollment) to determine initial eligibility for and interest in a study. The present animal model have a ethical issue , costly and variation in results .The *ex-vivo* model is the better alternative, but the current *ex-vivo* testicular model is unsuccessful due to a lack of long days, fully functional organs, seminiferous tubules, part of the testis, and so on. On the other hand, an *ex-vivo* model system takes less time to implement any evaluation techniques, is effective, aids in achieving reproducible results, and thus beneficial for future clinical studies. There are several *ex-vivo* testis models available from mice, humans, and hamsters, but none of them are raised from a fully functional entire organ testis, making maintaining an intact whole testis gland difficult. The earthworm is inexpensive, easily accessible, similar to higher animals and can be maintained easily. The earthworm, *E. eugeniae* has two pairs of testes in the pre-clitellum segment (one pair in each 10 and 11 segments) and which can be utilized for male reproductive toxicity studies by maintaining in *ex-vivo* conditions (Selo et al. 2021). To evaluate male reproductive toxicity, we

used an earthworm testis model and maintained it in an *ex-vivo* environment in accordance with the 3R rule (Replacement, Reduction, and Refinement).

1.2 Earthworm *Eudrilus eugeniae*

The earthworm *Eudrilus eugeniae* belongs to the phylum Annelida, class Clitellata, and family Eudrilidae. The worm is commonly referred to as the "African nightcrawler." The worm is an excellent model for cell and molecular biological experiments (Paul et al. 2021). Due to its amazing regeneration ability, the worm can be used to study the complex cell and molecular biological events of regeneration. *E. eugeniae* has several advantages as an experimental model for regenerative biology (Banik and Chaudhuri 2017). It is inexpensive and simple to keep the earthworm in the laboratory, whereas rearing fish, mice, and other animals is relatively expensive; 2. It has a faster growth rate, taking as little as 5 weeks to mature; 3. It attains 12mg body weight per day; 4. It reproduces rapidly; 5. It can tolerate temperature differences ranging from 15 to 30°C; because it has many well-developed organs such as the ovary, testis, seminal vesicle, prostate gland, and so on, it is possible to design regenerative biological studies of different organs (Chelladurai et al. 2021). Hence, the model outperforms both hydra and planarians (Reddy et al. 2019). They lack the well-developed organs that the worm does. *E. eugeniae* is a cost-effective model system for cell and molecular biological studies of regeneration and stem cell biology. The cellulite-dependent earthworm requires intact clitellum segments to survive and regenerate (Paul et al. 2022). It is a good source of protein and has a lot of pharmaceutical potential. This earthworm is endemic to tropical West Africa, but it is now common in warm areas due to vermicomposting. An adult worm has between 80 and 100 segments (Banik and Chaudhuri 2017). The following are the numbers and positions of all reproductive structures (Reinecke and Pieters, 1997): A pair of spermathecae and a pair of spermathecae aperture are present in 7th and 9th segments. Segment 10 consists of two testes and two sperm funnels and Segment 11 consists of two testes, two seminal vesicles, and two sperm funnels.

The vital organs of *E. eugeniae* are the mouth, simple brain, two pairs of hearts, testis, seminal vesicles, rudimentary ovary, oviduct, and auxiliary ovarian glands which are present in the first 13 segments of the earthworm (Sivasubramaniam 2021). The 13th to 18th segments contains the clitellum, a thick, cylindrical structure that resembles a collar and is crucial to reproduction. A pair

of prostate glands, the gut, and the anus are among the essential organs located in the posterior portion. The coelomic fluid, which also contains several cell types known as coelomic cells, contains all the internal organs (Yesudhason et al. 2018).

1.3 Male reproductive system of earthworm (Testis model)

The male reproductive system consists of up of testes, testis sacs, seminal vesicles, spermatric funnel, prostate glands, and other accessory glands.

Testis:

- a) Two pairs are present on the 10th and 11th segments.
- b) Each testis is made up of 4 to 8 finger-like projections.
- c) The testes contain spermatogonia cells in the lobules.
- d) They create sperm.

The earthworm, *Eudrilus eugeniae* has two pairs of testes in the pre-clitellum segment (one pair in each 10 and 11 segments) and which can be utilized for male reproductive toxicity studies by maintaining in *Ex-vivo* conditions. Clitellum plays a major role in earthworm reproduction and which is associated with specialized gland cells which contains mucous cells, albumin cells and cocoon secreting cells (Bhattacharjee and Chaudhuri 2002). Also, it is closely associated with male and female reproductive glands. Earthworm is hermaphrodite or monocious animals. Each pair is enclosed in the testis sac of that segment. They rise from the inner surface of the anterior wall of the testis sac.

1.4 Testicular toxicity studies

In recent years, the Food and Drug Administration (FDA) has withdrawn hundreds of drugs from the market due to male reproductive toxicity (Wesley et al. 2021). Male reproductive toxicity accessing Sperm movement, Sperm viability, Sperm tail break, Sperm head etc. Animal experiments tend to be utilised for male reproductive toxicology studies, which require long-term research experiments and large quantities of compounds (Working 1988). *Ex-vivo* model system, on the contrary hand, requires less time to implement any evaluation techniques, is effective, aids in achieving reproducible results, and thus beneficial for future clinical studies (Selo et al. 2021). *Ex-vivo* testis models from mice, humans, and hamsters are available, but none of them are raised from a fully functional entire organ testis, making maintaining an intact whole testis gland

is challenging. We successfully maintained the earthworm testis in *Ex-vivo* condition for a longer period of time (more than 2 weeks) either independently or in conjunction with tissues associated with testis (Iavicoli et al. 2013). The earthworm, *Eudrilus eugeniae* has two pairs of testes in the pre-clitellum segment (one pair in each 10 and 11 segments) and which are enclosed in testicular sacs. *Ex-vivo* whole organ testicular culture should be established by standardizing dissection techniques (intact dissection), medium conditions, and determining age factors of dissected testis obtained from the earthworm, *Eudrilus eugeniae* (Kalidas et al. 2015; Paul et al. 2021, 2022). To ascertain testicular toxicity *Ex-vivo* maintained earthworm testis gland using standard toxicants like testosterone, pramipexole dihydrochloride drug, and exposure to standard pharmacological drugs that cause male reproductive toxicity (FDA withdrawal drugs) (Sharpe 2001). Using histopathology, sperm analysis (number, shape, motility), microscopic variations, and standard molecular level markers such as wnt3a (marker for spermatogenesis, Spermatogonial stem cells), COX2 (marker for oxidative stress), H2AX (DNA damage response), and OCT4 (pluripotency regulator expression and protects embryonic stem cells in an undifferentiated state) to evaluate testicular toxicology. We also discovered that adding supporting supplements such as forskolin, and valproic acid (which converts pluripotent stem cells into spermatogonial stem cell-like cells) to testicular toxicology evaluation.

1.4.1 Testosterone

Males primary sex hormone and anabolic steroid is testosterone. In humans, testosterone plays an important role in the development of male reproductive tissues such as the testes and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass and the growth of body hair, as well as psychological characteristics in males such as increased aggression, sex drive, the desire to impress partners, and other courting behaviours.

1.4.2 Forskolin

Forskolin is a labdane diterpene derived from the plant *Coleus barbatulus*. Other names for this plant include pashanabhedhi, Indian coleus, makandi, HL-362, and mao hou qiao rui hua.

Forskolin, like many other members of the large diterpene class of plant metabolites, is derived from geranylgeranyl pyrophosphate. Forskolin is a root extract from the Indian plant *Coleus forskohlii*. It has been used for centuries in Ayurvedic medicine to treat diseases such as underactive thyroid, heart disease, and respiratory disorders.

1.4.3 Valproic acid

Valproic acid (VPA) is an anticonvulsant and mood stabiliser 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 sperm production and motility (Biermann et al. 2010).

1.4.4 Pramipexole dihydrochloride drug

Pramipexole dihydrochloride drug is used alone or in combination with other medications to treat the symptoms of Parkinson's disease (PD; a nervous system disorder that causes difficulties with movement, muscle control, and balance), such as shaking of parts of the body, stiffness, slowed movements, and balance problems (Selo et al. 2021).

CHAPTER - 2

2. REVIEW OF LITERATURE

2.1 *Ex-vivo* organ culture

Ex-vivo organ cultures are unique research models because they combine advantages of cell cultures and animal models. Being able to mimic *in vivo* situations using organ cultures provides an excellent opportunity to study cellular processes, molecular pathways, cell-cell interactions, structural and synaptic organization. Human and animal organ cultures are now well established, with sensitive, easy-to-manipulate experimental systems that raise barely any ethical concerns (Maliha et al. 2019).

Gassei et al.,(2014) maintained the testis model in *ex-vivo* conditions. The researchers isolated spermatogonial stem cells (SSCs) from rat testes and cultured them *ex - vivo* in a medium known as SSCM. The SSCs were cultured in SSCM for several weeks before forming the SC1 cell line, which exhibited characteristics of immature germ cells (Gassei et al. 2014).

Marcelo C. Leal et al. (2009) developed zebra fish primary testis tissue culture as a method for studying testis function *ex-vivo*. The study illustrated zebra fish testis tissue was developed in a medium-term *ex-vivo* organ culture system in order to create new tools for studying the regulation of testis physiology in teleost fish. It is observed that according to morphological, molecular, and immunohistochemical analyses, adding 100 nM 11-ketotestosterone to the system supported complete spermatogenesis. It was analyzed that acute androgen release from recently removed tissue was stimulated by forskolin (0.5 μ M), and the steroidogenic system's partial down-regulation was prevented. It assesses the effects of a wide range of substances on the two main functions of the testis, spermatogenesis and hormone production, in the current *ex-vivo* culture system (Leal et al. 2009).

Keith M. Goldstein et al. (2016) analyzed about use of a rat *ex-vivo* testis culture method to assess toxicity of select known male reproductive toxicants. It conducted a pilot study to assess the utility of the Durand *ex-vivo* rat seminiferous tubule culture model, which supports spermatogenesis through meiosis II, including the formation of round spermatids (Sutherland et al. 2016).

2.2 Testis

Richard M. Sharpe *et al.* (2001) demonstrate hormone and testis development, as well as the potential negative effects of environmental chemicals. This evidence for deteriorating human male reproductive health, particularly an increase in testicular cancer, points to disturbed (hormonal) foetal testis development. The similarities in outcomes and testicular cell-cell disruption between human testicular dysgenesis and foetal rat exposure to certain phthalates are highlighted, as are the pathways through which oestrogenic and (especially) anti-androgenic environmental chemicals may act to induce such changes (Sharpe 2001).

R.I. McLachlan *et al.* (2006) demonstrated that testicular biopsy is a critical assessment in reproductive practise, with diagnostic and prognostic significance for assisted reproductive technologies (ARTs) and the risk of testicular neoplasia. This study evaluate a large consecutive series of bilateral biopsies from infertile men and report (i) the frequency of bilateral or discordant patterns that supports the use of bilateral biopsy for comprehensive evaluation and (ii) a high prevalence of mixed patterns, particularly within the hypospermatogenesis classification, that helps account for reported TESE success. It propose a new diagnosis code for testicular biopsies that addresses the needs of ART clinicians while also allowing for data storage and retrieval of value in clinical practise and research (McLachlan and Quispel 2006).

2.3 Testosterone

Zhang *et al.* (2011) demonstrated testosterone maintenance in an *ex-vivo* testis model. The researchers cultured human foetal testis tissues *ex-vivo* and investigated the effects of testosterone and gonadotrophin stimulation on germ cells and somatic cells in this study. They discovered that testosterone supplementation could keep testosterone levels in the tissue stable for up to 7 days, which is critical for germ cell development and maturation (Zhang et al. 2011).

2.4 Valproic acid

Wannisa Sukhorum et l. (2016) analyzed about Changes in testicular function proteins and sperm acrosome status in rats treated with valproic acid. The study investigate the testicular proteins involved in testosterone synthesis and spermatogenesis, histopathology and sperm acrosome status in VPA-treated rats. They discovered that VPA treatment changes the expression of testicular

proteins responsible for spermatogenesis and testosterone production, resulting in male infertility (Sukhorum and Iamsaard 2017)

2.2 Pramipexole dihydrochloride drug

Zulfqar et al. 2020 analyzed about effect of Venlafaxine, Pramipexole, and Valsartan on Spermatogenesis in male rats. It was hypothesized that these drugs may affect the male fertility because of their long-term use in treatment of depression, hypertension, and Parkinson's diseases. Male rats were given venlafaxine, valsartan, and pramipexole at low- and high-dose levels orally once daily for 10 weeks. Results showed nonsignificant effect of venlafaxine on the sperm count, whereas a decreased sperm count was noted in all the treatment groups as compared to that of the control except valsartan at a low dose (Zulfqar et al. 2020).

CHAPTER - 3

AIM AND OBJECTIVE

3.1 AIM:

To maintain the earthworm testis model in *ex-vivo* condition and accessing male reproductive toxicity

3.2 OBJECTIVE:

- To establish functional earthworm, *Ex-vivo* whole organ testicular culture by standardizing dissection techniques (intact dissection) and medium conditions.
- Functional maintenance of the earthworm testis in *Ex-vivo* condition for a longer time duration (for 3 weeks) by supplemented with testosterone, valproic acid and froskolin.
- To determine testicular toxicity *Ex-vivo* maintained earthworm testis gland using standard toxicant pramipexole dihydrochloride (suspected FDA withdrawal drugs).
- Evaluating testicular toxicology and analyzing adverse effect of a toxicant or drugs using histopathology, sperm analysis (number, shape, motility), and by using standard molecular level markers like COX2 (marker for oxidative stress), H2AX (DNA damage response protein) and H3 (proliferation marker) and OCT4 (pluripotency regulator).

CHAPTER - 4

MATERIALS AND METHODS

4.1 Earthworm culture and maintenance:

Eudrilus eugeniae earthworms were obtained from SK Farms and Organics in Kongananchery village, Chengalpattu. Sexually matured *E. eugeniae* specimens were obtained from the Regeneration and Stem cell Biology Lab at the International Research Centre of Sathyabama Institute of Science and Technology. The worms were kept in a plastic tub filled with cow dung, organic waste, soil, and leaf litter. A weekly thrice feed of equal parts cow dung and jaggery was provided. The temperature in the incubation room was kept between 22-26°C (Chelladurai et al. 2020)

4.2 Dissection of Earthworm testis model:

Adult species of *E. eugeniae* were selected for dissection of the testis model. Two pairs of testis will be positioned in the earthworm's 10th and 11th segments. A sterile scalpel blade was used to dissect the testis in the ventral side of a worm. The cut was initially made along the worm's axis on the ventral side with a sterile scalpel. The testis is a minute white body close to the middle line of the testis sac that was carefully dissected out using the scalpel and fine forceps without disturbing the testis between the 10th and 11th segments. After dissection, the testis was washed thoroughly with ice-cold distilled water 1X PBS for 4 to 5 minutes to remove all the dust and dirt. (From this, every step should be done in an ice-cold condition).

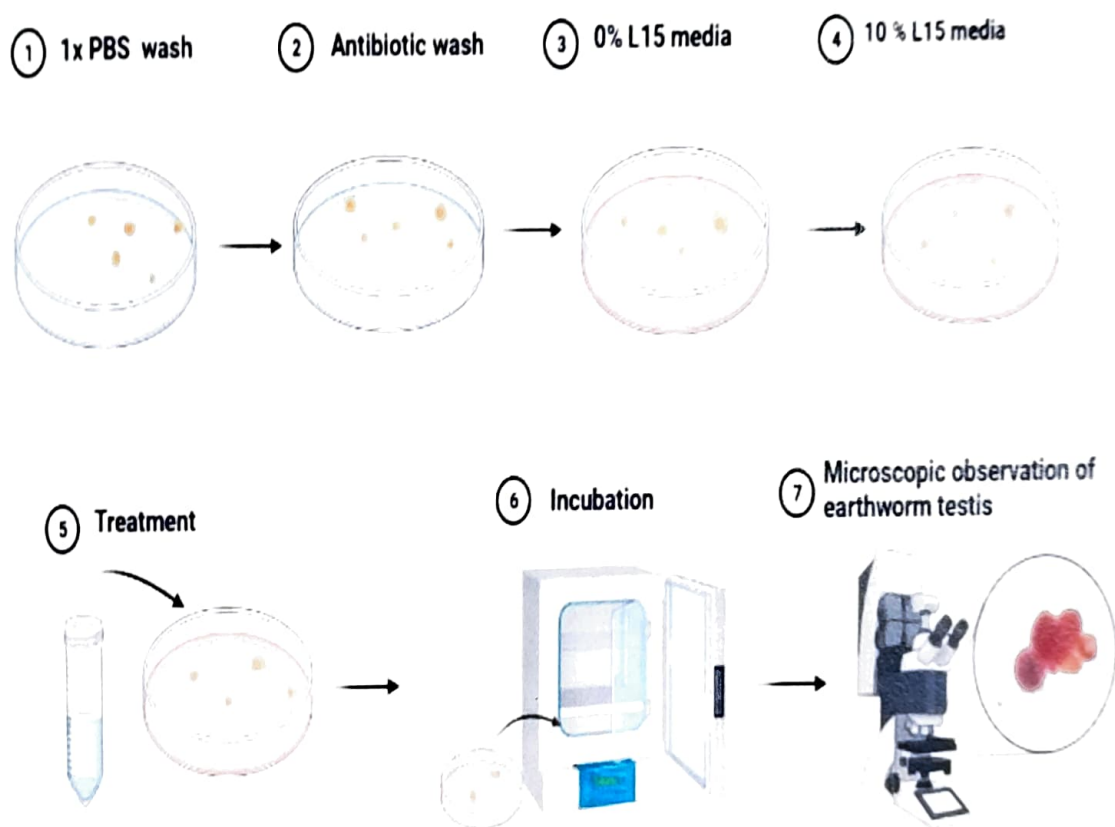
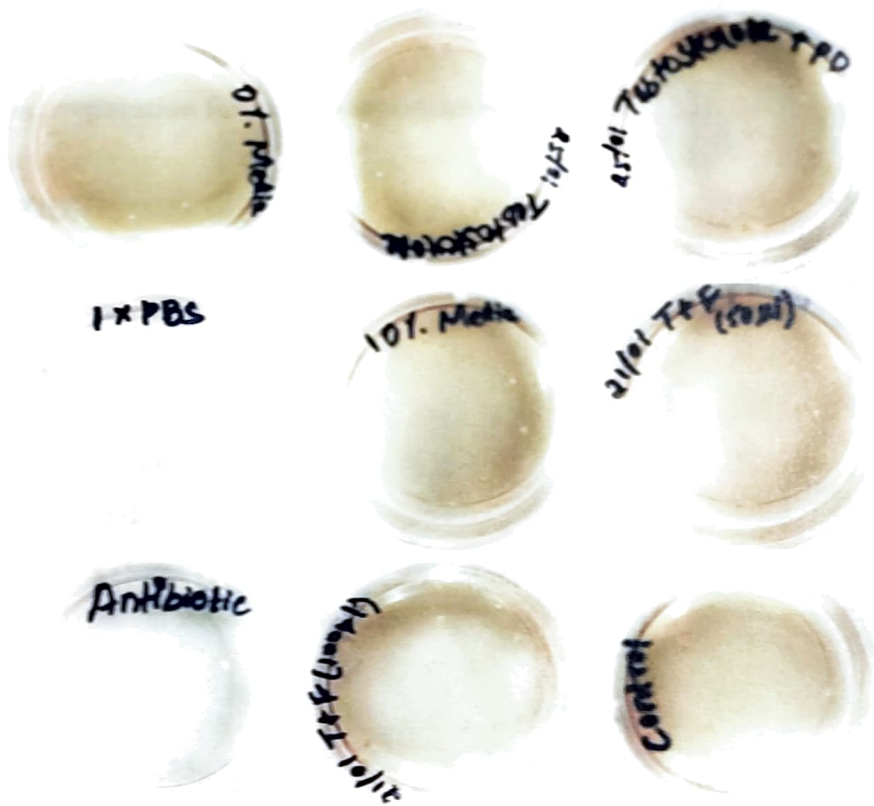


Fig 5.3 schematic illustration of *ex-vivo* maintenance of earthworm testis



Ex-vivo processing and maintenance of earthworm testis.

4.3 L15 media preparation for *ex-vivo* maintenance:

STERILIZATION:

- i. 121°C for 30 minutes.
- ii. Ethanol wash.

MEDIA PREPARATION

- i. L15 powder– 17.3g /L.
- ii. Streptomycin – 1g /L.
- iii. FBS (10%) – 100ml in 900ml medium.
- iv. Penicillin -1g/L.
- v. Amphotericin B – 1ml/L
- vi. Sodium carbonate – 3.75g/L

PROCEDURE:

- i. 1ml milli-Q water in screw cap bottle and sterilize it.
- ii. After sterilization keep it at room temperature for overnight.
- iii. Add 17.3g of L15 media.
- iv. Mix it well until soluble.
- v. Add streptomycin 1g, penicillin 1g, amphotericin- B1ml and add 3.75g of sodium carbonate. vi.
- pH should be adjusted to 7.2 to 7.4.
- vii. Filter with vacuum filtration unit.

4.4 Processing of Earthworm testis model for *Ex-vivo* maintenance:

The testis gland was washed twice in 4 to 5 minutes with ice-cold 1X PBS. Then the testis was transferred to a fresh Petri plate containing filtered ice-cold 1X PBS and washed for 5 to 7 minutes. (From this, all the processing steps should be done under sterile conditions). After washing, 1X PBS was removed, and an antibiotic solution (Streptomycin (1 µg/1 ml), Amphotericin B (1 µg/1 ml), Penicillin (1 µg/1 ml) were added and incubated for 7 to 10 minutes. Following incubation, the testis gland were transferred to a new plate containing serum-free L15 media and allowed to fix for 7 to 10 minutes. Finally, the testis was transferred to a new plate containing filtered 10% L15 serum media (10% FBS + L15 medium).

4.5 Chemical treatment

For ascertain testicular toxicity *ex-vivo* maintained earthworm testis gland using standard toxicants like testosterone, pramipexole dihydrochloride drug, and exposure to standard pharmacological drugs that cause male reproductive toxicity which was suspected by FDA. We also discovered that supplementing testicular toxicology with forskolin and valproic acid (which converts pluripotent stem cells into spermatogonial stem cell-like cells) improved the results. In comparison to the control (maintain in L15 media in an *ex-vivo* condition), chemically treated *ex-vivo* earthworm testis can be kept for a longer period of time (more than 2 weeks) in isolation or in conjunction with tissues associated with the testis. To determine testicular toxicology and analyse the adverse effects of a toxicant or drugs on the testis gland, sperm viability studies(number, shape, motility) performed with a hemocytometer. Before the sperm count can be calculated in hemocytometer, the testis gland must be squeezed in 1X PBS using a sterile needle.

4.5.1 Determining the Concentration of Sperm with a Hemocytometer

1. A hemocytometer is a device that counts the number of cells or particles in a sample. It is made of thick glass with two rails on each side. The rails are intended to support a coverslip that is 0.1 mm above a mirrored surface in the centre. A grid has been etched into this surface. Before you begin, clean the hemocytometer and the coverslip with a little water or alcohol, then dry with a kimwipe.
2. Next, we'll set up an incubation chamber (large petri dish with hemocytometer supports and wet paper in the bottom).
3. Dilute sperm with water. The objective is to kill sperm and dilute them sufficiently so that you can count 100-400 sperm cells in the 4 squares described below. The exact dilution must vary depending on the species and concentration of the ejaculate.
4. Place a coverslip on the hemocytometer after wetting supports (rails) with saliva or water. This helps to hold down the coverslip while loading the sperm
5. Place 10-15 μ l of the diluted sperm under the cover slip on each side of the hemocytometer.
6. Carefully place the hemocytometer in the prewetted chamber, close the lid and wait 5 minutes.
7. The hemocytometer should be removed without tilting, dried on the bottom if necessary, and set on a microscope. View the hemocytometer with a 40 X objective
8. Count the sperm in 4 of the squares as shown in the following images. Be sure you count 5 squares on each side of the hemocytometer and then use the average for your calculations.

4.6 Protein sample isolation:

Beta-Mercaptoethanol (β -ME-reducing agent) used for breaking of disulphide bonds of protein. 2X sample buffer were brought to room temperature before use. After that, 2X sample buffer were added with 5% of Beta-Mercaptoethanol (reducing agent) (stock 1). Samples were transferred into the fresh 1.5ml vial which has an equal amount of sample buffer at 4°C. Homogenizer was used for homogenization which was done manually by hands. After that samples were transferred into the dry bath at 100°C for 5 minutes, then centrifuge the samples at 10000 rpm for 5 minutes and supernatant was transferred to the fresh 1.5ml tube and stored in -20°C.

4.6.1 Lowry's Method

Before starting the experiment, prepare BSA stock solution along with Reagents A, B and C.

Table 4.3 Reagent preparation for Lowry's method

Reagents	Chemicals required	Quantity
BSA stock solution	Bovine Serum Albumin (BSA)	5 mg
	Distilled water	1 ml
Reagent A	Sodium hydroxide	25 mg
	Sodium bicarbonate (NaHCO_3)	50 mg
	Distilled water	5 ml
Reagent B	copper sulphate (CuSO_4)	25 mg
	Sodium potassium tartrate ($\text{C}_4\text{H}_4\text{O}_6\text{KNa}\cdot 4\text{H}_2\text{O}$)	50 mg
	Distilled water	5 ml
Reagent C	Reagent A	4 ml
	Reagent B	1 ml

Take a 96-well plate when reagent preparation is complete. Depending on the amount of samples, we will need one well for the blank, seven for the standards, and maybe more. Put 100 μl of distilled water into each well. The 7 standard wells should each have 5, 10, 15, 20, 25, 30, and 35 μl of distilled water removed from them respectively. 2 μl of distilled water should be taken out of the sample wells. Except for the blank and sample wells, add BSA solution to every well. Replace the distilled water withdrawn with BSA solution for the standard wells. Add 2 μl of the sample to the sample wells. Then add 100 μl Reagent C in all the wells and incubate for 10 minutes. Post incubation, add 10 μl of Folin-Ciocalteu reagent to all the wells. Make sure that dark conditions are maintained while adding Folin's reagent. Now keep this well plate on the shaker,

still in dark conditions for 30 minutes. A graph is plotted with the sample values in correlation with the standard values. These sample values can then further be utilized for SDS-PAGE and Western Blotting.

4.7 SDS-PAGE:

4.7.1. Principle:

SDS PAGE is an analytical technique which is used to separate the proteins based on their molecular weight. Electrophoresis is the study of the movement of charge molecules in an electric field. Their rate of movement depends on the strength of the field, net charge, size and shape of the molecules and also the ionic strength, viscosity and temperature of the medium in which the molecules are moving. Polyacrylamide gels are widely used for separating larger molecules like proteins. Based on the resistance of the gel matrix proteins are separated viz., smaller proteins generally have less resistance as well as larger proteins have more resistance. The molecular weight of protein was estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulfate (SDS) and a reducing agent β -Mercaptoethanol, it can bind with the protein backbone and denaturing effect of SDS is very strong and that disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol assists the protein denaturation by reducing all disulfide bonds (both inter-disulfide and intra-disulfide bonds). Polyacrylamide creates a mesh like matrix that helps in the separation of protein. During PAGE electrophoresis linear or primary structure of the proteins were stacked or arranged 14 in the proper way, once reached the separating gel, proteins started to separate based on their size.

4.7.2. Materials:

Eppendorf's; Micro-pipettes; Micro-tips; Centrifuge; Water bath; Vortex and Distilled water.

4.7.3. Chemical preparation of SDS-PAGE:

30% Acrylamide and 0.3% Bis acrylamide:

Acrylamide - 30g

N, N' Methylene Bisacrylamide - 0.3g

Make up into 100ml with distilled water.

4X Separating Buffer:

Tris HCL - 18.2g

Distilled water - 60ml

pH was adjusted to 8.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4g

Make up into 100ml with distilled water.

4X Stacking Buffer:

Tris Hcl - 6.05g

Distilled water - 40ml

pH was adjusted to 6.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4g

Make up into 100ml with distilled water.

2X Sample Buffer:

Tris stacking buffer (pH 6.8) - 25ml

Sodium Dodecyl Sulphate (SDS) - 4g

20% Glycerol - 20ml

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 - 100mg

Make up into 1ml with distilled water.

1X Running Buffer:

Tris Hcl - 3.94g

Glycine - 15.014g

SDS - 1g

Make up into 1L with distilled water.

Staining Solution:

Commassie Brilliant Blue R-250 - 200mg

Methanol - 50ml

Glacial Acetic acid - 7ml

Make up into 100ml with distilled water.

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

Destaining Solution:

Methanol - 30ml

Glacial Acetic acid - 7ml

Make up into 100ml with distilled water.

Table 4.1: Separating gel concentration

Sl. No	Stock Solution	5%	6%	7%	7.5%	8%	9%	10%	12%	13%	15%
1.	30% Acrylamide + 0.3% Bis-acrylamide (ml)	5.0	6.0	7.0	7.5	8.0	9.0	10.0	12.0	13.0	15.0

2.	4X Tris Separating Buffer (ml) pH 8.8	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
3.	Distilled water (ml)	17.5	16.5	15.5	15.0	14.5	13.5	12.5	10.5	9.5	7.5
4.	10% APS (μ l)	200	200	200	200	200	200	200	200	200	200

Table 4.2: *Stacking 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.		1.25	2.5	3.75	5.0

	4X Tris Stacking Buffer (ml) pH 6.8				
3.	Distilled water (ml)	3.05	5.0	7.5	10.0
4.	10% APS (μ l)	100	100	100	100
5.	TEMED (μ l)	10	10	10	10

4.7.4. Preparation of gel:

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

the bottom of the spacer were carefully removed and setup was fixed in the bottom chamber which carried 1X running buffer as well as upper chamber.

4.7.5. Sample loading and electrophoresis:

Protein sample of 60µg and the appropriate molecular markers were loaded in the wells. The apparatus was connected to the power pack with proper polarity and 5 to 6v/cm was applied. After electrophoresis, the glass plate assembly was taken from the buffer chamber. The spacer was gently twisted so that the upper glass plate pulled away from the gel.

4.7.6. Staining and Destaining the gel:

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

4.8 Immunoblotting

4.8.1. Buffer preparation

10X Transfer Buffer (Stock)

Glycine - 72.1g

Tris-base - 15.2g

Distiller water- 500ml

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

For Total volume 1 litre

100ml - 10X Transfer Buffer

200ml - Methanol

700ml - Distilled water

4.8.2. Running the gel:

Prepare 12% SDS gel and place it in SDS – PAGE setup. Pour 1X running buffer (100ml of 10X running buffer + 900ml of distilled water) into the setup. Protein ladder of 5 μ l is loaded at the first lane of the gel for the molecular weight determination. In other wells, the prepared protein samples were added (60 μ g/well) concentration. After loading all the samples, the setup was started to run at a 60V unit. The samples were let to run till it reaches the bottom-line.

4.8.3 Membrane transfer:

After the protein runs fully till the end, take off the glass slides from the setup and cut off the stacking gel. Then gently take the gel and keep it in the transfer butter for 5 minutes and simultaneously, cut the PVDF membrane by the same gel size. Soak the PVDF membrane in the methanol for 1 – 2 minute for activation of the membrane. Then, keep the membrane in 1X transfer butter until preparing the sandwich for blotting. Prepare blotting apparatus, sponge, and filter paper to arrange the sandwich setup and soak it in 1 X transfer butter before using.

4.8.4. Blocking

Blocking buffer was used to block all proteins which would bind with targetable and nontargetable proteins. 5% of Bovine serum albumin (BSA) with 20ml of 1X TBST buffer was prepared for Blocking and let it for 2 hours.

4.8.5. Primary Antibody

Primary antibodies COX2 (produced from Rabbit polyclonal Abcam, ab179800) and Anti β – actin (produced from mouse polyclonal Abcam, ab8226) was added in the 5% Bovine serum albumin (BSA) (3:5000 dilutions) and incubated overnight in cold conditions.

4.8.6. Secondary Antibody

Secondary antibody (Anti Rabbit IgG (Produced in goat) Abcam, AF568 – ab175471) was added in the 5% Bovine serum albumin (BSA) (1:1000 dilutions) and incubated for 2 hours at room

temperature. It could only bind with primary antibody COX2. (NOTE: starting from adding a secondary antibody, every step should be done in the dark condition as these antibodies are light sensitive and if subjected to light causes denaturation).

4.8.7 Gel imaging

Gel imaging has been carried out using the sophisticated BioRad ChemiDoc™ XRS+ imaging system for faster and more accurate western blot quantification. only stain-free enabled instrument capable of chemiluminescent, multiplex fluorescent, and routine gel imaging and it is used to quantify total protein for normalization purposes after transfer to the membrane. After the incubation with a secondary antibody for 2 hours has been completed, the PVDF membrane is washed with 1X PBST for every 5 minutes for 5 times. Then, ECL substrate solution was prepared by adding equal amount of ECL solution A and ECL solution B mixing together in (1:1 Ratio), after the secondary antibody process the blot was washed more than 5 times in the same box and transfer them into the new one with few washes to avoid patchy spots on the blot. Then, turn on the BioRad ChemiDoc™, and connect to the PC. The membrane is kept inside in the correct position after wiping the place provided for placing the membrane with tissue paper. Add the prepared ECL substrate over the membrane in drops / spraying covering all the areas of membrane.

4.9 Histopathology

4.8.1. Materials:

Tissue, screw cap bottle, clean albumenized glass slides, coupling jars, cover slip, forceps, formaldehyde, isopropyl alcohol, xylene, paraffin wax, haematoxylin and acidic dye eosin .

4.8.2. Reagent preparation:

50% Formaldehyde (Fixation)

Add 50ml of formaldehyde in 50ml of distilled water.

Isopropyl alcohol (Dehydration)

60% Isopropyl alcohol- 60ml of Isopropyl alcohol + 40ml of distilled water.

70% Isopropyl alcohol- 70ml of Isopropyl alcohol + 30ml of distilled water.

80% Isopropyl alcohol- 80ml of Isopropyl alcohol + 20ml of distilled water.

90% Isopropyl alcohol- 90ml of Isopropyl alcohol + 10ml of distilled water.

100% Isopropyl alcohol- 100ml of Isopropyl alcohol.

Wax preparation:

For preparation of wax, the melting temperature of wax should be between 58 – 60°C.

Albumin coat:

Egg white + equal amount of glycerol.

4.8.3. Tissue processing for histology:

After the gland was maintained in *ex-vivo* condition, the samples were stored in 10% formalin. Then after 1 day, the samples were washed with distilled water. Then for fixation, the samples were fixed with 50% formaldehyde solution for 24 hours at 50°C. Then wash the tissue with distilled water. After washing, the tissues were transferred into 60% isopropyl alcohol at 50°C for 1 hour. Again transfer the tissues into 70% isopropyl alcohol at 50°C for 1 hour. Then at 80% isopropyl alcohol at 50 °C for 1 hour, 90% isopropyl alcohol at 50°C for 1 hour, 100% isopropyl alcohol at 50°C for 1 hour and finally repeat again in 100% isopropyl alcohol at 50°C for 1 hour. Then, transfer the tissue into a cleaning solution, xylene for 30 – 45 minutes. Prepare wax-1, wax-2, wax-3. The tissues were immigrated in wax-1 overnight (Temperature supposed to be maintained in 58-60°C). After incubation tissues rottenly transferred to the second wax for 3 hours and after that tissues were placed on the final wax for 3 hours. Then the tissues were transferred into the boxes for sectioning by preparing the blocks by melting the paraffin wax and keeping the sample in horizontal position. After the preparation of wax blocks, the sectioning of the tissues can be done by using a microtome which is of 7-micron size. The 7µm sections were made and the ribbon model tissue with wax is kept in hot water for 5 seconds at 60°C. Then take off the tissue by albumin coated slide, and keep the slide in the slide warmer for 10 minutes to remove the water.

4.9.4. Tissue staining for histology (H & F staining):

First immerse the slides in xylene - 1 and 2 for 5 minutes to remove the water.

The slide should then be immersed in 100% isopropyl alcohol for three minutes to remove xylene -1 and 2. After that, wash the slide for 5 minutes under running water. The slide is then stained

with haematoxylin for 7 to 10 minutes. For about 10 minutes, wash the slide with tap water. The slides containing the tissue sections should then be washed with acid solution (99ml 70% isopropyl alcohol + 1ml distilled water). The slide should then be left in blooming tap water overnight. The slide should then be dipped in eosin stain for 30 seconds before being rinsed with tap water for a few seconds. The slide is then decolourize by immersing it in 100% isopropyl alcohol for 5 - 10 seconds prior then repeat xylene wash step. Finally, DPX mount (a mixture of distyrene, plasticizer, and xylene) should be used to mount the tissues on the slides.

4.10 Statistical analysis of *ex-vivo* maintained of earthworm testis gland :

To achieve statistical significance, all survival, testicular toxicity, and adverse effect of a toxicant or drug analyses were repeated three times. ANOVA was used to examine how the variance in the various groups varied. With the help of the statistical programme SPSS (version 22.0; IBM Corp., USA), the obtained data were examined, and the results were presented as mean SD. The p-value of 0.05 was used to determine whether the endpoints were statistically significant. With the aid of Image J, statistical data were examined. The band's length and width were measured and compared, and the results indicate that testicular toxicity. Measuring, normalizing, and manually calculating the lengths and widths. Microsoft Excel 2016 plotted each value on a bar graph.

CHAPTER - 5

RESULTS AND DISCUSSION

5.1 Morphology of earthworm, *E. eugeniae*

E. eugeniae grows quickly and is more productive than other earthworm species, and its large size makes it easier to handle and harvest (Dominguez et al., 2001). Sexually matured adult worms (Fig. 5.1A) were chosen and collected for this experiment from the Regeneration and Stem cell biology Lab, International Research Centre of Sathyabama Institute of Science and Technology. There are two sets of testicles. A pair is located on the 10th segment, and another pair is located on the 11th segment. These are located ventrally, below the intestine. Each pair is contained within that segment's testis sac. They come from the interior of the anterior wall of the corresponding testis sac. Testes are tiny digitate bodies with 4 to 8 digitate processes and a compact base. They come from the parietal coelomic epithelium. They produce spermatogonia, or sperm mother cells

5.2 Dissection and isolation of earthworm testis glands:

The worms were dissected (Fig. 5.2A), and the testis glands were isolated from the worms, which will be located in the 10th and 11th segments. Because the testis is soft tissue, sensible handling is required to avoid disturbing the testis glands (Fig. 5.2B). After isolation, the glands were washed to remove dust, blood, and nerve cords before being processed further.

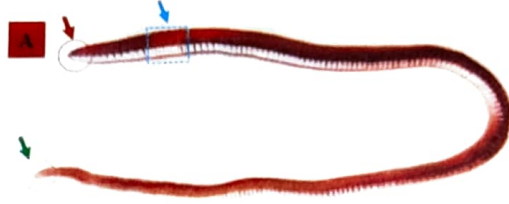


Fig 5.1 A. Adult earthworm, *E. eugeniae* B . Testosterone C. Forskolin D.Valproic acid F. Pramipexole dihydrochloride drug

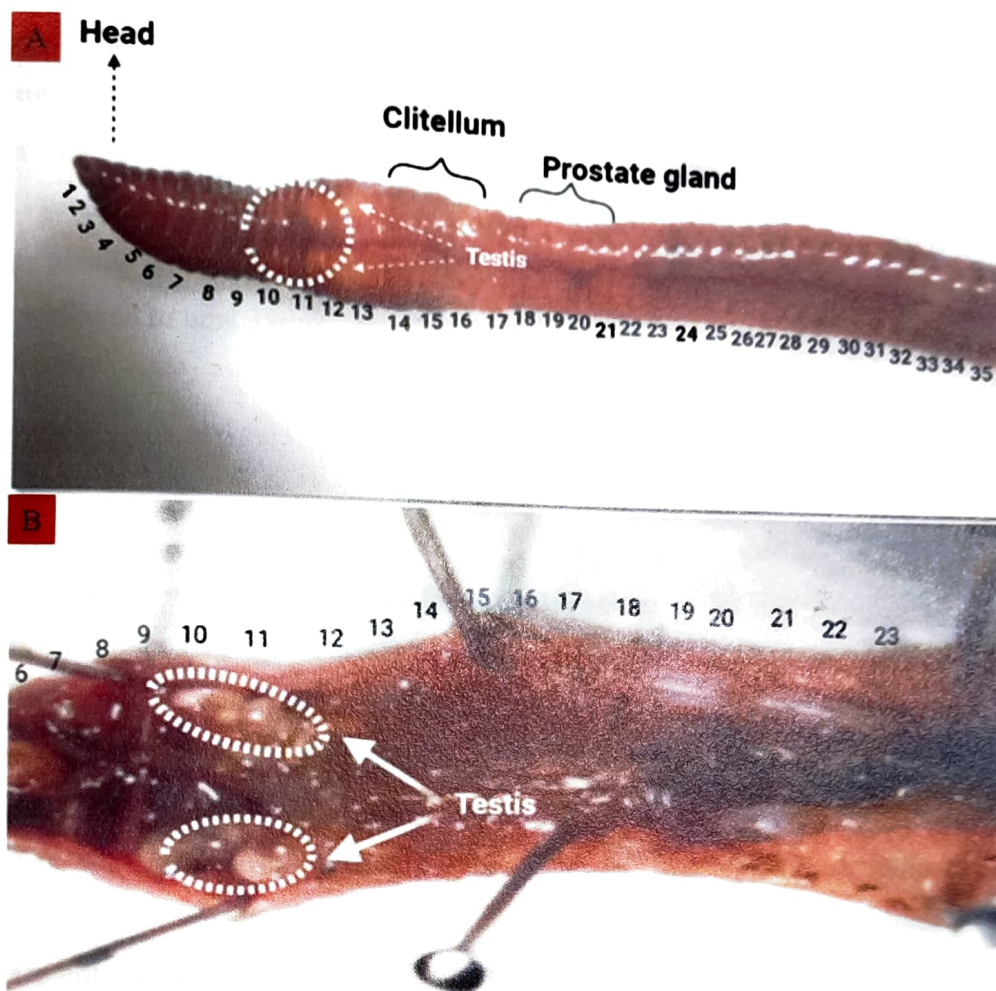


Fig 5.2 A Morphology of earthworm *E. eugeniae* B. Dissection of earthworm and the mar show testis (10th and 11th segment)

5.3 Processing of Earthworm testis gland for *Ex-vivo* maintenance:

After dissection all the testis glands were taken for processing (Fig. 5.3), initially washed with distilled water (Fig. 5.3A), then with 1X PBS thrice (Fig. 5.3B) (Fig. 5.3C) (Fig. 5.3D), then washed with 1% antibiotic solution (penicillin, streptomycin and amphotericin B) (Fig. 5.3E). After washing, glands were transferred to L15 0% serum free (Fig. 5.3F) media for adaptation, then finally transferred to new 35mm petri disk containing L15 10% serum media (Fig. 5.3G) for maintenance.

5.4 Augmentation of *ex-vivo* maintenance for long duration:

After *ex-vivo* processing of earthworm testis gland, we treated with the FDA withdrawn (suspected) drug like testosterone and pramipexole dihydrochloride to determine male reproductive toxicity. The untreated control was kept in the L15 media. We also observed that adding supporting supplements such as testosterone, forskolin and valproic acid will extend the *Ex-vivo* maintenance time even further. We calculated the sperm viability in 5th, 10th and 21st days, as compare to other drug testosterone maintain prolonged period and have high sperm motility. The control testis in L15 medium are able to maintain for only 10 days. The *ex-vivo* testis maintained along with the supplement of valproic acid increase its viability to up to 15 days and after that the organ starts to disintegrate. The *ex-vivo* testis maintained in supplemented with the testosterone maintained for 21 days. We also used testosterone in combination with forskolin (100µl and 50µl), but in 100µl concentration the organ disintegrates quickly, so we preferred the 50µl, which could last 16 days. From our collective results, it is observed that testosterone enhance the sperm viability for 3 weeks than any other supplements that are used in our studies.

Evaluating sperm viability using Sperm analysis:

Then, we calculated the sperm viability on the 5th, 10th and 21st days, comparing testosterone to other drugs that maintain prolonged periods and have high sperm motility. The testosterone was then treated with the drug pramipexole dihydrochloride, which reduced sperm motility.

4.5.2 Sperm viability calculation (% motility)

$$\frac{\% \text{ motility} = \text{Total sperm} - \text{Non motile sperm}}{\text{Total Sperm}} \times 100$$

Fresh testis gland

Total sperm = 218

Non motile =103

$$\begin{aligned} \% \text{ motility} &= \frac{218-103}{218} \times 100 \\ &= 0.5275 \times 100 \\ \% \text{ motility} &= 52.7 \end{aligned}$$

Control testis gland (5th day)

Total sperm = 273

Non motile = 127

$$\% \text{ motility} = \frac{273-127}{273} \times 100$$

Testosterone treated(5th day)

Total sperm = 920

Non motile = 220

$$\begin{aligned}\% \text{ motility} &= \frac{920-220}{920} \times 100 \\ &= 0.7608 \times 100\end{aligned}$$

% motility = 76.08

Testosterone treated(10th day)

Total sperm = 460

Non motile = 107

$$\begin{aligned}\% \text{ motility} &= \frac{460-107}{460} \times 100 \\ &= 0.7673 \times 100\end{aligned}$$

% motility = 76.73

Testosterone treated (21th days)

Total sperm = 371

Non motile = 52

$$\begin{aligned} \% \text{ motility} &= \frac{371 - 52}{52} \times 100 \\ &= 0.859 \times 100 \end{aligned}$$

% motility = 85.98

**Testosterone and pramipexole
dehydrochloride drug treated (10th day)**

Total sperm = 2102

Non motile = 960

$$\begin{aligned} \% \text{ motility} &= \frac{1142 - 960}{1142} \times 100 \\ &= 0.543 \times 100 \end{aligned}$$

% motility = 54.3

Valproic acid treated (5th day)

Total sperm = 90

Non motile = 25

$$\begin{aligned}\% \text{ motility} &= \frac{90 - 25}{90} \times 100 \\ &= 0.722 \times 100\end{aligned}$$

% motility = 72.2

**Testosterone and forskolin treated
(100 μ l)(5th day))**

Total sperm = 343

Non motile = 25

$$\begin{aligned}\% \text{ motility} &= \frac{343 - 25}{343} \times 100 \\ &= 0.9271 \times 100\end{aligned}$$

% motility = 92.71

Testosterone and forskolin treated (50 μ l) (5th day))

Total sperm = 820

Non motile = 162

$$\begin{aligned}\% \text{ motility} &= \frac{820 - 162}{820} \times 100 \\ &= 0.8024 \times 100\end{aligned}$$

% motility = 80.24

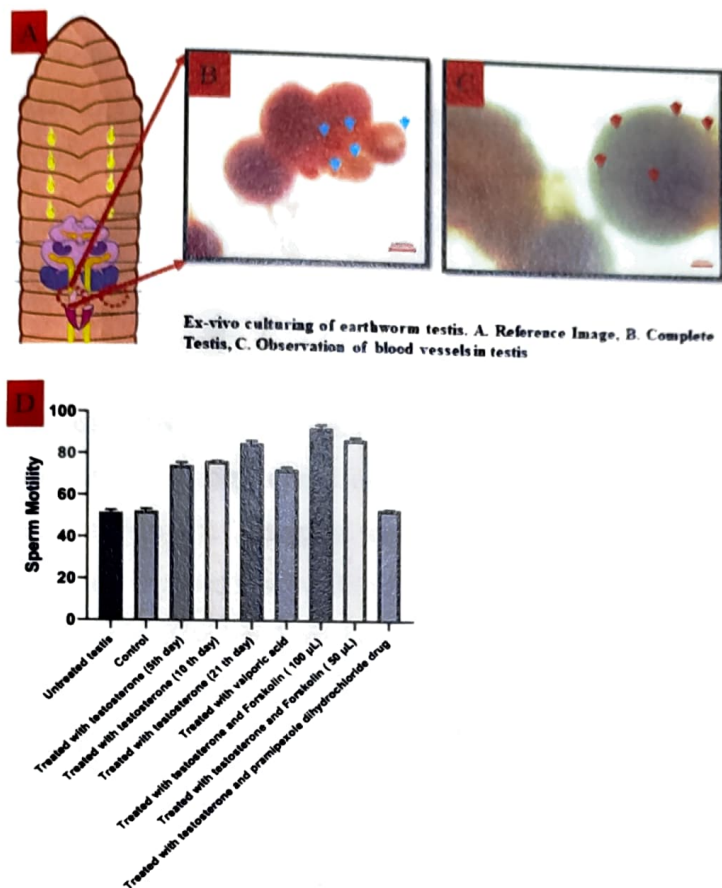


Fig 5.3 *Ex-vivo* culturing of earthworm testis A. Reference image B. Complete testis C. Observation of blood vessels in testis D. Statistical analysis of sperm viability

5.4 Histopathological analysis of testis condition:

The histological section of the control testis gland (Fig. 5.4A) maintained in an *ex-vivo* shows that the tissue was normal and have normal architecture. The testosterone-treated testis gland (Fig. 5.4B) shows some changes, including degeneration of some tissue, conversion to solid tissue, and increased hardness. Some changes occurred in the testosterone and pramipexole dihydrochloride drug treated testis gland (Fig. 5.4B) as compared to the testosterone treated testis gland such as testosterone treated was recovered, degeneration was reduced, and thickness and stiffness were reduced.

5.5 Western Blotting analysis of *ex-vivo* maintenance of testis gland:

To confirm the testicular toxicology condition, protein samples were isolated and subjected to protein expression analysis. In brief, samples were prepared from *ex-vivo* testis maintenance along with control and treated testis gland resolved in 12% SDS-PAGE gel, followed by western blotting analysis using standard molecular level markers such as H3 (proliferation marker), H2AX (DNA damage response protein, Apoptosis indicator), COX2 (marker for oxidative stress), and OCT4 (pluripotency regulator expression and protects embryonic stem cells in an undifferentiated state). To confirm the testicular toxicology condition, protein samples were isolated and subjected to protein expression analysis .control in lane 1 which is maintain *ex-vivo* condition ,treated with testosterone in Lane 2 and testosterone and pramipexole dihydrochloride treated in Lane 3 (Fig. 5.5) .Lane 1 represent control *ex -vivo* testis, Lane2 represent protein sample from *ex-vivo* testis supplemental with testosterone, Lane 3 represent protein sample from *ex-vivo* testis supplemental with testosterone and pramipexole dihydrochloride drug.

5.5.1 Molecular level characterization of testis gland using H3 antibody:

H3 antibody is a cellular proliferation marker with a molecular weight of 15kDa was anlaysis in the *ex-vivo* maintain testis model to access viability. In Control *ex -vivo* testis H3 shows normal expression pattern which are visualize with prominent protein bands. In testosterone treated *ex-vivo* testis the expression of H3 shows increase expression which represent increase in cellular

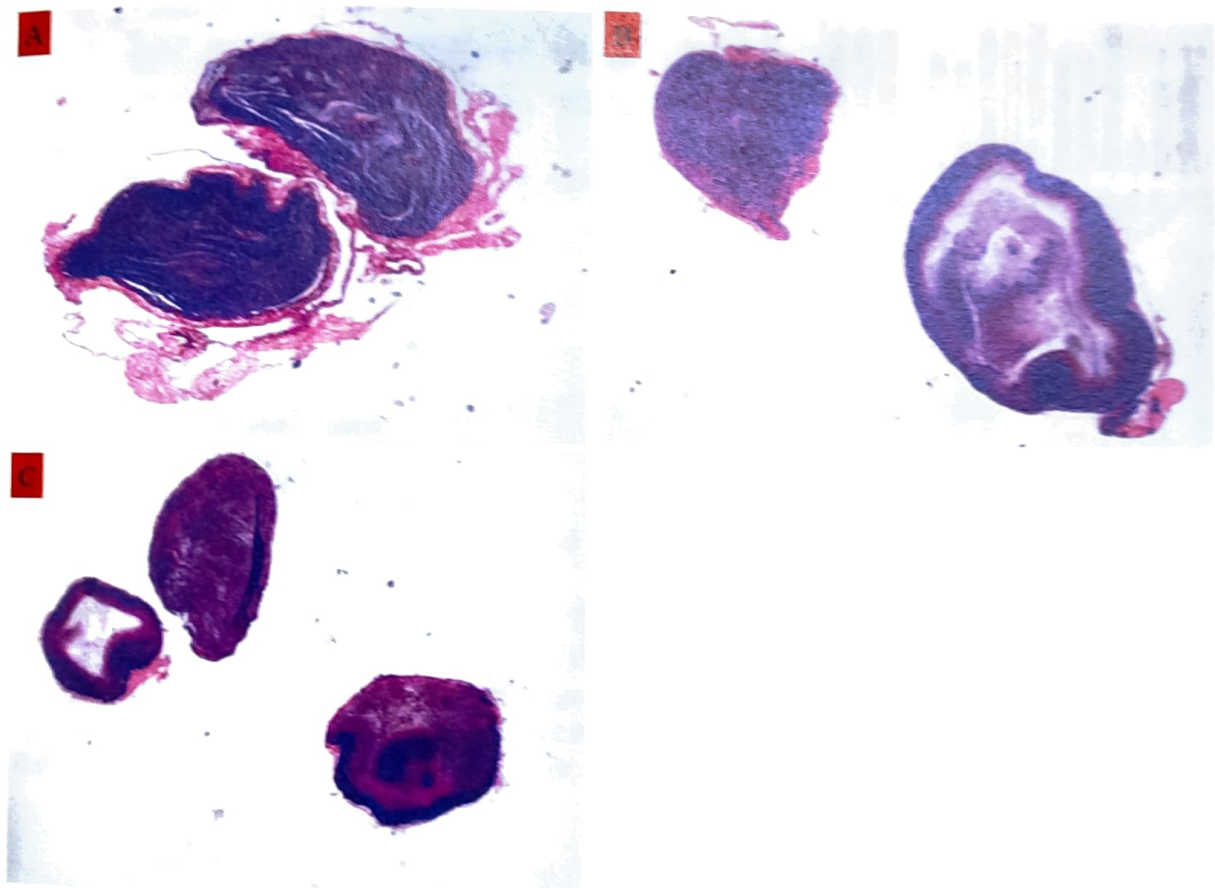
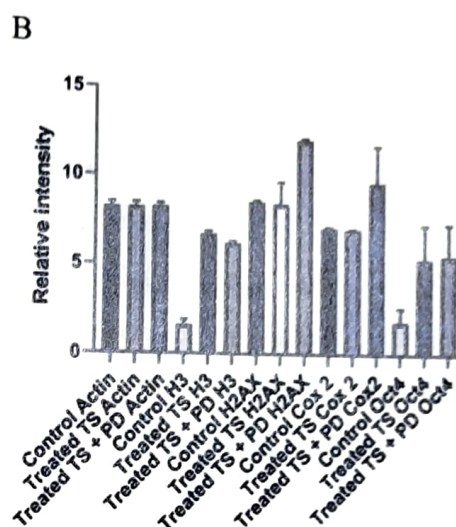
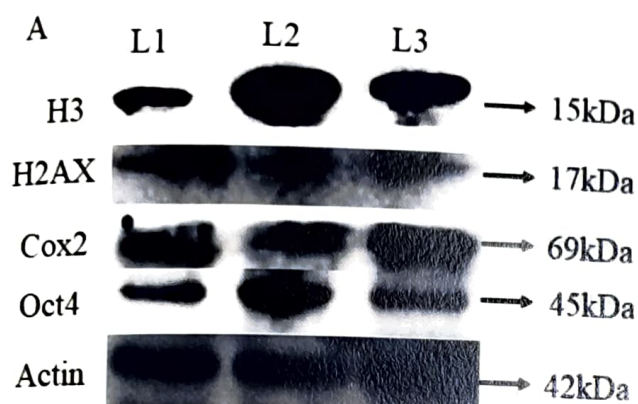


Fig 5.4 Histopathological analysis A. Control testis maintain in ex-vivo condition B. Testosterone treated testis C. Testosterone and Pramipexole dihydrochloride drug treated testis



L1 – Ladder 1 - Control Testis

L2 – Ladder 2 - Treated with testosterone

L3 – Ladder 3 – Treated with testosterone and Pramipexole dihydrochloride drug

Fig 5.5 A. Western blotting analysis B. Using ImageJ software and the band intensity was calculated and that have shown in the bar diagram, to analyze the statistical significance of protein expressions, experiments were repeated thrice and their values are projected as mean $\pm$ SD. p-values <0.05 were considered statistically significant.

proliferation and its viability, in the testosterone and drug treated sample (Pramipexole dihydrochloride drug) H3 shows reduce expression pattern and compare with the testosterone alone treated sample. The collective results with H3 shows that cellular proliferation is enhanced upon treatment with testosterone. H3 expression increased in testosterone-treated samples, which increased sperm count and proliferation. Because of the nature of the drug, H3 expression is decreased in drug treated sample.

5.5.2 Molecular level characterization of testis gland using H2AX antibody :

H2AX is a DNA damage response protein which also functions as an apoptotic indicator. It has a molecular weight of 17kDa. When treated with testosterone, H2AX decreased and the DNA damage response decreased, but when treated with testosterone and pramipexole dihydrochloride drug, H2AX increased and the DNA damage response increased. H2AX increase indicates apoptosis, so testosterone treated apoptosis is very low in comparison to the drug. H2AX is under pharmacological stress, so H2AX levels are increasing.

5.5.3 Molecular level characterization of testis gland using COX2 antibody:

COX2 is an inflammatory marker with a molecular weight of 69kDa. Control cannot maintain for a longer period of time, so COX2 expression is increased; however, in the testosterone-treated, sperm viability increased, time increased, and they were able to maintain for longer period of time for three weeks, so COX2 expression was reduced. COX2 expression increases after treatment with testosterone and pramipexole dihydrochloride, but not the level of control.

5.5.4 Molecular level characterization of testis gland using OCT4 antibody:

OCT 4 is a stem cell marker with a molecular weight of 38 kDa. Control can only be maintained for a limited time, so OCT4 is lower. If we treat for a longer period of time, the stem cell response will increase and we will be able to maintain for a longer period of time. When we treated with testosterone, sperm viability increased and we were able to maintain for a longer period of time because it increased the pluripotency of the stem cells, which increased OCT 4 expression. Muscles relax when treated with pramipexole dihydrochloride. It has more OCT4 expression than the control, but less than testosterone.

CHAPTER - 6

SUMMARY AND CONCLUSION

Even though earthworms are a simple organism, they have a number of molecular signals that control their growth, similar to those found in more complex organisms like humans. Earthworms play a critical role in the soil ecosystem. In the current study, we dissected, isolated, and kept the earthworm testis glands in an *ex-vivo* condition. Earthworm testis is soft tissue that is difficult to handle, but we successfully maintain the testis gland for 3 weeks. We also observed that adding supporting supplements such as testosterone, forskolin, valproic acid, and pramipexole dihydrochloride drug will extend the *Ex-vivo* maintenance time. In comparison to other drug treatments, testosterone can be used for a longer period of time and has a high sperm viability. Overall, this study shows that testosterone levels can be maintained in an *ex-vivo* testis model, which is important for studying the regulation of testosterone production and its effects on germ cells and somatic cells. The findings also suggest that high levels of gonadotrophin stimulation may be toxic to the developing human fetal testis.

CHAPTER - 7

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