

EXPLOITING THE mTOR PATHWAY AND ITS IMPACT IN EARTHWORM REGENERATION

Submitted in partial fulfillment of the requirements
for the award of

**Master of Science
in
Medical biotechnology and
Clinical research**

by

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MEDICAL BIOTECHNOLOGY AND CLINICAL RESEARCH

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of **Mr. GAJENTHIRAN E** who carried out the project entitled "**EXPLOITING THE mTOR PATHWAY AND ITS IMPACT IN EARTHWORM REGENERATION**" under my supervision from 2022-2023

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DECLARATION

I, **Mr. GAJENTHIRAN E (41101001)** hereby declare that the Project Report entitled "**EXPLOITING THE mTOR PATHWAY AND ITS IMPACT IN EARTHWORM REGENERATION**" done by me under the guidance of **Dr. S. Johnson Retnaraj Samuel**, Assistant Professor (Research), Center for Molecular and Nanomedical Sciences is submitted in partial fulfillment of the requirements for the award of Master of science biotechnology and clinical research.

DATE: 05.05.2023

PLACE: Chennai



SIGNATURE OF THE CANDIDATE

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ABSTRACT

mTOR (mechanistic target of rapamycin) is a protein kinase that plays a critical role in the regulation of cellular metabolism, growth, and survival. Given its critical role in the regulation of cellular metabolism and growth, mTOR has emerged as a promising target for the treatment of several diseases, including cancer, diabetes, and neurodegenerative disorders. *Perionyx excavatus*, commonly known as the blue worm or Indian blue worm, is a type of earthworm found in tropical and subtropical regions. The regeneration process in *Perionyx excavatus* is thought to occur through the activity of pluripotent stem cells, which are undifferentiated cells that have the potential to differentiate into various types of cells. Here we have injected rapamycin in different concentrations to regenerate earthworms. To identify the molecular mechanism of rapamycin, RT PCR was performed with the regeneration-specific gene TCTP. By western blotting, we found that rapamycin could down-regulate the TCTP, which indicate that rapamycin causes stress responses in regenerating earthworm. This study suggests that rapamycin can suppress the mTOR pathway in *P. excavatus* and slow down the regeneration growth kinetics by regulating the TCTP expression.

TABLE OF CONTENTS

S.NO	TITLE	PAGE NUMBER
4	Introduction	1
5	Review of Literature	3
6	Aim and Scope of Study	6
7	Materials and Methods	7
8	Results and Discussion	17
9	Conclusion	22
10	References	23

LIST OF ABBREVTIONS

PBS – Phosphate Buffered Saline

KCl – Potassium Chloride

APS – Ammonium per sulphate

TEMED - Tetramethylethylenediamine

NaOH – Sodium Hydroxide

SDS - PAGE – Sodium dodecyl sulfate and polyacrylamide gel electrophoresis

TBST – Tris Buffered saline with Tween 20

BSA – Bovine serum albumin

MTT- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

DMEM- Dulbecco's Modified Eagle Medium

DMSO- Dimethyl sulfoxide

CT – Cycle threshold

DPA – Days post amputation

LIST OF FIGURES

S.No	Description	Page No
1	Perionyx Excavatus	2
2	DNA-binding SYBR Green dye in RT-PCR. Fluorescence dramatically increases when SYBR Green binds to dsDNA.	13
3	Phenotypic observation of the effects of Rapamycin	18
4	Normalized gene expression of rapamycin treated samples	19
5	Protein expression of rapamycin treated samples and normalized expression of proteins	20
6	mTOR Pathway	21

CHAPTER-1

1. INTRODUCTION

Perionyx excavatus belongs to the phylum *Annelida*, class *Clitellata*, and family *Megascolecidae*. It is a species of earthworm that has adapted to live in tropical regions, particularly in Africa. One of the adaptations of *Perionyx excavatus* is its nocturnal behavior, which allows it to avoid the heat of the day and predators. This earthworm species also has a high tolerance for acidic conditions, making it well-suited for vermicomposting (Ravindran & Krishnamoorthy et. al., 2017). Additionally, it has a streamlined body shape with a uniform purple-grey shine and equally tapered posterior segments that enable it to move efficiently through the soil and organic matter. *Perionyx excavatus* has also been observed to exhibit behavioral adaptations, such as crawling away from potential predators and using its muscular body to move and burrow through soil. This species is also known for its ability to reproduce quickly and reach maturity at a young age, which is advantageous in environments with high rates of predation and competition for resources. Overall, the adaptations of *Perionyx excavatus* have allowed it to thrive in tropical regions and play an important role in soil health and nutrient cycling. (Edwards & Subler et. al., 2010).

Kingdom: *Animalia*

Phylum: *Annelida*

Class: *Clitellata*

Subclass: *Opisthopora*

Order: *Lumbricina*

Family: *Megascolecidae*

Genus: *Perionyx*

Species: *excavatus*

Scientific classification of *Perionyx excavatus*

The potent immunological disorder medication FK506 and rapamycin interfere with signal transduction pathways needed for T lymphocyte activation and growth. The distinct repressing effects of those medication on the T lymphocyte activation program mediate through the formation of pharmacologically active complexes with members of a family of intracellular receptors termed the FK506 binding proteins (Abraham et. al., 1996). Rapamycin was at first discovered as an antifungal substance made by *Actinomyces hygroscopicus* from a soil sample of Easter Island (also referred to as Rapa Nui). Afterward, rapamycin was found to treat immunological disorders and possess antiproliferative properties in mammalian cells (Jing li et. al., 2014)

Autophagy is a cellular degradation mechanism that transports cellular components to the lysosome for degradation and subsequent utilization of degradation products. This process involves several sequential steps, including sequestration, transport to lysosomes, degradation, and utilization of degradation products, as described by Mizushima in 2007. The central regulator of cell growth, mTOR, is a target that plays a crucial role at the intersection of pathways that collectively control the balance between cell growth and autophagy in response to various signals such as biological processes, protein, and stress (Jung et. al., 2010).

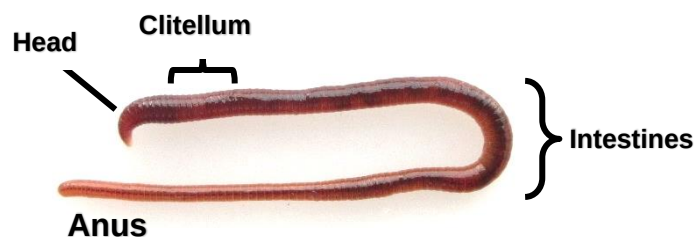


Fig. 1. *Perionyx excavatus*

CHAPTER-2

2. REVIEW OF LITERATURE

2.1 STUDIES ON EARTHWORM

Earthworms were members of phylum and class *Oligochaeta*, living in several strata of soil. Over 4400 species of earthworms have been found worldwide. Mainly they were burrowing animals and take advantage of dead organic matter, living microorganism, rotifers, nematodes, fungi, and alternative microorganisms (Edwards and Fletcher 1998).

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

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

2.2 RAPAMYCIN AND ITS SIGNIFICANCE

Rapamycin was an antifungal metabolite produced by *Actinomyces hygroscopicus* from an Easter Island soil sample (also known as Rapa Nui). Rapamycin was later discovered to treat the immunological disorder and have anti-proliferative properties in cancer cells, sparking interest in rapamycin's mode of action. Rapamycin was found to be a potent activator of S6K1, a serine/threonine kinase activated by a variety of agonists. At the same time, the target of rapamycin (mTOR) was discovered in yeast and animal cells (Chung et. al., 1992).

The mTOR signaling pathway in mammals is the master regulator of cell growth and metabolism. The mTOR pathway has been implicated in a wide range of

human diseases, including cancer, diabetes, obesity, medical specialty diseases, and genetic disorders. Rapamycin was also discovered to be extremely effective. (Sung et. al., 2010) used antibiotic B to treat general candidosis in mice. Aside from its historical use as an immunosuppressant, there is mounting pre-clinical evidence that rapamycin or rapamycin analogs increase life span in a variety of species, including flies and mice. In mice, the life-span-extending effect is observed even when treatment is not initiated until the mice are already old (Graziotto et. al., 2012).

2.3 RAPAMYCIN INCUDED mTOR INHIBITION:

Angus et. al., 2009 have reported the potent anti-immunological disorder action of rapamycin is usually reported to inhibition of growth factor-induced T lymphocyte proliferation. However, it's currently evident that the serine/threonine macromolecule enzyme class target of rapamycin (mTOR) has a vital role in the modulation of each innate and reconciling immune response.

Rapamycin does not directly inhibit mTOR however binds to its immunophilin, FK binding macromolecule (FKBP12). Rapamycin and FKBP12 then act with mTOR and inhibit its function, resulting in inhibition of cell growth and proliferation (Dutcher et. al., 2004). The class target of rapamycin, mTOR has been shown to connect the stimulation to macromolecule synthesis and cell cycle progression. However, activation of PI3K five and, probably, AKT/PKB, thought of to lie upstream of mTOR will defend cells from cell death evoked by stress. The function of mTOR in cell survival has not been established. To study the potential role of mTOR in growth cell survival, we tend to use the macrolide antibiotic rapamycin, which powerfully inhibits mTOR, prevents activation of T cells and inhibits the growth of normal cell lines and malignant cell lines in culture (Hosoi et. al., 1999).

The class target of rapamycin (mTOR) may be a downstream effector of the phosphatidylinositol 3-kinase (PI3K)/Akt (protein enzyme B) signal pathway, that mediates cell survival and proliferation. mTOR regulates essential signal-transduction pathways and is concerned with the coupling of growth stimuli with cell cycle progression. Mammalian target of rapamycin (mTOR) signal pathways within the management of cell survival and death. mTOR is the master regulator that integrates signals from external and internal signals, like growth factors, amino acids, aldohexose and energy to manage growth and metabolism. It

additionally inhibits autophagy under nutritive conditions (Dutcher et. al., 2011).

2.4 mTOR INHIBITION AND AUTOPHAGY:

Rapamycin induces autophagy by nutrient depletion via down-regulating the mammalian target of the rapamycin pathway. Autophagy is a degradation system that delivers cytoplasmic constituents to the cell organelles. Despite its simple process, recent progress has reported that autophagy plays important physiological and pathophysiological roles (Mizushima et. al., 2007). (Ravikumar et. al., 2010), This study has shown that cells underneath ER stress activate autophagosome formation, which ends in macroautophagy (autophagy). Since autophagy has a necessary role in promoting cellular- survival throughout starvation by “self-eating” components of the living substance and living thing organelles, it had been instructed that autophagy additionally features a crucial protecting role underneath ER stress. The activation of autophagy below rapamycin treatment would possibly contribute to a decrease in cell death (Kapuy et. al., 2014; Peter Nagy et. al., 2015).

The study has been reported on the role of autophagy in conserving the function of stem cells for the continual renewal of the internal organ epithelial tissue in *Drosophila*.

2.5 mTOR AND TCTP

Translationally controlled tumour protein (TCTP) is upregulated in a variety of cancer cell types, which is aided in mTOR activation. Recently, TCTP has been proposed to act as an indirect mTOR activator. While it is well known that mTOR regulates skeletal muscle mass, little is known about the role and regulation of TCTP in this post-mitotic tissue. Muscle TCTP and mTOR signalling are upregulated in a variety of mouse models (mdx mouse, mechanical load-induced hypertrophy, and denervation- and immobilization-induced atrophy), according to this study. Furthermore, the hypertrophic and atrophic increases in TCTP were caused in part by a rapamycin-sensitive mTOR-dependent mechanism. (Goodman et. al.,).

CHAPTER-3

3. AIM AND SCOPE OF THE STUDY

- To determine the rapamycin concentration for mTOR inhibition in *Perionyx excavatus*
- To study the effect of rapamycin mediated regeneration in the *Perionyx excavatus*
- To monitor the regeneration growth kinetics of rapamycin-treated *Perionyx excavatus* compared with untreated worms
- Investigating the molecular link of H2AX and TCTP expression levels in rapamycin-treated samples.
- Investigating the expression pattern of regeneration-specific genes in samples treated with rapamycin.

CHAPTER-4

4. MATERIALS AND METHODS

4.1 CULTURE AND MAINTENANCE OF EARTHWORM, *Perionyx excavatus*

Earthworm, *Perionyx excavatus* was reared from the stock that are maintained in Centre for Molecular and Nanomedical Sciences, International Research Centre, Sathyabama Institute of Science and Technology, Chennai, Tamilnadu, India. Earthworms were maintained according to the protocol of (Edwards, C. A. (1998), Dominguez, J., Edwards, C. A., & Subler, S. (2001)). Using cow dung and dry leaves, suitable environment for earthworms were created. Cow dung provides a source of organic matter and nutrients, while dry leaves provide carbon and help to maintain moisture in the soil.

4.2 SOLUBILITY OF RAPAMYCIN:

1mg of rapamycin tablet was crushed and dissolved in the 1ml of distilled water by vortexing. Rapamycin was injected to the earthworm at different concentrations (1 μ M, 3 μ M and 5 μ M).



Fig.4.1 Solubility of Rapamycin

4.3 RAPAMYCIN DOSAGE STANDARDIZATION:

The worms are given different doses of rapamycin to study the regeneration growth kinetics. Rapamycin was administered to earthworms via injection. It was injected into the worm's 16th segment, just below the clitellum. Rapamycin dosages of 1 μ M, 3 μ M and 5 μ M were used, as well as a control in which dH₂O was injected via insulin injection. Every worms were injected once a day for three days.

REGENERATION EXPERIMENTS:

After injecting rapamycin in the 16th segment located below the clitellum, the head part (10-12 segments) of the earthworm is amputated and placed in a separate plastic container to allow for regeneration. The earthworms were then left to grow for regeneration. Within five days of amputation, blastema formation was observed.

4.4 SDS PAGE

4.4.1 Requirements

- i. **Chemical Reagents** – Acrylamide/Bisacrylamide, SDS, 1.5 M Tris – HCl (pH – 8.8), 0.5 Tris – HCl (pH – 6.8), 2X Sample Buffer, 1X Running Buffer, 10% APS, TEMED, Staining and Destaining solution.
- ii. **Equipments** – Electrophoresis Unit, Power bank, Water bath or dry bath, Racker.

4.4.2 Reagent Preparation

i. 30% Acrylamide and 0.3% Bisacrylamide

Acrylamide - 30g/100mL

Bisacrylamide – 0.3g/100mL

Make 100mL with distilled water and store at 4°C in the dark.

ii. 4X Separating Buffer

Tris – HCl – 18.2g Distilled Water – 60mL

pH was adjusted to 8.8 with

10N NaOH SDS – 0.4g

Make up to 100mL with distilled water

iii. 4X Stacking Buffer

Tris – HCl – 6.05g

Distilled Water – 40mL

pH was adjusted to 6.8 with

10N NaOH Make up to 100mL

with distilled water

iv. 2X Sample buffer

Tris Stacking buffer (pH 6.8)

– 25mL SDS – 4g

Glycerol – 20mL (Should be added last and mixed well)

Makeup into 100mL with distilled water. During protein sample preparation

5% β -Mercaptoethanol and during loading 0.001% (w/v) of Bromo-phenol blue was added.

v. Ammonium per sulphate (APS)

APS – 100mg

Make up to 1mL with distilled water

vi. 1X Running Buffer

Tris – HCl – 3.94g

Glycine – 15.014g SDS – 1g

Makeup to 1L with distilled water

vii. Staining Solution

Coomassie Brilliant Blue – 200mg

Methanol – 50mL

Glacial Acetic acid – 7mL

Makeup to 100mL with distilled water. The above mixture was filtered using Whatman No. 1 filter paper.

viii. Destaining Solution

Methanol – 30mL

Glacial Acetic acid – 7mL

Make up to 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 (μl)	200	200	200	200	200	200	200	200	200	200
5.	TEMED (μl)	20	20	20	20	20	20	20	20	20	20

Table 4.2: Stacking gel concentration

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

4.5 Protein sample preparation:

In order to separate the protein samples, SDS-PAGE will be performed. The protein samples were prepared from the anterior blastema of the worm. The blastema was amputated and washed with the 1XPBS and it will be homogenized with 2X protein sample buffer and boiled in a boiling water bath for 10 min. The samples were then cooled, and stored at -20°C. The proteins present in the samples were estimated using Lowry's assay and then subjected for protein profile analysis using SDS-PAGE. The prepared protein samples were resolved in the 12% SDS-PAGE.

4.6 Preparation of gel:

The glass plates were cleaned assembled so that the longer glass plate laid first, then 2 spacers of 1.5 mm thickness was placed along the sides of the rectangular plate. The notched glass plate was placed on the top of the spacers so that the bottom ends of the spacers and glass plates were aligned and the whole setup was assembled on the stand. 1% agarose was prepared, boiled and it was used to seal the plates.

12% separating gel monomer solution was prepared and carefully poured into the glass plate assembly without forming air bubbles. The gel was overlaid with n-butanol so that it excludes the air. The separating gel was allowed to polymerase for 20 min. The butanol layer was removed and the gel was washed with distilled water.

Stacking gel monomer solution was then prepared and poured over the separating gel. The comb was then inserted and the gel was allowed to solidify for 10 min. After the gel gets solidified, the comb was removed carefully and the wells were washed with distilled water. The glass plate

assembly was then placed in the buffer chamber and running buffer was poured in both the upper and lower buffer chamber.

4.7 Sample loading and electrophoresis:

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

4.8 Staining and de-staining

The gel was carefully taken out from the glass plate and the stacking gel was removed. Separating gel was removed by gently grasping the two corners of the gel and placed in the container containing the coomassie brilliant blue R-250. The gel should be fully submerged in the staining solution for 2 hours by gentle agitation on a Rocker. Then it was washed with distilled water and transferred to destaining solution until protein bands were visualized clearly and the gel is documented.

4.9 Immunoblotting

12% gels were transferred in to the 1X Transfer Buffer for 15 minutes, Blotting apparatus was soaked in to the Transfer buffer, from the basement, sponges from the bottom onwards tissue papers and then gel was placed on it, after the carbinol activation of nylon membrane smooth surface was placed on the gel, then double tissue papers and sponge were applied and punched manually by hands, blotting apparatus was transferred in to the box which carries 1X transfer buffer with ice packs to maintaining the temperature at 4-8°C.

4.9.1 Blocking

5% of BSA Powder with 20 ml of 1X TBST buffer was prepared for Blocking. Blocking buffer is used to block all proteins, which are targetable and non-targetable proteins, also blocked.

4.9.2 Primary antibody:

Anti-H2AX (Abcam, ab50669; dilution, 1:5000), Anti-Wnt3a (Abcam, ab19925; dilution, 0.5:5000), and Anti-Lamin A ((L1293) (C-terminal) antibody produced in rabbit dilution, 0.5:5000). was added in the 5% BSA solution 0.5:5000 dilution.

4.9.3 Secondary antibody:

Secondary antibody H2AX-Anti Rabbit IgG HRP (ab150077) was added in the 5% BSA solution 0.5:5000 dilution.

4.9.4 Reagent required

i. Washing Buffer – 1X PBS + 0.1% Tween 20

Transfer buffer 25 mM Tris base 190 mM glycine 20% methanol

Check the pH and adjust to 8.3

4.10 Gene Expression using qPCR

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

Principle

Real-time detection of PCR products is made possible by incorporating a fluorescent molecule in the reaction that reflects an increase in DNA amount with a corresponding increase in fluorescent signal. The fluorescent chemistries used for this purpose include DNA-binding dyes and fluorescently labeled sequence-specific primers or probes. Fluorescence is monitored when amplicon occurs using specialized thermal cyclers that have fluorescence detecting modules. The amount of amplified product in each cycle is represented by the measured fluorescence. Real-time PCR has several advantages over traditional PCR, but its ability to estimate the beginning template copy number with precision and excellent sensitivity over a broad dynamic range is its key benefit. Results from real-time PCR might be quantitative or qualitative. Quantitative RT-PCR is also referred to as qPCR. Contrarily, traditional PCR is only semi-quantitative. Additionally, gel electrophoresis is not necessary for the evaluation of real-time PCR results, reducing experiment time and increasing throughput. Also, chances for contamination are very less because reactions are carried out and data are analyzed in a closed tube system.

SYBR green Dye

SYBR Green is the most commonly used double-stranded binding dye for RT-PCR. The dye exhibits minimal fluorescence when free in solution, but when bound to dsDNA, its fluorescence increases up to 1000-fold (Fig 1). In RT-PCR, its fluorescent signal increases in proportion to the amount of dsDNA generated during amplification. The excitation and emission maxima of SYBR Green dye are 494nm and 521 nm

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

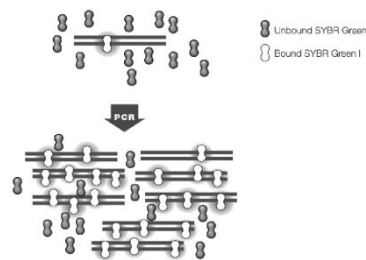


Figure 2. DNA-binding SYBR Green dye in RT-PCR. Fluorescence dramatically increases when SYBR Green binds to dsDNA.

Materials required for setting qPCR

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

Protocol for gene expression analysis using qPCR

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

Important points while designing primers

- Design the primers in such a way they have a GC content of 50-60%.

- It is best to have the melting temperature (T_m) between 50 and 65°C.
- Avoid repeats of Gs or Cs longer than 3 bases and place Gs and Cs on ends of primer.
- Check the sequence of forward and reverse primers to ensure no 3' complementarity to avoid primer-dimer formation.

Primer Dilution

- Centrifuge the primer tubes at 8000 rpm for 30sec.
- Dissolve the primer with appropriate ml of nuclease-free water which was mentioned in the primer datasheet.
- Vortex the tubes and allow them to set for 2 mins at room temperature.
- Keep the tubes in a boiling water bath for 10 min ant 60°C.
- Centrifuge the tubes at 8000 rpm for 30 sec.
- Aliquot it by taking 10 μ l of the primer and 90 μ l of Nuclease free water in a sterile centrifuge tube. Label properly and stored the tubes at -20 °C.

Preparation of reaction mixture

- Thaw the frozen 2X SYBR Green supermix, template DNA and primers on ice. Briefly vortex and spin the tubes to collect the contents at the bottom of the tubes.
- Add the components into each reaction tube as given in the Table 1. Briefly vortex each reaction mix, and spin the tubes at low speed in a microcentrifuge (2000 rpm for 2 min).

Table 1: Reaction Mix - for 10 μ l

Step	Volume	Final Concentration
Nuclease free water	1 μl	-
Forward Primer	1 μl	300 nM (100-500nM)
Reverse Primer	1 μl	300 nM (100-500nM)
2x SYBR Green super mix	5 μl	1x
Template DNA	2 μl	cDNA equivalent to 1pg to 100 ng of total RNA
Total reaction volume	10 μl	

Important Tips:

- Prepare 10% more reaction mix than required to compensate for the potential pipetting loss. Prepare triplicate samples as a master mix with a single addition of the sample template.
- Include no template control (NTC) for detecting possible contamination or primer-dimer formation.
- Load the reaction triplicates into the PCR tubes or microplates, and seal them properly. For better results, briefly spin the microplates or tubes in a microcentrifuge at low speed to remove any bubbles. If necessary, store the sealed reactions on ice or 4° C until start running the RT-PCR.
- Program the thermal cycler with recommended RT-PCR with or without the melt curve step (Table 2).
- Place the sealed reaction vessels in the thermal cycler block, and start running the RT-PCR protocol.

Table 2. RT-PCR protocol

Cycling Step	Temperature	Hold Time (min:sec)	No. of cycles
Initial denaturation and enzyme activation.	95 ° C	02:00	1
Denaturation	95° C	00:15	40
Annealing/ Extension	55-60° C	00:20	
Melt curve (Optional)	65-95° C (in 0.5 ° C increments)	00:05	1

qPCR Analysis Methods

It is important to choose reaction conditions for qPCR that will result in reliable and effective amplification for both relative and absolute measurement. The CT or Cq values obtained during qPCR are used for absolute and relative quantification.

In absolute quantification, the C_T or C_q values of test samples are compared to those of standards of known quantity using a standard curve. This analysis will provide the

copy number or concentration of the sample and this method is most widely used for the detection of pathogen or copy number analysis.

In relative quantification, the C_T or C_q values of the test samples are compared to those of the control samples. This analysis will yield the ratio of the relative amount ie the fold difference of the target gene in the test vs control samples and it is commonly used for gene expression studies. This analysis can be done using the software also we can calculate manually in an excel sheet using formulas.

CHAPTER-5

5. RESULTS AND DISCUSSION

5.1 DIFFERENT CONCENTRATION OF RAPAMYCIN INJECTION

Well matured worms were taken for the experiment. The drug rapamycin was injected to the earthworm, *Perionyx excavatus* at 1mM, 3mM, 5mM concentration and distilled water as control for three days. On the second day, the earthworms were amputated at the 10th segment in the anterior region. The experimental worms were maintained up to 10 days for observation. The amputated worm develops the blastema and it was measured with a scale every day and documented.

5.2 REGENERATION UPON INJECTION

Control and rapamycin injected worms were measured at different days. Rapamycin injected worms was compared with control worms. On the 6th day, significant difference was observed in the blastema of the earthworm. When comparing to 1mM and 3mM rapamycin concentration, 5mM concentration decreased the regeneration process based on the bud observation. For further molecular evidences, immunoblotting was carried out with the regenerated bud protein samples.

Perionyx excavatus earthworms have a segmented bright brown body with the clitellum located between the 13th and 17th segments. These earthworms can regenerate their body segments even in the absence of the clitellum. To observe the effects of rapamycin as a regeneration inhibitor, the earthworms in this experiment were amputated at the 10th or 11th segment. The results suggest that the degree of inhibition of earthworm regeneration was positively correlated with the dose of rapamycin administered, indicating the potential of rapamycin as a regeneration inhibitor. The pictures clearly demonstrate this phenomenon, despite the earthworm's natural regeneration ability. However, an increase in the level of rapamycin is proven as toxic to earthworms, resulting in their death. The study on earthworm's behavior has been ongoing for 120 hours post-amputation was monitored and documented using a Canon camera (Fig. 3).

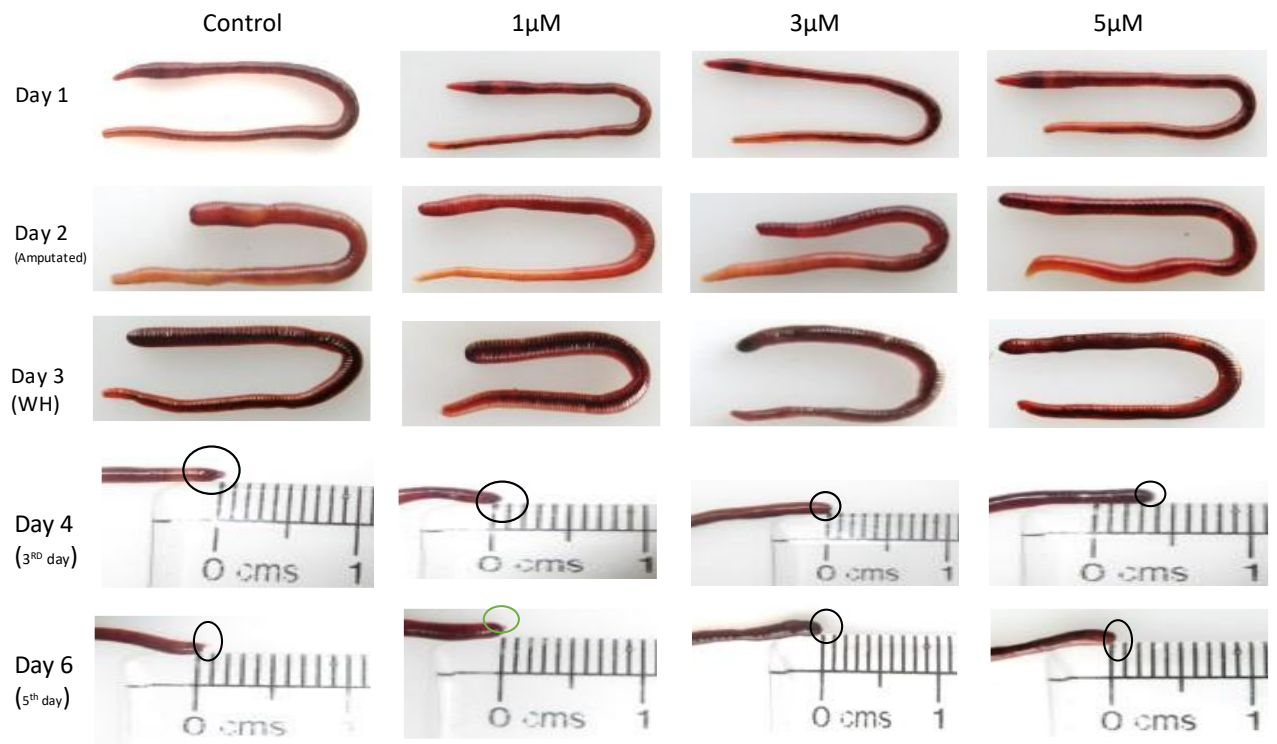


Fig. 3. Phenotypic observations of the effects of rapamycin

5.3 GENE EXPRESSIONS STUDIES

During regeneration, TCTP (Translationally Controlled Tumor Protein) is a protein that is highly conserved and plays an important role in cellular processes such as cell growth, proliferation, and differentiation, as well as tissue regeneration and wound healing. However, studies have shown that treating earthworms with rapamycin inhibits their regeneration capacity and also leads to a reduction in the expression levels of TCTP genes. To explore the potential mechanistic link between TCTP, mTOR signaling and muscle remodeling, we next examined whether mechanical overload (OV) would be associated with an increase in TCTP, and whether any increase in TCTP would occur in an mTOR kinase dependent manner (Fig. 4).

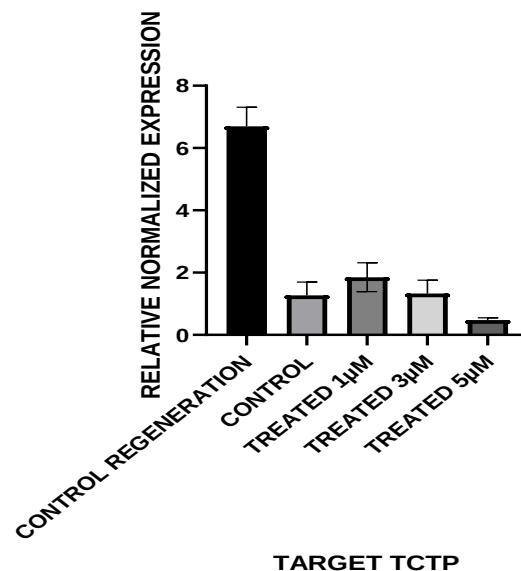
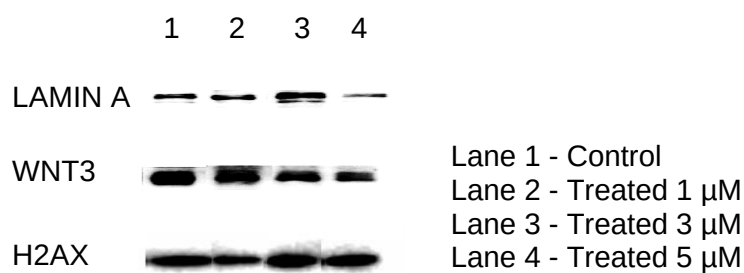


Fig. 4. Normalized gene expression of rapamycin treated samples

5.4 WESTERN BLOTTING ANALYSIS ON RAPAMYCIN TREATED AND CONTROL SAMPLES

To investigate the significance of TCTP in *P. excavatus* regeneration, protein expression studies on control and rapamycin-treated samples was conducted. The findings indicated that the expression of TCTP and H2AX proteins increased in rapamycin-treated samples compared to untreated samples, as illustrated in Fig 5. Additionally, the expression of Wnt 3 protein decreased in rapamycin-treated samples in comparison to the control. The TCTP protein had a molecular weight of 17 kDa, while the H2AX protein weighed 19kDa. Lamin A, with a molecular weight of 45kDa, served as the positive control. Fig 5 presented graphical representation of normalized values. In order to confirm the role of TCTP in preventing apoptosis and its involvement in morphallaxis during *P. excavatus* regeneration, the rapamycin was administered in combination with other substances and observed the expression of key proteins such as the H2AX apoptotic marker. The normalized values of protein expression were depicted in Fig 5. The intensity of protein expression was quantified using ImageJ software.



Lane 1 - Control
 Lane 2 - Treated 1 µM
 Lane 3 - Treated 3 µM
 Lane 4 - Treated 5 µM

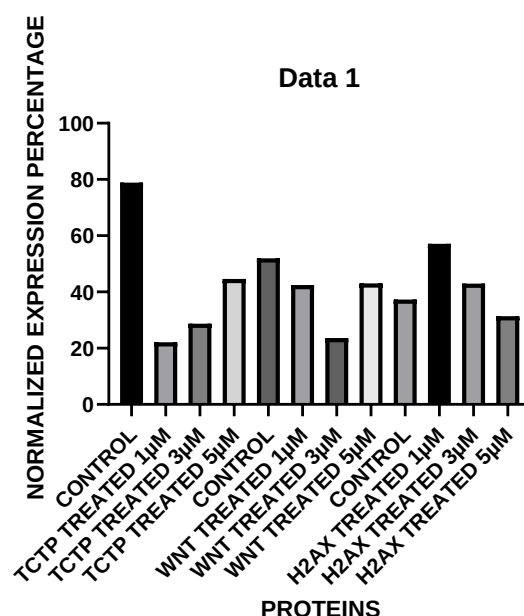


Fig. 5. Protein expression of rapamycin treated sample and normalized expression of proteins are shown in the graph

5.4 H2AX CORRELATION WITH mTOR PATHWAY

Saravi et al., 2020 reported that gene sets involved in G2/M checkpoint, DNA repair mTORC1 signaling were expressed on H2AX highly expressed tissue in Gene Set Enrichment Analysis. mTORC1 and mTORC2 control cellular growth, proliferation, and metabolism, all of which are required for optimal cellular function. However, rapamycin is a potent inhibitor of mTOR complex 1, it perturbs the mTOR pathway and alters the cellular functions (Fig 6). In this case blocking the mTOR pathway with 5 mM rapamycin, retard the regeneration growth kinetics but also alter the DNA damage proteins such as H2AX expression.

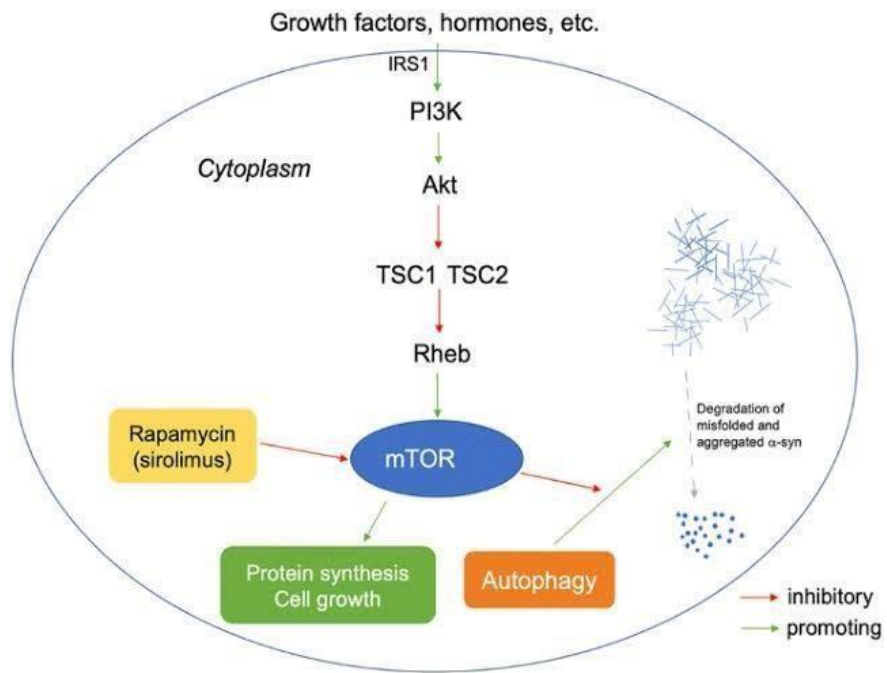


Fig. 6. mTOR pathway (28)

CHAPTER 6

CONCLUSION

- Rapamycin is a well known mTOR pathway inhibitor. It was observed that increasing concentration of rapamycin decreases the regeneration process.
- TCTP RNA level is increased in rapamycin treated samples. Interestingly, TCTP protein level was found decreasing upon increasing concentration of rapamycin. The molecular process behind the translation machinery of TCTP is yet to be identified.

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