Studying Retinal Regeneration in Type 2 Diabetes Mellitus Zebrafish Model, Developed Through Overfeeding

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Integrated BS-MS dual degree

Department of Biological Sciences

A dissertation submitted for the partial fulfilment of

BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali

April 2020

CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled **"Studying Retinal Regeneration in Type 2 Diabetes Mellitus Zebrafish Model, Developed Through Overfeeding"** submitted by Mr. Jithin R (Reg.No.MS15023) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr.Shashi Bhushan Pandit Dr. Kuljeet Singh Sandhu Dr. Rajesh Ramachandran (Supervisor)

Dated: 24 April 2020

DECLARATION

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rajesh Ramachandran at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bona fide record of original work done by me and all sources listed within have been detailed in the bibliography.

Jithin R
(Candidate)

Dated: April 24, 2020

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

ACKNOWLEDGEMENT

The MS thesis year was filled with some of the most memorable experiences which were both good and unfortunate and yet I was able to complete most of my thesis because of the help of many people who were there for me till the end. Firstly, I thank Dr.Rajesh Ramachandran, my supervisor for giving me valuable support and guidance throughout the year. I thank Mohammad Anwar Khursheed, my guide, for his suggestions and help in the beginning of my thesis and my second guide, Ms. Bindia Chawla, for her never-ending kind support and motivation. I also thank Dr Poonam Sharma for helping me in my thesis and my other Lab members, Mrs. Shivangi Gupta, Ms. Mansi Chaudhary, Ms. Sharanya Premraj, Mr. Ashish Sethi, Ms. Pooja Shukla who were always been there to help and gifted me lots of good memories. My lab life wouldn't have been more enjoyable without my fellow batchmates, Ziya, Athul, Jaskaran, Trirupa, Sapna and Ajay. Most importantly, I thank my parents, brother and my grandma for their infinite love and support.

I can't thank enough for the Zebrafishes, who sacrificed their life for us.

I also extend my gratitude to IISER Mohali and INSPIRE-DST for facilities and financial support.

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NOTATIONS

T2DM: Type 2 diabetes mellitus

MG: Muller glia

Insm1a: Insulinima-associated 1a

Dkk1: Dickkopf-related protein 1

Her4.1: hairy-related4, tandem duplicate1

Mycb: MYC proto-oncogene

Mmp9: Matrix metallopeptidase 9

Zic2b: zic family member2b

INL: Inner nuclear layer

ONL: Outer nuclear layer

GCL: Ganglionic cell layer

hpw: Hours post wound

mpi : Minutes post injury

dpi: Days post injury

hpi: Hours post injury

hpa: Hours post amputation

ABSTRACT

Tissue regeneration provides a way for restoring function to damaged and diseased tissues and organs[1]. Even though, mammals show very limited amount of regeneration capabilities in organs like skin, liver and skeletal muscles, they are unable to repair the damages caused in vital body parts and the Central Nervous System (CNS), especially retina, which is one of the most accessible part of CNS[1]. Irreparable tissue damages especially, in retina, heart, kidney, skin etc. are one of the major complications seen in Type 2 Diabetes Mellitus (T2DM) patients. The rising prevalence of T2DM and its complications worldwide, makes it one of the key research fields of the next decades. The Zebrafish is considered as one of the best model organsm to study regeneration as well as metabolic diseases like diabetes. Here, we are focusing on the major changes in the regenerative abilities of the Zebrafish caused due to the introduction of Type 2 Diabetes mellitus (T2DM). In this study, we developed T2DM model Zebrafishes by over feeding method. After developing a T2DM zebrafish model, we explored the areas of tissue regeneration by amputation of fin, skin and retinal injury. Our studies were mainly focused on the changes in retinal regeneration. We have shown that, overfeeding zebrafish from larval stage could cause obesity and weight gain along with persistent Hyperglycemia. We found that the retinal regeneration, as well as the fin regeneration and wound healing capabilities in T2DM adult zebrafish were impaired when compared with non-diabetic control group. We checked expression patterns of various regeneration associated genes like Insm1a, Dkk1, Her4.1, Mycb, Mmp9 and Zic2b. Later study on high-sugar diet fishes showed acute hyperglycemia, which also showed impaired fin and retinal regeneration. These results suggest that increase in blood glucose levels in zebrafish body can cause damage to processes like regeneration and wound healing.

CHAPTER 1: INTRODUCTION

Mammals have very limited regeneration capacity that occur in tissues and organs like skin, liver and skeletal muscle. Unlike mammals, regeneration is a common trait in vertebrates like zebrafish, axolotl, *Xenopus* frog etc. Among these animal models, Zebrafish, a teleost fish is one of the widely used models for regenerative studies. Zebrafish can regenerate almost all its organs such as heart, fin, liver and bone. Since organs like retina in zebrafish, are relatively simple in composition, easy accessibility to experimental manipulation and its similarity with mammalian retina, makes it an important system for studying CNS regeneration[2].

Retinal regeneration in zebrafish is mediated by muller glia cells, which upon injury re-enter cell cycle to acquire stem cell like properties forming Muller glia cell-derived progenitors, and thus they proliferate and re-differentiate to various retinal cell types and muller glia itself. Thus, by exploring the mechanisms of MG's reprogramming into stem cell will help us to bring strategies for stimulating retina regeneration in mammals.

Damaged neurons are the cause of blindness associated with retinal diseases like retinitis pigmentosa, macular degeneration, glaucoma and diabetic retinopathies[1]. Metabolic diseases like long term Type 2 Diabetes mellitus can give rise to complications such as neuropathy leading to diabetic retinopathy, limb amputations, slow wound healing capacity etc.

1.1 TYPE 2 DIABETES MELLITUS, OBESITY AND HYPERGLYCEMIA

T2DM is a chronic condition in which the body is unable to effectively use insulin to bring glucose into the cell. According to International diabetic federation, in 2019, approximately 463 million people were living with diabetes and by 2045 this figure is expected to rise to 700 million. T2DM is characterized by insulin resistance, this means that insulin does not works properly on liver, adipose tissue or skeletal muscles., thus glucose uptake by these organs get decreased. As a result, there will be high glucose level in blood for a longer period of time (Hyperglycemia). In liver, because of insulin resistance, glucose is not stored properly, instead it release more glucose in the attempt to supply glucose to other organs that needed Insulin resistance can cause beta cells in pancreas to do atrophy, this would further cause further problems and the person

suffering from diabetes need insulin injections to compensate. Insulin resistance occurs due to various number of factors like, genetic predisposition, bad eating habits and obesity (fig 1.1).

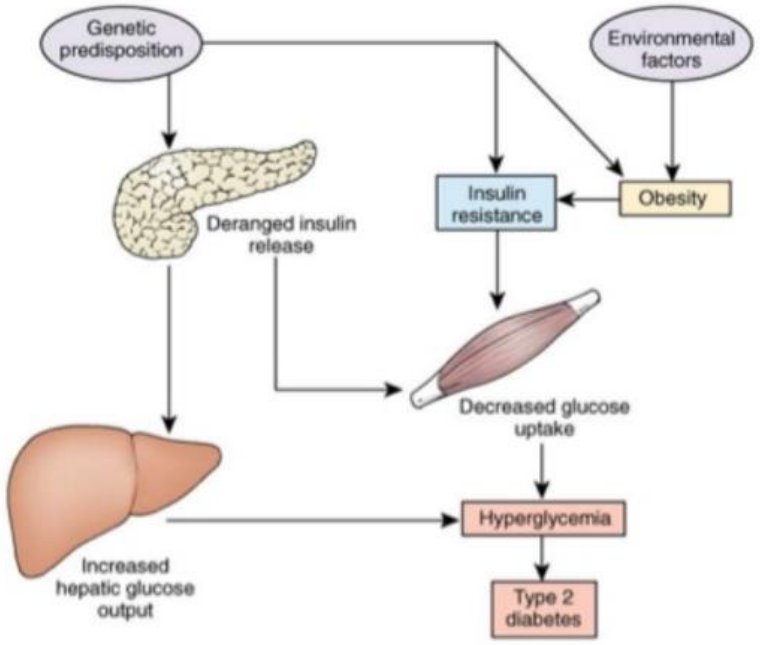

Fig 1.1: Factors causing type 2 diabetes mellitus: The diagram shows how obesity, genetic predisposition leads to hyperglycemia and thus T2D. (Image from: Everydayhealth .com website)

Some of the complications caused by T2DM:

1) Diabetic neuropathy: is a type of nerve damage, caused due to the damage of small blood vessels affected by high levels of glucose, due to diabetes. Diabetic neuropathy mainly damages the nerves in legs and feet.
2) Diabetic foot: High blood glucose levels in diabetic patients can cause diabetic neuropathy. As mentioned above, diabetic neuropathy often damages the nerves in legs and feet. This can cause complete loss of feelings in the feet. Hence, Cuts, sores or blisters in feet can cause ulcers and infections. Serious cases can lead to limb amputation.
3) Diabetic retinopathy: can damage blood vessels within the retinal tissue, causing the vessels to leak fluid and distort vision. Two types: Non proliferative (symptomless) and Proliferative (formation of new abnormal blood vessels in retina). Symptoms include, blurred vision, poor night vision, sudden loss of vision etc.

Zebrafish have been identified as an excellent model system for studying metabolic diseases like diabetes, because of high similarities in organ physiology, glucose homeostasis, pancreas structure and metabolism with that of mammals

1.2 RETINAL ARCHITECTURE, VARIOUS INJURY RESPONSES AND REGENERATION

a) Zebrafish Retinal Architecture

The zebrafish retina is divided into three main laminar layers: 1) Outer nuclear layer (ONL), 2) Inner nuclear layer (INL) and 3) Ganglion cell layer (GCL). Rod and cone photoreceptor cell bodies are located in the ON, which helps in sensing light and transmit the information to ganglion cells in GCL through the INL, which has cell bodies of bipolar (BP), horizontal (H), amacrine interneurons (A) and muller glia (MG) (Fig 1.2). This special MGs extend their processes out to all the layers and yet houses the cell body within the INL. This special anatomy of these cells allows them to monitor the retinal environment to sense any change in the retinal homeostasis. MGs also support neurons by releasing trophic factors, recycling neurotransmitters and controlling ionic balance in the extracellular space[3].It has been recently found that the MGs directly contribute to vision by acting as optical fibers to guide light to photoreceptors[1].

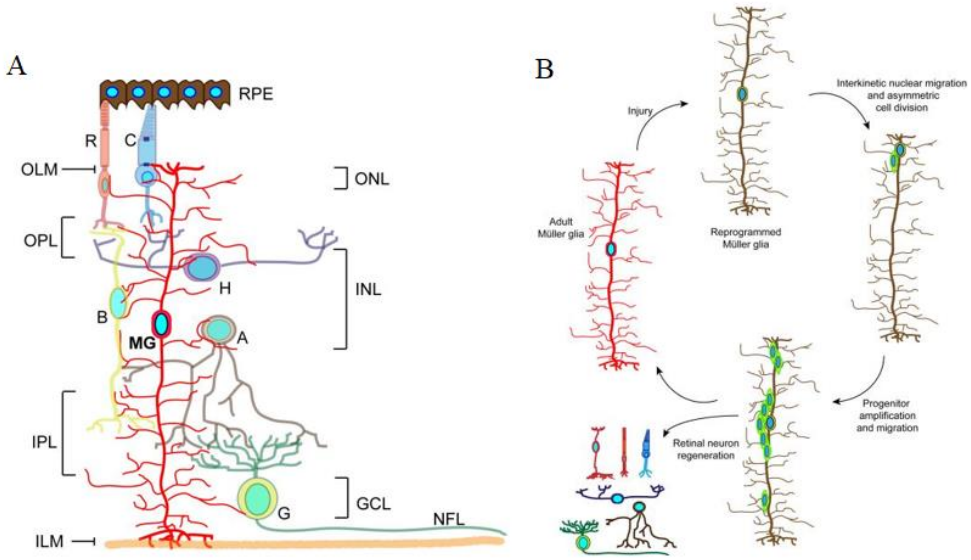

Fig 1.2: Anatomy of retina and formation of MGPCs after injury: (A) The different layers of retina: mainly, outer nuclear layer(ONL) ,Inner nuclear layer (INL) and ganglion cell layer (GCL). (B) Reprogramming of muller glia (explained below). (Image from: Goldman-Nat Rev Neurosci 2014)

b) Various Injury responses

1) MGs injury response:

In Zebrafish, retinal injury is often done in 4 ways, mechanical (needle poke method), by using toxic chemicals or genes, bright or UV light and laser aberration. After sensing retinal damage MGs in retina undergoes a gliotic response where they overexpress tubulin proteins like Gfap (glial fibrillary acid protein) and undergo hypertrophy. The gliotic response then gets convert to a regenerative response when the MGs get reprogrammed to have stem cell like properties. The main stages of regeneration are: a) Dedifferentiation of adult MGs into MGPCs b) Proliferation: Interkinetic nuclear migration to the ONL and asymmetric cell division. c) Redifferentiation: Migration of progenitors to various retinal layers and redifferentiation into various cell types.

Signal transduction in muller glia as a result of injury response:

Pathways like glycogen synthase kinase 3ß (Gsk3ß)–ß-catenin, Notch, Mapk–Erk and Jak–Stat signalling pathways regulate zebrafish retina regeneration. Suppression of pathways can also stimulate muller glia cell reprogramming, examples are MicroRNA (miRNA) let-7 signalling pathway and Dkk signaling pathway[3]. Notch signalling pathway also have an inhibitory role in zebrafish retina regeneration, notch target genes like her4 and other components like *deltaA, deltaB, deltaC, notch1* are induced by injury unlike other pathways which gets suppressed after injury[3]. Notch signalling helps muller glia to match the number of injury-responsive muller glia with the extent of retinal damage. Some of the early response genes include, ASCL1a, which activate lin28 expression, this is important regulator of tissue regeneration in mammals[3]. ASCL1A also regulate wnt signalling and regulates the expression of *insulinoma-associated 1a* (*insm1a*). It is a transcriptional repressor that affects both progenitor cell cycle exit and mullerglia cell reprogramming[3].

- Roles of Mycb during retina regeneration: Mycb activates ascl1a, which then regulates lin28 expression, which is important to induce various regeneration associated pathways[4]. Mycb also induces insm1a and inhibits her4.1[4].
- Role of Mmp9 during retinal regeneration: Mmp9 is highly induced in regenerating MG cells[5] and it is also essential during fin regeneration[6].
- Role of Zic2b during retina regeneration: Induction of zic2b in MGPCs triggers a proliferative phase mediated through shh signalinaling and can drive MGPCs towards differentiation[7].

2) Fin amputation response:

The zebrafish caudal fin is composed of several segmented bony rays and inter-ray mesenchymal tissue, enclosed by epidermis. Upon amputation of the caudal fin, a regenerative program gets activated and the fin fully regenerate all its tissues and structures within 2 weeks. At:

- 1-3 hpa : Epithilial cells migrate to cover and close wound.
- 18-24 hpa : an apical epidermal cap (AEC) along with a mass of undifferentiated mesenchymal cells called blastema underneath AEC is formed.
- 24 hpa: The blastema cells segregate into two morphologically indistinct compartments: a slowly proliferating distal blastema and a rapidly proliferating proximal blastema. The distal blastema contributes with daughter cells to the proximal blastema, which is a population of cells that migrate to new positions and differentiate to replace the lost tissues.
- 48hpa: The regeneration program gets installed and outgrowth continues until the original architecture is reconstituted.

3) Wound healing response:

In adult mammals, wound healing involves multiple steps including blood clotting, inflammation, re-epithilialization and granulation tissue formation and maturation. While adult zebrafish shows all steps of adult mammalian wound repair except external fibrin clot formation. In zebrafish wound re-epithelization is extremely rapid and initiates with

no apparent lag-phase, followed by immigration of inflammatory cells and the formation of granulation tissue, consisting of macrophages, fibroblasts, blood vessels and collagen[9]. Recent studies show that, one of the striking differences between mammalian and fish wound repair is that wound tissue remodeling in zebrafish is completed with minimal scarring. Zebrafish re-epithelialization occurs independently of inflammation and fibroblasts growth factors (FGF) signaling, but both are essential for granulation tissue formation and fibroblast requirement[9].

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CHAPTER 2: MATERIALS AND METHODS

2.1 T2DM zebrafish model development through overfeeding method

Wild type zebrafish larvae (~50hpf) was collected after breeding of wild type adult male and female fishes. The larvae have been separated into two equal groups of 40, one for control and the other for diabetic model. The control and experimental group fishes were further divided into groups of 10 in 3.5L tanks. The control groups were fed one time a day with commercially available fish food (FRIPPAK RACEWAY, RW+500, INVE technologies, Belgium) while the experimental groups were fed 4 times a day. This feeding method was followed from the larval stage and continued throughout approximately 3months of adult stage, until the organs were harvested for experiment. (FRIPPAK RACEWAY, RW+500, contains: Protein (46%), Lipid (7%), Fiber (3%) and moisture (9%).

Sucrose rich food for high sugar diet fishes: Preparation: 6g of normal fish food (FRIPPAK RACEWAY, RW+500) and 6g of sucrose crystals (SIGMA life science) were weighed. Sucrose crystals were allowed to melt in a beaker using a heater at ~190°C. Later this melted sucrose solution was mixed with the fish food and allowed to solidify. Small pellets were created and was given 4 times a day for a week to the high sugar diet experimental fish group.

2.2 Zebrafish Blood glucose test

For measuring the fasting blood glucose level of adult zebrafishes, Fishes were allowed to fast for overnight and blood were isolated from dorsal aorta on the next day using an indigenously developed blood isolation tube. The tube was made by connecting commercially available butterfly needle's tube to a glass capillary tube (1.0mm outer diameter), using parafilm. The tube had been sealed with tape to make it airtight.

For isolating the blood, the glass tube was coated with EDTA, in order to prevent fast coagulation of blood during isolation. The fishes were then anesthetized using Tricaine methanesulfonate. and dorsal aorta was located using microscope. After identifying the

dorsal aorta, blood was collected using the tube by suction method. Make sure to avoid air bubbles while sucking the tube.The collected blood in the glass tube was placed directly into the strip of glucometer (Dr.Morpen BG-03 Gluco One Glucometer) and the blood glucose level readings were obtained.

2.3 Wound healing test

Fish was anesthetized in Tricaine methanesulfonate and laid on a wet tissue paper. By using a sterilized razor blade, ~1cm length wound was introduced, onto the left flank directly anterior of dorsal fin. The fish was then released into an external water tank and was monitored for 15 days.

2.4 Fin amputation

Zebrafish were anesthetized using Tricaine methanesulfonate and the tail fins were cut using a sterilized razor blade immediately to the proximal branch point of the dermal rays within the fin. After 6dpa, the fishes were again anesthetized for taking the fin image. Fin images were obtained using the camera in a trinocular microscope.

2.5 Retina dissection for RNA isolation

1. Zebrafish was anesthetized using Tricaine.
2. Retina was injured using 30-gauge needle.
3. Eyes were harvested using forceps and needle, at the desired time. 1X PBS (Phosphate Buffered Saline) was used for harvesting retina and 4% paraformaldehyde (PFA) was used for tissue fixation. 4.Remove the lens for both isolating RNA/ tissue fixation.
5.Store the retina in trizol for RNA isolation.

2.6 Tissue fixation and sectioning

1. Isolated Eyes without lenses were kept in 4% PFA at 4℃ overnight.
2. On the Next day, following serial washings was given to the fixed tissue at RT for 45mins each on a rotor:

1ml of 5% sucrose

800µl of 5% and 400µl of 20% sucrose

500µl of 5% and 500µl of 20% sucrose

400µl of 5% and 800µl of 20% sucrose

1ml of 20% sucrose.

3. 500µl of OCT was added and allowed to rotate again for 30 min.

4. Then eyes were dipped firmly inside OCT, which was filled in small cubes made from aluminum foil and the samples were kept in -80℃ until sectioning.

5. Blocks were sectioned in cryostats (12µm thickness) and the sample sections were collected on super frost plus slides and dried overnight and was later stored in -20℃.

Composition of solutions used:

1.For 4% PFA in 1X Phosphate buffer:

2g PFA

5mL of 10X phosphate buffer

The volume was made up to 50mL with DEPC water.

Then dissolved by keeping in 65℃ and constant shaking

2. For 5% sucrose:

Dissolved 2.5g sucrose in 50mL of autoclaved water. Stored at -20℃.

3. For 20% sucrose:

Dissolve 10g sucrose in 50mL of autoclaved water. Store at -20℃.

2.7 Immunostaining

1. Slides kept in -20℃ was taken out and dried in 37oC for half an hour.

2. Slides were washed three times with 1xPBS, 10 min each time.

3. Then slides were dipped in 2N HCL/ 0.1mM Sodium Citrate (boiled at 100℃) for 20min.

4. After that slides were washed twice with Sodium borate (pH 8.5, 0.1M) for 10 min each.

5. Later, The tissues were blocked using 3% BSA in 1XPBST for one hour.

6. Primary Antibody diluted in 1%BSA /PBST (1:1000) was added carefully over the slides and kept in 4℃ overnight.

7. On next day, the slides were washed twice for 10 min each with 1xPBST.

8. Then secondary antibody diluted in 1%BSA in 1xPBST (1:1000) was overlaid carefully on the slides and kept in RT for 3 hours.
9. Then the slides were washed thrice with PBST for 10 min each.
10. After that, the slides were washed twice with water and allowed to dry for 1 hour.
11. Images of the section were obtained under a confocal microscope.

Microscopy: Bright field microscope (Zeiss) was used for dissecting retina.
Confocal microscope (Nikon) was used for fluorescence imaging.

2.8 RNA isolation

1. Retinas from -80℃ was thawed.
2. Tissues were homogenized using 200µl pipette.
3. 0.2 volumes (40µl) of chloroform were added and mixed the sample by moving the MCT up and down 15 times.
4. Then the samples were centrifuged at 10000 rcf for 10min at 4℃.
5. Using cut tips collect aqueous phase was slowly taken without disturbing the middle phase layer and was transferred into a fresh MCT.
6. Equal volume of Isopropanol was added and was placed on ice for 5 mins.
7. Then the samples were centrifuged at 10000rcf at 4℃ for 10min.
9. Supernatant was discarded and the pellet was washed with 80% alcohol (200µl) and centrifuged at 4℃ for 10 min at 7600 rcf..
10. After that the pellet were dissolved in DEPC treated water (10 µl) and stores at -80℃.
11. Presence of RNA was checked using agarose gel electrophoresis.and the amount of RNA was measured using nanodrop machine.

2.9 cDNA synthesis (Using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit)

For 15 µl reaction:
1. Following master mix was made:
Template RNA – 5µl

Oligo (dT) Primer - 0.75µl

Random Hexamer - 0.75µl

Nuclease free water -2.5µl

2. The above mix was put in 650C for 5min in PCR machine, after that the tube was immediately placed in ice for about 2 min.

3. After that the following mixture was added to the tube:

5X Reaction Buffer - 3µl

RiboLock RNase inhibitor - 0.75µl

10mM dNTP Mix – 1.5µl

RevertAid M-MuLV RT enzyme – 0.75µl

4. The above mixture was then kept in PCR machine with the following settings:

25℃- 5min

42℃ - 60min

70℃ - 5min

40℃ - infinite hold

5. cDNA was stored in -80o C for further use.

2.10 Reverse transcription Polymerase chain reaction (RT-PCR) using Taq Polymerase

The synthesized cDNA was diluted into 1:4 dilution (1µl cDNA+ 4µl Milli-Q water) and set reactions for 10µl.

20X buffer- 0.5µl

dNTP-1µl

forward primer and reverse primer(F+R) -0.5µl

template cDNA-0.7µl

MQ water -7.25µl

Taq polymerase -0.1µl

Cycling condition (25 cycles):

95℃-2min

95℃-20sec

60℃-30sec

72℃-30sec (Varies with genes)

72℃-5min

4℃-infinite hold

PCR product was checked using Agarose gel electrophoresis

2.11 Quantitative PCR (qPCR)

Before setting qPCR, already using Template was diluted to1:1 dilution and a 5 µl reaction was set in a qPCR machine:

KOD SYBR qPCR Master Mix mix- 2.5µl

Primers (F+R)-0.1 µl

Template - 1 µl

MQ water- 1.5µl

Data was analyzed and graph was plotted.

CHAPTER 3: RESULTS AND DISCUSSION

3.1Overfeeding leads to the development of obesity and overweight

One of the first step towards developing a type 2 diabetic zebrafish model was overfeeding. Overfeeding fishes 4 times a day with normal fish food had caused obesity in wild type fishes (fig3.1), which is characterized by increase in adipose tissue accumulation in belly area and increase in body weight as compared to the control fishes. This result indicate that overfed zebrafish develop obesity in a manner similar to those observed in other mammalian models as well as in humans, since mammals and zebrafish shares common pathophysiological pathways, organs and tissues.

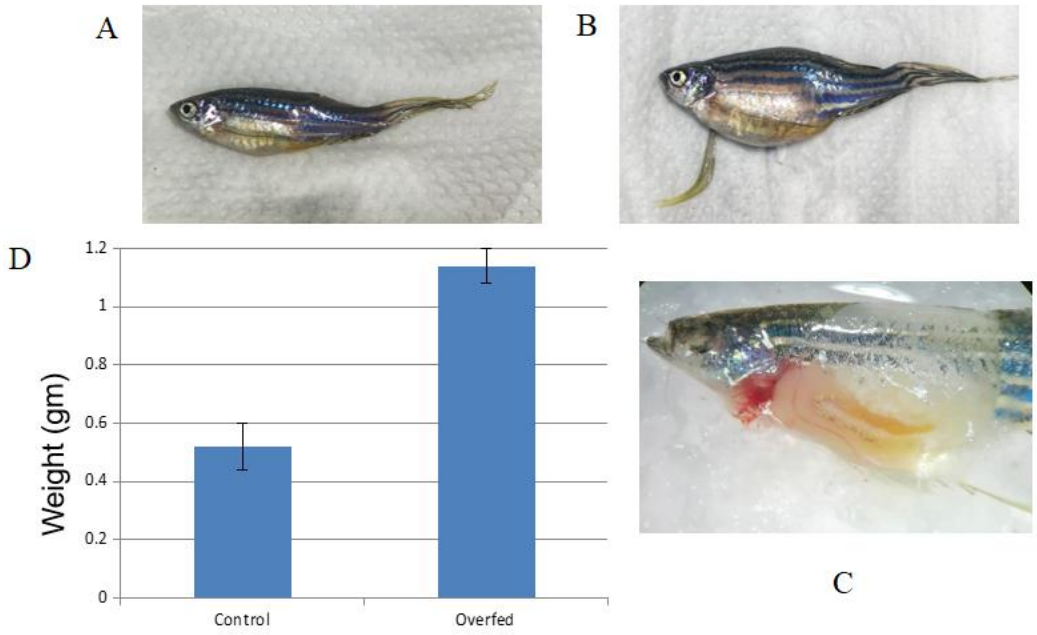


Fig3.1: Effect of overfeeding in zebrafish: (A) Control zebrafish (B) Overfed zebrafish developed obesity (C) Cross section of obese fish showing fat deposits (D) graph showing the weight comparison (in gm) between control and obese fish. Average weight of control fish : 0.52gm and average weight of overfed fish: 1.14gm.

3.2 Development of Hyperglycemia in Obese fishes

Measurement of fasting blood glucose level in obese fish group, showed that most of the fishes were hyperglycemic when compared to the control group, which suggests that the

model we developed are indeed showing complications of T2DM. Fig 3.2, shows the blood isolation process from the dorsal aorta of the zebrafish.

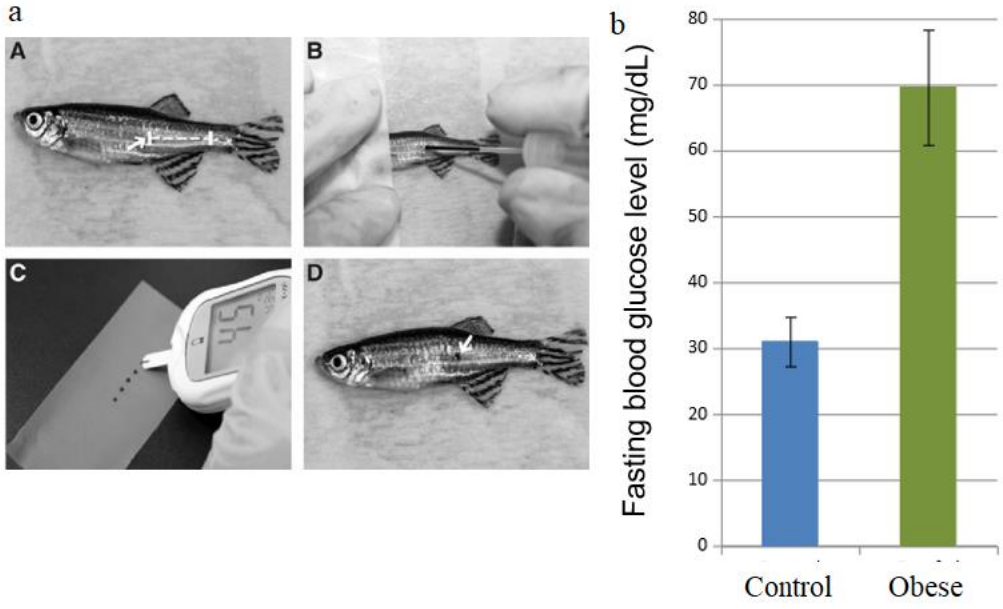


Fig3.2: Blood isolation from zebrafish and Hyperglycemia in obese fish: (a) Image showing the blood isolation method from the dorsal aorta of zebrafish using glass capillary and measurement of blood glucose level using a glucometer. (b)Obese fish showing hyperglycemia, average fasting blood glucose level: Control: 31mg/dL and for Obese fish: 69.6mg/dL (Image from: Liqing Zang- A novel reliable method for repeated blood collection from aquarium fish)

3.3 Limb regeneration is impaired in Hyperglycemic fish

It is known that people living with T2D have an increased risk of lower limb amputation due to ulcers or wounds that do not heal. The caudal fins of control and hyperglycemic fishes were amputated to assess the effect of limb regeneration. The result showed a significant reduction in blastema formation for the hyperglycemic fish than compared to the control fish after 6dpa.This result also suggests that hyperglycemia has direct impact on the limb regeneration capacity of zebrafish.

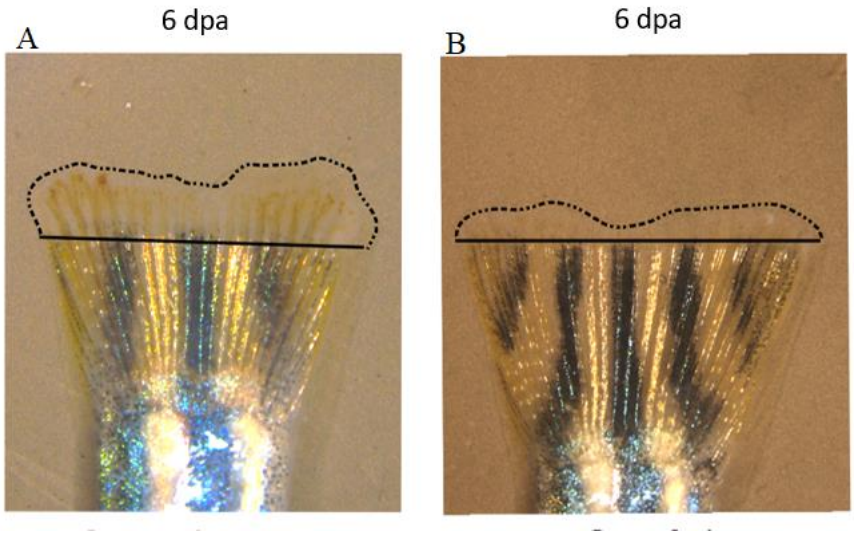


Fig3.3: Effect of hyperglycemia in fin regeneration: After 6dpa, blastema formation in hyperglycemic fish (B) was less than compared to the control fish (B).

3.4 Wound healing is impaired in Hyperglycemic fish and formation of Ulcers.

The wounds of hyperglycemic fishes healed very slowly when compared to the control fishes (fig3.4). This result suggests that high glucose in blood can cause a delayed response to injury and can also impair the function of immune cells in hyperglycemic fish. Delayed wound healing is commonly seen in patients with T2DM. Some of the fishes from the hyperglycemic batch showed ulcers in their body (fig3.5), after a long period of overfeeding. This can be because of their inability to heal sore due to poor blood circulation to that area and gradually may have got infected with bacteria to create Ulcers, which resembles to that of diabetic foot ulcers which are common in people with diabetes. These foot ulcers can be caused due to lack of pain caused because of nerve damage due to elevated blood glucose levels in blood for a longer period of time.

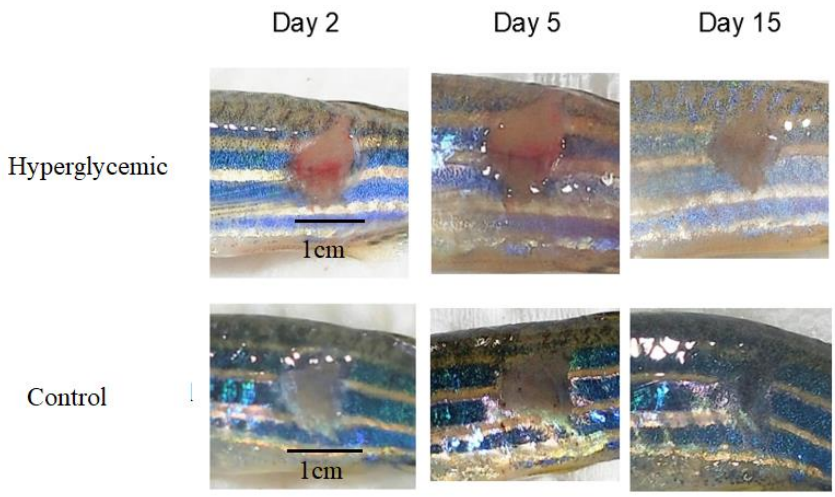


Fig3.4: Effect of hyperglycemia in wound healing process of zebrafish: Observation after 15dpw, showed reduced wound healing in hyperglycemic fish, while the wound of control fish was almost healed after 15dpw.

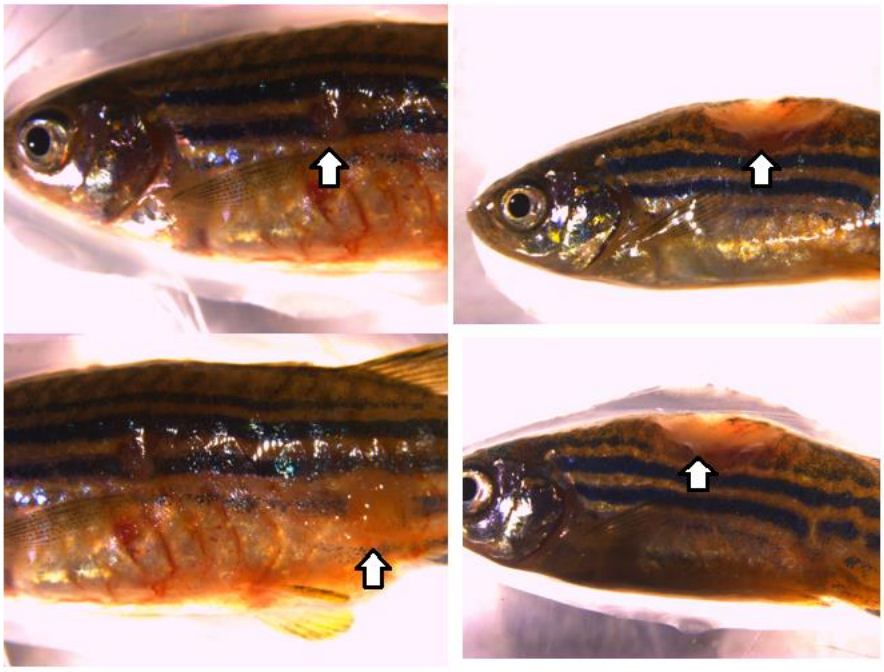


Fig3.5: Effect of long-term overfeeding: After overfeeding for more than 3 months, a few numbers of fishes had shown ulcer development on their body .

3.5 Retinal regeneration is impaired in Hyperglycemic fish

Reduction in PCNA+ve cells were observed in the hyperglycemic retina when compared to the control retina at 4dpi. The cell count data of PCNA+ve cells also showed the reduction of proliferating cells in hyperglycemic retina. PCNA is a molecule with a longer half-life and an ability to stay detectable even after cell-cycle exit. It is effectively used as an indicator of post-proliferative status.

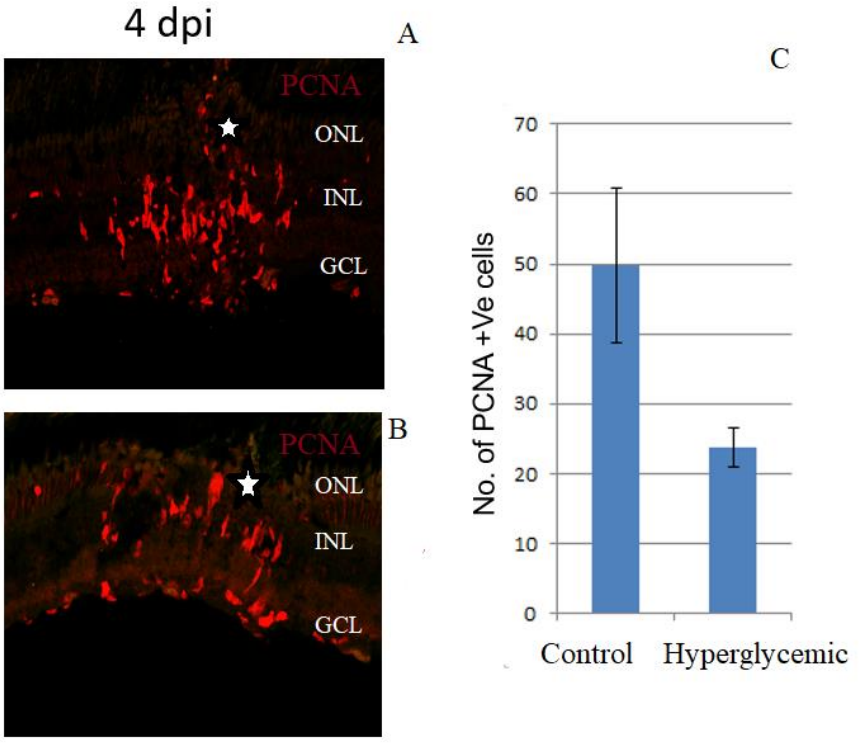


Fig3.6: Effect of hyperglycemia in zebrafish retinal regeneration: Reduction in PCNA+ve cells in hyperglycemic retina (B) after 4dpi, when compared to control retina (A). (C) PCNA+ve cell count, average cell count for control:49 and for hyperglycemic:23.

In order to further validate our result, we conducted the same experiment using transgenic fish that marks proliferating MGPCs. A 1016tuba1a: GFP transgenic zebrafish was used for this. This transgenic fish is well characterized to mark proliferating MG-derived progenitors after an injury (Fausett and Goldman, 2006)[8]. The result showed a decrease in GFP+ve cells in the hyperglycemic retina and increased number of GFP+ve cells in control retina. The cell count data also confirmed the reduction of GFP+ve cells in the hyperglycemic retina (fig 3.7).

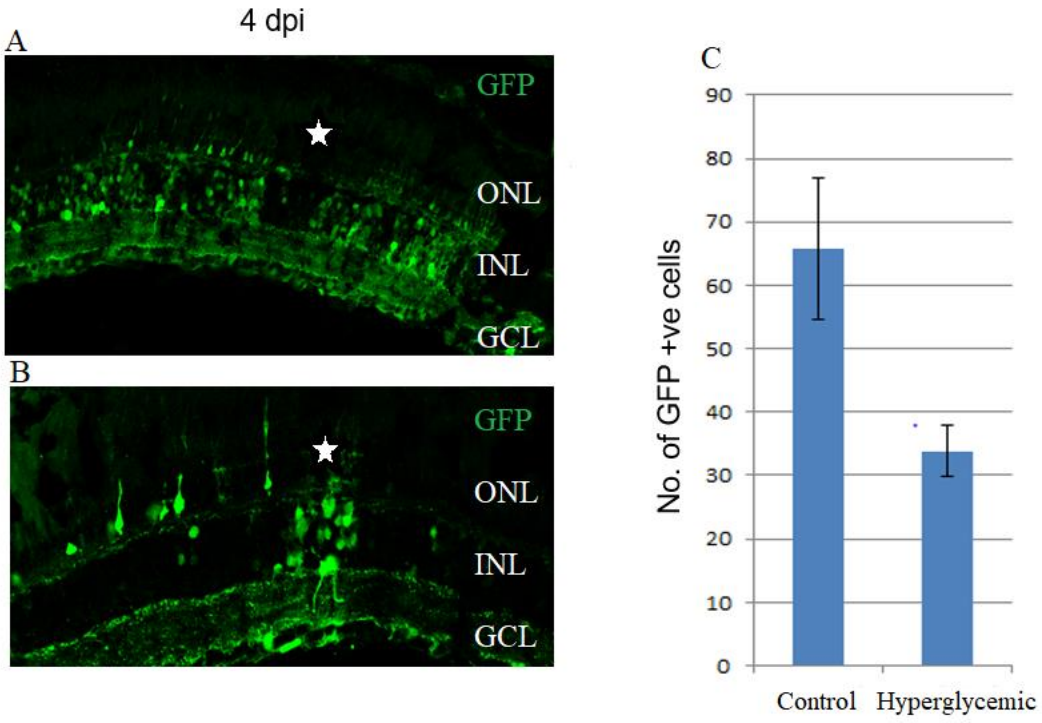


Fig3.7: Effect of hyperglycemia in overfed 1016tuba1a: GFP transgenic zebrafish: In comparison to Control (A), the number of GFP+ve cells has been seen to reduce after 4dpi (B). (C) GFP+ve cell count data, average cells for control:65, hyperglycemic:33.

3.6: Effect of hyperglycemia in the expression pattern of regeneration associated genes

In order to understand the effect of hyperglycemia in retinal regeneration. We tried to identify the change in expression pattern of some regeneration associated genes like Zic2b, Mmp9, Mycb, Insm1a, Her4.1 and dkk1b for hyperglycemic fish retina by using RT-PCR and qPCR data. We found that Mycb expression was maximum at early stages of regeneration in control retina (peak at 6hpi), and almost same early expression of Mycb was observed in hyperglycemic retina (peak at12hpi). Expression of Mmp9 was maximum at 1 dpi for control retina and for hyperglycemic retina, Mmp9 was expressed maximum at 4dpi. Zic2b was expressed in maximum at late stage of regeneration (peak at 4dpi) for control retina but Zic2b expression was maximum at 12 hpi, which is earlier than the control retina. Dkk1b expression for control retina was maximum at 1dpi, while Dkk1b expression for hyperglycemic fish was maximum at 4dpi. For Her4.1, control

retina showed late expression at 4dpi, while hyperglycemic retina showed an early expression at 12hpi. Finally, Insm1a expression peaked at 4dpi and started decreasing after that for control retina but in hyperglycemic retina, Insm1a expression continued to peak at late stage of regeneration (Peak at 7dpi). This result was further confirmed using qRT-PCR (fig:3.9). These shifts in expression of genes can be due to high glucose.

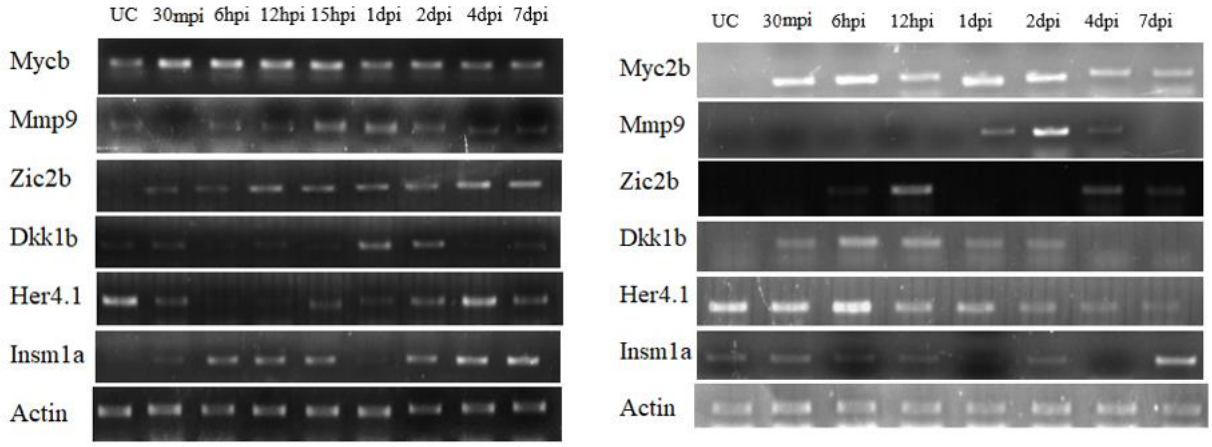
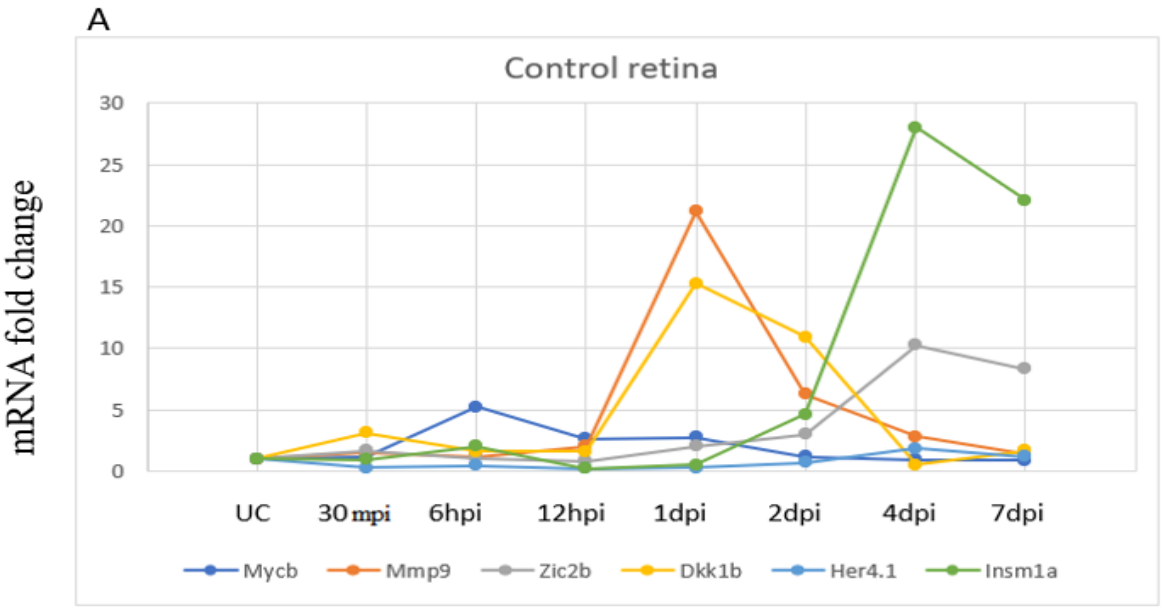


Fig:3.8: Changes in expression pattern of regeneration associated genes RT-PCR: (A)RT-PCR showing expressions of various genes in control and hyperglycemic retina (B) at various time points, starting from 30 mpi to 7dpi.



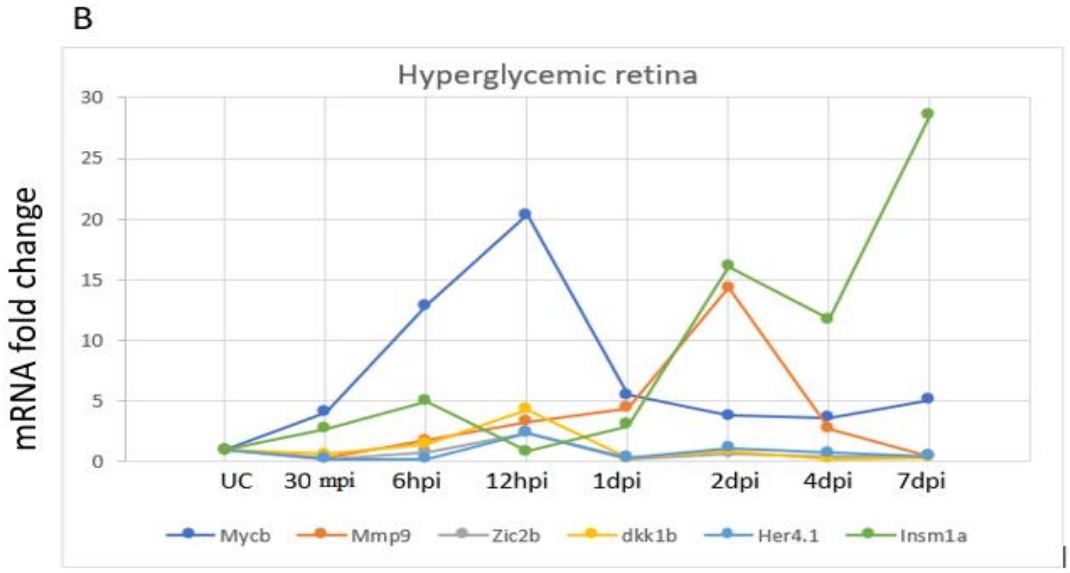


Fig3.9: mRNA levels of various regeneration associated genes: (A)qPCR data showing mRNA levels of various genes in control and hyperglycemic retina (B) at various time points, starting from 30 mpi to 7dpi.

3.7: High sugar diet causes acute hyperglycemia in zebrafish

In order to study the impact of glucose rich diet in regeneration, we performed regeneration experiments on high sugar diet fish model. Most of the results were similar to that of the diabetic ones.

Fishes fed with high sugar diet 4 times a day throughout a week has developed acute hyperglycemia, when compared to the control fishes, which has been fed with normal fish food. The fasting blood glucose level of these fishes were higher than the previous diabetic fishes (fig 3.10).

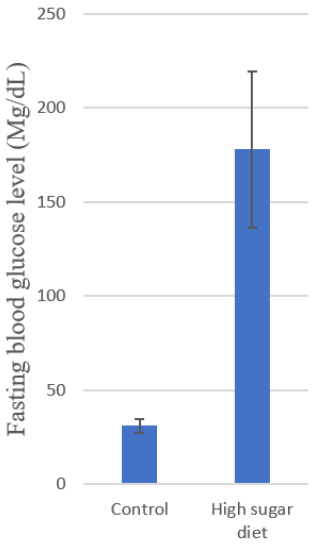


Fig 3.10: Effect of high sugar diet in zebrafish: High sugar diet showing acute hyperglycemia in experimental fishes. Control: 31mg/dL, High sugar diet: 178mg/dL

3.8: Limb regeneration impaired in high sugar diet fishes

The figure below shows that reduction in blastema formation for high sugar diet fish when compared to control fish fed with normal fish food. We got similar results for the T2DM fishes. The reduction in the blastema formation can be linked to the increased glucose levels of the fish.

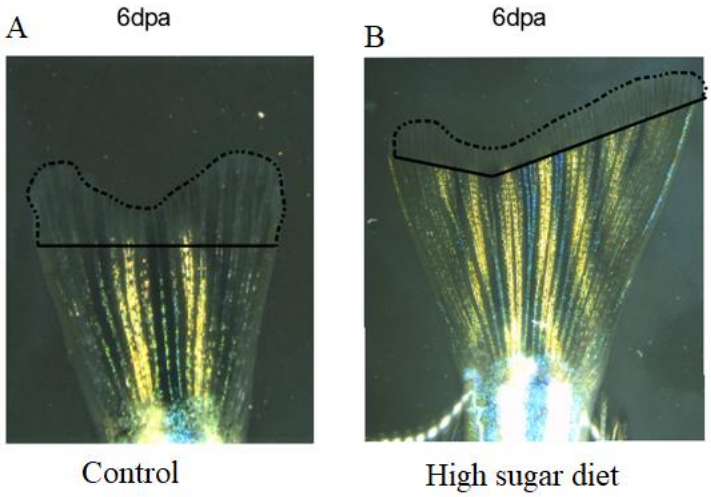


Fig3.11: Effect of high sugar diet on zebrafish fin regeneration: (A) Blastema formation in control fish's fin. (B) Fish fed with high sugar diet showed reduction in blastema formation after 6dpa.

3.9 Retinal regeneration impaired in high sugar diet fishes

Unlike the diabetic model, High sugar diet fishes showed a drastic reduction in PCNA+ve cells. Again, the increased amount of glucose in blood from the consumption of sucrose rich food, can be the possible reason for this result. The reduction in PCNA+ve cells was further confirmed with cell counting data.

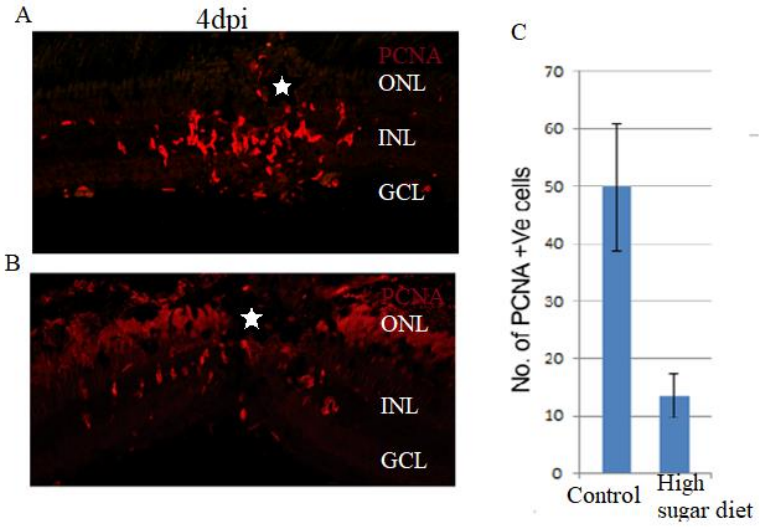


Fig3.12: Effect of high sugar diet on zebrafish retinal regeneration: (A) Increased number of PCNA+ve cells in control retina (B) reduction in PCNA+ve cells in High sugar diet retina. (C) PCNA+ve cell count: for control:49, high sugar diet:13.

3.10 Change in the reduction of Proliferating cells when feeding frequency was changed in high sugar-diet fishes

Reduction in PCNA+ve cells, when feeding frequency of high sugar diet was increased. When feeding frequency of high fat diet was increased from one time a day for one week (S1) to 4 times a day for one week (S4), a slight reduction in the PCNA+ve cells can be seen. Below graph shows the reduction in PCNA+ve cell number, as feeding frequency of high sugar diet is increased. This result suggest that the amount of glucose intake may have role in the retinal regeneration capability of zebrafish after injury.

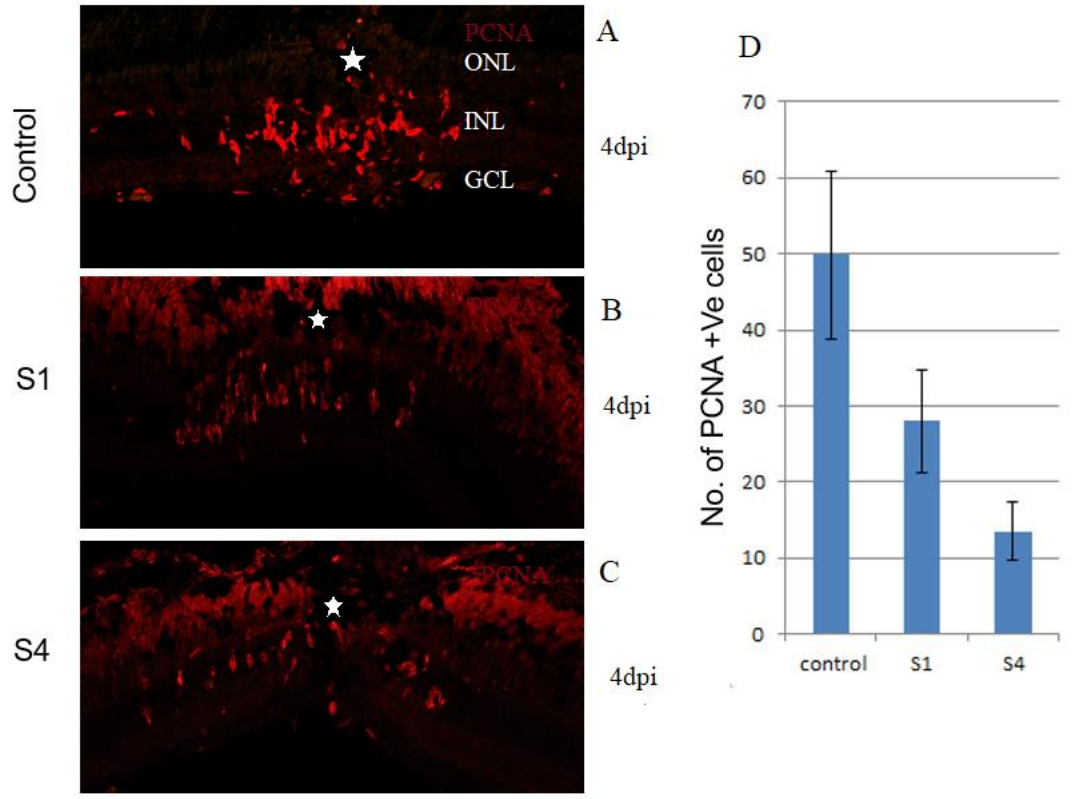


Fig3.13: Effect of High sugar diet frequency in retinal regeneration: S1 indicates fishes fed with high sugar diet, one time a day for 1 week, S4 indicates fishes fed with high sugar diet, 4 times a day for 1-week time interval. (A) Control retina, showing maximum regenerative cells, which was fed with normal fish food. (B and C): High sugar diet fishes fed for 4 times a day (s4) showing relatively fewer proliferating cells, when compared to S1. (D): Number of PCNA+ve cells Average for, control: 49, S1:28, S4:13.

CONCLUSION

In this study we had successfully developed a T2DM model zebrafish through overfeeding method, which showed various features and complications of that of a T2DM patient. Among them are Obesity, hyperglycemia, impaired wound healing capacity and ulcer development. We have shown that overfeeding wild type zebrafish from larval stage can induce obesity in later adult stage. It is important to note that fishes with obesity had shown increased fasting blood glucose level (hyperglycemia), which can be due to obesity. Obesity causes insulin resistance, which leads to decrease in uptake of blood glucose by organs like liver, adipose tissue and skeletal muscles, thus hyperglycemia. We also found that hyperglycemic fishes showed reduced blastema formation at 6dpa, as well as slow wound healing response even at 15dpw. We were also able to find ulcer developments in some of the adult hyperglycemic fishes. Complications like slower wound healing response, reduced blastema formation and ulcers can be because of diabetic neuropathy, developed due to hyperglycemia. All the above results suggest that our experimental fish groups have T2DM. So, we proceeded to investigate our main goal, that is to study the effect of hyperglycemia in retinal regeneration. 4 days post injury in hyperglycemic fish retina showed a reduction in PCNA+ve cells when compared to normal fish's injured retina. We were also able to find, reduced GFP+ve cells in hyperglycemic 1016tuba1a: GFP transgenic zebrafish. We were also able to find noticeable changes or shifts in the expression of some of the regeneration associated genes induced after retinal injury. This shifts in expression of most of the genes can be because of the effect of increased blood glucose level in the regions of retina. Further studies are needed to fully understand the effect of increased glucose in various pathways of zebrafish retinal regeneration. In another study, we were able to develop an acute hyperglycemic zebrafish model by feeding adult fishes with high sucrose food 4 times a day for a week. The hyperglycemic fishes developed through this method also showed impaired fin regeneration and retinal regeneration. Drastic reduction of PCNA+ve cells at 4dpi were seen in fishes fed with high sucrose rich food. We were also able to find increase in impairment of retinal regeneration with increase in feeding frequency of sucrose rich food.

In conclusion, we were able to develop T2DM zebrafish model using overfeeding method and were able to identify that increase in blood glucose level can damage or affect many regeneration processes like wound healing, limb regeneration and mainly retinal regeneration. Further study is needed to fully understand the effect of high glucose in retinal regeneration process.

FUTURE PERSPECTIVE

Investigation of various regeneration associated pathways that gets affected due to the high blood glucose level can open up many possibilities in the field of regeneration and diabetes biology, which can further help in creating effective therapies to treat diseases like diabetic retinopathy. In depth studies in regeneration associated signaling pathways would give a clear idea about the reason why we saw impairment of retinal regeneration in hyperglycemic fishes. RNA In-Situ hybridization of various regeneration associated genes are unexplored in this experiment. Investigation in pancreas of hyperglycemic fishes can give us information regarding beta cells. Effects of drugs like metformin (which are used as a medication for the treatment of T2DM) in hyperglycemic fishes are yet to be studied. Epigenetic aspects of retina regeneration with respect to hyperglycemia remains largely unknown and unexplored. Studying major signaling pathways of fin regeneration would help us to understand more about the influence of high blood glucose in limb regeneration. In addition, one can also explore how hyperglycemia affects various brain, heart and pancreas regeneration in zebrafish.

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PRIMERS USED

actin	RT	F: GCAGAAGGAGATCACATCCCTGGC
actin	RT	R: CATTGCCGTCACCTTCACCGTTC
her4.1	RT	F: GCTGATATCCTGGAGATGACG
her4.1	RT	R: GACTGTGGGCTGGAGTGTGTT
mmp9	RT	F: GGAGAAAACTTCTGGAGACTTG
mmp9	RT	R: CACTGAAGAGAAACGGTTTCC
mycb	RT	F: AGTAGTGACAGCGAATCCGATGACG
mycb	RT	R: ATGTGGCTCTCGAATTTAATCCGC
Her4.1	RT	F: GCTGATATCCTGGAGATGACG
her4.1	RT	R: GACTGTGGGCTGGAGTGTGTT
Zic2b	RT	F: CGCGGGTGTAGTGTCTTTACGCATTC
Zic2b	RT	R: GGGCACTTAAGGATCCCCGAAAATAC
dkk1b	RT	F: AATGACCCTGACATGATTCAGC
dkk1b	RT	R: AGGCTTGCAGATTTTGGACC
Insm1a	RT	F: GAAGGTCAATCTCCGAGTCTGAAATCC
Insm1a	RT	R: GCAGACGTATCTTGGTACAGAATCCAG