Diabetic Retinopathy:
Examining Pericyte-Endothelial Ratios Using Zebrafish and Rat Retinas

Laboratory Research and Background Literature Review

by

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I. Background: Diabetic Retinopathy

Diabetic retinopathy is the most common complication of diabetes mellitus, commonly referred to as diabetes – affecting up to 90 percent of individuals who have diabetes more than 10 years – and is the leading cause of adult blindness.[1] Hyperglycemia, or high blood glucose (sugar), characterizes diabetes. Hyperglycemia is a result of nonexistent or insufficient insulin production or non-receptiveness of cells to insulin. In type 1 diabetes, often referred to as juvenile diabetes, the body does not produce insulin – an individual's immune system destroys β-cells in the pancreas thereby inhibiting insulin production.[2] In type 2 diabetes, the most common form of diabetes, either the body produces too little insulin or the cells ignore the insulin.[2] Gestational diabetes occurs in women usually at 28 weeks of pregnancy or later.[2]

Insulin is a hormone that binds to cells to make them receptive to uptake of glucose from the blood. The pancreas releases insulin after an individual ingests sugars. Through metabolic processing, cells transform glucose into ATP (adenosine triphosphate) – the energy form necessary for cellular processes. In diabetes, without insulin or cell receptiveness of insulin, cells cannot uptake glucose and therefore cannot produce ATP.

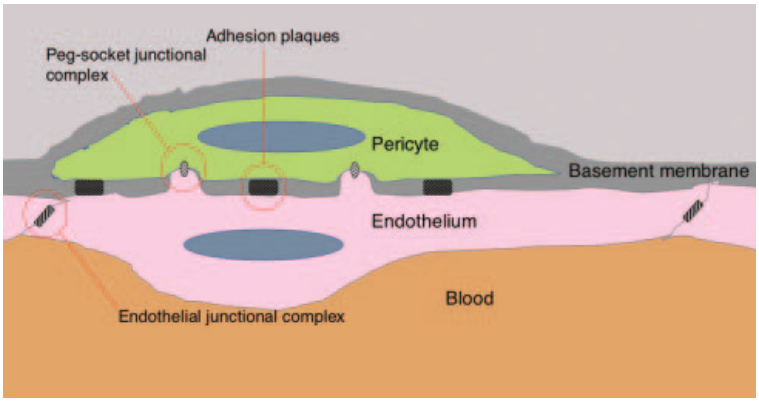
Diabetic retinopathy is diabetic-induced damage to the retina. It has two principal stages: nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). [3] Nonproliferative diabetic retinopathy can be subdivided into early NPDR – characterized histopathologically by pericyte loss and capillary basement membrane thickening and ophthalmoscopically by microaneurysms – and advanced NPDR.[3] Proliferative diabetic retinopathy (PDR) is characterized by neovascularization, or the proliferation of new blood vessels.[3]

II. Introduction: Endothelial-Pericyte cell interactions

Hyperglycemia causes changes in retinal vasculature (blood vessels) impairing vision and eventually resulting in blindness. The research focuses on two cells associated with the walls of vasculature: endothelial cells and pericyte cells. Normally both cells are found in about a 1:1 ratio. Loss of both cells due to aging is normal but significant loss of more pericyte cells than endothelial cells occurs diabetic retinopathy.

Endothelial cells form the inner lining of all blood vessels in the body. They provide an anticoagulant barrier between the vessel wall and blood. Pericyte cells are vascular mural cells embedded within the vascular basement membrane of microvasculature (i.e., capillaries, arterioles, and venules).[4] Endothelial cells and pericytes directly contact each other at certain points (Figure 1).[4] Pericytes are necessary components of microvasculature regulating development, stabilization, maturation, and remodeling.[4]

Figure 1: Pericyte-Endothelial physical interaction[4]



Loss of pericytes and the thickening of the capillary basement membrane are the earliest abnormalities seen histopathologically in diabetic retinopathy.[3] Great loss of pericytes results in shunting, or limiting blood flow through only a few capillaries. Microaneurysms occur as a result of weakened vasculature walls due to the loss of pericytes. Endothelial proliferation can occur in the few vessels receiving all the blood flow. Vessels not receiving blood flow lose all of their

remaining cells and cease to serve in a circulatory capacity.[5] Pericyte cells also seem to prevent neovasculogenesis and so without them new, unnecessary vessels form.

The research examines retinal vasculature of hyperglycemic zebrafish (*Danio rerio*) to observe pericyte and endothelial cells and their comparative ratios.

III. Methods

In 1960, Kuwabara and Cogan discussed "a new method of preparation in which the nonvascular components of the retina are digested prior to staining."[6] The new method was a 3% trypsin digest (0.3 grams of trypsin in 10 mL PBS) utilized in this project. Only fresh trypsin digest (only up to a week-old) was used. Periodic acid-Schiff (PAS) staining (no need for prior injection), with hemotoxylin as the counterstain, proved most effective for visualization of vessel walls and their cells' nuclei and thus was utilized for this project.[4]

This study utilized retinas of 8 previously dissected zebrafish eyes in paraformaldehyde fix (Fish 7, 8, 9, 10, 12, 13, 16, 17). The zebrafish were "hyperglycemic" – for 28 days prior to their sacrifice, the zebrafish were alternated every 24 hours between a (Deer Park) water environment and a 2% glucose environment.[7] One previously dissected rat eye was also used. The rat was not hyperglycemic.

The preparation for microscopy of each retina was a three-day process.

Day 1: Zebrafish

The dissected eye was removed from fix and placed under a dissecting microscope. The sclera was held by tweezers in order stabilize the eye. The center of the cornea was directly punctured using small surgical scissors and a radial incision was made. The lens was pushed out using a small probe. Three more evenly spaced radial incisions were made. The eye was flattened out. Using a small probe, the retina was gently and placed in a slide chamber.

The isolated retina was rinsed with 3 mL PBS (phosphate buffered saline) for 3 minutes. The chamber slide was shaken slightly so as to agitate the mixture. The PBS was removed and 3 mL trypsin digest (0.3 grams of trypsin in 10 mL PBS) was added. The chamber slide was left overnight on a stirrer.

Day 1: Rat

The dissected eye was removed from fix and placed under a dissecting microscope. The sclera was cut away from the eye. The center of the cornea was directly punctured using small surgical scissors and a radial incision was made. A circumferential cut was made. The lens was removed. Four side radial incisions were made. The eye was flattened out. Using a small probe, the retina was gently and placed in a well, with a cover slip on the bottom, of a six-welled plate.

The isolated retina was rinsed with 3 mL PBS (phosphate buffered saline) for 3 minutes. The chamber slide was agitated slightly. The PBS was removed and 3 mL of trypsin digest (0.3 grams of trypsin in 10 mL PBS) was added. The plate was left overnight on a rocker.

Day 2

The tryspin digest was removed. The retina was rinsed with 3 mL distilled water for 5 minutes. The water was removed. Sometimes at this point the retina was examined under the dissecting microscope to remove tissue that appeared to not be retinal. Three mL 0.5% periodic acid (0.05 grams of periodic acid in 10 mL of water) was added for 5 minutes. The periodic acid was removed and the retina was rinsed with distilled water for 3 minutes. The water was removed and 3 mL was added for 30 minutes. The Schiff's reagent was removed. The retina was washed in 2 mL luke warm Deer Park water for 5 minutes. The mixture was agitated and water was exchanged halfway through the 5 minutes. The retina was counterstained in 2 mL hemotoxylin for 3 minutes. The hemotoxylin was removed. The retina was washed in 2 mL luke warm Deer Park water for 5 minutes. The mixture was agitated and water was exchanged halfway through the 5 minutes. The retina was allowed to air dry overnight

Day 3: Zebrafish

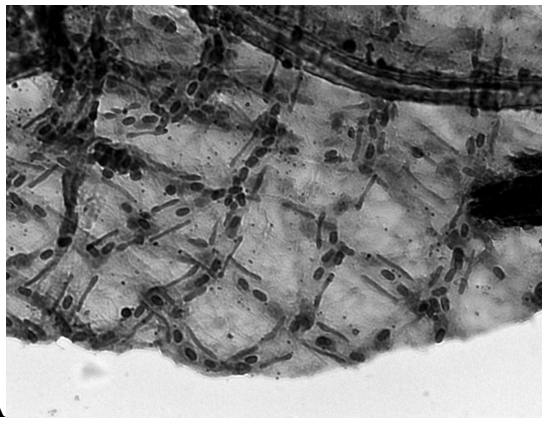
The chamber portion of the chamber slide was removed. Mounting gel was placed on the retina (mounting gel with DAPI for fish 10, 13, 16). The slide was cover-slipped and allowed to sit for 5 minutes before examination under the microscope.

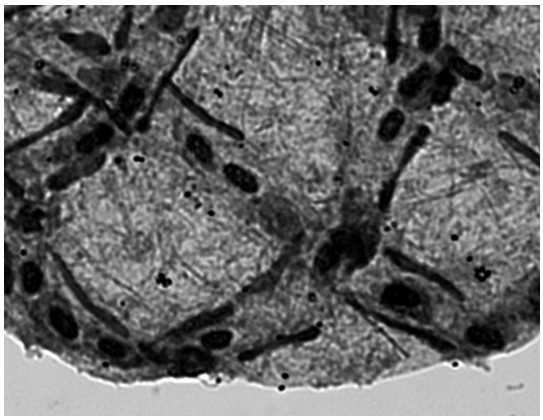
Day 3:Rat

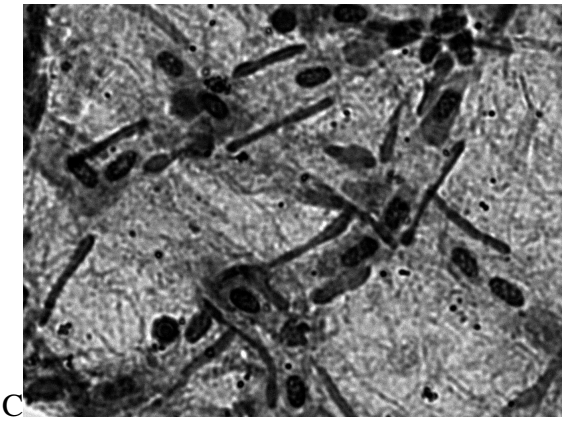
The cover slip at the bottom of the well was carefully removed. Mounting gel was placed on the retinal pieces on the cover slip. The cover slip was placed on a slide and allowed to sit for 5 minutes before examination under the microscope.

III. Results

Figure 2*: Control Zebrafish Vasculature

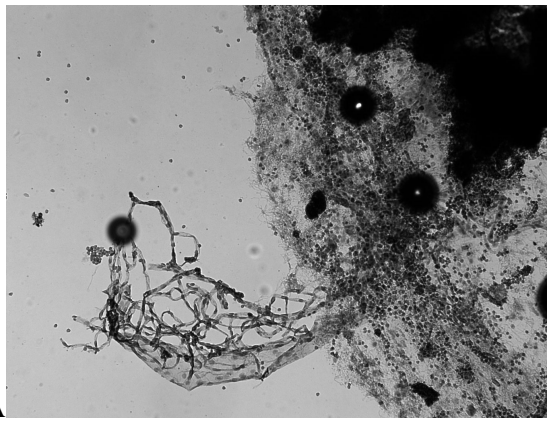
A 

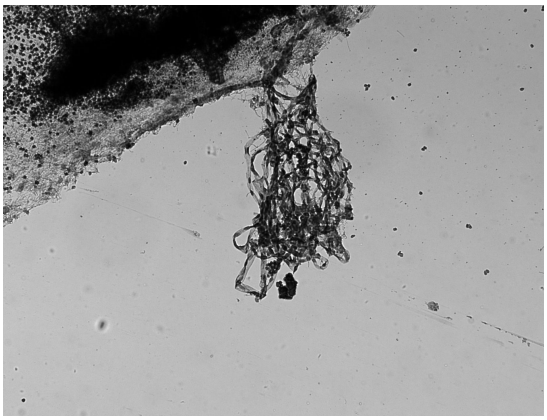
B 

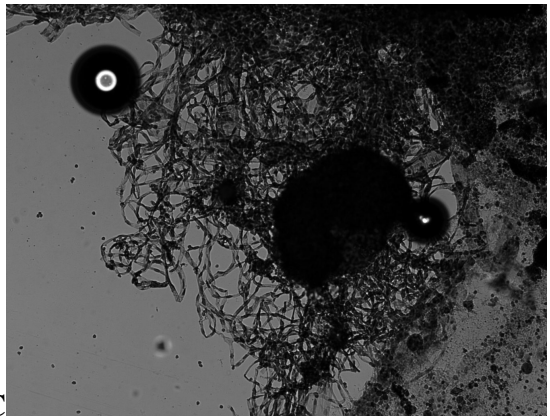
C 

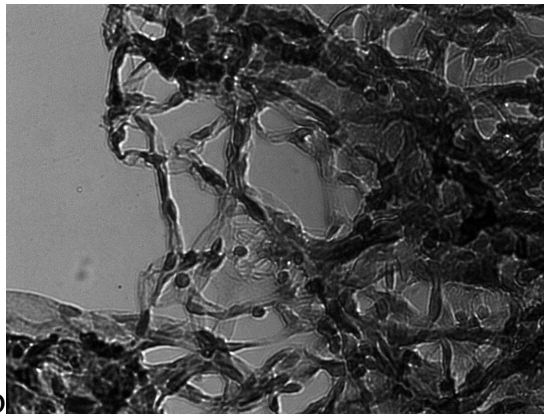
These images are of control (non-hyperglycemic) zebrafish vasculature. The endothelial cells are oblong and lighter than the pericytes which are oval and dark.[5] The cells make a clear pattern. A shows a piece of the vasculature seen at 40x magnification under Brightfield microscopy. B and C are both 100x magnification of different areas of the vasculature seen in A.

Figure 3*: Control Rat Vasculature

A 



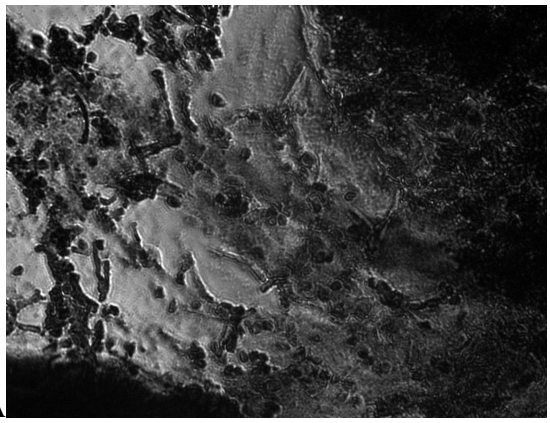
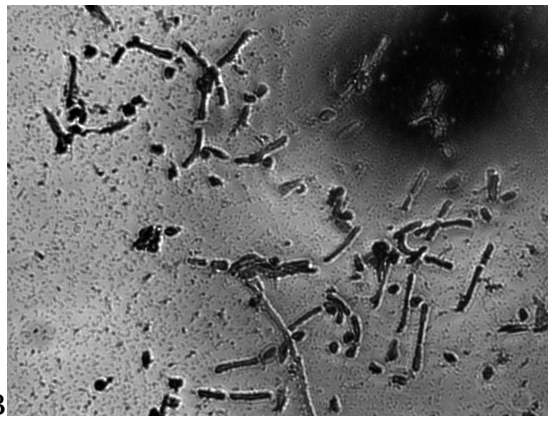
C 

D 

These images are of control (non-hyperglycemic) rat vasculature (all from the same rat). The endothelial cells are oblong and lighter than the pericytes which are oval and dark.[5] These images are very similar to human retinal vasculature tissue.[5,6] A-C show vasculature at 10x magnification under Brightfield microscopy. D is at 40x magnification

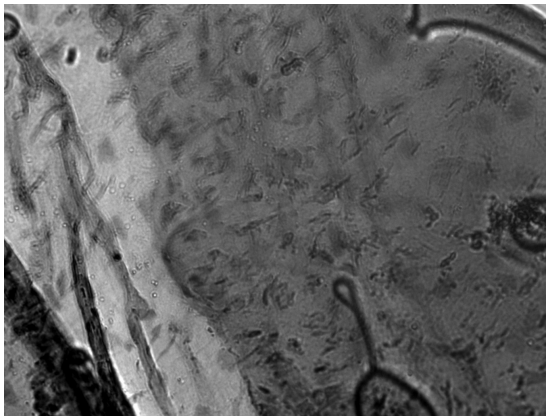
* Credit to Ruth Burley, MS Biology candidate at American University

Figure 4: Experimental Zebrafish Fish 17

A  B 

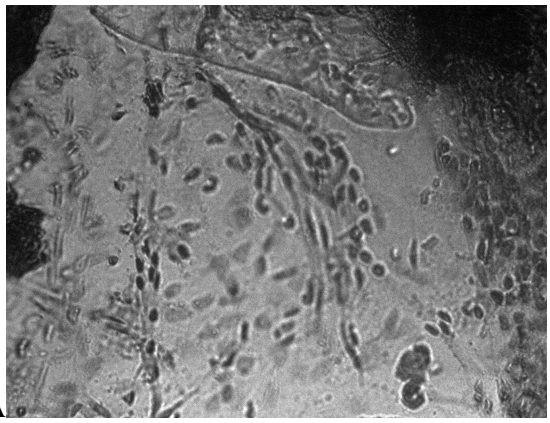
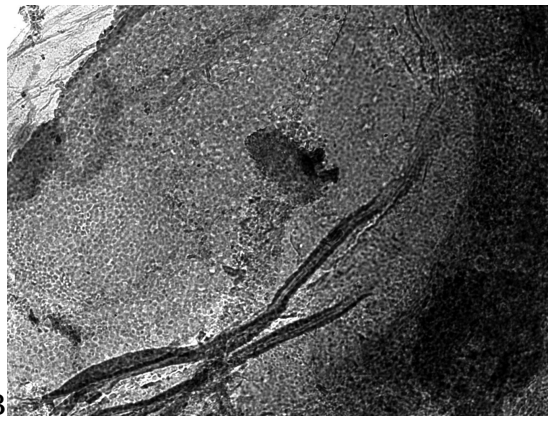
These images are of experimental zebrafish 17 vasculature. A and B are at 40x magnification under Brightfield microscopy. Neither A nor B show the clear pattern seen in Figure 2 or the lone vasculature without surrounding tissue seen in Figure 3. It appears the oblong, lighter cells are probably endothelial cells and the oval, dark cells are pericytes.[5] Cells in A and B appear indiscriminately placed. Cells in B appear to be free floating.

Figure 5: Experimental Zebrafish Fish 9



This is an image of experimental zebrafish 9 tissue at 40x magnification under Brightfield microscopy. The cells seen may be pericyte ghosts.

Figure 6: Experimental Zebrafish Fish 13

A  B 

There are images of experimental zebrafish 13 tissue. A is at 40x magnification under Brightfield microscopy and appears to be vasculature. B is at 10x magnification under Brightfield microscopy and displays small, compacted cells that were seen in isolated tissue of several experimental zebrafish.

V. Discussion

The diabetes epidemic in the US and in other developed countries necessitates greater pathophysiological understanding of the disease and its complications. Studies of histopathological disruptions – before the visible manifestation of diabetes' complications – require model organisms that can be dissected to examine the tissue at differing stages of the disease.

Model Organisms

Zebrafish are an established model of biological research – used as a classical development and embryological model beginning in the 1930s.[8] Their high fertility rate and simple living requirements make them conducive to laboratory study.[7] Due to their clear embryos and larvae and relatively quick development time, zebrafish are the chosen model for developmental biology.[6] Traditionally, preferring mammals because of greater structural and organ system similarity to humans, genetics studies have utilized mice or rats as model organisms.[6] However, since the sequencing of the complete zebrafish genome, zebrafish too have been used in genetics and mutation studies.[6] Increasingly, zebrafish are being used a model organisms for studying human disease pathogenesis such as cancer.[6]

Published research studying pericytes and their normal loss due to aging and abnormal loss due to diabetes began in 1960.[6] Studies from this time used human retinas; however, few institutions are able to obtain human tissue due to regulations and expense. In addition, human retinas in the primary stages of diabetic retinopathy – before observable manifestation of the disease – would be difficult to obtain since diabetes alone at this stage is not fatal. Therefore, the retinas of rats, dogs, cats, and mice, to a smaller extent, have been used to observe

histopathological changes as a result of diabetes. However, these changes have not been studied in zebrafish even though zebrafish are an established model in visual studies.[9]

Since zebrafish can be induced to become hyperglycemic, they have been proposed as a model organism in studying diabetic retinopathy. [5] Evidence suggests teleost (ray-finned) fish, particularly zebrafish, have β–cells or β–cell-like cells that produce insulin homologous to human insulin.[7] Like all teleost fish, zebrafish are considered glucose intolerant, referred to as pre-diabetes in humans, meaning high blood glucose levels are not transient but persist and can lead to chronic hyperglycemia.[10]

Today researchers study diabetes using selectively bred rodents, such as the non obese diabetic (NOD) mouse or bio breeding (BB) rat, who spontaneously develop the disease or drug injection, usually alloxan or streptozotocin, to destroy β-cells in order to induce hyperglycemia.[11] In contrast, inducing hyperglycemia in zebrafish only requires manipulation of their environment – not genetic or chemical modification. Water is constantly entering zebrafish though their gills. Since zebrafish live in freshwater, they have a higher salt contraction than their habitat and are constantly uptaking water – any solutes in the water also enter the fish..[7] Therefore, placing zebrafish every other day for a month in a 2% glucose solution for a month induced hyperglycemia. After the month, thinning of the inner plexiform layer (IPL) and inner nuclear layer (INL) were observed – this change suggested the hyperglycemia was persistent enough to cause significant tissue alteration.[7] Researchers did find a large range of blood glucose levels within the same experimental group.[7] Since average glucose levels remained at the hyperglycemic level and the amount of IPL thinning was similar in all the experimental fish researchers deemed zebrafish genetic variability as explanation for the range in blood glucose levels.[7]

Pathogenesis of Pericyte Loss

Hyperglycemia-induced pericyte apoptosis has been shown *in vivo* and *in vitro.*[12] Pathogenesis, or how diabetes specifically, at the molecular level, causes pericyte loss, during early diabetic retinopathy is unknown.[8] One hypothesis suggests accumulation of toxic products such as sorbitol or advanced glycation end products (AGEs) by the pericyte may lead to its loss.[8] Pericyte loss leaves capillaries weakened and allows for microaneurysm formation.[8] In addition to basement membrane thickening, there is also an increase in acellular-occluded, meaning closed, capillaries.[8] Pericyte loss, acellular- occluded capillaries, capillary basement thickening and microaneurysms, of early nonproliferative diabetic retinopathy set the stage for retinal hypoxia, or insufficient blood oxygen, leading to retinal hemorrhage and cotton-wool spots in advanced NPDR and eventually neovascularization in proliferative diabetic retinopathy.[3]

Researchers believe the activities of many elements in the retina leading to diabetic retinopathy development are stimulated by hyperglycemia. The vascular endothelial growth factor (VEGF), placenta growth factor and pigment epithelium-derived factor have all been implicated in diabetic retinopathy pathogenesis.[12] VEGF is a strong angiogenic (new blood vessel formation) promoter. Increased retinal VEGF levels have been associated with diabetes.[12] VEGF stimulates endothelial cells to degrade their extracellular matrix, move, replicate and form new blood vessels.[12]

Increased blood flow and blood flow distribution disparities are observed before histopathological changes; therefore, researchers are also studying the two classes of factors that regulate retinal blood flow: endothelium-derived relaxing factors (i.e., nitric oxide) and endothelium-derived contracting factors (i.e., endothelin).[12] The former inhibit smooth muscle cells and pericytes presumably allowing for unrestricted blood flow.[12] The latter stimulate

smooth muscle cells and pericytes presumably allowing for blood vessel constriction resulting in limiting blood flow.[12]

Platelet-derived growth factor PDGF-B plays a significant role in pericyte recruitment.[8] Measurements of pericyte numbers in nondiabetic and diabetic wild type and PDGF-B $^{+/-}$ mice (heterozygous for PDGF-B deletion – homozygous deletion mice were not viable) found 30% reduction in pericyte number in nondiabetic PDGF-B $^{+/-}$ mice compared with nondiabetic wild type mice.[8] However, there was only a slight increase in acellular capillaries and no observed microaneurysms in nondiabetic PDGF-B $^{+/-}$ mice compared with nondiabetic wild type mice. [8] Diabetic wild type and diabetic PDGF-B $^{+/-}$ mice experienced pericyte number reduction with greater than 50% loss in diabetic PDGF-B $^{+/-}$ mice. [8] Diabetic wild type mice showed a 2.5-fold increase in acellular capillaries, but no other abnormalities, compared to nondiabetic wild type mice. [8] Diabetic PDGF-B $^{+/-}$ mice showed a 3.5-fold increase in acellular capillaries compared to nondiabetic PDGF-B $^{+/-}$ and displayed microaneurysms. [8] This data confirms current understanding of large pericyte loss leading to other pathological capillary defects. [8]

Suggestions for future experiments

Zebrafish and rat retinal isolation required great care. Extracting retinal tissue from adjacent eye tissue, such as the pigment epithelium proved challenging. Erring on the side of caution, all tissue resembling retina, which has a “booger-like” appearance, as well as non-retinal vasculature not easily separated from the retina was incubated in the trypsin digest. In theory the trypsin digest should digest all nonvascular elements of the retina. However, all the stained tissue appeared to have more than just retinal vasculature and sometimes more than just retinal tissue. Even before examination under the microscope, under naked eye observation much of the tissue appears very dark. At times this is due to folding of more than a single tissue layer.

Much of the microscope examination revealed tissue that did not fit the ideal retinal vasculature isolations (Figures 2 and 3). The majority of tissue was too dark to observe. Cells resembling pericytes and endothelial cells were observed (Figures 4, 5, and 6A). Some cells greatly resembled pericytes and endothelial cells but there was not organization to their location (Figure 4). A significant amount of cells that did not resemble pericytes and endothelial cells was observed (Figure 6B).

With further practice, retinal isolation skills can be improved hopefully yielding better tissue results. The use of cell markers to identify pericyte and endothelial cells would be helpful in alleviating the uncertainty of the cell types being examined.

Conclusion

Research suggests strict glycemic control can delay and perhaps even prevent ~~the~~ diabetic retinopathy development.[12] In rats, for the first six weeks after it induction, hyperglycemia did not elicit pathological changes.[12] However, immediately after this point endothelial cell proliferation and swollen retinal vessels were observed therefore molecular-level changes must be occurring during the first six weeks of hyperglycemia.[12] Very early control of diabetes, preferably in the pre-diabetic stage, should delay the onset of diabetic retinopathy.

Acknowledgments

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References

[1] Feit-Leichman RA, et al. Vascular Damage in a Mouse Model of Diabetic Retinopathy: Relation to Neuronal and Glial Changes. *Investigative Ophthalmology and Visual Science*. 2005;46:4281-4287
[2] American Diabetes Association. <http://www.diabetes.org>
[3] Rosenblatt BJ and Benson WE. Chapter 30: Diabetic Retinopathy.
[4] Armulik A, Abramsson A, and Betsholtz C. Endothelial/Pericyte Interactions. *Circulation Research*. 2005;97:512-523.
[5] Kuwabara T and Cogan DG. Retinal Vasculature Patterns: VI: Mural Cells of the Retinal Capillaries. *Archives of Ophthalmology*. 1963;69:492-502
[6] Kuwabara T and Cogan DG. Studies of Retinal Vascular Patterns: Part 1. Normal Architecture. *Archives of Ophthalmology*. 1960;64:904-911.
[7] Gleeson M, Connaughton V, Arneson LS. Induction of hyperglycaemia in zebrafish (*Danio rerio)* leads to morphological changes in retina. *Acta Diabetological*: 2007;44:157-16.
[8] Hammes HP, et al. Pericytes and the Pathogenesis of Diabetic Retinopathy. *Diabetes*: 2002;51: 3107-3112.
[9] Bilotta J and Saszik S. The zebrafish as a model visual system. *International Journal of Developmental Neuroscience*. 2001;19:621-629.
[10] Moon T. Glucose intolerance in teleost fish: fact or fiction?. *Comparative Biochemistry and Physiology Part B*. 2001;129:243-249.
[11] Rees DJ and Alcolado JC. Animal models of diabetes mellitus. *Diabetic Medicine*. 2005;22:350-370.
[12] Cai J and Boulton M. The pathogenesis of diabetic retinopathy: old concepts and new questions. *Eye*. 2002;16:242-260.