AN EXAMINATION OF RETINAL VASCULATURE IN THE EARLY DIABETIC MOUSE

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Abstract:

Diabetic retinopathy, an eye disease caused by the long-term hyperglycemia that is characteristic of diabetes mellitus, is the leading cause of blindness in adults in the United States of America (CDC, 2013). With the increasing prevalence of diabetes mellitus, especially in younger adults, diabetic retinopathy is becoming a more-widespread problem. There are no cures for this form of retinopathy. In fact, the treatment options that are currently available, including surgical vitrectomy, anti-VEGF injections, and intraocular steroid injects, are not possible in the early stages of the disease, do not eliminate the ultimate risk of blindness, and come with their own, very serious, risks and side effects. Thus, it is necessary to characterize changes that are occurring in the retina in the early stages of diabetes in order to find possible therapeutic targets that will help to prevent blindness. This study focuses on the characterizing the vasculature of the retina in STZ-treated mice (a model for type 1 diabetes mellitus) compared to control mice. The lengths and diameters of capillary branches in each of three vascular plexuses were measured and analyzed for both conditions, which revealed no significant differences, neither qualitative nor quantitative.
INTRODUCTION/SIGNIFICANCE:

It is reported that 29.1 million people in the United States (9.3% of the population) have diabetes mellitus, including both type I and type II (National Diabetes Statistics Report, 2014). A common complication of both types of diabetes mellitus is diabetic retinopathy, which affected close to 5 million Americans in 2013 and is the leading cause of blindness in adults in the United States (CDC, 2013). Diabetic retinopathy is a progressive eye disease caused by changes in the vasculature of the retina (Figure 1), which result from the long-term hyperglycemia that is a feature of diabetes mellitus. The first stage of diabetic retinopathy is mild nonproliferative retinopathy, which is characterized by microaneurysms occurring in the retina. The second stage is moderate nonproliferative retinopathy, characterized by retinal blood vessel blockage, followed by severe nonproliferative retinopathy, in which there is an increase in the number of occluded blood vessels. The nonproliferative stages of diabetic retinopathy also show increased leakiness of vessels in the retina, vascular dilation, and macular edema, all of which can lead to blurred vision (Erikson et al., 2007). The fourth and final stage of diabetic retinopathy is proliferative retinopathy in which there is growth of new blood vessels and more pronounced leakiness and occlusion, which can ultimately lead to blindness of the subject (NIH, 2012).

Figure 1 – Stages of Diabetic Retinopathy
The progression of diabetic retinopathy has been well studied in humans, but it is difficult to assess microvascular changes that occur prior to the onset of retinopathy because quantifying and measuring live retinal capillaries requires the use of Fluorescein angiography, which is an invasive technique and is thus not clinically justified for use on patients who don’t exhibit signs of retinopathy. It is important, however, to characterize these early microvascular abnormalities because there is evidence that visual changes occur in the early stages of diabetes, prior to the onset of retinopathy, as well (Aung et al., 2013). These early microvascular abnormalities include decreased visual acuity, reduced color and contrast sensitivity, and increased electroretinogram (ERG) response times (for both rod-dominated and mixed rod and cone responses), all of which can occur as early as four weeks after the initial onset of diabetes, considerably prior to the onset of diabetic retinopathy, in humans and STZ-treated rats (Gualtieri et al., 2013; Aung et al., 2013). Studies have shown that these visual changes are not due to neuronal apoptosis in the cell layers of the retina (Moore-Dotson et al., 2015), but further research is necessary to reveal possible therapeutic targets to either prevent or delay the symptoms of diabetic retinopathy. This is extremely valuable as there are currently no curative treatments for diabetic retinopathy. In fact, most available treatments for diabetic retinopathy (including surgical vitrectomy, laser photocoagulation, anti-VEGF injections, and intraocular steroid injections) are not possible in the early stages of the disease and do not eliminate the ultimate risk of blindness (Robinson et al., 2012). Furthermore, these treatment options come with their own serious risks and side effects.
This research study aims to investigate, analyze, and compare the vasculature of the retinas of control mice with that of mice in the early stages of STZ-induced type I diabetes. In this case, ‘early stages’ is defined as six weeks after the initial onset of diabetes (this number was chosen because changes in visual acuity occur at four weeks in STZ-induced diabetic rats, Aung et al., 2013). The retinal vasculature will be qualitatively and quantitatively evaluated through measurements of length and diameter at three different depths (associated with the three vascular plexuses present in the retina). Changes such as an increase in the diameter of capillaries (Burns et al., 2014) and a decrease in the total length of capillaries due to capillary drop out (Tam et al., 2013), defined as a loss of capillaries which leads to ischemia and ultimately neovascularization in the later stages of retinopathy, would not be expected in this study as these occur in the mild nonproliferative stage of retinopathy. However, there could be changes occurring prior to retinopathy onset since there is a significant observed decrease in retinal blood flow after four weeks of STZ-induced hyperglycemia in rats (Yadav et al., 2011; Wang et al., 2010; Wang et al., 2011). This could be caused by a decreased diameter of capillaries or an increase in the total length of capillaries, both of which would reduce the rate of blood flow (Poiseuille’s Law), producing the changes previously seen. There are other possible mechanisms for the observed decrease in retinal blood flow, though, which could have nothing to do with the retina (a defect in systemic blood flow, for instance). Still, it is important to look at the microvasculature in early diabetes to see if there are abnormalities, and if these do cause visual changes, or to rule it out. The big picture of this study, the broader context, is that any changes that are observed and measured in the early stages of diabetes could reflect possible
targets for therapeutic treatment of diabetic retinopathy before blindness occurs in the subject and also reveal early vascular changes that have been previously unobserved in humans.

To study the effects of early diabetes on retinal microvasculature, an appropriate model of diabetes is necessary. In this study, mice with streptozotocin (STZ)-induced diabetes were used. STZ is a compound that induces type I diabetic pathology in the model rodents by causing damage to the β-cells of the pancreas, thus resulting in hyperglycemia and hypoinsulinemia (Graham et al., 2011). Mice treated with multiple, low-dose STZ injections induce hyperglycemia through apoptotic β-cell death mediated by activation of the immune system (Szkudelski, 2001). These models effectively mimic the early stages of diabetic retinopathy, including loss of capillaries and pericytes in the retina, vascular occlusion, and increased vascular permeability (Kern, 2009; Robinson et al., 2012), all of which begin to occur approximately six months after the onset of diabetes (Feit-Leichman et al., 2005). The later stages of diabetic retinopathy, including neovascularization, are not reproducible in these models (Robinson et al., 2012), partially due to the short lifespan of the animals, which does not allow the disease to progress to the proliferative stage. This is not an issue for the purposes of this study, however, as we are primarily interested in the impact of STZ-induced diabetes on changes that occur in the retina prior to the onset of diabetic retinopathy. Visual acuity and ERG tests showed that STZ-induced diabetes in mice effectively causes hyperglycemia and brings about visual abnormalities, including oscillatory potential delays, which mirror the defects seen in humans with diabetes (Pardue et al., 2014).
For the purposes of this study, it is important to understand how the vasculature of the retina arises and how it is structured. The retina is a highly metabolically active tissue so it requires a substantial blood supply, which it receives from two circulations: the choroidal circulation and the retinal circulation (Harris et al., 2013). The choroidal blood vessels, located between the retina and the sclera (Figure 2; Gariano et al., 2004), provide nutrients to the outer part of the retina, including the pigment epithelium, which leaves the outer nuclear layer (ONL), which consists of rod and cone photoreceptor cell bodies, avascular (Harris et al., 2013).

![Figure 2 – Schematic of the Eye](Picture from East Valley Ophthalmology (Cataracts)).

The retinal circulation, which supplies the remaining portion of the retina (Harris et al., 2013), stems directly from the central retinal artery (Erikson et al., 2007). In rodents, the vasculature that makes up the retinal circulation arises postnatally through the mechanism of sprouting angiogenesis, which starts in the first week of life (Okabe et al., 2014). First, the vasculature grows along the ganglion cell layer (GCL) to form the superficial vascular plexus (Figure 3; Okabe et al., 2014). Sprouting then occurs in the same plane of this primary plexus toward the periphery of the retina (Gerhardt et al., 2003) and then vertical sprouts invade the retina to establish a second plexus of vasculature, the deep plexus (Milde et al., 2013). The deep plexus extends through the
inner nuclear layer (INL), which consists of bipolar, horizontal, and amacrine cell nuclei, and slightly into the outer plexiform layer (OPL), which is a region of synapses between dendrites of cells in the INL and axons of rod and cone photoreceptor cell inner segments (Figure 3). A third and final plexus, named the intermediate vascular plexus, is formed between the superficial and deep plexuses (Figure 3), via vertical sprouting through the inner plexiform layer (IPL), which is a region of synapses between dendrites of ganglion cells and axons of cells in the INL. This formation of retinal vasculature is complete after three weeks of life and results in a planar, hierarchical, interconnected, and 3D vascular complex that encompasses the majority of the retina (Milde et al., 2013).

While past empirical studies have focused on the changes in vasculature that result in visual changes that occur in the later stages of diabetes, a comprehensive review of the literature revealed that no one has previously measured retinal vessel length or diameter after the onset of diabetes or in the early stages of the disease. It is
important to investigate any changes that occur in the early stages of diabetes, which could provide valuable insight into the initiation of the pathology of diabetic retinopathy and possible therapeutic targets to delay symptoms of the disease, and ultimately prevent blindness. This study aims to answer the question of whether visual changes and decreased retinal blood flow in early diabetes are caused by abnormalities in the quality, length, or diameter of blood vessels in the retina prior to any vascular changes due to retinopathy (which occur in the later stages of diabetes). It is hypothesized that there will be no qualitative or quantitative differences in these measurements between control mice and STZ-induced diabetic mice.

METHODS:

Mouse Models:

This study included 14 Wild type (WT) C57BL/6J mice, 14 transgenic Gus-GFP-C57BL/6J mice, and 14 transgenic Mito-CFP-P C57BL/6J mice. The transgenic Gus and Mito mice were primarily used in other experiments for their characteristic of specifically labeling subtypes of bipolar cells through expression of GFP and CFP (Breuinger et al., 2011; Schubert et al., 2008; Huang et al., 2003; Lin and Masland, 2005).

Seven mice from each type were injected with a solution containing 75 mg/kg of Streptozotocin (STZ; Sigma; St. Louis, MO) dissolved in 0.01 M sodium citrate while the remaining mice were injected only with a 0.01 M sodium citrate buffer solution when the mice were approximately five weeks old. Each mouse received three intraperitoneal injections over the course of three days (each after fasting for four hours prior to the
injection). The fasting levels of glucose in the blood and urine of each mouse were measured after injections on a weekly basis through tail-tipped blood tests, using an OneTouch UltraMini Monitoring System (LifeScan Inc; Milpitas, CA), and urine glucose strips (YD Diagnostics; Gyeonggi-do, South Korea), respectively. The mice were also weighed on a weekly basis. Mice were sacrificed using carbon dioxide at six weeks post-injection and the final measurements of blood glucose and weight, taken immediately before the mice were sacrificed, were analyzed to see if the mice injected with STZ were diabetic (defined as a blood glucose level above 200 mg/dL) compared to the control (Figure 3). Both the weight and blood glucose were statistically significant, determined using a two-tailed t-test (Microsoft Excel; p < 0.05).

**Figure 4 – STZ injections induce type I diabetes mellitus in mice**

Blood glucose and weight analysis revealed that STZ-treated mice had significantly higher levels of blood sugar (p < 0.05) and significantly lower weight (p < 0.05) compared to controls, indicating that STZ injections did induce diabetes in these mice.

**Immunohistochemistry:**

After sacrificing the mice, their eyes were surgically removed and 4 slits were made into the retina so that it would lay flat (Figure 5). The retinas were then fixed on filter paper for 30 minutes using 3% paraformaldehyde. Next, the retinas were removed
from the filter paper and treated overnight with 0.5% Triton-X (Sigma; St. Louis, MO). Tissues from WT mice were then placed in a 1:1,000 dilution of monoclonal anti-protein kinase C primary antibody (Sigma; St. Louis, MO) in 1.0% Phosphate Buffer Solution (PBS), 0.5% Triton-X (Sigma; St. Louis, MO), and donkey serum (Sigma; St. Louis, MO) for a total of five days (the tissue was placed on a rocker for the duration of this time in order to gently agitate the solutions). Tissues from Gus and Mito mice were labeled with anti-GFP, to stain for On and OFF bipolar cells, for also placed on a rocker for 5 days. All of the tissues were then washed with PBS three times for one hour each, after which they were stained with the anti-mouse secondary antibody, Alexa Fluor 488 (Invitrogen; Carlsbad, CA), which nonspecifically labels vasculature. Tissues were once again washed with PBS three times for one hour each.

Figure 5 – Methods Schematic
Four slits were made in the retina so it would lie flat on the microscope slide. 142.9 µm² images were taken approximately 500 µm from the optic nerve head in every direction (indicated by white squares) using a confocal microscope.
VectaShield (Vector Laboratories, Inc; Burlingame, CA) was then used to mount the tissues on microscope slides and clear fingernail polish was used to seal the slide covers. Four 142.86 µm by 142.86 µm images of the retina were taken, one in each region (Figure 5), at 500 µm from the head of the optic nerve using a Zeiss 510 confocal microscope with LSM 5.0 software. Images were captured in one-micron increments through the entire depth of the retina.

Data Analysis:

Each image was analyzed using ImageJ software. The freehand line tool was used to trace the length of each blood vessel and the straight-line tool was used to determine the diameter of each vessel. Measurements were made using the measure function and were performed throughout the multiple layers of retina that contained each vascular plexus (images show just one plane of these measurements). Vasculature observed before or within the GCL was considered part of the superficial vascular plexus, vessels measured in the IPL were considered part of the intermediate vascular plexus, and vasculature found throughout the INL and OPL were considered part of the deep vascular plexus (Figure 3).

To determine the length of blood vessels in each plexus, the length of every branch in the vascular layers was measured and summed together. The average diameter of blood vessels in each vascular plexus was determined by averaging four measurements from each branch of vasculature. The lengths and diameters from the four regions of each eye were averaged for each of three positions, corresponding to the three vascular plexuses in the retina (Figure 3). The averages of STZ and control mice were compared.
Diameter measurements from four WT tissues (including two control and two STZ-treated tissues) and five MITO tissues (including three control and two STZ-treated tissues) were excluded from analysis due to poor overall visualization of vasculature. Length measurements from the four GUS tissues (including two control and two STZ-treated tissues) were also excluded from analysis due to poor overall visualization of vasculature.

Statistics:

Error bars on all plots in this document are standard error. Three-Way ANOVAs (SigmaPlot) were utilized to determine the significance of interactions between the position, mouse line, and condition and interactions were deemed significant if $p < 0.05$. The Student-Newman-Keuls method was used for all pairwise multiple comparison procedures.

RESULTS:

Capillary Length:

In order to determine if the length of capillary blood vessels changes in early diabetes, the lengths of capillary branches were measured and totaled for each vascular plexus in WT and Gus mice. When the measured lengths from each vascular plexus are overlaid (Figure 6) there are no apparent qualitative differences in the overall vasculature between control and STZ-treated mice of any mouse line. However, there are apparent differences between the total lengths of blood vessels in each plexus for both conditions in WT and GUS mouse lines (Figures 7 & 8).
All capillaries in the vascular plexuses were traced, measured, and overlaid for one tissue of both the control and STZ conditions of every mouse line. Yellow = superficial vascular plexus, blue = intermediate vascular plexus, red = deep vascular plexus. There are no qualitative differences between the overall vasculature of control and STZ conditions regardless of mouse line.

Figure 6 – Overall vasculature
Figure 7 – Example of total length of capillaries in each vascular plexus of WT retinal tissues
These images are examples of individual planes that were measured. The yellow, red, and blue lines represent the length measurements. There were no qualitative differences in blood vessel length between control and STZ-treated tissues, though there were apparent differences in total length between each vascular plexus in the same condition.

Figure 8 – Example of total length of capillaries in each vascular plexus of GUS retinal tissues
These images are examples of individual planes that were measured. The yellow, red, and blue lines represent the length measurements. There were no qualitative differences in blood vessel length between control and STZ-treated tissues, though there were apparent differences in total length between each vascular plexus in the same condition.
There were also no quantitative differences between the length of capillaries of control and STZ-treated mice for the WT mouse line ($p = 0.705$), GUS mouse line ($p = 0.529$), or both groups together ($p = 0.477$). There were also no differences between conditions in any vascular plexus for both mouse lines ($p = 0.711, 0.502, 0.805$ for the superficial, intermediate, and deep vascular plexuses, respectively) (Figures 9 & 10). Additionally, there were no inter-mouse line differences ($p = 0.088$).

![Figure 9](image-url)  
**Figure 9** – Comparison of average capillary length in WT tissues  
There was no significant difference in the average capillary length between control and STZ-treated tissue in any vascular plexus ($p > 0.500$ in all instances).

![Figure 10](image-url)  
**Figure 10** – Comparison of average capillary length in GUS tissues  
There was no significant difference in the average capillary length between control and STZ-treated tissues in any vascular plexus ($p > 0.500$ in all instances).
There were significant differences between the vessel lengths in the superficial vascular plexus and the intermediate vascular plexus for WT mice, and also between blood vessel lengths in the superficial vascular plexus and deep vascular plexus for both WT mice and GUS mice in both the control and STZ-treated conditions ($p < 0.001$ in all cases). WT mice also showed a significant difference between vessel lengths in the intermediate and deep vascular plexuses ($p = 0.044$).

**Capillary Diameter:**

To determine if the diameter of capillary blood vessels changes in early diabetes, four diameter measurements were taken from every branch within all three vascular plexuses and averaged in two different groups of mice, WT and MITO. There were no qualitative differences in the appearance of the vascular plexuses between control and STZ-treated mice in either mouse line (Figures 11 & 12). However, the difference in the diameter of the capillaries in each plexus is qualitatively apparent in both conditions.

**Figure 11 – Example of capillary diameter measurements in each vascular plexus of WT retinal tissues**

These images are examples of individual planes that were measured. The red lines represent diameter measurements. There were no qualitative differences in blood vessel diameter between control and STZ-treated tissues, though there were apparent differences in the average diameter between each vascular plexus in the same condition.
There was not a significant difference between the diameters of vasculature in control and STZ-treated mice for WT mice \((p = 0.619)\), MITO mice \((p = 0.373)\), or both groups together \((p = 0.815)\). There were also no intergroup differences \((p = 0.060)\) or differences between conditions with respect to each vascular plexus for both groups \((p = 0.655, 0.287, 0.862\) for the superficial, intermediate, and deep vascular plexuses, respectively\) (Figures 13 & 14).

*Figure 12 – Example of capillary diameter measurements in each vascular plexus of MITO retinal tissues*

These images are examples of individual planes that were measured. The red lines represent diameter measurements. There were no qualitative differences in blood vessel diameter between control and STZ-treated tissues, though there were apparent differences in the average diameter between each vascular plexus in the same condition.
There were significant differences between the vessel diameters in the superficial vascular plexus and intermediate vascular plexus in both WT mice and MITO mice in both the control and STZ-treated conditions ($p < 0.05$ in all instances). There were also significant differences between the vessel diameters in the intermediate vascular plexus

**Figure 13 – Comparison of average capillary diameter in WT tissues**

There was no significant difference in the average diameter of capillaries between control and STZ-treated tissue in any vascular plexus ($p > 0.250$ in all instances).

There were significant differences between the vessel diameters in the superficial vascular plexus and intermediate vascular plexus in both WT mice and MITO mice in both the control and STZ-treated conditions ($p < 0.05$ in all instances). There were also significant differences between the vessel diameters in the intermediate vascular plexus
DISCUSSION:

The main results of this study can be summarized as: (1) STZ-induced diabetes mellitus does not affect the average length of capillary blood vessels in any of the three vascular plexuses compared to controls, and (2) STZ-induced diabetes mellitus does not affect the average diameter of capillary blood vessels in any of the three vascular plexuses compared to controls.

We did see differences in both capillary length and diameter between the vascular plexuses of each tissue, primarily between the superficial vascular plexus and the other two vascular plexuses. This is because the vascular plexuses are not identical. First, the superficial vascular plexus is associated with retinal astrocytes, which secrete fibronectin – an essential component for the progression of the plexus (Uemura et al., 2006). The intermediate and deep vascular plexuses, on the other hand, are formed via angiogenic sprouting due to VEGF signaling (Gerhardt et al., 2003) and are not associated with astrocytes. Furthermore, the regulation of the formation of the deep and intermediate vascular plexuses is dependent on the TSPAN12 protein. Deletion of TSPAN12 results in the lack of deep and intermediate vascular plexus formation while the development of the superficial vascular plexus remains intact (Junge et al., 2009). Another important thing to note is that the formation of vascular patterning relies on some of the same developmental cues used by neurons (like R-cadherin, for example), so it makes sense that more neurons will result in denser vasculature (Dorrel...
et al., 2002). Finally, the intermediate and deep vascular plexuses supply nutrients to the INL and IPL primarily – which are extremely metabolically active, meaning that they require more blood flow, which can be achieved both by increased number of vessels or branches, or increased diameter of vessels to provide nutrients more rapidly.

Our results also indicate that there are no microvascular changes occurring in the retina at 6 weeks post onset of diabetes, so there must be changes elsewhere that cause the visual defects noticed in early diabetes (Aung et al., 2013) and the decreased retinal blood flow seen after four weeks of hyperglycemia (Wang et al., 2011; Wang et al., 2012; Yadav et al., 2011). While recent studies have indicated that abnormalities such as cell death and impaired signaling in the neuroretina exist in diabetics prior to the onset of the microvascular changes characteristic of diabetic retinopathy (Antonetti et al., 2006; Barber et al., 2011; Curtis et al., 2009; Simo et al., 2010), these are typically identified shortly before the symptoms of retinopathy and are still occurring in the later stages of diabetes. So, these neuroretinal irregularities are likely to worsen the visual problems associated with the early stages of retinopathy, such as decreased color and contrast sensitivity and reduced electroretinography response (Gardner et al., 2011), and are not implicated in the visual changes that occur even earlier in diabetes (during the time span investigated in this study). Similar research into the neuroretina is necessary to determine what changes occur earlier on in diabetic patients to initiate the visual defects observed as early as four weeks after onset of diabetes (as demonstrated in Aung et al., 2013).

One change in the neuroretina that has been prominent in many recent studies is neurodegeneration. In fact, accelerated neurodegeneration has been shown to occur in
retinal ganglion cells of diabetic mouse models (induced via STZ-injections) at 8 months after the onset of diabetes (Kern et al., 2010). However, in even earlier stages of diabetes, such as 6-weeks post-onset (the time frame this study is interested in), no such neurodegeneration or neuronal apoptosis has occurred (Moore-Dotson et al., 2015).

In the future, it would be better to use tissues that are stained directly with an antibody that is selective for the vasculature, to better visualize the vasculature of the retina as a whole. Because the antibodies used in this experiment non-selectively stained blood vessels, certain portions of the vasculature were not easily visible. This was true for all tissues and every vascular plexus, so the overall results of this study do accurately characterize the retinal vasculature as a whole. However, it is the reason that some tissues were excluded from analysis.

Since this study determined that there are no changes in the retinal vasculature that originally stems from the optic artery, further research is necessary to identify what changes are occurring in the visual system to explain the decreased visual acuity of early diabetic patients. For instance, choroidal vessels, which are located between the retina and the sclera, supply the outer nuclear layer (including the photoreceptors) and the retinal pigment epithelium. These areas are extremely important in visual acuity and color sensitivity, two characteristics of vision that are affected in early diabetes, so it is definitely possible that changes in the choroidal vasculature could occur in early diabetes, even when changes in the retinal vasculature don’t. In fact, studies have shown that both choroidal and retinal vasculature are altered after prolonged diabetes and that the choroidal blood flow is affected earlier on in the pathology of diabetic
retinopathy compared to the retinal blood flow (Muir et al., 2012). Also, while studies have shown that there are no changes in the number of cells in the neuronal cell layers of the retina in early diabetes, research still needs to be done to investigate the impact of early diabetes on cell signaling including neurotransmitter release from these neurons. Finally, the visual system is composed of many different neural structures, which only begin with the retina. Thus, there could be changes or deficits in the cells of these structures, which could affect higher order processing, or even in the vasculature of these structures.

Overall, there is a great need for further research in this area of study to better characterize how diabetes affects the neuroretina and, consequently, vision. Only then will it be possible to find a therapeutic target before the retinal damage sustained due to diabetes becomes permanent and, ultimately, leads to blindness.
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