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M. Gleeson • V. Connaughton • L.S. Arneson

Induction of hyperglycaemia in zebrafish (*Danio rerio*) leads to morphological changes in the retina

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Abstract Diabetes affects over 16 million Americans yearly, resulting in hyperglycaemia and microvascular complications, including retinopathy, neuropathy and nephropathy. Animal models have been developed to examine the immunological aspects of type 1 diabetes and the pathogenic mechanisms associated with diabetic retinopathy, but the methods of diabetes induction raise concerns regarding these models. Zebrafish (*Danio rerio*) have been used extensively to study developmental processes and mutant zebrafish strains have been used to examine vision disease present in humans. In this paper, we have induced hyperglycaemia in zebrafish by alternately immersing the fish in glucose solution or water. Eyes from untreated fish or fish exposed to alternating glucose/water solutions for 28 days were dissected, sectioned and stained to visualise cell bodies in the retina. In untreated fish retinas, the inner plexiform layer (IPL) and inner nuclear layer (INL) were approximately the same thickness, whereas in fish repeatedly exposed to glucose solutions the IPL was approximately 55% the thickness of the INL. Both the IPL and INL were significantly reduced in retinas of treated fish, compared to untreated fish, similar to that seen in other animal models of diabetes and in diabetic patients. These results suggest that zebrafish may be used as an animal model in which to study diabetic retinopathy.

Key words Diabetes • Retinopathy • Zebrafish

M. Gleeson • V. Connaughton • L.S. Arneson (✉)
Department of Biology
American University
4400 Massachusetts Avenue NW
Washington, DC 20016, USA
E-mail: larneso@american.edu

Introduction

Diabetes mellitus affects over 16 million individuals in the USA each year and was the sixth leading cause of death in 2000 [1]. Type I, type II and gestational diabetes all lead to increased blood glucose levels due to either the loss of production or function of insulin, a hormone that promotes the transport of glucose from blood into cells. Increased blood glucose, or hyperglycaemia, leads to many complications, including cardiovascular and microvascular disease, periodontal disease and increased susceptibility to other diseases. The risk of developing microvascular complications such as neuropathy, nephropathy and retinopathy increases with poor glycaemic control.

Type I diabetes has been extensively studied in animal models. These models can be divided into two groups, one type in which the animal spontaneously develops diabetes similar to that seen in humans, and a second group in which diabetes is induced in animals by the introduction of a drug that destroys the insulin-producing beta cells. Two animal models exist that spontaneously develop diabetes, the non-obese diabetic (NOD) mouse [2] and the BB rat [3], although a new mouse strain ($Ins2^{Akita}$) has been identified that develops hyperglycaemia at an early

age [4]. These animals, especially the NOD mouse, have been used to identify genetic loci associated with increased susceptibility to diabetes, and to examine immunological aspects of type I diabetes, in which the beta cells are destroyed by an autoimmune response. Although it is likely that these animals develop microvascular complications associated with diabetic hyperglycaemia, they tend to have shorter life-spans due to the lack of treatment, and the onset and development of these complications have not been examined.

The second group of animal models includes mice and rat strains in which a drug, primarily either alloxan or streptozotocin, is administered to destroy the beta cells, resulting in hyperglycaemia [5, 6]. Microvascular complications of hyperglycaemia have primarily been examined in these models. However, these drugs are carcinogenic and may produce unexpected effects on the systems being studied. For example, streptozotocin may cause benign or malignant islet cell tumours [7] or kidney, lung, uterine or liver tumours following intraperitoneal injection [8].

Diabetic retinopathy, a microvascular complication seen with all forms of diabetes, is the leading cause of new cases of blindness and visual impairment in the USA [9], and affects approximately 75% of patients with diabetes for more than 15 years. The early stages of retinopathy, non-proliferative retinopathy, result in microangiopathy, leading to microaneurysms, the earliest clinical signs of diabetic retinopathy. The decreased blood flow, vessel blockage and increased leakage of blood from capillaries affects the microvascular circulation in the retina, leading to increased expression of vascular endothelial growth factor (VEGF), resulting in the formation of new blood vessels accompanied by fibroglial scaffolding, which forms new fibrovascular tissue from the retinal surface into the vitreal cavity. Additionally, diabetic retinopathy results in atrophy of ganglion cells and degeneration of the inner nuclear layer (INL) through apoptosis. Decreased neuronal cell number due to apoptosis contributes to morphological changes in the retinal structure and thickness associated with retinopathy. If untreated, vision loss will usually occur within two years after the onset of proliferative retinopathy [10, 11].

In this study we have begun to develop a new model organism to examine the secondary complications associated with poor glycaemic control in diabetes. We have chosen zebrafish as the model system due to the extensive use of zebrafish to study visual development and visual impairments similar to those seen in humans, such as night blindness [12]. The primary goal of this project was to develop zebrafish as a model in which to study diabetic retinopathy. To achieve this objective, we induced hyperglycaemia in zebrafish by immersion in a glucose solution and examined the retinal layers of hyperglycaemic fish for changes in morphology associated with diabetic retinopathy.

Materials and methods

Fish maintenance

The strains of zebrafish (*Danio rerio*) used were AB* (obtained from the National Institutes of Health) and wild-type (obtained from a local supplier, PetSmart). All fish were acclimated to constant laboratory conditions (14-h light:10-h dark photoperiod, diet, water, 28°C) for at least one week in stock aquaria before all experiments were conducted. Fish were fed Tetramin flakes daily, until the beginning of experiments. All fish used in these experiments were random adults from different clutches.

Survival studies

After acclimation, fish, either AB* or wild-type, were placed in a 1-l beaker containing 500 ml of various solutions of glucose (10%, 5%, 2% or 1%) (Sigma) in water (Deer Park) at room temperature. The water used in the experiments was identical (same mineral content) to that in the stock aquaria, except for the addition of glucose. Two fish were placed in each solution and were closely monitored for signs of stress (i.e., difficulty swimming or excessive gill movement). When stress was observed the fish were removed and placed into a recovery tank with 0% glucose solution. If the fish showed no signs of distress for the first four hours, they were allowed to remain in the glucose solution overnight. The time at which the fish were removed or found dead was considered to be the survival time.

Collection of blood glucose levels

Following incubation in one of the above glucose solutions, fish were anaesthetised in 0.04% Tricaine MS-222 (tricaine methanesulphonate) prior to decapitation. Fish were considered to be anaesthetised when all movement in the water had ceased. Fish were removed from the solution, patted dry with a Kimwipe and placed on a glass surface. Using a sharp blade, the head was completely removed by slicing behind the eyes. Immediately following decapitation, blood-glucose readings were collected by placing a glucometer test strip (One-Touch Ultra) directly on the punctured heart. Two readings were taken from each fish.

Induction of hyperglycaemia

The environment of the zebrafish was alternated every 24 h between a freshly prepared 2% glucose solution and a 0% glucose solution in water (Deer Park). The water used in these two solutions was identical, as well as identical to the water used in the stock aquaria. The fish were maintained at 28°C by placing 5-l containers (2 l liquid per container, 15 fish per container) in a water bath maintained at 28°C. The fish were not fed during the course of the experiment to prevent the increase of glucose concentration in the water. Five wild-type fish (n=64 for the experiment) were washed, anaesthetised, sacrificed, tested for

blood-glucose levels, and whole eyes dissected every day for the first four days of the experiment, then every third day until day 30. Four AB* fish (n=56 for the experiment) were used for each time point and data were collected every day for the first five days, then every third day until day 32.

Preparation of retinal tissue and staining

Whole eyes were removed from the fish following hemisection of the brainstem by a rostral-caudal incision through the brain. The eyes were then incubated in a 4% paraformaldehyde solution at 4°C for at least one week. Fixed whole eyes were allowed to equilibrate in a 30% sucrose solution in phosphate-buffered saline solution at 4°C overnight. Whole eyes were embedded in Tissue Freezing Medium (TFM) and 20-µm cryostat sections were made and collected on silanated slides (Amine). The slides were stored at -80°C until use for staining.

When labelling sections, tissue was allowed to come to room temperature. 4,6-Diaminidino-2-phenyloindole (DAPI) staining was used to examine the morphology of the cellular layers of the retinal tissues. DAPI (1:5000) was added to each section for 20 min followed by washing three times with phosphate-buffered saline. Slides were mounted with a coverslip with 50% glycerol in PBS before viewing. Sections were viewed using fluorescence microscopy (Olympus Bx51) and photographs were collected using a Nikon Coolpix 4500 digital cameral. All pictures were grey-scaled and viewed at 200%. Retinal layers from sections from fish sacrificed on days 0 and 28 were quantified. "C"-shaped sections from each fish were identified and the retinal area opposite the lens was photographed at 40x magnification. Pictures were transferred to Adobe Photoshop and a minimum of four measurements each were made of the ganglion layer (GL), inner plexiform layer (IPL) and INL for each section. The measurements were averaged for each section, and multiple sections for each fish were averaged to give a final retinal layer thickness for each fish.

Statistics

Statistical comparisons were made using the one-way Wilcoxon rank test (JMP 5.1.2, SAS). $p<0.05$ was considered to be significant.

Results

Survival in glucose solutions

We initiated our studies by determining the maximum glucose concentration in which zebrafish could survive for an indefinite period of time. Adult fish were placed in different percent glucose solutions and monitored for distress. Incubation in glucose solutions above 5% resulted in impairment or death within 4 h, whereas fish placed in 5% glucose solutions survived for approximately 36 h and fish in 1% glucose survived in excess of 14 days (data not shown). Based on these results, glucose solutions less than 5% were used in all subsequent studies.

Washing protocol

Examination of blood glucose required decapitation of the animal, potentially resulting in contamination of the samples by residual glucose solution on the external surface of the animal. Therefore, we developed a washing protocol to rinse off the external surface of the fish without allowing fish to equilibrate with their environment. Fish were incubated in a 1% glucose solution for 5 min then placed in a 0.04% Tricaine solution without glucose for either 1 or 2 min, followed by glucose testing of the exterior surface (scales) of the fish. A single wash for one minute was found to be sufficient to remove all residual glucose from the external surfaces of the fish (Table 1), and was used in all subsequent experiments prior to blood sample measurement.

Induction of hyperglycaemia

Constant immersion

Average resting blood glucose levels (±SE) in zebrafish were calculated as 74±8.5 mg/dl (n=15) (wild type) or 89±10.6 mg/dl (n=6) (AB*) (Table 2). To induce hyperglycaemia initially, we immersed the fish in a 1% glucose solution, as they readily take up substances from the water [13, 14]. We defined hyperglycaemia as three times greater than resting blood glucose levels (~200 mg/dl). In this protocol, two fish were sacrificed every 24 h to determine blood glucose levels. In this initial study we found that although continued immersion in a glucose solution does result in an increase in blood glucose levels, this increase is only transient and blood glucose levels decrease to val-

Table 1 Glucometer readings from fish scales after various wash protocols

Liquid	Glucometer reading (mg/dl)
Water	<20* (n=3)
Tricaine solution	<20* (n=3)
1% glucose	434±1.4 (n=2)
Scales after 2-min wash	<20* (n=3)
Scales after 1-min wash	<20* (n=3)
Scales, no wash	56±8.5 (n=2)

*All measurements were less than 20 mg/dl

Table 2 Average blood glucose levels in zebrafish

Wild-type		AB*	
Fish	Glucose (mg/dl)	Fish	Glucose (mg/dl)
1	66	1	56
2	44	2	63
3	52	3	127
4	60	4	100
5	79	5	87
6	29	6	99
7	37	Average	89
8	76	SE	10.6
9	132	Range	56–127
10	54		
11	53		
12	84		
13	118		
14	128		
15	105		
Average	74		
SE	8.5		
Range	29–132		

ues higher than normal, but not hyperglycaemic, despite constant immersion in 1% glucose (Fig. 1). Further, though increasing the environmental glucose concentration did result in increased blood glucose levels, it also increased mortality as discussed above (data not shown).

Fluctuating immersion

Blood glucose concentrations fluctuate dramatically in individuals with diabetes in response to dietary uptake and insulin injections. To model this fluctuation, we exposed zebrafish to either a 2% or 0% glucose solution, alternating between the two solutions every 24 h. Both wild-type and AB* fish were used in these experiments. Fish were exposed to alternating glucose solutions and 5 (wild type) or 4 (AB*) fish were sampled every 24 h for the first 4 (wild type) or 5 (AB*) days, followed by sampling every 3 days until day 30–32. The average blood glucose value following exposure to 2% glucose was hyperglycaemic, whereas average blood glucose values following incubation in 0% glucose approached resting levels (Fig. 2). The fish became hyperglycaemic following each exposure to 2% glucose for the duration of the study, although there was some decline in maximal blood glucose levels as the study progressed. These data show, however, that hyperglycaemia can be induced and maintained in zebrafish and that blood glucose levels in these conditions are similar to those reported by diabetic patients.

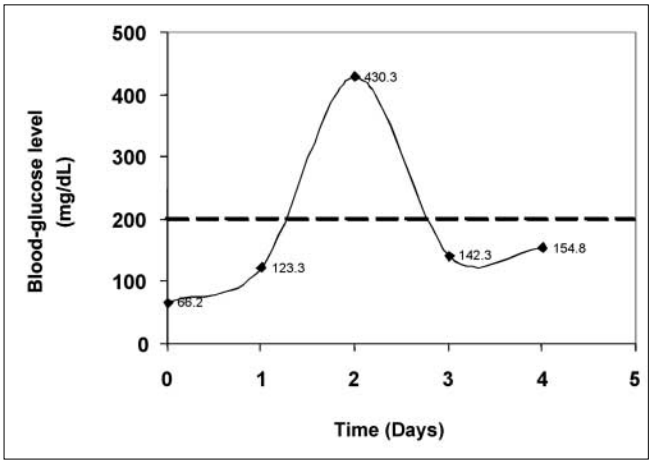

Fig. 1 Continual immersion in a 1% glucose solution induces transient hyperglycaemia in zebrafish. Zebrafish were placed in a 1% glucose solution, and two fish were sacrificed every 24 h to determine blood glucose levels (mg/dl). Average values are given. The dashed line is at 200 mg/dl and any values above this level are considered to be hyperglycaemic

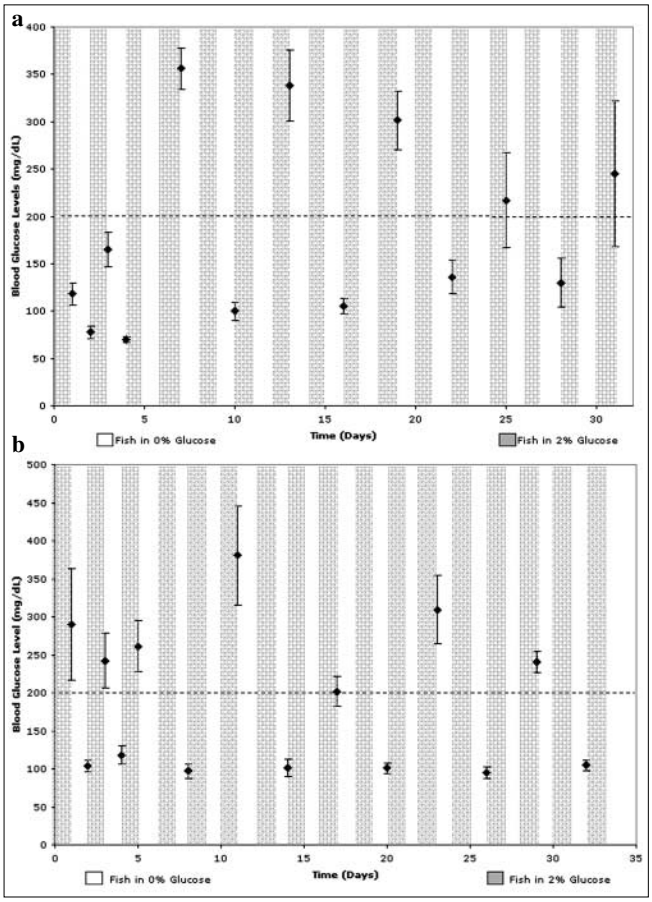

Fig. 2 *Blood glucose levels fluctuate depending on glucose levels in environment*. Wild-type (**a**) or AB* (**b**) zebrafish were placed in glucose solutions alternating between 2% (shaded days) and 0% glucose (unshaded) for up to 30 days. Five (wild type) or four (AB*) fish were sacrificed every 24 h for 4 (wild type) or 5 (AB*) days to determine blood glucose values. Average values are given for each sample and standard errors of the mean are indicated. The dashed line is at 200 mg/dl. Any values above this line are considered to be hyperglycaemic

Effects of chronic hyperglycaemia on retinal morphology

Retinal sections from control zebrafish (day 0) and fish sacrificed at the conclusion of the 30-day experiment were examined following DAPI staining of nuclei for changes in gross morphology. The thickness of the proximal retinal layers was examined and compared between the untreated and hyperglycaemic fish (Fig. 3). The most striking difference between the control and treated fish was the change in thickness of the IPL. The IPL thickness significantly decreased approximately 60% from day 0 (n=4) to day 28 (n=3) (p<0.05). Although the thickness of the INL also decreased over time, the relationship between the IPL and INL within an individual also changed between control and treated fish. In fish at day 0, the thickness of the IPL and INL was not significantly different; however, by day 28 the IPL was significantly thinner than the INL (p<0.05), with the IPL being approximately 55% the thickness of the INL (Fig. 3). These results indicate significant IPL thinning over time by comparing different individuals, as well as by comparing relative retinal layer thickness within an individual.

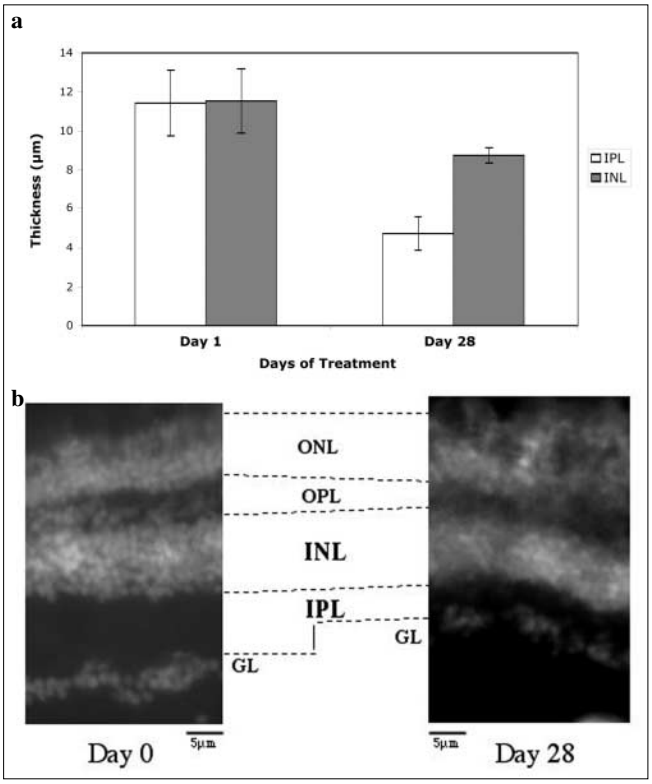

Fig. 3 *Chronic hyperglycaemia induces retinal morphology changes in the zebrafish. Eyes were dissected from control fish or fish exposed to alternating 0%/2% glucose solutions for up to 30 days. Retinal sections were stained with DAPI, and the thickness of the IPL was determined. Multiple sections per eye per fish were examined and measured for IPL and INL thickness*. **a** Average thickness of IPL and INL for all fish on days 0 or 28. Error bars indicate standard error (n=4, day 0; n=3, day 28; p<0.05). **b** Representative sections of fish retina at days 0 or day 28. Individual retinal layers are indicated

Discussion

Various animal models have been developed to examine diabetic retinopathy. Most models utilise primates, small mammals, mice or rats, in which diabetes has been chemically induced through the injection of streptozotocin or alloxan. Although rodents share very similar retinal physiology with humans, the chemicals used to induce insulin-dependent diabetes have side effects, including kidney, lung and liver tumours (streptozotocin) or liver and kidney necrosis (alloxan), which illustrate the lack of specificity of the drugs and may affect blood glucose levels [15].

In this paper, we propose the zebrafish (*D. rerio*) as a new model for use in the study of diabetic retinopathy. We transiently induced hyperglycaemia by immersing zebrafish in a glucose solution. After immersion in alternating 2% and 0% glucose solutions for approximately 30 days, the thickness of the IPL and INL is decreased, consistent with previous results from mice with spontaneous hyperglycaemia [16], streptozotocin-induced diabetes in rats [17, 18] and mice [19], and in diabetic humans [19–21]. The reduced thickness of the IPL suggests atrophying of the processes between neurons in the retina. As the IPL is more affected by hyperglycaemia in this study, it seems likely that degeneration of the processes occurs prior to the loss of cell bodies in the inner retina.

Zebrafish have been widely used to examine molecular and genetic aspects of developmental biology, in part due to their high fertility rate, rapid rate of development and simple husbandry requirements [22]. More recently, this species has become extensively used to examine the physiological processes of the retina required for vision, and behavioural tests examining visual and neural health have been developed [23–26]. Zebrafish mutants are currently being developed to examine human visual diseases, such as night blindness [12, 23]. The genomic sequence of *D. rerio* has also been solved, allowing targeted genetic and mutational analyses [27].

Zebrafish are freshwater teleosts that regulate their internal water and total solute concentrations [28]. They operate hyperosmotically, a strategy of osmoregulation that involves the continuous gain of water as a result of a higher internal concentration of salt compared to their freshwater environment. The constant influx of water results in the uptake of other molecules, including glucose, from their environment. Thus, zebrafish are an excellent candidate for use in early chemical screening for toxicity [13, 14, 29] and drug efficacy, as administration in large numbers would consist of merely adding the compound to the aquarium water.

To be useful in the study of human disease, an animal model must parallel the normal physiology of humans in the targeted tissue or organ, as well as the abnormal changes in physiology associated with the disease being studied. Previous studies have shown that teleosts in general, and zebrafish specifically, have endocrine islet tissue containing hormone-producing cells such as β cells, and that these cells converge in a central area corresponding to the islet of Langerhans. Teleost insulin is homologous to human insulin, produced at levels equivalent to those in humans [30], and is functional in mice [31]. Zebrafish also express a glucose transporter molecule homologous to GLUT 1 [32], and contain additional genomic sequences with high homology to glucose transporter molecules (Lowe and Arneson, personal observation). Other molecules necessary in the insulin signalling pathway, including insulin receptor a and b [33] and a tyrosine-kinase substrate [34], are also expressed in zebrafish. Thus, current data suggest that zebrafish respond to glucose uptake by insulin production and release, resulting in an insulin signalling pathway that promotes glucose transport into cells.

Hyperglycaemia has been previously induced in teleost fish following isletectomy (goby, *Gillichthys mirabilis*) [35] or immersion in alloxan (goldfish, *Carassius auratus*) [15]. However, isletectomy removed all exocrine as well as endocrine functions, and alloxan immersion resulted in transient hyperglycaemia, but with significant systemic toxicity [36].

One caveat of the work presented in this paper is the high variability in blood glucose levels between fish in the same experimental group. Although some specific strains of zebrafish are primarily used for experimentation, extensively inbred strains of zebrafish with very similar genetic profiles do not exist [37]. Thus, it is likely that natural genetic variation in levels of insulin and glucose transporter expression and/or efficiency of osmoregulation resulted in variation in blood glucose levels. An examination of interclutch variability in control glucose levels could be used to screen for a genetic element potentially associated with predisposition to hyperglycaemia and its complications. Similar variability in glucose levels between fish was also seen in the outbred strain, AB*, used in the experiments. However, despite the variability, average values of assayed fish following immersion in 2% glucose remained hyperglycaemic for the duration of the study and similar levels of decreased IPL thickness were seen in different fish.

Overall, these data indicate that zebrafish can be induced to become hyperglycaemic, and can be maintained indefinitely with repeated hyperglycaemic spikes occurring every 24 h. Chronic hyperglycaemia results in significant thinning of the IPL and INL, as seen previously in other model systems, consistent with onset of diabetic retinopathy. The simple husbandry requirements, functional and morphological similarity to human systems [38–40], and ability to take up solute from their aqueous environment make zebrafish an ideal model in which to study the mechanisms and pharmacological treatments of diabetic retinopathy.

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