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Limb Regeneration is Impaired in an Adult Zebrafish Model of Diabetes Mellitus

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Abstract

The zebrafish (*Danio Rerio*) is an established model organism for the study of developmental processes, human disease and tissue regeneration. We report that limb regeneration is severely impaired in our newly developed adult zebrafish model of type I diabetes. Intraperitoneal streptozocin injection of adult, wild type zebrafish results in a sustained hyperglycemic state as determined by elevated fasting blood glucose values and increased glycation of serum protein. Serum insulin levels are also decreased and pancreas immunohistochemistry revealed a lesser amount of insulin signal in hyperglycemic fish. Additionally, the diabetic complications of retinal thinning and glomerular basement membrane thickening (early signs of retinopathy and nephropathy) resulting from the hyperglycemic state were evident in streptozocin injected fish at three weeks. Most significantly, limb regeneration, following caudal fin amputation, is severely impaired in diabetic zebrafish. Nonspecific toxic effects outside the pancreas were not found to contribute to impaired limb regeneration. This experimental system using adult zebrafish facilitates a broad spectrum of genetic and molecular approaches to study regeneration in the diabetic background.

Keywords

limb regeneration; zebrafish; diabetes

INTRODUCTION

The world prevalence of diabetes mellitus among adults (aged 20-79 years) is 6.4%, affecting 285 million adults, and will increase to 7.7%, and 439 million adults by 2030 (1). DM is a disease of metabolic dysregulation, initially diagnosed as hyperglycemia, that ultimately results in blood vessel damage leading to complications such as cardiovascular disease and stroke, retinopathy, nephropathy, neuropathy, and impaired wound healing. These complications persist even after normoglycemia is achieved though pharmaceutical intervention.

One objective of regenerative medicine is to define the permissive/inhibitory factors that allow for the full restoration of diseased or damaged tissue, and to translate this knowledge into pharmaceutical therapies to stimulate regeneration in humans. For regenerative medicine to be applied in patients with diabetes mellitus any alteration to these signals must

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be defined within the diabetic background. To date this has not been possible as animal models of diabetes mellitus with tissue regeneration capacity have not been reported.

The zebrafish has become a premier model organism to study vertebrate development due to the optical clarity of the embryo, high fecundity, short generation time, and an array of tools for gene manipulation (2). Recently, zebrafish models of human disease have been established spanning a wide range of human pathologies including genetic disorders and acquired diseases as many cellular processes are highly conserved throughout vertebrate evolution (3-13). From a clinical perspective, the most important contribution the study of a model organism can achieve is the improvement of diagnosis and treatment for a disease. The extensive conservation of genetic pathways and cellular physiology among the vertebrates and the capacity to perform high throughput drug screenings have led zebrafish to be successfully used for human pharmaceutical discovery (14).

Here we report that the diabetogenic drug, streptozocin, induces a type I diabetic state in the regeneration competent zebrafish (*Danio rerio*) that results in a broad spectrum of complications characteristic of diabetes mellitus. Streptozocin injection results in sustained hyperglycemia, increased non-enzymatic protein glycation, reduced insulin production, glomerular basement membrane thickening, and thinning of the retina. A significant impairment of tissue regeneration occurs in this model as a result of decreased cell proliferation in the regenerating limb of hyperglycemic fish. This model provides a unique opportunity to study diabetes mellitus in a regeneration competent organism.

MATERIALS AND METHODS

Zebrafish Husbandry

Wild-type zebrafish (*Danio rerio*) were maintained under standard laboratory conditions using a light schedule of 14 h on and 10 h off at a temperature of 28.5°C (15). Fish were fed twice daily using a combination of dry food and brine shrimp (Brine Shrimp Direct, Ogden UT). Wild type, age matched zebrafish (4 to 6 months) with an equal gender ratio were used for all experiments. All procedures were performed as described in the 'Principles of Laboratory Animal care' (NIH publication no. 85-23, revised 1985); and approved IACUC animal protocol 08-19.

Streptozocin Injection

Fish were anesthetized by placing them in 1:1000 2-phenoxyethanol (Sigma, P1126, St. Louis, MO) for 1-2 minutes. For intraperitoneal injection an insulin syringe with a 28.5 gauge needle was used to deliver 0.3% Streptozocin (Sigma, S0130, St. Louis, MO) solution in 5mM citrate buffer, pH 5.0 to a dose of 350 mg/kg (70 -150 microliters dependent on weight). Control fish were injected with a like volume of citrate buffer. For direct caudal fin injection the same concentration was used and the injection was performed with a Harvard Apparatus micro-injector. Timing of injections is discussed in the results section and following injection the fish were maintained at 25°C for the duration of the diabetes induction and maintenance phases.

Blood Glucose, Glycated Serum Protein, Serum Albumin and Alkaline Phosphatase Activity Assays

Fish were fasted for 24 hours prior to blood collection and were euthanized with 1:500 2-phenoxyethanol prior to blood collection. Blood was collected using a micropipettor following excision of the zebrafish head at the level of the anterior heart. One to two microliters of blood was collected from each fish. For blood glucose determinations, the blood sample was added to 5 microliters of normal saline and the Quantichrom Glucose

Assay (Bioassay Systems, DIGL-200, Hayward, CA) was performed to determine blood glucose. Blood glucose was assayed one (W1), two, (W2) or three weeks (W3) from the time of the first STZ injection. Serum glycated protein, alkaline phosphatase activity, and albumin detection were performed with serum collected from the pooled blood of four fish per sample and the assays were performed in quadruplicate (16 fish total per assay). Pooled whole blood samples were added to 5 microliters of normal saline and centrifuged at 3000rpm for 10 minutes to separate serum. The Glycated Serum Protein Enzymatic Assay (Diazyme, DZ112A-K, Dresden, Germany), BCP albumin assay Kit (Bioassay systems, DIAP-250 Hayward, CA), and alkaline phosphatase activity (Bioassay systems, DALP-250 Hayward, CA) assays were performed per the manufacturer's protocol.

Serum Insulin Detection

Undiluted or a 1:5 dilution of pooled serum from four hyperglycemic and control fish (W3) was spotted onto a nitrocellulose membrane and probed for the presence of insulin (Sigma, I551, 1:200, St Louis, MO) overnight at 4°C. The membrane was washed 3 times, incubated for 1 hour at room temperature with an HRP-conjugated secondary antibody, (Millipore AP108P, Bellerica, MA) and washed again 3 times. The membrane was then processed for chemiluminescence detection (Pierce Cat. # 34080, Rockford, IL) and the signal was detected by autoradiography.

Pancreas Immunohistochemistry

Following blood collection, fish were fixed in 4% paraformaldehyde for one week at 4°C, washed in PBS three times, and placed in a 30% sucrose solution overnight at 4°C. Samples were frozen in OCT embedding medium (Tissue-Tek, Torrance, CA) and serial 10 micron sections were made in the transverse plane. The sections were made in the anterior to posterior direction and the transition between the esophagus and intestine was the landmark used to locate pancreas tissue. The primary islet was identified by its anterior location and large size using phase contrast microscopy. Sections were incubated in 1:200 insulin primary antibody (Sigma, I5510, St. Louis, MO) and 1:4000 glucagon (Sigma, G2654, St. Louis, MO) primary antibody in 0.025% Triton X-100 phosphate buffered saline overnight at 4°C. This was followed by incubation with secondary antibodies (Abcam, Ab6769 and Ab6585, Cambridge, MA) at room temperature for one hour at a dilution of 1:1000 and finally counterstained with DAPI (Roche, 236276, Indianapolis, IN) for 30 minutes. Images (20X for phase contrast, 40X for immuno-fluorescence) were collected with a Nikon Eclipse 80i (Nikon, Melville, NY) microscope equipped with a digital camera and NIS Elements software.

Retina, and Kidney Histology and Electron Microscopy

Zebrafish eyes and kidneys were harvested from 8 euthanized hyperglycemic and control fish (W3) and fixed in 4% paraformaldehyde solution for 24 hours at 4°C. Additional fixation of kidney tissue for electron microscopy was done with osmium tetroxide and samples were embedded in epon resin. One micron sections of the fixed retinas were stained with toluidine blue and images were collected using a Qimaging camera and NIS-Elements software for morphometric analysis. The optic nerve was utilized as the mid-point and 6 equidistant measurements were taken from dorsal side and 6 from the ventral side for a total of 12 measurements from which the average was derived. Transmission electron microscopy was performed on the kidney samples and 40000X images of three distinct areas of each glomerulus were collected for analysis. The mean thickness of the basement membrane for each glomerulus was determined by drawing 15 non-overlapping rectangles (250 nm length) of the GBM area and the average of the 15 measurements was taken as the thickness for each glomerulus.

Fin Regeneration

Fin regeneration studies were performed as previously described (16). Zebrafish were anesthetized with 2-phenoxyethanol and tail fins were cut with a scalpel immediately proximal to the proximal branch point of the dermal rays within the fin. Following amputation, fish were incubated at 33°C for the indicated periods of time. The fish were again anesthetized, and images of the regenerating fins were taken at 24, 48, and 72 hours with a Qimaging camera and NIS-Elements software (Nikon, Melville, NY) and were printed for morphometric analysis. The area of regeneration was traced 5 independent times to ensure accuracy and the dorsal ventral length of the fin at the level of the amputation was also determined. The area of regeneration was divided by the dorsal ventral length of the fin to normalize the amount of regeneration for fish of different sizes.

Cell Proliferation and Death Analysis

For analysis of caudal fins, 8 hyperglycemic and control fish had their fins amputated and were allowed to regenerate for 72 hours. At this time, the fish were euthanized and the fin was amputated immediately distal to the pelvic girdle and fixed in 4% paraformaldehyde for one week at 4°C, washed in PBS three times, and placed in a 30% sucrose solution overnight at 4°C. Samples were frozen in OCT embedding medium (Tissue-Tek, Torrance, CA) and serial 10 micron sections were made in the longitudinal plane through the whole fin. Adjacent sections (5 per fin, ranging from the dorsal to ventral side) were utilized for determining the presence of cell death (apoptosis) via the *in situ* cell death detection kit (Roche, 11684795910, Indianapolis, IN). Alternatively, slides were assayed for cell proliferation via indirect immuno-fluorescence utilizing a proliferating cell nuclear antigen antibody (PCNA) (Sigma, P8825, St. Louis, MO) or a phospho-histone H3 antibody (Ser10) (Cell Signaling, 9701, Boston, MA). For apoptosis assays of body tissues, fish were fixed in 4% paraformaldehyde for one week at 4°C following blood collection, washed in PBS three times, and placed in a 30% sucrose solution overnight at 4°C. Samples were frozen in OCT embedding medium (Tissue-Tek, Torrance, CA) and serial 10 micron sections were made in the transverse plane. Sections were then identified that contained kidney, liver, intestine and pancreas. The above assay was then utilized to determine the incidence of apoptotic cells.

STZ Fin Injection

Adult zebrafish were anesthetized and injected with 350 mg/kg of STZ (buffer contained dye for visualization purposes) between each of the dermal rays at the proximal branch point in the dorsal half of the fin with a PLI-90 injector (Harvard Biosciences, Inc., MA). The same series and time course for induction of hyperglycemia was followed. The caudal fin was then amputated after week 3 at the distal portion of the injected tissue to ensure that the regenerating tissue was exposed to the drug. The fish were then returned to the 33°C tank. Images of the fins were collected at 24, 48 and 72 hr post-amputation, each fin was photographed and the area of both the dorsal and ventral fin re-growth was measured. The percentage of regeneration was calculated as $D/V \times 100$ (where D was the area of re-growth on the dorsal side and V was the area of re-growth on the ventral side).

Statistical Analysis

Student's independent t tests were used to compare STZ glucose values, fin regeneration, retinal layer thickness, GBM thickness differences, glycated serum protein values, and liver function tests to controls. Pearson correlation coefficient and linear regression analysis was performed to compare blood glucose values to area of regeneration. All statistics were performed using Microsoft Excel 2003 and Sigma Stat version 3.5 (Aspire software, Ashburn, VA).

RESULTS

Streptozocin Injection Results in a Sustained Increase in Fasting Blood Glucose Levels

Experimental models of type I diabetes can be created by destruction of pancreatic beta cells resulting from the administration of the diabetogenic drug streptozocin (STZ) (17). To initiate this study, the normal fasting blood glucose levels (FBGLs¹) of adult zebrafish were determined by fasting the fish for 24 hours followed by euthanasia and cardiac puncture. The FBGLs were immediately determined and yielded a value of 58.85 $\pm$ 4.79 mg/dL (n=15) (Fig. 1A, UN). Zebrafish were administered three intraperitoneal injections (350mg/kg) of STZ or buffer (as control) on alternating days (W1), and subsequent booster injection/s were administered on a weekly basis to yield the W2 and W3 groups. The FBGLs were determined one week after the final injection. The dose of STZ administered was based on a previous report where hyperglycemia was induced in the teleost *Oreochromis niloticus* (18). The average FBGL of control injected fish was 61.94 $\pm$ 5.60 (n=287) which is within the error of untreated fish indicating that the injection procedure alone did not alter FBGLs (Fig. 1A, GC). In contrast, injection of the zebrafish with STZ induced a normal distribution of FBGL levels ranging from approximately 60 mg/dl to 650 mg/dL with a mean value of 306.3 $\pm$ 34 mg/dL, (n=17) one week following administration of the initial dose/s (Fig. 1A, W1). Furthermore, the hyperglycemic state was maintained throughout the duration of this study as the glucose concentration for the two week group (307.9 $\pm$ 37.3 mg/dL, n=13) or three week group (311.6 $\pm$ 42.1 mg/dL, n=213) (Fig. 1A, W2 and W3) was not statistically different from the week one group. This data indicates that hyperglycemia can be induced and sustained in the zebrafish by intraperitoneal injection of streptozocin.

Increased Non-Enzymatic Glycation Occurs in W3 Hyperglycemic Zebrafish

One of the methods used to determine the magnitude of glycemic control in human patients during the previous 2-4 week period involves detection of the level of non-enzymatically glycated serum proteins, fructosamines (19). Here we utilized the enzyme based fructosamine quantification assay (Diazyme) to document the presence of non-enzymatic glycation products in the serum of hyperglycemic zebrafish. As this test required more serum than we could obtain from a single fish, we pooled serum from four STZ or control injected fish (W3) to perform the assay. From our results, there is a 320 % (p < 0.01, n = 3 pools, 12 fish total) increase in the amount glycated protein in the serum from STZ treated zebrafish (Fig 1B). This not only documents that non-enzymatic glycation occurs, but also supports our data demonstrating that the STZ treated zebrafish were in a persistent hyperglycemic state for 2-4 weeks.

Decreased Insulin Accompanies Hyperglycemia in Streptozocin Treated Zebrafish

STZ treatment of regeneration incompetent organisms such as rodents causes insulinopenia due to ablation of the pancreatic beta-cells (20). In order to verify a reduction in blood insulin levels, undiluted or a 1:5 dilution of pooled serum from four hyperglycemic and control fish (W3) was spotted onto a nitrocellulose membrane and probed for the presence of insulin. The undiluted signal from STZ-treated fish (Fig. 2A, bottom row) is comparable with the 1:5 dilution of the serum from control fish (Fig. 2A, top row) indicating a reduction of approximately 80 % in the insulin levels of STZ-treated zebrafish.

The zebrafish pancreas is similar in basic structure (containing both endocrine and exocrine components), and cellular makeup to the mammalian pancreas (21). It consists of 4 distinct regions that lie along the three lobes of the intestine in which four kinds of islets (principal islets, Brockmann bodies, diffusely existing islets, and single beta-cells) can be distinguished (22). The largest islet (approximately 300 μ M wide and 500 μ M long) is always observed at the dorsal-anterior position along the first intestinal lobe and may

develop from the principle islet observed in embryonic stages (21). In order to further substantiate a decrease in insulin production, we performed immuno-fluorescence microscopy on pancreatic tissue from control and diabetic fish. Nine control and hyperglycemic zebrafish (W3) were sacrificed, their FBGLs determined, and serial 10 μ m transverse cryo-sections were made. The sections were observed initially by phase contrast microscopy to identify pancreas tissue (PI Fig 2Bi and 2Bii) using the intestine (IN, Fig. 2Bi and 2Bii) as a stationary landmark. These samples were then processed by double indirect immuno-fluorescence to visualize insulin (red), glucagon (green), and were counter-stained with DAPI in order to visualize nuclei (blue). Epi-fluorescence microscopy indicated that the insulin signal from control fish was consistently much greater than that of hyperglycemic fish (compare red signal from Fig. 2Biii and 2Biv). In contrast, the glucagon signal (green) did not vary significantly between the two groups (compare green signal from Fig. 2Biii and 2Biv). We have presented the data obtained from the largest islet; however, similar differences were seen across all pancreatic tissue and in combination with the immunoblot data show reduced insulin production in STZ treated zebrafish.

Diabetic Complications are Seen in Hyperglycemic Zebrafish

Thickening of the glomerular basement membrane (GBM) is the earliest detectable feature of diabetic kidney disease (23). In order to assay for the presence of this complication in hyperglycemic zebrafish, glomeruli from eight control and hyperglycemic zebrafish (W3) were imaged via transmission electron microscopy (Fig 3A & 3B). The glomeruli were easily identifiable due to the presence of podocytes (P), the GBM (G) and endothelial cells (EC). The mean area for each glomerulus was determined by drawing 15 non-overlapping rectangles (250 nm length) of the GBM area. This analysis revealed that there was a 61 % increase (Control: 1056 $\pm$ 29.5 units, STZ: 1695 $\pm$ 93.5 units, $p < 0.00001$) in the thickness of the GBM in the hyperglycemic fish.

Diabetes mellitus is the leading cause of adult onset blindness; early changes in humans and animal models include retinal thinning (24). The retina consists of multiple identifiable layers of specialized cells and previous studies of STZ-induced diabetes mellitus in rats showed that within three weeks of hyperglycemia the thickness of the inner plexiform layer (IPL), and photoreceptor layer (PSL) were reduced (24). Additionally, cone receptor dysfunction, and neuronal damage independent of vascular defects, were documented in zebrafish exposed to high glucose concentrations in their water (25). Eight hyperglycemic and control zebrafish (W3) were euthanized, FBGLs determined, and eyes were removed for analysis. One micron sections that included all of the retina layers were obtained and images from the thin sections were printed for morphometric analysis (Fig. 4A & B). This analysis revealed an 18.7 % (Control: 43.67 $\pm$ 1.52, STZ = 35.5 $\pm$ 2.02, $p < 0.005$) and 26.5 % (IPL control = 39.2 $\pm$ 1.28 and IPL STZ = 28.8 $\pm$ 1.15, $p < 0.000001$) decrease in the retinal PSL and IPL layers respectively in hyperglycemic zebrafish when compared to control (Fig. 4C). From this data we conclude that early retinal changes occur in hyperglycemic zebrafish and this provides further evidence that a true diabetic state has been achieved.

Limb Regeneration is Impaired in Hyperglycemic Zebrafish

Adult zebrafish can regenerate many structures including the heart, retina, spinal cord, pancreas, and fins (26;27). The caudal fin has become a popular model for studying the molecular mechanisms regulating regeneration due to its accessibility for amputation and its relatively simple structure (16).

In order to assess the effect of hyperglycemia on limb regeneration, caudal fins of control and hyperglycemic zebrafish (W1, W2 and W3) were amputated immediately proximal to

the most anterior branch point in the lepidotrichia of the fin. Images were collected at 24, 48, and 72 hours post amputation using NIS elements imaging software and images were printed for morphometric analysis (Fig. 5 A & B). The newly regenerated tissue was traced 5 independent times using a digitizing pad and image J software, yielding the area of regeneration. The average area was recorded as the amount of regeneration in each animal. In order to normalize for the differences in initial fin size, this area was divided by the dorsal/ventral length of the fin at the amputation site. We have expressed the amount of fin regeneration in hyperglycemic zebrafish as a percent of control fin regeneration to illustrate the differences that were observed (Fig. 5C). After one week of hyperglycemia there was no statistical difference in fin regeneration between control and hyperglycemic zebrafish at 24, 48, or 72 hours following amputation (101.0 %, 106.6 % and 100 % respectively). However, after 2 weeks of hyperglycemia, zebrafish exhibited a statistically significant reduction at all three time points (79.1 %, 61.0 % and 57.6 %) which was further reduced by a longer duration of hyperglycemia (3 weeks) (43.6 %, 52.8 % and 48.6 %).

As described earlier, we encountered a range in FBGLs of treated fish and as such we performed a linear regression and a Pearson Product Moment coefficient analysis to determine the relationship between FBGL of each STZ injected fish and the amount of fin regeneration. This analysis resulted in a correlation coefficient of -0.92 and an R square value of 0.86 which documents an inverse relationship between the increase in FBGLs and fin regeneration ability. Collectively, this indicates that the extent of sustained hyperglycemia has a direct impact on the regenerative capacity of the zebrafish and the amount of this reduction can be correlated to the duration of hyperglycemia.

Cell Proliferation is Decreased in Diabetic Regenerating Fins

Tissue regeneration requires the coordination of cell proliferation and apoptosis (28) and we hypothesized that the reduction documented above could be due to alterations in either of these two processes. In order to address this we performed indirect immuno-fluorescence and TUNEL assays on $10\ \mu\text{M}$ cryo-sections of regenerating fins 72 hours following amputation. Through initial visual analysis no difference could be detected in the number or distribution of apoptotic cells (Fig. 5A panels v and vi); however, there appeared to be a marked decrease in the number of proliferating cells in the hyperglycemic fish (Fig. 5A panels i - iv). In order to quantify this difference, proliferative cells were counted ($n = 8$ fins for each group) and expressed as a percentage of the total number of cells (Fig. 5B). When all of the cells of the proliferative unit were examined there was a 48 % decrease in the number of PCNA positive proliferating cells (Control: $56.8\ \% \pm 3.4$ versus STZ $29.7\ \% \pm 2.9$, $p < 0.01$), and a 39.3 % decrease (Control: $34.9\ \% \pm 3.0$ versus STZ $21.2\ \% \pm 2.5$, $p < 0.01$) when examined by phospho-histone H3 (Ser 10). When only the blastema was considered this reduction was 39.1 % (Control: $64.1\ \% \pm 5.3$ versus STZ $39.6\ \% \pm 2.5$, $p < 0.01$) and 28.5 (Control: $29.9\ \% \pm 1.9$ versus STZ $21.4\ \% \pm 1.4$, $p < 0.01$) for each antigen respectively. Therefore it appears that hyperglycemia does not cause increased cell death but does decrease the proliferative competency of the regenerating limb.

STZ-Treatment Does Not Result in Non-Specific Damage

Although STZ is known to be selectively toxic to pancreatic beta cells, we wanted to ensure that non-specific harmful effects of the drug were not confounding our results. During the course of our work we encountered drug-treated fish that appeared to be resistant to the hyperglycemia inducing effects of STZ treatment as has been previously reported in rodents (29). When these fish (W3) were assayed for their regenerative capacity a range of 96.8% -105.9 % ($n=18$) was determined. This demonstrates that the reduction of regeneration is directly correlated with the hyperglycemic state of the zebrafish and not simply correlated

with the presence of STZ which suggests that the reduction in regeneration is not due to non-specific toxic effects of the drug.

We further addressed this by directly injecting the caudal fin with the same concentration and injection series of STZ used above to induce and maintain the hyperglycemic state. The dorsal side of the fin was injected between each ray at the proximal lepidotrichia branch point with 350 mg/kg of STZ or buffer only (as control). The fins were then amputated and the fish were allowed to regenerate their fins. When regenerative growth was examined, no differences were detected between the dorsal and ventral sides of STZ versus control injection (supplemental Fig. 1A). This data clearly indicates that STZ exposure to caudal fin tissue does not reduce fin regeneration rate.

It has been reported that intravenous STZ injection (350mg/kg) of *Talapia* resulted in several indices of general toxicity including; severe hepatic failure characterized by hepatic necrosis, jaundice, and ascites accumulation (18). To address this, the commonly used human liver function tests; serum albumin and serum alkaline phosphatase activity assays were employed (30). Serum from 16 control and STZ treated fish were analyzed for each assay and no statistically significant differences were detected indicating that liver damage was not present (supplemental Fig 1. B and C). Additionally, we have not encountered any indication of gross tissue or cell damage during histological examination. To rule out possible damage leading to cell death we examined the presence of apoptotic cells in kidney, intestine, liver and pancreas. The results from this indicate that the only tissue where an increase in apoptosis is observed is the pancreas (supplemental fig. 1D). We did not observe any weight gain or loss, significant behavioral changes or noticeable distress of the STZ treated zebrafish. Collectively, the data indicate that we do not encounter non-specific effects from STZ using this injection regimen.

DISCUSSION

Type I diabetes mellitus is caused by autoimmune destruction of the insulin producing pancreatic beta islet cells and results in insulinopenia. Type II diabetes mellitus, at least initially, develops from insulin resistance of body tissues. The diabetogenic drug STZ causes ablation of the pancreatic beta-cells and has been used extensively to generate animal models of type I diabetes (20). However, to date, this has not been reported for an organism capable of regeneration. In this study, we have used STZ to generate a type 1 diabetes mellitus model in zebrafish. This provides a unique opportunity to study regeneration in the context of diabetes mellitus.

We report the normal FBGL of zebrafish to be 58.85 $\pm$ 4.79 mg/dL which is supported by the control injected fish values in this study and is virtually identical to the recently reported value of 57.4 $\pm$ 4.1 mg/dL (31). Induction of hyperglycemia following STZ injection (350 mg/kg) raised the FBGLs to approximately 300 mg/dL which is similar to values seen in rodent models(32). In contrast, this value is considerably higher than that reported in Moss et al. (31) where a single injection of 1g/kg in zebrafish only raised the FBGLs to approximately 125 mg/dL and the cause of this difference is not clear. Of equal importance, the hyperglycemic state was maintained during the course of this study. When this work was initiated, we hypothesized that the adult zebrafish would be capable of pancreatic regeneration resulting in a restoration of normal glucose levels which since has been reported to occur within 7 days of a single STZ injection (31). To prevent regeneration and allow for a prolonged hyperglycemic state, we incorporated weekly booster injections into our injection protocol. This booster strategy successfully results in a consistent maintenance of the hyperglycemic state for a prolonged period of time and we have extended this duration to 90 days.

Sustained hyperglycemia results in the non-enzymatic glycation (NEG) of blood proteins and ultimately formation of advanced glycation end-products (AGE) in humans and animal models of diabetes mellitus (33). The formation and accumulation of AGEs occurs naturally during the aging process but accumulate at an accelerated rate in patients with diabetes mellitus. There is a growing body of evidence implicating AGEs and their receptor (RAGE) in the pathogenesis of diabetic vascular complications (33). Here we have shown a 320 % increase in the amount of NEG proteins in the serum from hyperglycemic zebrafish which further supports our data that STZ treated zebrafish maintained a hyperglycemic state for the duration of the study. We hypothesize that the increased fructosamines will lead to increased AGE formation and therefore provide a new model to study the relationship between AGE formation and diabetic complications.

As described earlier, diabetes mellitus type I is caused by the loss of pancreatic beta cells resulting in reduced insulin production. Our approaches to document a reduction of insulin show that the relative serum insulin levels are decreased in STZ treated zebrafish and this is due to a relative decrease in production from insulin producing beta-cells. We conclude that using STZ we have generated a drug induced zebrafish model of type I diabetes that results in sustained hyperglycemia with reduced insulin secretion by the endocrine pancreas.

Diabetic nephropathy (DN) is a major complication of diabetes mellitus and one of the leading causes of death among patients with diabetes (34). Thickening of the glomerular basement membrane (GBM) is the earliest detectable morphological feature of diabetic kidney disease. It has been suggested that the early changes in GBM thickness and content ultimately affect the filtration properties of the kidney leading to increased urinary albumin and eventually to gross proteinuria (23). The precise pathogenic mechanisms involved in the initiation and progression of diabetic nephropathy remain incompletely understood. STZ-induced diabetic nephropathy occurs within 4-6 weeks in rodents as assessed by a significant increase in proteinuria (35). After careful examination of the literature, it is not clear when GBM thickening occurs in rodent models as most GBM determinations are performed approximately 1 year following onset (genetic or drug induced) of the disorder. However, when GBM thickening has been assayed, a 15-20 % increase in the GBM thickness has been reported (29;36). Here we have shown that after 3 weeks, the hyperglycemic zebrafish kidney GBM is 161 % of its normal thickness and thus may represent an accelerated model to study diabetic kidney disease.

Another debilitating complication of diabetes mellitus is retinopathy leading to blindness. The retina is composed of multiple layers and previous studies utilizing STZ treatment of rats showed that within three weeks the thickness of the inner plexiform layer (IPL), and photoreceptor layer (PSL) were reduced by 9.9 %, and 18.9 % respectively when compared to control animals (37). In another study in which hyperglycemia was induced in zebrafish by the addition of glucose to aquarium water, the inner plexiform layer thickness was decreased by a remarkable 60 % when compared to control animals at 28 days (38). In our hyperglycemic zebrafish we saw a 26.5 % and 18.7 % decrease in the IPL and PSL layers respectively when compared to controls. From this data we conclude that early retinal changes observed in hyperglycemic fish are similar to those seen in other model organisms.

One goal of regenerative medicine is to stimulate humans to replace lost or damaged tissues through regeneration. A significant body of zebrafish research in the last 10 years has involved determining the signals and mechanisms that are responsible for regeneration with the goal of application in human disease and injury. However, until this report, these types of studies could not be performed in the context of diabetic pathology. The caudal fin is a popular model for studying the molecular mechanisms regulating regeneration due to its accessibility and its relatively simple structure. Following amputation, zebrafish fins

regenerate through three overlapping steps: wound healing (0-24 hours), blastema formation (24-48 hours), and outgrowth (72 + hours), with full restoration of the amputated appendage complete in seven to fourteen days. We examined caudal fin amputation and re-growth as our assay to determine the effect of hyperglycemia on regeneration. From this data it is clear that one week of hyperglycemia is insufficient to affect regeneration as no difference was observed when compared to controls. However, by 2 weeks, a significant reduction at 24, 48, and 72 hrs following amputation was documented (79.1 %, 61.0 % and 57.6 %, of control values respectively) which was further exacerbated when we analyzed fish that were hyperglycemic for 3 weeks (43.6 % 52.8 % and 48.6 % of control values). Using fluorescence microscopy we have also characterized that the regeneration defect is due to a reduced number of proliferating cells and we are currently working to identify the mechanism of the proliferation defect. The pattern of gene expression revealed using microarray analysis has shown that the process of regeneration in different tissues share a number of universal similarities. Therefore, the signals identified for caudal fin regeneration may have broader application.

Complications of diabetes mellitus result in reduced longevity and quality of life. In diabetic patients, these complications persist and progress even after successful pharmaceutical re-establishment of normoglycemia due to a phenomenon termed metabolic memory (39). One current focus of diabetes mellitus research is the reintroduction of beta islet cells through transplantation (allograft or xenograft) or the generation of islets from resident or transplanted stem cells (40). While islet transplantation will alleviate the need for insulin treatment, it can be expected that harmful complications will persist and progress in post-transplantation patients. It is therefore necessary to understand the altered molecular mechanisms sensitive to metabolic memory if regenerative medicine is to be successfully applied to prevent or reverse diabetic complications. As previously discussed, the zebrafish is able to regenerate a functional pancreas and can therefore model the post transplantation environment. Furthermore, we have observed that normoglycemia can be re-established following cessation of STZ administration. In contrast, caudal fin regeneration remains impaired to the same extent as it was during the acute hyperglycemic state which demonstrates the presence of metabolic memory (data not shown, manuscript in preparation). Therefore, the model presented here will allow for studying the mechanisms of metabolic memory with the goal of ameliorating persistent diabetic complications.

In summary, we have shown the utility of streptozocin to induce diabetes mellitus in the adult zebrafish and that treated fish develop complications similar to those seen in humans and other model organisms. Most importantly, we have characterized a reduction in the regenerative capacity of these diabetic zebrafish and are able, for the first time, to study the regeneration process in the presence of diabetic pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract.* 2010; 87:4–14. [PubMed: 19896746]

2. Amsterdam A, Hopkins N. Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. *Trends Genet.* 2006; 22:473–78. [PubMed: 16844256]
3. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet.* 2007; 8:353–67. [PubMed: 17440532]
4. Bassett D, Currie PD. Identification of a zebrafish model of muscular dystrophy. *Clin Exp Pharmacol Physiol.* 2004; 31:537–40. [PubMed: 15298547]
5. Childs S, Weinstein BM, Mohideen MA, Donohue S, Bonkovsky H, Fishman MC. Zebrafish dracula encodes ferrochelatase and its mutation provides a model for erythropoietic protoporphyria. *Curr Biol.* 2000; 10:1001–4. [PubMed: 10985389]
6. Piotrowski T, Ahn DG, Schilling TF, Nair S, Ruvinsky I, Geisler R, Rauch GJ, Haffter P, Zon LI, Zhou Y, Foott H, Dawid IB, Ho RK. The zebrafish van gogh mutation disrupts *tbx1*, which is involved in the DiGeorge deletion syndrome in humans. *Development.* 2003; 130:5043–52. [PubMed: 12952905]
7. Sun Z, Amsterdam A, Pazour GJ, Cole DG, Miller MS, Hopkins N. A genetic screen in zebrafish identifies cilia genes as a principal cause of cystic kidney. *Development.* 2004; 131:4085–93. [PubMed: 15269167]
8. Gerull B, Gramlich M, Atherton J, McNabb M, Trombitas K, Sasse-Klaassen S, Seidman JG, Seidman C, Granzier H, Labeit S, Frenneaux M, Thierfelder L. Mutations of *TTN*, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. *Nat Genet.* 2002; 30:201–4. [PubMed: 11788824]
9. Berghmans S, Jette C, Langenau D, Hsu K, Stewart R, Look T, Kanki JP. Making waves in cancer research: new models in the zebrafish. *Biotechniques.* 2005; 39:227–237. [PubMed: 16116796]
10. Langenau DM, Feng H, Berghmans S, Kanki JP, Kutok JL, Look AT. Cre/lox-regulated transgenic zebrafish model with conditional myc-induced T cell acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A.* 2005; 102:6068–73. [PubMed: 15827121]
11. van der Sar AM, Appelmelk BJ, Vandenbroucke-Grauls CM, Bitter W. A star with stripes: zebrafish as an infection model. *Trends Microbiol.* 2004; 12:451–7. [PubMed: 15381194]
12. Darland T, Dowling JE. Behavioral screening for cocaine sensitivity in mutagenized zebrafish. *Proc Natl Acad Sci U S A.* 2001; 98:11691–6. [PubMed: 11553778]
13. Liu FJ, Wang JS, Theodorakis CW. Thyrotoxicity of sodium arsenate, sodium perchlorate, and their mixture in zebrafish *Danio rerio*. *Environ Sci Technol.* 2006; 40:3429–36. [PubMed: 16749717]
14. Mandrekar N, Thakur NL. Significance of the zebrafish model in the discovery of bioactive molecules from nature. *Biotechnol Lett.* 2009; 31:171–9. [PubMed: 18931972]
15. Westerfield M, Doerry E, Kirkpatrick AE, Driever W, Douglas SA. An on-line database for zebrafish development and genetics research. *Semin Cell Dev Biol.* 1997; 8:477–88. [PubMed: 9441953]
16. Thummel R, Bai S, Sarras MP Jr, Song P, McDermott J, Brewer J, Perry M, Zhang X, Hyde DR, Godwin AR. Inhibition of zebrafish fin regeneration using in vivo electroporation of morpholinos against *fgfr1* and *msxb*. *Dev Dyn.* 2006; 235:336–46. [PubMed: 16273523]
17. Ganda OP, Rossini AA, Like AA. Studies on streptozotocin diabetes. *Diabetes.* 1976; 25:595–603. [PubMed: 132382]
18. Wright JR Jr, Abraham C, Dickson BC, Yang H, Morrison CM. Streptozotocin dose-response curve in tilapia, a glucose-responsive teleost fish. *Gen Comp Endocrinol.* 1999; 114:431–40. [PubMed: 10336831]
19. Armbruster DA. Fructosamine: structure, analysis, and clinical usefulness. *Clin Chem.* 1987; 33:2153–63. [PubMed: 3319287]
20. Rees DA, Alcolado JC. Animal models of diabetes mellitus. *Diabet Med.* 2005; 22:359–70. [PubMed: 15787657]
21. Li Z, Wen C, Peng J, Korzh V, Gong Z. Generation of living color transgenic zebrafish to trace somatostatin-expressing cells and endocrine pancreas organization. *Differentiation.* 2009; 77:128–34. [PubMed: 19281772]
22. Chen S, Li C, Yuan G, Xie F. Anatomical and histological observation on the pancreas in adult zebrafish. *Pancreas.* 2007; 34:120–5. [PubMed: 17198193]

23. Jefferson JA, Shankland SJ, Pichler RH. Proteinuria in diabetic kidney disease: a mechanistic viewpoint. *Kidney Int.* 2008; 74:22–36. [PubMed: 18418356]
24. Aizu Y, Oyanagi K, Hu J, Nakagawa H. Degeneration of retinal neuronal processes and pigment epithelium in the early stage of the streptozotocin-diabetic rats. *Neuropathology.* 2002; 22:161–70. [PubMed: 12416555]
25. Alvarez Y, Chen K, Reynolds AL, Waghorne N, O'Connor JJ, Kennedy BN. Predominant cone photoreceptor dysfunction in a hyperglycaemic model of non-proliferative diabetic retinopathy. *Dis Model Mech.* 2010; 3:236–45. [PubMed: 20142328]
26. Akimenko MA, Mari-Beffa M, Becerra J, Geraudie J. Old questions, new tools, and some answers to the mystery of fin regeneration. *Dev Dyn.* 2003; 226:190–201. [PubMed: 12557198]
27. Poss KD, Keating MT, Nechiporuk A. Tales of regeneration in zebrafish. *Dev Dyn.* 2003; 226:202–10. [PubMed: 12557199]
28. Makino S, Whitehead GG, Lien CL, Kim S, Jhavar P, Kono A, Kawata Y, Keating MT. Heat-shock protein 60 is required for blastema formation and maintenance during regeneration. *Proc Natl Acad Sci U S A.* 2005; 102:14599–604. [PubMed: 16204379]
29. Lerco MM, Macedo CS, Silva RJ, Pinheiro DO, Spadella CT. The number of podocyte and slit diaphragm is decreased in experimental diabetic nephropathy. *Acta Cir Bras.* 2006; 21:87–91. [PubMed: 16583060]
30. Tajiri K, Shimizu Y. Practical guidelines for diagnosis and early management of drug-induced liver injury. *World J Gastroenterol.* 2008; 14:6774–85. [PubMed: 19058303]
31. Moss JB, Koustubhan P, Greenman M, Parsons MJ, Walter I, Moss LG. Regeneration of the Pancreas in Adult Zebrafish. *Diabetes.* 2009; 58(8):1844–51. Epub 2009 Jun 2. [PubMed: 19491207]
32. Kasselman LJ, Veves A, Gibbons CH, Rutkove SB. Cold exposure exacerbates the development of diabetic polyneuropathy in the rat. *Exp Diabetes Res.* 2009;827943. 2009. Epub 2010 Jan 14. [PubMed: 20130819]
33. Yamagishi S, Nakamura K, Matsui T, Ueda S, Fukami K, Okuda S. Agents that block advanced glycation end product (AGE)-RAGE (receptor for AGEs)-oxidative stress system: a novel therapeutic strategy for diabetic vascular complications. *Expert Opin Investig Drugs.* 2008; 17:983–96.
34. Chiarelli F, Gaspari S, Marcovecchio ML. Role of growth factors in diabetic kidney disease. *Horm Metab Res.* 2009; 41(8):585–93. Epub 2009 May 18. [PubMed: 19452424]
35. Breyer MD, Tchekneva E, Qi Z, Takahashi T, Fogo AB, Harris RC. Examining diabetic nephropathy through the lens of mouse genetics. *Curr Diab Rep.* 2007; 7:459–66. [PubMed: 18255011]
36. Gross O, Beirowski B, Harvey SJ, McFadden C, Chen D, Tam S, Thorner PS, Smyth N, Addicks K, Bloch W, Ninomiya Y, Sado Y, Weber M, Vogel WF. DDR1-deficient mice show localized subepithelial GBM thickening with focal loss of slit diaphragms and proteinuria. *Kidney Int.* 2004; 66:102–11. [PubMed: 15200417]
37. Aizu Y, Katayama H, Takahama S, Hu J, Nakagawa H, Oyanagi K. Topical instillation of ciliary neurotrophic factor inhibits retinal degeneration in streptozotocin-induced diabetic rats. *Neuroreport.* 2003; 14:2067–71. [PubMed: 14600499]
38. Gleeson M, Connaughton V, Arneson LS. Induction of hyperglycaemia in zebrafish (*Danio rerio*) leads to morphological changes in the retina. *Acta Diabetol.* 2007; 44:157–163. [PubMed: 17721755]
39. Ceriello A, Ihnat MA, Thorpe JE. Clinical review 2: The “metabolic memory”: is more than just tight glucose control necessary to prevent diabetic complications? *J Clin Endocrinol Metab.* 2009; 94:410–15. [PubMed: 19066300]
40. Yechoor V, Chan L. Minireview: {beta}-Cell Replacement Therapy for Diabetes in the 21st Century: Manipulation of Cell Fate by Directed Differentiation. *Mol Endocrinol.* Mar 10.2010 Epub ahead of print.

Figure 1. Streptozocin injection results in sustained hyperglycemia

A. Graphic representation of fasting blood glucose concentration. UN: uninjected zebrafish, 58.85 ± 4.79 mg/dL (n= 15), C: 61.94 ± 5.60 (n=287), W1: 306.3 ± 32 mg/dL, n = 17, zebrafish that only received injections for one week, W2: 307.9 ± 37.3 mg/dL n = 13, zebrafish that received first week injections plus one booster, W3: 311.6 ± 42.1 mg/dL, n =224, zebrafish that received first week injections plus two boosters. A Student's t-test of statistical significance $p < 0.00001$ was obtained when W1, W2 and W3 were compared to C and UN. **B.** Graphic comparison of serum fructosamine levels in control and STZ treated zebrafish. The serum from 4 fish were pooled and the experiment was repeated 3 times (total n =12). The optical density measurements (output of the assay) indicate a 320 % increase ($p < 0.05$) of fructosamines in STZ treated zebrafish.

Figure 2. Streptozocin injection results in insulinopenia

A. Analysis of insulin levels in the blood shows a decrease in the amount of insulin in STZ (W3) treated fish. An undiluted (U) or a 1:5 dilution (1:5) of pooled serum samples from control (C) and hyperglycemic fish (H), probed with anti-insulin antibody. **B.** Phase contrast images of control (i) and W3 (ii) fish are shown where P indicates the pancreas tissue, IN marks the intestine and a 100 micrometer scale bar is included. Representative immunofluorescence microscopy images of the same pancreas tissue indicating the presence of glucagon (green), insulin (red), and DAPI (blue) for control (iii) and W3 (iv) fish are shown. The scale bar indicates 50 micrometers.

Figure 3. Hyperglycemic fish show thickening of the glomerular basement membrane at three weeks

A. A 40,000X Image of a glomerulus from a control injected zebrafish. The podocytes (P), endothelial cells (E) and glomerular basement membrane (G) are indicated on the figure and the inset scale bar indicates 500 nm. **B.** A 40,000X image of a glomerulus from a STZ injected fish showing increased thickness of the glomerular basement membrane. The letter designations are as in panel A. **C.** Graphic comparison of the mean values of glomerular basement membrane thickness (15 measurements for each). The bars represent the mean thickness measurement of all GBMs in each group with the error bar representing the standard error. Control: 1056 $\pm$ 29.5 units, STZ: 1695 $\pm$ 93.5 ($p < .000005$).

Figure 4. Hyperglycemic fish show retina layer thinning

Representative images of retina from (W3) control and STZ injected fish. **A.** Control fish retina; the thickness of the photoreceptor layer (PSL) is indicated by the yellow bar, the thickness of the inner plexiform layer (IPL) is indicated by the red bar and the white scale bar indicates 200 micrometers. **B.** Hyperglycemic fish retina with PSL and IPL designations as in panel A. **C.** Graphic representation comparing the thickness (arbitrary units) of the IPL and PSL layers of hyperglycemic (black bars) and control (white bars) fish. The bars represent the mean thickness measurement of all retinas in each group ($n = 8$ for each) with the error bar representing the standard error. PSL control = 43.67 ± 1.52 , PSL STZ = 35.5 ± 2.02 , IPL control = 39.2 ± 1.28 and IPL STZ = 28.8 ± 1.15 . $p < 0.005$, PSL and $p < 0.0000001$, IPL.

Figure 5. Hyperglycemic fish have a reduced rate of caudal fin regeneration

A. Caudal fin image from a control injected fish showing a normal amount of regenerative growth 72 hours post amputation. The area of regeneration was determined and the original cut line (white line in image) was used to normalize fin size differences. **B.** A 72 hour post amputation caudal fin from a W3 hyperglycemic fish showing a smaller area of regenerated tissue. **C.** Graphic presentation of the relative regeneration rate of hyperglycemic zebrafish as compared to control fish at week 1 (white bars) week 2 (grey bars) and week 3 (black bars). Week 1: 101.0 %, 106.6 % and 100 % for 24, 48 and 72 hours respectively, n= 10 for control and 14 for hyperglycemic fish. Week 2: 79.1 %, 61.0 % and 57.6 % for 24, 48 and 72 hours respectively, n= 11 for control and 9 for hyperglycemic fish. Week 3: 43.6 %, 52.8 % and 48.6 % for 24, 48 and 72 hours respectively, n= 32 for control and 19 for hyperglycemic fish. When values within an experiment were statistically compared using a Student's t test a $p < 0.05$ or less was obtained.

Figure 6. Reduced Proliferation is Observed in the Regenerating Caudal Fins of Hyperglycemic Fish

A. Representative fluorescence images of the regenerative area for cell proliferation and apoptosis assays are presented as indicated below. Adjacent 10 micrometer sections of W3 control (i,iii and v, n = 8) or STZ injected fish (ii,iv and vi n = 8) were processed with either anti-PCNA antibody (i and ii), phosphor-histone H3 (Ser10) (H3P) (iii and iv) or processed via the TUNEL assay to detect apoptosis (v and vi). A white 50 micrometer scale bar is included. **B.** Quantification of proliferation in regenerating fins. The number of PCNA or (H3P) positive cells (n = 12 for each group) was determined and is expressed as a percentage of the total number of cells (determined by DAPI staining). PCNA:Epithelium + Blastema: control 56.8 % $\pm$ 3.4, STZ 29.7 % $\pm$ 2.9, Blastema only: control 64.1 % $\pm$ 5.3, STZ 39.6 $\pm$ 2.5. $p < .01$ for both. H3P: Epithelium + Blastema: control 34.9 % $\pm$ 3.0, STZ 21.2 % $\pm$ 2.5. Blastema only: control 29.9 % $\pm$ 1.9, STZ 21.4 $\pm$ 1.4, $p < 0.01$. $p < .01$ for both