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Acute effects of hyperglycemia in a chemical-genetic cell ablation model

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Abstract

Diabetes Mellitus (DM) is a debilitating disease characterized by elevated blood glucose levels, known as hyperglycemia. Glucose, the energy source for metabolism, is transported to the cells of the body by the hormone insulin. Without the proper formation and utilization of insulin, DM occurs in two forms; Type I DM is characterized by the loss β -cells which produce insulin, whereas, Type II DM is characterized by insulin resistance in which the body does not respond to the insulin that is produced. Regardless of DM subtype, 50% of patients suffer with mild to severe forms of nerve damage known as diabetic peripheral neuropathy (DPN). In the quest for therapeutic techniques, we are first trying to determine if zebrafish are an appropriate model organism for studying DPN as they are easier to observe than other animals such as mice, rats and humans. Their ability to serve as an appropriate model is dependent on them showing signs of peripheral nerve damage, as seen in humans, when rendered hyperglycemic. This was examined by exploiting a transgenic zebrafish line, *Tg(ins;nfsB-mcherry)*. In this line, insulin producing β -cells of the pancreas are destroyed using the prodrug metronidazole (MTZ) and visualized by the fluorescent protein, mcherry, which was inserted into its genome. To assess possible peripheral nerve changes, we crossbred the transgenic lines, *Tg(nkx2.2a;megfp)* and *Tg(nbt;dsred)*, into the *Tg(ins;nfsB-mcherry)* line to allow us to visualize the perineurial glia (GFP), the motor axons (DsRed) and the β -cells (mcherry) in the offspring. The offspring were raised for 5 days post fertilization (dpf) in egg water and then treated with MTZ for 48 hours. Using epifluorescent microscopy, peripheral nerve disruption was observed in two ways; (1) fewer motor nerves had perineurium (connective tissue that is important in maintaining the integrity of nerves) associated with them and (2) when the perineurium was present, it often appeared to improperly wrap the motor nerve. In addition, the motor axons of the MTZ-treated

group also appear to be defasciculated and the neuromasts, mechanosensory hair cells, appear unhealthy compared to the control. This suggests that the sensory and motor systems of zebrafish are influenced by acute hyperglycemia. Future work will include a small molecule drug screen that may be able to provide insight on the molecular mechanisms underlying hyperglycemia-induced degeneration of the peripheral nerve.

Introduction

Diabetes Mellitus (DM) is a debilitating disease characterized by high circulating blood glucose levels (hyperglycemia). In the 2014 National Diabetes Statistics Report (jointly produced by the CDC, NIH and ADA), DM was documented as the seventh leading cause of death in the United States in 2010¹. This epidemic has led to \$245 billion in costs in 2012 with 9.3% of the population suffering from the disease¹.

The origin of this deleterious condition can be attributed to two forms of DM, Type I or Type II DM. In both forms, hyperglycemia is caused by disrupted insulin signaling. Insulin is a hormone produced by the β -cells of the pancreas and promotes the absorption of glucose from the blood into the body's cells². Once inside the cell, glucose is broken down to provide the body with the energy needed to sustain a healthy life. Type I DM is caused by the loss of insulin producing β -cells through a destructive T cell-mediated autoimmune response³. Type II DM, is caused by the downregulation of insulin receptors (also known as insulin resistance). Therefore, although insulin is being secreted from the pancreas (usually in appropriate amounts) it is unable to exert an effect due to the lack of insulin receptors⁴.

A full understanding of how DM manifests in both Type I and II is incomplete as different combinations of genetic and environmental factors play a role. The current accepted treatment practice is to bring blood glucose levels to a normal range; in Type I DM this means undergoing tight control by monitoring glucose levels and delivering insulin injections when appropriate. Practicing tight control requires maintaining a blood glucose level between 70 and 130 mg/dL before meals, less than 180 mg/dL two hours after meals and having glycated hemoglobin (A1c) level at 7 percent or less⁵. Doing so prevents exacerbating any injuries or complications related to the disease. In Type II DM this means monitoring glucose levels, but

since insulin is not typically administered more attention must be given to lifestyle habits such as nutrition, physical activity and management of stress⁶. If left unmanaged, patients may develop life-threatening conditions, the two most serious being Diabetic Ketoacidosis (DKA) and hyperosmolar hyperglycemic state (HHS)⁷. In DKA, when glucose is not being metabolized, the body will use fat and muscle energy stores⁷. The byproduct of these reactions is ketones, acids that become lethal at high concentrations⁷. Symptoms associated with this condition are excessive thirst, frequent urination, nausea and vomiting, abdominal pain, fatigue, shortness of breath and confusion⁷. If emergency care is not taken after the onset of symptoms, the condition will lead to coma or death⁷. While patients with HHS experience overlapping symptoms, it is a state of severe dehydration and must first be treated by attempting to compensate for water loss⁸.

In the past decade, the prevalence of people with diabetes has continually grown and it has put them at a much higher risk for myocardial infarction, stroke and limb amputation⁹. It is also now a leading cause of blindness, end stage renal disease and several neuropathies (peripheral, proximal, autonomic and focal neuropathies)⁹. Peripheral neuropathy is the most common complication associated with DM; ~50% of people with diabetes have mild to severe forms of peripheral nerve damage¹⁰. The condition can affect motor and/or sensory nerves of the patient. Motor nerves allow the brain to communicate with muscles and glands and damage can lead to weakness or sharp pain¹¹. Sensory nerves have receptors that allow individuals to detect pressure, changes in temperature, pain and chemical stimuli. Symptoms of sensory nerve damage typically include numbness, tingling, and pain in the hands or feet¹².

Of particular interest in our lab, is diabetic peripheral neuropathy (DPN). Currently, treatment for DPN is limited to temporary pain relief illustrating a strong need for continued research. As 50% of people suffering from both type 1 and 2 DM have DPN, the annual costs of

DPN and its complications in the United States is \$10.9 billion¹³. The vertebrate peripheral nerve is composed of axons (which send the signal), Schwann cells (that make up the insulating myelin sheath), the endoneurium (surrounding myelinated nerve fibers), perineurial cells (which ensheath fascicles or bundles of nerve fibers) and the epineurium (that surrounds several fascicles to form the outermost layer of a nerve)¹⁴. How this structure is breaking down is unclear. In order to have translational research, the model system must mimic physiological conditions of humans with DPN. The mouse model is currently the most well-established model of DPN, but there are several disadvantages that create a need for alternative methods of study. The most common method of Type I DM induction in mice is with treatment of the drug streptozotocin (STZ)¹⁵. While STZ is a reliable method of β - cell destruction, it can also be toxic to other organs in the body¹⁵. Non-obese diabetic (NOD) mice serve as an autoimmune model as their lymphocytes infiltrate the β -cells by 1 month; however, drugs that treat NOD mice have been ineffective in humans¹⁵. Pancreatectomies have also been performed in larger mammals, but are rather invasive and frequently result in gastrointestinal issues¹⁵.

Zebrafish are an ideal animal model because they are vertebrates and are genetically similar to humans¹⁶. In addition, zebrafish are also easy to house, have a high fecundity, and genetic changes can be introduced with ease. There are also many resourceful tools that are applicable *in vivo* with zebrafish, such as microscopy. Here, genes of interest can be tagged with fluorescent proteins and because the zebrafish embryos are optically transparent, we are able to watch (in real-time) these fluorescent genes of interest interact. For example, we can visualize cells that express *olig2:dsred* (motor axons tagged red) and cells that express *sox10:gfp* (Schwann cells tagged green) in order to watch the interactions between the axon and the Schwann cells in the peripheral nervous system. Most importantly, there is strong morphological

conservation of gene expression involved in pancreatic development between zebrafish and humans¹⁷ and previous data recapitulates diabetic complications in zebrafish¹⁸⁻²¹. A study done on limb regeneration in zebrafish showed that the injection of streptozocin, a β -cells toxin, resulted in hyperglycemia²⁰. Associated symptoms seen in this study included retinal thinning and glomerular basement membrane thickening, early signs of retinopathy and nephropathy²⁰. Other diabetic symptoms found in zebrafish following glucose-induced hyperglycemia include, cone photoreceptor disruption, dilated and thickened blood vessels in central retina, altered cardiac development, and increased cortisol levels²²⁻²⁴. Following caudal fin amputation, diabetic zebrafish are also seen to have impaired fin regeneration compared to a metabolically healthy group²¹. We will exploit this model system to determine how hyperglycemia manifests itself in the peripheral nervous system.

Preliminary data from our lab suggest that hyperglycemia induces degeneration of motor axons in zebrafish (Fig. 1), making them an effective model for studying diabetic neuropathy. It is unclear which component, the axons, the Schwann cells or the perineurial sheath, degenerates first. Because axons and Schwann cells are dependent on one another for growth signals, it could be that the loss of myelin is actually indicative of the loss of Schwann cells and without the growth signals from the Schwann cells, the axon deteriorates secondarily.

Another possibility is that the perineurium is damaged and without its protective capacity in place, leads to the destruction of axons. The blood-nerve-barrier consists of the endoneurial microvessels and the tightly jointed, multilayered perineurium²⁵. It is relatively impermeable in order to protect the nervous system from the flow of potentially toxic substances present in the blood plasma²⁵. Thus, its disruption is a clear sign of infection or inflammation. Knowing the initial target of damage will potentially give insight into the molecular mechanism responsible

for axonal degeneration. To date, there is no published work on the effect of hyperglycemia-induced DPN in zebrafish, however, our lab has preliminary data suggesting that motor axons (Fig. 1), sensory neurons (Fig. 2) and the perineurium (Fig. 3) are all disrupted in response to high blood glucose levels.

The current study will utilize the chemical-genetic cell ablation model which induces β -cell death, to observe the axonal and perineurial glial response to hyperglycemia. The transgenic zebrafish line (*Tg(ins;nfsB-mcherry)*) has DNA from *Escherichia coli* that was amplified and introduced into the zebrafish genome using the *ins* promoter¹⁸. The *E. coli* gene, *nfsB*, is then expressed in the β -cells of the pancreas¹⁸. *NfsB* allows for the expression of the enzyme nitroreductase, which converts the added prodrug, metronidazole, to a cytotoxin, resulting in β -cell destruction. *NfsB* is fused to a fluorescent protein, mCherry, which allows the visualization of β -cell loss¹⁸. To further examine the degeneration process, this line was crossed with *Tg(nkx2.2a;megfp)* and *Tg(nbt;dsred)*, in order to visualize the motor axons (DsRed) and perineurial glia (GFP) of the peripheral nerve following β -cell ablation. To ensure that the treated group actually becomes hyperglycemic a glucose assay was performed after treatment (Fig. 4). A high blood glucose concentration affirms that this model of induction is effective in rendering the zebrafish hyperglycemic. We hypothesize that hyperglycemia in this zebrafish model will result in axonal degeneration as seen in the glucose induction model. Furthermore, we hypothesize that hyperglycemia is deleterious to the health of the perineurium in this chemical-genetic cell ablation model as well.

Materials and Methods

Danio rerio (Zebrafish) Care

Zebrafish are kept in 3L and 10L standing water tanks under a 14:10 hour light:dark cycle. Environmental control consists of maintaining an average 24-28°C water temperature, 6.0-8.0 pH, 50-150 mg/L CaCO₃, a conductivity of 300-1500 μS, and a hardness of 30-300+ mg/L. Adult zebrafish (>1 month) are fed brine and TetraMin tropical flakes 2x/day and larvae (<30 dpf) are fed hatchfry 2x/day. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Zebrafish Husbandry

The transgenic lines, *Tg(nkx2.2a: megfp)*, *Tg(nbt:dsred)* and *Tg(ins;nfsB-mcherry)* were placed in breeding chambers in a 1:1 male to female ratio overnight. The following day embryos were collected and placed into petri dishes while the adult fish were placed back into their respective tanks. From the embryos collected, those that were unfertilized or dead were removed from the dishes so that the development of the healthy, fertilized embryos would not be harmed. The healthy embryos were then raised in embryo medium at 28.5°C.

Chemical-Genetic Cell Ablation Model

Three lines of transgenic fish were crossed, *Tg(nkx2.2a:mEGFP)*, *Tg(nbt:dsred)* and *Tg(ins;nfsB-mcherry)* to produce offspring that have fluorescently tagged perineurial glia, motor axons and pancreatic β-cells respectively. Hyperglycemic induction is accomplished by employing the *Tg(ins;nfsB-mcherry)* line. In this line, (*Tg(ins;nfsB-mcherry)*) zebrafish express the *E. coli* gene, *NfsB* which codes for the enzyme nitroreductase, in the β-cells of the pancreas.

After treatment with metronidazole (MTZ), nitroreductase, converts the prodrug into a cytotoxin resulting in β -cell ablation (similar to Type I DM). The fusion of NfsB to a fluorescent protein, mCherry, allows the visualization of the β -cell loss.

Hyperglycemic Induction

At 5dpf the larvae were examined using epifluorescent microscopy and those that expressed perineurial glia, motor axons, and pancreatic β -cell fluorescence were used for experimentation. The treated group was raised in egg water until 5dpf then treated with 10mM MTZ and incubated in the dark for 48 hours, replacing the media with fresh MTZ solution at the 24-hour mark. It is essential that incubation is done in the dark, as MTZ degrades with exposure to the light. The control group was raised in egg water until 7dpf. At 7 dpf both groups were visualized *in vivo* using epifluorescence microscopy. The control group was used as a comparison for how healthy β -cells should appear. Treated fish that experienced β -cell death, as seen by loss of mcherry+ fluorescence in the pancreatic islet, were then further examined for peripheral nerve damage.

Histology

Both the control and treated groups were then anesthetized and fixed in 4% PFA for two hours at room temperature. Following fixation, the fish were mounted in agar and placed in a 30% sucrose solution for at least 24 hours and not exceeding one week. Using a cryostat, the fish were then sliced into 15 μ m thick cross-sections, situated onto microscope slides and coverslipped. The sucrose solution used in the previous step serves as a cryoprotectant to protect the tissue from freezing damage that could occur during the sectioning process. After the slides

have been given ample amount of time to dry, analysis of the peripheral nervous system can begin. Using the SPOT RT/KE (Diagnostic Instruments Inc., Sterling Heights, MI) camera connected to the epifluorescent microscope, images taken under appropriate filters can be merged to create a comprehensive picture of transgenes present in the fish.

Glucose Assay

To affirm that the experimental design leads to hyperglycemia, a glucose assay (Glucose Bioassay, VWR) was conducted. After performing hyperglycemic induction following the protocol used in this study, samples of 20 fish per group were collected and flash frozen at -80°C . Samples were then thawed in assay buffer, homogenized and centrifuged at 13,000 rpm for 1 minute. Thereafter the supernatant was collected and pellet discarded. A glucose standard was diluted to $1\text{nmol}/\mu\text{l}$ and 1,2,4,6,8,10 μl were added to a series of wells. The well volume was then brought up to 50 μl with Glucose Assay Buffer. 16 μl of sample was added to 34 μl assay buffer. A reaction mix consisting of 46 μl Glucose Assay Buffer, 2 μl Glucose Probe and 2 μl Glucose Enzyme Mix per sample was added to each well. The plate was incubated at 37°C in the dark for 30 minutes. Following incubation, absorbance was measured at 570 nm in a microplate reader and analyzed with Spectromax pro. Glucose Enzyme Mix oxidizes glucose and creates a product that reacts with a dye to produce a pink color that reads at 570nm and is directly proportional to the concentration of glucose.

Specific Aim 1: Motor axons degenerate following acute hyperglycemia

We hypothesize that *Tg(nkx2.2a: megfp);Tg(nbt:dsred);Tg(ins;nfsB-mcherry)+* zebrafish will experience motor axon degeneration following MTZ-induced cytotoxicity of β -cells. DPN is defined as peripheral nerve damage caused by hyperglycemia. The addition of MTZ raises blood glucose concentration by ablating insulin producing β -cells of the pancreas. We thereby expect to observe motor axon damage similar to that seen in previous data with the 60 mM glucose treated zebrafish (Fig.1). The purpose of this experiment is to provide another form of evidence that zebrafish rendered hyperglycemic experience similar DPN phenotypes to human patients.

Results

In this study, we observe that the motor axons of the MTZ-treated group appear to be defasciculated (Fig. 5). Surprisingly, we also observed that the neuromasts appeared unhealthy compared to the control (Fig. 5, inset) Neuromasts are sensory organs on the lateral line of zebrafish, which allow fish to determine the direction and rate of water movement¹⁷. Neuromasts consist of mechanosensory hair cells that are innervated by sensory neurons localized in the cranial ganglion¹⁷. Mechanoreception is the ability to sense external stimuli such as touch, pressure, and vibration¹⁷. While there is no direct correlation, since there is no human homologue, DPN patients also exhibit sensory damage. The data found in this study suggests that the motor and sensory systems in zebrafish are influenced by acute hyperglycemia making it an appropriate model organism.

Specific Aim 2: The perineurium is disturbed following acute hyperglycemia

We hypothesize that *Tg(nkx2.2a: megfp);Tg(nbt:dsred);Tg(ins;nfsB-mcherry)*+ zebrafish will experience a disturbance in the perineurium following MTZ-induced cytotoxicity of β -cells. Knowing that motor axon degeneration occurs in this model of hyperglycemic induction, we wanted to find out what structures were involved. Inside of the outermost layer of the nerve, the epineurium, is the perineurium which wraps nerve fascicles containing the individual nerve fibers. This structure protects the delicate axons from ionic influx. In preliminary data where zebrafish were treated with 60 mM glucose, the motor axons were not properly ensheathed by the perineurium (Fig. 3). We want to strengthen the support for the zebrafish model by seeing if DPN phenotype can be replicated in zebrafish treated with metronidazole.

Results

The control group maintained healthy motor nerves seen by axons appropriately wrapped by perineurial glia (Fig. 6A). Peripheral nerve disruption was observed in the MTZ-treated group in two ways; (1) fewer motor nerves had perineurium associate with them and (2) when the perineurium was present, it often appeared to improperly wrap the motor nerve (Fig.6B).

Discussion:

The significance of this work is that the established chemical-genetic cell ablation model of hyperglycemic induction in zebrafish mimics the symptoms of DPN. Genetic techniques are a valuable asset by targeting only the cells of interest, β -cells, and eliminating confounding variables that may disturb other pathways in the body. Here, motor axons are seen

defasciculating in larval zebrafish following the MTZ treatment. Motor nerves are responsible for carrying signals from the brain or spinal cord to muscles and glands. Without this relay of impulses, muscle strength declines and movements such as walking are greatly impaired. Additionally, fewer motor nerves had perineurium associated with them and when the perineurium was present, it often appeared to improperly wrap the motor nerve. The perineurium is an essential component of the blood nerve barrier and regulates permeability in order to prohibit the transfer of inappropriate substance to the nerve fibers. Neuromasts, mechanosensory organs of zebrafish are seen as unhealthy in larval zebrafish following the MTZ treatment. Together, this data suggest that both the motor and sensory systems are influenced by acute hyperglycemia in zebrafish. Demonstrating the effects associated with DM in this study provides a framework for future research into the disease.

Future work will examine the time course and molecular mechanisms underlying hyperglycemia-induced degeneration of the peripheral nerve. More markers of peripheral nerve components need to be studied in order to gain a complete picture of the underlying molecular causes of DPN. What components are affected first; the axons, the Schwann cells or the perineurial glia? Because axons, Schwann cells and perineurial glia are dependent on one another for growth signals, a more thorough time course of degeneration may allow a better understanding of the onset of DPN and possible clinical therapies. To check for the reversibility of DPN symptoms and potentially narrow down the molecular pathways involved, a small molecule drug screen will be performed. This process involves treating the fish with MTZ and then putting them in a well plate with each well containing a different drug.

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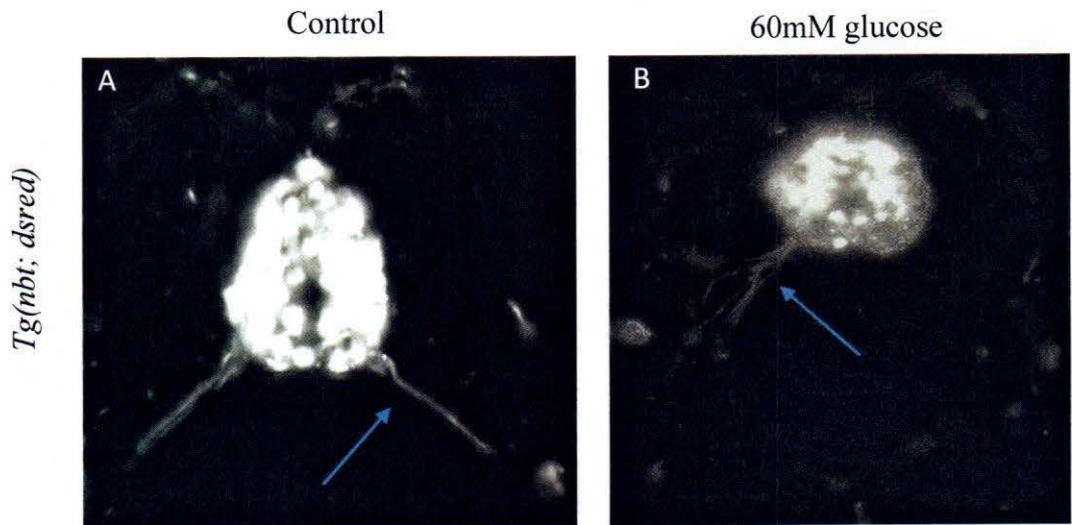


Figure 1. Motor axons (arrows) appear defasciculated (B) in zebrafish treated with 60 mM glucose solution for 5 days compared to control (A).

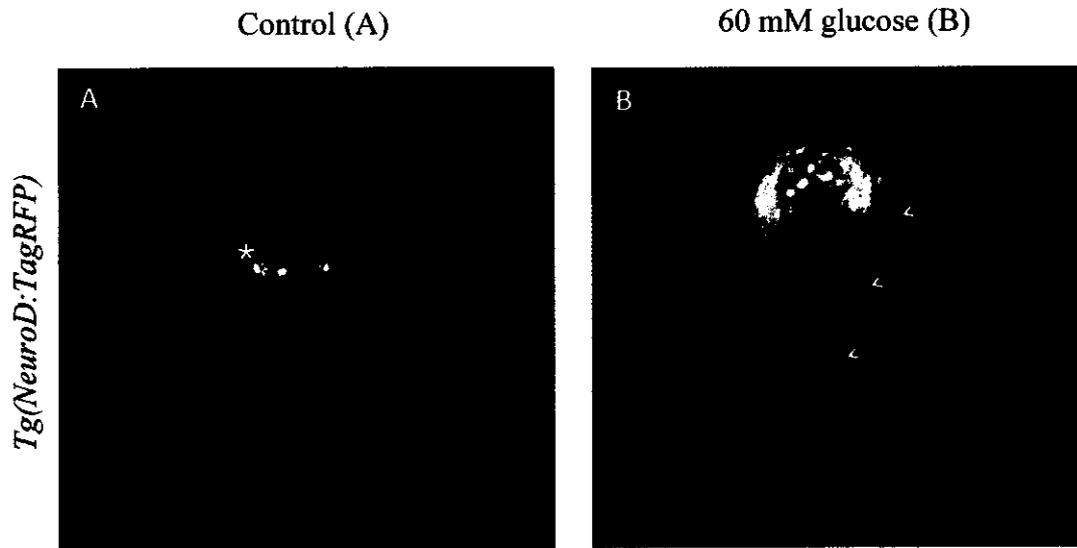


Figure 2. Sensory neurons in zebrafish (10dpf) treated with 60mM glucose for 5 days appear unhealthy and migrate away from the dorsal root ganglion of the spinal cord. An asterisk in the control and arrows in the experimental marks the sensory neurons, which are clustered appropriately around the spinal cord in the control (A) but have migrated along an axon in the 60mM glucose treated group (B).

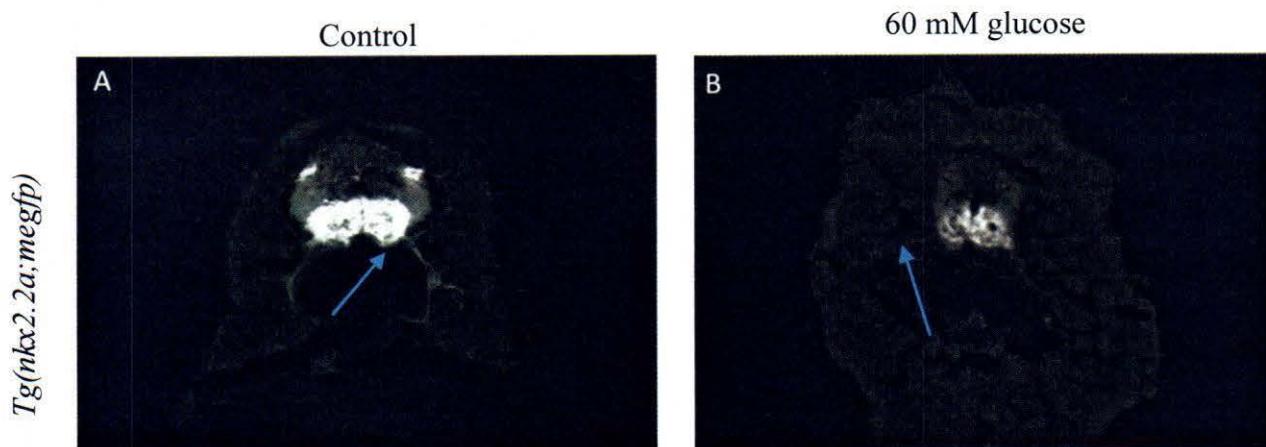


Figure 3. The control group (A) has normal perineurial glial cells that cover and protect the axons while the treated group (B), has perineurial extensions that are not properly ensheathing the motor nerve as observed in the control group.

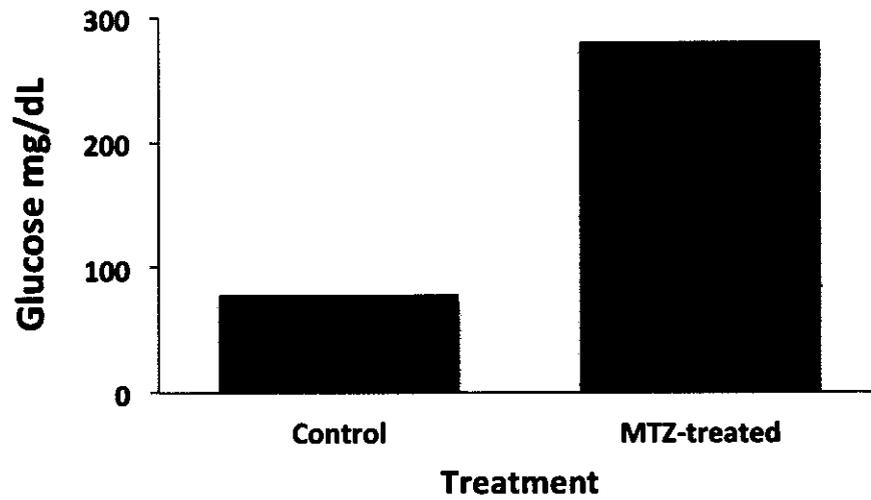


Figure 4. Glucose levels of *Tg(Ins:nsfB-mCherry)* control and MTZ-treated groups were assessed at 7dpf using a glucose assay (Glucose Bioassay, VWR). n=20/group, run in duplicate

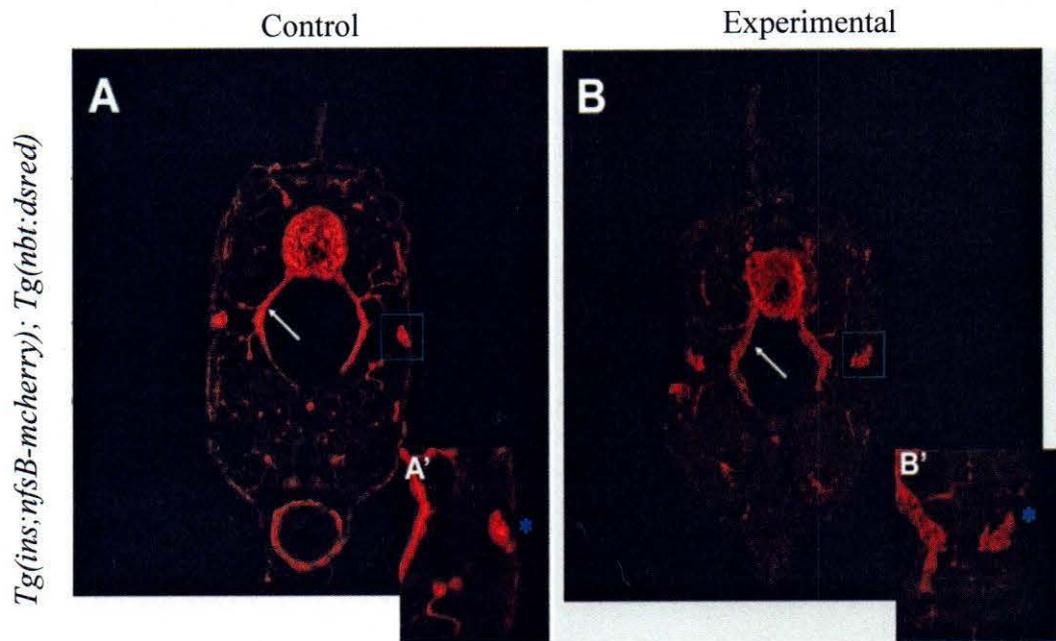


Figure 5. The axons (arrows) appear to be defasciculating in the MTZ-treated group (B) compared to the control (A). The neuromast (inset; asterisks) also appears unhealthy in the MTZ-treated group treated (B') compared to the control (A').

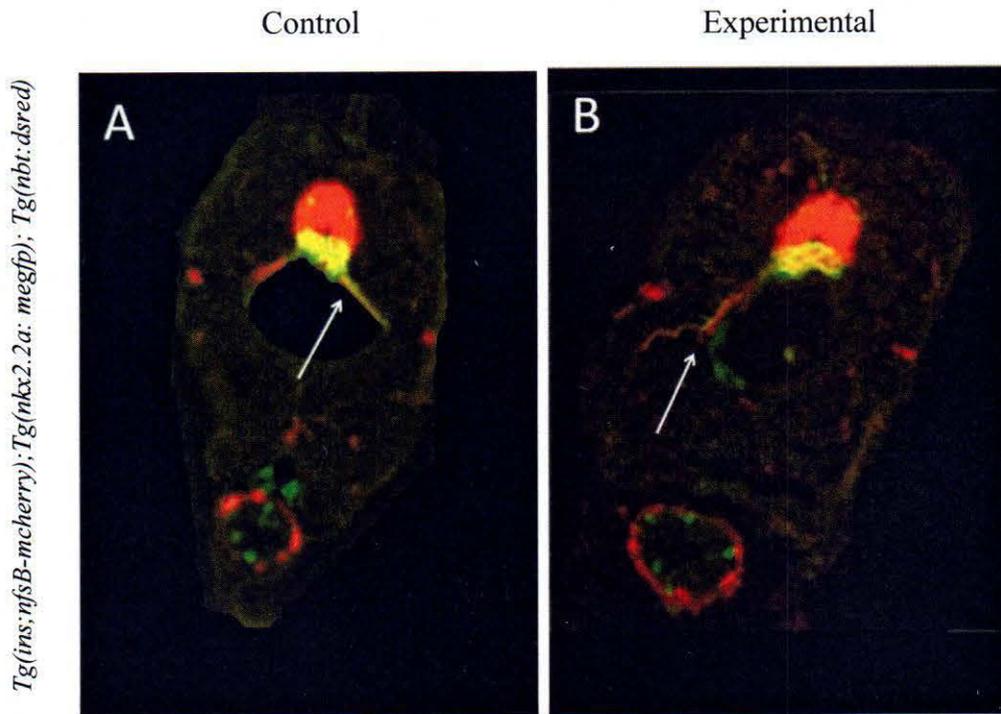


Figure 6. The control group (A) represents healthy nerve fibers (A; arrow) sheathed by the perineurium (GFP) while the MTZ-treated group (B) shows the perineurium (B; arrow) detaching itself from the axons (RFP).