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Alternate Immersion in Glucose to Produce Prolonged Hyperglycemia in Zebrafish

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Citation

McCarthy, E., Rowe, C.J., Crowley-Perry, M., Connaughton, V.P. Alternate Immersion in Glucose to Produce Prolonged Hyperglycemia in Zebrafish. *J. Vis. Exp.* (), e61935, doi:10.3791/61935 (2020).

Date Published

October 19, 2020

DOI

10.3791/61935

URL

jove.com/t/61935

Abstract

Zebrafish (*Danio rerio*) are an excellent model to investigate the effects of chronic hyperglycemia, a hallmark of Type II Diabetes Mellitus (T2DM). This alternate immersion protocol is a noninvasive, step-wise method of inducing hyperglycemia for up to eight weeks. Adult zebrafish are alternately exposed to sugar (glucose) and water for 24 hours each. The zebrafish begin treatment in a 1% glucose solution for 2 weeks, then a 2% solution for 2 weeks, and finally a 3% solution for the remaining 4 weeks. Compared to water-treated (stress) and mannitol-treated (osmotic) controls, glucose-treated zebrafish have significantly higher blood sugar levels. The glucose-treated zebrafish show blood sugar levels of 3-times that of controls, suggesting that after both four and eight weeks hyperglycemia can be achieved. Sustained hyperglycemia was associated with increased Glial Fibrillary Acidic Protein (GFAP) levels in retina and decreased physiological responses, as well as cognitive deficits suggesting this protocol can be used to model disease complications.

Introduction

Zebrafish (*Danio rerio*) are quickly becoming a widely used animal model to study both disease and cognition[1]. The ease of genetic manipulation and embryonic transparency through the early developmental stages, make them a prime candidate to study human diseases with a known genetic basis. For example, zebrafish have been used to study Holt-Oram syndrome, cardiomyopathies, glomerulocystic kidney disease, muscular dystrophy, and diabetes mellitus (DM) among other diseases[1]. In addition, the zebrafish model is ideal because of the species' small size, ease of maintenance, and high fecundity[2,3].

The zebrafish pancreas is both anatomically and functionally similar to the mammalian pancreas[4]. Thus, the unique characteristics of size, high fecundity, and similar endocrine structures make zebrafish a suitable candidate for studying DM-related complications. In zebrafish, there are two experimental methods used to induce the prolonged hyperglycemia that is characteristic of DM: an influx of glucose (modeling Type 2) and cessation of insulin secretion

(modeling Type 1)[5,6]. Experimentally, to stop insulin secretion, pancreatic β-cells can be chemically destroyed using either Streptozotocin (STZ) or Alloxan injections. STZ has been used successfully in rodents and zebrafish, resulting in complications associated with retinopathy[7,8,9], cognitive impairments[10], and limb regeneration[11]. However, in zebrafish, β-cells regenerate after treatment, causing "booster injections" of STZ to be necessary to maintain diabetic conditions[12]. Alternatively, the pancreas of the zebrafish can be removed[6]. These are both highly invasive procedures, due to the multiple injections, and extensive recovery time.

Conversely, hyperglycemia can be induced noninvasively through exposure to exogenous glucose. In this protocol, fish are submerged in a highly concentrated glucose solution for 24-hours[5,13] or continually for 2-weeks[14,15,16]. Exogenous glucose is taken up transdermally, by ingestion, and/or across the gills resulting in elevated blood sugar levels. Since this ~~non-nvasive~~ technique does not directly manipulate insulin levels, it cannot claim to induce Type 2 DM. However, it can be used to examine complications induced by hyperglycemia, which is one of the main symptoms of Type 2 DM.

Recently, the zebrafish mutant *pdx1*$^{-/-}$ was developed by manipulating the pancreatic and duodenal homeobox 1 gene, a gene linked to the genetic cause of Type 2 DM in humans. Using this mutant, researchers have been able to replicate pancreatic development disruption, high blood sugar, and study hyperglycemia-induced diabetic retinopathy[17,18].

In this paper, we describe a noninvasive hyperglycemia induction method that uses an alternating immersion protocol. This protocol maintains hyperglycemic conditions for up to 8 weeks with subsequent complications observed. In brief, adult zebrafish are placed in a sugar solution for 24 hours and then a water solution for 24 hours. As opposed to continuous immersion in external glucose solutions, alternating days between sugar and water mimics the rise and fall of blood sugar in diabetes. An alternating glucose protocol additionally allows hyperglycemia to be induced for longer periods of time, as the zebrafish are not as able to compensate for the high external glucose conditions. As proof of principle, we provide data showing that hyperglycemia induced using this protocol alters retinal chemistry and physiology.

Protocol

All procedures were approved by the Institutional Animal Care and Use Committee at American University.

1. Preparing the Solution Tanks

1. Obtain six tanks, two for each experimental group (glucose, mannitol, and water). Label one of the two tanks 'housing tank' (it will house the fish) and label the other 'solution tank' (it will hold the solution).
   NOTE: The mannitol treatment group is the osmotic control, and the water treatment group is a handling/stress control. It is important to keep the tanks, airlines/airstones, lids, and cleaning supplies separate for each treatment group
2. Use a 2 L tank if the total number of fish used is less than 20. Use a 4 L tank if the total number of fish used is more than 20.
   NOTE: Use an N of 5-10 per treatment group per sampling time point.
3. Keep the tanks in a water bath at 28-29 °C to maintain water temperature.
4. On Day 1, place the fish into their respective treatment solutions (glucose, mannitol, water) for 24-hours ('Water

to Treatment'). On Day 2, transfer the fish from their treatment solutions to water for 24-hours ('Treatment to Water'). On Day 3, transfer the fish from water to treatment solutions ('Water to Treatment'). This alternating exposure continues for the remainder of the experiment (**Figure 1**). Transfer water-treated control fish from water to water daily.

5. Ensure that the fish are fed and transferred within the same 2-hour window each day throughout the duration of the experiment.

2. Preparing the fish

1. Use adult zebrafish (4 months – 1 year)[5].
2. Feed the fish ground Tetramin flakes daily upon arrival to the lab.
3. Record the pH and the temperature of all the tanks and record the general condition of the fish.

3. Transferring fish

1. Transfer fish in each treatment group from the housing tank to the corresponding solution tank using a standard fish net.
2. Place the tank containing the fish back in the water bath, replace the airstone and tank lid. This tank is now the 'housing tank' and the tank that previously held the fish is now the 'solution tank'.
3. Discard the old solution and clean the tank, along with the tank lids, airlines, airstones, and nets to prevent buildup of glucose and mannitol.
   NOTE: Do not wash items with soap. Use water and a dedicated scrub brush/sponge for each treatment condition to properly clean the tanks.
4. Dry the newly cleaned 'solution tanks' with a paper towel. Prepare the solutions for the following day using this tank. Ensure the other items are dried and separated by appropriate treatment groups.
   NOTE: Keep a log of what solutions the fish are being transferred out of and into each day, as well as the solutions that are prepared for the following day. For example: Fish transferred from glucose to H_2O, new 1% glucose solution prepared for tomorrow.

4. Post-transfer solution preparation

1. Preparing sugar solutions
   1. Fill each solution tank with 2 L (or 4 L) of System Water (system water is defined as water that has been treated with the correct ratio of salt solution).
   2. Measure the correct amount of glucose and mannitol (see step 5 below) using a top loading scale and separate weigh boats for each chemical.
   3. Add the weighed glucose or mannitol aliquot to the appropriate, cleaned solution tank, which contains only system water.
   4. Stir the glucose and mannitol solutions with separate glass stir rods until the sugars are completely dissolved.
   5. Return solution tanks to the water bath and cover with their corresponding lids.
2. Preparing water solutions
   1. Fill experimental tanks (2 L or 4 L) with System Water.
   2. Return these 'solution tanks' to the water bath and cover with their corresponding lids.

5. Changing percentages

1. Maintain the fish in a 1% solution during the first 2-weeks of treatment: 40 g of glucose or mannitol in a 4 L tank.
2. Maintain the fish in a 2% solution during weeks 3 and 4 of treatment: 80 g of glucose or mannitol in a 4 L tank.
3. Maintain the fish in a 3% solution for the final 4 weeks of treatment: 120 g of glucose or mannitol in a 4 L tank.

6. Measuring blood glucose levels

1. Anesthetize fish 2 at a time in a 0.02% Tricaine solution.
2. Decapitate the fish directly behind the gills using a razorblade.
3. Measure blood sugar value.
   NOTE: We use a blood glucose meter (e.g., Freestyle Lite) to measure blood glucose and place the test strip directly on the exposed heart (cardiac blood sample).
4. Dissect the wanted tissue from the fish (brain, muscle, etc.).
5. Store collected tissue by flash freezing on dry ice and storing in a -80 °C freezer, fixing in 4% paraformaldehyde, or placing in a buffer solution for immediate use.

Representative Results

Using this protocol (**Figure 1**), blood sugar values are significantly elevated after both 4-weeks and 8-weeks of treatment (**Figure 2A**), with hyperglycemia defined as 3x the control averages from both water-treated and mannitol-treated groups. Water-treated controls are transferred in and out of water daily, providing a stress/handling control. Mannitol serves as an osmotic control in in vitro glucose studies[19,20], as it is a 6-carbon sugar like glucose but is not taken up by cells. To be consistent with those studies, and other studies in zebrafish[21], we administered mannitol in the same concentrations as glucose to determine if observed effects were due to the high osmolarity resulting from glucose exposure or a glucose-specific effect.

Blood sugar is measured by anesthetizing the fish using 0.02% Tricaine until gill movements have slowed, and then decapitating. Blood glucose levels, measured with a blood glucose meter (**Figure 2B**), are determined from placing the glucometer test strip directly on the punctured heart (i.e., cardiac blood).

Retinal tissue collected after 4-weeks of hyperglycemia displays an increase in Glial Fibrillary Acidic Protein (GFAP) levels (**Figure 3A**). GFAP expression is observed in Muller glial cells in the retina, which are altered in diabetic retinopathy[22,23]. Increased GFAP content and/or immunoreactivity patterns are also observed in STZ-induced diabetic rats[24,25,26,27,28], *pdx1*$^{-/-}$ mutant fish[17], and in retinas from diabetic humans[29]. This increase in GFAP is associated with an increase in nuclear factor Kappa B (NF-kB) levels (**Figure 3B**)[30], suggesting the hyperglycemia induced in zebrafish using the alternate immersion protocol triggers an inflammatory response and reactive gliosis. ERG recordings after 4-weeks of treatment identified a decreased response in glucose-treated retinas compared to mannitol-treated controls (**Figure 4A**). Amplitudes of both a-wave (photoreceptor) and b-wave (bipolar cells) components are decreased in hyperglycemic fish (**Figure 4B**). These altered ERG responses are correlated to specific changes in red and/or green cones[30,21], which appear particularly sensitive to hyperglycemic insult. Altered ERG responses are also observed in animal models of diabetes[31,32,33,34,35] and diabetic humans. Glucose-treated zebrafish also show decreased cognitive performance (see Rowe et al., 2020, in

this issue), suggesting prolonged hyperglycemia also leads to cognitive function deficits which is also reported in older diabetic patients.

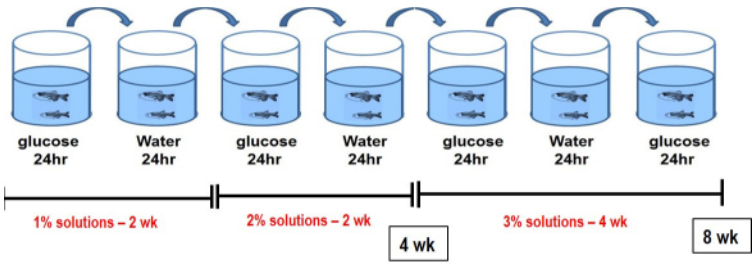

Figure 1: Schematic of the alternate immersion protocol. This is a visual representation of the transfer process. Fish are maintained in 1% solution for 2-weeks, 2% solution for 2 weeks, and then 3% solution for the remaining 4 weeks. Each day, fish are transferred into either sugar or water solution. The ~~water~~ control ~~treatment transfers~~ fish in and out of water (0% glucose - handling control) every 24 hours or into and out of mannitol (osmotic control), with mannitol concentrations paralleling those used for glucose. We have measured blood glucose levels, performed experiments, and collected tissue after 4 and 8 weeks of treatment (boxed). Please click here to view a larger version of this figure.

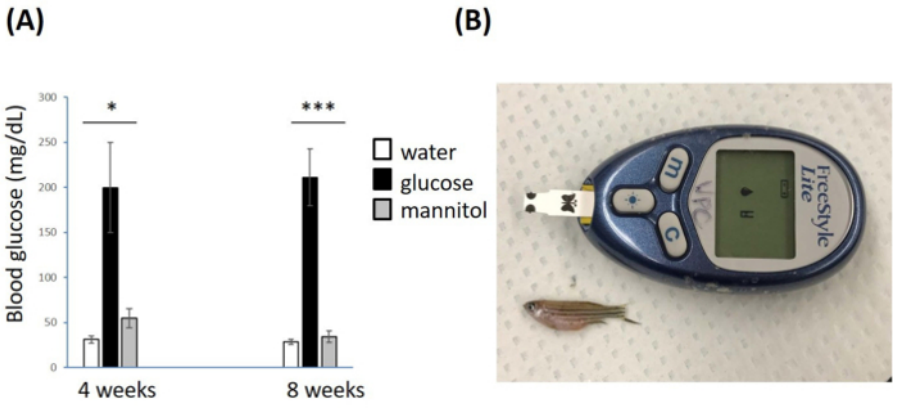

Figure 2: Blood Glucose Levels are elevated after 4 and 8 weeks of treatment. (**A**) The glucose-treated fish have more than 3x the amount of blood sugar compared to water- and mannitol-treated control fish, a significant increase ($p = 0.029$ at 4-weeks; $p < 0.001$ at 8-weeks). This means that after both 4- and 8-weeks the zebrafish treated with glucose were hyperglycemic. Data were collected from $n = 5$ mannitol treated fish, $n = 8$ glucose-treated fish, and $n = 3$ water control fish at 4 weeks; $n = 5$ mannitol-treated fish, $n = 10$ glucose-treated fish, and $n = 7$ water treated fish at 8 weeks. (**B**) A visual representation of a zebrafish and the Freestyle Lite Blood Glucose Meter that we use to measure blood glucose levels. Blood sugar levels are measured from a cardiac blood sample after the fish are anesthetized in a 0.02% tricaine solution and decapitated. Values are mean ± SE. Asterisks denote significant differences where * = $p < 0.05$; *** = $p < 0.001$. Please click here to view a larger version of this figure.

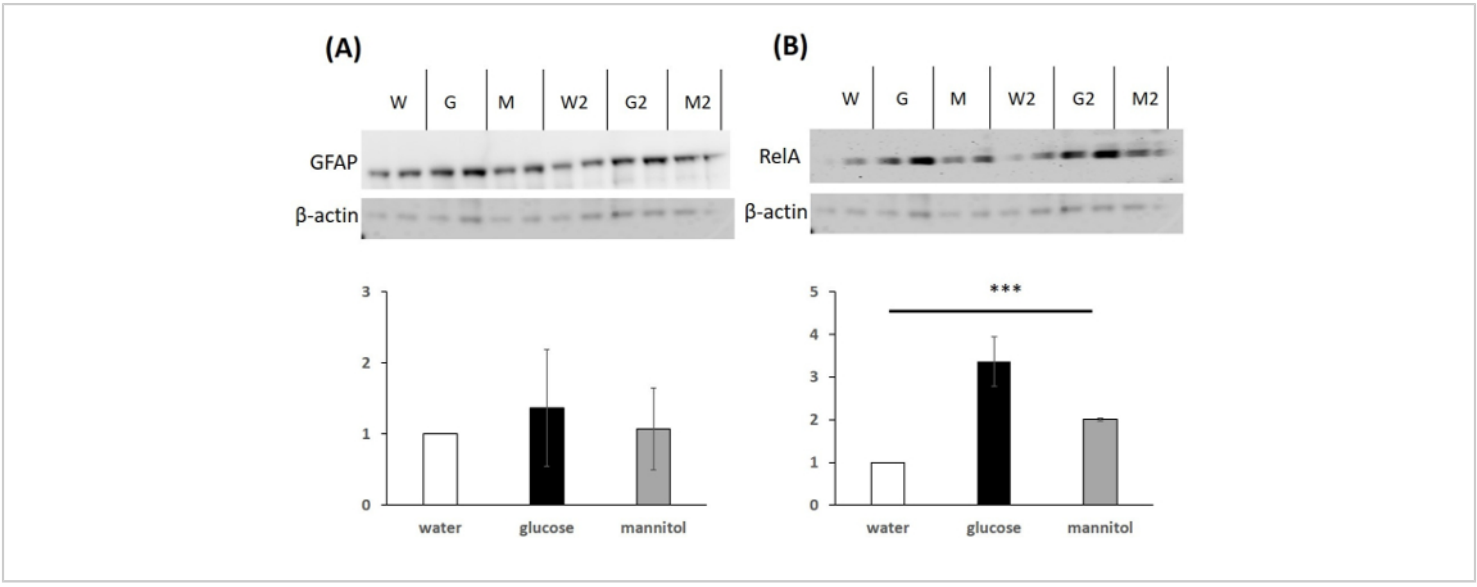

Figure 3: GFAP levels are increased in glucose-treated zebrafish. Glucose-treated zebrafish have increased levels of (**A**) glial fibrillary acidic protein (GFAP; 1:1000) and (**B**) Rel-A (NfK-B; 1:1000) as determined from densitometry analysis of Western blots. β-actin (1:1000) served as the loading control. The increase in Rel-A levels was significant ($p < 0.003$, asterisks). W = water-treated control, G = glucose-treated, M = mannitol-treated. W2, G2, M2 are replicates for W, G, and M. This suggests that there is insult to the retina in hyperglycemic zebrafish that causes reactive gliosis. (Modified from **Figure 7**, Tanvir et al., 2018[30], published originally under the terms of a CC-BY license). Please click here to view a larger version of this figure.

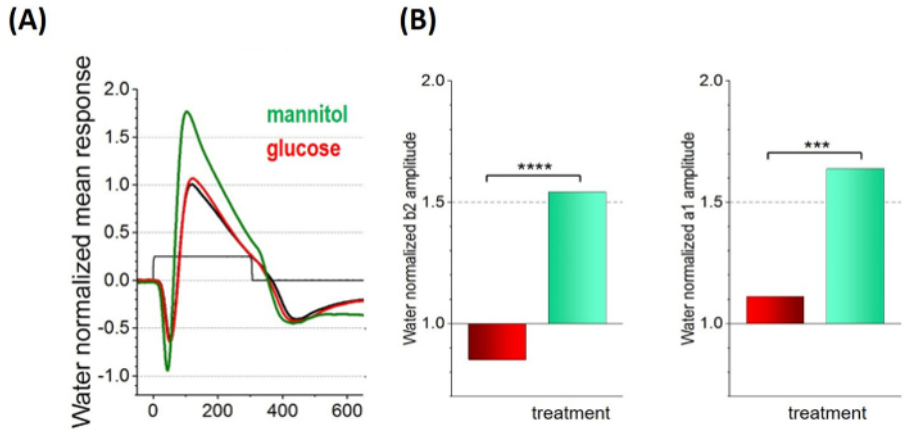

Figure 4: ERG response is reduced in glucose-treated fish. (**A**) Representative ERG traces from glucose-treated (red), mannitol-treated (green), and water-treated (black) zebrafish retinas evoked with a 570nm stimulus on a red adapting background. The glutamate receptor blocker CNQX (50μM) was present in the bath solution to isolate photoreceptor and ON bipolar cell responses. Units: y-axis = μV, x-axis = time. The square wave pulse reflects the duration of the light pulse. (**B**) Quantifying changes in response amplitudes. Mean normalized response amplitudes for photoreceptor a-waves (a1 amplitude, right), measured as the peak downward deflection at the start of the square light pulse, and bipolar cell b-waves (b2 amplitude, left), measured from trough to peak during the light pulse. Both values were significantly reduced in glucose- vs. mannitol-treated tissues ($p < 0.001$ for a1; $p < 0.0001$ for b2; $n = 14$ eyes per treatment). Values were normalized to the water-treated control. This is consistent with other diabetic animal models (such as rodents) and in humans with diabetes. (Taken from Figure 4, Tanvir et al. 2018[30], published originally under the terms of a CC-BY license). Please click here to view a larger version of this figure.

Discussion

Diabetes is a nationwide problem. Studies show that by 2030, an estimated 400 million people will have some form of diabetes. In rodent models, Type 2 DM is studied using genetic manipulation. In rats, the Zucker diabetic fatty rats (ZDF), and the Otsuka Long-Evans Tokushima fatty rats (OLETF), are providing more information on the effects of Type 2 DM[10]. In addition, high fat diets have been used in rodent to induce hyperglycemia. This mirrors the noninvasive procedure proposed in this paper. Using our noninvasive protocol, we can induce hyperglycemia for up to 8-weeks, therefore simulating prolonged hyperglycemia in otherwise healthy zebrafish.

After blood sugar is measured, tissue (retina and brain) can be collected for subsequent analysis. Physiological differences, such as electroretinogram (ERG) recordings, can be measured directly from retinal eye cups[21,30]. Behavioral responses (i.e., optomotor responses or cognitive differences (Rowe et al., 2020) can be assessed prior to blood sugar measurement. For Western Blot determination of protein levels, tissue is placed into RIPA buffer or flash frozen and stored at -80 °C. Due to differences in size/protein

content, several retinas may have to be pooled prior to analysis. For immunocytochemistry, tissue is fixed in a 4% paraformaldehyde solution for 24 hours and then equilibrated in a 30% sucrose solution prior to until frozen sectioning (20 µm).

To optimize results, carefully weigh out both mannitol and glucose, and make sure that the solutions are thoroughly mixed. It is also very important to keep an active schedule of the transfer days, to make sure sugar and water are alternating daily and at the same time of day. Furthermore, make sure to carefully measure out the water as too little or too much may change the concentration of the solution. Lastly, carefully monitor the pH and the temperature of the solutions, as transferring fish into extreme solutions can shock the fish and cause mortality. There has been no evidence that prolonged glucose exposure or high concentrations of glucose, when exposed gradually, has toxic effects on zebrafish. If the protocol is followed correctly, no extraneous deaths of the zebrafish should be expected.

While this is a proven method of inducing hyperglycemia, a limitation of this protocol is that one cannot ascertain that the fish are hyperglycemic until after they are sacrificed. Another limitation is that we do not assess the pancreas or insulin levels and, therefore, we cannot claim to induce Type 2 Diabetes, only that we induce hyperglycemia. However, this is also what makes this procedure better than competing methods: it is non-invasive. We have observed complications of prolonged hyperglycemia induced by this protocol that are reported in rodent models and diabetic individuals. In the future, it is possible that we can use this procedure to look at therapeutic techniques that help alleviate symptoms of DM, such as retinopathy.

In summary, this noninvasive alternating immersion protocol is an effective way to induce hyperglycemia for up to 8 weeks. This method is a powerful tool that allows for the complications of chronic hyperglycemia to be examined and subsequent determination of therapeutic treatments. It also offers the opportunity to compare biomedical research findings across model organisms.

Disclosures

The authors declare no conflicts of interest.

Acknowledgments

We would like to acknowledge VPC, CJR, and MCP for the development of this protocol. EMM received financial support from the American University College of Arts and Sciences Graduate Student Support to carry out this research.

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