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TITLE:

Alternate Immersion in Glucose to Produce Prolonged Hyperglycemia in Zebrafish

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KEYWORDS:

Danio rerio, *Hyperglycemia*, Glucose, Retinopathy, Alternate Immersion

SUMMARY:

This protocol noninvasively induces hyperglycemia in zebrafish for up to 8 weeks. Using this protocol, an in-depth study of the adverse effects of hyperglycemia can be made.

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[7-9], 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[14-16]. 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

1.1 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

1.2 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.

1.3 Keep the tanks in a water bath at 28-29 °C to maintain water temperature.

1.4 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.

1.5 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

2.1 Use adult zebrafish (4 months – 1 year)[5].

2.2 Feed the fish ground Tetramin flakes daily upon arrival to the lab.

2.3 Record the pH and the temperature of all the tanks and record the general condition of the fish.

3. Transferring fish

3.1 Transfer fish in each treatment group from the housing tank to the corresponding solution tank using a standard fish net.

3.2 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'.

3.3 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.

3.4 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

4.1 Preparing sugar solutions

4.1.1 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).

4.1.2 Measure the correct amount of glucose and mannitol (see step 5 below) using a top loading scale and separate weigh boats for each chemical.

4.1.3 Add the weighed glucose or mannitol aliquot to the appropriate, cleaned solution tank, which contains only system water.

4.1.4 Stir the glucose and mannitol solutions with separate glass stir rods until the sugars are completely dissolved.

4.1.5 Return solution tanks to the water bath and cover with their corresponding lids.

4.2 Preparing water solutions

4.2.1 Fill experimental tanks (2 L or 4 L) with System Water.

4.2.2 Return these 'solution tanks' to the water bath and cover with their corresponding lids.

5. Changing percentages

5.1 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.

5.2 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.

5.3 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

6.1 Anesthetize fish 2 at a time in a 0.02% Tricaine solution.

6.2 Decapitate the fish directly behind the gills using a razorblade.

6.3 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).

6.4 Dissect the wanted tissue from the fish (brain, muscle, etc.).

6.5 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[24-28], *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[31-35] 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 LEGENDS

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).

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 * = $p < 0.05$; *** = $p < 0.001$.

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).

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).

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.

ACKNOWLEDGEMENTS

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DISCLOSURE

The authors declare no conflicts of interest.

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Figure

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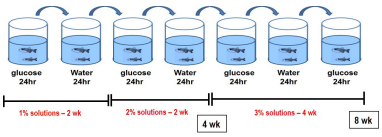

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Figure

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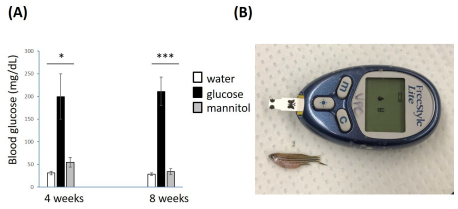

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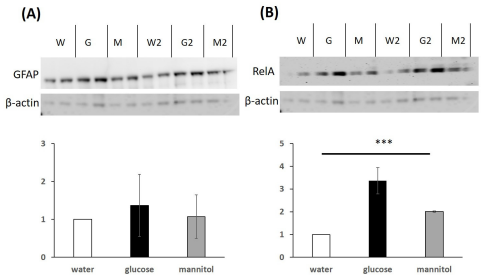

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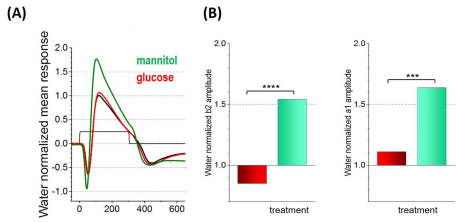

McCarthy et al Figure 4

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Airline Tubing	petsmart	5291863	This can be used in the tank to circulate air
Airpump	petsmart	5094984	This can be used in the tank to circulate air
Airstones	petsmart	5149683	This can be used in the tank to circulate air
D-glucose	Sigma	G8270-5KG	
D-mannitol	Acros Organics	AC125340050	
Freestyle Lite Meter	Amazon	B01LMOMLTU	
Freestyle Lite Strips	Amazon	B074ZN3H2Z	
Net	petsmart	5175115	
Tanks	Amazon	B0002APZO4	

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22 September 2020

Benjamin Werth, Senior Science Editor
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Dear Dr. Werth,

I have uploaded a revised version of our manuscript entitled "**Alternate Immersion in Glucose Produces Prolonged Hyperglycemia in Zebrafish**". We thank the reviewers for their careful read and thoughtful comments on the document. We have addressed all the comments, which are highlighted red in the revised draft. We have also provided detailed answers to each comment on the following pages.

In particular, we have revised the title and added significantly more detail within the manuscript, to better describe the protocol. We have also added a section explaining how blood sugar measurements are collected. We have also replaced the graph in Figure 3 and proofread all the figures and captions for consistency and clarity. As a result of these significant edits, we feel the manuscript is more thorough and the protocol more descriptive.

Please let us know if any further revision is needed.

Thank you for your time.

Sincerely,

Elizabeth McCarthy

Elizabeth McCarthy B.S.
Graduate Student at American University

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Response:

Thank you. We have proofread the revised manuscript.

2. none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

Response:

We have corrected the line spacing and font as indicated.

3. Please make the title concise. It cannot be a sentence and the focus must be on the protocol being presented. e.g., Use of external glucose solution as an alternate immersion solution to produce diabetes like complication.

Response:

We have edited the title to now read: Alternate immersion in glucose produces prolonged hyperglycemia in zebrafish

4. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

Response:

The abstract is 151 words falling within the 150-300 word range

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Response:

An ethics statement has been added.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Response:

We have edited our protocol section using the imperative tense.

7. Please ensure you answer the “how” question, i.e., how is the step performed?

Response:

We have edited our protocol as needed to address this.

8. 1.4: How many fish per tank or per experimental condition is used?
Response:
We have added this information as a Note in the protocol (section 1.2)

9. How do you treat the control group?
Response:
We have added this information in section 1.4

10. Please ensure all steps are action steps containing all specific details associated with the step. Please ensure all steps are written in order.
Response:
We have edited/revised the protocol as needed to ensure all details are provided. The steps are written in order.

11. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response:
The part to be filmed is highlighted yellow.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Response:
Figures 3 and 4 have been taken from a previous publication. We have attributed credit to the original publication in both figure captions, which is allowed under an open access CC-BY license. The link to the policy is https://dmm.biologists.org/content/rights-permissions. We have also placed it in a Word doc and uploaded it as indicated.

13. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.
Response:
We have revised and edited the figure legends as appropriate to address this.

14. As we are a methods journal, please ensure that the Discussion explicitly cover the following

in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response:
This information has been included in the discussion

15. Figure 2: What does the error bar represent?
Response:
The data presented are means +/- SE. We have added this information to the figure legend, as well as the N's used to calculate those values.

16. Figure 3 please include error bars.
Response:
We have revised Figure 3 to include error bars and the original Western Blot images.

17. Please sort the materials table in alphabetical order.
Response:
We have made this correction.

Reviewers' comments:

Reviewer #1:

Overall this is a useful protocol. However, demonstration of the impact of the hyperglycemia on physiology or pathology is not very strong. Inclusion of additional protocol details would be helpful.

Response:

An additional section has been added to the protocol referring to the measuring of blood glucose levels.

Graphs need proper labeling and indications of statistical significance.

Response:

We have revised and/or replaced the graphs, and edited their captions, to include this information.

Minor Concerns:

Title and Abstract: The claim of 'diabetes-like complications' is overstated. The increase of GFAP is not significant. Cognitive deficits are not demonstrated.

Response:

The title has been altered

Introduction:

(1) There are also genetic models of diabetes in zebrafish. This should be mentioned. Diabetic zebrafish with a mutation in pdx1 develop signs of diabetic retinopathy (Wiggenhauser 2020, DOI:10.2337/db19-0873 and Ali 2020, DOI:10.1167/iovs.61.2.43).

Response:

We have edited the introduction (lines 71-74) to include information about the pdx1 mutation.

(2) Line 69: "Since this non-invasive technique does not directly manipulate insulin levels so it can be used to model complications of Type 2 DM."

This statement is confusing in its logic (and its grammar). Type 2 DM is characterized by changes in insulin levels.

Response:

We have edited this sentence to reflect that we cannot claim to induce T2DM, but that we are simply looking at complication due to hyperglycemia, a hallmark of T2DM. (lines 68-70)

Protocol:

1.2 What is the maximum number of fish for treatment in a 4L tank?

Response:

The maximum number of fish we have treated in a 4L tank is ~30.

Since elevating glucose is the major outcome, it would be useful to explain the method for measurement of blood glucose in more detail.
Response:
We have added an additional section to the protocol describing how blood glucose levels are measured in the fish.

Results:
GFAP levels (Figure 3) show a small increase by Western blot (what is the p-value?). Were samples analyzed at 4 weeks or 8 weeks?
Response:
This graph shows a sample taken at 4-weeks, as does Figure 4. We have revised figure 3 to include error bars and indicate significance.

Immunohistochemistry is mentioned in the results, but no immunohistochemistry is shown. GFAP labeling of retinal sections would provide information about cellular localization of increased GFAP expression and support the relevance of the hyperglycemia for diabetic retinopathy.
Response:
Immunohistochemistry has been removed from the results section, this data has not yet been collected.

What was the duration of treatment for the samples analyzed by ERG (Fig. 4)? Is there a change between 4 weeks and 8 weeks of glucose treatment? The change caused by mannitol treatment is highly significant, compared to the small change in the glucose treated samples relative to water-treated controls.
Response
ERGs were measured after 4-weeks of treatment. We don't have data from the 8-week time point yet (that is the focus of current and future studies).
Figure 4 has been replaced and the caption edited to include information about significance level, N's and other details of the recording procedures.

(Lines 181-183) This information could be expanded as a detailed step-by-step description and included in the Protocol section. Is retina tissue dissected or is the eye cup used? How are protein extracts prepared? How many eyes/retinas are needed to give a detectable signal?
Response
A section has been added to the protocol to reflect this information.

(Lines 188-190) GFAP expression was also increased in the pdx1 mutant zebrafish DR model (Ali et al, 2020).
Response:

The sentence was edited to reflect this.

The results section should be related to shown experimental data. Additional methods (cognitive responses) that can be potentially applied should be moved to the discussion.
Response
We have made the indicated change to the text.

Figures:
Fig. 2A. The graph is lacking units on the y-axis and labeling of the conditions on the x-axis.
Response:
We have uploaded a new version of the figure with the indicated labels.

Fig. 2B. This figure does not provide much useful information.
Response:
There is no data in this figure, that is true. However, we include it here because it shows that a standard blood glucose meter can be used with the fish, something that is not hampered by the small size of zebrafish.

Fig. 3. Are the changes shown significant? Was there a loading control? How many samples were analyzed? (Pooled or biological replicates?) Can a picture of the gel be included?
Response:
We have revised Figure 3 to include the original blots, and the loading control.

Fig 4. The significance level for the glucose treated samples in missing. (Are changes significant relative to water control and/or mannitol?)
Response
Figure 4 has been replaced and the caption edited to include information about significance level, N's and other details of the recording procedures.

Discussion:
A few additional points could be addressed:
Are there toxicities associated with longer glucose treatments and/or exposure to higher glucose concentrations? What is the survival using the protocol described?
Response:
This concern was addressed by adding a sentence to the end of paragraph 2 in the discussion (lines 303-305)

The effect of mannitol on the ERG response is much greater than the effect of glucose. Is there a possible explanation for this?
Response:

We do not know why the ERG response in mannitol treated fish was so large. It may reflect an osmotic effect. Interestingly, ERG responses in glucose treated fish was significantly reduced compared to mannitol, suggesting a glucose, but not osmotic, effect.

Materials:
What is the source for the GFAP antibody?
Response:
The GFAP antibody is from Sigma Aldrich and the Rel-A antibody is from Anaspec. We have included this information in the document.

Reviewer #2:

The authors here demonstrate a non-invasive technique for inducing hyperglycemia in adult zebrafish. The fish are immersed in glucose solution or system water on alternating days for several weeks. A similar technique is already established in in the system and a video-accompanied article will be a good resource for researchers.

Thank you.

I have a few concerns which, if addressed to, can surely make the article stronger.

1. Line 46: 'mental processes' should maybe read 'behavioral studies'. I think by 'developmental transparency', the authors mean transparency of early embryonic stages.

Response:

Line 46/47 Mental processes was changed to "cognition" to be clearer. "Developmental transparency" was expanded on to be clearer.

2. Line 62: Why do the authors call 'STZ treatment' as an invasive method?

Response:

STZ treatment is seen as an invasive procedure, due to the injection, and possible booster injections, needed to successfully induced DM. The sentence has been changed to reflect this (line 63).

3. It will be useful if authors explain why alternate immersion is better than continuous immersion. In other words, what the strengths of this particular method are in comparison to continuous immersion method.

Response:

This is now addressed at the end of the introduction.

4. Authors must replace 'water' with 'system water' where applicable to avoid confusion for the readers.

Response:

An addendum has been added to reflect what system water refers to, as it is treated water and not DI water. (protocol 4.1.1)

5. It is unclear what processes are carried out for water-treated animals. Are the control (water-treated) animals also transferred from one tank to another on a daily basis? If yes, please mention that in step #1.4.

Response:

Step 1.4 was changed to reflect that control zebrafish are similarly transferred daily.

6. Figure 2: Mention number of fish used to calculate the error.

Response

The N's for the different treatment groups are provided in the caption for Figure 2.

7. Figure 3: Original western blot image should be provided. The graph has no error bars. Why?
Response:
We have revised this figure to include the original Western Blot and a graph with error bars.

8. Figure 4: axes labels are missing. Include Tanvir et al in the reference list.
Response:
We replaced figure 4 for one that is clearer and indicated what the units for the axes are in the caption. The y-axis is the voltage (in uV) and the x-axis is time (in ms). For 4b, 'treatment' has been added to the x-axis.

9. Line 176: Cite reference for how to obtain cardiac blood to measure glucose.
Response:
The phrasing of this in line 176 has been altered to reflect how blood samples were taken.

10. Rationale and reference for use of mannitol as osmotic control should be provided.
Response:
Mannitol is used as an osmotic control in cell culture studies with high glucose concentrations (Chen et al., 2013; Costa et al., 2013) and in other studies with zebrafish (Alvarez et al., 2010). We use it here to be consistent with these studies and have added this information to the manuscript.

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link for permissions regarding McCarthy et al Figures 3 and 4