# Acute and Chronic Models of Hyperglycemia in Zebrafish: A Method to Assess the Impact of Hyperglycemia on Neurogenesis and the Biodistribution of Radiolabeled Molecules

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Anne-Claire Dorsemans[1], Christian Lefebvre d'Hellencourt[1], Imade Ait-Arsa[2], Emmanuelle Jestin[1,2], Olivier Meilhac[1,3], Nicolas Diotel[1]

[1],, UMR 1188 Diabète athérothombose Térapies Réunion Océan Indien (DéTROI), Saint-Denis de La Réunion, France

[2]RIPA (Radiochimie et Imagerie du Petit Animal), Cyclotron de la Réunion Océan Indien CYROI

[3]CHU de La Réunion

Correspondence to: Nicolas Diotel at nicolas.diotel@univ-reunion.fr

## Abstract

Hyperglycemia is a major health issue that leads to cardiovascular and cerebral dysfunction. For instance, it is associated with increased neurological problems after stroke and is shown to impair neurogenic processes. Interestingly, the adult zebrafish has recently emerged as a relevant and useful model to mimic hyperglycemia/ diabetes and to investigate constitutive and regenerative neurogenesis. This work provides methods to develop zebrafish models of hyperglycemia to explore the impact of hyperglycemia on brain cell proliferation under homeostatic and brain repair conditions. Acute hyperglycemia is established using the intraperitoneal injection of D-glucose (2.5 g/kg bodyweight) into adult zebrafish. Chronic hyperglycemia is induced by immersing adult zebrafish in D-glucose (111 mM) containing water for 14 days. Blood-glucose-level measurements are described for these different approaches. Methods to investigate the impact of hyperglycemia on constitutive and regenerative neurogenesis, by describing the mechanical injury of the telencephalon, dissecting the brain, paraffin embedding and sectioning with a microtome, and performing immunohistochemistry procedures, are demonstrated. Finally, the method of using zebrafish as a relevant model for studying the biodistribution of radiolabeled molecules (here,[$^{18}$F]-FDG) using PET/CT is also described.

## Introduction

Hyperglycemia is defined as excessive blood glucose levels. Although it could reflect a situation of acute stress, hyperglycemia is also a condition that often leads to a diagnosis of diabetes, a chronic disorder of insulin secretion and/or resistance. In 2016, the number of adults living with diabetes has reached 422 million worldwide, and each year, 1.5 million people die from this disease, making it a major health problem[1]. Indeed, uncontrolled diabetes leads to several physiological disorders affecting the cardiovascular system, kidneys, and the peripheral and central nervous systems.

Interestingly, acute and chronic hyperglycemia may alter cognition and contribute to both dementia and depression[2,3,4,5,6]. In addition, the admission of patients with hyperglycemia has been associated with worse functional, neurological, and survival outcomes after ischemic stroke[7,8,9,10,11]. It was also shown that hyperglycemia/diabetes affect adult neurogenesis, a process leading to the generation of new neurons, by impacting neural stem cell activity and neuronal differentiation, migration, and survival[2,12].

In contrast to mammals, teleost fish, like zebrafish, display intense neurogenic activity throughout the whole brain and exhibit an outstanding capacity for brain repair during adulthood[13,14,15,16]. Notably, such capacities are possible due to the persistence of neural stem/progenitor cells, including radial glia and neuroblasts[17,18,19]. In addition, the zebrafish has recently emerged as a model for studying metabolic disorders, including obesity and hyperglycemia/diabetes[20,21,22].

Although the zebrafish is a well-recognized model of hyperglycemia and neurogenesis, few studies have investigated the impact of hyperglycemia on brain homeostasis and cognitive function[12,23]. To determine the impact of hyperglycemia on constitutive and injury-induced brain cell proliferation, a model of acute hyperglycemia was created through the intraperitoneal injection of D-glucose. In addition, a model of chronic hyperglycemia was reproduced through the immersion of fish in water supplemented with D-glucose[12]. Zebrafish exhibit many advantages in research. They are cheap, easy to raise, and transparent during the first stages of development, and their genome has been sequenced. In the context of this work, they also display several additional advantages: (1) they share similar physiological processes with humans, making them a critical tool for biomedical research; (2) they allow for the quick investigation of the impact of hyperglycemia on brain homeostasis and neurogenesis, given their widespread and strong neurogenic activity; and (3) they are an alternative model, allowing for the

reduction of the number of mammals used in research. Finally, zebrafish can be used as a model for testing the biodistribution of radiolabeled molecules and potential therapeutic agents using PET/CT.

The overall goal of the following procedure is to visually document how to set up models of acute and chronic hyperglycemia in zebrafish, use zebrafish to assess brain remodeling in hyperglycemic conditions, and monitor radiolabeled molecules (here, [$^{18}$F]-FDG) using PET/CT.

### Protocol

Adult wildtype zebrafish (*Danio rerio*) were maintained under standard photoperiod (14/10 h light/dark) and temperature (28 °C) conditions. All experiments were conducted in accordance with the French and European Community Guidelines for the Use of Animals in Research (86/609/EEC and 2010/63/EU) and were approved by the local Ethics Committee for animal experimentation.

## 1. Establishing a model of acute hyperglycemia in zebrafish

1. Prepare a stock solution of tricaine (MS-222) by dissolving 400 mg of tricaine powder in 97.9 mL of water and 2.1 mL of 1 M Tris/HCl buffer (pH 9). Adjust the pH to 7 and aliquot for storage at -20 °C.
2. Prepare an anesthetic for the adult zebrafish by putting 5 mL of tricaine (stock solution) into 100 mL of fish water (final tricaine concentration: 0.02%).
3. Transfer one zebrafish (3 to 6 months) from its tank to the anesthetic until it stops moving.
4. Remove the fish using a cut pipette and quickly dry it on absorbent tissue paper.
5. Weigh the fish.
6. Prepare a syringe of D-glucose dissolved in 1X PBS and inject 50 µL of D-glucose (2.5 g/kg bodyweight).
   NOTE: For instance, a 0.6 g fish will receive 50 µL of 3% D-glucose/PBS solution.
7. Put the fish on its back and insert the needle of the syringe into the intraperitoneal cavity.
8. Slowly inject the D-glucose/PBS solution and then place the fish back in the water.
9. Check on the fish until it recovers completely. Return it to its tank until the required time for performing the blood glucose measurement is reached.
   NOTE: The blood glucose is measured 1.5 h after the injection.

## 2. Establishing a model of chronic hyperglycemia in zebrafish

1. Prepare a 2-L tank of clean fish water.
2. Dissolve 40 g of D-glucose into the 2 L of fish water for a final D-glucose concentration of 111 mM.
3. Immerse 5 to 7 adult zebrafish in the D-glucose-containing water.
4. Replace the D-glucose fish water every 2 days in order to avoid the growth of bacteria or other micro-organisms.
5. After 14 days of treatment, place the fish briefly in fresh water to remove the D-glucose outside the body prior to taking the blood glucose-level measurement.

## 3. Measuring blood glucose levels in zebrafish

1. Cover the fish with ice for rapid euthanasia.
   NOTE: Do not use an overdose of tricaine, as this induces wide variations in blood glucose levels. Do not leave the fish in the ice for too long, as this will cause the blood to clot.
2. Wipe the fish with absorbent tissue paper to remove all water and to avoid any blood dilution during the blood glucose measurement.
3. Remove an eye using dissecting forceps and wait until the eye cavity is filled with blood.
4. Put a test strip on a glucometer and insert the strip into the eye cavity.
5. Measure the blood glucose levels.

## 4. Analyzing brain cell proliferation following hyperglycemia

1. Solutions and buffers: requirements and preparation
   1. Prepare 1 L of 1X PBS by adding 100 mL of 10X PBS to 900 mL of distilled $H_2O$ ($dH_2O$) and mix.
   2. Prepare 1X PBS with 0.2% detergent (PBS-T; see the table of materials).
      NOTE: To prepare 1 L of PBS-T, add 2 mL of detergent (see the table of materials) to 1 L of PBS.
   3. Prepare an ethanol series (100% x 2; 95%; 85%; 70%; 50%, and 30%) and 0.85% NaCl.
   4. Prepare blocking buffer: PBS-T containing 0.5% to 1% milk powder.
      NOTE: To prepare 200 mL of blocking buffer, add 2 g of milk powder to 200 mL of PBST.
   5. Prepare antigen retrieval buffer, sodium citrate.
      NOTE: To prepare 1 L of sodium citrate buffer, add 2.94 g of sodium citrate trisodium salt dehydrate to 1 L of $dH_20$. Adjust the pH to 6 prior to filling to 1 L.
   6. Prepare a 4% paraformaldehyde-PBS buffer, adding 4 g of paraformaldehyde to 100 mL of 1X PBS. Warm it under agitation at 58-60 °C until it dissolves completely.

2. Sample preparation for immunohistochemistry: fixation and dehydration
   1. After measuring the blood glucose level, separate the head from the body by cutting the head behind the gills.
      Note: Alternatively, the brain can be directly extracted and frozen for other experiments, such as mRNA extraction.
   2. Fix the heads overnight at 4 °C in 4% paraformaldehyde dissolved in PBS (PFA-PBS).

3. The next day, briefly wash the heads in PBS.
4. Dissect the brains carefully using a microscope. Rinse the fixed heads with PBS and use a needle to secure a head under a dissecting microscope. Remove the eyes and the top of the skull with forceps. Carefully extract the brain and place it in 1X PBS.
5. Successively wash the fixed brains with 1X PBS for 30 min, 0.85% NaCl for 30 min, 70% EtOH/0.85% NaCl (v/v) for 15 min, 70% EtOH for 15 min (twice), 85% EtOH for 20 min, 95% EtOH for 20 min, and 100% EtOH for 20 min (twice). Incubate overnight in a final 100% EtOH solution.
   NOTE: Dehydrated brains can remain for several months in 100% EtOH at 4 °C before paraffin embedding.

3. Sample preparation for immunohistochemistry: tissue preparation for paraffin embedding
   1. Place the brains in a small glass beaker.
      NOTE: Do not use a plastic receptacle, as toluene can damage some plastics.
   2. Remove the ethanol and replace it with toluene, as the brains must be totally recovered by toluene. Perform two 30-min baths of toluene.
   3. Remove the toluene and place the brains in an embedding cassette. Put the closed cassette in melted paraffin series beakers at 58-60 °C (30 min in each paraffin beaker). Turn on the warming inclusion forceps. At the end of the paraffin baths, take out the cassette and pour some liquid paraffin into a mold.
   4. Pour the melted paraffin into a mold and place the brain inside. Orient the brain using the warming inclusion forceps, using the anteroposterior orientation of the brain as a guide. Let the paraffin harden on the cooling part of the embedding machine.
   5. For technical reasons, position the brains along the anteroposterior axis. After unmolding the paraffin block, crop it and fix it on a cassette, with the melted paraffin in the proper orientation to allow for transversal sectioning.
   6. Insert the paraffin block into the arm of the microtome. Cut 50 µm-thick sections until the brain sample level is reached. Trim the paraffin block to obtain a trapeze and adjust the sectioning thickness to 7 µm. Collect the paraffin ribbons using paintbrushes and place them on black paper. Cut them gently every 3 to 4 sections.
   7. Put a slide on a warming plate and cover it with $dH_2O$ water.
   8. Gently position the cut ribbons on the water. Remove the water with absorbent tissue paper when the paraffin ribbons are sufficiently spread out/unfolded. Remove the last drops mechanically. Remove the water from the slides and let them dry for at least 3 h on the warming plate at around 30-40 °C.

4. Immunohistochemistry procedure
   1. Remove the paraffin wax by placing sections in three containers of xylene for 7 min each.
   2. Rehydrate the sections by putting the slides in two containers of 100% EtOH for 2 min each, followed by baths of 95% EtOH, 85% EtOH, 70% EtOH, and 30% EtOH for 30 s each. Finally, briefly put the slides in $dH_2O$ and twice in PBS for 5 min each.
   3. Perform antigen retrieval by incubating the sections in citrate buffer in a microwave (2 min at 500 W) until the buffer starts to boil. Let the slides recover at room temperature for 15 min.
   4. Wash three times in PBST, 5 min each, and block the sections for 45 min in PBST containing 1% milk. Incubate overnight with primary antibodies (*e.g.* proliferating cell nuclear antigen (PCNA) for proliferative cell staining; 1/100) diluted in blocking buffer (*i.e.* PBST, 1% milk).
   5. Wash three times in PBST, 5 min each, and incubate the sections for 90 min with appropriate secondary antibodies (*e.g.* goat anti-mouse Alexa Fluor 488; 1/200) diluted in blocking buffer and with DAPI (1/500).
   6. Wash three times in PBST, 5 min each, and mount the slides with fluorescent mounting medium (see the table of materials).
   7. Analyze the staining using an epifluorescence and/or confocal microscope.
   8. Quantify the brain cell proliferation on at least three successive brain sections of a region of interest in at least three distinct animals.
      NOTE: Alternatively, brain embedding can be done in agarose to proceed to vibratome sectioning and free-floating immunohistochemistry, as previously described[24].

5. Studying the impact of hyperglycemia on brain repair mechanisms
   NOTE: Alternatively, the investigation of brain repair under acute and chronic hyperglycemia can be performed after stab wound injury of the telencephalon, as previously described[24,25,26]. Briefly:
   1. Anesthetize adult zebrafish with tricaine.
   2. Place the fish under a dissecting microscope with light.
   3. Hold the fish with one hand.
   4. With the other hand, insert a 30G syringe vertically through the skull into the medial region of the right telencephalic hemisphere.
   5. Place the fish back into fresh fish water, D-glucose-supplemented water (111 mM), or control fish water. Allow the fish to survive for 7 days post-injury.
      NOTE: Refer to step 4 for the rest of the procedure.

## 5. Imaging the biodistribution of radiolabeled molecules by PET/CT in zebrafish: Fluorodeoxyglucose ([18F]-FDG) to analyze glucose metabolism

1. Prepare a 50-µL syringe containing 20 MBq of a saline solution of [18F]-FDG.
2. Place the syringe behind the radiation protective screen.
3. Anesthetize an adult zebrafish with tricaine.
4. Place the fish behind a radiation protective screen.
5. Inject [18F]-FDG into the intraperitoneal cavity. Wipe the injection site with a small piece of tissue paper to prevent the detection of residual [18F]-FDG that could leak out of the fish belly.
6. Use a radioisotope calibrator to measure the remaining activity contained on the syringe and the tissue to calculate the exact injected dose.
7. Place the fish on a new, small piece of absorbent tissue soaked with tricaine and gently wrap it up. Place the fish with the absorbent tissue on the bed of the PET/CT imager.

8. Insert the bed into the PET/CT imaging system and start the acquisition.
9. Proceed to conduct the PET/CT acquisition.
10. At the end of the procedure, place the fish back into a small amount of fresh fish water.
    NOTE: Alternatively, after the [18F]-FDG injection, the fish can be put back into a small amount of fresh water for 10 min to 1 h to facilitate the diffusion of [18F]-FDG before anesthetizing the fish once again for PET/CT imaging.

## Representative Results

Using the procedures described in this article, the intraperitoneal injection of D-glucose (2.5 g/kg bodyweight) was performed on adult zebrafish and led to a significant increase in blood glucose levels 1.5 h after injection (**Figure 1A**). 24 h post-injection, the blood glucose levels were similar between D-glucose and PBS-injected fish[12]. For chronic treatment, zebrafish were immersed in D-glucose water (111 mM) and became hyperglycemic at the end of their 14 days of treatment (**Figure 1B**), as was previously shown[12,22].

To investigate the impact of hyperglycemia on brain cell proliferation, PCNA immunohistochemistry was performed on zebrafish brains following the induction of acute and chronic hyperglycemia. Although acute hyperglycemia did not impact brain cell proliferation[12], chronic hyperglycemia induced a significant decrease in neural stem cell proliferation along the ventricle, as previously shown by Dorsemans and colleagues (2016). Indeed, the number of PCNA-positive cells was reduced in the subpallium (Vv/Vd), the pallium (Dm), and the regions surrounding the lateral and posterior recess of the caudal hypothalamus (LR/PR) (**Figure 2**).

Injury-induced neurogenesis was also studied after the mechanical injury of the telencephalon under acute and chronic hyperglycemia. As previously described after brain injury in zebrafish, a first parenchymal proliferation of microglial cells and oligodendrocytes occurred, followed by a strong up-regulation of proliferation at the ventricular layer 7 days after the injury[25,27,28,29,30]. Acute hyperglycemia did not modulate the initial step of proliferation in the brain parenchyma. In contrast, chronic hyperglycemia impaired brain cell proliferation along the telencephalic ventricles 7 days after injury (**Figure 3**).

The zebrafish model is also interesting for monitoring the biodistribution of radiolabeled molecules using PET/CT imaging. Here, [18F]-FDG was intraperitoneally injected into adult zebrafish. After 30 min, PET/CT acquisition shows that the glucose is distributed not only at the site of injection, but also in the head of the fish, including the brain, and along the spinal cord (**Figure 4**).

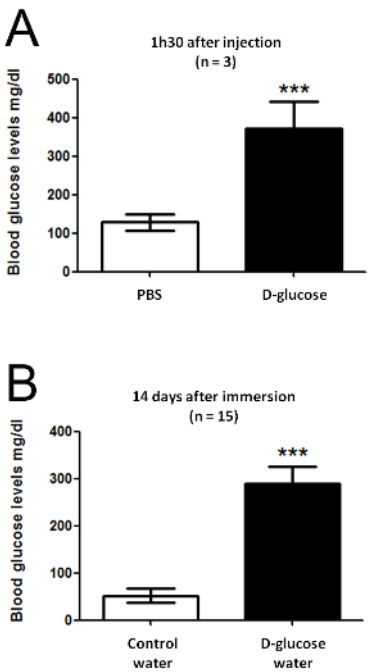



**Figure 1: Acute and chronic models of hyperglycemia in zebrafish**
**A:** The intraperitoneal injection of D-glucose (2.5 g/kg bodyweight) results in a significant increase in blood glucose levels 1.5 h after the injection (n = 3).
**B:** The immersion of zebrafish in D-glucose water (111 mM) for 14 days results in a significant increase in blood glucose levels (n = 15). Please click here to view a larger version of this figure.

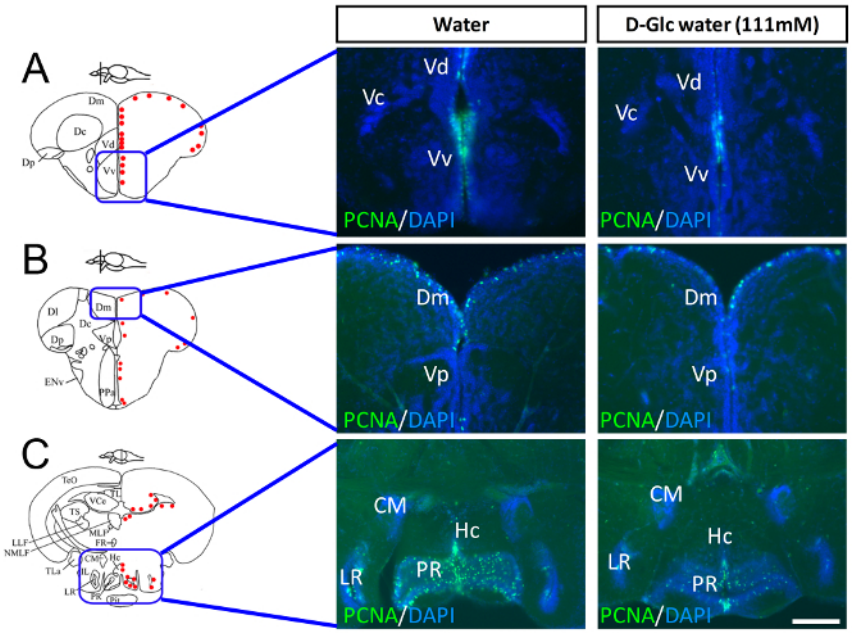
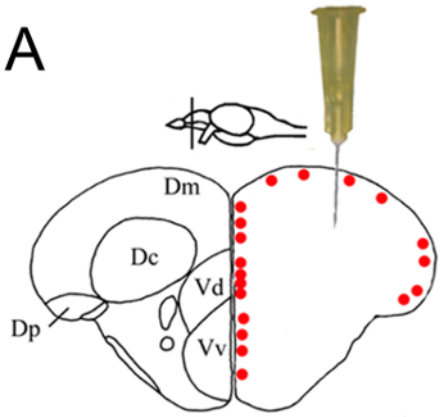



**Figure 2: Chronic hyperglycemia impairs brain cell proliferation after 14 days of treatment**
Proliferative cells are labeled in green with a PCNA antibody. Cell nuclei are counterstained with DAPI (blue). Chronic hyperglycemia decreases brain cell proliferation after 14 days of treatment in the subpallium **(A)**, in the pallium **(B),** and in the caudal hypothalamus around the lateral and posterior recess of the ventricle **(C)**. Scale bar: 120 µm (A and B), 200 µm (C). Please click here to view a larger version of this figure.



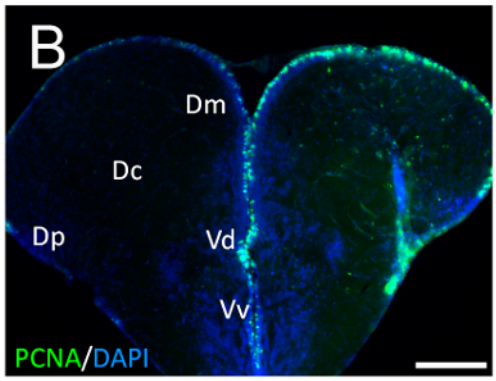




**Figure 3: Stab wound injury of the telencephalon upregulates brain proliferation at 7 days post-lesion**
**A:** Schematic overview of a transversal section of the zebrafish telencephalon at the level indicated in the upper sagittal. Schema have been taken from the zebrafish brain atlas[31]. The red dots indicate proliferating cells[32,33]. The needle indicates the site of the lesion.
**B:** PCNA (green) immunohistochemistry 7 days after brain injury shows a strong upregulation of proliferation along the brain ventricle in the injured telencephalon. Scale bar: 200 µm. Please click here to view a larger version of this figure.

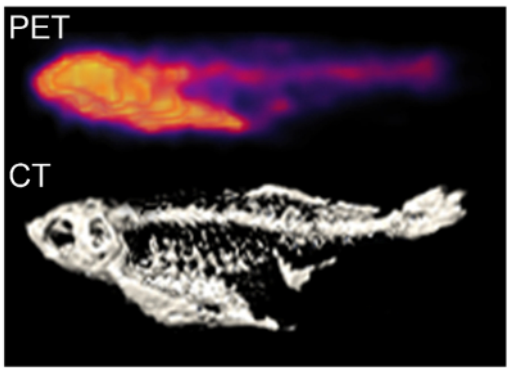



**Figure 4: PET/CT imaging of [18F]-FDG (20 MBq injected) 30 min after the intraperitoneal injection**
Representative images of PET/CT imaging show a wide distribution of [18F]-FDG in the body of the zebrafish, including the head, the brain, and the spinal cord. Please click here to view a larger version of this figure.

## Discussion

This work describes various methods to establish acute and chronic models of hyperglycemia in zebrafish. The main advantages of these procedures are that: (1) they allow for a reduction in the number of mammals used for research, (2) they are simple to set up and quick to implement, and (3) they are economical. Therefore, such models allow for the investigation of the impact of hyperglycemia on a large number of animals to study its impact on different physiological processes, including atherothrombosis, cardiovascular dysfunctions, retinopathies, blood-brain barrier leakage, and constitutive and regenerative neurogenesis. This work describes how to proceed with investigations on the effects of hyperglycemia on brain cell proliferation under normal or injury-induced conditions.

One critical limitation of the chronic hyperglycemia procedure is that, in some experiments, some fish do not display hyperglycemia after the chronic immersion in D-glucose water (111 mM for 14 days). The percentage of responsive and non-responsive fish has been previously estimated by Dorsemans and colleagues (2016) to be 83% versus 17%, respectively. It is possible that fish display individual susceptibilities according to their age, sex, and capacity to compensate for hyperglycemia by making more pancreatic β-cells[34,35]. For acute hyperglycemia, the blood glucose levels are quite homogeneous 1.5 h after the injection, demonstrating the robustness of the method.

A critical step of this procedure concerns blood-glucose-level measurements. The quantity of blood that fills the eye cavity is, in rare cases, too low to allow for the loading of the glucometer test strip. In addition, the fish should not stay on ice for too long in order to avoid blood coagulation. However, they should remain on ice for a time sufficient to ensure the induction of anesthesia and the death of the animals. It is also important to mention that, for acute hyperglycemia, the volume of D-glucose injected should be altered to account for the size of the fish. The 50-µL intraperitoneal injection is designed for a medium-sized fish (0.5 g). Indeed, a small fish might not be able to receive a 50-µL intraperitoneal injection and the volume of injection must be reduced to prevent animal suffering and to avoid solution being pushed straight back out by pressure.

Another critical step is the reproducibility of the stab wound injury of the adult telencephalon; which requires some technical experience. Additionally, counting should be performed on three successive sections of a region of interest and in at least three animals. Automated counting in larger brain areas can reveal important information concerning the global effect of hyperglycemia on the process of neurogenesis.

Another reason to use zebrafish is for the ability monitor the biodistribution of radiolabeled molecules using PET/CT. Here, [18F]-FDG was used, and its distribution throughout the zebrafish body was demonstrated, notably including the brain and the spinal cord. Such techniques are of particular interest when determining the delivery and bioaccumulation of potential therapeutic agents in *in vivo* models. This technique also represents an alternative method to investigate the ability of some molecules to cross through the blood-brain barrier and to determine their potential effects on the central nervous system under physiological or pathophysiological conditions. Indeed, hyperglycemia and hypoglycemia are known to modulate blood-brain barrier permeability[36].

One critical limitation of PET/CT imaging in zebrafish after intraperitoneal injection is the necessity to anesthetize the fish in order to avoid any movement during the acquisition. Such anesthesia could strongly reduce the heart rate and therefore the radiotracer biodistribution. To solve this problem, fish can be injected and allowed to recover in fresh water for a few minutes or hours, depending the imaging protocol and the half-life of the radioisotope used. In addition, the intraperitoneal injection could result in the strong accumulation of signal in the peritoneal cavity.

To conclude, this work described efficient methods to establish models of hyperglycemia in zebrafish and to monitor radiolabeled-molecule distribution. Such approaches could open a field of research relating to the investigation of the impact of metabolic disorders on brain homeostasis and on the biodistribution of potential therapeutic agents.

## Disclosures

No potential conflicts of interest were disclosed.

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