Simplified Protocol for Production of Persistent Hyperglycemic Zebrafish Model for Type II Diabetes Mellitus Studying

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder characterized by extremely elevated blood sugar, i.e. hyperglycemia (HG). This hyperglycemia is caused by either defective insulin production, marking DM type 1 (T1DM), or impaired biological effects of insulin by developing insulin resistance, marking DM type 2 (T2DM). Zebrafish *Danio rerio* provides an excellent model for testing T2DM therapies, having a great resemblance to humans' metabolic pathways related to T2DM development. In the current study, two different glucose immersion protocols were tested to induce persistent HG in zebrafish *Danio rerio*. The first included prolonged immersion of the fish in gradually elevating glucose concentrations, i.e. 45 mM, 90 mM, and 200 mM glucose monohydrate. The second was through prolonged immersion of fish in a fixed concentration of glucose, i.e., 90 mM. Both were followed by a recovery period of fish in glucose-free waters. Glucose and triglycerides were assessed in the livers of immersed fish after i) full-time immersion in glucose, and ii) the recovery period in glucose-free waters. Only the "fixed-dose" immersion protocol resulted in significant HG and hypertriglyceridemia (HTG) after the glucose immersion. A recovery period in glucose-free waters could make glucose-challenged fish recover from neither HG nor HTG. The results indicated the feasibility of fixed immersion of adult zebrafish in 90 mM of glucose solution in the induction of persistent HG and HTG, rather than the "gradually elevating" glucose immersion protocol. The identified induction protocol can be recommended for testing different therapies aiming to combat T2DM by affecting both HG and HTG.

INTRODUCTION

State diabetes mellitus (DM) is a common endocrine disorder that affects more than 537 million adult people worldwide according the most recent data of the International Diabetes Federation (**IDF, 2021**). It is caused by the loss of capability of insulin to perform its regulatory role in promoting glucose uptake by tissue, owing to a damage in the producing β islets of Langerhans in the pancreas, or due to a developed resistance of tissues against insulin itself (**Ismail, 2009**). This results in a deleterious increase in the

concentrations of blood glucose, resulting in severe damage to numerous body systems and organs, particularly blood vessels, eyes, kidney, heart and nerves **(Ismail, 2009)**. Based on the cause of insulin role loss, DM has been classified into two types, insulin-dependent DM (type I, T1DM) and non-insulin-dependent DM (type II, T2DM). Type I diabetes is an autoimmune disease characterized by a local inflammatory reaction in and around β islets that is followed by selective destruction of insulin-secreting cells, whereas type II diabetes is characterized by peripheral insulin resistance and subsequent impaired insulin secretion **(Arora *et al.*, 2009)**.

Multiple risk factors are known for T2DM, including genetic, metabolic and environmental factors **(Galicia-Garcia *et al.*, 2020)**. Many of them interact to promote the development of symptoms of this metabolic syndrome early in life. However, and despite some of these factors being non-modifiable, e.g. individual genetic predisposition, family history, etc., still countless other risk factors are modifiable enough to prevent disease development. These modifiable factors include obesity, low physical activity and an unhealthy diet **(Galicia-Garcia *et al.*, 2020)**.

Obesity, i.e. a body mass index (BMI) equal to or above 30 kg/m^2, is probably the major risk factor in T2DM development **(Bellou *et al.*, 2018)**. An inverse relationship was elucidated between the age of T2DM diagnosis and BMI **(Galicia-Garcia *et al.*, 2020)**. Although the exact mechanism by which obesity is implicated in T2DM development is still under investigation, the relation between both metabolic dysfunctions is well established. Obesity develops as a result of overconsumption of carbohydrates and fats, that are primarily stored as glycogen and fats, mainly triglycerides. Excessive amounts of glucose are metabolized in adipocytes to fatty acids and glycerol-3-phosphate, both combine to produce triglycerides. Meanwhile, fats, absorbed as triglycerides and cholesterol, are transported in chylomicrons through lymphatic tissues to various tissues. With the help of insulin, fats are transported to muscle cells for functional energy and adipocytes for storage as fat **(Malone & Hansen, 2019)**. The main energy stores for muscle functions are fatty acids and glucose, with the excess being stored in fat cells for the next use. One of the major perturbances leading to obesity is excessive nutrition, which leads finally to improper oxidation of fatty acids and oxidative stress. One of the consequences of this is the induction of oxidation-induced inactivation of glucose transporter GLUT4 **(Malone & Hansen, 2019)**. Thus, individuals with excessive fat storage also have higher insulin levels than normal, and this happens when insulin resistance and T2DM appear **(Malone & Hansen, 2019).**

Zebrafish, nowadays known as a preferable animal model, was first introduced as an laboratory animal by **Streisinger *et al.* (1981)**. They described zebrafish as a favorable model for developmental studies due to its transparency of embryo and remarkable fecundity. After a while, large-scale genetic studies demonstrated that zebrafish conserve ortholog genes which get disrupted in a state of illness similar to humans **(Haffter *et al.*, 1996; Barbazuk *et al.*, 2000)**. In a short time, zebrafish were converted to a frequent

model of disease and drug discovery simultaneously with the progression of genetic methods **(Dooley & Zon, 2000; Nasevicius & Ekker, 2000)**. They drew a lot of attention to be a proper model for metabolic diseases **(Wang *et al.*, 1998).**

A variety of key features made zebrafish superior as an experimental model in numerous scenarios, viz. having a mammalian-like pancreas specifications **(Zon and Peterson, 2005)**, possessing mammalian such as glucose regulation **(Jurczyk *et al.*, 2011)**, in addition to the similarity of nearly 70% of genes to human **(Howe *et al.*, 2013)**. Additionally, whole organ transcriptomic and proteomic analyses were possible due to relatively the small size of zebrafish genome **(Junker *et al.*, 2014; Nolte *et al.*, 2015)**. Moreover, zebrafish digestive system, adipose tissues, and skeletal muscle are physically human-like **(Schlegel & Stainier, 2007)**. Furthermore, blood circulation in vessels and neuronal and hormonal systems develop and become functional at the early stages of development, making zebrafish favorable for early metabolic studies **(Kimmel *et al.*, 1995, Lakstygal *et al.*, 2019; Pourghadamyari *et al.*, 2019)**.

Therefore, the current study aimed to compare two different systems for the induction of persistent, non-recoverable hyperglycemia in zebrafish adults, as a way to promote any future research work aiming to use it as a model for different therapeutics related to T2DM disease prevention.

MATERIALS AND METHODS

Experimental fish obtaining and laboratory animal care

Zebrafish *Danio rerio* (Fig. 1)(n=50) mixed-sex adults were obtained from commercial pet shops in Shebin El-Kom City, Egypt (Fig. 1). They were maintained at normal water temperatures (22±2°C) and light: dark cycle (12h:12h) for two weeks before initiating the experiment. The fish were fed once a day with diet for tropical fish aquaria, encompassing 23% crude protein, 3% crude fat, 1.5% crude fiber, 8.0% and 0.7% moisture and phosphorus. The feeding ratio was 2% of the total body mass on a daily basis. They were maintained in 14L plastic aquaria (Fig. 2), equipped with mechanical/biological filters and heaters to keep fixed temperature at 22ºC. 50% of the water was renewed daily after cleaning the aquaria to remove wastes. The aquaria were daily observed for mortality eventual occurrence. Feeding and aquarium cleaning/dose compensation proceeded in the same way during the subsequent glucose challenge experiments to keep the aquaria of all groups as clean as possible from any organic contaminants. All maintenance and experimental procedures on zebrafish were approved by the Institutional Animal Care and Use Committee (IACUC) Faculty of Science, Menoufia University (approval code: MUFSFGE823).

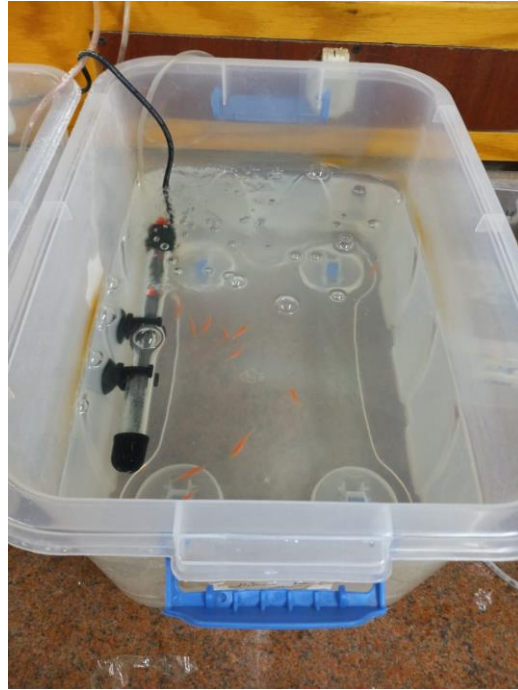**Fig. 1.** Zebrafish *Danio rerio* applied in the current study

Fig. 2. Example of a 14L- aquarium applied for experimentation with zebrafish

Hyperglycemia induction

For the induction of persistent hyperglycemia in zebrafish, two different protocol were compared. The first included a glucose stepwise elevation approach, where a gradual increase of glucose level was applied on the experimental fish. The second was through applying single dose of glucose throughout the experimental period. Details of both protocols are as follows:

Stepwise elevating protocol

The procedures included in this protocol followed those of **Mohammadi *et al.* (2021)**. A group of zebrafish (n= 20 , weight: 1.8±0.4 g, Length: 1.5±0.5 cm) was placed in a 5-L aquarium containing 45mM of glucose (D-Glucose "Dextrose monohydrate", MF: $C_6H_{12}O_6.H_2O$, Cat. No. G002, PIOCHEM, Egypt) solution in dechlorinated tap water (herein referred to as G-group). Another group (n = 20) was maintained until the end of the experiment in a 5-L aquarium without glucose to serve as the negative control (termed herein C-group). The G-group was maintained in its 45mM glucose for 4 days.

Next, this group of fish was gently transferred into a new aquarium containing 90mM glucose, and fish specimens were kept for 4 further days. Later on, G-group fish were transferred to a third aquarium containing 200mM glucose and kept there for a further 4 days. However, for the death of 7 experimental fish, the experiment was repeated to set the glucose incubation times to be 4 days at 45mM glucose, followed by 7 days at 90mM glucose concentration.

Finaly, G-group fish was transferred in day 12 from the onset of the experiment to glucose-free aquarium, containing only dechlorinated tap water (Fig. 2). Meanwhile, C-group was simultaneously transferred among 3 glucose-free aquaria filled with dechlorinated tap waters to simulate any possible stress the G-group may have been suffering during its transfer among aquaria with different glucose concentration. Fish were maintained in the glucose-free aquaria for further 4 days, i.e. until day 15 of the experiment. Sampling was proceeded in the next day.

Fixed-concentration protocol

The G-group in this experiment (n= 10, weight: 1.8±0.4 g, Length: 1.5±0.5 cm) was placed in a 4-L aquarium containing 90mM of glucose (D-Glucose "Dextrose monohydrate", MF: $C_6H_{12}O_6.H_2O$, Cat. No. G002, PIOCHEM, Egypt) solution in water. The C-group was maintained in a glucose-free 4-L aquarium. Both groups were incubated in their aquaria for 13 days. Next day, i.e. day 14 of the onset of the experiment, all fish were transferred into new 4 L-aquaria (n=10 zebrafish/aquarium) containing glucose-free, dechlorinated tap waters, and maintained there for further 4 days, i.e. until day 18. Feeding was normally proceeded with the same initial rations. Sampling was proceeded in the next day.

Tissue sampling and measurements

Fish were collected at two time points (n=5/group/time point). The first point was directly after the transfer from glucose to glucose-free water in both exposure protocols, whereas the next point was after the experiment was concluded. Fish from each group were euthanized by placing in ice-cold, glucose-free waters (2 – 4 °C). Each fish was dissected, and the entire livers were separately collected and preserved using the same method mentioned in the study of **Galal-Khallaf *et al.* (2022)**. Liver tissues of the experimental fishes were removed to sterile 1.5mL tubes containing 500μL of ice-cold phosphate buffer (KH_2PO_4, pH=7.2, 10mM). These tubes were separately and immediately homogenized. The homogenates were then centrifuged at 10,000g for 10 min at 4ºC. The supernatants were transferred to new sterile 1.5mL tubes. These new tubes were stored in a –80ºC until measuring glucose, triglycerides, and insulin concentrations.

Assessment of energetic metabolites levels
Measurement of glucose levels was carried out using a commercial kit (Spinreact, cat. No. 1001191). In addition, triglycerides were measured using a commercial kit from Spinreact (Cat. no. 1001310). In both cases, KH_2PO_4 (10 mM, pH 7.2) was used as a blank. Homogenates of hyperglycemia-induced and control zebrafish were applied in triplicate in a 96-well microplate for each treatment. Intra-specific errors were calculated as the standard error of means of measurements among the triplicate of the same sample. After the addition of the reagents to the sample, negative control, blank, and standard curve wells, the plates were read at 492nm in the spectrophotometric plate reader (TECAN, infinite F50, Mannedorf, Switzerland).

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RESULTS

Mortality of experimental fishes was only found in the "stepwise elevating" glucose challenge protocol in the G-group, where 7 fishes died out the 20 applied in the experimental group. Repeating this protocol with only 45mM glucose for 4 days, 90ppm for 7 days, then transferring to blank waters resulted in no mortalities. Meanwhile, "fixed-concentration" dosing protocol fulfilled by incubating fishes with 90 mM glucose for 12 days continuously, then incubating them for further 4 days in glucose-free water resulted in zero mortalities.

Standard curves for both glucose and triglycerides resulted in regression coefficients (r^2) higher than 0.9, between the serial dilutions and absorbance values (Fig. 3a, b). For glucose, the assay was proven to be linear at least between 100 and 0.16mg/dL (Fig. 3a). For triglycerides, the assay was proven linear between 200 & 0.32mg/ dL at least (Fig. 3b).
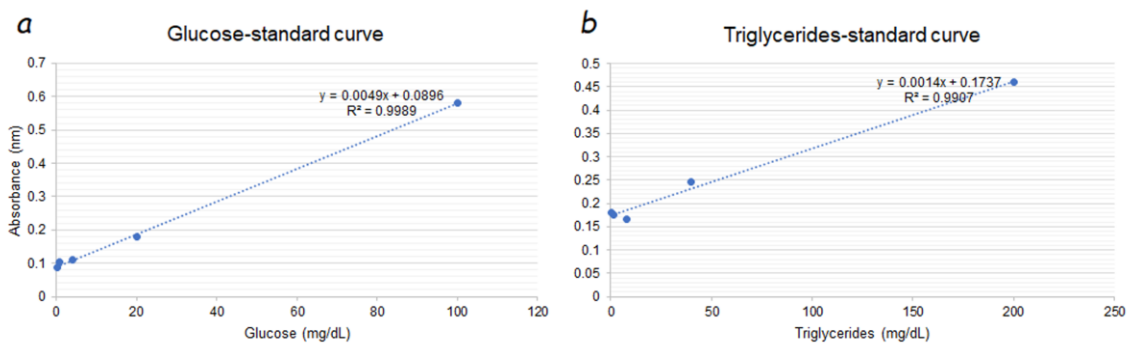

Fig. 3. Linearity of glucose (a) and triglycerides (b) standard curves. The standard curves in the current study were made by 1/5 seral dilutions from the initial glucose and triglycerides standard of 100 mg/dL.

The patterns of glucose and triglycerides also varied strongly between the two applied hyperglycemia induction protocols. For the "stepwise elevating" protocol, no significant difference could be found. Glucose I livers of fish in the G-group that were sampled on day 12, after their transfer from glucose-containing to glucose-free water, showed no significant difference with the negative control groups collected in the same day (Fig. 4A). Likewise, individuals of the same group, left in glucose-free waters to recover from possible hyperglycemia for further 3 days, showed significantly no different levels, with respect to the negative control group at the same day (Fig. 4A). For triglycerides, different pattern was obtained. The G-group at day 12 showed a slight, yet non-significant increase in comparison to the negative control group (Fig. 4A). The G-group at day 15 exhibited significantly elevated liver TG levels, with respect to the C-group in the same day (Fig. 4B). However, still this latter elevation is very modest despite being significant (about 2- 4mg/ dL only) (Fig. 4B).
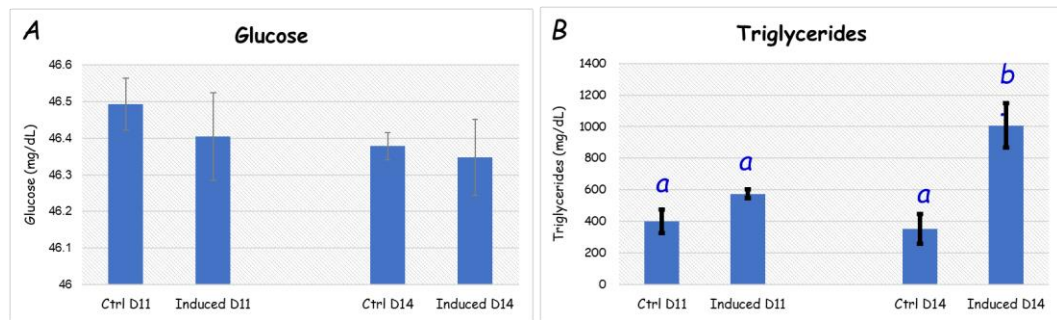

Fig. 4. Level of glucose (A) and triglycerides (B) in the livers of fish subjected to stepwise elevating glucose levels. Letters (a, b) above bars refer to significant difference among different experimental fish. "Ctrl" abbreviation refers to the negative control group of fish that was incubated in glucose-free water. "Induced" refers to fish that were incubated in glucose-containing water.

Meanwhile, the experiment where fish were kept in 90mM glucose concentration for longer time period, i.e. 14 days, resulted in more statistically significant results (Fig. 5). Glucose levels were significantly higher in the G-group at day 14 than the C-group. Similar pattern was found for both groups at day 18, i.e. 4 days after keeping fish in glucose-free waters (Fig 5A). Even a slight, yet non-significant, increase was found in G-group in day 18 over glucose levels of the same group at day 14 (Fig. 5A). Triglycerides showed similar pattern to glucose levels, being G-group with significantly higher TG levels than the negative control group in both day 14 and day 18 (Fig. 5B). Moreover, TG levels in G-group in day 18 were significantly higher than their levels in day 14, even 4 days after glucose withdrawal from aquarium waters (Fig. 5B).
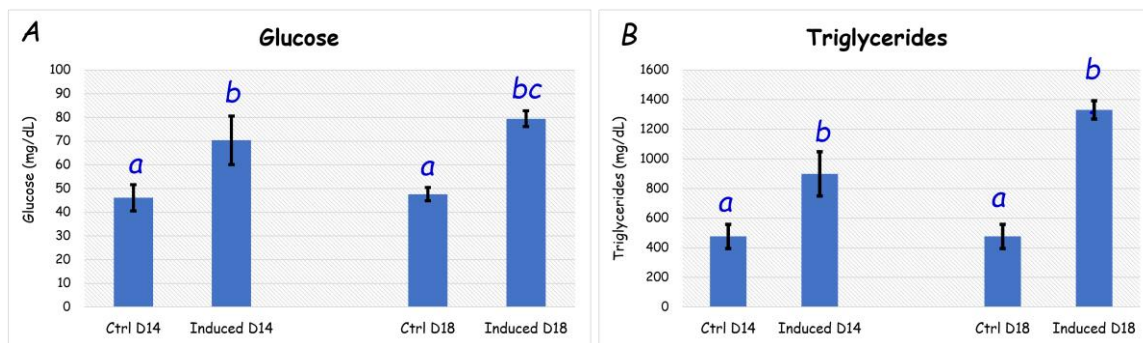
Fig. 5. Level of glucose (A) and triglycerides (B) in the livers of fish subjected for 14 days TO a fixed glucose concentration of 90mM. (Ctrl) abbreviation refers to the negative control group of fish that was incubated in glucose-free water. "Induced" refers to fish that were incubated in glucose-containing water.

DISCUSSION

Zebrafish is continuously gaining popularity as a model of choice for studies of DM induction and treatment **(Gleeson *et al.* 2007)**. Easiness in care, relatively short life span, and transcriptome similarity with higher vertebrates, including human, all recommended its use in this field of investigation **(Schlegel & Stainier, 2007)**. Glucose immersion method was identified as one of the main methods for the induction of T2DM in zebrafish **(Cao *et al.*, 2023)**.

The two applied protocols were associated with very different patterns of metabolic alterations. In the "stepwise elevating" glucose challenge protocol, hepatic glucose values did not vary significantly. Only a non-significant decrease was found in G-group after 10 days of exposure, and in both C- and G-groups after keeping the fish for 3 days in glucose-free waters. However, "fixed-concentration" protocol resulted in significantly elevated levels of glucose after 13 day of fish immersion in 90mM glucose. This induced hyperglycemia persisted even after glucose withdrawal keeping fish in normal waters for further 4 days. It's noteworthy to mention that, glucose levels identified in the control groups in the current study were all in the known ranges for this metabolite in adult zebrafish under normal, non-stressed conditions. For example, **Xi *et al.* (2023)** recorded normal glucose levels of about 40mg/ dL in adult zebrafish fed normal diet. Additionally, **Lai *et al.* (2022)** identified glucose levels of about 50- 70mg/ dL in normal adults that increase to about 150- 200mg/ dL in adult fish subjected to lipolysis impairment and metabolic syndrome.

Previous studies showed that feeding zebrafish with continuously high carbohydrate diets increases body glucose levels for prolonged times, as indicated in the study of **Dandin *et al.* (2022)**. It would be expected that glucose withdrawal in both induction protocols would reduce the high blood levels through the recovery of insulin response **(Longkumer *et al.*, 2020)**. Increase in glucose levels in response to high

carbohydrate diets was found to be traced back to several causes including hepatic glycolysis induction through activating the expression of liver glycolysis-related genes, and hence significantly elevating glucose levels (**Xi *et al.*, 2023**). However, a non-treated elevation of glucose in liver can be a symptom of an increased insulin resistance, which can lead to the loss of insulin role in inhibiting liver glucose production as one of the key manifestations of T2DM (**Kotronen *et al.*, 2008; Maddison *et al.*, 2015; Mathur *et al.*, 2016; Guerra & Gastaldelli, 2020**). Being this persistent hyperglycemia noted only in the "fixed-concentration" protocol, it can be suggested that this protocol can provide better hyperglycemia zebrafish model than the other protocol we applied herein, i.e. the "Stepwise elevating" protocol.

In addition, triglycerides levels in the negative control groups in the current study were similar to the previously identified ones for zebrafish adults. For TG, a normal value of 292.36 ± 111.28mg/ dL was identified for adult fish without access to eggs and 457.64 ± 43.78mg/ dL for fish with access to eggs (**Pedroso *et al.*, 2012**). Besides, normal TG values were found to be around 200- 400mg/ dL for male and female zebrafish (**Jin *et al.*, 2023**). Yet, some variations in these levels were identified, for example **Lai *et al.* (2022)** recorded TG levels of about 100-200mg/ dL in adult zebrafish. This may suggest some dependence of these levels on other factors, including for example different diet contents, feeding regimes, or others. Moreover, triglycerides showed variable patterns among the groups assessed in the current work for the applied two hyperglycemia induction protocols. This took place with a clear, significant tendency to accumulate in the livers of fishes in the G-groups under both induction protocols at the last time points, i.e. D11 for the "Stepwise elevating" and D14 in the "fixed-concentration" protocols. This aligns well with the known accumulation of hepatic triglycerides as a strong overfeeding or toxicity marker, being an excess diet containing carbohydrates as a strong stimulator for lipogenesis (**Zhao *et al.*, 2021**; **Xi *et al.*, 2023**).

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CONCLUSION

Zebrafish stock applied in the current study showed better survival and clearer metabolic alterations to glucose exposure as a fixed-dose protocol for HG and HTG induction than to stepwise elevating glucose dose protocol. This fixed-dose protocol can be then strongly recommended for testing the effects of different drugs on the treatment of HG and HTG.

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Arabic Summary

بروتوكول مبسط لانتاج نموذج من سمك الزيبرا مصاب بارتفاع السكر المستديم بغرض دراسة داء السكري.

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داء السكري (DM) هو اضطراب أيضي يتميز بارتفاع نسبة السكر في الدم بشكل كبير، فيما يعرف بالهايبرجلايسيميا.ويحدث هذا الارتفاع الفائق إما بسبب خلل في إنتاج الأنسولين، وهو مايميز النوع الاول لداء السكري، أو بسبب انخافض قدرة الانسولين على ممارسة تاثيراته الخافضة لمستوى الجلوكوز بالدم وذلك بسبب تطوير الانسجة المختلفة مقاومة للأنسولين، وهو مايحدث في النوع الثاني لداء السكري. وفي حالة النوع الثاني، يعد عرضي ارتفاع نسبة سكر الدم والدهون الثلاثية من عوامل الخطورة الاساسية التي تسبق الاصابة بهذا الداء. توفرأسماك الزيبرا نموذجًا ممتازًا لاختبار علاجات النوع الثاني من داء السكري، حيث يظهر هذا النوع من الاسماك تشابها كبيرا في المسارات الأيضية المتعلقة بتطور الداء مثيلاتها لدى البشر. في الدراسة الحالية، تم اختبار بروتوكولين مختلفين لاحداث ارتفاع مستمر لمستوى السكر الدموي في اسماك الزيبرا من النوع دانيو ريريو وذلك بغمر الطور البالغ لهذه الاسماك في مياه تحتوي على الجلوكوز بطريقتين مختلفتين اشتملت الأولى الغمر لفترات طويلة في تركيزات متصاعدة تدريجيا من الجلوكوز، وهي 45 مللي مولار، و 90 مللي مولار، و 200 مللي مولار، بينما اشتملت الطريقة الثانية غمر الأسماك لفترة طويلة في تركيز ثابت من الجلوكوز، وهو 90 مللي مولار .وأعقب كلاهما فترة تعافي للأسماك في المياه الخالية من الجلوكوز .تم تقييم الجلوكوز والدهون الثلاثية في كبد الأسماك المغمورة بعد 1) الغمر في الجلوكوز، و2) فترة التعافي في المياه الخالية من الجلوكوز. أظهرت النتائج قدرة بروتوكول الغمر في "الجرعة الثابتة" على احداث زيادة كبيرة في نسبة سكر الدم و الدهون الثلاثية، ورغم ان فترة التعافي المعطاة للاسماك بنقلها الى مياه خالية من الجلوكوز كانت قادرة على جعل الأسماك تتعافى من ارتفاع تلك المعاملات الايضية، الا ان المجموعة المعرضة للغمر الثابت في 90 مللي مولار من محلول الجلوكوز احتفظت بنسب عالية بشكل ملموس من سكر الدم والدهون الثلاثية بالمقارنة بالمجموعة الضابطة التي لم تلق اي كمية من الجلوكوز في مياه تربيتها.، في حين ان المجموعة المعرضة لبروتوكول "الجرعات التصاعدية" لم تظهر الا ارتفاع ملموس في نسب الدهون الثلاثية فقط وليس سكر الدم وذلك بعد فترة التعافي فقط، لذا فان الدراسة الحالية توصي باستعمال بروتوكول "الجرعة الثابتة" من الجلوكوز لاحداث ارتفاع مستمر في سكر الدم والدهون الثلاثية، مما يمكن استعماله لاحقا في اختبار علاجات مرض السكر التي تهدف بشكل اساسي لمعالجة هذين العرضين، والذان يسبقان بشكل ثابت الاصابة بداء السكري من النوع الثاني.