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Persistent impaired glucose metabolism in a zebrafish hyperglycemia model

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ABSTRACT

Diabetes mellitus (DM) affects over 10% of the world's population. Hyperglycemia is the main feature for the diagnosis of this disease. The zebrafish (*Danio rerio*) is an established model organism for the study of various metabolic diseases. In this paper, hyperglycemic zebrafish, when immersed in a 111 mM glucose solution for 14 days, developed increased glycation of proteins from the eyes, decreased mRNA levels of insulin receptors in the muscle, and a reversion of high blood glucose level after treatment with anti-diabetic drugs (glimepiride and metformin) even after 7 days of glucose withdrawal. Additionally, hyperglycemic zebrafish developed an impaired response to exogenous insulin, which was recovered after 7 days of glucose withdrawal. These data suggest that the exposure of adult zebrafish to high glucose concentration is able to induce persistent metabolic changes probably underlined by a hyperinsulinemic state and impaired peripheral glucose metabolism.

1. Introduction

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by high levels of blood sugar, termed hyperglycemia (SBD, 2009). According to the International Diabetes Federation (IDF), in the year 2012, the estimated number of adults living with diabetes had soared to 371 million or 8.5% of the global adult population (IDF, 2012). New projected data suggest that until 2030 there will be 552 million people with diabetes; this means for that every 10 s approximately three more people will be diagnosed with diabetes (IDF, 2012).

Chronic blood sugar elevation is the major biochemical diagnostic parameter that is seen in the two major forms of diabetes: Type 1 diabetes or insulin-dependent, caused by autoimmune destruction of insulin producing pancreatic β-cells, and Type 2 diabetes or non-insulin dependent characterized by insulin insensitivity (Harris and Zimmet, 1997).

Zebrafish has been established as a good animal model to understand physiological and pathological conditions in vertebrates (Maddison and Chen, 2012; Asaoka et al., 2013; Stewart et al., 2014). Olsen et al. (2010) described the diabetes model in adult zebrafish via streptozocin which induces pancreactic cell death, promoting diabetic complications such as fasting elevated blood glucose values, increased glycation of serum protein, retinopathy, decreased serum insulin levels and impaired regeneration in damaged areas (Oka et al., 2010; Olsen et al., 2010). Other studies described an obesity model in adult zebrafish, revealing that the induction of obesity is able to promote hypertriglyceridemia and hyperglycemia, as common pathophysiological pathways similar with those found in mammalians (Gleeson et al., 2007; Oka et al., 2010). Adult zebrafish easily absorb molecules from water, due to an ability to regulate their internal water and total solute concentrations (Moyle and Cech, 2000). They operate hyperosmotically, a strategy of osmoregulation that involves the continuous gain of water as a result of a higher internal concentration of salt compared to their freshwater environment. The constant influx of water results in the uptake of molecules from their environment. In this way, an easy model of hyperglycemia was proposed by immersing zebrafish in a glucose solution, being capable of generating diabetic retinopathy similar to humans (Ferraris and Ahearn, 1984; Bogé and Péres, 1990; Gleeson et al., 2007; Tseng et al., 2009; Alvarez et al., 2010). In zebrafish, the glucose absorption/uptake occurs through a glucose transporter, called GLUT, expressed in the

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gills (GLUT 1–3, 6, 8, and 10–13) and intestine (GLUT 5 and 9) (Tseng et al., 2009). Here we described that glucose overload in living water of fish is able to induce symptoms related to the pathophysiology of DM, which are persistent after a glucose withdrawal period and sensitive to anti-diabetic drugs.

2. Material and methods

2.1. Animals

Zebrafish (*Danio rerio*) adult wild type (Tübingen background; 3–5 cm) of both sexes were acclimated for at least 14 days in the experimental room. Animals were housed in groups of 15 fish in 5-L thermostated (28 ± 2 °C) tanks, kept under constant chemical, biological and mechanical water filtration and aeration (7.20 mg O_2/L). Fish were maintained under a 14 h/10 h day/night photoperiod cycle, fed three times a day with commercial flakes containing 48% protein, 8% fat, and 2% fiber (TetraMin™, NC, USA) (Siccardi et al., 2009) and supplemented with live brine shrimp. All fish used in these experiments were randomly chosen from different clusters. The animals were submitted to hypothermia by exposure to ice water, flocked, and followed by decapitation as the endpoint. Anesthesia with Tricaine MS-222 was done only in experiments using intraperitoneal injection as described below. All protocols were approved by the Institutional Animal Care Committee (12/00310 — CEUA PUCRS) and followed Brazilian legislation, the guidelines of the Federal Council of Veterinary Medicine (CFMV), and the Canadian Council for Animal Care (CCAC) "Guide on the Care and Use of Fish in Research, Teaching, and Testing".

2.2. Drugs

Treatment concentrations were as follows: Tricaine MS-222 (0.1 g/mL; ethyl 3-aminobenzoate methanesulfonate salt, Fluka™); glucose in three concentrations: 55.5, 111 and 166 mM (w/v) (Nuclear™); human insulin (0.01, 0.1 and 1 U/kg; Novolin™); metformin chloridate (10 μM; Merck™) and glimepiride (100 μM; Merck™).

2.3. Induction of hyperglycemia

Groups of 15 adult animals were placed in 5-L aquaria containing different glucose solutions (55.5, 111 and 166 mM), which were diluted in water and maintained during 14 days at room temperature. The feeding schedule and general maintenance procedures were as already described in the previous section. The solutions of glucose were exchanged three times per week to avoid contamination with opportunistic microorganisms. The animals were placed in each solution and were monitored for signs of stress such as difficulty of swimming or excessive gill movement (Pedroso et al., 2012). Control and 55.5 and 111 mM glucose-treated groups showed no signs of stress during all treatment with low mortality (30% and 20%, respectively), while 166 mM glucose-treated animals appeared to be more affected by the treatment, demonstrating 40% mortality. Control group animals were maintained in 5-L aquariums with normal water for the same period and conditions as the glucose-treated groups. For the following experiments, we chose the 111 mM glucose solution since the survival was the highest and the profile of blood glucose levels was similar to those published by Gleeson et al. (2007).

2.4. Withdrawal of glucose

In order to verify whether the effects caused by 111 mM glucose solution treatment would be persistent, we kept a group of 15 fish in glucose-free water for an additional 7 days (washout group, no glucose added). During this period the animals were maintained at the same conditions of welfare described in the previous section. Control group animals were maintained in 5-L aquariums with water for the same period and were handled as a washout group.

2.5. Determination of blood glucose levels

For all tests with determination of blood glucose levels, before the beginning of blood collection procedures, fish were fasted for 12 h and placed for 15 min in an aquarium with water without glucose to avoid contamination of the glucometer strip (Gleeson et al., 2007). The procedure of euthanasia occurred after hypothermia induction, which contributes to reduce the variability of blood glucose analyses (Eames et al., 2010). Immediately, the tail was cut and blood-glucose readings were taken by placing a glucometer test strip (One-Touch Ultra, Accu Check) directly on the docked tail. The blood glucose levels were measured throughout the treatment for 14 days at all concentrations tested (55.5, 111 and 166 mM glucose-treated groups) and after 7 days of glucose withdrawal (washout group) (Gleeson et al., 2007).

2.6. Determination of glycated proteins from zebrafish eyes

One of the methods to monitor glycemic control in humans is through the levels of non-enzymatic glycation of proteins, such as the determination of fructosamine levels (Armbruster, 1987). We utilized the enzyme based fructosamine quantification assay (Biotechnical™, RS, Brazil), with minor adaptation, to document the presence of non-enzymatic glycation products from zebrafish eyes from all groups. Zebrafish eyes were removed and washed in water and afterwards placed in 500 μL of saline and homogenized. Following the manufacturer's instructions, 50 μL samples were added to the 1 mL of reagent containing 200 nmol/L carbonate buffer, and 0.25 nmol/L Blue nitrotetrazol which were incubated for 10–15 min at 37 °C. The quantification of fructosamine was determined spectrophotometrically at 520 nm and the concentration was expressed as μmol/L.

2.7. Treatment with anti-diabetic metformin and glimepiride drugs

Glucose-treated and washout animals were submitted to the anti-diabetic metformin or glimepiride. We dissolved metformin chloridate in water of fish to a final concentration of 10 μM. The collection of blood samples was performed after 4 days of treatment (Elo et al., 2007; Polakof et al., 2011; Polakof and Comte, 2012). For treatment with glimepiride, we dissolved glimepiride in DMSO (final concentration 0.0005%) and added to water to a final concentration of 100 μM. The collection of blood samples was performed after 24 h of treatment, due to the results found by Elo et al. (2007). Blood glucose levels were measured with a blood glucometer as described. Saline was used as control for the metformin group and 0.0005% (v/v) DMSO solution was used as control for the glimepiride group. Metformin, glimepiride, DMSO and saline were freshly prepared on the experimental day.

2.8. Gene expression analysis by quantitative real time RT-PCR (RT-qPCR)

The expression of isoforms of insulin receptor genes (*insra-1*, *insra-2*, *insrb-1* and *insrb-2*) was analyzed by a quantitative real time reverse transcription polymerase chain reaction (RT-qPCR) assay. TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) was employed to isolate total RNA from zebrafish skeletal muscle in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50 for reference genes *EF1α*, *Rpl13α* and *insra-1* and 1:100 for *insra-2*, *insrb-1*, *insrb-2*), containing SYBR® Green I 0.2 times diluted (Invitrogen), 100 μM dNTP, 1 × PCR buffer, 3 mM $MgCl_2$, 0.25 U

Platinum® Taq DNA polymerase (Invitrogen) and 200 nM of each reverse and forward primers (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing, and 15 s at 72 °C for elongation. At the end of the cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System sequence detection software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR version 2012.3 software (http://LinRegPCR.nl) and the stability of the references genes, *EF1α*, and *Rpl13α* (*M-value*) and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Oggier et al., 2010; Nery et al., 2011).

2.9. Response to insulin

Glucose and washout groups were checked for the responsiveness to insulin. Fifteen animals of each group (control, 111 mM glucose-treated and washout) were placed in water without glucose for 24 h before the beginning of the experiment, in order to prevent interference on the fasting regimen. After that, fish were anesthetized in 0.1 mg/mL of Tricaine MS-222 prior the collection of blood. Fish were considered to be anesthetized when they presented lack of motor coordination, reduced respiration rate and no response to external stimuli (Phelps et al., 2009). We utilized Novolin™ Insulin Human 100 U/mL in three different doses: 0.01 U, 0.1 U and 1 U with volume adjusted to the fish body weight (injection volume was 10 μL) in a 20 mL/kg regimen. Intraperitonial injections were conducted using a 3/10-mL U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16″) × 31 G short needles (Becton Dickinson and Company, New Jersey, USA) according to the protocol established by Phelps et al. (2009). The needle was inserted parallel to the spine into the midline of the abdomen, posterior to the pectoral fins. The injection procedure was conducted to guarantee that the animals did not spend more than 10 s out of the water. After the injection, the animals were placed in a separate tank with highly aerated unchlorinated tap water (28 ± 2 °C) to facilitate animal recovery from anesthesia. Saline was used as control. Both insulin and saline were freshly prepared on the experimental day. All the animals had recovered after 2–3 min following the injection. The glycemia was accessed 4 h after insulin injection as suggested by previous studies using zebrafish (Olsen et al., 2010). The insulin-treated animals and controls had its tail cut and the blood collected (Pedroso et al., 2012). Animals were gently placed in a water-soaked gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen) (Phelps et al., 2009).

2.10. Statistical analysis

Data were expressed as mean ± standard error of mean. The data from insulin response experiments were analyzed by student's *t*-test. All other data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's considering $P < 0.05$ as statistically different for biochemical and mRNA expression experiments in adult zebrafish.

3. Results

3.1. Chronic treatment with glucose induces persistent hyperglycemia

Our results demonstrated that the immersion in all glucose solution tested (Fig. 1) provoked up to a 4–5 times increase in blood glucose levels until the 14th day of treatment in relation to control animals (2.99 ± 0.18 mM) [55.5 mM – $F_{(6;\ 100)} = 25.39$, 111 mM – $F_{(6;\ 100)} = 25.57$, 166 mM – $F_{(6;\ 101)} = 34.72$]. We observed that 20% of the animals treated with 166 mM glucose died during the treatment, and the next analyses were made with only the 111 mM glucose treated animals, which is in accordance to the zebrafish model of hyperglycemia described by Gleeson et al. (2007). In order to verify if the effects caused by treatment of 14 days of immersion in 111 mM glucose solution would be persistent, we performed a glucose withdrawal. Our results demonstrated that after 7 days of glucose withdrawal, there was a strong reduction (68%) on blood glucose levels (from 13.52 ± 1.29 mM to 4.26 ± 0.31 mM) when compared to the 111 mM glucose group, but still almost 2 times higher than the control group (2.48 ± 0.17 mM) [$F_{(2;\ 43)} = 69.03$, $p < 0.001$] (Fig. 2). These data show that hyperglycemia can be induced and maintained in zebrafish.

3.2. Hyperglycemia increases glycation of proteins from zebrafish eyes

In order to demonstrate that treatment with glucose for 14 days was causing persistent glycation of proteins, we analyzed the levels of fructosamine from zebrafish eyes during glucose treatment and after glucose withdrawal. Our results demonstrated that the treatment with 111 mM glucose for 14 days was able to increase (41%) fructosamine levels from zebrafish eyes and also demonstrated that this effect was prolonged up to 7 days of glucose withdrawal, demonstrating an increase (40%) in the fructosamine levels from zebrafish eyes in comparison to the control group (80.78 ± 8.54 μmol/L) [$F_{(2;\ 17)} = 6.369$, $p < 0.05$] (Fig. 3).

3.3. Anti-diabetic drugs reduce blood glucose levels from hyperglycemic zebrafish

To evaluate if hyperglycemic zebrafish would be responsive to the anti-diabetic drugs, we treated the animals with glimepiride

Table 1
Primer sequences for RT-qPCR experiments included in the study.

Sequence	Forward	Reverse	Accession number (mRNA)	Amplicon size (bp)
EF1α[*]	5′-CTGGAGGCCAGCTCAAACAT-3′	5′-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3′	ENSDART00000023156	86
Rpl13α[*]	5′-TCTGGAGGACTGTAAGAGGTATGC-3′	5′-AGACGCACAATCTTGAGAGCAG-3′	NM_212784.1	147
insra-1[**]	5′-CAACATGCCCCCTCACCACT-3	5′-CGACACACATGTTGTTGTG-3′	NM_001142672	215
insra-2[**]	5′-GGAGCCCCACTCGTCTAACAAA-3′	5′-CGCCGTTGTGAATGACGTATTC-3′	–	193
insrb-1[**]	5′-GACTGATTACTATCGCAAGGG-3′	5′-TCCAGGTATCCTCCGTCCAT-3′	NM_001123229	190
insrb-2[**]	5′-CCACCGCCAACCCTAAAGGA-3′	5′-TTGCGATAGTAATCAGTC TCGTAAAT-3′	–	170

[*] Tang et al., (2007).
[**] Toyoshima et al., (2008).

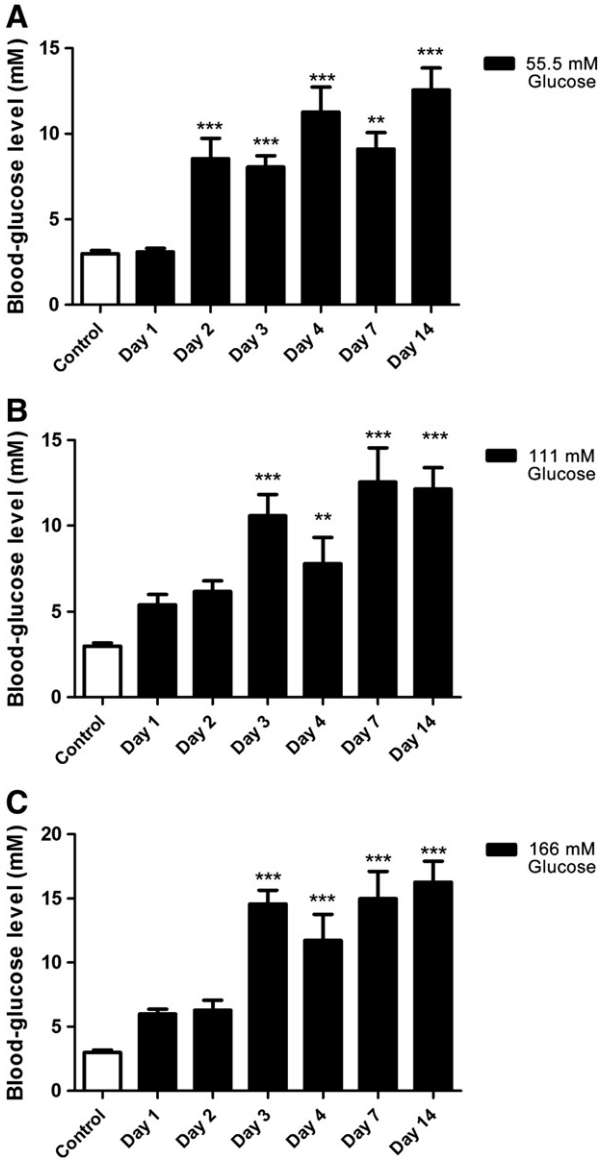

Fig. 1. Effects of glucose treatment up to 14 days on blood glucose levels. (A) 55.5 mM glucose, (B) 111 mM glucose and (C) 166 mM glucose. At least nine zebrafish were used for each group. Data are expressed as mean ± SEM of nine independent experiments. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. ***$p < 0.001$ and **$p < 0.01$.

and metformin. Our results demonstrated that the treatment with 100 μM glimepiride was able to reduce 64% of the blood glucose levels provoked by 111 mM glucose treatment (from 8.71 ± 2.22 mM to 4.43 ± 0.56 mM) [$F_{(3;\ 13)} = 63.68$; $p < 0.001$] (Fig. 4A), and 63% in the glucose washout group (from 5.23 ± 0.32 mM to 1.94 ± 0.16 mM) [$F_{(3;20)} = 53.48$; $p < 0.001$] (Fig. 4B). The treatment with 10 μM of metformin was able to reduce 51% of the blood glucose levels provoked by 111 mM glucose treatment (from 9.21 ± 1.12 mM to 4.69 ± 1.77 mM) [$F_{(3,49)} = 99.72$; $p < 0.0001$], and 45% in the glucose washout group (from 5.20 ± 0.55 mM to 2.36 mM ± 0.66 mM) [$F_{(3,39)} = 47.77$; $p < 0.0001$] (Fig. 4C and D, respectively). The anti-diabetic drugs alone did not affect the control glycemia (Fig. 4).

3.4. Effect of hyperglycemia on insulin receptors expression in skeletal muscles of zebrafish

To evaluate if the hyperglycemia could alter the relative gene expression of insulin receptors in skeletal muscles, real-time quantitative

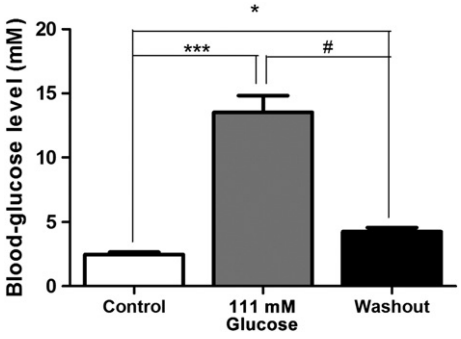

Fig. 2. Effect of glucose washout on hyperglycemic zebrafish. At least thirteen zebrafish were used for each group. Data are expressed as mean ± SEM of ten independent experiments. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. The # represents a significant difference from the 111 mM glucose group. *$p < 0.05$ and ***$p < 0.001$ and #$p < 0.001$.

PCR analyses were performed. Our results demonstrated that the expression levels of insulin receptor subunit genes (*insra-1*, *insrb-1* and *insrb-2*) were increased (*insra-1* (97.5%) [$F_{(2;\ 17)} = 8.040$; $p < 0.05$], *insrb-1* (48%) [$F_{(2;16)} = 6.288$; $p < 0.01$] and *insrb-2* (44.5%) [$F_{(2;16)} = 5.92$; $p < 0.05$]) in the washout group when compared to the control group (Fig. 5 A, C and D). However, there were no statistical relevant changes in *insra-2* gene (Fig. 5B).

3.5. Impaired response to insulin

As the above analyses assemble possible information on insulin response impairment, we submitted glucose-treated animals to an injection of insulin in three different doses: 0.01 U/kg, 0.1 U/kg and 1 U/kg. Our results demonstrated that 0.01 U/kg and 0.1 U/kg doses were unable to reverse the hyperglycemic effect caused by the treatment with 111 mM glucose solution or to reduce glucose blood level of the control group (data not shown). However, the 1 U/kg dose was able to decrease 20–32% of the basal blood glucose levels of the control group (Fig. 6A: from 2.80 ± 0.18 mM to 2.27 ± 0.09 mM and Fig. 6B: from 2.01 ± 0.14 mM to 1.36 ± 0.1 mM) [$p < 0.05$ and $p < 0.01$, respectively], while the same dose was unable to decrease the hyperglycemia from 111 mM glucose treated animals (Fig. 6A). In the washout group (Fig. 6B), the 1 U/kg dose was able to decrease 39% of the blood glucose levels (from 4.01 ± 0.32 mM to 2.58 ± 0.32 mM) [$p < 0.01$]. This protocol requires a prior anesthesia with tricaine, which could be the reason for the variability of the blood glucose levels from 111 mM glucose-treated animals (4.53 ± 0.68 mM) as discussed by Eames et al. (2010).

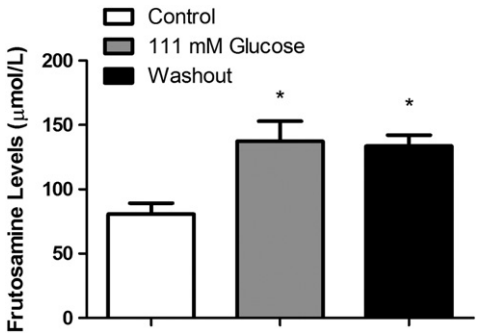

Fig. 3. Effect of 111 mM glucose treatment for 14 days and glucose washout of 7 days on fructosamine levels in zebrafish's eyes. A pool of three zebrafish's eyes were used for each group. Data are expressed as mean ± SEM of five independent experiments. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. *$p < 0.05$.

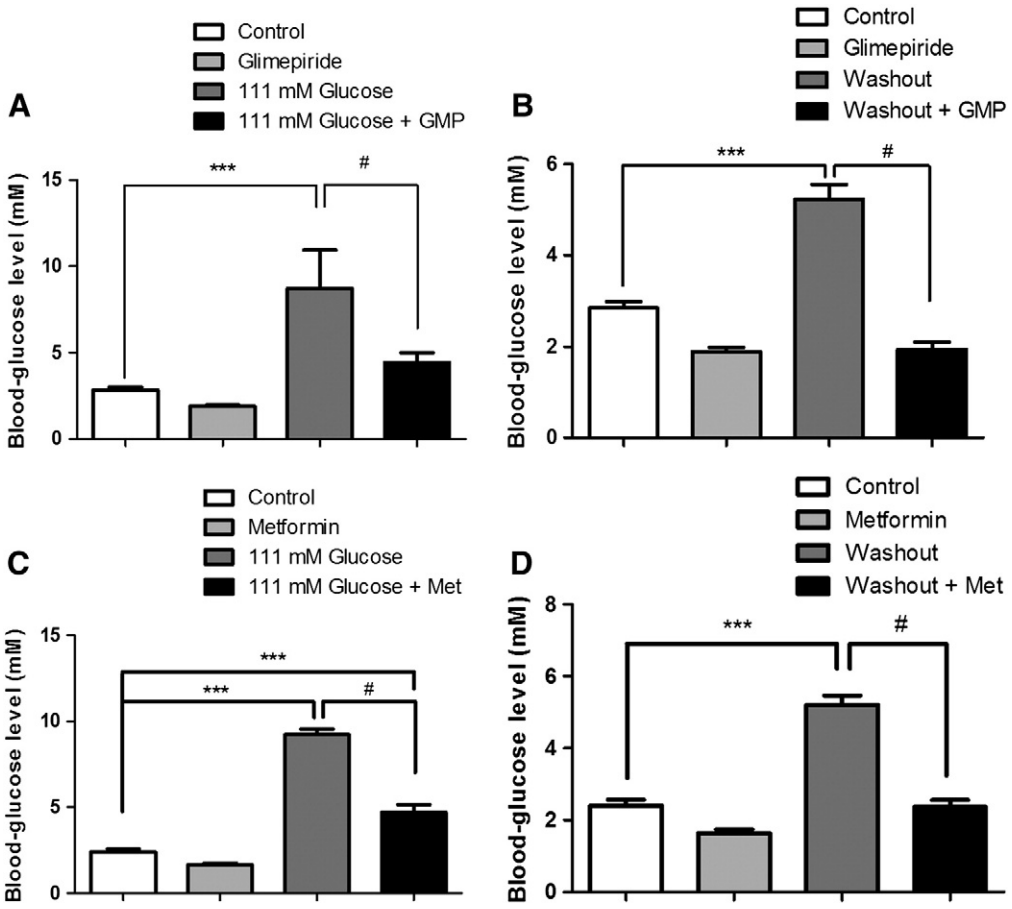

Fig. 4. Effect of anti-diabetic drug administration on 2% glucose treated and washout groups. Effect of glimepiride (100 μM) (A) and metformin (10 μM) (C) on blood glucose levels of zebrafish exposed to 111 mM glucose. Effect of glimepiride (100 μM) (B) and metformin (10 μM) (D) on blood glucose levels of zebrafish after a glucose washout period. At least ten zebrafish were used for each group. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. The # represents a significant difference from the 2% glucose group. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$ and $^{\#}p < 0.01$.

4. Discussion

In the present study, we described persistent features of impaired glucose metabolism in adult zebrafish exposed to a solution of 111 mM glucose during 14 days. The lowest mortality was seemed in animals treated with 111 mM of glucose (20%). However, the high mortality observed in 55.5 and 166 mM glucose-treated animals can be related to reduced oxygenation and/or osmotic stress.

In this work we reached similar high blood glucose levels as the former work designed by Gleeson et al. (2007). Here, all glucose solutions promoted high blood glucose levels after 48–72 h after exposure. After the glucose withdrawal by 7 days, the blood glucose levels were still elevated, but lower than the 111 mM glucose group. However, as the blood glucose levels of the washout group remained significantly higher than the control group, a long time course observation should be interesting to observe when/if levels return to normal.

In order to verify if persistent hyperglycemia could change a classical parameter of DM diagnostic, as glycosylation of proteins, we evaluated the fructosamine levels from zebrafish eyes. Our results demonstrated that there is an increase of fructosamine levels from zebrafish eyes treated with 111 mM glucose, which persists in the washout group. Although, traditionally glycosylated hemoglobin (HbA1c) has been used to monitor glycemic control in DM, unfortunately we could not analyze the HbA1c levels in blood zebrafish considering the needed volume of blood for this procedure. However, several studies have demonstrated that fructosamine and HbA1c levels have been shown as an appropriate index of glycemic control in DM patients (Juraschek et al., 2012; Lee et al., 2013).

To assist in defining the mechanisms involved in persistent hyperglycemia developed for this model, we used two normoglycemic drugs with different mechanisms: metformin chloridate (biguanide) and glimepiride (sulphonylurea). Our results demonstrated that the metformin treatment by 4 days was able to decrease the blood glucose levels in hyperglycemic zebrafish. Metformin is often recommended as the first line drug in patients with type 2 diabetes (Nathan et al., 2009; ADA, 2013), because this oral medication has an effect on the regulation of glucose uptake, gluconeogenesis, glycolysis and glycogen synthesis (McIntyre et al., 1991; Shaw et al., 2005). Metformin increases the activity of the insulin receptor and enhances glucose uptake via increased translocation of glucose transporters, such as GLUT-1 and GLUT-4 to the plasma membrane (Gunton et al., 2003; Yang and Holman, 2006). As a result, metformin enhances the insulin-mediated suppression of gluconeogenesis (Bailey and Turner, 1996). However the progressive nature of type 2 diabetes, leads to a decline in endogenous insulin secretion, thus the use of a second drug, such as glimepiride, is suggested.

The glimepiride treatment, proved to be effective for lowering blood glucose levels in zebrafish after 24 h of exposure. The main mechanism of action of glimepiride is on the activation of ATPase-dependent potassium channels in β cells of the pancreas which stimulates insulin release (Campbell, 1998), leading to a decrease in hexokinase binding to porin proteins and an increase in the expression of hexokinase mRNA (Hällsten et al., 2002). Several studies demonstrated that glimepiride is able to stimulate insulin secretion, but these studies also demonstrated important extrapancreatic effects, such as activation of glucose transport and glucose-metabolizing enzymes leading to stimulation of

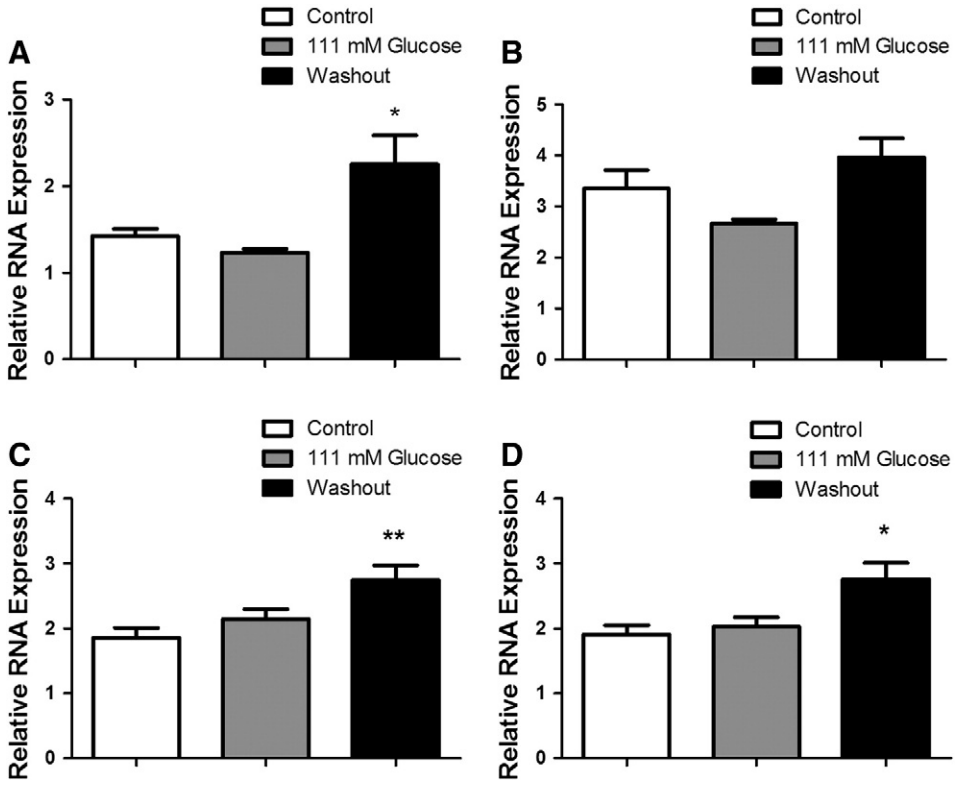

Fig. 5. Effect of 111 mM glucose treatment for 14 days and washout of 7 days on (A) *insra-1*, (B) *insra-2*, (C) *insrb-1* and (D) *insrb-2* gene expression in the skeletal muscle of zebrafish. At least four zebrafish were used for each group. Data are expressed as mean ± SEM of experiments performed in quadruplicate. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. *$p < 0.05$ and **$p < 0.01$.

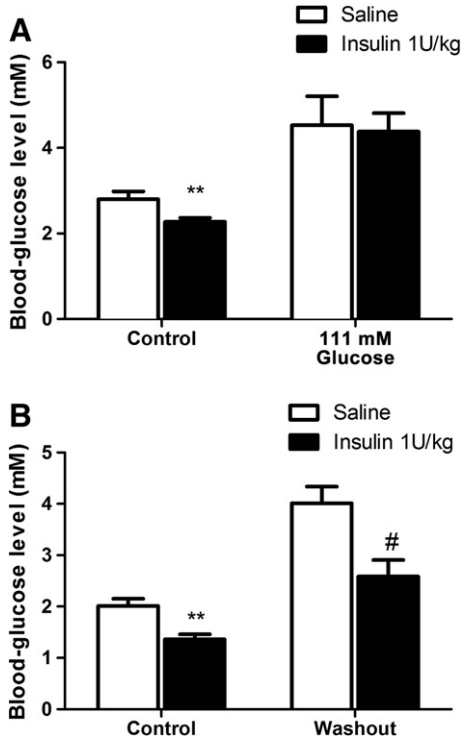

Fig. 6. Effect of insulin administration (1 U/kg) on blood glucose levels from the 111 mM glucose treated group (A) and the washout group (B). At least eleven zebrafish were used for each group. Data are expressed as mean ± SEM. Data were analyzed by *t*-test. The * represents a significant difference from the control–saline group. The # represents difference from the washout–saline group. **$p < 0.01$ and #$p < 0.01$.

glycogen and lipid synthesis, downregulation of the cAMP regulatory cascade, and inhibition of lipolysis (Briscoe et al., 2010). According to Muller et al. (1995), glimepiride seems to exert pleiotropic effects via two independent mechanisms: the stimulation of specific protein phosphatases responsible for the regulation of GLUT-4 translocation and glycogen synthase/glycerol3-phosphate acyltransferase activity; and the stimulation of cAMP-specific phosphodiesterase (Muller and Wied, 1994; Muller et al., 1995; Muller and Geisen, 1996).

If the successful effects of glimepiride were related to increased insulin secretion, this lowering of blood glucose levels should be mimicked by exogenous insulin. We injected insulin in the control, 111 mM glucose-treated and washout animals. Exogenous insulin was able to reduce blood glucose levels in all groups except in those from the glucose-treated group. The insulin resistance state is related to an impaired insulin action and it is believed that this resistance leads to hyperinsulinemia when β-islets produce a large amount of insulin in an effort to control the blood glucose level (Pessin and Saltiel, 2000; Ye, 2013). Even though an insulin level measurement should be mandatory in this approach, we failed in the collection of the required blood sample using an immunoenzymatic assay for quantitative determination of insulin from serum (Insulin SYM Kit, Symbiosis Diagnostica LTDA, São Paulo, Brazil). Gathering such information, the glimepiride effects observed could be more related to extrapancreatic effects than its well-known effect of stimulating insulin secretion.

The insulin responsive tissues express insulin receptors (IRs) at the cell surface plasma membrane. After the IR has been activated, they generate second messengers that activate a cascade of phosphorylation–dephosphorylation reactions resulting in the stimulation of intracellular glucose metabolism (Kahn et al., 1993; Whitehead et al., 2000). Dysfunctions of the IR and components of signaling cascade result in insulin resistance that leads to type 2 diabetes mellitus (Tseng et al., 2009). The

IRs of zebrafish were identified and characterized by Toyoshima et al., who demonstrated two isoforms of the IRs in zebrafish, *Insra* and *Insrb*, which are homologous to human IRs (Toyoshima et al., 2008). Several proteins have been identified in zebrafish as components of glucose metabolism, as glucose transports (GLUTs) and phosphoenolpyruvate carboxykinase (PEPCK), which have similar regulation patterns and activity as seen in mammalian counterparts (Taniguchi et al., 2006; Elo et al., 2007).

Interestingly, our results demonstrated that the mRNA expression levels of *insra-1*, *insrb-1* and *insrb-2* were significantly increased in the washout group, promoting a classical mechanism known as up-regulation, which could explain the partial reduction of glycemia in the washout group and the rescue of response to exogenous insulin. Other studies demonstrated that the kinetics of the insulin signaling via *insra* is similar to the mammalian insulin signaling cascades, suggesting that the signaling pathways downstream of the zebrafish IR are the same as found in other vertebrates (Stumvoll et al., 1995; Toyoshima et al., 2008). During early development of zebrafish the two IRs appear to play differently, whereas *insra* is critical for brain development and for general growth, *insrb* is essential for proper heart development (Stumvoll et al., 1995; Toyoshima et al., 2008).

5. Conclusions

In summary, our data demonstrated that the exposure of zebrafish to glucose diluted in water during 14 days promoted persistent high blood glucose levels, glycation of proteins of the eyes and impaired response to exogenous insulin. The hyperglycemic state was counteracted by biguanide and sulphonylurea drugs, probably by the increase of peripheral tissue response to insulin and not by pancreatic secretion of insulin, since exogenous insulin was ineffective. The glucose withdrawal of 7 days was able to partially recover some consequences of glucose exposure, probably by the recovery of insulin response, through an increase of insulin receptors in muscle. This study attempts to establish zebrafish as a valuable model for the study of targets applicable to diabetes. Zebrafish may be an ideal model to study the different mechanisms affected by hyperglycemia, as well as to understand the link between the central nervous system function and the alterations in the endocrine system, such as in DM.

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