High Glucose-Induced ROS-Accumulation in Zebrafish Larvae Leads to Mitochondria-Mediated Apoptosis

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Abstract

In recent decades, diabetes mellitus has become a major chronic disease threatening human health worldwide, and the age of patients tends to be younger; however, the pathogenesis remains unclear, resulting in many difficulties in its treatment. As an ideal model animal, zebrafish can simulate the processes of human diabetes well. In this study, we successfully established a model of diabetic zebrafish larvae in a previous work. Furthermore, transcriptome analysis was completed, and the results suggested that 10.59% of differentially expressed genes (DEGs) related to the apoptosis pathway need to be considered. Then, glucose-induced developmental toxicity, reactive oxygen species (ROS) accumulation, antioxidant system function, apoptosis and mitochondrial dysfunction were measured in zebrafish larvae. We hope that this study will provide valuable reference information for type 2 juvenile diabetes treatment.

1. Introduction

Diabetes mellitus (DM), as a metabolic disease induced by hyperglycaemia over the course of a lifetime, is caused by multiple causes. Long-term hyperglycaemia in patients with diabetes will widely lead to chronic damage and dysfunction of various tissues of the body, especially the eyes, kidney, heart, blood vessels and nerves, thus seriously impairing human health(1–3). As a global epidemic disease, the prevalence of DM is increasing year by year and has become one of the main causes of death and disability. Therefore, it has attracted the attention of many researchers and has always been an important topic all over the world, but the aetiology of diabetes has not been fully clarified(4). In the past 30 years, the prevalence of DM has shown a trend of younger age as the number of young people with type 2 diabetes mellitus (T2DM) has increased significantly, which has gradually become a threat to the health of young people(5, 6).

Animal models have become an important tool for human disease research, as these animal models can not only intuitively reflect the pathophysiological changes of human disease and be used to study the mechanism of diseases but can also be used to develop new therapeutic drugs through high-throughput screening(7–9). At present, diabetes and its complications have been widely studied using animal diabetes models, among which the most commonly used animals are mice and rats, but no animal models can completely represent the clinical symptoms of human diabetes.

Bony fish have endogenous glands that are homologous to human glands and contain hormone-secreting cells that produce a variety of hormones, including insulin, for metabolic regulation(10). Zebrafish, which show 87% genetic homology with humans, is an ideal vertebrate model organism with a small size, large spawning capacity, short growth cycle and simple breeding of adult fish, which can effectively improve experimental efficiency(11, 12). In the study of the function of endocrine glands and the mechanisms of glucose metabolism, zebrafish display their own characteristics and advantages and have been applied to the study of human diabetes and other metabolic diseases. The major organs related to metabolic regulation, such as the pancreas and some insulin-sensitive peripheral tissues,
namely, the liver and muscles, are evolutionarily conserved in zebrafish (13, 14). Furthermore, some key mechanisms related to glucose metabolism regulation are very similar to those of other mammals, making zebrafish a good research model of glucose metabolism regulation used in human diabetes research (15). Carbohydrates are an important component in the diet of zebrafish, leading to the growth rate of zebrafish being proportional to their dietary content (16). Some important genes related to glucose metabolism, such as hexokinase and glucose transporter genes, are actively expressed in zebrafish. In contrast, the deletion of these genes can cause a series of serious neural defects in zebrafish embryos. Therefore, glucose metabolism is an essential part of metabolism in zebrafish, and zebrafish is an ideal model for diabetes-related research (17–19).

In this work, zebrafish larvae were raised with glucose treatment to successfully establish a model of diabetic zebrafish larvae. Based on this model, we focused on the mechanism of developmental toxicity of zebrafish larvae induced by glucose treatment. This study represents not only a warning for the long-term high intake of sugar in adolescents resulting in type 2 diabetes mellitus but also provides valuable information for the treatment of juvenile type 2 diabetes.

2. Material And Methods

2.1 Zebrafish maintenance and growth conditions

Wild-type zebrafish (AB line, Danio rerio) and transgenic zebrafish (cms Tg/+ AB) were maintained at the Institute of Life Sciences, College of Life and Environmental Sciences, Wenzhou University. The zebrafish were maintained in a circulatory system with dechlorinated tap water (pH 6.9–7.3) in an enclosed fish house (14 h/10 h light/dark cycle) at a constant temperature of 28 ± 0.5°C. Embryo culture medium was prepared as a 20 L solution containing 70 g of NaCl, 200 ml of 0.5% KCl, 200 ml of 1% CaCl₂, 200 ml of 0.25% NaHCO₃, 26 ml of methylene blue and 19.8 L of ddH₂O. Fertilized eggs were collected and incubated at 28 °C in an incubator.

2.2 Hatchability and survival rate statistics and blood glucose monitoring

Fertilized eggs were transferred into 6-well cell culture plates filled with 10 ml of embryo culture medium, where every 3 wells made up one group, and every well contained 30 eggs. They were raised in embryo culture medium containing 0 (control), 0.5%, 1%, 1.5%, 2% and 2.5% glucose and mannitol. All of the solutions in different groups were changed twice a day. After 5 days, hatchability was counted, and the zebrafish larvae were raised until the survival rate was counted on the tenth day. Additionally, dead zebrafish larvae were removed using a straw in a timely manner.

2.3 Establishment of the model of type 2 diabetic zebrafish larvae
Fertilized eggs were raised in embryo culture medium containing 1% glucose in a container at a constant temperature of 28 ± 0.5°C in an enclosed fish house (14 h/10 h light/dark cycle). The culture medium was changed twice a day, and hatchlings were raised with paramecium combined with special feed for zebrafish larvae until the tenth day. Blood glucose levels of zebrafish larvae were measured at 2 hours and 12 hours after a meal following the instructions of the glucose test kit (Art. No: A154-1-1, Nanjing Jiancheng Bioengineering Institute).

2.4 Spinal curvature and organ oedema analysis

Zebrafish larvae exposed to 1.0% glucose solution with spinal curvature and organ oedema were picked out and placed on a slide for observation using a fluorescence microscope.

2.5 Nanopore full-length transcript analysis

Zebrafish larvae from the control and glucose exposure groups were used for nanopore full-length transcriptomic analysis (third-generation sequencing technology). A total of 250 zebrafish larvae per replicate (n = 3 replicates/treatment) were sampled, and the major work of transcriptomic sequencing was accomplished by Biomarker Technologies Company (Beijing, China). Appropriate differentially expressed genes (DEGs) were selected and analysed using DESeq2 software with FDR = 0.05 and FC = 2.

2.6 Quantitative real-time PCR (RT-PCR)

The RNA-Seq results were verified via RT-qPCR. Total RNA was extracted from the control and glucose treatment groups in triplicate, and the differentially expressed genes (DEGs) were detected by RT-PCR according to the PrimeScript™ RT MASTER MIX kit (TaKaRa, Jiangshu), where β-actin, an endogenous reference gene, was used as the housekeeping gene. The primers and RT-PCR results are listed in Supplemental Table 1 and Fig. 1, respectively.

2.7 Acridine orange (AO) and reactive oxygen species (ROS) staining

Apoptotic cells were stained with AO in vivo as follows: zebrafish larvae were transferred into 0.01 M PBS containing 5 µg/mL AO and incubated for 30 minutes in the dark at 25 °C. Then, they were washed clean with deionized water three times, and apoptotic cells were observed using a fluorescence microscope.

ROS contents were detected using a DCFH-DA fluorescent probe. Zebrafish larvae were incubated in 0.01 M PBS containing 10 µM DCFH for 20 minutes in the dark at 37 °C, washed with deionized water three times and observed using a fluorescence microscope.

2.8 Detection of apoptosis, mitochondrial function, intracellular Ca²⁺, ROS and malondialdehyde (MDA) content in zebrafish larvae
Thirty zebrafish larvae were digested with digestive enzymes to prepare cell suspensions using a KeyGen tissue dissociation kit (KeyGen BioTECH, Jiangshu). Apoptosis of zebrafish larvae was detected by measuring the fluorescence intensity of suspended cells stained with AO and propidium iodide (PI) using flow cytometry (FCM).

Mitochondrial function was investigated by direct detection of mitochondrial fluorescence in transgenic zebrafish (cms Tg/+ ) using a fluorescence microscope. Mitochondria were extracted from thirty washed zebrafish larvae according to a mitochondrial extraction kit (Solarbio, SM0020-50T), and mitochondrial membrane potential was measured using FCM with a JC-1 fluorescence probe (Solarbio, M8650).

Intracellular Ca$^{2+}$ and ROS were stained with Fluo-4 AM probe and DCFH-DA fluorescent probe, respectively, and fluorescence was assessed via FCM.

The MDA content was measured as previously described(20).

### 2.9 Detection of the activities of superoxide dismutase (SOD), catalase (CAT) and caspase 3

Zebrafish larvae (0.1 g) were washed with ddH$_2$O and ground into cytoplasmic homogenate with 1 ml of 0.01 mM PBS solution. Intracellular SOD activities in zebrafish larvae were measured according to the Total Superoxide Dismutase Assay Kit with WST-8 (S0101S, 100 times) purchased from Beyotime Biotechnology Company.

The activities of CAT and caspase 3 were detected as previously described (21).

### 2.10 Statistics

The hatchability, survival rate, relative ROS content, SOD activity, CAT activity, Ca$^{2+}$ fluorescence intensity, blood sugar, MDA content, early apoptosis, late apoptosis, apoptosis rate, caspase 3 activity and JC-1 monomer fluorescence ratio were statistically analysed by one-way ANOVA. Dunnett’s test was performed to determine the mean differences between the control and exposure treatment groups (SPSS Statistics 25.0). Significant differences versus control are indicated as “*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001”.

### 3. Results

#### 3.1 High glucose exposure resulted in a low survival rate of zebrafish larvae

Usually, prolonged high blood sugar induced by the intake of too much sugar takes a toll on the body of both human beings and animals, and hyperglycaemia-induced sugar toxicity and osmotic stress often occur simultaneously(22, 23). Based on this information, to explore the effects of glucose toxicity and minimize the interference of osmotic stress, a suitable concentration of glucose treatment needs to be
verified. On the one hand, fertilized eggs were incubated in embryo culture medium containing 0% (control), 0.5%, 1%, 1.5%, 2% and 2.5% glucose and mannitol to explore the influence on the hatchability of zebrafish larvae on the fifth day, and the results showed that there was almost no influence on the hatchability of zebrafish larvae treated with both glucose and mannitol in the concentration range from 0–2.5% (Fig. 1a, b). On the other hand, survival rates were counted for zebrafish larvae that were raised in solutions as described above, and the results suggested that high glucose exposure resulted in a significant decrease in the survival rate at concentrations ranging from 0.5–2.5% (Fig. 1c), while mannitol exposure led to a significant decrease in the survival rate at concentrations ranging from 1.0–2.5% (Fig. 1d). Therefore, the concentration of glucose exposure in this work was determined to be 1.0% solution.

3.2 Hyperglycaemia induced by glucose exposure caused spine malformation and organ oedema in zebrafish larvae

To determine the blood glucose level, the blood glucose of zebrafish larvae was detected at 2 hours and 12 hours after a meal. The results showed that the blood glucose content in zebrafish larvae exposed to glucose was nearly three times as high as that in the control at both 2 hours and 12 hours after a meal (Fig. 2a, b).

Because the toxic symptoms of spinal curvature and oedema of tissues or organs induced by glucose exposure resulted in the death of zebrafish larvae, the effects of glucose exposure on zebrafish larvae were analysed on the tenth day, and the result showed that the activity of almost all larvae was suppressed by glucose exposure. Furthermore, compared with the control group (Fig. 2c), the majority of them appeared to have symptoms of spinal curvature (Fig. 2e, g, i), oedema of tissues or organs (Fig. 2k, h) or both (Fig. 2d, f, j, l).

3.3 Most of the DEGs were related to the apoptosis pathway in the transcriptomic analysis

Mapped data were obtained from clean data filtered using DESeq2 software with the parameters FDR = 0.05 and FC = 2 on a BMK cloud net. On the basis of RNA-seq, a total of 253 differential genes were detected, 187 of which were downregulated and 66 of which were upregulated. According to the above data, a thermogram of differential gene expression was generated, which was related to the zebrafish larval response to sugar toxicity (Fig. 3a).

Combining the significant KEGG pathways and the number of DEGs, the peroxisome, carbon metabolism, apoptosis and phototransduction signalling pathways were suggested to be some of the most significantly enriched pathways, which were usually activated in animals with hyperglycaemia. Compared with the genome-wide background, the DEGs with significant enrichment of more than 5% were selected, and they were related to the peroxisome pathway (5.88%), carbon metabolism pathway (7.06%), phototransduction pathway (7.06%) and the largest number of apoptosis pathways (10.59%) (Fig. 3b).
3.4 Glucose exposure activated the ROS-accumulation-induced apoptosis in zebrafish larvae in vivo

According to the RNA-seq data, the apoptosis pathway of DEGs needs to be explored preferentially. Under stress conditions, the structure and function of mitochondria, where ROS are primarily produced, need to be evaluated to determine whether they are normal. First, ROS accumulation was detected with a DCFH-DA fluorescent probe, and the results showed that glucose exposure increased the accumulation of ROS concentrated in visceral areas much more than that in the control group (Fig. 4a, b). Furthermore, the function of mitochondria was checked using transgenic zebrafish larvae (cms Tg/+ AB), whose mitochondria with normal function glowed bright green under a fluorescence microscope but were faint green when dysfunctional. The results showed that glucose exposure significantly impaired the function of mitochondria in zebrafish larvae compared to the control (Fig. 4c, d). Finally, apoptotic cells were stained with AO dye in vivo, and more apoptotic cells were located in the head and abdomen of zebrafish larvae treated with glucose than in the control larvae (Fig. 4e, f).

3.5 High blood glucose significantly reduced intracellular Ca\textsuperscript{2+} and increased apoptosis

Because Ca\textsuperscript{2+} signalling plays an important role in the processes of both apoptosis and autophagy(24), the intracellular Ca\textsuperscript{2+} content was detected, and it was found that the intracellular free Ca\textsuperscript{2+} content was significantly lower in glucose-treated zebrafish larvae than in control zebrafish larvae (Fig. 5a, c). To explore the intracellular redox state in zebrafish larvae, intracellular ROS accumulation was measured, and glucose exposure observably improved the ROS content compared to the control (Fig. 5b, f). Based on the detection of apoptosis in vivo, apoptotic cells were measured using FCM, and the results showed that there were many more cells in the early stage of apoptosis in glucose-treated zebrafish larvae than in the control, and although there were still more cells in the late stage of apoptosis in zebrafish larvae exposed to glucose than in the control larvae, the number of late apoptotic cells was relatively low (Fig. 5d, e, g, h). Therefore, in this phase, although a large number of cells had undergone the process of apoptosis, most of them were at the early stage in glucose-treated zebrafish larvae (Fig. g, h, i).

3.6 The antioxidant system was weakened by glucose exposure in zebrafish larvae

The activities of key enzymes of the antioxidant system, which scavenge excess ROS in normal cells, were detected in zebrafish larvae after glucose exposure. Compared with the control, the SOD activity showed a significant decline in glucose-treated zebrafish larvae (Fig. 6a), while the CAT activity was significantly improved (Fig. 6b). To analyse the levels of lipid peroxidation in cells, the MDA content was tested, and the results suggested that glucose exposure caused a significant elevation of MDA in zebrafish larvae compared to the control (Fig. 6c).
3.7 Effects of glucose exposure on the apoptosis of zebrafish larvae

The activity of caspase 3, as a key enzyme of the apoptosis pathway, was measured, and the results showed that glucose exposure significantly improved the activity of caspase 3 in zebrafish larvae (Fig. 7a). Mitochondria are recognized as the initiator of apoptosis, so the mitochondrial membrane potential was tested, and the results suggested that glucose exposure caused prominent depolarization of mitochondrial membrane potential (Fig. 7b).

4. Discussion

In recent decades, a high-sugar diet has become an important factor that induces T2DM, which is increasingly seen in adolescents(25, 26). Although hyperglycaemia can cause severe developmental toxicity in adolescents, the mechanisms remain unclear. Here, we provided evidence that glucose exposure resulted in mitochondria-mediated apoptosis causing developmental toxicity in zebrafish larvae. We expect that these results will provide valuable information for elucidating the molecular mechanism of juvenile T2DM and for developing potential therapeutics.

4.1 Glucose exposure resulted in developmental toxicity in zebrafish larvae

Usually, there are two stressors, namely, glucose toxicity and osmotic stress, in animals with high blood sugar levels(27, 28). To study glycotoxicity separately and minimize osmotic stress interference, zebrafish larvae were simultaneously exposed to glucose and mannitol, and analysis of the results suggested that 1% glucose solution was the best choice for exposure (Fig. 1). Regarding the phenotype of zebrafish larvae, glucose exposure directly led to hyperglycaemia (Fig. 2a, b), and the larvae thus exhibited typical symptoms of developmental toxicity, such as curvature of the spine and swelling of the organs (Fig. 2d-k), which might be caused by nerve or muscle damage resulting from toxic accumulation or abnormal apoptosis of cells(29–31).

4.2 Hyperglycaemia activated the apoptosis pathway of zebrafish larvae

To fully understand the influence of glucose exposure at the transcriptional level in zebrafish larvae, nanopore full-length transcriptomic analysis was completed. Much information can be gained from the main significantly enriched KEGG pathways of glucose-exposed zebrafish larvae. In cellular processes, 5.88% and 10.59% of DEGs were related to peroxisome and apoptosis pathways, respectively, suggesting that peroxidation had occurred in the membrane system and that apoptosis was activated in glucose-exposed zebrafish larvae (Fig. 8), which were verified in subsequent experiments. For instance, membrane lipid peroxidation was detected by the MDA content (Fig. 6c), and apoptosis was verified at three levels,
including vital staining with AO (Fig. e, f), apoptotic cell detection using FCM (Fig. 5d, e) and caspase 3 activity measurement (Fig. 7a).

In total, 3.53% of DEGs were related to the MAPK, FoxO and calcium signalling pathways, which are involved in environmental information processing (Fig. 8), implying that zebrafish larvae were living under stress conditions that usually resulted in ROS accumulation, as verified in Fig. 4a, b and Fig. 5b, f. Additionally, intracellular free Ca$^{2+}$, as a second messenger, was detected, and the results showed that its content dropped significantly (Fig. 5a, c), which might have resulted from the inhibition of ATP-dependent Ca$^{2+}$ channels on the membrane system caused by disturbance of energy metabolism. Low levels of intracellular free Ca$^{2+}$ might also weaken the zebrafish larval response to environmental stress and result in a reduction in stress tolerance$^{(32–34)}$.

Hyperglycaemia is usually classified as a metabolic disease$^{(35, 36)}$. In this study, there were a number of DEGs related to metabolism, and some of them had greater than 5% enrichment, such as drug metabolism-other enzymes; glycine, serine and threonine metabolism; glycolysis/gluconeogenesis; and carbon metabolism (Fig. 8). The insulin and adipocytokine signalling pathways were typical features of T2DM, and it is worth noting that 7.06% of the DEGs being related to phototransduction suggested that high blood glucose severely impaired the visual development of zebrafish larvae (Fig. 8), which might be caused by abnormal apoptosis-induced optic nerve and microvascular injury.

### 4.3 Excessive ROS accumulation impairs the structure of mitochondria, resulting in abnormal apoptosis

In humans, hyperglycaemia-induced ROS accumulation usually results in oxidative stress, which is the main factor in diabetes$^{(37, 38)}$. Here, we detected the ROS contents in zebrafish in vivo and found that glucose exposure significantly increased ROS accumulation (Fig. 4a, b), which could also be verified in a follow-up experiment in which there was a marked rise in the intracellular ROS content in glucose-exposed zebrafish larvae (Fig. 5b, f). In both animals and plants, excessive ROS can be removed by the antioxidant system, while excessive intracellular ROS accumulation usually means that the antioxidant system has been damaged$^{(39–41)}$. In this work, the detection of SOD and CAT activities suggested that the antioxidant system was disordered (Fig. 6a, b) and that the intracellular system was observably peroxidated under glucose treatment conditions (Fig. 6c). A study also found that the more ROS that accumulated, the more apoptotic cells there were in zebrafish larvae exposed to glucose (Fig. 4, 5). Therefore, excessive ROS accumulation might be closely associated with apoptosis in zebrafish larvae.

Because mitochondria are not only the main site of ROS production but also the regulatory centre of apoptosis$^{(42)}$, detection of the structure and function of mitochondria was necessary, and the results showed that hyperglycaemia damaged mitochondrial function (Fig. 4c, d) and structure (Fig. 7b). When this occurred, apoptotic pathways were activated (Fig. 3b, 7a), and a large number of apoptotic cells appeared, most of which were in the early stages of apoptosis in zebrafish larvae (Fig. 5d, e, g, h), thus leading to severe developmental toxicity or even individual death.
5. Conclusion

In recent years, T2DM, as a health-threatening disease, has become an epidemic among teenagers due to the intake of excessive amounts of sugar; however, the pathogenesis remains unclear. As a model animal whose genome has high homology with humans, zebrafish were studied to explore the mechanism of T2DM. In this study, a model of type 2 diabetic zebrafish larvae was successfully established, and glucose-induced developmental toxicity, ROS accumulation, antioxidant system function, DEGs, apoptosis and mitochondrial dysfunction were measured. The evidence suggests that glucose exposure leads to mitochondria-mediated apoptosis, resulting in developmental toxicity in zebrafish larvae. We expect that this study will provide valuable reference information for the treatment of type 2 juvenile diabetes, thus reducing T2DM-induced damage to health during development.

Declarations

Author Contributions


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Conflicts of interest

The authors declare no conflict of interest.

References


Tables

Table 1 is available in the Supplementary Files Section.

Figures

**Figure 1**

Effects of glucose and mannitol treatment on the hatchability and survival rate of zebrafish larvae. The hatchability and survival rate of zebrafish larvae exposed to embryo culture medium containing glucose and mannitol at concentrations ranging from 0% to 2.5% were analysed on the fifth and ninth days, respectively. Significant differences between the control and glucose exposure groups are indicated by asterisks (* P< 0.05, ** P< 0.01, ***P< 0.001 and ****P< 0.0001).

**Figure 2**

High blood glucose led to curvature of the spine and oedema of tissues or organs in zebrafish larvae. The blood glucose content in zebrafish larvae was tested at 2 hours and 12 hours after a meal (a, b). Compared with normally developed zebrafish larvae (c), glucose exposure resulted in curvature of the spine (d, e, f, g, i, j, l) and oedema of the tissues or organs (k-l). Significant differences between the control and glucose exposure groups are indicated by asterisks (***P< 0.001 and ****P< 0.0001).

**Figure 3**

Heatmap of the transcriptional level of genes and KEGG pathway enrichment analysis of DEGs. A heatmap obtained from the hierarchical clustering of DEGs (a) and KEGG enrichment pathway analysis was generated on a BMK cloud net (http://www.biocloud.net/) belonging to Biomarker Technologies Company, where the RNA-seq was completed.
Figure 4

Effects of glucose exposure on zebrafish larvae measured in different manners. ROS and apoptotic cells were stained with the DCFH-DA fluorescent probe and AO dye, respectively (a, b). The mitochondrial fluorescence of transgenic zebrafish (cmsTg/+) treated with glucose displayed a lower fluorescence intensity (d) than the controls (c).

Figure 5

Intracellular Ca\(^{2+}\) and ROS contents and apoptosis of zebrafish larvae were detected using FCM. Intracellular Ca\(^{2+}\) and ROS were measured using FCM with a Fluo-4 AM probe and DCFH-DA fluorescent probe, respectively (Fig. 5 a, b), and the fluorescence signal was analysed (Fig. 5 c, f). Apoptotic cells were detected using FCM with AO dye combined with PI dye simultaneously (Fig. 5 d, e), and the fluorescence signal was analysed (Fig. 5 g, h, i). Significant differences between the control and glucose exposure groups are indicated by asterisks (* P< 0.05, ** P< 0.01, ***P< 0.001 and ****P< 0.0001).

Figure 6

The antioxidant system was detected in zebrafish larvae. SOD activity and CAT activity were detected, and the data were analysed in a and b, respectively. The intracellular MDA content was detected in c. Significant differences between the control and glucose exposure groups are indicated by asterisks (* P< 0.05 and ** P< 0.01).

Figure 7

Apoptosis was detected in zebrafish larvae. The key enzyme of apoptosis (a) and the mitochondrial membrane potential (b) were measured. Significant differences between the control and glucose exposure groups are indicated by asterisks (** P< 0.01).

Figure 8

The main significantly enriched KEGG pathways of DEGs related to different pathways in zebrafish larvae exposed to glucose.
Supplementary Files

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- Table1.xlsx