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Methodological approaches for inducing diabetes-like metabolic conditions in zebrafish larvae

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ABSTRACT

Zebrafish represent a widely used model for studying metabolic disorders due to their genetic similarity to humans, low cost of breeding and suitability for high-throughput screening. This review presents current methodological approaches for inducing diabetes-like metabolic conditions in zebrafish larvae, focusing on both glucose-based exposure (immersion in high-glucose solutions) and chemical induction methods using compounds such as streptozotocin and alloxan. The main advantages and limitations of each protocol are summarized, including variations in exposure duration, developmental stage and compound concentration. Standardized and validated protocols remain crucial for enhancing comparability across studies and for enabling reliable preclinical testing of antidiabetic compounds. The zebrafish larval model continues to offer a promising and flexible system for investigating the mechanisms underlying diabetes and related metabolic dysfunctions.

INTRODUCTION

Diabetes mellitus represents one of the most formidable global health challenges of the 21st century. As of 2021, over 500 million adults are estimated to live with diabetes worldwide, and this is expected to rise to 783 million by 2045, driven largely by increasing rates of obesity, sedentary lifestyles and aging populations [1]. The disease imposes a tremendous socio-economic burden, being a leading cause of morbidity, premature mortality and healthcare expenditure due to its chronic complications, including cardiovascular disease, kidney failure, neuropathy, and retinopathy. The pathogenesis of diabetes is multifactorial and involves complex interactions between genetic, immunologic and environmental factors. In type 1 diabetes (T1D), autoimmune-mediated destruction of pancreatic β-cells leads to absolute insulin deficiency. In contrast, type 2 diabetes (T2D) is characterized by insulin resistance, progressive β-cell dysfunction and (often) hyperinsulinemia in early stages [2]. Both forms of the disease involve intricate signaling networks and organ crosstalk, contributing to metabolic dysregulation and systemic complications.

Animal models have been pivotal in advancing our understanding of diabetes and in preclinical drug development. Models such as the streptozotocin (STZ)-induced and alloxan (ALX)-induced diabetic rodent and genetically modified strains like the NOD (non-obese diabetic) and db/db (mutation in the gene encoding leptin receptor) mice have elucidated numerous aspects of disease mechanisms and therapeutic targets [3]. However, no single model can fully replicate the complexity of human diabetes, including immune-mediated processes, β-cell heterogeneity and long-term complications. This underscores the urgent need for new, refined animal models that can bridge the gap between mechanistic research and clinical translation. Such models should better reflect the immune, metabolic and vascular aspects of diabetes to enhance the predictive validity of preclinical studies and support the development of more effective therapies.

ZEBRAFISH AS A DIABETIC MODEL

Among emerging animal models in diabetes research, the zebrafish stands out as a highly promising system due to its unique advantages. Its optical transparency during embryonic and larval stages, for example, allows for in vivo visualization of pancreatic development and islet function. Zebrafish are cost-effective to maintain, produce large numbers of offspring, and develop rapidly, making them ideal for high-throughput drug screening and genetic manipulation studies [4]. The zebrafish pancreas begins forming by 24 hours post-fertilization (hpf), with endocrine cell types: insulin-producing β-cells, glucagon-producing α-cells and

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ghrelin-secreting ε-cells, detectable within the first day of development. These endocrine cells organize into islet-like clusters that share structural and functional homology with mammalian islets. Zebrafish larvae begin producing insulin early and can regulate glucose homeostasis via mechanisms analogous to mammals, including β-cell insulin secretion in response to hyperglycemia [5,6]. Zebrafish also possess key genes involved in glucose and lipid metabolism, and several studies have confirmed that both lipid storage and mobilization pathways are conserved in this species [7,8].

Chemical Induction of Diabetes-Like Conditions

In preclinical diabetes research, STZ and ALX are among the most widely used diabetogenic agents for inducing experimental diabetes in rodent models. Both compounds destroy pancreatic β-cells, resulting in insulin deficiency and persistent hyperglycemia, thereby mimicking aspects of human diabetes. STZ, a nitrosourea compound and glucose analog, enters β-cells via the GLUT2 transporter and exerts its cytotoxic effect primarily through DNA methylation and the generation of reactive oxygen species (ROS), leading to cell death. ALX, on the other hand, induces β-cell death through a redox cycling mechanism that produces superoxide radicals and hydrogen peroxide, overwhelming the cells' antioxidant defenses. A major limitation of both STZ and alloxan is their chemical instability in aqueous solutions. STZ is especially unstable at physiological or alkaline pH and must be freshly prepared in cold 0.1 M citrate buffer (pH 4.5) immediately before injection. The solution should be kept on ice and used within 15 minutes to maintain potency. Alloxan is usually dissolved in normal saline at neutral pH just before use. Both agents degrade rapidly and require precise pH control and temperature management to ensure consistent dosing and reproducibility in experimental outcomes [9,10].

The chemical induction of diabetic phenotype in zebrafish larvae is most commonly achieved through exposure to ALX, apart from transgenic models that use, for example, metronidazole (nitroreductase system). The larval stage chosen for such experiments typically ranges from 3 to 6 days post-fertilization (dpf), when the endocrine pancreas is sufficiently developed and larvae remain optically transparent. ALX is applied at concentrations between 100 µM and 600 µM, with exposure durations varying from as short as 15 minutes to as long as 72 hours. A summary of the protocols for induction of the diabetic phenotype with ALX can be found in Table 1. In each of these protocols, pancreatic β-cell damage was inferred from reduced islet size and impaired glucose uptake visualized via the fluorescent glucose analog 2-NBDG. ALX-induced damage consistently resulted in a significant reduction in pancreatic islet area (often by 30-40%) and the intensity of 2-NBDG fluorescence, interpreted as diminished glucose uptake capacity.

Beyond imaging endpoints, several studies included evaluation of biochemical and molecular markers to substantiate the diabetic phenotype [11-16]. ALX-exposed larvae showed increased oxidative stress, evidenced by elevated intensity of fluorescence of DCFDA (reactive oxygen species), DPPP (lipid peroxidation) and acridine orange (apoptosis), intravitally imaged. Alongside reductions in antioxidant enzymes activity in tissue, including superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH). These alterations were accompanied by increased levels of lactate dehydrogenase (LDH), nitric oxide (NO), and malondialdehyde (MDA), all hallmarks of cellular damage. Importantly, the study by Nayak et al. also measured whole-larval glucose concentrations, showing a significant elevation in glucose levels following ALX exposure - effects that were reversed by glimepiride, an antidiabetic drug from the sulfonylurea group. Furthermore, mRNA expression analysis revealed upregulation of pro-inflammatory and diabetic stress genes such as tnf-α, il-1β, nfκb, and gsk3, and downregulation of genes associated with insulin signaling and glucose transport, including glut2, sirt1, ppar-γ, and insa. All these parameters were normalized following glimepiride treatment, which highlights its validity as a pharmacological control [16].

Notably, only one study has successfully utilized STZ in zebrafish larvae, injecting a saturated STZ (pH 4.4) into the pericardial space of 9-dpf larvae using the microinjection technique [17]. It should be emphasized here that zebrafish larvae are housed in a pH range of 6.5-8 [18]. STZ exposure led to significant β-cell apoptosis, elevated glucose levels and altered gene expression (ins↓, glut2↓, pck↓, casp3a↑, pck1↑), mimicking diabetic pathology [17]. Although effective, this method is logistically challenging, as biochemical or molecular testing requires pooling of numerous larvae, making manual injections labor-intensive. In a more recent study, zebrafish larvae at 3-dpf were exposed to 100 µg/mL STZ for 24 hours in E3 medium, resulting in elevated glucose levels and ROS. This exposure also increased the activity of antioxidant enzymes, including CAT, as well as SOD, and upregulated the expression of glutathione-related

Table 1. Overview of experimental strategies employed to chemically induce a diabetic phenotype in zebrafish larvae via ALX immersion. In the presented studies, glimepiride served as the sole positive control to validate the efficacy of the induction protocols

Larval age	Concentration and exposure time	2-NBDG concentration and incubation time	Outcome	Glimperide concentration [µM]	References
5 dpf	100 µM for 6h	25 µM for 12h	pancreatic islet size↓, 2-NBDG uptake↓	5 µM for 1h	[11]
6 dpf	600 µM for 3h	40 µM for 0.5h	pancreatic islet size↓	10 µM for 3h	[12]
6 dpf	600 µM for 3h	40 µM for 0.5h	pancreatic islet size↓	2 µM for 2h	[14]
4 dpf	a) 500 µM for 24h b) 500 µM for 72h	25 µM for 1h	a) ROS↑, O^-_2↑, glutathione↓, apoptosis↑, lipid peroxidation↑ b) 2-NBDG uptake↓, glucose concentration↑, mRNA expression of insulin↓ and PEPCK↑	10 µM for a) 24h b) 72h	[15]
5 dpf	100 µM for 15 min.	40 µM for 0.5h	pancreatic islet size↓; 2-NBDG uptake↓; mRNA expression of GCK↓, GCKR↑, GLIS3↑, CDKN2B↓	No concentration stated, 12h	[13]
embryos	500 µM for 72h	no concentration stated, 3h incubation	glucose concentration↑, ROS↑, SOD↓, CAT↓, GSH↓, lipid peroxidation↑, MDA↑, LDH↑, NO↑, apoptosis↑, tnf-α↑, il-1β↑, nfκb↑, gsk3↑, glut2↓, sirt1↓, ppar-γ↓, insa↓	10 µM for 72h	[16]

enzymes: glutathione S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR), confirming oxidative stress response activation [19].

Additionally, a hybrid diabetic model was developed by exposing 3-dpf zebrafish larvae to a combination of STZ (50 μg/mL ≈ 188 μM) and high-glucose solution (40 mM) for 48 hours, simulating sustained hyperglycemia. While the primary outcomes of the study centered on cardiac function, including ejection fraction, heart rate and end-diastolic volume, the authors also incorporated key metabolic assessments to validate the diabetic phenotype. Specifically, glucose concentrations were shown to increase in both glucose-only and STZ+glucose-exposed larvae, with the latter inducing a more pronounced hyperglycemic effect. Importantly, insulin levels were elevated in the glucose-only group, but not in the STZ+glucose group. At the molecular level, gene expression analysis revealed that insulin transcripts were upregulated by glucose alone, but not in the STZ+glucose condition, while phosphoenolpyruvate carboxykinase (pck1) (a marker of gluconeogenesis), was upregulated in both treatments, further confirming systemic metabolic disturbance. Despite these characterizations, no antidiabetic drugs were employed in this initial study [20].

In their subsequent work, the same model was used to assess the cardioprotective effects of SGLT2 inhibitors (empagliflozin and sotagliflozin). Although these agents are clinically validated antidiabetic drugs, the study focused exclusively on cardiovascular endpoints without re-evaluating metabolic parameters such as glucose levels, insulin production or metabolically relevant gene expression [21]. No other studies to date have used STZ via immersion in larvae, likely due to the instability of the compound in aqueous environments. It appears that despite the lack of stability of ALX and STZ, their use in the zebrafish larvae model appears to be effective.

GLUCOSE-INDUCED DIABETES-LIKE PHENOTYPES IN ZEBRAFISH LARVAE

To induce a diabetic phenotype in zebrafish larvae without chemically invasive methods, two widely used strategies have been used: glucose immersion and nutritional overfeeding. The immersion of adult zebrafish in glucose-enriched water has become a well-established method for inducing a diabetic phenotype that mimics key features of T2D mellitus in humans. This non-invasive experimental protocols most often employ glucose concentrations ranging from 55.5 mM to 166 mM (equivalent to approximately 1-3% glucose w/v), with exposure periods lasting from 2 to 4 weeks. Adult fish subjected to such treatment consistently develop hyperglycemia. Importantly, this hyperglycemic state persists beyond the exposure period in some models. In addition, adult zebrafish exhibit secondary metabolic disturbances, including increased activity of hepatic enzymes and signs of systemic stress. The translational validity of this model is further underscored by its responsiveness to antidiabetic drugs such as metformin and glimepiride, which effectively reduce glucose levels and restore biochemical markers to near-normal ranges [22,23]. Furthermore, changes in antioxidant enzyme activity and inflammatory gene expression in the glucose-immersion diabetic model were reported [24,25].

In contrast, the application of glucose immersion to zebrafish larvae remains a developing area of research, characterized by broader methodological diversity and less consensus on optimal conditions. Table 2 summarizes the methods used to induce a diabetic phenotype in zebrafish larvae through glucose exposure. Larval studies use glucose concentrations of 40-166 mM (0.75%-3% w/v), typically given from 3 to 10 dpf. Chen et al., for instance, exposed larvae to 2% glucose for 10 days, which significantly raised whole-body glucose levels; this effect was partially reversed by sitagliptin treatment [26].

Table 2. Summary of the use of glucose immersion to induce diabetic phenotypes and related complications

Larval age	Glucose concentration	Antidiabetic drug used	Main outcome	References
3-10 dpf	110 mM	sitagliptin (250 μM)	glucose↑, glucagon↑, insulin↓; reversed by drug	[26]
4 dpf	40 mM	metformin (10 μM), glipizide (250 μM)	pepck↓, insulin↓	[30]
0-10 dpf	55 mM	-	ROS↑, apoptoza↑, caspase3↑, MDA↑, CAT↑, SOD↓	[27]
3-6 dpf	130 mM	-	glucose↑, retinal vessel diameter↑, vegf↑	[31]
3-6 dpf	90-160 mM	-	glucose↑, retinal vessel diameter↑, vegf↑, IL6↑, IL1β↑, TNFα↑, STAT3↑	[29]
0-10 dpf	55 mM	-	glucose↑, ROS↑, LPO↑, apoptosis↑, caspase3↑, IL1β↑, TNFα↑, IFN↑	[32]
0-5 dpf (injection) 2-5 dpf (immersion)	15 nM 277 mM	-	glucose↑	[33]

At the molecular level, several studies have investigated the downstream metabolic, oxidative, and transcriptional consequences of glucose immersion in zebrafish larvae. Immersion of 96 hpf larvae in 40 mM glucose for 24-48 hours resulted in the upregulation of preproinsulin and concurrent downregulation of phosphoenolpyruvate carboxykinase (PEPCK), a key gluconeogenic enzyme. These changes were reversed by co-treatment with metformin or glipizide [27]. Glucose-rich environments cause oxidative and inflammatory stress in zebrafish larvae. Exposure to 1% glucose increases ROS, activates caspase-3, raises MDA levels, lowers SOD activity, and raises CAT activity, all indicating redox imbalance and mitochondria-driven apoptosis [27]. Further supporting the metabolic relevance of the larval model, it was reported that larvae exposed to 3% glucose in combination with a high cholesterol diet (HCD) for 10 days developed hallmark features of metabolic syndrome, including increased glucose, triglycerides, and cholesterol, as well as vascular lipid deposition, endothelial thickening, and inflammatory cell infiltration. Although glucose levels were not pharmacologically reduced in that study, co-treatment with pioglitazone or metformin improved vascular morphology and blood flow, suggesting partial recovery of the pathological phenotype [28].

Ocular effects similar to diabetic retinopathy have been observed in zebrafish larvae exposed to glucose. In research conducted by Jung et al., larvae were subjected to

increasing glucose concentrations (up to 135 mM) from 3 to 6 days post-fertilization, leading to hyaloid-retinal vessel dilation, disruption of tight junctions and elevated expression levels of VEGF and nitric oxide. These outcomes were reduced when anti-VEGF treatment (ranibizumab) or nitric oxide synthase inhibitors were administered [30]. A model described by Lee et al. used 130 mM glucose and reported elevations in the inflammatory markers IL-6, STAT3, IL-1β, and TNF-α. Treatment with aflibercept (a VEGF inhibitor) was associated with normalization of vessel morphology and reduction of inflammation [29].

REFINING THE LARVAL ZEBRAFISH MODEL FOR HYPERGLYCEMIA RESEARCH: CURRENT LIMITATIONS AND FUTURE DIRECTIONS

Although larval zebrafish have emerged as a valuable model for studying early-onset hyperglycemia and pancreatic dysfunction, several methodological and interpretive challenges limit their broader translational relevance. One major issue is the lack of standardization across experimental protocols. Studies vary widely in glucose concentration, exposure duration, developmental stage at treatment initiation and the presence of metabolic co-stressors such as high-fat or high-cholesterol diets. These inconsistencies hinder cross-study comparisons. Another significant limitation is the insufficient validation of the hyperglycemic state. Whole-body glucose levels are not often quantified directly, and the use of surrogate markers, such as 2-NBDG uptake or islet area, while convenient, provides only indirect measures of metabolic status. These imaging-based metrics are prone to variability due to differences in dye uptake, photobleaching and imaging conditions.

The transient nature of glucose-induced hyperglycemia in larvae further complicates model interpretation. Due to their high surface-to-volume ratio and efficient excretion systems, zebrafish larvae may rapidly clear exogenous glucose, raising doubts about the persistence and physiological relevance of the induced diabetic phenotype. To date, no studies have assessed whether hyperglycemia persists following glucose withdrawal in larvae, although similar investigations with success have been conducted in adult zebrafish. As a result, longitudinal data on glycemic dynamics during the larval stage remain absent. This gap limits our ability to distinguish between acute responses and sustained metabolic dysregulation.

Moreover, the inconsistent use of pharmacological controls across studies limits validation of the model's relevance to human diabetes. Many experiments omit antidiabetic agents to confirm the reversibility of hyperglycemia, weakening the overall interpretive framework. Similarly, comprehensive metabolic evaluations are infrequently performed, leaving the model's pathophysiological relevance insufficiently characterized.

To enhance the utility of the larval zebrafish model, future research should focus on the establishment of standardized, well-validated protocols for glucose immersion, with clear definitions of exposure parameters that reliably induce stable and quantifiable metabolic effects. Incorporating pharmacological interventions, along with time-resolved measurements of glucose clearance and gene expression, will be crucial for verifying model robustness. Expanding phenotypic assessments to include vascular, behavioral and organ-specific endpoints could further enrich the model's applicability to reflect systemic complications of diabetes. These improvements will be essential for fully realizing the potential of zebrafish larvae as a scalable, high-throughput platform for metabolic disease modeling and therapeutic screening.

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