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—Review—

The promise of zebrafish as a model of metabolic syndrome

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Abstract: Metabolic syndrome is a cluster including hyperglycaemia, obesity, hypertension, and hypertriglyceridaemia as a result of biochemical and physiological alterations and can increase the risk of cardiovascular disease and diabetes. Fundamental research on this disease requires validated animal models. One potential animal model that is rapidly gaining in popularity is zebrafish (*Danio rerio*). The use of zebrafish as an animal model conveys several advantages, including high human genetic homology, transparent embryos and larvae that allow easier visualization. This review discusses how zebrafish models contribute to the development of metabolic syndrome studies. Different diseases in the cluster of metabolic syndrome, such as hyperglycaemia, obesity, diabetes, and hypertriglyceridaemia, have been successfully studied using zebrafish; and the model is promising for hypertension and cardiovascular metabolic-related diseases due to its genetic similarity to mammals. Genetic mutation, chemical induction, and dietary alteration are among the tools used to improve zebrafish models. This field is expanding, and thus, more effective and efficient techniques are currently developed to fulfil the increasing demand for thorough investigations.
Key words: diabetes, hypertension, metabolic syndrome, obesity, zebrafish

Introduction

Since the 1970s, the use of *Danio rerio*, commonly known as zebrafish, as an animal model has spread widely among various universities and research centres. The model has shed new light into various research fields. George Streisinger, a molecular biologist from the University of Oregon, was the first scientist to use zebrafish as a tool to study the nervous system. This vertebrate animal was found to be less complicated than mice [16]. The zebrafish is an animal model that was established to be an ideal experimental model for sev-

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eral biological disorders [20].

The use of zebrafish as an animal model provides several advantages. Housing is less cumbersome relative to other animal models, allowing cost efficiency. The rapid egg production and early developmental morphology of this small organism make it one of the favourite animal models for drug screening. One mating can produce approximately 300 eggs in each conjugation in one night. The zebrafish embryo can reach the early larval stage after 3 days' post-fertilisation (dpf) and become an adult at 3 months' post-fertilisation. Their small eggs can be placed into the wells of plates and survive for approximately 7 days without feeding or changing the water. These characteristics are beneficial, as several drugs can be screened in a short period of time. Furthermore, the size of this animal, which is relatively small, reduces the quantity of tested drug/plant extract, which makes it possible to test a small amount of compound(s). Zebrafish share 70% homology to mammals and 84% similarity to mammalian disease genes. Gene similarity leads to the use of zebrafish as a model for studying gene functions and mammalian disorders, such as metabolic syndromes [21, 23]. Oka *et al.* [48] reported that the zebrafish shares similar pathological characteristics with humans since the same genes are affected, including *IL-6*, *IL-1β* and *APOH* in the coagulation pathway and *SREBF1*, *PPARα/γ*, *NR1H3* and *LEP* in lipid metabolism. In particular, zebrafish embryos and larvae have similarly coded genes for carbohydrate metabolism to mammals [78]. In addition, zebrafish larvae have adipocytes that store lipids and other specific cells involved in lipid metabolism [26]. The pancreas of zebrafish also has a structure similar to that of mammals, with two parts: endocrine and exocrine. The endocrine cells of zebrafish were found to have three types of cells, α, β, and δ, which release insulin, glucagon and somatostatin, respectively. The exocrine gland contains three types of cells, ductal cells, acinar cells, and centroacinar cells [8, 42]. This discovery has initiated the use of zebrafish as a model for studying different metabolic disorders associated with glucose metabolism.

Nevertheless, apart from these advantages, the solubility of testing compounds is an obstacle for certain studies using zebrafish embryo, whereby the tested compounds or drug are preferably water soluble, or at least soluble in 1% DMSO or acetone [3]. However, it will not be a limiting factor when an adult zebrafish is used since force-feeding can be applied and enabling the evaluation of whole part of the sample. Apart from it, the volume of zebrafish blood is very limited; however, it can be overcome through the use of high-end analytical instrument such liquid-chromatography tandem mass-spectrometry (LC-MS) for analysis of the metabolites. This technique is very sensitive enabling analysis of small quantity of the sample. LC-MS time of flight (LC-MS-TOF) has been utilized extensively in metabolomics and proteomics approach using zebrafish [12, 71]. Finally, the small size of zebrafish causes difficulties in dissection and thus requires more effort and skill to extract the organs compared to other conventional animal models. Although certain organs in zebrafish are very fragile, the isolation of those organs for histology is possible [80].

Metabolic Syndrome

In 1988, Gerald Reaven was the first scientist who gave the term syndrome X to various clusters of abnormal disorders [17]. Since then, syndrome X has been the focus of many researchers and defined differently. Later, the term syndrome X was changed to metabolic syndrome, although the definition of metabolic syndrome varies and is still unclear. According to the International Diabetes Federation (IDF) definition, the patient at risk of metabolic syndrome has been diagnosed with obesity and at least two of the four following disorders: high fasting blood glucose (≥100 mg/dl), high blood pressure (systolic BP ≥130 or diastolic BP ≥85 mmHg), high triglycerides (≥150 mg/dl), and a decrease in high-density lipoprotein (HDL <40 mg/dl in males, <50 mg/dl in females) [69]. However, the World Health Organization (WHO) has suggested different definitions, i.e., metabolic syndrome is an abnormal glucose level with at least two of the following health problems: high arterial pressure (≥140/90 mmHg), high plasma triglyceride (≥150 mg/dl), abdominal obesity body mass index (BMI >30 kg/m^2) and microalbuminuria [67]. Notably, the European Group for the Study of Insulin Resistance (EGIR) changed the term to insulin resistance syndrome. According to the EGIR, a person with insulin resistance syndrome has a high level of plasma insulin with two of the following factors: raised plasma glucose but not diabetic, raised triglycerides (≥150 mg/dl) and/or reduced high-density lipoproteins (HDL <110 mg/dl), high blood pressure (>130/85 mmHg), and hypertriglyceridaemia (≥150 mg/dl) with a low amount of HDL in both

genders. According to all mentioned definitions, the four diseases (diabetes mellitus, high blood pressure, obesity, and high triglyceride levels) are clearly shown to be related to each other [27].

Zebrafish as a Model to Study Metabolic Syndrome

Zebrafish as a diabetic model

Diabetes mellitus (DM) is a prolonged state of high blood glucose levels. A great deal of complexity is associated with this metabolic illness and is primarily manifested among DM patients [79]. The hormones that are responsible for maintaining the blood glucose level within the optimal range are insulin, glucagon, and somatostatin. These hormones are released from the endocrine part of the pancreas [55]. The similarity between the zebrafish and human pancreas has been avidly studied. The zebrafish pancreas can be divided into two important parts: the exocrine and endocrine segments. The exocrine segment contains three major types of cells: ductal, acinar, and centroacinar cells. In contrast, the endocrine part is separated into islets that contain three types of cells: beta cells that produce insulin, alpha cells that secrete glucagon, and delta cells that give rise to somatostatin [8, 42]. Thus, from this point, humans and zebrafish can be concluded to be morphologically comparable. Similarly, the normal blood glucose level in zebrafish (50–75 mg/dl) is close to the normal human blood glucose level range (70–120 mg/dl) [75].

PCR analyses of both adult and larval zebrafish showed that the same genes that are regulated by carbohydrates have been detected in mammals. The pancreas of zebrafish is largely composed of exocrine tissue organized in acini. Likewise, it has the same function and structure as the mammalian counterpart in glucose homeostasis [28]. Zebrafish have the ability to transcribe all genes related to gluconeogenesis and lipolysis after 4 dpf, such as cytosolic phosphoenol pyruvate carboxykinase, glucose-6-phosphatase, fatty acid synthase, acetyl-coA carboxylase, glucose-6-phosphate dehydrogenase, glycogen synthase, and glycogen phosphorylase [13]. At 4 dpf, pancreas is still immature but it is functioning [40]. To exploit this advantage, the larvae were treated with an overdose of insulin, resulting in insulin resistance [38]. Numerous investigations have documented the relationship between insulin resistance and obesity in metabolic syndrome. Forn-Cuní *et al.* [14] reported the intersection between the non-alcoholic fatty liver of zebrafish and humans. Both zebrafish liver genes of atp5e and atp5gb3b play a significant role in insulin resistance associated with obesity. Moreover, Malle *et al.* [35] reported that systemic monovalent 1 (MV1) can activate NF-ƙB–inducing kinase (NIK). The NF-ƙB pathway is associated with β cell dysfunction, which causes insulin resistance. Song and Cone [66] reported that overexpression of agouti-related protein (*AgRP*) increases the energy storage rate, causing overweight zebrafish.

Essentially, the most difficult part of inducing diabetes in zebrafish is the rapid recovery of β cells. Because of β cell damage, centroacinar cells are stimulated to multiply and separate into β cells. In addition, α cells can also contribute to supplanting β cells [4]. Although numerous studies discussed diabetes in zebrafish before 2007, the primary trial for the ablation (removal) of β cells from the pancreas of zebrafish was carried out by Pisharath *et al.* [51]. Indeed, the objective of this study was primarily to establish a zebrafish diabetic model to test the ability of zebrafish as a diabetic model.

Table 1 presents the various studies that have used zebrafish as a model for obesity and diabetes, which are related directly to metabolic syndrome. The food, environmental, and chemical inducers and their doses are indicated in Table 1. Pisharath *et al.* [51] submerged zebrafish larvae in metronidazole with the goal of ablating the β cells. The outcome demonstrated that at 10 mM, the drug can completely decimate the β cells. Around the same time, another study took one step forward by the work carried out by Elo *et al.* [13], who demonstrated that the antidiabetic drug glipizide can decrease the glucose level in adult zebrafish after exposure to 25% glucose in Me_2SO. The main diabetic model utilising zebrafish was conceived by Gleeson *et al.* [19] when they screened diverse groups of glucose-utilising adult zebrafish. The blood glucose level was significantly increased after immersing the adult zebrafish in the glucose solution. Connaughton *et al.* [10] increased the duration of the hyperglycaemic state of adult zebrafish from 2 to 8 months by increasing the glucose solution from 1 to 3% and the exposure time to two months. Similarly, alloxan monohydrate has demonstrated a successful outcome in raising the blood glucose level. Submerging the fish in 300 mg alloxan/100 ml H_2O and 400 mg alloxan/100 ml H_2O solution for 30 min as well as treatment with 1% glucose solution led to an augmentation of blood

Table 1. Reported zebrafish models developed for obesity, hyperlipidemia, diabetes, and hyperglycemia

Disorders	Establishment of the model	Reference
Obesity/ hyperlipidemia	The feeding of adult zebrafish for 3 times per day with freshly hatched artemia (60 mg cysts/fish/day) for 8 weeks.	48
	Feeding of adult zebrafish for 6 weeks with high fat diet (HFD) which contains 20% lard, and 80% basal diet. Each tank of 8 fish received 80 mg/day of HFD.	41
	Continuous light exposure of larvae zebrafish for 24 h. Light source used= Beams Work Power LED 200 (10.000K daylight, 200 lumen).	32
	Immersion of larvae zebrafish in 1 μM rosiglitazone or 10 μM T0070907 or 20 μM phenylephrine.	68
Diabetes/ hyperglycemia	Immersion of adult zebrafish in 1% water glucose solution for 30 mins, followed by immersion in water for 1 hour, and subsequently immersed in 300 mg alloxan/100 ml H_2O for 30 min.	64
	Single injection of adult zebrafish with 1 g/kg of streptozotocin.	45
	Multiple injections of streptozotocin to the adult zebrafish: Each injection= 350 mg/kg. Week 1= 3 injections (day 1, 3, 5), week 2= 1 injection (day 12), week 3= 1 injection (day 19).	25
	Immersion of adult zebrafish in 25% glucose in Me_2SO.	13
	Immersion of adult zebrafish in a 111 mM glucose solution for 14 days.	6
	Immersion of larvae zebrafish in 10 mM metronidazole.	51
	Feeding adult male AB strain zebrafish with high fat diets for 8 weeks for three times per day. The food was mixed artemia (5 mg artemia) with egg yolk powder contain 59% fat, 32% proteins, and 2% carbohydrates.	34
	Feeding the adult zebrafish with normal food which contain for 8 weeks for 6 feeds per day contain 120 mg for each feed. The food contains 11% crude fat, 51% crude protein, 2.3% crude calcium, 1.5% phosphorous, 15% ash, 3% crude fiber, and 6.5% moisture.	76

glucose level [64].

Routinely, to induce diabetes in rodents, researchers utilised diabetogenic medications, for example, alloxan or streptozotocin (STZ). Olsen *et al.* [49] developed another convention when 0.3% STZ solution was infused into zebrafish at 350 mg/kg body weight. The STZ-injected fish showed altered glucose levels, certain diabetes complications reported in this investigation, and increased serum non-enzymatic glycated protein levels compared to the healthy fish. However, insulin levels increased by 80%. More profound, a high blood glucose level influences the limb regeneration of zebrafish. This outcome can be seen following two weeks of high-glucose exposure. Later, the same research group recognised 71 factors hindering the caudal balance recovery of diabetic zebrafish [58]. Although the two investigations focused on multiple aspects of the zebrafish diabetic model, a need for a clear STZ injection procedure was demonstrated. Therefore, Intine *et al.* [25] gave a full clarification of the protocol for establishing a diabetes model by infusing 5 injections (350 mg/kg BW) of STZ over 3 weeks. Additionally, 5% of mortality was found in this investigation. Moss *et al.* [45] also used STZ to observe the regeneration process of zebrafish pancreas. This study further demonstrated that STZ can effectively destroy β cells.

Typically, high-fat and glucose regimens are the typical approaches used to induce type 2 diabetes in rodents. Accordingly, a similar method was used to induce type 2 diabetes in zebrafish. The overfeeding of adult zebrafish with a high-fat diet promotes certain health issues, including hyperglycaemia and fat accumulation in the liver [34]. Similarly, overfeeding with normal zebrafish food for 8 weeks/6 feeds/day promoted increases in glucose and insulin. In addition, overfeeding induces impaired tolerance to glucose. Treatment with metformin and glimepiride could reverse the damaging effect of overfeeding in the body of zebrafish [76]. Furthermore, the high-glucose diet increases the levels of insulin, glucagon, and phosphoenolpyruvate carboxykinase. These 3 metabolites are directly correlated with type 2 diabetes [13].

Notably, the non-enzymatic glycated proteins are initially more pronounced when the glucose level is elevated in the circulatory system. Fructosamine is one of these glycated proteins whose level was raised by 41% in a

diabetic zebrafish model. The focal neurological system complexity related to diabetes was a part of this investigation [6]. This conclusion was reached after an experiment in which the fish were immersed in 111 mM glucose for 14 days. The results demonstrated that the action of acetylcholinesterase had increased in the diabetic fish, unlike the control aggregate, and acetylcholinesterase was the main focus of this research to investigate the impact of diabetes on the cerebrum. This protein is situated in the myoneural intersection and separates acetylcholine and different esters. Acetylcholinesterase alteration in diabetes can cause certain neurological maladies [7]. A similar convention was employed by Alvarez *et al.* [1], who examined the impact of hyperglycaemia and its relation to retinopathy. The retinal vessels in 2-year-old fish were apparently thickened by aggravation of retinal vein obstruction. Related to diabetic complications, hyperglycaemia influences the level of cortisol in zebrafish hatchlings. Therefore, the diabetic zebrafish model provides better insight into the disorder. However, further diabetes studies in zebrafish are still warranted to derive further key information [52].

Zebrafish as an obesity and hyperlipidaemia model

Obesity is a metabolic disorder that is becoming highly prevalent in developed and developing countries. This lipid metabolism disorder disrupts the energy balance and is associated with genes and environment [54]. Previously, obesity was most likely associated with different genes, such as those encoding the leptin receptor, melanocortin-4-receptor *Mc4r*, pro-hormone convertase 1, and pro-opiomelanocortin genes [31]. The increasing prevalence of obesity has switched the focus of genotyping from a single gene to polygenic obesity [2]. Genome-wide association studies (GWASs) aided in creating the human obesity genome map, and the 12th update indicated the involvement of 253 quantitative trait loci [54]. The recent trend of obesity has brought the role of the environment into the light. Therefore, obesity in humans is often researched based on monozygotic and dizygotic twin studies [11].

Energy balance and metabolism are related to the leptin and insulin pathways, and are thus intercalated with other metabolic disorders. The brain and hormonal activities also affected food uptake and in turn, contributed to metabolic disorder [9]. Obesity is detrimental on a large scale as it increases the risk of other metabolic disorders such as hyperlipidaemia, cardiovascular diseases, type 2 diabetes, and cancer [15].

The increase in obesity has further highlighted the need to develop animal models. Monogenic and polygenic mutations have been used in rodent models. Mouse and rat models are the most common obesity models with a single gene causing a mutation in leptin, the leptin receptor, and insulin signalling using *ob/ob* and *db/db* mice [22], as well as Zucker *fa/fa* and Wistar-Kyoto rat models [39].

Polygenic models have been studied through diet-induced and age-related obesity [29]. Western human and high-fat diets have been found to affect leptin and insulin signalling in rats. These protocols show the role of the hypothalamus during obesity development [18]. Various mouse models, including New Zealand obese mice, elucidated the type 2 diabetes state and revealed its linkage to metabolic disorders [61]. The age-related obesity model is more suitable for mice, because it mimics the late onset of obesity in humans [77]. In the human obesity genome map, 119 genes have been recognised to be associated with obesity. Therefore, various genetic modification models in animals have been made [74]. The rodents are also often exposed to mutagens by radiation to alter genes related to adiposity for a faster random model, but this procedure is often not economical and does not help characterise energy homeostasis as a basis of measurement [24].

Non-rodent models, namely, chickens and pigs, are less common but have also been induced through high-fat diets [44]. Obesity is often diagnosed in dogs, which could be an upcoming model [50]. Non-human primates are evolutionarily closer, defining a role of epigenetics; thus, they have also helped in such studies, but a further exploration into the role of the brain in energy homeostasis and food intake was not investigated. Ethical complications, cumbersome processes, and high cost and maintenance have caused researchers to consider other models. Zebrafish, as a member of the lower metazoan family, are similar in terms of organs and adipose tissues [62]. It also has similar signalling pathways of leptin [43] and melanocortin [21] linked to the neuronal endocrine pathways [48].

As observed in humans, the mutation of *Mc4r* results in the obese state, whereby the same gene also plays a prime role in zebrafish. The zebrafish model has been used to study the genes involved in obesity, revealing the overexpression of *zAgRP*, which acts as an antagonist to the melanocortin receptor and in turn causes adipocyte

hypertrophy [66]. The overexpression of genes related to insulin signalling *Akt1* is able to induce obesity. This finding has allowed a novel anti-obesity study at the larval zebrafish stage [57]. Furthermore, chemical mutagens such as rosiglitazone, phenylephrine, and T0070907 at varying concentrations, along with diet, have been used to create a larval zebrafish model for studying this metabolic disorder successfully, as shown in Table 1. Beyond chemical mutagens, interesting new insights into the environmental role in obesity have been found, and a group of researchers have obtained promising results by altering the circadian rhythm [5]. Kopp *et al.* [32] found that prolonged exposure of the larval zebrafish to light increases the number of adipocytes by seven-fold, but it did not affect fatty acids.

As seen in rodents, the zebrafish model of diet-induced obesity is also an upcoming option focusing on the external factors contributing to obesity. Overfeeding of high-fat foods, such as artemia, 60 mg/day for 8 weeks, to adult zebrafish has been proven to play a role in the lipid metabolism pathway. This finding was deduced by comparing diet-induced obesity mouse and rat models and humans [48]. A fat-rich diet model of adult zebrafish has also been used for natural anti-obesity compound studies. Meguro *et al.* [41] tested the effect of green tea extracts on the diet-induced obesity model. The zebrafish model of obesity is an excellent alternative to the previous study models and is expected to be exploited further, as studies will utilise this model because it is beneficial, informative, and cost-effective.

Zebra fish as a high blood pressure model

High blood pressure or hypertension is a noteworthy general medical issue. Hypertension is characterised by high volumes of blood in the blood supply routes, where the blood flows between the heart and every organ in the body. Blood pressure can be grouped into systolic and diastolic pressures. The estimation of the pulse is communicated in terms of systolic pressure (when the heart pumps the blood) over diastolic pressure (between the heart beats). Hypertension management is not completely understood; consequently, further studies are greatly needed, which has given rise to a newly found commitment to manage hypertension in a better way.

The renin-angiotensin-aldosterone system (RAAS) is the hormone system that regulates blood pressure. As the blood flows, high sodium levels affect the circulation. In this circumstance, the renin that is discharged from the kidney alters the conversion of angiotensin I (Ang I) to angiotensin II (Ang II) by the enzyme called angiotensin-converting enzyme (ACE), which is secreted from the lung [63]. The increase in Ang II causes the tubular epithelial cells of the adrenal gland to discharge more aldosterone, promoting the reabsorption of sodium particles from tubular liquid and excreting potassium particles in the urine. In vascular endothelial cells, Ang II can cause vasoconstriction of arterioles by reducing the synthesis of nitric oxide (NO) [60, 70]. ACE plays another role in inhibiting the production of NO by breaking down bradykinin, which is required for the synthesis of NO [36]. The pituitary gland is also affected by Ang II through its binding to angiotensin II receptor type-1 (AT_1), causing the reabsorption of water in kidneys and triggering the stimulus for thirst in the cerebrum. This process results in urging the individual to drink more water, contributing to the decreased concentration of salt in the blood [53]. The renin gene was reported in the hereditary material of zebrafish, and it begins to appear 24-h post-fertilisation and exhibits 53% similarity to human renin [56].

NOSTRIN is another gene associated indirectly with high blood pressure and is expressed in zebrafish. The knockdown of this gene can affect the retinal blood vessels by increasing the clearance of protein from the serum in the vessels, resulting in obvious damage to both glomerular endothelial cells and the glomerular basement membrane in the kidney. Furthermore, this damage alters salt absorption into blood [30].

Another system regulating blood pressure was reported by Marek-Trzonkowska *et al.* [37], who demonstrated a connection between hypertension and angiogenesis. Angiogenesis is the development of new blood vessels, and the initiation of this procedure could reduce blood pressure [46]. The restraint of delta-like 4 (DII4)-Notch by fortification of microRNA-30a (miR-30a) can prompt angiogenesis in zebrafish [65]. The flowers of *Panax notoginseng* were found to be able to repair the damaged blood vessels by accelerating angiogenesis in zebrafish embryos [73]. This plant is utilised as a part of traditional Chinese medicine for the treatment of hypertension [72]. In a related study, Lai *et al.* [33] discovered a new gene that can control angiogenesis. They found that the knockdown of the WNK lysine-deficient protein kinase 1 (*WNK1*) gene in zebrafish can affect the angiogenesis process. Overexpression of this gene was reported to increase blood pressure in humans [47]. Re-

cently, a coiled-coil domain containing 80 (*CCDC80*) was identified as the gene associated with pulmonary arterial hypertension, and this gene was discovered to exist in the genetic material of zebrafish. The knockout of this gene resulted in inhibition of NO synthesis, further contributing to the increase in the size of the arterial blood supply. The effect of NO synthesis inhibition can be seen through the decrease in diameter of the ventral artery. Moreover, the regulation of NO synthesis relies upon the cGMP-dependent protein kinase. These outcomes vividly demonstrate that the zebrafish has a similar pulmonary artery regulatory process to that observed in mammals [59]. Although zebrafish cardiovascular models-related metabolic syndrome has not been completely established, the evidential genetic similarity of zebrafish to mammals will support the establishment of the model through genetic mutation, chemical induction, and dietary alteration.

Conclusion

The zebrafish model is on the rise due to its good reproducibility, simplicity, and cost effectiveness. Models for metabolic syndrome, including hyperglycaemia, obesity, and hyperlipidaemia, have been developed through different methods of induction, such as genetic mutation, chemical induction, or dietary alteration. Although zebrafish model related to hypertension and cardiovascular disease has not been completely established, it is promising to be developed due to its genetic similarity to mammals.

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