Original Article

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Intermittent Fasting Modulates the Glycogen Level in Zebrafish (*Danio rerio*) and their Next Generation

Biochemistry Section

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ABSTRACT

Introduction: Zebrafish (*Danio rerio*) has become the best model organism to study the evolutionary biological process and human developmental studies. The liver glycogen plays a vital role in maintaining cellular metabolism, accumulation of glycogen in liver affects the enzymes related to glycogen metabolism.

Aim: Impact of intermittent fasting, refeed and overfeeding in glycogen homeostasis on Zebrafish (*Danio rerio*) and their F_1 generation.

Materials and Methods: The present in-vivo study demonstrates the effect of intermittent fasting on glycogen storage in zebrafish and their F_1 generation. The study was conducted at Department of Biochemistry and Biotechnology, Annamalai University, Chidambaram, Tamil Nadu, India. The duration of study was carried out for one month (December, 2021) for both parental and their F_1 generation (April, 2022) groups. The F_1 generation fishes involved after its matured (three months). The zebrafish (AB strain) were randomised and split into five experimental groups such as control, overfed, 12 hours, 24 hours, and 48 hours intermittent fasting. The F_1 generation from each group was treated as same as parenting groups. The physiological and histological changes were observed in the study group. Significant results were evaluated as $p<0.05$ values turkey's method was used.

Results: The fasting and overfeeding significantly affects the physiological condition like body weight, length and Body Mass Index (BMI). The parental control and their F_1 have a BMI of 0.042±0.04 g/cm² and 0.041±0.04 g/cm². The maximum fasting treated groups (48 hours) of both parent and their F_1 generation shows reduced BMI such as 0.032±0.03 g/cm² and 0.030±0.04 g/cm². The over feed group shows a BMI of 0.053±0.05 g/cm² and 0.052±0.05 g/cm². The result demonstrates that the food-deprived groups and their F_1 generation showed less glycogen storage in histological observation. The refeed and overfed groups and their F_1 generation exhibit more glycogen accumulation in the liver. The result confers normal regulation of glycogen synthase and glycogen synthase kinase 3 in normally in control and fasting groups as well as in their F_1 generation. Conversely, the overfeeding and refeed groups show modulated glycogen activity in both parent and their F_1 generation.

Conclusion: Glycogen accumulation leads too many diseases and it also affects the generations. The frequent fasting may help to minimise glycogen accumulation and BMI level reduces the complications of disorders related to glycogen homeostasis.

Keywords: Glycogen synthase kinase-3, Glycogen synthase, Overfeed

INTRODUCTION

Glycogen is a multibranched polysaccharide of glucose it has a role in the storage and redistribution of lipids, proteins, and carbohydrates [1]. Liver is an essential organ that helps the body to adjust dietary changes in both eating and fasting periods [2,3]. Liver glycogen is needed for blood glucose regulation. Insulin stimulates glycogen formation in hepatocytes, which has a substantial direct effect on them [4,5]. Glycogen synthase, which catalyses the addition of glucose to the glycogen chain, and glycogen phosphorylase, which catalyses the breakdown of glycogen to release glucose-1-phosphate, are the two essential enzymes that control the glycogen metabolism that occurs in the liver [6]. The primary enzyme in glycogen production, Glycogen Synthase (GS), is triggered by the allosteric stimulator Glucose-6-Phosphate (G6P), as well as by dephosphorylation after insulin inactivates GS kinase-3. Given that glycogen synthesis is crucial for maintaining glucose homeostasis, decreased glycogenic activity is predicted to encourage the accumulation of intracellular G6P levels [7]. The fasting/feeding response to glycogen accumulation helps for the better understanding of complications related to high glycogen storage in the liver [8]. Nutritional status has an impact on a variety of different pathways, such as those involved in bile acid metabolism, iron metabolism, immunological responses, circadian rhythms, and stress responses. Dietary regulation of hepatic transcription is essential for physiology, and metabolic disorders are characterised by disruptions of these pathways. Future research is anticipated to uncover additional links and deepen our understanding of this crucial physiologic response in progeny with the continuous development of novel techniques and genetic models [9].

The zebrafish has become a popular model organism for developmental biology, neurology, and molecular genetics [10]. Currently, zebrafish are being suggested as a viable model organism for research on nutrition and development [11-13]. Utilised to study human metabolic illnesses, with a focus on diabetes and obesity. Zebrafish's lipid metabolism networks are highly similar to those of humans, according to a comparative transcriptome analysis of visceral adipose tissue from zebrafish, mice, rats, and humans [14]. Overfeeding caused significant hepatic steatosis, implying that a high-calorie diet increased fat accumulation in the liver [5]. This model could help researchers to better understand the relationship between obesity and hepatic injury, moreover it provides a viable option for developing useful therapeutic products in the field of biomedical research. The zebrafish share similar characteristic features to human condition like overweight/obese pathologies [15,16]. This study also may helps to understand the impact of overweight on cognitive impairments and that adult neurogenesis is involved in memory [17-19]. In this study, authors have analysed the changes in glycogen levels of food-deprived and overfeeding zebrafish and their F_1 generation. The physical parameters and glycogen accumulation in liver of both conditions were observed in parent and F_1 generation.

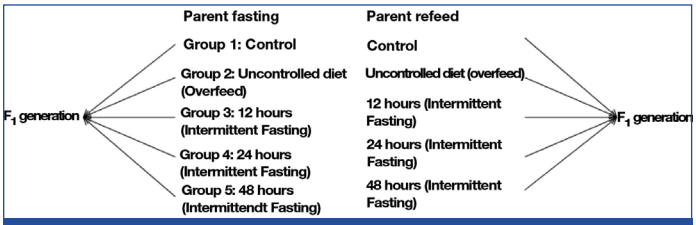
MATERIALS AND METHODS

The present in-vivo study was conducted in Department of Biochemistry and Biotechnology, Annamalai University, Chidambaram, Tamil Nadu, India, experiment's was carried out over four weeks.

Fish Maintenance

Adult zebrafish (three months) were obtained from a finites aquarium in Chidambaram, Tamil Nadu, India. The fishes were kept seven days for acclimatisation under laboratory condition. The water condition was monitored frequently during study period. The experiments were carried out in Department of Biochemistry and Biotechnology, Annamalai University, Chidambaram, Tamil Nadu, India.

The fishes were maintained under 14-hour light: 10-hour dark cycle at 28°C with water quality monitored using a well-proven method with around 10 fish per 4-L tank [20]. Zebrafish were divided into five dietary groups [Table/Fig-1]. In each experimental design, 50 fishes were used (n=10) which so in total for this experiment, 200 fishes were used as mentioned, and were refeed three times a day with feed containing moisture, crude protein, crude fat, crude fibre, carbohydrate, and ash. For this study, the experimental conditions were maintained for four weeks and the condition has given for F_1 generation zebrafish.

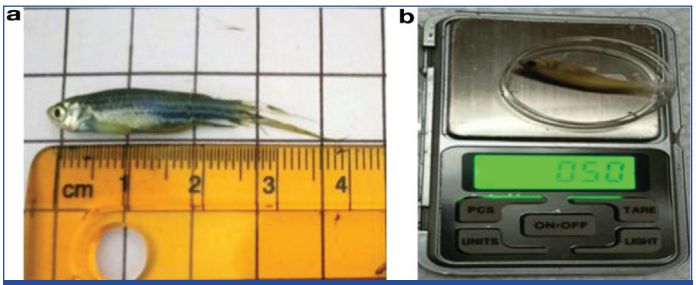


[Table/Fig-1]: The schematic representation of grouping and study plan.

Measurement of Physical Parameters

The weight and length of zebrafish were measured frequently during the study period. The length and weight of each zebrafish body were assessed to calculate BMI [Table/Fig-2]. The BMI was calculated by measuring body weight (g) divided by the square of the body length (cm^2) [21]. [Table/Fig-2] shows the length and weight measurement of zebrafish used in this study.

$$BMI = \frac{\text{Body weight (g)}}{\sqrt{\text{length (CM)}}}$$



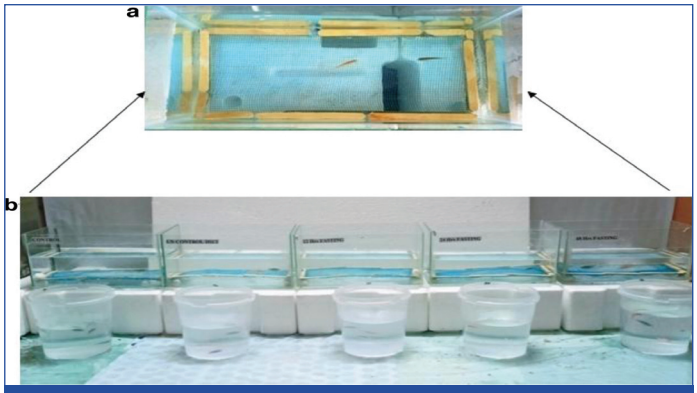
[Table/Fig-2]: a) Length measurement of zebrafish b) Weight measurement of zebra during the study period.

Breeding and Larval Maintenance

The breeding and larval maintenance of zebrafish is represented in [Table/Fig-3a,b]. The fishes were taken from experimental groups and kept in the breeding tank. The eggs were collected after a certain period with a dropper. The embryos were rinsed and examined under a microscope. Fertilised eggs were incubated for 72 hours at 28.5 °C until the F_1 larvae hatched and after 5 dpf (day's post fertilisation) feeding starts to grow. After three months, embryos were developed into sexually mature adults used for the experimental study [22].

Artemia Cultivation for Live Feed

The *artemia nauplii* (brine shrimp) was used as a feed for Larvae after fertilisation (dpf) (5 days) [23]. Artemia cysts were purchased from a local pet store and cultivated in salt water with a motor for aeration and focus light for temperature maintenance. After 18-36 hours, the cysts were hatched and grew as adult reddish brown artemia which was given as feed to F_1 generation.



[Table/Fig-3]: a) Top view of each tank setup of breeding b) The figure shows experimental parent groups for breeding for F_1 generation development and the identical methodology for cultivation.

Histology and Tissue Sectioning

The fish liver was fixed using 10% formalin solution and embedded in blocks. Then transversely sections were cut and stained with Haemotoxylin and Eosin (H&E). The glycogen vacuolation detection and tissue grading were performed on the parent and F_1 offspring. To determine the relative amount of glycogen in hepatocyte cytoplasm, two consecutive sections of whole fish were stained for the Periodic Acid–Schiff (PAS). In the glycogen determination, the PAS-stained slide served as a negative control [24].

STATISTICAL ANALYSIS

In terms of statistical analysis, the mean±SEM was utilised to represent all of the data to compare various groups; a one-way Analysis of Variance (ANOVA) was used, followed by a Duncan's Multiple Range Test (DMRT) test for control, fasting and refeed group comparisons. A p-value <0.05 was considered statistically significant in all groups.

RESULTS

Effects of Intermittent Fasting and Feed on the Physical Measurement of the Zebrafish

The physiological changes of each experimental group of fishes were analysed to differentiate the control, intermittent fasting, and feeding groups. Body weight was found to be significantly higher in the overfeed and refeed fish groups compared to the control and fasting groups. The initial mean body weight of the zebrafish was roughly found to be 0.40-0.55 g. The BMI of the overfeed group was 0.60±0.05 (BMI 0.053±0.05) and the control group was 0.55±0.04 (BMI=0.042±0.04), respectively. Similarly, the BMI values of 0.54±0.04 (BMI=0.039±0.04), 0.52±0.04 (BMI=0.034±0.03), and 0.50±0.03 (BMI=0.032±0.03) were observed in food-deprived groups. The results show loss of weight and BMI in 12 hours, 24 hours, and 48 hours of intermittent fasting. The physical parameters were seen to be changed in zebrafish. This same parent group, process of feeding three fasting experimental groups increased body weight and BMI shown in [Table/Fig-4].

Physical Parameter Comparison of Parent and their F_1 Generation

Physical parameter was measured in F_1 generation of each group. It was observed that when compared to parent group, the body weight of the F_1 generation groups was slightly changed, in the control group (F_1 0.53±0.04, BMI=0.041±0.04) and in overfed group (0.57±0.04 (BMI=0.052±0.05). It was also noticed there was a decrease in body weight and BMI level of three fasting groups 12 hours (0.52±0.04, BMI=0.037±0.04) 24 hours (0.48±0.04,

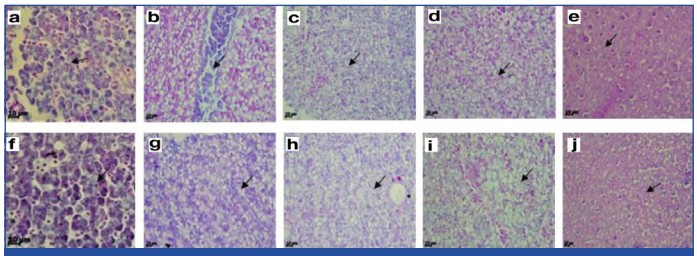
Parent fasting	Weight (g)	p-value	Length (cm)	p-value	BMI (g/cm²)	F_1 generation	Weight (g)	p-value	Length (cm)	p-value	BMI (g/cm²)
Control	0.55±0.04	0.002	3.6±0.27	0.004	0.042±0.04	**Control**	0.53±0.04	0.002	3.5±0.27	0.001	0.041±0.04
Uncontrolled diet (overfeeding)	0.60±0.05	0.07	3.3±0.25	0.002	0.053±0.05^	**Uncontrolled diet (overfeeding)**	0.57±0.04	0.009	3.2±0.25	0.002	0.052±0.05
12 hours fasting	0.54±0.04	0.001	3.7±0.28	0.004	0.039±0.04	**12 hours fasting**	0.52±0.04	0.001	3.6±0.27	0.001	0.037±0.04^
24 hours fasting	0.52±0.04	0.003	3.8±0.29	0.004	0.034±0.03	**24 hours fasting**	0.48±0.04	0.001	3.7±0.28	0.002	0.033±0.03^
48 hours fasting	0.50±0.04	0.005	3.9±0.30	0.004	0.032±0.03	**48 hours fasting**	0.46±0.04	0.003	3.8±0.29	0.005	0.030±0.04^
Parent refeeding											
Control	0.56±0.04	0.004	3.5±0.27	0.003	0.045±0.05	**Control**	0.55±0.05	0.004	3.5±0.27	0.004	0.047±0.05
Uncontrolled diet (overfeeding)	0.61±0.05	0.004	3.1±0.24	0.006	0.066±0.07^	**Uncontrolled diet (overfeeding)**	0.63±0.05	0.008	3.1±0.24	0.004	0.066±0.07
12 hours fasting	0.57±0.04	0.004	3.6±0.27	0.004	0.041±0.04^	**12 hours fasting**	0.58±0.05	0.004	3.7±0.28	0.004	0.045±0.05^
24 hours fasting	0.53±0.04	0.004	3.7±0.28	0.004	0.042±0.04^	**24 hours fasting**	0.60±0.05	0.006	3.8±0.29	0.003	0.046±0.05^
48 hours fasting	0.51±0.04	0.004	3.8±0.29	0.004	0.035±0.03^	**48 hours fasting**	0.61±0.05	0.07	3.9±0.30	0.004	0.039±0.04^

[Table/Fig-4]: Parent group of the fasting and feeding period calculated parameters and their first generation (^, v) these symbols represent BMI increased and decreased, the values are expressed mean±SEM and p<0.05 are considered significant in the all groups.

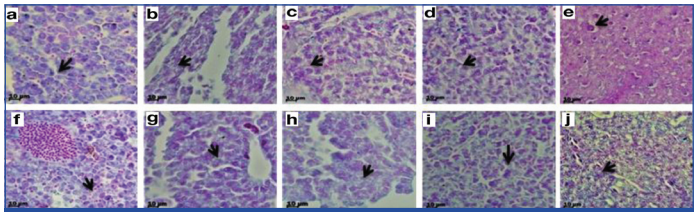
BMI=0.033±0.03), and 48 hours (0.46±0.04, BMI=0.030±0.04) [Table/Fig-4]. Conversely, the refeed body weight and BMI of the control (0.55±0.05 BMI=0.047±0.05), Overfed group (0.63±0.05 BMI=0.066±0.07), 12 hours of fasting (0.58±0.05, BMI=0.045±0.05), 24 hours (0.60±0.05, BMI=0.046±0.05) and 48 hours (0.61±0.05, BMI=0.039±0.04) of F_1 generation increased, respectively [Table/Fig-4].

Liver Histology and PAS Staining

The histology results show amount of glycogen accumulation in liver of experimental group fish. There was decreased amount of hepatic glycogen vacuolation observed in fasting groups and in their F_1 generation [Table/Fig-5a-j]. Conversely, the refeed increased level of glycogen deposition observed in control, overfeed group [Table/Fig-6 a-e] and also in their F_1 generation [Table/Fig-6f-j]. The tissues were stained with PAS and the decreases in glycogen vacuolation were observed. Despite a difference in PAS staining intensity between the low glycogen exposure and intermittent fasting in the glycogen levels were dramatically reduced in the group exposed to intermittent intermittent fasting and its offspring.



[Table/Fig-5]: Glycogen hepatocyte vacuolation was observed using a 40X magnification position visible microscope and the PAS staining technique. The arrow mark detected vacuolated and non-vacuolated. Glycogen vacuolation is continually limited in the liver of parent control a) 12 hours, b) 24 hours, c) 48 hours, d) overfed, and e) food deprived and their F_1 generation (f-j).



[Table/Fig-6]: Glycogen level was detected by the PAS staining method. The parent refeeding (a-e), and (f-j) their first generation hepatocellular vacuolation compared to parent and their generation to continually increased in the refeed and overfed groups.

DISCUSSION

Given its functions in controlling whole-body energy metabolism, digesting dietary nutrients, and preserving blood glucose levels, the liver serves as a focal point for the coordination of fasting-feeding transitions [4]. The liver's metabolism of glycogen plays a role in controlling blood glucose levels. It involves the conflicting reactions of the enzymes Glycogen Synthase (GYS2) and Phosphorylase (PYGL). When an animal switches between a fed and fasted condition, the liver is the principal organ responsible for maintaining metabolic equilibrium. Fasting causes the liver to produce glucose and ketone bodies, deplete its supply of glycogen, and accumulate triacylglycerol [25,26]. Glycogen breakdown or gluconeogenesis from glycerol, amino acids, or Tricarboxylic Acid (TCA) cycle intermediates are the two mechanisms by which hepatic glucose is produced. A previous study found that feed-restricted male mice perform better physically, have improved insulin sensitivity, and had lower levels of cholesterol and lipoprotein [27-29]. Present study was parent of fasting group BMI level reduced compared than feeding group. However, during the refeeding period earlier research, fish fed a high-carbohydrates diet had much greater plasma glucose and glycogen levels than fish fed a low-carbohydrate diet. In comparison to zebrafish that were fed normally, the overfed animals had higher BMIs, hypertriglyceridaemia, and hepatosteatosis. Zebrafish males and females responded to overnutrition in a similar manner [30]. Evidences from the past studies mostly reported intermittent fasting has been studied in animal models and human research to give an outlook on the health benefits [31]. Authors explicitly demonstrated that a four weeks considerably reduced BMI measurements in both parent and F_1 generation whereas fed alone resulted in significantly larger rises than fasting group participants. It is crucial to understand the immunophysiological reactions triggered by overfeeding-induced obesity.

When calorie intake is reduced, the liver's storage of glycogen during feeding conditions serves as a type of glucose reserve that can be used. Glycogen storage higher in overfed and feeding groups to be continuously increased their generation activation of glycogen synthase, a fundamental enzyme of glycogenesis (glycogen synthesis), whereas glycogen phosphorylase, a key enzyme of glycogenolysis, is repressed (glycogen breakdown) [32]. The histology of liver stained using PAS staining showed depletion in glycogen reserves during intermittent fasting utilising the glycogen reserves for survival [24]. However, the glycogen reserves reversed back upon refeeding the parent group subjected to intermittent fasting. More glycogen accumulation observed in overfeed group and it followed in their F_1 generation [33]. Conversely the fasting group exhibit less amount of accumulation of glycogen in both parent and F_1 generation. Under fasting conditions, dephosphorylated and active GSK-3 phosphorylate and inactivate glycogen synthase, leading to the inhibition of hepatic glycogen synthesis [34]. The glycogen synthase that appears to be most prevalent is the gene product (Gsy2). Both the phosphorylated (less active) and the dephosphorylated (active) versions of the Gsy1 and Gsy2 isoforms exist, and both have activities that are regulated allosterically. A

glycogen synthase kinase enzyme is inhibited by G6P, which also acts as a direct activator and regulates the phosphorylation state of this enzyme [35].

In response to food intake, increased insulin signalling activates the cell's Akt, which then phosphorylates and inactivates GSK-3, activating glycogen synthase. Additionally, this enzyme is allosterically activated by higher G6P concentrations, which increases its catalytic activity in feeding situations [36, 37]. According to some research, GSK-3 may control insulin resistance development and regulate glucose metabolism without affecting glycogen production [35]. Also GSK-3 has been linked to the development of neurodegenerative illnesses like Alzheimer disease [38]. Additionally connect human psychiatric disorders like schizophrenia and bipolar disorder is GSK-3 deregulation. The modulation of synaptic plasticity and memory is facilitated by GSK-3. Investigation into how GSK-3 affects the onset of obesity and its associated metabolic problems, such as insulin resistance, in adipose tissue is ongoing [39,40].

This study observed the BMI alterations as well as increased glycogen storage in the liver. Evidence from the previous study created a consistent overfeeding zebrafish model that led to various metabolic problems, including increased body weight, BMI, blood glucose levels, and liver steatosis. Previous researches discussed above on behavioural changes in zebrafish under control, fasting, refeed, and overfeed conditions showed, zebrafish that had been overfeed and refeed had lipid accumulation and glycogen storage in their liver tissue [41-45]. This study was an experimental proof that intermittent fasting can cause glycogen utilisation via glycogen synthase enzyme and regulate glycogen synthase kinase-3 enzyme outreached to obesity, diabetes and neurological disease related conditions. In the current study, it can be said that intermittent fasting had most benefits when compared to the BMI, fasting glucose. Insulin levels gradually decreased over time, implying that long-term CR (caloric restriction) and physical exercise have great benefits on the health of obese patients. This study shows a direct connection between human overeating behaviour and changes in liver glycogen, which may increase your risk of obesity-related health problems.

Limitation(s)

In the future, authors will test obese patients for the GSK-3 enzyme to determine the gene expression level and to find ways to prevent genetic disease which is at the moment, a limitation of this study.

CONCLUSION(S)

The findings of study have shown that the zebrafish is an ideal model to study intermittent fasting. The result confers the similar physiological and histological responses to the dietary interventions throughout the generation. The present study illustrates intermittent fasting repercussions of dietary modifications that contained the most positive reactions from the parent and its F_1 generation. Thus intermittent fasting was necessary to keep GS and GSK-3 under control. The risk of obesity, diabetes, neurodegenerative and cardiovascular diseases were reduced during frequent fasting conditions. The overfeed and refeeded group and its descendent generation continued to increase body parameters composition and glycogen vacuoles in liver histology tests. According to findings, controlled diet may help to prevention of genetic problems dietary related disorders.

Author's contribution: The BE conceptualise the work and wrote final version of the manuscript. ST did all the experimental work and wrote the draft version of the manuscript. UD helped in the experimental work.

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