ABSTRACT

Obesity leads to non-alcoholic fatty liver disease (NAFLD), where fat gets deposited in hepatocytes; progresses gradually into non-alcoholic steato-hepatitis (NASH) and Hepato-Cellular Carcinoma (HCC). Treatment of NAFLD is expensive owing to non-availability of biomarkers and therapeutic targets for diagnosis and treatment. Objective: We aim to generate NAFLD zebrafish model by High Cholesterol Diet (HCD) and analyse histologically the effects of HCD on NAFLD and NASH induction. Methods: Zebrafish were fed HCD (6-8%) for 8-16 weeks for inducing NAFLD and confirmed by morphological and histological analyses of liver. Results: Feeding HCD for 16 weeks induced symptoms of NAFLD, confirmed by fatty liver morphology, significantly (P<0.05) increased hepato-somatic index (HSI; 0.659 ± 0.0545) and histological analyses of liver sections showed degenerated hepatocytes, dense accumulation of LDs and hepatic membrane dis-integrity confirming its progression into NASH (>18 weeks) while, controls fed with regular diet had less LDs and remained healthy. Conclusion: we show that zebrafish can be used as a NAFLD model using less expensive HCD feeding displaying symptoms as in humans. Identification of novel therapeutic targets and biomarkers in NAFLD may help early diagnostics and develop personalized therapeutics for patients. Advantages and disadvantages of using zebrafish model to investigate NAFLD are discussed.

KEYWORDS: high-cholesterol diet, NAFLD, NASH, fatty liver, hepato-somatic index, zebrafish.
were reported recently but only in humans and rodents.\textsuperscript{[5, 6, 7]}

In mammals, free fatty acids (FFA) and triglycerides (TGs) are released into the hepatocytes where β-oxidation of fatty acids occur in mitochondria resulting in TG accumulation in liver when fed in excess.\textsuperscript{[10]} TGs may be stored as lipid droplets in hepatocytes leading to NASH while molecular factors like G Protein Coupled Receptors (GPCRs) that controls feeding and body weight\textsuperscript{[14]}, elevated leptin levels.\textsuperscript{[17]} However, our knowledge on the etiology and patho-physiological mechanisms underlying NAFLD, NASH and HCC still remains obscure.\textsuperscript{[6]} So far, only mammalian and rodent models have provided clues into the origin hence, identification of novel biomarkers and functional therapeutic targets were required. Hence, for delineating molecular pathways involved in NAFLD, vertebrate models like zebrafish\textsuperscript{[15, 18, 19]} and tetrads\textsuperscript{[20, 21]} may offer an inexpensive alternative.

Zebrafish, \textit{Danio rerio} Hamilton\textsuperscript{[21]} has become an excellent vertebrate model for studying metabolic disorders given its genetic homology (>75\%) and visceral organs similarity to humans; optically translucent embryos and larvae that remain amenable to genome manipulation and editing technologies, short generation time (48 hr embryonic development and <4 months for sexual maturity) and its robustness as a high-throughput screening model for identifying novel therapeutic targets in metabolic disorders.\textsuperscript{[18, 3, 9]} Hence, in the present study, our objectives were to (i) induce NAFLD with high cholesterol diet (ii) confirm liver inflammation and hepatocyte degeneration by morphological and histological analyses and (ii) generate NAFLD zebrafish model for elucidating biomarkers expressed during progression into NASH.

**MATERIAL AND METHODS**

**Zebrafish maintenance**

Wild type zebrafish were purchased (M/S. Aqua Gardens, Madurai) and homozygous clonal population of zebrafish were raised following established protocols\textsuperscript{[18]} under constant environmental conditions (12:12- Light: Dark (LD) and 28 ± 2°C) as required.\textsuperscript{[22, 23]} Controls and experimental fishes were raised in separate glass tanks preventing microbial contamination. Controls were fed pelleted diet while experimental zebrafish with cholesterol filled pellets twice a day and blood worms during breeding. About 1/3 volume of water in the rearing tanks were changed every other day to remove debris and unfed diet.

**High Cholesterol Diet**

Pelleted feed (Aqua Feed, Madurai) were stored inside refrigerator (4°C) during experiments. For HCD, Cholesterol (4-8\%) (Sigma, USA) was dissolved in diethyl ether, mixed with pellets and finally incubated in egg yolk solution for 4 hr; pellets were allowed to air dry under sterile conditions in an incubator (Labmate, India). Pelleted feed were stored in -20°C deep freezer (Bluestar, India) in metal container sealed with tinfoil to prevent bacterial contamination and cholesterol degradation. Experimental zebrafish were fed cholesterol supplemented diet while controls with regular pellets following previously established protocols.\textsuperscript{[23]}

**Feeding schedule**

For confirming obesity, male and female zebrafish were weighed prior to and after feeding experiments and fed for 16-18 weeks. Briefly, for measuring weight and length, fish were anesthetised with Clove oil (0.5\%), wiped dry with cotton and weighed in an electronic balance (Shimadzu, Japan) and body length measured with scale (from the snout to the tail). Hepato-Somatic Index (HSI) of each fish was calculated by dividing liver weight/square of body length.\textsuperscript{[3]}

**Histology**

Liver from both control and experimental zebrafish were cross sectioned using a cryotome, for further analyses. Briefly, control and obese zebrafish were sacrificed and livers fixed in Bouins fixative overnight followed by washing in grades of alcohol following established protocols.\textsuperscript{[3, 9]} Control and fatty livers were cross sectioned (10μM) and stained with haematoxylin for detecting lipid droplet (LD) deposition in hepatocytes and altered cellular morphology following established protocols.\textsuperscript{[19]} Lipid droplets and diet-induced morphological changes in non-alcoholic fatty liver were imaged under microscope (Lawrence & Mayo, India).

**RESULTS**

**Confirmation of NAFLD**

Diet-induced NAFLD zebrafish exhibited morphological and cytological symptoms similar to humans and mice.\textsuperscript{[20]} Livers were enlarged after 8 weeks of feeding and noticeable after >14 weeks of continuous HCD feeding in experimental zebrafish (Fig 1B). Liver inflammation was marked and visible to naked eye after 16 weeks of feeding with fish exhibiting reddish swollen livers visible through their lower abdomen (Fig.1B). NAFLD symptoms like liver enlargement as evidenced by increased HSI (0.959 ± 0.245) were significantly (P<0.05) higher in zebrafish after14 weeks of HCD feeding, compared to the controls (0.613 ± 0.0329). Zebrafish fed cholesterol filled pellets gained more weight in < 9 weeks of feeding as confirmed by their significantly higher BMI values than the controls\textsuperscript{[3]} similarly, HSI also increased with HCD feeding after 14 weeks.
Energy homeostasis is an interesting arena of research which underlies most metabolic disorders in humans, especially in adipocyte accumulation and obesity.[5] Earlier studies from our lab confirmed the ability of high cholesterol diet on inducing obesity in zebrafish after 6-8 weeks of feeding.[5] Further, increase in numbers of specific members of the phylum Firmicutes in gut microbes accelerated obesity by increasing lipid uptake and metabolism in zebrafish intestine as confirmed by our gut microbial transplantation experiments but negatively affecting liver morphology.[13, 9] Though caloric restriction and physical exercise in humans showed positive effects in reducing adiposity, comorbidities like NAFLD and NASH and cardiac disorders remain fatal owing to lack of molecular biomarkers for early detection and treatment.[6, 7] In the present study, we confirm that HCD feeding in zebrafish not only induced obesity but also mimicked early symptoms of NAFLD both morphologically and cytologically as in humans hence may serve as humanized NAFLD model for elucidating molecular pathways underlying pathogenesis. Very recently, researchers have uncovered genetic factors putatively responsible for NAFLD induction such as Melanocortin 4 receptor (MC4R).[24] Reactive Oxygen species (ROS) generation[26] and elevated levels of leptin in rodent models of fatty liver disease.[25] Similarly, membrane bound transcription factors like Sterol Regulatory Element Binding proteins (SREBPs) and Peroxisome Proliferator Activated Receptors (PPARs), the master transcriptional regulator of lipogenesis in humans may indirectly regulate expression of several lipogenic genes and downstream targets responsible for NAFLD induction in vertebrates.[27, 28] Hence, detailed transcriptome profiling of NAFLD zebrafish model may help identify genes and molecular pathways dysregulated, which is presently lacking.

However, in the present study, extending feeding trials beyond 16 weeks in zebrafish showcased earlier symptoms of NASH such as liver cirrhosis and fibrosis (data not shown). Generating zebrafish models for NAFLD by inexpensive HCD feeding and HCC may be beneficial for developing targeted therapeutics for treating NASH and HCC in humans, for which, presently there is no cure.[18] NASH pathogenesis follows a 2 step process in which firstly, increased hepatic accumulation of triglycerides (TG) happen. These TGs are generated by esterification of free fatty acids (FFA); FFA are derived from dietary lipids, or fatty acids released from adipocytes and or de novo lipogenesis (DNL) as demonstrated in zebrafish.[18] TGs are stored as lipid droplets observed in obese zebrafish liver hepatocytes were significantly more in number than control and livers remain enlarged even after completion of feeding trials for ~ 2weeks, displaying progressive NASH phenotype as observed in mice models.[17] Hence, high cholesterol feeding may also lead to non-alcoholic steato-hepatitis (NASH) assuring the role of zebrafish as a model for understanding the etiology and molecular mechanisms regulating NAFL or NASH.

DISCUSSION

Histological analyses of liver cross sections confirmed reduced deposition of lipid droplets in the control hepatocytes with normal HSI while, zebrafish fed HCD had increased HSI and exhibited ballooning of hepatocytes due to increased LDs deposition, highlighting early symptoms of non-alcoholic fatty liver disease (NAFLD) similar to mice and humans (Fig 2B).[25] Control hepatocytes showed distinct cell membrane and uniform hepatocyte morphology (Fig. 2A). However, NAFLD zebrafish hepatocytes displayed disoriented cell membrane and remain enlarged as a result of accumulation of more LDs (indicated by arrows) thus phenocopying symptoms of NAFLD, as in humans (Fig. 2B).

Figure 1. Representative samples of control (A) and (B) NAFLD zebrafish model showing fatty liver symptoms. Arrows indicate enlarged liver characteristic of liver inflammation in NAFLD model and Progressive NASH(B) and normal liver in control (A).

Figure 2. Cross sections of the Liver: (A) Fatty liver showing degenerated hepatocytes and (B) control. Arrows indicate marked nuclear membrane in controls and degenerate hepatocytes in NAFLD zebrafish liver.

Histopathology of hepatocytes

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droplets in hepatocytes or released into blood stream as very low density lipoproteins (VLDL). However in NASH patients suffering from hyper-insulinemia, up-regulation of SREBP1c, results in increased expression of most lipogenic genes creating imbalance in lipid output affecting lipid homeostasis culminating in hepatic steatosis.[28] Our present study also support the above conclusions but further studies are being carried out to determine the major molecular players in NASH progression in zebrafish.

Histological analyses of obese zebrafish liver confirmed increased deposition of LDs after >12 weeks post HCD feeding compared to the control, similar to mouse models and humans. In the present study, histological analyses of the liver sections confirmed increased accumulation of TGs in the HCD fed zebrafish hepatocytes leading to liver inflammation (Figure 1B). Further, progression of NAFLD into NASH is attributed to the action of liver Kupfer cells that secrete inflammatory cytokines i.e. increased levels of Tumour Necrosis Factor α (TNFα) leading to NASH.[3] Prolonged release of inflammatory cytokines and adipokines by adipose may also accelerate progression of NASH into HCC.[32] The present study demonstrates HCDs ability to induce NAFLD in zebrafish that displayed related co-morbidities, similar to humans. Further, histological analyses of the liver tissues from obese zebrafish showed disoriented hepatic cell membrane and enlargement by accumulation of more LDs resembling symptoms of NAFLD, as in humans (Fig 1B). However, further studies are required to understand the functional role of major regulatory transcription factors and micro RNAs (miRNAs) that act on downstream targets in NASH and HCC.

From the present study, we show that consumption of HCD in zebrafish leads to development of fatty liver, NAFLD and NASH, displaying symptoms similar to humans. Hence, generating inexpensive zebrafish models of NAFLD may lead us to get deeper insights on dysregulated molecular pathways and targets in NASH and HCC for identifying novel therapeutic targets and therapy for humans. We conclude that zebrafish can serve as an excellent model for NAFLD, NASH, Hepatic cirrhosis and HCC and may pave way for developing novel diagnostic markers and targeted therapeutics. Further studies on transcriptome profiling of humanized NAFLD or NASH zebrafish model may unravel novel genetic biomarkers and downstream targets affected and help increase our understanding of pathophysiology underlying NASH and HCC progression, which is currently limited.

CONCLUSION
Our present study demonstrates the feasibility of generating a NAFLD zebrafish model by inexpensive high-cholesterol feeding protocol, phenocopying NAFLD and NASH symptoms. However, future studies for deeper understanding of the role dietary lipids, biochemical pathways and dysregulated genes in NAFLD patho-physiology may pave way for developing novel biomarkers for early diagnosis and therapeutics in humans.

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