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Animal Model of Non-Alcoholic Steatohepatitis in Histology Department Sohag University

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is characterized by the deposition of triglycerides in the hepatocytes causing their injury resulting in non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis, as a healing response, and may progress to hepatocellular carcinoma (HCC). We aimed to establish an animal model for NAFLD/NASH by the use of a high-fat diet. A total number of 30 adult male mice were categorized into two groups; control, high-fat diet (HFD) 15 animals each. The livers of these animals were excised and processed for histological examination for the progress of NAFLD/NASH changes. At the end of the experiment, the mice were slaughtered at 11, 13, 15, and 17-week intervals. Liver specimens and blood samples from all animal groups were collected. The liver specimens were processed and stained with hematoxylin & eosin. Blood samples were processed for liver functions. Our results demonstrate that NASH was established 17 weeks after high-fat diet administration.

Keywords: Animal Model, Non-Alcoholic Steatohepatitis, high-fat diet

Introduction:

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease and its incidence is quickly growing across the world. It affects 10- 24% of the population ([1]).

NAFLD is a sequence of events started by the deposition of lipid within the liver cells (simple steatosis) that may lead to a spectrum of histopathological findings. Approximately 30- 40% of patients with simple steatosis develop non-alcoholic steatohepatitis (NASH). The body responds to the injured liver by a process of fibrosis, as a part of the healing process, with subsequent cirrhosis, which may be complicated by hepatocellular carcinoma [(2)].

The absence of adequate animal models limits NAFLD research in humans. The optimal NAFLD animal model should represent all characteristics of human NAFLD etiopathogenesis as well as the usual histological findings of its various phases [(3)].

There was no such animal model till today. As a result, researchers aimed to develop an appropriate model of NAFLD that mimicked at least the most relevant pathogenic and histological aspects of NAFLD. The selection of an appropriate animal model for this disease may aid in improving knowledge of its complicated etiology and the development of relevant treatment methods.

Materials and methods

1-Experimental design

30 Adult male Balb/c mice; about 2 months old with an average weight of 25 gm, were purchased from Assuit Experimental Animal Facility, Assiut University, Egypt. Animals were housed in Sohag University Animal House with free access to water and chow. They were maintained in ventilated metal cages on a 12-h light/12-h dark cycle at room temperature (22 ± 2 °C) and humidity (50 ± 10%). They were acclimatized to this environment for one week before the experiment. This study was approved by the ethics committee of the Faculty of Medicine at Sohag University. The animal protocol was approved by the Laboratory Animal Care and Use Committee of Sohag University Faculty of Medicine.

Animals were randomly divided into two groups of 15 animals each:

Group I: control group, fed standard diet (SD)

Group II: fed high-fat diet (HFD) (71% fat, 11% carbohydrates, 18% protein) for 17 weeks.

2. Body weight and liver weight coefficient

The animals were sacrificed after being anesthetized, and a dose of 100mg/kg body weight of ketamine was injected intraperitoneally [(4)]**.** The liver was resected and meticulously weighed at different time points (11, 13, 15, and 17 weeks) after HFD feeding to evaluate structural changes and the starting time of NAFLD or NASH development. The liver tissues were removed and weighed The liver-to-body weight coefficient was calculated for each animal as the ratio of the liver (wet weight mg) to body weight (g) in all groups [(5)]**.**

3. Biochemical studies

Intracardiac blood samples were immediately taken after scarification from all animals and processed for assessment of serum liver enzymes (ALT & AST) and lipid profile [cholesterol, triglycerides (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL)].

4. Histological studies

Liver samples were taken from the right lobes of all animals studied and promptly fixed in a 10% formalin solution, embedded in paraffin blocks, cut to 5m thickness, mounted on glass slides, and stained with hematoxylin and eosin.

5- Statistical analysis:

Animal body weight, Liver weight coefficient, liver enzymes and, Plasma FFA levels of animals
of both groups were estimated at the time end of the experiment.
All data were expressed as mean ± standard error (SEM). Data analyses were performed using
SPSS software (version 16.00; SPSS Inc., Chicago, Illinois (|USA) with a statistical significance
of $P<0.05$. Graphs were drawn in the GraphPad Prism software (version 6).

Results

Animal body weight:

The animal body weight increased significantly in HFD groups as compared to the matching control groups. from the 5^{th} week. The mean animal body weights in all groups of the experiment were summarized in table 1 and Fig. 1.

Liver weight coefficient:

The liver weight coefficient increased significantly in HFD groups as compared to the comparable control groups. which were progressive with the increase of the

duration of HFD ingestion. The mean liver weight coefficients in all groups of the experiment were summarized in table 1 and Fig.1.

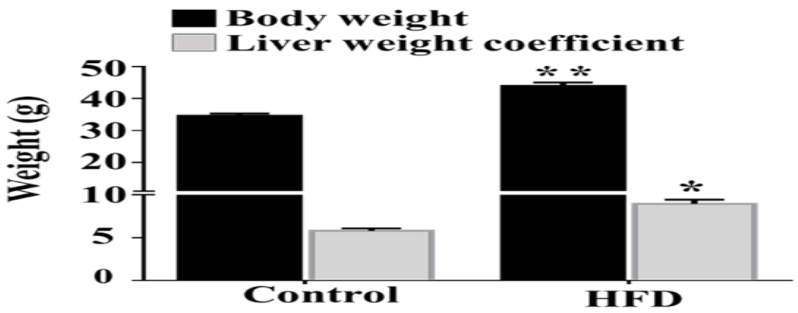

Fig. 1: Liver weight coefficient

Table (1): Body weight and Liver weight coefficient

Weight	Liver weight coefficient of control animals (mean±SD)	Liver weight coefficient of HFD animals (mean±SD)	P-value
Body weight	33.1±0.8	42.3±1.2*	< 0.01
Liver weight coefficient	5.6±0.4	8.9±0.3*	< 0.05

* significant compared to the control at the corresponding time point (P value<0.05).

Biochemical results:

A significant increase in the liver enzymes and plasma free fatty acids FFA (cholesterol, TG, and LDL-C) with no significant changes in the HDL in HFD groups as compared to that of the corresponding control groups were observed and it was directly proportional with the increase in the duration of HFD ingestion. The mean liver enzymes in all groups of the experiment were summarized in table 2, Fig. 2, and the mean plasma FFA in all groups of the experiment were summarized in table 3 and Fig. 3.

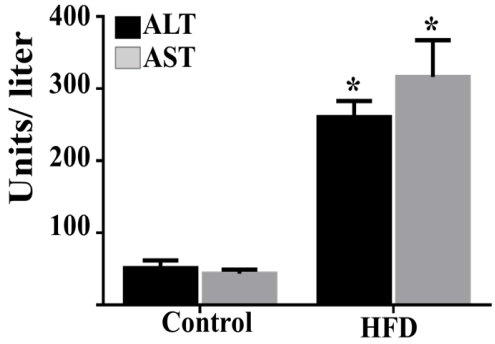

Fig. 2: liver enzymes

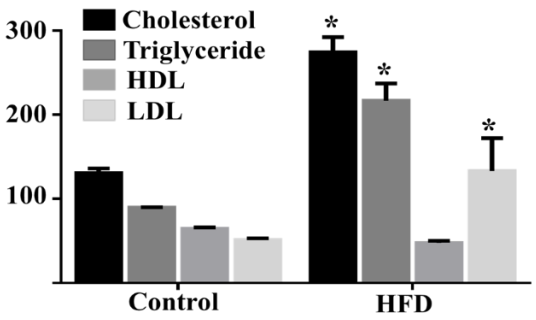

Fig. 3: Plasma FFA levels

Table (2): liver enzymes

Liver enzymes	Control	HFD	P value
ALT	51.0±5.4	260.1±11.1	< 0.05
AST	43.5±2.7	316.7±25.7	< 0.05

Table (3): Plasma FFA levels

FFA in plasma	Control	HFD	P value
Cholesterol	130.5±5.5	274.1±18.1	< 0.05
Triglyceride	89.5±5.0	216.5±20.5	< 0.05
HDL	64.5±2.5	47.1±5.0	> 0.05
LDL	51.5±2.0	133.7±39.0	< 0.05

Histological results:

All of the animals in Group I had comparable outcomes. The H&E-stained liver slices revealed hepatocyte plates in the hepatic lobules spreading from the central vein to the periphery, with blood sinusoids in between. Hepatocytes possessed acidophilic vacuolated cytoplasm, a vesicular nucleus, and some were binucleated (Fig.4a).

At the 11th week of HFD ingestion: The architecture of the hepatic lobules was found to be more or less normal in the investigated liver sections. cellular infiltrateions and apoptotic changes in the form of highly acidophilic cytoplasm and pyknotic nuclei were observed. The nuclei were of different sizes some of them were enlarged, while others were shrunk (Fig. 4b).

At the 13th week of HFD ingestion: The same results but cellular infiltrations and apoptotic changes were more prominent. Most hepatocytes appeared with rarified cytoplasm (Fig. 4c).

At the 15th week of HFD ingestion: The same results with signs of fatty degeneration in hepatocytes were more obvious. There was congestion in the central vein and sinusoidal congestion. Macro and microvesicular steatosis was seen in many hepatocytes (Fig. 4d).

At the the17th week of HFD ingestion: There were congestion and dilatation in the portal veins. Inflammatory cellular infiltrations were observed especially around the ballooned hepatocytes as well as within the portal areas. Many hepatocytes showed both macrovesicular and microvesicular steatosis mainly in zone III. Ballooning degeneration of most hepaticytes was observed with rarified acidophilic cytoplasm. Mallory-Denk bodies were frequently observed as acidophilic inclusions around the nuclei, especially of ballooned hepatocytes. Some hepatocytes appeared with highly acidophilic cytoplasm and pyknotic nuclei. Glycogenated nuclei were observed in some hepaticytes (Fig. 4e).

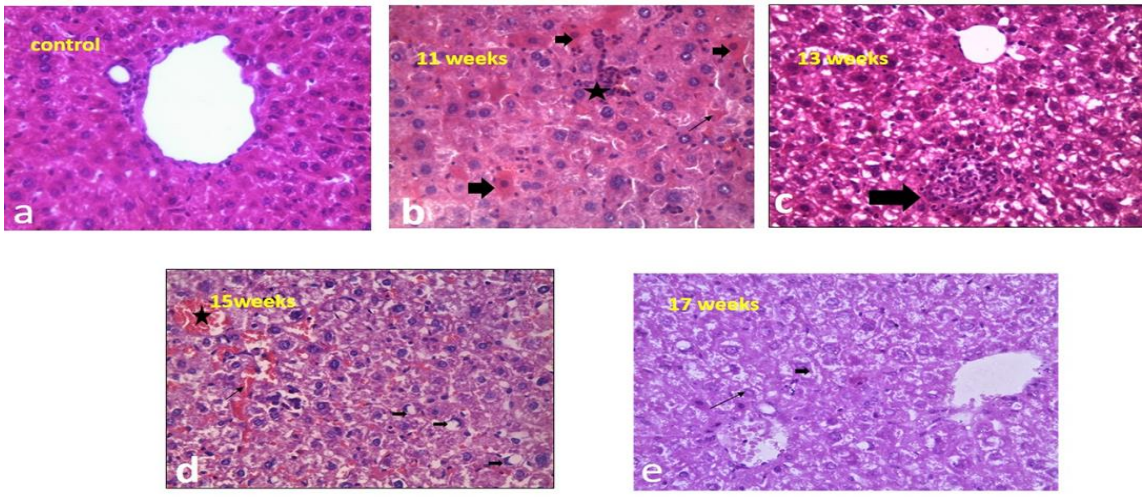

Fig .4: Photomicrographs of liver sections from animals of control groups and HFD groups.
(a) control liver with normal architecture and hepatocytes radiating from the central vein with acidophilic cytoplasm and central rounded vesicular nucleus. (b) HFD group at 11th-week changes in form of inflammatory cellular infiltration (star), apoptotic hepatocytes (thick arrows), and sinusoidal congestion(arrow). (c) HFD group at 13th-week changes in form of inflammatory cellular infiltration (thick arrow) and rarified cytoplasm of most of the hepatocytes. (d) HFD group at 15th-week changes in form of macrovesicular steatosis (thick arrows), sinusoidal congestion (thin arrow), and central vein congestion (star). (e) HFD group at 13th-week changes in form of macro and microvesicular steatosis, central vein congestion, Mallory-Denk bodies (thick arrow), and glycogenated nuclei (thin arrow) (H and E ×400)

Table (4) : nonalcoholic fatty liver disease activity score (NAS score) (14).

Item	Definition	Score
Steatosis	< 5%	0
	5%-33%	1
	> 33%-66%	2
	> 66%	3
Lobular inflammation	No foci	0
	< 2 foci per 200 × field	1
	2-4 foci per 200 × field	2
	> 4 foci per 200 × field	3
Ballooning	None	0
	Few balloon cells	1
	Many cells/prominent ballooning	2

Discussion :

NAFLD is considered a hepatic hallmark of metabolic syndrome ([6]. NAFLD has been on the rise in recent years, along with rising rates of obesity, hyperlipidemia, and type 2 diabetes [7]. NAFLD/ NASH is recognized as the most prevalent chronic liver disease globally, with an estimated incidence of around 20% of all people having NAFLD and 2% to 3% of individuals having NASH [8]. Because the pathophysiology of NASH is unknown, therapies for NASH other than dietary and exercise modifications have yet to be adequately identified [9].

The use of humans in NAFLD/NASH research has significant constraints concerning time, sample collection, and ethical considerations **[10]**. Animal models of NAFLD/NASH provide information on disease pathophysiology as well as the treatment effects of different medicines. These animal models must accurately mimic both the histology and pathogenesis of human NAFLD/NASH **[11]**. Many animal models of NAFLD/ NASH have been developed which do not reflect the full spectrum of the disease in humans, but they can give an idea about the pathogenesis of NAFLD/NASH and in searching for curative treatment for this disease.

We believe that in the future, animal models that fully mirror the histology and pathophysiology of human NAFLD/NASH will be produced and that employing these models will expand knowledge about the etiology and therapy of NAFLD/NASH. Animal models of NAFLD/NASH are divided into three types: genetic models, nutritional models, and models that combine genetic and nutritional components **[11]**.

Genetic models such as SREBP-1c transgenic mice, Ob/ob mice, Db/DB mice, KK-Ay mice, PTEN null mice, Peroxisome proliferator-activated receptor-α knockout Mice PPAR-α knockout mice, Acyl-coenzyme A oxidase null mice AOX null mice, and Methionine adenosyltransferase-1A null micMAT1A null mice. Nutritional models such as Methionine and choline-deficient diet, High fat, Cholesterol, and cholate enriched diet and Fructose enriched diet **[11]**.

Despite advances in noninvasive clinical assessments and image-based NAFLD diagnosis, histological assessment of biopsy specimens remains the gold standard for diagnosing NAFLD/NASH **[12]**. Steatosis, lobular inflammation, and hepatocellular ballooning are all required for NASH diagnosis; fibrosis is also commonly noted. Hepatocellular glycogenated nuclei, lipogranulomas, acidophil bodies, Mallory -Denk bodies, iron deposition (in hepatocytes or sinusoidal lining cells), and Megamitochondria are further histological abnormalities seen in NASH (in hepatocytes) **[13]**.

Diagnosis of NASH based on nonalcoholic fatty liver disease activity score (NAS score) -table 4 - if NAS score $\geq$5 it is diagnosed as NASH but if $\leq$5 it is not NASH **[14]**.

In our study, we used a nutritional mice model of a high-fat diet as follows (71% of energy obtained from fat, 11% from carbohydrates, and 18% from proteins) for 17 weeks. from the 5th week, there was an increase in the coefficient of both animal body weight and liver weight. Also, there was a marked elevation in liver enzymes and free fatty acids. From the 8th week, hepatocellular changes started to appear in form of central vein and sinusoidal congestion, ballooned hepatocytes, macrovesicular and microvesicular steatosis, glycogenated nuclei, Mallory-Denk bodies, and inflammatory cellular infiltrations.

So according to (NAS score) our results show NAS score = 6 so our animal model success reflects the histopathological changes of NASH and we hope to do further study to test the possible protective and therapeutic agents for NASH by using this animal model for NASH.

References:

1. Vernon G, Baranova A, Younossi Z. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Alimentary pharmacology & therapeutics. 2011;34(3):274-85.

2. Yki-Järvinen H. Nutritional modulation of nonalcoholic fatty liver disease and insulin resistance: human data. Current Opinion in Clinical Nutrition & Metabolic Care. 2010;13(6):709-14.

3. Clapper JR, Hendricks MD, Gu G, Wittmer C, Dolman CS, Herich J, et al. Diet-induced mouse model of fatty liver disease and nonalcoholic steatohepatitis reflecting clinical disease progression and methods of assessment. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2013;305(7): G483-G95.

4. Vanheule E, Geerts AM, Van Huysse J, Schelfhout D, Praet M, Van Vlierberghe H, et al. An intravital microscopic study of the hepatic microcirculation in cirrhotic mice models: the relationship between fibrosis and angiogenesis. International journal of experimental pathology. 2008;89(6):419-32.

5. Abdelmegeed MA, Choi Y, Godlewski G, Ha S-K, Banerjee A, Jang S, et al. Cytochrome P450-2E1 promotes fast food-mediated hepatic fibrosis. Scientific reports. 2017;7(1):1-12.

6. Abdelmalek MF, Diehl AM. Nonalcoholic fatty liver disease as a complication of insulin resistance. Medical Clinics of North America. 2007;91(6):1125-49.

7. Neuschwander-Tetri BA. Nonalcoholic steatohepatitis and metabolic syndrome. The American journal of the medical sciences. 2005;330(6):326-35.

8. Harrison SA, Torgerson S, Hayashi PH. The natural history of nonalcoholic fatty liver disease: a clinical histopathological study. Am J Gastroenterol. 2003;98(9):2042-7.

9. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. Science. 2011;332(6037):1519-23.

10. Schattenberg J, Galle P. Animal models of non-alcoholic steatohepatitis: of mice and man. Digestive diseases. 2010;28(1):247-54.

11. Hebbard L, George J. Animal models of nonalcoholic fatty liver disease. Nature Reviews Gastroenterology & hepatology. 2011;8(1):35-44.

12. Takahashi Y, Soejima Y, Fukusato T. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World journal of gastroenterology: WJG. 2012;18(19):2300.

13. Takahashi Y, Fukusato T. Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World journal of gastroenterology: WJG. 2014;20(42):15539.

14. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41(6):1313-21.