Comparison of Non-Alcoholic Fatty Liver Disease (NAFLD) Model using Diet-Induced NAFLD Mice with Genetically Modified Mice

(Perbandingan Model Penyakit Hati Berlemak (NAFLD) menggunakan Tikus NAFLD Diet-Teraruh dengan Tikus Terubah Suai secara Genetik)

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ABSTRACT

Prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing steadily every year affecting all population both Western and Asian countries. The current treatments available for NAFLD are non-conclusive warranting newer effective pharmacological agents. Newly formulated agents require prior testing using animal models. However, in developing countries, these models are often costly. The possibility of using more affordable animal model in local settings should be investigated. In this study, ten Institute of Cancer Research (ICR) and seven B6.Cg-LepOb/J leptin-knockout (JAX) male mice were recruited. Five ICR and all JAX mice were subjected to high-fat diet (60% kcal fat) and remaining ICR mice were given standard diet (SD) for six weeks. Body weight and food intake were measured weekly while abdominal circumference, random blood glucose and liver span were measured at the end of the HFD study. Livers collected were subjected to histology assessment. Compared to ICR group, JAX group presented with significantly higher body weight (58 ± 0.72, $p<0.05$), larger body weight changes (16.57 ± 0.81, $p<0.05$), more HFD intake (197.14 ± 0.812, $p<0.05$) and larger abdominal circumference (11.79 ± 0.34: $p<0.05$). Liver from JAX group appeared with general steatosis and presentation of high-grade panacinar steatosis, low number of lobular inflammations and minimal fibrosis. Liver of ICR mice showed Zone 3 steatosis with high number of lobular inflammations without fibrosis. The NAFLD characteristics presented in JAX group suggested that B6.Cg-LepOb/J mice developed characteristics of NAFLD resembling human while ICR is suitable NAFLD model resembling human population resilient towards NAFLD.

Keywords: Animal model; B6.Cg-LepOb/J strain; high-fat diet; histology; Institute of Cancer Research strain; NAFLD

ABSTRAK

Kelaziman penyakit hati berlemak atau dikenali sebagai penyakit hati berlemak bukan alkohol (NAFLD) meningkat setiap tahun dalam kalangan populasi negara Barat dan Asia. Pada masa ini, rawatan bagi penyakit ini adalah tidak khusus, lalu mendorong kepada penyelidikan dalam mendapatkan agen rawatan yang berkesan. Rumusan agen rawatan yang baharu perlu dikaji menggunakan model haiwan. Penggunaan model haiwan dalam kalangan negara membangun

membabitkan kos yang tinggi. Oleh itu, penyelidikan menggunakan model haiwan mengikut tetapan tempatan perlu dikaji. Dalam kajian ini, sepuluh ekor tikus jantan dan tujuh ekor tikus jantan B6.Cg-LepOb/J tanpa leptin (JAX) diambil daripada Institut Penyelidikan Kanser (ICR). Lima daripada tikus ICR dan kesemua tikus JAX diberi diet lemak tinggi (HFD) mengandungi 60% kcal daripada lemak selama enam minggu, dan selebihnya tikus ICR diberi diet piawai (SD). Berat badan dan pengambilan diet diukur setiap minggu manakala ukur lilit abdomen, bacaan glukosa darah rawak dan panjang hati diukur pada akhir tempoh kajian. Hati yang diperoleh daripada setiap tikus menjalani analisis histologi. Berbanding dengan kumpulan ICR, kumpulan JAX menunjukkan berat badan lebih berat (58 ± 0.72, p<0.05), perubahan berat badan lebih ketara (16.57 ± 0.81, p<0.05), pengambilan HFD lebih banyak (197.14 ± 0.812, p<0.05) dan ukur lilit abdomen lebih besar (11.79 ± 0.34: p<0.05). Analisis histologi kumpulan JAX menunjukkan kehadiran lemak (steatosis) dalam hati bertaraf tinggi, bilangan radang lobular dan parut yang rendah. Hati daripada kumpulan ICR menunjukkan gred 3 steatosis dan bilangan radang lobular yang tinggi. Ciri-ciri NAFLD yang ditonjolkan oleh kumpulan JAX menunjukkan model ini lebih mirip ke ciri-ciri NAFLD manusia manakala kumpulan ICR menunjukkan ciri-ciri ke arah manusia yang mempunyai kerintangan terhadap NAFLD.

Kata kunci: Diet lemak tinggi; histologi; model haiwan; NAFLD; strain B6.Cg-LepOb/J; strain Institut Penyelidikan Kanser

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is increasing steadily for the past 20 years in its prevalence and globally affecting all ages (Younossi et al. 2019). According to Younossi et al. (2016), the collective prevalence of individuals with NAFLD globally diagnosed by imaging was at 25%. The increasing prevalence of NAFLD has surpassed other common liver diseases including alcoholic fatty liver disease (AFLD) and hepatitis (Estes et al. 2018). Treating NAFLD remains a challenge due to unavailability of specific therapy including FDA approved drugs in reversing the condition (Chalasani et al. 2018; Hung & Bodenheimer 2018). The gold-standard of treatment recommended for NAFLD currently is reducing body weight through exercise and dietary modification (EASL 2016). However, these options would result in lower compliance among patients as these suggestions require patient-directed motivation. Other options involve radical treatment consisting of surgical intervention such as bariatric surgery to promote weight loss where only half of NAFLD patients showed improvement (Fakhry et al. 2019; Salsamendi et al. 2015).

Pharmacological interventions introduced to NAFLD patients often recommended in patients who have developed features of Non-alcoholic Steatohepatitis (NASH) as the signs and symptoms of NAFLD often overlooked (Sanyal et al. 2010). Treating NAFLD using pharmacological approaches include the use of antioxidant agent primarily vitamin E and insulin sensitizer such as pioglitazone (Leoni et al. 2018; Sanyal et al. 2010). However, these treatments often resulted in debate on their benefits over the target population in terms of efficacy and consistency in clinical outcome (Sridharan et al. 2018). Therefore, newer drugs with better efficacy and specificity for NAFLD should be investigated compared to readily available treatments for NASH.

Testing of newly formulated drugs, be it synthetic or plant-based requires prior animal studies and completion of human randomized clinical trial before the treatment is made available in the market. Animal models, specifically NAFLD animal model, can be derived in several methods to investigate the outcome and adverse effects of newly formulated NAFLD drugs.

NAFLD animal models have been fundamentally described in few articles where rodents are often opted for the model especially mice and rat (Hebbard & George 2011; Solinas et al. 2014; Van Herck et al. 2017). These models can be established through either dietary or chemically induced or genetically modified animal or in combination. In terms of dietary induced NAFLD, several diets are available to promote excessive deposition of lipids into the liver affecting *de novo* lipogenesis (DNL), increasing liver fatty acid storage (Duarte et al. 2014) or causing metabolic dysregulation to regulate lipid metabolism in the liver (Machado et al. 2015). As an example, high-fat diet (HFD, 60% kcal from fat) supplied to mice for several weeks would result in features of NAFLD and metabolic syndrome (MetS) developed in these mice such as high body weight, steatotic liver and high blood glucose level suggesting obesity, fatty liver and type 2 diabetes (T2DM), respectively (Clapper et al. 2013; Fraulob et al. 2010). Studies have reported that NAFLD characteristics can be easily achieved using HFD but formation of MetS and T2DM in NAFLD-affected mice required combination of Methionine and Choline Deficient (MCD) diet (Zhang et al. 2018) or Choline-deficient (CD) diet (Chiba et al. 2016). However, both MCD and CD diets would promote rapid progression of NAFLD to NASH compared to HFD only which would require extended period to develop it (Hebbard & George 2011).

Chemically-induced NAFLD animal model could be accomplished by introducing Streptozotocin (STZ)

via intraperitoneally or subcutaneously to destroy the pancreatic islets to promote insulin insensitivity and obesity whereby both are risk factors of NAFLD (Ma et al. 2018). Other options include carbon tetrachloride (CCl_4) (Domitrovic et al. 2009) and diethylnitrosamine (DEN) (Wu 2016). Consideration on using chemicals to induce NAFLD in animal should be applied cautiously as these chemicals often resulted in aggressive form of NAFLD involving rapid development of NASH and leading to development of hepatocellular carcinoma (HCC) (Liu et al. 2013).

Genetically modified NAFLD animal model consisting deleting specific genes related to DNL or insulin sensitivity. Among the options available, specifically mice strain, are *ob/ob* mice, *db/db* mice, yellow-obese agouti (Ay) mice, $CD36^{-/-}$, Galactin-3 knockout mice and few others (Nagarajan et al. 2012). Leptin-deficient mice, known as *ob/ob* mice is the favoured genetically-modified NAFLD model due to its advantage in providing a window to induce NASH or severe form of NAFLD through chemical or diet (Kristiansen et al. 2016). These mice would appear with NAFLD characteristics such as obesity, high serum glucose level, hyperinsulinemia and unsatiated appetite. NASH could be induced in these *ob/ob* mice by introducing MCD or CD diet or STZ in attaining NASH or HCC animal model (Brix et al. 2002).

Apart from selecting the appropriate NAFLD model to achieve study objectives, other several factors should be considered as well. Among the factors are cost and accessibility of the desired NAFLD animal model, especially genetically modified mice. In developing countries, accessibility to genetically modified mice remained fairly limited due to its high cost, often require importing from other countries and prolonged time in transportation rendering studies conducted to identify affordable NAFLD animal model (Yahaghi et al. 2019). Therefore, an easily accessible and affordable NAFLD animal model should be studied suitable to local environment and availability of laboratory animal supplier. This study comprises of using Institute of Cancer Research (ICR) male mice, which is an affordable and locally attainable dietary-induced NAFLD model in comparison to the imported genetically-modified leptin-knockout B6.Cg-LepOb/J mice. Both animal models were subjected to a high-fat diet (HFD) in order to promote NAFLD and NAFLD animal model parameters were investigated in these two models.

Materials and Methods

Animals and Experimental Procedures

Animal studies were conducted under the approval of Committee on Animal Research and Ethics, Universiti Teknologi MARA (UiTM) (Approval code: UiTM CARE 251/2018 (3/8/2018)). This study comprised of two groups of Institute of Cancer Research (ICR) mice (*Mus musculus musculus*) and one group of leptin-knockout B6.Cg-LepOb/J male mice (*Mus musculus musculus*). All ten ICR male mice at six-weeks old were purchased from a local animal laboratory supplier (Selangor, Malaysia) while the seven JAX male mice at six-weeks old were purchased from Jackson Laboratory (Maine, United States of America). Ten ICR mice (n=10; ICR NAFLD model) were randomly divided into the two groups while seven B6.Cg-LepOb/J mice (n=7; JAX NAFLD model) were designated as the third group. The first group consisted of ICR mice on standard diet (n=5, ICR-SD group) designated as control group and another five ICR mice were subjected to high-fat diet (HFD) (n=5, ICR-HFD group). The third group consisted of JAX mice were subjected to HFD (n=7, JAX group). The HFD given to the designated mice were of 60% kcal (composition of diet in supplement 1) from fat *ad libitum* (Altromin, Germany).

Before study was conducted, all ICR mice were acclimatized for a week while the JAX mice acclimatized for two weeks. The difference in acclimatization period was due to consideration of the transportation time taken and stress developed throughout the process (Obernier & Baldwin 2006). The ICR mice were purchased locally subjected to local environment and climate with transportation time taken only for an hour. The JAX mice were imported from USA, exposed to long hours of flight and originated from different climate. Therefore, it was a necessity for acclimatizing for additional one week. During acclimatization and study period, all mice were housed individually using plastic cage with metal covering. The room containing these mice was controlled with ambient temperature fixed to 24 °C ± 1. Humidity was controlled using de-humidifier (Panasonic, Japan) at 50% ± 5. Room lighting was set to be adhere to strict 12 h light and dark cycle. These mice were provided with reverse-osmosis (RO) water through water a bottle fixed onto the cages.

The study was conducted for six weeks. Dietary intake was measured daily, and total weekly dietary intake was calculated by the sum of daily intake for each mouse on last day of each week. The mean dietary intake for each group was derived from the total dietary of each mouse in the respective group. At the end of the study, all mice were euthanized by rapid cervical dislocation according to the Animal Welfare Act 2015 (Act 772), Law of Malaysia. Mice were exposed laparatomically and the liver was dissected and measured for liver span. Each lobe from every liver was incised and stored in 10% neutral buffered formalin (NBF; R&M Chemicals, Malaysia) for histology processing.

ANTHROPOMETRIC INDICES

Body weight and food intake were recorded once a week after acclimatization. At the end of the study, abdominal circumference, liver span and tail-blood random blood glucose (RBG) were measured in all mice. Abdominal circumference was determined by measuring the largest zone on each mouse using measuring tape as demonstrated by Gerbaix et al. (2010). Liver span was measured by measuring the length from the edge of left lobe to the edge of right lobe as demonstrated by Wang et al. (2016). Random blood glucose (mmol/L) was assessed by using glucometer using tail-blood after euthanization.

LIVER HISTOPATHOLOGY

Liver tissue was sampled from left, right and middle lobe of each mouse and were kept in NBF for 72 h where the formalin was replaced every 24 h. Total of 15 liver tissues were sampled in each ICR group (ICR-SD and ICR-HFD groups) and 21 in JAX group. Then, all liver tissues were processed by the tissue processor (Sakura, Europe). Once processed, the tissues were embedded into the cassette using a paraffin-embedding machine (Thermo Scientific, Germany). The embedded tissues were allowed to cool for a day at room temperature before being trimmed and sectioned at 5 µm thick. The sectioned tissues were fixed into microscope glass slides (Sail, China). The tissues were stained with Hematoxylin and Eosin (H&E; Merck, Germany) and Masson Trichrome (MT; Bio-Optical, Italy). The sections were assessed by resident histopathologists via single blinded assessment on the severity of fatty liver based on the criteria of NAFLD Activity Score (NAS) previously reported (Kleiner et al. 2005; Liang et al. 2014) as outlined in Table 1. Apart from that, gross liver appearance was inspected as well during euthanization.

TABLE 1. NAFLD Activity Score (NAS) criteria

Item	Definition
Steatosis grade	Evaluation of parenchymal involvement by steatosis based on low to medium power observation
	<5%
	5% - 33%
	>33% - 66%
	>66%
Location	Predominant distribution pattern
	Zone 3
	Zone 1
	No specific zone
	Panacinar
Microvesicular steatosis	Connecting patches
	Not present
	Present
Fibrosis stage	None
	Perisinusoidal or periportal
	Mild, zone 3, perisinusoidal
	Moderate, zone 3, perisinusoidal
	Portal or periportal
	Bridging fibrosis
	Cirrhosis
Lobular inflammation at 200x magnification	No foci
	<2 foci
	2-4 foci
	>4 foci
Liver cell ballooning	None
	Few balloon cells
	Prominent ballooning

Table was adapted and modified from Kleiner et al. (2005)

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS (Version 26, IBM, USA). Reflecting the number of samples per group which low number of sample size would not amount to normality test as described by Ricci et al. (2020), non-parametric test such as Kruskal-Wallis test was employed to compare means between groups. All values are reported as mean and standard error (mean ± SEM). The General Linear Model was selected to compare the means of the interval data collected (weekly weight and dietary intake). Statistically significant cut-off value was decided to be less than 0.05 (p<0.05).

RESULTS

ANTHROPOMETRIC INDICES AND BIOLOGICAL PARAMETERS

The mean body weight of ICR-SD, ICR-HFD and JAX group at the beginning and end of study are shown in Figure 1(A). ICR-SD group body weight at the beginning of study was 33.2 ± 0.37 g while ICR-HFD recorded at 33.6 ± 1.75 g. JAX group body weight was 41.43 ± 1.57 g at the beginning of study. At the end of the study, ICR-SD group body weight was 39.4 ± 1.806 g while ICR-HFD recorded at 47.80 ± 4.22 g. JAX group body weight at the end of study was 58.00 ± 0.724 g. Using the General Linear Model, body weight of mice between groups showed significant weight gain difference at the beginning and end of study where JAX group body weight was larger compared to ICR-SD and ICR-HFD (p<0.05, Figure 1(A)). In addition, comparison between ICR-SD and ICR-HFD only showed that the body weight of ICR-HFD measured at the beginning and end of study were significantly higher (p<0.05, Figure 1(A)). The total body weight changes throughout the study recorded in ICR-SD group was 4.6 ± 1.63 g while ICR-HFD recorded with 14.2 ± 2.71 g. In JAX group, the total body weight changes recorded was 16.57 ± 0.812 g. The total body weight changes between the groups were significant where ICR-HFD (209%) and JAX (260%) groups had significant body weight changes compared to ICR-SD respectively (p<0.05, Figure 1(B)).

The mean of weekly total food intake at the beginning (week 1) and end of study (week 6) between groups are illustrated in Figure 1(C). In terms of standard diet intake, ICR-SD group recorded 7.4 ± 0.68 g while ICR-HFD consumed 22.4 ± 1.17 g of HFD. In JAX group, consumption of HFD was recorded at 38.14 ± 2.1 g at the beginning of study. At the end of study week, ICR-SD

group consumed a total of 8 ± 0.63 g of standard diet while ICR-HFD consumed 15 ± 1.64 g of HFD. In JAX group, total of 24 ± 1.75 g of HFD were consumed. General Linear Model analysis showed significant differences between groups where the JAX group consumed food significantly higher compared to ICR-SD and ICR-HFD (p<0.05, Figure 1(C)). ICR-HFD consumed significant amount of food compared to ICR-SD (p<0.05, Figure 1(C)). The total intake of the standard diet consumed by ICR-SD group was 52.6 ± 1.29 g and the total intake of HFD recorded in ICR-HFD group was 124.6 ± 5.81 g. In JAX group, 197.14 ± 1.55 g of HFD consumption was recorded. The total of food consumed between groups was significant where JAX group consumed significantly higher compared to ICR-SD (73.2%) and ICR-HFD (37.8%) group (p<0.05, Figure 1(D)). Comparison between ICR-SD and ICR-HFD only showed that the total food consumed by ICR-HFD was significantly higher (p<0.05, Figure 1(D)).

Abdominal circumference measured in ICR-SD group was 8.3 ± 3 and ICR-HFD abdominal circumference was measured at 10.8 ± 0.49 cm. In JAX group, abdominal circumference was measured at 11.79 ± 0.34 cm. There was a significant difference between groups where the abdominal circumference of JAX group was larger compared to ICR-SD group (p<0.05) but not with ICR-HFD group (p > 0.05) (Figure 1(E)). ICR-HFD had significantly larger abdominal circumference compared to ICR-SD (p<0.05, Figure 1(E)). Lastly, random blood glucose sampled from tail blood was measured at 7.44 ± 0.70 mmol/L in ICR-SD group, 10.44 ± 1.49 mmol/litre in ICR-HFD group and 13.24 ± 0.74 in JAX group. Statistical analysis showed random blood glucose level was significantly higher in JAX group when compared to ICR-SD group only (p>0.05, Figure 1(F)). However, no significant different on random blood glucose were observed between ICR-SD and ICR-HFD only (p>0.05, Figure 1(F)).

LIVER HISTOPATHOLOGY

On gross liver appearance, ICR-HFD group showed patches of steatosis denoted by yellowish areas on each lobe compared to JAX group where the liver appeared generally yellow and swollen (Figure 2). Liver span measured was significantly larger (p<0.05, Figure 1(G)) in ICR-HFD group (3.2 ± 0.2 cm) and JAX group (4.31 ± 0.14 cm) compared to ICR-SD mice (2.5 ± 0.16 cm). Representative liver histology slides based on haematoxylin and eosin (H&E) staining are displayed in
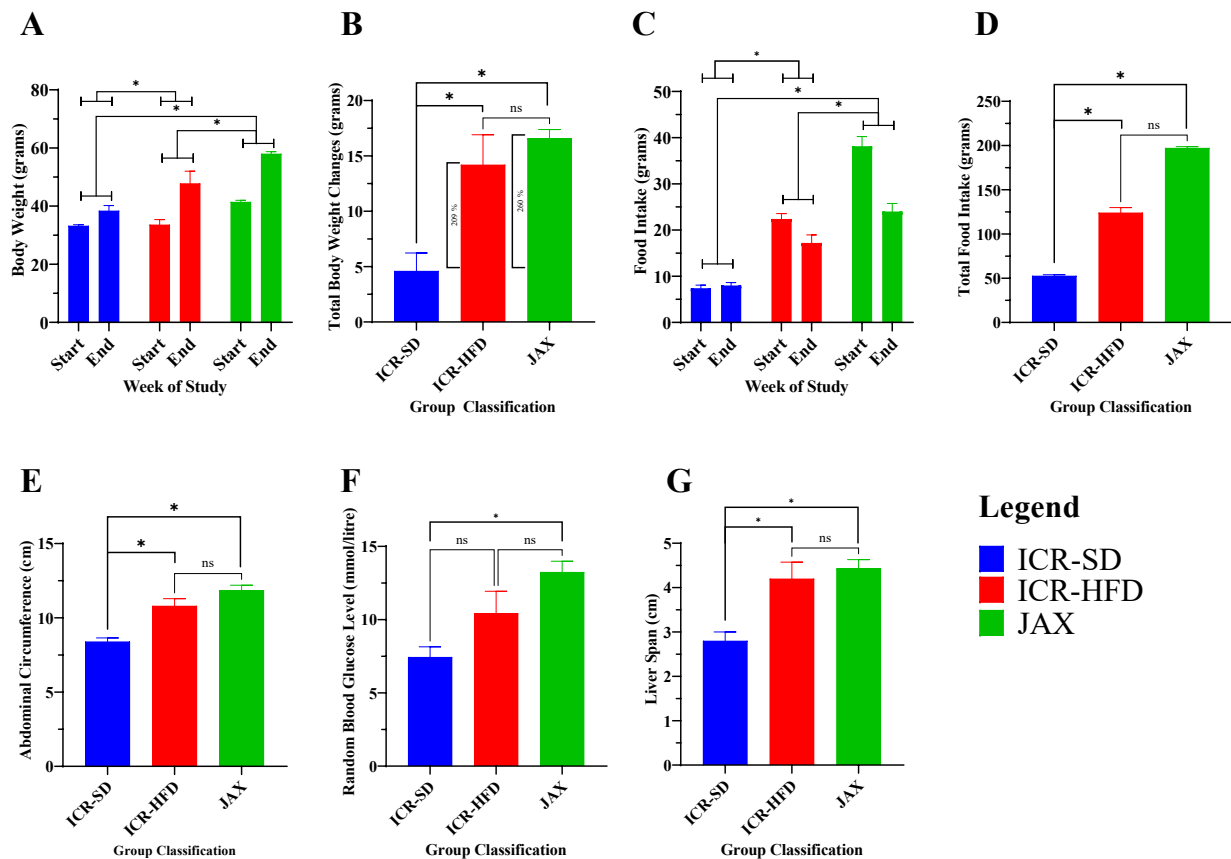
FIGURE 1. Illustration of the (A) body weight recorded at the beginning and end of study, (B) total body weight changes, (C) food intake at the beginning and end of study, (D) total food intake, (E) abdominal circumference, (F) random blood glucose level, and (G) liver span measurement

*:p < 0.05; ns = not significant

Figure 3 and Masson Trichrome (MT) staining in Figure 4. The NAFLD Activity Score (NAS) was assessed in all groups and presented in Table 2. Both ICR-HFD and JAX liver sections showed presence of macrovesicular and microvesicular steatosis which were not evident in ICR-SD group. In terms of steatosis grade, 20% (n=3) from ICR-HFD group showed low-grade steatosis and 80% (n = 12) showed mid-grade (5 - 33%) steatosis. In JAX group, 95% of samples (n = 20) presented with high-grade steatosis (>66%) and one sample (5%) showed >33-66% steatosis grade. In addition, all samples (n = 15) in ICR-HFD group presented with Zone 3 steatosis while 57% (n = 12) in JAX group presented with panacinar steatosis, 14% (n = 3) with azonal steatosis and 29% (n = 6) with Zone 3 steatosis. Lobular inflammation foci

in ICR-HFD group presented in one of the samples (7%) to have 2-4 foci of inflammation and 60% of samples (n=9) had <2 foci of inflammation. Thirty-three percent (33%) of ICR-HFD group had not presented with foci of inflammation (n = 5). In JAX groups, <2 inflammation foci were seen in 29% of the samples (n = 6) while 71% (n = 17) observed with absence of inflammatory foci. In ICR-HFD group, liver fibrosis was not observed in all samples. However, in JAX group, 14% of the samples (n = 3) showed perisinusoidal or periportal fibrosis and not present in other samples (86%, n = 19). Other histology features such as hepatocyte ballooning and Mallory-Denk bodies were absent in both ICR-HFD and JAX groups. Based on histology findings, JAX group showed higher NAS compared to ICR.
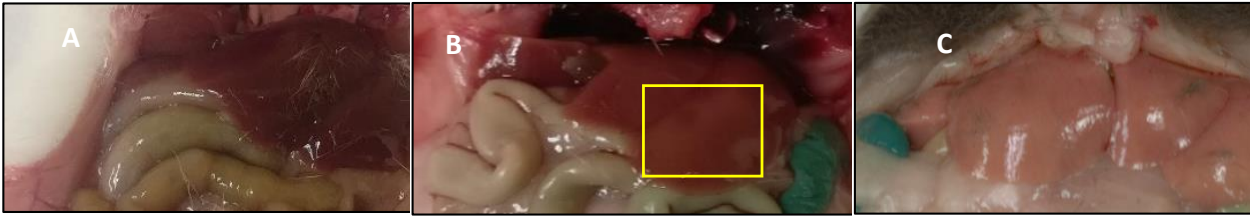
FIGURE 2. Representative images of gross liver appearance of (A) ICR-SD group, (B) ICR-HFD group, and (C) JAX group. The liver of ICR-SD appeared dark red with sharp liver edges. The liver from ICR-HFD group showed yellow patch (□) denoting areas of steatosis while JAX (C) showed liver appeared to be generally pale-yellow and swollen
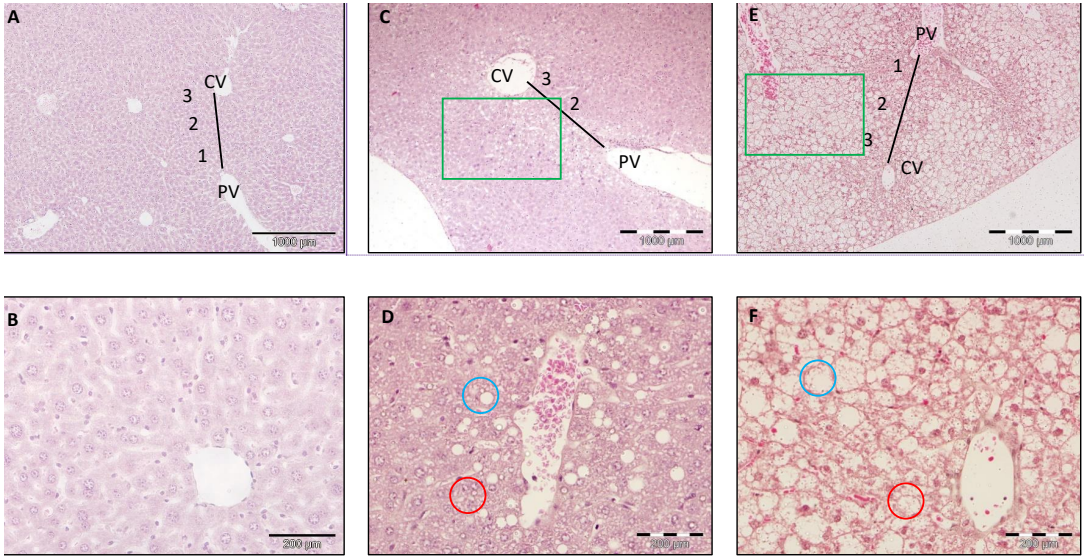

FIGURE 3. Representative histology sections of liver from (A & B) ICR-SD group, (C & D) ICR-HFD, and (E & F) JAX using haematoxylin and eosin (H&E) staining. Compared to ICR-SD group, the livers from (C) ICR-HFD and (E) JAX presented with areas of steatosis (□). At higher magnification (40x), macrovesicular (○) and macrovesicular (○) steatosis can be observed in (D) ICR-HFD and (F) JAX
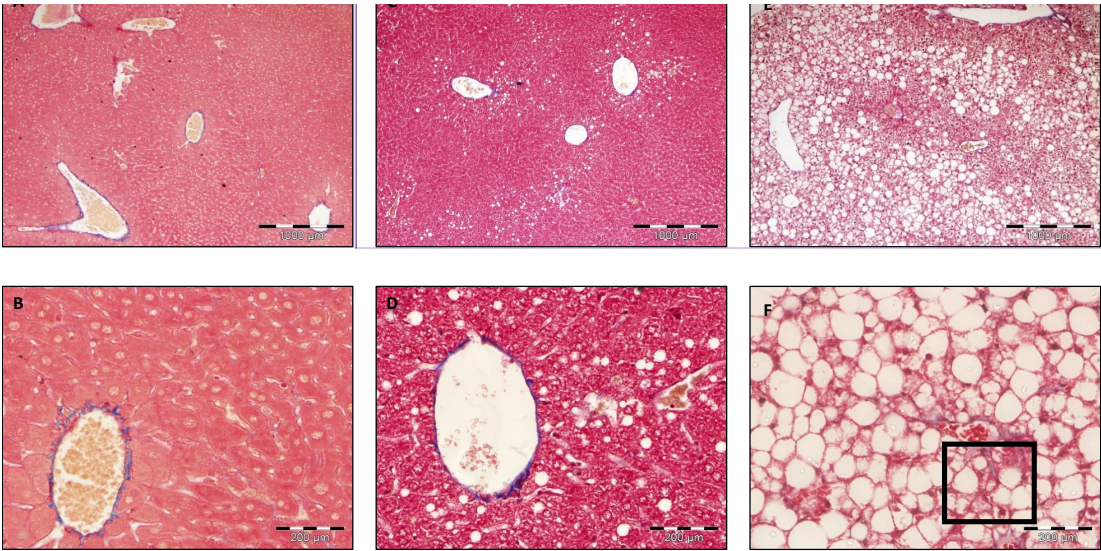

FIGURE 4. Representative histology sections of liver from (A & B) ICR-SD group, (C & D) ICR-HFD, and (E & F) JAX using Masson Trichrome (MT) staining. Fibrosis demarcated by purple staining was observed around the periportal region (F; □) in JAX liver sections. Fibrosis was not observable in (A & B) ICR-SD and (C & D) ICR-HFD groups

TABLE 2. NAFLD Activity Score (NAS) of ICR-SD, ICR-HFD and JAX

Item	Definition	Group		
		ICR-SD	ICR-HFD	JAX
Steatosis	<5%	100%	20%	0%
	5% - 33%	0%	80%	0%
	>33% - 66%	0%	0%	5%
	>66%	0%	0%	95%
Location	Zone 3	100%	100%	29%
	Zone 1	0%	0%	0%
	Azonal	0%	0%	14%
	Panacinar	0%	0%	57%
Fibrosis	None	100%	100%	91%
	Perisinusoidal or periportal	0%	0%	9%
	Perisinusoidal and portal/ periportal	0%	0%	0%
	Bridging fibrosis	0%	0%	0%
	Cirrhosis	0%	0%	0%
Lobular inflammation	No foci	53%	33%	71%
	< 2 foci	33%	60%	29%
	2 – 4 foci	13%	7%	0%
	> 4 foci	0%	0%	0%
Hepatocyte ballooning	None	100%	100%	100%
	Few balloon cells	0%	0%	0%
	Prominent ballooning	0%	0%	0%

Lobe: L=left lobe, R= right lobe, M=median lobe. Grade of steatosis: 0=5%, 1=5%-33%, 3= >66%. Location: 0= Zone 3, 1=Zone 1, 2=Azonal, 3=Panacinar. Microvesicular steatosis: 0=Not present, 1=present. Fibrosis stage 0=none, 1=periportal or perisinusoidal, 1A=mild, zone 3, perisinusoidal, 1B=moderate, zone 3, perisinusoidal, 1C=portal or periportal, 2=perisinusoidal and portal or periportal, 3=bridging fibrosis, 4=cirrhosis. Inflammation: 0=no foci, 1=<2 foci, 2=2-4 foci, 3=>4 foci. Microgranulomas: 0=absent, 1=present. Large lipogranulomas: 0=absent, 1=present. Portal inflammation: 0=none to minimal, 1=greater than minimal. Ballooning: 0=none, 1=few balloon cells, 2=prominent. Acidophil bodies: 0=none, 1=many. Pigmented macrophages: 0=none, 1=many. Megamitochondria: 0=none, 1=many. Mallory hyaline: 0=none, 1=many. Glycogenated nuclei: 0=none, 1=many. Scoring is based on the NAS outlined by (Kleiner et al. 2005)

DISCUSSION

This study focused on comparing the characteristics of non-alcoholic fatty liver disease (NAFLD) developed in two types of animal model. The first model consisted of dietary induced NAFLD model using ICR male mice at six-weeks old while the second model consisted of genetically-modified and dietary induced NAFLD model using the leptin-knockout B6.Cg-LepOb/J (JAX) male mice at six-weeks old on 60% high-fat diet (HFD). Both of these models were compared to ICR male mice at six-weeks old provided with a standard diet throughout this study. In addition, this study measures anthropometric indices such as body weight changes during study, food intake, abdominal circumference, liver span and also measured biological parameters such as random blood glucose sampled from tail blood during euthanization.

Liver was sampled from each lobe to assess histologically from all groups. Based on this study, the ICR mice are economical for most of the researchers from developing countries compared to the imported JAX mice. Apart from that, the time period for availability of JAX mice to arrive at our facility was time-consuming compared to ICR which was easily delivered upon purchasing.

Male mice were selected as the preferred gender in this study in order to represent the human NAFLD population. Many epidemiological studies have shown male being the predominant gender to develop NAFLD (Sayiner et al. 2016), even reported mostly in male children and adolescents (Anderson et al. 2015; Pawar et al. 2016). The lower prevalence of NAFLD in the female population is contributed by the protective effect of oestrogen (Besse-Patin et al. 2017; Palmisano et al. 2017) providing evidence the female mice are most likely not suitable as NAFLD animal model. In fact, few animal studies had suggested the role of oestrogen receptor as a target for therapy in downregulating *de novo* lipogenesis (B'Chir et al. 2018; Guillaume et al. 2019).

At the end of the study, both ICR-HFD and JAX group gained substantial weight after HFD compared to ICR-SD. Similar finding in terms of body weight using ICR mice fed with HFD was described by Li et al. (2020). The ICR-HFD gained twice the body weight of ICR-SD group. The high body weight gain was most likely contributed by the 60% kcal from fat originating from the provided HFD which similarly reported by Avtanski et al. (2019) and Li et al. (2020). In addition, the body weight observed higher in JAX group was most likely contributed by the nullified leptin resulting in lower activity of lipolysis, energy expenditure and increase in DNL activities (Yasmeen et al. 2018). In comparison to ICR-HFD, in which leptin is being circulated in their blood, some degree of control in weight gain and energy expenditure are preserved (Knuth et al. 2014). Based on our findings, JAX mice subjected to HFD would be more reflective of NAFLD over ICR-HFD mice as majority of the individuals with NAFLD are obese primarily central obesity). This is further supported by the high measurement of waist circumference among JAX mice recorded at the end of the study. Random blood glucose showed these mice were diabetic as well, though further tests such as HbA1c is warranted. Nevertheless, combining the obesity feature, large waist circumference and hyperglycemia in the JAX group, these mice would most likely develop Metabolic Syndrome (MetS) and NAFLD is manifestation of this syndrome (Kulkarni et al. 2015). Compared to the ICR group, the JAX model

represents a higher similarity of NAFLD in the human population with the features of MetS.

The development of fatty liver in ICR-HFD and JAX was not only supported by the anthropometric indices but both gross and microscopic features of their liver promoted by the high free fatty acid derived from the provided HFD which was also demonstrated by Wang et al. (2016). At gross, liver appeared with yellow infiltration in both ICR-HFD and generalized pale-yellow in the JAX group. The yellow infiltration suggests high fat deposition due to increase in DNL activity accompanied by decrease in lipolysis activity. Similar findings were presented in other similar animal model (Kristiansen et al. 2016). However, histologically, the JAX group provided higher degree of similarities of histologic features of NAFLD in human population compared to ICR-HFD group. The human NAFLD histologic features were compared with mice in study demonstrated by Kleiner et al. (2005). Compared to the histology findings illustrated by Kleiner et al. (2005), histologic features presented in JAX group such as combination of macrovesicular and microvesicular steatosis encompassing all zones and high NAS reflected the resemblances of human NAFLD. This is less likely seen in the ICR-HFD group. Nevertheless, the histologic features presented in ICR-HFD with the presence of both microvesicular and macrovesicular steatosis despite being metabolically active suggest that the presence of leptin is important to establish the characteristics of NAFLD similar to human NAFLD. If this situation is to be applied in humans, it is proposed that individuals who practice high-fat diets on a daily basis are predisposed and increase the risk of developing NAFLD (Chan et al. 2015).

The limitation of this study was the lack of understanding on the response of JAX mice towards the high-fat diet as this is not well reported by others. Throughout the study, these mice developed constipation which required manual evacuation and highly susceptible to injury due to the hardened stool. Therefore, precaution should be taken in handling these mice models in inducing NAFLD through a high-fat diet.

CONCLUSION

B6.Cg-LepOb/J mice developed NAFLD resembling to human NAFLD condition in consideration of the metabolic and histologic features compared to ICR mice. Further investigation is necessary to fully elucidate the biochemical profile of this model especially its effect when subjected to established and new formulation of therapy. The ICR-HFD is recommended model in

providing insights of newly formulated therapy prior to moving to the costly genetically-modified B6.Cg-LepOb/J mice.

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