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Original Article

Energy intake correlates with the levels of fatty acid synthase and insulin-like growth factor-1 in male and female C57BL/6 mice

Anup S Ramdhave[1], Shreesh Ojha[2], Mukesh Nandave[1]

[1]SPP School of Pharmacy & Technology Management, SVKM's NMIMS, Mumbai, India; [2]Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, UAE

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Abstract: Emerging evidence suggests that, dysregulation of fatty acid synthase (FASN) and insulin-like growth factor-1 (IGF-1) could play a vital role in pathology of various diseases. Our aim was to determine the changes in FASN and IGF-1 levels concomitant to long term feeding of HFD in either sex. Male and female mice were fed either HFD or LFD for a period of 16 weeks. During this period, physiological, biochemical, and histological parameters were evaluated. Mice fed with HFD showed a significant gain in body weight, body mass index, energy intake, and abdominal circumference. These changes were accompanied by compromised glucose and insulin tolerance, hyperinsulinemia, dyslipidemia, elevated plasma IL-6, and TNF-α concentration. Histologically, hepatocytes showed an elevated fat accumulation, appended by an increase in plasma activities of liver enzymes. Pancreas showed upsurge in number of β-cells with subsequent increase in size of islet implying its compromised state. While the kidney showed mild tubulointerstitial fibrosis indicating initiation of kidney impairment. These metabolic perturbations were related to the energy intake which was higher in males as compared to females. This led to a proportional rise in plasma as well as liver FASN and IGF-1 in HFD fed mice. Within both sexes, mice fed with HFD developed features of non-alcoholic steatohepatitis (NASH), hyperinsulinemia, dyslipidemia, impaired glucose and insulin tolerance but the magnitude of these abnormalities was found to be less in female mice. This variation in magnitude could be attributed to the difference in energy intake and ultimately its effect on FASN and IGF-1 levels.

Keywords: Fatty acid synthase, insulin-like growth factor-1, high-fat diet, C57BL/6 mice

Introduction

The increased prevalence of metabolic syndrome (MetS) continues to be a major global health issue [1]. MetS is also known to predispose various disorders which includes type 2 diabetes [2, 3], cardiovascular disease [4-6], non-alcoholic fatty liver disease (NAFLD) [7, 8], polycystic ovarian syndrome (PCOS) [9, 10], and even cancer [11-14]. The most common factors contributing in development of MetS and related disorders are: high-fat, caloric-dense diets, sedentary lifestyles, increased urbanization and psychosocial stress [15].

Emerging evidence suggest that, fatty acid synthase (FASN) catalyzed *de novo* fatty acid synthesis plays a vital role in the pathology of MetS by aggravation of insulin resistance [16, 17]. FASN is also known to modulate the expression of key genes involved in the glucose-insulin axis [16]. One of the key genes involved is insulin-like growth factor binding protein-1 (IGFBP-1). The elevated insulin and body fat are associated with decreased IGFBP-1 levels, which ultimately lead to increase in circulating insulin-like growth factor-1 (IGF-1) levels [18, 19]. Reversibly, this increase in IGF-1 and insulin level is also known to further up regulate the transcription of FASN gene [20, 21].

Historically, FASN gene has been investigated as a possible oncogene [22, 23]. Thus, there is a misconception that increased concentrations of circulating FASN can be observed solely during progression of human malignancies. However, Menendez et al. [16] hypothesised that, while dysfunction of glucose/lipid meta-

bolism is an early hallmark in most human malignancies, increased concentrations of circulating FASN might also occur in MetS related disorders in which insulin resistance is prominent.

To date, there are no animal studies which have demonstrated up-regulation of FASN and subsequent IGF-1 level in metabolically challenged mice with absence of neoplasms. Thus, our aim was to determine the changes in FASN and IGF-1 levels concomitant to long term feeding of HFD in age-matched male and female mice.

Insulin-resistant conditions such as obesity, type 2 diabetes, and cancer arise from a common FASN-driven "lipogenic state" [15] along with IGF-1. Thus, change in FASN and IGF-1 levels in humans could be used in assessment of disease progression from obesity and type 2 diabetes to cancer.

Material and methods

Animals and diet

Male and female C57BL/6 mice, 5-6 weeks old (National Institute of Nutrition, Hyderabad, India), were housed in a temperature-controlled (21 ± 2°C) room with 12 hour light-dark cycle. The experimental protocol was approved by Institutional Animal Ethical Committee as per the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Forest and Environment, Government of India, New Delhi, India (Permit No.: CPCSEA/IAEC/SPTM/P-06/2014). Prior to the experiment, both male and female mice were acclimatized for at least 1 week and were randomly assigned into two groups based on diet. During the acclimatization period, study animals were maintained on LFD consisting of 10% calories from fat (D12450B; Research Diets Inc., NJ, USA) while during study phase, animals were maintained on measured quantities of LFD or HFD consisting of 60% calories from fat (D12492; Research Diets Inc., NJ, USA). The animals had similar weight range (± 2 g) within both the sexes before initiation of the study.

Physiological parameters estimation

Food intake and body weight was measured weekly throughout the study. Energy intake was calculated based on the food intake, wherein the energy consumed per gram of HFD and LFD was 5.24 kcal and 3.85 kcal respectively. Abdominal circumference at lumbar region and body length from nose to anus were determined at 0, 4, 8, 12, and 16th week. BMI was calculated as body weight (g)/square of body length (cm).

Glucose and insulin tolerance test

Oral glucose (OGTT) and intraperitoneal insulin tolerance test (IPITT) were performed as described by Gallou-Kabani *et al.* [24], at 8th and 16th week. For OGTT, animals were fasted for a period of 14-16 hours while for IPITT, animals were fasted for a period of 4 hours. Before initiating the tests, 0 minute blood samples were withdrawn to estimate fasting glucose levels. For OGTT, glucose (Loba Chemie, Mumbai, India) was administered by an oral gavage (2 g/kg) and for IPITT, insulin (Human Actrapid®, 40 IU/ml, Novo Nordisk) was injected intraperitoneally (1 IU/kg). Plasma glucose was then measured according to manufacturers' recommendations using an ERBA Mannheim® commercial kit (Code No. 120200) at 30, 60, and 120 minutes for both the tests.

Biochemical analysis

Blood samples were collected via retro-orbital puncture after 8 and 16 weeks of feeding, plasma was separated and maintained at -20°C until further analysis. Activities of enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) and concentrations of glucose, total cholesterol, triglycerides (TG) high-density lipoprotein (HDL), and creatinine were measured in plasma. The low-density lipoprotein (LDL) was obtained via the Friedwald's formula. Plasma activity of enzyme and concentration of these analytes was measured by ERBA CHEM 7 Analyser (ERBA Diagnostics Mannheim GmbH, Germany) with ERBA Mannheim® commercial kits: ALT, 120207; AST, 120204; ALP, 120190; Cholesterol, 120194; TG, 120211; HDL, 120-664; and Creatinine, 120246. Plasma insulin (ELM-Insulin, RayBiotech, Inc. GA, USA), IL-6 (ELM-IL6-001, RayBiotech, Inc. GA, USA), TNF-α (KB2052, Krishgen Biosystems, Mumbai, India), IGF-1 (ELM-IGF1, RayBiotech, Inc. GA, USA) and FASN (DL-FASN-Mu, Wuxi Donglin Sci & Tech Development Co. Ltd., Jiangsu, PRC) levels were estimated by commercially avail-

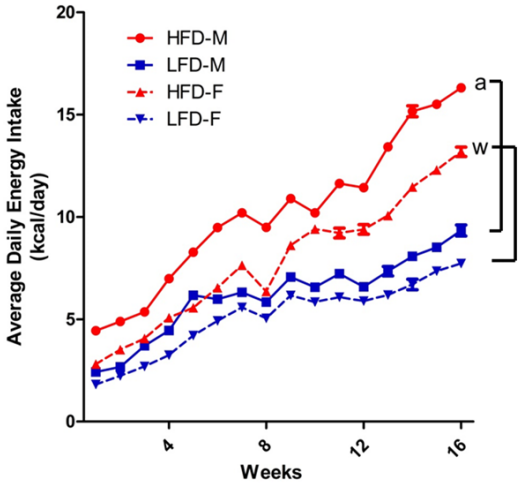

Figure 1. Average daily energy intake change with time for both groups. Male mice fed with high-fat diet (HFD-M; n=33), male mice fed with low-fat diet (LFD-M; n=14), female mice fed with high-fat diet (HFD-F; n=34) and female mice fed with low-fat diet (LFD-F; n=11) fed for 16 weeks. Values are Mean ± SEM, wherein labelled means without a common letter differ, P<0.05.

able ELISA kits according to the manufacturers' protocol using ERBA Microscan ELx800. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as (fasting plasma glucose × fasting plasma insulin/22.5) to assess insulin resistance. The fasting plasma glucose values of OGTT at 0 minute were considered for HOMA-IR calculations.

Hepatic lipid analysis

Approximately 200 mg of frozen liver from each group (n=6) was homogenized, and lipids were extracted using Folch's method [25]. The triglyceride levels in the liver were measured using ERBA Mannheim® commercial kit as described above.

Histological analysis

At the end of 16 week study, 6 representative mice from each group were deeply anesthetized (intraperitoneal sodium pentobarbital, 50 mg/kg) and blood samples were quickly withdrawn by cardiac puncture. These samples were then centrifuged (4000 rpm for 15 min at 4°C), plasma was separated and stored individually at -20°C until biochemical analyses were performed. Fat pads (gonadal, retroperitoneal) were carefully removed and weighed. Portions of liver, pancreas, heart, and kidney were formalin fixed (formaldehyde 10% w/v in 0.1 M phosphate buffer, pH 7.2) and paraffin embedded for H&E staining. The abnormalities in histological samples of mice fed with HFD were graded as: no abnormality detected (NAD), minimal (+), mild (++), moderate (+++) or severe (++++) changes.

The quantitative histological analysis was performed with ImageJ 1.48v (NIH, USA). A histogram of liver fat vacuoles size was plotted by initially converting the image in binary 8-bit mode, followed by calibration of the image. The image was then adjusted for its threshold and the particles size were analysed. The diameter (μm) of each vacuole was calculated using the formula (Area=3.14 × radius square).

Histology of pancreas was quantified by measuring the circumference of pancreatic islet in mice fed with HFD and LFD. The histology image was converted into binary 8-bit mode, followed by its calibration. Longer (A) and shorter (B) diameters of pancreatic islet were measured using freehand line. Circumference of the islet was calculated by formula [3.14 × Square Root of 2 × ((A/2) square + (B/2) square)].

Western blot analysis

Liver was homogenized in an extraction buffer as described by Lee [26], and 100 μg of protein were fractionated by SDS-PAGE on a 10% gel, and transferred to a PVDF membrane. Appropriate blocking solutions (5% BSA/5% non-fat milk) were added to the membrane for 1 hour. The membrane was incubated overnight with an anti-IGF1 (PA1746, Boster Biological Technology Ltd., USA) or anti-FASN (HPA006461, Sigma Aldrich, USA) diluted at 1:500. The immune complexes were detected by using the ECL western blotting detection kit. The ImageJ program was used for the densitometric analysis.

Data analysis

Data are displayed as mean ± SEM. Differences between the HFD and LFD were analysed with Student *t* test (GraphPad Prism 5, San Diego, CA, USA) and a *P* value ≤0.05 was considered as statistically significant.

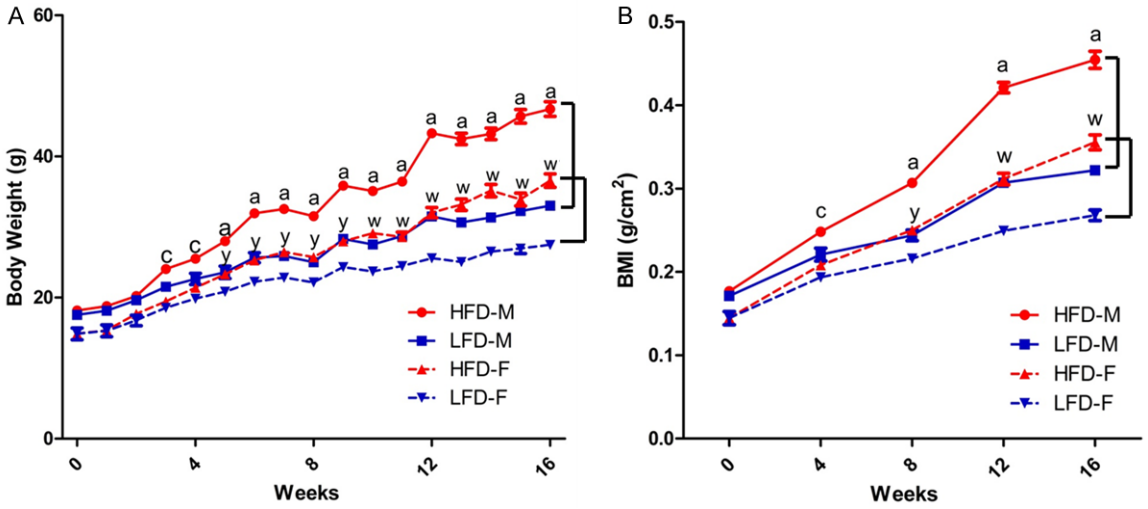

Figure 2. Male vs. female body weight and BMI changes with time. A. Body weight. B. BMI. Male mice fed with high-fat diet (HFD-M; n=33), male mice fed with low-fat diet (LFD-M; n=14), female mice fed with high-fat diet (HFD-F; n=34) and female mice fed with low-fat diet (LFD-F; n=11) fed for 16 weeks. Values are Mean ± SEM, wherein labelled means without a common letter differ, P<0.05.

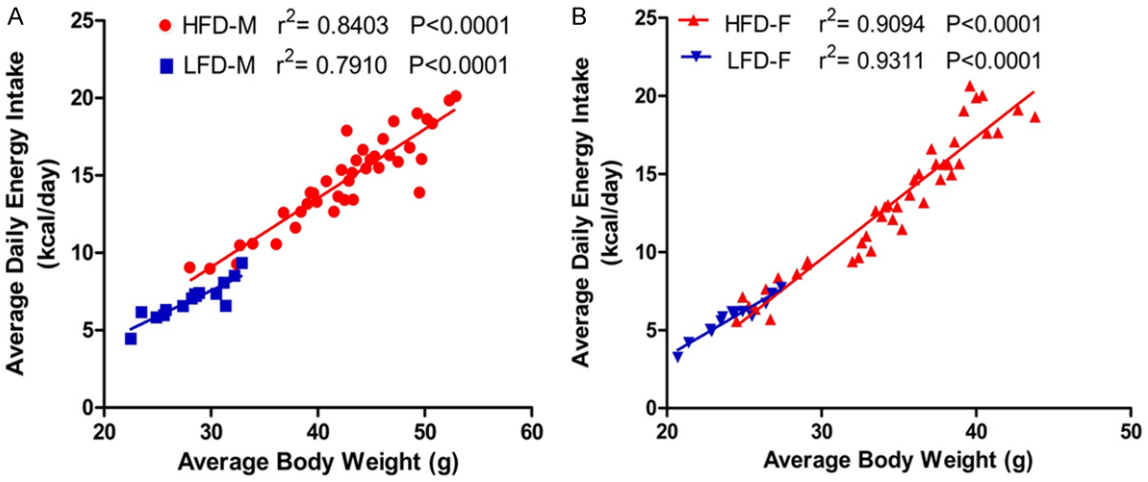

Figure 3. Correlation of energy intake and body weight. A. Male C57BL/6 mice. B. Female C57BL/6 mice.

Results

Physiological parameters estimation

Food and energy intake: Among both the sexes, the difference in daily food intake was not significant between mice fed with either HFD or LFD (data not shown). However in case of energy intake, there was a significant difference (all P<0.0001) between mice fed with HFD and LFD among both sexes (**Figure 1**).

Body weight and BMI

During 16 weeks of feeding, the average gain in body weight of HFD-M mice was 28.54 g and 21.69 g for HFD-F, versus 15.45 g for LFD-M and 12.63 g for LFD-F (**Figure 2A**).

The average change in BMI from week 0 to week 16 was found to be 0.28 g/cm^2 for HFD-M and 0.21 g/cm^2 in HFD-F, versus 0.15 g/cm^2 for LFD-M and 0.12 g/cm^2 for LFD-F (**Figure 2B**).

The energy intake during 16 weeks of study was significantly correlated with body weight among mice fed with HFD or LFD (**Figure 3A**, **3B**). The correlation was found to be stronger in female mice fed with either HFD or LFD wherein, HFD-F (r^2=0.90, P<0.0001), LFD-F (r^2=0.93, P<0.0001), HFD-M (r^2=0.84, P<0.0001) and LFD-M (r^2=0.79, P<0.0001). Based on physio-

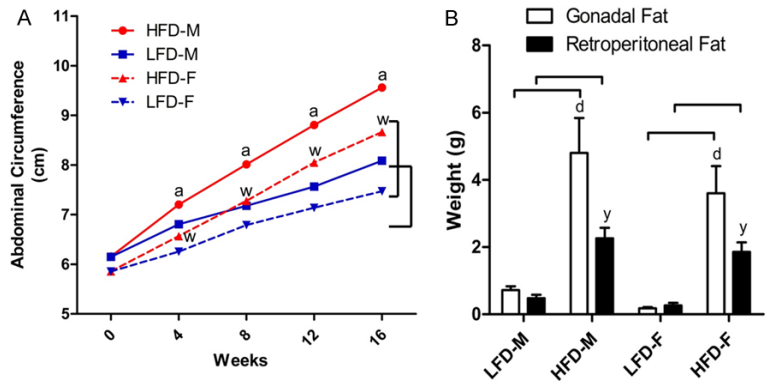

Figure 4. Male vs. female abdominal circumference changes and female fat pads weights with time. A. Abdominal circumference changes. Male mice fed with high-fat diet (HFD-M; n=33), male mice fed with low-fat diet (LFD-M; n=14), female mice fed with high-fat diet (HFD-F; n=34) and female mice fed with low-fat diet (LFD-F; n=11) fed for 16 weeks and; B. Fat pads weights at 16th week. n=6. Values are Mean ± SEM, wherein labelled means without a common letter differ, P<0.05.

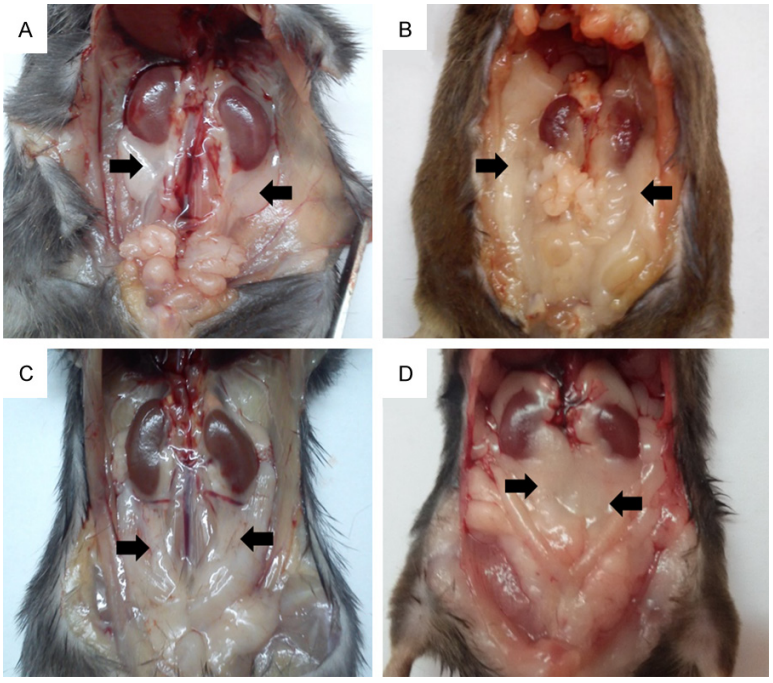

Figure 5. Fat pads of mice fed with LFD and HFD. A. Retroperitoneal fat of male mice fed with low-fat diet (LFD-M). B. Retroperitoneal fat of male mice fed with high-fat diet (HFD-M). C. Retroperitoneal fat of female mice fed with low-fat diet (LFD-F). D. Retroperitoneal fat of female mice fed with high-fat diet (HFD-F).

logical parameters HFD-M=5 and HFD-F=7 were found to be resistant to HFD.

Abdominal circumference and fat pads

The average gain in abdominal circumference from week 0 to week 16 was 3.41 cm for HFD-M and 2.81 cm for HFD-F, versus 1.93 cm for LFD-M and 1.62 cm for LFD-F (**Figure 4A**).

Gonadal fat pads were significantly heavier (P<0.05) in mice fed HFD, compared to mice fed with LFD. Likewise, the retroperitoneal fat were also significantly heavier (P< 0.01) in mice fed with HFD (**Figures 4B**, **5A-D**).

OGTT

At 8th week (**Figure 6A**), glucose intolerance was seen in both the sexes fed with HFD. For males on HFD, there was a significant difference (P= 0.0431) in plasma glucose at 0 minute when compared with LFD. Further, there was a significant difference (P< 0.01) in clearance of plasma glucose at 30 minutes, and (P≤0.001) at 60, and 120 minutes. For females on HFD, the difference in plasma glucose level at 0 minute was not significant (P=0.24). But, there was a significant difference (P<0.01) in clearance of plasma glucose at 30 and 60 minutes, and (P=0.0204) at 120 minutes.

At 16th week (**Figure 6B**), glucose intolerance was distinctly evident in both sexes fed with HFD. For males on HFD, there was a significant difference (P<0.001) in plasma glucose at 0 minute when compared with LFD. Further, there was a significant difference (P<0.001) in clearance of plasma glucose at 30 and 60 minutes, while (P≤0.0001) at 120 minutes. For females on HFD, there was a significant difference (P=0.0059) in plasma glucose at 0 minute. Further, there was a significant difference (P<0.001) in clearance of plasma glucose at 30, 60, and 120 minutes, when compared with mice fed with LFD. Thus, the time-course

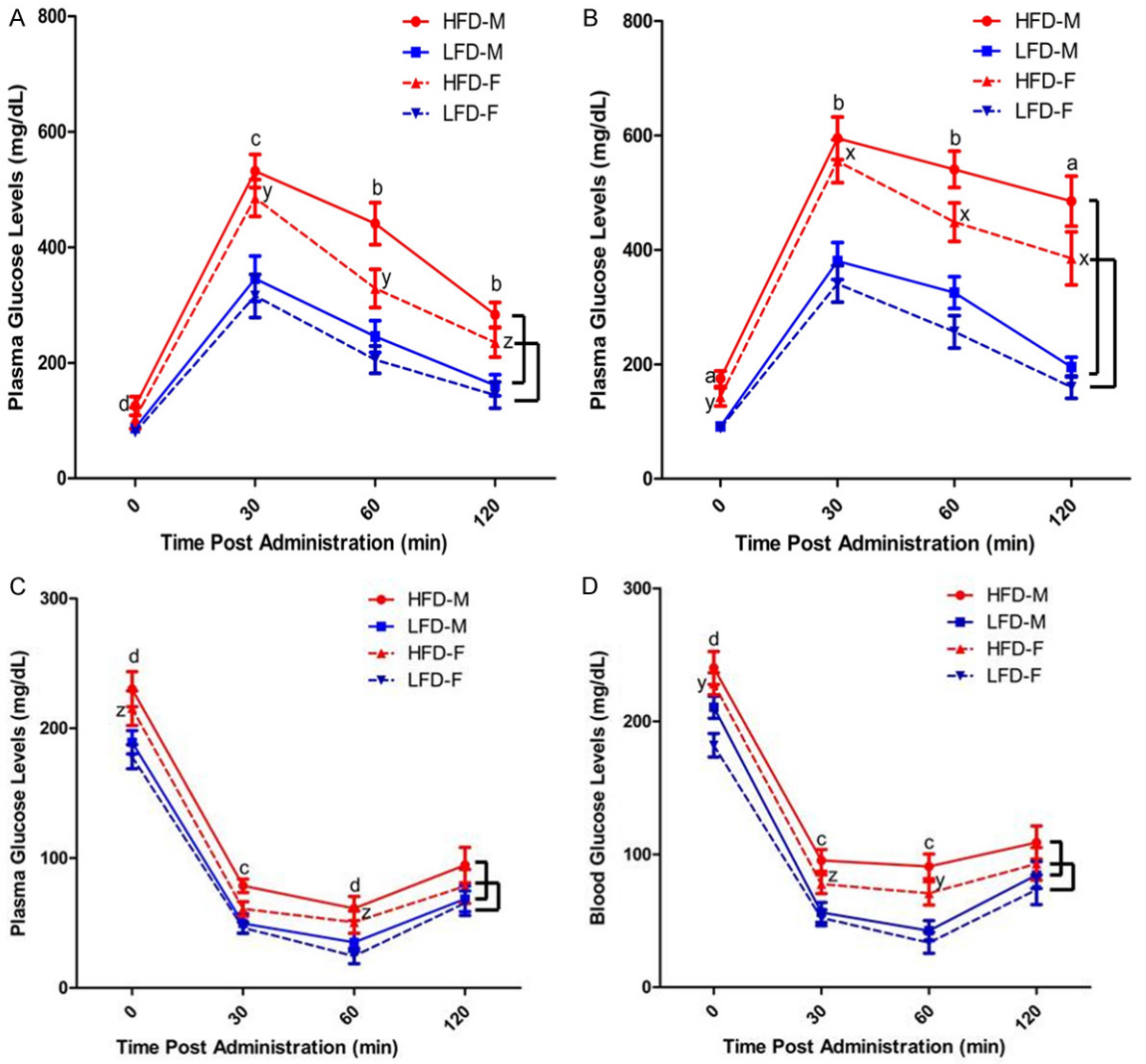

Figure 6. Plasma glucose concentration measured during glucose (OGTT) and insulin tolerance test (IPITT). A. OGTT at 8th week in male and female mice fed with HFD and LFD (n=7) and. B. OGTT at 16th week in male and female mice fed with HFD and LFD (n=7). C. IPITT at 8th week in male and female mice fed with HFD and LFD (n=7). D. IPITT at 16th week in male and female mice fed with HFD and LFD (n=7). Values are Mean ± SEM, wherein labelled means without a common letter differ, P<0.05.

of glucose clearance in mice fed with HFD was delayed as compared to mice fed with LFD, implying them as glucose intolerant.

IPITT

As demonstrated by plasma glucose levels at 8th week (**Figure 6C**), insulin resistance was observed more in males as compared to females fed with HFD. For males fed with HFD, there was a significant difference (P=0.0262) in plasma glucose at 0 minute. Further, there was a significant difference (P=0.0077) in plasma glucose at 30 minutes and (P=0.0462) at 60 minutes, but the difference at 120 minutes was not significant when compared with LFD. Females on HFD also demonstrated a significant difference in plasma glucose at 0 minute (P=0.0407) and 60 minutes (P= 0.0277), but the difference at 30 minutes (P=0.06) and 120 minutes (P=0.44) were not significant when compared with LFD.

At 16th week (**Figure 6D**), insulin resistance was apparently observed in both sexes fed with HFD. For males on HFD, there was a significant difference (P<0.01) in plasma glucose at 0 minute. Further, there was a significant difference

Table 1. Metabolic parameters of male and female C57BL/6 mice fed with either HFD or LFD at 8th week[1]

Parameter	Groups		HFD-M vs. LFD-M	Groups		HFD-F vs. LFD-F
	HFD-M	LFD-M	*P*-value	HFD-F	LFD-F	*P*-value
Fed Glucose (mg/dL)	268.43 ± 14.14	196.62 ± 16.98	P<0.01	253.28 ± 14.40	189.35 ± 16.48	P<0.05
Fasting Plasma glucose (mg/dL)	125.16 ± 16.12	87.35 ± 4.41	P<0.05	104.31 ± 18.39	79.73 ± 7.01	NS
HDL (mg/dL)	27.35 ± 3.96	46.63 ± 4.94	P<0.01	31.94 ± 4.77	51.98 ± 5.35	P<0.05
LDL (mg/dL)	129.72 ± 15.07	51.98 ± 12.38	P<0.01	103.85 ± 13.78	38.60 ± 11.87	P<0.01
TG (mg/dL)	141.53 ± 16.17	79.42 ± 10.02	P<0.01	109.31 ± 11.75	68.62 ± 7.913	P<0.05
Cholesterol (mg/dL)	185.41 ± 18.27	114.5 ± 16.32	P<0.05	167.62 ± 16.90	104.34 ± 14.80	P<0.05

[1]Values are Mean ± SEM, n=8. HFD-M, male mice fed with high-fat diet; LFD-M, male mice fed with low-fat diet; HFD-F, female mice fed with high-fat diet; LFD-F, female mice fed with low-fat diet; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides.

Table 2. Metabolic parameters, hepatic and renal function of male and female C57BL/6 mice fed with either HFD or LFD at 16th week[2]

Parameter	Groups		HFD-M vs. LFD-M	Groups		HFD-F vs. LFD-F
	HFD-M	LFD-M	*P*-value	HFD-F	LFD-F	*P*-value
Fed Plasma glucose (mg/dL)	279.24 ± 11.31	207.24 ± 14.07	P<0.001	270.66 ± 12.5	194.19 ± 13.96	P<0.01
Fasting Plasma glucose (mg/dL)	175.13 ± 13.28	91.32 ± 3.23	P<0.001	143.16 ± 15.83	88.35 ± 4.42	P<0.01
HOMA-IR	298.50 ± 30.84	91.41 ± 10.30	P<0.01	196.79 ± 22.93	75.94 ± 9.13	P<0.01
HDL (mg/dL)	22.54 ± 3.21	41.69 ± 3.66	P=0.001	26.58 ± 3.50	46.21 ± 4.78	P<0.01
LDL (mg/dL)	172.29 ± 22.03	64.48 ± 13.99	P<0.001	141.72 ± 21.61	48.60 ± 11.57	P<0.01
TG (mg/dL)	276.73 ± 37.07	101.99 ± 19.23	P<0.001	249.88 ± 34.70	92.25 ± 17.73	P<0.001
Cholesterol (mg/dL)	250.18 ± 24.75	126.57 ± 18.71	P<0.001	218.28 ± 22.72	109.26 ± 15.09	P<0.001
AST (IU/L)	168.30 ± 17.84	79.89 ± 9.94	P<0.001	155.79 ± 15.15	84.53 ± 10.78	P<0.01
ALT (IU/L)	78.82 ± 9.34	33.06 ± 6.66	P<0.001	64.64 ± 8.85	25.35 ± 5.09	P<0.01
ALP (IU/L)	292.01 ± 30.82	174.82 ± 16.96	P<0.01	302.41 ± 34.02	203.84 ± 21.05	P<0.05
Creatinine (mg/dL)	0.499 ± 0.13	0.276 ± 0.05	NS	0.421 ± 0.10	0.226 ± 0.04	NS

[2]Values are Mean ± SEM, n=10. HFD-M, male mice fed with high-fat diet; LFD-M, male mice fed with low-fat diet; HFD-F, female mice fed with high-fat diet; LFD-F, female mice fed with low-fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

in plasma glucose levels at 30 minutes (P= 0.0041) and 60 minutes (P=0.0016), but the difference at 120 minutes was not significant (P=0.15). For females on HFD, there was a significant difference in plasma glucose at 0 minute (P<0.05), 30 minutes (P=0.0171) and 60 minutes (P=0.0082), whereas the difference was not significant (P=0.27) at 120 minutes when compared with LFD.

Biochemical analysis

At 8th week (**Table 1**), within males there was a significant rise (P=0.0058) in fed state plasma glucose level of mice. Similarly within females, there was a significant rise (P=0.0112) in plasma glucose level of mice fed with HFD as compared to LFD. Lipid profile analysis revealed that among males, mice fed with HFD had a significant difference (P<0.01) in HDL, LDL, and TG level as compared to mice fed with LFD. Difference in cholesterol level was also significant (P<0.05) in male mice fed with HFD. Whereas in females, mice fed with HFD had a significant difference (P<0.05) in HDL, triglyceride, and cholesterol level as compared to mice fed with LFD. LDL was also significantly elevated (P<0.01) in HFD-F.

At 16th week (**Table 2**), there was a significant difference in fed state plasma glucose levels of HFD-M (P=0.0009) and HFD-F (P=0.0011) when compared to their respective counterparts. In terms of HOMA-IR, within both the sexes, mice fed with HFD had developed a significant insulin resistance (P<0.01) when compared with mice fed with LFD. Lipid profile analysis revealed that among males, mice fed with

Table 3. Plasma insulin and cytokines levels of male and female C57BL/6 mice fed with either HFD or LFD at 16th week[3]

Parameter	Groups		HFD-M vs. LFD-M	Groups		HFD-F vs. LFD-F
	HFD-M	LFD-M	*P*-value	HFD-F	LFD-F	*P*-value
Insulin (μIU/ml)	38.35 ± 3.41	22.52 ± 2.72	P<0.01	30.93 ± 2.33	19.34 ± 1.69	P<0.05
IL-6 (pg/ml)	82.34 ± 19.65	39.97 ± 5.62	P<0.05	56.98 ± 14.22	27.68 ± 4.35	P<0.05
TNF-α (pg/ml)	63.93 ± 17.61	28.48 ± 7.55	P<0.05	46.22 ± 11.34	19.46 ± 4.21	P<0.05
IGF-1 (pg/ml)	1754.25 ± 157.27	1221.56 ± 91.94	P<0.05	1593.85 ± 142.33	1142.68 ± 74.10	P<0.05
FASN (ng/ml)	5.35 ± 0.58	2.57 ± 0.26	P<0.05	4.05 ± 0.44	2.17 ± 0.18	P<0.05

[3]Values are Mean ± SEM, n=6. HFD-M, male mice fed with high-fat diet; LFD-M, male mice fed with low-fat diet; HFD-F, female mice fed with high-fat diet; LFD-F, female mice fed with low-fat diet; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IGF-1, Insulin-like growth factor-1; FASN, Fatty acid synthase.

HFD had a significant difference (P≤0.001) in HDL, LDL, TG and cholesterol level as compared to mice fed with LFD. Whereas in females, mice fed with HFD had a significant difference (P<0.001) in TG and cholesterol level as compared to mice fed with LFD. Difference in HDL and LDL was also significant (P=0.0039 and 0.0013 respectively) in female mice fed with HFD. Liver function implying tests such as AST and ALT level were significantly elevated (P< 0.001) in male mice fed with HFD as compared LFD. Further there was also a significant difference (P=0.0037) in ALP level in male mice fed with HFD as compared LFD. In female mice fed with HFD, there was a significant difference (P<0.01) in AST and ALT level as compared to mice fed with LFD. A significant difference (P=0.0241) in ALP level was also observed in female mice fed with HFD. The difference in creatinine level was not significant in either sex of mice, irrespective of diet fed.

Within both the sexes, there was a significant escalation in insulin level of mice fed with HFD (P<0.01 for HFD-M and P<0.05 for HFD-F) when compared with mice fed with LFD. The levels of IL-6 and TNF-α, were significantly elevated (P<0.05) in mice fed with HFD, implying development of pro-inflammatory condition. Concomitant with these metabolic abnormalities, IGF-1 and FASN level were also found to be proportionally elevated (P<0.05) in mice fed with HFD (**Table 3**).

Hepatic lipid analysis

Among both the sexes, there was a significant gain (P<0.01) in liver weight of mice fed with HFD as compared to LFD (**Figure 7A**, **7C-F**). The hepatic triglyceride level revealed that there was a significant (P<0.01) deposition of triglycerides in mice fed with HFD as compared to LFD (**Figure 7B**).

Histological examination

Histological analysis of liver sections revealed that the mice fed with HFD had moderate fatty changes in terms of fat vacuoles in hepatocytes as compared to mice fed with LFD. Further, mild focal swelling, granular, and vacuolar changes in cytoplasm of hepatocytes were observed in mice fed with HFD. Also, there were mild degenerative changes in hepatocytes along with moderate necrotic changes accompanied by minimal bile duct hyperplasia. These abnormalities were absent in mice fed with LFD. There was no infiltration of mononuclear cells (MNC) or microgranuloma lymphoid aggregation observed in mice fed with either HFD or LFD (**Figure 8A-E**; Table S1). As computed by ImageJ, the vacuole size (mean ± SEM) was found to be 30.49 ± 1.48 and 27.76 ± 1.59 in HFD-M and HFD-F, respectively. The vacuole size distribution in liver sections of HFD-M and HFD-F is depicted in **Figure 8C**, **8F**.

Mice fed with HFD revealed mild changes in pancreatic islets, which had larger diameter as compared to mice fed with LFD. This was further confirmed by ImageJ software. There was a significant difference (P<0.01) in circumference of LFD-M and HFD-M, which was found to be 1162.56 ± 65.61 and 1750.14 ± 94.65 respectively. In case of females too, there was a significant difference (P<0.05) in circumference of LFD-M and HFD-M, which was found to be 1004.99 ± 74.02 and 1524.18 ± 110.82 respectively. There was no infiltration of MNC in mice fed with either HFD or LFD. Further, mice fed with HFD-M showed mild degenerative and

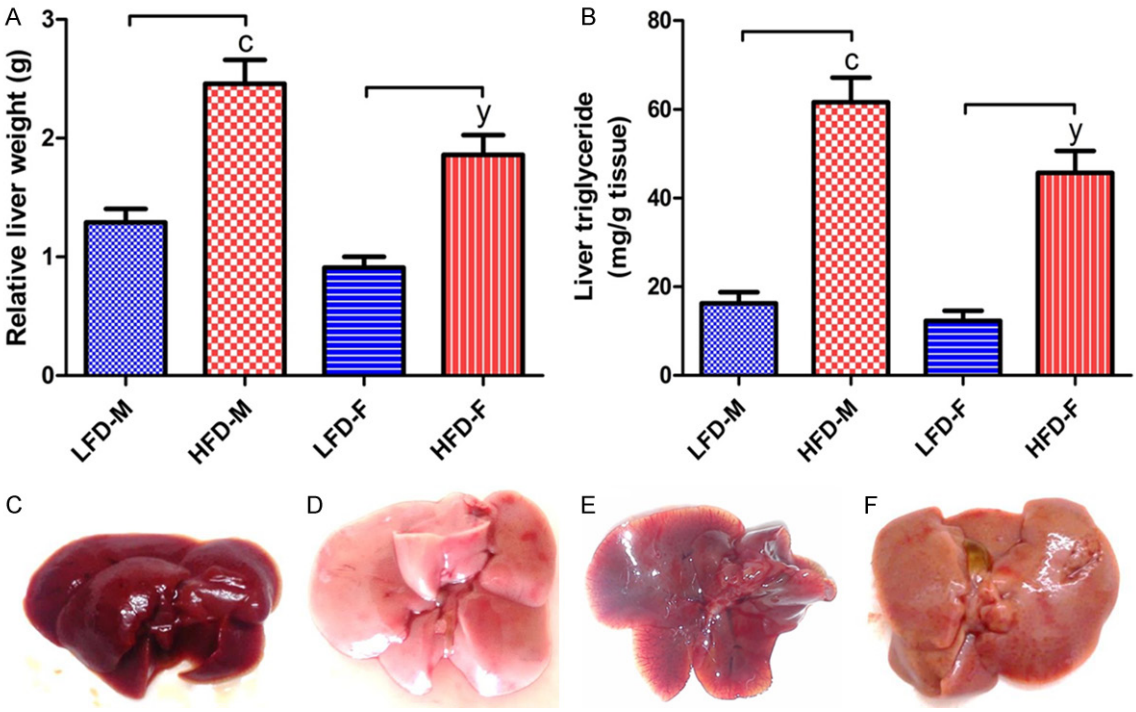

Figure 7. Hepatic lipid analysis showing non-alcoholic steatohepatitis at 16th week. A. Relative liver weight determination (n=6). B. Liver triglyceride determination (n=6). C. Liver of male mice fed with low-fat diet (LFD-M). D. Liver of male mice fed with high-fat diet (HFD-M). E. Liver of female mice fed with low-fat diet (LFD-F). F. Liver of female mice fed with high-fat diet (HFD-F). Values are Mean ± SEM, wherein labelled means without a common letter differ, $P<0.05$.

necrotic changes in β-cell of islet, whereas HFD-F showed minimal changes for the same (**Figure 9A-D**; Table S2).

Histological analysis of kidney showed that there were mild focal changes in glomerular size, lysis of glomerular tuft, atrophy, and hypercellularity in mice fed with HFD. Further, there was mild tubulointerstitial fibrosis along with necrotic changes in tubular epithelium. This was also accompanied by mild infiltration of MNC, focal tubular swelling, vacuolar changes in renal tubules, tubular degeneration, sloughing of tubular epithelium, and presence of cast in lumen of tubules in mice fed with HFD (**Figure 10A-D**; Table S3).

Histology of heart showed mild degenerative changes in cardiac fibres, loss of striations, and swelling of cardiac fibres in mice fed with HFD. Further, there were minimal necrotic and coagulative changes of cardiac muscle with loss of nucleus. These abnormalities were not seen in mice fed with LFD. There was no infiltration of MNC in mice fed with either HFD or LFD (**Figure 11A-D**; Table S4).

Western blot analysis

Our western blot analysis revealed that HFD increases FASN level by approximately 2.5-fold in male mice while 2-fold in female mice, when compared to their respective LFD counterparts (**Figure 12A**, **12C**). Similarly in case of IGF-1, mice fed with HFD had increased IGF-1 level by approximately 2-fold in male mice while 1.5-fold in female mice, when compared to their respective LFD counterparts (**Figure 12A**, **12B**). This phenomenon was observed in spite of fact that, male and female mice were age-matched and were fed with same diet for a period of 16 weeks.

Discussion

In this model, the high-fat in diet supplied surplus energy (60% kcal from fat) than required by the animal. This surplus energy enhanced the hyperplasia as well as the size of adipocytes [27]. Thus, mice fed with HFD displayed a gradual increase in body weight, BMI, abdominal fat deposition, and abdominal circumfer-

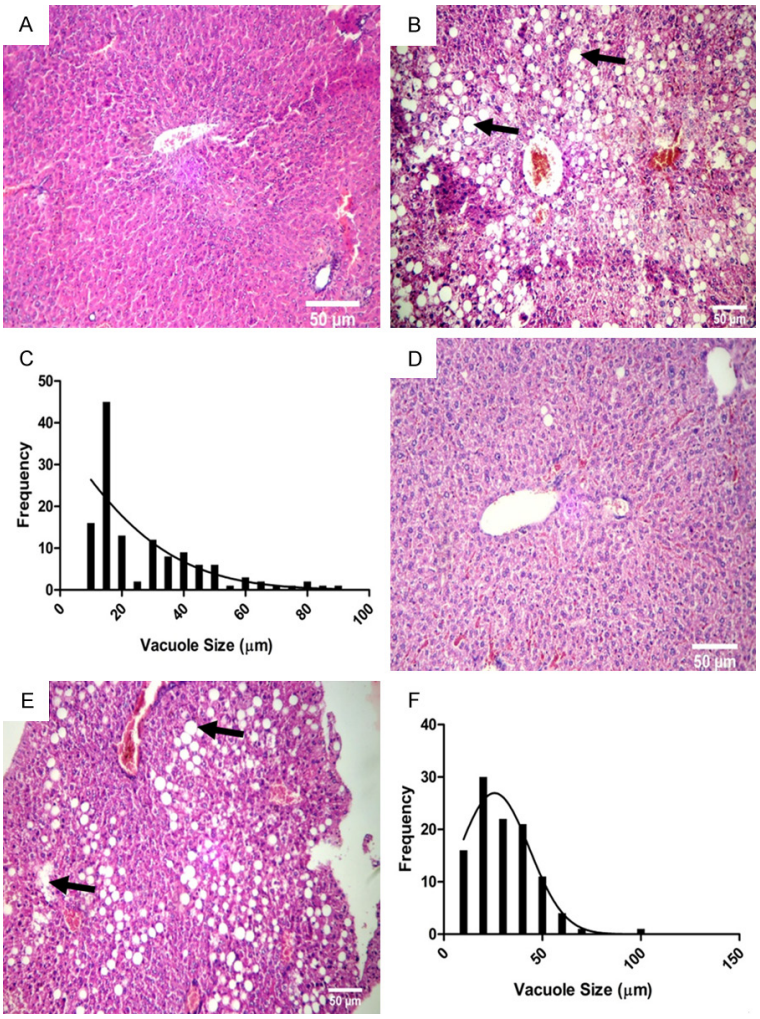

Figure 8. Fat deposition in liver showing non-alcoholic steatohepatitis at 16th week. Hematoxylin and eosin staining of hepatocytes (×40) showing hepatocytes with enlarged fat vacuoles (marked with arrow). A. Male mice fed with low-fat diet (LFD-M). B. Male mice fed with high-fat diet (HFD-M). C. Histogram of vacuole size in male mice fed with high-fat diet (HFD-M). D. Female mice fed with low-fat diet (LFD-F). E. Female mice fed with high-fat diet (HFD-F). F. Histogram of vacuole size in female mice fed with high-fat diet (HFD-F).

ence. There were sex specific variations observed in physiological parameters in mice fed with HFD. We found that the physiological parameters monitored from week 0 to week 16 were augmented in mice fed with HFD as compared to mice fed with LFD, but the extent of augmentation was lower in HDF-F when compared with HDF-M. The possible mechanism includes aggravation of insulin resistance by both IGF-1 and FASN which depends on the energy intake [16]. In our study, this effect was seen to be proportional. As observed, HFD fed male and female mice had FASN up-regulation by 2.5-fold and 2-fold, respectively. Similarly, HFD fed male and female mice had IGF-1 up-regulation by 2-fold and 1.5 fold, respectively at the end of 16th week. The HOMA-IR index at 16th week supported our hypothesis, wherein HFD-M had an index of 298.50 ± 30.84 and HFD-F had an index of 196.79 ± 22.93. Other factors which may have contributed differences includes estrogen level [28], sex-specific leptinlevels[29,30]anddifferences in gross locomotor activity [31], although these parameters were not evaluated in this study. In agreement to Koza *et al.* [32, 33], our data demonstrates a significant correlation between energy intake and average body weight.

NAFLD reflects hepatic manifestation of MetS [34]. Our data supports Day and James [35] "two-hit hypothesis" which explains the development of NAFLD, and the progression from simple steatosis to non-alcoholic steatohepatitis (NASH). The "first hit" is the escalated lipid storage in hepatocytes and insulin resistance which are the key pathogenic factor for the progression of hepatic steatosis. The "second hit" leads to hepatocyte injury, inflammation, and fibrosis. Factors which are responsible for the second hit are oxidative stress, proinflammatory cytokines, adipokines, and mitochondrial dysfunction. Our data showed that the mice fed with HFD had moderate fatty changes in terms of fat vacuoles in hepatocytes supporting the "first hit" hypothesis. This was associated with marked changes in hepatic TG level along with plasma LDL, HDL, TG and cholesterol level in mice fed with HFD. Further, the biomarkers of liver function such as AST, ALT and ALP were also significantly elevated in mice fed with HFD suggesting impaired hepatocytes functioning. Mice fed with HFD had aug-

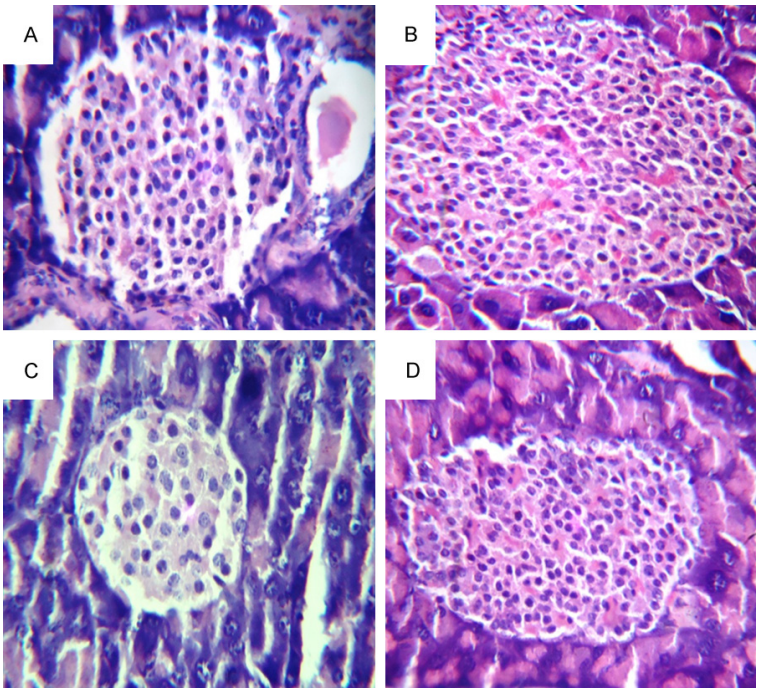

Figure 9. Hematoxylin and eosin staining of pancreas (×40) showing increased islet size at 16th week. A. Male mice fed with low-fat diet (LFD-M). B. Male mice fed with high-fat diet (HFD-M). C. Female mice fed with low-fat diet (LFD-F). D. Female mice fed with high-fat diet (HFD-F).

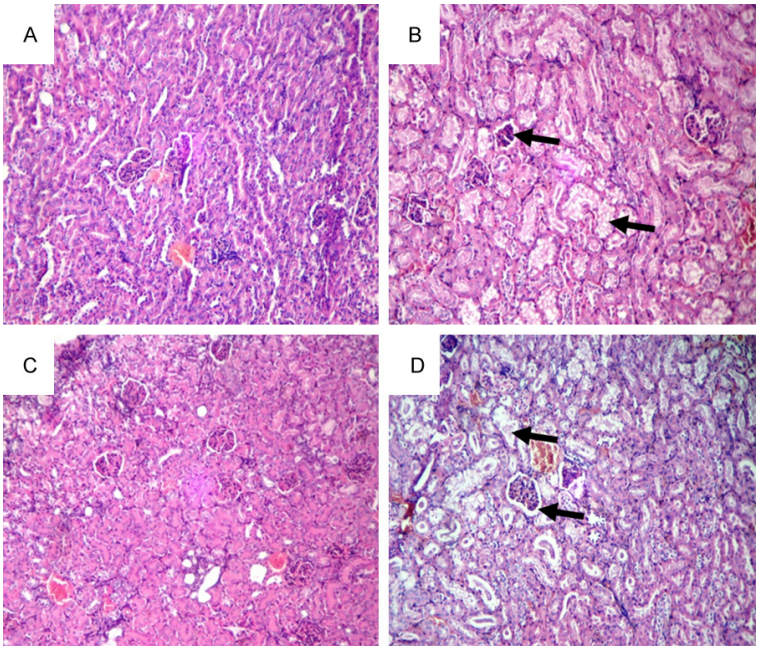

Figure 10. Mild tubulointerstitial fibrosis of kidney showing initiation of kidney impairment at 16th week. Hematoxylin and eosin staining of kidney (×40) showing pathological changes (marked with arrow). A. Male mice fed with low-fat diet (LFD-M). B. Male mice fed with high-fat diet (HFD-M). C. Female mice fed with low-fat diet (LFD-F). D. Female mice fed with high-fat diet (HFD-F).

mentation in insulin resistance as evidenced by OGTT, IPITT, HOMA-IR and plasma insulin level; however the magnitude of insulin resistance was less in female mice fed with HFD. Again, this difference may be due to variation in energy intake as discussed above, which ultimately had its effect on FASN and IGF-1 levels. Infiltration of MNC or microgranuloma lymphoid aggregation was absent in mice fed with HFD suggesting that "second hit" of the hypothesis did not initiate. Furthermore, the hypothesis was supported by our data which showed mild escalation in proinflammatory cytokines such as IL-6 and TNF-α. Other parameters like oxidative stress, adipokines level, and mitochondrial dysfunction were not performed.

The functional state of the pancreas plays a remarkable role in development of the basic components of MetS that includes hyperinsulinemia, insulin resistance, and impaired glucose tolerance. Inversely, the existing metabolic changes such as obesity and dyslipidemia aggravate the impairment of pancreatic functions [36]. Further, as described by Dr. Reaven in his Banting lecture [37], "deterioration of glucose tolerance can only be prevented if the β-cell is able to increase its insulin secretory response and maintain a state of chronic hyperinsulinemia". Mice fed with HFD showed significant impairment in glucose tolerance. To compensate this impairment, pancreatic cells underwent hypertrophy in terms of enlarged islet diameter and increased number of β-cells within the islets. These altera-

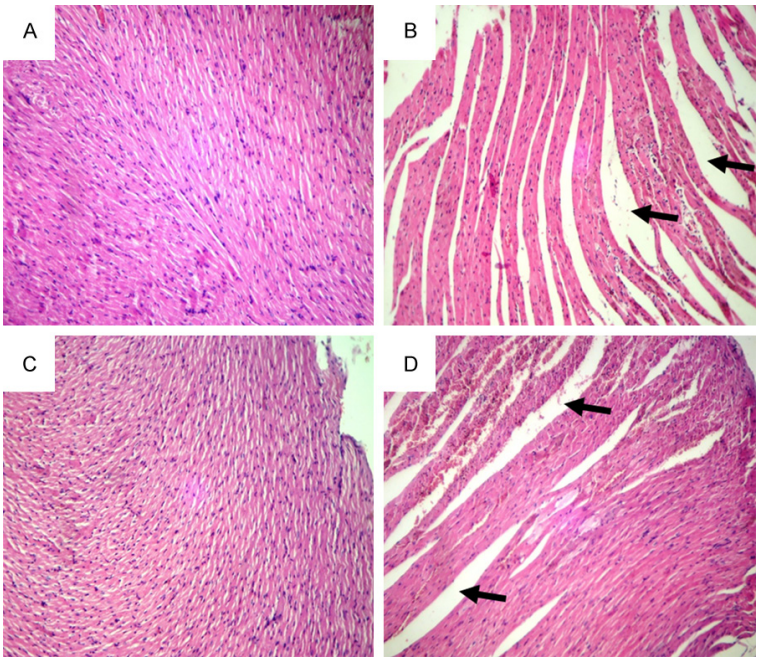

Figure 11. Hematoxylin and eosin staining (×40) of heart at 16th week. A. Male mice fed with low-fat diet (LFD-M). B. Male mice fed with high-fat diet (HFD-M). C. Female mice fed with low-fat diet (LFD-F). D. Female mice fed with high-fat diet (HFD-F).

tions led to state of chronic hyperinsulinemia. Ai *et al.* have correlated enlarged islet mass with existence of insulin resistance [38]. As demonstrated by IPITT and HOMA-IR, mice fed with HFD developed a significant insulin resistance. The functions of pancreas were also compromised by dyslipidemia condition as evidenced by LDL, HDL, TG, and cholesterol levels. Overall our data suggests that the mice were in pre-diabetic stage, which may have progressed to severe metabolically altered state on continued feeding of the HFD diet.

Since MetS is a cluster of several metabolic abnormalities, it is difficult to identify the exact cause for perturbations in glomerular hemodynamics. It has been demonstrated by Chagnac *et al.* [39] that obesity is associated with increased renal plasma flow (RPF) and glomerular filtration rate (GFR). Henegar *et al.* [40] also demonstrated that a feeding HFD to dogs causes metabolic abnormalities which led to structural changes in the kidney. As seen from our histology data, there was mild tubulointerstitial fibrosis in the kidney of mice fed with HFD diet, suggesting that kidney damage was initiated. This hypothesis was supported by the data of GFR assessment by plasma creatinine levels, which showed non-significant difference in mice fed with HFD and LFD.

In agreement with Noyan-Ashraf *et al.* [41], our data suggests the absence of cardiac remodelling, despite of the metabolic perturbations and significant increase in body weight in mice fed with HFD. Histologically heart showed mild degenerative changes in cardiac fibres, loss of striations and swelling of cardiac fibres in mice fed with HFD.

To summarize, chronic positive energy balance in male and female mice led to increase in body weight, BMI and central adiposity. These physiological changes caused concomitant rise in plasma glucose and lipid levels, resulting in compensatory hyperinsulinemia conjugated with pro-inflammatory conditions. Insulin resistance in major organ such as liver and adipose tissue is known to produce higher free fatty acid via up-regulation of FASN [16, 17]. Increase in FASN, insulin level and body fat together are known to decrease binding protein of IGF-1, leading to increased bioavailability of IGF-1 [18, 19]. Rise in IGF-1 and insulin, is known to reversibly up-regulate transcription of FASN gene [20, 21]. Overall, it appears to be a vicious cycle between insulin resistance, FASN and IGF-1 which aggravates each other. Ultimately, these metabolic abnormalities are known to pre-dispose other metabolically related disorders such as type 2 diabetes, NAFLD and even cancer [16, 18, 20].

Conclusion

In conclusion, prolong exposure of HFD in age-matched male and female C57BL/6 mice showed variations in physical development wherein, male mice had greater body weight, BMI, central adiposity, and energy intake as compared to female mice. Further, both male and female C57BL/6 mice fed with HFD developed features of NASH, hyperinsulinemia, dys-

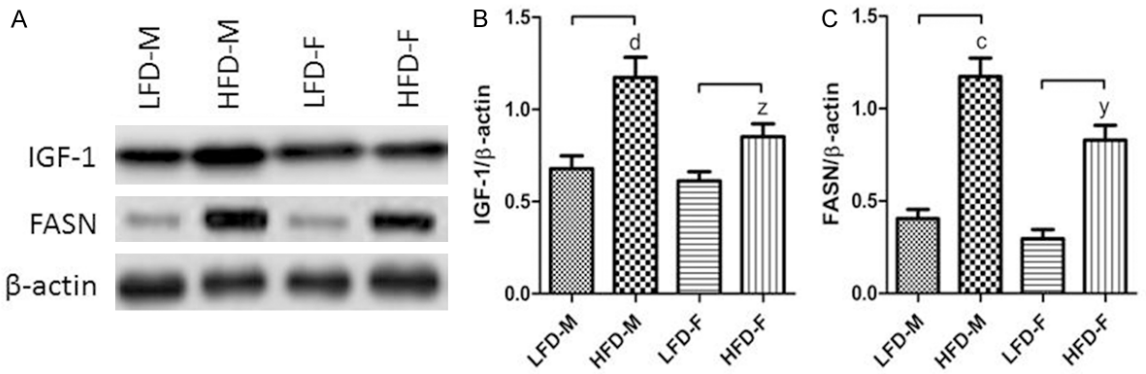

Figure 12. Western blot analysis of IGF-1 and FASN in male and female C57BL/6 mice. A. Protein level of IGF-1 and FASN. B. Densitometry plots of IGF-1. C. Densitometry plots of FASN. Values are mean ± SEM, wherein labelled means without a common letter differ, $P<0.05$.

lipidemia, impaired glucose and insulin tolerance but the magnitude of these abnormalities was found to be less in female mice. This variation in magnitude could be attributed to the difference in energy intake and ultimately its effect on FASN and IGF-1 levels. Researchers should thus take into consideration this correlation between energy intake and changes in levels of FASN and IGF-1 among both the sexes in diet induced metabolic abnormalities.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Mukesh Nandave, Department of Pharmacology, SPP School of Pharmacy & Technology Management, SVKM'S NMIMS, Vile Parle (W), Mumbai-400056, India. Tel: +91-2242332000; Fax: +912226185422; E-mail: mukeshnandave@gmail.com; Shreesh Ojha, Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, P O Box - 17666, United Arab Emirates University, Al Ain, UAE. Tel: - 971-3-7137524, Fax: - 971-3-7672033; E-mail: shreeshojha@uaeu.ac.ae

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Table S1. Histological analysis of liver[4]

Group (n=6)	Sinusoidal congestion and haemorrhages	Focal cellular swelling, granular and vacuolar changes in cytoplasm of hepatocytes	Degenerative changes in hepatocytes	Necrotic changes in hepatocyte, and loss of nuclei	Infiltration of mononuclear cells (MNC), Microgranuloma, lymphoid aggregation	Bile duct hyperplasia	Fatty changes in hepatocytes	Overall pathological grade (Lesion Score)
HFD-M	++	++	++	+++	NAD	+ (focal)	+++	Moderate (+++)
LFD-M	+ (focal)	+ (focal)	NAD	NAD	NAD	NAD	NAD	NAD
HFD-F	++	++	++	+++	NAD	+ (focal)	++	Moderate (+++)
LFD-F	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD

[4]Histological grading as per pathologist: No Abnormality Detected (NAD), Minimal changes (+), Mild changes (++), Moderate changes (+++), Severe changes (++++).

Table S2. Histological analysis of pancreas[5]

Group (n=6)	Loss of histological architecture of pancreas	Congestion, haemorrhages in exocrine and endocrine glands	Increased number of β-cells in islet, and subsequent change in islet size	Degenerative and necrotic changes in β-cells of islets	Infiltration MNC, Microgranuloma, lymphoid aggregation	Overall pathological grade (Lesion Score)
HFD-M	NAD	+ (focal)	+++	++	NAD	Moderate (+++)
LFD-M	NAD	NAD	NAD	NAD	NAD	NAD
HFD-F	NAD	+ (focal)	+++	+	NAD	Moderate (+++)
LFD-F	NAD	NAD	NAD	NAD	NAD	NAD

[5]Histological grading as per pathologist: No Abnormality Detected (NAD), Minimal changes (+), Mild changes (++), Moderate changes (+++), Severe changes (++++).

Table S3. Histological analysis of kidney[6]

Group (n=6)	Congestion and haemorrhages in renal parenchyma	Focal tubular swelling, granular and vacuolar changes in renal tubules	Tubular degeneration, sloughing of tubular epithelium, presence of cast in lumen of tubules	Necrotic changes of tubular epithelium	Infiltration of MNC	Glomerular changes (Size, Atrophy, hypercellularity, etc)	Tubulo-interstitial fibrosis	Overall pathological grade (Lesion Score)
HFD-M	++	++	++	++	++	+ (focal)	++	Mild (++)
LFD-M	+ (focal)	NAD	NAD	NAD	NAD	NAD	NAD	NAD
HFD-F	++	++	++	+	+	+	++	Mild (++)
LFD-F	+ (focal)	NAD	NAD	NAD	NAD	NAD	NAD	NAD

[6]Histological grading as per pathologist: No Abnormality Detected (NAD), Minimal changes (+), Mild changes (++), Moderate changes (+++), Severe changes (++++).

Table S4. Histological analysis of heart[7]

Group (n=6)	Congestion and haemorrhages in between cardiac muscle fiber	Break in length of cardiac muscle fibers	Degenerative changes in cardiac fibers, loss of striations, swelling of cardiac fibers	Necrotic and coagulative changes of cardiac muscle, loss of nucleus	Infiltration MNC	Overall pathological grade (Lesion Score)
HFD-M	++	++	++	+	NAD	Mild (++)
LFD-M	NAD	NAD	NAD	NAD	NAD	NAD
HFD-F	+	+	+	+	NAD	Minimal (+)
LFD-F	+ (focal)	NAD	NAD	NAD	NAD	NAD

[7]Histological grading as per pathologist: No Abnormality Detected (NAD), Minimal changes (+), Mild changes (++), Moderate changes (+++), Severe changes (++++).