



Research Article

Comparison of Three Different Diet-Induced Non Alcoholic Fatty Liver Disease Protocols in Rats: A Pilot Study

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ABSTRACT

Background: There are many methods for inducing non-alcoholic fatty liver disease (NAFLD) in experimental animals. Due to the diversity of these methods and different variables involved in choosing the appropriate one, this study aimed to examine the effect of three different diets on development of NAFLD in rats.

Methods: Twelve rats were divided to receive a standard, high fat high fructose (HFHFr), high cholesterol high fructose (HCHFr) or high fat high sucrose diet (HFHS); with access to tap water, fructose or sucrose solutions. The liver histopathological and biochemical assessments were examined after 40 and 60 days.

Results: According to the histological findings, after 60 days of dietary exposures, all three experimental groups showed evidence of fatty changes; however a higher grade of ballooning and NAFLD activity score was found in the HFHFr compared with the other groups. Furthermore, all three diets induced a non-significant increase in serum liver enzymes relative to the control diet.

Conclusion: This study indicates that HFHFr diet induce higher grade of hepatic steatosis and ballooning degenerations after 60 days in comparison with the other groups. So HFHFr diet can be considered as a suitable method for inducing of fatty liver for nutritional and pharmacological studies.

Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) which considered as an important manifestation of the metabolic syndrome and obesity, is the most common form of chronic liver disease in the Western world.¹ This disease is a spectrum of fat-associated liver conditions ranging from steatosis to steatohepatitis which can lead to fibrosis, cirrhosis, and even hepatocellular carcinoma.² NAFLD is defined as the accumulation of lipids within hepatocytes, primarily in the form of triglycerides, without alcohol abuse and with the exclusion of other known causes of steatosis such as total parenteral nutrition, rapid weight loss, acute starvation, abdominal surgery (extensive small bowel resection, biliopancreatic diversion, jejunoileal bypass), drugs or toxins (amiodarone, tamoxifen, glucocorticoids, estrogen, antiretroviral agents, tetracycline), abetalipoproteinemia, lipodystrophy, and Wilson's disease).^{3,4} Excessive lipid accumulation is caused by increasing in the mobilization of fatty acids from adipose tissue, hepatic synthesis of fatty acids, triglyceride production and reducing in fatty acid

oxidation due to imbalanced influx vs. removal of lipids in the liver.²

The prevalence of the disease has increased during the last decades probably because of the increased detection rate and risk factors and the growing epidemic of obesity and diabetes. Key risk factors including obesity, insulin resistance, sedentary lifestyle and altered dietary pattern, as well as genetic factors and disturbances of the intestinal barrier function have been identified in the recent years.^{5,6} Estimates of the global prevalence of NAFLD ranges from 6.3% to 33% with average of 20% in the general population, based on variety of assessment methods,⁷ and 30–50% in patients with diabetes.⁸

The pathogenesis of NAFLD has not been completely clarified, but a currently popular hypothesis, the “two-hit” hypothesis, proposed by Day et al. (1998) is widely accepted as the pathogenesis of NAFLD/NASH (Nonalcoholic Steatohepatitis). Accordingly, NASH development requires a double hit, the ‘1st hit’ causes lipid accumulation in hepatocytes, and the ‘2nd hit’ causes inflammation and fibrosis.⁹ Fat accumulation in

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the liver is associated with metabolic disorders related to central obesity and insulin resistance.¹⁰

Nowadays, we still do not have an effective and consensus therapeutic option for NAFLD except lifestyle modification including weight reduction diets and exercise.⁶ Due to limitations of human studies on NAFLD/NASH (such as ethical limitations in administering drugs, use of liver biopsy and the long period of development and progression of disease), animal models can give definitive information, not only in clarifying the pathogenesis of NAFLD but also in examining therapeutic effects of numerous agents. Several studies have been aimed to produce an ideal animal model of NAFLD by different methods like genetic, dietary, and combination models.^{11,12} There is no ideal animal model, so it is important to select the best method that conforms to the aim of the study regarding the period of feeding, composition of diets, age, strain and sex of rats, method of feeding and others.

Kučera *et al.* (2011) demonstrated that Wistar rats provided with a high fat diet are more sensitive to steatosis than Sprague-Dawley rats.¹² In contrast, it was reported that 14 weeks high saturated fat feeding did not induce hepatic steatosis and NASH in Wistar rats.¹³ Experimental studies with carbohydrate-enriched diet (e.g., high-sucrose or high-fructose) are recognized as other models of NAFLD. Accumulating evidence has linked these types of diet to the development of NAFLD in humans as well as animal models.¹⁴ In addition, it has been reported that a westernized diet, a combination of both fat and fructose, is more effective in progressing advanced stages of NAFLD than just

feeding a fructose or fat rich diet⁶. Somewhat contrary to these results, Kawasaki *et al.* reported that the macrovesicular steatosis grade, liver to body weight ratio and hepatic triglyceride concentration were significantly higher in the high-fructose group (70%) than in the cornstarch (70%), high-sucrose (70%), high-fat (15%), and high-fat (15%) high-fructose (50%) groups.¹⁵

Since, the components of the experimental diets, have not been exactly mentioned in some studies and due to several models for inducing of NAFLD in laboratory animals and inconsistent results, we aimed to conduct an experimental pilot study to evaluate the effect of three different diets, similar to human diet, on the development of non-alcoholic fatty liver disease in rats to choose low-cost and appropriate method for developing NAFLD in this strain of animal, as a reference in future studies.

Material and Methods

Animals and diets

Twelve Male Wistar rats, obtained from Urmia Medical Sciences University, were housed at a temperature of 20-23°C with a 12-h light-dark cycle. They were randomly divided into 4 groups with three rats in each group. Group 1 (control) received the standard diet and tap water during the experimental periods; group 2 (HFHFr) received the High Fat High Fructose diet;¹⁶ group 3 (HCHFr) were given High Cholesterol High Fructose;^{17,18} and group 4 (HFHS) received High Fat High Sucrose diet.^{19,20} The Composition of modified diets and water has been shown in Tables 1 and 2, respectively

Table 1. Macronutrient composition and energy contents of the experimental diets.

Constituent	Control	HFHFr	HCHFr	HFHS
Macronutrients (% by weight)				
Carbohydrate	52.8	47.9	52	55.6
<i>Starch</i>	52.8	30.4	52	21.6
<i>Sucrose</i>	0	0	0	30
<i>Fructose</i>	0	17.5	0	4
Fat	5	27.8	6.3	27.5
<i>Soybean oil</i>	5	2.8	5.9	2.5
<i>Hydrogenated oil</i>	0	5	0	5
<i>Sheep tallow</i>	0	20	0	20
<i>Cholesterol</i>	0	0	0.4	0
Protein	20	11.5	19.7	8.2
Macronutrients (% Kcal)				
Carbohydrate	59.7	37.3	57.3	43.4
Fat	14.5	53	17.9	49.9
Protein	25.8	9.7	24.8	6.7
Energy (Kcal/g)	3.1	4.72	3.18	4.88

HFHFr: High Fat High Fructose Diet, HCHFr: High Cholesterol High Fructose Diet, HFHS: High Fat High Sucrose Diet.

The periods of 40^{19,21,22} and 60^{16,18} days were chosen because it was defined as sufficient to induce hepatic steatosis in other studies. All group-specific diets were custom prepared in our laboratory. The Control diet

was prepared by thoroughly mixing powdered rat feed (21 Beiza animal feed company, Shiraz). HFHFr and HCHFr groups had continuous access to a separate bottle with 20% fructose (Cologrin, Germany). For the

HFHS group, drinking water was augmented with 2.5% fructose and 16% sucrose, which was very similar to the High Fructose Corn Syrup-55 (HFCS55) regarding its composition with 55% fructose and 45% glucose. To monitor the health of the animals, weekly body weight, food, and water measurements were taken. Energy intake (kcal per day) was calculated from the caloric value of each diet. This study was approved by the Ethics Committee of Urmia Medical Sciences University.

Table 2. Composition and energy contents of the rodent available waters.

Constituent	Control	HFHFfr	HCHFfr	HFHS
Fructose (g/l)	0	200	200	25
Sucrose (g/l)	0	0	0	160
Energy (Kcal/ml)	0	0.8	0.8	0.74

HFHFfr: High Fat High Fructose Diet, HCHFfr: High Cholesterol High Fructose Diet, HFHS: High Fat High Sucrose Diet.

Sample preparation, Biochemical assay

For biochemical studies, rats were anesthetized with a mixture of ketamine–xylazine (80 and 10 mg/kg, respectively, i.p.). Then, blood samples were taken via portal vein and collected in tubes and centrifuged (3000 rpm, 10 min) to obtain Serum. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), Alkaline Phosphatase (ALP) and Gamma Glutamyl Transferase (GGT) levels were determined by standard enzymatic techniques and using an automatic biochemical analyzer.

Liver histology

After 40 days, one rat of each group was killed and other rats were sacrificed at the end of 60 days for pathophysiological evaluations. After blood sampling, the livers were removed immediately and washed with physiological saline; fragments of liver tissue were cut and being kept in solution of 10% buffered formaldehyde. Formalin-fixed and paraffin-embedded tissue were processed for hematoxylin and eosin staining, in order to semi-quantitatively assessment of the fatty degenerations using the NAFLD activity score (NAS). The histological features were graded according to percentage of distributions while pathologists were blinded regarding experimental groups. Scores for steatosis (score 0 to 3, S₀: <5%; S₁: 5%-33%; S₂: 33%-66%; S₃: >66%), lobular inflammation (score 0 to 3, I₀: No foci; I₁: <2 foci per 200× field; I₂: 2-4 foci per 200× field; I₃: >4 foci per 200 × field), and ballooning (score 0 to 2, B₀: None; B₁: few balloon cells; B₂: many cells/prominent ballooning), were also summed to calculate the NAS score (ranging from 0 to 8)^{23,24}. Paraffin blocks were also stained by periodic-acid Schiff (PAS) for detection of excessive hepatic glycogen.

Statistical analysis

The results are presented as the mean±SD. The statistical significance of differences between groups was determined by Kruskal-Wallis H test and comparisons between two groups were analyzed via Mann-Whitney U test. The level of probability was set at p≤0.05 as statistically significant.

Results

Body weights, food, water and caloric intake

Data of body weights and average daily food, water and caloric intake are shown in Figure 1 and 2, respectively. There were no significant differences in initial (P-value=0.076) and final (P-value=0.083) body weights between groups. However after 40 days, the body weights in HCHFfr and HFHS rats were significantly higher and lower than other groups, respectively (P-value=0.05). Additionally, Compared with control rats, average daily food and water intake in other three groups and caloric intake in HFHFfr and HFHS groups were significantly lower (P-value=0.05).

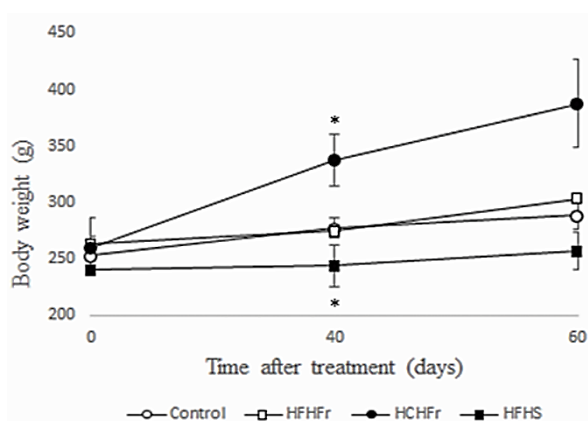


Figure 1. Effect of experimental diets on body weight. HFHFfr: High Fat High Fructose Diet, HCHFfr: High Cholesterol High Fructose Diet, HFHS: High Fat High Sucrose Diet. *P=0.05 versus other groups. Data are mean±SD.

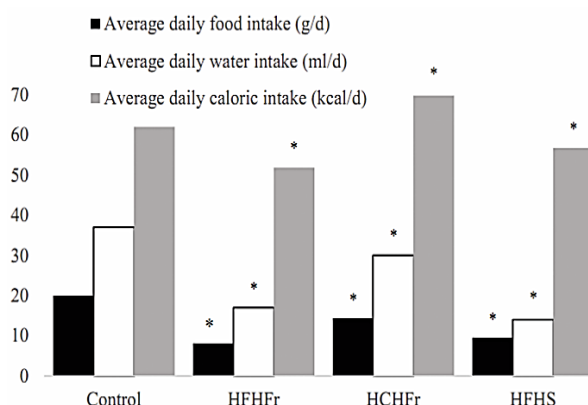


Figure 2. Effect of experimental diets on average daily food, water and energy intake. HFHFfr: High Fat High Fructose Diet, HCHFfr: High Cholesterol High Fructose Diet, HFHS: High Fat High Sucrose Diet. *P=0.05 versus control group.

Biochemical indicators of liver function

The effect of the various diets on liver enzymes are shown in Figure 3. After 60 days, all three

experimental diets induced a non-significant increase in serum aminotransferase levels relative to the control diet (P -value > 0.05).

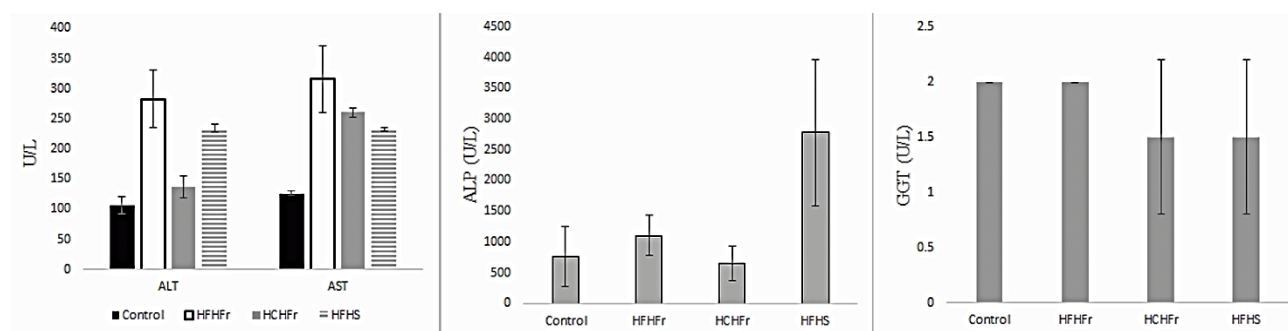


Figure 3. Effect of experimental diets on liver enzymes. HFHFr: High Fat High Fructose Diet, HCHF: High Cholesterol High Fructose Diet, HFHS: High Fat High Sucrose Diet, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline Phosphatase, GGT: Gamma Glutamyl Transferase. Data are mean \pm SD.

Hepatic histology assessment

As shown in Figure 4, there was no evidence for fat deposition in the sections of livers obtained from control group (Figure 4 A); but the histology of the livers from all other three groups showed evidence of fatty changes; although fatty degeneration was higher

in HFHFr and HFHS groups versus HCHF group (Figure 4 B-G). The severity of NAFLD was assessed by the NAS index following H&E staining. According to Table 3, there was a significantly higher ballooning and NAS scores in HFHFr rats than the other two groups after 60 days.

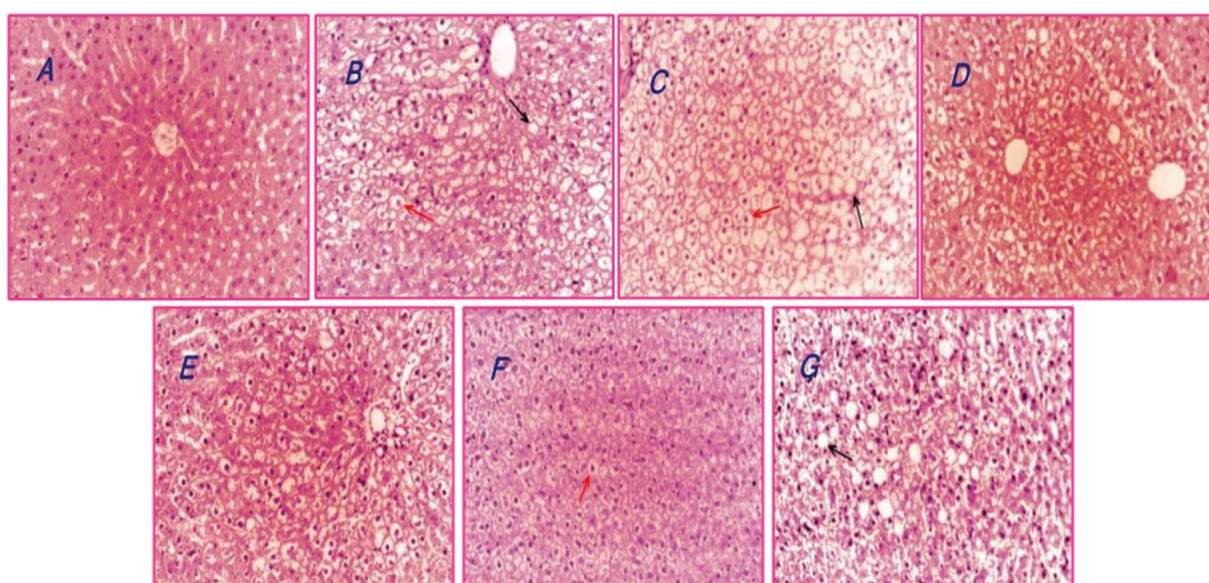


Figure 4. Histopathological features after haematoxylin–eosin staining of liver sections from a representative rat of each group ($\times 200$), panel A-G: (A) control diet; (B) high-fat–high-fructose, 40d; (C) high-fat–high-fructose, 60d; (D) high-fat–high-Cholesterol, 40d; (E) high-fat–high Cholesterol, 60d; (F) high-fat–high-sucrose, 40d; (G) high-fat–high- sucrose, 60d. Black and red arrows indicate steatosis and ballooning, respectively.

Table 3. Histopathological assessment of liver fat, hepatocyte ballooning, lobular inflammation and NAFLD activity score (NAS) in Different Dietary Groups.

	Control	HFHFr, 40d	HFHFr, 60d	HCHF, 40d	HCHF, 60d	HFHS, 40d	HFHS, 60d
Liver fat score	0.00 \pm 0.00	1.5 \pm 0.7*	1.75 \pm 0.5**†	0.5 \pm 0.7	0.5 \pm 0.57	1 \pm 0*	1.25 \pm 0.35*
Ballooning score	0.00 \pm 0.00	1.5 \pm 0.7*	2 \pm 0*†	1.25 \pm 0.35*	1 \pm 0*	1.5 \pm 0.7*	1 \pm 0*
Lobular inflammation score	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
NAFLD activity score (NAS)	0.00 \pm 0.00	3 \pm 1.4*	3.75 \pm 0.5*†	1.75 \pm 1.06*	1.5 \pm 0.57*	2.5 \pm 0.7*	2.25 \pm 0.35*

HFHFr: High Fat High Fructose Diet, HCHF: High Cholesterol High Fructose Diet, HFHS: High Fat High Sucrose Diet. $P < 0.05$ versus Control group, * $P < 0.05$ versus other groups, † $P < 0.05$ versus HCHF group. Values are expressed as mean \pm SD.

However, a higher grade of macro and microvesicular steatosis was only found in the HFHF_r compared with the HCHF group. Livers of all groups were also stained by PAS to differentiate excessive deposition of glycogen from fatty changes (Figure 5). As shown,

control rats showed normal glycogen storage patterns (Figure 5 A) and other groups revealed minimal PAS positivity in these sections. In this figure intracellular red granules represent glycogen deposition, while intracellular vacuoles reflect fatty changes.

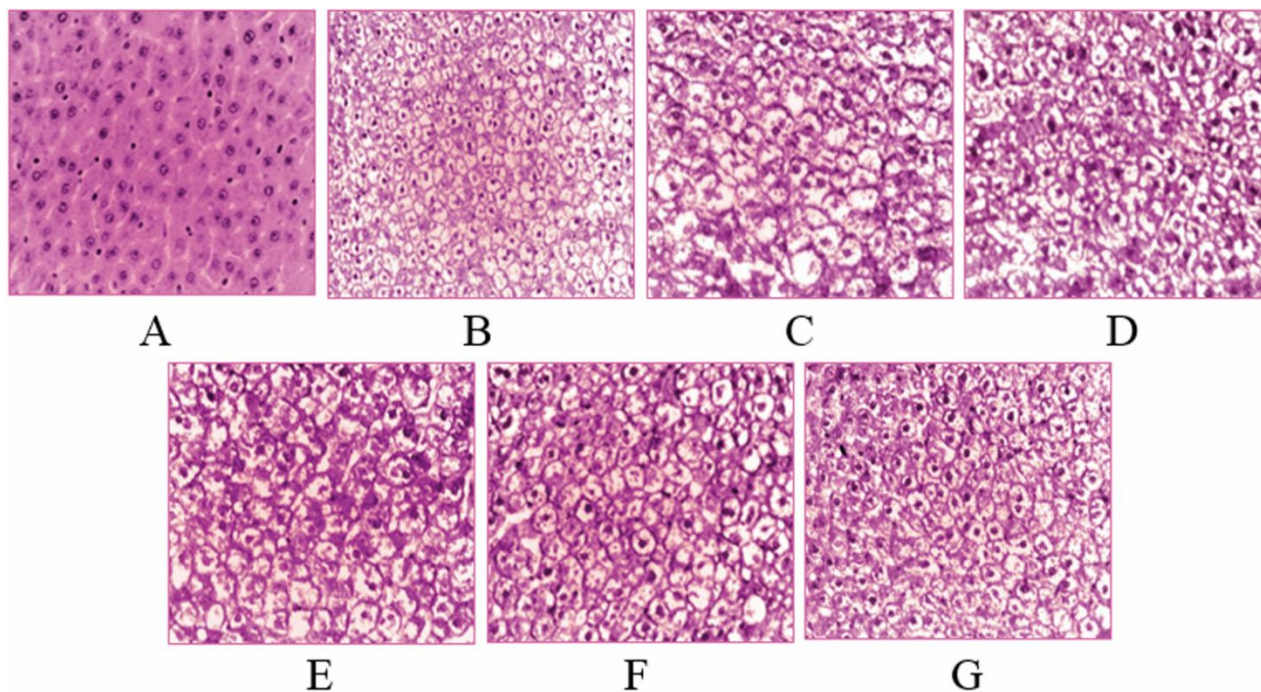


Figure 5. Histopathological features after PAS staining of liver sections from a representative rat of each group (×200), panel A-G: (A) control diet; (B) high-fat–high-fructose, 40d; (C) high-fat–high-fructose, 60d; (D) high-fat–high-Cholesterol, 40d; (E) high-fat–high-Cholesterol, 60d; (F) high-fat–high-sucrose, 40d; (G) high-fat–high-sucrose, 60d.

Discussion

In the present experimental pilot study, the effects of three different types of diets on inducing NAFLD were compared in rats after 40 and 60 days. As confirmed by histological findings, our three protocols led to the development of NAFLD in rats, especially after 60 days of dietary exposures, with the higher ballooning and NAS score in HFHF_r rats.

Animal models of NAFLD are essential tools for studying the pathogenesis and treatment of NAFLD. There are many studies suggest various types of dietary methods for developing NAFLD in experimental animals with different results. Kučera et al. (2011) demonstrated that Wistar rats provided with a high fat diet (olive, corn and safflower oil) are more sensitive to developing steatosis in comparison with Sprague-Dawley rats. In their study, it was shown that Wistar or Sprague-Dawley rats feeding with a high-fat diet (71 % kcal fat) for 3 or even 6 weeks caused steatosis without any significant changes in the serum activities of ALT and AST, however a medium-fat diet (35 % kcal fat) for 3 or 6 weeks, induced significant microvesicular steatosis in Wistar but not in Sprague-Dawley rats.¹² In contrast, in 2007, Romestaing et al. found that chronic administration of a high saturated fat diet (67% of calories from coconut or butter) for 14 weeks, did not

induce hepatic steatosis and NASH in 21 day-old wistar rats¹³. In another study, adult rats provided with diet containing 60% of calories from fat for 5 weeks, showed elevated body weights and liver enzymes levels as well as steatosis.²² These inconsistent results could be due to the design of the studies and different variables included the quantity and quality of dietary fat, the fatty acid and other dietary compositions, age of animals, period of feeding and route of fructose or sucrose administration, such as in the diet¹⁹ or water.^{20,25}

Fructose, sucrose, HFCS or cholesterol-enriched diets or a westernized diet are other dietary models to induce NAFLD in laboratory animals.¹¹ Some evidences, but not all, suggest that a westernized diet may lead to a severe stage of NAFLD than just feeding a fructose or fat rich diet.^{6,15} Recently, Fakhoury-Sayegh et al. have shown that consumption of a high fat diet (51%) for 16 weeks in wistar rats results in a higher percentage of steatosis than other diets composed of 61% sucrose or fructose. Although the high fructose group showed significantly higher levels of serum alanine aminotransferase and triglycerides.²⁶

In this study, we did not observe any significant differences in final body weights and liver enzyme levels between groups that may be because of small

sample size. Somewhat contrary to our results, Zhang *et al.* (2014) who used a diet containing 15% fat, 15% sucrose and 2% cholesterol for 8 weeks, as a dietary model of NAFLD, reported increased food intake, body and liver weights as well as macrovesicular steatosis compared with control group. However, in consistent to our study, those authors were unable to detect any significant increase in the ALT and AST levels.²⁷ In the other study by Sadi *et al.* (2014), microvesicular steatosis and the decrease of caloric intake and body weights was reported in wistar rats provided with drinking water containing 20% HFCS (56% fructose and 37% glucose) for 12 weeks; however any significant differences in the plasma levels of liver enzymes (ALT and AST) was not observed.²⁸

The limitations of the present study include small sample size and not measurement of some related biochemical markers due to financial limitations; but the strengths of this study outweigh these limitations. Variety of methods, inconsistent results and not precisely mention of dietary compositions in previous studies, were the reasons for doing this pilot research to evaluate the effect of three affordable different types of diets, similar to human diet, on the development of NAFLD in rats to make a suitable method for other studies.

Conclusion

In summary, this study compared the effects of three different types of diets on inducing non-alcoholic fatty liver disease in rats after 40 and 60 days, to identify an affordable animal model for NAFLD. As a result, hepatic histology assessments showed evidence of steatosis and ballooning degenerations in all three experimental groups, especially in HFHF rats and after 60 days.

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Conflict of interests

The authors claim that there is no conflict of interest.

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