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• BASIC RESEARCH •

Effects of low-calorie diet on steatohepatitis in rats with obesity and hyperlipidemia

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Abstract

AIM: To evaluate the effects of low calorie diet (LCD) on nonalcoholic steatohepatitis (NASH) in rats with obesity and hyperlipidemia.

METHODS: 29 Sprague-Dawley (SD) rats were randomly divided into three groups. The animals in control (*n*=9) and NASH group (*n*=10) were fed on standard rat diet and high fat diet respectively for 12 weeks, ten rats in LCD group were fed on high fat diet for 10 weeks and then low calorie diet for 2 weeks. At the end of the experiment, body weight, abdominal adipose content, liver function, and hepatopathological changes were examined to evaluate the effect of different feeding protocols on the experimental animals.

RESULTS: There was no death of animal in the experimental period. All rats in the NASH group developed steatohepatitis according to liver histological findings. Compared with the control group, body weight (423.5±65.2 *vs* 351.1±43.0 g, *P*<0.05), abdominal adipose content (14.25±1.86 *vs* 9.54±1.43, *P*<0.05), liver index (3.784±0.533 *vs* 2.957±0.301 %, *P*<0.01), total serum cholesterol (1.60±0.41 *vs* 1.27±0.17 mmol/L,*P*<0.05) and free fatty acids (728.2±178.5 *vs* 429.2±96.7 mmol/L, *P*<0.01), serum alanine aminotransferase (1 257.51±671.34 *vs* 671.34±118.57 nkat/L, *P*<0.05) and aspartic aminotransferse (2 760.51±998.66 *vs* 1 648.29±414.16 nkat/L, *P*<0.01) were significantly increased in the NASH group. Whereas, when rats were fed on LCD protocol, their body weight (329.5±38.4 g, *P*<0.01), abdominal adipose content (310.21±1.52 g, *P*<0.05), liver index (3.199±0.552 %, *P*<0.05), and serum alanine aminotransferase (683.03±245.49 nkat/L, *P*<0.05) were significantly decreased, and the degree of hepatic steatosis (*P*<0.05) was markedly improved compared with those in the NASH group. However, no significant difference was found in serum lipid variables and hepatic inflammatory changes between the two groups.

CONCLUSION: LCD might play a role in the prevention and treatment of obesity and hepatic steatosis in SD rats, but it exerts no significant effects on both serum lipid disorders and hepatic inflammatory changes.

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INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a hepatic disorder with the histopathological features of alcohol-induced liver disease that occurs in individuals who do not consume a large amount of alcohol. In recent years it has been believed to be a progressive liver disease that can lead to cirrhosis and even hepatocellular carcinoma. Unfortunately, up to the present its pathogenesis remains unknown. An empirical management of this disease in clinical practice, in which weight is reducted by a low-calorie diet (LCD), has been recommended to treat those patient with overweight and obesity. However, inappropriate caloric restrictions would lead to metabolic disorder, even promote hepatic portal inflammation, fibrosis, bile stasis and focal necrosis[1-8]. In the present study, we established a rat model of NASH with overwight/obesity and hyperlipidemia by chronically feeding high-fat diet to evaluate the protective effects of LCD on the metabolic changes of this disease to provide experimental evidence for the NASH treatment strategy.

MATERIALS AND METHODS

Animals

Male Spraque-Dawley rats weighing 140-160 g obtained from Shanghai Experimental Animal Center (Shanghai, China) were used in the present study. The rats were housed in plastic cages with a wire-mesh to isolate them from a hygienic bed and exposed to a 12-hour controlled light cycle. The rats were given free access to food and water under controlled humidity (55 %) and temperature (23+/-1 ℃). All protocols for animal experimentation and maintenance were approved by the Animal Ethics Committee in our university and conformed to the highest international standards of humane care.

Reagents

Cholesterol was from Huamei Company (Shanghai, China). Lard oil was prepared in our laboratory. Alanine aminotransferase (ALT) and aspartic aminotransferase (AST) assay kits were purchased from Sheneng Company (Shanghai). Free fatty acid (FFA), triglycerides (TG) and total cholesterol (TCH) assay kits were obtained from Zhicheng Company (Shanghai). Albumin (A) and total protein (TP) assay kits were provided by Shanghai Institution of Bio-products. Rabbit polyclonal anti-human lysozyme antibody was from Shanghai Biogenex Company. Mouse anti-human α-smooth muscle actin (α-SMA) was from Dako Company (Carpinteria, CA, USA). The second antibody for immunochemistry assay was from American Antibody Company (Greenwich, USA).

Experimental protocol

After fed on standard rat diet for one week, Spraque-Dawley rats were randomly divided into three groups. Animals in the control (*n*=9) and NASH group (*n*=10) were fed on standard

rat diet and high fat diet (a standard diet supplemented with 10 % lard oil and 2 % cholesterol) respectively for 12 weeks, while the rats in the LCD group (n=10) were fed on high fat diet for 10 weeks and then on low-calorie diet (70 kcal/kg/day accounting for 1/3 of the daily needs of a healthy rat) for 2 weeks. One rat of NASH group was harvested at week 10 for the demonstration of hepatopathological changes. The animals were weighed before experiment and one day prior to sacrifice. Blood samples were obtained by aorta abdominalis puncture at the time of sacrifice, and the resulting serum was stored at -20 ℃ until analysis. Meanwhile, liver samples were rapidly excised, weighed and frozen at -70 ℃, or fixed in 4 % buffered formaldehyde solution until use.

Blood biochemical analyses

Serum biochemical parameters such as ALT, AST, A, TP, TG, TCH and FFA were automatically analyzed with a multifunctional biochemistry analyzer Olympus AU1000.

Histopathological examination

Hepatic sections were prepared and stained with hematoxylin and eosin (H&E) for routine histopathological examination. Some sections were stained with VG carbazotic acid for detection of fibrosis. Ultromicrotomy was performed for transmission electron microscopy (JEM-1200EX, Japan). Hepatocytes involved in lobular fatty infiltration were counted in H&E stained sections. The severity of steatosis was graded on the basis of the extent of parenchyma involved. Grade 1 (+): <33 % of hepatocytes were involved. Grade 2(++): 33 % to 66 % of hepatocytes were involved. Grade 3(+++): >66 % of hepatocytes were involved. Normal(-): no hepatocytes were involved[4,9]. Knodell histological activity index (HAI) and modified HAI by Tailin Wang were used to determine hepatic necroinflammatory activity[9-11] scored by the severity of portal inflammation (P), intralobular inflammation(L), piecemeal necrosis (PN) and bridging necrosis (BN). The score from 1 to 4 was in accordance with the severity of lesions and the total score was calculated as P+L+2 (PN+BN). The number of Kupffer's cells and activated hepatic stellate cells was determined by immunohistochemistry using lysozyme and α-SMA antibody respectively. All samples were evaluated blindly by the same pathologist and confirmed by the other researcher.

Statistics

Data were expressed as mean ± SD unless otherwise specified. The Student t test was used to test individual differences. Rank samples were analyzed by Rank-sum test. Rate comparison was analyzed by u test. A value of P<0.05 was considered to be statistically significant.

RESULTS

General information

During the experimental period, the body weight of the rats fed on high-fat diet increased quickly. By the end of the 10th week, the body weight of high-fat fed rats was significantly increased compared with the controls. At the same time,we randomly harvested one of the high-fat fed rats for hepatopathological examination, which showed liver steatosis with mild intralobular inflammation. Biochemical analysis indicated that serum TCH, FFA, ALT, AST levels in this rat were higher than normal. However, rats in LCD group fed on low-calorie diet were fretful and inflammable, their bellicose and body weight stopped increasing.No animal died during the experimental period.

Body and liver weight changes

At the end of the experiment, the body weight of animals in the NASH group was 20 % higher than that in the control group (t=2.281, P<0.05). The liver index (liver weight/body weight ×100 %) and the abdominal adipose content in this group were also significantly increased compared with the controls (t=4.097 and 2.891, P<0.01and 0.05 respectively). Compared with the NASH group, the body weight,liver index and abdominal adipose content in the LCD group decreased significantly (t=3.928, 2.411, 2.632. P<0.01, 0.05, 0.05 respectively) (Table 1).

Table 1 Changes of body weight and liver index

Groups	n	Body weight/g	Liver index 100 %	Abdominal adipose/g
Control	9	351.1±43.0	2.957±0.301	9.54±1.43
NASH	10	423.5±65.2[a]	3.784±0.533[b]	14.25±1.86[a]
LCD	10	329.5±38.4[c]	3.199±0.552[d]	10.21±1.52[d]

[a]P<0.05, [b]P<0.01 *vs* control. [c]P<0.01, [d]P<0.05 *vs* NASH group.

Changes of serum lipids and glucose

At the end of the experiment, serum TCH and FFA in the NASH group were significantly higher than those in the controls (t=2.242 and 4.462; P<0.05 and 0.01 respectively), whereas serum TG level remained unchanged. Compared with the NASH group, serum TCH level in the LCD group was significantly increased (t=2.152, P<0.05) and FFA level was only slightly increased (P>0.05), whereas TG level was significantly decreased (t=4.435, P<0.001), even signicantly less than that in the control group (P<0.001), with a trend of decreased blood glucose (Table 2).

Table 2 Changes of major plasma lipid parameters

Groups	n	TG mmol/L	TCH mmol/L	FFA mmol/L
Control	9	0.63±0.22	1.27±0.17	429.2±96.7
NASH	10	0.62±0.10	1.60±0.41[a]	728.2±178.5[b]
LCD	10	0.39±0.13[c]	2.04±0.50[d]	771.3±124.4

[a]P<0.05, [b]P<0.01 *vs* control. [c]P<0.001, [d]P<0.05 *vs* NASH group.

Liver function

At the end of 12 weeks, serum ALT and AST levels were significantly increased in the NASH group compared with those in the controls (t=2.576 and 3.103, P<0.05 and 0.01 respectively). Compared with the NASH group, serum ALT level in LCD group was significantly decreased (t=2.541, P<0.05), whereas serum AST level only displayed a decreasing trend in plasma (P>0.05). There were no significant differences in plasma albumin levels and albumin-globulin ratio among these groups of rats (Table 3).

Table 3 Alternations of some biochemical variables in rat liver function

Groups	n	ALT nkat/L	AST nkat/L	A g/L	A/G
Control	9	671.34±118.57	1648.29±414.16	25.13±4.61	0.71±0.11
NASH	10	1257.51±671.34[a]	2760.51±998.66[b]	27.40±2.04	0.73±0.08
LCD	10	683.03±245.49[c]	2344.68±539.41	24.51±4.69	0.71±0.16

[a]P<0.05, [b]P<0.01 *vs* control. [c]P<0.05 *vs* NASH group.

Hepatopathological manifestations

At the end of the experiments, no specific findings were observed during the hepatohistological examination in the

controls. Under light microscope, sections stained with H&E in the NASH group showed moderate to severe macrovesicular steatosis which was diffusely distributed throughout the liver lobule, and parenchymal inflammation with both acute and chronic inflammatory cells accompanying focal necrosis. In 80 % of the samples, mild portal inflammation was noted, compared with lobular inflammation, and 20 % samples were accompanied by piecemeal necrosis. The score of HAI was significantly higher than that in the controls (3.4±2.1 *vs* 0.8±0.8, t=3.461, P<0.01) (Figures 1, 2). No obvious liver fibrosis was found in VG carbazotic acid stained sections. Immunohistochemical analysis showed that lysozyme and α-SMA positively stained cells in the NASH group were significantly increased compared with the controls.

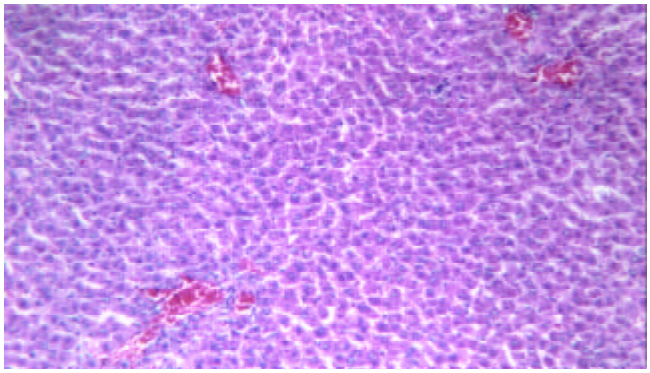

Figure 1 Light microscopy for control liver tissue, normal liver histology. H&E×100.

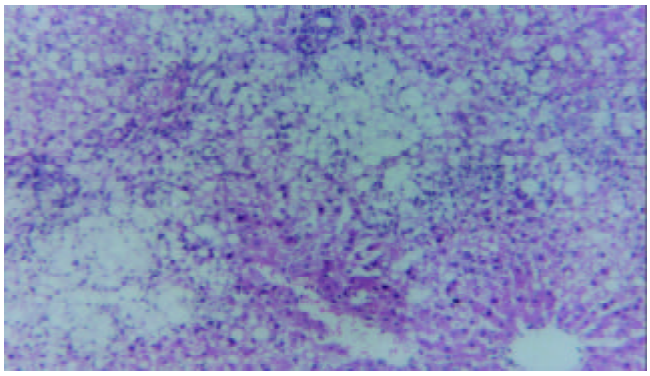

Figure 2 Light microscopy for liver tissue from a 12-week treated rat in NASH group, severe macrovesicular steatosis with mixed parenchymal inflammation and spotty focal necrosis. H&E×100.

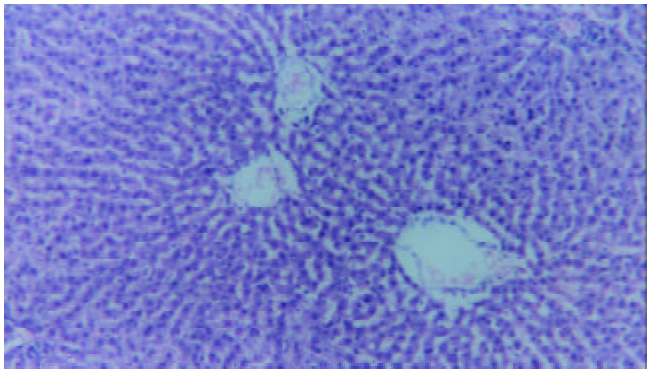

Figure 3 Light microscopy for liver tissue from a rat treated with LCD during 12-week experiment, the pathological changes of liver were obviously improved compared with the NASH group. H&E×100.

Compared with the NASH group, the liver steatosis in the LCD group was significantly reduced (P<0.05) (Figure 3, Table 4). However the score of HAI only had a trend of decrease (2.5±1.0 *vs* 3.4±2.1, P>0.05). There were no differences in the number of positive cells stained by lysozyme and α-SMA and liver fibrosis on VG stained sections between LCD and NASH groups. The liver histological findings were almost normalized in 1 sample of the LCD group.

Table 4 Severity of hepatic steatosis in rats of different groups

Groups	*n*	-	+	++	+++
Control	9	9			
NASH	10		3	6	1
LCD	10	3	5	2	

Rank sum test: P<0.05.

DISCUSSION

Non-alcoholic steatohepatitis (NASH) can be defined pathologically as severe steatohepatitis that is not resulted from alcohol,drug or any other singly identifiable causes. NASH is becoming a common liver disease and probably has a similar risk of progression to cirrhosis as chronic hepatitis C. No treatment has been yet proven to be efficient. Those who are overweight and suffer from NASH should be considered to employ a weight reduction program. Diet is an important component of weight-reduction regimen[1-8]. However, no controlled studies are available as for the value of diet in the management of NASH, further researches are needed to evaluate the effect of diet modalities on NASH either by clinical trial or by animal experiment[1,2,5,12].

In a recent study, liver tests and fatty infiltration were significantly improved in 15 obese patients with NASH treated with a restricted diet (25 kcal/kg· day) plus exercise for months. Improvement in the degree of inflammation and fibrosis was also achieved in some patients[13]. However, in another report, five obese patients stopped eating for some time and lost 14-30 kg within 1 month. Hepatic fat content decreased in three of them, but fibrosis became more prominent in four out of the five patients[14]. In addition, in another series, 41 morbidly obese patients with NASH had a median weight loss of 34 kg during the treatment with a very low calorie formula diet (388 kcal/day). The liver fat infiltration was also significantly improved. However, a fifth of the patients, particularly those had more pronounced reduction of liver fat and faster weight loss, developed mild portal inflammation or fibrosis[15]. It is well known that rapid weight reduction would lead to excessive fat catabolism, and marked elevation of FFA and lack of essential amino acids in serum and liver, which might finally induce or aggravate steatohepatitis and liver fibrosis[16-20].So, the adequate rate and degree of weight reduction remain to be established. Further studies are necessary to determine the appropriate caloric restrictions and the formula for obese patients with NASH[21-23].

No ideal animal model has yet been established for NASH research[24-31]. We have therefore established a model of this disease in rats by continuous feeding on a diet rich in fat and cholesterol for 12 weeks[17-19]. These animals were overweight and showed abnormal increase of abdominal fat (standing for trunk obesity), as well as markedly elevated levels of serum TCH, FFA and aminotransferase. Moderate to severe steatosis combined with intralobular inflammation and spotty necrosis was found in their hepatopathological examinations. Although fibrosis was absent on VG staining, we found that hepatic stellate cells and Kupffer cells were activated and proliferated,

suggesting that liver fibrosis might be inevitable[32,33]. Our subsequent research also demonstrated that feeding on a high fat diet for 24 weeks could induce steatohepatitis with liver fibrosis[34,35]. This animal model was easily established with low mortality (0 %) and high reproductive rate (100 %). Furthermore, this model was similar to that of the human disease, suggesting that this rat model is suitable for investigating the pathogenesis and prevention and treatment of NASH[30,33]. However, our model has some shortcomings. Firstly, the hepatopathological changes in this rat model were not entirely consistent with those in patients with NASH. Specifically, zone 3 involvement was not dominant. Moreover, no Mallory Hyaline bodies were found in sections stained by H&E. Secondly, NASH is often associated with hypertriglyceridemia which was not observed in this model.

The weight reduction diets recommended for NASH with obesity are slimming, low-calorie diet (LCD) and very-low calorie diet (VLCD). Slimming diets involve caloric intake of 1 200-1 800 kcal per day for adults, which is slightly less than that of normal diet, while LCD involves an intake of 600-1 200 kcal per day for adults and VLCD involves a caloric intake of 200-600 kcal per day[5,8,36]. Patients with moderate or severe obesity are usually put on LCD for weight reduction. In contrast, VLCD is seldom used clinically because of severe complications[5,8,36,37]. In our study, we took a caloric intake protocal for the animal model that belongs to LCD according to caloric calculation (70 kcal/kg· day *vs* 210 kcal/kg· day for rats).

While the rats fed on a high fat diet for 10 weeks were overweight and developed hyperlipidemia and fatty liver, a subsequent 2 weeks on LCD made both of their overweight and hyperlipidemia alleviated. In contrast,an additional two weeks on the high fat diet led to the development of more severe obesity,hyperlipidemia and steatohepatitis. These findings suggest that altering a high fat or high calorie diet to LCD may have markedly positive effects on obesity, hyperlipidemia and combined fatty liver, while continuation on the fat-rich diet may lead to the development of steatohepatitis. Since the rats in our LCD group developed hypercholesterolemia and hypoglyceridemia with a trend to increase serum FFA. Some of their liver samples were still found to have hepatocyte necrosis and inflammatory cell infiltration, indicating that LCD therapy for 2 weeks may be not quite enough to reverse steatohepatitis.

In summary, this study indicates that it might be difficult to resolve steatohepatitis by merely short-term LCD therapy, long-term appropriate diet control or concurrent administration of medications that can directly reduce the severity of liver damage may be reasonable alternatives for the treatment of NASH patients with obesity[2,3,5,23,24].

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