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• *BRIEF REPORTS* •

Effects of ursodeoxycholic acid and/or low-calorie diet on steatohepatitis in rats with obesity and hyperlipidemia

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Abstract

AIM: To evaluate the effects of ursodeoxycholic acid (UDCA) and/or low-calorie diet (LCD) on a rat model of nonalcoholic steatohepatitis (NASH).

METHODS: Fifty-five Sprague-Dawley rats were divided into five groups. The control group (n = 9) was fed with standard rat diet for 12 wk, NASH group (n = 10) was fed with high-fat diet consisted of normal diet, 10% lard oil and 2% cholesterol for 12 wk, UDCA group (n = 10) was fed with high-fat diet supplemented with UDCA at a dose of 25 mg/(kg • d) in drinking water for 12 wk, LCD group (n = 10) was fed with high-fat diet for 10 wk and then LCD for 2 wk, and UDCA+LCD group (n = 15) was fed with high-fat diet for 10 wk, followed by LCD+UDCA for 2 wk. At the end of the experiment, body weight, serum biochemical index, and hepatopathologic changes were examined.

RESULTS: Compared with the control group, rats in the NASH group had significantly increased body weight, liver weight, and serum lipid and aminotransferase levels. All rats in the NASH group developed steatohepatitis, as determined by their liver histology. Compared with the NASH group, there were no significant changes in body weight, liver weight, blood biochemical index, the degree of hepatic steatosis, and histological activity index (HAI) score in the UDCA group; however, body and liver weights were significantly decreased, and the degree of steatosis was markedly improved in rats of both the LCD group and the UDCA+LCD group, but significant improvement with regard to serum lipid variables and hepatic inflammatory changes were seen only in rats of the UDCA+LCD group, and not in the LCD group.

CONCLUSION: LCD might play a role in the treatment of obesity and hepatic steatosis in rats, but it exerts no significant effect on both serum lipid disorders and hepatic inflammatory changes. UDCA may enhance the therapeutic effects of LCD on steatohepatitis accompanied by obesity and hyperlipidemia. However, UDCA alone is not effective in the prevention of steatohepatitis induced by high-fat diet.

Key words: UDCA; LCD

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INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is a hepatic disorder with histologic features of alcoholic hepatitis, occurring in individuals who do not consume significant amount of alcohol. In spite of the high prevalence of hepatic steatosis and of the potential of NASH to progress to fibrosis and cirrhosis, no effective specific treatment is available[1-7]. Management of this condition today is empirical. In NASH patients with comorbid obesity, weight reduction is recommended, chiefly by means of a low-calorie diet (LCD). An inappropriate diet, however, may lead to metabolic disorders, and may even promote portal inflammation, fibrosis, bile stasis or focal necrosis[8-13]. Ursodeoxycholic acid (UDCA) has cytoprotective, anti-apoptotic, membrane stabilizing, anti-oxidant and immunomodulative effects[14]. So UDCA has been recommended in the treatment of NASH and in the prevention of cholelithiasis during weight reduction. Clinical trials of UDCA in NASH therapy, however, have yielded ambiguous results[12-14].

In the present study we determined the effects of UDCA and/or LCD administration on NASH in rats chronically fed with high-fat diet.

MATERIALS AND METHODS

Reagents

Cholesterol was purchased from the Department of Reagents, Huamei Drug Store (Shanghai, China). Lard oil was prepared in our laboratory. UDCA powder (99.9% purity) was kindly provided by the Shanghai 1st Chinese Herb Pharmaceutical Company (Shanghai). Alanine aminotransferase (ALT) and aspartic aminotransferase (AST) assay kits were purchased from Sheneng Company

(Shanghai). Assay kits for free fatty acids (FFA), triglycerides (TG) and total cholesterol (TCH) were from Zhicheng Company (Shanghai), while assay kits for albumin (A) and total proteins (TP) were from the Shanghai Institution of Bio-products (Shanghai). Rabbit polyclonal anti-human lysozyme antibody was purchased from Shanghai Biogenex Company, and mouse anti-human α-smooth muscle actin (α-SMA) was from Dako (Carpinteria, CA). The second antibody for immunochemical assays was from the American Antibody Company (Greenwich, CT).

Animal diet and experimental protocol

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.

Fifty-five male Sprague-Dawley rats weighing 140-160 g were obtained from the Shanghai Experimental Animal Center (Shanghai, China). After being fed with standard rat chow for one week, Sprague-Dawley rats were randomly divided into five groups. Control rats (n = 9) were fed with standard rat diet for 12 wk. Rats of NASH group (n = 10) were fed with high-fat diet (i.e., standard diet supplemented with 10% lard oil and 2% cholesterol) for 12 wk. Animals in the UDCA group (n = 10) were fed with high-fat diet supplemented with UDCA (25 mg/(kg • d) in drinking water) for 12 wk. Rats in the LCD group (n = 10) were fed with high-fat diet for 10 wk and then fed with LCD (70 kcal/(kg • d)) accounting for 1/3 of the daily needs of a healthy rat of that age for 2 wk. Rats in the UDCA+LCD group (n = 15) were fed with high-fat diet for 10 wk and then fed with LCD+UDCA (25 mg/(kg • d)) for 2 wk. Animals were maintained in separate cages and provided with unrestricted amounts of food and water. The cages were kept in temperature-and humidity-controlled rooms, which were maintained on a 12-h light/dark cycle.

The animals were weighed on d 0, 10th wk of the experiment and one day before killing. All rats were killed at the end of wk 12, except for one of the rats in the NASH group, which was killed at the end of wk 10 for the demonstration of hepatopathologic changes. At the time of killing the rats were free from food at least 12 h, blood was obtained by aorta abdominalis puncture, and the resulting serum was stored at -20 ℃ until analysis. Meanwhile, liver sample was rapidly excised and weighed, tissue samples were snap frozen and stored at -70 ℃ until analysis, or were fixed in 4% buffered formaldehyde solution until use.

Blood biochemical analyses

Serum ALT, AST, A, TP, TG, TCH, and FFA were assayed biochemically using an Olympus AU1000 and automated procedures.

Histologic studies

Hepatic sections were stained with hematoxylin and eosin (H&E) for routine histology or with VG carbazotic acid for detection of fibrosis. Ultramicrotomy was performed for transmission electron microscopy (JEM-1200EX, Japan). Hepatocytes associated with fat infiltration into the lobules were counted in H&E stained sections.

The severity of steatosis was graded on the basis of the extent of involved parenchyma. Samples scored as+were those in which fewer than 33% of the hepatocytes were affected; samples scored as ++ were those in which 33-66% of the hepatocytes were affected; samples scored as +++ were those in which more than 66% of the hepatocytes were affected; and samples scored as -- were those in which no hepatocytes were affected[15-17]. Modified Knodell histological activity index (HAI) was used to determine hepatic necroinflammatory activity 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 cells and activated hepatic stellate cells was determined immunochemically using lysozyme and α-SMA antibody respectively. All samples were evaluated blindly by the same pathologist.

Statistics

Data were expressed as mean±SD unless otherwise specified. The Student's t test was used to analyze individual differences. Rank samples were analyzed by the Rank-sum test. Rate comparison was analyzed by the 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 rats fed on high-fat diet increased quickly. By the end of the 10th wk, the body weight of high-fat fed rats was significantly higher than 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 nonspecific intralobular inflammation. Biochemical analyses indicated that serum TCH, FFA, ALT, and AST levels in this rat were higher than normal. Rats in the LCD and LCD+UDCA groups were fretful, inflammable, and bellicose, and their weight stopped increasing. No animal died during the experimental period.

Changes in body and liver weight

At the end of the 12-wk experimental period, the rats in the NASH group were 20% heavier than those in the control group ($P<0.05$). Liver index (liver wet weight/body weight×100%) in the NASH group was also significantly higher than in the controls ($P<0.01$). In the UDCA group, the changes in body weight and HAI score were similar to the NASH group. Compared with the NASH group, the body weight ($P<0.01$) and liver index ($P<0.05$) in the LCD group were significantly lower, while the changes in the UDCA+LCD group were similar to those in the LCD group (Table 1).

Changes in serum lipids

After 12 wk, serum TCH ($P<0.05$) and FFA ($P<0.01$) in the NASH group were significantly higher than those in the control group, whereas serum TG levels were equivalent. The changes of blood lipid variables in the UDCA group

Table 1 **Changes of body weight and liver index**

Group	*n*	Body weight (g)	Liver index (%)
Control	9	351.1±43.0	2.957±0.301
NASH	10	423.5±65.2[a]	3.784±0.533[b]
UDCA	10	426.5±26.6	3.476±0.351
LCD	10	329.5±38.4[d]	3.199±0.552[c]
LCD+UDCA	15	310.3±38.6[d]	3.113±0.466[c]

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

Table 3 **Alterations of some biochemical variables in rat liver function**

Group	*n*	ALT (u/L)	AST (u/L)	A (g/L)	A/G
Control	9	40.2±7.1	98.7±24.8	25.13±4.61	0.71±0.11
NASH	10	75.3±40.21[a]	165.3±59.8[b]	27.40±2.04	0.73±0.08
UDCA	10	66.0±49.4	139.5±54.52	7.49±1.78	0.66±0.09
LCD	10	40.9±14.7[c]	140.4±32.3	24.51±4.69	0.71±0.16
UDCA+LCD	15	40.1±8.8[c]	111.8±22.9[c]	25.3±1.34	0.71±0.07

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

were similar to those in the NASH group. Serum TCH in the LCD group was significantly higher than that in the NASH group ($P<0.05$), and FFA in the LCD group was inclined to increase as compared to the NASH group, while TG was significantly decreased ($P<0.001$). Compared with the LCD group, serum lipid disorders in the UDCA+LCD group had a tendency to improve (Table 2).

Changes in liver function

At the end of the 12 wk, serum ALT ($P<0.05$) and AST ($P<0.01$) levels were significantly higher in the NASH group than in the controls. While serum AST was slightly lower in the UDCA group than that in the NASH group, the difference was not statistically significant. In the LCD group, serum ALT level was significantly lower than in the NASH group ($P<0.05$), whereas serum AST level only displayed a decreasing trend in plasma ($P>0.05$). Compared with the LCD group, serum AST level was significantly lower in the UDCA+LCD group ($P<0.05$), while serum ALT level was about the same in the both groups. There were no significant differences in serum albumin levels and albumin-globulin ratio among these groups of rats (Table 3).

Changes in liver histology

At the end of the 12 wk, we observed no specific histologic alterations in the control livers. Under light microscope, sections stained with H&E in the NASH group showed steatosis, which was predominantly macrovesicular and more or less diffusely distributed throughout the liver lobule, and parenchymal inflammation with both acute and chronic inflammatory cells accompanied with focal necrosis. In 80% of the samples, portal inflammation was noted, which was mild compared with lobular inflammation; and 20% samples accompanied with piecemeal necrosis. The score of HAI in the NASH group was significantly higher than in the controls ($P<0.01$). No obvious liver fibrosis was found in VG carbazotic acid-stained tissue sections. Immunohistochemical analysis showed that the number of cells positive for lysozyme and α-SMA was significantly higher in the NASH group than in the controls. Electron microscopic examination showed that, in the NASH group, liver cell nuclei were squeezed to cell ambitus by large lipid droplets, and that there was mitochondrial swelling; proliferation of hepatic stellate cells and Kupffer cells was also found.

Compared with the NASH group, there were no alterations in hepatopathologic findings of rats in the UDCA group. However, the degree of steatosis in the LCD group was significantly reduced ($P<0.05$). Both the degree of steatosis and the score of HAI in the UDCA+LCD group were a little lower than in the LCD group, but obviously lower than the NASH group ($P<0.05$). Significantly fewer cells positive for lysozyme and α-SMA were observed in the UDCA+LCD group than in the NASH group, but these parameters did not differ among the other groups. Histologically, the livers of four rats in the UDCA+LCD group and one in the LCD group were almost normalized, but this difference was not significant (Tables 4 and 5). There still existed mild, nonspecific intralobular inflammation in the rat of the LCD group with hepatic steatosis regressed.

DISCUSSION

Because NASH may lead to progressive hepatic fibrosis and eventually cirrhosis and because its prevalence appears to be increasing, it is necessary to develop effective therapies for NASH. Currently, multiple therapies have been recommended for NASH. However, as is usual in such instance, when many modes of therapy have been proposed, there is no single, well-established consensus regarding effective therapy[1-13].

In our study, while the rats fed on a high-fat diet for 10 wk were overweight and developed hyperlipidemia and fatty liver (this was confirmed by our another study)[18,19], a subsequent 2 wk on LCD made both of their overweight and hyperlipidemia and hepatic steatosis alleviated. In contrast, an additional 2 wk of the high-fat diet led to the development of more severe obesity, hyperlipidemia and steatohepatitis. These findings suggest that altering a high-fat diet to LCD may have markedly positive effects on obesity,

Table 2 **Changes of major plasma lipid parameters**

Group	*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]
UDCA	10	0.59±0.13	1.60±0.43	629.0±229.6
LCD	10	0.39±0.13[d]	2.04±0.50[c]	771.3±124.4
UDCA+LCD	15	0.51±0.17[e]	1.93±0.27[c]	605.6±152.2

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

Table 4 **Hepatic steatosis in groups of rats**

Group	*n*	-	+	++	+++
Control	9	9			
NASH	10		3	6	1
UDCA	10	1	3	4	2
LCD	10	3	5	2	
UDCA+LCD	15	8	1	4	2

Table 5 HAI in groups of rats

Group	*n*	HAI
Control	9	0.8±0.8
NASH	10	3.4±2.1
UDCA	10	3.0±1.3
LCD	10	2.5±1.0
UDCA+LCD	15	1.9±1.1

hyperlipidemia and combined fatty liver, while continuation on the fat-rich diet may lead to the development of steatohepatitis[20]. Although there was no liver fibrosis, we found activation and proliferation of hepatic stellate cells and Kupffer cells, suggesting that liver fibrosis might be inevitable[18,19]. Subsequent research demonstrated that feeding with a high-fat diet for 24 wk could induce steatohepatitis with liver fibrosis[18,19]. Since the rats in our LCD group developed hypercholesterolemia and hypotriglyceridemia with a trend to increase of serum FFA level, some of their liver samples were still found to have hepatocyte necrosis and inflammatory cell infiltration, indicating that merely short-term LCD therapy may be not quite enough to reverse steatohepatitis and serum lipid disorders[20,21]. Concurrent administration of medications with potential hepatoprotective effects may be reasonable alternatives for the treatment of NASH with obesity.

UDCA is a bile acid with multiple potential mechanisms of action, including immunomodulatory effects, displacement of toxic hydrophobic bile salts from the bile acid pool, lipid-altering properties, and direct cytoprotective effects[22-26]. UDCA was first used as an empiric treatment in NASH in a 66-year-old woman. The patient experienced normalization of serum liver enzyme levels after treatment of UDCA for 1 year[27]. In a pilot study of 40 patients with NASH established by liver biopsy and the exclusion of other causes of hepatitis, 24 patients were treated with UDCA (13-15 mg/(kg • d)) for 1 year. There was no significant change in body weight. There was a statistically significant decrease in levels of ALT, alkaline phosphatase, gamma-glutamyltranspeptidase and in improvement in histologic grade of hepatic steatosis. Sixteen patients with hypertriglyceridemia were placed on clofibrate (2 g/d) for 12 mo and did not have any benefit[28]. Recently, McCullough recommended that until more information is provided, it seems appropriate to treat comorbid diseases in patients with NASH and to implement UDCA[29]. However, our findings suggest that UDCA alone did not have significant effects on rats with NASH induced by high-fat diet. Compared with the NASH group, no alterations in serum biochemistry or liver histology were observed in the UDCA group. This was in accordance with the new results of a randomized, placebo-controlled trial completed by the UDCA/NASH study group of USA, which showed that UDCA (13-15 mg/(kg • d)) was not associated with improved liver biochemistries or liver histology in patients with NASH when compared to placebo, and the weight was stable in the two groups[30,31].

In addition, in our study UDCA was able to ameliorate blood lipid disorders, facilitate regression of hepatic steatosis, inflammation and necrosis, and significantly decrease serum AST level when given together with LCD therapy. So, the addition of UDCA was more efficacious than LCD alone. If this is the case, the following possible mechanisms of UDCA in improving the effects of LCD on steatohepatitis can be proposed in light of the previous study[32-34]. In rats, Tabouy and colleagues showed that the preferential shift of the metabolic pathway of fatty acids from esterification to oxidation due to the ethanol-induced liver mitochondrial damage, and subsequent fat accumulation, may be prevented by UDCA by way of improving ATP synthesis with protection of mitochondrial morphology[32]. In addition to this membrane-protective effect of UDCA, whether this drug directly mediates a shift in fatty acid metabolism from esterification to oxidation is also worth examining. A recent rat study, in which the effect of UDCA on alcohol-induced steatosis and lipid peroxidation was examined, suggests that UDCA might reduce steatosis and lipid peroxidation[33,34].

In summary, our findings suggest that UDCA alone is not effective for the treatment of NASH rats and that short-term LCD alone does not usually resolve NASH. Concurrent administration of UDCA and LCD, however, may enhance the curative effects and reduce the side effects of rapid weight reduction. Thus, while UDCA may not directly reduce or reverse steatohepatitis independently of weight loss, this medication might complement the diet of patients with NASH.

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