The Seoul Journal of Medicine
Vol. 34, No. 3: 187-194, September 1993

An Antiproteinase Inhibits High Carbohydrate-Diet Induced Gallstone Formation in Hamsters

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=Abstract=**High carbohydrate(CHO) diet plays a role in pigment gallstone formation, though the mechanism is not yet clarified. We postulated that high CHO diet induces gallstone formation through a mechanism whereby high CHO diet poorly stimulates cholecystokinin(CCK) release and causes relative bile stasis. The purpose of our study was to examine whether camostat mesilate(camostat), oral antiproteinase increasing CCK release, inhibits gallstone formation from high CHO diet in hamsters. Fifty six hamsters were divided into 3 groups(G): I(n = 18) was fed normal rat chow(43% CHO), II(n = 19) was fed a high CHO diet(65% CHO), III(n = 19) was fed 0.2% camostat high CHO diet. The animals were sacrificed after 8 weeks. Stones were checked grossly and gallbladder bile was analysed. Gallstones had developed in 11.1% of G-I, 84.2% of G-II, and 26.3% of G-III($p < 0.05$: II vs III). Concentrations of cholesterol, phospholipid, calcium, bilirubin and bile acid were low in G-III. Pancreatic weight, reflecting chronic status of CCK level, of G-III was greater than other groups and that of G-II was smaller than that of G-I. In conclusion, camostat inhibits high CHO diet induced gallstone formation in hamsters and the possible mechanism is that camostat recovers the low CCK release by high CHO diet. This study suggests that high CHO diet is associated with pigment stone formation through the mechanism that high CHO diet causes poor CCK release.**

Key Words: *Gallstone, Cholecystokinin, Antiproteinase*

INTRODUCTION

The mechanism of pigment gallstone formation, especially that of calcium bilirubinate stones has not been clearly defined. It seems to be related to bile stasis and biliary infection rather than changes in bile composition. It has been suggested that high carbohydrate(CHO) diet plays a role in pigment gallstone formation. Pigment stone is more prevalent in southeast Asian countries where the main meal is composed of a high CHO diet(Park and Kim 1988). Epidemiologic studies in Japan have shown that the changing pattern in the prevalence rate of pigment gallstones is related to the amount of CHO consumption in food(Nakayama and

Received June 1993, and in final form august 1993.
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Miyake 1970; Nagase *et al.* 1978; Nagase *et al.* 1980). High CHO diet induces pigment gallstone formation in hamsters and prairie dogs(Cohen *et al.*1987; Rege and Nahrwold 1987; Suh *et al.* 1993). However, it has not been clarified how a CHO diet makes gallstones.

Many investigators have demonstrated how gallbladder contractility in response to food intake is decreased in patients with gallstone disease(Maugdal *et al.* 1980; Thompson *et al.* 1982; Fischer *et al* 1982; Forgcas *et al.* 1984 Pomeranz *et al.* 1985). Well preserved gallbladder responsiveness to cholecystokinin(CCK) and decreased CCK release in response to duodenal fat in gallstone disease suggest that chronic gallbladder stasis caused by low CCK release is associated with gallstone pathogenesis(Fischer *et al.* 1982; Masclee *et al.* 1989).

CCK stimulates gallbladder contraction and relaxation of the sphincter of Oddi. It also stimulates pancreatic enzyme secretion and pancreatic growth(Mainz *et al.* 1973; Folsch *et al.* 1978; Solomon *et al.* 1978). CCK release is stimulated by intraduodenal digested food material, especially protein and fat. CHO is a relatively weak stimulator compared to protein and fat(Walsh 1987).

CCK release is negatively controlled by a feedback mechanism. Decreasing intraduodenal bile salt by cholestyramine increases CCK release and intraduodenal bile salt decreases CCK release(Gomez *et al.* 1988). Trypsin inhibits CCK release and trypsin inhibitor increases CCK release(Brand *et al.* 1981; Liddle *et al.* 1984; Yonezawa 1984; Owyang *et al.* 1986; Goke *et al.* 1986; Watanabe *et al.* 1992).

Camostat($C_{20}H_{20}N_4O_5.CH_3SO_3H$), a synthetic antiprotease, inhibits the activities of trypsin, plasmin, kallikrein and thrombin (Muramutu *et al.* 1972; Tamura *et al.* 1977). Camostat is metabolized to Foy-305, a potent protease inhibitor. Oral camostat 600 mg blocks most of the trypsin activity for 90 minutes in humans(Adler *et al.* 1988). Trypsin inhibitors stimulate pancreatic secretion and pancreatic growth(Goke *et al.* 1986; Otsuki *et al.* 1987). Experimental studies have shown that these actions are mediated by CCK(Mainz *et al.* 1973; Folsch *et al.* 1978; Solomon *et al.* 1978; Watanabe *et al.* 1992). Decreased CCK release causes pancreatic atrophy and exogenous CCK administration reverses this effect(Gomez *et al.* 1990). Camostat 100 mg/kg/day increases CCK release and induces pancreatic hypertrophy (Muramuto *et al.* 1972; Mainz *et al.* 1973; Solomon *et al.* 1978; Otsuki *et al.* 1985; Goke *et al.* 1987; Keim *et al.* 1988).

CHO is a weak stimulator for CCK release compared to protein and fat. We postulated that high CHO diet induces gallstone formation through the mechanism that high CHO diet poorly stimulates CCK release and causes relative bile stasis. The purpose of our study was to examine whether camostat inhibits gallstone formation from high CHO diet in hamsters through the mechanism of increasing endogenous CCK release.

MATERIALS AND METHODS

Fifty six male syrian golden hamsters weighing 98 to 130 gm were used in this study. After a 1 week acclimatization period, the hamsters were divided into 3 groups: Group I (n = 18) was fed normal rat chow, Group II (n = 19) was fed a high CHO diet, Group III (n = 19) was fed 0.2% camostat containing a high CHO diet. Diet compositions for each group are shown in Table 1. Since a hamster fed about 50 gm/kg/day, 0.2% camostat diet would be administered camostat 100 mg/kg/day. Body weight change and amount of food consumption were monitored throughout the experimental period. Hamsters were sacrificed after 8 weeks of feeding after 24 hours fasting. They were sacrificed alternatively within a short period of time to minimize diurnal variation. After weighing and anesthetizing the hamsters with ketamine(100 mg/kg) and diazepam(0.15 mg/kg), the abdomen was widely opened. Gallbladder bile was taken with an insulin syringe and stored at –40°C until analysis. Presence or absence of stone was checked grossly and the pancreas and left kidney(as a control) were care-

fully isolated and wet weights were measured. Gallstones were qualitatively analysed by infrared spectroscopy (Diffraction grating Infrared Spectrophotometer, Perkin Elmer FT-IR Spectrophotometer 1725). Gallbladder bile was analysed using kits. Total bilirubin was measured by the method of Michelsson(1981) using T-bilirubin reagents(Kyokuto Pharm. Japan), calcium was measured by the o-cresolphthalein complexon method of Aderegg (1954) using Calcium Reagent(Gilford Systems, Ohio, USA), phospholipid was measured by the method using phospholipase D(Dryer *et al.* 1956) (Phospholipase B test: Wako, Japan). Cholesterol was measured by the method of Roeschlau(1974) using cholesterol oxidase(TC-V: Kyokuto Pham., Japan) and total bile acid was measured by the method of Turley (1977) using 3α hydroxysteroid dehydrogenase (Kyokuto Pharm., Japan).

Chi-square test, Wilkoxon Rank Sum and t-test were used for statistical analysis.

RESULTS

All the hamsters had been alive and healthy without diarrhea throughout the experimental period. Group III appeared to eat less compared to the other two groups as shown in the amount of diet consumption per body weight (Table 2). Group III failed to gain weight through the experimental period (Fig.1). Gallstones developed in 11.1%(2/18) of Group I, 84.2%(16/19) of Group II, and 26.3%(5/19) of Group III($p<0.05$: II vs I, III). High CHO diet increased stone incidence

Table 1. Composition of 3 types of diet for the 3 groups

	Group I(%)	Group II(%)	Group III(%)
Carbohydrate	42.6	65.1	64.9
Corn	29.8	–	–
Wheat	12.8	–	–
Rice	–	58.5	58.3
Sucrose	–	6.6	6.6
Protein	24.2	12.7	12.7
Fat	6.4	3.1	3.1
Fiber	4.1	1.1	1.1
Water	8.9	8.7	8.7
Miscellaneous	13.8	9.3	9.3
Camostat	–	–	0.2

Group I: normal rodent diet. Group. Ii: high-carbohydrate diet, Group. III: high-carbohydrate diet + camostat mesilate

Table 2. Diet consumption during the experimental period(gm/kg/day)

	Group I(n = 18)	Group II(n = 19)	Group III(n = 19)
1st week	56.9	60.1	54.3
2nd week	49.4	52.7	39.8
3rd week	43.9	43.0	44.1
4th week	45.3	45.8	39.0
5th week	50.0	48.9	48.9
6th week	58.2	53.9	52.1
7th week	56.2	47.1	56.2
Average	51.4 ± 2.2	50.2 ± 2.2	47.7 ± 2.6

and camostat reversed the effect of this lithogenic diet.

Stones were black-colored and 0.1 to 1 mm in size (Fig.2). Lamellated structure, as seen on human calcium bilirubinate stones, was noted in some stones. Infrared spectroscopy revealed that the major components of these stones were calcium phosphate, calcium bilirubinate, cholesterol and calcium palmitate and the pattern was similar to human calcium bilirubinate stone (Fig.3). Samples from 2 to 3 hamsters(0.1-0.2 ml from each) were pooled and analysed (Table 3). No differences were noted between Group I and Group II except for phospholipid content, which was lower in Group II($p < 0.05$). Concentrations of all components were quite low in Group III compared to the other two groups.

The pancreatic weight of Group III was greater than the other groups and that of Group II was smaller than that of Group I, whereas kidney weight was similar in each group (Table 4). Representative sections of pancreatic tissues from each group are shown in Fig 4. Acinar size and density of zymogen granules were increased in Group III.

DISCUSSION

Camostat(0.2%) in the diet reversed the increased incidence of gallstone from a high CHO diet in hamsters. Since we have not established the CCK assay yet, plasma CCK level could not be shown here. However, it was clearly demonstrated in this study that a high CHO diet decreased and camostat remarkably increased pancreatic wet weight. It was evident in the histologic findings as well. Camostat stimulates pancreatic growth through the CCK-mediated mechanism(Muramuto *et al.* 1972; Mainz *et al.* 1973; Solomon *et al.* 1978; Otsuki *et al.* 1985; ; Goke *et al.* 1986; Rausch *et al.* 1987; Keim *et al.* 1988). Pancreatic weight in this study reflected the chronic effect of a high CHO diet and camostat on the plasma CCK level. Therefore, decreased pancreatic weight in Group II means that high CHO (or low protein and fat) diet stimulate CCK release less, and pancreatic hypertrophy in Group III means that camostat induced chronically elevated CCK release. These facts suggest that relative gallbladder stasis due to weak CCK release by high CHO diet plays a role in the high CHO diet induced gallstone formation. However, in order to prove this, it is essential to show that exogenous CCK administration also inhibits stone formation. CCK antagonist reverses the effect of camostat as well as the consistent level of plasma CCK.

The dosage of camostat in this study

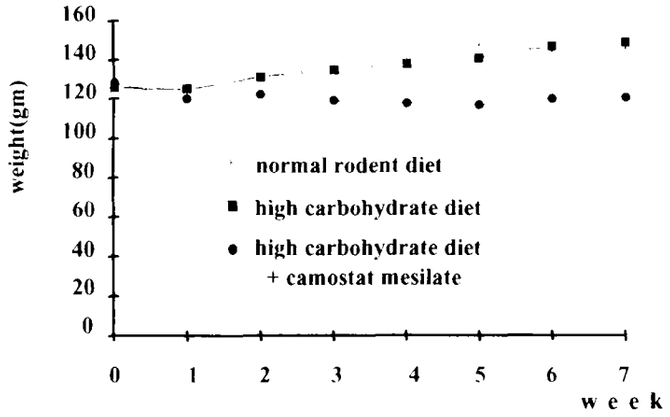


Fig. 1. Body weight change during the experiment. Body weight of Group III hamsters was stationary, while the other two groups of hamsters gained weight.

Fig. 2. Gross feature of gallstones in hamsters.

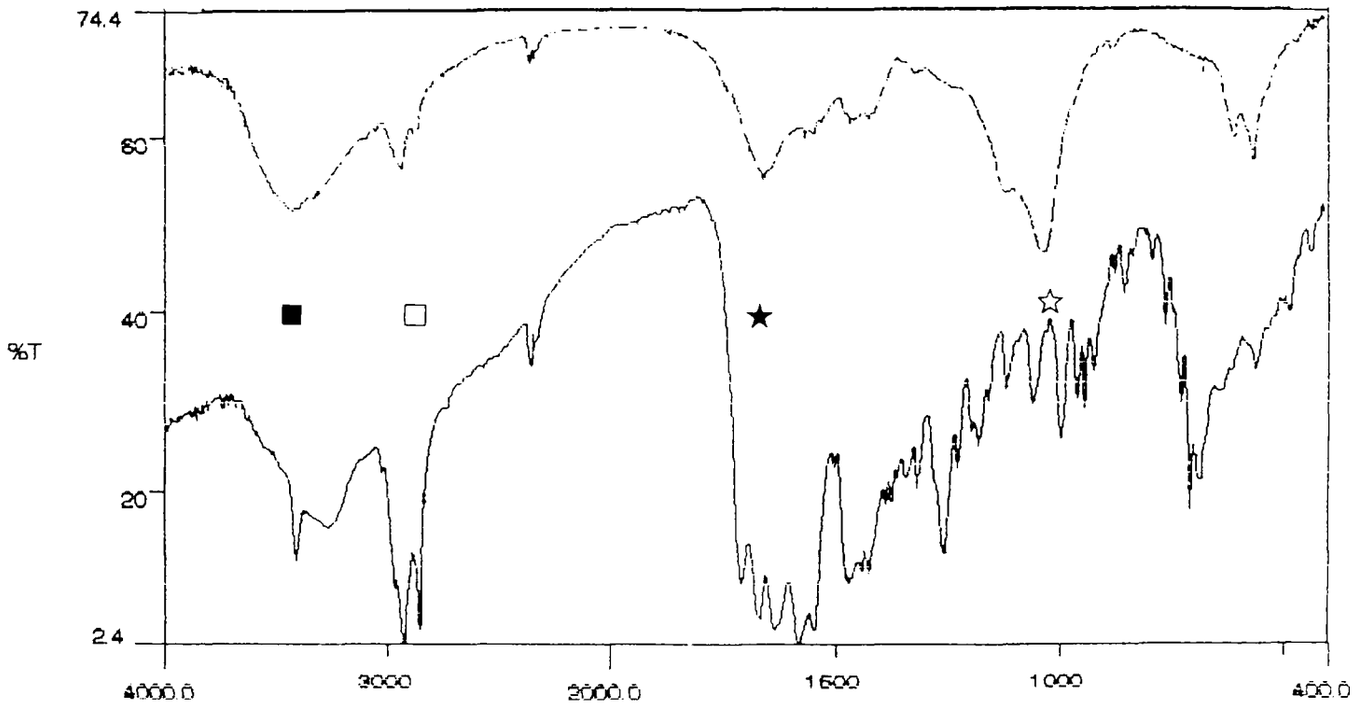


Fig. 3. Infrared absorption spectrum of gallstones. The pattern is similar to that of human calcium bilirubinate stone.

Table 3. Gallbladder bile composition (Mean ± SE)

	Group I (N = 7)	Group II (N = 8)	Group III (N = 8)
Cholesterol(mg/dl)	111.9 ± 8.9	128.7 ± 12.3	95.0 ± 3.4***
Phospholipid(mg/dl)	1682 ± 107	1323 ± 67*	645 ± 55**
Total Calcium(mg/dl)	5.3 ± 0.4	5.6 ± 0.6	4.0 ± 0.2**
Bilirubin(mg/dl)	28.7 ± 2.7	22.3 ± 1.6	10.6 ± 0.5**
Bile acid(mmol/L)	100.6 ± 6.6	93.3 ± 7.9	74.3 ± 3.4**

* p-value < 0.05 vs group I, ** p-value < 0.05 vs groups I and II, *** p-value < 0.05 vs group II

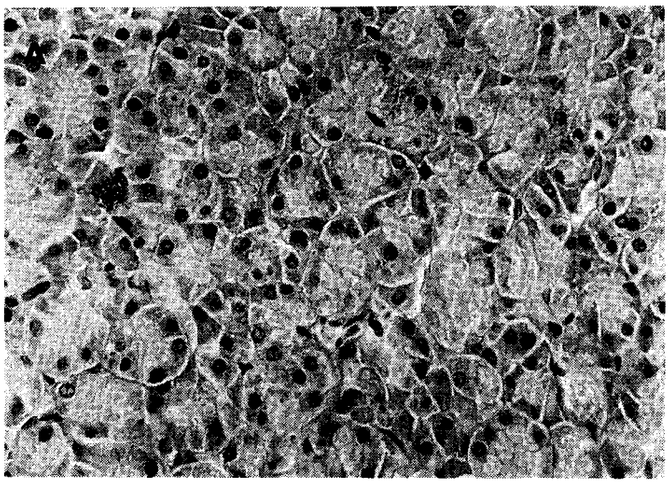
Table 4. Pancreas weight and kidney weight per body weight (Mean ± SE)

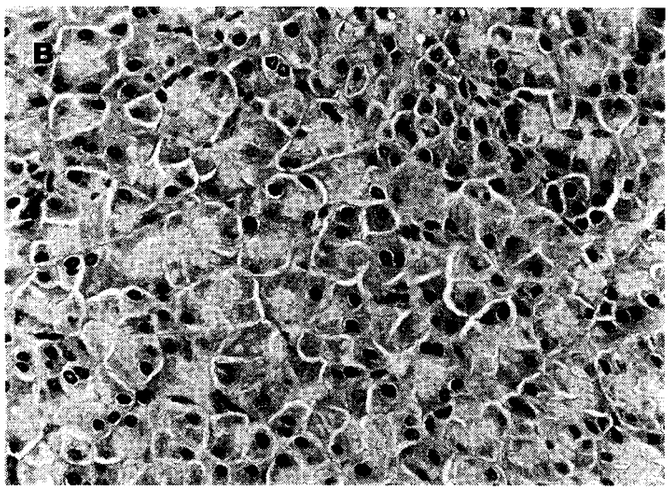
	Group I	Group II	Group III
pancreas weight(mg/gm)	2.9 ± 0.13	2.5 ± 0.08*	4.1 ± 0.13**
kidney weight (mg/gm)	3.9 ±0.09	4.0 ± 0.07	3.8 ± 0.11

* p-value < 0.05(vs group I), ** p-value < 0.001(vs groups I and II)

appears to be too high. Camostat made the animals eat less and fail to gain weight. The authors believe that it is because increased CCK from camostat stimulates the satiety center in hamsters, which is also one of the important actions of CCK(Gibbs *et al.* 1973; Stacher *et al.* 1979; Peikin 1989; Reidelberg *et al.* 1989). We need to investigate whether camostat induces weight loss in the adult animals and humans, whether diet restriction has any effect on this lithogenic diet model and if low dose camostat still has the same effect on gallstone formation without change in the diet amount.

The infrared spectroscopic pattern of the





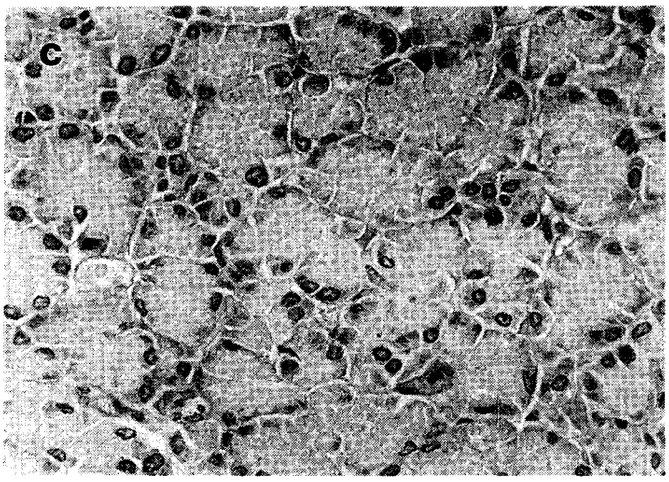


Fig. 4. Microscopic features of pancreas (PAS staining, x400). A: Group I, B: Group II, C: Group III. Diameter of acinar glands and the density of zymogen granules are much greater in Group III than those in the other two groups.

stone was very similar to that of human calcium bilirubinate. As it is hard to determine just how this stone from hamsters resembles the human one, the results of this study can only be a postulation. At least we can say that the stones are quite different from human cholesterol stones. The most important reason for the high proportion of pigment stones in Asian countries is the high proportion of bile duct, especially intrahepatic duct stones in this area. Considering gallbladder stone, the difference between Asian and western countries is not so remarkable as before.

Bile composition was not changed principally by high CHO diet. Further investigation about the significance of the difference in phospholipid concentration is needed. In Group III all the components measured were remarkably low compared to the other groups, in other words, camostat diluted gallbladder bile. Since the fasting duration was very similar in each group and hamsters belonging to different groups were killed alternatively, no significant physiologic factor could be responsible for this phenomenon. Malnutrition, (which might be related to less feeding) may explain the decreased cholesterol concentration, but not the changes in the other components. Maintenance of high CCK level by camostat increases bile flow and gallbladder contractility and, in turn, the gallbladder has not enough time for bile concentration. The concentrations of components in Group III may be close to those of hepatic bile, though not measured in this study. The mechanism of this effect needs to be explored.

Clinical application of antiproteinase has been limited to pancreatitis and disseminated intravascular coagulation so far, although it has not been well established yet. We are now planning to try antiproteinase in gallstone disease. Antiproteinase may possibly prevent pigment stone recurrence after surgery for bile duct stones through the mechanism mediated by increased CCK release. Bile acid dissolution therapy for cholesterol stones has been tried with disappointing results. Oral bile acid may inhibit CCK release through the feedback mechanism and cause relative bile stasis.

Combination with antiproteinase should eliminate this possible unfavorable effect of oral bile acid therapy and increase the success rate of oral dissolution therapy.

Camostat inhibits high CHO diet induced gallstone formation in hamsters. The possible mechanism is that camostat recovers the low CCK release from the high CHO diet. This study suggests that a high CHO diet is associated with pigment stone formation through the mechanism that high CHO diet causes poor CCK release. Antiproteinase is a possible future candidate for a prophylactic agent against recurrent pigment stones and for a supplementary agent of dissolution therapy for cholesterol stones in humans.

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