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Short communication

Dose–response efficacy and long-term effect of the hypocholesterolemic effect of octadecylpectinamide in rats

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ABSTRACT

The dose–response efficiency and long-term effect of the hypocholesterolemic effect of octadecylpectinamide was examined in female rats fed diets containing cholesterol at 10 g/kg. In our first experiment, amidated pectin supplied at 20 g/kg, 40 g/kg and 60 g/kg significantly decreased serum cholesterol from 3.32 μmol/ml (control) to 1.23 μmol/ml in a dose-dependent manner. In a second experiment, the hypocholesterolemic effect of amidated pectin supplied at 20 g/kg persisted after 3 months of feeding. In both experiments, the amidated pectin significantly decreased the concentrations of cholesterol in hepatic tissue and triacylglycerols in serum. The serum concentration of aspartate aminotransferase significantly increased in rats fed amidated pectin at 60 g/kg for 4 weeks, and at 20 g/kg for 3 months. In conclusion, amidated pectin at a low dose and used for a period shorter than 3 months might be considered as an effective hypocholesterolemic and lipid-lowering agent that may substitute typical antilipidemic drugs.

1. Introduction

An elevated level of serum cholesterol plays an important role in the development of coronary heart disease. At present, the major hypercholesterolemic drugs are statins, inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA reductase, which is the rate-limiting enzyme of cholesterol biosynthesis. However, because a significant percentage of patients receiving statins suffer from side effects or do not respond well to the therapy, novel approaches to develop new non-statin hypolipidemic drugs are relevant. Non-statin strategies include both the preparation of new hypolipidemic drugs, e.g., new inhibitors of cholesterol biosynthesis, and the use of drugs that have a long history of clinical use, such as bile acid sequestrants, phytosterols, ezetimibe, niacin and fibrates (Costet, 2010; Rozman & Monostory, 2010). The bile acid sequestrants cholestyramine and colestipol are synthetic resins that bind bile acids in the small intestine and interrupt their enterohepatic circulation (Farmer & Gotto, 1995). In contrast, hydrophobic amidated pectin binds neutral sterols, whereas its affinity for bile acids is low. In female rats fed a cholesterol-containing diet, amidated pectin decreased the concentration of cholesterol in the serum and liver and increased the faecal excretion of neutral sterols. The observed hypercholesterolemic effect was similar to that of cholestyramine and greater than that of psyllium (Marounek, Volek, Skřivanová, & Tůma, 2010a; Marounek, Volek, Skřivanová, & Tůma, 2010b), though rather high dietary concentrations of amidated pectin were used in our previous experiments (50 g/kg and 60 g/kg, respectively). Therefore, the aim of the present study was to compare the cholesterol-lowering effect of amidated pectin in female rats when supplied at 20 g/kg, 40 g/kg and 60 g/kg diet for 4 weeks. In a subsequent experiment, we tested a hypothesis that the hypercholesterolemic effect of amidated pectin would exist after three months of feeding, and that amidated pectin has no negative effect on the health of the animals.

2. Materials and methods

2.1. Materials

The amidated pectin was *N*-octadecylpectinamide with a degree of substitution of 60% prepared by the heterogeneous aminodealkoxylation of highly methoxylated citrus pectin with *N*-octadecylamine (Synytsya et al., 2004). The product of the reaction was washed with 80% ethanol, petrolether, 0.1 M HCl in ethanol and again with 80% ethanol. The rat diet ST-1 was supplied by Velaz Ltd. (Lysolaje, Czech Republic), protected palm fat AkoFeed Gigant 60 was obtained from AarhusKarlshamn Sweden AB (Prague, Czech Republic), and the other chemicals were purchased from Sigma–Aldrich (Prague, Czech Republic).

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Table 1
Composition of control and experimental diets (g/kg).

Ingredient	Diet				
	1[a]	2[a]	3	4	5
Cholesterol	0	10	10	10	10
Amidated pectin	0	0	20	40	60
Palm fat	60	50	50	50	50
Cellulose	60	60	40	20	–
Diet ST-1[b]	880	880	880	880	880

[a] Control diets without amidated pectin.
[b] The rat diet ST-1 ingredients were soybean meal, meat and bone meal, fish meal, wheat, maize, oats, wheat bran, limestone, dicalcium phosphate, salt, and supplements of vitamins, trace elements and amino acids. The diet contained crude protein, fibre, fat and ash at 208 g/kg, 41 g/kg, 19 g/kg, and 54 g/kg, respectively. The contents of cholesterol, vitamin E and vitamin A were 280 mg/kg, 75 mg/kg, and 6 mg/kg, respectively.

2.2. Animals and diets

Thirty female Wistar rats at approximately 6 weeks of age weighing approximately 170 g were used in the first experiment. The rats were housed individually in a temperature- and humidity-controlled room. The vivarium was maintained on a 12 h light: 12 h dark daily photoperiod cycle at a temperature of 22 ± 1 °C and a relative humidity of 55–65%. The rat diet ST-1 was supplemented with microcrystalline cellulose and palm fat at 60 g/kg (control diet, no. 1). After 4 weeks, the rats were randomly divided into 5 groups of 6 animals each. The experimental diets no. 2–5 were supplemented with cholesterol at 10 g/kg and amidated pectin at 0 g/kg, 20 g/kg, 40 g/kg, and 60 g/kg, respectively (Table 1). Food and water were available *ad libitum*. The duration of the experiment was 4 weeks, and the rats received 4 g of feed 4 h before they were killed (Spielman, Stangl, & Eder, 2008). The rats were killed by decapitation after anaesthesia by the inhalation of Isofluran (Nicholas Piramal India Ltd., London, UK).

In the subsequent experiment, 15 female rats of the same age were fed the ST-1 diet supplemented with microcrystalline cellulose and palm fat (diet no. 1 in Table 1). At the age of 10 weeks, the rats were randomly divided into 3 groups of 5 animals each. The experimental diets were supplemented with cholesterol at 10 g/kg (diet 2) or with cholesterol and amidated pectin at 10 g/kg and 20 g/kg, respectively (diet 3). The rats were killed 3 months after the start of the experiment.

2.3. Sampling

Mixed blood samples were collected from each animal at the time of sacrifice. The samples were taken into 2 ml tubes without an anticoagulant and allowed to stand at room temperature for 30 min. The sera were separated by centrifugation at 3000 rpm for 20 min, stored at +4 °C, and analyzed the subsequent day. The livers were excised, and one half of each liver was fixed in buffered neutral formaldehyde for histological examination (Pearse, 1968); the second half was frozen and kept at −40 °C until analysis. In the first experiment, the faeces of the rats fed diets 1, 2, and 6 were collected during the last week and stored frozen until analysis.

2.4. Analyses

The analyses of the feed were performed as described previously (Marounek, Volek, Skřivanová, & Czauderna, 2012). An attempt was made to determine the residual octadecylamine in amidated pectin using the method of Evtushenko, Ivanov, and Zaitsev (2002). The levels of serum total cholesterol, LDL-cholesterol and triacylglycerols were determined using commercial kits BIO-LA-TESTs supplied by Erba – Lachema Inc. (Brno, Czech Republic).

Table 2
Effect of cholesterol and amidated pectin at 20 g/kg, 40 g/kg, and 60 g/kg diet on growth and feed intake in female rats.[a] The diets were fed for 4 weeks.

Diet	1	2	3	4	5		
Cholesterol (g/kg)	0	10	10	10	10	SEM	P
Amidated pectin (g/kg)	0	0	20	40	60		
Initial weight (g)	206	211	210	209	209	2.6	0.500
Final weight (g)	230	232	231	228	229	4.0	0.989
Weight gain (g)	24	21	21	19	20	2.3	0.896
Feed intake (g/day)	16.7	17.1	16.2	16.4	16.3	0.2	0.411

[a] Six rats per group.

The total hepatic and faecal lipids were extracted with 2:1 chloroform–methanol and determined gravimetrically (Folch, Lees, & Sloane-Stanley, 1957). Hepatic cholesterol was assayed as follows: liver tissue (3 g) with 0.3 mg of 5α-cholestan (the internal standard) was extracted with 2:1 chloroform–methanol in IKA® DI 25 basic Yellow line homogenizer (supplied by Neo Tec Ltd., Prague). The extract was filtered, washed with physiological saline, mixture methanol–chloroform–water (48:3:47, v/v/v), dried on dry sodium sulphate and evaporated. The hepatic lipids were saponified with 50 ml of 2 M ethanolic KOH at 100 °C 1 h, then 100 ml of distilled water were added and the emulsion was three times extracted with diethyl ether. The extract was washed with distilled water and the cholesterol was derivatised using pyridine–hexamethyldisilazane–trimethylchlorsilane (2.5:0.5:0.2, v/v/v) silylation reagent for 10 min at room temperature. The silyl derivatives were quantified using a gas chromatograph GC 7890 A-system (Agilent Technologies, Inc., Santa Clara, USA) equipped with an 30 m SAC-5 capillary column (Supelco, Bellefonte, USA) operated isothermally. Column and injection heater were maintained at 285 °C, detector at 300 °C.

A standard histological technique was used to detect possible pathomorphological changes of the liver tissue, whereby haematoxylin-stained paraffin sections were prepared and examined microscopically (Pearse, 1968).

2.5. Statistics

The data were statistically analyzed by a one-way analysis of variance using the GLM procedure of SAS, version 8.2 (SAS Institute, Cary, NC, USA). The results were expressed as the mean and the SEM, and all differences were considered non-significant at $P > 0.05$.

3. Results

No treatment effect on the growth and feed intake of the rats in the first experiment was observed (Table 2). The serum and hepatic cholesterol concentrations in the rats fed the basal diet were 1.70 μmol/ml and 5.45 μmol/g, respectively (Table 3). Cholesterol supplementation significantly increased the serum and hepatic cholesterol concentrations to 3.32 μmol/ml and 16.74 μmol/g, respectively, and non-significantly increased the serum triacylglycerols from 1.63 μmol/ml to 2.35 μmol/ml. The effect of cholesterol supplementation on LDL-cholesterol was more pronounced than that on total cholesterol. In the rats fed the cholesterol-containing diets, amidated pectin significantly decreased the serum cholesterol in a dose-dependent manner from 3.32 μmol/ml (control) to 1.23 μmol/ml, and significantly decreased the hepatic cholesterol level. Furthermore, the hepatic cholesterol correlated significantly with the serum total cholesterol ($r = 0.774$; $P < 0.001$). At the highest concentration tested (60 g/kg), amidated pectin significantly increased the activity of aspartate aminotransferase and increased the concentration of total lipids in the faeces.

The lowest dietary concentration of amidated pectin tested was chosen for the second experiment. The feed intake and weight

Table 3
Effect of cholesterol and amidated pectin at 20 g/kg, 40 g/kg, and 60 g/kg diet on the serum and hepatic concentrations of cholesterol, serum triacylglycerols and aminotransferases, liver weight and faecal concentrations of total lipids in female rats.[a] The diets were fed for 4 weeks.

Diet	1	2	3	4	5		
Cholesterol (g/kg)	0	10	10	10	10	SEM	P
Amidated pectin (g/kg)	0	0	20	40	60		
Serum concentrations							
Total cholesterol (μmol/ml)	1.70[b]	3.32[a]	1.73[b]	1.57[b]	1.23[b]	0.15	<0.001
LDL-cholesterol (μmol/ml)	0.02[c]	1.09[a]	0.17[b]	0.17[b]	0.12[b]	0.08	<0.001
Triacylglycerols (μmol/ml)	1.63[a,b]	2.35[a]	1.46[a,b]	1.36[a,b]	1.00[b]	0.16	0.040
AST (nkat/ml)	2.90[a,b]	2.66[b]	3.93[a,b]	3.80[a,b]	4.17[a]	0.19	0.027
ALT (nkat/ml)	0.96	0.87	1.00	1.00	1.05	0.03	NS
Hepatic cholesterol (μmol/g)	5.45[c]	16.74[a]	8.57[b]	6.69[c]	7.17[c]	0.76	<0.001
Weight of liver (g/100 g)	3.57	3.58	3.39	3.73	3.76	0.07	NS
Faecal lipids (mg/g)	24.2[a]	37.1[b]	ND	ND	56.2[c]	3.90	<0.001

Values in the same row with different superscripts differ significantly.
NS – not significant, ND – not determined.
[a] Six rats per group.

Table 4
Effect of cholesterol and amidated pectin at 20 g/kg diet on growth and fed intake in female rats[a]. The diets were fed for 3 months.

Diet	1	2	3		
Cholesterol (g/kg)	0	10	10	SEM	P
Amidated pectin (g/kg)	0	0	20		
Initial weight (g)	218	215	215	2.2	NS
Final weight (g)	274	278	262	3.1	NS
Weight gain (g)	56[a,b]	63[a]	47[b]	3.9	0.034
Feed intake (g/day)	17.6[a]	17.8[a]	15.8[b]	0.29	<0.001

Values in the same row with different superscripts differ significantly.
NS – not significant.
[a] Five rats per group.

gain of the rats fed the amidated pectin at 20 g/kg for 3 months were significantly reduced (Table 4). In this experiment, the feed intake and weight gain significantly correlated in the last two months of the trial ($r = 0.653$; $P = 0.008$), but not in the first month ($r = 0.068$; $P = 0.825$). In the rats fed the cholesterol-containing diets, amidated pectin significantly decreased the serum and hepatic cholesterol, serum triacylglycerols and content of total lipids in the hepatic tissue (Table 5). Amidated pectin combined with cholesterol significantly increased the activity of aspartate aminotransferase in the serum. Although liver weight was significantly increased in the rats fed the cholesterol-containing diets, histological analysis showed no pathophysiological changes in the livers of any group (data not shown). The concentration of residual octadecylamine in amidated pectin was below the level of quantification using the method of Evtushenko et al. (2002), i.e. below 1.5 mg/g.

4. Discussion

In the present study, the hypocholesterolemic effect of amidated pectin supplied at 20 g/kg diet on female rats was similar to that observed in previous experiments using diets supplemented with amidated pectin at 50 g/kg and 60 g/kg (Marounek et al., 2010a, 2010b). The hypocholesterolemic effect of amidated pectin did not decrease after 3 months of feeding. In the first experiment, there was no effect of amidated pectin on growth, whereas the growth of the rats fed amidated pectin for 3 months was significantly decreased in the second experiment, which can be explained, at least in part, by the lower feed intake. Greater faecal losses of fat in rats fed amidated pectin were observed also in our previous experiment (Marounek et al., 2010a). Aspartate aminotransferase was significantly increased in the serum of the rats fed amidated pectin at 60 g/kg diet for 4 weeks, and in the serum of the rats fed amidated pectin at 20 g/kg diet for 3 months. These results may indicate a possible negative effect of amidated pectin on hepatic cells, in spite of the fact that no hepatic pathophysiology was apparent in either the present or previous experiment (Marounek et al., 2010a). It is noteworthy that adverse side effects have been reported for most hypolipidemic drugs (Farmer & Gotto, 1995; Knodel & Talbert, 1987). Amidated pectin is an efficient sorbent of lipids, thus its large dietary concentration and long-term intake may be associated with the malabsorption of lipophilic vitamins; indeed, the possibility of the depletion of fat-soluble vitamins during the long-term use of cholestyramine has been reported by Vroonhof, van Rijn, and van Hattum (2003). In the present study, amidated pectin decreased the concentration of cholesterol and triacylglycerols in serum and the concentration of cholesterol and total lipids in

Table 5
Effect of cholesterol and amidated pectin at 20 g/kg on the serum concentrations of cholesterol, triacylglycerols and aminotransferases, hepatic concentrations of cholesterol, and total lipids and liver weight in female rats[a]. The diets were fed for 3 months.

Diet	1	2	3		
Cholesterol (g/kg)	0	10	10	SEM	P
Amidated pectin (g/kg)	0	0	20		
Serum concentrations					
Total cholesterol (μmol/ml)	2.08[b]	3.16[a]	1.38[b]	0.24	0.002
LDL-cholesterol (μmol/ml)	0.15[b]	0.98[a]	0.06[b]	0.16	0.015
Triacylglycerols (μmol/ml)	1.08[b]	1.78[a]	0.82[b]	0.14	0.004
AST (nkat/ml)	2.55[b]	2.95[a,b]	3.55[a]	0.17	0.041
ALT (nkat/ml)	1.07	1.04	1.16	0.04	NS
Hepatic concentrations					
Cholesterol (μmol/g)	6.36[b]	14.38[a]	6.56[b]	1.03	<0.001
Total lipids (mg/g)	67.5[b]	113.6[a]	65.0[b]	6.76	<0.001
Weight of liver (g/100 g)	3.08[b]	3.59[a]	3.81[a]	0.11	0.007

Values in the same row with different superscripts differ significantly.
NS – not significant.
[a] Five rats per group.

hepatic tissue and increased the concentration of lipids in faeces. In summary, hydrophobic amidated pectin might be considered a clinically effective hypocholesterolemic and lipid-lowering agent used alone or in combination with other lipid-lowering drugs in cases in which a combined therapy is recommended (Knopp et al., 1993; Reiner, 2010; Shanes, 2012).

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