

## LONG-TERM HYPOCHOLESTEROLEMIC EFFECT OF AMIDATED ALGINATE IN RATS

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*The effect of octadecylamide of alginic acid on blood serum and hepatic cholesterol, and the faecal output of fat and sterols was examined in female rats fed diets containing cholesterol and palm fat at 10 and 50 g/kg, respectively for 10 weeks. Amidated alginate, supplied at 10 and 20 g/kg, significantly decreased serum cholesterol from 5.25 to 2.99 and 2.39  $\mu\text{mol/ml}$ , respectively, and decreased hepatic cholesterol from 30.7 to 12.3 and 9.4  $\mu\text{mol/g}$ , respectively. Amidated alginate increased the faecal output of fat and at higher dosing significantly decreased faecal output of bile acids. Faecal output of bile acids and hepatic cholesterol significantly correlated ( $r = 0.791$ ;  $P < 0.001$ ). The results of the present experiment showed that hypocholesterolemic effect of amidated alginate persisted within 10 weeks of feeding.*

**Key words:** cholesterol, blood serum, liver, faeces, fat, bile acids.

Lipid-lowering agents are used with the purpose of amelioration of hyperlipoproteinemia in order to prevent arterial disease. The clinical expression of arterial disease can be delayed by modifying plasma lipoproteins using systemic or non-systemic agents having hypocholesterolemic properties [1]. The latter hypocholesterolemic agents are anion-exchange resins which bind bile acids and increase their faecal excretion [2], and ezetimibe, a cholesterol absorption inhibitor that targets uptake at the jejunal enterocyte brush border [3]. An alternative to bile acid sequestrants are hydrophobic sorbents of neutral sterols: amidated pectin [4], amidated celluloses [5, 6], and amidated alginate [7, 8]. Amidated alginate is an effective cholesterol-lowering agent and sorbent of dietary fat. However, this suggests that the long-term intake of amidated alginate may cause the malabsorption of lipophilic micronutrients. The possibility of depletion of vitamins K and D during the long-term use of cholestyramine (a bile acid sequestrant) has been reported [9, 10].

In this study we tested a hypothesis that the hypocholesterolemic effect of amidated alginate would

exist in rats within 10 weeks of feeding. The effect of amidated alginate on growth of rats, feed intake, and weight of liver and hepatic aminotransferases was examined as well. The present experiment extends our previous study in which amidated alginate was fed to rats for three weeks [7].

### Material and Methods

Sodium alginate from brown algae was purchased from Sigma-Aldrich (a low-viscosity product no. A2158, Prague, Czech Republic). Alginic acid was prepared by washing sodium alginate with 1:1 mixture of ethanol and 4 M HCl, pure ethanol, acetone, and dried in air. Alginic acid was esterified with methanol containing concentrated sulphuric acid. The methyl ester of alginic acid was amino-dealkoxylated with n-octadecylamine [11]. The product of the reaction was filtered, washed with petroleum ether and pure ethanol. Its degree of amidation, calculated according to Taubner et al. [12] on basis of organic elemental analysis was 94.3%. 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). Sylon HTP kit (hexamethyldisilazane-trimethylchlorosilan-pyridine 3:1:9) was purchased from Supelco (Bellefonte, USA). Isolithocholic acid, 12-ketolithocholic acid, norcholic acid,  $\alpha$ -,  $\beta$ -,  $\omega$ -muricholic acids, and  $\beta$ -sitostanol were purchased from Steraloids Inc. (Newport, USA). Other sterols were supplied by Sigma-Aldrich (Prague, Czech Republic).

Twenty-eight female Wistar rats, approximately 6 weeks old, were used. The rats were housed individually in a temperature- and humidity-controlled room. The ST-1 rat diet was supplemented with palm fat and microcrystalline cellulose at 60 and 40 g/kg, respectively (basal diet, no. 1). After 4 weeks, the rats were randomly divided into 4 groups of 7 rats each. The diets no. 2, 3, 4 were supplemented with cholesterol at 10 g/kg, at the expense of palm fat. The diets no. 3, 4 were supplemented with amidated alginate at 10 and 20 g/kg, respectively, at the expense of cellulose (Table). Diets and water were available ad libitum. The experiment duration was 10 weeks, and then rats were sacrificed by decapitation, after anaesthesia by the inhalation of isofluran (Nicholas Piramal India Ltd., London, UK). The rats received 4 g of feed 4 h before they were sacrificed [13].

In the course of the experiment, body weight and feed intake were measured. The study was approved by the Ethics Committee of the Institute of Animal Science and the Central Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic.

Mixed blood samples were collected from each rat at the time of slaughter. The samples were taken into tubes without anticoagulant and allowed to stand for 30 min. The sera were separated by centrifugation, stored at +4 °C, and analysed the subsequent day. The livers were excised and kept at -40 °C until analysis. Faeces were collected during the last 5 days of the experiment, weighed, pooled and stored frozen until analysis.

The serum cholesterol, triacylglycerols, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using commercial kits (BioVendor Ltd., Brno, Czech Republic). The total hepatic and faecal lipids were extracted with 2:1 chloroform-methanol and measured gravimetrically [14]. Hepatic cholesterol was saponified with 2M ethanolic KOH (1 h at 100 °C), extracted with ethyl ether, derivatised using trimethylchlorosilane and hexamethyldisilazane (Sigma-Aldrich, Prague, Czech Republic), and determined using gas chromatograph equipped with SAC-5 capillary column

Composition of control diet and experimental diets (g/kg)

Ingredient	Diet			
	1 <sup>a</sup>	2 <sup>a</sup>	3	4
Cholesterol	0	10	10	10
Palm fat	60	50	50	50
Amidated alginate	0	0	10	20
Cellulose	40	40	30	20
Diet ST-1 <sup>b</sup>	900	900	900	900

<sup>a</sup>Control diets without amidated alginate. <sup>b</sup>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, ash and cholesterol at 196, 40, 24, 41, and 0.28 g/kg.

(Supelco, Bellefonte, USA). The 5 $\alpha$ -cholestane at 0.5 mg/ml was used as the internal standard.

Faecal sterols were determined in freeze-dried samples chromatographically after butylation and silylation with Sylon HTP according to Batta et al. [15]. Norcholic acid was used as the internal standard. Gas chromatograph Focus – GC coupled with MS model ITQ 700 (Thermo Scientific, Illinois, 60060, USA) was used and equipped with the Thermo TR-5MS SQC column. Helium was the carrier gas. The neutral sterols and bile acids were identified on the basis of retention times by comparing to standards. The linear detector responses were confirmed for variable amounts of individual neutral sterols and bile acids injected onto the column. The dry matter (DM) of faeces was determined by oven drying the faeces at 105 °C.

The data were analysed 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 Means  $\pm$  SD. Significant differences ( $P < 0.05$ ) were identified using Tukey's test. The Pearson correlation coefficient was used as a measure of the dependence between pairs of observations.

## Results and Discussion

Body weight and feed intake did not differ among treatment groups (Fig. 1). Supplementation of diets with cholesterol significantly increased total serum cholesterol, hepatic fat, as well as cholesterol concentration in the liver tissue. In rats fed diets supplemented with cholesterol, amidated algi-

nate significantly decreased serum cholesterol and hepatic cholesterol concentration. The serum total cholesterol and hepatic cholesterol were significantly correlated ( $r = 0.888$ ;  $P < 0.001$ ). Amidated alginate at 20 g/kg non-significantly increased activity of aminotransferases.

Amidated alginate at lower dosing (10 g/kg diet) significantly increased faecal output of fat, and at higher dosing (20 g/kg diet) significantly decreased faecal output of bile acids (Fig. 2). Faecal output of bile acids significantly correlated with hepatic cholesterol ( $r = 0.791$ ;  $P < 0.001$ ). In rats fed diets containing cholesterol serum cholesterol negatively correlated with the faecal output of cholesterol ( $r = -0.496$ ;  $P = 0.022$ ).

The hypocholesterolaemic effect of amidated alginate in rats fed cholesterol- and fat-enriched diets for 10 week was stable and similar to that observed

in previous experiment in which amidated alginate was fed for three weeks [7]. The faecal output of cholesterol and serum cholesterol negatively correlated, which suggests that the faecal loss of cholesterol was important for the hypocholesterolemic effect of amidated alginate. Amidated alginate significantly decreased the faecal concentration of bile acids, presumably due to lower synthesis of bile acids in the liver in response to the lower availability of substrate cholesterol. The faecal output of bile acids and cholesterol concentration in hepatocytes were significantly correlated.

There were no significant signs of toxicity of amidated alginate (feed intake, weight of liver, activity of aminotransferases); however, the possibility of depletion of fat-soluble vitamins from the intestinal lumen at long-term treatment and high dosing of amidated alginate cannot be excluded.

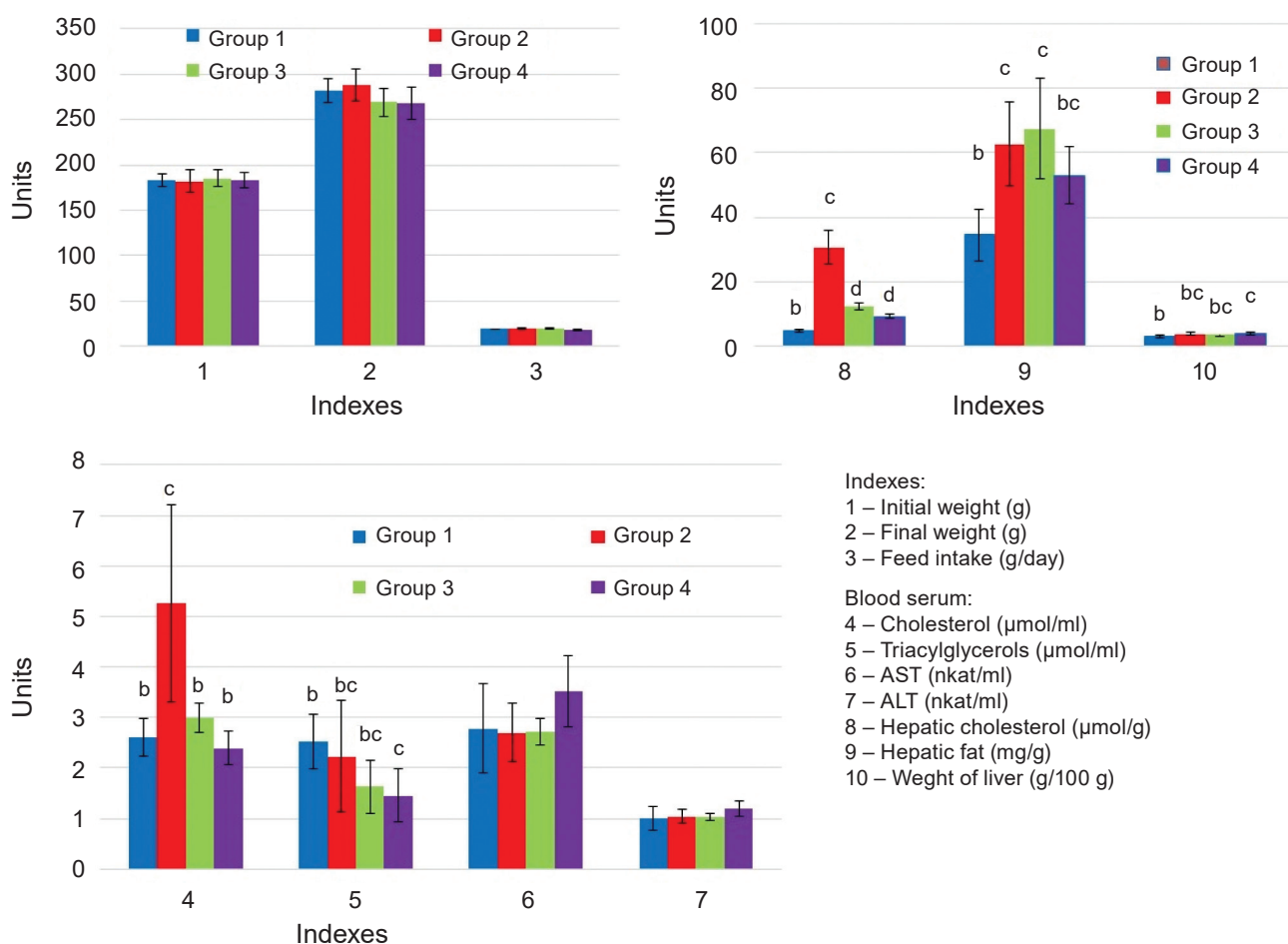


Fig. 1. Effect of cholesterol and amidated alginate on growth, fed intake, serum and hepatic lipids, aminotransferases, and liver weight in rats <sup>a</sup> (a Seven female rats per group. b-d Values in the same index-set with different letters differ significantly)

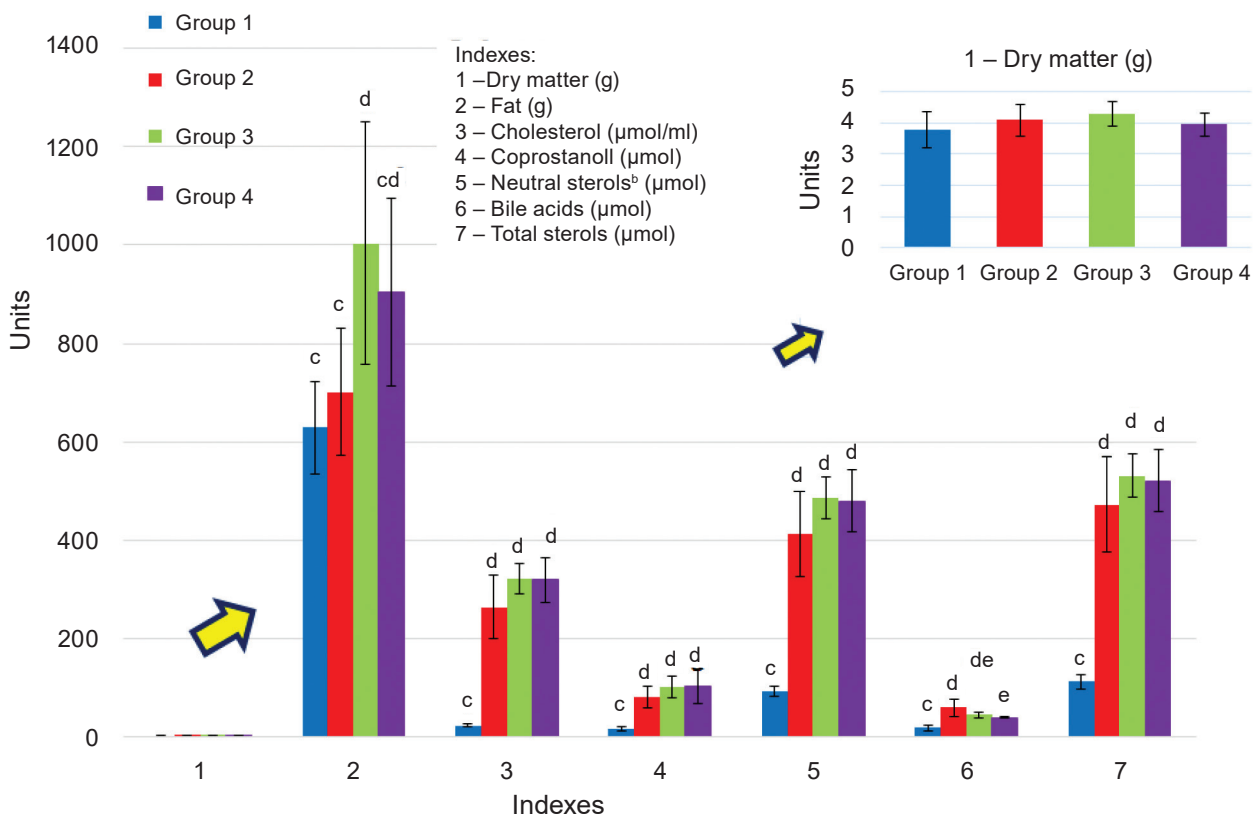


Fig. 2. Effect of cholesterol and amidated alginate on daily faecal output of dry matter, fat, and sterols in rats <sup>a</sup> (a Seven female rats per group. b Including plant sterols. c-e Values in the same index-set with different letters differ significantly)

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### ДОВГОСТРОКОВИЙ ГІПОХОЛЕСТЕРИНЕМІЧНИЙ ЕФЕКТ АМІДОВАНОГО АЛЬГІНАТУ В ЩУРІВ

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Вплив октадециламідів альгінової кислоти на холестерол сироватки крові й печінки, фекальне виділення жиру і стеролів було дослідже-

но у самок щурів, яких утримували на раціонах, що містять холестерол і пальмовий жир відповідно по 10 і 50 г/кг маси тіла тварин протягом 10 тижнів. Амідований альгінат, який давали по 10 і 20 г/кг, значно зменшував рівень холестеролу в сироватці крові від 5,25 до 2,99 і 2,39 мкмоль/мл та знижував рівень холестеролу в печінці від 30,7 до 12,3 і 9,4 мкмоль/г відповідно. Амідований альгінат збільшував фекальний вихід жиру і за вищої дози значно зменшував фекальний вихід жовчних кислот. Фекальне виділення жовчних кислот і холестеролу печінки вірогідно корелювало ( $r = 0,791$ ,  $P < 0,001$ ). Результати експерименту показали, що гіпохолестеринемічний ефект амідованого альгінату зберігається впродовж 10 тижнів годування.

**Ключові слова:** холестерол, сироватка крові, печінка, фекалії, жири, жовчні кислоти.

## ДОЛГОСРОЧНЫЙ ГИПОХОЛЕСТЕРИНЕМИЧЕСКИЙ ЭФФЕКТ АМИДИРОВАННОГО АЛЬГИНАТА У КРЫС

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Влияние октадециламида альгиновой кислоты на холестерол сыворотки крови и печени, фекальное выделение жира и стеролов было исследовано у самок крыс, которых содержали на рационах, содержащих холестерол и пальмовый жир соответственно по 10 и 50 г/кг массы тела животных в течение 10 недель. Амидированный альгинат (подаваемый по 10 и 20 г/кг) значительно уменьшал уровень холестерина в сыворотке крови от 5,25 до 2,99 и 2,39 мкмоль/мл и снижал уровень холестерина в печени от 30,7 до 12,3 и 9,4 мкмоль/г соответственно. Амидированный альгинат увеличивал фекальный выход жира и при более высокой дозе значительно уменьшал фекальный выход желчных кислот. Фекальное выделение желчных кислот и холестерина печени достоверно коррелировало ( $r = 0,791$ ,  $P < 0,001$ ). Результаты настоящего эксперимента показали, что гипохолестеринемический эффект амидированного альгината сохраняется в течение 10 недель кормления.

**Ключевые слова:** холестерол, сыворотка крови, печень, фекалии, жиры, желчные кислоты.

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