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Influence of dietary tender cluster beans (*Cyamopsis tetragonoloba*) on biliary proteins, bile acid synthesis and cholesterol crystal growth in rat bile

Chikkanna K. Raghavendra, Krishnapura Srinivasan *

Department of Biochemistry and Nutrition, CSIR – Central Food Technological Research Institute, Mysore 570 020, India

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ABSTRACT

Tender cluster beans (CBs; *Cyamopsis tetragonoloba*) are observed to possess anti-lithogenic potential in experimental mice. Formation of cholesterol gallstones in gallbladder is controlled by procrystallizing and anticrystallizing factors present in bile in addition to supersaturation of cholesterol. This study aimed at evaluating the influence of CB on biliary glycoproteins, low molecular weight (LMW) and high molecular weight (HMW) proteins, cholesterol nucleation time, and cholesterol crystal growth in rat hepatic bile. Groups of rats were fed for 10 weeks with 0.5% cholesterol to render the bile lithogenic. Experimental dietary interventions were: 10% freeze-dried CB, 1% garlic powder or their combination. Incorporation of CB into HCD decreased the cholesterol saturation index in bile, increased bile flow and biliary glycoproteins. Dietary CB prolonged cholesterol nucleation time in bile. Electrophoresis of biliary proteins showed the presence of high concentration of 27 kDa protein which might be responsible for the prolongation of cholesterol nucleation time in the CB fed group. Proteins of 20 kDa and 18 kDa were higher in CB treated animals, while the same were less expressed in HCD group. Biliary proteins from CB fed animals reduced cholesterol crystal growth index which was elevated in the presence of proteins from HCD group. Cholesterol-7α-hydroxylase and cholesterol-27-hydroxylase mRNA expression was increased in CB treated animals contributing to the bile acid synthesis. Thus, the beneficial anti-lithogenic effect of dietary CB which primarily is due to reduced cholesterol saturation index was additionally affected through a modulation of the nucleating and anti-nucleating proteins that affect cholesterol crystallization.

1. Introduction

Cholesterol gallstone (CGS) disease is a gastroenterological disorder wherein various factors are involved in the CGS formation in the gallbladder. Even if the same degree of super saturation of cholesterol is observed in the bile of a normal individual and a CGS afflicted individual, appearance of cholesterol crystallization will be dissimilar in the bile of the CGS patient when compared to that of the normal individual [1]. Cholesterol gallstone formation involves procrystalizing and anticrystalizing factors viz., calcium, bilirubin, high molecular weight (HMW) proteins, low molecular weight (LMW) proteins and increased cholesterol saturation index, cholesterol: phospholipid ratio and expression of genes involved in bile acid synthesis. Cholesterol-7α-hydroxylase and cholesterol-27-hydroxylase are major rate-limiting enzymes in bile acid synthesis. Bile acid synthesis pathway is important to remove excess of cholesterol from liver. Recently, we have reported that tender cluster beans (*Cyamopsis tetragonoloba*) and garlic (*Allium sativum*) have the potential to reduce the formation of cholesterol gallstone in experimental mice [2]. Tender cluster beans are considered as soluble dietary fiber-rich vegetable which obstruct the cholesterol absorption in the intestine and also produce short chain fatty acids (SCFA) by large intestinal microbial flora. These SCFA have an impact of reducing endogenous cholesterol synthesis, fatty acid and very low density lipoproteins [3]. Garlic acts through its active principle diallyl disulfide, inactivating enzymes and substrates containing thiol groups in an exchange reaction; increases hydrolysis of triacylglycerols through induction of lipase activity; and

Abbreviations: AIN, American Institute of Nutrition; CGS, cholesterol gallstones; CSI, cholesterol saturation index; CYP7A, cholesterol-7α-hydroxylase; CYP27, cholesterol-27-hydroxylase; HCD, high cholesterol diet; HMW, high molecular weight; LMW, low molecular weight; NADPH, nicotinamide adenine dinucleotide phosphate – reduced; NT, nucleation time; SCFA, short chain fatty acids.

* Corresponding author. Tel.: +91 821 2514876; fax: +91 821 2517233.
E-mail address: ksri.cftri@gmail.com (K. Srinivasan).

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reduces the biosynthesis of triacylglycerols as NADPH is made unavailable for the process. The hypocholesterolemic effect of garlic stems in part from decreased hepatic cholesterogenesis [4].

Besides these, cluster beans and garlic may also have an effect on biliary proteins and thus on cholesterol nucleation time in bile. Hence the present study investigates biliary proteins in the bile of experimental rats maintained on these dietary interventions and also cholesterol nucleation time in a model bile system. HMW proteins and LMW proteins were separated on gel permeation chromatography and by electrophoresis. These proteins were appropriately inserted and checked for the nucleation time and appearance of cholesterol crystals in rat bile of different dietary groups and in a model bile system. This particular investigation was undertaken to observe the impact of these two food ingredients on proteins present in the bile that are likely to influence cholesterol crystallization in a supersaturated bile, cholesterol crystal growth in model bile system and also expression of CYP7A and CYP27 genes.

2. Experimental

2.1. Chemicals

Cholesterol, bile salts, dipalmitoyl phosphatidylcholine, triolein, 3α-hydroxysteroid dehydrogenase, standard bile acids, 7β-hydroxycholesterol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium azide, acrylamide, *bis*-acrylamide, tetramethyl ethylenediamine (TEMED), ammonium persulphate, protein markers, DNA ladder, TRI-reagent, diethyl pyrocarbonate (DEPC), RNA-later, urethane and alpha cellulose were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Intramedic PE-10 tubing was purchased from Thomas Scientific Inc. (Swedesboro, NJ, USA). Dithiothreitol was purchased from Fluka Chemie (Buchs, Switzerland) and solvents were obtained from SISCO Research laboratory (Mumbai, India). All solvents used were of analytical grade. All vitamins, DL-methionine and choline chloride were from Himedia (Mumbai, India). SYBR green master mix, oligos, RT-PCR plates from Bio-Rad laboratories Inc. (Hercules, CA, USA). Tender cluster beans, garlic bulbs, refined groundnut oil and cane sugar were procured from the local market.

2.2. Animals and diets

Animal study was carried out with due approval from the Institutional Animal Ethics Committee (CFTRI, Mysore). Wistar albino rats (OUTB/Swiss albino/Ind/CFTRI) raised at our experimental animal production facility weighing 90 ± 5 g were used. Rats (64 No.) were divided into eight groups (n = 8) and were housed in polypropylene cages with four animals per cage and maintained at 25 ± 2 °C with humidity 65 ± 5% with 12 h cycles of day and night. AIN-76 semi-purified diets were prepared by mixing ingredients in a mechanical mixer. Basal AIN-76 diet consisted of: sucrose 65%, casein 20%, cellulose 5%, AIN-76 mineral mix 3.5%, AIN-76 vitamin mix 1%, **dl**-methionine 0.3%, choline chloride 0.2% and refined peanut oil 5%. Lithogenic diet (High cholesterol diet, HCD) was prepared by supplementing 0.5% cholesterol and 0.125% bile salts (1:1 mixture of sodium cholate and sodium deoxycholate) making it isoenergetic by varying sucrose concentration. Test diets were prepared by incorporating freeze-dried tender cluster bean (CB) powder 10% or freeze-dried garlic (1%) or their combination in the lithogenic and basal control diet at the expense of sucrose. These eight groups of animals, viz., Control, HCD, CB (10%), Garlic (1%) and CB + Garlic (10% + 1%) with Basal control as well as lithogenic and given free access to animals to respective diets and water for a duration of 6 weeks. Animal weights were recorded every week till the end of the experiment.

2.3. Bile cannulation and biliary lipid profile

After feeding regimen of 6 weeks animals were fasted overnight and anesthetized with ethyl urethane (1.2 g/kg body weight). Cannulation of bile duct was done and bile was collected for 3 h at 37 °C using incandescent light. After collection of bile the volume was measured and stored for further analysis. Animals were sacrificed and liver was excised stored in RNA-later for the gene expression studies.

Biliary lipids were extracted by using chloroform and methanol (2:1 v/v ratio) and the upper methanolic layer was used for the estimation of bile acids. Lower phase of chloroform was used for the quantification of phospholipid and cholesterol [5]. Bile acids were quantified by using 3α-hydroxysteroid dehydrogenase and standard bile acid [6]. Cholesterol was estimated [7] and phospholipid content was quantified by using ferrous ammonium thiocyanate and reference standard dipalmitoyl phosphatidylcholine [8]. Cholesterol saturation index was calculated by using total lipids and lecithin/bile acid ratio and also cholesterol: phospholipid ratio was also calculated [9].

2.4. Cholesterol nucleation time in different groups of rat bile

Fresh bile sample collected from rats of different groups which were fed with dietary interventions were mixed in different ratios viz., 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100 and incubated for 21 days at 37 °C. An aliquot of these mixed bile was withdrawn at different time intervals and observed under polarized microscope for any precipitation and appearance of cholesterol monohydrate crystals. The day on which any crystal appeared was considered as the cholesterol nucleation time of that particular bile sample. After the incubation of 21 days the bile samples were extracted and lipid profile was done. The cholesterol saturation index was calculated by using lipid composition.

2.5. Preparation of model bile and cholesterol monohydrate crystals (seed crystals)

Model bile was prepared by using predetermined CSI values. Cholesterol and phosphotidyl choline in chloroform and sodium taurocholate in methanol were mixed in different proportions to get desired CSI, flushed in under nitrogen, shaken for 2 h at 37 °C, the organic solvent was evaporated under stream of nitrogen, then lyophilized. Mixture of lipid was made up to required volume by the addition of HEPES buffer saline containing 140 mm NaCl and maintained pH-7.45. The suspension was incubated with shaking at 100 rpm maintained at 55 °C for 6 h or until microscopically homogenous. The clear solution was filtered through 0.22 μm Millipore filter, flushed with nitrogen. The solution was incubated at 37 °C for 15 min prior to use in the crystal growth study [10].

2.6. Cholesterol crystal growth assay

Cholesterol crystal growth in the model bile was measured as described by [11]. Aliquots of filtered (0.22 μm) aqueous solutions of the effectors of interest (both LMW and HMW) were inserted into vials equipped with Teflon-lined screw caps, lyophilized and resolubilized in 40 μL TBS, control vials contained equal volume of TBS and equilibrated at 37 °C. Aliquots (400–500 μL) of model bile equilibrated at 37 °C were distributed to each vial 15 min after model bile filtration. Cholesterol monohydrate crystals were prepared by dissolving 5 g of crystalline cholesterol in 400 mL of 95% ethanol at 60 °C and kept at 4 °C for 3 days. After 3 days the large cholesterol crystals were harvested by using 0.45 μm filters and then suspended in 100 mL water, sonicated for 60 s and