Dr. J.L. Turumin, MD, PhD, DMSci.

The role of cholestanol in the pathogenesis of cholesterol gallstones.
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Jacob L. Turumin
Department of Experimental Surgery, Irkutsk Institute of Surgery,
Scientific Centre of Reconstructive-Restorative Surgery,
Siberian Division, Russian Academy of Medical Sciences, Irkutsk, Russia.

Abstract
The results allow proposing a novel hypothesis and concept of cholesterol gallstone pathogenesis in man: cholestanol may be responsible for the cholesterol supersaturation of gallbladder bile, aseptic inflammation of the gallbladder mucus and consecutive hypersecretion of glycoprotein mucin, gallbladder hypomotility as well as for the heterogenic nucleation process and precipitation of cholesterol monohydrate crystals. Collectively, those data may suggest that cholestanol is likely to play an important role in cholesterol gallstone formation and the cholestanol/cholesterol ratio may be an initializing factor in this process. An increased ratio of cholestanol to cholesterol in bile may be a marker of cholesterol gallstone disease before cholesterol gallstone formation.

Key words: Gallbladder; Cholesterol; Cholestanol; Cholesterol monohydrate crystals; Bile acids; Precipitation; Gallstone disease.

The pathogenesis of cholesterol gallstones in man
The pathogenesis of cholesterol gallstones in man includes five key elements [1, 2]:
1. Supersaturation of bile with cholesterol;
2. Aseptic inflammation of the gallbladder mucosa;
3. Mucin hypersecretion by the gallbladder mucosa;
4. Gallbladder hypomotility with bile stasis;
5. Heterogenic precipitation of cholesterol monohydrate crystals.

Cholesterol supersaturation of gallbladder bile in man results from: a) the increase of hydroxymethylglutarylcoenzyme A reductase activity (HMG-CoA-reductase), increasing endogenous cholesterol biosynthesis in the liver [3]; b) the decrease of cholesterol-7α-hydroxylase activity, decreasing cholesterol transformation into bile acids [4]. These factors, on the one hand, increase biliary cholesterol secretion, and on the other hand decrease cholesterol solubilization by simple bile salt micelles and mixed bile salt-lecithin micelles, hence increasing the already high cholesterol saturation index (CSI) in the gallbladder bile [5].

In animals fed a lithogenic diet, cholesterol supersaturation of gallbladder bile stimulates hypersecretion of gallbladder mucin [6], one of the key components of biliary sludge [7, 8] and cholesterol gallstone matrix [9].

Arachidonyl lecithin [10, 11], arachidonic acid, prostaglandins and lyssolecithin [12, 13, 14] – the traditional mediators of aseptic inflammation of the gallbladder mucosa – increase gallbladder mucin hypersecretion. The addition of salicylates [15] and hydrocortisone (16) in the lithogenic diet results in inhibition of mucin hypersecretion and formation of cholesterol gallstones in the gallbladder.

Though cholesterol in human gallbladder bile is solubilized by simple bile salt micelles, mixed bile salt-lecithin micelles and unilamellar phospholipid vesicles [17, 18, 19], precipitation of cholesterol monohydrate crystals apparently originates only from aggregated multilamellar phospholipid vesicles [20, 21, 22]. These form by aggregation of unilamellar phospholipid vesicles, a process governed by the balance of nucleation-promoting and nucleation-inhibiting agents [23, 24, 25]. Cholesterol monohydrate crystals then heterogeneously nucleate by epitaxy on other crystals such as calcium salts [26, 27], which leave similar crystal lattice structure and dimensions.
A period of time is necessary for primary nucleation of cholesterol monohydrate crystals, the hypomotility of the gallbladder [28, 29], characteristic of patients with cholesterol gallstones [30], plays an important role.

In 1979 P. Boldrini [31] suggested that cholestanol in bile may play a role in pathogenesis of cholesterol gallstones diseases in man. In 1980 Ilias et al. [32] demonstrated that along with cholesterol, cholestanol is the only other biliary sterol (cholesterol precursors, its metabolites and plan sterols) consistently present in cholesterol gallstones. In 1983 Valantinas [33] reported finding cholestanol-cholesterol-dihydrate crystals in the duodenal bile of 77% of patients with cholecystitis who had minor cholesterol supersaturation. In 1986 Miettinen et al. [34] stated that, after ultracentrifugation of gallbladder bile containing cholesterol gallstones, the content of cholestanol was higher in the sediment (73%) than in the supernatant (23%). Koopman et al. [35] proposed that the ratio of concentrations of cholestanol/cholesterol is a long-term parameter of hepato-biliary function. Krikshtopaitis et al. [36] demonstrated that the cholestanol/cholesterol ratio may increase up to 2.4 times in cholesterol gallstone patient’s bile.

Collectively, those data may suggest that cholestanol is likely to play a role in cholesterol gallstone formation and the cholestanol/cholesterol ratio may be an initializing factor in this process [37, 38, 39].

Cholestanol (5α-cholestan-3β-ol) accompanies cholesterol in serum [35] and bile [34]. It derived from two cholestanol pools: 1. exogenous; 2. endogenous. Exogenous cholestanol is found in some foods of animal origin, chiefly dairy products as eggs, and in the liver [32]. Up to 0.5 mg/day may be absorbed by the intestine [32]. Endogenous cholestanol derives from the metabolism of cholesterol in the liver. This amounts normally for about 12 mg/day [40].

There are 2 possible ways of cholestanol biosynthesis by microsomal hepatic enzymes:
1. "direct" or "classic" pathway: cholesterol → 4-cholesten-3-one → 5α-cholestan-3-one → 5α-cholestan-3β-ol (cholestanol) [40];
2. "7α-hydroxylation/dehydroxylation” pathway: cholesterol → 7α-hydroxycholesterol → 7α-hydroxy-4-cholesten-3-one → cholest-4,6-dien-3-one → 4-cholesten-3-one → 5-cholesten-3-one → 5α-cholestan-3β-ol (cholestanol) [41, 42].

The increase of biliary cholestanol in patients with cholesterol gallstones obviously is the result of "direct" biosynthesis activation caused by a decreased cholesterol transformation into primary bile acid intermediates [43].

A possible role of cholestanol for the biliary supersaturation
The experiments on model animals fed with cholestanol demonstrated increased concentrations of cholesterol in the liver [44] and a 2.6 fold increase of the HMG-CoA reductase activity [45]. Additionally, cholestanol inhibits cholesterol-7α-hydroxylase activity [46]. Supersaturation may even be caused by the high content of exogenous cholesterol and cholestanol administration of the lithogenic diet [32, 47]. All three mechanisms result in biliary supersaturation.

A possible role of cholestanol in aseptic inflammation of the gallbladder wall
The experiments on animals fed with cholestanol demonstrated, that exogenous cholestanol may cause inflammation of the gallbladder and bile ducts [44]. Rabbits fed with cholestanol demonstrated gallstone formation, the stones consisted mainly of calcium allodeoxycholate [48]. Taking the high correlation between arachidonic acid and deoxycholate in patients with cholesterol gallstones into account [10] we assume that cholestanol may influence aseptic gallbladder wall inflammation via the increase of deoxycholate in bile.

A possible role of cholestanol in hypersecretion of the gallbladder mucosa
Cholestanol fed in animals induced mucin hypersecretion of the gallbladder mucosa [49].
This could be confirmed by roentgen-cholecystography “in vivo” in rabbits with dietary induced calcium alldexocholate lithogenesis [48, 50].

A possible role of cholestanol in gallbladder hypomotility
Lately, some authors demonstrated that a weak reaction of muscular gallbladder wall fibres on CCK stimulation resulted in gallbladder hypomotility in gallstone patients [30]. Earlier, Boldrini [51] advanced a hypothesis that cholestanol or C-C-2W may cause degradation of aortic smooth muscle cells. This process could occur in human gallbladder muscular fibres as well and result in a more pronounced decline of gallbladder contractility.

A possible role of cholestanol in heterogenic cholesterol monohydrate crystal precipitation
Cholestanol is the only saturated sterol present in the bile of patients with cholesterol gallstones [32]. It is the only invariable sputnik of cholesterol in human cholesterol gallstones when compared to other sterols (cholesterol precursors, its metabolites and plant sterols) [32, 34]. Ultracentrifugation of gallbladder bile from patients with cholesterol gallstones reveals cholestanol to be concentrated higher in the sediment (73%) than in the supernatant (27%). This may be interpreted that cholestanol may participate in the primary precipitation of cholesterol within the gallbladder of patients with cholesterol gallstones [34]. Cholestanol solubility in polar and non-polar solvents is less than half of that of cholesterol [51, 52]. Among all sterols present in bile and cholesterol gallstones only cholestanol monohydrate can form poorly soluble equimolare complexes (C-C-2W) with cholesterol monohydrate [31, 51, 52]. Therefore, C-C-2W may be a centre for heterogenous cholesterol monohydrate crystal nucleation according to an epitaxial mechanism [52]. The presented data demonstrate that the cholestanol/cholesterol ration is the highest in the centre of cholesterol gallstones, compatible with the suggested role of cholestanol in cholesterol nucleation. Biliary sludge and the nucleus of cholesterol gallstones consist of calcium bilirubinate granules, human gallbladder mucin and cholesterol monohydrate crystals [2, 7, 8, 9]. Therefore, the sequence of primary precipitation in hydrophobic domains [53, 54, 55] of a human gallbladder could be the following: calcium bilirubinate precipitation → cholesterol monohydrate precipitation [56], i.e. more hydrophobic particles precipitate first. Taking the greater hydrophobicity of cholestanol into account when compared to cholesterol one can assume the following sequence:

1). Precipitation of calcium bilirubinate granules;
2). Precipitation of C-C-2W or cholestanol-cholesterol (C-C) microcrystals, which may become the heterogenic centre for the nucleation of cholesterol monohydrate crystal [52].

The analysis of literature data [1, 2, 31, 32, 33, 34, 35, 36, 45, 46, 47, 48, 49, 50, 51, 52] and the results of our experiments [37, 38, 39] allow to propose a novel concept of cholesterol gallstone pathogenesis in man: cholestanol is responsible for the cholesterol supersaturation of gallbladder bile, aseptic inflammation of the gallbladder mucus and consecutive hypersecretion of glycoprotein mucin, gallbladder hypomotility as well as for the heterogenic nucleation process and precipitation of cholesterol monohydrate crystals.

It is known that cholesterol gallbladder bile supersaturation is caused by hepatic cholesterol hypersecretion or bile salt hyposecretion as well as both mechanisms combined [3, 4]. Primary cholestanol increase in the liver is caused by exogenic and endogenic factors. It is known that food rich in cholesterol and cholestanol promotes the cholestanol concentration of the liver [32, 47]. Dietary cholesterol increases the cholestanol by the factor of 2.6 [44]. The increased activity of microsomal enzyme 3β-hydroxy-steroid-Δ^5-dehydrogenase which regulates cholestanol biosynthesis in the liver is the endogenic cause of its higher concentration [40]. This increased cholestanol content activates HMG-CoA reductase activity on a cellular level and additionally, it stimulates biosynthesis by increasing the concentration of its precursors [34, 45]. Cholestanol interacting with cholesterol-7α-hydroxylase inhibits this enzyme up to 35% of its initial activity and thus delays biosynthesis or primary bile acids [46]. The increased cholesterol biosynthesis and its decreased metabo-
Lipid into primary bile acids cause a way [40, 42]. This “vicious circle” will maintain the increase in cholestanol concentration even further. Clinically this may result in the increased cholestanol concentration and cholestanol/cholesterol ratio in the hepatic bile and in higher activity of serum aminotransferases [44]. In the gallbladder bile the cholesterol saturation index lies above 1.0 because of the increased cholesterol biosynthesis and the partial inhibition of the bile acids formation. The increase of cholesterol saturation in the micellar phase promotes vesicular cholesterol fraction formation. The increase of cholestanol concentration in the gallbladder bile and higher proportions of secondary bile acids are likely to promote redistribution and accumulation of cholestanol in unilamellar phospholipid vesicles which by diffusion into the gallbladder mucus may cause aseptic inflammation and promote glycoprotein mucin secretion. It is assumed that glycoprotein mucin hypersecretion represents a protective response of the gallbladder mucosa to the diffused toxic cholestanol. This assumption corresponds to the data obtained in experiments on rabbits [49]. As cholestanol is more hydrophobic than cholesterol it can decrease membrane fluidity of the gallbladder wall smooth muscle cells. The decrease of smooth muscular membrane fluidity promotes the decrease of gallbladder wall motility, possibly via CCK receptor interactions. This hypothesis is in accordance with the experimental data. Rabbits receiving cholestanol food exhibit hypomotility of the gallbladder wall, proved by cholecystography “in vivo” [50].

**Cholestanol in unilamellar cholesterol-unsaturated phospholipid vesicles** is likely to have a hydrogen bind with lecithin. As cholesterol saturation of unilamellar vesicles occurs, cholestanol seems to be replaced by cholesterol and occurs in a free state: cholestanol-lecithin + free cholesterol $\rightarrow$ cholesterol-lecithin + free cholestanol.

This assumption was proved in model bile solutions when cholesterol bound hydrogen with a lesser amount of lecithin than cholestanol. Free cholestanol molecules form cholestanol hydrophobic domains together with excessive cholesterol which possesses no hydrogen bindings with lecithin. The increase of domains rich in cholestanol-cholesterol on the surface and in the inner bilayer of unilamellar phospholipid vesicles promotes the contact between free cholestanol and cholesterol molecules: cholestanol-cholesterol-rich domains of the unilamellar phospholipid vesicle surface contact with molecules of water promoting hydration of these molecules with the formation of cholestanol monohydrate-cholesterol monohydrate-rich domains. Aggregation of unilamellar phospholipid vesicles causes the formation of multilamellar phospholipid vesicles and an overlay of cholestanol monohydrate-cholesterol monohydrate-rich domains of one bilayer on the other. It’s known that a single block of cholesterol monohydrate crystals contains 8 in-built molecules [57]. Whereas for the formation of cholestanol-cholesterol-dihydrate complex 1 molecule of cholestanol monohydrate and 3 molecules of cholesterol monohydrate are enough [53]. Structuration of hydrogen bindings between neighboring bilayers of cholestanol monohydrate-cholesterol monohydrate-rich domains will form special lattice of the future cholestanol-cholesterol-dihydrate crystal, i.e. to form primary heterogenic centres of nucleation and precipitation of cholesterol monohydrate crystals [58].

**The precipitation process of cholestanol-cholesterol-dihydrate crystals may follow 2 ways:**

1. in pores of the viscous elastic gel like mucin layer of the gallbladder wall mucosa;
2. in the gallbladder bile during interaction of unilamellar phospholipid vesicles with nucleation-promoting proteins. As cholestanol-cholesterol-dihydrate is less soluble than cholesterol monohydrate, it nucleates first and forms the primary heterogenic nucleation centres of cholesterol monohydrate crystals [52].

The performed nucleation and precipitation of a single cholestanol-cholesterol-dihydrate crystal promotes subsequent nucleation and precipitation of the other crystals of cholestanol-cholesterol-dihydrate and cholesterol monohydrate and lathosterol-cholesterol-dihydrate [32, 34]. Our experiments proved that the cholestanol content is highest in the centre of cholesterol gallstones [38, 59].
Conclusion
The proposed concept includes the ideas of M.C. Carey (1988) and P. Boldrini (1979) about cholesterol gallstone disease pathogenesis in man. In addition, our studies show that an increased ratio of cholestanol to cholesterol in bile may be a marker of cholesterol gallstone disease before cholesterol gallstone formation.

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Web-site: http://www.drturumin.com
e-mail: drjacobturumin@yahoo.com


